SUPPLEMENTAL INFORMATION

Potassium acts through mTOR to regulate its own secretion

Brief title: K^+ regulates mTOR

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Supplemental Methods

Materials

Dulbecco's modified Eagle medium (DMEM) with high glucose (4.5g/Liter) (CCFAA005) and K^+ free DMEM with high glucose (4.5g/Liter) (CCFDA003) and penicillin-streptomycin were purchased from the UCSF Cell Culture Facility. Fetal bovine serum (FBS) was from Atlanta Biologicals; L-Glutamine, Trypsin and Collagen I were from Corning. Polyethyleneimine "MAX" (MW 40,000) transfection reagent was from Polysciences, Inc (Cat # 24756). GSK650394 were from Tocris; AZD8055 was from SelleckChem; rapamycin,amiloride benzamil were from Sigma-Aldrich. PP242 was kind gift from Kevan Shokat, UCSF. VU0134992 was provided by Dr. Sujay Kharade and Prof. Jerod S. Denton (Vanderbilt University Medical Center). Bradford Assay Reagent was purchased from ThermoFisher (Cat# 1856209). ECL Western Blotting Detection reagent was obtained from GE Heathcare.

Benzamil injections

Benzamil is a specific ENaC antagonist (1). 0.2 µg/g BW benzamil (0.4mg/ml dissolved in 0.9 % saline with 0.5 % DMSO) (Sigma-Aldrich) or vehicle (5 % DMSO in 0.9 % saline) was administrated via intraperitoneal (i.p.) injections 15 minutes prior to gavage. This dose has been validated to inhibit ENaC efficiently and specific (2) and has been tested in a dose-response experiment on NMRI mice showing that the selected dose fully inhibits ENaC-mediated sodium reabsorbtion (3). Thus, benzamil-induced natriuresis is a quantitative measurement of functional ENaC-mediated Na^+ transport *in vivo*.

Spot urine in metabolic cages

Before gavage, the mice were weighed to adjust electrolyte and benzamil dose. Post gavage aminals were placed in metabolic balance cages (Techoplast, Italy). In the metabolic cages, parafilm covered the collection funnel. Because of the hydrophobic nature of parafilm, it was possible to collect small volume urine samples using a micropipette. Urine samples collected during handling of the mice prior to gavage were used as baseline values. Following the gavage urine samples were collected and the time of collection was noted, as has been previously described (3).

KCl infusion

Mice were given a single water load (25 µl/g BW) as gastric gavage to secure stable diuresis throughout the experiment. Subsequently the mice were anesthetized with a single i.p. bolus injection (0.1ml/10g BW) containing ketamine (10 mg/ml) and xylazin (1 mg/ml) (Aarhus University Hospital general pharmacy). Anesthesia was maintained by repetitive bolus injection with a third of the induction dose every 30 min. The mice were placed on a heating plate with thermostatic control that kept body temperature at 38°C. Next, an intravenous catheter was inserted in one of the tail veins and continuous infusion of 300 mOsm NaCl solution (saline) at a rate of 4µl/g BW/h via a perfusion pump (Harvard apparatus, USA) was started. The lower part of the abdomen was then incised, and the urinary bladder was identified and catheterized. Urine was collected every 5 minutes with calibrated glass capillaries and stored at -20 ºC for later flame photometry. The mice were randomly divided in two groups. After 30 minutes the NaCl infusion was switched to a 300 mOsm KCl infusion at a rate of 4 µl/g BW/h in one group. In the second group the saline infusion was continued throughout the experiment. The perfusion rates were

adapted from the study by Choi and colleagues (4). Simultaneously with changes of infusion solution all animals received an i.p. bolus injection of benzamil (0.2 µg/g BW). To evaluate the benzamil-sensitive Na^+ excretion, urinary Na^+ concentration was determined by flame photometry and Na^+ excretion rates were calculated.

