Ubiquitin-specific protease USP2-45 acts as a molecular switch to promote $\alpha_2\delta-1$-induced downregulation of Ca$_v$1.2 channels

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Abstract Availability of voltage-gated calcium channels (Ca$_v$) at the plasma membrane is paramount to maintaining the calcium homeostasis of the cell. It is proposed that the ubiquitylation/de-ubiquitylation balance regulates the density of ion channels at the cell surface. Voltage-gated calcium channels Ca$_v$1.2 have been found to be ubiquitylated under basal conditions both in vitro and in vivo. In a previous study, we have shown that Ca$_v$1.2 channels are ubiquitylated by neuronal precursor cell-expressed developmentally downregulated 4 (Nedd4-1) ubiquitin ligases, but the identity of the counterpart de-ubiquitylating enzyme remained to be elucidated. Regarding sodium and potassium channels, it has been reported that the action of the related isoform Nedd4-2 is counteracted by the ubiquitin-specific protease (USP) 2-45. In this study, we show that USP 2-45 also de-ubiquitylates Ca$_v$ channels. We co-expressed USPs and Ca$_v$1.2 channels together with the accessory subunits $\beta_2$ and $\alpha_2\delta-1$, in tsA-201 and HEK-293 mammalian cell lines. Using whole-cell current recordings and surface biotinylation assays, we show that USP2-45 specifically decreases both the amplitude of Ca$_v$ currents and the amount of Ca$_v$1.2 subunits inserted at the plasma membrane. Importantly, co-expression of the $\alpha_2\delta-1$ accessory subunit is necessary to support the effect of USP2-45. We further show that USP2-45 promotes the de-ubiquitylation of both Ca$_v$1.2 and $\alpha_2\delta-1$ subunits. Remarkably, $\alpha_2\delta-1$, but not Ca$_v$1.2 nor $\beta_2$, co-precipitated with USP2-45. These results suggest that USP2-45 binding to $\alpha_2\delta-1$ promotes the de-ubiquitylation of both Ca$_v$1.2 and $\alpha_2\delta-1$ subunits, in order to regulate the expression of Ca$_v$1.2 channels at the plasma membrane.

Keywords Calcium channels · Ubiquitin-specific proteases 2-45 · Ubiquitylation · $\alpha_2\delta-1$ Subunit

Abbreviations
USPs Ubiquitin-specific proteases
Ca$_v$ Voltage-gated calcium channel

Introduction

Ubiquitylation is a post-translational modification which has been shown to regulate the availability of both intracellular and membrane proteins [1, 35]. Ubiquitin, a small protein of 8 kDa, is attached by ubiquitin ligases onto lysine residues of target proteins. Equally important is the reverse process operated by de-ubiquitylases (DUBs) [29, 32]. The ubiquitylation and therefore the fate of the targeted proteins result from the balanced action of both families of enzymes. A multitude of ubiquitin ligases and de-ubiquitylases have already been...
identified, and one current challenge is to identify their associated targets. The human genome encodes for more than 100 de-ubiquitylases [29, 32]. They have been classified into five families according to the active sites used to perform the de-ubiquitylation process: cysteine or zinc active sites. The zinc active site is found in JAB1/MPN/MOV34 metalloenzymes, and the cysteine active site is found in ubiquitin C-terminal hydrolase, ovarian tumour proteases, Machado-Josephin domains and ubiquitin-specific proteases (USPs) [29, 32].

Interestingly, USP2-45 had been shown to de-ubiquitylate the epithelial sodium channel (ENaC) [38] and regulate its expression at the plasma membrane. The same research group further reported that USP2-45 physically interacts with the neuronal precursor cell-expressed developmentally downregulated 4 (Nedd4-2) ubiquitin ligases [30] known to ubiquitylate ENaC [24, 44]. Moreover, both USP2-45 and USP2-69 counteracted the Nedd4-2-mediated ubiquitylation of cardiac potassium channels [26]. We have previously shown that another closely related isoform, Nedd4-1, ubiquitylates Ca\textsubscript{1.2} channels [36]. In spite of Nedd4-1 and Nedd4-2 having selective targets [24, 36], these studies suggest that there may be a reciprocity between the two families of Nedd4 ubiquitin ligases and USP de-ubiquitylases, which lead us to firstly investigate USP2-45 as a potential regulator of Ca\textsubscript{1.2} channels. USP2-45 is one of the two isoforms encoded by the USP2 gene which is alternatively spliced to give USP2-45 (45 kDa) and USP2-69 (69.5 kDa) [29]. Both splice variants share an identical catalytic core, but USP2-69 has a longer N-terminal domain which may possibly regulate USP2-69 subcellular localization or act as an auto-inhibitory domain [30]. Gousseva and Baker have shown that USP2-45 and USP2-69 messenger RNAs (mRNAs) are expressed together in mouse testis, skeletal muscle and heart [22]. USP2-45 mRNA is also present in the brain, liver and kidney [22]. Increased expression of USP2 was reported in prostate tumours [31]. Tissue expression of USP2 can also be modulated by signalling pathways, as shown by Fakitsas et al. [15] who demonstrated that aldosterone induces the expression of USP2-45 in the cortical collecting duct of the kidney.

