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Up-Regulation of the Inwardly Rectifying K^+ Channel Kir2.1 (KCNJ2) by Protein Kinase B (PKB/Akt) and PIKfyve

Carlos Munoz · Ahmad Almilaji · Iwan Setiawan · Michael Föller · Florian Lang

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Abstract The inward rectifier K^+ channel Kir2.1 contributes to the maintenance of the resting cell membrane potential in excitable cells. Loss of function mutations of KCNJ2 encoding Kir2.1 result in Andersen-Tawil syndrome, a disorder characterized by periodic paralysis, cardiac arrhythmia, and dysmorphic features. The ubiquitously expressed protein kinase B (PKB/Akt) activates the phosphatidylinositol-3-phosphate-5-kinase PIKfyve, which in turn regulates a variety of carriers and channels. The present study explored whether PKB/PIKfve contributes to the regulation of Kir2.1. To this end, cRNA encoding Kir2.1 was injected into *Xenopus* oocytes with and without additional injection of cRNA encoding wild type PKB (PKB), constitutively active ^{T308D,S473D}PKB or inactive ^{T308A,S473A}PKB. Kir2.1 activity was determined by two-electrode voltage-clamp. As a result, PKB and ^{T308D,S473D}PKB, but not ^{T308A,S473A}PKB, significantly increased Kir2.1-mediated currents. The effect of PKB was mimicked by coexpression of PIKfyve but not of ^{S318A}Pikfyve lacking the PKB phosphorylation site. The decay of Kir2.1-mediated currents after inhibition of channel insertion into the cell membrane by brefeldin A (5 μM) was similar in oocytes expressing Kir2.1 + PKB or Kir2.1 + PIKfyve to those expressing Kir2.1 alone, suggesting that PKB and PIKfyve influence channel insertion into rather than channel retrieval from the cell membrane. In conclusion, PKB and PIKfyve are novel regulators of Kir2.1.

Keywords Cardiomyocytes · Cell membrane potential · Heart · KCNJ2 · Neurons · PIKfyve

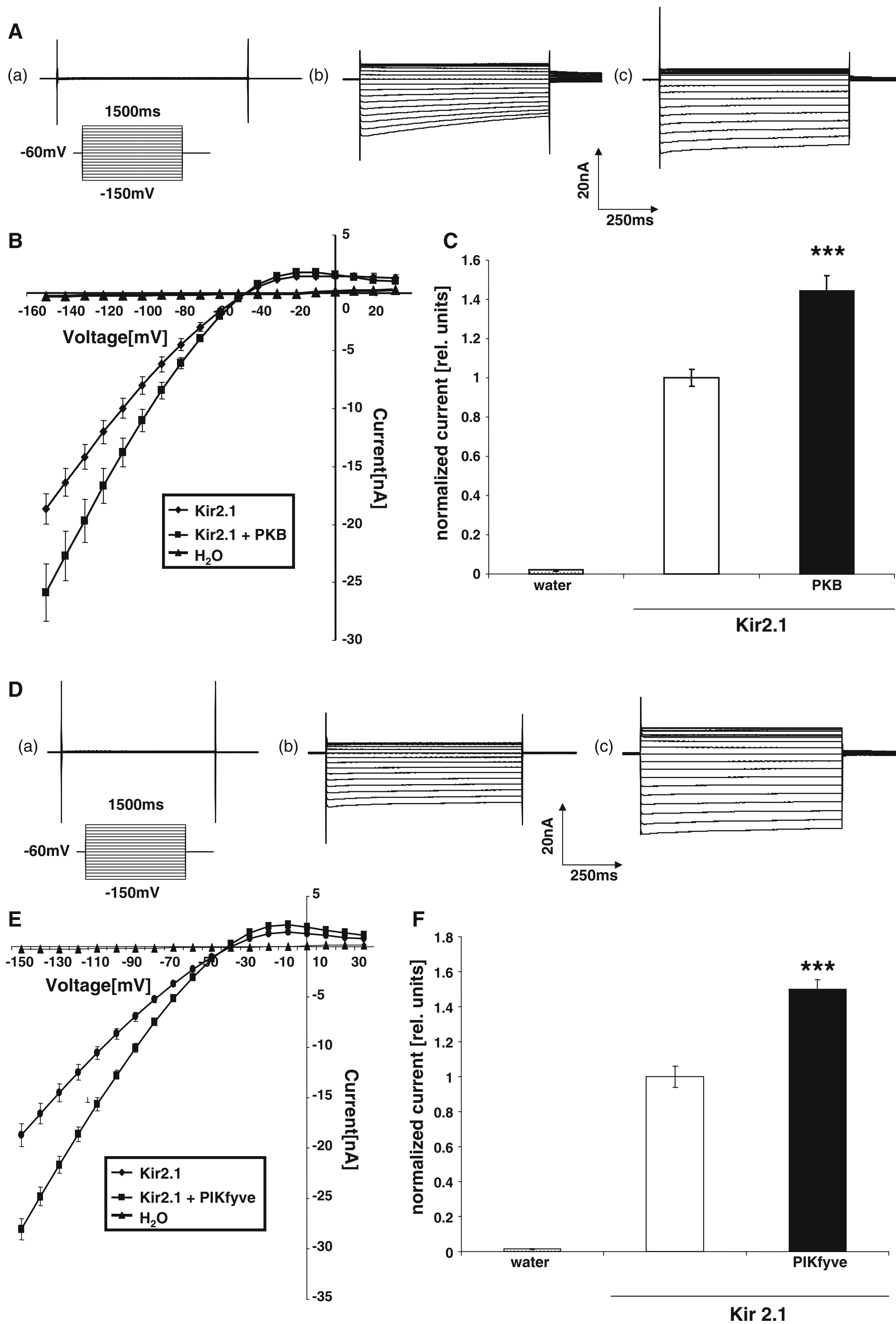
C. Munoz · A. Almilaji · M. Föller · F. Lang (✉)
Department of Physiology, University of Tübingen, Gmelinstr 5, 72076 Tübingen, Germany
e-mail: florian.lang@uni-tuebingen.de

