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SPAK and OSR1 Sensitivity of Voltage-Gated K^+ Channel Kv1.5

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Abstract SPS1-related proline/alanine-rich kinase (SPAK) and oxidative stress-responsive kinase 1 (OSR1) are potent regulators of several transporters and ion channels. The kinases are under regulation of with-no-K(Lys) (WNK) kinases. The present study explored whether SPAK and/or OSR1 modify the expression and/or activity of the voltage-gated K^+ channel Kv1.5, which participates in the regulation of diverse functions including atrial cardiac action potential and tumor cell proliferation. cRNA encoding Kv1.5 was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding wild-type SPAK, constitutively active ^{T233E}SPAK, WNK insensitive ^{T233A}SPAK, catalytically inactive ^{D212A}SPAK, wild-type OSR1, constitutively active ^{T185E}OSR1, WNK insensitive ^{T185A}OSR1, and catalytically inactive ^{D164A}OSR1. Voltage-gated K^+ channel activity was quantified utilizing dual electrode voltage clamp and Kv1.5 channel protein abundance in the cell membrane utilizing chemiluminescence of Kv1.5 containing an extracellular hemagglutinin epitope (Kv1.5-HA). Kv1.5 activity and Kv1.5-HA protein abundance were significantly decreased by wild-type SPAK and ^{T233E}SPAK, but not by ^{T233A}SPAK and ^{D212A}SPAK. Similarly, Kv1.5 activity and Kv1.5-HA protein abundance were significantly down-regulated by wild-type OSR1 and ^{T185E}OSR1, but not by ^{T185A}OSR1 and ^{D164A}OSR1. Both, SPAK and OSR1 decrease cell membrane Kv1.5 protein abundance and activity.

Keywords K^+ channel · Oxidative stress-responsive kinase 1 · SPS1-related proline/alanine-rich kinase · WNK

Introduction

SPS1-related proline/alanine-rich kinase (SPAK) (Castaneda-Bueno and Gamba 2010; Rafiqi et al. 2010; Yang et al. 2010) and oxidative stress-responsive kinase 1 (OSR1) (Lin et al. 2011; Villa et al. 2008) are related kinases contributing to the regulation of ion transport and blood pressure (Gagnon and Delpire 2012). The kinases are regulated by the with-no-K(Lys) (WNK) kinases (Glover et al. 2011; O'Reilly et al. 2003; Rafiqi et al. 2010; Vitari et al. 2005; Vitari et al. 2006), which are again regulators of transport and blood pressure (Flatman 2008; Furgeson and Linas 2010; Kahle et al. 2010; Uchida 2010; Wilson et al. 2001). SPAK and OSR1 have initially been shown to up-regulate the NaCl (NCC) and Na^+,K^+,$2Cl^-$ (NKCC) cotransporters (Delpire and Gagnon 2006; 2008; Gagnon and Delpire 2010; Gagnon and Delpire 2012; Gimenez 2006; Glover and O'Shaughnessy 2011; Huang et al. 2008; Kahle et al. 2010; Lin et al. 2011; Mercier-Zuber and O'Shaughnessy 2011; Richardson and Alessi 2008; Richardson et al. 2011; Villa et al. 2008; Vitari et al. 2005; Vitari et al. 2006). Specific genetic defects of WNK kinases lead to Gordon's syndrome, a monogenic disease characterized by hypertension and hyperkalaemia (Achard et al. 2001; Capasso et al. 2005; Glover et al. 2011; O'Reilly et al. 2003).

Little is known about the role of SPAK and OSR1 in the regulation of ion channels (Falin et al. 2011; Falin et al. 2009; Miyazaki and Strange 2012; Park et al. 2010). The

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present study explored, whether SPAK and/or OSR1 may modify the activity of the voltage-gated K^+ channel Kv1.5, a channel implicated in a variety of functions including the proliferation and migration of normal and tumor cells (Comes et al. 2013; Felipe et al. 2012), repolarization in cardiac atria (Bilodeau and Trotter 2009; Brendel and Peukert 2003; Gonzalez et al. 2010; Tamargo et al. 2009), and regulation of pulmonary artery smooth muscle cell activity (Archer et al. 2008; Bonnet et al. 2007).

