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Regulation of the Ca^{2+} Channel TRPV6 by the Kinases SGK1, PKB/Akt, and PIKfyve

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Abstract The serum- and glucocorticoid-inducible kinase SGK1 and the protein kinase PKB/Akt presumably phosphorylate and, by this means, activate the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve (PIP5K3), which has in turn been shown to regulate transporters and channels. SGK1-regulated channels include the Ca^{2+} channel TRPV6, which is expressed in a variety of epithelial and nonepithelial cells including tumor cells. SGK1 and protein kinase B PKB/Akt foster tumor growth. The present study thus explored whether TRPV6 is regulated by PIKfyve. TRPV6 was expressed in *Xenopus laevis* oocytes with or without additional coexpression of constitutively active ^{S422D}SGK1, constitutively active ^{T308D,S473D}PKB, wild-type PIKfyve, and ^{S318A}PIKfyve lacking the SGK1 phosphorylation site. TRPV6 activity was determined from the current (I_{Ca}) resulting from TRPV6-induced Ca^{2+} entry and subsequent activation of Ca^{2+}-sensitive endogenous Cl^- channels. TRPV6 protein abundance in the cell membrane was determined utilizing immunohistochemistry and Western blotting. In TRPV6-expressing oocytes I_H was increased by coexpression of ^{S422D}SGK1 and by ^{T308D,S473D}PKB. Coexpression of wild-type PIKfyve further increased I_H in TRPV6 + ^{S422D}SGK1-expressing oocytes but did not significantly modify I_{Ca} in oocytes expressing TRPV6 alone. ^{S318A}PIKfyve failed to significantly modify I_{Ca} in the presence and absence of ^{S422D}SGK1. ^{S422D}SGK1 increased the TRPV6 protein abundance in the cell membrane, an effect augmented by additional expression of wild-type PIKfyve. We conclude that PIKfyve participates in the regulation of TRPV6.

Keywords Phosphatidylinositol-3-phosphate-5-kinase · Serum- and glucocorticoid-inducible kinase · SGK1 · PKB · Akt · Ca^{2+} channel

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TRPV6, a member of the transient receptor potential channel family TRPV, is expressed in a wide variety of epithelial and nonepithelial tissues including several tumor cells (Fukushima et al. 2009; Prevarskaya et al. 2007; Semenova et al. 2009; Stumpf et al. 2008; Takumida et al. 2009). The channel is involved in epithelial transport (Thyagarajan et al. 2009; van de Graaf et al. 2006) and the regulation of diverse functions including cell proliferation (Bolanz et al. 2008; Heine et al. 2008; Lallet-Daher et al. 2009; Lehen'kyi et al. 2007).

Previous studies utilizing the heterologous expression in *Xenopus laevis* oocytes revealed that TRPV6 is regulated by the serum- and glucocorticoid-inducible kinase SGK isoforms SGK1 and SGK3 (Bohmer et al. 2007). SGK1 was effective by enhancing the abundance of the TRPV6 protein in the plasma membrane. The mechanism underlying this effect remained, however, elusive. In theory, SGK1-dependent regulation may involve the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve (Seebohm et al. 2007), a kinase generating phosphatidylinositol 3,5-bisphosphate [$PI(3,5)P_2$] (Ikonomov et al. 2002; Sbrissa et al. 1999, 2002, 2004). PIKfyve regulates endosomal transport (Ikonomov et al. 2001, 2003, 2006; Rusten et al. 2006; Rutherford et al. 2006) and plays a critical role in the regulation of the glucose carrier GLUT4 (Berwick et al. 2004; Watson and Pessin 2006; Welsh et al. 2005), the

Na^+-glucose cotransporter SGLT1 (Shojaiefard et al. 2007), the creatine transporter CreaT (Strutz-Seebohm et al. 2007b), the K^+ channel KCNQ1/KCNE1 (Seebohm et al. 2007), and the Cl^- channel ClC_2 (Klaus et al. 2009).

