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SPAK-Sensitive Regulation of Glucose Transporter SGLT1

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Abstract The WNK-dependent STE20/SPS1-related proline/alanine-rich kinase SPAK is a powerful regulator of ion transport. The study explored whether SPAK similarly regulates nutrient transporters, such as the Na^+-coupled glucose transporter SGLT1 (SLC5A1). To this end, SGLT1 was expressed in *Xenopus* oocytes with or without additional expression of wild-type SPAK, constitutively active ^{T233E}SPAK, WNK-insensitive ^{T233A}SPAK or catalytically inactive ^{D212A}SPAK, and electrogenic glucose transport determined by dual-electrode voltage-clamp experiments. Moreover, Ussing chamber was employed to determine the electrogenic glucose transport in intestine from wild-type mice ($spak^{wt/wt}$) and from gene-targeted mice carrying WNK-insensitive SPAK ($spak^{tg/tg}$). In SGLT1-expressing oocytes, but not in water-injected oocytes, the glucose-dependent current (I_g) was significantly decreased following coexpression of wild-type SPAK and ^{T233E}SPAK, but not by coexpression of ^{T233A}SPAK or ^{D212A}SPAK. Kinetic analysis revealed that SPAK decreased maximal I_g without significantly modifying the glucose concentration required for halfmaximal I_g (K_m). According to the chemiluminescence experiments, wild-type SPAK but not ^{D212A}SPAK decreased SGLT1 protein abundance in the cell membrane. Inhibition of SGLT1 insertion by brefeldin A (5 μM) resulted in a decline of I_g, which was similar in the absence and presence of SPAK, suggesting that SPAK did not accelerate the retrieval of SGLT1 protein from the cell membrane but rather down-regulated carrier insertion into the cell membrane. Intestinal electrogenic glucose transport was significantly lower in $spak^{wt/wt}$ than in $spak^{tg/tg}$ mice. In conclusion, SPAK is a powerful negative regulator of SGLT1 protein abundance in the cell membrane and thus of electrogenic glucose transport.

Keywords Glucose transport · Intestine · Oocytes · Mice · WNK

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Introduction

SPAK (SPS1-related proline/alanine-rich kinase) is a powerful regulator of ion transport and blood pressure (Castaneda-Bueno and Gamba 2010; Rafiqi et al. 2010; Yang et al. 2010). The activity of SPAK is governed by WNK (with-no-K[Lys]) kinases (Glover et al. 2011; O'Reilly et al. 2003; Rafiqi et al. 2010; Vitari et al. 2005), which are similarly involved in the regulation of ion transport and blood pressure (Flatman 2008; Furgeson and Linas 2010; Kahle et al. 2010; Uchida 2010; Wilson et al. 2001). SPAK and the related oxidative stress-responsive kinase 1 (OSR1) kinase specifically upregulate the NaCl cotransporter (NCC) and the Na^+,K^+,$2Cl^-$ cotransporter (NKCC2) (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 et al. 2011; Villa et al. 2008; Vitari et al. 2005). Mutations of genes encoding WNK kinases cause Gordon's syndrome, a genetic disease characterized by hypertension and hyperkalaemia (Achard et al. 2001; Capasso et al. 2005; Glover et al. 2011; O'Reilly et al. 2003). SPAK is expressed in several epithelia including intestine (Ushiro et al. 1998) and more recent observations revealed the capacity of OSR and/or SPAK to modify the function of further carriers, such as Na^+-coupled phosphate transport (Pathare et al. 2012a, b), and Na^+/H^+ exchanger (Pasham et al. 2012b). The kinases may thus participate in the regulation of further epithelial transport processes. To the best of our knowledge, however, nothing is known about a role of SPAK in the regulation of nutrient transport.

