Deubiquitylating enzyme USP2 counteracts Nedd4-2–mediated downregulation of KCNQ1 potassium channels

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BACKGROUND KCNQ1 (Kv7.1), together with its KCNE β subunits, plays a pivotal role both in the repolarization of cardiac tissue and in water and salt transport across epithelial membranes. Nedd4/Nedd4-like (neuronal precursor cell–expressed developmentally downregulated 4) ubiquitin–protein ligases interact with the KCNQ1 potassium channel through a PY motif located in the C terminus of KCNQ1. This interaction induces ubiquitylation of KCNQ1, resulting in a reduced surface density of the channel. It was reported recently that the epithelial sodium channel is regulated by the reverse process—deubiquitylation—mediated by USP2 (ubiquitin-specific protease 2).

OBJECTIVE In this article, we investigated whether deubiquitylation may regulate KCNQ1 channel complexes.

METHODS In this study, we used electrophysiology, biochemistry, and confocal microscopy.

RESULTS Electrophysiological investigations of KCNQ1/KCNE1 proteins coexpressed with USP2-45 or USP2-69 isoforms and Nedd4-2 in *Xenopus laevis* oocytes and mammalian cells revealed that both USP2 isoforms counter the Nedd4-2–specific downregulation of I_{Ks}. Biochemical studies showed that the total and surface-expressed KCNQ1 protein was more abundant when coexpressed with USP2 and Nedd4-2 as compared with Nedd4-2 alone. Western blotting revealed partial protection against covalent attachment of ubiquitin moieties on KCNQ1 when USP2 was coexpressed with Nedd4-2. Coimmunoprecipitation assays suggested that USP2 can bind to KCNQ1 independently of the PY motif. Immunocytochemistry confirmed that USP2 restores the membrane localization of KCNQ1.

CONCLUSION These results demonstrate that USP2 can be a potent regulator of KCNQ1 surface density. USP2, which is well expressed in many tissues, may therefore be important in controlling the KCNQ1 channel dynamics in vivo.

KEYWORDS I_{Ks}; Kv7.1; minK; Potassium channels; Ubiquitylation; Deubiquitylation; Membrane protein regulation

ABBREVIATIONS CA = changed to alanine; **DUB** = deubiquitylating enzyme; **ENac** = epithelial sodium channel; **Nedd4** = neuronal precursor cell–expressed developmentally downregulated 4; **USP2** = ubiquitin-specific protease 2

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Introduction

KCNQ1 potassium channel complexes play an important role in a number of tissues, including heart, inner ear, stomach, colon, and kidney.[1] The channel associates with KCNE β subunits that enforce prominent changes in the KCNQ1(minK) current kinetics, which is likely one of the reasons for KCNQ1/KCNE complexes being capable of participating in several different tissue functions. KCNQ1 current kinetics is also regulated by sympathetic input through a Protein kinase A (PKA)-mediated phosphorylation, inducing an increased current level.[2] While the kinetic properties of KCNQ1 have been investigated in detail, the regulatory mechanisms underlying surface expression and localization have only been touched upon.[3]

For a number of ion channels, surface expression has been shown to be regulated by ubiquitylating enzymes of the Nedd4/Nedd4 (neuronal precursor cell–expressed developmentally downregulated 4)–like family[4] and we have demonstrated how ubiquitylation is an important regulatory mechanism for the KCNQ1 channel.[5] Ubiquitylation is mediated by an enzymatic cascade, transferring ubiquitin moieties from the E1 to E2 enzymes and then further on to the E3 ubiquitin–protein ligases.[6–8] Nedd4/Nedd4-like proteins, constituting

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one of the E3 enzyme families, often interact with PY motifs and thereupon catalyze covalent attachment of ubiquitin moieties to the target protein. The best-described ion channel regulated by Nedd4/Nedd4-like ubiquitylation is the epithelial sodium channel (ENaC). Mutations in the PY motifs of the protein subunits constituting ENaC have been found to cause Liddle's syndrome, which is a disease characterized by severe hypertension due to an imbalance in the sodium and potassium homeostases.[9–14] Mutated PY motifs lead to an increased apical density of the ENaC in the renal tubules, which causes an increased absorption of sodium. Hence, Nedd4/Ned4-like–mediated ubiquitylation is necessary to adjust the surface expression of the ENaC.

KCNQ1 is also regulated by at least 3 of the Nedd4/Nedd4-like ubiquitin ligases—Nedd4-1, Nedd4-2, and WWP2—through a PY motif–dependent mechanism.[5] They bind to a PY motif located in the distal part of the C-terminal tail of KCNQ1. This induces ubiquitylation, which is followed by a removal of KCNQ1 channel complexes from the cell surface and a reduced total cellular amount of KCNQ1.

