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Inhibition of Connexin 26 by the AMP-Activated Protein Kinase

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Abstract Connexins provide intercellular connections that allow passage of ions and small organic molecules. They clamp the cell membrane potential and cellular ion composition to that of neighboring cells. The cell membrane potential and ion composition of an energy-depleted cell could thus be maintained despite its compromised Na^+/K^+ activity. By the same token, however, the breakdown of ion gradients in that cell imposes an additional challenge to the neighboring cells, which may jeopardize their survival. Thus, timely closure of connexins may be critically important for the survival of those cells. Energy depletion stimulates the AMP-activated protein kinase (AMPK), a serine/threonine kinase that senses energy depletion and stimulates several cellular mechanisms to enhance energy production and to limit energy utilization. The present study explored whether AMPK regulates connexin 26. To this end, cRNA encoding connexin 26 was injected into *Xenopus* oocytes with and without additional injection of wild-type AMPK ($\alpha1\beta1\gamma1$), of the constitutively active $^{\gamma R70Q}$AMPK ($\alpha1\beta1\gamma1$[R70Q]) or of the inactive mutant $^{\alpha K45R}$AMPK ($\alpha1$[K45R]$\beta1\gamma1$). Connexin 26 activity was determined in dual-electrode voltage-clamp experiments. Moreover, connexin 26 abundance was determined in the oocyte cell membrane by chemiluminescence and confocal microscopy. As a result, connexin 26-mediated current and connexin 26 protein abundance were significantly decreased by coexpression of $^{\gamma R70Q}$AMPK and, to a lower extent, by wild-type AMPK but not by $^{\alpha K45R}$AMPK. In conclusion, AMPK is a potent regulator of connexin 26.

Keywords Cx26 · AMPK · Ischemia · Inner ear

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Introduction

Gap junctions provide intercellular connections for the passage of ions, metabolites and other small organic molecules between neighboring cells (Scemes et al. 2009). Connexin 26 is involved in diverse functions including embryonic cortical development (Elias and Kriegstein 2008), formation of keratinocytes of the skin (Arndt et al. 2010; Lee and White 2009) and tumor growth (Nojima et al. 2007). It is particularly important for the function of the inner ear (Chan et al. 2010; Grifa et al. 1999; Lee and White 2009; Mohamed et al. 2010).

The intercellular connections are further important for cell survival (Decrock et al. 2009). The impact of connexins on cell survival may be particularly important during energy depletion. Open connexins clamp the cell membrane potential and ion composition of a given cell to that of neighboring cells. Accordingly, neighboring cells could maintain the cell membrane potential and ion composition of an energy-depleted cell despite its ATP depletion and, thus, compromised Na^+/K^+ activity. Without the support of the neighboring cells, energy depletion may eventually lead to breakdown of ionic gradients with subsequent swelling and necrotic death of the energy-depleted cell (Lang et al. 1998). Thus, the neighboring cells could

support the survival of the energy-depleted cells. The assistance of the neighboring cells requires, however, that their Na^+/K^+ activity is increased, leading to enhanced energy consumption of those cells, which may in turn jeopardize their survival. Thus, their survival may depend on the timely closure of connexins to energy-depleted cells. Accordingly, adequate regulation of connexin function may be decisive for cell survival during energy depletion. Along those lines, connexin 43 (Cx43) expression is decreased during ischemia (Brezillon et al. 1997; De Vuyst et al. 2011; Lu et al. 2009; Miura et al. 2010) and regulation of Cx43 impacts on ischemic injury (Schulz and Heusch 2006). Ischemia is followed by dephosphorylation of Cx43 and uncoupling of gap junctions (Turner et al. 2004). Conversely, beta-adrenergic receptor activation results in stimulation of PKCε with subsequent Cx43 phosphorylation and increased gap junctional conduction (Duquesnes et al. 2010). Moreover, Cx43 is regulated by excessive glucose concentrations (Hills et al. 2009; Yu et al. 2010). Cx40 is inhibited by ischemia with abrupt reoxygenation via oxidant- and PKA-dependent signaling (Bolon et al. 2005). Cx26 is similarly downregulated by ischemia (Morizane et al. 2005; Ulfig 2004). Knowledge of the signaling that regulates connexin function during energy depletion has remained, however, limited.

