Original Paper

SPAK and OSR1 Dependent Down-Regulation of Murine Renal Outer Medullary K⁺ Channel ROMK1

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Key Words

K⁺ channel • Oxidative stress-responsive kinase 1 • Renal tubule • SPS1-related proline/alanine-rich kinase • WNK

Abstract

Background/Aims: The kinases SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1) participate in the regulation of the NaCl cotransporter NCC and the Na⁺,K⁺,2Cl⁻ cotransporter NKCC2. The kinases are regulated by WNK (with-no-K[Lys]) kinases. Mutations of genes encoding WNK kinases underly Gordon's syndrome, a monogenic disease leading to hypertension and hyperkalemia. WNK kinases further regulate the renal outer medullary K⁺ channel ROMK1. The present study explored, whether SPAK and/or OSR1 have similarly the potential to modify the activity of ROMK1. **Methods:** ROMK1 was expressed in *Xenopus* oocytes with or without additional expression of wild-type SPAK, constitutively active T233E SPAK, catalytically inactive D212A SPAK, wild-type OSR1, constitutively active T185E OSR1 and catalytically inactive D164A OSR1. Channel activity was determined utilizing dual electrode voltage clamp and ROMK1 protein abundance in the cell membrane utilizing chemiluminescence of ROMK1 containing an extracellular hemagglutinin epitope (ROMK1-HA). **Results:** ROMK1 activity and ROMK1-HA protein abundance were significantly down-regulated by wild-type SPAK and T233E SPAK, but not by D212A SPAK. Similarly, ROMK1 activity and ROMK1-HA protein abundance were significantly down-regulated by wild-type OSR1 and T185E OSR1, but not by D164A OSR1. **Conclusion:** ROMK1 protein abundance and activity are down-regulated by SPAK and OSR1.

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Bernat Elvira and Carlos Munoz contributed equally and thus share first authorship

Introduction

SPAK (SPS1-related proline/alanine-rich kinase) [1-3] and the related OSR1 (oxidative stress-responsive kinase 1) [4, 5] are both kinases participating in the regulation of ion transport and thus blood pressure. Activity of those kinases is under control of WNK (with-no-K[Lys]) kinases [1, 6-9], which are similarly powerful regulators of renal tubular ion transport and blood pressure [10-14]. SPAK and OSR1 up-regulate the NaCl (NCC) and Na⁺,K⁺,2Cl⁻ (NKCC) cotransporters [4-7, 10, 15-23]. Mutations of genes encoding WNK kinases underly Gordon's syndrome, a monogenic disease leading to hypertension and hyperkalaemia [8, 9, 24, 25]. WNK kinases further regulate the renal outer medullary K⁺ channel ROMK [26, 27]. They do so by interacting with intersectin [21] and influencing clathrin-mediated endocytosis [26, 28]. Whether or not ROMK1 is, in addition, regulated by SPAK and/or OSR1 has, to the best of our knowledge, never been reported.

The present study thus explored, whether SPAK and/or OSR1 may modify the activity of the renal outer medullary K⁺ channel ROMK1.

Materials and Methods

Ethical Statement

All experiments conform with the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985) and were conducted according to the German law for the welfare of animals and the surgical procedures on the adult *Xenopus laevis* were reviewed and approved by the respective government authority of the state Baden-Württemberg (Regierungspräsidium) prior to the start of the study (Anzeige für Organentnahme nach §6).

Constructs

Constructs encoding wild-type ROMK1 or ROMK1 containing an extracellular hemagglutinin epitope (ROMK1-HA) [29], wild-type SPAK, constitutively active ^{T233E}SPAK, catalytically inactive ^{D212A}SPAK [6, 30], wild-type OSR1, constitutively active ^{T185E}OSR1, and catalytically inactive ^{D164A}OSR1 [31], were used for generation of cRNA as described previously [32]. The constructs were a kind gift from Dario Alessi (University of Dundee).

