Original Paper

Regulation of CIC-2 Activity by SPAK and OSR1

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Key Words

Cl⁻ channels • Cell volume regulation • WNK • Voltage clamp • Oocytes

Abstract

Background/Aims: SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1) are powerful regulators of diverse transport processes. Both kinases are activated by cell shrinkage and participate in stimulation of regulatory cell volume increase (RVI). Execution of RVI involves inhibition of Cl⁻ channels. The present study explored whether SPAK and/or OSR1 regulate the activity of the Cl⁻ channel CIC-2. **Methods:** To this end, CIC-2 was expressed in *Xenopus laevis* oocytes with or without additional expression of wild type SPAK, constitutively active SPAK^{T233E}, WNK1 insensitive inactive SPAK^{T233A}, catalytically inactive SPAK^{D212A}, wild type OSR1, constitutively active OSR1^{T185E}, WNK1 insensitive inactive OSR1^{T185A}, and catalytically inactive OSR1^{D164A}. Cl⁻ channel activity was determined by dual electrode voltage clamp. **Results:** Expression of CIC-2 was followed by the appearance of a conductance (G_{Cl}), which was significantly decreased following coexpression of wild-type SPAK, SPAK^{T233E}, wild type OSR1 or OSR1^{T185E}, but not by coexpression of SPAK^{T233A}, SPAK^{D212A}, OSR1^{T185A}, or OSR1^{D164A}. Inhibition of CIC-2 insertion by brefeldin A (5 μM) resulted in a decline of G_{Cl} which was similar in the absence and presence of SPAK or OSR1, suggesting that SPAK and OSR1 did not accelerate the retrieval of CIC-2 protein from the cell membrane. **Conclusion:** SPAK and OSR1 are powerful negative regulators of the cell volume regulatory Cl⁻ channel CIC-2.

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Introduction

SPAK (SPS1-related proline/alanine-rich kinase) [1-3] and OSR1 (oxidative stress-responsive kinase 1) [4, 5] are related kinases involved in the regulation of ion transport and thus blood pressure. The activity of SPAK and OSR1 is controlled by WNK (with-no-K[Lys]) kinases [1, 6-9], which are again involved in the regulation of ion transport and

blood pressure [10-14]. Along those lines, mutations of genes encoding WNK kinases may lead to hypertension and hyperkalaemia [7, 8, 15, 16]. Carriers upregulated by SPAK and OSR1 include the Na⁺-Cl⁻ cotransporter and the Na⁺,K⁺,2Cl⁻ cotransporter [4-6, 9, 10, 17-25]. Moreover, OSR1 and/or SPAK may modify further transport systems, including Na⁺ coupled glucose transporter SGLT1 [26], Na⁺ coupled phosphate transport [27, 28], and Na⁺/H⁺ exchanger [29]. SPAK and OSR1 are activated by cell shrinkage and the kinases are involved in regulatory cell volume decrease [30]. Cell volume regulation is in part accomplished by Cl⁻ channels [31-33] including the ubiquitously expressed inwardly rectifying Cl⁻ channel ClC-2 [33, 34]. Cell shrinkage leads to inhibition of Cl⁻ channels thus curtailing cellular Cl⁻ loss [35, 36].

The present study explored whether SPAK and/or OSR1 modify the activity of the Cl⁻ channel ClC-2. To this end, cRNA encoding ClC-2 was injected into *Xenopus laevis* oocytes with or without cRNA encoding wild-type SPAK, WNK1 insensitive SPAK^{T233A}, constitutively active SPAK^{T233E}, catalytically inactive SPAK^{D212A}, wild-type OSR1, WNK1 insensitive inactive OSR1^{T185A}, constitutively active OSR1^{T185E}, and catalytically inactive OSR1^{D164A} [9]. ClC-2 activity in those oocytes was estimated from cell membrane conductance, which was quantified by dual electrode voltage clamp.

Materials and Methods

Constructs

Constructs encoding wild-type human ClC-2 [37, 38], wild-type SPAK, WNK1 insensitive inactive SPAK^{T233A}, constitutively active SPAK^{T233E}, and catalytically inactive SPAK^{D212A}, wild-type OSR1, WNK1 insensitive inactive OSR1^{T185A}, constitutively active OSR1^{T185E}, and catalytically inactive OSR1^{D164A} [9] were used for generation of cRNA as described previously [39, 40]. All mutants were kindly provided by Dario Alessi.