I. Setiawan
Department of Physiology, Faculty of Medicine, Universitas Padjadjaran, Banding, Indonesia

M. Föller
Campbell Family Institute for Breast Cancer Research, Ontario Cancer Institute, University Health Network (UHN), 620 University Avenue, Toronto, ON M5G 2C1, Canada

Introduction

Inwardly rectifying K^+ channels of the Kir2 (*KCNJ*, IRK) subfamily participate in the maintenance of the resting membrane potential in cardiac myocytes, skeletal muscle, and neurons (Hibino et al. 2010; Lopatin and Nichols 2001). In cardiomyocytes Kir2.1 accounts for the inwardly rectifying K^+ current I_{K1} (Liu et al. 2001a; Preisig-Müller et al. 2002; Tristani-Firouzi and Etheridge 2010). Gain-of-function mutations of Kir2.1 result in atrial fibrillation (Preisig-Müller et al. 2002; Xia et al. 2005). Loss-of-function mutations of Kir2.1 underlie Andersen-Tawil syndrome (ATS), a rare familial disorder characterized by potassium-sensitive periodic paralysis, ventricular arrhythmias and dysmorphic features including syndactyly and altered face shapes (Andersen et al. 1971; Plaster et al. 2001; Sansone et al. 1997; Tawil et al. 1994). Kir2 channels are regulated by mitochondria (Collins and Larson 2002; Lodge and Normandin 1997) and contribute to ischemic preconditioning (Diaz et al. 2004). Kir2.1 is regulated by arachidonic acid (Liu et al. 2001b), cholesterol (Romanenko et al. 2004), PKC (Zitron et al. 2004), tyrosine phosphorylation (Henry et al. 1996; Wischmeyer et al. 1998), AKAP79 (Dart and Leyland 2001), Rho (Jones 2003), TNF-α (Vicente et al. 2004), Chapsyn 110 (Leyland


A
(a)
(b)
(c)
1500ms
-60mV
-150mV
20nA
250ms
B
Voltage[mV]
Current[nA]
Kir2.1
Kir2.1 + PKB
H_2O
C
normalized current [rel. units]
***
water
PKB
Kir2.1
D
(a)
(b)
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1500ms
-60mV
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20nA
250ms
E
Voltage[mV]
Current[nA]
Kir2.1
Kir2.1 + PIKfyve
H_2O
F
normalized current [rel. units]
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water
PIKfyve
Kir 2.1