In order to test for an effect of SPAK or OSR1 on Kv1.5 channel activity, Kv1.5 was expressed in *Xenopus* oocytes without or with additional expression of the kinases. The voltage-gated K^+ current was determined in those oocytes by dual electrode voltage clamp.

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 *Xenous 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 36).

Constructs

Constructs encoding mouse wild-type Kv1.5 or Kv1.5 containing an extracellular hemagglutinin epitope (Kv1.5-HA) (Mia et al. 2012), wild-type SPAK, constitutively active ^{T233E}SPAK, WNK insensitive ^{T233A}SPAK, catalytically inactive ^{D212A}SPAK (Pathare et al. 2012b; Vitari et al. 2005), wild-type OSR1, constitutively active ^{T185E}OSR1, WNK insensitive ^{T185A}OSR1 and catalytically inactive ^{D164A}OSR1 (Pathare et al. 2012a), were used for generation of cRNA as described previously (Hosseinzadeh et al. 2013a; Warsi et al. 2013). The constructs were a kind gift from Dario Alessi (University of Dundee).

Voltage Clamp in Xenopus Oocytes

Xenopus oocytes were prepared as previously described (Alesutan et al. 2012; Henrion et al. 2012). 2.5 ng cRNA encoding Kv1.5 and 10 ng of cRNA encoding wild-type, constitutively active or inactive kinase were injected on the same day after preparation of the oocytes. The oocytes were maintained at 17 °C in ND96, a solution containing (in mM): 88.5 NaCl, 2 KCl, 1 $MgC1_2$, 1.8 $CaC1_2$, 5 HEPES, and 5 sodium pyruvate ($C_3H_3NaO_3$). Gentamycin (100 mg/l), Tetracycline (50 mg/l), Ciprofloxacin (1.6 mg/l), and Theophiline (90 mg/l) were added and pH was adjusted to pH 7.4 (Almilaji et al. 2013b; Hosseinzadeh et al. 2012). The voltage clamp experiments were performed at room temperature 3 days after the first injection (Almilaji et al. 2013a; Bogatikov et al. 2012). Kv1.5 channel currents were elicited every 20 s with 3 s pulses from −80 to +50 mV in 20 s increments of 10 mV steps from a holding potential of -100 mV. The data were filtered at 2 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) (Hosseinzadeh et al. 2014; Hosseinzadeh et al. 2013b; Munoz et al. 2013). The control superfusate (ND96) contained (in mM): 93.5 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 2.5 NaOH, and 5 HEPES (pH 7.4). 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 (Pakladok et al. 2014; Shojaiefard et al. 2012; Warsi et al. 2014).

Detection of Kv1.5-HA Cell Surface Expression by Chemiluminescence

To determine Kv1.5-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 (Pakladok et al. 2013).

Statistical Analysis

Data are provided as mean ± SEM, n represents the number of oocytes investigated. As different batches of oocytes may yield different results, comparisons were always made within a given oocyte batch. 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 tested whether SPAK (SPS1-related proline/alanine-rich kinase) and/or OSR1 (oxidative stress-responsive kinase 1) modifies the activity of the voltage-gated K^+ channel Kv1.5. To this end, cRNA encoding Kv1.5 was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding either wild-type or mutant SPAK, or cRNA encoding wild-type or mutant OSR1. Subsequent dual electrode voltage clamp experiments were performed to determine the K^+ conductance (G_K) in those oocytes. As shown in Fig. 1, G_K was negligible in water-injected oocytes indicating that the oocytes did not express appreciable voltage-gated K^+ channels. In contrast, sizable voltage-gated K^+ currents were observed in oocytes expressing Kv1.5. The additional coexpression of wild-type SPAK was followed by a significant decrease of G_K.

Further experiments explored, whether the effect of wild-type SPAK on Kv1.5 was modified by mutations affecting kinase activity. As illustrated in Fig. 2, the effect of wild-type SPAK was mimicked by the constitutively active ^{T233E}SPAK, but not by the WNK insensitive inactive ^{T233A}SPAK, and not by the catalytically inactive ^{D212A}SPAK. Coexpression of ^{T233E}SPAK significantly decreased G_K in Kv1.5 expressing oocytes. In contrast, coexpression of neither ^{T233A}SPAK nor ^{D212A}SPAK significantly modified G_K.

