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Original Paper

SGK3 Sensitivity of Voltage Gated K^+ Channel $K_{v1.5}$ (KCNA5)

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Key Words
Serum & glucocorticoid inducible kinase • K^+ channel • Nedd4-2 • Na^+/K^+ ATPase • Ouabain

Abstract
Background: The serum & glucocorticoid inducible kinase isoform SGK3 is a powerful regulator of several transporters, ion channels and the Na^+/K^+ ATPase. Targets of SGK3 include the ubiquitin ligase Nedd4-2, which is in turn a known regulator of the voltage gated K^+ channel $K_{v1.5}$ (KCNA5). The present study thus explored whether SGK3 modifies the activity of the voltage gated K^+ channel KCNA5, which participates in the regulation of diverse functions including atrial cardiac action potential, activity of vascular smooth muscle cells, insulin release and tumour cell proliferation. ***Methods:*** cRNA encoding KCNA5 was injected into *Xenopus* oocytes with and without additional injection of cRNA encoding wild-type SGK3, constitutively active ^{S419D}SGK3, inactive ^{K191N}SGK3 and/or wild type Nedd4-2. Voltage gated K^+ channel activity was quantified utilizing dual electrode voltage clamp. ***Results:*** Voltage gated current in KCNA5 expressing *Xenopus* oocytes was significantly enhanced by wild-type SGK3 and ^{S419D}SGK3, but not by ^{K191N}SGK3. SGK3 was effective in the presence of ouabain (1 mM) and thus did not require Na^+/K^+ ATPase activity. Coexpression of Nedd4-2 decreased the voltage gated current in KCNA5 expressing *Xenopus* oocytes, an effect largely reversed by additional coexpression of SGK3. ***Conclusion:*** SGK3 is a positive regulator of KCNA5, which is at least partially effective by abrogating the effect of Nedd4-2.

Introduction

The serum & glucocorticoid inducible kinase isoform SGK3 up-regulates diverse transporters [1-5], the Na^+/K^+ ATPase [6] and channels [4, 5, 7, 8] including Ca^{2+} channels [7, 9] and voltage-gated K^+ channels [4, 10, 11]. The cardiac channels regulated by SGK3 include $K_V11.1$ channels, which play an important role in the repolarization phase of the cardiac action potential [11]. The related kinase SGK1 has previously been shown to regulate the voltage gated K^+ channel KCNA5 [12], a channel implicated in a variety of functions including

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the proliferation and migration of normal and tumour cells [13, 14], repolarization in cardiac atria [15-18], pulmonary artery smooth muscle cell activity [19, 20], and insulin release [12].

KCNA5 is a target of the ubiquitin ligase Nedd4-2 , which is in turn a target of SGK isoforms [21]. Along those lines, SGK1 is effective by inhibiting Nedd4-2 and thus increases KCNA5 protein abundance in the cell membrane [22]. Conversely Nedd4-2 decreases the KCNA5 protein abundance in the cell membrane and ablation of the SGK1 phosphorylation sites in the Nedd4-2 protein significantly blunted the effect of the kinase on KCNA5 protein abundance in the cell membrane [22].

The present study explored, whether SGK3 similarly modifies the activity of KCNA5. To this end, KCNA5 was expressed in *Xenopus* oocytes without or with additional expression of wild type SGK3, constitutively active ^{S419D}SGK3, or inactive ^{K191N}SGK3. Additional experiments were performed in oocytes expressing wild type Nedd4-2. The voltage gated K^+ current was determined in those oocytes by 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 and the surgical procedures on the adult *Xenous laevis* frogs were reviewed and approved by the respective government authority of the state Baden-Württemberg (Regierungspräsidium) prior to the start of the study (Anzeige für Organentnahme nach §36).

Constructs

Constructs encoding mouse wild-type KCNA5 [23, 24], wild type SGK3 [25], constitutively active ^{S419D}SGK3 [26], inactive ^{K191N}SGK3 [26] and wild type Nedd4-2 [2] were used for generation of cRNA as described previously [27-29].

