PKC and AMPK regulation of Kv1.5 potassium channels

Martin Nybo Andersen#, Lasse Skibsbye#, Chuyi Tang, Frederic Petersen, Nanna MacAulay, Hanne Borger Rasmussen, and Thomas Jespersen*
Danish National Research Foundation Center for Cardiac Arrhythmia; Dept. of Biomedical Sciences; Faculty of Health and Medical Sciences; University of Copenhagen; Copenhagen, Denmark

#These authors contributed equally.

The voltage-gated Kv1.5 potassium channel, conducting the ultra-rapid rectifier K⁺ current (IKur), is regulated through several pathways. Here we investigate if Kv1.5 surface expression is controlled by the 2 kinases PKC and AMPK, using Xenopus oocytes, MDCK cells and atrial derived HL-1 cells. By confocal microscopy combined with electrophysiology we demonstrate that PKC activation reduces Kv1.5 current, through a decrease in membrane expressed channels. AMPK activation was found to decrease the membrane expression in MDCK cells, but not in HL-1 cells and was furthermore shown to be dependent on co-expression of Nedd4–2 in Xenopus oocytes. These results indicate that Kv1.5 channels are regulated by both kinases, although through different molecular mechanisms in different cell systems.

Introduction

Kv1.5 potassium channels are expressed in a number of cell types where they have been suggested to be involved in several physiological functions, including insulin secretion and smooth muscle contraction. In cardiac atria currents conducted through Kv1.5 channels underlie the ultra-rapid delayed rectifier potassium current IKur, which plays an important role in the early repolarization of the atrial action potential. Reduced expression of Kv1.5 has been linked to inherited atrial fibrillation, while pharmacological blockade of IKur has been suggested to be anti-arrhythmic. Kv1.5 channels are regulated by auxiliary proteins and by secondary modifications e.g. phosphorylation and ubiquitylation. Kv1.5 β-subunits alter both voltage-dependence of activation and slow and fast inactivation components while KChIP subunits reduce the cell surface expression of Kv1.5 channels without changing current kinetics. Protein kinase A has been reported to down-regulate Kv1.5 current through phosphorylation of Kvβ1.3, an accessory subunit which has a profound effect on the Kv1.5 current kinetics. Several studies have revealed that protein kinase C (PKC) modify the current kinetics of Kv1.5 through phosphorylation of either Kvβ1.2 or Kvβ1.3. Furthermore, it has been reported that SGK1 upregulates Kv1.5 current through phosphorylation of either Kvβ1.2 or Kvβ1.3. Furthermore, it has been reported that SGK1 upregulates Kv1.5 current through a downregulation of the neural precursor cell expressed developmentally downregulated protein 4–2 (Nedd4–2), which is an E3 type ubiquitin ligase.
processes following polarization of epithelial Madin Darby Canine Kidney (MDCK) cells. Polarization of MDCK cells triggers a PKC activity which probably through LKB1 activates AMPK leading to increased activity of Nedd4-2.20 For the Kv7.1 channel, the activation of Nedd4-2 results in an increased ubiquitylation, which leads to internalization and degradation.21-24

In this study we investigate whether an observed reduction in Kv1.5 surface expression following MDCK polarization is mediated by PKC activation and/or by the downstream mechanisms of AMPK activation though Nedd4-2 ubiquitylation, ultimately leading to reduced Kv1.5 current. Since this could have implications for the regulation of native Kv1.5 currents and thus, among other electrophysiological responses, the transient cardiac atrial repolarization, we furthermore investigated PKC and AMPK activation in the atrial derived HL-1 cell line.

**Materials and Methods**

**DNA constructs**

Rat Kv1.5 and human Nedd4-2 were expressed from the plasmids pXOOM-rKv1.5 25 and pXOOM-hNedd4-2.23 pDsRed2-ER was purchased from Clontech Laboratories, CA, USA.

**Antibodies and chemicals**

The antibodies used in this study were rabbit polyclonal anti-Kv1.5 (1:50, APC-004, Alomone Labs), Alexa Fluor®488-conjugated donkey anti-rabbit IgG (1:200, Invitrogen), Alexa Fluor®647-conjugated phalloidin (1:200, Invitrogen) and 4’,6-diamidino-2-phenylindole (DAPI, 1:300, Invitrogen).

The activators used were as follows: 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR), 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranosyl-5’-monophosphate (ZMP), 2-O-tetradecanoylphorbol-13-acetate (PMA) and 2-chloro-5-[5’-([5,4,5-dimethyl-2-nitropheny]-2-furanyl)methylene]-4,5-dihydro-4-oxo-2-thiazolyl]amino]benzoic-acid (PT1). All compounds were purchased from Sigma-Aldrich (Copenhagen, DK) except PT1 which was obtained from Tocris Bioscience (Bristol, UK).

