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# The Glycolytic Enzymes Glyceraldehyde 3-Phosphate Dehydrogenase and Enolase Interact with the Renal Epithelial $K^+$ Channel ROMK2 and Regulate its Function

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**Key Words**

Epithelial potassium channels • Potassium homeostasis • Kir1.1 • Metabolic channelling

**Abstract**

Background/Aims: ROMK channels mediate potassium secretion and regulate NaCl reabsorption in the kidney. The aim was to study the functional implications of the interaction between ROMK2 (Kir1.1b) and two glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and enolase-$\alpha$, which were identified as potential regulatory subunits of the channel complex. Methods: We performed a membrane yeast-two-hybrid screen of a human kidney cDNA library with ROMK2 as a bait. Interaction of ROMK2 with GAPDH and enolase was verified using GST pull-down, co-immunoprecipitation, immunohistochemistry and co-expression in *Xenopus* oocytes. Results: Confocal imaging showed co-localisation of enolase and GAPDH with ROMK2 in the apical membrane of the renal epithelial cells of the thick ascending limb. Over-expression of GAPDH or enolase-$\alpha$ in *Xenopus* oocytes markedly reduced the amplitude of ROMK2 currents but did not affect the surface expression of the channels. Co-expression of the glycolytically inactive GAPDH mutant C149G did not have any effect on ROMK2 current amplitude. Conclusion: Our results suggest that the glycolytic enzymes GAPDH and enolase are part of the ROMK2 channel supramolecular complex and may serve to couple salt reabsorption in the thick ascending limb of the loop of Henle to the metabolic status of the renal epithelial cells.



## Introduction

It is increasingly recognized that the specificity of signalling at the cell membrane is dependent on local subcellular concentration gradients caused by the limited mobility of intracellular ions, metabolites, second messengers and regulatory enzymes in the sub-membrane zone [1, 2]. This "metabolic compartmentation" or "metabolic channelling" is especially important in supramolecular signalling complexes, which may include channels, transporters, receptors, anchoring proteins, protein kinases and/or phosphatases [3-6]. In ion channel research, the coupling between energy metabolism and electrical activity of the cells has received a lot of attention. The best studied examples are the ATP-sensitive

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potassium channels ($K_{ATP}$-channels), which are inhibited by intracellular ATP and activated by intracellular Mg-ADP. $K_{ATP}$-channels are octamers consisting of four ion channel subunits (Kir6.1 or Kir6.2) and four regulatory subunits (SUR1 or SUR2) belonging to the ATP-binding cassette (ABC protein) superfamily [7]. The regulation of cardiac $K_{ATP}$-channels has been extensively studied [1, 8] and it has been proposed that the channels physically interact with metabolically active enzymes such as adenylate kinase [9], lactate dehydrogenase [10], creatine kinase [10, 11], and pyruvate kinase [12]. The open probability of $K_{ATP}$-channels is modulated not only by ATP and Mg-ADP but also by various intermediates of carbohydrate or fatty acid metabolism produced in the close proximity of the membrane [1, 2, 12, 13].

Much less is known about the metabolic regulation of the renal potassium channels of the Kir1.1 subfamily, also denoted ROMK channels because these **K**$^+$ channels have first been cloned from the **R**enal **O**uter **M**edulla [14, 15]. Alternative splicing generates three ROMK isoforms, ROMK1 (Kir1.1a), ROMK2 (Kir1.1b) and ROMK3 (Kir1.1c) that differ in their N-terminus. The three ROMK splice variants are all localized to the apical membrane of renal epithelial cells, but they have different functional roles. ROMK2 channels are expressed in the thick ascending limb (TAL) of Henle's loop, where they form the small-conductance (35 pS) renal $K^+$ channels responsible for recycling potassium across the apical membrane. $K^+$-efflux through these channels is required to maintain avid NaCl reabsorption through the NKCC2 co-transporter and thus represents an important element of the urinary concentrating mechanism. ROMK2 channels are also expressed in the distal convoluted tubule and in the cortical collecting duct (CCD), where they contribute to the fine-tuning of potassium balance. Loss-of-function mutations in the ROMK gene cause Bartter syndrome, a hereditary salt-wasting nephropathy [16, 17].

Recent findings suggest that the ROMK channels in the TAL, like $K_{ATP}$-channels, might be regulated by an ABC protein, the cystic fibrosis transmembrane regulator (CFTR), and that the interaction with CFTR may render the channel sensitive to intracellular ATP [18, 19]. However, there are some important differences between classical $K_{ATP}$-channels and ROMK channels: the interaction between ROMK channels and CFTR is likely to be indirect [20] and regulated by protein kinase A [19], and the sensitivity of ROMK to intracellular ATP is much lower than that of cardiac or pancreatic $K_{ATP}$-channels [14]. In our present study, we have identified two glycolytic enzymes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase, as accessory subunits of ROMK2. Co-expression of ROMK2 with GAPDH or enolase-α in *Xenopus* oocytes resulted in a decrease in channel activity without influencing the trafficking of the channels to the cell surface. Co-expression of ROMK2 with a glycolytically inactive mutant of GAPDH [21] did not decrease channel activity. Our results suggest that GAPDH and enolase-α form an integral part of the ROMK2 channel macromolecular complex. The close association of the channel with two glycolytic enzymes may serve to couple the energy metabolism of renal epithelial cells with $K^+$ secretion into the lumen of the distal sections of the nephron.

## Materials and Methods

*Ethical approval*

For experiments involving *Xenopus* oocytes adult female African clawed frogs (*Xenopus laevis*) were used. The frogs were anaesthetised by putting them in water containing 1g/l tricaine. Stage-V oocytes were obtained from ovarian lobes. Anaesthesia and operation were carried out in accordance with the principles of German legislation with approval of the animal welfare officer of the Medical Faculty of Marburg University under the governance of the Regierungspräsidium Giessen (the regional veterinary heath authority).

*Molecular cloning and mutagenesis*

Full length ROMK2 and GAPDH or enolase-α was cloned into membrane yeast two hybrid bait (pBT3C and pBT3N) and prey (pPR3C and pPR3N) vectors (MoBiTec Molecular Biotechnology, Göttingen, Germany) as described previously [22], using SfiI sites, such that the insert is in-frame to the downstream and upstream reporter cassette, respectively. All constructs used for studies in *Xenopus* oocyte were subcloned between the 5' and 3' UTR of the *Xenopus* β-globin gene in the modified pOG1 vector to increase expression efficiency. Complementary RNA transcripts were synthesised with the mMessage mMachine kit (Ambion, Huntingdon, UK). For surface quantification, a plasmid with ROMK2 containing an external hemagglutinin (HA) epitope tag was used [22]. For GST fusion proteins, cDNA encoding the carboxyl (164-372aa) and amino terminal amino acids (1-57aa) of ROMK2 were PCR amplified and subcloned in the glutathione *S*-transferase (GST) expression vector, pGEX4T-1 (GE Healthcare, Piscataway, NJ) via EcoRI and SalI restriction sites. A catalytically inactive mutant of GAPDH (C149G) was made as described by Tisdale et al. [21].