Voltage clamp in Xenopus oocytes

Xenopus oocytes were prepared as previously described [30-32]. 2.5 ng cRNA encoding KCNA5 and 10 ng of cRNA encoding wild-type SGK3, constitutively active ^{S419D}SGK3 , inactive ^{K191N}SGK3 or wild type Nedd4-2 were injected on the same day after preparation of the oocytes. Where indicated oocytes expressing KCNA5 without or with additional expression of SGK3 were treated with 1 mM ouabain for 10 minutes before measurement and ouabain was superfused during the measurement. KCNA5 activation kinetics were determined from peak current quantification during a -60 mV test-pulse following 200ms pre-pulses from -50 to +50 mV. Determination of KCNA5 inactivation was based on a two-pulse protocol containing a 1 s pre-pulse from potentials of -60 to +40 mV followed by a 200 ms test-pulse to +70 mV. Normalized test-pulse peak currents were plotted versus pre-pulse voltage and fitted to the Boltzmann equation yielding $V_{1/2}$ for channel activation and inactivation. The oocytes were maintained at 17°C in ND96A, a storage solution containing (in mM): 88.5 NaCl, 2 KCl, 1 $MgC1_2$, 1.8 $CaC1_2$, 5 HEPES (pH 7.5), 5 sodium pyruvate ($C_3H_3NaO_3$), Gentamycin (100 mg/l), Tetracycline (50 mg/l), Ciprofloxacin (1.6 mg/l), Theophylline (90 mg/l) [33-36]. The voltage clamp experiments were performed at room temperature 3 days after the first injection [37-39]. KCNA5 channel currents were elicited every 20 s with 2 s pulses from -80 to +50 mV in 20 second increments of 10 mV steps from a holding potential of -100 mV. The data were filtered at 1 kHz 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) [40-42]. The control superfusate (ND96B) contained (in mM): 93.5 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 2.5 NaOH and 5 HEPES, pH 7.4. The flow rate of the superfusion was approx. 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s [43, 44].

Statistical analysis

Data are provided as means ± SEM, n represents the number of oocytes investigated. As different batches of oocytes may yield different results, comparisons were always made within a given oocyte batch. 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 (Tukey test or Kruskal-Wallis test) or t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored whether the serum & glucocorticoid inducible kinase isoform SGK3 participates in the regulation of the voltage gated K^+ channel KCNA5. To this end, cRNA encoding KCNA5 was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding wild-type SGK3 and the peak K^+ current (I_K) quantified in those oocytes utilizing dual electrode voltage clamp. As illustrated in Fig. 1, I_K was negligible in water-injected oocytes indicating that the oocytes did not express appreciable voltage gated K^+ channels. In contrast, sizable voltage gated K^+ currents were observed in *Xenopus* oocytes expressing KCNA5. The additional coexpression of wild-type SGK3 was followed by a