TRPV6 is regulated by phosphatidylinositol 4,5-bisphosphate (Thyagarajan et al. 2008), but nothing is known about its regulation by PIKfyve or $PI(3,5)P_2$. The present study explored whether PIKfyve is involved in the SGK1-dependent regulation of TRPV6. To this end, TRPV6 was expressed in *Xenopus* oocytes with or without PIKfyve and/or constitutively active ^{S422D}SGK1.

Materials and Methods

For the generation of cRNA, constructs were used encoding wild-type human TRPV6 (Bohmer et al. 2007), wild-type PIKfyve (Berwick et al. 2004; Strutz-Seebohm et al. 2005), mutated ^{S318A}PIKfyve lacking the SGK1 phosphorylation consensus sequence (Berwick et al. 2004; Strutz-Seebohm et al. 2007b), constitutively active human ^{S422D}SGK1 (Boehmer et al. 2008b), and constitutively active human ^{T308D,S473D}PKB (Klaus et al. 2008). The cRNA was generated as described previously (Boehmer et al. 2008a). ^{S422D}SGK1 and PKB cDNA were kindly provided by Sir Philip Cohen, College of Life Sciences, and Sir James Black Centre, University of Dundee; the PIKfyve cDNA, by Jeremy M. Tavaré, University of Bristol.

For analyzing TRPV6 activity by dual-electrode voltage clamping, *Xenopus* oocytes were prepared as previously described (Strutz-Seebohm et al. 2007a). Oocytes were injected with 25 ng TRPV6, 7.5 ng ^{T308D,S473D}PKB, and/or 5 ng wild-type PIKfyve or mutant ^{S318A}PIKfyve cRNA. Where indicated, 7.5 ng cRNA encoding constitutively active ^{S422D}SGK1 was injected separately or together with PIKfyve or ^{S318A}PIKfyve and H_2O as a control.

All experiments were performed at room temperature 4–5 days after injection of the respective cRNAs unless otherwise stated. The currents were determined in two-electrode voltage-clamp experiments utilizing a pulse protocol of a 5-s linear voltage ramp from −150 to +50 mV. The intermediate holding potential between the voltage ramps was −60 mV.

The data were filtered at 1 kHz and recorded with a GeneClamp 500 amplifier, a DigiData 1322A A/D-D/A converter, and the pClamp 9.0 software package for data acquisition and analysis (Axon Instruments, USA). The bath solution contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.4. Oocytes were kept in modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, and 5 mM HEPES, pH 7.4, supplemented with 25 μg/ml gentamycin. The flow rate of the superfusion was ~20 ml/min and a complete exchange of the bath solution was reached within about 10 s. Calcium influx via TRPV6 elevates the intracellular calcium concentration, leading to a secondary activation of a calcium-sensitive chloride conductance. The calcium-induced current I_{Ca} was taken as a measure of TRPV6 activity in these experiments. To this end, induced peak currents were recorded following the described pulse protocol. Arrows in the original tracings indicate the values taken for evaluation.

For immunohistochemistry and Western blotting oocytes were devitellinized and injected with 7.5 ng ^{S422D}SGK1, 5 ng PIKfyve (wild-type or S318A mutant), or 27 ng TRPV6 (hemagglutinin [HA]) cRNA containing an extracellular HA epitope between amino acid 374 and amino acid 375 as indicated. The activity of HA-tagged TRPV6 was confirmed by two-electrode voltage-clamp experiments. After washing with PBS, oocytes were permeabilized at room temperature for 30 min by incubation in PBS containing 0.2% TritonX-100. In the following the oocytes were fixed in 4% paraformaldehyde at room temperature for 2 h. They were blocked in PBS containing 10% normal goat serum for 2 h at room temperature. Oocytes were subsequently incubated for 1 h at 37°C with 100 ng/ml primary rat monoclonal anti-HA antibody (clone 3F10; Roche, Mannheim, Germany). After three washes with 1% TBS-goat, oocytes were incubated for 30 min with 1 μg/ml secondary, Cy5-conjugated H&L goat anti-rat IgG (Abcam, Cambridge, UK), washed with TBS, and rinsed with distilled water. The oocytes were subsequently analyzed by a fluorescence laser scanning confocal microscope (LSM 510; Zeiss, Germany).