*Split-ubiquitin membrane yeast two hybrid assay*

The split-ubiquitin membrane yeast-two-hybrid assay (MoBiTec, Göttingen, Germany) was used to identify proteins that interact with ROMK2. The C-terminal half of ubiquitin (Cub), along with an artificial transcription factor (TF) was fused

to the C terminus of ROMK2 (Fig. 1A). Yeast strains expressing the bait were transformed with a human kidney cDNA library (prey) carrying a mutant of the N-terminal half of ubiquitin (Nub-G) which has a low affinity for the Cub moiety. Interaction between bait and prey proteins brings Cub and Nub-G together and activates the transcription factor complex. This complex enters the nucleus and activates the reporter genes, resulting in histidine-prototroph cells that are able to grow on medium lacking histidine and turn blue in a β-galactosidase assay. A ROMK2 bait vector for type I proteins (pBT3C, MoBiTec) was used for screening. Identification of positive clones, recovery of library plasmids and identification of prey sequences were performed according to the manufacturer's guidelines. In brief, the *S. cerevisiae* reporter strain NMY51 was transformed with the ROMK2 bait plasmid and the correct expression of the bait was verified by Western blot using LexA mouse monoclonal antibody (Santa Cruz Biotechnology). To verify the correct topology of the bait and the correct function of the screen ROMK2 was also co-transformed with positive (pAI-Alg5) and negative (pDL2-Alg5) control preys supplied by the manufacturer (MoBiTec, Göttingen, Germany). pAI-Alg5 contains the entire open reading frame of the yeast resident ER protein Alg5 fused to wild-type Nub moiety, which has a high affinity for Cub (positive control), whereas pDL2-Alg5 contains Alg5 fused Nub-G, which has a low affinity for Cub (negative control). The highest level of selection stringency for screening was applied by using a selection medium lacking leucine, tryptophan, histidine and adenine (-LWHA).

The yeast strains expressing the bait were transformed with human kidney cDNA library (Molecular Biotechnology). Protein-protein interaction was determined by growth on -LWHA plates. Positive colonies were further verified using the second marker β-galactosidase. The nucleotide sequence of the positive colonies was determined by sequencing, and the identity of the encoded putative interacting protein was determined by data base search (Blastx).

*Immunohistochemistry*

Immunohistochemical double labelling procedures were performed using mouse anti-GAPDH (1:500), mouse anti-enolase (1:500) (Abcam, UK) and rabbit anti-ROMK (1:500) (Alomone Laboratories, Jerusalem, Israel). Mice were anesthetized by an injection of pentobarbital sodium (0.06 mg/g body wt ip). The abdominal cavity was opened and kidneys were perfused retrogradely through the abdominal aorta using 3% paraformaldehyde and processed for cryosectioning. Cryosections were treated with 0.5% Triton X-100 in PBS for 30 min for better antigen retrieval; unspecific binding was blocked with 5% milk powder in PBS for 30 min. The primary antibodies (diluted in 5% milk powder) were sequentially applied for 1 h at room temperature, followed by application of appropriate secondary fluorescent Cy3-, Cy2-conjugated antibodies (1:300) (Dianova, Hamburg, Germany) for 2 h. Sections were washed, coverslipped with PBS-glycerol, and analyzed in a Leica DMRB microscope equipped with a SPOT 32 camera and MetaView 3.6a software (Diagnostic Instruments; Universal Imaging) or a Zeiss confocal microscope (LSM 5 Exciter).

*GST-pull-down studies*

Glutathione-S-transferase (GST) and GST fusion proteins were expressed in the BL-21 bacterial strain by induction with β-D-thiogalactopyranoside as described previously [23] and incubated with glutathione-sepharose 4B beads for 1 h at 4°C. GST or GST fusion proteins were then incubated for 3 h at 4°C with HEK293 cell lysate. The beads were washed five times with 1 ml of pulldown buffer (50 mm Tris-HCl, pH 7.4, 150 mm NaCl, 5 mm EDTA, 0.1% Triton X-100). Bound proteins were eluted by boiling in SDS gel sample buffer, resolved on an SDS gel by electrophoresis and visualized by autoradiography. The following antibodies were used (all from Santa Cruz Biotechnology, UK): anti GST (1:1000), anti GAPDH (1:1000), anti enolase (1.1000) primary antibodies and anti rabbit HRP conjugate (1:10000) secondary antibody.

*Co-Immunoprecipitation*

Immunoprecipitations were carried out using Immunoprecipitation Kit - Dynabeads® Protein G (Invitrogen) following the manufacturer's instructions. Briefly, Protein G Dynabeads were coated with mouse anti -His antibodies (GE Healthcare) under rotation for 1-2 hr at 4°C. Flp-In-293/ROMK2 cells stably expressing His tagged ROMK2 [22] were used in this study. Triton X-100 lysates prepared from HEK293 cells or Flp-In-293/ROMK2 were used for immunoprecipitations. Antibody coated beads were incubated with the lysates under rotation for 2-6 hr at 4°C. Dynabeads coated with antigen-antibody complex were washed extensively (4x) with the wash buffer supplied by the manufacturer. The proteins on Dynabeads were eluted by boiling at 95°C for 5 min in SDS sample loading buffer and separated on a 10 or 12 % SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose membrane and probed with either rabbit anti GAPDH (1:1000) (Santa Cruz Biotechnology) or rabbit anti enolase (1:1000) (Santa Cruz Biotechnology) or anti His (1:1000) (GE Healthcare) antibody. The membrane was washed and incubated with HRP-conjugated secondary antibody (1:10,000), and the bands were detected using the SuperSignal kit (Pierce).

*Voltage-clamp measurements*

Defolliculated *Xenopus laevis* oocytes were obtained as described previously [22] and were injected with 50 nl nuclease free water containing cRNA (10 pg of ROMK2 and 1 or 10 ng of GAPDH, enolase-α or barttin), and then stored at 16°C in ND96 solution containing (mM) 96 NaCl, 2 KCl, 1 MgCl2, 1,8 CaCl2 and 5 HEPES (pH 7.4), supplemented with 100 μg/ml gentamycin and 2.5 mM sodium pyruvate. Two to three days following injection, two-electrode voltage-clamp measurements were performed at room temperature with a Gene Clamp 500 amplifier (Axon Instruments, Union City, USA) as described previously [22]. To test whether the resting membrane potential was large enough and to confirm oocyte viability, initial electrode impalements were made in standard ND96 solution. Membrane voltage was clamped over the range -100 to +60 mV in 20 mV steps. In order to describe the intrinsic rectifying properties of the expressed channels, symmetrical potassium conditions were established by replacing the bath solution with KD96

solution containing (mM): 98 KCl, 1 $MgCl_2$, 1,8 $CaCl_2$ and 5 HEPES (pH 7.4). $K^+$ currents were recorded in bath solution containing KD96. Experiments were repeated in at least three different batches of oocytes derived from different frogs. Since the ROMK2 current amplitude varied between batches the data of each batch were normalised, i.e. the mean ROMK2 current amplitude measured under control conditions was taken as 1 and the relative current magnitude measured in the presence of overexpressed glycolytic enzymes was determined. The normalised data from the different batches were combined.

