Downregulation of Kv1.5 K⁺ Channels by the AMP-Activated Protein Kinase

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Key Words
Kv1.5 • AMPK • Ischemia • Energy depletion

Abstract
Background: The voltage gated K⁺ channel Kv1.5 participates in the repolarization of a wide variety of cell types. Kv1.5 is downregulated during hypoxia, which is known to stimulate the energy-sensing AMP-activated serine/threonine protein kinase (AMPK). AMPK is a powerful regulator of nutrient transport and metabolism. Moreover, AMPK is known to downregulate several ion channels, an effect at least in part due to stimulation of the ubiquitin ligase Nedd4-2. The present study explored whether AMPK regulates Kv1.5.

Methods: cRNA encoding Kv1.5 was injected into Xenopus oocytes with and without additional injection of wild-type AMPK (α1β1γ1), of constitutively active γR70Q AMPK (α1β1γ1(R70Q)), of inactive mutant αK45R AMPK (α1(K45R)β1γ1), or of Nedd4-2. Kv1.5 activity was determined by two-electrode voltage-clamp. Moreover, Kv1.5 protein abundance in the cell membrane was determined by chemiluminescence and immunostaining with subsequent confocal microscopy.

Results: Coexpression of wild-type AMPKWT and constitutively active AMPKγR70Q, but not of inactive AMPKαK45R, significantly reduced Kv1.5-mediated currents. Coexpression of constitutively active AMPKγR70Q further reduced Kv1.5 K⁺ channel protein abundance in the cell membrane. Coexpression of Nedd4-2 similarly downregulated Kv1.5-mediated currents.

Conclusion: AMPK is a potent regulator of Kv1.5. AMPK inhibits Kv1.5 presumably in part by activation of Nedd4-2 with subsequent clearance of channel protein from the cell membrane.
Introduction

The voltage-gated K⁺ channel Kv1.5 contributes to the maintenance of the cell membrane potential in a wide variety of tissues, such as pancreatic β-cells [1-3], brain [4], macrophages [5], dendritic cells [6, 7], heart [4, 8-11], skeletal muscle [12], as well as smooth muscles in vessels [13, 14], intestine [15], and airways [16]. In the heart, Kv1.5 generates the ultra-rapid delayed rectifier current (I₉₋₂) [17] and thus contributes to the repolarization of the cardiac action potential [18-20]. Kv1.5 is highly expressed in atrial myocytes [21] and loss-of-function Kv1.5 mutations may cause atrial fibrillation [22]. Kv1.5 is thus an attractive target for the treatment of atrial arrhythmias [13, 23-32]. Kv1.5 is further expressed in several tumor cells and participates in the regulation of adhesion, proliferation and sensitivity to apoptosis [33-39]. Thus, Kv1.5 has been considered a potential target against tumor growth [40].

Kv1.5 activity is downregulated by hypoxia, an effect at least partially explained by 15-HETE formation [41]. Hypoxia is further expected to stimulate the AMP-activated protein kinase (AMPK), a kinase sensitive to cytosolic AMP/ATP concentration ratio and thus the energy status of the cell [42, 43]. AMPK inhibits energy-utilizing mechanisms, including protein synthesis, gluconeogenesis and lipogenesis [43-45] and stimulates energy providing mechanisms, such as cellular glucose uptake, glycolysis, fatty acid oxidation and enzymes required for ATP production [43, 44, 46-66]. Thus, AMPK restores cellular ATP levels [45] and protects against cell death during energy depletion [45, 67, 68].