The decrease of Kv1.5 channel activity following expression of SPAK in Kv1.5 expressing oocytes may have been due to inactivation of channel protein or due to decrease of channel protein abundance in the cell membrane. In order to discriminate between those two possibilities, chemiluminescence was employed for the quantification of Kv1.5 protein abundance in the cell membrane. To this end, Kv1.5 was tagged with an extracellular hemagglutinin epitope (Kv1.5-HA) and the protein detected with an antibody directed against this epitope. As shown in Fig. 3, coexpression of wild-type SPAK decreased the Kv1.5 protein abundance in the cell membrane of *Xenopus* oocytes.

Similar experiments addressed the effects of OSR1 on Kv1.5 channel activity and protein abundance. As shown in Fig. 4, the voltage-gated K^+ current was in Kv1.5

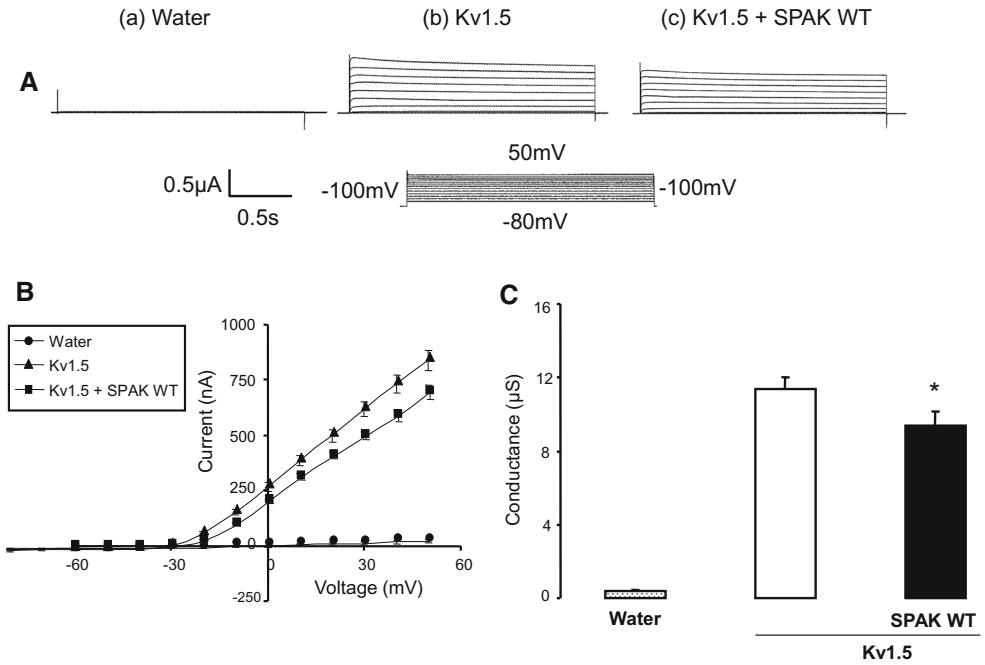

Fig. 1 Coexpression of wild-type SPAK decreased the K^+ current in Kv1.5-expressing *Xenopus* oocytes. **A** Representative original tracings showing currents in *Xenopus* oocytes injected with water (*a*), expressing Kv1.5 alone (*b*) or expressing Kv1.5 with additional coexpression of wild-type SPAK (*c*). The voltage protocol is shown (not to scale). Currents were activated by depolarization from −80 to +50 mV in 20 s increments of 10 mV steps from a holding potential of −100 mV. **B** Arithmetic mean ± SEM (n = 14–19) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus* oocytes injected with water (*black circles*) or expressing Kv1.5 without (*black triangles*) or with (*black squares*) additional coexpression of wild-type SPAK. **C** Arithmetic mean ± SEM (n = 14–19) of the conductance calculated by linear fit of I/V-curves shown in **B** between 20 and 50 mV in *Xenopus* oocytes injected with water (*dotted bar*), or expressing Kv1.5 without (*white bar*) or with (*black bar*) additional coexpression of wild-type SPAK. *$p < 0.05$ indicates statistically significant difference from oocytes expressing Kv1.5 alone