*Surface expression analysis*

Surface expression of HA-tagged ROMK2 channels in *Xenopus* oocytes was analysed two days after injection with the cRNA (1 ng/oocyte of ROMK2 alone or together with 10 ng of GAPDH or enolase-α) as described previously [22, 24]. Oocytes were incubated for 30 min in ND96 solution containing 1% BSA at 4°C to block non-specific binding of antibodies. Subsequently, oocytes were incubated for 60 min at 4°C with 1 mg/ml rat monoclonal anti-HA antibody (clone 3F10, Roche Pharmaceuticals, Basel, Switzerland) in 1% BSA/ND96, washed 6x at 4°C with 1% BSA/ND96 and incubated with 2 mg/ml peroxidase-conjugated affinity-purified F(ab)2 fragment goat anti-rat immunoglobulin G antibody (Jackson ImmunoResearch, West Grove, PA, USA) in 1% BSA/ND96 for 60 min. Oocytes were washed thoroughly, initially in 1% BSA/ND96 (at 4°C for 60 min) and then in ND96 without BSA (at 4°C for 15 min). Individual oocytes were placed in 20 µl SuperSignal Elisa Femto solution (Pierce, Chester, UK) and, after an equilibration period of 10 s, chemiluminescence was quantified in a luminometer (Lumat LB9507, Berthold Technologies, Bad Wildbad, Germany). With each construct surface expression of 15 oocytes was analysed in one experiment, and at least three experiments (~ 40 oocytes) were carried out. The luminescence produced by water injected oocytes or oocytes expressing WT-ROMK without HA epitope was used as a reference signal (negative control). To verify equal expression of all HA-tagged ROMK2 fusion proteins in *Xenopus* oocytes, total protein was extracted from 10 oocytes for each construct as described previously [22], and subjected to protein electrophoresis and immunoblotting. Nitrocellulose membranes were blocked overnight at room temperature in Tris Buffered Saline with 0.1 % Tween 20 (TBS-T) containing 5 % non-fat milk (NFM). Rat anti-HA monoclonal antibody (Roche, Germany; 1:1000) and secondary goat anti-rat HRP conjugate antibodies (Jackson ImmunoResearch, West Grove, PA, USA; 1:5000) were diluted in 5% NFM, incubated for 1 h each. Following each antibody incubation the membrane was washed three times for 10 min in TBS-T buffer. Bound antibodies were assayed using ECL plus western blotting detection system (Amersham Biosciences, UK).

*Single-channel recordings in HEK293 cells*

Single-channel measurements were carried out as described previously [25, 26]. Data acquisition was performed with an Axopatch 200B amplifier (Axon Instruments) and A/D converter (PCI-MIO 16-XE-10, National Instruments) and data evaluation and acquisition software developed in our laboratory (PC.DAQ1.1). The sampling rate was 10 kHz; the filter cut-off frequency was 2 kHz. The pipette solution contained (mM): 60 KCl, 65 K-glutamate, 5 EGTA, 2 $MgCl_2$, 3 $K_2ATP$, 0.2 $Na_2GTP$ and 5 HEPES (pH 7.2 with KOH) in HEK293 cells stably expressing ROMK2 channels. The pipette resistance was ~4 MΩ. The bath solution contained 135 NaCl, 10 HEPES, 10 Glucose, 5 KCl, 2 Na-Pvruvat, 1 $MgCl_2$, 1 $CaCl_2$, 0.33 Na $H_2PO_4$ (pH 7.4 with NaOH). The temperature was 22-23°C.

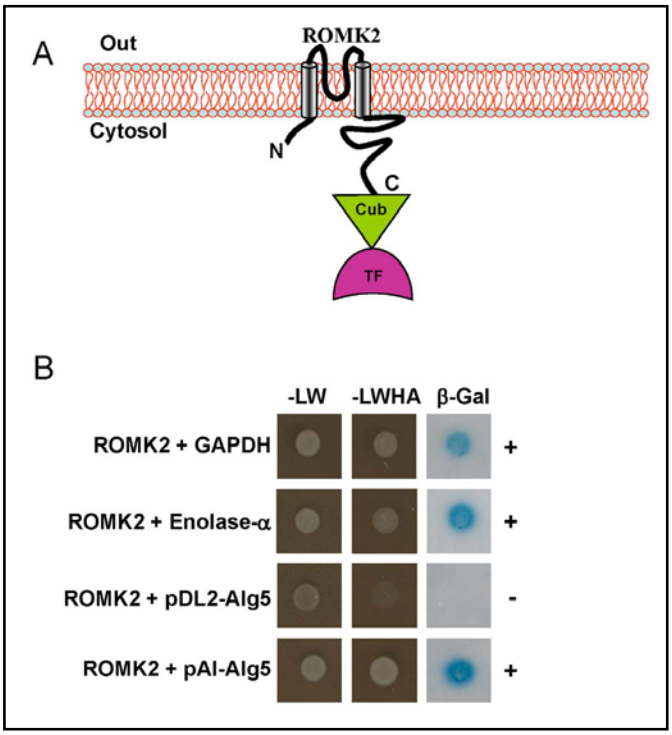


**Fig. 1.** ROMK interacts with GAPDH and enolase-α. (A) Membrane yeast two hybrid construct used in this study. ROMK2, fused to the C-terminal half of ubiquitin (Cub) along with an artificial transcriptional factor (TF) and a downstream reporter cassette, was used as bait. (B) Yeast strains showing positive interaction between ROMK2 and GAPDH or enolase-α. Shown are plates with selective drop-out medium lacking leucine and tryptophan (-LW), indicating the transformation of both bait and prey vectors; with selective drop-out medium lacking leucine, tryptophan, histidine and adenine (-LWHA), indicating the expression of reporter genes *HIS3* and *ADE2*; and with β-galactosidase as a second reporter assay, indicating positive interaction. ROMK2 was also co-transformed with positive (pAI-Alg5) and negative (pDL2-Alg5) control preys supplied by the manufacturer (see Methods).

*Statistical Tests*

Data are reported as means ± S.E.M. Statistical significance was determined using Student's *t*-test. n the Figures, statistically significant differences to control values are marked by asterisks (*$P$ <0.01, **$P$ <0.001 and ***$P$ <0.0001); *n.s.* indicates non-significant differences ($P$ > 0.05).

