Original Paper

SPAK and OSR1 Sensitive Cell Membrane Protein Abundance and Activity of KCNQ1/E1 K⁺ Channels

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Key Words

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

Abstract

Background/Aims: KCNQ1/E1 channels are expressed in diverse tissues and serve a variety of functions including endolymph secretion in the inner ear, cardiac repolarization, epithelial transport and cell volume regulation. Kinases involved in regulation of epithelial transport and cell volume include SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1), which are under control of WNK (with-no-K[Lys]) kinases. The present study explored whether KCNQ1/E1 channels are regulated by SPAK and/or OSR1. **Methods:** cRNA encoding KCNQ1/E1 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 KCNQ1/E1 channel protein abundance in the cell membrane utilizing chemiluminescence of KCNQ1/E1 containing an extracellular Flag tag epitope (KCNQ1-Flag/E1). **Results:** KCNQ1/E1 activity and KCNQ1-Flag/E1 protein abundance were significantly enhanced by wild-type SPAK and T233E SPAK, but not by T233A SPAK and D212A SPAK. Similarly, KCNQ1/E1 activity and KCNQ1-Flag/E1 protein abundance were significantly increased by wild-type OSR1 and T185E OSR1, but not by T185A OSR1 and D164A OSR1. **Conclusions:** SPAK and OSR1 participate in the regulation of KCNQ1/E1 protein abundance and activity.

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Introduction

K⁺ channel subunits KCNE1 or KCNQ1 are expressed in several tissues including heart [1, 2], skeletal muscle [3], stria vascularis of the inner ear [4], renal proximal tubule [5], gastric parietal cells [6-8], intestine [5, 6, 8-11] and hepatocytes [12-14]. KCNQ1/E1 are critically important for hearing [4] and cardiac repolarisation [1, 2, 15]. Loss of function mutations of

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KCNQ1/E1 are associated with hearing loss and cardiac arrhythmia [1, 2, 16]. KCNQ1 gene variants are further associated with diabetes [17, 18]. Gene targeted mice lacking KCNQ1 suffer from severe impairment of hearing [19, 20], of gastric acid secretion [20, 21], and of intestinal electrolyte and substrate transport [22]. KCNQ1 channels further participate in cell volume regulation [13, 14, 23-25].

Kinases involved in cell volume regulatory ion transport include SPAK (SPS1-related proline/alanine-rich kinase) [26-28] and OSR1 (oxidative stress-responsive kinase 1) [29, 30]. The kinases further participate in the regulation of epithelial ion transport and blood pressure [31]. The kinases are under control of WNK (with-no-K[Lys]) kinases [26, 32-35], which are similarly implicated in the regulation of ion transport and blood pressure [36-40]. SPAK and OSR1 are powerful stimulators of NaCl (NCC) and Na⁺,K⁺,2Cl⁻ (NKCC) cotransporters [29-33, 36, 41-49] as well as several ion channels [50-53]. Genetic defects of WNK kinases may result in Gordon's syndrome, which is characterized by hypertension and hyperkalemia [34, 35, 54, 55].

The present study explored, whether SPAK and/or OSR1 participate in the regulation of KCNQ1/E1 K⁺ channels. To this end, KCNQ1/E1 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 and the channel protein abundance in the cell membrane utilizing chemiluminescence.

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. 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 36).

Constructs

Constructs encoding human wild-type KCNQ1/KCNE1 [56] human wild-type KCNQ1-Flag carrying an extracellular Flag tag epitope [57], human wild-type SPAK, constitutively active T233E SPAK, WNK insensitive T233A SPAK, catalytically inactive D212A SPAK [32], human wild-type OSR1, constitutively active T185E OSR1, WNK insensitive T185A OSR1 and catalytically inactive D164A OSR1 [58], were used for generation of cRNA as described previously [59, 60]. The constructs were a kind gift from Dario Alessi (University of Dundee).