Effects of AMPK include downregulation of ion channels, such as the epithelial Na⁺ channel ENaC [69-72], the inwardly rectifying K⁺ channel Kir2.1 [73] and the outwardly rectifying K⁺ channel KCNQ1/KCNE1 [74]. AMPK is at least partially effective by stimulation of the ubiquitin ligase Nedd4-2 [70]. The ubiquitin ligase labels channel and carrier proteins for subsequent proteasomal degradation [75]. Channel proteins targeted by Nedd4-2 include the Na⁺ channels EnaC [75, 76] and Nav1.6 [77], the Ca²⁺ channels Orai [78] and TRPV6 [79], as well as the K⁺ channels KCNE1/KCNQ1 [74, 80, 81], KCNQ2/3 and KCNQ3/5 [82], HERG [83] Kir2.1 [73], Kv4.3 [84], Kv7.1 [85] and Kv1.5 [86], Carriers targeted by Nedd4-2 include the Na⁺,Cl⁻ Cotransporter NCC [87], the phosphate transporter NaPiIIb [88], the glutamate transporter EAAT2 [89], the glucose transporter SGLT1 [90], and the myo-inositol transporter SMIT1 [91].

The present study explored whether Kv1.5 K⁺ channels are regulated by AMPK. To this end, voltage-gated current was determined in Xenopus oocytes expressing Kv1.5 with or without additional coexpression of wild-type, of constitutively active or of inactive mutant AMPK. Additional experiments were performed to elucidate whether AMPK influences Kv1.5 K⁺ channel protein abundance in the cell membrane and whether Kv1.5-mediated currents are modified by the ubiquitin ligase Nedd4-2.

Materials and Methods

Constructs

For generation of cRNA, constructs were used encoding wild-type human Kv1.5 [92], Kv1.5-HA containing an extracellular hemagglutinin epitope [93], wild-type human AMPKα1-HA, AMPK β1-Flag, AMPK γ1-HA [94], constitutively active AMPKγ1K45R-HA [95], kinase dead mutant AMPK α1K45R-HA [72], and wild-type human Nedd4-2 [86]. The constructs were used for the generation of cRNA as described previously [96].

Voltage clamp in Xenopus oocytes

Xenopus oocytes were prepared as previously described [97-99], cRNA encoding Kv1.5 (2.5 ng) was injected with or without 4.6 ng of cRNA encoding either AMPKα1-HA + AMPKβ1-Flag + AMPKγ1-HA (AMPKγ1), or AMPKα1-HA + AMPKβ1-Flag + AMPKγ1K45R-HA (AMPKγ1K45R), or AMPKα1-HA + AMPKβ1-Flag + AMPKγ1K45R-HA + AMPKβ1-Flag + AMPKγ1-HA (AMPKγ1K45R) with or without 10 ng cRNA encoding Nedd4-2 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 Kv1.5 channel currents were elicited every 20 s with 2 s pulses from -80 mV to +50 mV applied from a holding potential of -100 mV. Pulses were applied in 10 mV increments. The data were filtered at 1 kHz and recorded with a Digidata 1322A A/D-D/A converter and Chart V.4.2 software for data acquisition and analysis (Axon Instruments) [100]. The analysis of the data was performed with Clampfit 9.01 (Axon Instruments) software.

Detection of Kv1.5 cell surface expression by chemiluminescence

Oocytes expressing HA-tagged Kv1.5 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, USA). 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.

Immunocytochemistry and confocal microscopy

After 4% paraformaldehyde fixation for at least 12 hours, 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, fixed in acetone/methanol (1:1) for 15 min at room temperature, washed in PBS and blocked for 1 hour in 1% bovine serum albumin in PBS. The primary antibody used was rat monoclonal anti-HA antibody (diluted 1:100; clone 3 F10; Boehringer, Biberach, Germany). Incubation was performed in a moist chamber overnight at 4°C. The binding of primary antibody was visualized with an anti-rat Alexa488-conjugated antibody (diluted 1:200; Invitrogen, Carlsbad, CA). Next, oocytes were analyzed by a fluorescence laser scanning microscope (LSM 510; CarlZeiss MicroImaging, Göttingen, Germany) with A-Plan 40x/1.2W DICIII. Brightness and contrast settings were kept constant during imaging of all oocytes in each injection series.

