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The KCNQ1 potassium channel is down-regulated by ubiquitylating enzymes of the Nedd4/Nedd4-like family

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Abstract

Objective: The voltage-gated KCNQ1 potassium channel regulates key physiological functions in a number of tissues. In the heart, KCNQ1 α-subunits assemble with KCNE1 β-subunits forming a channel complex constituting the delayed rectifier current I_{Ks}. In epithelia, KCNQ1 channels participate in controlling body electrolyte homeostasis. Several regulatory mechanisms of the KCNQ1 channel complexes have been reported, including protein kinase A (PKA)-phosphorylation and β-subunit interactions. However, the mechanisms controlling the membrane density of KCNQ1 channels have attracted less attention.

Methods and results: Here we demonstrate that KCNQ1 proteins expressed in HEK293 cells are down-regulated by Nedd4/Nedd4-like ubiquitin-protein ligases. KCNQ1 and KCNQ1/KCNE1 currents were reduced upon co-expression of Nedd4-2, the isoform among the nine members of the Nedd4/Nedd4-like family displaying the highest expression level in human heart. *In vivo* expression of a catalytically inactive form of Nedd4-2, able to antagonize endogenous Nedd4-2 in guinea-pig cardiomyocytes, increased I_{Ks} significantly, but did not modify I_{K1}. Concomitant with the reduction in current induced by Nedd4-2, an increased ubiquitylation as well as a decreased total level of KCNQ1 proteins were observed in HEK293 cells. Pull-down and co-immunoprecipitation experiments showed that Nedd4-2 interacts with the C-terminal part of KCNQ1. The Nedd4/Nedd4-like-mediated regulation of the KCNQ1 channel complexes is strictly dependent on a PY motif located in the distal part of the C-terminal domain. When this motif was mutated, the current and ubiquitylation levels were unaffected by Nedd4-2, and Nedd4-2 proteins were neither pulled-down nor co-immunoprecipitated.

Conclusions: These results suggest that KCNQ1 internalization and stability is physiologically regulated by its Nedd4/Nedd4-like-dependent ubiquitylation. This mechanism may thereby be important in regulating the surface density of the KCNQ1 channels in cardiomyocytes and other cell types.

Keywords: KCNQ1; KCNE1; WWP2; qPCR; Ubiquitin; Nedd4

This article is referred to in the Editorial by J. Ehrlich (pages 6–7) in this issue.

1. Introduction

The delayed rectifier potassium current I_{Ks} plays a pivotal role in the human heart. This slowly activating current peaks at the end of the cardiac action potential, and together with

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I_{Kr} (ERG1) and I_{K1} (Kir2.x), it repolarizes the membrane of cardiac cells. Hence, the I_{Ks} amplitude partly determines the cardiac action potential duration. The molecular components underlying I_{Ks} are the voltage-gated KCNQ1 channel assembled with the KCNE1 (minK) β-subunit [1,2]. The importance of functional I_{Ks} channels is underlined by the fact that reduction of the current caused by mutations in either *KCNQ1* or *KCNE1* may cause congenital long QT syndrome, and life-threatening arrhythmia [3]. KCNQ1 channels are not only found in the heart, but also in epithelial tissues, such as kidney and intestine, where they are involved in salt and water transport across the epithelia [4].

Within the past few years, a number of ion channels have been shown to be regulated by Nedd4/Nedd4-like ubiquitin-protein ligases [5]. Nedd4/Nedd4-like proteins bind to, ubiquitylate, and are thought to modulate the internalization of certain channel subunits bearing a so-called PY motif found in their intracellular C-terminal domains. Ubiquitin is a 76-residue protein which, upon covalent attachment to lysines of target membrane proteins, signals internalization and/or degradation by lysosomes or proteasomes [6]. Proteins can either be monoubiquitylated (one ubiquitin per lysine), multiubiquitylated (several monoubiquitylated sites on one target protein), or polyubiquitylated (chains of ubiquitin attached to one lysine) [7]. Protein ubiquitylation is achieved by specific ubiquitin-protein ligase enzymes named E3s [6]. The Nedd4/Nedd4-like proteins are E3 enzymes comprising nine different members encoded in the human and mouse genomes. This family is characterized by a C-terminal catalytic domain homologous to E6–AP–COOH terminus (HECT) domain, 2 to 4 WW domains, promoting interaction with PY motifs of target proteins, and an N-terminal, calcium-dependent, lipid-binding domain [6]. The tissue expression pattern of the Nedd4/Nedd4-like genes/proteins has been analyzed for some of the nine members of the family [8–11], and it has been found that Nedd4-2 is well-expressed in kidney. However, a comprehensive characterization of the relative tissue expression levels of all nine members of the family has not yet been performed.

The epithelial sodium channel ENaC, which plays an important role in body sodium homeostasis, is the most extensively investigated ion channel undergoing ubiquitylation [5]. Through an interaction with a PY motif in the C-terminal region of ENaC subunits, Nedd4-2 mediates ubiquitylation of the channel, thus enhancing its internalization [12]. The ClC-5 chloride transporter, which is contributing to the acidification of the endocytic pathway, is also regulated by Nedd4/Nedd4-like proteins [13]. Recently our group reported that Nedd4/Nedd4-like proteins are capable of down-regulating the $Na_v1.5$ sodium channel, which mediates the fast depolarization of the cardiac action potential [14]. As for ENaC, Nedd4-2-induced down-regulation of $Na_v1.5$ depends on a functional PY motif located in the C-terminal domain.

In this report, we investigated the regulatory role of Nedd4/Nedd4-like ubiquitin-protein ligases on KCNQ1 and KCNQ1/KCNE1 channels. The obtained results suggest that ubiquitylation of KCNQ1 protein is a way to regulate the protein expression and membrane density of KCNQ1 complexes. This regulatory mechanism may thereby prove important in understanding the dynamics of physiological functions such as the termination of the cardiac action potential, and regulation of body electrolyte homeostasis.

2. Materials and methods

2.1. DNA constructs

pXOOM–hKCNQ1 and pBi–KCNE1–KCNQ1, which includes an EMCV–IRES element, ensuring expression of both human KCNE1 and human KCNQ1 from the same mRNA transcript, have been described previously [15]. Similarly, pcDNA3.1–hNedd4-2, pcDNA3.1–hNedd4-1, and pcDNA3.1–hWWP2–T7 were described earlier [16]. KCNQ1 PY motif, Nedd4–2CS, and Nedd4–1CS mutant constructs were generated using the QuickChange Mutagenesis Kit (Stratagene) and pIRES–CD8–hNedd4-2 plasmids (wt/CS) were generated from pIRES–CD8 and pcDNA3.1–hNedd4-2 (wt/CS). GST–KCNQ1 constructs were generated by PCR amplification of the 150 bp encoding the C-terminal fragment (amino acids 627–676) of hKCNQ1 followed by insertion into pGEX–4T1.