Deubiquitylating enzymes (DUBs), through removal of ubiquitin from target proteins, have been proven to participate in many important processes in the cell, for example, protein degradation, DNA repair, endocytosis, and signal transduction. Their best understood function to date is to counteract the targeting of proteins for degradation by the 26S proteasome through removal of covalently attached ubiquitin moieties/chains.[15–17] Ubiquitin-specific proteases (USPs) are a large family of DUBs belonging to cysteine proteases, where USP2 is one of the best described members.[18–20] It has been shown that USP2 is present in the form of 2 splicing variants of 69 and 45 kDa (also named USP2a and USP2b, respectively) and that it is expressed on the mRNA level in the heart, as well as in most other tissues, such as skeletal muscle, testis, brain, liver, spleen, pancreas, and kidney. Furthermore, it has been found on the protein level in kidney and testis.[21–25] In addition, the expression of USP2 has been linked to both cell differentiation and cancer.[19,26–28]

Recently, it was found that ubiquitylation of the ENaC can be antagonized by the USP2-45 enzyme.[24,29] Aldosterone-dependent upregulation of USP2-45 in the murine distal nephron correlates with an increase in transepithelial sodium transport through the ENaC, and in cellular systems a USP2-specific deubiquitylating effect on the ENaC was established.

In this article we describe the antagonizing effect of USP2 on the Nedd4-2–mediated downregulation of KCNQ1. We find by electrophysiology, biochemistry, and confocal microscopy that both USP2-45 and USP2-69 counter the Nedd4-2–dependent ubiquitylation and thereby restore the plasma membrane localization of KCNQ1 potassium channels.

Material and methods

See Supplementary Material.

Results

USP2 antagonizes the Nedd4-2–mediated effect on KCNQ1 current amplitude

In order to investigate the effect of USP2 on Nedd4-2–dependent downregulation of I_{Ks}, we carried out 2-electrode voltage clamp measurements on *Xenopus laevis* oocytes. As previously reported in mammalian cells, Nedd4-2 enforced a drastic reduction in I_{Ks}, which is formed by KCNQ1/KCNE1 proteins. Dose–response experiments, with increasing amounts of Nedd4-2 cRNA, together with a constant amount of KCNQ1 (1 ng) and KCNE1 (0.2 ng) cRNA, established that with 0.2 ng or more of Nedd4-2 cRNA a drastic reduction in the current amplitude was obtained (Figure 1A). This amount was therefore used in the following experiments also including USP2, which was co-injected at the same molar ratio as KCNQ1. Oocytes coexpressing KCNQ1/KCNE1 and Nedd4-2 demonstrated the same apparent current kinetics but revealed a ~65% reduction in the absolute current amplitude (measured at the end of the 5-second depolarizing step at +40 mV) (Figures 1B and 1C). However, when KCNQ1/KCNE1 proteins were coexpressed with Nedd4-2 and USP2-45 or USP2-69 isoforms, these DUBs counteracted the Nedd4-2–specific downregulation of I_{Ks}. In contrast to previous observations with the ENaC, coexpression of the 2 isoforms of USP2 in the present study did not increase the KCNQ1/KCNE1-mediated current in *Xenopus* oocytes per se.[24] Similar results were obtained when only KCNQ1, and not KCNQ1/KCNE1, were expressed in oocytes (data not shown).

In order to complement the data obtained from oocytes, mammalian HEK293 cells were used for whole-cell patch clamping (Figures 1D and 1E). When Nedd4-2 was coexpressed with wild-type USP2-69, the current density was similar to control, as observed in oocytes, showing the protective effect of USP2-69 on Nedd4-2–dependent downregulation of the KCNQ1/KCNE1 current. To test whether the action of USP2-69 was mediated by its protease activity, we created a mutant where one of the catalytically active amino acids in the cysteine protease motif—cysteine 67—was changed to alanine (CA).[18,19,21] When Nedd4-2 was coexpressed with catalytically inactive USP2-69 CA, a ~50% reduction in current amplitude was observed, confirming that the catalytically active site is a requirement for the action of USP2. Similar results were obtained with HEK293 cells expressing KCNQ1 without KCNE1 (data not shown).

USP2 protects against Nedd4-2–dependent decrease in the cellular KCNQ1 protein

The covalent attachment of ubiquitin moieties signals downregulation of KCNQ1 channels.[5] Whether USP2 reverse the Nedd4-2–mediated ubiquitylation was investigated by the analysis of total cellular lysate, as well as membrane fractions from HEK293 cells. By performing Western blotting of the soluble fraction of lysed HEK cells, we observed a drastic reduction in the KCNQ1 protein level, which was caused by Nedd4-2 (Figure 2A). Both USP2-45 and USP2-69 restored the KCNQ1 protein level almost to

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