Voltage clamp in *Xenopus* oocytes

Xenopus oocytes were prepared as previously described [61, 62]. 4.5 ng cRNA encoding KCNQ1, 1.5 ng cRNA encoding KCNE1 and 10 ng 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, a solution containing (in mM): 88.5 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES, 5 sodium pyruvate (C₃H₃NaO₃), Gentamycin (100 mg/l), Tetracycline (50 mg/l), Ciprofloxacin (1.6 mg/l), Theophylline (90 mg/l) and pH 7.4 [63, 64]. The voltage clamp experiments were performed at room temperature 3 days after the first injection [65, 66]. KCNQ1/E1 channel currents were elicited every 3 s with 5 s pulses from -120 to +80 mV in 8 second increments of 20 mV steps from a holding potential of -80 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) [67-69]. The control superfusate (ND96-B) contained (in mM): 93.5 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 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 [70-72].

Detection of KCNQ1-Flag/E1 cell surface expression by chemiluminescence

To determine KCNQ1-Flag/E1 cell surface expression by chemiluminescence, the oocytes were incubated with primary monoclonal mouse anti-Flag antibody (1:200, Sigma Aldrich) and subsequently

with secondary, HRP-conjugated anti-mouse IgG antibody (1:2500, GE Healthcare Life Sciences). Individual oocytes were placed in 96 well plates with 20 μ l 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 [73, 74].

Statistical analysis

Data are provided as means $\pm$ 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 explored the putative influence of SPAK (SPS1-related proline/alanine-rich kinase) and/or OSR1 (oxidative stress-responsive kinase 1) on the function of KCNQ1/E1 K^+ channels.

In a first series of experiments cRNA encoding KCNQ1/E1 was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding wild-type SPAK. The K^+ currents in those oocytes were determined by dual electrode voltage clamp experiments. As illustrated in Fig. 1, the K^+ currents were negligible in water-injected oocytes or in oocytes expressing SPAK alone, indicating that the oocytes did not express appreciable

Fig. 1. Coexpression of wild-type SPAK increased the K^+ current in KCNQ1/E1-expressing *Xenopus* oocytes. A: Representative original tracings showing currents in *Xenopus* oocytes injected with water (a), expressing SPAK alone (b), expressing KCNQ1/E1 alone (c) or expressing KCNQ1/E1 with additional coexpression of wild-type SPAK (d). The voltage protocol is shown (not to scale).

Currents were activated by depolarization from -120 to +80 mV in 8 second increments of 20 mV steps from a holding potential of -80 mV. B: Arithmetic means $\pm$ SEM ($n = 20-30$) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus* oocytes injected with water (black circles), expressing SPAK alone (black diamonds), expressing KCNQ1/E1 alone (black squares) or expressing KCNQ1/E1 with SPAK (black triangles). C: Arithmetic means $\pm$ SEM ($n = 20-30$) 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 SPAK alone (grey bar) or expressing KCNQ1/E1 (right bars) without (white bar) or with (black bar) additional coexpression of wild-type SPAK. ** ($p < 0.01$) indicates statistically significant difference from oocytes expressing KCNQ1/E1 alone.

Fig. 2. The effect of wild-type SPAK on KCNQ1/E1 was mimicked by constitutively active $T233E$ SPAK but not by WNK insensitive $T233A$ SPAK or catalytically inactive $D212A$ SPAK. **A:** Representative original tracings showing currents in *Xenopus* oocytes injected with water (a) expressing KCNQ1/E1 alone (b), or expressing KCNQ1/E1 together with constitutively active $T233E$ SPAK (c), with WNK insensitive $T233A$ SPAK (d) or with catalytically inactive $D212A$ SPAK (e). **B:** Arithmetic means $\pm$ SEM ($n = 22-35$) 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 KCNQ1/E1 alone (black squares) or expressing KCNQ1/E1 together with constitutively active $T233E$ SPAK (black triangles), inactive $T233A$ SPAK (black diamonds) or catalytically inactive $D212A$ SPAK (white squares). **C:** Arithmetic means $\pm$ SEM ($n = 22-35$) 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 KCNQ1/E1 alone (white bar), expressing KCNQ1/E1 with constitutively active $T233E$ SPAK (black bar), or expressing KCNQ1/E1 with inactive $T233A$ SPAK (dark grey bar), or $D212A$ SPAK (light grey bar). *** ($p < 0.001$) indicates statistically significant difference from oocytes expressing KCNQ1/E1 alone.