The present study thus explored whether SPAK influences the function of the glucose carrier SGLT1 (Wright and Turk 2004). To this end, cRNA encoding SGLT1 was injected into *Xenopus* oocytes with or without cRNA encoding wild-type SPAK, WNK1-insensitive ^{T233A}SPAK, constitutively active ^{T233E}SPAK, and catalytically inactive ^{D212A}SPAK (Vitari et al. 2005). Glucose transport was quantified by determination of glucose-induced current in two-electrode voltage-clamp experiments and protein abundance of SGLT1 was determined by chemiluminescence. Moreover, glucose-induced current was determined in Ussing chamber experiments of distal jejunum isolated either from gene-targeted mice expressing SPAK resistant to WNK-dependent activation (*spak*$^{tg/tg}$) or from mice expressing wild-type SPAK (*spak*$^{wt/wt}$).

Materials and Methods

Constructs

Constructs encoding human SGLT1 (Alesutan et al. 2012), wild-type SPAK, WNK1-insensitive inactive ^{T233A}SPAK, constitutively active ^{T233E}SPAK, and catalytically inactive ^{D212A}SPAK (Vitari et al. 2005), were used for generation of cRNA as described previously (Hosseinzadeh et al. 2013a, 2013b).

Voltage Clamp in *Xenopus* Oocytes

Xenopus oocytes were prepared as previously described (Henrion et al. 2012; Hosseinzadeh et al. 2014). Where not indicated otherwise, 10 ng cRNA encoding SGLT1 was injected on the first day and 10 ng of cRNA encoding wild-type SPAK, ^{T233A}SPAK, ^{T233E}SPAK, or ^{D212A}SPAK was injected on the second day or the same day after oocyte preparation (Hosseinzadeh et al. 2012; Pathare et al. 2012a). The oocytes were maintained at 17 °C in ND96-A solution containing (in mM): 88.5 NaCl, 2 KCl, 1 MgC12, 1.8 CaC12, 5 HEPES, tetracycline (Sigma, 0.11 mM), ciprofloxacin (Sigma, 4 µM), gentamycin (Refobacin, 0.2 mM), theophylin (Euphylong, 0.5 mM), sodium pyruvate (Sigma, 5 mM). pH was adjusted to 7.5 by addition of NaOH (Warsi et al. 2014, 2013). In order to discriminate between enhanced insertion of SGLT1 into the cell membrane and delayed retrieval of SGLT1 from the membrane, experiments were performed utilizing brefeldin A (Sigma-Aldrich Chemie, Steinheim, Germany) (Almilaji et al. 2013a). Brefeldin A (5 µM) was added to the culture medium 24 h after cRNA injection (for total 48 h incubation with brefeldin A) or 48 h after cRNA injection (for total 24 h incubation with brefeldin A). The voltage-clamp experiments were performed at room temperature 3 days after injection (Munoz et al. 2013; Pakladok et al. 2014). Two-electrode voltage-clamp recordings were performed at a holding potential of −60 mV. The data were filtered at 10 Hz and recorded with a Digidata A/D–D/A converter and Clampex 9.2 software for data acquisition and analysis (Axon Instruments) (Bogatikov et al. 2012; Shojaiefard et al. 2012). 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. Glucose was added to the solutions at a concentration of 2 mM, unless otherwise stated. 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 (Almilaji et al. 2013b; Dermaku-Sopjani et al. 2013).

Detection of SGLT1 Cell Surface Expression by Chemiluminescence

Defolliculated oocytes were incubated with rabbit polyclonal anti-SGLT1 antibody (diluted 1:1000, Millipore, Billerica, MA, USA) and subsequently with secondary goat anti-rabbit HRP-conjugated antibody (1:1000, Cell Signaling Technology, MA, USA). After staining individual oocytes were placed in 96-well plates with 20 µl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). The chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader; Perkin Elmer, Jügesheim, Germany) by integrating the signal over a period of 1 s. Results display normalized arbitrary light units, which are proportional to the detector voltage (Mia et al. 2012; Pakladok et al. 2013).