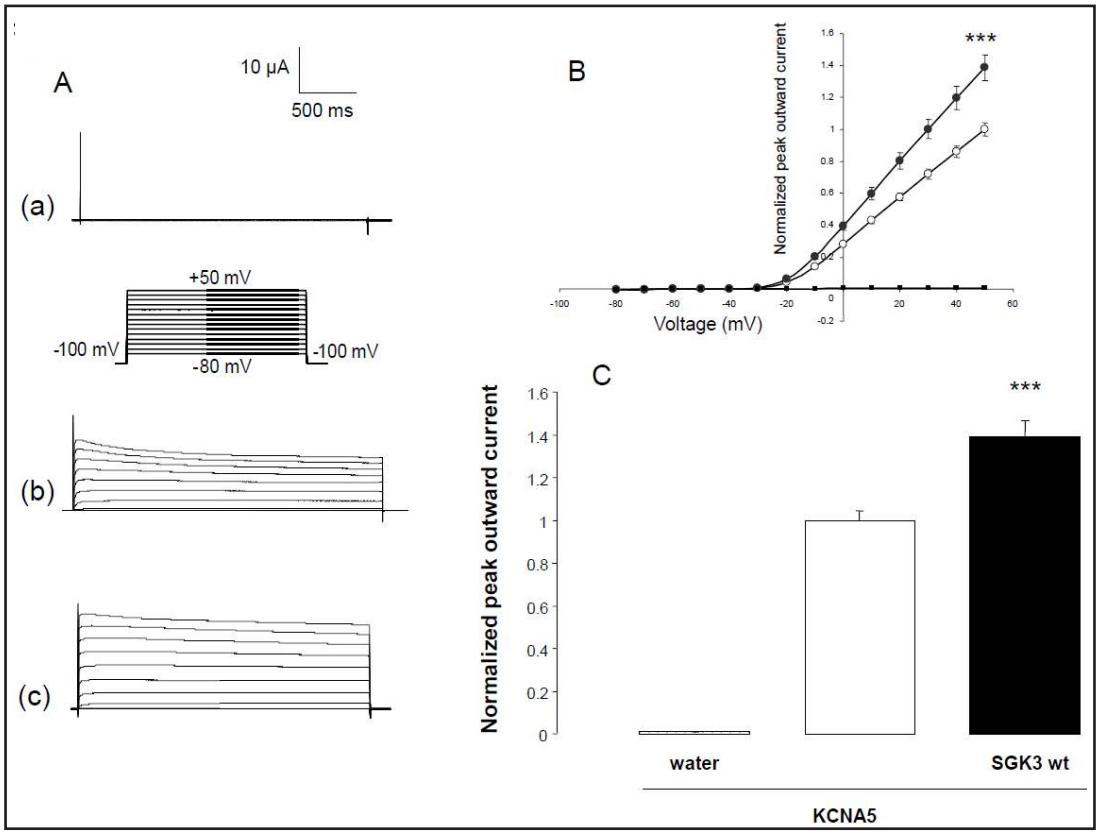

Fig. 1. Coexpression of wild type SGK3 increases the K^+ current in KCNA5-expressing *Xenopus* oocytes. A: Representative original tracings showing currents in *Xenopus* oocytes injected with water (a), expressing KCNA5 alone (b) or expressing KCNA5 with additional coexpression of wild-type SGK3 (c). Currents were activated by depolarization from -80 to +50 mV in 20 second increments of 10 mV steps from a holding potential of -100 mV. B: Arithmetic means ± SEM (n = 6 - 26) of the normalized depolarization-induced KCNA5 peak current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus* oocytes injected with water (black squares) or expressing KCNA5 without (white circles) or with (black circles) additional coexpression of wild-type SGK3. Peak currents were normalized to the mean peak current at +50 mV in *Xenopus* oocytes injected with cRNA encoding KCNA5 alone. C: Arithmetic means ± SEM (n = 6 - 26) of the normalized KCNA5 -peak current at +50 mV in *Xenopus* oocytes injected with water (dotted bar), or expressing KCNA5 without (white bar) or with (black bar) additional coexpression of wild-type SGK3. *** ($p<0.001$) indicates statistically significant difference from oocytes expressing KCNA5 alone (ANOVA-one way).

