# Up-regulation of hypertonicity-activated myo-inositol transporter SMIT1 by the cell volume-sensitive protein kinase SGK1

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**Mechanisms of regulatory cell volume increase following cell shrinkage include accumulation of organic osmolytes such as betaine, taurine, sorbitol, glycerophosphorylcholine (GPC) and myo-inositol. Myo-inositol is taken up by the sodium-myo-inositol-transporter SMIT1 (SLC5A3) expressed in a wide variety of cell types. Hypertonicity induces the transcription of the SMIT1 gene upon binding of the transcription factor tonicity enhancer binding protein (TonEBP) to tonicity responsive enhancers (TonE) in the SMIT1 promoter region. However, little is known about post-translational regulation of the carrier protein. In this study we show that SMIT1 is modulated by the serum- and glucocorticoid-inducible kinase SGK1, a protein genomically up-regulated by hypertonicity. As demonstrated by two-electrode voltage-clamp in the *Xenopus* oocyte expression system, SMIT1-mediated myo-inositol-induced currents are up-regulated by coexpression of wild type SGK1 and constitutively active $^{S422D}$SGK1 but not by inactive $^{K127N}$SGK1. The increase in SMIT1 activity is due to an elevated cell surface expression of the carrier while its kinetic properties remain unaffected. According to the decay of SMIT1 activity in the presence of brefeldin A, SGK1 stabilizes the SMIT1 protein in the plasma membrane. The SGK isoforms SGK2, SGK3 and the closely related protein kinase B (PKB) are similarly capable of activating SMIT1 activity. SMIT1-mediated currents are decreased by coexpression of the ubiquitin-ligase Nedd4-2, an effect counteracted by additional coexpression of SGK1. In conclusion, the present observations disclose SGK isoforms and protein kinase B as novel regulators of SMIT1 activity.**

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Cell volume constancy and cytosolic ion homoeostasis are prerequisites for proper cell function. They are challenged by exposure to extracellular anisotonicity or by alterations of cytosolic osmolarity (Lang *et al.* 1991, 1998). Rapid equilibration of osmotic gradients serves to drive water into or out of the cell thereby re-establishing initial cell shape and volume (Macknight, 1988; Macknight *et al.* 1994; Waldegger *et al.* 1998*b*). Regulatory volume decrease (RVD) after cell swelling and regulatory volume increase (RVI) after cell shrinkage are achieved acutely by a variety of membrane transport systems and ion channels importing or exporting primarily small ions including sodium, potassium and chloride (Waldegger *et al.* 1998*b*; Lang *et al.* 1998).

Cell volume regulatory uptake of inorganic ions during cell shrinkage may compromise protein stability and affect neuronal electrical excitability (Somero, 1986). To avoid untoward effects of cell volume regulation with inorganic ions, cells additionally make use of non-pertubing uncharged osmolytes (Yancey *et al.* 1982; Somero, 1986). These include betaine, taurine, sorbitol, glycerophosphorylcholine (GPC) and myo-inositol that passively diffuse through selective ion channels and/or are cotransported by specific membrane carriers (Lang *et al.* 1991).

Transport of myo-inositol across the outer cell membrane is facilitated by the sodium myo-inositol transporter SMIT1 (SLC5A3) (Berry *et al.* 1995). SMIT1 is expressed in various tissues investigated and couples concentrative myo-inositol uptake to the sodium gradient (Kwon *et al.* 1992; Hager *et al.* 1995). At hypertonicity, SMIT1 expression is rapidly stimulated leading to increased myo-inositol uptake from the extracellular space (Kwon *et al.* 1992). The significance of SMIT1 in cell protection is illustrated by the observation that

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pharmacological SMIT1 inhibition leads to severe renal failure in rats (Kitamura *et al.* 1998).