Ussing Chamber Experiments

All animal experiments were conducted according to the German law for the welfare of animals and according to the guidelines of the American Physiological Society and were

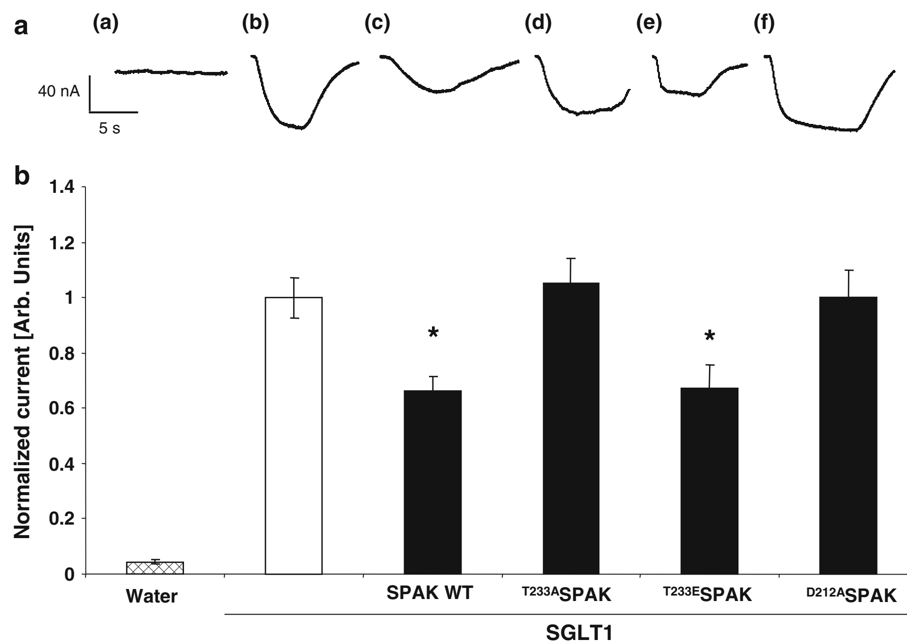

Fig. 1 Effect of wild-type SPAK, constitutively active ^{T233E}SPAK, WNK-insensitive inactive ^{T233A}SPAK, or catalytically inactive ^{D212A}SPAK on electrogenic glucose transport in SGLT1-expressing *Xenopus laevis* oocytes. **a** Representative original tracings showing glucose (2 mM)-induced current (*Ig*) in *Xenopus* oocytes injected with water (*a*), or expressing SGLT1 alone (*b*), or with additional coexpression of wild-type SPAK (*c*), WNK-insensitive ^{T233A}SPAK (*d*), constitutively active ^{T233E}SPAK (*e*) or catalytically inactive ^{D212A}SPAK (*f*). **b** Arithmetic means ± SEM (n = 15–21) of glucose (2 mM)-induced current (I_g) in *Xenopus* oocytes injected with water (dotted bar), expressing SGLT1 alone (*white bar*), or expressing SGLT1 together with wild-type SPAK (1st *black bar*), WNK-insensitive ^{T233A}SPAK (2nd *black gray bar*), constitutively active ^{T233E}SPAK (3rd *black bar*), or catalytically inactive ^{D212A}SPAK (4th *black bar*). * indicates statistically significant ($p < 0.05$) difference from *Xenopus* oocytes expressing SGLT1 alone (Tukey test)

approved by local authorities (Regierungspräsidium Tübingen). Experiments were performed using intestinal segments from 16-week-old female gene-targeted mice expressing SPAK resistant to WNK-dependent activation (*spak*$^{tg/tg}$) and in mice expressing wild-type SPAK (*spak*$^{wt/wt}$) (Pathare et al. 2012b). The mice were fed a control diet (1314, Altromin, Heidenau, Germany) and had free access to tap drinking water.