◀ **Fig. 1** Coexpression of wild type PKB or of wild type PIKfyve increased the inwardly rectifying current in Kir2.1-expressing *Xenopus* oocytes. **A** Original tracings illustrating currents determined in *Xenopus* oocytes injected with water (**a**), or expressing Kir2.1 without (**b**) or with wild type PKB (**c**). **B** Arithmetic means ± SEM ($n = 10$–19) of the current as a function of voltage in *Xenopus* oocytes injected with water (*triangles*) or expressing Kir2.1 without (*circles*) or with additional expression of PKB (*squares*). **C** Arithmetic means ± SEM ($n = 10$–19) of the normalized current at −150 mV in *Xenopus* oocytes injected with water (*first bar*) or expressing Kir2.1 without (*second bar*) or with (*third bar*) additional expression of PKB. ***indicates statistically significant ($p < 0.001$) difference from oocytes expressing Kir2.1 alone. **D** Original tracings illustrating currents determined in *Xenopus* oocytes injected with water (**a**), or expressing Kir2.1 without (**b**) or with PIKfyve (**c**). **E** Arithmetic means ± SEM ($n = 10$–27) of the current as a function of voltage in *Xenopus* oocytes injected with water (*triangles*) or expressing Kir2.1 without (circles) or with additional expression of PIKfyve (*squares*). **F** Arithmetic means ± SEM ($n = 10$–27) of the normalized current at −150 mV in *Xenopus* oocytes injected with water (*first bar*) or expressing Kir2.1 without (*second bar*) or with (*third bar*) additional expression of PIKfyve. ***indicates statistically significant ($p < 0.001$) difference from oocytes expressing Kir2.1 alone

and Dart 2004), filamin-A (Sampson et al. 2003), PSD95 (Nehring et al. 2000), SAP97, CASK, Veli, and Mint1 (Leonoudakis et al. 2004).

Kir2.1 channels are further regulated by phosphatidylinositol 4,5-bisphosphate PI(4,5)P_2 (Donaldson et al. 2003; Rohács et al. 1999; Soom et al. 2001; Xiao et al. 2003). The related phosphatidylinositol 3,5-bisphosphate (PI(3,5)P_2) is generated by the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve (Hill et al. 2010; Ikonomov et al. 2002; Sbrissa et al. 1999, 2002, 2004). PIKfyve has been shown to regulate endosomal transport (Hill et al. 2010; Ikonomov et al. 2001, 2003, 2006; Rusten et al. 2006; Rutherford et al. 2006). As a result, PIKfyve has been shown to play 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. 2007), glutamate transporters (Alesutan et al. 2010; Gehring et al. 2009b), Cl^- channels (Gehring et al. 2009a; Klaus et al. 2009), Ca^{2+} channels (Sopjani et al. 2010; Tsuruta et al. 2009) and the K^+ channel KCNQ1/KCNE1 (Seebohm et al. 2007). PIKfyve is phosphorylated and thus activated by protein kinase B (PKB/Akt), which is well known to regulate glucose carriers through activation of PIKfyve (Hill et al. 2010).

The present study explored the regulation of Kir2.1 by PIKfyve and PKB. To this end, cRNA encoding Kir2.1 was injected into *Xenopus* oocytes either without or with additional injection of cRNA encoding wild type or mutated PIKfyve and/or PKB. Channel activity was subsequently determined by the dual electrode voltage clamp.

Methods

Constructs

For generation of cRNA (Dërmaku-Sopjani et al. 2011) constructs were used encoding wild type human Kir2.1 (Ureche et al. 2008), wild type PIKfyve, mutated ^{S318A}PIKfyve lacking the PKB phosphorylation consensus sequence (Berwick et al. 2004; Seebohm et al. 2007), wild type PKB, constitutively active ^{T308D,S473D}PKB, single mutants ^{T308A}PKB or ^{S473A}PKB and inactive ^{T308A,S473A}PKB (Klaus et al. 2008). The constructs were used for the generation of cRNA as described previously (Mohamed et al. 2010; Strutz-Seebohm et al. 2011). PKB cDNA was 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.