Plasma electrolyte and aldosterone measurements and tissue harvest

45 minutes post gavage, mice were anesthetized using ketamine (10 mg/ml) and xylazin (1 mg/ml) administered by i.p. injections (5.7 µl/g BW). One hour post gavage and upon absence of reaction to painful stimuli (pinching the tip of the tail) blood was sampled by retroorbital puncture using a heparinized capillary tube (Radiometer, Denmark). Blood was analyzed immediately in an ABL80 blood gas and osmolyte analyzer (Radiometer, Denmark). Subsequently the thorax was cut open and whole blood was extracted from the right atrium of the heart by a heparinized syringe (Pico, Radiometer, Denmark) mounted with to 0.4 x 19 mm needle (27G, Microlance BD). The blood was immediately transferred from the syringe to BD Microtainer PST™ LH Tubes (BD Diagnostics) and centrifuged at 4600 g for 2 minutes. The plasma was transferred to clean tubes and put on ice. Samples were kept at -80°C until use. Plasma aldosterone was measured using an ELISA kit (DRG Aldosterone ELISA kit, DRG diagnostics, Germany) in accordance to the product manual. Plasma was diluted 1:4 in standard solution provided in the kit in order to be within detection range.

After blood collection, mice were perfused with 20 ml PBS via the left atrium of the heart. The kidneys were then removed, decapsulated and then snap-frozen in liquid nitrogen and kept at -80°C until use.

Patch-clamp and whole cell current measurement in native CCD

Animals were anesthetized and killed by cervical dislocation. Kidneys were then removed. The collecting tubules were dissected from the kidney slices and placed into normal K^+ buffer (5 mM). The isolated CCDs were split open and prepared for apical membrane patch clamp, as previously described (5). An Axon 200A amplifier was used to measure the whole-cell Na^+ currents in CCD. The pipette solution contained 125 mM KGluconate, 15 mM KCl, 2 mM MgATP, 1 mM EGTA 10 mM HEPES (pH 7.4) and the bath solution contained 130 mM NaGluconate, 10 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). After a high-resistance seal (>2 GΩ) was formed, the membrane capacitance was monitored until the whole cell patch configuration was formed. The membrane potential was clamped at -60 mV with 140 mM Na^+/5 mM K^+ in the bath (extracellular solution) and 140 mM K^+ in the pipette (intracellular solution). Under such experimental conditions, an inward current was observed and addition of 0.1 mM amiloride largely abolished the inward current. The amiloride-sensitive cation current was defined as ENaC activity. The bath solution was changed to 1 mM by replacing 4 mM of KCl with NaCl. Measurements were made after currents reached a steady state (15-20 min after change of bath). The currents were low-pass filtered at 1 kHz and digitized by an Axon interface with 4 kHz sampling rate (Digidata 1440A). Data were analyzed using the pClamp software system 9.0 (Axon).

Cell culture, transfection and Treatment

For transfection and K^+ regulation experiments, HEK293 cells, HEK-293T $SIN1^{-/-}$ and HEK-293T $WNK1^{-/-}$ were seeded on 10-cm dishes and allowed to grow overnight at 37°C in a humidified incubator with 5% CO_2. They were then transfected with 1.5μg of pMO/Flag/mSGK1 (Flag-

epitope at N-terminal of mouse SGK1), 0.5μg of SIN1-V5, 0.5μg of WNK1 (1-491) WT, 0.5μg of WNK1 (1-491) K233M or the empty vector using Polyethylenimine (PEI). 36 hours post-transfection and 16 hours prior to the experiments, cells were transferred to serum-free DMEM and then adapted to DMEM with 1 mM [K^+] for 2 hours. At t=0, KCl or equimolar Choline Chloride or sodium chloride was added to raise the medium [K^+] and incubated for 1 h, after which cells were lysed and prepared for western blot. In some experiments cells were adapted to 5 mM [K^+], and then medium [K^+] was changed, as indicated, after which cells were lysed and prepared for Western blot.