For analysis of electrogenic intestinal glucose transport, jejunal segments were mounted into a custom made mini-Ussing chamber with an opening of 0.00769 cm^2. Under controlled conditions, the serosal and luminal perfusate contained (in mM): 115 NaCl, 2 KCl, 1 $MgCl_2$, 1.25 $CaCl_2$, 0.4 KH_2PO_4, 1.6 K_2HPO_4, 5 Na pyruvate, 25 $NaHCO_3$, (pH 7.4, NaOH). Where indicated, glucose was added to the luminal perfusate at the indicated concentrations (the substances were from Sigma, Schnelldorf, Germany, or from Roth, Karlsruhe, Germany).

In all Ussing chamber experiments the transepithelial potential difference (Vt) was determined continuously and the transepithelial resistance (Rt) was estimated from the voltage deflections (ΔVt) elicited by imposing test currents (I_t). The resulting Rt was calculated according to Ohm's law (Hosseinzadeh et al. 2013a; Rexhepaj et al. 2010).

Statistical Analysis

Data are provided as mean ± SEM, n represents the number of oocytes or intestinal segments investigated. All voltage-clamp experiments were repeated with at least 2–3 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 two-tailed unpaired *t* test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored whether SPAK influences the function of SGLT1. To this end, cRNA encoding SGLT1 was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding wild-type SPAK, constitutively active ^{T233E}SPAK, WNK-resistant ^{T233A}SPAK, or catalytically inactive ^{D212A}SPAK. Electrogenic glucose transport was estimated from the glucose-induced inward current (I_g) utilizing dual-electrode voltage clamp (TEVC). Following addition of 2 mM glucose, no appreciable I_g was observed in water-injected *Xenopus* oocytes, indicating that

Xenopus oocytes do not express appreciable electrogenic glucose transport (Fig. 1). In contrast, addition of 2 mM glucose generated a large I_g in *Xenopus* oocytes expressing SGLT1 (SLC5A1). The additional expression of wild-type SPAK or constitutively active ^{T233E}SPAK was followed by a significant decrease of I_g in SGLT1-expressing *Xenopus* oocytes. However, additional expression of WNK-insensitive inactive ^{T233A}SPAK or catalytically inactive ^{D212A}SPAK did not modify I_g in SGLT1-expressing *Xenopus* oocytes (Fig. 1a, b).

Additional experiments were performed in order to test whether SPAK influences the maximal transport rate of SGLT1 or carrier affinity to glucose. To this end, *Xenopus* oocytes expressing SGLT1 alone or together with SPAK were exposed to glucose concentrations ranging from 1 μM to 20 mM (Fig. 2). Kinetic analysis of the glucose-induced currents yielded a maximal current of 325 ± 16 nA ($n = 9$) in *Xenopus* oocytes expressing SGLT1 alone. Coexpression of SPAK significantly ($p < 0.001$) decreased the maximal current to 214 ± 8 nA ($n = 9$). Calculation of the glucose concentration required for half maximal current (K_M) yielded values of 641 ± 99 μM in oocytes expressing SGLT1 alone and of 798 ± 159 μM in oocytes expressing both, SGLT1 and SPAK, values are not significantly different. Thus, coexpression of SPAK decreased SGLT1 activity at least in part by decreasing the maximal current.

The decreased maximal electrogenic glucose transport in SGLT1-expressing oocytes following coexpression of SPAK could have resulted from decreased carrier protein abundance in the plasma membrane. Thus, chemiluminescence was employed in order to determine the SGLT1 protein abundance in the cell membrane of oocytes injected with water, expressing SGLT1 alone, expressing SGLT1 together with wild-type SPAK or expressing SGLT1 together with catalytically inactive ^{D212A}SPAK. As shown in Fig. 3, the coexpression of SPAK was followed by a significant decrease of SGLT1 protein abundance within the oocyte cell membrane. In contrast, the coexpression of catalytically inactive ^{D212A}SPAK did not alter SGLT1 protein abundance in the oocyte cell membrane.

