A Novel Pathway of Epithelial Sodium Channel Activation Involves a Serum- and Glucocorticoid-inducible Kinase Consensus Motif in the C Terminus of the Channel’s α-Subunit*

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Aldosterone-induced serum- and glucocorticoid-inducible kinase isoform 1 (SGK1) contributes to the regulation of the epithelial sodium channel (ENaC), the activity of which is critical for long-term blood pressure control. Aldosterone-induced SGK1 is thought to enhance ENaC surface expression by phosphorylating Nedd4-2 and thereby preventing ENaC retrieval and degradation. In outside-out membrane patches of Xenopus laevis oocytes heterologously expressing ENaC, amiloride-sensitive ENaC currents were enhanced by phosphatase inhibitors and were dependent on cytosolic Mg2⁺. This indicates that a kinase is involved in channel regulation. Indeed, recombinant constitutively active SGK1, included in the pipette solution, caused a sustained 2- to 3-fold increase of ENaC currents. Deletion of the C terminus of αENaC largely reduced the stimulatory effect of SGK1, whereas stimulation by SGK1 did not require the presence of the C termini of the β- or γ-subunits. Replacing the serine residue Ser621 of the SGK1 consensus motif in the C terminus of the α-subunit by an alanine specifically abolished the stimulatory effect of SGK1. Our findings indicate that SGK1 can stimulate ENaC activity independently of an inhibition of Nedd4-2-mediated channel retrieval. This defines a novel regulatory pathway likely to be relevant for aldosterone-induced stimulation of ENaC in vivo.

The appropriate regulation of the epithelial sodium channel (ENaC) in the kidney is critically important for the maintenance of body sodium balance and hence for long-term regulation of arterial blood pressure (1). Indeed, two human genetic diseases provide direct evidence that molecular dysfunction of ENaC has severe effects on arterial blood pressure. Loss-of-function mutations of ENaC cause urinary sodium loss, hyperkalemia, and low blood pressure in patients with pseudohyposaldosteronism type 1 (2). In contrast, gain-of-function mutations of ENaC are found in patients with so-called Liddle’s syndrome (pseudohyperaldosteronism) and result in increased renal sodium re-absorption, hypokalemia, and severe arterial hypertension (3).

ENaC is composed of three subunits called α, β, and γ (4). The C termini of the ENaC subunits each contain a proline-rich PXXY (PY) motif, which is believed to be important for interaction with the ubiquitin-protein ligases Nedd4 and Nedd4-2, promoting the ubiquitination, endocytosis, and proteasomal degradation of the channel (5–8). The functional importance of the PY motif was recognized in Liddle’s syndrome where mutations and/or deletions of the PY motif in β or γ ENaC reduce the endocytic retrieval of ENaC from the membrane (9, 10). This results in an increase in the number of ENaC channels in the membrane, which in turn is thought to cause hyperabsorption of Na⁺ and hypertension in patients with Liddle’s syndrome (11, 12).

The most important hormone to regulate ENaC activity is the mineralocorticoid aldosterone. The effects of aldosterone include transcriptional, translational, and post-translational modifications of ENaC and involve a complex system of aldosterone-induced and/or aldosterone-repressed regulatory proteins (13). Despite impressive progress in this field of research the molecular mechanisms that mediate the stimulatory effect of aldosterone on ENaC activity remain incompletely understood (14). However, there is a growing body of evidence that the serum- and glucocorticoid-inducible kinase isoform 1 (SGK1) is an important contributing factor in the signal transduction cascade of aldosterone action on epithelial sodium transport (15). In primary cultures of rabbit renal collecting duct cells (16) and in rat distal nephron, aldosterone was shown to stimulate the expression of SGK1 mRNA (17). This effect correlates well with the stimulatory effect of aldosterone on sodium transport and appears to be mediated by the mineralocorticoid receptor (18). Immunohistochemical studies have shown that in the renal collecting duct SGK1 is not expressed in intercalated cells but in principal cells consistent with a specific co-expression of SGK1 and ENaC (19). SGK1 mRNA and protein levels are also increased by aldosterone in distal colon (17, 18, 20). These findings suggest that SGK1 is a mediator of aldosterone action in classic mineralocorticoid target tissues.

SGK1 was the first aldosterone-induced gene shown to up-regulate ENaC-mediated sodium transport. This was initially demonstrated by co-expression experiments in Xenopus laevis oocyte (16, 18, 21, 22) and more recently in cultured renal epithelial cells (23, 24). SGK1 is thought to be an important molecular target that integrates multiple endocrine inputs regulating epithelial sodium transport (24, 25). The recent development of SGK1 null mice has confirmed that the presence of...
SGK1 is important for the maintenance of sodium balance, since these animals develop pseudohypoaldosteronism when kept on a low sodium diet (26).