Voltage clamp in *Xenopus laevis* oocytes

Xenopus laevis oocytes were prepared as previously described [41]. Where not indicated otherwise, 15 ng cRNA encoding ClC-2 were injected on the first day and 10 ng cRNA encoding SPAK, SPAK^{T233A}, SPAK^{T233E}, SPAK^{D212A}, OSR1, OSR1^{T185A}, OSR1^{T185E}, or OSR1^{D164A} were injected on the second day or the same day after preparation of the oocytes [27, 42]. The oocytes were maintained at 17°C in ND96-A solution containing (in mM): 88.5 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 2.5 NaOH 5 HEPES, 5 sodium pyruvate (C₃H₃NaO₃) pH 7.4, gentamycin (100 mg/l), tetracycline (50 mg/l), ciprofloxacin (1.6 mg/l), and theophiline (90 mg/l) [43]. Where indicated, brefeldin A (5 µM) was added to the respective solutions [44]. The voltage clamp experiments were performed at room temperature 3 days after injection. Two-electrode voltage-clamp recordings [45] were obtained utilizing a pulse protocol of 10 s pulses from -140 mV to +40 mV in 20 mV increments. The intermediate holding voltage was -60 mV. The data were filtered at 2 kHz, and recorded with a DigiData 1322A converter and the pClamp 9.2 software for data acquisition and analysis (Axon Instruments, USA) [46]. The superfusate (ND96) 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 [47, 48].

Statistical analysis

Data are provided as means ± SEM, n represents the number of oocytes. 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. Results with p < 0.05 were considered statistically significant.

Results

The present study explored whether SPAK (STE20/SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1) influence the activity of ClC-2 Cl⁻ channels. To this end cRNA encoding ClC-2 was injected into *Xenopus laevis* oocytes with

Fig. 1. Effect of wild-type SPAK coexpression on Cl⁻ conductance in ClC-2-expressing *Xenopus laevis* oocytes. A: Representative original tracings showing currents in *Xenopus laevis* oocytes injected with DEPC water (a), as well as in oocytes expressing ClC-2 without (b) or with (c) additional coexpression of wild type SPAK. B: Arithmetic means ± SEM (n = 10-31) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus laevis* oocytes injected with water (circles), expressing ClC-2 alone (squares) or expressing ClC-2 together with wild type SPAK (triangles). C: Arithmetic means ± SEM (n = 10-31) of the conductance calculated by linear fit of I/V-curves shown in B between -140 mV and -80 mV in *Xenopus laevis* oocytes injected with water (dotted bar), expressing ClC-2 alone (white bar) or expressing ClC-2 together with wild type SPAK (black bar). *(p<0.05) indicates statistically significant difference to expression of ClC-2 alone.

or without additional injection of cRNA encoding SPAK or OSR1 and the cell membrane conductance determined utilizing dual-electrode voltage-clamp. In water injected oocytes the cell membrane conductance was low (Fig.1). As illustrated in Fig. 1, expression of ClC-2 resulted in a marked increase of cell membrane conductance. As shown in Fig. 1, the cell membrane conductance of ClC-2 expressing *Xenopus laevis* oocytes was significantly decreased by additional expression of wild type SPAK. Similarly, coexpression of OSR1 was followed by a significant decrease of cell membrane conductance in ClC-2 expressing *Xenopus laevis* oocytes (Fig. 2).

The effect of wild type SPAK was mimicked by the constitutively active mutant SPAK^{T233E}. Accordingly, the conductance was significantly lower in *Xenopus laevis* oocytes expressing ClC-2 together with SPAK^{T233E} than in *Xenopus laevis* oocytes expressing ClC-2 alone (Fig. 3). In contrast, ClC-2 activity was not significantly modified by WNK1 insensitive inactive SPAK^{T233A} or by catalytically inactive SPAK^{D212A} (Fig. 3).

The effect of wild type OSR1 was mimicked by the constitutively active mutant OSR1^{T185E}. Again, coexpression of OSR1^{T185E} significantly decreased cell membrane conductance of ClC-2 expressing *Xenopus laevis* oocytes and the conductance was significantly lower in *Xenopus laevis* oocytes expressing ClC-2 together with OSR1^{T185E} than in *Xenopus laevis* oocytes expressing ClC-2 alone (Fig. 4). In contrast, ClC-2 activity was not significantly modified by coexpression of WNK1 insensitive inactive OSR1^{T185A} or by coexpression of catalytically inactive OSR1^{D164A} (Fig. 4).