Our study investigates whether USP2 regulates voltage-gated calcium Ca\textsubscript{1.2} channels. These widely expressed channels constitute the main pathway for calcium entry into vascular and cardiac myocytes and are a major target for the treatment of cardiovascular diseases [27, 40]. Ca\textsubscript{1.2} channels also contribute to neuron excitability and are involved in the control of gene transcription [13]. In addition to the main pore-forming Ca\textsubscript{1.2} subunit, Ca\textsubscript{1.2} channels also contain accessory subunits: β and α\textsubscript{2}δ, which regulate both the gating properties and trafficking of the channels [9, 41, 17, 7, 6]. Ca\textsubscript{1.2} channels are predominantly associated with β\textsubscript{2} [10, 43] and α\textsubscript{2}δ-1 [5, 28] in the heart. We, and others, have previously shown that these three Ca\textsubscript{1.2} subunits are ubiquitylated in vitro and in vivo [25, 36]. Here, we found that both USP2-45 and USP2-69 regulate cloned cardiac Ca\textsubscript{1.2} channels expressed in mammalian cell lines. The main difference between the two USP2 isoforms resides in their N-terminal domain which was suggested to direct target specificity [30] as reported for ENaC channels which are not sensitive to USP2-69 [15]. The similar reduction of Ca\textsubscript{v} currents obtained with either splice variant suggests that Ca\textsubscript{v} channels are the target of both isoforms and that the minimum sequence required for Ca\textsubscript{v} current reduction resides within the short variant form of USP. Hence, we focussed our investigation on USP2-45-induced regulation of Ca\textsubscript{v} channels.

Our study reveals a new role for α\textsubscript{2}δ-1 subunits in binding USP2-45 and promoting the de-ubiquitylation of both α1 and α\textsubscript{2}δ-1 subunits. USP2-45-induced Ca\textsubscript{v} regulation leads to a decrease of Ca\textsubscript{1.2} channels available at the plasma membrane.

**Methods**

**DNA constructs**

Rabbit Ca\textsubscript{1.2} (cardiac isoform α1c) (P15381.1), β\textsubscript{2b} (P54288) and α\textsubscript{2}δ-1a (P13806) complementary DNAs (cDNAs) subcloned into pcARDHE, pBH17 and pCA1S, respectively, were gifts from Dr. G.S. Pitt (Department of Medicine, Division of Cardiology, Duke University Medical Center, Durham, NC, USA). Mouse USP2-45 (NM 198091) with and without S-tag and the mutant USP2-45C67A cDNAs subcloned into pcDNA3.1 were gifts from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland).

**Transfections**

For electrophysiological studies, T25-cm\textsuperscript{2} flasks of tsA-201 cells were transiently co-transfected using Fugene\textsuperscript{®} 6 mix reagent (Roche Diagnostics, IN, USA) with 0.3 μg of each subunit of voltage-gated calcium channel (Ca\textsubscript{1.2}, β\textsubscript{2} and α\textsubscript{2}δ-1 subunits; ratio 1:1:1) and 1.0 μg of other constructs or empty vector. An equivalent amount of pcDNA3.1 DNA was added to the transfection mix to compensate for the absence of DNA encoding Ca\textsubscript{v} subunits or USPs when omitted. All transfections included 0.5 μg of cDNA encoding CD8 antigen as a reporter gene. Anti-CD8 beads (Dynal\textsuperscript{®}, Oslo, Norway) were used to identify transfected cells, and only decorated cells were analysed. For biochemistry experiments, T75-cm\textsuperscript{2} flasks of HEK-293 cells were transfected using Lipofectamine LTX\textsuperscript{®} (Invitrogen, Basel, Switzerland) according to the manufacturer’s instructions. The ratio of cDNAs/Lipofectamine LTX\textsuperscript{®} was 10 μg cDNAs/30 μL Lipofectamine. The ratio of the different constructs was similar to those used in patch clamp experiments. In biochemistry experiments, an additional...
control was performed by using the empty vector pcDNA3.1 only in Ca,,-untransfected cells. Cells were used 48 h after transfection.