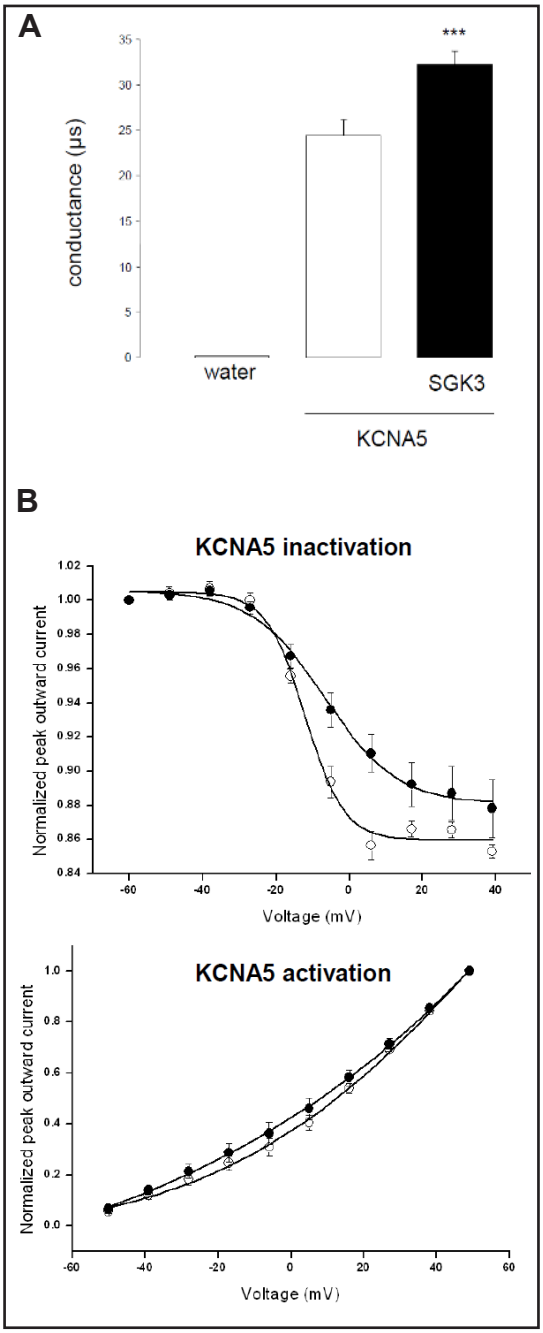

Fig. 2. Coexpression of wild type SGK3 increases the K^+ conductance but does not significantly modify channel kinetics in KCNA5-expressing *Xenopus* oocytes. A. Arithmetic means ± SEM (n = 6 - 26) of the conductance analyzed between 20 mV and 50 mV in *Xenopus* oocytes injected with water (dotted bar), or expressing KCNA5 without (white bar) or with (black bar) additional coexpression of wild-type SGK3. B. KCNA5 kinetics in *Xenopus* oocytes expressing KCNA5 without (white circles) or with (black circles) additional coexpression of wild-type SGK3. KCNA5 activation kinetics was determined from peak current quantification during a -60 mV test-pulse following 200ms pre-pulses from -50 to +50 mV. Determination of KCNA5 inactivation was based on a two-pulse protocol containing a 1 s pre-pulse from potentials of -60 to +40 mV followed by a 200 ms test-pulse to +70 mV. *** ($p<0.001$) indicates statistically significant difference from oocytes expressing KCNA5 alone.

significant increase of I_K. As illustrated in Fig. 2, expression of wild-type SGK3 increased the K^+ conductance and did not shift the KCNA5 kinetics.

Further experiments explored, whether the effect of wild-type SGK3 on KCNA5 was modified by mutations affecting kinase activity. As illustrated in Fig. 3, the effect of wild type SGK3 was mimicked by the constitutively active ^{S419D}SGK3, but not by the inactive ^{K191N}SGK3, i.e. coexpression of ^{S419D}SGK3 but not of ^{K191N}SGK3 significantly increased I_K in KCNA5 expressing oocytes.

Several K^+ channels have been shown to be sensitive to Na^+/K^+ ATPase activity [45]. Thus, the up-regulation of KCNA5 by SGK3 could have been secondary to the known stimulation of Na^+/K^+ ATPase activity by the kinase [6]. In order to test for a putative role of the Na^+/K^+ ATPase activity in the up-regulation of KCNA5 channel activity by SGK3, experiments were repeated in the presence of Na^+/K^+ ATPase inhibitor ouabain (1 mM). Oocytes expressing KCNA5 without or with additional expression of SGK3 were treated with 1 mM ouabain for 10 minutes before measurement and ouabain was superfused during the measurement. As illustrated in Fig. 4, even in the presence of ouabain, coexpression of SGK3 significantly increased I_K in *Xenopus* oocytes expressing KCNA5.

Additional experiments addressed the putative involvement of the ubiquitin ligase Nedd4-2 in the SGK3 sensitive regulation of KCNA5. As illustrated in Fig. 5, coexpression of Nedd4-2 was followed by a marked and significant decrease of I_K in *Xenopus* oocytes expressing KCNA5. The down-regulation of I_K was almost completely reversed by the additional coexpression of SGK3.

