Akt Mediates the Effect of Insulin on Epithelial Sodium Channels by Inhibiting Nedd4-2*

Il-Ha Lee, Anuwat Dinudom, Angeles Sanchez-Perez, Sharad Kumar, and David I. Cook

From the Discipline of Physiology, School of Medical Science, Faculty of Medicine, University of Sydney, Sydney, New South Wales 2006 and Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, Frome Road, Adelaide, South Australia 500, Australia

The epithelial sodium channel (ENaC) plays an important role in transepithelial Na⁺ absorption; hence its function is essential for maintaining Na⁺ and fluid homeostasis and regulating blood pressure. Insulin is one of the hormones that regulates activity of ENaC. In this study, we investigated the contribution of two related protein kinases, Akt (also known as protein kinase B) and the serum- and glucocorticoid-dependent kinase (Sgk), on insulin-induced ENaC activity in Fisher rat thyroid cells expressing ENaC. Overexpression of Akt1 or Sgk1 significantly increased ENaC activity, whereas expression of a dominant-negative construct of Akt1, Akt1K179M, decreased basal activity of ENaC. Inhibition of the endogenous expression of Akt1 and Sgk1 by short interfering RNA not only inhibited ENaC but also disrupted the stimulatory effect on ENaC of insulin and of the downstream effectors of insulin, phosphatidylinositol 3-kinase and PDK1. Conversely, overexpression of Akt1 or Sgk1 increased expression of ENaC at the cell membrane and overcame the inhibitory effect of Nedd4-2 on ENaC. Furthermore, mutation of consensus phosphorylation sites on Nedd4-2 for Akt1 and Sgk1, Ser342 and Ser428, completely abolished the inhibitory effect of Sgk1 and Akt1 on Nedd4-2 action. Together these data suggest that both Akt and Sgk are components of an insulin signaling pathway that increases Na⁺ absorption by up-regulating membrane expression of ENaC via a regulatory system that involves inhibition of Nedd4-2.

Transepithelial Na⁺ absorption across the kidney distal collecting duct, the distal colon, and the ducts of the exocrine glands occurs via the epithelial sodium channels (ENaC) and is tightly regulated by hormones such as aldosterone, insulin, and arginine vasopressin. These hormones play an important role in Na⁺ and fluid homeostasis, and their activities are critical in the regulation of blood pressure. Comprehensive knowledge of the signal transduction pathways that underlie the effect of these hormones on ENaC is therefore essential for our understanding of the development of homeostatic abnormalities such as essential hypertension.

Insulin is a peptide hormone that manifests a stimulatory effect on Na⁺ transport in a variety of epithelia (1–5). The immediate natrieric effect of insulin is attributed to an increase in the open probability of ENaC (5, 6) or an increase in the number of active ENaC at the apical membrane (4, 7). Acting through insulin receptor substrate-1, insulin activates phosphatidylinositol 3-kinase (PI3K), a heterodimeric enzyme that catalyzes the formation of phosphatidylinositol 3,4,5-trisphosphate. This activation is essential for mediating several insulin responses, and PI3K has been identified as integral for mediating the effect of insulin on ENaC (8). For instance, insulin treatment increases colocalization of PI3K with ENaC, thereby promoting translocation of ENaC to the apical membrane (4), whereas inhibition of PI3K by LY294002 prevents insulin-induced translocation of the channel (4) and attenuates the insulin-induced current attributed to ENaC (9). In addition, the initial phospholipid product catalyzed by PI3K, phosphatidylinositol 3,4,5-trisphosphate, is known to have a direct stimulatory effect on ENaC (5, 10); thus its action may account for part of the natrieric effect of insulin.

Less well understood, however, is the downstream signaling pathway linking PI3K to the final activation of ENaC. The insulin signaling cascade after PI3K involves translocation of several effector proteins that harbor phospholipid-specific binding motifs. These effector proteins include Akt, a group of protein kinases composed of three isoforms, Akt1, Akt2, and Akt3, that belong to the “cAMP-dependent, cGMP-dependent, protein kinase C” family. At the cell membrane, Akt is phosphorylated by the phosphoinositide-dependent protein kinase, PDK1 (11), and activated Akt is believed to be the central mediator of insulin signaling with profound effects on several physiological events (12–16). Despite the key role of Akt in insulin signaling, a recent study suggested that Akt plays no part in insulin-induced ENaC regulation (17). This conclusion was based on the observation that overexpression of a constitutively active Akt1 mutant (Akt1T308D/S473D) failed to potentiate either the basal or the insulin-induced Na⁺ currents (17).