Electrophysiology

Whole-cell currents were measured at room temperature (22–23 °C) using an Axopatch200B amplifier (Axon Instruments, Union City, CA, USA). TsA-201 cells were replated 24 h post-transfection onto 35-mm plastic Petri dishes and recorded from 24 h post-replating. The internal pipette solution was composed of (in mM) 60 CsCl, 70 cesium aspartate, 1 MgCl2, 10 HEPES, 11 EGTA and 5 Mg-ATP, pH 7.2 with CsOH. The external solution contained (in mM) 130 NaCl, 5.6 KCl, 5 to 20 BaCl2, 1 MgCl2, 10 HEPES and 11 D-glucose, pH 7.4 with NaOH. The osmolality, assessed using a Löser micro-osmometer (Giessen, Marburger, Germany), was 295 mOsm l⁻¹ for both the internal solution and the 5 mM BaCl2 external solution and 325 mOsm l⁻¹ for the 20 mM BaCl2 external solution. In experiments performed without β subunits which are known to promote Ca, currents, we used the higher 20 mM BaCl2 solution to compensate for the lack of β subunits which greatly reduced Ca, currents. 5 mM BaCl2 was used for all other experiments. Data were analysed using pClamp software, version 9.2 (Axon Instruments, Union City, CA, USA), and Origin software, version 7.5 (OriginLab® corporation, Northampton, MD, USA). Barium current densities (pA/pF) were calculated by dividing the peak current by the cell capacitance. I-V relationship (IV) were fitted with the following equation I=(g(Vh−Vrev))/(1+exp((Vh−V50)/k)), in which I is the normalized peak current density (pA/pF) at a given holding potential (Vh), V50,act is the voltage at which half of the channels are activated, k is the slope factor, Vrev is the reversal potential and g is the conductance. The maximal conductance Gmax was calculated from the maximal peak current density.

Western blots

T75-cm² flasks of HEK-293 cells were lysed in 1.0 ml of lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 10 % glycerol, 1 % triton, 1 mM EGTA supplemented with 10 mM N-ethylmaleimide and protease inhibitors). Protein concentration was systematically determined by performing a Bradford assay (Coo protein dosage kit; Interchim, Montluçon, France). Eighty micrograms of proteins were loaded on an SDS-PAGE gel. Protein transfer was done with the dry system transfer iBlot® from Invitrogen (Invitrogen, Basel, Switzerland). Immunoblotting was accomplished by using the SNAP id® system of Millipore (Millipore, Zug, Switzerland). Fluorescent secondary antibodies were used, and detection was realized using the LICOR system® (Lincoln, USA). The intensity of the bands was quantified with the Odyssey software (LICOR).

Surface biotinylation assay

HEK-293 cells were washed twice with PBS 1×, 48 h after transfection, and then treated for 30 min at 4 °C with 4 ml non-permeant biotin per T75-cm² flasks (1 mg/ml; EZ link Sulfo-NHS-SS-Biotin; Pierce, Rockford, USA). Biotin binds to the lysine residues of proteins exposed to the extracellular medium. Cells were washed three times with cold PBS 1× containing 0.2 M glycine and lysed with 1 ml/dish of lysis buffer. The cells were solubilized for 1 h on a wheel at 4 °C and centrifuged for 30 min at 20,000g at 4 °C. Supernatants were recovered, and protein concentrations were quantified by the Bradford method. One hundred micrograms of the lysates were used to assess the transfection efficiency. Proteins inserted at the plasma membrane were then selectively pulled down with 50 μl of streptavidin sepharose beads (GE Healthcare Europe, Glattrugg, Switzerland) added to 1 mg of total proteins before incubation for 2 h on a wheel at 4 °C. The beads were washed five times with lysis buffer, and beads were resuspended in 2.5× sample buffer (Invitrogen, Basel, Switzerland). Eluted proteins were analysed by Western blot.

Immunoprecipitation

Transiently transfected HEK-293 cells in P100 plates were harvested after 48-h incubation and lysed with 1× cold Ubi lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EGTA, pH 8.0, 10 % glycerol, 1× EDTA-free complete protease inhibitor cocktail (Roche, Mannheim, Germany); 2 mM N-ethylmaleimide/NEM (Sigma-Aldrich, St. Louis, MO, USA); 10 mM iodoacetamide/IAA (Sigma-Aldrich, St. Louis, MO, USA)) containing 1 % Triton X-100 for 1 h at 4 °C. Cell lysates were then centrifuged at 16,000g at 4 °C for 15 min. Two milligrams of the supernatant (lysatse) was incubated at 4 °C for 24 h with anti-Ca, 1.2 channel subunits antibodies. One volume of 1× cold Ubi lysis buffer without Triton X-100 (to obtain a final concentration of 0.5 % Triton X-100) was also added in the mix. On the next day, the lysate-antibody mix was transferted to a microcentrifuge tube containing 50 μg (1:1 beads to lysis buffer ratio) of Protein G Sepharose beads (GE Healthcare, Uppsala, Sweden) which were previously washed three times with 1× cold Ubi lysis buffer containing 0.5 % Triton X-100. After adding fresh 1× EDTA-free complete protease inhibitor cocktail, the mix was incubated overnight at 4 °C. The beads were subsequently washed five times (4 °C; 3,000 rpm) with 1× cold Ubi buffer containing 0.3 % Triton X-100 before elution with 50 μl of 2× NuPAGE sample buffer with 100 mM DTT at 37 °C for 30 min. These samples are designated as immunoprecipitation (IP) fractions. The input fractions were resuspended with 4× NuPAGE sample buffer with 100 mM DTT to give a concentration of 1 mg/ml and incubated at 37 °C for 30 min. All lysis and incubation steps, except elution in sample buffer, were performed in the dark.