Voltage clamp in *Xenopus* oocytes

Xenopus oocytes were prepared as previously described [33, 34]. 5 ng cRNA encoding ROMK1 and 10 ng of cRNA encoding wild-type, constitutively active or inactive kinase were injected on the second day after preparation of the oocytes [35]. The oocytes were maintained at 17°C in ND96-A solution containing (in mM): 88.5 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES, tetracycline (Sigma, 0.11 mM), ciprofloxacin (Sigma, 4 μM), gentamycin (Refobacin, 0.2 mM) and theophyllin (Euphyllong, 0.5 mM) as well as sodium pyruvate (Sigma, 5 mM) were added to the ND96, pH was adjusted to 7.5 by addition of NaOH. The voltage clamp experiments were performed at room temperature 4 days after the first injection [36]. ROMK1 channel currents were elicited every 20 s with 3 s pulses from -160 mV to +60 mV applied from a holding potential of -80 mV. Pulses were applied in 20 mV increments. The data were filtered at 1 kHz and recorded with a Digidata A/D-D/A converter (1322A Axon Instruments) and Clampex 9.2 software for data acquisition and analysis (Axon Instruments) [37]. The control superfusate (ND96) contained (in mM): 93.5 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂ and 5 HEPES, pH was adjusted to 7.4 by addition of NaOH. The flow rate of the superfusion was approx. 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s.

Detection of ROMK1-HA cell surface expression by chemiluminescence

To determine ROMK1-HA cell surface expression by chemiluminescence, the oocytes were incubated with mouse monoclonal anti-HA antibody conjugated to horseradish peroxidase (1:1000, Miltenyi Biotec Inc, CA, USA). Individual oocytes were placed in 96 well plates with 20 μl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer, Juegesheim, Germany) by integrating the signal over a period of 1 s. Results display normalized arbitrary light units. Integrity of the measured oocytes was assessed by visual control after the measurement to avoid unspecific light signals from the cytosol [38].

Fig. 1. Coexpression of wild type SPAK decreased the K^+ current in ROMK1-expressing *Xenopus* oocytes. **A:** Representative original tracings showing currents in *Xenopus* oocytes injected with water (1), expressing ROMK1 alone (2) or expressing ROMK1 with additional coexpression of wild-type SPAK (3).

The voltage protocol is shown (not to scale), whereby cells were held at -80 mV and voltage steps were applied each 20 s in 20 mV increments for 3 s from -160 mV to +60 mV. **B:** Arithmetic means $\pm$ SEM ($n = 11-20$) of the normalized K^+ current (I_K) in *Xenopus* oocytes injected with water (dotted bar), or expressing ROMK1 without (white bar) or with (black bar) additional coexpression of wild-type SPAK. For normalization, the individual currents at -20 mV were divided by the mean current at -20 mV of oocytes expressing ROMK1 alone. **C:** Arithmetic means $\pm$ SEM ($n = 11-20$) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus* oocytes injected with water (grey triangles) or expressing ROMK1 without (white squares) or with (black squares) additional coexpression of wild-type SPAK. * ($p < 0.05$) indicates statistically significant difference from oocytes expressing ROMK1 alone.

Statistical analysis

Data are provided as means $\pm$ SEM, n represents the number of oocytes investigated. All voltage clamp experiments were repeated with at least 3 batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA (Tukey test or Kruskal-Wallis test) or t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored whether SPAK (SPS1-related proline/alanine-rich kinase) and/or OSR1 (oxidative stress-responsive kinase 1) participate in the regulation of the renal outer medullary K^+ channel ROMK1. To this end, cRNA encoding ROMK1 was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding either wild-type or mutant SPAK, or encoding wild-type or mutant OSR1. The K^+ current (I_K) was determined utilizing dual electrode voltage clamp. As illustrated in Fig. 1, I_K was low in water-injected oocytes but markedly increased by expression of ROMK1. The additional coexpression of wild-type SPAK resulted in a significant decrease of I_K .