Fig. 2. Effect of wild-type OSR1 coexpression on Cl⁻ conductance in ClC-2-expressing *Xenopus laevis* oocytes. A: Representative original tracings showing currents in *Xenopus laevis* oocytes injected with DEPC water (a), as well as in oocytes expressing ClC-2 without (b) or with (c) additional coexpression of wild type OSR1. B: Arithmetic means ± SEM (n = 16-34) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus laevis* oocytes injected with water (circles), expressing ClC-2 alone (squares) or expressing ClC-2 together with wild type OSR1 (triangles). C: Arithmetic means ± SEM (n = 16-34) of the conductance calculated by linear fit of I/V-curves shown in B between -140 mV and -80 mV in *Xenopus laevis* oocytes injected with water (dotted bar), expressing ClC-2 alone (white bar) or expressing ClC-2 together with wild type OSR1 (black bar). ** (p < 0.01) indicates statistically significant difference to expression of ClC-2 alone.

At least in theory, SPAK and OSR1 could have decreased ClC-2 channel activity by accelerating the retrieval of channel protein from the cell membrane. In order to test this possibility, *Xenopus laevis* oocytes expressing ClC-2 with or without SPAK were treated with 5 μM brefeldin A, a substance disrupting insertion of new channel protein into the cell membrane. As illustrated in Fig. 5A, the decline of conductance in the presence of brefeldin A was similar in oocytes expressing ClC-2 together with SPAK and oocytes expressing ClC-2 alone. The same observations were made in *Xenopus laevis* oocytes expressing ClC-2 with or without OSR1. As shown in Fig. 5B, the decline of conductance in the presence of brefeldin A was again similar in oocytes expressing ClC-2 together with OSR1 and in oocytes expressing ClC-2 alone. Thus, neither SPAK nor OSR1 accelerated the retrieval of channel protein from the cell membrane.

Discussion

The present study reveals that the WNK-dependent STE20/SPS1-related proline/alanine-rich kinase SPAK and the oxidative stress-responsive kinase OSR1 are both powerful negative regulators of the ubiquitously expressed Cl⁻ channel ClC-2. The kinases do not significantly modify the decline of the current following inhibition of channel insertion into the cell membrane indicating that the kinases are not effective by accelerating the clearance

Fig. 3. Effect of expression of constitutively active SPAK^{T233E}, inactive SPAK^{T233A} and catalytically inactive SPAK^{D212A} on Cl⁻ conductance in CIC-2-expressing *Xenopus laevis* oocytes. A: Representative original tracings showing currents in *Xenopus laevis* oocytes injected with DEPC water (a), as well as in oocytes expressing CIC-2 without (b) or with additional coexpression of constitutively active SPAK^{T233E} (c), inactive SPAK^{T233A} (d) and catalytically inactive SPAK^{D212A} (e). B: Arithmetic means ± SEM (n = 18-25) 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 CIC-2 alone (black squares) or expressing CIC-2 together with constitutively active SPAK^{T233E} (black triangles), inactive SPAK^{T233A} (white diamonds) and catalytically inactive SPAK^{D212A} (white squares). C: Arithmetic means ± SEM (n = 18-25) of the conductance calculated by linear fit of I/V-curves shown in B between -140 mV and -80 mV in *Xenopus laevis* oocytes injected with water (dotted bar), expressing CIC-2 alone (white bar) or expressing CIC-2 together with constitutively active SPAK^{T233E} (black bar), inactive SPAK^{T233A} (dark grey bar) and catalytically inactive SPAK^{D212A} (light grey bar). *** (p < 0.001) indicates statistically significant difference to expression of CIC-2 alone.

of channel protein from the cell membrane. CIC-2 downregulation is observed following coexpression of wild type SPAK and OSR1 as well as constitutively active SPAK^{T233E} and OSR1^{T185E} but not following coexpression of WNK1 insensitive inactive SPAK^{T233A} and OSR1^{T185A} or catalytically inactive SPAK^{D212A} or OSR1^{D164A}. Accordingly, kinase activity is required for the observed effects.

