Cell Surface Expression of the ROMK (Kir 1.1) Channel Is Regulated by the Aldosterone-induced Kinase, SGK-1, and Protein Kinase A*

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The Kir1.1 (ROMK) subtypes of inward rectifier K⁺ channels mediate potassium secretion and regulate sodium chloride reabsorption in the kidney. The density of ROMK channels on the cortical collecting duct apical membrane is exquisitely regulated in concert with physiological demands. Although protein kinase A-dependent phosphorylation of one of the three phosphoryceptors in Kir1.1, Ser-44, also a canonical serum-glucocorticoid-regulated kinase (SGK-1) phosphorylation site, controls the number of active channels, it is unknown whether this involves activating dormant channels already residing on the plasma membrane or recruiting new channels to the cell surface. Here we explore the mechanism and test whether SGK-1 phosphorylation of ROMK regulates cell surface expression. Removal of the phosphorylation site by point mutation (Kir1.1, S44A) dramatically attenuated the macroscopic current density in Xenopus oocytes. As measured by antibody binding of external epitope-tagged forms of Kir1.1, surface expression of Kir1.1 S44A was inhibited, paralleling the PKA dependency of ROMK channel surface density to the same level as the phosphorylation mimic mutation, Kir1.1 S44D. In vitro phosphorylation assays revealed that Ser-44 is a substrate of SGK-1 phosphorylation, and expression of SGK-1 with the wild type channel increased channel density. Moreover, the stimulatory effect of SGK-1 was completely abrogated by mutation of the phosphorylation site. In conclusion, SGK-1 phosphorylation of Kir1.1 drives expression on the plasmalemma. Because SGK-1 is an early aldosterone-induced gene, our results suggest a possible molecular mechanism for aldosterone-dependent regulation of the secretory potassium channel in the kidney.

Extracellular potassium homeostasis, maintained by the regulation of renal potassium excretion, is dependent on the activity of weakly inward rectifying "small conductance" potassium channels (SK) that are expressed on the apical membrane of epithelial cells in the distal nephron (1, 2). Encoded by the ROMK (Kir 1.1 or KCNJ1) gene (3, 4), these Kir channels are thought to be the major, but not exclusive (5, 6), route for potassium transport into the tubule lumen and constitute a final regulated component of the potassium secretory machinery of the kidney (7, 8). Indeed, aldosterone, vasopressin, and other factors precisely regulate SK activity, controlling potassium excretion in accord with the demands of potassium balance. Because ROMK channels normally exhibit a very high open probability, near unity, physiologic augmentation of channel activity, as controlled by hormones and dietary potassium (9), is achieved largely by regulated changes in the number of active channels on the plasma membrane.

Although the precise molecular mechanisms responsible for physiological augmentation of ROMK channel surface density have remained unclear, a growing body of evidence has pointed to an important role of protein kinase A (PKA) phosphorylation. Like the native secretory channel (10), ROMK activity is dependent on direct PKA phosphorylation (11, 12), possibly facilitated by A-kinase-associated proteins (13). In fact, activation of ROMK by PKA is thought to underlie the regulation of renal potassium transport by vasopressin (14). All three PKA phospho-acceptor sites in ROMK, embedded within the cytoplasmic NH₂- (Ser-44) and COOH termini (Ser-219 and Ser-313), must be phosphorylated for full channel function (15). Interestingly, single channel experiments revealed that the different PKA phospho-acceptors regulate the channel through different mechanisms (15). Phosphorylation of the two COOH-terminal sites are required to maintain the channel in a high open probability state (15), controlling both pH-dependent gating (16) and phosphatidylinositol 4,5-bisphosphate-dependent activation of the channel (17). Phosphorylation of the NH₂-terminal site, on the other hand, has no effect on channel open probability. Instead, it appears to control the number of active channels on the cell surface (15), reminiscent of the way vasopressin affects the activity of the native secretory channel (14). Just how this is achieved remains a fundamental question in the field. In particular, it is unknown whether phosphorylation of the serine 44 residue simply switches a pre-existing pool of inactive channels on the membrane into an active gating mode or drives cell surface expression by a vesicular trafficking process.

Close inspection of the NH₂-terminal PKA site in ROMK1 reveals that it also falls within a canonical serum- and glucocorticoid-regulated kinase (SGK1) phosphorylation sequence (recognized by a REXXX/S/P) (18, 19), suggesting that the channel, and serine 44 in particular, might also be a target of SGK1. This observation has potentially important physiologi-

cine; EGF, enhanced green fluorescent protein; WT, wild type; MOPS, 4-morpholinepropanesulfonic acid; ENaC, epithelial Na⁺ channel; NHERF, sodium-hydrogen exchange regulatory factor; RLU, relative light units.