The stimulatory effect of SGK1 occurs in the absence of an increase in ENaC expression levels and appears to be due, at least in part, to increasing the surface expression of ENaC. Recent evidence suggests that the increase in surface expression of ENaC is mediated by PY motif-dependent binding of SGK1 to Nedd4-2 and its subsequent phosphorylation. This has been reported to result in an inhibition of Nedd4-2-mediated ubiquitination, endocytic retrieval, and proteasomal degradation of ENaC, thereby increasing the number of functional ENaC channels in the plasma membrane (27, 28). However, there is disagreement as to whether the stimulatory effect of SGK1 on ENaC surface expression is primarily mediated by a decrease in removal from or an increase in translational to the plasma membrane (23, 29).

Finally, in addition to its stimulatory effect on ENaC surface expression, SGK1 may also increase ENaC open probability (30).

ENaC phosphorylation by kinases and dephosphorylation by phosphatases (31) has long been thought to contribute to ENaC regulation (1). Aldosterone, insulin, and protein kinases A and C have been shown to increase in vivo phosphorylation of the C termini of the β- and γ-subunits of ENaC (32). Moreover, the C termini of ENaC subunits expressed as glutathione S-transferase fusion proteins were found to be phosphorylated by cytosolic fractions derived from rat colon (33). This phosphorylation is thought to involve at least three different types of kinases, including the extracellular-regulated kinase (34) and casein kinase 2 (35).

In the present report we used outside-out membrane patch recordings to functionally confirm the involvement of kinases and phosphatases in the regulation of ENaC heterologously expressed in X. laevis oocytes. More specifically, we demonstrated a stimulatory effect of recombinant and constitutively active SGK1 on ENaC currents and identified the importance of an SGK consensus motif in the α-subunit for mediating this effect. These results suggest that SGK1 can directly stimulate ENaC activity independently of its effects on Nedd4-2-mediated channel retrieval.

EXPERIMENTAL PROCEDURES

Molecular Biology—The full-length cDNAs encoding the three subunits of wild-type (wt) rat ENaC (α-, β-, and γ-ENaC) (4) were in pGEM-HE. Those encoding the truncated rENaC subunits αrescue, βrescue, and γrescue (36) and the mutant subunit β525IC (37) were in pS5D and were a gift of Drs. Bernard C. Rossier and Laurent Schild (Lausanne, Switzerland). Linearized plasmids were used as templates for cRNA synthesis using either T7 (wt rat) and T3 (mutant) RNA polymerases (mMessage mMACHINE, Ambion, Austin, TX). To replace the serine Ser283 in the SGK consensus motif of rat αENaC by an alanine (αSer283A-ENaC) or by aspartate (αSer283D-ENaC), site-directed mutagenesis was performed using T7 and Sp6 as flanking primers. To generate αSer283A-ENaC, a mutagenic forward primer with the sequence 5′-CGG AGC CGG TAC TGG GCC CCA GGA CGA GGG GCC-3′ and a reverse primer with the sequence 5′-GCG CCC TGG TCG TGG GCC GTA CCG GCC GGT CCG-3′ were used to introduce a triplet mutation from TCT to nucleotides 1861–1863 to GGC. To generate αSer283D-ENaC, a mutagenic forward primer with the sequence 5′-CGG AGC CGG TAC TGG GCC CCA GGA CGA GGG GCC-3′ and a reverse primer with the sequence 5′-GCG CCC TGG TCG TGG GTC CCA GTA CCG GCC CCG-3′ were used to introduce a triplet mutation from TCT to nucleotides 1861–1863 to GAC. Mutations were confirmed by sequence analysis.

Isolation of Oocytes, Injection of cRNA, and Two-Electrode Voltage Clamp Experiments—Isolation and injection of X. laevis oocytes and two-electrode voltage clamp experiments were performed essentially as described previously (38). Defolliculated stage V–VI oocytes were injected with cRNA using 0.2 or 0.5 ng of cRNA per ENaC subunit. To prevent Na+ overloading (39) injected oocytes were kept in “low sodium” modified Barth’s saline (in mM: 1 NaCl, 40 KCl, 60 N-methyl-D-glucamine (NMDG)-Cl, 0.3 CaCl2, 0.4 CaCl2, 0.8 MgSO4, adjusted to pH 7.4 with HCl, supplemented with 10 units/ml sodium penicillin, and 10 μg/ml streptomycin sulfate).