The genomic regulation of SMIT1 and other organic osmolyte transporters involves the transcription factor tonicity enhancer binding protein (TonEBP) (Miyakawa *et al.* 1999). TonEBP is activated by hypertonicity and binds to tonicity responsive enhancers (TonE) on the SMIT1 promoter region thereby inducing transcription of the carrier protein (Rim *et al.* 1997; Miyakawa *et al.* 1999). Tonicity responsive elements were also identified in the promotor region of other osmoprotective carriers including the betaine transporter BGT1 (SLC6A12) (Takenaka *et al.* 1994) and the taurine transporter TAUT (SLC6A6) (Ito *et al.* 2004). These elements are also present in the aldose reductase gene (Ferraris *et al.* 1996). Binding of TonEBP to the promotor region of osmosensitive genes upon hypertonic conditions was therefore considered to be a general mechanism to protect cells from shrinkage. However, a recent study on osmoprotective gene regulation in the brain demonstrated that in various non-neuronal cells, i.e. astrocytes, oligodendrocytes, ependymocytes, microglial and endothelial cells, TonEBP is neither expressed at basal conditions nor at prolonged hypertonicity (Maallem *et al.* 2006*a*,*b*) despite the fact that robust expression and hypertonic up-regulation of osmoprotective genes was reported to occur in most of those cell types. These include astrocytes (Strange *et al.* 1994; Bitoun & Tappaz, 2000), endothelial cells (Wiese *et al.* 1996) and macrophages (Zhang *et al.* 1996; Denkert *et al.* 1998). Beyond that, little is known about post-translational regulation of the carrier. It is tempting to speculate that the carrier protein is modulated by phosphorylation. A candidate protein kinase is the serum- and glucocorticoid-inducible kinase SGK1 that belongs to the AGC family of serine and threonine kinases. SGK1 is a downstream kinase in the phosphatidylinositol 3-kinase (PI3K) signalling pathway (Kobayashi *et al.* 1999) which has been shown to modulate a variety of ion channels and transporters (reviewed in Lang *et al.* 2006). SGK1 was initially cloned from a rat mammary tumour line (Webster *et al.* 1993) and later identified to be an early gene up-regulated by hypertonicity (Waldegger *et al.* 1997, 1998*a*). The kinase has been detected in a wide variety of species including shark and *Caenorhabditis elegans*. In mammals, SGK1 is expressed in virtually all tissues tested. However, transcript levels vary profoundly among different cell types of any given tissue (reviewed in Lang *et al.* 2006).

SGK1 and SMIT1 are coexpressed in various organ systems including kidney (Kwon *et al.* 1992; Hou *et al.* 2002; McCormick *et al.* 2005) and brain (Kwon *et al.* 1992; Warntges *et al.* 2002). To investigate a putative regulation of the myo-inositol transporter SMIT1 by the hypertonicity-induced protein kinase SGK1, both proteins were coexpressed in the *Xenopus* oocyte system. Functional studies were performed by two-electrode voltage-clamp and tracer flux measurements and the myo-inositol-induced inward current and [$^3$H]myo-inositol uptake were taken as a measure for SMIT1 activity. The study provides new insights into the signalling of cell volume regulatory events upon shrinkage of cells by increased extracellular osmolarity.

## Methods

### Expression in *Xenopus laevis* oocytes

cRNA encoding wild type or haemagglutinin (HA)-tagged SMIT1 (Hager *et al.* 1995), wild type human Nedd4-2 (Debonneville *et al.* 2001), the respective SGK protein kinase constructs (Waldegger *et al.* 1997; Kobayashi *et al.* 1999) and protein kinase B (Alessi *et al.* 1996) have been synthesized as described (Wagner *et al.* 2000). Dissection of *Xenopus laevis* ovaries, collection and handling of the oocytes has been described in detail elsewhere (Wagner *et al.* 2000). Briefly, female *Xenopus laevis* frogs were anaesthetized by submersion in an aqueous ice-cold 0.1% 3-aminobenzoic acid ethyl ester solution (Sigma, St Louis, MO, USA) and placed on ice for surgery. A small abdominal incision was carried out (0.5–1 cm) and small pieces of the ovaries were removed. Afterwards the cut was sewn with a reabsorbable suture. Frogs were kept in drug-free water until reflexes were fully recovered. After two surgeries, frogs were killed humanely at the end of the experiments by cervical dislocation. All experiments were carried out according to guidelines of the animal welfare committee of the University of Tuebingen, Germany.

Oocytes were injected with 7.5 ng of the respective kinase cRNA and/or 5 ng Nedd4-2 cRNA or water on the first day after preparation of the oocytes and subsequently with 25 ng SMIT1 cRNA.

Myo-inositol-evoked currents were highly variable between different batches of oocytes. For data display, currents were normalized to the arithmetic mean of the control group (cells solely injected with SMIT1 cRNA) of each preparation and modulation of SMIT1 activity was reported as percentage changes.

### Electrophysiological studies

All experiments were performed at room temperature 5 days after injection. 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 GeneClamp 500 amplifier, a DigiData 1300 A/D–D/A converter and the pCLAMP 9.0 software package for data acquisition and analysis (Axon Instruments, USA). The control solution (superfusate-ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM

Hepes, pH 7.4. Myo-inositol was added to the solutions at the indicated concentrations. The final solutions were titrated to the pH indicated using HCl or NaOH. The flow rate of the superfusion was 20 ml $min^{-1}$ and a complete exchange of the bath solution was reached within about 10 s.

