Original Paper

Negative Regulation of the Creatine Transporter SLC6A8 by SPAK and OSR1

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Key Words

CreaT • Oxidative stress-responsive kinase 1 • SPS1-related proline/alanine-rich kinase • WNK

Abstract

Background/Aims: Transport regulation involves several kinases including 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 SPAK and/or OSR1 participate in the regulation of the creatine transporter CreaT (SLC6A8), which accomplishes Na⁺ coupled cellular uptake of creatine in several tissues including kidney, intestine, heart, skeletal muscle and brain. **Methods:** cRNA encoding SLC6A8 was injected into *Xenopus laevis* 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. Transporter activity was determined from creatine (1 mM) induced current utilizing dual electrode voltage clamp. **Results:** Coexpression of wild-type SPAK and of ^{T233E}SPAK, but not of ^{T233A}SPAK or of ^{D212A}SPAK was followed by a significant decrease of creatine induced current in SLC6A8 expressing oocytes. Coexpression of SPAK significantly decreased maximal transport rate. Coexpression of wild-type OSR1, ^{T185E}OSR1 and ^{T185A}OSR1 but not of ^{D164A}OSR1 significantly negatively regulated SLC6A8 activity. OSR1 again decreased significantly maximal transport rate. **Conclusions:** Both, SPAK and OSR1, are negative regulators of the creatine transporter SLC6A8.

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Introduction

With-no-K[Lys] kinases (WNK) [1-5] and their downstream kinases SPS1-related proline/alanine-rich kinase (SPAK) [1, 6, 7] and oxidative stress-responsive kinase 1 (OSR1) [8, 9] participate in the regulation of transport and blood pressure [10-14]. Both, SPAK and

OSR1 up-regulate the Na^+Cl^- (NCC) and $\text{Na}^+\text{K}^+\text{2Cl}^-$ (NKCC) cotransporters [2, 3, 8-10, 15-23] as well as Na^+ coupled phosphate transport [24]. Genetic defects affecting the activity of WNK kinases may lead to Gordon's syndrome, a monogenic disorder characterized by hyperkalaemia and hypertension [4, 5, 25, 26]. SPAK/OSR1 participates in a wide variety of further functions including neuroexcitability [27-29].

To the best of our knowledge, nothing is known about effects of WNK, SPAK and/or OSR1 on the activity of the creatine transporter CreaT (SLC6A8), a member of the superfamily of Na^+Cl^- coupled transporters for neurotransmitters [30-32] and organic osmolytes [33, 34]. SLC6A8 is expressed in a wide variety of tissues, such as kidney, small intestine, heart, skeletal muscle, brain and retina [35-38]. Genetic disorders affecting SLC6A8 lead to mental retardation with seizures [39-56].

In order to test whether SPAK or OSR1 may influence SLC6A8 activity, cRNA encoding SLC6A8 was injected into *Xenopus laevis* oocytes without or with additional injection of cRNA encoding SPAK or OSR1. The creatine induced current was determined utilizing 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.

Constructs

Constructs encoding bovine wild-type SLC6A8 (CreaT) [31], human wild-type SPAK, constitutively active ^{T233E}SPAK, WNK insensitive ^{T233A}SPAK, catalytically inactive ^{D212A}SPAK [2, 24], human wild-type OSR1, constitutively active ^{T185E}OSR1, WNK insensitive ^{T185A}OSR1 and catalytically inactive ^{D164A}OSR1 [57] were used for generation of cRNA as described previously [58, 59]. The constructs were a kind gift from Dario Alessi (University of Dundee).

Voltage clamp in *Xenopus laevis* oocytes

Xenopus laevis oocytes were prepared as previously described [60, 61]. 15 ng cRNA encoding SLC6A8 and 10 ng of cRNA encoding wild-type, constitutively active, WNK insensitive or inactive kinase of either SPAK or OSR1 were injected on the same day after preparation of the oocytes. The oocytes were stored at 17°C in incubation medium ND96, a solution containing 88.5 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, 2.5 mM NaOH and 5 mM sodium pyruvate ($\text{C}_3\text{H}_3\text{NaO}_3$), supplemented with 100 mg/l Gentamycin, 50 mg/l Tetracycline, 1.6 mg/l Ciprofloxacin and 90 mg/l Theophiline (pH 7.4). [62, 63]. Solutions were sterilized through 0.22 μm filter. The voltage clamp experiments were performed at room temperature 4 days after the first injection [64, 65]. Carrier activity was estimated from substrate (1 mM creatine) induced current at a holding potential of -60 mV [66]. The data were filtered at 10 Hz 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 oocytes recording chamber was continuously perfused with control ND96 solution containing (in mM): 93.5 NaCl, 2 KCl, 1.8 CaCl_2 , 1 MgCl_2 , 5 HEPES, 2.5 NaOH (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]. For kinetic analysis the creatine induced-current (I_{creat}) was plotted against the respective creatine concentration (s) and maximal current (I_{max}) as well as concentration required for halfmaximal current (k_m) calculated using the equation $I_{\text{CreaT}} = I_{\text{max}} \cdot s / (k_m + s)$.