Outside-out Macropatch Recordings—After a brief (1–2 min) exposure to hypertonic NMDG-Cl bath solution (in mM: 95 NMDG-Cl, 2 KCl, 1 MgCl2, 1 CaCl2, 200 sucrose, 10 HEPEG adjusted to pH 7.4 with Tris), oocytes were permeabilized with detergent and transferred to a bath chamber on a Leica DM IRB inverted microscope (Leitz Microsystems UK Ltd., Milton Keynes, UK). A computer-controlled EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) was used as described previously (38) to perform conventional outside-out membrane patch recordings (40). It was usually possible to obtain a second patch from the same oocyte used for control experiments. Alternatively, a patch from another oocyte of the same batch served as control. For each experimental series oocytes from at least three different batches were used. Patch pipettes were pulled from borosilicate glass capillaries (catalog no. 1155150, inner diameter 0.87 mm, outer diameter 1.5 mm, Hilgenberg, Masfeld, Germany) using a DMZ-Universal puller (Zeitz Instrumente, Munich, Germany) and had a tip diameter of about 5–7 μm after fire polishing to obtain macropatches. Unless stated otherwise pipettes were filled with potassium gluconate pipette solution (in mM: 90 potassium gluconate, 5 NaCl, 2 Mg-ATP, 2 EGTA, and 10 mm HEPEG adjusted to pH 7.4 with Tris). A NaCl-free NMDG-Cl bath solution (in mM: 95 NMDG-Cl, 2 KCl, 1 MgCl2, 1 CaCl2, 10 HEPEG adjusted to pH 7.4 with Tris) was the standard bath solution at the beginning of each experiment. In NaCl bath solution, NMDG-Cl was replaced by 95 mM NaCl. Downward current deflections correspond to cell membrane inward currents, i.e. movement of positive charge from the extracellular side to the cytoplasmic side. Outside-out patches were routinely held at a holding pipette potential of −70 mV, which was close to the reversal potential of Cl− (ECl = −77.2 mV) and K+ (EK = −97.4 mV) under our experimental conditions. I-V plots were obtained from voltage step protocols, and ΔNmax values were obtained by subtracting the currents in the presence of amiloride (2 μM) from the corresponding currents prior to addition of amiloride.

Single Channel Recordings in Outside-out Patches—Single channel recordings were performed essentially as described previously (38, 41). Bath and pipette solutions were identical to those used for recordings in outside-out macropatches. The pipette had a tip diameter of about 1 μm after fire polishing. Single channel current data were initially filtered at 250 Hz and sampled at 1 kHz. Data were analyzed using the program “Patch for Windows” written by Dr. Bernd Letz (HEKA Elektronik, Lambrecht/Pfalz, Germany). Using channel traces re-filtered at 15 Hz, channel activity was estimated from binned amplitude histograms as the product NP_o, where N is the number of channels and P_o is single channel open probability. The program for calculating NP_o, from integration of the areas under the peaks of amplitude histograms uses the following equation (42): 

\[ \text{NP}_o = \int_{-\infty}^{\infty} f(x) \, dx \]

where \( f(x) \) is the number of channels and \( x \) is the mean single-channel current and where \( j \) refers to the \( j^{th} \) current amplitude bin, and \( r \) ranges from 1 to the total number of bins; \( n_r \) is the number of events within bin \( j \); \( \Delta_j \) is \( j^{th} \) current amp quantum, \( \Delta_{max} \) is the total number of events in the population; \( \text{NP}_o \) is the mean single-channel current and \( j \) is single channel open probability bin of \( \text{NP}_o \).

Solutions and Chemicals—Recombinant constitutively active SGK1(1–60, S422D) was purchased from Biomol GmbH (Hamburg, Germany) as 2-μg vials in 50 μl of stock containing as main components 50 mM Tris-HEPES, 0.1 mM EGTA, 0.1% mercaptoethanol, 500 mM NaCl, and 270 mM sucrose. NSGK1 pipettes were prepared on the day of the experiment by adding 2 μl of the SGK1 stock solution to 1 ml of the potassium-gluconate pipette solution giving a final SGK1 concentration of 80 ng/ml. To preserve SGK1 activity, the pipette solution was supplemented with dithiothreitol (1 mM). For control experiments the pipette solution also contained dithiothreitol (1 mM) in the vehicle control, while maintaining the main content of the SGK1 stock solution as indicated above. In addition control experiments were performed using heat-inactivated SGK1 stock solution, which had been incubated at 68 °C for 45 min. Okadaic acid was purchased from Sigma-Aldrich (Taufkirchen, Germany) and was dissolved in Me2SO as a stock solution with a concentration of 0.1 mM. Recombinant protein phosphatase type 2 PP2A was obtained from Merck Biosciences GmbH (Schwalbach, Germany) as a stock solution containing 1 mg/ml PP2A. Okadaic acid and PP2A were added to the pipette solution to give final concentrations of 100 mM and 1 mg/ml, respectively. Appropriate amounts of Me2SO or of a buffer corresponding to the main components...
ENaC Activation Involves SGK Consensus Motif in α-Subunit

Fig. 1. Amiloride-sensitive Na+ currents can be recorded in outside-out macropatches of X. laevis oocytes heterologously expressing ENaC. A, representative current (I) trace from an outside-out patch recorded at a holding potential (V_{hold}) of −70 mV. As indicated by the bars, bath solution was changed from a Na+−free (NMDG-Cl) to a Na+−containing (NaCl) solution with or without amiloride (Ami) in a concentration of 2 μM. B, IV plots were derived from voltage step protocols performed during the experiment shown in A in the absence (open circles) and presence (filled circles) of amiloride. C, average IV plot of the amiloride-sensitive current (ΔI_{Ami}) from similar experiments (n = 22). Vertical bars represent S.E. values. The dotted line represents a GHK fit of the data for a Na+−selective channel with a predicted Na+ equilibrium potential (E_{Na}) of 75.1 mV.