**Transient expression in MDCK cells**

MDCK (strain II) cells were grown in DMEM (Invitrogen) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 10% FBS (Sigma-Aldrich) at 37°C in a humidified atmosphere with 5% CO2.

MDCK cells were transfected with 2 μg of pXOOM-rKv1.5 (in co-transfections with pDsRed2-ER 1 μg of each plasmid were used) using Lipofectamine and Plus Reagent (Invitrogen) according to manufacturer’s protocol. After transfection, the cells were plated on glass coverslips (12 mm in diameter, Thermo Scientific).

**Transient expression in HL-1 cells**

HL-1 cells were grown in Claycomb medium (Sigma-Aldrich, Copenhagen, DK) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-Glutamine, 0.1 mM Norepinephrine and 10% FBS (Sigma-Aldrich) at 37°C in a humidified atmosphere with 5% CO2. HL-1 cells were transfected in suspension using SilectFect (Bio-Rad laboratories) and plated on glass coverslips (12 mm in diameter, Thermo Scientific) or in T25 Flasks (Thermo Scientific).

**Calcium switch experiment**

MDCK cells expressing pXOOM-rKv1.5 and DsRed2-ER were plated on glass coverslips and the calcium switch experiment was performed as previously described.20

**Immunofluorescence**

HL-1 and MDCK cells grown on glass coverslips were fixed in 4% paraformaldehyde in PBS for 15 and 30 minutes at room temperature, respectively. Blocking was performed by a 30-minute incubation with 0.2% fish skin gelatin in PBS supplemented with 0.1% Triton X-100 (PBST). The cells were incubated for 1 hour in primary antibodies diluted in PBST. Secondary antibodies were diluted in PBST and applied for 45 minutes. The coverslips were mounted in Prolong Gold (Invitrogen).

**Confocal microscopy and imaging**

Laser scanning confocal microscopy was performed using the Zeiss LSM 780 confocal system. Images were acquired using a 63× oil immersion objective, NA 1.4 with a pinhole size of 1 and a pixel format of 1024×1024. Line averaging was used to reduce noise. Sequential scanning was employed to allow the separation of signals from the individual channels. Images were adjusted using the Zen 2011 software (Carl Zeiss).

**cRNA generation and oocytes injection**

cRNA was generated from linearized pXOOM clones using the Ambion T7 m-Message Machine kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. cRNA concentrations were determined using the ND-1000 NanoDrop UV spectrophotometer and its integrity was confirmed by gel electrophoresis. cRNAs were stored at −80°C until injection. *Xenopus Laevis* surgery and oocyte treatment were done as previously described.26 Before cRNA injection the oocytes were kept at 19°C for at least 24 hours in Kulori solution consisting of (in mM) 4 KCl, 90 NaCl, 1 MgCl2, 1 CaCl2, 5 HEPES, pH 7.4. 50 nl of cRNA (250 pg of KCNA5, 50 pg of Nedd4-2) was injected using a Nanoject microinjector from Drummond (Drummond Scientific, Broomall, PA, USA). The oocytes were kept at 19°C for 2 to 3 d before the measurements were performed. 50 nl buffer solution (0.1 M NH4Cl, 0.1 M NaOH) alone or with diluted ZMP (84.5 ng) was injected one hour before the measurements.

**Electrophysiology**

**Oocyte experiments**

Two-electrode voltage-clamp recordings were performed in *Xenopus* Oocytes at room temperature under continuous superfusion with Kulori-buffer. The data was recorded using a Dagan CA-1B amplifier (Minneapolis, MN, USA), a HEKA EPC-9 interface and HEKA Pulse v8.54 software (HEKA Elektronik, Lambrecht/Pfalz, Germany). Electrodes pulled from borosilicate glass...
capillaries using a Sutter Instrument Co. model P-97 puller were filled with 2.5 M KCl and had a resistance of 0.3 – 0.6 MΩ. The currents were analyzed by applying a standard step protocol with pulses from −80 to +60 mV (over 4000 ms) with 20 mV increments from a holding potential of −80 mV. Only oocytes with membrane potential more negative than −35 mV were used. Oocytes were treated with 100 nM PMA, 84.5 ng ZMP or vehicle for 30 min – 1 hour before current recordings took place.