A candidate signaling molecule regulating connexin function during energy depletion is the AMP-activated protein kinase (AMPK), which senses the cytosolic AMP/ATP concentration ratio and thus the energy status of the cell (Towler and Hardie 2007; Winder et al. 2000). AMPK stimulates a variety of functions prone to increase cellular ATP generation (McGee and Hargreaves 2008), including cellular glucose uptake, glycolysis, fatty acid oxidation and enzymes required for ATP production (Carling 2007; Guan et al. 2008; Horie et al. 2008; Jensen et al. 2007; Jessen et al. 2003; Lei et al. 2005; Li et al. 2004; Luiken et al. 2004; MacLean et al. 2002; Natsuizaka et al. 2007; Ojuka et al. 2000; Park et al. 2009; Walker et al. 2005; Winder et al. 2000; Winder and Thomson 2007; Zheng et al. 2001). In addition, AMPK inhibits a number of energy-utilizing mechanisms, such as protein synthesis, gluconeogenesis and lipogenesis (Carling 2007; McGee and Hargreaves 2008; Winder and Thomson 2007). The stimulation of glucose uptake is due to activation of the facilitative glucose carriers GLUT1, GLUT2, GLUT3 and GLUT4 (Guan et al. 2008; Jessen et al. 2003; Lei et al. 2005; Li et al. 2004; Luiken et al. 2004; MacLean et al. 2002; Natsuizaka et al. 2007; Ojuka et al. 2000; Park et al. 2009; Walker et al. 2005; Winder et al. 2000; Zheng et al. 2001). The effects of AMPK confer some protection against cell death during energy depletion (Foller et al. 2009; Hardie 2004; McGee and Hargreaves 2008). Nothing is known, however, about the regulation of connexins by AMPK.

The present study explored whether AMPK regulates Cx26. To this end, the voltage-gated current and membrane abundance were determined in *Xenopus* oocytes expressing Cx26 with or without wild-type, constitutively active and inactive AMPK.

Methods

Constructs

For generation of cRNA, constructs were used encoding wild-type human Cx26-HA (Haack et al. 2006); wild-type human Cx30 (Grifa et al. 1999); wild-type AMPKα1-HA, AMPKβ1-Flag and AMPKγ1-HA (Fraser et al. 2007); constitutively active ^{R70Q}AMPKγ1-HA (Hamilton et al. 2001); and kinase dead mutant ^{K45R}AMPKα1-HA (Hallows et al. 2003).

Voltage Clamp in *Xenopus* Oocytes

Xenopus oocytes were prepared as previously described (Boehmer et al. 2008). cRNA (4.6 ng) encoding either AMPKα1-HA + AMPKβ1-Flag + AMPKγ1-HA (^{WT}AMPK), AMPKα1-HA + AMPKβ1-Flag + ^{R70Q}AMPKγ1-HA ($^{\gamma R70Q}$AMPK) or ^{K45R}AMPKα1KD-HA + AMPKβ1-Flag + AMPKγ1-HA ($^{\alpha K45R}$AMPK) was injected on the first day and 12.5–23 ng Cx26 cRNA or 10 ng Cx30 cRNA on the second day after preparation of the *Xenopus* oocytes. All experiments were performed at room temperature 2 days after the second injection. In two-electrode voltage-clamp experiments, the cell membrane potential was changed from −140 to +60 mV in 40-mV increments for 20 s. The intermediate holding potential was −60 mV. The steady-state current at +60 mV was taken as a measure of hemichannel activity. Currents were filtered at 10 Hz and recorded with a Digidata AD/DA interface digital to analog converter and pClamp software for data acquisition and analysis (Axon Instruments, Foster City, CA). The bath solution (ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES (pH 7.4). The final solution was titrated to pH 7.4.

Immunohistochemistry

After 4% paraformaldehyde fixation for at least 12 h, oocytes were cryoprotected in 30% sucrose, frozen in mounting medium and placed on a cryostat. Sections were collected at a thickness of 8 µm on coated slides and stored at −20°C. For immunostaining, sections were dehydrated at room temperature, fixated in acetone/methanol (1:1) for 15 min at room temperature, washed in PBS and blocked for 1 h in 5% bovine serum albumin in PBS. The primary

antibody used was rabbit anti-GJB2 antibody (diluted 1:100; Abnova, Heidelberg, Germany). Incubation was performed in a moist chamber overnight at 4°C. The binding of the primary antibody was visualized with an anti-rabbit FITC-conjugated antibody (diluted 1:500; Invitrogen, Carlsbad, CA). Next, oocytes were analyzed by a fluorescence laser scanning microscope (LSM 510; Carl Zeiss MicroImaging, Göttingen, Germany) with A-Plan 20x/0.48 Ph2. Brightness and contrast settings were kept constant during imaging of all oocytes in each injection series. The fluorescence intensity reflecting Cx26 membrane abundance was quantified by ZEN2009 software (Carl Zeiss MicroImaging).