Further experiments explored whether the effect of wild-type SPAK on ROMK1 was mimicked by the constitutively active $T233E$ SPAK and/or the catalytically inactive $D212A$ SPAK. As illustrated in Fig. 2, similar to coexpression of wild-type SPAK, coexpression of $T233E$ SPAK significantly decreased I_K . In contrast, catalytically inactive $D212A$ SPAK was without significant effect on I_K (Fig. 2).

The effect of SPAK may have been due to inactivation of channel protein or due to down-regulation of channel protein in the cell membrane. Chemiluminescence was thus employed

Fig. 2. The effect of wild type SPAK on ROMK1 was mimicked by constitutively active $T233E$ SPAK but not by inactive $D212A$ SPAK.

A: Representative original tracings showing currents in *Xenopus* oocytes injected with water (1) expressing ROMK1 alone (2), expressing ROMK1 together with

constitutively active $T233E$ SPAK (3) or expressing ROMK1 with catalytically inactive $D212A$ SPAK (4). **B:** Arithmetic means $\pm$ SEM ($n = 9-19$) of the normalized I_K in *Xenopus* oocytes injected with water (dotted bar), expressing ROMK1 alone (white bar), expressing ROMK1 with constitutively active $T233E$ SPAK (1st black bar) or expressing ROMK1 with inactive $D212A$ SPAK (2nd black bar). For normalization, the individual currents at -20 mV were divided by the mean current at -20 mV of oocytes expressing ROMK1 alone. ** ($p < 0.01$) indicates statistically significant difference from oocytes expressing ROMK1 alone.

Fig. 3. SPAK decreased ROMK1-HA protein abundance within the oocyte membrane. Arithmetic means $\pm$ SEM ($n = 80-110$) of normalized ROMK1-HA chemiluminescence in *Xenopus* oocytes injected with water (dotted bar), expressing ROMK1-HA alone (white bar) or expressing ROMK1-HA together with wild-type SPAK (black bar). For normalization, the chemiluminescence was divided by the chemiluminescence of oocytes expressing ROMK1-HA alone. *** ($p < 0.001$) indicates statistically significant difference from oocytes expressing ROMK1-HA alone.

to quantify the ROMK1-HA protein abundance in the cell membrane. To this end, ROMK1 was tagged with an extracellular hemagglutinin epitope (ROMK1-HA) and the protein detected with an antibody directed against this epitope. As illustrated in Fig. 3, coexpression of wild-type SPAK decreased the ROMK1 protein abundance in the cell membrane of *Xenopus* oocytes.

Similar experiments were performed to elucidate the effects of OSR1 on ROMK1 channel activity and protein abundance. As illustrated in Fig. 4, I_K in ROMK1 expressing oocytes was significantly decreased following coexpression of wild-type OSR1. The effect of wild-type OSR1 was again mimicked by coexpression of constitutively active $T185E$ OSR1 (Fig. 5). In contrast, the catalytically inactive $D164A$ OSR1 did not significantly modify I_K .

Fig. 4. Coexpression of wild type OSR1 decreased the K^+ current in ROMK-expressing *Xenopus* oocytes. **A:** Representative original tracings showing currents in *Xenopus* oocytes injected with water (1), expressing ROMK1 alone (2) or expressing ROMK1 with additional co-expression of wild-type OSR1 (3). **B:** Arithmetic means $\pm$ SEM (n = 13-21) of

the normalized I_K in *Xenopus* oocytes injected with water (dotted bar), or expressing ROMK1 without (white bar) or with additional coexpression of wild-type OSR1 (black bar). For normalization, the individual currents at -20 mV were divided by the mean current at -20 mV of oocytes expressing ROMK1 alone. **C:** Arithmetic means $\pm$ SEM (n = 13-21) of the I/V relationship in *Xenopus* oocytes injected with water (grey triangles) or expressing ROMK1 without (white circles) or with additional coexpression of wild type OSR1 (black circles). ** (p<0.01) indicates statistically significant difference from oocytes expressing ROMK1 alone.