The other candidate for the effector proteins downstream of PI3K that regulate ENaC is Sgk. This kinase is known to be a key mediator of the effect of the hormone aldosterone on ENaC. Aldosterone acts through Sgk1 to increase ENaC activity by a
mechanism that involves inhibition of the ubiquitin protein ligase, Nedd4-2 (18–20), a ubiquitin-protein isopeptide ligase enzyme that modulates several membrane proteins via a ubiquitin-dependent mechanism. Nedd4-2 binds its tryptophan-rich WW domains to the proline-rich PY motifs on its target protein causing ubiquitination of the substrate. Current studies suggest that phosphorylation of Nedd4-2 by Sgk1 prevents Nedd4-2 from interacting with and down-regulating the activity of ENaC (18, 19). There are two important observations that support the notion that Sgk1 may be the key mediator of the insulin signaling pathway that links P13K with ENaC activation. First, many physiological effects of insulin, such as translocation of GLUT1 and stimulation of NHE3, which are mediated by P13K, are Sgk1-dependent (21, 22). Additionally, insulin cannot further increase ENaC activity in cells that overexpress Sgk1 (17). Surprisingly, given the significant role of Sgk1 in mediating the effect of both aldosterone and insulin on ENaC activity, Sgk1 knock-out mice did not display any alteration of Na+ homeostasis except when challenged with a low salt diet (23). The lack of influence of Sgk1 on Na+ and fluid homeostasis seen in the Sgk1 knock-out mice suggests the presence of a redundant regulatory pathway or pathways that can mediate the effect of these hormones on ENaC in the absence of Sgk1. To determine whether an alternative effector protein mediates the effect of insulin on ENaC, we reinvestigated the contribution of Akt to insulin-induced ENaC activity. Our strategy was to determine whether alteration of the level of expression of Akt could modify the stimulatory effect of insulin on ENaC. The roles of P13K, PDK1, and Sgk1 on ENaC activity and the regulatory mechanism that underlies the impeding effect of Akt and Sgk1 on the activity of Nedd4-2 action and hence ENaC regulation were also investigated.

EXPERIMENTAL PROCEDURES

**DNA Constructs**—Mouse α-, β-, and γ-ENaC in pBluescript were the gifts from Thomas R. Kleyman (University of Pittsburgh). These clones contain FLAG tags at the C-terminal end. The cDNA were subcloned into pcDNA3.1. A wild-type Akt1 (wtAkt1), a constitutively active myristoylated Akt1 (Akt1Myr), and a dominant-negative Akt1 mutant (Akt1K179M) were obtained from Philip N. Tsichlis (Tufts-New England Medical Center). The cDNA for a constitutively active PI3K (PI3Kp110*) was provided by Lawrence M. Pfeffer (University of Tennessee). The cDNA of ENaC, were the gift from Dr. Christoph Korbmacher (University of Erlangen-Nürnberg). Both cell lines were transfected in Ham’s Dulbecco’s modified Eagle’s/F-12 media with 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C; however, the media for FRT cells contained 5% fetal bovine serum whereas the media for M1 cells contained 10% fetal bovine serum and 10 mM dexamethasone. Cells were seeded onto permeable filter supports (Millicell PCF, 0.4-μm pore size, Millipore). One day after seeding, FRT cells were cotransfected with cDNA of α-, β-, and γ-mENaC-FLAG (0.7 μg/ml) together with cDNA of Akt1, Akt2, Akt3, Sgk1, P13K, PDK1 or Nedd4-2 (3 μg/ml) or siRNA against Akt1, Akt2, Akt3, Sgk1, or Nedd4-2 (0.5 μg/ml). All siRNAs were obtained from Qiagen, Australia. In short, cDNA or siRNA were mixed with Lipofectamine-2000 (Invitrogen) in Opti-MEM reduced serum media (Invitrogen) and incubated for 20 min before being transferred to the apical side of the monolayer and further incubated for 6 h. The transfection medium was then replaced with Ham’s Dulbecco’s modified Eagle’s/F-12 media containing fetal bovine serum, antibiotics, and amiloride (10 μM).

**Western Blot**—Two days after transfection, FRT cells were washed twice with phosphate-buffered saline and lysed in lysis buffer containing (in mM) 50 Tris–HCl, 150 NaCl, 10 EDTA, 10% glycerol, 1% Triton X-100 plus Complete Protease Inhibitor mixture (Roche Applied Science). After the protein concentration of each lysate was determined, an equal amount of protein lysate was loaded onto a 12% SDS-polyacrylamide gel. Following electrophoresis, the protein was transferred to a nitrocellulose membrane and incubated overnight with goat anti-Akt1 or goat anti-Sgk1 polyclonal antibody (Santa Cruz Biotechnology) or anti-FLAG M2 monoclonal antibody (Sigma). The blots were washed to remove unbound antibodies before incubating with a horseradish peroxidase-conjugated secondary antibody. The blots were then washed with a TBS buffer containing 0.1% Tween 20, and the proteins of interest were visualized.