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Expression of GST-S5a fusion proteins in *Escherichia coli* bacteria was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside for 4 h at 29 °C. Cells were harvested by centrifugation and resuspended in lysis buffer (200 mM Tris pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.5 % Igepal). Supernatant from 15 min of centrifugation at 13,000g (4 °C) was incubated 1 h in the presence of GSH-sepharose beads at 4 °C. Beads were then washed three times with lysis buffer and used in pull-down experiments. One milligram of total protein (HEK-293 lysates) was added to 50 µg of GST-S5A beads and incubated for 2 h at 4 °C. After washing the beads three times with lysis buffer, precipitated proteins were eluted with sampling buffer (Invitrogen, Basel, Switzerland) and analysed by Western blot.

**Pull down of ubiquitylated proteins**

Pull down of S-tagged USP2-45

One milligram of HEK-293 cells lysate was incubated for 1 h at 4 °C with 1 µl of biotinylated S-protein (Merck biosciences, Darmstadt, Germany), followed by 1-h incubation at 4 °C with streptavidin sepharose beads (GE Healthcare Europe, Glattbrugg, Switzerland). The beads were washed five times with lysis buffer, and beads were resuspended in 2.5× sample buffer (Invitrogen, Basel, Switzerland). Eluted proteins were analysed by Western blot.

**Antibodies**

Antibody against Ca$_{v}$1.2 (ACC003; Alomone, Jerusalem, Israel) was used at a dilution of 1/200. Antibody against β$_{2b}$ (ab54920; Abcam, Cambridge, UK) was used at a dilution of 1/200. Antibody against α$_{2}$δ-1 (ab2864; Abcam, Cambridge, UK) was used at a dilution of 1/1,000. FK2 antibody was used at the dilution of 1/500 (PW8810, Enzo Life Sciences, Lausanne, Switzerland). USP2 antibody used at a dilution of 1/500 was a gift from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland). USP2 antibody used at a dilution of 1/500 was a gift from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland). USP2 antibody used at a dilution of 1/500 was a gift from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland). USP2 antibody used at a dilution of 1/500 was a gift from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland). USP2 antibody used at a dilution of 1/500 was a gift from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland). USP2 antibody used at a dilution of 1/500 was a gift from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland). USP2 antibody used at a dilution of 1/500 was a gift from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland). USP2 antibody used at a dilution of 1/500 was a gift from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland). USP2 antibody used at a dilution of 1/500 was a gift from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland). USP2 antibody used at a dilution of 1/500 was a gift from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland).

Two-tailed Student’s *t* test was used to compare two groups of data. One-way ANOVA was used to compare three or more groups. Data are represented as mean±SEM. *P*<0.05 was considered significant.

Results

Ca$_{v}$1.2 currents are downregulated by USP2-45

We expressed Ca$_{v}$1.2 together with the accessory subunits β$_{2}$ and α$_{2}$δ-1 in tsA-201 cells and assessed the effect of the two USP2 variants and USP15 on Ca$_{v}$ currents. Figure 1 shows that co-expressing either USP2-69 (Fig. 1a, b) or USP2-45 (Fig. 1c, d) reduced whole-cell Ca$_{v}$ current densities (respectively by 83±7 %, *n* = 10; *p* < 0.05 for USP2-69 at 0 mV; and 74±8 %, *n* = 19; *p* < 0.05 for USP2-45 at 0 mV). In contrast, USP15, a related de-ubiquitylase [29], had no effect (Fig. 1a, b), suggesting that Ca$_{v}$ channels are selectively regulated by USP2 de-ubiquitylases. The decrease in current amplitude was not caused by a shift in the current-voltage relationship (Fig. 1b, d) or inactivation properties of the channels (not shown). In particular, the midpoint of voltage-dependent activation (*V*$_{50,act}$), calculated by fitting the current-voltage relationship as indicated in ‘Methods’, was not significantly altered by USP2-69 (−7±3 mV versus −10±1 mV in control; non-significant; NS) or USP2-45 (−9±4 mV versus −10±1 mV in control; NS), hence the amplitude of the effect of either USP2 splice variant can be directly compared at the same voltage. Interestingly, both USP2-69 and USP2-45 significantly increased the slope factor value (*k* = −9.1±0.9 in USP2-69-transfected cells versus *k* = −5.9±0.3 in control; *P* < 0.01; and *k* = −9.0±1.1 in USP2-45-transfected cells versus *k* = −5.9±0.4 in control; *P* < 0.05) which suggests that de-ubiquitylation of the channels may decrease their voltage-sensitivity. Figure 1c, d shows that the catalytically inactive mutant USP2-45C67A failed to regulate Ca$_{v}$1.2 channels. Current densities recorded in USP2-45C67A-transfected cells were similar to control (control 38±5 pA/pF at 0 mV, *n* = 16; and USP2-45C67A 37±12 pA/pF at 0 mV, *n* = 11; NS), confirming that the effect of USP2-45 is mediated by its de-ubiquitylating activity. Further experiments were conducted with the USP2-45 isofrom only.