Detection of Cx26 Cell Surface Expression by Chemiluminescence

Defolliculated intact oocytes were incubated with 1 μg/ml primary rat monoclonal anti-HA antibody (clone 3 F10; Boehringer, Biberach, Germany) and 2 μg/ml secondary, peroxidase-conjugated goat anti-rat antibody (Cell Signaling, Danvers, MA). Individual oocytes were placed in 96-well plates with 20 μl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). The chemiluminescence of single oocytes was quantified in a luminometer (WalterWallac2 plate reader; Perkin Elmer, Jügesheim, Germany) by integrating the signal over a period of 1 s. Results display normalized arbitrary light units, which are proportional to the detector voltage. The intensity of the chemiluminescence signal is proportional to the number of bound anti-HA antibodies. This method therefore allows quantification of the Cx26 surface expression.

Statistical Analysis

Data are provided as means ± SEM, with n representing the number of experiments. All oocyte experiments are 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

AMPK Inhibited Voltage-Gated Outward Currents in Cx26-Expressing *Xenopus* Oocytes

In Cx26-expressing, but not in water-injected, *Xenopus* oocytes, depolarization triggered a current (I_{CX}) without rectification (Fig. 1). Coexpression of wild-type AMPK (AMPKα1 + AMPKβ1 + AMPKγ1) was followed by a significant decrease of I_{CX} by 64.1 ± 3% ($n = 13$) at +60 mV (Fig. 1).

The Constitutively Active $^{\gamma R70Q}$AMPK, but not the Inactive Mutant $^{\alpha K45R}$AMPK, Mimicked the Effect of Wild-Type AMPK

As shown in Fig. 2, the I_{CX} of Cx26-expressing *Xenopus* oocytes was significantly decreased by coexpression of the constitutively active mutant $^{\gamma R70Q}$AMPK (AMPKα1-HA + AMPKβ1-Flag + ^{R70Q}AMPKγ1-HA). In contrast, coexpression of the inactive form of $^{\alpha K45R}$AMPK (^{K45R}AMPKα1KD-HA + AMPKβ1-Flag + AMPKγ1-HA) did not significantly modify I_{CX} in Cx26-expressing *Xenopus* oocytes. Thus, kinase activity is required for the effect of AMPK on Cx26 activity. In order to test whether AMPK similarly downregulates other connexins expressed in the inner ear, further experiments were performed with Cx30, the other main connexin of nonsensory cochlea cells. The current measured in Cx30-expressing oocytes was 0.84 ± 0.09 μA ($n = 23$). The coexpression of constitutively active $^{\gamma R70Q}$AMPK significantly increased the Cx30-mediated current to 1.44 ± 0.09 μA ($n = 31$). Thus, the effect of AMPK on connexins is not uniform.

AMPK Decreased Connexin 26 Protein Abundance in the Cell Membrane

A decrease of the slowly activating outward current could have resulted from a decrease of Cx26 protein abundance in the cell membrane. To test this possibility, the Cx26 protein abundance was determined by chemiluminescence and by confocal microscopy in *Xenopus* oocytes injected with water, in oocytes expressing Cx26 alone and in *Xenopus* oocytes expressing Cx26 together with constitutively active AMPK and the kinase dead mutant of AMPK. As shown in Fig. 3, according to chemiluminescence, the Cx26 cell surface expression in *Xenopus* oocytes injected with cRNA encoding Cx26 was significantly decreased by the coexpression of constitutively active $^{\gamma R70Q}$AMPK. Confocal microscopy confirmed the results obtained from chemiluminescence (Fig. 4).

Discussion

The present study reveals a completely novel regulator of gap junctions. The AMP-activated kinase AMPK downregulates Cx26 and thus uncouples energy-depleted cells from the neighboring cells. Notably, coexpression of AMPK rather increased the activity of Cx30, indicating that AMPK does not generally downregulate connexins.