Fig. 3. SPAK increased KCNQ1-Flag/E1 protein abundance within the oocyte membrane. Arithmetic means $\pm$ SEM ($n = 86-95$) of normalized KCNQ1-Flag/E1 chemiluminescence in *Xenopus* oocytes injected with water (dotted bar), expressing KCNQ1-Flag/E1 alone (white bar) or expressing KCNQ1-Flag/E1 together with wild-type SPAK (black bar). For normalization, the chemiluminescence was divided by the chemiluminescence of oocytes expressing KCNQ1-Flag/E1 alone. ** ($p < 0.01$) indicates statistically significant difference from oocytes expressing KCNQ1-Flag/E1 alone.

voltage gated K^+ channels. In contrast, large voltage gated K^+ currents were observed in oocytes expressing KCNQ1/E1. The additional coexpression of wild-type SPAK was followed by a significant increase of the voltage gated K^+ currents.

In a next series of experiments cRNA encoding KCNQ1/E1 was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding constitutively active $T233E$ SPAK or cRNA encoding WNK insensitive inactive $T233A$ SPAK, or cRNA encoding catalytically inactive

Fig. 4. Coexpression of wild-type OSR1 increased the K⁺ current in KCNQ1/E1-expressing *Xenopus* oocytes. **A:** Representative original tracings showing currents in *Xenopus* oocytes injected with water (a), expressing OSR1 alone (b), expressing KCNQ1/E1 alone (c) or expressing KCNQ1/E1 with additional coexpression of wild-type OSR1 (d). The voltage protocol is shown (not to scale). Currents were activated by depolarization from -120 to +80 mV in 20 second increments of 20 mV steps from a holding potential of -80 mV. **B:** Arithmetic means $\pm$ SEM ($n = 21-32$) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus* oocytes injected with water (black circles), expressing OSR1 alone (black diamonds), expressing KCNQ1/E1 alone (black squares) or expressing KCNQ1/E1 with OSR1 (black triangles). **C:** Arithmetic means $\pm$ SEM ($n = 21-32$) 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 OSR1 alone (grey bar) or expressing KCNQ1/E1 (right bars) without (white bar) or with (black bar) additional coexpression of wild-type OSR1. * ($p < 0.05$) indicates statistically significant difference from oocytes expressing KCNQ1/E1 alone.

^{D212A}SPAK. As illustrated in Fig. 2, the voltage gated K⁺ currents in KCNQ1/E1 expressing oocytes was significantly increased by coexpression of ^{T233E}SPAK, but neither by coexpression of ^{T233A}SPAK, nor by coexpression of ^{D212A}SPAK.

A next series of experiments tested, whether the increase of KCNQ1/E1 channel activity following expression of SPAK in KCNQ1/E1 expressing oocytes was paralleled by increase of channel protein abundance in the cell membrane. In order to quantify KCNQ1/E1 protein abundance in the cell membrane, chemiluminescence was employed. To this end, KCNQ1/E1 was tagged with an extracellular Flag tag epitope (KCNQ1-Flag/E1) allowing detection of the channel protein with an antibody directed against this epitope. As illustrated in Fig. 3, coexpression of wild-type SPAK significantly increased the KCNQ1-Flag/E1 protein abundance in the cell membrane of *Xenopus* oocytes.

An additional series of experiments elucidated the effect of OSR1 on KCNQ1/E1 channel activity and protein abundance. As illustrated in Fig. 4, the voltage gated K⁺ current was in KCNQ1/E1 expressing oocytes significantly enhanced by the coexpression of wild-type OSR1. As shown in Fig. 5, the effect of wild-type OSR1 on the voltage gated K⁺ current in KCNQ1/E1 expressing oocytes was mimicked by constitutively active ^{T185E}OSR1 but neither by the WNK insensitive inactive ^{T185A}OSR1 nor by the catalytically dead ^{D164A}OSR1 (Fig. 5).

The effect of wild-type OSR1 expression on KCNQ1-Flag/E1 protein abundance in the cell membrane of KCNQ1-Flag/E1 expressing *Xenopus* oocytes was quantified by