Voltage Clamp in *Xenopus* Oocytes

Xenopus oocytes were prepared as previously described (Böhmer et al. 2010; Rexhepaj et al. 2010). cRNA encoding Kir2.1 (10 ng) was injected with or without additional injection of 10 ng cRNA encoding wild type or mutated PKB and/or Pikfyve on the day of preparation of the *Xenopus* oocytes. All experiments were performed at room temperature 3 days after injection. In two-electrode voltage-clamp experiments Kir2.1 channel currents were elicited every 20 s with 1 s pulses from −150 mV to +30 mV applied from a holding potential of −60 mV. Pulses were applied in 10 mV increments. The oocytes were maintained at 17 °C in a solution containing 88.5 mM NaCl, 2 mM KCl, 1.8 mM $CaC1_2$, 1 mM $MgC1_2$, 5 mM HEPES, 0.11 mM tetracycline, 4 μM ciprofloxacin, 0.22 mM gentamycin (Refobacin), 0.5 mM of the oocyte maturation inhibitor theophylline (Euphylong) (Bravo et al. 1978; O'Connor and Smith 1976), as well as 5 mM sodium pyruvate. The pH was adjusted to 7.4 by addition of NaOH. Brefeldin A was used to discriminate between enhanced insertion of Kir2.1 into the cell membrane and delayed retrieval of Kir2.1 from the cell membrane. Brefeldin A inhibits the trans-Golgi network (TGN) thereby preventing the insertion of newly synthesized channel proteins into the membrane (Kirkbride et al. 2012; Klausner et al. 1992). In detail, cRNA encoding Kir2.1 and PKB were injected on the day of preparation of the oocytes. Brefeldin A (5 μM, Sigma, Schnelldorf, Germany) was added to the culture medium 24 h later (for a total 48 h incubation with brefeldin A) or 48 h later (for a total 24 h incubation with brefeldin A), All electrophysiological recordings were performed 72 h after the cRNA injection. Control superfusate was composed of 88 mM NaCl, 10 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, titrated

to pH 7.4 by NaOH. The data were filtered at 1 kHz and recorded with Digidata 1322A A/D-D/A converter and Clampex V.4.2 software for data acquisition and analysis (Axon Instruments, Union City, CA, USA) (Eckey et al. 2010). The analysis of the data was performed with Clampfit 9.2 (Axon Instruments) software (Hosseinzadeh et al. 2011).

Statistical Analysis

Data are provided as means ± SEM; n represents the number of oocytes. All oocyte experiments were repeated with at least 3 batches; 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

To possibly uncover an effect of protein kinase B (PKB) on Kir2.1, cRNA encoding Kir2.1 was injected with or without cRNA encoding PKB into *Xenopus* oocytes. As a result, an inwardly rectifying current (I_{Kir}) was observed in Kir2.1-expressing, but not in water-injected *Xenopus* oocytes (Fig. 1). Coexpression of PKB was followed by a significant increase in I_{Kir} (Fig. 1A–C). Similarly, coexpression of Pikfyve significantly enhanced I_{Kir} (Fig. 1D–F).

The effect of wild type PKB was mimicked by constitutively active ^{T308D,S473D}PKB but not by the catalytically inactive mutant ^{T308A,S473A}PKB (Fig. 2). Coexpression of the ^{T308A}PKB mutant, but not of the ^{S473A}PKB mutant resulted in a significant increase in I_{Kir} (Fig. 2). Accordingly, the phosphorylation at S473 was critically important for the effect of PKB on Kir2.1 whereas the phosphorylation at T308 appeared to be dispensable.

A further series of experiments explored whether the effect of PKB involved phosphatidylinositol-3-phosphate-5-kinase (PIKfyve). Again, I_{Kir} was significantly higher in *Xenopus* oocytes coexpressing PKB than in *Xenopus* oocytes expressing Kir2.1 alone (Fig. 3). Importantly, the additional coexpression of PIKfyve, but not of ^{S31}PIKfyve lacking the PKB phosphorylation site led to a further significant increase in I_{Kir} (Fig. 3).

Stimulation of Kir2.1 by PKB or PIKfyve could have resulted from accelerated insertion of channel protein into the cell membrane or delayed retrieval of channel protein from the cell membrane. To discriminate between those two possibilities, voltage clamp experiments were performed in the absence and presence of brefeldin A (5 μM), an inhibitor of protein insertion into the cell membrane. As shown in Fig. 4, brefeldin A treatment was followed by a decay of the current in *Xenopus* oocytes expressing Kir2.1 with PKB (Fig. 4A) or PIKfyve (Fig. 4B) which was similar to the decay of the current in oocytes expressing Kir2.1 alone. This observation indicates that PKB is not primarily effective by delaying channel retrieval from the cell membrane.