USP2-45 decreases the availability of Ca$_{v}$1.2 channels at the plasma membrane

A reduction of Ca$_{v}$ current density may reflect a decrease in the number of channels trafficked to the plasma membrane. Hence, we examined the effect of USP2-45 on the plasma membrane expression of Ca$_{v}$ channels by performing surface biotinylation assays. Figure 2a, c shows that USP2-45 reduced the amount of biotinylated Ca$_{v}$1.2, whereas the surface abundance of α$_{2}$δ-1 and co-precipitated β$_{2}$ subunits were not affected. Remarkably, Western blots performed on the corresponding whole-cell lysates showed that USP2-45 decreased the protein amount of all three Ca$_{v}$ subunits (Fig. 2b, d). It is noteworthy that the cytosolic β$_{2}$ is pulled down together with the biotinylated Ca$_{v}$1.2 (Fig. 2a). Interestingly, the amount of
coprecipitated $\beta$ suggested that the proportion of $\beta$ associated with Ca v1.2 may be increased in USP2-45-transfected cells (by 120±32%, $n=4$; $p<0.05$ when calculating the ratio of co-
precipitated $\beta$ divided by the amount of biotinylated Ca v1.2). The same comparison cannot be made regarding $\alpha_2$δ-1, which is known to traffic to the cell surface independently of Ca v1.2 [18, 33]. Overall, these experiments showed that USP2-45 decreases the amount of Ca v1.2 channels inserted at the plasma membrane and that this downregulation is correlated with a reduction of all three (pore-forming and auxiliary) Ca v proteins.

USP2-45 promotes the de-ubiquitylation of both Ca v1.2 and $\alpha_2$δ-1 subunits.

Next, we examined the effect of USP2-45 on Ca v ubiquitylation. We immunoprecipitated Ca v1.2 and detected its level of ubiquitylation by using an FK2 anti-ubiquitin

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**Fig. 1** Effect of USP de-ubiquitylases on Ca v1.2 currents. a Representative whole-cell current traces and b corresponding current-voltage (I-V) relationships in Ca v1.2/ $\beta_2$/ $\alpha_2$δ-1 channels transfected alone with control (white circle (control)), or with USP2-69 (black square) or USP15 (white square) in tsA-201 cells. The maximal conductance and reversal potential were calculated with the I-V fit described in ‘Methods’. $G_{\text{max}}$ was specifically decreased in USP2-69 (0.3±0.2 nS/pF versus 0.8±0.1 nS/pF in control; $P<0.05$), but not in USP15-transfected cells (0.8±0.2 nS/pF; NS versus control), whilst $V_{\text{rev}}$ was not modified by either USP2-69 (48±3 mV versus 54±3 mV in control; NS) or USP15 (46±3 mV; NS versus control). The values for $V_{\text{50,act}}$ and slope factors are indicated in the results section. One-way ANOVA statistical analysis compared control with USP2-69 or USP15 (*$p<0.05$). The number of cells is indicated in parentheses. a Representative whole-cell current traces and b corresponding current-voltage relationships in tsA-201 cells transfected with Ca v1.2/ $\beta_2$/ $\alpha_2$δ-1 channels alone (white circle (control)), or with USP2-45 (black triangle) or the catalytically inactive mutant USP2-45 C67A (white triangle). $G_{\text{max}}$ was decreased by USP2-45 (0.4±0.1 nS/pF versus 0.8±0.1 nS/pF in control; $P<0.05$), but not by USP2-45 C67A (0.8±0.2 nS/pF; NS versus control), whilst $V_{\text{rev}}$ was not modified by either USP2-45 (44±4 mV versus 54±3 mV in control; NS) or USP2-45 C67A (52±1 mV; NS versus control). The values for $V_{\text{50,act}}$ and slope factors are indicated in the results section. One-way ANOVA statistical analysis compared control with USP2-45 (*$p<0.05$) and USP2-45 C67A with USP2-45 (#$p<0.05$). In all experiments, 5 mM BaCl$_2$ was used to record Ca v currents.
antibody, which recognizes both mono- and poly-ubiquitinated proteins. We found that USP2-45 reduced the ubiquitylation status of Ca\textsubscript{v}1.2 (Fig. 3a). Figure 3c shows a significant reduction in the ratio of intensity of the signal detected with the FK2 anti-ubiquitin antibody relative to the total amount of immunoprecipitated Ca\textsubscript{v}1.2, suggesting that USP2-45 de-ubiquitylates Ca\textsubscript{v}1.2 subunits. By contrast, USP2-45 did not significantly alter the ubiquitylation status of immunoprecipitated \( \beta_2 \) subunits (Fig. 3c). Because of the lack of sensitivity of the \( \alpha_2\delta-1 \) antibody, together with the downregulating effect of USP2-45, the amount of immunoprecipitated subunits was low and detection of
ubiquitin on α2δ-1 was too weak to be reliably analysed using the FK2 antibody (not shown). Hence, we used an alternative approach and performed pull-down experiments using GST-S5a fusion proteins, which recognize poly-ubiquitylated proteins [12]. The specificity of the GST-S5a was demonstrated in our previous works [36]. In cells transfected with the channels only, GST-S5A pulled down all three Ca, subunits, whereas no signal was recovered in cells transfected with the empty vector pcDNA3.1 only (Fig. 4a). This result is in line with the fact that the three subunits are tonically ubiquitylated in basal conditions [25, 36]. Most importantly, Fig. 4a shows that the amount of Ca,1.2 and α2δ-1 subunits recovered with GST-S5a was drastically reduced in cells co-transfected with USP2-45 compared to control cells transfected with the channels only. As expected from the experiments shown in Fig. 2b, d, USP2-45 also decreased the amount of proteins recovered in the corresponding whole-cell lysates (Fig. 4b, d).