Statistical analysis

Data are provided as means ± SEM, n represents the number of experiments. All experiments were repeated with at least three 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

In *Xenopus* oocytes expressing Kv1.5, but not in water-injected *Xenopus* oocytes, depolarization triggered an outwardly directed current ($I_{\text{out}}$), which was significantly decreased by coexpression of wild-type AMPK<sup>WT</sup> (AMPK<sub>α</sub>1 + AMPK<sub>β</sub>1 + AMPK<sub>γ</sub>1). At +50 mV, $I_{\text{out}}$ was in average 28.5 ± 4.8% (n = 12-15) lower in *Xenopus* oocytes expressing Kv1.5 together with wild-type AMPK than in *Xenopus* oocytes expressing Kv1.5 alone (Fig. 1B). Fig. 1C illustrates the IV curve in *Xenopus* oocytes expressing Kv1.5 alone and in *Xenopus* oocytes expressing Kv1.5 together with wild-type AMPK. At each holding voltage, the current was lower in *Xenopus* oocytes expressing Kv1.5 together with wild-type AMPK than in *Xenopus* oocytes expressing Kv1.5 alone.

As illustrated in Fig. 2, coexpression of the constitutively active AMPK<sup>R70Q</sup> (AMPK<sub>α</sub>1 + AMPK<sub>β</sub>1 + AMPK<sub>γ</sub>1<sup>R70Q</sup>) similarly decreased the outward current in Kv1.5-expressing *Xenopus* oocytes. In contrast, the inactive mutant AMPK<sup>K45R</sup> (AMPK<sub>α</sub>1<sup>K45R</sup> + AMPK<sub>β</sub>1 + AMPK<sub>γ</sub>1) did not significantly modify $I_{\text{out}}$. Accordingly, AMPK kinase activity was required for the inhibitory effect on Kv1.5 currents (Fig. 2).

Additional experiments were performed to test whether Kv1.5 is regulated by the AMPK-sensitive ubiquitin ligase Nedd4-2. To this end, Kv1.5 was expressed with or without wild-type Nedd4-2. As shown in Fig. 3, Nedd4-2 indeed decreased Kv1.5-dependent currents (Fig. 3).
Fig. 1. Coexpression of AMPK decreased K⁺ current in Kv1.5-expressing Xenopus oocytes. A. Original tracings of the current following depolarization from -80 to +50 mV in Xenopus oocytes injected with water (a), expressing Kv1.5 without (b) or with additional coexpression of wild-type AMPK (c). B. Arithmetic means ± SEM (n = 12-15) of K⁺ current at +50 mV in Xenopus oocytes injected with water (H₂O, dotted bar), or expressing Kv1.5 without (white bar) or with additional coexpression of wild-type AMPK (AMPK_WT, dark grey bar). *** (p<0.001) indicates statistically significant difference from the value obtained in Xenopus oocytes expressing Kv1.5 alone. C. Current as a function of voltage in Xenopus oocytes injected with water (H₂O, closed squares), or expressing Kv1.5 without (Kv1.5, open circles) or with additional coexpression of wild-type AMPK (Kv1.5 + AMPK, closed circles).

Fig. 2. Constitutively active AMPKγR70Q but not inactive mutant AMPKαK45R decreased K⁺ current in Kv1.5-expressing Xenopus oocytes. A. Original tracings of the current following depolarization from -80 to +50 mV in Xenopus oocytes injected with water (a) or expressing Kv1.5 without (b) or with additional coexpression of inactive mutant AMPKαK45R (c) or of constitutively active AMPKγR70Q (d). B. Arithmetic means ± SEM (n = 12-19) of the normalized outwardly rectifying K⁺ current at +50 mV in Xenopus oocytes injected with water (dotted bar), expressing Kv1.5 without (white bar) or with additional coexpression of inactive mutant AMPKαK45R (AMPKαK45R, light grey bar) or of constitutively active AMPKγR70Q (AMPKγR70Q, black bar). *, *** (p<0.05, p<0.001) indicate statistically significant difference from the value obtained in Xenopus oocytes expressing Kv1.5 alone. C. Current as a function of voltage in Xenopus oocytes expressing Kv1.5 without (Kv1.5, open circles) or with additional coexpression of inactive mutant AMPKαK45R (Kv1.5 + AMPKαK45R, closed triangles) or of constitutively active AMPKγR70Q (Kv1.5 + AMPKγR70Q, closed rombi). For comparison, water injected oocytes (H₂O, closed squares) are shown.
The observed decrease of Kv1.5 currents could reflect reduced channel activity or a decline of channel protein abundance in the cell membrane. In order to estimate protein abundance of Kv1.5 channels, chemiluminescence was employed using an HA-tagged Kv1.5 construct. Fig. 4B illustrates the chemiluminescence of *Xenopus* oocytes injected with water (H₂O, dotted bar), expressing Kv1.5 without (white bar) or with additional coexpression of wild-type Nedd4-2 (Nedd4-2, black bar). *** (p<0.001) indicates statistically significant difference from respective value obtained in *Xenopus* oocytes expressing Kv1.5 alone.