**Electrophysiology Experiments**—After the monolayer became confluent, normally within 2–3 days after transfection, the Millipore PCF insert was transferred to a modified Ussing chamber. Apical and basolateral surfaces of the monolayer were simultaneously perfused with a solution containing (in mM) 130 NaCl, 1 CaCl₂, 1 KCl, 1 MgCl₂, 5 glucose, 10 HEPES, pH 7.4, maintained at 37 °C. Experiments were carried out under open circuit conditions (24). Transepithelial resistance was measured by applying short (1 s) repetitive 10-μA current pulses across the epithelium. The transepithelial potential differences (VTₑ) were measured with reference to the luminal side of the epithelium, and equivalent short circuit current was calculated according to Ohm’s law. Amiloride-sensitive equivalent short circuit current (Iₑₑ) was determined as the change in current following addition of amiloride (10 μM) to the apical bathing solution. Data were normalized by dividing the amiloride-sensitive short circuit current by that observed in control cells transfected with ENaC studied on the same day and reported as relative amiloride-sensitive equivalent short circuit current (Iₑₑ_relative). Data for each experiment were obtained from at least three different batches of cells and are reported as mean ± S.E. with the number of experiment in parentheses. Statistical significance was assessed using Student’s t test.

**Surface Expression of ENaC**—FRT cells were transfected with FLAG-tagged α-, β-, and γ-mENaC. Two days after transfection, the cells were washed three times with ice-cold phosphate-buffered saline and then incubated for 30 min in 5 ml of cell-impermeant Sulfo-NHS-SS-Biotin solution (0.5 mg/ml; Pierce) at 4 °C. The reaction was stopped by quenching with Tris-buffered saline. The cells were then solubilized in lysis
buffer, and the lysate was centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant was collected and mixed with 250 μl of NeutrAvidin™ slurry (Pierce) before incubating for 60 min at room temperature with gentle rocking. After incubation, the sample was centrifuged at 1,000 rpm for 2 min. The precipitant at room temperature with gentle rocking. After incubation, the sample was centrifuged at 1,000 rpm for 2 min. The precipitant was extracted using RNeasy mini kit (Qiagen). The RT-PCR total RNA was extracted from each sample and reverse-transcribed. The RT product, containing (in mm) 5 MgCl₂, 1 dNTPs, 0.1 unit/μl Taq DNA polymerase, and 10 pmol of Akt1, Akt2, or Akt3 primers together with 10 pm glyceraldehyde-3-phosphate dehydrogenase primers as a control, was amplified for 30 cycles. Each PCR cycle consisted of 1 min each of denaturation at 95 °C, annealing at 55 °C, and extension at 72 °C. The following primers were used: Akt1 (forward, 5′-GCT GAT GGA CTC AAA CGG CA-3′; reverse, 5′-CCC GAA GTG CGT TAT CTT GA-3′), Akt2 (forward, 5′-CCC TTC TAC AAC CAG GAC CA-3′; reverse, 5′-AGA ACT GGG GGA AGT GTG TG-3′), Akt3 (forward, 5′-CTT CAA GAT GTG GAC TTA CCT-3′; reverse, 5′-ATG ATG GGT GTG TGT AGA TGC ATC-3′), and glyceraldehyde-3-phosphate dehydrogenase (forward, 5′-TGG CCT TCC GTG TTC CTA CC-3′; reverse, 5′-TGG CCT TCC GTG TTC CTA CC-3′). A 10-μl aliquot of the PCR product was then separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

**RESULTS**

Akt Up-regulates Activity of ENaC—We first established the basal characteristics of FRT cells transfected with ENaC in an Ussing chamber under open circuit conditions. Fig. 1A shows representative tracings of the transmembrane potential of an FRT monolayer measured under open circuit conditions. The confluent FRT monolayer exhibited a basal transmembrane potential difference (Vₜₑ) of 7.04 ± 0.92 mV (n = 25) with a transepithelial resistance (Rₑ) of 4.96 ± 0.46 kilo-ohms/cm² (n = 25), corresponding to an equivalent short circuit current of 1.96 ± 0.35 μA-cm² (n = 25). Addition of amiloride (10 μM) to the mucosal side reduced Vₑ to 2.49 ± 0.43 mV (n = 25) and increased Rₑ to 5.81 ± 0.53 kilo-ohms/cm² (n = 25), corresponding to a calculated amiloride-sensitive equivalent short circuit current (Iₑ-amil) of 1.44 ± 0.34 μA-cm² (n = 25). Next, we investigated whether ENaC expressed in our FRT cell system can be up-regulated by Sgk1, a known activator of ENaC. Consistent with a previous report (19), overexpression of wtSgk1 caused a 2-fold increase in ENaC activity (Fig. 1B). To determine the role of Akt in ENaC regulation, we investigated whether overexpression of Akt would increase basal activity of ENaC by cotransfecting ENaC and wtAkt1 into FRT cells. Akt1