To correct for the reduction of Ca, proteins and determine the relative change in ubiquitylation of each subunit, we calculated the ratio of ubiquitylated versus total (ubiquitylated and non-ubiquitylated) proteins. The effect of USP2-45 was expressed as a percentage of change from the control value (Fig. 4c). USP2-45 significantly decreased the ubiquitylation of Ca,1.2 and α2δ-1 but not β2 subunits. Altogether, these results indicate that both Ca,1.2 and α2δ-1, but not β2, are regulated by the USP2-45 de-ubiquitylase.

The α2δ-1 subunit is essential for the regulation of Ca, channels by USP-45

In support of a direct effect of USP2 on the channels, we found that USP2-45 co-immunoprecipitated with α2δ-1 but not with Ca,1.2 subunits (Fig. 5). As expected, we found no evidence for interaction of USP2-45 with β2 subunits (Fig. 5) which are not targeted by this de-ubiquitylase (Fig. 4a, c). These results suggest that USP2-45 binds only to the auxiliary subunit α2δ-1 and that this interaction is sufficient to promote the de-ubiquitylation of both α2δ-1 and Ca,1.2. To confirm the involvement of α2δ-1, we assessed the effect of USP2-45 on channels expressed without α2δ-1 or β subunits. We show that, in the absence of α2δ-1, USP2-45 did not alter Ca,1.2/β2 current densities (reduced by 9±16 % at +10 mV, n=19; NS; Fig. 6a), nor the current-voltage relationship: $V_{50,act}$ for Ca,1.2/β2 channels was not significantly different between control (0.8±1.0 mV, n=11) and USP2-45-transfected cells (−2.6±0.9 mV, n=9; NS). By contrast, USP2-45 still reduced Ca,1.2/α2δ-1 current densities in the absence of β (by 56±19 % at +20 mV, n=9; p<0.05; Fig. 6b), again without altering the current-voltage relationship ($V_{50,act}$=−6.8±1.6 mV, n=6 in control, versus $V_{50,act}$=−7.7±4.6 mV, n=3; NS; in USP2-45-transfected cells), confirming that, unlike α2δ-1, β subunits are not required for regulation of Ca, channels by USP-45. Altogether, these data demonstrate
Discussion

In this study, we identify the de-ubiquitylase USP2-45 as a novel regulator of Cav channels. We show that USP2-45 binds to α₂δ-1, but not Ca₅.1,2 subunits, and that α₂δ-1 is required for USP2-45-induced Ca₅.1,2 downregulation. This result suggests that the first critical event is the binding of USP2-45 to the auxiliary α₂δ-1 subunit which may act as an anchor allowing for USP2-45 to de-ubiquitylate Ca₅.1,2 channels. USP2-45 promotes the de-ubiquitylation of both Ca₅.1,2 and α₂δ-1 subunits, reducing the availability of Ca₅.1,2 channels at the plasma membrane. This result is intriguing because de-ubiquitylation is most often associated with stabilization of proteins at the cell surface [15, 38]. Nonetheless, USP2-45 was also reported to induce the degradation of a mineralocorticoid receptor, by disrupting its association with a stabilizing partner which preferentially interacts with the ubiquitylated form of the receptor [16]. Overexpression of USP2 was also shown to reduce p53 stability [39], and another de-ubiquitylase USP8 (also called UBPY) promotes epithelial growth factor receptor degradation [4, 32, 37]. De-ubiquitylation may also