### Tracer flux studies

SMIT activity was also assessed by [$^3$H]myo-inositol uptake. Oocytes were microinjected with 25 ng of SMIT cRNA alone or together with $^{S422D}$SGK1 (7.5 ng) or with $H_2O$ (control oocytes). After 5–6 days, measurements of $Na^+$-dependent [$^3$H]myo-inositol uptake were performed. [$^3$H]myo-inositol uptake was initiated by transferring oocytes to a ND96 solution containing 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes (pH 7.4), 96 mM NaCl (ND96-Na) or 96 mM ChCl (ND96-Ch), 10 $\mu$Ci $ml^{-1}$ [$^3$H]myo-inositol (14 Ci $mmol^{-1}$, Amersham, Freiburg, Germany) and 300 $\mu$M of unlabelled myo-inositol. After incubation for 30 min at room temperature with [$^3$H]myo-inositol (linear range of uptake), uptake was terminated by washing the oocytes four times with ice-cold ND96-Ch. Oocytes were individually transferred into scintillation vials and dissolved by adding 200 $\mu$l of 10% SDS. Radioactivity incorporated into the cells was measured with a liquid scintillation counter.

### Site directed mutagenesis

The haemagglutinin (HA)-tagged SMIT1 was generated by two-stage PCR site-directed mutagenesis using the primers: SMIT1-HA,s: 5′-GAA TGG GCA GCA GGC CTA CGA CGTA CCA GAT TAC GCT TGT CCC TAG CCA TCC-3′; SMIT1-HA,as: 5′GGA TGG CTA GGG ACA TAG CGT AAT CTG GTA CGT CGT AGC CTG CTG CCC ATTC-3′. The HA-tagged SMIT1 mutant was sequenced to verify the presence of the desired mutation.

### Detection of cell surface expression by chemiluminescence

Defolliculated oocytes were first injected with SGK1 cRNA or water and 1 day later with SMIT1 (HA), which contains an HA epitope inserted extracellularly. Oocytes were incubated with 1 $\mu$g $ml^{-1}$ primary rat monoclonal anti-HA antibody (clone 3F10, Boehringer, Biberach, Germany), and 2 $\mu$g $ml^{-1}$ secondary, peroxidase-conjugated affinity-purified F(ab′)2 goat anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, USA). Individual oocytes were placed in 100 $\mu$l of ND96 and 20 $\mu$l of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, USA), and chemiluminescence was quantified in a luminometer (Victor, Perkin Elmer, Wellesley, USA) integrating the signal over a period of 1 s. Results display normalized relative light units (RLU).

### Statistical analysis

Data are provided as means ± S.E.M., $n$ represents the number of oocytes investigated. All data were tested for significance using ANOVA, and only results with $P < 0.05$ were considered as statistically significant.

## Results

In order to investigate whether the SGK1 protein kinase plays a role in the regulation of the sodium-myo-inositol-transporter SMIT1, the carrier was expressed in *Xenopus laevis* oocytes and myo-inositol-induced currents were taken as a measure for SMIT1 activity in the presence and absence of the respective protein kinase. *Xenopus* oocytes injected with SMIT1 showed an inward current of 16 ± 2 nA ($n = 136$) when superfused with 2 mM myo-inositol (Fig. 1). Coexpression of SGK1 increased the current to 220 ± 13% ($n = 153$) of the control value. As shown in Fig. 1, myo-inositol-induced currents were low in oocytes solely injected with water (0.1 ± 0.2 nA, $n = 29$) or in cells injected with SGK1 in the absence of SMIT1 (0.4 ± 0.1 nA, $n = 3$).

To demonstrate that the observed increase of myo-inositol-evoked currents in SMIT1-expressing cells by SGK1 is mediated by sodium-dependent transport, we performed isotope uptake studies with these cells. Uptake of radiolabelled [$^3$H]myo-inositol was activated upon coexpression of SGK1 to 186 ± 6%, $n = 39$, compared to oocytes solely expressing the SMIT1 transporter. In

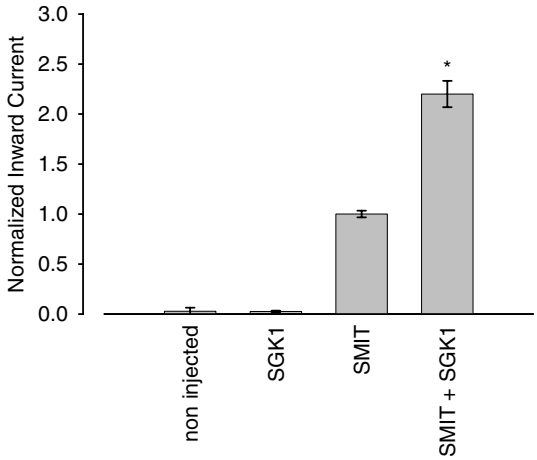


**Figure 1. Stimulation of myo-inositol-evoked currents by the serum- and glucocorticoid-inducible kinase SGK1**
Non-injected oocytes or oocytes solely injected with the SGK1 protein kinase did not display any myo-inositol-sensitive current. Arithmetic means ± S.E.M. of myo-inositol (2 mM)-evoked currents. * indicates statistically significant difference ($P < 0.05$) to oocytes injected with SMIT1 alone.