Fig. 5. The effect of wild type OSR1 on KCNQ1/E1 was mimicked by constitutively active T185E OSR1 but not by WNK insensitive T185A OSR1 or catalytically inactive D164A OSR1. A: Representative original tracings showing currents in *Xenopus* oocytes injected with water (a), expressing KCNQ1/E1 alone (b), expressing KCNQ1/E1 together with constitutively active T185E OSR1 (c) or expressing KCNQ1/E1 with WNK insensitive T185A OSR1 (d) or catalytically inactive D164A OSR1 (e). B: Arithmetic means $\pm$ SEM ($n = 15-33$) 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 KCNQ1/E1 alone (black squares) or expressing KCNQ1/E1 together with constitutively active T185E OSR1 (black triangles), WNK insensitive T185A OSR1 (black diamonds) or catalytically inactive D164A OSR1 (white squares). C: Arithmetic means $\pm$ SEM ($n = 15-33$) 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 KCNQ1/E1 alone (white bar), expressing KCNQ1/E1 with constitutively active T185E OSR1 (black bar), or expressing KCNQ1/E1 with WNK insensitive T185A OSR1 (dark grey bar), or inactive D164A OSR1 (light grey bar). *** ($p < 0.001$) indicates statistically significant difference from oocytes expressing KCNQ1/E1 alone.

Fig. 6. OSR1 increased KCNQ1-Flag/E1 protein abundance within the oocyte membrane. Arithmetic means $\pm$ SEM ($n = 77-92$) of normalized KCNQ1-Flag/E1 chemiluminescence in *Xenopus* oocytes injected with water (dotted bar), expressing KCNQ1-Flag/E1 alone (white bar) or expressing KCNQ1-Flag/E1 together with wild-type OSR1 (black bar). For normalization, the chemiluminescence was divided by the chemiluminescence of oocytes expressing KCNQ1-Flag/E1 alone. *** ($p < 0.001$) indicates statistically significant difference from oocytes expressing KCNQ1-Flag/E1 alone.

chemiluminescence. As illustrated in Fig. 6, coexpression of OSR1 significantly enhanced the KCNQ1-Flag/E1 protein abundance in the cell membrane.

Discussion

The present study discloses SPAK/OSR1 sensitivity of KCNQ1/E1 K⁺ channels. Coexpression of either SPAK or OSR1 increases the KCNQ1/E1 channel protein abundance in the cell membrane and thus enhances the respective K⁺ current in KCNQ1/E1 expressing oocytes. In theory, SPAK/OSR1 could delay the retrieval of channel protein from the cell membrane or accelerate the insertion of new channel protein into the cell membrane. The effects of wild-type SPAK is mimicked by constitutively active ^{T233E}SPAK but not by WNK insensitive ^{T233A}SPAK or catalytically inactive ^{D212A}SPAK. The effect of wild-type OSR1 is mimicked by constitutively active ^{T185E}OSR1, but not by WNK insensitive ^{T185A}OSR1 or catalytically inactive ^{D164A}OSR1. Accordingly, activation by WNK kinases of SPAK or OSR1 is required for the effect on KCNQ1/E1 activity. This observation does not necessarily mean that SPAK and OSR1 are effective by directly phosphorylating the channel protein. Instead, the kinases may be effective by phosphorylating other regulators of the channel protein thus indirectly modifying its regulation.

In theory, the kinases could influence KCNQ1/E1 channel by modifying cell volume and/or cytosolic K⁺ concentration. SPAK and OSR1 stimulate the NaCl cotransporter as well as the Na⁺,K⁺,2Cl⁻ cotransporter and inhibit KCl cotransporters thus fostering cellular K⁺ uptake [29-33, 36, 41-49]. The cellular accumulation of KCl with osmotically obliged water leads to cell swelling [75-77]. KCNQ1 channels have previously been shown to be sensitive to cell swelling [13, 14, 23-25].

The present study did not elucidate the functional significance of SPAK and OSR1 sensitive KCNQ1/E1 function. KCNQ1/E1 channels serve a variety of functions, including cardiac repolarisation [1, 2, 15], hearing [4, 19, 20], renal tubular transport [5], gastric acid secretion [6-8, 20, 21], intestinal transport [5, 6, 8-11, 22], hepatocyte function [12-14] and metabolism [17, 18]. The effect of SPAK and OSR1 on KCNQ1/E1 is, however, small and is not expected to disrupt any of the KCNQ1/E1 sensitive functions. Additional experimental effort is needed to define the biological significance of SPAK/OSR1-sensitive regulation of KCNQ1/E1.

In conclusion, SPAK and OSR1 have both the potential to up-regulate KCNQ1/E1 protein abundance in the cell membrane, an effect possibly participating in the regulation of cell volume, excitability, epithelial transport and metabolism.

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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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