For Western blotting, the membrane abundance of TRPV6 was analyzed by surface biotinylation. After incubating the oocytes in 1 mg/ml EZ-Link-Sulfo-NHS-Biotin (Pierce Protein Research Products, Rockford, IL, USA), oocytes were washed five times for 10 min in ND96. The cells were homogenized with a pestle in 1% Triton X-100, 100 mM NaCl, 20 mM Tris–HCl, pH 7.4, and Complete Protease Inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) and incubated for 1 h at 4°C on a rotator. Afterwards, cells were centrifuged and the protein concentration was measured by Bradford assay. Three hundred to five hundred micrograms of labeled protein was collected by rotating the lysates overnight at 4°C together with Neutravidin-coated agarose beads (Pierce Protein Research).

For immunoblotting, the proteins were electrotransferred onto a PVDF membrane and blocked with 5% BSA in TBS-0.1% Tween 20 (TBST) at room temperature for 1 h. Then the membrane was incubated with 0.5 ng/ml primary rat monoclonal anti-HA antibody (clone 3F10;

Roche). After washing (TBST) the blots were incubated with secondary anti-rat antibody conjugated with horseradish peroxidase (HRP; 1:1,000; Cell Signaling, Danvers, MA, USA) for 1 h at room temperature. After washing antibody binding was detected with the ECL detection reagent (Amersham, Freiburg, Germany). Bands were quantified with Quantity One Software (Biorad, München, Germany).

Where applicable, data are provided as mean ± SE, and *n* represents the number of oocytes investigated. All experiments were repeated with at least two batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA, and results with $P < 0.05$ were considered statistically significant.

Results

The first series of experiments explored whether the serum- and glucocorticoid-inducible kinase SGK1 stimulates the TRPV6-dependent current. In all experiments the cell membrane current was low in water-injected *Xenopus* oocytes (110.1 ± 4.1 nA; $n = 64$). Expression of TRPV6 increased the current in *Xenopus* oocytes to 264.9 ± 6.4 nA ($n = 88$). The current was further increased by additional expression of the constitutively active SGK1 mutant ^{S422D}SGK1 (Fig. 1a, b). Coexpression of the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve did not significantly modify the current in *Xenopus* oocytes expressing TRPV6 alone but significantly increased the current in *Xenopus* oocytes expressing both ^{S422D}SGK1 and TRPV6 (Fig. 1a, b). The Ca^{2+} dependence of the measured currents was confirmed by exposing oocytes to 5 μM Ca^{2+} ionophore ionomycin at defined extracellular Ca^{2+} concentrations. As shown in Fig. 1c, the current indeed increased in ionomycin-treated oocytes with increasing extracellular Ca^{2+} concentrations.

To disclose a possible effect of temperature on TRPV6-dependent currents in *Xenopus* oocytes, a further series of experiments was performed at room temperature and at 37°C. As a result, expression of TRPV6 resulted in a mean current of 1,542 ± 44 nA ($n = 4$) at room temperature, compared to a current of 2,803 ± 212 nA ($n = 4$) at 37°C. Coexpression of SGK1 and PIKfyve enhanced the TRPV6-dependent currents to 2,992 ± 700 nA ($n = 5$) at room temperature and to 4,442 ± 1,022 nA ($n = 5$) at 37°C. Accordingly, enhanced temperature increased the absolute current but did not affect the stimulating effect of SGK1 and PIKfyve.