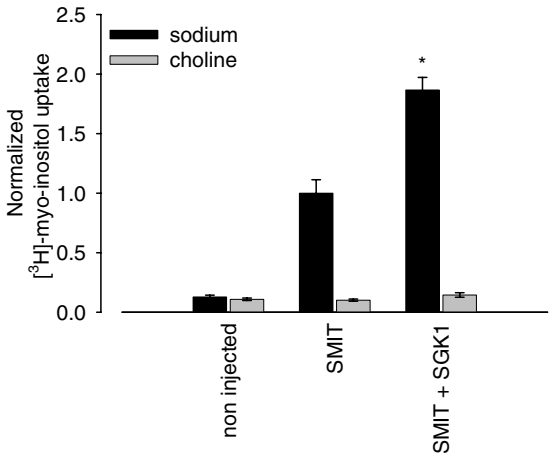


**Figure 2. Stimulation of myo-inositol uptake by the serum- and glucocorticoid-inducible kinase SGK1**
Uptake of radiolabelled [$^3$H]myo-inositol is activated upon coexpression of SGK1 in SMIT1-injected oocytes. Arithmetic means ± S.E.M. of [$^3$H]myo-inositol tracer fluxes. * indicates statistically significant difference ($P < 0.05$) to oocytes injected with SMIT1 alone.

$Na^+$-free ND96 (ND96-Ch) solution, [$^3$H]myo-inositol uptake in SMIT1-expressing oocytes was not significantly different from the transport measured in non-injected oocytes (Fig. 2).

SGK1 shares ∼80% homology with its isoforms SGK2 and SGK3 and the PKB. When coexpressed with SMIT1 in *Xenopus* oocytes, the respective kinases were similarly capable of activating myo-inositol-evoked currents (Fig. 3). Compared to control cells solely injected with SMIT1, SGK2 activated the current to 180 ± 19% ($n = 44$) and SGK3 to 168 ± 11% ($n = 42$). Likewise the constitutively active PKB (T308D, S473D) stimulated SMIT1 activity to 136 ± 23% ($n = 24$).

To explore whether modulation of SMIT1 currents depends on SGK1–3 kinase catalytical activity and is not due to non-phosphorylation-dependent protein–protein interaction, constitutively active and inactive mutants of the respective kinases were coexpressed with SMIT1 in *Xenopus* oocytes. While the constitutively active SGK1 ($^{S422D}$SGK1) increased myo-inositol-induced currents to 215 ± 13% ($n = 116$) of the control value, no increase of current was observed upon coinjection of the kinase-dead SGK1 ($^{K127N}$SGK1). In $^{K127N}$SGK1 coexpressing oocytes, the SMIT1-mediated currents were not significantly different to the currents in cells solely injected with the SMIT1 transporter (94 ± 17%, $n = 15$). Similarly, the constitutively active $^{S356D}$SGK2 (181 ± 12% of control, $n = 52$) and $^{S419D}$SGK3 (159 ± 10% of control, $n = 50$) significantly increased the SMIT1-mediated current. In contrast, coexpression of the inactive $^{K64N}$SGK2 (100 ± 13% of control, $n = 9$) and $^{K191N}$SGK3 (112 ± 23% of control, $n = 8$) did not significantly affect SMIT1 currents (Fig. 4).

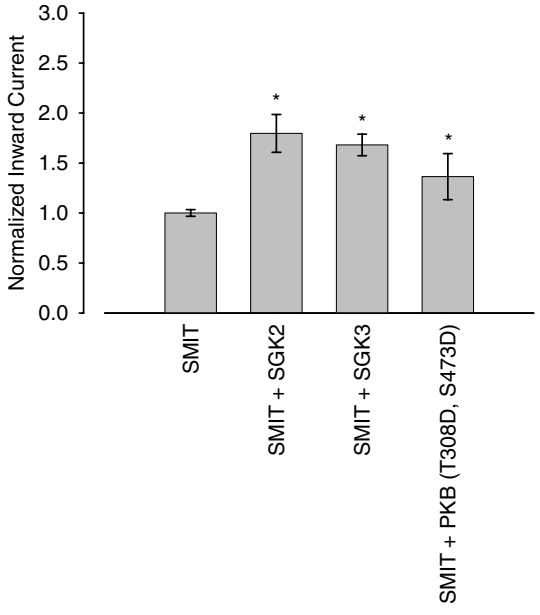


**Figure 3. Stimulation of the sodium myo-inositol transporter SMIT1 by SGK isoforms and PKB**
The SGK isoforms 2 and 3 and to a lesser extent the protein kinase B were similarly capable of up-regulating SMIT1 currents in *Xenopus* oocytes. Arithmetic means ± S.E.M. of myo-inositol (2 mM)-evoked currents. * indicates statistically significant difference ($P < 0.05$) to oocytes injected with SMIT1 alone.