SPAK could impair SGLT1 protein abundance either by down-regulating carrier protein insertion into the cell membrane or by accelerating retrieval of carrier protein from the cell membrane. In order to discriminate between those two possibilities the SGLT1-expressing *Xenopus* oocytes were treated with 5 μM brefeldin A. As illustrated in Fig. 4, the glucose-induced current declined in the presence of brefeldin A at a similar rate in oocytes expressing SGLT1 alone and in oocytes expressing SGLT1 together with SPAK. After 24 h treatment with brefeldin A, I_g was similarly low in oocytes expressing SGLT1 together with SPAK as in oocytes expressing SGLT1 alone. Thus, SPAK did not appreciably accelerate the retrieval of SGLT1 protein from the cell membrane but rather down-regulated SGLT1 by inhibiting carrier insertion into the cell membrane.

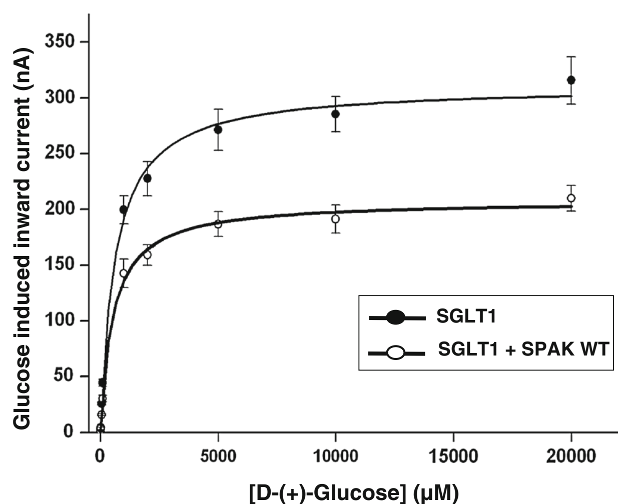

Fig. 2 Effect of wild-type SPAK on the kinetics of electrogenic glucose transport in SGLT1-expressing *Xenopus laevis* oocytes. Arithmetic means ± SEM ($n = 9$) of glucose-induced current (I_g) as a function of glucose concentration in *Xenopus* oocytes expressing SLC5A1 (SGLT1) alone (*black circles*) or together with wild-type SPAK (*white circles*). The values were fitted to Michaelis–Menten equation

To test whether downregulation of SGLT1 by SPAK plays a role in vivo, glucose-induced current was measured in jejunal tissue utilizing Ussing chamber experiments. As illustrated in Fig. 5, the glucose-induced current was significantly higher in gene-targeted mice expressing WNK-insensitive SPAK (*spak*$^{tg/tg}$) than in intestine from their wild-type littermates (*spak*$^{wt/wt}$).

Discussion

The present study reveals that the WNK-dependent STE20/SPS1-related proline/alanine-rich kinase SPAK is a powerful negative regulator of the high affinity Na^+-coupled glucose transporter SGLT1, a nutrient carrier expressed predominantly in the brush border of the small intestine and the proximal tubule within the kidney (Wright and Turk 2004). The carrier couples the uphill reabsorption of glucose to the downhill movement of Na^+ across the plasma membrane. SPAK is at least partially effective by decreasing the SGLT1 protein abundance in the cell membrane. Possibly, SPAK interferes with the insertion of the carrier into the cell membrane. Along those lines, the differences of electrogenic glucose transport between *Xenopus* oocytes expressing SPAK and SGLT1 and *Xenopus* oocytes expressing SGLT1 alone were dissipated by prevention of carrier insertion with brefeldin A.