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**FIGURE 1. Akt increases ENaC activity.** A, representative recordings of transepithelial potential (Vₑ) of FRT cells coexpressing ENaC and wtAkt1 or Akt1 siRNA (5′-ATGGAGTGTGGACAGTGAA-3′ showing the response to 10 μM amiloride and wtAkt1 or Akt1 siRNA (5′-ATGGAGTGTGGACAGTGAA-3′) showing the response to 10 μM amiloride. B, relative amiloride-sensitive short circuit current (Iₑ-amil) in FRT cells cotransfected with ENaC and wtAkt1 or Akt1 siRNA (5′-ATGGAGTGTGGACAGTGAA-3′) showing the response to 10 μM amiloride and wtAkt1 or Akt1 siRNA (5′-ATGGAGTGTGGACAGTGAA-3′) showing the response to 10 μM amiloride. C, Western blot of FRT cells untransfected with ENaC in an Ussing chamber under open circuit conditions. The confluent FRT monolayer exhibited a basal transepithelial potential difference (Vₑ) of 7.04 ± 0.92 mV (n = 25) with a transepithelial resistance (Rₑ) of 4.96 ± 0.46 kilo-ohms/cm² (n = 25), corresponding to an equivalent short circuit current of 1.96 ± 0.35 μA-cm² (n = 25). Addition of amiloride (10 μM) to the mucosal side reduced Vₑ to 2.49 ± 0.43 mV (n = 25) and increased Rₑ to 5.81 ± 0.53 kilo-ohms/cm² (n = 25), corresponding to a calculated amiloride-sensitive equivalent short circuit current (Iₑ-amil) of 1.44 ± 0.34 μA-cm² (n = 25). Next, we investigated whether ENaC expressed in our FRT cell system can be up-regulated by Sgk1, a known activator of ENaC. Consistent with a previous report (19), overexpression of wtSgk1 caused a 2-fold increase in ENaC activity (Fig. 1B). To determine the role of Akt in ENaC regulation, we investigated whether overexpression of Akt would increase basal activity of ENaC by cotransfecting ENaC and wtAkt1 into FRT cells. Akt1
protein expression was confirmed by Western blot (Fig. 1C), and amiloride-sensitive Na\(^+\) current was measured as an assay of ENaC function (Fig. 1B). In cells overexpressing Akt1, the amiloride-sensitive Na\(^+\) current was increased by 50\% compared with that of the cells expressing ENaC alone. Interestingly, overexpression of a constitutively active Akt1 mutant, Akt1\(_{K179M}\), which was expected to have a greater stimulatory effect on ENaC than wtAkt1, not only failed to activate ENaC but also reduced basal ENaC activity.

We then asked whether activity of Akt1 or of Sgk1 is essential for maintaining the basal activity of ENaC. If Akt1 or Sgk1 are indispensable, inhibition of the activity of these kinases should have an adverse effect on the amiloride-sensitive Na\(^+\) current. To answer this question, we cotransfected FRT cells with an siRNA against Akt1 or an siRNA against Sgk1. The effectiveness of the siRNAs in preventing expression of the corresponding kinases was then confirmed by Western blot (Fig. 1C). Inhibition of Akt1 or Sgk1 expression significantly diminished activity of ENaC. Consistent with this finding, inhibition of Akt1 activity by overexpressing a dominant-negative Akt1 mutant (Akt1\(_{K179M}\)) also significantly reduced activity of ENaC (Fig. 1B). Together, the data indicate that activity of Akt and Sgk is essential for maintaining the basal activity of ENaC.

The Roles of Sgk1 and Akt1 in Insulin-induced ENaC Activity—Although it has been reported previously that Sgk1, but not Akt1, is involved in the mechanism by which insulin activates ENaC activity (17), our finding that activity of both Akt1 and Sgk1 not only sustains the basal activity of ENaC, but also stimulates the channel, led us to reinvestigate the possible involvement of Akt1 in the insulin signaling pathway that modulates ENaC activity. We first determined whether inhibition of Akt1 and Sgk1 expression perturbed insulin-induced ENaC activity. We did this by cotransfecting FRT cells with either an siRNA against Akt1 or an siRNA against Sgk1. In a control experiment, insulin-treated cells exhibited the 2-fold increase in amiloride-sensitive Na\(^+\) current. Transfection of an siRNA against Akt1 or an siRNA against Sgk1 rendered the cells insensitive to insulin challenge and decreased basal ENaC activity to only 50\% of that observed in the nontreated cells (Fig. 1B and Fig. 2A). Moreover, when FRT cells were simultaneously transfected with both Akt1 and Sgk1 siRNAs, activity of ENaC was almost completely abolished. The significance of Akt1 in mediating insulin-induced ENaC current was then confirmed in M1 mouse collecting duct cells natively expressing ENaC (17). In agreement with our observations in FRT cells, siRNA against Akt1 also inhibited insulin-induced ENaC activity in M1 cells. Together, these data indicate that endogenous Akt1 and Sgk1 activities are required for the stimulatory effect of insulin on the channel.