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The abbreviations used are: SK, small conductance potassium channel; A-kinase; PKA, protein kinase A; SGK-1, serum-glucocorticoid-regulated kinase; HRP, horseradish peroxidase; BSA, bovine serum albumin; HA, hemagglutinin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; EGFP, enhanced green fluorescent protein; WT, wild type; MOPS, 4-morpholinepropanesulfonic acid; ENaC, epithelial Na⁺ channel; NHERF, sodium-hydrogen exchange regulatory factor; RLU, relative light units.
cal ramifications. SGK1, a member of the PKB/Akt family of serine/threonine kinases (20), is an immediate early aldosterone-induced gene product (21, 22) in the renal collecting duct (22–24), which has been shown recently to regulate epithelial Na⁺ channel (ENaC) cell surface expression (25) and, consequently, act as a key mediator of the early aldosterone effect on sodium transport in the distal nephron. Conceivably, SGK1 might also control the number of functional ROMK channels at the plasmalemma and explain how aldosterone regulates renal potassium secretion. It should be pointed out that an early test of this idea, using a co-expression assay with the Xenopus orthologue of SGK-1 and the mammalian ROMK channel, failed to detect a permissive action of the kinase (26). Nevertheless, it has remained uncertain whether the negative result was a consequence of the xenogeneic nature of the experimental design, or the requirement of A-kinase-associated protein-like proteins, or the high level of basal phosphorylation state of ROMK in oocytes. Interestingly, a recent report that SGK activation of the sodium-hydrogen exchanger-3 is facilitated by the sodium-hydrogen exchange regulatory factor-2 (NHERF-2) (27), a PDZ protein found in the collecting duct (28) that directly interacts with ROMK,² provided further incentive to evaluate if mammalian SGK-1 regulates the ROMK channel.

The goals of the present study were to determine the mechanism by which phosphorylation of serine 44 in ROMK controls channel activity and to determine whether this residue is a substrate for the aldosterone-induced kinase, SGK-1. Here we show that SGK-1 phosphorylates serine 44 to drive cell surface expression, providing a potential mechanistic explanation for aldosterone-dependent regulation of the secretory potassium channel.

# MATERIALS AND METHODS

**Molecular Biology—**A hemagglutinin (HA) epitope tag was introduced into the extracellular loop of ROMK by overlap expansion PCR at position 113 of ROMK1. Both ends of the epitope were flanked by two glycine residues to enhance accessibility and flexibility of the extracellular HA tag, creating a sequence which reads 5'-EGPYGPYDVPDPYAGGYP. ROMK1 serine 44 was changed to alanine or aspartic acid by site-directed mutagenesis. The mouse SGK-1 cDNA, encoding the entire reading frame, was amplified from reverse-transcribed mouse kidney RNA with the PCR by using SGK-1-specific primers (5'-AGCGCTCTGGGAGGAGGTA-3', and 5'-AGACACGAGGAAAACTTC-3'). All constructs used for studies in Xenopus oocytes were subcloned between the 5'- and 3'- untranslated regions of the Xenopus β-globin gene in the modified pSD64 vector to increase expression efficiency (29). This vector also contains a polyadenylation sequence in the 3'-untranslated region (daA23dC30). With the exception of EGFP-ROMK, all constructs used for mammalian expression were subcloned into pcDNA 3.1+ (Invitrogen). EGFP was engineered onto the NH₂ terminus of HA-ROMK by subcloning the epitope-tagged Kir1.1 in-frame with EGFP in the pEGFP-C1 (Clontech). The sequence of all amplified or modified cDNAs was confirmed by dye termination DNA sequencing (University of Maryland School of Medicine Biopolymer Core).

**cRNA Synthesis—**Complementary RNA was transcribed in vitro in the presence of capping analogue G(5)ppp(5)'G from linearized plasmid containing the cDNA of interest using SP6 RNA polymerase (mMessage, Ambion Inc.). cRNA was purified by phenol/chloroform extraction and precipitated with ammonium acetate/isopropanol alcohol. Yield was quantified spectrophotometrically and confirmed by agarose gel electrophoresis.

**Transient Transfection and Injection—**Oocytes from female Xenopus laevis (Xenopus Express, Homosassa, FL) were isolated and maintained using the standard procedures as described previously (30). Briefly, frogs were anesthetized with 0.15% 3-aminobenzoate, and a partial oophorectomy was performed through an abdominal incision. Oocyte aggregates were manually dissected from the ovarian lobes and then incubated in OR-2 medium (92.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES, pH 7.5) containing collagenase (type 3, Worthington) for 2 h at room temperature to remove the follicular layer. After extensive washing with collagenase-free OR-2, oocytes were stored at 10°C in OR-3 medium (50% Leibovitz's medium, 10 mM HEPES, pH 7.4). 12–24 h later, healthy looking Dunmont stage V–VI oocytes were pneumatically injected with 50 nl of diethyl pyrocarbonate-treated water containing 0–1 ng of cRNA and then stored in OR-3 medium at 19°C for 2–6 days.

**Electrophysiology—**Whole cell currents in Xenopus oocytes were monitored using a two-microelectrode voltage clamp as described previously (30, 31). Briefly, oocytes were bathed in a 45 mM K solution (45 mM KC1, 45 mM N-methyl-D-glucamine-Cl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, pH 7.4). Voltage sensing and current injecting microelectrodes had resistances of 0.5–1.5 MΩ when backfilled with 3 M KCl. Once a stable membrane potential was attained, oocytes were voltage clamped to a holding potential of −20 mV, and currents were recorded during 500-ms voltage steps, ranging from −100 to +40 mV in 20-mV increments. Data were collected using an ITC16 analogue to digital, digital to analogue converter (Instrutech Corp.), and filtered at 1 kHz and digitized on line at 2 kHz using Pulse software (HEKA Electronik) for later analysis. ROMK currents are taken as the barium-sensitive inward current (2 mM barium acetate) as we have done before (31, 32).