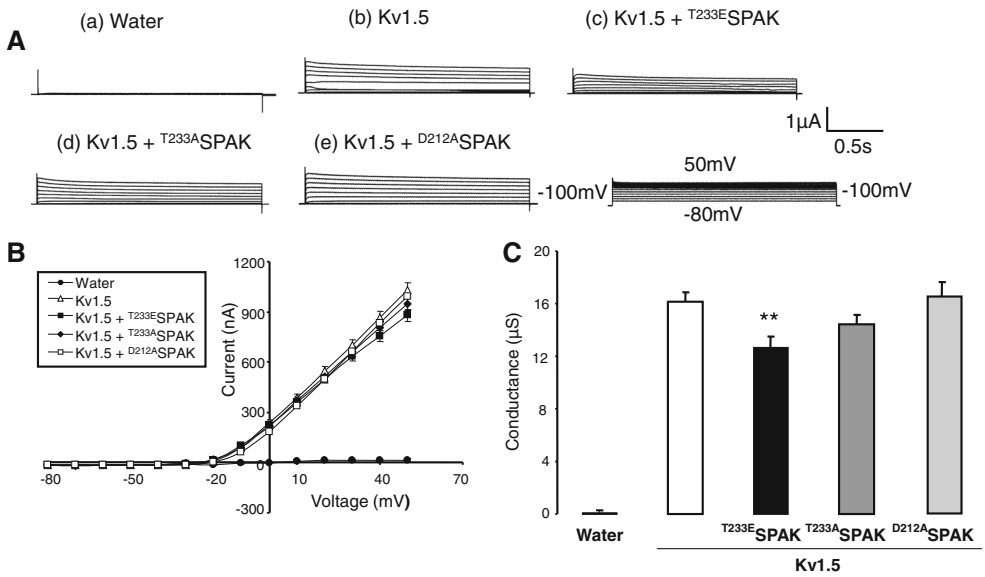

Fig. 2 The effect of wild-type SPAK on Kv1.5 was mimicked by constitutively active ^{T233E}SPAK but not by inactive ^{T233A}SPAK or ^{D212A}SPAK. **A** Representative original tracings showing currents in *Xenopus* oocytes injected with water (*a*) expressing Kv1.5 alone (*b*), expressing Kv1.5 together with constitutively active ^{T233E}SPAK (*c*), or expressing Kv1.5 with inactive ^{T233A}SPAK (d) or ^{D212A}SPAK (*e*). **B** Arithmetic mean ± SEM ($n = 17$–28) of the current (*I*) as a function of the potential difference across the cell membrane (*V*) in *Xenopus laevis* oocytes injected with water (*black circles*), expressing Kv1.5 alone (*white triangles*) or expressing Kv1.5 together with constitutively active ^{T233E}SPAK (*black squares*), inactive ^{T233A}SPAK (*black diamonds*) and catalytically inactive ^{D212A}SPAK (*white squares*). **C** Arithmetic mean ± SEM ($n = 17$–28) of the conductance calculated by linear fit of I/V-curves shown in **B** between 20 and 50 mV in *Xenopus* oocytes injected with water (*dotted bar*), expressing Kv1.5 alone (*white bar*), expressing Kv1.5 with constitutively active ^{T233E}SPAK (*black bar*), or expressing Kv1.5 with inactive ^{T233A}SPAK (*dark gray bar*), or ^{D212A}SPAK (*light gray bar*). $^{**}p < 0.01$ indicates statistically significant difference from oocytes expressing Kv1.5 alone

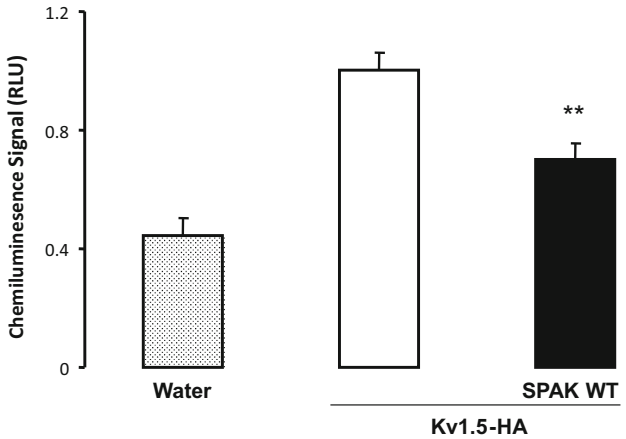

Fig. 3 SPAK decreased Kv1.5-HA protein abundance within the oocyte membrane. Arithmetic mean ± SEM ($n = 87$–91) of normalized Kv1.5-HA chemiluminescence in *Xenopus* oocytes injected with water (*dotted bar*), expressing Kv1.5-HA alone (*white bar*) or expressing Kv1.5-HA together with wild-type SPAK (*black bar*). For normalization, the chemiluminescence was divided by the chemiluminescence of oocytes expressing Kv1.5-HA alone. $^{**}p < 0.01$ indicates statistically significant difference from oocytes expressing Kv1.5-HA alone