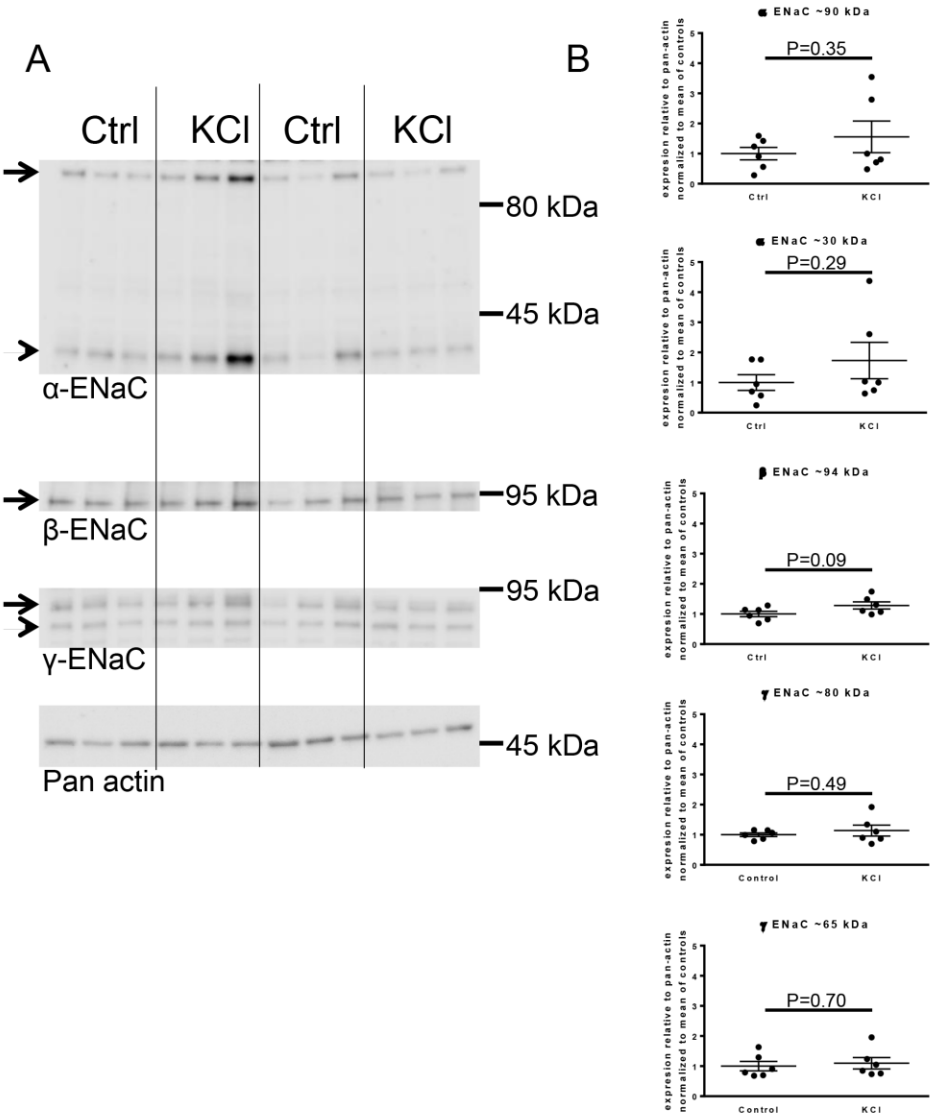
Immunoblotting

Cultured cells: For Western blot analysis to determine protein expression levels, cells were lysed using lysis buffer containing 40 mM HEPES (pH 7.5), 120 mM NaCl, 50 mM NaF, 10 mM Sodium Pyrophosphate, 10 mM glycerophosphate, 1 mM EDTA (pH 8), 1% Triton-X-100 with complete protease inhibitor cocktail and PhoSTOP phosphatase inhibitor. After centrifugation, supernatant was collected, and protein concentration was determined using Bradford assay. 40 μg of total protein from each cell extract supernatant was electrophoresed on 7.5% polyacrylamide Gels and transferred onto PVDF membranes (ThermoFisher Scientific) using a Transblot system (Bio-Rad). The blots were then probed with primary antibodies rabbit anti-pSGK1 (S422), anti-phospho-p70 S6K(t389) overnight at 4°C, or rabbit anti-pAKT(S473), anti-pAKT(T308), anti-SGK1, anti-SIN1, mouse anti-tubulin for 1 h at room temperature followed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1: 5000 dilution) or anti-mouse IgG (1:20,000) for 1 h at room temperature. The signals were visualized using ECL western blotting detection system working solution according to the manufacturer's instructions. Each experiment was repeated more than

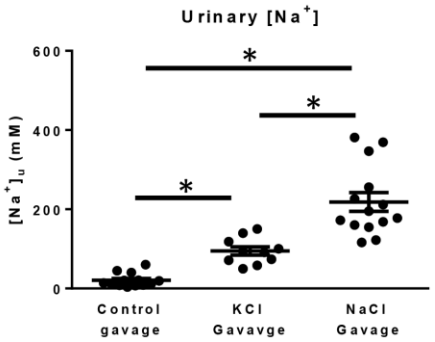
three times. Loading control experiments were also carried out by Western blotting using the antibodies to α-tubulin. Densitometry of the band intensities were performed using Image J software.