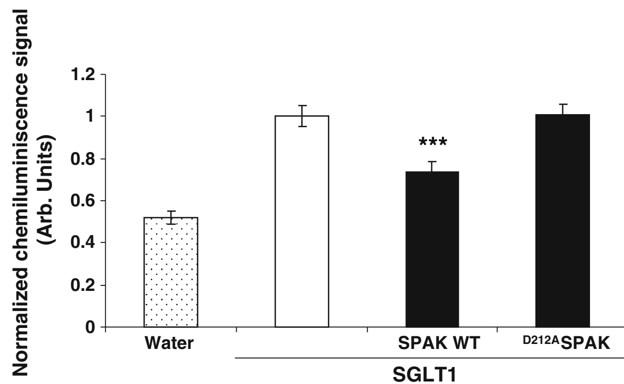

Fig. 3 Effect of SPAK on SGLT1 protein abundance in the *Xenopus laevis* oocyte cell membrane. Arithmetic means ± SEM ($n = 96$–117) of SGLT1 protein abundance determined by chemiluminescence in *Xenopus* oocytes injected with water (*dotted bar*) or expressing SGLT1 alone (*white bar*), or together with wild-type SPAK (1st *black bar*) or inactive ^{D212A}SPAK (2nd *black bar*). *** indicates statistically significant ($p < 0.001$) difference from *Xenopus* oocytes expressing SGLT1 alone (Kruskal–Wallis test)

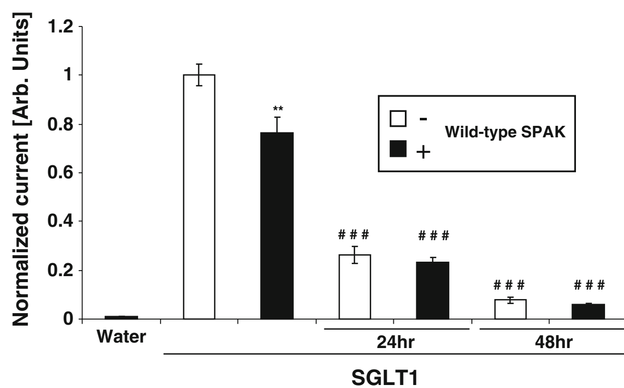

Fig. 4 Decline of electrogenic glucose transport in the presence of brefeldin A in oocytes expressing SGLT1 with or without SPAK. Arithmetic means ± SEM ($n = 5$–14) of glucose (2 mM)—induced current (I_g) in *Xenopus* oocytes injected with cRNA encoding SGLT1 without (SGLT1, *white bars*) or with (SGLT1 + SPAK, *black bars*) wild-type SPAK and exposed to 5 µM brefeldin A for the indicated time periods. $^{**}p < 0.01$ indicates statistically significant difference from the absence of SPAK; $^{\#\#\#}p < 0.001$ indicates statistically significant difference from the absence of brefeldin A (Tukey test)

The present study demonstrates that both wild-type and constitutively active ^{T233E}SPAK, but not the catalytically inactive mutant ^{D212A}SPAK (Vitari et al. 2005) downregulated SGLT1. Thus, kinase activity is apparently required for the observed effect. Moreover, the effect obviously required WNK1-sensitive activation of SPAK, as the WNK-insensitive ^{T233A}SPAK (Vitari et al. 2005) was not capable to downregulate SGLT1 activity. Along those lines, mice carrying the WNK1-insensitive ^{T243A}SPAK instead of wild-type SPAK had higher intestinal SGLT1 activity than wild-type mice.