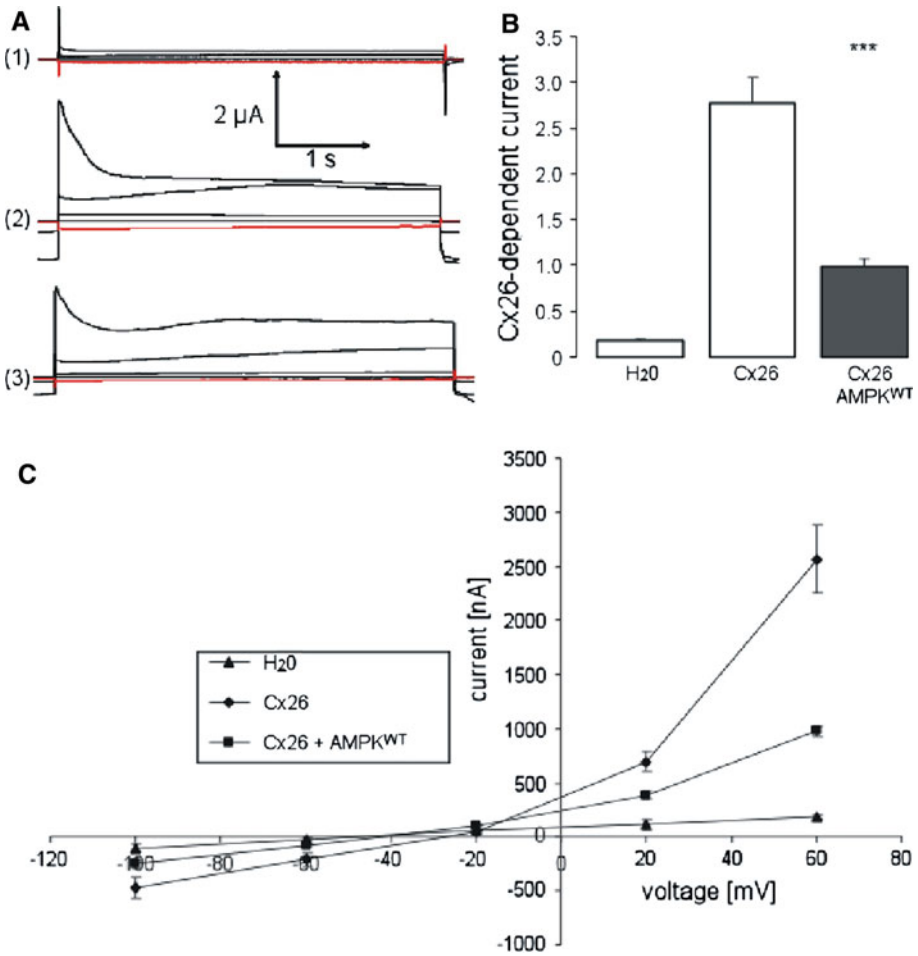

Fig. 1 Coexpression of AMPK decreased voltage-gated outward current in Cx26-expressing *Xenopus* oocytes. **a** Original tracings of the current induced by depolarization from −140 mV to −100, −60, −20, +20 and +60 mV in *Xenopus* oocytes injected with water (*1*) and expressing Cx26 alone (*2*) or Cx26 together with wild-type AMPK (*3*). **b** Arithmetic means ± SEM of depolarization-induced current (I_{CX}) at +60 mV in *Xenopus* oocytes injected with water ($n = 7$), with Cx26 alone ($n = 12$) and with Cx26 together with wild-type AMPK (AMPKα1 + AMPKβ1 + AMPKγ1) ($n = 13$). ***Statistically significant difference from currents measured in oocytes expressing Cx26 alone ($P < 0.001$). **c** *I–V* curve of the measurements shown in **b**. Arithmetic means ± SEM ($n = 7$–13)

AMPK is at least partially effective through a decrease of Cx26 protein abundance in the plasma membrane. The present study did not aim to elucidate the underlying mechanism. AMPK is known to regulate ion channels in part by stimulation of Nedd4-2 (neuronal precursor cells expressed developmentally downregulated), which ubiquitinates its target proteins, thus preparing them for degradation (Almaca et al. 2009; Alzamora et al. 2010; Bhalla et al. 2006; Carattino et al. 2005). Thus, at least in theory, AMPK may be effective through stimulation of Nedd4-2 with subsequent ubiquitination and proteasomal degradation of the Cx26 channel protein. The mechanism accounting for the upregulation of Cx30 may result from enhanced insertion of the channel protein into the cell membrane, as has been shown for the glucose carrier SGLT1 (Sopjani et al. 2010).