**Patch clamp**

Kv1.5 current was recorded in HL-1 cells transiently transfected with 0.5 µg pXOOM-rKv1.5 and 0.2 µg pcDNA3-eGFP as a reporter gene, using siLentFect lipid reagent, according to the manufacturer’s instructions. Whole cell patch-clamp experiments were performed at room temperature (22°C-25°C) 2–3 d after transfection. Extracellular solution was perfused at 1 ml/min. The extracellular solution consisted of (in mM) NaCl 140; KCl 4; CaCl₂ 2; MgCl₂ 1; HEPES 10; and D-Glucose 10 (pH 7.3 with NaOH); the intracellular (pipette) solution contained (in mM) KCl 140; Na₂ATP 1; EGTA 2; HEPES 10; CaCl₂ 0.1; MgCl₂ 1; and D-Glucose 10 (pH 7.4 with KOH). Borosilicate glass pipettes were pulled on a DPZ-Universal puller (Zeitz Instrumente, Martinsried, Germany). The pipettes had a resistance of 2.0-2.5 MΩ when filled with intracellular solution. The series resistance in the whole-cell configuration was compensated (80%). The seal resistance in all experiments was greater than 1.0 GΩ. Currents were recorded by application of voltage-step protocols detailed in the figures legends. Data analyses were performed as previously described.²⁷ HL-1 cells were treated with 100 nM PMA or vehicle for 20 min, applied directly to the extracellular solution before current recordings took place.

**Data Analysis**

All data are presented as mean ± standard error of the mean (SEM). Statistical analysis in Figure 2 and Figure 3 was done using One-way analysis of variance (ANOVA) with Bonferroni post hoc test. P-values < 0.05 were considered statistically significant. GraphPad Prism 5 (GraphPad Software, USA) was used for statistical analysis. In figures, statistical significance is denoted by * = P < 0.05, ** = P < 0.01, *** = P < 0.001.

**Results**

Kv1.5 channels are known to be expressed in various epithelial cells.²⁸,²⁹ It has previously been demonstrated that both PKC and AMPK activation lead to a reduction of Kv1.5 currents in different cell systems.¹³,¹⁹ In order to investigate whether this reduction in current levels could be due to removal of Kv1.5 channels from the plasma membrane we took advantage of the epithelial MDCK cell system, in which we previously have reported an activated PKC-AMPK-Nedd4–2 pathway during MDCK cell polarization.²⁰,²³ The polarization process can be controlled by the calcium switch assay (please see materials and methods and²⁰). Briefly, MDCK cells were co-transfected with Kv1.5 and a marker for endoplasmatic reticulum (DsRed-ER) and grown in low calcium DMEM medium, which prevents initiation of the polarization process. Two days after transfection, the low calcium medium was changed to normal DMEM medium, thereby beginning the polarization process. As illustrated in Figure 1A, initiation of MDCK cell polarization resulted in the removal of surface expressed Kv1.5 channels. To examine if the removal of surface expressed channels could be due to activation of PKC and/or AMPK we repeated the experiment, but instead of changing the low calcium medium to normal medium, we added 100 nM PMA (a potent activator of PKC) or the AMPK activators AICAR (500 µM) and PT1 (200 µM) to MDCK cells kept in low calcium medium. The two AMPK activators were chosen as they activate AMPK by 2 different mechanisms; AICAR activates AMPK by an indirect increase in the intracellular AMP concentration through the AMP analog ZMP and PT1 has a direct mode of action near the auto-inhibitory domain of the AMPK α-subunit.³⁰ Both PKC (Fig. 1B) and AMPK (Fig. 1C) activation lead to removal of surface expressed Kv1.5 channels as also observed upon initiation of the polarization process. As shown in Figure 1, Kv1.5 appears to accumulate in the endoplasmic reticulum 3 hours into the polarization process and after 3 hours of drug treatment (Fig. 1A, B and C).

As Kv1.5 is expressed in the atria,³¹ we investigated if activation of PKC and AMPK also would reduce surface-expression of the channel in the atrial cell line HL-1.³² Kv1.5 transfected HL-1 cells were treated with 100 nM PMA to activate PKC or with 1 mM AICAR or 200 µM PT1 to activate AMPK. Untreated cells expressed Kv1.5 both at the cell surface and in intracellular structures resembling the ER (Fig. 2A). Membrane expression was reduced after PMA treatment (Fig. 2A, 30 min PMA and 3 h PMA) and instead vesicular structures containing Kv1.5 had appeared (Fig. 2A, 30 min PMA). These observations could be speculated to be due to endocytosis of the channel protein. Surprisingly activation of AMPK by either of the 2 activators did not alter the localization of Kv1.5 channels as surface expression was still observed after AMPK activation (Fig. 2B).