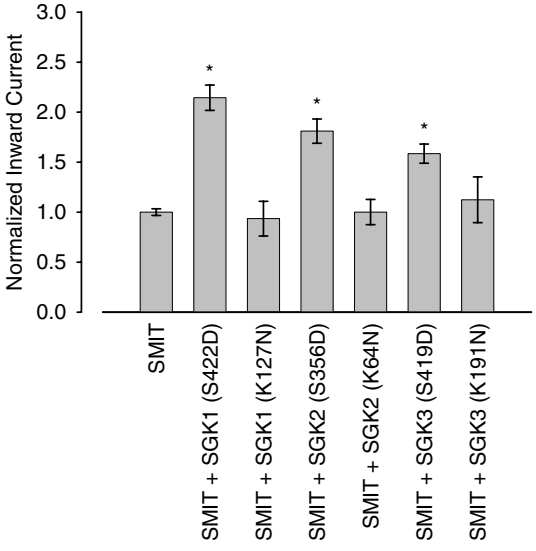


**Figure 4. Up-regulation of SMIT1 by the SGK isoforms critically depends on the catalytical activity of the respective protein kinases**
While coexpression of constitutively active SGK1 (S422D), SGK2 (S356D) and SGK3 (S419D) activated SMIT1, coexpression of inactive SGK1 (K127N), SGK2 (K64N) and SGK3 (K191N) did not affect SMIT1-mediated currents in *Xenopus* oocytes. Arithmetic means ± S.E.M. of myo-inositol (2 mM)-evoked currents. * indicates statistically significant difference ($P < 0.05$) to oocytes injected with SMIT1 alone.

The molecular mechanism of SGK action on the SMIT1 transporter may either be due to a direct phosphorylation of its target protein or by phosphorylation and thereby inactivation of the ubiquitin ligase Nedd4-2, an effect described in previous reports, i.e. for the epithelial sodium channel ENaC (Debonneville *et al.* 2001) or the excitatory amino acid transporter EAAT1 (Boehmer *et al.* 2003). To test whether SMIT1 activity is modulated by the ubiquitin ligase Nedd4-2 both proteins were coexpressed in *Xenopus* oocytes. Indeed, Nedd4-2 coinjection reduced myo-inositol currents to $64 \pm 11\%$ ($n = 19$) of control while expression of the inactive Nedd4-2 (C962S) mutant did not affect the currents significantly ($116 \pm 11\%$, $n = 18$). The inhibiting effect of the ubiquitin ligase was partially reversed by further coexpression of $^{S422D}$SGK1 ($99 \pm 14\%$ of control, $n = 9$). $^{S422D}$SGK1 expression in this series of experiments stimulated the SMIT1 currents to $167 \pm 3\%$ ($n = 6$) of control, a value comparable to the coexpression of $^{S422D}$SGK1 together with the inactive Nedd4-2 (C962S) and SMIT1 ($181 \pm 20\%$, $n = 9$) (Fig. 5).

The observed activation of SMIT1 currents may either be due to an increased cell surface expression of the transporter or to a modulation of the carriers substrate affinities. To clarify, whether substrate affinities are modulated upon SGK1 or SGK3 coexpression, dose–response curves were determined upon coinjection of the respective kinases. The apparent half-maximal substrate concentrations were similar upon the coinjection of SMIT1 with the protein kinases SGK1 ($40 \pm 5\ \mu$M, $n = 11$) or SGK3 ($48 \pm 11\ \mu$M, $n = 6$) as upon expression of SMIT1 alone ($59 \pm 5\ \mu$M, $n = 8$). Calculated $V_{max}$ values from regression fits with the modified Hill equation equalled $1.8 \pm 0.2$ ($n = 11$) for coexpression of SMIT1 with SGK1, $2.0 \pm 0.4$ ($n = 6$) for coexpression of SMIT1 with SGK3 as compared to $1.0 \pm 0.2$ ($n = 8$) for expression of SMIT1 alone (Fig. 6 and Table 1).