Values reported in the text are the barium-sensitive inward currents at −100 mV. Cation permeability ratios (PNa/PK) were estimated from the change in reversal potential induced by replacing extracellular potassium with an equivalent concentration of sodium as we have done before (30).

**Surface Expression—**Plasmalemmal expression of the external HA-tagged ROMK1 channel was measured in single oocytes following procedures outlined by Zerangue et al. (33) with slight modifications. In these studies, oocytes were washed twice in cold OR-2 medium, fixed with 4% formaldehyde in OR-2 for 15 min at 4°C, and washed 3 times in OR-2. To block spurious antibody binding, oocytes were then incubated for 1 h at 4°C in OR-2 containing 1% bovine serum albumin (BSA). Exposed HA epitopes on the surface of intact oocytes were labeled with a rat monoclonal anti-HA antibody (Roche Applied Science, 1 µg/ml, 3F10, 1% BSA, 4°C overnight), and then oocytes were washed at 4°C with OR-2 and incubated with HRP-coupled goat anti-rat (The Jackson Laboratories, 1 µg/ml, 1% BSA, 4°C, 1 h). Cells were washed twice for 1 h at 4°C in OR-2 containing 1% BSA then incubated in OR-2 medium without BSA. Individual oocytes were placed in 50 µl of enhanced chemiluminescence substrate (Amersham Biosciences) and incubated for 1 min at room temperature. Luminescence from single oocyte was measured for 10 s in a Sirius luminoimeter and reported as relative light units per s.

**Western Blot Analysis—**Oocytes were processed following the protocol described by Kamsteeg and Deen (34) to isolate proteins from total membrane. In brief, oocytes were washed twice in homogenization buffer (80 mM sucrose, 5 mM MgCl₂, 5 mM Na₂HPO₄, 1 mM EDTA, 20 mM Tris, pH 7.4) containing a protease inhibitor mixture (5 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 5 µg/ml peptatin A) and homogenized by triturating with a 25-gauge needle. Membrane proteins and nuclei, homogenates were spun twice at low speed (100 × g) for 10 min. Supernatants were then spun at high speed (14,000 × g) for 20 min at 4°C to collect the total membrane fraction. Pellets were washed once in the homogenization buffer and spun at top speed again for 10 min and then placed in solubilization buffer (4% sodium deoxycholate, 20 mM Tris, pH 8.0, 5 mM EDTA, 10% glycerol, containing the protease inhibitors) and rocked for 2 h at 37°C. Particular material was pelleted (14,000 × g for 20 min at 4°C), and the solubilized proteins in the supernatant were resolved by SDS-PAGE and transferred to nitrocellulose membranes.

Blots were blocked in Tris-buffered saline with Tween 20 (0.1%) (TBS-T) containing 5% non-fat dry milk for 1 h at room temperature. Rat anti-HA monoclonal antibody (3F10, Roche Applied Science) was diluted in 5% non-fat dry milk to 0.1 µg/ml and incubated at room temperature for 1 h, washed for 15 min in TBS-T, incubated with goat anti-rat antibody coupled to horseradish peroxidase (HRP) (1:5000, 5% non-fat dry milk), and washed extensively for 20 min in TBS-T. Bound antibody was then revealed using enhanced chemiluminescence reagent (Amersham Biosciences) and fluorography (X-Omat, Eastman Kodak Co.). Fluorographs were assessed by densitometry using NIH Image. Integrated optical density of each band is reported in arbitrary units where the control is considered to be 1 unit.

**Immunofluorescence of COS-7—**Cells, grown on glass coverslips, were fixed by incubation with 4% (v/v) paraformaldehyde (4% PFA) in PBS for 10 min, washed with PBS, and permeabilized with 0.1% Triton X-100 for 10 min. Cells were blocked with 10% FBS in PBS for 1 h and then incubated with antibodies overnight at 4°C. Primary antibodies were detected with a 1:100 dilution of an anti-rat antibody coupled to Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen). After washing, cells were mounted in a solution of Fluorescein Diacetate (10 µg/ml) in PBS. Images were captured with a Zeiss Axiophot microscope equipped with a QImaging Retiga EX digital camera.