Statistical analysis

Data are provided as means $\pm$ SEM, n represents the number of oocytes investigated [73]. 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 or t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Fig. 1. Coexpression of SPAK decreases electrogenic creatine transport in SLC6A8 expressing *Xenopus laevis* oocytes. A: Representative original tracings showing creatine (1 mM) - induced current (I_{creat}) in *Xenopus laevis* oocytes injected with water (a) or expressing SLC6A8 without (b) or with (c) additional coexpression of wild-type SPAK. B: Arithmetic means ± SEM (n = 11 - 23) of I_{creat} in *Xenopus laevis* oocytes injected with water (white bar), or expressing SLC6A8 without (black bar) or with wild-type SPAK (grey bar). *** (p<0.001) indicates statistically significant difference from the absence of SPAK.

Fig. 2. Coexpression of SPAK decreases maximal electrogenic creatine transport in SLC6A8-expressing *Xenopus laevis* oocytes. A: Representative original tracings showing the current induced by increasing concentrations of creatine (from 3 μM to 2 mM) in *Xenopus laevis* oocytes expressing SLC6A8 without (upper panel) or with (lower panel) additional coexpression of wild-type SPAK. B: Arithmetic means ± SEM (n = 6-8) of I_{creat} as a function of creatine concentration in *Xenopus laevis* oocytes expressing SLC6A8 without (black circles), or with (white circles) additional coexpression of wild-type SPAK.

Results

The present study explored whether SPAK (SPS1-related proline/alanine-rich kinase) and/or OSR1 (oxidative stress-responsive kinase 1) are able to modify the function of the creatine transporter CreaT (SLC6A8). To this end, cRNA encoding SLC6A8 was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding wild-type SPAK, mutant SPAK, wild-type OSR1 or mutant OSR1. Creatine induced-current (I_{creat}) was determined utilizing dual electrode voltage clamp. As shown in Fig. 1, no appreciable current was observed in water-injected oocytes indicating that the oocytes did not express significant endogenous electrogenic creatine transport. Creatine exposure of SLC6A8 expressing oocytes was, however followed by a sizable I_{creat} . The additional coexpression of wild-type SPAK significantly decreased the I_{creat} in SLC6A8 expressing *Xenopus laevis* oocytes.

In order to determine whether SPAK coexpression modifies the maximal I_{creat} or the affinity of the carrier, *Xenopus laevis* oocytes expressing SLC6A8 without or with additional expression of SPAK were exposed to creatine at concentrations ranging from 3 μM to 2 mM. As illustrated in Fig. 2, the I_{creat} was a function of the extracellular creatine concentration. Kinetic analysis revealed that the maximal creatine induced current was significantly (p<0.01) higher in *Xenopus laevis* oocytes expressing SLC6A8 alone (18.4 ± 2.6 nA, n = 6-8) than in *Xenopus laevis* oocytes expressing SLC6A8 together with SPAK (7.6 ± 1.0 nA, n = 6-7). The concentration required for halfmaximal creatine induced current was highly variable and not significantly different between *Xenopus laevis* oocytes expressing SLC6A8 alone (192 ± 102 mM, n = 6-8) and *Xenopus laevis* oocytes expressing SLC6A8 together with SPAK (29 ± 22 mM, n = 6-7).

Further experiments explored the effect of SPAK mutants on the I_{creat} in SLC6A8 expressing *Xenopus laevis* oocytes. To this end the SPAK mutants were coexpressed in SLC6A8 expressing *Xenopus* oocytes. As illustrated in Fig. 3, the effect of wild-type SPAK was

Fig. 3. The effect of SPAK on SLC6A8 is mimicked by active T233E SPAK but not by WNK insensitive T233A SPAK or inactive D212A SPAK. A: Representative original tracings showing I_{creat} in *Xenopus laevis* oocytes injected with water (a), or expressing SLC6A8 without (b) or with additional coexpression of constitutively active T233E SPAK (c), WNK1 insensitive T233A SPAK (d), or catalytically inactive D212A SPAK (e). B: Arithmetic means $\pm$ SEM ($n = 12 - 30$) of I_{creat} in *Xenopus laevis* oocytes injected with water (white bar), or expressing SLC6A8 without (black bar) or with constitutively active T233E SPAK (light grey bar), WNK insensitive T233A SPAK (middle grey bar) or catalytically inactive D212A SPAK (dark grey bar). *** ($p < 0.001$, indicates statistically significant difference from oocytes expressing SLC6A8 alone).

Fig. 4. Coexpression of OSR1 decreases electrogenic creatine transport in SLC6A8 expressing *Xenopus laevis* oocytes. A: Representative original tracings showing I_{creat} in *Xenopus laevis* oocytes injected with water (a) or expressing SLC6A8 without (b) or with (c) additional coexpression of wild-type OSR1. B: Arithmetic means $\pm$ SEM ($n = 10 - 30$) of I_{creat} in *Xenopus laevis* oocytes injected with water (white bar), or expressing SLC6A8 without (black bar) or with (grey bar) wild-type OSR1. *** ($p < 0.001$, indicates statistically significant difference from the absence of OSR1).