To investigate whether SMIT1 current activation correlates with an increased cell surface abundance of the transporter, a haemagluttinin (HA) tag was inserted into an extracellular loop of the protein. A chemiluminescence assay was used to quantify SMIT1 expression at the outer membrane by a primary HA antibody coupled to peroxidase. The emitted light

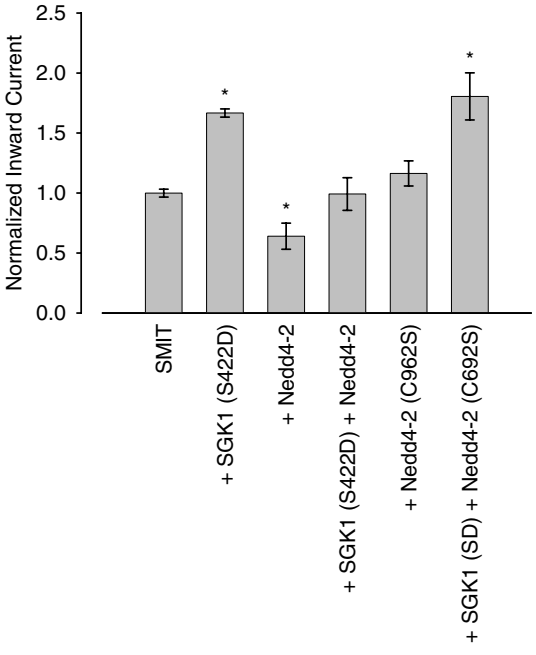


**Figure 5. Regulation of SMIT1 by the ubiquitin ligase Nedd4-2**
Coexpression of wild type Nedd4-2, but not of the catalytically inactive Nedd4-2 (C962S), significantly down-regulated myo-inositol-induced currents. SGK1 counteracted the inhibiting Nedd4-2 effect and activated SMIT1 currents when coexpressed with inactive Nedd4-2 (C962S). Arithmetic means ± S.E.M. of myo-inositol (2 mM)-evoked currents. * indicates statistically significant difference ($P < 0.05$) to oocytes injected with SMIT1 alone.

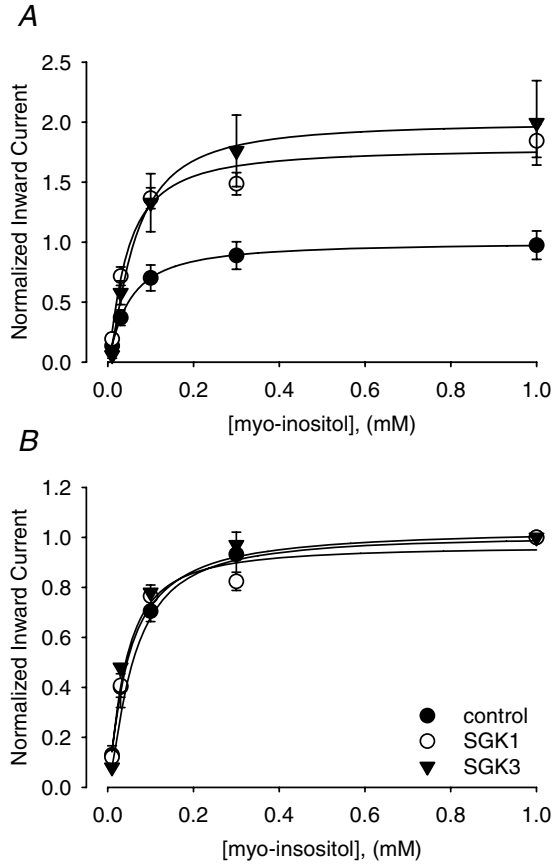


**Figure 6. Kinetics of myo-inositol-evoked currents in SMIT1 expressing *Xenopus laevis* oocytes**
While coexpression of SGK1 and SGK3 increased $V_{max}$ of the carrier (see Table 1 and *A*), SMIT1 substrate affinities for myo-inositol remained unaffected (see Table 1 and *B*). Arithmetic means ± S.E.M. of myo-inositol (2 mM)-evoked currents. Data are normalized to the maximum value of the control group (cells solely injected with SMIT) (*A*) or to maximum value of the respective group injected with SMIT alone, SMIT + SGK1 or SMIT + SGK3 (*B*).

**Table 1. Kinetics of myo-inositol-evoked currents in SMIT1-expressing *Xenopus laevis* oocytes**

| | $K_m$ | $P$ | $V_{max}$ | $P$ | $n$ |
|---|---|---|---|---|---|
| SMIT | 59 ± 5 | — | 1.0 ± 0.2 | — | 8 |
| SMIT + SGK1 | 40 ± 5 | n.s. | 1.8 ± 0.2 | < 0.05 | 11 |
| SMIT + SGK3 | 48 ± 11 | n.n. | 2.0 ± 0.4 | < 0.05 | 6 |

While coexpression of SGK1 and SGK3 increased $V_{max}$ of the carrier, SMIT1 substrate affinities for myo-inositol were not significantly affected. Arithmetic means ± S.E.M. of myo-inositol (2 mM)-evoked currents. Data are normalized to the maximum value of the control group (oocytes solely expressing SMIT1). $V_{max}$ and $K_m$ values were calculated with the modified Hill equation. $P$ values are relative to the control group (oocytes solely expressing SMIT1).