**Outgrowth Experiment—**Xenopus oocytes were cultured in the presence of 1 µM 20β-estradiol (E2) for 2 days. Oocytes were then washed in ice-cold modified Ringer's solution (144 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 5.5 mM MgCl₂, 1.2 mM Na₂HPO₄, 5 mM HEPES, pH 7.4) and fixed with 4% formaldehyde in Ringer's solution for 15 min at 4°C. One group of cells was 

⁴ D. Yoo and P. A. Wellin, unpublished observations.
permeabilized with 0.1% Triton X-100 for 30 min at room temperature and washed three times in the modified Ringer's solution before blocking. Non-permeabilized cells were not treated with Triton. Both groups where blocked with 1% BSA in the modified Ringer's solution for 30 min at room temperature and labeled with primary antibody in 0.1% BSA (rat anti-HA (Roche Applied Science) at 10 ng/ml; mouse anti-PDI (Qiagen). Serum- and glucocorticoid-regulated kinase-1 or actin cDNAs were separately amplified in parallel real time PCR's, containing an aliquot of the reverse-transcribed reaction, 0.2× SYBR Green I (Molecu- lar Probes), 5 μM each of forward and reverse primers, and 1/10 of either serum- and glucocorticoid-regulated kinase-1 (SGK)-specific primers (forward 5′-GCCATGTTTACFCTAGGCTC-3′; reverse 5′-CTTCGCCCTCGTCTCCACACG-3′) or actin-specific primers (forward 5′-CCATGTTTACFCTAGGCTC-3′; reverse 5′-CTTCGCCCTCGTCTCCACACG-3′) in separate wells using the Bio-Rad iCycler real time PCR detection system. Each sample was amplified in triplicate. Specificity of the amplified product was deter- mined using melting curve analysis and agarose gel electrophoresis. For quantification, real time PCR of serially diluted SGK cDNA was carried out as a positive control and to establish an optimal threshold cycle detection level; threshold cycle values, Ct, of the test samples were then measured from cycle-dependent product amplification curves (Bio- Rad software package iCycler version 3.0a). The change in SGK tran- script, reported as the fold induction over the actin control, was calcu- lated using the 2(–ΔΔCt) method (35).

RESULTS

Surface Expression Assay—Phosphorylation of the serine 44 residue regulates ROMK either by activating dormant chan- nels already residing on the plasma membrane or by recruiting new channels to the cell surface, two mechanisms that can be distinguished readily by differences in cell surface expression. Accordingly, we incorporated a hemagglutinin epitope tag into the extracellular loop of the ROMK channel so that plasma- lemma channel levels could be quantified accurately by anti- body binding. When expressed in Xenopus oocytes, HA-tagged ROMK carried weakly inward-rectifying, K+ selective (P0/PNa > 15) and barium-sensitive currents identical to those observed for the wild type channel (Fig. 1). To measure relative amounts of surface HA-tagged ROMK, we employed a method that com- bines enzyme-linked immunosorbent assay with the sensitivity and linearity of analytical luminometry as originally described by Zerangue et al. (33). In this assay, exposed HA epitopes on the surface of intact oocytes were labeled with a monoclonal antibody to HA and then with an HRP-conjugated secondary antibody. Anti- body bound to the cell surface was quantified by luminometer measurements of single oocyte chemiluminescence. As shown in Fig. 2, the luminescence response is linear over a wide range of expression levels, permitting an accurate appraisal of rela- tive ROMK channel density at the cell surface.

Phosphorylation of Serine 44 Controls ROMK Expression at the Plasmalemma—To determine the mechanism by which phosphorylation of the serine 44 residue regulates ROMK, we employed the assay, described above, to measure to cell surface channel density and the two-microelectrode voltage clamp to measure the macroscopic potassium channel current activity in Xenopus oocytes separately injected with equal amounts of either wild type, HA-tagged ROMK, or HA-tagged ROMK channels bearing phosphorylation null (S44A) or phosphorylation mimic (S44D) mutations (Fig. 3). Xenopus oocytes are uniquely suited for these studies because endogenous levels of PKA generally support high constitutive activity of the ROMK channel (11). Replacement of the critical serine with alanine (S44A) caused a dramatic fall in macroscopic current and a parallel reduction in cell surface expression, without change in total cellular abundance of ROMK protein, revealing that the phospho-acceptor, serine 44, is required for ROMK expression at the plasmalemma. This finding is confirmed and extended by the observation that channels bearing the phosphorylation...
mimic mutation (S44D) exhibited a greater macroscopic current and cell surface channel density than the wild type channel. Although the S44D mutation tended to produce a slightly larger surface channel density than functional activity, it was not statistically significant, indicating that surface expression and channel activity increase in parallel. Western blot analysis confirmed that the response was not related to dramatically different protein expression levels; no differences in band density could be detected between the different groups (band density of WT, 1 unit; S44A, 0.86 ± 0.13 units; S44D, 0.9 ± 0.15 units). Collectively, these data indicate that a high degree of basal phosphorylation drives cell surface expression of ROMK in Xenopus oocytes. As inferred by the enhanced activity and surface expression of S44D channels, a small pool of wild type ROMK must exist in an intracellular compartment in a non-phosphorylated form, available for recruitment to the plasma membrane.