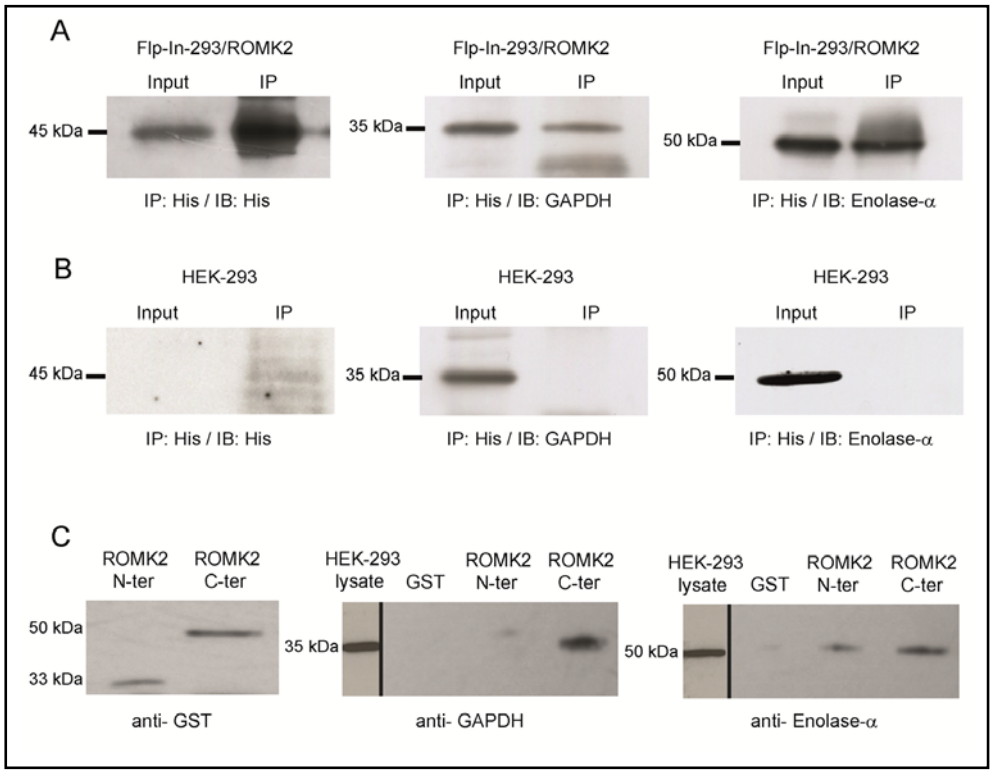


**Fig. 2.** Co-immunoprecipitation and GST pulldown of GAPDH and enolase-α with ROMK2. (A) Cell lysates of Flp-In-293/ROMK2 stable cells expressing His tagged ROMK2 were immunoprecipitated (IP) with anti-His antibody, and immunoblotted (IB) with anti-GAPDH or anti-enolase antibodies. (B) HEK293 cell lysate was used as a negative control. (C) GST fusion proteins containing the amino (N) and carboxy (C) terminus of ROMK2 were recombinantly expressed in bacteria and immobilized on glutathione-sepharose beads. HEK293 cell lysate was used in the pull-down assays. The proteins bound in the pull-down assay were blotted and stained with anti-GAPDH and anti-enolase antibodies.

## Results

*Identification of two glycolytic enzymes as ROMK2 interacting proteins*

In a quest for novel regulatory subunits of the renal potassium channel ROMK, we performed a yeast-two-hybrid screen with a human kidney cDNA library using ROMK2 (the ROMK isoform which shows the highest expression along the TAL) as bait. We used the split-ubiquitin variant of the yeast-two-hybrid system because it enables the use of full length integral membrane proteins as baits and because it preferentially screens for interactions with integral membrane proteins and membrane associated proteins (see Methods). The screening of about 1000 clones revealed two putative interacting proteins: the glycolytic enzymes GAPDH and enolase-α. The interaction between these two enzymes and ROMK2 was confirmed by a direct yeast-two-hybrid assay using ROMK2 as bait and GAPDH or enolase as prey. In both cases a *his*$^+$*/ade*$^+$*/lacZ*$^+$ phenotype was obtained (Fig. 1B), indicating robust interaction.

To further verify the interaction we performed co-immunoprecipitation studies using cell lysates from a cell line stably expressing His-tagged ROMK2 (Flp-In-293 cell line, Invitrogen). Lysates from HEK293 cells were used as a negative control. We detected GAPDH and enolase-α protein in the anti-His immunoprecipitates of Flp-In-293/ROMK2 cells expressing His tagged ROMK2, as illustrated in Fig. 2A; (typical result of two independent experiments). No GAPDH or enolase-α protein was detected in the anti-His immunoprecipitates of HEK293 cells (Fig. 2B).

To test whether the intracellular regions of ROMK2 can interact with native GAPDH and enolase at physiological expression levels we employed a glutathione-S-transferase (GST) pull-down assay. GST alone, GST fused to the C-terminus of ROMK2 (amino acids 164-372), and GST fused to the N-terminus of ROMK2 (amino acids 1-57) were conjugated to glutathione-Sepharose beads and incubated with the lysates of HEK293cells. GAPDH and enolase, which are endogenously expressed in HEK293 cells, were detected at an apparent molecular size of 35 and 50 kDa respectively (Fig. 2C; n = 3 experiments). The GST-fusion protein with the C-terminus of ROMK2 captured GAPDH efficiently, whereas the N-terminus did not appear to interact with GAPDH. GST-fusion proteins with either the N-terminus or with the C-terminus of ROMK2 captured enolase; the interaction of the C-terminus of ROMK2 with enolase was more robust than the interaction of the N-terminus (Fig. 2C*)*. Control experiments showed that purified GST did not capture either enolase or GAPDH in this assay (Fig. 2C*)*. Thus the interaction with the two glycolytic enzymes appears to be mediated by the intracellular regions of ROMK2. Both the N- and the C-terminal intracellular region of ROMK2 channel are involved

in the interaction with enolase, whereas the C-terminal region of ROMK2 appears to be responsible for the interaction with GAPDH.

To obtain more information on the possible co-localisation of ROMK2 with the glycolytic enzymes in the intact kidney we performed immunohistochemical experiments on cryosections from mouse kidney. Confocal imaging of enolase and GAPDH showed a broad distribution of both proteins in renal epithelia (Fig. 3A,B). The sections through the thick ascending limb (TAL) show that enolase and GAPDH could be found in the cytosol of the epithelial cells as well as in the basolateral and apical membranes. Interestingly, the intensity of the staining of both enolase and GAPDH was highest at the apical side of the cells (left column, green arrows). ROMK-2 channels were exclusively localised to the apical membrane of the epithelial cells (middle column, red arrows), although some of the cells were apparently not labelled (white arrows), as has been observed previously [27, 28]. The cross sections through the tubules marked with an asterisk (upper panels of Fig. 3A and B) are also shown at higher resolution (lower panels). It can be seen that the glycolytic enzymes and ROMK channels were partially co-localised at the apical side of the TAL (yellow arrows). Very similar results were obtained with rat kidney sections (n = 2 experiments; not illustrated). Thus, it appears that in native cells the bulk of GAPDH and enolase is localised to the cytosol, but a distinct fraction of the enzymes is associated with the apical membrane.