Discussion

The present observations demonstrate that protein kinase B (PKB) up-regulates the inwardly rectifying current generated by the K^+ channel Kir2.1. PKB is activated by a signaling involving phosphoinositide-3-kinase (PI3 K) and

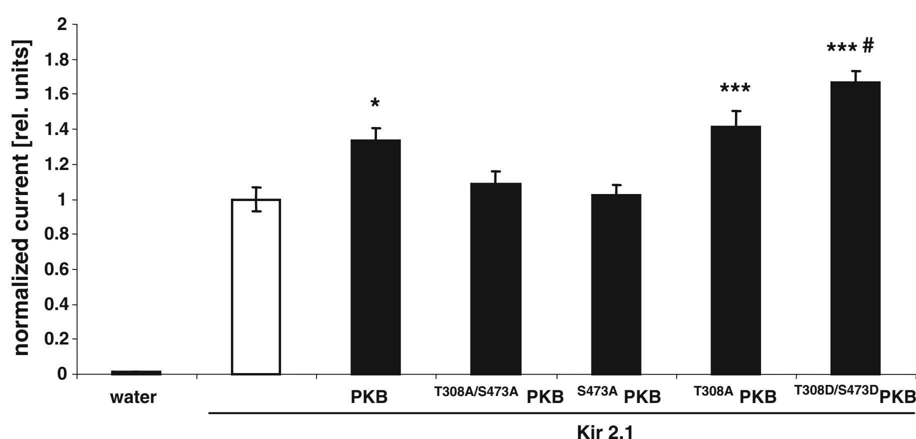

Fig. 2 Effect of wild type PKB was mimicked by constitutively active ^{T308D,S473D}PKB but not by inactive ^{T308A,S473A}PKB. Arithmetic means ± SEM (n = 14–22) of the normalized current at −150 mV in *Xenopus* oocytes injected with water (*first bar*), or expressing Kir2.1 without (*second bar*), or with wild type PKB (*third bar*), with inactive ^{T308A,S473A}PKB (*fourth bar*), with the single mutants ^{S473A}PKB (*fifth bar*) and ^{T308A}PKB (*sixth bar*) or with constitutively active ^{T308D,S473D}PKB (*seventh bar*). *, ***indicate statistically significant ($p < 0.05$, $p < 0.001$) difference from oocytes expressing Kir2.1 alone. # indicates statistically significant ($p < 0.05$) difference from coexpression of Kir2.1 together with wild type PKB

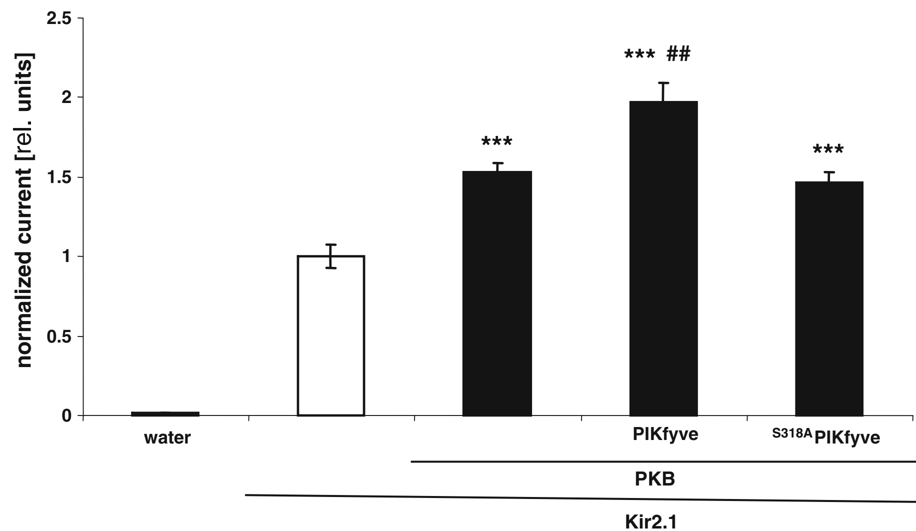

Fig. 3 Effect of wild type PKB was mimicked by PIKfyve. Arithmetic means ± SEM ($n = 14$–20) of the normalized current at -150 mV in *Xenopus* oocytes injected with water (*first bar*), or expressing Kir2.1 without (*second bar*) or with wild type PKB either without (*third bar*) or with additional coexpression of wild type PIKfyve (*fourth bar*) or of PKB-resistant ^{S318A}PIKfyve (*fifth bar*). ##indicates statistically significant difference ($p < 0.01$) from coexpression of PKB alone, *** indicates statistically significant difference ($p < 0.001$) from the expression of Kir2.1 alone