SGK-1 Is a Potential Physiological Regulator of ROMK in Potassium Adaptation

This hypothesis predicts that phosphorylation of serine 44 by an exogenous kinase should increase wild type ROMK activity to the same extent as the phosphorylation mimic mutation, S44D. Analysis of the ROMK primary structure revealed that serine 44 not only lies within one of three PKA phosphorylation sites but is also uniquely embedded within an SGK phosphorylation consensus sequence, recognized by the RXXRXX(S/T) motif (18, 19). Furthermore, we found that the abundance of SGK-1 transcript is rapidly increased in the mouse kidney following augmentation of dietary potassium intake (Fig. 4). Because the response is coincident with the early increase in ROMK channel density in the renal collecting duct (36), SGK-1 might be a physiological mediator of ROMK in the potassium adaptation response, further prompting us to test whether that mammalian form of SGK-1 controls ROMK activity by direct phosphorylation of serine 44.

SGK-1 Regulates ROMK Activity and Surface Expression

In light of a recent report (27) that SGK activation of the sodium-hydrogen exchanger-3 is facilitated by NHERF-2,
tested whether SGK-1 regulates ROMK activity in an NHERF-2-dependent manner. In these studies, summarized in Fig. 5, SGK-1 and NHERF-2 cRNA was injected separately or together into oocytes with the ROMK channel cRNA (750 pg each), and macroscopic potassium currents (at −100 mV in 45 mM K⁺) were measured. Data are normalized to the mean wild type current, $I_{WT}$, measured in the absence of either SGK-1 or NHERF-2. Western blots of total HA-tagged protein in the total oocyte membrane fraction are shown below the plot. Data represent a mean ± S.E. of at least 25 oocytes from five frogs. *p < 0.05.

Fig. 3. Phosphorylation of serine 44 drives surface expression of ROMK. *Xenopus* oocytes injected with either WT, phosphorylation null mutant S44A, or phospho-mimic mutant S44D, ROMK cRNA (750 pg) were assayed for both functional expression (top), measured as the macroscopic K⁺ current at −100 mV in 45 mM K⁺, and surface expression (bottom), as measured by whole cell luminescence. Data are normalized to the mean wild type current, $I_{WT}$, and mean wild type surface expression, RLU$_{WT}$. Each column represents the mean ± S.E. of at least 20 oocytes for functional expression and 50 oocytes for surface expression from four frogs. Western blots of HA-tagged protein in the total oocyte membrane fraction are shown below the plots.

FIG.3 . Phosphorylation of serine 44 drives surface expression of ROMK. Xenopus oocytes injected with either WT, phosphorylation null mutant S44A, or phospho-mimic mutant S44D, ROMK cRNA (750 pg) were assayed for both functional expression (top), measured as the macroscopic K⁺ current at −100 mV in 45 mM K⁺, and surface expression (bottom), as measured by whole cell luminescence. Data are normalized to the mean wild type current, $I_{WT}$, and mean wild type surface expression, RLU$_{WT}$. Each column represents the mean ± S.E. of at least 20 oocytes for functional expression and 50 oocytes for surface expression from four frogs. Western blots of HA-tagged protein in the total oocyte membrane fraction are shown below the plots.

Fig. 4. An increase in dietary potassium augments SGK-1 transcript abundance in the kidney. Mice, maintained on a standard rodent chow, were placed on a high potassium diet containing 10% KCl for 0.5, 1, 2, and 4 days, and SGK-1 transcript abundance in the kidney was assessed by real time PCR. Data are reported as fold SGK-1 induction, assessed by normalizing SGK-1 transcript abundance to β-actin (b-actin) mRNA levels. Within 12 h, representing the first potassium-rich meal, a 2.1-fold induction of SGK-1 transcript was detected. Mean ± S.E. (n = 6).

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FIG.4 . An increase in dietary potassium augments SGK-1 transcript abundance in the kidney. Mice, maintained on a standard rodent chow, were placed on a high potassium diet containing 10% KCl for 0.5, 1, 2, and 4 days, and SGK-1 transcript abundance in the kidney was assessed by real time PCR. Data are reported as fold SGK-1 induction, assessed by normalizing SGK-1 transcript abundance to β-actin (b-actin) mRNA levels. Within 12 h, representing the first potassium-rich meal, a 2.1-fold induction of SGK-1 transcript was detected. Mean ± S.E. (n = 6).

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FIG.5 . SGK-1 enhances ROMK activity. SGK-1 and NHERF-2 cRNA was injected separately or together into oocytes with the ROMK channel cRNA (750 pg each), and macroscopic potassium currents (at −100 mV in 45 mM K⁺) were measured. Data are normalized to the mean wild type current, $I_{WT}$, measured in the absence of either SGK-1 or NHERF-2. Western blots of total HA-tagged protein in the total oocyte membrane fraction are shown below the plot. Data represent a mean ± S.E. of at least 25 oocytes from five frogs. *p < 0.05.

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FIG.6 . SGK-1 increases plasmalemma expression of ROMK. Macroscopic potassium currents, $I_{WT}$, and cell surface expression, RLU/RLU$_{WT}$, were measured as above in oocytes injected with HA-ROMK alone, co-injected with HA-ROMK cRNA and SGK-1 cRNA, or injected with cRNA encoding HA-ROMK bearing the phospho-mimic mutation, S44D (750 pg each). Data are normalized to the mean wild type current, $I_{WT}$, or surface luminescence (RLU$_{WT}$) and represent a mean ± S.E. of cohorts of at least seven oocytes. *p < 0.05.