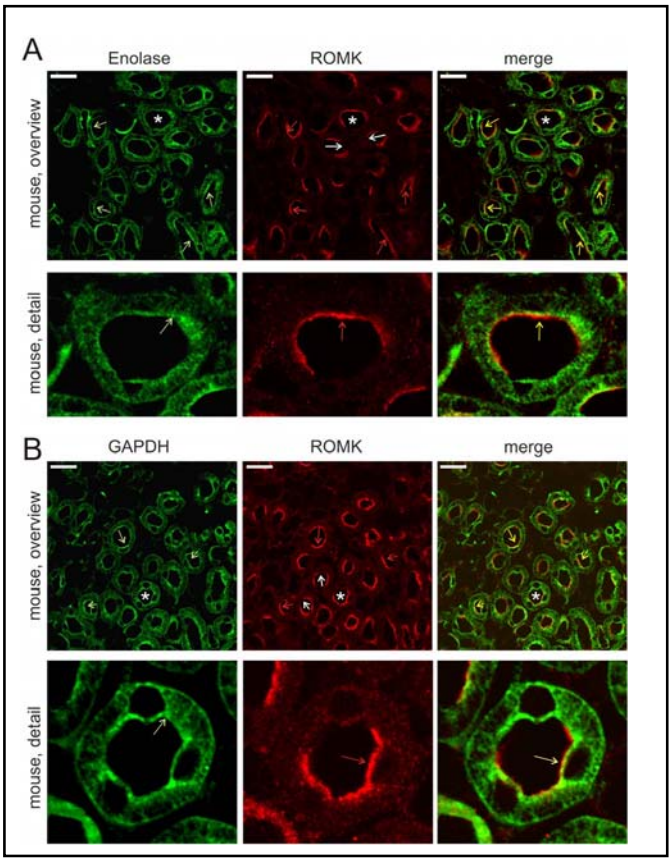


**Fig. 3.** Immunohistochemical labelling of ROMK2, enolase and GAPDH in renal epithelial cells of the thick ascending limb and the collecting duct. Confocal imaging of immunostained cryosections of mouse kidney. (A) Co-localization of enolase-α and ROMK in TAL and collecting duct. (B) Co-localization of GAPDH and ROMK in TAL and collecting duct. The scale bars are 100 µm. The tubules marked with an asterisk are shown at 3.5× higher magnification in the lower rows of (A) and (B). Note strong apical enolase and GAPDH signals in epithelial cells of the TAL. These data are typical for two experiments on mouse kidney sections.

*Functional consequences of the interaction between glycolytic enzymes and ROMK2*

To investigate the functional relevance of the interaction of ROMK2 with GAPDH and enolase-α, we co-expressed the channel and the glycolytic enzymes in *Xenopus* oocytes and measured ROMK currents with the two-electrode voltage clamp technique. With normal $K^+$ concentration in the bath solution (2 mM), expression of ROMK2 channels induced outwardly rectifying currents reversing near the calculated potassium equilibrium potential (not illustrated). In the presence of symmetrical $K^+$ concentrations across the membrane, expression of ROMK2 induced currents with weak inward rectification (Fig. 4A,B), as expected for ROMK channels [29]. When ROMK2 cRNA (10 pg) was co-injected with GAPDH (1 ng) or enolase-α (1 ng) into *Xenopus* oocytes a significantly decreased ROMK current amplitude was observed (Fig. 4A-C). Co-expression of ROMK2 with both GAPDH and enolase-α was associated with an even further decrease in current amplitude (Fig. 4C, red bars).

To find out whether the amount of cRNA injected influenced the effects of GAPDH and enolase-α on ROMK current we repeated this experiment with a higher amount of cRNA (10 ng/oocyte) coding for GAPDH and enolase-α (Fig. 4C, green bars). Under these conditions the reduction in current amplitude caused by GAPDH was somewhat more pronounced ($p < 0.01$), but the reduction in current amplitude caused by co-expression of enolase-α or of both enzymes was very similar (compare red and green bars in Fig. 4C). Co-expression of ROMK2 with barttin (10 ng cRNA/oocyte) did not influence ROMK currents, indicating that co-injection of additional cRNA *per se* had no measurable effect. Finally, co-expression of a glycolytically inactive GAPDH mutant (C149G) with ROMK2 did not cause a decrease in

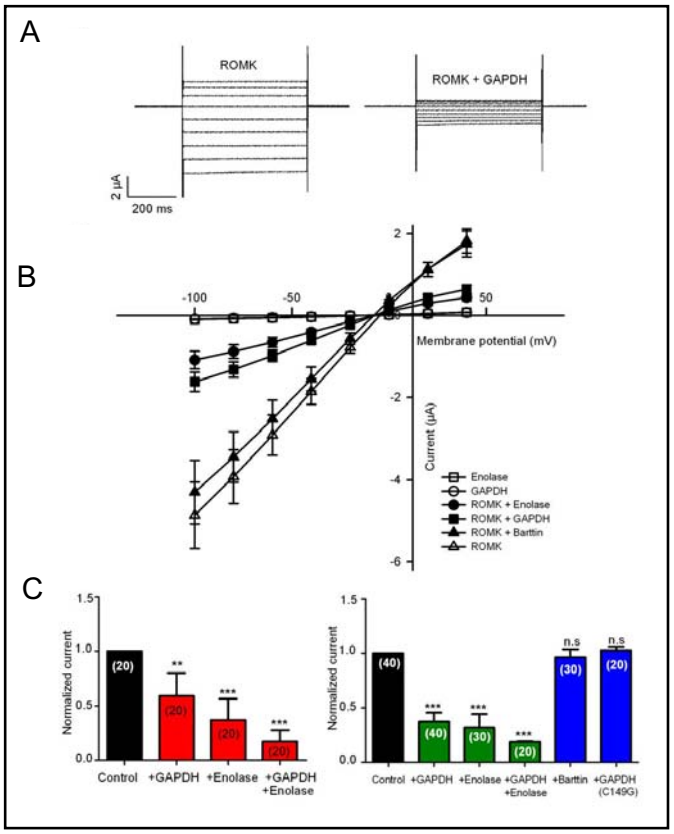


**Fig. 4.** Co-expression of ROMK2 with GAPDH, enolase-α or glycolytically inactive GAPDH in *Xenopus* oocytes. (A) Representative whole cell currents from *Xenopus* oocytes injected with ROMK2 alone (left) or co-injected with ROMK2 plus GAPDH. (B) ROMK2 current-voltage relationships measured under various conditions (see key). (C) Statistics of the effect of co-expression of GAPDH, enolase-α, barttin and an enzymatically inactive mutant of GAPDH on ROMK current amplitude. Currents with and without co-injection of glycolytic enzymes were measured in the same batches of oocytes at -100 mV and normalised. The amount of ROMK cRNA injected was 10 pg throughout. In the left panel 1 ng cRNA of GAPDH or enolase-α (or 1 ng of either enzyme) was injected (red bars). In the right panel 10 ng cRNA of GAPDH or enolase-α (or 10 ng of either enzyme) was injected green bars) The blue bars show that co-injection of barttin (10 ng) or GAPDH$^{C149G}$ had no significant effect. The error bars represent S.E.M; the number of oocytes from which the data were obtained is indicated in brackets; $^{**}P$ <0.01, $^{***}P$ <0.001 Vs ROMK2, *n.s*, not significant.