2.2. Real-time PCR

cDNA synthesis of human brain/heart/kidney total RNA (Ambion®, Inc, TX, USA) were accomplished with M–MuLV reverse transcriptase (Q-BIOgene, CA, USA) using hexamer random primers. Real-time PCR of 40 ng (RNA-to-cDNA) in 20 µl was performed with the Taqman® 2xPCR master mix on an ABI PRISM 7500 Sequence Detector (Applied Biosystems, CA, USA). Cyclophilin A was used as an internal control (VIC-TAMRA probes). Additional information is found in the online data supplement.

2.3. Transfection and homogenization of HEK293 cells

HEK293 cells were transiently transfected using Lipofectamine 2000™ (Invitrogen, USA) according to manufacturer's instructions. The following DNA amounts were used; Pull-down assays: 5 µg/P100 dish of Nedd4-2, Nedd4-1, or WWP2; immunoprecipitation: 0.6 µg KCNQ1 or KCNQ1/KCNE1, 2.4 µg Nedd4/Nedd4-like in P60 dishes. pcDNA3 was used for control conditions in order to have equal DNA transfection amounts. Two to three days after transfection, cells were solubilized by 30 min rotation at 4 °C in lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Tx-100, 10% glycerol, 1 mM EGTA and Complete protease inhibitor cocktail (Roche, Switzerland). Soluble fractions were recovered in supernatants after 15 min of centrifugation at 18,000 ×*g*.

2.4. Pull-down assays

Pull-down assays with GST fusion proteins (GST–$KCNQ1_{627–676}$ (wt, PA, or YA)) and soluble fractions (~2 mg) of either Nedd4-2, Nedd4-1, or WWP2 transfected HEK293 cells were performed as previously described [16].

2.5. Western blotting

Antibodies against KCNQ1 (C20, Santa Cruz Biotechnology, CA, USA), Nedd4-2 (A27, described in [17]), Nedd4-1 (5606, described in [17]), WWP2-T7 (Sigma, MS, USA), and actin (Sigma, MS, USA) were incubated 2 h, RT in 5% BSA TBS-Tween buffer containing the specific antibodies (1/500 dilution). Anti-ubiquitin monoclonal FK2 antibody from Affiniti Research (Exeter, UK) was used in a 1/500 dilution with 1% milk and incubated 6–12 h at 4 °C.

2.6. Immunoprecipitation

Soluble fractions from transfected HEK293 cells were incubated for 2 hours by rotation at 4 °C with anti-KCNQ1 (C20, Santa Cruz). After addition of protein-G-Sepharose beads (Amersham Bioscience, Sweden), incubation followed for 1 hour. Following three washes of the beads in lysis buffer, IP-fractions were analyzed by Western blotting. For the co-IP assays of KCNQ1 and Nedd4-2 three washings were performed with the following buffer: 50 mM TRIS, pH 7.5, 500 mM NaCl, 2% Tx-100, 1 mM DTT, 1 mM PMSF.

2.7. Transfection of guinea-pig cardiomyocytes

100 μg pIRES–CD8–hNedd4-2 (wt/CS) or pIRES–CD8 alone were complexed with tetronic 304 (5%), a poloxamine provided by BASF (Mount Olive, NJ, USA), just before injection in guinea-pig myocardium, as previously described with PE6400 [18]. After subcutaneous atropine injection (0.1 mg/kg, atropine sulfate, Aguettant, France), guinea-pigs (300 g) were anesthetized with isofluran (3%) and tracheally ventilated (Harvard Rodent Ventilator, Harvard Apparatus, MS, USA). After left lateral thoracotomy, the injection (50 μL) was performed into the left ventricular free wall with a 100-μl Hamilton syringe. Four to eight days later, ventricular myocytes were dissociated by standard enzymatic digestion.

2.8. Electrophysiology

HEK293 cells were co-transfected with 0.2 μg KCNQ1 or KCNQ1/KCNE1, 0.6 μg Nedd4/Nedd4-like constructs, and 0.8 μg cDNA encoding CD-8. Transfected HEK293 cells and myocytes were detected with Dynabeads M-450 CD8 (Dynal Biotech, Norway). Experiments were performed under whole-cell patch-clamp configuration at room temperature for HEK293 cells and at 37 °C for myocytes. Pipettes had a resistance between 1.5 and 3.5 MΩ. Composition of solutions is found in the online data supplement.

2.9. Analysis of data

Data are represented as mean ± SEM. Two-tailed Student *t* test was used to compare the means.

3. Results

It has been previously shown that several types of ion channels sharing a C-terminal PY motif as a common feature are regulated by Nedd4/Nedd4-like ubiquitin-protein ligases [5]. KCNQ1 orthologs of various species also contain a stretch of residues in the distal domain of the C-terminal tail (9–17 residues from the carboxy-terminal of the human sequence, see Fig. 1A and B) conforming to a PY consensus

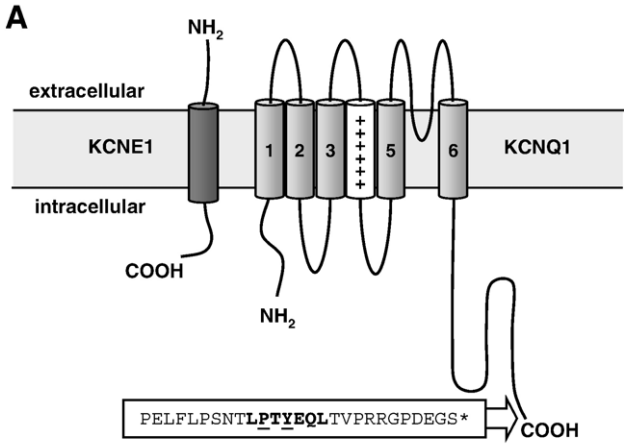

Fig. 1. KCNQ1 contains a C-terminal PY motif. (A) Topology of the six-transmembrane domain KCNQ1 protein (right), containing intracellular located N- and C-termini, a voltage-sensing transmembrane domain (in white). The one-transmembrane domain β-subunit KCNE1 is shown on the left. Four KCNQ1 α-subunits assemble to form the basic channel. The KCNQ1 protein sequence of the distal part of the C-terminal bears a PY motif (bold characters), which potentially interacts with the WW domains of Nedd4/Nedd4-like proteins. PY motifs are defined according to the following sequence: L/P–P–x–Y–x–x–Φ, where L is a leucine, P is a proline, Y is a tyrosine, x any amino acid, and Φ a hydrophobic amino acid [25,26]. Residues mutated in this study are underlined. (B) Alignment of the C-terminal peptide sequence of KCNQ1 of five animal species using T-coffee (www.ch.embnet.org/software/TCoffee.html).