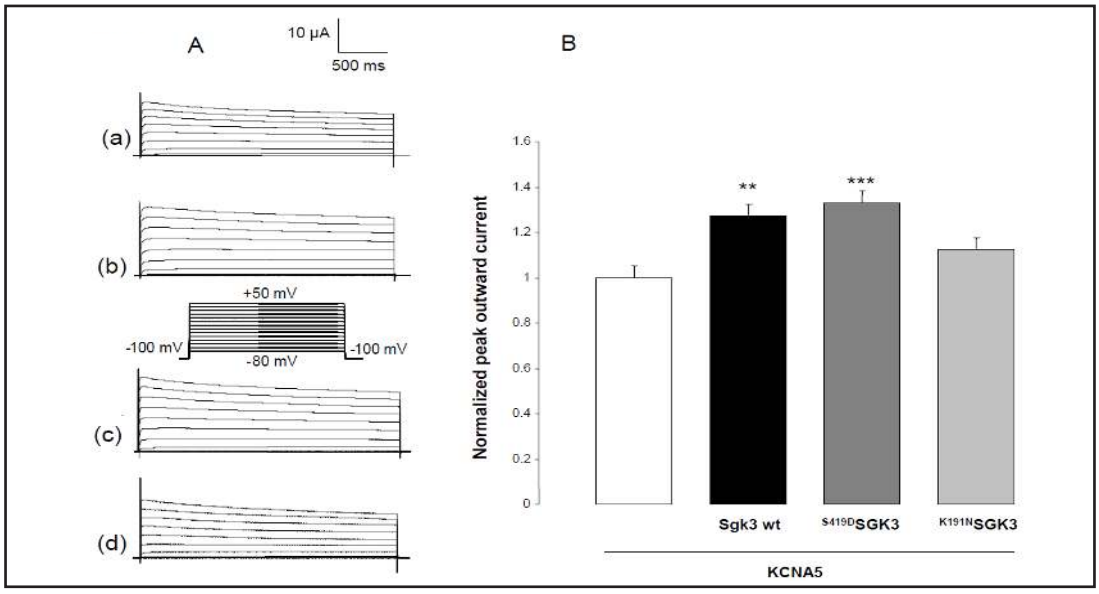

Fig. 3. The effect of wild type SGK3 on KCNA5 was mimicked by constitutively active ^{S419D}SGK3 but not by inactive ^{K191N}SGK3. A: Representative original tracings showing currents in *Xenopus* oocytes expressing KCNA5 alone (a), expressing KCNA5 together with wild-type SGK3 (b), expressing KCNA5 together with constitutively active ^{S419D}SGK3 (c), or expressing KCNA5 with inactive ^{K191N}SGK3 (d). B: Arithmetic means ± SEM (n = 17) of the normalized KCNA5 -peak current at +50 mV in *Xenopus laevis* oocytes expressing KCNA5 alone (white bar) or expressing KCNA5 together with wild type SGK3 (black bar), with constitutively active ^{S419D}SGK3 (dark grey bars), or with inactive ^{K191N}SGK3 (light grey bar). ** ($p<0.01$), *** ($p<0.001$) indicates statistically significant difference from oocytes expressing KCNA5 alone (ANOVA-one way).