Fig. 6. SGK-1 increases plasmalemma expression of ROMK. Macroscopic potassium currents, $I_{WT}$, and cell surface expression, RLU/RLU$_{WT}$, were measured as above in oocytes injected with HA-ROMK alone, co-injected with HA-ROMK cRNA and SGK-1 cRNA, or injected with cRNA encoding HA-ROMK bearing the phospho-mimic mutation, S44D (750 pg each). Data are normalized to the mean wild type current, $I_{WT}$, or surface luminescence (RLU$_{WT}$) and represent a mean ± S.E. of cohorts of at least seven oocytes. *p < 0.05.
related to dramatically different protein expression levels; no differences in band density could be detected between the different groups (band density of the ROMK only, 1 unit; ROMK + NHERF-2, 1.03 ± 0.16 units; ROMK + NHERF-2 and SGK-1, 0.99 ± 0.12 units; ROMK + SGK-1, 1.03 ± 0.04 units). The particular batch of oocytes, in which SGK-1 had no effect on ROMK, was notable in that macroscopic current density in the presence of constitutively active recombinant SGK-1. Reactions were either run in vitro, or in the presence and absence of constitutively active recombinant SGK-1. Autoradiography revealed a phosphorylated 45-kDa species in reactions containing SGK protein but not in those in which SGK or ROMK was omitted. Western blotting of the immunoprecipitate with anti-HA antibodies confirmed the identity of the phosphorylated 45-kDa protein as HA-ROMK.

Thus, ROMK is indeed a substrate for SGK phosphorylation. To determine whether SGK phosphorylates serine 44, we carried out in vitro phosphorylation assays using a synthetic peptide corresponding to the wild type ROMK NH2-terminal residues, 34–48, and a mutant NH2-terminal peptide (residues 34–48) so that the apparent Km could be determined (Fig. 8). SGK phosphorylation was compared in parallel reactions with PKA, well known to phosphorylate Ser-44 in the physiological regulation of the ROMK channel. As shown in Fig. 8, phosphorylation exhibited by each kinase for the ROMK peptide (SGK-1, 0.50 pmol min⁻¹; PKA, 0.18 pmol min⁻¹). Lines are best fits to the Michaelis-Menten equation. Data represents the mean ± S.E. of three separate experiments.

As a further test of SGK-1 specificity, phosphorylation reaction velocity was measured as a function of peptide concentration. Velocity data are normalized to the maximum velocity, Vmax, of ROMK peptide phosphorylation exhibited by each kinase for the ROMK peptide (SGK-1, 0.50 pmol min⁻¹; PKA, 0.18 pmol min⁻¹). Lines are best fits to the Michaelis-Menten equation. Data represents the mean ± S.E. of three separate experiments.

whether SGK-1 regulates ROMK1 directly, we first tested whether ROMK1 is a substrate for SGK-1 phosphorylation. In these studies, HA-ROMK1 was transfected into COS-7 cells, immunopurified on anti HA-bound protein G-Sepharose beads, and incubated with [γ-32P]ATP in the presence and absence of constitutively active recombinant SGK-1. Fig. 7A shows a typical study. Autoradiography revealed a phosphorylated 45-kDa species in reactions containing SGK protein but not in those in which SGK or ROMK was omitted. Western blotting of the immunoprecipitate with anti-HA antibodies confirmed the identity of the phosphorylated 45-kDa protein as HA-ROMK. Thus, ROMK is indeed a substrate for SGK phosphorylation. To determine whether SGK phosphorylates serine 44, we carried out in vitro phosphorylation assays using a synthetic peptide corresponding to the wild type ROMK NH2-terminal residues, 34–48, and a mutant NH2-terminal peptide (residues 34–48) so that the apparent Km could be determined (Fig. 8). SGK phosphorylation was compared in parallel reactions with PKA, well known to phosphorylate Ser-44 in the physiological regulation of the ROMK channel. As shown in Fig. 8, phosphorylation exhibited by each kinase for the ROMK peptide (SGK-1, 0.50 pmol min⁻¹; PKA, 0.18 pmol min⁻¹). Lines are best fits to the Michaelis-Menten equation. Data represents the mean ± S.E. of three separate experiments.

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values are comparable with the micromolar affinities reported for most well known PKA substrates (see Ref. 38 for address to web-based catalog) such as pyruvate kinase (39) (1–20 μM), providing strong support for SGK-1 specificity.

SGK-1 Activation of ROMK Requires Serine 44—If phosphorylation of serine 44 is the mechanism by which SGK stimulates ROMK, mutation of the phosphorylation site should abrogate the stimulatory effect of SGK. To test this hypothesis, macroscopic channel activity was measured under voltage clamp as above in Xenopus oocytes expressing either wild type, phosphorylation null (S44A), or phospho-mimic (S44D) ROMK channels in the presence and absence of SGK-1. As shown in Fig. 9, removal of the phosphoacceptor, S44A, completely abrogated the stimulatory effect on the channel. Furthermore, the phosphorylation mimic mutation increased channel activity to a level that was identical to the SGK-1-stimulated wild type channel. Moreover, SGK-1 co-expression did not cause a further significant augmentation of S44D ROMK channel activity.