The effect of PIKfyve was disrupted by mutation of the SGK1 phosphorylation site. As shown in Fig. 2, stimulation

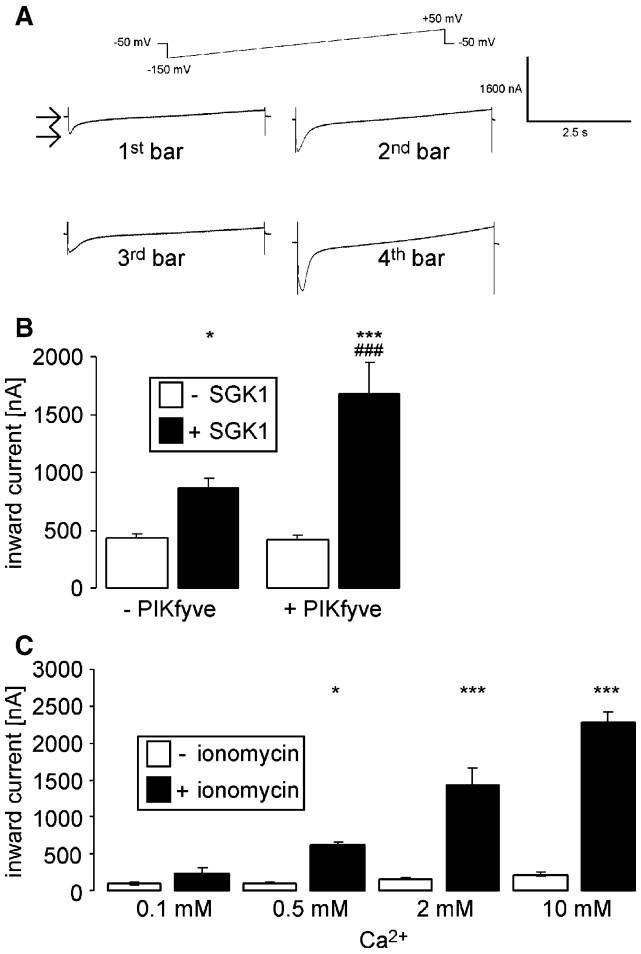

Fig. 1 Coexpression of PIKfyve stimulates the currents in TRPV6- and SGK1-expressing *Xenopus* oocytes. **a** The *upper panel* shows the applied two-electrode voltage-clamp protocol; the *lower panels* depict original voltage-clamp recordings corresponding to the *bars* in **b**. *Arrows* indicate the induced peak currents. **b** Arithmetic mean ± SE (n = 17–27) of currents in *Xenopus* oocytes injected with TRPV6 without (*open bar*) or with (*closed bars*) expression of constitutively active ^{S422D}SGK1 without (*left bars*) or with (*right bars*) additional expression of PIKfyve. Statistically significant differences from the respective current in *Xenopus* oocytes without expression of ^{S422D}SGK1: $*P < 0.05$ and $***P < 0.001$. Statistically significant difference from the respective current in *Xenopus* oocytes without expression of PIKfyve: $^{###}P < 0.001$. **c** Arithmetic mean ± SE (n = 4–5) of currents in *Xenopus* oocytes exposed to bath solution without (*open bars*) and with (*filled bars*) 5 μM Ca^{2+} ionophore ionomycin at the indicated extracellular Ca^{2+} concentration. Significant differences from the absence of ionomycin: $*P < 0.05$ and $***P < 0.001$

of TRPV6-induced current by PIKfyve was abrogated by replacement of serine 318 with alanine, the only SGK1 phosphorylation consensus sequence within the PIKfyve protein (^{S318A}PIKfyve). The observed current was similar in *Xenopus* oocytes expressing TRPV6 together with ^{S318A}PIKfyve and *Xenopus* oocytes expressing TRPV6 alone (Fig. 2). In *Xenopus* oocytes coexpressing TRPV6 together with constitutively active ^{S422D}SGK1, the

additional coexpression of ^{S318A}PIKfyve did not further increase the current (Fig. 2).

TRPV6 was similarly stimulated by protein kinase B PKB/Akt. Coexpression of the constitutively active PKB/Akt mutant ^{T308D,S473D}PKB significantly increased the current in TRPV6-expressing *Xenopus* oocytes (Fig. 3). Coexpression of the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve significantly increased the current in *Xenopus* oocytes expressing both ^{T308D,S473D}PKB and TRPV6 (Fig. 3). Accordingly, the current was significantly higher in *Xenopus* oocytes expressing TRPV6 together with PKB and PIKfyve than in *Xenopus* oocytes expressing TRPV6 with PKB alone, pointing to activation of coexpressed PIKfyve by PKB. Again, ^{S318A}PIKfyve failed to significantly modify the TRPV6-dependent current in oocytes expressing ^{T308D,S473D}PKB (Fig. 3).