Fig. 3. The ubiquitin ligase Nedd4-2 down-regulated K⁺ current in Kv1.5-expressing *Xenopus* oocytes. A. Original tracings of the current following depolarization from -80 to +50 mV in *Xenopus* oocytes injected with water (a), expressing Kv1.5 without (b) or with additional coexpression of Nedd4-2 (c). B. Arithmetic means ± SEM (n = 12-19) of the normalized K⁺ current at +50 mV in *Xenopus* oocytes injected with water (H₂O, dotted bar), expressing Kv1.5 without (white bar) or with additional coexpression of wild-type Nedd4-2 (Nedd4-2, black bar). *** (p<0.001) indicates statistically significant difference from respective value obtained in *Xenopus* oocytes expressing Kv1.5 alone.

Fig. 4. Coexpression of AMPK decreased the Kv1.5-HA protein abundance within the plasma membrane of *Xenopus* oocytes. A. Confocal images reflecting Kv1.5-HA membrane protein abundance in *Xenopus* oocytes injected with water (H₂O), expressing Kv1.5-HA without (Kv1.5-HA) or with additional coexpression of wild-type AMPK (Kv1.5-HA + AMPKWT), of inactive mutant AMPKαK45R (Kv1.5-HA + AMPKαK45R) or of constitutively active AMPKγR70Q (Kv1.5-HA + AMPKγR70Q). B. Arithmetic means ± SEM (n = 48-60) of Kv1.5-HA protein abundance in the plasma membrane of *Xenopus* oocytes injected with water (H₂O, dotted bar), expressing Kv1.5-HA without (white bar) or with additional coexpression of wild-type AMPKWT (AMPKWT, dark grey bar), of inactive mutant AMPKαK45R (AMPKαK45R, light grey bar) or of constitutively active AMPKγR70Q (AMPKγR70Q, black bar). *** (p<0.001) indicates statistically significant difference from respective value obtained in oocytes expressing Kv1.5-HA alone.
mutant AMPK\textsuperscript{αK45R} or with constitutively active AMPK\textsuperscript{γR70Q}. As a result, the cell surface expression of the Kv1.5-HA channel protein in Kv1.5-HA expressing \textit{Xenopus} oocytes was significantly decreased following coexpression of constitutively active AMPK\textsuperscript{γR70Q}, but not following coexpression of inactive mutant AMPK\textsuperscript{αK45R}. Coexpression of wild-type AMPK tended to decrease Kv1.5-HA protein abundance in the cell membrane, an effect, however, not reaching statistical significance. The effect of AMPK\textsuperscript{αR70Q} on chemiluminescence was seemingly larger than the effect of AMPK\textsuperscript{γR70Q} on the current. However, the experiments have been done in different batches of oocytes precluding safe conclusions from comparisons of current and chemiluminescence. In any case, AMPK downregulated both, current and channel protein in the cell membrane. Confocal microscopy of Kv1.5 expressing oocytes again revealed decreased channel protein abundance following coexpression of AMPK thus confirming the results obtained with chemiluminescence (Fig. 4A).