Fig. 5. The effect of wild type OSR1 on ROMK1 was mimicked by constitutively active $T185E$ OSR1 but not by inactive $D164A$ OSR1. **A:** Representative original tracings showing currents in *Xenopus* oocytes injected with water (1), expressing ROMK1 alone (2), expressing ROMK1 together with constitutively active $T185E$ OSR1 (3) or expressing ROMK1 with catalytically inactive $D164A$ OSR1 (4). **B:** Arithmetic means

$\pm$ SEM (n = 10-19) of the normalized I_K in *Xenopus* oocytes injected with water (dotted bar), expressing ROMK1 alone (white bar), expressing ROMK1 with constitutively active $T185E$ OSR1 (1st black bar) or expressing ROMK1 with inactive $D164A$ OSR1 (2nd black bar). For normalization, the individual currents at -20 mV were divided by the mean current at -20 mV of oocytes expressing ROMK1 alone. * (p<0.05) indicates statistically significant difference from oocytes expressing ROMK1 alone.

Fig. 6. OSR1 decreased ROMK1-HA protein abundance within the oocyte membrane. Arithmetic means $\pm$ SEM (n=106-110) of ROMK1-HA chemiluminescence in *Xenopus* oocytes injected with water (dotted bar), expressing ROMK1-HA alone (white bar) or expressing ROMK1-HA together with wild-type OSR1 (black bar). *** ($p < 0.001$) indicates statistically significant difference from oocytes expressing ROMK1 alone.

Again, chemiluminescence was employed to quantify the effect of wild-type OSR1 on ROMK1-HA protein abundance in the cell membrane of *Xenopus* oocytes. As illustrated in Fig. 6, coexpression of OSR1 decreased the ROMK1-HA protein abundance in the cell membrane.

Discussion

The present study reveals that both, SPAK and OSR1 are powerful potential regulators of ROMK1. Coexpression of wild-type or constitutively active SPAK or OSR1 leads to significant down-regulation of ROMK1. In contrast, ROMK1 is not significantly modified by the inactive SPAK and OSR1 mutants. Thus, kinase activity is required for the effect of SPAK and OSR1 on ROMK1.

The effects of SPAK and OSR1 on ROMK1 may contribute to the regulation of renal K^+ elimination. Inhibition of ROMK1 channels in the luminal cell membrane are expected to decrease renal tubular K^+ secretion and thus to foster renal K^+ retention. The kinases could further regulate renal K^+ excretion indirectly by stimulating the the NaCl cotransporter and the $Na^+, K^+, 2Cl^-$ cotransporter [4-7, 10, 15-23]. Enhanced NKCC2 activity fosters K^+ retention by cellular uptake of luminal K^+ . Beyond that, the stimulation of NKCC and NCC leads to renal tubular Na^+ retention, which decreases the delivery of Na^+ to the principal cells of the distal nephron and thus curtails Na^+ reabsorption and K^+ secretion in the terminal nephron segment [39, 40]. However, preliminary observations did not reveal significant alterations of renal K^+ excretion in mice expressing WNK insensitive SPAK (not shown). It must be kept in mind that SPAK and OSR may replace each other in the regulation of ROMK. Moreover, further kinases participating in the regulation of ROMK may prevent appreciable alterations of renal K^+ elimination following altered function of SPAK and/or OSR1. Nevertheless, the *in vivo* significance of SPAK and/or OSR sensitive regulation of ROMK remains uncertain.

WNK kinases could modify renal K^+ elimination in several ways, i.e. by direct regulation of ROMK1 [21, 26-28, 40], by modifying the serum & glucocorticoid inducible kinase [40], and by regulating SPAK and OSR1 [1, 6-9]. The hyperkalemia of Gordon's syndrome [8, 9, 24, 25] may thus result from several complex ramifications of WNK dependent transport regulation rather than exclusively from direct influence on ROMK1 channel activity.

Conclusion

SPAK and OSR1 have both the potential to down-regulate ROMK1 and thus might contribute to the regulation of renal K^+ elimination.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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