It is widely accepted that, in most cell types, insulin treatment typically results in activation of PI3K and recruitment of PDK1 to the cell membrane that, in turn, activates several downstream mediators of the PI3K signaling pathway, including Akt and Sgk (26). Previous studies indicated that PI3K might have an important signaling role in mediating the stimulatory effect of insulin on ENaC (4, 9, 27). In agreement with these findings, we found that overexpression of either a dominant-negative PI3K (PI3K\(_{A p85}\)) or a dominant-negative PDK (myc-PDK1-KD) abolished the stimulatory effect of insulin on ENaC activity in FRT cells transfected with 3 subunits of mENaC-FLAG alone or cotransfected with siRNA directed against Akt1, Sgk1, or combined Akt1 and Sgk1 siRNAs. The cells were treated without (control) or with insulin (100 nm) for 2 h prior to the experiment. B, relative amiloride-sensitive short circuit current of M1 mouse collecting duct cells treated without (−) or with (+) insulin (100 nm) for 2 h prior to the experiment. Dexamethasone was removed from the media 22 h prior to insulin treatment. To investigate the role of Akt1 on the insulin-induced amiloride-sensitive current, M1 cells were seeded onto permeable filter supports. One day after seeding, the cells were transfected with Akt1 siRNA (0.5 \(\mu\)g/ml) using Lipofectamine-2000 (Invitrogen).

C, relative amiloride-sensitive short circuit current from a similar set of transfected FRT cells as in A that had been cotransfected with PI3K\(_{A p85}\) or wtPDK1.
Insulin Activates ENaC via Akt

cascade that regulates ENaC, we first investigated whether PI3K and PDK1 have a stimulatory effect on ENaC, and we then determined whether the effect of these kinases on ENaC would be diminished in the absence of Akt1 or Sgk1. In this experiment, we increased PI3K and PDK1 activity by transfecting FRT cells with either a constitutively active PI3K (PI3Kp110*) or a wild-type PDK1 (wtPDK1) and then silenced Sgk1 and Akt1 activity by cotransfecting the cells with either Sgk1 siRNA or Akt1 siRNA. Overexpression of PI3Kp110* or wtPDK1 significantly increased ENaC activity to the same magnitude of that observed in insulin-treated cells (Fig. 2C). In addition, the ability of PI3Kp110* and wtPDK1 to up-regulate ENaC was abolished by Sgk1 and Akt1 siRNAs.

In agreement with the previous experiment (Fig. 2A), together Akt1 siRNA and Sgk1 siRNA exhibit an additional inhibitory effect on basal ENaC activity in cells overexpressing PI3Kp110* or wtPDK1. Taken together, these data confirm that endogenous activity of Akt1 and Sgk1 is essential for maintaining basal ENaC activity and that these kinases are important components of the insulin-induced PI3K/PDK signaling cascade that controls activity of ENaC in FRT cells.

Sgk1 and Akt1 Inhibit Nedd4-2 Action—It has been suggested that Sgk1 increases Na+ absorption by modulating surface expression of ENaC (18, 28, 29). To investigate whether the stimulatory effect of Sgk1 and Akt1 observed in our study is attributable to an increase in expression of ENaC at the cell membrane, we cotransfected cDNA of PI3Kp110* or wtPDK1. Taken together, these data confirm that inhibitory effect of basal ENaC was abolished by Sgk1 and Akt1 observed in our study is attributable to an increase in expression of ENaC at the cell membrane.
siRNA decreased ENaC surface expression, whereas coexpression of both Akt1 siRNA and Sgk1 siRNA displayed an additional inhibitory effect (Fig. 3A). In addition, Akt1 and Sgk1 had no effect on the relative levels of the cleaved and noncleaved ENaC subunits. For example, in control ENaC-expressed FRT cells, the density of the 95-kDa band as a percentage of the total FLAG-tagged proteins is 76.3 ± 12.4% (n = 3), which is not significantly different from that in cells cotransfected with Akt1 siRNA, 68.2 ± 7.40% (n = 3), or in cells cotransfected with Sgk1 siRNA, 72.4 ± 4.9% (n = 3). The data are consistent with both Akt1 and Sgk1 having an important role in sustaining ENaC expression in the plasma membrane.

Expression of ENaC at the cell membrane is controlled by a mechanism that involves binding of the ubiquitin protease ligase Nedd4 or Nedd4-2 to the PY motifs at the C terminus of the β and γ subunits of ENaC, which subsequently lead to ubiquitin-dependent internalization of the channel (31). Previous studies suggested that Sgk1 increases activity of ENaC by enhancing phosphorylation of Nedd4-2, thereby rendering Nedd4-2 unable to interact with, and regulate, ENaC (18, 19). To investigate further the role of Akt1 and Sgk1 on membrane expression of ENaC, we first asked whether Akt1 and Sgk1 have an inhibitory effect on Nedd4-2-induced down-regulation of ENaC activity. We did this by transfecting the FRT cells with a wild-type Nedd4-2 construct to increase Nedd4-2 activity. We then elevated Akt1 or Sgk1 activity by cotransfecting cells with either wtSgk1 or wtAkt1. Fig. 4A shows that overexpression of Nedd4-2 decreased the activity of ENaC by 50%. Conversely, overexpression of either Akt1 or Sgk1 overcame this inhibitory effect of Nedd4-2 on ENaC. We further confirmed that the effect Akt1 and Sgk1 on ENaC is Nedd4-2-dependent by silencing Nedd4-2 expression with an siRNA against Nedd4-2 and then overexpressed Akt1 or Sgk1 in these cells. Depletion of Nedd4-2 expression by siRNA caused a 2-fold increase in ENaC activity. Moreover, when Nedd4-2 expression was depleted, overexpression Akt1 and Sgk1 had no stimulatory effect on ENaC activity. Therefore, this finding is consistent with Akt1 and Sgk1 exerting their effect on ENaC by inhibiting Nedd4-2.