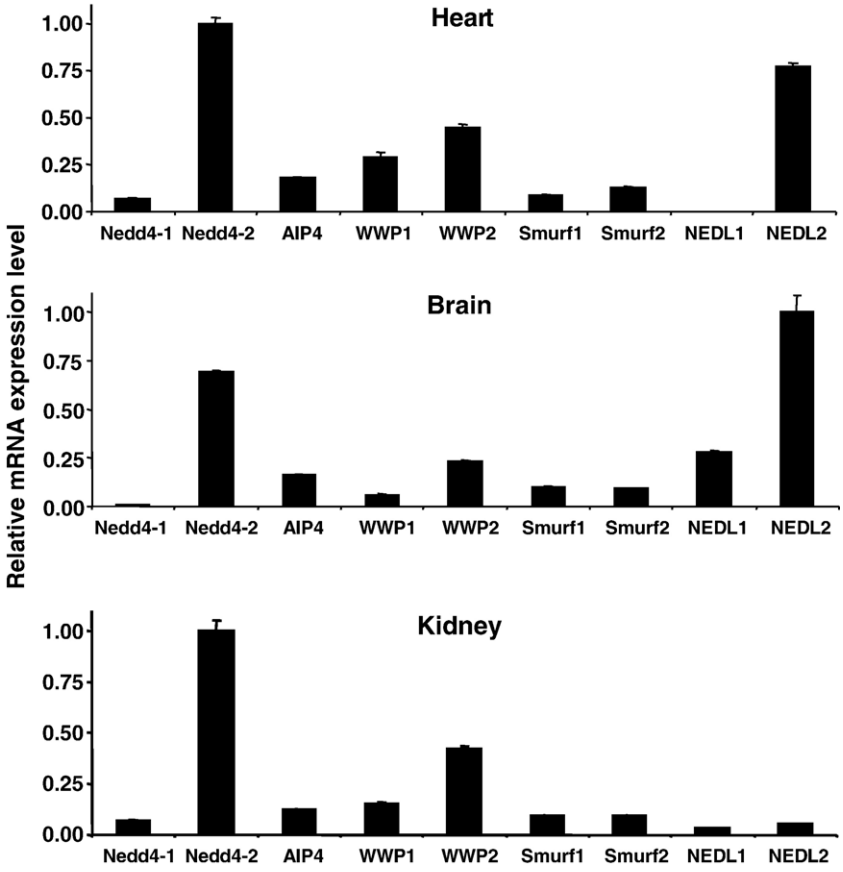

Fig. 2. mRNA quantification (qPCR) of the nine members of the Nedd4/Nedd4-like family. Taqman® real-time PCR analyses of Nedd4/Nedd4-like mRNA's isolated from human heart, brain, and kidney. The expression levels were normalized to the highest expressing gene in each tissue. All values are an average of 9 quantification measurements obtained from at least two different reverse transcriptase synthesis and three different real-time PCR runs.

motif [5]. This feature is exclusively found in the KCNQ1 subfamily, since the C-termini of the other members of the KCNQ family (KCNQ2–5) do not contain any sequence conforming to the PY motif consensus (not shown). Furthermore, the KCNE1 modulatory β-subunit does not contain any PY motif either (not shown). Because of the presence of this PY motif in KCNQ1, we investigated whether this channel may be regulated by Nedd4/Nedd4-like enzymes.

3.1. Quantification of Nedd4/Nedd4-like mRNA expression

KCNQ1 is well expressed in heart and kidney. We might hypothesize that, if Nedd4-like proteins are involved in the regulation of this channel, the isoforms which are expressed at the highest levels in the above-mentioned tissues will be the most likely candidates regulating KCNQ1. Therefore, in order to establish which of the 9 different Nedd4/Nedd4-like proteins could play a physiological role in human heart and kidney, real-time RT-PCR (Taqman®) analyses were performed (Fig. 2). qPCR analyses were also carried out on brain cDNA in order to have a more comprehensive view of the variability of expression. Total human mRNA from whole organs (Ambion®) were reverse transcribed into cDNA and analyzed with primer sets spanning at least one intron. All analyses were performed on cDNA from the 3′ end of the genes as several splice variants have been reported in the 5′ end of some of the mRNA's encoding Nedd4/Nedd4-like proteins [19]. Nedd4-2 has the highest mRNA expression level in heart and kidney, while NEDL2 shows the highest level in brain. Nedd4-2 is also highly expressed in brain and NEDL2 likewise in heart. WWP2 shows an intermediate expression level in all three organs, while Nedd4-1 shows a low abundance in all three organs, compared to Nedd4-2 and NEDL2. These results led us to primarily focus on the potential of Nedd4-2 to regulate KCNQ1.

3.2. Nedd4/Nedd4-like proteins down-regulate KCNQ1 and KCNQ1/KCNE1 currents

The effects of Nedd4-2 on KCNQ1 and KCNQ1/KCNE1 currents in transfected HEK293 cells were investigated by whole-cell patch-clamping. Applying voltage-steps to cells expressing KCNQ1 channels evoked relatively slowly activating potassium currents at potentials more positive than −40 mV (Fig. 3A). Co-expression of KCNE1 drastically altered the kinetics of the KCNQ1 channel. As