RESULTS

ENaC Currents Can Be Recorded in Outside-Out Macropatches from X. laevis Oocytes Heterologously Expressing ENaC—In oocytes injected with ENaC cRNA, channel expression was routinely confirmed by using two-electrode voltage clamp measurements, which revealed amiloride (2 μM)-sensitive currents (∆I_{Ami}) averaging 10.3 ± 1.1 μA at a holding potential of −60 mV (n = 43 in 14 batches of oocytes). From these oocytes it was possible to obtain stable outside-out macropatches in about 50% of attempts. Fig. 1A shows a typical current recording starting about 5 min after patch excision. In the absence of extracellular Na+ in NMDG-Cl bath solution, only a minor inward current component was initially observed. Changing to NaCl bath solution resulted in an immediate increase of the inward current consistent with the occurrence of a current component carried by Na+ influx. After an initial transient peak this Na+−current component relaxed to a slightly lower quasi-steady-state current. The current peak with subsequent relaxation is a well known phenomenon and is most likely due to so-called Na+ self-inhibition by extracellular Na+ thought to reduce the open probability of ENaC through interaction with an extracellular Na+ binding site (43). As shown in the current trace in Fig. 1A, application of amiloride, in a concentration of 2 μM known to specifically inhibit ENaC, instantaneously inhibited the Na+ inward current component and the effect was readily reversible upon washout of amiloride. In similar experiments as the one shown in Fig. 1A, ∆I_{Ami} averaged 364 ± 88 pA (n = 22 in 9 batches of oocytes) at a holding potential of −80 mV. Amiloride-sensitive currents could be recorded in all successful outside-out patches from ENaC-expressing oocytes. Moreover, in about 60% of cases it was possible to obtain a second patch with amiloride-sensitive currents from the same oocyte to serve as a direct control experiment. We never detected amiloride (2 μM)-sensitive currents in outside-out patches of non-injected control oocytes (n = 12). Fig. 1B shows I/V plots obtained from voltage step protocols performed in the absence and presence of amiloride during the experiment shown in Fig. 1A. A subtracted I/V plot representing the average ∆I_{Ami} values of similar experiments is shown in Fig. 1C. A Goldman-Hodgkin-Katz (GHK) fit of these data reveals that ∆I_{Ami} is highly Na+ selective as expected for a current mediated by ENaC.

ENaC Activity Is Stimulated by Phosphatase Inhibitors and Is Dependent on the Presence of Cytosolic Mg2+.—Representative current recordings from outside-out patches are shown using a control pipette solution (A), a pipette solution containing 1 μg/ml of the phosphatase inhibitor PPI2 (B), or a nominally Mg2+−free pipette solution containing 10 mM EDTA (C). Experiments were performed as illustrated in Fig. 1, but current responses resulting from voltage step protocols were omitted from the traces for clarity. In D, average normalized amiloride-sensitive current data (∆I_{Ami} (%)) of similar experiments as shown in A–C are plotted versus time, including data from experiments using 100 nM phosphatase inhibitor okadaic acid (OA) in the pipette solution.

Fig. 2. ENaC currents are stimulated by phosphatase inhibitors and are dependent on the presence of cytosolic Mg2+. Representative current recordings from outside-out patches are shown using a control pipette solution (A), a pipette solution containing 1 μg/ml of the phosphatase inhibitor PPI2 (B), or a nominally Mg2+−free pipette solution containing 10 mM EDTA (C). Experiments were performed as illustrated in Fig. 1, but current responses resulting from voltage step protocols were omitted from the traces for clarity. In D, average normalized amiloride-sensitive current data (∆I_{Ami} (%)) of similar experiments as shown in A–C are plotted versus time, including data from experiments using 100 nM phosphatase inhibitor okadaic acid (OA) in the pipette solution.
I/H9004, this resulted in a substantial increase of nant SGK1 (80 ng/ml) was included in the pipette solution. As activity in outside-out patches, constitutively active recombinant SGK1 was shown (same experiments as in C). The GHK fits (dotted lines) of the data demonstrate that activation by SGK1 does not alter the Na+ selectivity of the currents.

Recombinant SGK1 Stimulates ENaC Currents in Outside-out Membrane Patches. Representative current traces of a control experiment with heat-inactivated enzyme (A) or with 80 ng/ml constitutively active recombinant SGK1 (B) are shown. Normalized data from similar experiments are summarized in C. In D, average I/V plots of ΔJ_Ami measured 5 or 28 min after patch excision with a pipette solution containing SGK1 are shown (same experiments as in C). The GHK fits (dotted lines) of the data demonstrate that activation by SGK1 does not alter the Na+ selectivity of the currents.

The stimulatory effect of SGK1 is preserved in the presence of colchicine and of low cytosolic Ca2+. A, to inhibit microtubule-dependent recycling, colchicine (20 μM) was included in the pipette solution with SGK1 (filled triangles) or without SGK1 (open circles). B, to prevent Ca2+-dependent channel insertion into the membrane, a nominally Ca2+-free pipette solution containing 10 mM EGTA with SGK1 or without SGK1 was used in a nominally Ca2+-free bath solution containing 1 mM EGTA.