In the next series of experiments immunohistochemistry was applied to determine whether ^{S422D}SGK1 or PIKfyve influences the TRPV6 protein abundance in the plasma membrane. As illustrated in Fig. 4a, the protein abundance

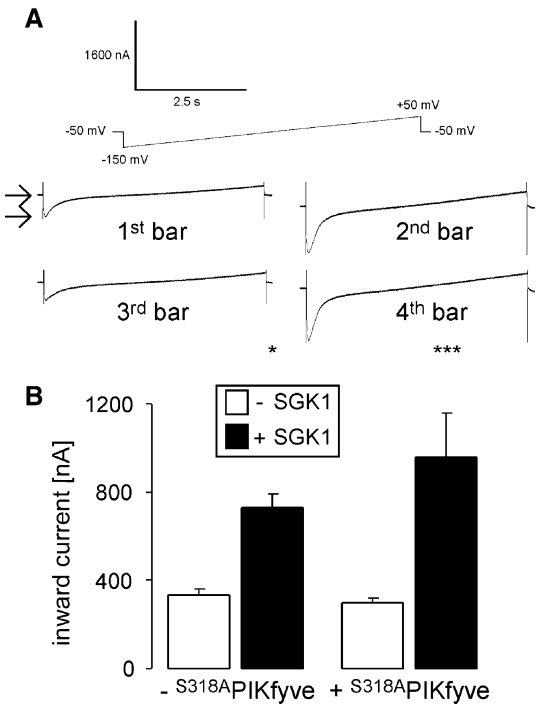

Fig. 2 Replacement of serine318 by alanine abrogates the stimulating effect of PIKfyve on TRPV6. **a** The *upper panel* shows the applied two-electrode voltage-clamp protocol; the *lower panels* depict original voltage-clamp recordings corresponding to the *bars* in **b**. *Arrows* indicate the induced peak currents. **b** Arithmetic mean ± SE (n = 13) of currents in *Xenopus* oocytes injected with TRPV6 without (*open bars*) or with (*filled bars*) expression of constitutively active ^{S422D}SGK1 without (*left bars*) or with (*right bars*) additional expression of mutated ^{S318A}PIKfyve. Statistically significant difference from the respective current in *Xenopus* oocytes without expression of ^{S422D}SGK1: $^{*}P < 0.05$ and $^{***}P < 0.001$