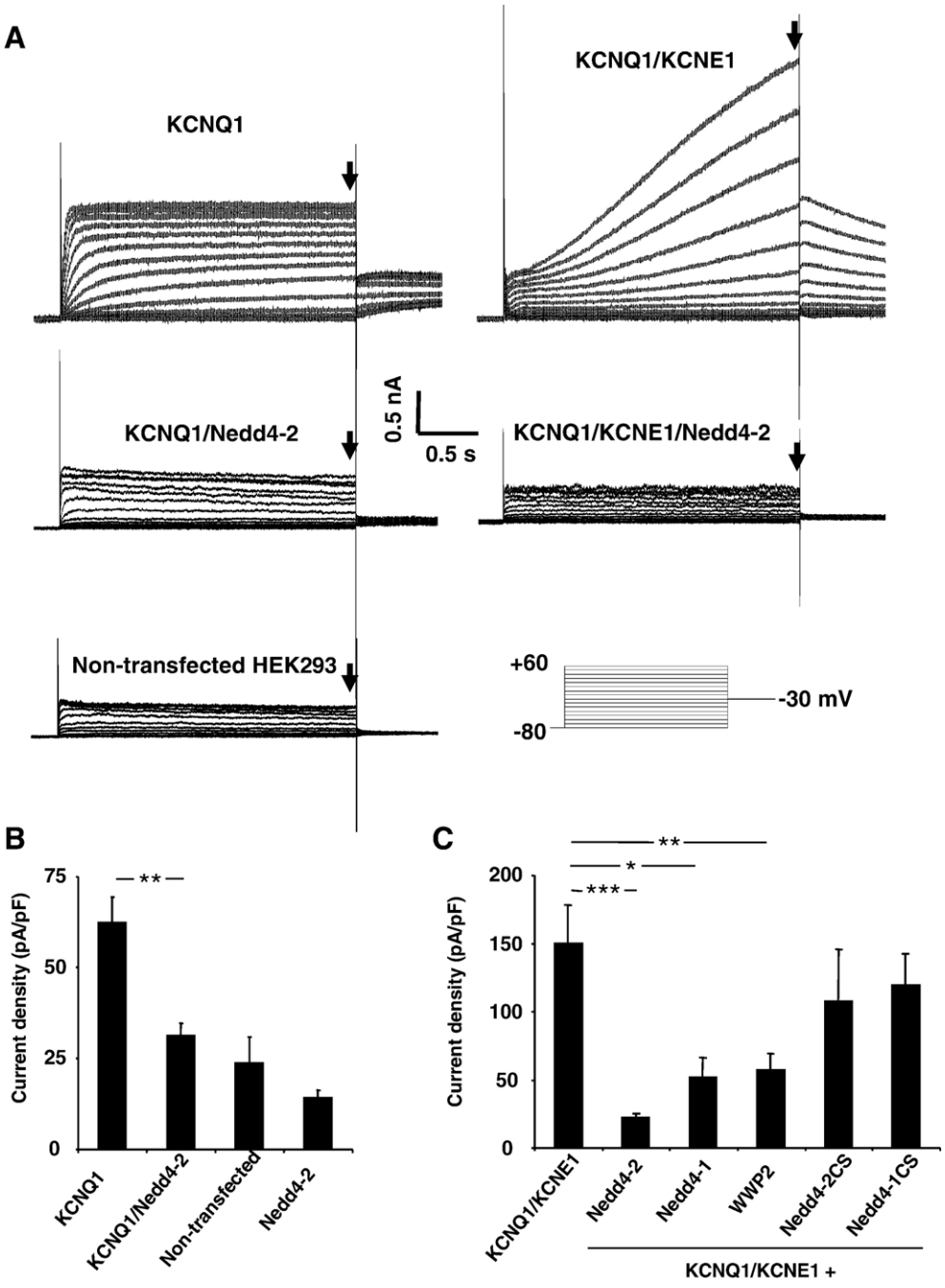

Fig. 3. Nedd4/Nedd4-like regulation of KCNQ1 and KCNQ1/KCNE1 currents. (A) Representative traces obtained from whole-cell patch-clamping of HEK293 cells expressing the indicated proteins. Increasing voltage-steps were applied according to the protocol depicted in the inset. (B, C) Peak current densities were obtained at the end of the 2-s pulse to 60 mV in HEK293 cells expressing the indicated proteins (indicated by arrows). KCNQ1 currents are significantly down-regulated by Nedd4-2, and KCNQ1/KCNE1 currents are significantly down-regulated by Nedd4-2, Nedd4-1, and WWP2. The obtained data are from at least two independent transfections; $n = 10–19$: *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

it can be viewed in Fig. 3A, the KCNQ1/KCNE1 channel complex, which underlies the cardiac I_{Ks}, mediated extremely slowly activating and deactivating potassium currents at potentials more positive than −20 mV.

Cells co-expressing KCNQ1 and Nedd4-2 in a 3-fold molar excess demonstrated a ~50% reduction in the absolute current amplitude (measured at the end of the 2-s depolarizing step to 60 mV), but no alteration — in the activation and deactivation properties (data not shown), as compared to cells expressing only KCNQ1 channels (Fig. 3B). However, since HEK293 endogenous channels [20] conduct a sizable outward current in non-transfected and Nedd4-2-transfected cells, the Nedd4-2-mediated reduction of the KCNQ1 current was in fact larger, namely ~75%, if