SGK-1 Phosphorylation-dependent ROMK Plasmalemma Expression in Mammalian Cells—Our studies above in Xenopus oocytes indicate that high levels of basal ROMK phosphorylation drive cell surface expression in this system. These observations contrast mammalian expression systems where active PKA is normally low and ROMK is inefficiently expressed on the plasmalemma (40). In fact, as shown in COS-7 cells, ROMK largely accumulates in an intracellular compartment resembling the endoplasmic reticulum (Fig. 10). Because the closely related channel, Kir 2.1 (41), is largely expressed on the plasmalemma in the same cells using identical transfection and detection conditions, it is unlikely that the intracellular expression of ROMK is an artifact of overexpression. Moreover, co-localization of the ROMK channel with protein-disulfide isomerase reveals that ROMK is largely expressed in the endoplasmic reticulum.

To determine whether SGK-1 phosphorylation increases cell surface expression in a system where the channel is expressed in the endoplasmic reticulum under basal conditions, COS-7 cells were transfected with a double-tagged ROMK construct, containing EGFP on the NH2 terminus and the extracellular HA epitope, in the absence and presence of SGK-1. The EGFP tag permits detection of ROMK-transfected cells under non-permeabilized conditions, and the external epitope allows detection of plasmalemma channel expression by HA antibody binding. As shown in Fig. 11, SGK-1 co-transfection dramatically increased the number of cells that express ROMK on the cell surface. This effect was absolutely dependent on serine 44 (Fig. 11C). Removal of the phosphoacceptor, S44A, completely abrogated the stimulatory effect of SGK-1 on plasmalemma channel expression. Furthermore, the phosphorylation mimic mutation increased cell surface expression to a level that was identical to the SGK-1-stimulated wild type channel.

DISCUSSION

The density of active ROMK channels on the renal cortical collecting duct apical membrane is exquisitely controlled by hormones and other factors, helping to ensure that renal potassium excretion matches dietary intake and potassium homeostasis is maintained (7). Down-regulation of ROMK channel density in states of potassium deprivation is thought to involve Src-dependent channel endocytosis (42, 43), whereas phosphorylation of one of the three PKA phosphorylation sites...
in ROMK (12), serine 44, has been implicated in physiological augmentation of active ROMK channel density (15). In the present study, we elucidate the molecular mechanism and identify a pathway for the hormonal regulation of the channel by vasopressin and aldosterone. By measuring cell surface density and functional activity of wild type and phosphorylation mutant ROMK channels, we discovered that phosphorylation of serine 44 dramatically increases functional channel density by recruiting or retaining new channels on the plasma-lemma rather than by activating silent channels already present on the plasma membrane. The observation implies that vasopressin, and other procedures that cause PKA-mediated phosphorylation of serine 44, enhance the number of ROMK channels at the cell surface by phosphorylation-dependent intracellular trafficking processes, reminiscent of the way vasopressin controls the AQP-2 water channel in the collecting duct (44). Moreover, our discovery that serine 44 is also a substrate for SGK-1 illuminates a potential molecular mechanism for the regulation of ROMK density by dietary potassium.

A series of adaptive changes in the collecting duct principal cell take place in response to an increase in dietary potassium, allowing a more effective and enhanced excretion of potassium after an acute potassium load (7). Although it is generally believed that the response, called potassium adaptation, depends on elevated aldosterone levels and other synergistic factors, triggering an increase in apical macroscopic potassium conductance (45) and parallel increase in the number of active SK channels on the apical plasmalemma (2, 8, 46), the molecular mechanisms and pathways connecting aldosterone to the channel have remained vague. At most, observations that the increase in SK density can be observed on the cortical collecting duct apical membrane soon after dietary potassium load is increased (~6 h) (8), and without changes in ROMK transcript abundance (47), have suggested that the response, at least in the acute phase, is mediated by a post-translational modification of a pre-existing pool of channels or closely associated proteins.

Now, following recent discoveries that aldosterone rapidly induces the SGK-1 kinase (21, 22) to control the epithelial sodium channel (ENaC) density (25), the present study provides evidence that SGK-1 may also serve as an arbitrator of the aldosterone-dependent limb of potassium adaptation process, directly regulating ROMK surface expression. We do not want to suggest our findings rule out the role of other yet-to-be identified signaling pathways in potassium adaptation, rather our data point to a potential role of SGK-1. First, SGK-1 transcript in the kidney is rapidly induced by dietary potassium loading, precisely correlating with the early increase in ROMK channel density as measured in the renal collecting duct by Palmer and Frindt (36). Second, the ROMK channel contains a single consensus site for phosphorylation by SGK-1 (serine 44), recognized by the sequence RXRXX(S/T) (18, 19). As predicted...
by this observation, in vitro phosphorylation studies revealed that ROMK serine 44 is indeed a substrate for phosphorylation by SGK-1. In fact, we found that SGK-1 phosphorylates the Ser-44 site in ROMK with an affinity that is identical to that exhibited by PKA. Third, co-expression of SGK-1 with ROMK1 stimulated channel activity and surface expression to about the same level observed with channels bearing a phosphorylation mimic mutation, S44D. Finally, the stimulatory effect of SGK-1 was completely abrogated by removal of the critical phosphorylation acceptor, serine 44.