The effect of SPAK/OSR1 on CIC-2 may participate in the regulation of cell volume, as inhibition of Cl⁻ channels interferes with Cl⁻ exit thus leading to hyperpolarization of the cell membrane with subsequent decrease of K⁺ exit. The inhibition of KCl exit prevents further loss of osmotically obliged water. Cell shrinkage is well known to inhibit cell volume regulatory Cl⁻ channels [35, 36]. CIC-2 has been shown to be regulated by cell volume [32] and down regulation of CIC-2 could well participate in the SPAK/OSR1 sensitive regulation of cell volume.

Cl⁻ channels participate in the regulation of cell proliferation [49]. Activation of Cl⁻ channels fosters cell shrinkage, a prerequisite for triggering oscillations of cytosolic Ca²⁺ activity in proliferating cells [50].

Fig. 4. Effect of expression of constitutively active OSR1^{T185E}, inactive OSR1^{T185A} and catalytically inactive OSR1^{D164A} on Cl⁻ conductance in ClC-2-expressing *Xenopus laevis* oocytes. **A:** Representative original tracings showing currents in *Xenopus laevis* oocytes injected with DEPC water (a), as well as in oocytes expressing ClC-2 without (b) or with additional coexpression of constitutively active OSR1^{T185E} (c), inactive OSR1^{T185A} (d) and catalytically inactive OSR1^{D164A} (e). **B:** Arithmetic means ± SEM (n = 21-25) 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 ClC-2 alone (black squares) or expressing ClC-2 together with constitutively active OSR1^{T185E} (black triangles), inactive OSR1^{T185A} (white diamonds) and catalytically inactive OSR1^{D164A} (white squares). **C:** Arithmetic means ± SEM (n = 21-25) of the conductance calculated by linear fit of I/V-curves shown in B between -140 mV and -80 mV in *Xenopus laevis* oocytes injected with water (dotted bar), expressing ClC-2 alone (white bar) or expressing ClC-2 together with constitutively active OSR1^{T185E} (black bar), inactive OSR1^{T185A} (dark grey bar) and catalytically inactive OSR1^{D164A} (light grey bar). * (p < 0.05) indicates statistically significant difference to expression of ClC-2 alone.

Cl⁻ channels further participate in the regulation of cell volume during apoptosis [51-61] and SPAK/OSR1 sensitive ClC-2 activity could - at least in theory - counteract apoptotic cell shrinkage. ClC-2 Cl⁻ channels particularly impact on survival of male germ cells and photoreceptors [62].

SPAK/OSR1 sensitive ClC-2 activity may further participate in the regulation of cytosolic Cl⁻ activity, cell membrane potential and thus excitability of neurons [63]. ClC-2 participates in the regulation of pulmonary chloride and water secretion, a prerequisite for fetal lung development [64]. Clearly, further experiments are required to fully elucidate the physiological functions dependent on SPAK/OSR1 sensitive regulation of ClC-2.

Conclusion

SPAK and OSR1 both downregulate ClC-2 and thus contribute to the regulation of this ubiquitously expressed Cl⁻ channel.

Fig.5. Effect of brefeldin A on ClC-2 channel activity with or without coexpression of SPAK or OSR1. A: Arithmetic means $\pm$ SEM (n = 18-30) of conductance calculated by linear fit of I/V-curves between -140 mV and -80 mV in *Xenopus laevis* oocytes injected with ClC-2 alone (ClC-2, white bars) or expressing ClC-2 together with SPAK (black bars) prior to (left bars, 0h) and following (middle and right bars) incubation with brefeldin A (5 μ M) for 16 h or 24 h. B: Arithmetic means $\pm$ SEM (n = 16-30) of conductance calculated by linear fit of respective I/V-curves between -140 mV and -80 mV in *Xenopus laevis* oocytes injected with ClC-2 alone (ClC-2, white bars) or expressing ClC-2 together with OSR1 (black bars) prior to (left bars, 0h) and following (middle and right bars) incubation with brefeldin A (5 μ M) for 16 h or 24 h. *** (p<0.001) indicates statistically significant difference from expression of ClC-2 alone. # (p<0.05), ## (p<0.01) and ### (p<0.001) indicate statistically significant difference from absence of brefeldin A.

Disclosure Statement

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

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