Fig. 5 USP2-45 binds to α₂δ-1 subunits. Pull-down experiments were performed on HEK-293 cells using biotinylated S-protein which recognizes an S-tag epitope inserted into USP2-45. Western blots show that USP2-45 co-precipitated with α₂δ-1 but not Ca₅.1,2 nor β₂ subunits (n=3)
serve to allow the recycling of ubiquitin moieties attached to the proteins prior to degradation, as reported for epithelial growth factor receptors [3, 37]. In addition, the binding of USP2-45 to \( \alpha_2\delta-1 \) may disrupt the chaperone role of this subunit towards Ca\( v_{1.2} \), leading to the reduction of Ca\( v_{1.2} \) surface expression. The conserved amount of membrane-associated \( \alpha_2\delta-1 \) subunits in USP2-45-transfected cells, revealed by surface biotinylation assays, could be explained by the fact that these subunits are expressed at the cell surface as both single units and complexed with the main pore-forming Ca\( v_{1.2} \) \( \alpha_1 \) subunits [18, 33]. It is expected that the remaining fraction of \( \alpha_2\delta-1 \) subunits, which have not yet been sent for degradation, continues to be efficiently targeted alone to the plasma membrane, independently of Ca\( v_{1.2} \). Noteworthily, \( \alpha_2\delta-1 \) is described as both transmembrane proteins [34] and extracellular glycosylphosphatidylinositol (GPI)-anchored proteins [11] and may coexist in the two forms [11]. Because USP2-45 specifically binds to \( \alpha_2\delta-1 \), one can assume that the cytosolic USP2-45 recovered in surface biotinylation assays is co-precipitated with the biotinylated \( \alpha_2\delta-1 \) subunits. Importantly, this result suggests that at least a fraction of \( \alpha_2\delta-1 \) is a transmembrane protein, accessible for and able to retain the binding of the cytosolic USP2-45. Remarkably, our study identifies USP2-45 as a novel binding partner for \( \alpha_2\delta-1 \). To date, apart from \( \alpha_1 \) (the pore-forming subunit itself), the only other known \( \alpha_2\delta-1 \)-interacting proteins were components of the extracellular matrix called thrombospondins (TSP1, TSP2 and TSP4) [14, 21] and a subunit of a mitochondrial ATP

**Fig. 6** \( \alpha_2\delta-1 \) is necessary for the USP2-45-induced decrease of Ca\( v_{1.2} \) currents. **a, b** Representative whole-cell current traces and corresponding bar graphs showing that USP2-45 failed to regulate Ca\( v_{1.2} \) channels in the absence of \( \alpha_2\delta-1 \) subunits (**a**), whereas USP2-45 still decreases Ca\( v_{1.2} \) currents in the absence of \( \beta_2 \) (**b**). tsA-201 cells were transfected with Ca\( v_{1.2}/\beta_2 \) alone (white circle, control) or together with USP2-45 (black circle) in **a** and Ca\( v_{1.2}/\alpha_2\delta-1 \) alone (white square (control)) or together with USP2-45 (black square) in **b**. As expected because of their known involvement in Ca\( v \) trafficking, the absence of either subunit decreased Ca\( v \) current densities (compared to Fig. 1). The lack of \( \beta \) subunit in particular dramatically decreased Ca\( v \) currents, despite the charge carrier being increased to 20 mM BaCl\( _2 \) in order to reliably quantify the decrease caused by USP2-45 on Ca\( v_{1.2}/\alpha_2\delta-1 \) channels. The data show the amplitude of the current densities recorded at 20 mV which generates the maximal current in the absence of \( \beta \). As for prior experiments, 5 mM BaCl\( _2 \) was used to record Ca\( v_{1.2}/\beta_2 \) currents. Currents densities are compared at 10 mV which generates the maximal current in the absence of \( \alpha_2\delta-1 \). The number of cells is indicated in parentheses. NS non-significant. *\( p<0.05 \) when compared with respective control.
The αδ-1 subunit also binds to the anti-allodynic drug gabapentin used for the treatment of neuropathic pain [20].

One intriguing finding of our study is that although the Ca\(\text{v}1.2\) subunit β is not itself de-ubiquitylated by USP2-45, the total amount of β available in USP2-45-transfected cells is reduced together with Ca\(\text{v}1.2\) and αδ-1. One possible explanation is that USP2-45 preferentially regulates Ca\(\text{v}1.2\) when associated with both accessory subunits β and αδ-1, which may then be concomitantly sent for degradation. Alternatively, an excess of free β may be degraded consequently to USP2-45-induced degradation of Ca\(\text{v}1.2\). In favour of this hypothesis, a recent report showed that β subunits which fail to associate with Ca\(\text{v}2.2\) are degraded by the proteasome [42]. Importantly, both β and αδ-1 can independently promote Ca\(\text{v}1.2\) insertion [9, 17], but only αδ-1-associated channels are sensitive to USP2-45. Hence, the fraction of Ca\(\text{v}\) channels insensitive to USP2-45 and able to reach the plasma membrane would be mostly composed of Ca\(\text{v}1.2/\beta\) channels. In favour of this model, we found that the fraction of Ca\(\text{v}\) channels expressed at the plasma membrane of USP2-45-transfected cells is slightly enriched in Ca\(\text{v}1.2/\beta\).