To investigate whether the inhibitory effect of Akt1 and Sgk1 on Nedd4-2 seen in our study involves Nedd4-2 phosphorylation, we generated two Nedd4-2 mutants, one with Ser342 and the other with Ser428 mutated to alanine. These two mutated serines are reported to be Sgk1 phosphorylation sites on Nedd4 or Nedd4-2 (18, 19). When coexpressed with ENaC, the inhibitory effect of Nedd4-2Ser342A on ENaC is 2-fold greater than the inhibitory effect of Nedd4-2Ser428A on ENaC. In addition, Nedd4-2Ser342A was almost twice as effective as Nedd4-2Ser428A in inhibiting ENaC. The inhibitory effect of Nedd4-2Ser342A on ENaC was only partially reversed by overexpression of Akt1 and Sgk1. Interestingly, neither Akt1 nor Sgk1 had any effect on the inhibitory effect of Nedd4-2Ser428A on ENaC. Taken together, these data indicate that phosphorylation of Ser342 and Ser428 of Nedd4-2 is required for mediation of the inhibitory effects of Akt1 and Sgk1 on Nedd4-2 action and that phosphorylation of the Ser428 is an essential step in negative regulation of Nedd4-2 function.

Expression of Endogenous Akt Isoforms in FRT Cells—Akt proteins are serine/threonine kinases that are broadly expressed in most organs and tissues. Although the role of Akt in insulin signaling has been extensively studied, the relative contribution of specific Akt isoforms to insulin signaling is not well understood. To investigate the role of Akt isoforms in insulin-induced ENaC activity, we first determined expression of Akt isoforms in FRT cells and then explored whether suppression of endogenous expression of each Akt isoform by isoform-specific siRNA would alter the response of ENaC to insulin treatment. The abundance of Akt mRNA expression was determined by performing RT-PCR with total RNA extracted from FRT cells using Akt1-, Akt2-, and Akt3-specific primers. Expression of mRNA for all three Akt isoforms was detected in FRT cells using Akt1-, Akt2-, and Akt3-specific primers. However, the specific Akt siRNAs diminished endogenous expression of each Akt isoform by isoform-specific siRNA would alter the response of ENaC to insulin treatment. The abundance of Akt mRNA expression was determined by performing RT-PCR with total RNA extracted from FRT cells using Akt1-, Akt2-, and Akt3-specific primers. Expression of mRNA for all three Akt isoforms was detected (Fig. 5A). We then demonstrated that expression of isoform-specific Akt siRNAs diminished endogenous expression of corresponding Akts (Fig. 5B) and caused a 50% reduction of basal ENaC current in FRT cells (Fig. 5C).

![Graph showing the effect of Akt1 and Sgk1 on ENaC activity](image)

**FIGURE 4.** Akt1 and Sgk1 regulate the activity of ENaC by a Nedd4-2-dependent mechanism. A, effect of wtAkt1 or wtSgk1 on the relative amiloride-sensitive short circuit current in FRT cells expressing mENaC alone or together with wtNedd4-2 or Nedd4-2 siRNA (5’-AACCACAACAACCACTACAG-3’). B, effect of wtAkt1 or wtSgk1 on the amiloride-sensitive short circuit current in FRT cells expressed ENaC alone or together with Nedd4-2Ser342A or Nedd4-2Ser428A mutants.
Akt isoforms were unable to sustain ENaC activity at the specific Akt siRNA, the other two, nontargeted, endogenous isoform-specific. Conversely, in the presence of an isoform-tive current suggested that the effect of Akt on ENaC is not comparable inhibitory effect on amiloride-sensitive (Fig. 1)
of the insulin signaling pathway in FRT cells. basal ENaC activity and that they are all involved in mediation of all three Akt isoforms are required for the maintenance of activity on ENaC. Together, the data indicate that the activity of all three Akt isoforms are required for the maintenance of basal ENaC activity and that they are all involved in mediation of the insulin signaling pathway in FRT cells.

DISCUSSION
Insulin plays an important role in the regulation of renal Na⁺ absorption (32, 33) by increasing ENaC activity (4, 7, 34). This study provides evidence to support the role of Akt and Sgk1 in maintaining basal activity of ENaC and to support their role as intermediaries of the insulin signaling pathway at a point downstream to PI3K/PDK1. Both kinases modulate the basal activity of ENaC, most likely by supporting ENaC membrane expression at a normal level. Silencing endogenous expression of either Akt1 or Sgk1 by siRNAs reduced this supporting role of these kinases and, consequently, reduced expression of ENaC at the cell membrane (Fig. 3A), hence diminishing ENaC activity (Fig. 1B). In addition, the ability of isoform-specific Akt siRNAs to produce a comparable inhibitory effect on amiloride-sensitive current suggested that the effect of Akt on ENaC is not isoform-specific. Conversely, in the presence of an isoform-specific Akt siRNA, the other two, nontargeted, endogenous Akt isoforms were unable to sustain ENaC activity at the basal level. Together with an additive inhibitory effect of isoform-specific Akt siRNAs on ENaC, these data indicate a contribution of each Akt isoform to modulating ENaC function.