To investigate whether the PKC induced reduction of Kv1.5 surface expression could be detected functionally, we performed patch-clamp measurements on HL-1 cells expressing Kv1.5 (Fig. 2C). Following a voltage-step protocol Kv1.5 currents appeared at potentials more depolarized to −20 mV. The Kv1.5 currents arising from HL-1 cells show a relatively fast activation with a minor inactivation component, similar to native Kv1.5 currents recorded in cardiomyocytes.³³ In agreement with the imaging data, PMA treatment significantly reduced the Kv1.5 current (Fig. 2D).

To investigate whether Nedd4–2 is involved in the observed responses to AMPK and PKC activation, 2-electrode voltage-clamp current recordings in *Xenopus laevis* oocytes expressing Kv1.5 with or without Nedd4–2 were performed (Fig. 3). We have previously
reported AMPK activation to stimulate the activity of co-expressed Nedd4–2, leading to removal of Kv7.1 channels from the cell surface.23 Further, it has recently been demonstrated that AMPK overexpression in Xenopus oocytes reduces Kv1.5 current levels.19 As Kv1.5 is regulated by Nedd4–2,17,19 we therefore hypothesized that Nedd4–2 is involved in the observed PKC/AMPK regulation leading to reduced Kv1.5 membrane expression and current levels. Figure 3 shows representative Kv1.5 current recordings from oocytes expressing either Kv1.5 or Kv1.5/Nedd4–2 with or without PMA treatment. Expression of Nedd4–2 (1:8 molar ratio to Kv1.5 cRNA) resulted in a 3-fold reduction in the current amplitude without affecting the Kv1.5 current kinetics (Fig. 3E). Application of PMA resulted in an approximately 4-fold current reduction in both Kv1.5 and Kv1.5/Nedd4–2 expressing oocytes (Fig. 3E). Similar experiments were conducted using the AMPK activator ZMP (Fig. 3F). The AMP analog ZMP activates AMPK by increasing the AMP/ATP ratio and has previously been used in Xenopus oocyte experiments.23,34 Contrary to PKC activation, AMPK activation did not lead to a direct effect on the Kv1.5 current expressed in oocytes. However, when Nedd4–2 was co-injected we observed a reduction in Kv1.5 current and this current reduction was even more pronounced in ZMP treated oocytes. These observations indicate that in Xenopus oocytes PKC activation has a direct inhibitory effect on Kv1.5 circumventing the Nedd4–2 pathway, while AMPK activation primarily regulates Kv1.5 through the Nedd4–2 pathway (Fig. 4).

Discussion

A very tight regulation of ion channel membrane expression is crucial in the control of many physiological functions. This is the case in atrial electrophysiology, where Kv1.5 channels constitute the pore forming subunit of the ultra-rapid delayed rectifier current (I_Kur). We recently reported dynamic changes in ion channel surface expression during polarization of the epithelial MDCK cell line. In the current study, we observed that the surface expression of exogenously expressed Kv1.5 channels is reduced in response to initiation...
of MDCK cell polarization. For Kv7.1 we have demonstrated that MDCK cell polarization leads to channel endocytosis through activation of a PKC-AMPK-Nedd4–2 pathway.20,23 Similarly, we here observe that a reduction in Kv1.5 membrane expression could be induced by stimulating either PKC or AMPK. Furthermore, we observed accumulation of Kv1.5 in the endoplasmic reticulum, which could be due to the synthesis of new channels, analogous to what we previously have reported for the Kv7.1 potassium channel.20,23 As AMPK is a well-known activator of the Nedd4-like ubiquitylating enzyme family (which is a group of E3 enzymes that tag surface proteins for internalization through attachment of ubiquitin moieties to their target proteins) we investigated whether Nedd4–2 can reduce the current levels of Kv1.5 and whether PKC and AMPK reduces Kv1.5 surface expression through Nedd4–2. PKC activation induced a ~75% reduction in both oocytes expressing only Kv1.5 and in oocytes co-expressing Kv1.5 and Nedd4–2, as compared to non-stimulated controls. In contrast, activation of endogenous AMPK did not reduce the current level in oocytes expressing only Kv1.5. Mia and colleagues 19 reported a small reduction in Kv1.5 current when co-expressing AMPK enzymes. Similar discrepancy between an effect of overexpressing WT AMPK and activating endogenous AMPK in relation to the current amplitude of Kv7.1 has previously been reported.22,23 In agreement with previous reports we found that co-expression of Nedd4–2 produced a pronounced reduction in Kv1.5 current levels.19 In oocytes co-expressing Kv1.5 and Nedd4–2, a ~50% current reduction was observed. Activation of AMPK further reduced the Kv1.5 current to ~25%. These results indicate that in Xenopus oocytes a reduction in Kv1.5 current amplitude can be induced both by an AMPK-Nedd4–2 pathway and by a PKC pathway operating independently of Nedd4–2. The PKC pathway might operate directly on Kv1.5 or through auxiliary subunits such as KChIPs or Kvβ subunits, which are both known to interact with Kv1.5.9-11 In fact, co-expression of KChIP2 subunits has been demonstrated to affect the surface expression of Kv1.5.11 Furthermore, PKC activation can result in phosphorylation of the Kvβ1.2 subunit which leads to a relatively slow time dependent reduction in Kv1.5.