It should be pointed out that although aldosterone might be necessary for potassium adaptation, it is not sufficient to increase SK density on the CCD apical membrane (48). Indeed, elevation of aldosterone levels by osmotic minipumps or dietary sodium restriction has no effect on potassium secretory channel activity in the rat cortical collecting duct, even though the increase in SK channel density that is normally observed in response to dietary potassium loading (1, 46) requires intact adrenal glands and presumably elevated aldosterone levels. To explain these observations, Palmer and Frindt (48) reasonably proposed that the potassium adaptation response requires unidentified kalliuretic factor(s), which work synergistically with aldosterone to augment SK channel density. The identification of SGK-1 as a regulator of ROMK surface expression provides a clue as to how this might work. Although aldosterone induces the expression of SGK-1 (22, 24), the kinase must be subsequently activated by phosphorylation (20), involving 3-phosphoinositide-dependent kinases 1 and 2 (18) or the mitogen-activated protein kinase family member extracellular signal-regulated protein kinase 5 (49, 50), and perhaps other unidentified kinases. Being sequentially activated at transcriptional and post-transcriptional levels, SGK-1 is poised to integrate the effects of aldosterone and a number of extracellular signals, such as those that might emanate from the kalliuretic factor, and thereby play a key role in regulating ROMK channel density in potassium adaptation.

Our results with mouse SGK-1 and ROMK-1 clearly differ from those initially reported by Chen et al. (21), who failed to detect a stimulatory effect of the Xenopus SGK-1 on ROMK2, a splice variant that lacks the first 19 amino acids of ROMK1. Furthermore, while the present paper was under revision, Yun et al. (51) reported that SGK-1 does not directly act by itself but instead synergizes with the Na+/H+ exchange regulating factor-2 to stimulate ROMK. Although there are a number of methodological differences between these studies and ours that could potentially account for the disparity, we believe that the most likely explanation lies in a variable degree of basal ROMK phosphorylation in Xenopus oocytes. This concept has precedence with the AQP2 water channel in which high, but variable, levels of channel phosphorylation in Xenopus oocytes can yield contrasting effects of exogenous cAMP on the PKA-dependent surface expression response (52, 53). It is well known that Xenopus oocytes are developmentally arrested at the first meiotic prophase by high intracellular cAMP and active PKA levels and that meiotic maturation depends on a progesterone- and cAMP and active PKA. Although oocytes were not exposed to progesterone to lower cAMP in the present study, we did fortuitously overexpress ROMK at a level that was nearly an order of magnitude greater than those reported earlier, presumably increasing the probability that some ROMK channels escape phosphorylation by endogenous PKA and thus be available for SGK-1. Indeed, as inferred from the increased activity and surface expression of ROMK channels bearing the phosphorylation mimic mutation, S44D, compared with the wild type channel, a small population of wild type channels must exist in a non-phosphorylated form, open for phosphorylation by exogenous SGK-1, at the expression levels in the present study. Although augmentation averaged 40–50% of the basal activity, similar to what was observed with SGK-1, it is important to note that some variation between batches of oocytes was observed, presumably a consequence of variable basal phosphorylation levels. In cases where maximum phosphorylation of ROMK has all ready been achieved by endogenous PKA, no effect of SGK-1 will be observed, even though SGK-1 is capable of phosphorylating and regulating the channel.

Just how phosphorylation of serine 44 controls cell surface expression, regulating ROMK traffic to and/or from the plasmalemma, remains to be determined. Phosphorylation-dependent intracellular trafficking processes are thought to be coordinated by phosphorylation-dependent interaction or dissociation of intracellular trafficking machinery. For instance, recent studies indicate that SGK-1 phosphorylation of the NEDD4–2 ubiquitin ligase inhibits the PY-WW domain (26)-mediated NEDD-4 interaction with the epithelial sodium channel, preventing channel ubiquitination, endocytosis, and degradation and thereby increasing ENaC surface expression (25). Unlike ENaC, ROMK does not have the capacity to bind to NEDD4–2 through a PY-WW interaction, making it unlikely that SGK-1-dependent regulation of ROMK is similar to ENaC. Instead, our observations in COS cells, where ROMK channels are usually inefficiently processed to the plasmalemma and reside predominantly in the endoplasmic reticulum until SGK-1 co-expression, provide reason to speculate that phosphorylation of the serine 44 residue in ROMK may regulate ROMK traffic from the endoplasmic reticulum. Intriguingly, serine 44 juxtaposes a putative endoplasmic reticulum retention signal, XR, similar to those initially identified in other Kir-type channels (33).

In summary, phosphorylation of ROMK at serine 44 by PKA or SGK-1 is necessary for cell surface expression of the channel. The observation may provide a mechanistic explanation for vasopressin and aldosterone-dependent regulation of the secretory potassium channel in the kidney.

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Cell Surface Expression of the ROMK (Kir 1.1) Channel Is Regulated by the Aldosterone-induced Kinase, SGK-1, and Protein Kinase A
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