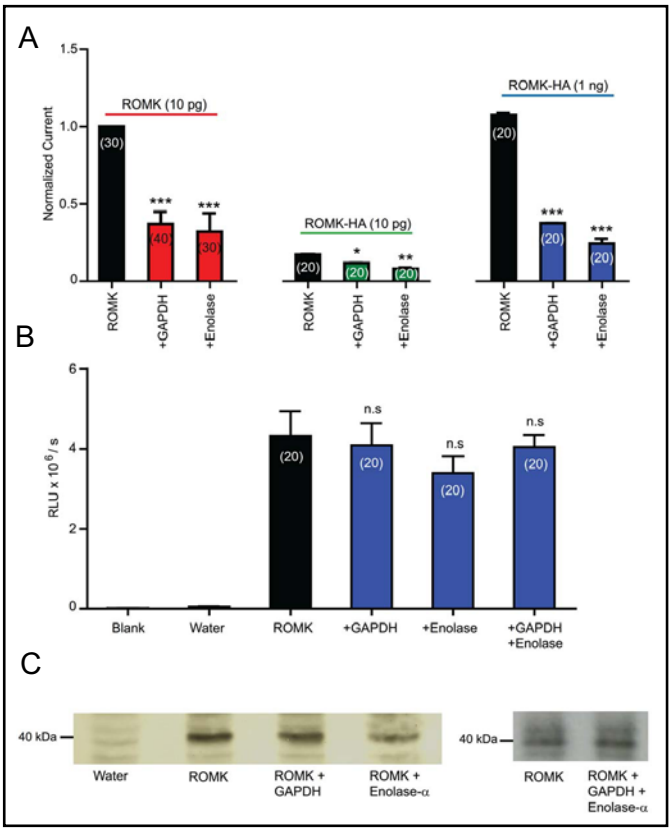


**Fig. 5.** Effects of GAPDH and enolase-α on currents and surface expression of ROMK2 channels. (A) Normalised currents measured at -100 mV in *Xenopus* oocytes 48 h after injection of 10 pg wt ROMK2 cRNA, 10 pg HA-tagged ROMK2 cRNA, and 1 ng HA-tagged ROMK2 cRNA (black bars). The reduction in ROMK current observed after co-expression of the channel with GAPDH (1 ng/oocyte) or with enolase-α (1 ng/oocyte) is indicated by the red, green and blue bars for the different conditions. The error bars represent S.E.M; the number of oocytes from which the data were obtained is indicated in brackets. (B) Effects of co-expression of GAPDH or enolase-α or both on the surface expression of HA-tagged ROMK2 channels as determined by a luminometric assay (see Methods); 1 ng ROMK2 cRNA was injected; *n.s.* indicates a change that is not statistically significant ($p > 0.05$). Non-injected and water injected oocytes were taken as controls to assess antibody specificity. (C) Western blots of HA-tagged ROMK2 protein in total oocyte lysates. No major changes in protein expression were detected.

ROMK current (Fig. 4C, blue bar), in contrast to wild type GAPDH. This finding suggests that the enzymatic activity of GAPDH may play a role in the modulation of ROMK currents.

*Effect of glycolytic enzymes on ROMK2 surface expression*

To test the possibility that the decrease in ROMK2 current amplitude observed in the presence of glycolytic enzymes was due to a change in surface expression we introduced a hemagglutinin (HA)-tag in the first extracellular loop of ROMK2 and studied the effect of GAPDH or enolase-α on the surface expression of the channels with a luminometric technique (see Methods). When injected at identical concentrations (10 pg/oocyte), HA-tagged ROMK2 produced a much lower current amplitude than WT-ROMK2 (Fig. 5A, compare black bars). However, the reduction in current amplitude caused by

co-expression of GAPDH or enolase-α could still be observed. When injected at a higher concentration (1 ng/oocyte), HA-tagged ROMK2 yielded currents identical to those observed for the WT-ROMK2 (10 pg). Under these conditions the decrease in channel activity caused by co-expression of GAPDH or enolase-α was similar to that observed with wild-type ROMK2 (Fig. 5A, compare red and blue bars). Thus, it appears that the addition of the HA-tag impedes the trafficking of ROMK2 channels to the surface membrane but does not change the properties of the channels or the modulation of the channels by glycolytic enzymes. These findings are consistent with previous studies showing that addition of an HA tag does not alter the functional properties of ROMK channels [22, 30, 31].

The surface expression of ROMK2 channels expressed in *Xenopus* oocytes was measured using a luminometric technique described previously [22]. When oocytes were injected with 1 ng cRNA coding for HA-tagged ROMK2 channels a robust luminescence signal could be measured which was not observed in oocytes expressing wild-type ROMK2 without HA-epitope (data not shown). Co-expression of GAPDH or enolase-α, or both together, produced no significant change in the surface expression of ROMK2 (Fig. 5B). Western blots confirmed approximately equal expression of all HA-tagged ROMK2 proteins in *Xenopus* oocytes (Fig. 5C), although in the case of enolase there was a trend to partial reduction of protein expression. Taken together, our results suggest that the decrease in ROMK current amplitude observed after co-expression with the glycolytic enzymes GAPDH or enolase-α was not due to a decrease in the surface expression of the channel.

*Effect of glycolytic enzymes on ROMK2 single-channel conductance*

To test for changes in single-channel properties we carried out cell-attached measurements of single ROMK2 channels in HEK293 cells stably expressing ROMK2 [22] with and without transient transfection of GAPDH. Figure 6A shows typical cell-attached measurements in a permanent cell line expressing ROMK2 channels. The amplitude of single-channel currents became larger when positive pipette potentials were applied. The corresponding all-point histograms are shown in Fig. 6B. The single-channel conductance was determined from single-channel current-voltage relations (Fig. 6C). There was no significant change in single-channel conductance when the cells stably expressing ROMK2 channels were (i) not transfected with any glycolytic enzymes, (ii) transiently transfected with the active (wild-type) form of GAPDH, or (iii) transfected with the glycolytically inactive form of GAPDH (C149G mutant).