The conflicting data between the effect of wild-type Akt1 and the constitutively active Akt1 mutants, Akt1Myr in this study and Akt1T308D/S473D in another study (17), on ENaC activity is somewhat surprising and deserves careful consideration. Although both forms of the constitutively active Akt1 mutants have been used successfully in mimicking Akt1 effects (12, 35), Akt1Myr not only failed to increase ENaC activity but had an adverse effect on the channel (Fig. 1B). In agreement with this finding, A6 cells stably transfected with Akt1T308D/S473D exhibited neither an increase in the base-line ENaC current nor a greater response to insulin, aldosterone, or AVP than seen in the control cells (17). An apparent increase in phosphorylation of GSK3, a known substrate of Akt, in Akt1T308D/S473D-transfected cells has ruled out the possibility that the transfected Akt1T308D/S473D was ineffective. Given that Akt has a profound effect on several signal transduction pathways, we speculate that hyperactivity of Akt in the constitutively active Akt1-expressing cells may activate multiple signaling pathways, one of which had a negative effect on ENaC.

The cellular mechanism that underlies insulin-induced Akt and Sgk1 regulation of ENaC function involves phosphorylation of Ned4-2. Previous studies indicated that Sgk1 inhibits ENaC by selectively phosphorylating Ned4-2 at two specific sites comparable with Ser^342 and Ser^428 in hNedd4-2, thereby preventing the interaction between Ned4-2 and ENaC (18, 19). Here we provide further evidence to indicate that Akt1 increases ENaC activity by inducing phosphorylation of these same serines on Ned4-2. An increase in the inhibitory effect on ENaC of Ned4-2S342A and Ned4-2S428A (Fig. 4C) may be attributable to a higher affinity of unphosphorylated Ned4-2 to the channel, hence stronger inhibition. Interestingly, the inhibitory effect of Ned4-2S428A on ENaC was greater than that of Ned4-2S342A, and its action could not be altered by Akt1 or Sgk1. Apparently, phosphorylation of Ser^428 must be one of the most critical steps in regulating the effect of Ned4-2 on ENaC.

The regulatory mechanism of insulin that involves Akt, Sgk1, and Ned4-2 modulates activity of several membrane proteins other than ENaC. For instance, a similar mechanism mediates the effect of insulin on the Na⁺-coupled amino acid trans-
porter, SN1 (36), and the Na\textsuperscript{+}-coupled glucose transporter, SGLT1 (13). It is unclear why both kinases, Akt and Sgk, are required for mediating the same natrierific effect of insulin. Such a redundant system, however, could provide a safeguard against homeostatic aberration when one of the kinases becomes ineffective, which might also explain, at least in part, why Sgk1 knock-out mice exhibit normal Na\textsuperscript{+} renal handling and remain normotensive while maintained on a normal low salt diet (23). Furthermore, by using both Akt and Sgk as its effectors, insulin may be able to amplify its effect and simultaneously modulate several membrane transporters with different kinase specificities to provide adequate homeostatic regulation. For instance, insulin increases translocation of two different glucose transporters, GLUT4 via Akt (12) and GLUT1 via Sgk (21).

In conclusion, our data suggest that by activating the classical PI3K and PDK1 pathway, insulin increases ENaC activity by an Akt- and Sgk-dependent mechanism. Phosphorylation of Nedd4-2 by Akt or Sgk reduces the ability of Nedd4-2 to suppress surface expression of ENaC, consequently allowing Na\textsuperscript{+} absorption to increase.