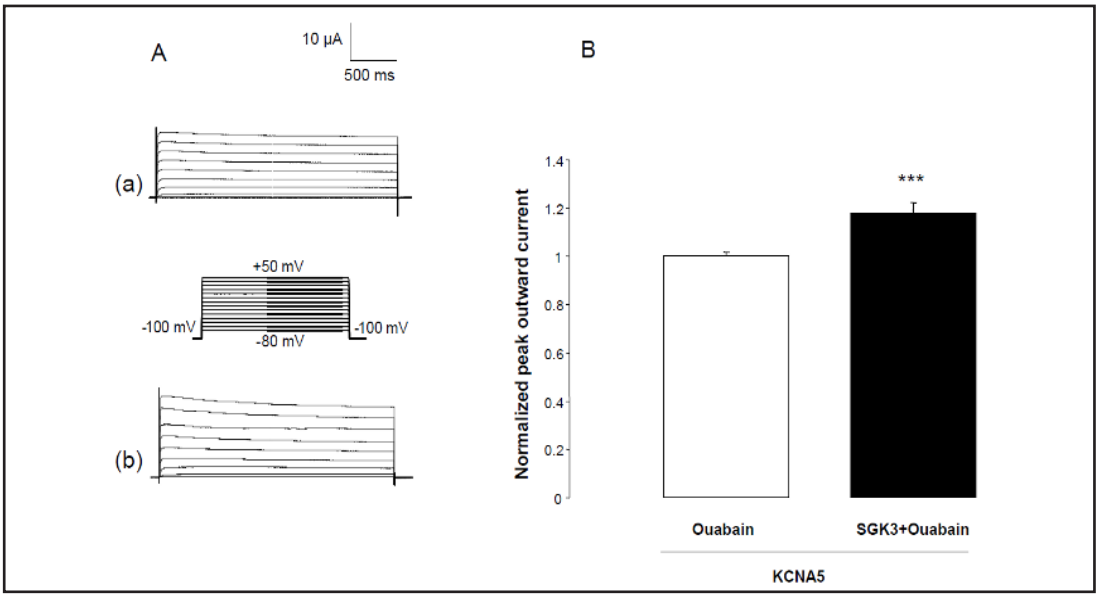

Fig. 4. SGK3 increases the K^+ current in ouabain treated KCNA5-expressing *Xenopus* oocytes. A: Representative original tracings showing currents in ouabain (1 mM) treated *Xenopus* oocytes expressing KCNA5 alone (a) or expressing KCNA5 together with wild-type SGK3 (b). Currents were activated by depolarization from -80 to +50 mV in 20 second increments of 10 mV steps from a holding potential of -100 mV. B: Arithmetic means ± SEM (n = 19) of the normalized KCNA5 -peak current at +50 mV in ouabain treated *Xenopus* oocytes expressing KCNA5 without (white bar) or with (black bar) additional coexpression of wild-type SGK3. *** ($p<0.001$) indicates statistically significant difference from ouabain treated oocytes expressing KCNA5 alone (ANOVA-one way).