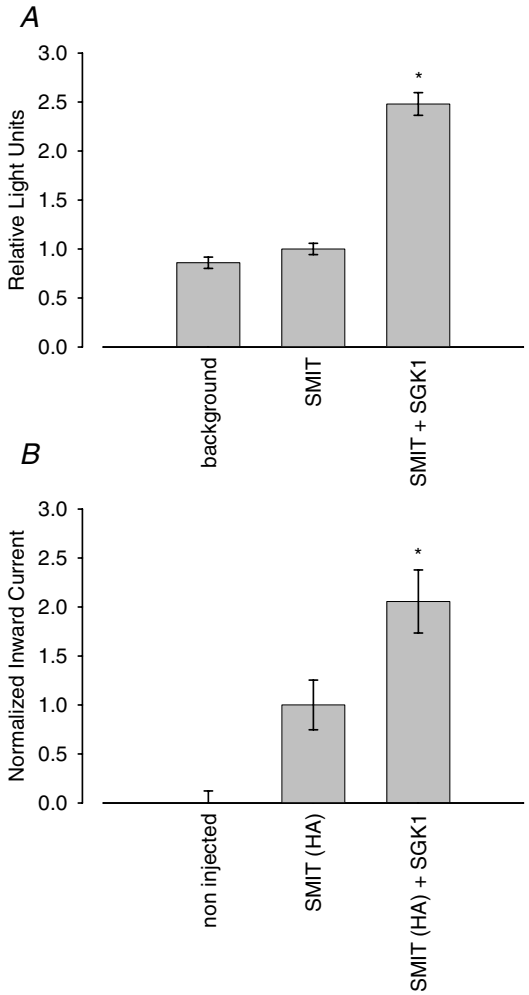


**Figure 7. Cell surface abundance of SMIT1 is up-regulated by SGK1**

*A*, arithmetic means ± S.E.M. of protein abundance as reflected by chemiluminescence of SMIT1 (HA). * indicates statistically significant difference ($P < 0.05$) to oocytes injected with SMIT1 (HA) alone. *B*, extracellular insertion of haemagluttinin into the SMIT1 sequence does not affect up-regulation of the transporter by SGK1. Arithmetic means ± S.E.M. of myo-inositol (2 mM)-evoked currents. * indicates statistically significant difference ($P < 0.05$) to oocytes injected with SMIT1 (HA) alone.

directly corresponds to the amount of protein at the cell surface. To test for functionality of the newly generated SMIT1 (HA) constructs and their comparative activation by SGK1 relative to the wild type SMIT1, cells used for the chemiluminescence assay were tested for myo-inositol-sensitive currents in parallel. As depicted in Fig. 6, 2 mM substrate generated inward currents in SMIT1 (HA)-expressing oocytes which were significantly higher in oocytes additionally expressing SGK1 (206 ± 32%, $n = 13$) than in oocytes expressing SMIT1 (HA) alone (100 ± 25%, $n = 13$). The chemiluminescence assay revealed that the increase in carrier cell surface expression correlates with the observed increase in currents upon SGK1 coinjection. Non-injected control oocytes display a background fluorescence of 0.9 ± 0.1 relative light units (RLU) ($n = 11$) while the signal from cells expressing SMIT1 alone equals 1.0 ± 0.1 RLU ($n = 11$). Coexpression of the SGK1 protein kinase significantly increased the light signal to 2.5 ± 0.1 RLU ($n = 11$) (Fig. 7).

The increase in SMIT1 cell surface expression might be the result of an induced carrier insertion into the membrane or a decreased removal of the carrier protein. To distinguish between these two mechanisms, SMIT1 activity was quantified after different time points of brefeldin A (BFA) administration. This drug inhibits secretory mechanisms by inhibiting vesicle formation at the Golgi apparatus and thus prevents further insertion of carrier protein into the cell membrane. As demonstrated in a previous study from this lab, BFA (5 μM) treatment does not affect viability of the oocytes (Yun *et al.* 2002). Administration of BFA gradually decreased myo-inositol-evoked currents in SMIT1-expressing oocytes. Compared to control cells solely injected with the SMIT1 carrier, the decrease in