The AMPK-dependent regulation of Cx26 may impact on the function of the inner ear, where gap junctions participate in the itinerary of K^+ recycling from the sensory cells during the mechanosensory transduction process back to the endolymph (Kikuchi et al. 2000; Oesterle and Dallos 1990; Sacchi et al. 1985; Zhao et al. 2006). Connexin subunits expressed in the mammalian inner ear include Cx26, Cx30, Cx31 and Cx43 (Ahmad et al. 2003; Forge et al. 1999; Kikuchi et al. 1995, 2000; Lautermann et al. 1998; Lopez-Bigas et al. 2002; Xia et al. 2000; Zhao et al. 2005). GJB2, GJB3 and GJB6, the genes coding for Cx26, Cx31 and Cx30, respectively, are expressed in the organ of Corti; and GJB2 and GJB6 are expressed in the lateral wall of the cochlea (Kikuchi et al. 2000; Lopez-Bigas et al. 2002; Matsunami et al. 2006; Xia et al. 1999, 2000).

Mutations of Cx26 are the most common cause of hereditary childhood deafness (Denoyelle et al. 1998; Haack et al. 2006; Palmada et al. 2006; Zelante et al. 1997).

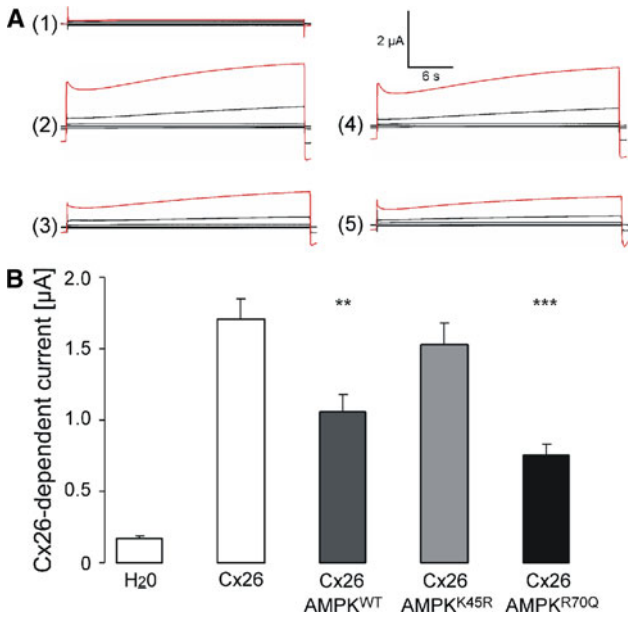

Fig. 2 The constitutively active $^{\gamma R70Q}$AMPK, but not the inactive mutant $^{\alpha K45R}$AMPK, mimicked the effect of wild-type AMPK. **a** Original tracings of the current induced by depolarization from −140 mV to −100, −60, −20, +20 and +60 mV in *Xenopus* oocytes expressing Cx26 alone (*2*) or Cx26 together with wild-type AMPK (*3*), with inactive $^{\alpha K45R}$AMPK (^{K45R}AMPKα1KD-HA + AMPKβ1-Flag + AMPKγ1-HA) (*4*) or with constitutively active $^{\gamma R70Q}$AMPK (AMPKα1-HA + AMPKβ1-Flag + ^{R70Q}AMPKγ1-HA) (*5*) or injected with water only (*1*). **b** Arithmetic means ± SEM of depolarization-induced current (I_{CX}) at +60 mV in *Xenopus* oocytes injected with water ($n = 22$), in *Xenopus* oocytes injected with Cx26 alone ($n = 30$) and in *Xenopus* oocytes injected with Cx26 together with wild-type AMPK ($n = 13$), with inactive $^{\alpha K45R}$AMPK ($n = 12$) or with constitutively active $^{\gamma R70Q}$AMPK ($n = 21$). Statistically significant difference from currents measured in oocytes expressing Cx26 alone: $^{**}P < 0.01$, $^{***}P < 0.001$