**Discussion**

The present study reveals a novel function of AMP-activated protein kinase, i.e. the regulation of the voltage-gated K\textsuperscript{+} channel Kv1.5. The AMP-activated protein kinase decreases the channel protein abundance in the cell membrane. AMPK may thus decrease K\textsuperscript{+} conductance and repolarization.

The effect of AMPK is mimicked by coexpression of the ubiquitin ligase Nedd4-2. According to previous observations [70], AMPK phosphorylates Nedd4-2 thus fostering the interaction of the ubiquitin ligase with the epithelial Na\textsuperscript{+} channel ENaC [69-71]. As AMPK, Nedd4-2 and Kv1.5 are widely expressed, the AMPK and Nedd4-2 sensitivity of endogenous Kv1.5 may play a role in the regulation of a variety of cells. Moreover, Nedd4-2 may similarly contribute to the AMPK induced down-regulation of the inwardly rectifying K\textsuperscript{+} channel Kir2.1 [73] and the outwardly rectifying K\textsuperscript{+} channel KCNQ1/KCNE1 [74].

In contrast to its effect on ENaC, Kir2.1, KCNQ1/KCNE1 and Kv1.5, AMPK stimulates the activity of the facilitative glucose carriers GLUT1, GLUT2, GLUT3 and GLUT4 and thus increases the cellular uptake of glucose [47, 50, 53-58, 60, 63-65]. Stimulation of cellular glucose uptake provides substrate, which is subsequently utilized by AMPK stimulated glycolysis [43, 44]. AMPK further enhances ATP generation by stimulation of fatty acid oxidation and expression of enzymes required for ATP production [43, 44].

The inhibitory effect of AMPK on K\textsuperscript{+} channels is expected to depolarize the cell membrane with decrease of the driving force for electrogenic HCO\textsubscript{3}\textsuperscript{-} exit leading to cytosolic alkalization, which enhances the flux though glycolysis [101]. The alkalization inhibits Na\textsuperscript{+}/H\textsuperscript{+} exchangers [102], which curtails Na\textsuperscript{+} entry and thus decreases the requirement for energy-consuming Na\textsuperscript{+} extrusion by the Na\textsuperscript{+}/K\textsuperscript{+} ATPase [102]. On the other hand, AMPK stimulates the Na\textsuperscript{+}/H\textsuperscript{+} exchanger [103], which contributes to cytosolic alkalization. Inhibition of K\textsuperscript{+} channels is further expected to limit cellular K\textsuperscript{+} loss during impaired function of Na\textsuperscript{+}/K\textsuperscript{+} ATPase in energy-depleted cells. Cellular K\textsuperscript{+} loss is well known to stimulate suicidal cell death or apoptosis [104-108]. Apoptosis is further sensitive to cytosolic pH and fostered by cytosolic acidification [109].

The depolarization is, however, expected to foster Cl\textsuperscript{-} entry, which may eventually lead to deleterious cell swelling [110, 111]. In the heart, inhibition of K\textsuperscript{+} channels compromises maintenance of the cell membrane potential and repolarization thus jeopardizing cardiac function. Thus, inhibition of K\textsuperscript{+} channels in energy depletion may be considered a double edged sword.

AMPK is not only activated by energy depletion, but in addition by an increase in cytosolic Ca\textsuperscript{2+} activity [42], by a decrease of O\textsubscript{2} levels [112] and by exposure to nitric oxide [113]. Thus, AMPK may mediate effects of Ca\textsuperscript{2+} activity, hypoxia and nitric oxide on Kv1.5 activity.

In conclusion, the present observations disclose an inhibitory effect of the AMP-activated kinase on voltage gated K\textsuperscript{+} channels Kv1.5. The effect presumably participates in
the regulation of Kv1.5 channel activity during energy depletion, hypoxia, excessive cytosolic Ca\(^{2+}\) activity, and exposure to nitric oxide.

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**Declaration of Interests**

The authors of this manuscript declare that they have neither financial nor any other conflicts of interests.

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