The Effect of SGK1 Is Preserved in the Presence of Colchicine and of Low Cytosolic Ca2+.—An SGK1-mediated increase in ENaC currents may be due to an increase in channel open probability or an increase in the number of channels in the plasma membrane. In outside-out macropatches some cytoskeletal elements may remain attached to the excised plasma membrane. Thus, some of the machinery required for ion channel trafficking may be functional even in excised patches. Channel trafficking to the plasma membrane is likely to involve the microtubule system, whereas the final fusion step of sub-membranous vesicles containing ion channels with the plasma membrane is likely to be calcium-dependent. To test a possible involvement of the microtubule system, we investigated the effect of SGK1 on ENaC currents in the presence of 20 μM colchicine, which was included in the pipette solution and has previously been used in similar concentrations to inhibit microtubule-dependent recycling (44). As shown in Fig. 4A, colchicine had no effect on ENaC currents in control experiments and did not prevent the stimulatory effect of SGK1, which was fully preserved in the presence of colchicine. To investigate the calcium dependence of ENaC stimulation by SGK1, we tested the effect of 10 mM EGTA included in a nominally calcium-free pipette solution, which should maximally reduce the cytosolic and sub-membranous free Ca2+ concentration without having a major effect on the free Mg2+ concentration. In these experi-
iments we also used a nominally calcium-free bath solution containing 1 mM EGTA to prevent Ca\(^{2+}\) influx from the extracellular solution. As shown in Fig. 4B this resulted in a continuous rundown of $\Delta J_{\text{Ami}}$ in control experiments to less than 50% of the initial current value indicating that a minimal cytosolic Ca\(^{2+}\) concentration is probably required to maintain stable ENaC currents. Importantly, even in the presence of 10 mM EGTA in the pipette solution and in the absence of extracellular Ca\(^{2+}\) a substantial stimulatory effect of SGK1 was preserved (Fig. 4B), indicating that Ca\(^{2+}\)-dependent mechanisms are unlikely to be essential for mediating the effect of SGK1. Taken together these findings suggest that the stimulatory effect of SGK1 observed in outside-out patches is not due to an increase in microtubule-mediated or calcium-dependent channel insertion into the plasma membrane.

**Single Channel Recordings Demonstrate That SGK1 Increases the Number of Apparent Channels in the Patch**—If the stimulatory effect of SGK1 is not mediated by insertion of new channels in the plasma membrane, it is likely to be mediated by an effect of SGK1 on channel open probability ($P_o$). In this case SGK1 may either uniformly stimulate $P_o$ of all ENaC channels present in the plasma membrane or, alternatively, may activate silent channels that are thought to be present in the plasma membrane of ENaC-expressing oocytes (11). To investigate this issue we performed single channel recordings in outside-out patches in the presence or absence of SGK1 in the pipette solution. We were able to detect amiloride-sensitive single channel current events in outside-out patches with a single channel current amplitude of 0.67 ± 0.03 pA ($n = 11$) at a holding potential of −100 mV. This corresponds to a single channel conductance of 6.7 ± 0.3 pS ($n = 11$) typical for ENaC.

Importantly, we observed a gradual increase of the number of apparent single channel current levels in experiments in which SGK1 was included in the pipette solution ($n = 4$). This is illustrated by the current traces shown in Fig. 5, which were recorded in the same patch at various times after patch excision. In the particular experiment shown in Fig. 5, we observed maximally two channel levels at the beginning of the experiment. Amplitude histograms revealed $N_{p_o}$ values of 1.13 for the current trace starting 4 min after excision and of 0.95 for the current trace starting 8 min after excision, corresponding to a $P_o$ of 0.57 and 0.48, respectively, assuming that only two channels were active in the patch during this period. These estimates for $P_o$ are in good agreement with the average $P_o$ of 0.5 reported for ENaC in the native collecting duct (45). In the traces starting 12 and 16 min after patch excision, additional channel levels appeared and $N_{p_o}$ increased to 3.84 and 4.23, respectively. Dividing these $N_{p_o}$ values by the maximal number of channels observed during the corresponding recording period we can estimate $P_p$ values of 0.64 and 0.53, respectively. Importantly, in matched control experiments without SGK1 in the pipette solution stable single channel currents or continuous channel rundown was observed ($n = 6$). Taken together, these findings indicate that SGK1 does not uniformly increase the $P_o$ of ENaC channels that are already active in the plasma membrane. Indeed, the gradual appearance of additional channel levels suggests that SGK1 may convert silent ENaC channels resident in the plasma membrane (11) to active channels with a single channel $P_o$ of about 0.5–0.6. Although this is a plausible interpretation, it has to be noted that our single channel recordings cannot distinguish between the possibility of an activation of silent channels resident in the plasma membrane and the insertion of new channels into the plasma membrane.