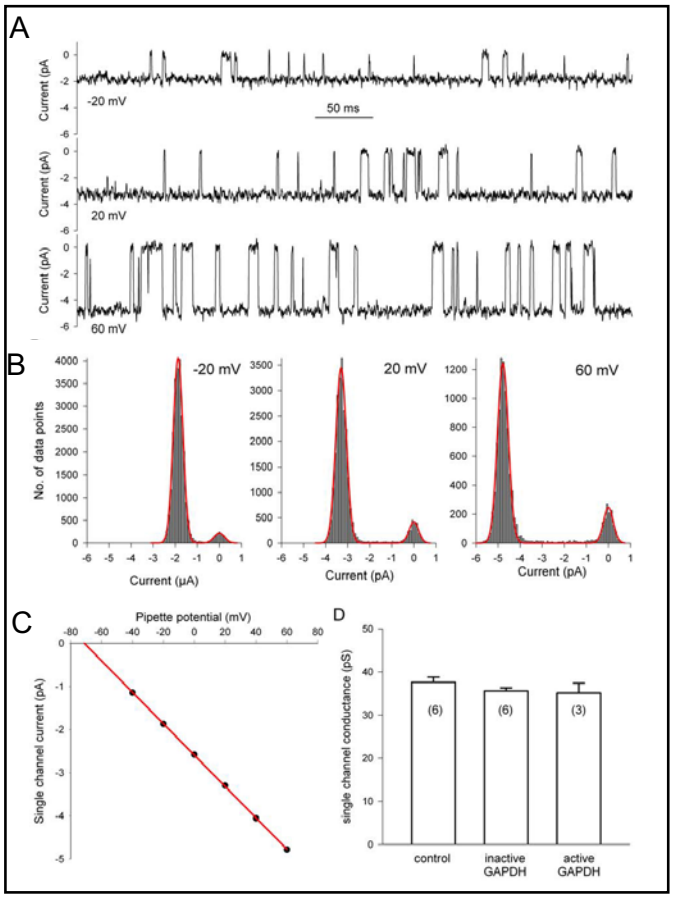


**Fig. 6.** Effects of GAPDH on the conductance of ROMK2 channels. (A) Single ROMK2 channels measured in cell-attached patches in HEK293 cells transfected with ROMK2. The pipette potential is indicated below the traces. (B) All-point histograms of the currents shown in panel (A). (C) Single-channel current voltage relation. (D) Conductances of ROMK2 channels with and without co-transfection of GAPDH or GAPDH$^{C149G}$.

## Discussion

We have obtained evidence that two key glycolytic enzymes, GAPDH and enolase-α, can bind to the renal potassium channel ROMK2 and pursued the question how this interaction can modulate renal function. The following findings support the idea that the interaction between the channel and the two enzymes may indeed take place in intact cells and may be functionally relevant in the regulation of ion homeostasis: (1) Our membrane yeast two hybrid screen identified GAPDH and enolase-α as proteins interacting with ROMK2. (2) Our co-

immunoprecipitation studies showed that ROMK2 can interact with native (non-transfected) GAPDH and enolase-α. (3) Our GST-pulldown studies showed that the C-terminus of ROMK2 can interact with native GAPDH and that both the C and the N-terminus of ROMK2 can interact with native enolase-α. (4) Immunohistochemistry experiments in sections of mouse and rat kidney showed that GAPDH and enolase were co-localised with ROMK2 channels in the apical membrane of renal epithelial cells of the TAL. (5) Our voltage-clamp measurements in *Xenopus* oocytes showed a decrease in the current amplitude of ROMK2 when the channel was co-expressed with GAPDH or enolase-α. (6) Co-expression of a glycolytically inactive mutant of GAPDH (C149G) had no effect on the amplitude of ROMK currents. (7) Our surface expression studies in *Xenopus* oocyte showed that co-expression of GAPDH or enolase-α had no effect on the surface expression of ROMK. (8) Our single channels measurements in HEK293 cells suggest that over-expression of GAPDH has no effect on the single-channel conductance of ROMK2 channels.

The decrease in ROMK2 current amplitude observed after co-expression with GAPDH or enolase-α could be due to (i) a reduction of the number of channels at the cell surface membrane, (ii) a change in single-channel properties, or (iii) a change in channel gating. Since surface expression and single-channel conductance were found to be unchanged our results suggest, by exclusion, that over-expression of GAPDH or enolase-α caused a decrease in open probability of the channels. Unfortunately, we did not succeed in demonstrating this directly in HEK293 cells over-expressing GAPDH. It turned out that the variability in the number of channels per patch was too large to allow a reliable comparison of single-channel kinetics under different experimental conditions. Thus, the detailed mechanisms by which GAPDH and enolase-α modulate the gating of ROMK channels require further investigation.

The simplest interpretation of our results is that ROMK2 might be part of a supramolecular complex that directs metabolic intermediates to regulatory sites, which may either be localised to the channel protein itself or to accessory proteins. In cytoskeletal proteins, effects of GAPDH which are independent of its catalytic activity have been described [21, 32]. Thus, an alternative interpretation of our present findings is that a conformational change of the potassium channel protein brought about by the binding of GAPDH and/or enolase-α was responsible for the change in open probability of ROMK2. However, in view of the finding that metabolically inactive GAPDH had no effect on ROMK2 current amplitude, we consider this possibility unlikely.

The potassium recycling across the apical membrane of epithelial cells is essential for salt reabsorption in the TAL and thus plays an important role in urine concentration. The salt reabsorption in the TAL usually occurs against a large osmotic gradient and requires a high energy turnover, as indicated by the numerous mitochondria present in the epithelial cells lining the TAL. In view of the large energy expenditure of these cells a coupling between energy status (turnover rate of GAPDH and enolase) and open probability of ROMK channels could be physiologically very relevant. It could serve to match salt reabsorption in the renal medulla and oxygen supply via the *vasa recta*.

In conclusion, previous work has shown that two scaffolding proteins, an A-kinase anchoring protein and the $Na^+/H^+$ exchange regulatory factor 2 interact with ROMK channels (reviewed in [13]). Our present study shows that the supramolecular complex surrounding ROMK channels also includes GAPDH and enolase-α. These glycolytic enzymes may modulate the open probability of the channels, probably by local production of metabolic intermediates. The open probability of ROMK channels is an important determinant of the amount of salt reabsorption by the epithelial cells in the TAL. Our results suggest that ROMK channels, via their association with glycolytic enzymes, may serve to couple salt reabsorption to the metabolic status of the epithelial cells in the TAL of Henle's loop.

## Abbreviations

ROMK2 (renal outer medulla potassium channel splice variant 2); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); GST (glutathione *S*-transferase).


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## References


1 Zingman LV, Alekseev AE, Hodgson-Zingman DM, Terzic A: ATP-sensitive potassium channels: metabolic sensing and cardioprotection. J Appl Physiol 2007;103:1888-93.

2 Dhar-Chowdhury P, Malester B, Rajacic P, Coetzee WA: The regulation of ion channels and transporters by glycolytically derived ATP. Cell Mol Life Sci 2007;64:3069-83.

3 Hulme JT, Scheuer T, Catterall WA: Regulation of cardiac ion channels by signaling complexes: role of modified leucine zipper motifs. J Mol Cell Cardiol 2004;37:625-31.

4 Catterall WA, Few AP: Calcium channel regulation and presynaptic plasticity. Neuron 2008;59:882-901.

5 Doupnik CA: GPCR-Kir channel signaling complexes: defining rules of engagement. J Recept Signal Transduct Res 2008;28:83-91.