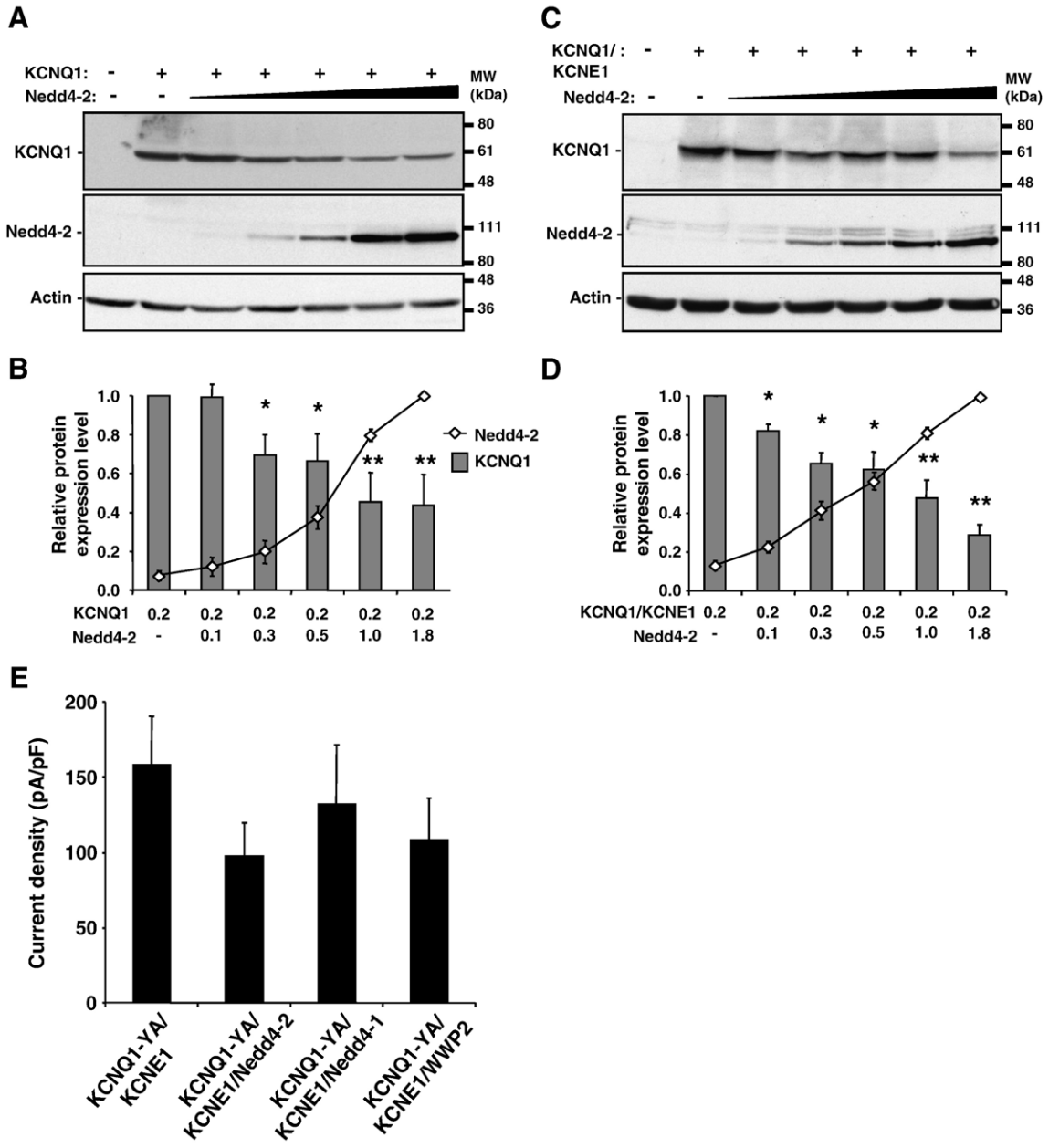

Fig. 4. Nedd4-2 mediated decrease of the cellular KCNQ1 protein amount. (A, C) Western blot experiments of total cellular lysate from cells transfected with the same amount of KCNQ1 or KCNQ1/KCNE1 DNA (0.2 μg) and with an increasing amount of Nedd4-2 DNA (up to 9-fold molar excess). (B, D) Quantification of the KCNQ1 and Nedd4-2 protein levels. The KCNQ1 and Nedd4-2 protein values were normalized to the expression level measured in cells only expressing KCNQ1 and expressing 1.8 μg Nedd4-2, respectively. The obtained values represent the average of five gels from four different transfections. With a 9-fold excess of Nedd4-2, reductions in the KCNQ1 protein levels of 56% and 72% for cells transfected with KCNQ1 or KCNQ1/KCNE1, respectively, were measured; $^{*}p<0.05$, $^{**}p<0.005$. (E) KCNQ1/KCNE1 channels with mutated PY motif are not down-regulated by Nedd4/Nedd4-like proteins. Whole-cell patch-clamp of HEK293 cells transfected with KCNQ1 with mutated PY-motif (tyrosine mutated to alanine; KCNQ1-YA) and KCNE1. Co-expression of Nedd4-2, Nedd4-1, and WWP2 did not significantly reduce the current densities. $n=8$–19 cells.

the background currents are subtracted (HEK293 expressing only Nedd4-2).

After having established that Nedd4-2 regulates the KCNQ1 current, further electrophysiological analyses of Nedd4/Nedd4-like mediated-regulation of the KCNQ1/KCNE1 current were performed. Co-expression of Nedd4-2 with KCNQ1/KCNE1 lead to a more than 6-fold reduction in the total current density (Fig. 3A and C). Similarly, Nedd4-1 and WWP2, two of the other Nedd4/Nedd4-like proteins with mRNA expressed at low and intermediate levels in human heart, respectively, also significantly down-regulated the KCNQ1/KCNE1 currents. As with KCNQ1, no alteration — in the activation and deactivation kinetics was found when Nedd4/Nedd4-like proteins were co-expressed with KCNQ1/KCNE1 (data not shown). We then investigated whether the enzymatic activity of the Nedd4/Nedd4-like