**The Stimulatory Effect of SGK1 Is Not Due to a Uniform Increase in ENaC Open Probability**—To confirm the finding of our single channel data that SGK1 increases the apparent number of ENaC channels in the patch without having a major effect on the single channel open probability, we performed additional experiments using the S518C mutant of the $\beta$-subunit of rat ENaC ($\beta_{518C}$). This mutant channel can be converted from a channel with a normal open probability to a channel with an open probability of nearly one by exposing the channel to the sulfhydryl reagent MTSET, which destabilizes the channel’s closed state (37).

In outside-out patch recordings from oocytes expressing the mutant channel application of MTSET increased ENaC currents as expected by a factor of about two consistent with an increase in $P_o$ from about 0.5 to 1 (Fig. 6A and C). We also demonstrated that the mutant channel was stimulated by recombinant SGK1 to a similar degree as the wild-type channel (Fig. 6B). Importantly, after stimulation of ENaC by SGK1 the subsequent exposure to MTSET resulted in an additional increase of ENaC currents by a factor of about two (Fig. 6B and C). This demonstrates that MTSET also increases the open probability of ENaC channels from about 0.5 to 1 after they have been activated by SGK1. This finding is nicely consistent with our single channel data demonstrating that SGK1 does not uniformly increase the open probability of ENaC channels but increases the number of apparent ENaC channels in the patch.

The effect of MTSET on the $\beta_{518C}$ mutant ENaC has been
The hatched bars indicate that the C-terminal truncations were analogous to the original Liddle's syndrome mutation of the β-subunit resulting in the loss of all PY-motifs (36) (with SGK1, n = 10; without SGK1, n = 6). B, presence of SGK1 stimulation was confirmed in matched control experiments performed in oocytes from the same batches as used in A but expressing wild-type ENaC (WT-ENaC) rather than αβγ-ENaC (with SGK1, n = 6; without SGK1, n = 4).

Deletion of the C-terminal Sgk1 Consensus Motif in the α-Subunit Is Critical for the Stimulatory Effect of SGK1—To determine the relative importance of the C terminus of the different subunits for the stimulatory SGK1 effect, we performed an additional series of experiments in which we tested the effects of C-terminal truncations of individual ENaC subunits. As shown in Fig. 8 the stimulatory effect of SGK1 was largely preserved when the C termini of βENaC or γENaC were deleted (Fig. 8, B and C). However, in ENaC channels with a C-terminally truncated α-subunit the stimulatory effect of SGK1 was largely abolished. In humans C-terminal truncations of either the β- or the γ-subunit are known to cause Liddle's syndrome. Because Liddle's syndrome is thought to occur because the mutations reduce the PY motif-dependent Nedd4-2-mediated retrieval of ENaC, our finding of a preserved stimulatory SGK1 effect in ENaC channels with C-terminally truncated β- and γ-subunits indicates that in outside-out macropatches ENaC stimulation by SGK1 is not due to an inhibition of Nedd4-2-mediated channel retrieval.

The C-terminal Sgk1 Consensus Motif in the α-Subunit Is Required for ENaC Stimulation—Our findings suggest that the stimulatory effect of SGK1 may be mediated by a direct effect of SGK1 on ENaC. Sequence analysis of the ENaC subunits indicated the presence of two SGK consensus motifs corresponding to a sequence of RRXX(S/T) (Fig. 9) (27, 46). One consensus motif (RRKKS) is localized in the extracellular loop of the γ-subunit at the amino acid position 178–183 shortly after the first transmembrane domain. This motif is unlikely to be involved in mediating the SGK1 effect, because SGK1 acts from the cytosol. The other consensus motif is localized in the C terminus of the rat α-subunit at amino acid position 616–621 just after the second transmembrane domain and is well conserved in mammals. To test whether this SGK1 consensus motif is involved in mediating the effect of SGK1, we mutated its serine to an alanine changing the motif from 616RSRYWS to 616RSRYWA. Two-electrode voltage clamp experiments confirmed that the mutated channel was functional and that its expression resulted in amiloride-sensitive whole cells currents that averaged 11.1 ± 1.0 μA (n =
ENaC Activation Involves SGK Consensus Motif in α-Subunit

**DISCUSSION**

The key findings of the present study are the following: (i) ENaC channel activity in outside-out macropatches is enhanced by phosphatase inhibitors and is dependent on the presence of the SGK1 consensus site in the C terminus of ENaC, and (ii) the C-terminal truncations of ENaC (P646 stop) and of γENaC (F606 stop) result in deletions of the PY motifs and are analogous to the rat equivalent of the original Liddle's syndrome mutation in ENaC.

8) and were well within the range of currents observed with wild-type ENaC. However, as shown in Fig. 10, in outside-out patches the stimulatory effect of SGK1 was completely absent in ENaC channels with a mutated SGK1 consensus site in the α-subunit. Similarly, SGK1 failed to stimulate ENaC currents in outside-out patches when the serine residue 621 of the α-subunit was mutated to an aspartate (αS621D-ENaC; n = 10).

These findings indicate that in outside-out macropatches the stimulatory effect of SGK1 on ENaC currents is critically dependent on the presence of the SGK1 consensus site in the C terminus of αENaC.