expressing oocytes significantly decreased by coexpression of wild-type OSR1. The effect of wild-type OSR1 was again mimicked by constitutively active ^{T185E}OSR1 but neither by the WNK insensitive inactive ^{T185A}OSR1 nor by the catalytically inactive ^{D164A}OSR1 (Fig. 5).

Chemiluminescence was again employed to quantify the effect of wild-type OSR1 expression on Kv1.5-HA protein abundance in the cell membrane of Kv1.5-HA expressing *Xenopus* oocytes. As shown in Fig. 6, coexpression of OSR1 significantly decreased the Kv1.5-HA protein abundance in the cell membrane.

Discussion

The present study identifies a novel target of SPAK and OSR1, i.e., the voltage-gated K^+ channel Kv1.5. SPAK and OSR1 both down-regulate Kv1.5. The kinases are at least partially effective by decreasing the channel protein abundance in the cell membrane. In theory, SPAK/OSR1 could expedite the retrieval of channel protein from the cell membrane or impede the insertion of new channel protein into the cell membrane.

In *Xenopus* oocytes expressing Kv1.5, the coexpression of wild-type and constitutively active SPAK and OSR1 leads to significant down-regulation of Kv1.5. In contrast, the inactive SPAK and OSR1 mutants did not significantly modify Kv1.5 channel activity. Accordingly, kinase activity is required for the effect of SPAK and OSR1 on