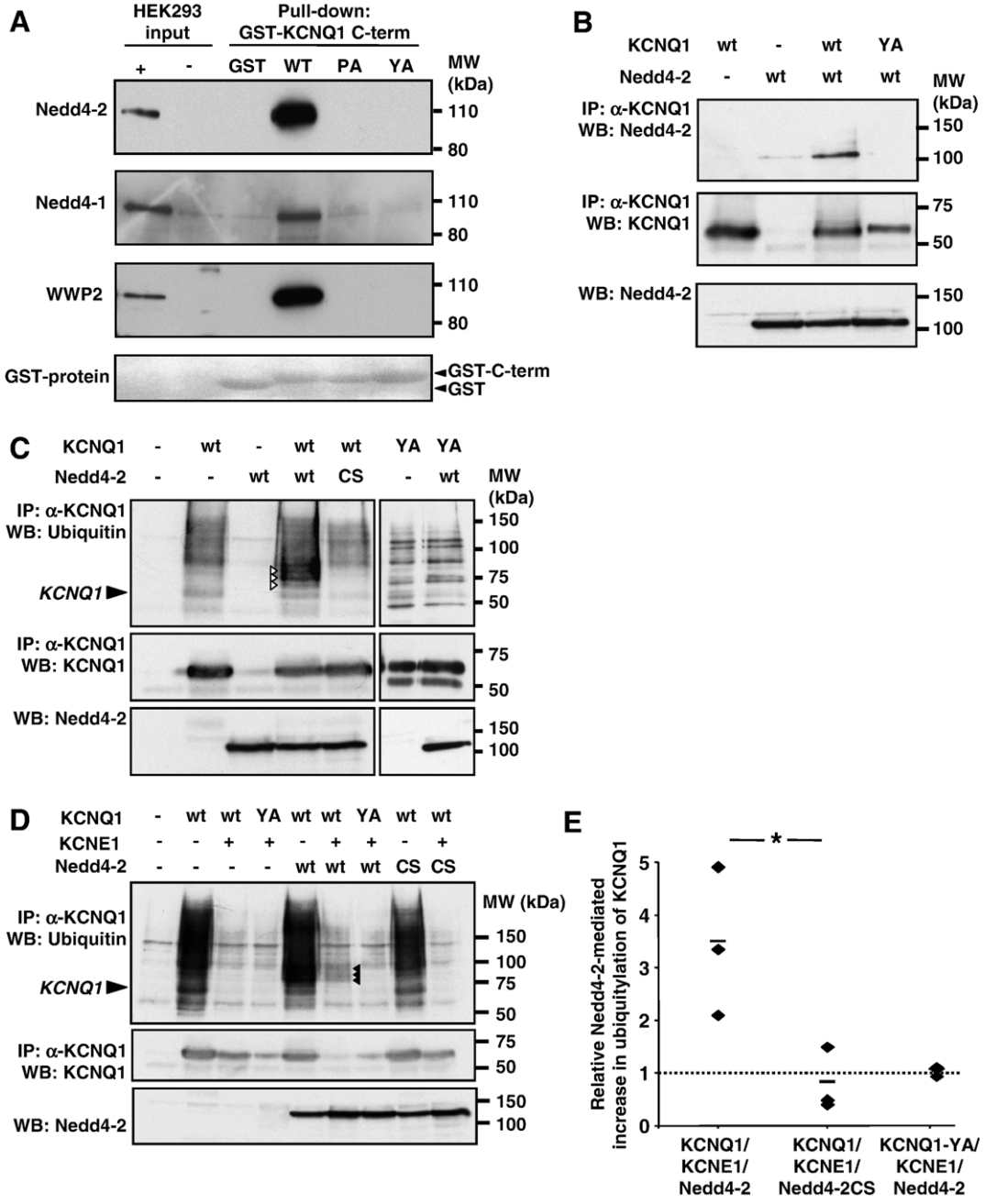

Fig. 5. Nedd4/Nedd4-like proteins interact with the KCNQ1 C-terminal PY-motif and Nedd4-2 increases ubiquitylation of wt KCNQ1. (A) Pull-down assay using GST proteins fused to the last 50 residues of the KCNQ1 C-terminal were performed using Nedd4-2, Nedd4-1, and WWP2-expressing HEK293 cell lysates. WT KCNQ1 C-terminal binds all three Nedd4/Nedd4-like proteins. In contrast, GST-fusion proteins with mutated PY motif (proline to alanine (PA) and tyrosine to alanine (YA)) did not pull-down the three Nedd4/Nedd4-like proteins. GST-fusion proteins are shown (Ponceau staining) as a control for equal loading (bottom panel). (B) Nedd4-2 and KCNQ1 proteins co-immunoprecipitate. The upper panel shows a Western blot (WB) of Nedd4-2 proteins co-immunoprecipitated with KCNQ1. The middle panel is a staining of the immunoprecipitated (IP) KCNQ1 proteins. The lower panel confirms the expression of Nedd4-2. Lysates of HEK293 cells transiently transfected with wild type (wt) and PY motif mutated (YA) KCNQ1, and wt Nedd4-2 were used. (C and D) Nedd4-2 induces increased ubiquitylation of KCNQ1 (C) and KCNQ1/KCNE1 complexes (D). Immunoprecipitations (IP) of KCNQ1 proteins expressed in HEK293 cells. The upper panels show Western blots (WB) of ubiquitylated KCNQ1 proteins after IP of the proteins indicated above the images. The middle panels are immunoblots of immunoprecipitated KCNQ1. The lower images confirm the expression of Nedd4-2 (WB of input). HEK293 cells transiently transfected with KCNQ1 either wild type (wt) or with a mutated PY motif (YA), and Nedd4-2, either wild type (wt) or catalytically inactivated (CS), were used. The expected locations of non-ubiquitylated KCNQ1 (►) as well as the position of bands with increased intensity upon Nedd4-2 expression (white and black arrow heads, C and D) are indicated. (E) The relative KCNQ1 ubiquitylation level as compared to the amount of KCNQ1 proteins expressed in KCNQ1/KCNE1 transfected HEK293 cells. The lower part of the ubiquitylation signal (the area covering the bands indicated by arrow heads) were quantified and divided by the measured KCNQ1 intensity. The values obtained with KCNQ1(wt/YA)/KCNE1/Nedd4-2(wt/CS) were normalized with respect to the control conditions (without Nedd4-2 (wt/CS)) and the relative increase/decrease was plotted. The horizontal bars indicate the average values obtained from 3 independent experiments. The ubiquitylation level is 3.5-fold increased for wt KCNQ1/KCNE1 when Nedd4-2 is co-expressed; $*p<0.05$.

ubiquitylation enzymes was responsible for the observed reduction in current amplitude by substituting the active enzyme with a catalytically inactive form of Nedd4-2 or Nedd4-1, bearing the C801S (Nedd4-2CS) or C867S (Nedd4-1CS) mutations [21]. Cells co-expressing KCNQ1/KCNE1 and Nedd4-2CS or Nedd4-1CS did not show current levels significantly different from controls, indicating that enzymatic activity of these Nedd4/Nedd4-like proteins is necessary for the down-regulation of KCNQ1/KCNE1. I_{Ks} was down-regulated by Nedd4-2 in COS-7 cells in a similar manner as observed in HEK293 cells (data not shown), suggesting that Nedd4/Nedd4-like induced regulation could take place in a variety of cell types.

3.3. Nedd4-2 decreases the cellular amount of KCNQ1 protein

It is known that Nedd4/Nedd4-like proteins may signal internalization by promoting ubiquitylation of the target proteins, e.g. ion channels. Whether such a presumed internalization of KCNQ1 leads to storage or degradation of KCNQ1 protein was analyzed by quantifying KCNQ1 amount from cells expressing increasing amounts of Nedd4-2 (Fig. 4A-D). For cells expressing KCNQ1 channels, a significant reduction of the total cellular amount of KCNQ1 protein was observed when Nedd4-2 was expressed in a 1.5-fold excess, and a 56% reduction was found with a large excess of Nedd4-2 (Fig. 4B). For cells expressing KCNQ1/KCNE1, a significant reduction in KCNQ1 protein amount was observed when half the molar Nedd4-2 DNA amount was transfected as compared to the amount of transfected KCNQ1/KCNE1 DNA (Fig. 4D). A 72% reduction in the KCNQ1 protein amount was observed with a large excess of Nedd4-2 transfected DNA.

3.4. Nedd4/Nedd4-like proteins interact with the C-terminal PY motif of KCNQ1

In order to investigate the potential role of the C-terminal PY motif of KCNQ1, the tyrosine residue in the motif was mutated to an alanine residue (KCNQ1-YA). Electrophysiological measurements using this construct co-expressed with KCNE1 revealed kinetics (not shown) and current densities similar to wt channels (Fig. 4E). Co-expression of Nedd4-2, Nedd4-1, and WWP2 yielded no significant reduction in the current amplitude, suggesting that the PY motif is implicated in the Nedd4/Nedd4-like mediated down-regulation. Even though KCNQ1/KCNE1 current density was not significantly decreased upon Nedd4-2, Nedd4-1, or WWP2 co-expression, a trend towards lower current levels can be observed (Fig. 4E). This current reduction may in part be explained by a Nedd4/Nedd4-like-mediated reduction in the endogenous current level as shown in Fig. 3B.

Biochemical interaction between KCNQ1 and the three Nedd4/Nedd4-like proteins (i.e. Nedd4-2, Nedd4-1, and WWP2) was explored by pull-down assays, using GST proteins coupled to the KCNQ1 C-terminal tail (Fig. 5A). Binding of all three Nedd4/Nedd4-like proteins to the wt C-terminal tail of KCNQ1 was observed. In contrast, if either the proline or the tyrosine of the PY motif were mutated into an alanine (PA or YA, see Fig. 1A), none of the E3 enzymes could be recovered in the precipitated fractions. We analyzed whether this interaction also took place in a cellular context by investigating co-immunoprecipitation of proteins expressed in HEK293 cells (Fig. 5B). This experiment revealed an interaction between wt KCNQ1 and Nedd4-2. KCNQ1 proteins with mutated PY motif (YA mutant) did not co-immunoprecipitate Nedd4-2. Hence, these results confirm the importance of the PY motif in the Nedd4/Nedd4-like mediated regulation of KCNQ1, and also show that the proteins are interacting with each other in cells.