phosphoinositide dependent kinase (PDK1). Apparently, the signaling leading to PKB activation is active in oocytes, as expression of wild type PKB is effective without exogenous stimulation of the PI3 K pathway. PKB is in turn known to phosphorylate PIKfyve (Hill et al. 2010) and thus to regulate PIKfyve-sensitive carriers and ion channels (Alesutan et al. 2010; Gehring et al. 2009a, 2009b; Hill et al. 2010; Klaus et al. 2009; Shojaiefard et al. 2007; Sopjani et al. 2010; Strutz-Seebohm et al. 2007; Tsuruta et al. 2009). Our observations suggest that PIKfyve stimulates the Kir2.1 channel protein insertion into the cell membrane. The brefeldin A experiments suggest that PKB and PIKfyve do not interfere with the clearance of channel protein from the cell membrane. The enhanced protein abundance in the cell membrane is thus presumably due to accelerated insertion of channel protein into the cell membrane. PIKfyve is known to phosphorylate phosphatidylinositol-3-phosphate (PtdIns3*P*) resulting in the formation of phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2) (Sbrissa et al. 1999). Phosphatidylinositides participate in the regulation of membrane trafficking (de Lartigue et al. 2009; Ikonomov et al. 2009; Morris and Smyth 2007; Zheng and Bobich 2004). The present observations do, however, not rule out additional mechanisms involved in the stimulating effect of PKB on Kir2.1, such as direct phosphorylation of the channel protein or phosphorylation of other signaling molecules governing Kir2.1 activity.

An up-regulation of Kir2.1 channels is expected to hyperpolarize the cell membrane. In excitable cells, the stimulation of Kir2.1 thus counteracts depolarization and fosters repolarization. Because of its inwardly rectifying property, Kir2.1 is particularly important for the most terminal phase of repolarization in cardiomyocytes and is the dominating conductance maintaining the resting cell membrane potential (Tristani-Firouzi and Etheridge 2010). Loss of function mutations of Kir2.1 bear the risk of ventricular arrhythmias (Tristani-Firouzi and Etheridge 2010). Kir2.1 deficiency further impairs repolarization of skeletal muscle cells leading to potassium-sensitive periodic paralysis (Andersen et al. 1971; Plaster et al. 2001; Sansone et al. 1997; Tawil et al. 1994).

In epithelial cells inwardly rectifying K^+ channels maintain the electrical driving force for electrogenic transport systems. Accordingly, stimulation of the K^+ channels augments Na^+-coupled transport of glucose (Dieter et al. 2004), amino acids (Boehmer et al. 2005, 2006; Böhmer et al. 2010; Palmada et al. 2005), creatine (Shojaiefard et al. 2005; Strutz-Seebohm et al. 2007), organic osmolytes (Klaus et al. 2008), and phosphate (Bhandaru et al. 2011). In proximal renal tubules activation of Kir2.1 and subsequent hyperpolarization further increases the electrical driving force for electrogenic HCO_3^- exit across the basolateral cell membrane leading to cytosolic acidification and subsequent stimulation of the apical Na^+/H^+ exchanger (Lang and Rehwald 1992). Accordingly, enhanced Kir2.1 activity increases Na^+ entry and thus increases the demand for Na^+ extrusion through the Na^+/K^+ ATPase (Lang and Rehwald 1992).

In any cell type stimulation of K^+ channels results in K^+ exit, which may, during energy depletion and thus impaired function of Na^+/K^+ ATPase, lead to significant cellular K^+ loss, and cellular K^+ depletion, which in turn stimulates suicidal cell death (Becker et al. 2007; Bortner and Cidlowski 2004; Föller et al. 2006; Schneider et al. 2007; Shimizu et al. 2006). The hyperpolarization further drives Cl^- exit across the cell membrane, which leads to cellular loss of KCl and osmotically obliged water thus leading to cell shrinkage (Lang et al. 1986, 1998).

The HCO_3^- exit after hyperpolarization favors the development of cytosolic acidification, which has been shown to accelerate suicidal cell death (Lupescu et al. 2009) and to impair glycolysis (Boiteux and Hess 1981).