Kidney lysates: Half kidneys were homogenized in 1 ml lysis buffer using a tissuelyser (Qiagen) for 30 seconds and centrifuged at 1000 g for 15 min at 4°C. The supernatant was aspirated and protein concentrations were measured using Pierce™ BCA Protein Assay Kit (Thermo Scientific). To ensure that the amount of protein loaded was in the linear detection range for each used antibody western blots were performed with 5, 10, 15, 20, 25 µg protein loaded per sample(3). Equal loading (15 µg) was obtained by normalizing to densitometry of a commassie blue staining. All samples were run on criterion™ TGX™ Precast Gels (Bio-Rad, Copenhagen, Denmark). Proteins were transferred to PVDF membranes using a wet transfer system with plate electrodes (Bio-Rad). Membranes were blocked in 5 % milk in PBS-T for 1-hour. After washing 1 x 15 minutes and 2 x 5 minutes in PBS-T, the membranes were incubated overnight with primary antibodies (AB) (see table S3), washed 1 x 15 minutes and 2 x 5 minutes in PBS-T and incubated with secondary goat anti-rabbit AB (DakoCytomation, Glostrup, Denmark) for one hour. After another wash cycle, the membranes were developed using Clarity™ Western ECL substrate (Bio-Rad) in an ImageQuant LAS 4000 mini (GE Healthcare Life Science). Then, membranes were stripped and incubated with a pan-actin AB (Cell Signaling Technology, MA, USA) as reference protein. All images were analyzed using Image Studio™ Lite (Li-Cor Biosciences, NB, USA). Solutions: Lysis buffer: 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 mM leupeptin, 1 mM Pefabloc (Sigma-Aldrich) and phosSTOP phosphatase inhibitor cocktail (1 tablet in 10ml, Roche Diagnostics, Manheim, Germany) adjusted to pH 7.2. PBS: 137 mM NaCl, 10 mM Na_2HPO_4, 1.8

mM KH_2HPO_4, 2.7 mM KCl. Adjusted to pH 7.4. PBS-T: PBS including 0.1 % Tween 20 (Sigma-Aldrich).

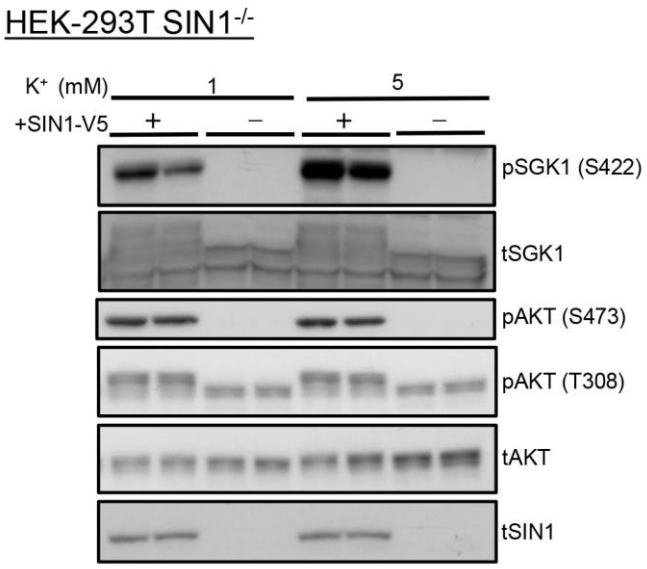


Supplemental Figure 1. ENaC subunit abundance one hour post control or KCl gavage. (A) Immunoblot showing expression of α-, β- and γ-ENaC full length (arrows) and proteolytically-cleaved α- and γ-ENaC (arrow heads). **(B)** Quantification of immunoblot density of α-, β- and γ-ENaC full length and proteolytically-cleaved α- and γ-ENaC normalized against pan-actin. Please note that no alteration in expression total or cleavage of any ENaC subunits. n=6 per group, P-values were obtained by Student's T-test as indicated in Figure.