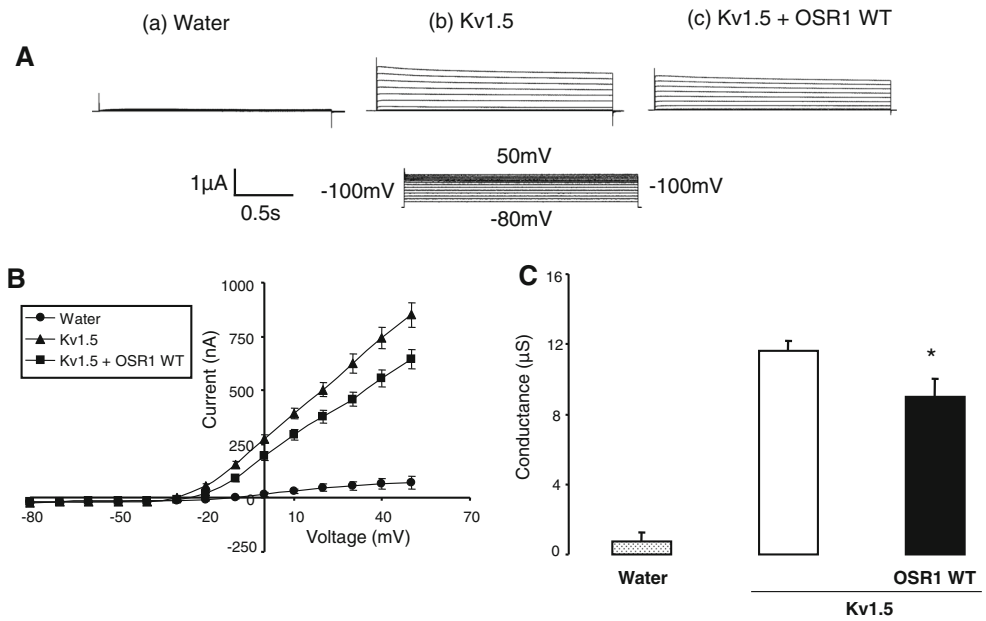

Fig. 4 Coexpression of wild-type OSR1 decreased the K^+ current in Kv1.5-expressing *Xenopus* oocytes. **A** Representative original tracings showing currents in *Xenopus* oocytes injected with water (*a*), expressing Kv1.5 alone (*b*) or expressing Kv1.5 with additional coexpression of wild-type OSR1 (*c*). Currents were activated by depolarization from −80 to +50 mV in 20 s increments of 10 mV steps from a holding potential of −100 mV. **B** Arithmetic mean ± SEM ($n = 14$–18) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus* oocytes injected with water (*black circles*) or expressing Kv1.5 without (*black triangles*) or with (*black squares*) additional coexpression of wild-type OSR1. **C** Arithmetic mean ± SEM (n = 14–18) of the conductance calculated by linear fit of I/V-curves shown in **B** between 20 and 50 mV in *Xenopus* oocytes injected with water (*dotted bar*), or expressing Kv1.5 without (*white bar*) or with (*black bar*) additional coexpression of wild-type OSR1. $*p < 0.05$ indicates statistically significant difference from oocytes expressing Kv1.5 alone

Kv1.5. The present observations do not allow the conclusion, however, that the kinases phosphorylate the channel protein. Instead, the kinases may be effective by phosphorylating regulators of the channel protein thus indirectly modifying its regulation.

The present observations further do not allow any safe conclusions about the in vivo significance of SPAK/OSR1 sensitive Kv1.5 regulation. It is nevertheless tempting to speculate that the inhibition of the Kv1.5 channels by SPAK/OSR1 participates in the machinery regulating cell volume. SPAK/OSR1 up-regulate the NaCl cotransporter and the Na^+,K^+,$2Cl^-$ cotransporter thus stimulating cellular ion uptake (Delpire and Gagnon 2006; 2008; Gagnon and Delpire 2010; Gimenez 2006; Glover and O'Shaughnessy 2011; Huang et al. 2008; Kahle et al. 2010; Lin et al. 2011; Mercier-Zuber and O'Shaughnessy 2011; Richardson and Alessi 2008; Richardson et al. 2011; Villa et al. 2008; Vitari et al. 2005; Vitari et al. 2006), which in turn increases cell volume (Hoffmann 2011; Hoffmann et al. 2009; Lang 2007). The simultaneous inhibition of K^+ channels could limit the cellular loss of ions. Kv1.5 channels have indeed been implicated in cell volume regulation (Barfield et al. 2005b; Barfield et al. 2005a; Felipe et al. 2012; Felipe et al. 1993; Yeung and Cooper 2008).

SPAK/OSR1 sensitive regulation of Kv1.5 may further impact on cell proliferation and survival of tumor cells (Comes et al. 2013; Leanza et al. 2014; Leanza et al. 2012). SPAK and OSR1 have indeed been implicated in the regulation of cell proliferation and cell death (Gagnon and Delpire 2012). It is further tempting to speculate that the activation of OSR1 by oxidative stress leads to depolarization of excitable cells due to down-regulation of Kv1.5 or similarly SPAK/OSR1 sensitive K^+ channels. SPAK and OSR1 are again known to modify neuronal excitability (Gagnon and Delpire 2012). However, it must be kept in mind that the effect on Kv1.5 is small and may not be sufficient to significantly interfere with Kv1.5 dependent cellular functions. Moreover, SPAK and OSR1 may primarily be effective by regulating NaCl (NCC) and Na^+, K^+,$2Cl^-$ (NKCC) cotransporters and/or KCl cotransporters (Gagnon and Delpire 2012). Clearly, additional experiments will be required to define the significance of SPAK/OSR1-sensitive regulation of Kv1.5.