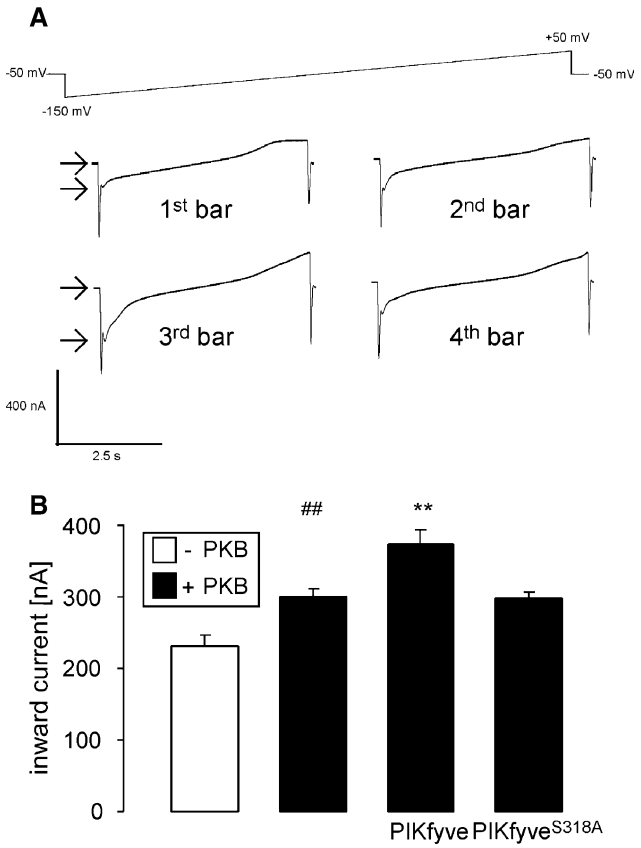

Fig. 3 Coexpression of PIKfyve stimulates the currents in TRPV6- and PKB-expressing *Xenopus* oocytes. **a** The *upper panel* shows the applied two-electrode voltage-clamp protocol; the *lower panels* depict original voltage-clamp recordings corresponding to the *bars* in **b**. *Arrows* indicate the induced peak currents. **b** Arithmetic mean ± SE (n = 17–21) of currents in *Xenopus* oocytes injected with TRPV6 without (*open bar*) or with (*filled bars*) expression of constitutively active ^{T308D,S473D}PKB without or with additional expression of PIKfyve (*middle right bar*) or mutated ^{S318A}PIKfyve (*outer right bar*). Statistically significant difference from the respective current in *Xenopus* oocytes without expression of ^{T308D,S473D}PKB: $^{\#\#}P < 0.01$. Statistically significant difference from the respective current in *Xenopus* oocytes without expression of PIKfyve: $^{**}P < 0.01$

of TRPV6 in the cell membrane of *Xenopus laevis* oocytes expressing TRPV6 was indeed enhanced by additional expression of ^{S422D}SGK1 and PIKfyve (Fig. 4a). The results of immunhistochemistry were confirmed by Western blot analysis of biotinylated surface proteins in *Xenopus laevis* oocytes (Fig. 4b, c)

Discussion

The present paper confirms that the serum-and glucocorticoid-inducible kinase SGK1 stimulates the TRPV6 channel, an observation reported previously (Bohmer et al. 2007). More importantly, the present observations reveal a novel regulator of TRPV6, i.e., the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve. Somewhat