Fig. 5. Coexpression of OSR1 decreases maximal electrogenic creatine transport in SLC6A8-expressing *Xenopus laevis* oocytes. A: Representative original tracings showing the current induced by increasing concentrations of creatine (from 3 μM to 2 mM) in *Xenopus laevis* oocytes expressing SLC6A8 without (upper panel) or with (lower panel) additional coexpression of wild-type OSR1. B: Arithmetic means $\pm$ SEM ($n = 5 - 7$) of I_{creat} as a function of creatine concentration in *Xenopus laevis* oocytes expressing SLC6A8 without (black circles), or with (white circles) additional coexpression of wild-type OSR1.

mimicked by the constitutively active T233E SPAK but neither by the WNK insensitive T233A SPAK nor by the catalytically inactive D212A SPAK. Coexpression of T233E SPAK significantly decreased the I_{creat} in SLC6A8 expressing oocytes.

Further experiments addressed the effect of OSR1 on SLC6A8 activity. As shown in Fig. 4, the additional coexpression of wild-type OSR1 significantly decreased the I_{creat} in SLC6A8 expressing *Xenopus laevis* oocytes.

Fig. 5 displays the I_{creat} as a function of the extracellular creatine concentration in *Xenopus* oocytes expressing SLC6A8 alone and in *Xenopus laevis* oocytes expressing SLC6A8 together

with OSR1. Kinetic analysis revealed that the maximal I_{creat} was significantly (p<0.001) higher in *Xenopus laevis* oocytes expressing SLC6A8 alone (23.9 ± 2.1 nA, n = 5-6) than in *Xenopus laevis* oocytes expressing SLC6A8 together with OSR1 (8.2 ± 0.7 nA, n = 7). The concentration required for halfmaximal creatine induced current was significantly higher in *Xenopus* oocytes expressing SLC6A8 alone (152 ± 53 mM, n = 5-6) than in *Xenopus* oocytes expressing SLC6A8 together with OSR1 (26 ± 14 mM, n = 7) (p<0.05).

As illustrated in Fig. 6, the effect of wild-type OSR1 was mimicked by constitutively active $T185E$ OSR1 and less by WNK insensitive $T185A$ OSR1 but not by the catalytically inactive $D164A$ OSR1.

Discussion

The present study discloses a novel function of SPAK and OSR1, i.e. the negative regulation of the creatine transporter SLC6A8. Coexpression of SPAK or OSR1 is followed by a decrease of the maximal creatine transport in SLC6A8 expressing oocytes. Transporters similarly negatively regulated by SPAK and OSR1 include KCl cotransporters [27, 74]. On the other hand SPAK and OSR1 positively regulate the Na^+, Cl^- (NCC) and $Na^+, K^+, 2Cl^-$ (NKCC) cotransporters [2, 3, 8-10, 15-23] as well as Na^+ coupled phosphate transport [24]. The molecular requirements for negative or positive regulation by SPAK and OSR1 are incompletely understood.

The effect of wild type SPAK and OSR1 on SLC6A8 activity was mimicked by the constitutively active mutants. SLC6A8 activity was not affected by the kinase dead mutants, indicating that functional kinases are required for the SPAK/OSR1 sensitive regulation of SLC6A8. SLC6A8 activity tended to be decreased by the WNK insensitive $T233A$ SPAK mutant and was significantly decreased by the WNK insensitive $T185A$ OSR1 mutant. Those observations could point to some WNK independent activity of SPAK and/or OSR1. The observations do not necessarily indicate that the carrier is directly phosphorylated by the kinases. Instead, the kinases could phosphorylate and thus modify the function of other signaling molecules involved in the regulation of the carrier.

The present study did not address the *in vivo* significance of SPAK/OSR1 sensitive creatine transport activity. The carrier plays an important role in the regulation of neuronal function and neuroexcitability [39-56]. The WNK-SPAK/OSR1 kinase pathway is similarly

involved in the regulation of neuronal excitation [27, 74]. SPAK/OSR1 modify, however, neuroexcitability at least in part by activation of NKCC dependent Cl^- uptake and inhibition of KCC dependent Cl^- exit leading to increase of intracellular chloride concentration [27, 74]. The intracellular Cl^- concentration is in turn a critical determinant of the effects of GABA on cell membrane potential and neuronal excitation [27, 74]. WNK kinases are activated following decrease of intracellular Cl^- concentration and they trigger cellular Cl^- uptake by activating SPAK/OSR1 [27]. Disorders involving deranged SPAK/OSR activity include arterial hypertension and enhanced neuronal activity leading to epilepsy, spasticity, neuropathic pain, schizophrenia, and autism [27].

SPAK/OSR1 further contribute to cell volume regulation by triggering cellular NaCl uptake due to activation of the Na^+, Cl^- cotransporter and the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter [2, 3, 8-10, 15-23]. We do not know, whether SLC6A8 is regulated by cell volume. The SLC6A8 related organic osmolyte transporters are up-regulated rather than negatively regulated by cell shrinkage [33, 34].

Conclusion

SPAK and OSR1 are capable to negatively regulate the creatine transporter SLC6A8.

Disclosure Statement

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

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