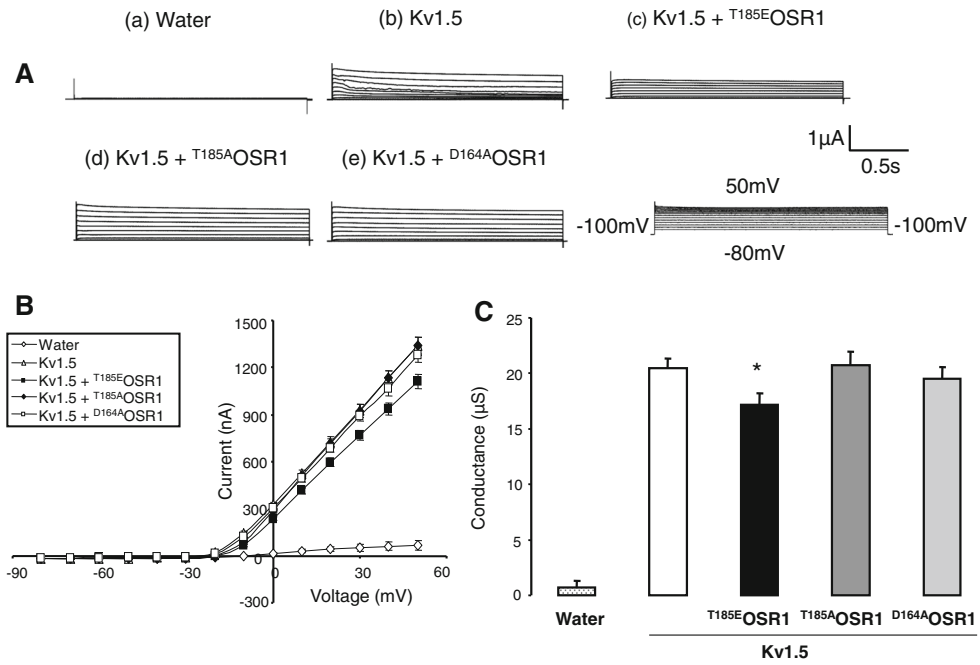

Fig. 5 The effect of wild-type OSR1 on Kv1.5 was mimicked by constitutively active ^{T185E}OSR1 but not by inactive ^{T185A}OSR1 or ^{D164A}OSR1. **A** Representative original tracings showing currents in *Xenopus* oocytes injected with water (*a*), expressing Kv1.5 alone (*b*), expressing KV1.5 together with constitutively active ^{T185E}OSR1 (*c*) or expressing Kv1.5 with inactive ^{T185A}OSR1 (*d*) or ^{D164A}OSR1 (*e*). **B** Arithmetic mean ± SEM ($n = 14$–25) of the current (*I*) as a function of the potential difference across the cell membrane (V) in *Xenopus laevis* oocytes injected with water (*white diamond*), expressing Kv1.5 alone (*white triangles*) or expressing Kv1.5 together with constitutively active ^{T185E}OSR1 (*black squares*), inactive ^{T185A}OSR1 (*black diamonds*) and catalytically inactive ^{D164A}OSR1 (*white squares*). **C** Arithmetic mean ± SEM ($n = 14$–25) of the conductance calculated by linear fit of I/V-curves shown in B between 20 mV and 50 mV in *Xenopus* oocytes injected with water (*dotted bar*), expressing Kv1.5 alone (*white bar*), expressing Kv1.5 with constitutively active ^{T185E}OSR1 (*black bar*), or expressing Kv1.5 with inactive ^{T185A}OSR1 (*dark gray bar*), or ^{D164A}OSR1 (*light gray bar*). $^{*}p < 0.05$ indicates statistically significant difference from oocytes expressing Kv1.5 alone

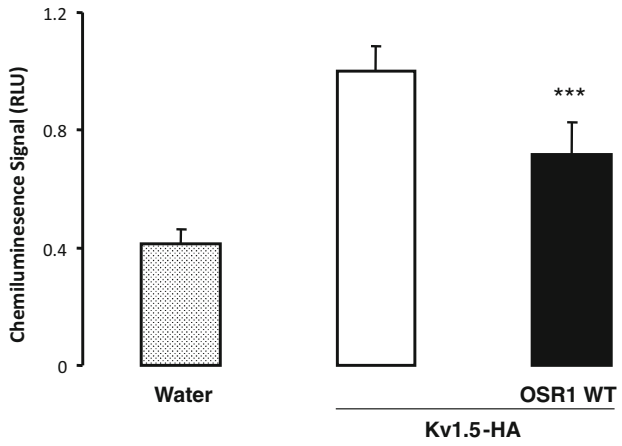

Fig. 6 OSR1 decreased Kv1.5-HA protein abundance within the oocyte membrane. Arithmetic mean ± SEM ($n = 72$–87) of Kv1.5-HA chemiluminescence in *Xenopus* oocytes injected with water (*dotted bar*), expressing KV1.5-HA alone (*white bar*) or expressing Kv1.5-HA together with wild-type OSR1 (*black bar*). $^{***}p < 0.001$ indicates statistically significant difference from oocytes expressing Kv1.5 alone

In conclusion, SPAK and OSR1 have both the potential to down-regulate Kv1.5, an effect possibly contributing to the regulation of cell membrane potential and cell volume.

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

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