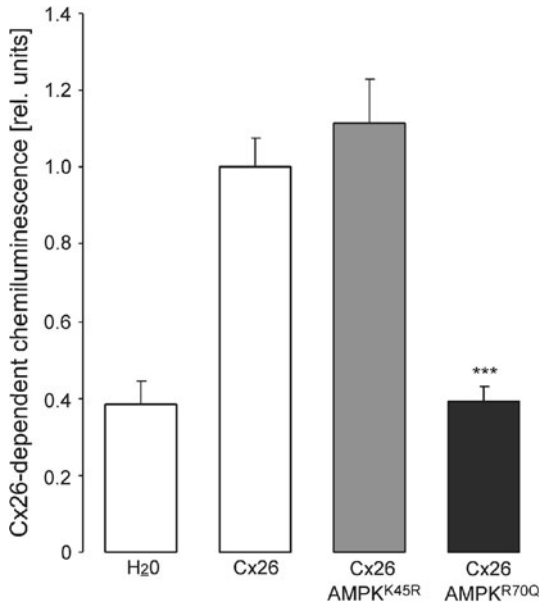

Fig. 3 Coexpression of AMPK decreases the Cx26 protein abundance within the plasma membrane of oocytes. Surface expression–dependent intensity of the chemiluminescence signal. Arithmetic means ± SEM of the values obtained from *Xenopus* oocytes injected with water ($n = 72$), in *Xenopus* oocytes injected with Cx26 alone ($n = 69$) and in *Xenopus* oocytes injected with Cx26 together with inactive $^{\alpha K45R}$AMPK ($n = 49$) or with constitutively active $^{\gamma R70Q}$AMPK ($n = 70$). ***Statistically significant difference from values obtained in oocytes expressing Cx26 alone ($P < 0.001$)

The mutations can prevent the exchange of organic solutes but permit the free exchange of ions (Kudo et al. 2003; Zhang et al. 2005). Cx26 in supporting cells of the organ of Corti is apparently particularly important for the transcellular movement of glutamate. In mice that lack Cx26, glutamate-buffering supporting cells near the inner hair cells undergo apoptosis, most likely as a consequence of their inability to distribute glutamate via gap junctions to neighboring supporting cells expressing glutamine synthase (Cohen-Salmon et al. 2002; Eybalin et al. 1996).

In contrast to downregulating Cx26, AMPK stimulates cellular glucose uptake by the glucose carriers GLUT1, GLUT2, GLUT3, GLUT4 and SGLT1 (Guan et al. 2008; Jessen et al. 2003; Lei et al. 2005; Li et al. 2004; Luiken et al. 2004; MacLean et al. 2002; Natsuizaka et al. 2007; Ojuka et al. 2000; Park et al. 2009; Sopjani et al. 2010; Walker et al. 2005; Winder et al. 2000; Zheng et al. 2001). The glucose uptake provides the cell with fuel. In addition, AMPK stimulates glycolysis, fatty acid oxidation and expression of enzymes required for ATP production (Carling 2007; Winder and Thomson 2007). AMPK thus triggers several mechanisms aimed at increasing the cellular ATP content.

If those mechanisms fail to restore the ATP content of the cell, the energy-depleted cell is exposed to the risk of undergoing necrotic cell death. ATP deficiency compromises the function of the Na^+/K^+ ATPase, leading to cellular K^+ loss and cellular Na^+ accumulation, which eventually lead to cell swelling (Lang et al. 1998). Excessive cell swelling may jeopardize the integrity of the cell membrane, leading to irreversible necrotic cell death (Lang et al. 1998). The cellular K^+ loss may further trigger suicidal cell death (Becker et al. 2007; Bortner and Cidlowski 2004; Foller et al. 2006; Schneider et al. 2007; Shimizu et al. 2006).

The uncoupling of the energy-depleted cell from its neighbors disrupts the support of those cells. If the energy depletion affects a single cell, it could be supported by ion fluxes through the gap junctions, which maintain the K^+ and Na^+ gradients and the potential difference across the cell membrane and thus prevent swelling of the defective cells. Accordingly, closure of the gap junctions by the energy-depleted cell is expected to accelerate the death of the affected cell. By the same token, however, the