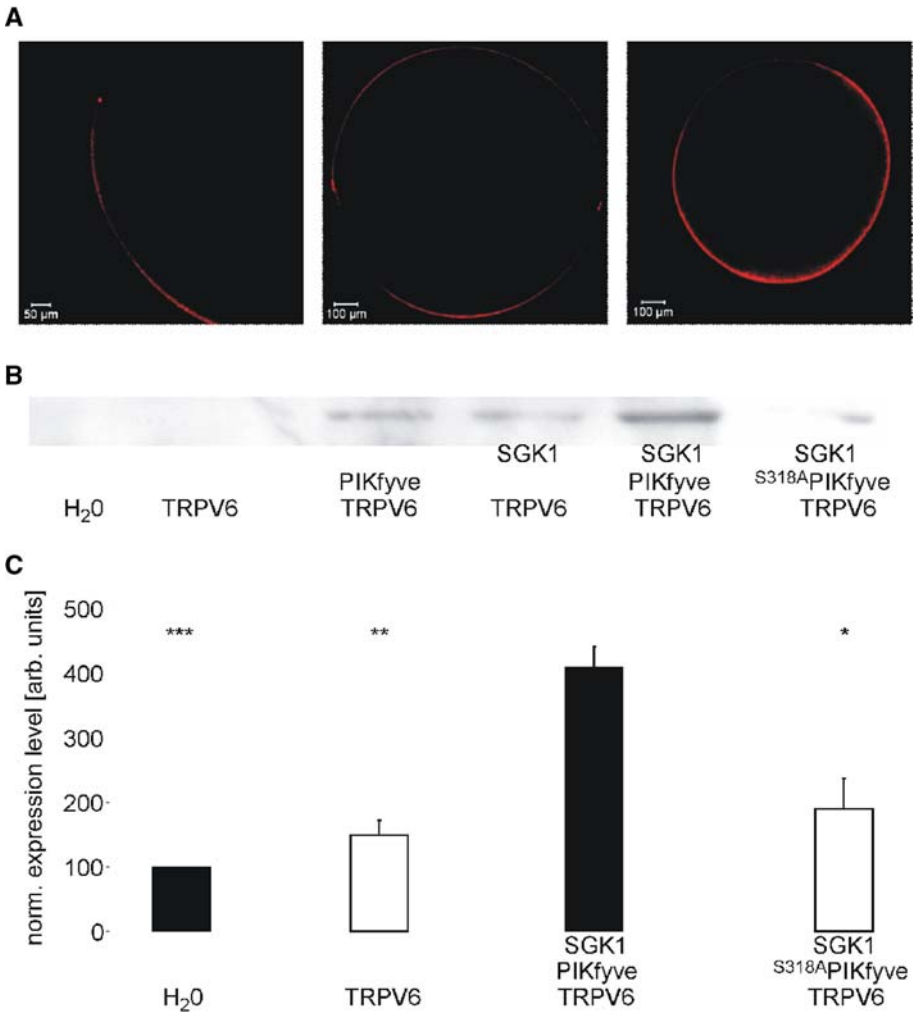

Fig. 4 Coexpression of PIKFyve enhances the TRPV6 protein abundance within the plasma membrane of oocytes expressing TRPV6 and SGK1. **a** Immunohistochemistry of surface TRPV6 protein abundance in *Xenopus* oocytes expressing TRPV6 alone (*left panel*), or together with ^{S422D}SGK1 (*middle panel*), or together with both ^{S422D}SGK1 and PIKfyve (*right panel*). **b** Western blot analysis of surface TRPV6 protein expression in *Xenopus* oocytes injected with water (*lane 1*), expressing TRPV6 alone (*lane 2*), together with PIKfyve (*lane 3*), together with ^{S422D}SGK1 (*lane 4*), together with both ^{S422D}SGK1 and PIKfyve (*lane 5*), or together with both ^{S422D}SGK1 and ^{S318A}PIKfyve (*lane 6*). **c** Quantitative densitometric analysis of the Western blot analysis of surface TRPV6 protein expression in *Xenopus* oocytes. Arithmetic mean ± SE (n = 5). Expression level data were normalized to the expression level of H_2O-injected oocytes in each of the five experiments. Thus, the standard deviation of the first bar is 0. Significant difference from expression of TRPV6 together with both ^{S422D}SGK1 and PIKfyve: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

surprisingly, PIKfyve did not stimulate TRPV6 when expressed without SGK1. The observations could be interpreted as evidence that PIKfyve requires activation by SGK1 to become effective. The stimulation of other carriers or channels by PIKfyve, however, did not require coexpression of SGK1 pointing to sufficient activation by an endogenous kinase (Klaus et al. 2009; Seebohm et al. 2007; Shojaiefard et al. 2007; Strutz-Seebohm et al. 2007b). Unpublished immunofluorescence data indeed revealed expression of endogenous SGK1 and PIKfyve in *Xenopus* oocytes. As PKB is an ubiquitously expressed protein, it is highly likely that it is similarly expressed in *Xenopus* oocytes. Possibly, the ability of endogenous SGK1 to activate PIKfyve is sensitive to the coexpressed carrier or channel protein. Moreover, the possibility cannot be ruled out that the expression and activity of endogenous SGK1 are variable. It should be further kept in mind that the observed current is a function of Ca^{2+} entry through TRPV6 and subsequent activation of Ca^{2+}-sensitive Cl^- channels. Accordingly, the observed current may be a complex function of TRPV6 activity. In any case, in the presence of SGK1, PIKfyve is a potent additional stimulator of TRPV6. SGK1 phosphorylates PIKfyve at serine in position 318, an effect believed to activate PIKfyve (Seebohm et al. 2007). Along those lines, the SGK1-resistant PIKfyve mutant ^{S318A}PIKfyve failed to stimulate

TRPV6 even in the presence of SGK1. Obviously, protein kinase B PKB/Akt is similarly capable to stimulate PIKfyve. Again, PIKfyve stimulated TRPV6 in the presence of ^{T308D,S473D}PKB, whereas mutant ^{S318A}PIKfyve failed to do so.