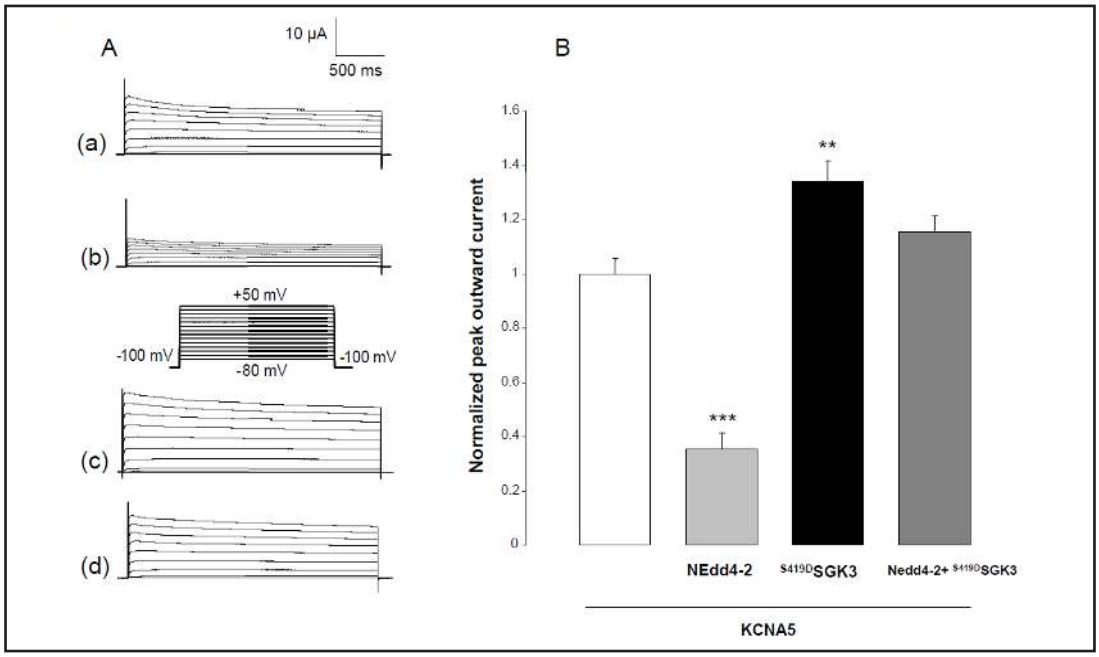

Fig. 5. SGK3 reverses the decline of K^+ current in KCNA5-expressing *Xenopus* oocytes following coexpression of Nedd4-2. A: Representative original tracings showing currents in *Xenopus* oocytes expressing KCNA5 alone (a) or expressing KCNA5 with additional coexpression of Nedd4-2 (b) with additional coexpression of constitutively active ^{S419D}SGK3 (c), or with additional coexpression of both, ^{S419D}SGK3 and Nedd4-2 (d). Currents were activated by depolarization from -80 to +50 mV in 20 second increments of 10 mV steps from a holding potential of -100 mV. B: Arithmetic means ± SEM (n = 16 - 18) of the normalized KCNA5 -peak current at +50 mV in *Xenopus* oocytes expressing KCNA5 alone (white bar) or expressing KCNA5 with additional coexpression of Nedd4-2 (light grey bar), with additional coexpression of constitutively active ^{S419D}SGK3 (black bar), or with additional coexpression of both, ^{S419D}SGK3 and Nedd4-2 (dark grey bar). *** ($p<0.001$), ** ($p<0.01$) indicates statistically significant difference from oocytes expressing KCNA5 alone (ANOVA-one way).

Discussion

The present study discloses a positive effect of the serum & glucocorticoid inducible kinase isoform SGK3 on the voltage gated K^+ channel KCNA5. Coexpression of SGK3 leads to up-regulation of the voltage gated current in KCNA5 expressing *Xenopus* oocytes. The effect of wild type SGK3 is mimicked by the constitutively active ^{S419D}SGK3, but not by the inactive mutant ^{K191N}SGK3. Thus, kinase activity is apparently required for the effect of SGK3 on KCNA5. The observation does not necessarily indicate that SGK3 phosphorylates the KCNA5 channel protein. Instead, SGK3 could exert its effect on KCNA5 by phosphorylating regulators of the channel protein thus indirectly modifying its regulation. Such a regulator is Nedd4-2, an ubiquitin ligase ubiquitinating target proteins thus tagging them for degradation [46]. KCNA5 is a known target of Nedd4-2 [22, 23].

The present observations do not allow any safe conclusions about the *in vivo* significance of SGK3 sensitive KCNA5 regulation. The effect of SGK3 on KCNA5 is small and may not be sufficient to significantly interfere with KCNA5 dependent cellular functions. Moreover, the effect of SGK3 is shared by SGK1 [12, 22] which could thus easily replace SGK3. Functions sensitive to K^+ channel activity include cell volume regulation [47-49]. Cell volume sensitive K^+ channels include KCNA5 channels [14, 50-53]. SGK3 sensitive regulation of KCNA5 may in addition foster cell proliferation and survival of tumour cells [13, 54, 55]. SGK3 has been shown to support survival and proliferation of some tumour cells [56-63]. Further functions of KCNA5 include repolarization of atrial myocardial cells [15-18], pulmonary arterial vascular dilatation [19, 20], and inhibition of insulin release [12]. Clearly, additional

experimental evidence is required shedding light on the significance of SGK3-sensitive regulation of KCNA5.

In conclusion, the serum & glucocorticoid inducible kinase isoform SGK3 up-regulates the voltage gated K^+ channel KCNA5, an effect possibly contributing to the regulation of cell membrane potential, cell volume and cell proliferation.

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Disclosure Statement

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

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