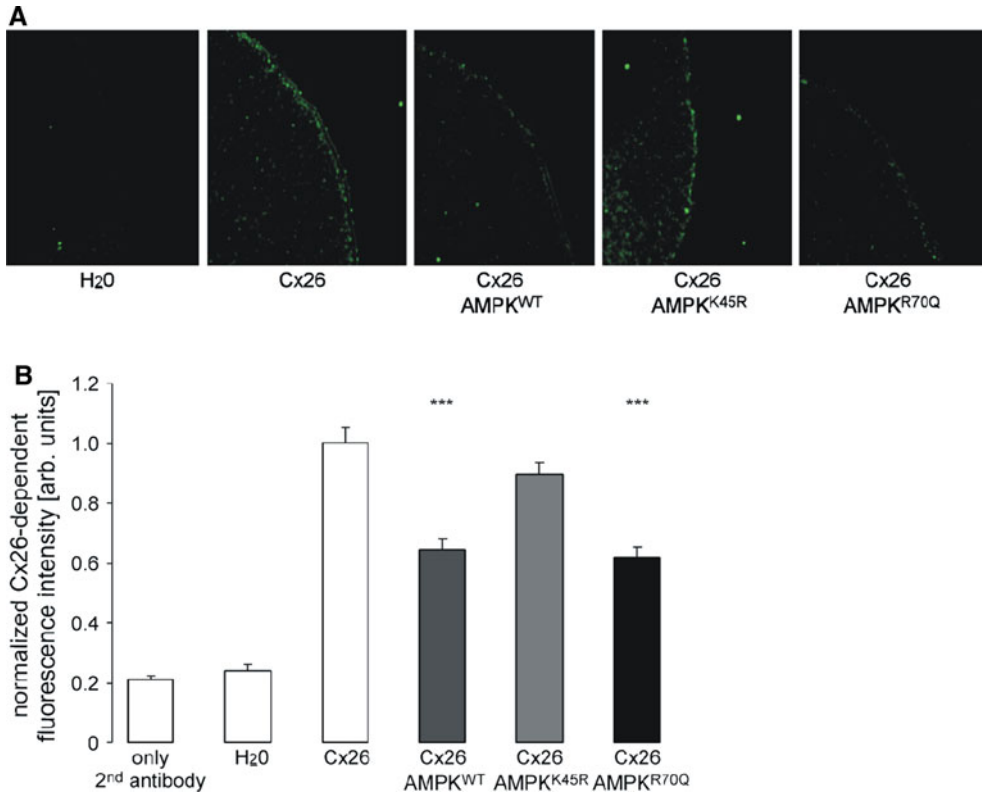

Fig. 4 Confocal microscopy of Cx26 protein abundance within the plasma membrane of oocytes. **a** Confocal images of Cx26 protein abundance in the plasma membrane of *Xenopus* oocytes injected with water, with Cx26 alone and with Cx26 together with wild-type AMPK, inactive $^{\alpha K45R}$AMPK or constitutively active $^{\gamma R70Q}$AMPK. **b** Arithmetic means ± SEM of the normalized Cx26-dependent surface fluorescence intensity of *Xenopus* oocytes injected with water ($n = 8$ images of two oocytes), with Cx26 alone ($n = 12$ images of three oocytes) and with Cx26 together with wild-type AMPK ($n = 12$ images of three oocytes), inactive $^{\alpha K45R}$AMPK ($n = 12$ images of three oocytes) or constitutively active $^{\gamma R70Q}$AMPK ($n = 12$ images of three oocytes). *First bar* depicts the surface fluorescence intensity of *Xenopus* oocytes injected with Cx26 alone and incubated only with the secondary antibody. ***Statistically significant difference from values obtained in oocytes expressing Cx26 alone ($P < 0.001$)

uncoupling protects the other cells, which may prevent similar swelling and subsequent death of those cells, if they are able to maintain their own ionic gradients but are not able to support their defective neighboring cells. Notably, AMPK is further stimulated by an increase in cytosolic Ca^{2+} activity (Towler and Hardie 2007), which may occur when the membrane of a cell is leaky and which similarly closes gap junctions. Persistent coupling of leaky cells may jeopardize the survival of the neighboring cells without rescuing the irreversibly defective cell.

AMPK is further activated by decrease of O_2 levels (Evans et al. 2005) and by exposure to nitric oxide (Lira et al. 2007). Both are thus expected to downregulate Cx26 activity.

In conclusion, the present observations reveal a novel regulator of Cx26, which may disrupt the coupling of cells during hypoxia, energy depletion, excessive cytosolic Ca^{2+} activity and exposure to nitric oxide.

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