The key findings of the present study are the following: (i) ENaC channel activity in outside-out macropatches is enhanced by phosphatase inhibitors and is dependent on the presence of cytosolic Mg$^{2+}$ indicating the involvement of a kinase in channel regulation, (ii) recombinant constitutively active SGK1 included in the pipette solution causes a sustained increase of the ENaC current to a level about 2- to 3-fold that of its initial value, (iii) deletion of the C terminus of the α-subunit of ENaC largely reduces the stimulatory effect of SGK1, whereas ENaC stimulation by SGK1 does not require the presence of the C termini of the β- or γ-subunit, and (iv) replacing the serine of the SGK consensus motif in the C terminus of the α-subunit by an alanine specifically abolishes the stimulatory effect of SGK1.

These findings define a novel pathway by which SGK1 acti-
ENaC Activation Involves SGK Consensus Motif in α-Subunit

...vates ENaC. This pathway is likely to contribute to the aldosterone-induced stimulation of ENaC in vivo. So far it has been thought that the main effect of aldosterone-induced SGK1 is mediated by its inhibitory action on Nedd4-2. Indeed, phosphorylation of Nedd4-2 by SGK1 has been reported to reduce its ability to interact with ENaC thereby preventing ubiquitination, retrieval, and proteasomal degradation of the channel (27, 28). Furthermore, SGK1 has been shown to increase surface expression of ENaC, which is consistent with the concept that it reduces the rate of ENaC retrieval by inhibiting the interaction of Nedd4-2 and ENaC. However, it has been demonstrated that the stimulatory effect of SGK1 on ENaC whole cell currents and surface expression was preserved in oocytes expressing mutated ENaC channels consisting of αβγ-subunits that were C-terminally truncated (29). Under these conditions all PY motifs of the channel were missing preventing an interaction of ENaC with the WW domains of Nedd4-2. Thus, in these experiments the stimulatory effect of SGK1 could not be due to an inhibition of Nedd4-2-mediated channel retrieval. Moreover, in the same study it was demonstrated that the rate of channel endocytosis was similar in oocytes co-expressing ENaC and SGK1 compared with control oocytes expressing ENaC alone. Recently, another group has reported that a point mutation (Y618A) in the β-subunit of ENaC, which is known to impair regulation of ENaC by Nedd4, had no significant effect on the stimulatory effect of SGK1 on ENaC currents (18). Taken together, these findings argue against an exclusive effect of SGK1 on the rate of Nedd4-2-mediated channel retrieval. Indeed, it has been proposed that the stimulatory effect of SGK1 on ENaC currents and channel surface expression is probably due to an increased channel insertion rate rather than to an inhibition of channel retrieval (23, 29).

Our experiments using outside-out patch recordings cannot resolve the question of whether SGK1 enhances channel insertion or reduces channel retrieval in the intact oocyte, because these processes are unlikely to be fully operational in the outside-out patches used in our study. Interfering with the microtubular system by using colchicine or preventing calcium-mediated vesicle insertion by using high concentrations of calcium buffer in the pipette solution did not prevent the stimulatory effect of SGK1 in outside-out macropatches. This suggests that, in outside-out macropatches, channel trafficking and insertion of additional channels into the plasma membrane are unlikely to contribute to the SGK1-mediated increase in ENaC currents. On the other hand inhibition of Nedd4-2-mediated channel retrieval is also unlikely to contribute to the effect of SGK1 in our experiments for the following reasons. First, channel retrieval from the plasma membrane and subsequent intracellular processing also involve the microtubule system, but colchicine failed to affect ENaC stimulation by SGK1. Second, although Na+ feedback inhibition is thought to be mediated by an activation of Nedd4-2-mediated ENaC retrieval (47), we can assume that under our experimental conditions the Nedd4-2 pathway is largely suppressed due to the low cytosolic Na+ concentration (5 mM) of the pipette solution. Third, the finding that SGK1 stimulation of ENaC was not dependent on the presence of the C terminus of the β- or γ-subunit also argues against an involvement of the Nedd4-2 pathway, because Nedd4-2 is thought to interact with the PY motifs of these subunits. Thus, the stimulatory effect of SGK1 in outside-out membrane patches is probably not due to an inhibition of Nedd4-2-mediated channel retrieval.

Our single channel recordings suggest that SGK1 recruits silent ENaC channels resident in the plasma membrane (11) and/or promotes channel insertion rather than uniformly increasing single channel P_o of ENaC channels that are already active. Our experiments, using MTSET as a tool to increase the open probability of mutant P_o, ENaC channels (37), demonstrated that the stimulatory effect of MTSET is preserved in SGK1-treated patches and was similar to the effect in non-treated patches. This is consistent with the interpretation that SGK1 increases the number of active channels in the patch without having a major effect on the open probability of the individual channels. Taken together our data suggest that in addition to increasing channel surface expression (29) SGK1 increases the number of active channels in the plasma membrane possibly by converting silent channels into an active state. This interpretation is consistent with a recent report that co-expression of SGK1 increased ENaC whole cell currents 2.5-fold but channel surface expression only 1.6-fold, indicating a dual effect on the number of channels present in the membrane and on their activity (30). This concept of a dual effect of SGK1 is also supported by our finding that a C-terminal truncation of αENaC largely inhibits the stimulatory effect of SGK1 in outside-out membrane patches probably by impeding the SGK1-induced conversion of silent into active channels, whereas in the intact oocyte the C-terminal truncation of all three subunits does not prevent a stimulatory effect of SGK1 on ENaC surface expression (29).