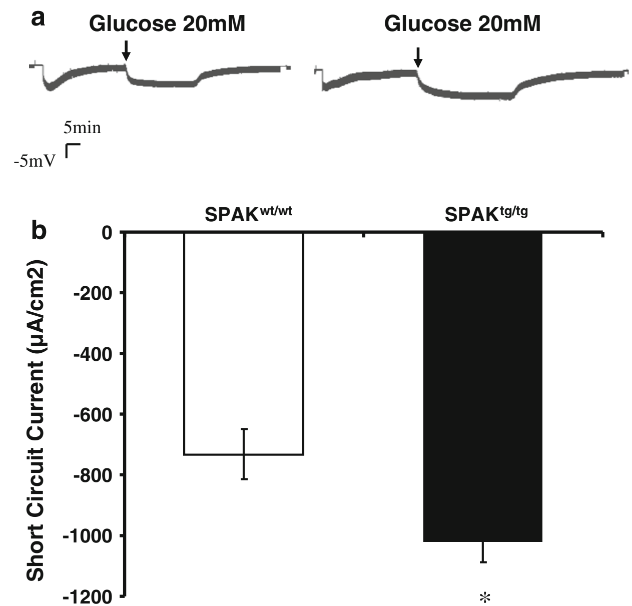

Fig. 5 Glucose-sensitive transepithelial current in jejunum from spak$^{wt/wt}$ and spak$^{tg/tg}$ mice. **a** Representative original tracings of the transepithelial jejunal potential difference in *spak*$^{wt/wt}$ and *spak*$^{tg/tg}$ mice. *Arrows* highlight the addition of glucose (20 mM). **b** Arithmetic means ± SE ($n = 6$) of the glucose-sensitive equivalent short-circuit current in jejunum from *spak*$^{wt/wt}$ (*white bar*) and *spak*$^{tg/tg}$ mice (*black bar*). $^{*}p < 0.05$ indicates statistically significant difference from *spak*$^{wt/wt}$ (unpaired *t* test)

The regulation of SGLT1 by SPAK impacts on intestinal electrogenic glucose transport. Multiple mechanisms are known regulating intestinal glucose transport. According to previous observations SGLT1 could be regulated by altered transcription (Martin et al. 2000), mRNA stability (Loflin and Lever 2001), transporter protein abundance in the plasma membrane (Hirsh and Cheeseman 1998), and transporter activity (Vayro and Silverman 1999). Regulators of SGLT1 activity include cytosolic Na^+ activity (Kusche-Vihrog et al. 2009), insulin (Stumpel et al. 1996), insulin-like growth factors (Lane et al. 2002), glucagon-like peptide 2 (Cheeseman 1997), cholecystokinin (Hirsh and Cheeseman 1998), adrenergic innervation (Ishikawa et al. 1997), lipopolysaccharides (Amador et al. 2008), and carbohydrate-rich diet (Ferraris and Diamond 1989). Kinases involved in the regulation of SGLT1 activity include AMP-activated kinase (AMPK) (Sopjani et al. 2010), protein kinase C (Veyhl et al. 2003), phosphatidylinositol-3-phosphate-5-kinase PIKfyve (PIP5K3) (Shojaiefard et al. 2007), glycogen synthase kinase 3 (Rexhepaj et al. 2010), oxidative stress-regulated kinase (OSR) (Pasham et al. 2012a), B-RAF (Pakladok et al. 2012), Tau tubulin kinase 2 (Alesutan et al. 2012), Janus-activated kinase (JAK2) (Hosseinzadeh et al. 2011), phosphatidylinositol (PI) 3 kinase (Rexhepaj et al. 2007), PIK3-regulated

kinases, phosphoinositide-dependent kinase 1 (PDK1) (Artunc et al. 2006), protein kinase B (PKB/Akt) (Dieter et al. 2004; Kempe et al. 2010), and serum- and glucocorticoid-regulated kinases (SGK1, SGK3) (Nasir et al. 2010; Schwab et al. 2008). Further studies will be required to define the specific role of SPAK in the regulatory network determining electrogenic glucose transport and the functional states in which the regulation of SPAK takes a leading part. The comparison between untreated mice expressing either WNK-insensitive SPAK (*spak*$^{tg/tg}$) or wild-type SPAK (*spak*$^{wt/wt}$) clearly indicated that under unstimulated control conditions SPAK does play a significant role for electrogenic intestinal glucose transport.

In conclusion, SPAK inhibits SGLT1 and thus contributes to the complex regulatory network of this important nutrient carrier.

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