3.5. Co-expression of Nedd4-2 increases KCNQ1 ubiquitylation

Nedd4/Nedd4-like-mediated down-regulation of ion channels has been shown to involve their ubiquitylation [5]. Whether this is also the case for KCNQ1 was investigated by immunoprecipitating KCNQ1 proteins followed by detection of ubiquitylated proteins by Western blot (Fig. 5C and D). HEK293 cells expressing KCNQ1, but not control cells or cells only expressing Nedd4-2, revealed endogenous KCNQ1 ubiquitylation (Fig. 5C and D), i.e. a "smeary" signal when using an anti-ubiquitin antibody. This phenomenon was primarily seen for proteins with sizes of more than 90 kDa, indicating that, under these conditions, several ubiquitin molecules are covalently attached to KCNQ1. Co-expression of Nedd4-2 with either KCNQ1 (Fig. 5C) or KCNQ1/KCNE1 (Fig. 5D) resulted in an increased level of ubiquitylation of KCNQ1. Specifically, the most pronounced increase in the ubiquitylation pattern was observed as three distinct bands just above the expected size of KCNQ1 (see arrow heads in Fig. 5C and D). As it can be seen, there was approximately the same distance between each of the three bands; this distance represents a difference in the molecular size which is within the range of one single ubiquitin-protein (~8 kDa), suggesting that this ladder may represent covalent attachments of an increasing amount of ubiquitin moieties on KCNQ1. From three independent experiments with KCNQ1/KCNE1, a 3.5-fold average increase in Nedd4-2-mediated ubiquitylation was observed for proteins found between 65 and 85 kDa (Fig. 5E). As expected, expression of neither Nedd4-2CS nor KCNQ1-YA altered the level of KCNQ1 ubiquitylation (Fig. 5E), confirming that the increase in ubiquitylation is strictly dependent on Nedd4-2 enzymatic activity as well as an intact PY motif. Concomitantly, co-expression of Nedd4-2 with KCNQ1-YA did not alter the total cellular amount of KCNQ1 proteins, which is visualized on the middle panel of Fig. 5C, showing that the PY motif is also crucial in the Nedd4-2 mediated down-regulation of KCNQ1 protein.

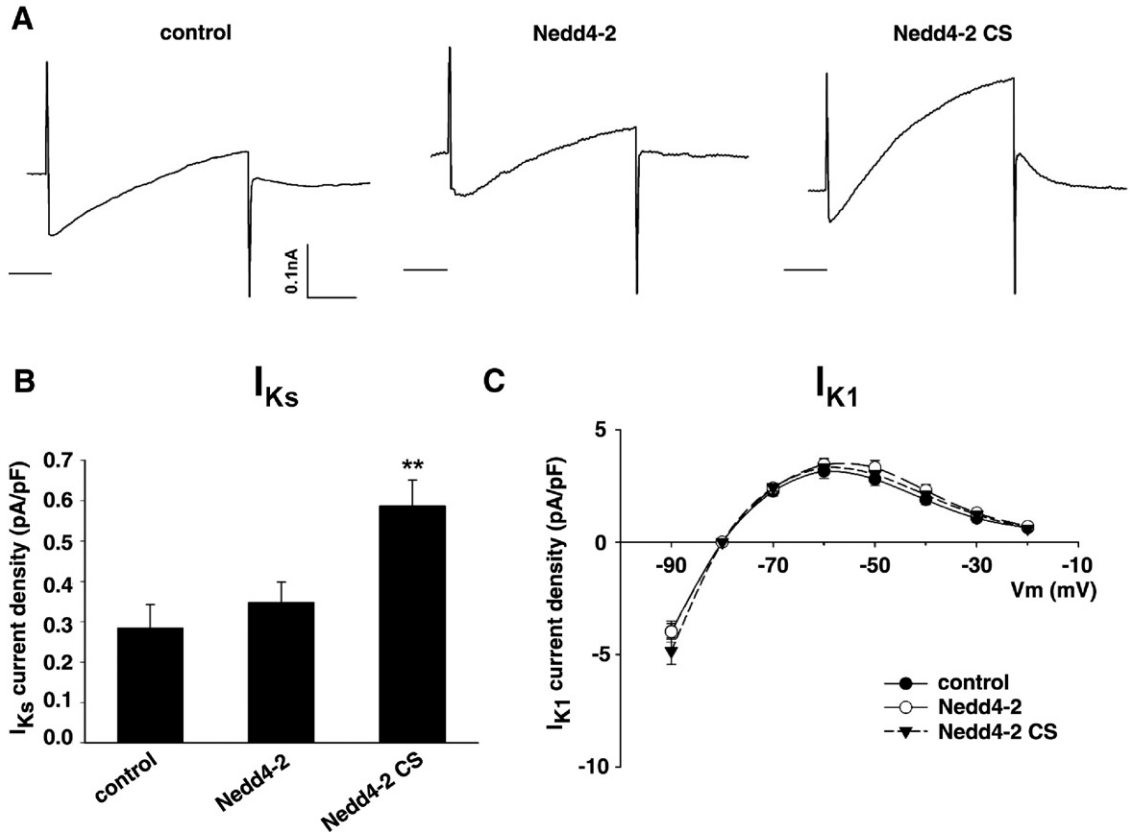

Fig. 6. Nedd4-2CS increases I_{Ks} of guinea-pig ventricular myocytes. (A) Representative current traces obtained from guinea-pig ventricular myocytes transfected with pIRES–CD8 (control) or pIRES–CD8–Nedd4-2 (wt or CS). The cells were depolarized from −40 mV to +40 mV. Tail current densities were measured at −40 mV after 1-s depolarization and are summarized in (B) (control, $n=11$ from 5 animals; Nedd4-2, $n=13$ from 6 animals, and Nedd4-2CS, $n=9$ from 4 animals), $^{**}p<0.01$, lines represent zero current. (C) I_{K1} densities from transfected guinea-pig ventricular cells (control, $n=11$, Nedd4-2, $n=11$; Nedd4-2CS, $n=8$) vs. potential. Voltage steps were applied from −60 mV to various potentials, and I_{K1} was measured at the end of a 500-ms pulse. No significant difference was observed between the different conditions.

3.6. I_{Ks} of guinea-pig ventricular myocytes is modulated by inactive Nedd4-2

Finally, we investigated whether Nedd4-2 could modulate I_{Ks} in guinea-pig cardiomyocytes. *In vivo* expression of transgenes could be achieved in adult animals by using a new synthetic transfection reagent complexed with the plasmids [18]. The mix was injected in the left ventricular free wall 4–8 days before isolating and patching the cells. CD8 was used as reporter gene to identify cardiomyocytes effectively expressing the transgenes. Only myocytes selected by the magnetic field and still associated with CD8 beads were used for patch-clamp experiments.