How then does SGK1 affect ENaC function in the plasma membrane of outside-out patches? Our findings clearly demonstrate the functional importance of the C terminus of the α-subunit and more specifically of the SGK consensus motif. In this context it should be pointed out that the C-terminal truncation of the α-subunit used in our study did not ablate the SGK consensus motif, which is found in the initial portion of the C terminus between the second transmembrane domain and the site at which the C terminus was truncated. Interestingly, as shown in Fig. 8A, the C-terminal truncation of the α-subunit did not completely abolish the stimulatory effect of SGK1 on ENaC currents. This may be due to the fact that the SGK motif remains intact and still allows a partial, albeit very small, activation of the channel. Thus, the presence of the SGK1 motif in the α-subunit is not sufficient for ENaC activation but in addition requires an intact C terminus. In contrast, direct mutation of the SGK consensus motif completely prevented SGK1-mediated ENaC activation even in the presence of an intact C terminus. Taken together these findings indicate that, although the consensus motif is the critical site for the stimulatory effect of SGK1 on ENaC, the more distal C terminus, including the PY motif, is also functionally important for ENaC activation. It is tempting to speculate that phosphorylation of the serine residue within the SGK consensus motif results in a conformational change of the more distal C terminus leading to an activation of ENaC.

As stated in the introduction, phosphorylation and dephosphorylation of ENaC seem to play a major role in the regulation of channel activity. Our finding that phosphatase inhibitors increase ENaC currents in outside-out patches of oocytes is in agreement with a recent report of the stimulatory effect of okadaic acid on ENaC-mediated transepithelial sodium transport in A6 cells (31). The Mg2+ dependence of ENaC activity further confirms the presence of a kinase in the outside-out macropatch. Taken together, our findings demonstrate that a kinase-mediated phosphorylation step is important for maintaining or stimulating ENaC activity. ENaC phosphorylation in Madin-Darby canine kidney cells, stably transfected with the three subunits of ENaC, has been shown to involve the C termini of the β- and γ-subunits, whereas no phosphorylation of the α-subunits was found (32). In A6 cells a baseline phosphorylation of the α- and β-subunits of ENaC was detected, whereas the γ-subunit was found to be either weakly phosphorylated or not phosphorylated (48). More recently, in vitro studies have confirmed the phosphorylation of certain threo-
nine and serine residues of the β- and γ-subunits, whereas no significant phosphorylation was found in the C terminus of the α-subunit (35–35). In this context it is interesting to note that SGK1 has been reported to physically interact with the C-terminal tails of α-ENaC and β-ENaC in vitro. However, there was no evidence that this association resulted in ENaC phosphorylation (25). Thus, as far as we know, specific phosphorylation of the serine residue 621 within the SGK consensus motif of α-ENaC has not yet been reported. Nevertheless, according to the functional analysis of our present study it is likely but not yet proven that phosphorylation of this serine residue plays an important role in the process of SGK1-mediated ENaC activation. It is not yet clear whether SGK1 directly mediates the phosphorylation of this residue or whether this requires an additional not yet identified SGK1-dependent kinase. Indeed, the SGK consensus motif is not uniquely specific for SGK1 and may also be phosphorylated by other kinases like, e.g., protein kinase B known to mediate many of the metabolic actions of insulin (46). Thus, the emerging picture of ENaC regulation by various phosphorylation and dephosphorylation events may turn out to be highly complex, involving a network of interacting kinase cascades. Indeed, following induction of its expression by aldosterone, SGK1 itself requires activation by a pathway that involves the phosphatidylinositol 3-kinase and the phosphoinositide-dependent kinases 1 and 2 (46, 49). Protein kinase A has also been reported to activate SGK, which may contribute to the stimulatory effect of vasopressin on Na+ transport (50).

In conclusion our study provides evidence for a novel pathway of SGK1 stimulation of ENaC involving the C terminus of the α-subunit and, more specifically, a serine residue in its SGK consensus motif close to the second transmembrane domain. Thus, in addition to the known stimulatory effect of aldosterone, SGK1 itself requires activation by a pathway that involves the phosphatidylinositol 3-kinase and, more specifically, a serine residue in its SGK consensus motif in the C terminus of α-ENaC and 3-phosphoinositide-dependent kinases 1 and 2 (46, 49). Protein kinase A has also been reported to activate SGK, which may contribute to the stimulatory effect of vasopressin on Na+ transport (50).
A Novel Pathway of Epithelial Sodium Channel Activation Involves a Serum- and Glucocorticoid-inducible Kinase Consensus Motif in the C Terminus of the Channel's α-Subunit

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