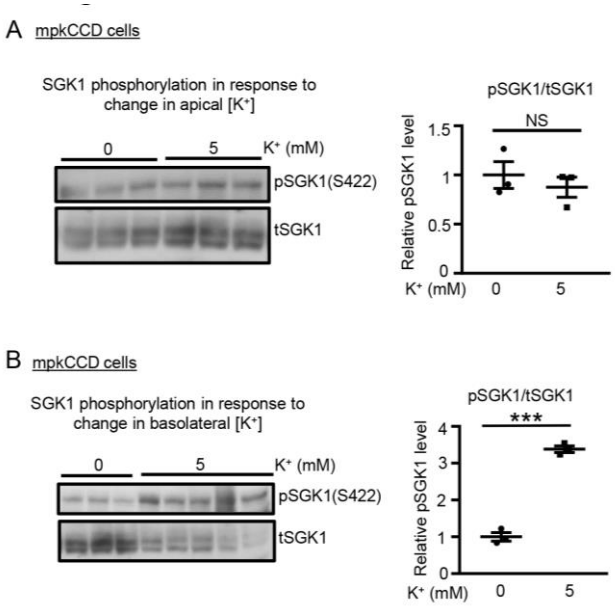


Supplemental Figure 2. Urinary Na^+ concentration following control, KCl and NaCl gavage.

Urinary Na^+ concentration in spot urines collected 90 to 120 minutes post gavage. Urinary Na^+ concentration is elevated following KCl as compared to control gavage but even further elevated following NaCl gavage. * indicates $P<0.05$ by 1way ANOVA, n= 10-14 in each group.



Supplemental Figure 3. Extracellular [K^+] effect on SGK1 phosphorylation is mTORC2-dependent. Western blot analyses of whole cell extract derived from HEK-293T cells with CRISPER/Cas9 mediated knockout of SIN1, in response to the changes in the extracellular [K^+]. SIN1 knockout abrogated the stimulatory effect of extracellular [K^+] on SGK1 S422 phosphorylation. Phosphorylation of AKT at S473 (mTORC2-dependent phosphorylation) and at T308 (PDK1-dependent phosphorylation) were unaffected by changes in [K^+]



Supplemental Figure 4. Reducing basolateral [K^+] inhibits SGK1 S422 phosphorylation. mpkCCD cells were adapted to 5 mM [K^+], and then either apical (A) or basolateral (B) [K^+] was changed to 0 mM for 1 h, as shown, after which cells were lysed and prepared for Western blot. This experiment is essentially the inverse of Figure 6. Data shown are means ±SEM from three independent experiments. *** $P<0.001$ by two-tailed Student's T-test. NS, Not significant.

Supplemental Table 1 . mTOR activity is required for the effects of extracellular [K^+] on ENaC activity in native cortical collecting duct.

	pA/cell	**pA/cell**	**pA/cell**	**pA/cell**	**pA/cell**	**Mean±SEM**
5 mM K^+	340	430	380	400	390	388±15 pA
5 mM K^+ +AZD8055	275	200	180			218±30 pA
1 mM K^+	290	320	250	310	280	290±12pA
1 mM K^+ +AZD8055	215	230	190			213±12 pA

Measurements of amiloride sensitive Na^+ current in native CCD measured by whole cell patch clamp in presence or absence of mTORC inhibitor AZD8055. pA, picoamperes.

Supplemental Table 2. Primary antibodies dilution, source and validation reference

Antibody	Host	Dilution	Source	Reference
pSGK1	Rabbit	1000	sc-16745, Santa Cruz Bio.	(6)
tSGK1	Rabbit	2000	5188, Sigma	(6)
pAKT (S473)	Rabbit	2000	4060, CST	
pAKT (T308)	Rabbit	2000	2965, CST	
tAKT	Rabbit	2000	9272, CST	
c-Myc	Mouse	5000	M4439, Sigma	
Phospho-p70S6K(t389)	Rabbit	1000	9234, CST	(7)
SIN1	Mouse	1000	05-1044 Millipore	(8)
Tubulin	Mouse	5000	T9026 Sigma	(9)
α-ENaC	Rabbit	1000	Loffing	(10)
β-ENaC	Rabbit	1000	Loffing	(11)
γ-ENaC	Rabbit	7500	Loffing	(11)
Pan actin	Mouse	10000	Sigma	

Supplemental References

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