Figure 2. PKC but not AMPK activation reduces Kv1.5 surface expression in HL-1 cells. (A and B) Confocal scans of HL-1 cells transiently transfected with Kv1.5 cDNA and treated with PMA (100 nM), AICAR (1 mM) or PT1 (200 μM). As illustrated, surface expression of Kv1.5 channels was reduced in response to PKC activation (A). This response was not observed with either of the 2 AMPK activators (B). Cytoskeletal actin staining was used as a marker for the membrane. Scalebar, 10 μm. (C) Representative traces of Kv1.5 currents recorded in HL-1 cells before and after treatment with PMA. Protocol shown in the insert. (D) Current-voltage relationship of currents recorded in Kv1.5 transfected and untransfected HL-1 cells. PMA significantly down regulated the current level by 74%. Numbers of cells are: Kv1.5 (n=12); Kv1.5+PMC (n=13); untransfected (n=8); untransfected+PMA (n=10).
current,\textsuperscript{13,14} which could indicate that the channels are removed from the plasma membrane like we observe in this study. Based on these reports it would in future studies therefore be of interest to address how important auxiliary subunits are for the regulatory pathways we have investigated in the present study. As Kv1.5 channels are important in atrial electrophysiology we investigated whether PKC and AMPK activation also impacts the localization of Kv1.5 channels when expressed in the atrial muscle cell line HL-1. As observed in MDCK cells, PKC activation leads to a reduction in Kv1.5 surface expression and reduced current amplitude in these cells. In contrast, activation of AMPK was without effect on Kv1.5 localization, suggesting that the AMPK-Nedd4–2 pathway is not an

\textbf{Figure 3.} Two-electrode voltage clamp recordings of \textit{Xenopus laevis} oocytes expressing Kv1.5 +/− Nedd4–2 following PKC and AMPK activation. (A-D) Representative Kv1.5 current recordings following a step protocol. (E) PKC activation by PMA resulted in a drastic Kv1.5 current reduction both in the presence and absence of co-expressed Nedd4–2. (E) Similar experiments were conducted using the AMPK activator ZMP (100 nM). Contrary to PKC activation, AMPK activation did not affect Kv1.5 current levels when Nedd4–2 was not co-expressed. However, when Nedd4–2 was co-injected, leading to a down-regulation of Kv1.5 surface current, ZMP induced a further current reduction. The number of cells in each group was (n>10).
important regulator of Kv1.5 in HL-1 cells. Whether this is due to lack of expression of Nedd4-like proteins interacting with Kv1.5, expression of different endogenous β-subunit or an AMPK subunit configuration not sustaining this pathway or other undisclosed mechanisms cannot be answered through the results obtained in this study.

**Conclusion**

PKC activation leads to a reduction in surface expression of the voltage-gated potassium channel Kv1.5 in both the epithelial MDCK cells and in atrial HL-1 cells and resulted in reduced currents in *Xenopus* oocytes. The regulation of Kv1.5 by the AMPK-Nedd4–2 pathway was found to be highly dependent on the cell system as we observed reduced current in *Xenopus* oocytes and reduced membrane expression in MDCK cells, but not in HL-1 cells. Our *Xenopus* oocyte data furthermore indicate an involvement of Nedd4–2 in the AMPK, but not the PKC mediated decrease in Kv1.5 current. Thereby, this study reveals a new cell specific aspect in the regulation of Kv1.5, which may have important implications on several physiological functions including cardiac atrial repolarization.

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**Figure 4.** Schematic illustration of the PKC and AMPK pathways resulting in Kv1.5 downregulation. The polarization of MDCK cells triggers activation of PKC and AMPK kinases. PKC activation can be mimicked by PMA. We suggest this activation of PKC can impact Kv1.5 channels through 2 pathways, depending on which the cell system used. This might be through AMPK activation (that can be mimicked by PT1, AICAR and ZMP), which will activate Nedd4 ubiquitylating enzymes or by another so far undisclosed mechanism. Activation of both pathways results in a reduced Kv1.5 surface expression.
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