In cardiomyocytes, the mean tail I_{Ks} density of cells transfected with inactive Nedd4-2CS was significantly increased (2.1-fold) compared to the value measured in control conditions (cells transfected with pIRES-CD8 alone) (Fig. 6A and B). Activation and deactivation kinetics of I_{Ks} were not modified by Nedd4-2 (wt or mutated) overexpression (table, supplemental data). Such a protective effect mediated by Nedd4-2CS against active Nedd4/Nedd4-like proteins, in this case endogenously expressed, was also observed in culture cells where we found that co-expression of Nedd4-2CS together with KCNQ1, KCNE1, and Nedd4-2 provided protection against down-regulation (data not shown). The observed Nedd4-2CS-induced increase of the I_{Ks} current was specific since the I_{K1} current was not altered in any conditions (Fig. 6C). However, under these conditions wt Nedd4-2 overexpression did not significantly modify the I_{Ks} density, suggesting that cardiac I_{Ks} channels are already heavily regulated by Nedd4/Nedd4-like proteins as only overexpression of the catalytically inactive variant of Nedd4-2 modulates I_{Ks}.

4. Discussion

The presented results reveal that KCNQ1 and KCNQ1/KCNE1 channels expressed in HEK293 cells can be down-regulated by Nedd4/Nedd4-like ubiquitin-protein ligases. A similar Nedd4/Nedd4-like-dependent regulation of I_{Ks} could also be demonstrated in guinea-pig myocytes. In HEK293 cells, this decreased current level is followed both by a reduced quantity of the total cellular amount of KCNQ1 protein, and by

an increased ubiquitylation of the remaining KCNQ1 proteins. Furthermore, by performing pull-down and co-immunoprecipitation experiments, Nedd4-2 was found to bind to the PY motif of the C-terminal domain of KCNQ1. Mutating this PY motif abolished all the observed Nedd4-2-mediated biochemical and functional effects. The presented results are consistent with a mechanism where: i) Nedd4/Nedd4-like proteins interact with the KCNQ1 channel complexes through the C-terminal located PY motif, ii) this interaction promotes ubiquitylation of the channels, iii) the cellular machinery recognizes the ubiquitylated channels which leads to internalization, and iv) the internalized channels are degraded.

4.1. Nedd4/Nedd4-like expression

The relative expression level of Nedd4/Nedd4-like genes in human heart, brain, and kidney was determined by real-time PCR quantifications. Measurement of mRNA levels may provide an indication of the amount of the corresponding protein, but does not necessarily reflect the protein abundance of the implicated gene. We found a relatively high expression level of Nedd4-2 in all three organs, and of NEDL2 in heart and brain. The other 7 members of the Nedd4/Nedd4-like family showed an intermediate or low expression level as compared to Nedd4-2. Nedd4-2 is by far the most studied protein, and is now known to interact with four ion channels [5] through PY motifs. However, whether relatively low and high expression profiles reflect the importance of the individual Nedd4/Nedd4-like proteins or whether other parameters, such as cellular and subcellular localization, are more important is so far unknown and remains to be further investigated.

4.2. Interaction with the PY motif

The PY motifs of ENaC subunits and most Na_v channels conform to the sequence **P**PxY, which is in contrast to the PY motif of KCNQ1 that has the less common **L**PxY sequence. Recently, Kanelis and co-workers [22] have reported that the PY motif of the *drosophila* commissureless transmembrane protein, required for neuromuscular synaptogenesis, bears **L**PxY residues interacting with dNedd4. Interestingly, changing the leucine (**L**PXY) into a proline (**P**PXY), thereby creating a more "classical" PY motif, resulted in a 2-fold reduction in the dNedd4 binding affinity, showing that different subtypes of PY motifs can interact with Nedd4/Nedd4-like proteins. Furthermore, it could be speculated that alterations in the PY motif sequence could not only play a role in the degree of Nedd4/Nedd4-like regulation, but could also be important in differentiating between the different Nedd/Nedd4-like proteins. An example of such a differential regulation is illustrated by Nedd4-1 that has been shown to negatively regulate the KCNQ1/KCNE1 current (this study), but not the $Na_v1.5$ current [14]. While the peptide sequence defining the PY motif of KCNQ1 is identical among several animal species, the flanking regions are not conserved (Fig. 1B). This suggests that the interaction with Nedd4/Nedd4-like proteins mediated with the PY motif is important in controlling the membrane density of KCNQ1 proteins throughout the animal kingdom. The role of KCNE1 in Nedd4-2-mediated KCNQ1 down-regulation merits further investigations since we observed a trend, albeit not statistically significant, of a more pronounced effect when KCNE1 was co-expressed.

4.3. Ubiquitylation of KCNQ1

The analyses of the ubiquitylation pattern of KCNQ1 revealed that the channel protein expressed in HEK293 cells undergo endogenous ubiquitylation, producing a fraction of the KCNQ1 proteins with a size above 90 kDa, whereas the main fraction of KCNQ1 has a size of ~60 kDa, under our experimental conditions. The nature of the endogenous ubiquitin-protein ligase(s) responsible for this ubiquitylation is unknown. Also, the cellular mechanisms involved do not seem to overlap with that of Nedd4-2, since the inactivated Nedd4-2-CS, which could potentially antagonize any endogenous activity, neither altered the ubiquitylation pattern nor the current density. It may be speculated that this background ubiquitylation is due to KCNQ1 over-expression in HEK293 cells, which may lead to ER-associated degradation [23]. Co-expression of Nedd4-2 resulted in a significant increase in the ubiquitylation level, in particular of three discrete bands with a molecular size ranging from ~65 to ~85 kDa. Since these bands seem to form a ladder just above the expected size of non-ubiquitylated KCNQ1, it can be suspected that the observed Nedd4-2-mediated ubiquitylation of KCNQ1 channel complexes represent a sequential covalent attachment of single ubiquitin molecules. This finding is actually in contrast to the previously reported PY motif-dependent polyubiquitylation of ENaC and $Na_v1.5$ [14]. The reason for this difference is unknown and further studies are needed to specifically address this interesting question.

5. Conclusions

I_{Ks} is one of the important currents in determining the length of the cardiac action potential in humans. This notion is clearly exemplified in patients suffering from short and long QT syndromes — increased and decreased I_{Ks}, respectively, caused by mutations in either *KCNQ1* or *KCNE1* [4]. Short term regulation of I_{Ks}, e.g. as a response to exercise, is primarily controlled through sympathetic stimulation modifying the channel kinetics [24]. In contrast to this, mechanisms underlying long-term regulation of the I_{Ks} channel in the heart are largely unknown. In this study, we have presented results revealing a new regulatory mechanism of KCNQ1 and KCNQ1/KCNE1 (I_{Ks}) channels. The fact that Nedd4-2, and other Nedd4/Nedd4-like ligases, modulates KCNQ1 channels in culture cells, and I_{Ks} in cardiomyocytes, after *in vivo* expression, suggests a physiological significance of this finding. This Nedd4/

Nedd4-like-mediated regulation could be important in the constitutive control of the KCNQ1/KCNE1 membrane density, and/or may play a role under specific pathophysiological conditions that remain to be determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2007.01.008.

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