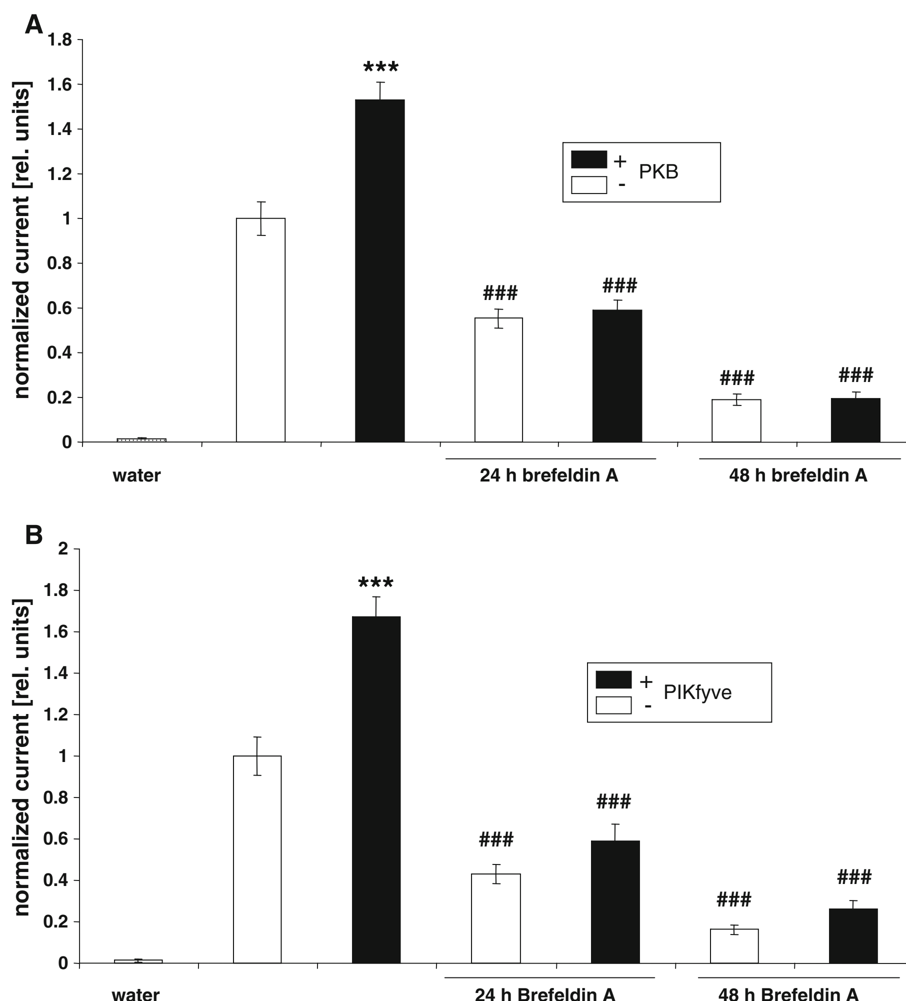

Fig. 4 Effect of brefeldin A in Kir2.1-expressing *Xenopus* oocytes with or without PKB or PIKfyve coexpression. **a** Arithmetic means ± SEM ($n = 12$–22) of the normalized current at -150 mV in *Xenopus* oocytes expressing Kir2.1 without (*white bars*) or with additional expression of wild type PKB (*black bars*) and treated for 24 h (*fourth and fifth bars*) or 48 h (*sixth and seventh bars*) with or without 5 μM brefeldin A. ***indicates statistically significant ($p < 0.001$) difference from the expression of Kir2.1 alone; ###indicates statistically significant ($p < 0.001$) difference from the absence of brefeldin A. **b** Arithmetic means ± SEM ($n = 12$–20) of the normalized current at -150 mV in *Xenopus* oocytes expressing Kir2.1 without (*white bars*) or with additional expression of wild type PIKfyve (*black bars*) and treated for 24 h (*fourth and fifth bars*) or 48 h (*sixth and seventh bars*) with or without 5 μM brefeldin A. ***indicates statistically significant ($p < 0.001$) difference from the expression of Kir2.1 alone; ### indicates statistically significant ($p < 0.001$) difference from the absence of brefeldin A

In conclusion, the present observations show that PKB in conjunction with PIKfyve activates Kir2.1 channels. This process is expected to impact on the excitability of excitable cells, on transport across epithelial cells as well as on cell volume and survival of a wide variety of cells.

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