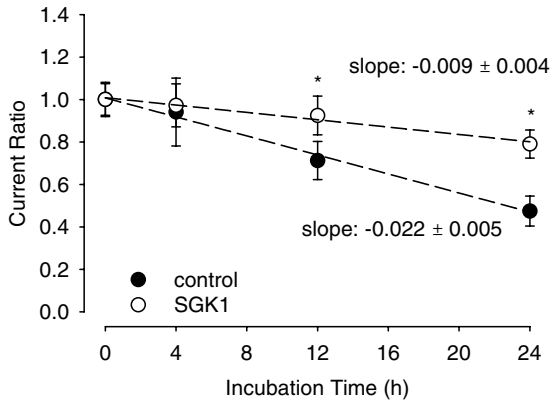


**Figure 8. Coexpression of SGK1 stabilizes the SMIT1 carrier in the oocyte membrane**

Myo-inositol-evoked currents in brefeldin A (5 μM)-treated oocytes at the indicated time points. Arithmetic means ± S.E.M. of myo-inositol (2 mM)-evoked currents. * indicates statistically significant difference ($P < 0.05$) between oocytes injected with SMIT1 alone and coexpression of SMIT1 together with SGK1.

currents was less rapid in cells coinjected with SMIT1 and the SGK1 protein kinase. While control oocytes showed a current ratio of $0.7 \pm 0.1$, $n = 12$ at $t = 12$ h, SGK1-coinjected cells displayed a significantly higher current ratio at this time point ($0.9 \pm 0.1$, $n = 20$). Twenty four hours after BFA incubation the difference in current ratios was even more prominent ($0.5 \pm 0.1$, $n = 17$, in the control group compared to $0.8 \pm 0.1$, $n = 20$, in SGK1-coinjected cells) (Fig. 8).

## Discussion

The present study reveals that SMIT1 is activated by the serum- and glucocorticoid-inducible kinase SGK1, its isoforms 2, 3 and the closely related PKB. The present observations thus disclose a novel mechanism of SMIT1 regulation. As SGK1 is strongly up-regulated by both isotonic and hypertonic cell shrinkage (Waldegger *et al.* 1997, 1998*a*), the kinase could indeed participate in the up-regulation of SMIT1 activity during regulatory cell volume increase. Indeed, SMIT1 is colocalized with SGK1 in various organs including kidney (Kwon *et al.* 1992; Hou *et al.* 2002; McCormick *et al.* 2005) and brain (Kwon *et al.* 1992; Warntges *et al.* 2002). This study investigated regulation of SMIT1 activity in the *Xenopus* oocyte expression system under isotonic conditions.

SMIT1 activation by SGK is critically dependent on the phosphorylation processes as the catalytically inactive kinase isoforms are not capable of stimulating myo-inositol currents. The molecular mechanism of SMIT1 up-regulation may be due to direct phosphorylation of the transporter or by phosphorylation of an intermediate protein by SGK1. One known SGK1 substrate that is involved in membrane transport regulation is the ubiquitin ligase Nedd4-2 (Debonneville *et al.* 2001; Boehmer *et al.* 2006; Rajamanickam *et al.* 2007). SGK1 phosphorylates and abolishes interaction of Nedd4-2 with its target thereby decreasing its removal from the plasma membrane (Debonneville *et al.* 2001). The ubiquitin ligase is endogenously expressed in *Xenopus* oocytes and thus expression of SGK1 alone may be capable of activating membrane transport as described for the epithelial sodium channel ENaC (Debonneville *et al.* 2001) and several excitatory amino acid transporters (Boehmer *et al.* 2003, 2005, 2006; Rajamanickam *et al.* 2007). The present study shows that Nedd4-2 down-regulates SMIT1 activity, an effect that is compensated by further expression of SGK1. However, the SMIT1-induced current is significantly larger in oocytes coexpressing SMIT1 with constitutively active $^{S442D}$SGK1 and inactive $^{C692S}$Nedd4-2 than in oocytes coexpressing SMIT1 with constitutively active $^{S442D}$SGK1 and wild type Nedd4-2. Thus, $^{S442D}$SGK1 did not fully inactivate Nedd4-2 and $^{S442D}$SGK1 may be effective by mechanisms other than Nedd4-2 phosphorylation. Nevertheless, expression of SGK1 in the *Xenopus* oocyte system leads to phosphorylation of intrinsic Nedd4-2 (Dieter *et al.* 2004).

The observed increase in SMIT1 activity is due to an increased abundance of the transporter at the cell membrane as revealed by the chemiluminescence assay using the SMIT1 (HA) construct. Upon brefeldin A administration myo-inositol currents in oocytes injected with SGK1 and SMIT1 together decrease less rapidly compared to cells injected solely with the transporter. Obviously, the observed increase in SMIT1 activity is at least partially due to stabilization of SMIT1 in the outer membrane with enhanced dwelling time of the carrier in the cell membrane.

In summary, this study indicates that SGK1 and its isoforms modulate SMIT1 function by increasing its plasma membrane abundance without affecting transport kinetics. SGK1 might partially be effective through phosphorylation and thus inhibition of the intrinsic ubiquitin ligase Nedd4-2. This novel mechanism of SMIT1 regulation at the post-transcriptional level might provide new insights into the signalling of cell volume regulation upon hypertonic stress.

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