PKB- and SGK1-dependent regulation of TRPV6 presumably involves phosphorylation of PIKfyve by SGK1, PIKfyve-dependent formation of $PI(3,5)P_2$ and subsequent $PI(3,5)P_2$-dependent docking of TRPV6-containing membrane vesicles to the plasma membrane. Besides the putative stimulation of carrier protein insertion into the cell membrane, PIKfyve could enhance the protein abundance in the cell membrane by delaying the retrieval of channel protein from the membrane. Effects on transcription would not be apparent in the heterologous expression system. The present observations do not rule out additional PKB-, SGK1-, and/or PIKfyve-dependent mechanisms in the regulation of TRPV6.

PIKfyve has previously been shown to participate in the SGK1-dependent activation of the Cl^- channel ClC_2 (Klaus et al. 2009) and the K^+ channel KCNQ1/KCNE1 (Seebohm et al. 2007). As revealed by the present paper, PIKfyve is further involved in the SGK1-dependent regulation of the Ca^{2+} channel TRPV6. SGK1 stimulates additional ion channels (Lang et al. 2006), such as the Cl^- channels CFTR (Sato et al. 2007; Shaw et al. 2008), and ClC-Kb (Embark et al. 2004). Whether or not the regulation of those channels similarly involves PIKFyve remains to be determined.

The present experiments in the *Xenopus* oocyte system reveal that regulation of TRPV6 involves SGK1 and PIKfyve in the respective overexpressing cells. The present observations do not allow the prediction that this regulation plays a role under physiological, unchallenged conditions. As a matter of fact, even though SGK1 stimulates the electrogenic Na^+-glucose cotransporter SGLT1, electrogenic glucose transport is not significantly different between SGK1 knockout mice ($sgk1^{-/-}$) and their wild-type littermates ($sgk1^{+/+}$) under control conditions (Grahammer et al. 2006). Only challenging them with glucocorticoid treatment reveals a difference between $sgk1^{-/-}$ and $sgk1^{+/+}$ mice (Grahammer et al. 2006). Similarly, the stimulating effect of SGK1 on the Na^+/H^+ exchanger (Grahammer et al. 2006) and the peptide transporter (Boehmer et al. 2008b; Rexhepaj et al. 2009) requires the upregulation of SGK1 by glucocorticoids in vivo. Moreover, the stimulating effect of SGK1 on KCNQ1 becomes relevant for gastric acid secretion only following upregulation of SGK1 by glucocorticoids (Sandu et al. 2007), PPARgamma agonists (Rotte et al. 2009b), or APC deficiency (Rotte et al. 2009a). The ENaC-stimulating effect of SGK1 becomes relevant for renal Na^+ excretion only following exposure to a salt-deficient diet, unmasking the limited ability of the $sgk1^{-/-}$ mice to decrease their urinary Na^+ output (Wulff et al. 2002). In all those and further cases (Lang et al. 2006), the *Xenopus* oocyte system predicted a function which could indeed be identified as being SGK1-dependent under appropriate in vivo conditions.

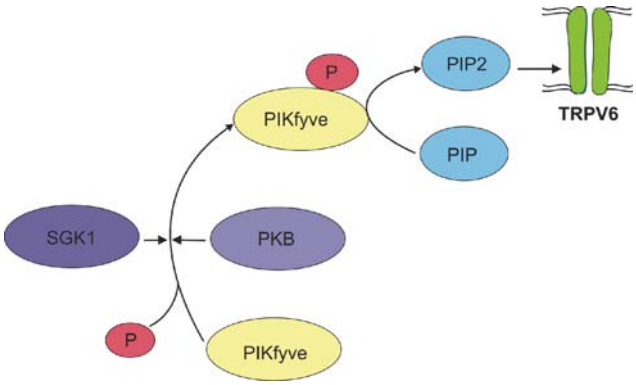

Fig. 5 Scheme illustrating the SGK1- and PKB/Akt-dependent regulation of TRPV6

In conclusion, SGK1 stimulates TRPV6, an effect at least partially accomplished by activation of PIKfyve (Fig. 5).

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