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REFERENCES

1. Nizet, A., Lefebvre, P., Luyckx, A., and Crabbe, J. (1976) Curr. Probl. Clin. Biochem. 6, 262–271
2. Blazer-Yost, B. L., Cox, M., and Furlanetto, R. (1989) Am. J. Physiol. 257, C612–C620
3. Herrera, F. C. (1965) Am. J. Physiol. 219, 819–824
4. Blazer-Yost, B. L., Esterman, M. A., and Vlahos, C. J. (2003) Am. J. Physiol. 284, C1645–C1653
5. Tong, Q., Gamper, N., Medina, J. L., Shapiro, M. S., and Stockand, J. D. (2004) J. Biol. Chem. 279, 22654–22663
6. Marunaka, Y., Hagiwara, N., and Tohda, H. (1992) Am. J. Physiol. 263, F392–F400
7. Blazer-Yost, B. L., Liu, X., and Helman, S. I. (1998) Am. J. Physiol. 274, C1373–C1379
8. Cohen, P. (2002) Nat. Cell Biol. 4, E127–E130
9. Record, R. D., Froelich, L. L., Vlahos, C. J., and Blazer-Yost, B. L. (1998) Am. J. Physiol. 274, E611–E617
10. Helms, M. N., Liu, L., Liang, Y. Y., Al-Khalili, O., Vandewalle, A., Saxena, S., Eaton, D. C., and Ma, H. P. (2005) J. Biol. Chem. 280, 40885–40891
11. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Galfrey, P. R., Reese, C. B., and Cohen, P. (1997) Curr. Biol. 7, 261–269
12. Cong, L. N., Chen, H., Li, Y., Zhou, L., McGibbon, M. A., Taylor, S. I., and Quon, M. J. (1997) Mol. Endocrinol. 11, 1881–1890
13. Dieter, M., Palmada, M., Rajamanickam, J., Aydin, A., Busjahn, A., Boehmer, C., Luft, F. C., and Lang, F. (2004) Obes. Res. 12, 862–870
14. van der Heide, L. P., Kamal, A., Artola, A., Gispen, W. H., and Ramakers, G. M. (2005) J. Neurochem. 94, 1158–1166
15. Mora, A., Sakamoto, K., McMans, E. J., and Alessi, D. R. (2005) FEBS Lett. 579, 3632–3638
16. Stambolic, V., and Woodgett, J. R. (2006) Trends Cell Biol. 16, 461–466
17. Arteaga, M. F., and Canessa, C. M. (2005) Am. J. Physiol. 289, F90–F96
18. Debonneville, C., Flores, S. Y., Kamynina, E., Plant, P. J., Tauxe, C., Thomas, M. A., Munster, C., Chraibi, A., Pratt, J. H., Horisberger, J. D., Pearce, D., Loffing, J., and Staub, O. (2001) EMBO J. 20, 7052–7059
19. Snyder, P. M., Olson, D. R., and Thomas, B. C. (2002) J. Biol. Chem. 277, 5–8
20. Rauh, R., Dinudom, A., Fotia, A. B., Paulides, M., Kumar, S., Korbmacher, C., and Cook, D. I. (2006) Pfluegers Arch. 452, 299–299
21. Palmada, M., Boehmer, C., Akel, A., Rajamanickam, J., Jeyaraj, S., Keller, K., and Lang, F. (2006) Diabetes 55, 421–427
22. Fuster, D. G., Bobulescu, I. A., Zhang, J., Wade, J., and Moe, O. W. (2006) Am. J. Physiol. 292, F577–F585
23. Wulff, P., Vallon, V., Huang, D. Y., Volkl, H., Yu, F., Richter, K., Jansen, M., Schlunz, M., Klingel, K., Loffing, J., Kauselmann, G., Bosl, M. R., Lang, F., and Kuhl, D. (2002) J. Clin. Invest. 110, 1263–1268
24. Kunzelmann, K., Beesley, A. H., King, N. J., Karupiah, G., Young, J. A., and Cook, D. I. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10282–10287
25. Kim, I. S., Lee, M. Y., Lee, I. H., Shin, S. L., and Lee, S. Y. (2000) Biochim. Biophys. Acta 1496, 333–340
26. Tessier, M., and Woodgett, J. R. (2006) J. Cell. Biochem. 98, 1391–1407
27. Shane, M. A., Nozijger, C., and Blazer-Yost, B. L. (2006) Gen. Comp. Endocrinol. 147, 85–92
28. Alvarez de la Rosa, D., Zhang, P., Naray-Fejes-Toth, A., Fejes-Toth, G., and Canessa, C. M. (1999) J. Biol. Chem. 274, 37834–37839
29. Loffing, J., Zecevic, M., Feraillle, E., Kaissling, B., Asher, C., Rossier, B. C., Firestone, G. L., Pearce, D., and Verrey, F. (2001) Am. J. Physiol. 280, F675–F682
30. Zhou, R., Patel, S. V., and Snyder, P. M. (2007) J. Biol. Chem. 282, 20207–20212
31. Malik, B., Price, S. R., Mitch, W. E., Yue, Q., and Eaton, D. C. (2006) Am. J. Physiol. 290, F1285–F1294
32. DeFronzo, R. A., Cooke, C. R., Andres, R., Faloona, G. R., and Davis, P. J. (1975) J. Clin. Invest. 55, 845–855
33. Nizet, A., Lefebvre, P., and Crabbe, J. (1997) Pfluegers Arch. 323, 11–20
34. Record, R. D., Johnson, M., Lee, S., and Blazer-Yost, B. L. (1996) Am. J. Physiol. 271, C1079–C1084
35. Ahmed, N. N., Grimes, H. L., Bellacosa, A., Chan, T. O., and Tsichlis, P. N. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3627–3632
36. Boehmer, C., Okur, F., Setiawan, I., Broer, S., and Lang, F. (2003) Biochem. Biophys. Res. Commun. 306, 156–162