6 Dai S, Hall DD, Hell JW: Supramolecular assemblies and localized regulation of voltage-gated ion channels. Physiol Rev 2009;89:411-52.

7 Inagaki N, Gonoi T, Clement JPt, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, Bryan J: Reconstitution of $I_{KATP}$: an inward rectifier subunit plus the sulfonylurea receptor. Science 1995;270:1166-70.

8 Nichols CG: KATP channels as molecular sensors of cellular metabolism. Nature 2006;440:470-6.

9 Carrasco AJ, Dzeja PP, Alekseev AE, Pucar D, Zingman LV, Abraham MR, Hodgson D, Bienengraeber M, Puceat M, Janssen E, Wieringa B, Terzic A: Adenylate kinase phosphotransfer communicates cellular energetic signals to ATP-sensitive potassium channels. Proc Natl Acad Sci USA 2001;98:7623-8.

10 Crawford RM, Budas GR, Jovanovic S, Ranki HJ, Wilson TJ, Davies AM, Jovanovic A: M-LDH serves as a sarcolemmal K(ATP) channel subunit essential for cell protection against ischemia. EMBO J 2002;21:3936-48.

11 Crawford RM, Ranki HJ, Botting CH, Budas GR, Jovanovic A: Creatine kinase is physically associated with the cardiac ATP-sensitive $K^+$channel in vivo. FASEB J 2002;16:102-4.

12 Dhar-Chowdhury P, Harrell MD, Han SY, Jankowska D, Parachuru L, Morrissey A, Srivastava S, Liu W, Malester B, Yoshida H, Coetzee WA: The glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase, triose-phosphate isomerase, and pyruvate kinase are components of the $K_{ATP}$ channel macromolecular complex and regulate its function. J Biol Chem 2005;280:38464-70.

13 Liu GX, Hanley PJ, Ray J, Daut J: Long-chain acyl-coenzyme A esters and fatty acids directly link metabolism to $K_{ATP}$ channels in the heart. Circ Res 2001;88:918-24.

14 Welling PA, Ho K: A comprehensive guide to the ROMK potassium channel: form and function in health and disease. Am J Physiol Renal Physiol 2009;297:F849-63.

15 Boim MA, Ho K, Shuck ME, Bienkowski MJ, Block JH, Slightom JL, Yang Y, Brenner BM, Hebert SC: ROMK inwardly rectifying ATP-sensitive $K^+$ channel. II. Cloning and distribution of alternative forms. Am J Physiol 1995;268:F1132-40.

16 Derst C, Konrad M, Köckerling A, Karolyi L, Deschenes G, Daut J, Karschin A, Seyberth HW: Mutations in the ROMK gene in antenatal Bartter syndrome are associated with impaired $K^+$ channel function. Biochem Biophys Res Commun 1997;230:641-5.

17 Simon DB, Karet FE, Rodriguez-Soriano J, Hamdan JH, DiPietro A, Trachtman H, Sanjad SA, Lifton RP: Genetic heterogeneity of Bartter's syndrome revealed by mutations in the $K^+$ channel, ROMK. Nat Genet 1996;14:152-6.

18 McNicholas CM, Guggino WB, Schwiebert EM, Hebert SC, Giebisch G, Egan ME: Sensitivity of a renal $K^+$ channel (ROMK2) to the inhibitory sulfonylurea compound glibenclamide is enhanced by coexpression with the ATP-binding cassette transporter cystic fibrosis transmembrane regulator. Proc Natl Acad Sci USA 1996;93:8083-8.

19 Lu M, Leng Q, Egan ME, Caplan MJ, Boulpaep EL, Giebisch GH, Hebert SC: CFTR is required for PKA-regulated ATP sensitivity of Kir1.1 potassium channels in mouse kidney. J Clin Invest 2006;116:797-807.

20 Yoo D, Flagg TP, Olsen O, Raghuram V, Foskett JK, Welling PA: Assembly and trafficking of a multiprotein ROMK (Kir 1.1) channel complex by PDZ interactions. J Biol Chem 2004;279:6863-73.

21 Tisdale EJ, Kelly C, Artalejo CR: Glyceraldehyde-3-phosphate dehydrogenase interacts with Rab2 and plays an essential role in endoplasmic reticulum to Golgi transport exclusive of its glycolytic activity. J Biol Chem 2004;279:54046-52.

22 Renigunta A, Renigunta V, Saritas T, Decher N, Mutig K, Waldegger S: Tamm-Horsfall glycoprotein interacts with renal outer medullary potassium channel ROMK2 and regulates its function. J Biol Chem 2011;286:2224-35.

23 Renigunta V, Yuan H, Zuzarte M, Rinne S, Koch A, Wischmeyer E, Schlichthörl G, Gao Y, Karschin A, Jacob R, Schwappach B, Daut J, Preisig-Müller R: The retention factor p11 confers an endoplasmic reticulum-localization signal to the potassium channel TASK-1. Traffic 2006;7:168-81.

24 Margeta-Mitrovic M: Assembly-dependent trafficking assays in the detection of receptor-receptor interactions. Methods 2002;27:311-7.

25 Li XT, Dyachenko V, Zuzarte M, Putzke C, Preisig-Müller R, Isenberg G, Daut J: The stretch-activated potassium channel TREK-1 in rat cardiac ventricular muscle. Cardiovasc Res 2006;69:86-97.

26 Rajan S, Preisig-Müller R, Wischmeyer E, Nehring R, Hanley PJ, Renigunta V, Musset B, Schlichthörl G, Derst C, Karschin A, Daut J: Interaction with 14-3-3 proteins promotes functional expression of the potassium channels TASK-1 and TASK-3. J Physiol 2002;545:13-26.

27 Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A, O'Rourke B: Cytoprotective role of $Ca^{2+}$- activated $K^+$ channels in the cardiac inner mitochondrial membrane. Science 2002;298:1029-33.

28 Watts SW. Endothelin receptors: what's new and what do we need to know? Am J Physiol Regul Integr Comp Physiol 2010;298:R254-60.

29 Ho K, Nichols CG, Lederer WJ, Lytton J, Vassilev PM, Kanazirska MV, Hebert SC. Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. Nature 1993;362:31-8.

30 Yoo D, Kim BY, Campo C, Nance L, King A, Maouyo D, Welling PA. Cell surface expression of the ROMK (Kir 1.1) channel is regulated by the aldosterone-induced kinase, SGK-1, and protein kinase A. J Biol Chem 2003;278:23066-75.

31 Konstas AA, Koch JP, Tucker SJ, Korbmacher C. Cystic fibrosis transmembrane conductance regulator-dependent up-regulation of Kir1.1 (ROMK) renal K+ channels by the epithelial sodium channel. J Biol Chem 2002;277:25377-84.

32 Tisdale EJ, Azizi F, Artalejo CR. Rab2 utilizes glyceraldehyde-3-phosphate dehydrogenase and protein kinase C{iota} to associate with microtubules and to recruit dynein. J Biol Chem 2009;284:5876-84.