In our study, we exogenously expressed cloned cardiac Ca\(\text{v}\) channels and USP in mammalian cell lines but the extent of such regulation and the role of αδ-1 in the de-ubiquitylation of Ca\(\text{v}\) channels in cardiac myocytes remain to be investigated. The αδ-1 subunits are also associated with Ca\(\text{v}2.2\) channels which are predominantly expressed in neurons [8]. αδ-1 was reported to control synaptic release probability by regulating presynaptic Ca\(\text{v}2.2\) channel availability [23]. Ca\(\text{v}2.2\) is also ubiquitylated [2]. Hence, it would be worth assessing whether αδ-1 enables USP2-45, which is known to be expressed in the brain [22], to also de-ubiquitylate neuronal Ca\(\text{v}2.2\) channels.

Overall, our results argue for a dual role of αδ on Ca\(\text{v}\) trafficking: increasing (as previously shown [16, 7, 17]) or decreasing Ca\(\text{v}\) expression at the plasma membrane. We propose that USP2-45 acts as a switch to redirect the action of αδ towards a decrease in Ca\(\text{v}\) channels availability, thus revealing a new role both for αδ and USP2-45 in calcium signalling.

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**Ethical standards** All experiments comply with the current laws of the countries (England, Switzerland) in which they were performed.

**Conflict of interest** The authors declare that they have no conflict of interest.

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**References**

1. Abriel H, Staub O (2005) Ubiquitylation of ion channels. Physiol (Bethesda) 20:398–407. doi:10.1152/physiol.00333.2005

2. Altier C, Garcia-Caballero A, Simmons B, You H, Chen L, Walcher J, Tedford HW, Hermosilla T, Zamponi GW (2011) The Cavbeta subunit prevents RFP2-mediated ubiquitination and proteasomal degradation of L-type channels. Nat Neurosci 14(2):173–180. doi:10.1038/nn.2712

3. Alwan HA, van Zeele EJ, van Leeuwen JE (2003) Ligand-induced lysosomal epidermal growth factor receptor (EGFR) degradation is preceded by proteasome-dependent EGFR de-ubiquitination. J Biol Chem 278(37):35781–35790. doi:10.1074/jbc.M301262200

4. Alwan HA, van Leeuwen JE (2007) UBPY-mediated epidermal growth factor receptor (EGFR) de-ubiquitination promotes EGFR degradation. J Biol Chem 282(3):1658–1669. doi:10.1074/jbc.M604711200

5. Angelotti T, Hofmann F (1996) Tissue-specific expression of splice variants of the mouse voltage-gated calcium channel alpha2/delta subunit. FEBS Lett 397:331–337

6. Bernstein GM, Jones OT (2007) Kinetics of internalization and degradation of N-type voltage-gated calcium channels: role of the alpha2 subunit. Cell Calcium 41:27–40

7. Canti C et al (2005) The metal-ion-dependent adhesion site in the presynaptic Ca2+ channel subunit. Cell Calcium 41:336–349

8. Catterall WA, Perez-Reyes E, Snutch TP, Striessnig J (2005) Kinetics of internalization and degradation of N-type voltage-gated calcium channels: role of the α2δ-1 subunit. Cell Calcium 41:27–40

9. Chien AJ, Zhao X, Shirakov RE, Puri TS, Chang CF, Sun D, Rios E, Hosey MM (1995) Roles of a membrane-localized beta subunit in the formation and targeting of functional L-type Ca2+ channels. J Biol Chem 270(50):30036–30044

10. Colecraft HM, Alseikhan B, Takahashi SX, Chaudhuri D, Mittman S, Yegnasubramanian V, Alvania RS, Johns DC, Marbán E, Yue DT (2002) Novel functional properties of Ca2+ channel beta subunits revealed by their expression in adult rat heart cells. J Physiol 541(Pt 1):435–452

11. Davies A, Kadurin I, Alvarez-Laviada A, Douglas L, Nieto-Rostro M, Bauer CS, Pratt WS, Dolphin AC (2010) The alpha2delta subunits of voltage-gated calcium channels form GPI-anchored proteins, a posttranslational modification essential for function. Proc Natl Acad Sci U S A 107(4):1654–1659. doi:10.1073/pnas.0908735107

12. Deveraux Q, Ustrell V, Pickart C, Rechsteiner M (1994) A 26 S protease subunit that binds ubiquitin conjugates. J Biol Chem 269(10):7059–7061

13. Dolmetsch R (2003) Excitation-transcription coupling: signaling by ion channels to the nucleus. Science’s STKE: signal transduction knowledge environment 2003(166):PE4. doi:10.1126/stke.2003.166.pe4

14. Eroglu C, Allen NJ, Susman MW, O’Rourke NA, Park CY, Orkan E, Chakraborty C, Mulinyawe SB, Annis DS, Huberman AD, Green EM, Lawler J, Dolmetsch R, Garcia KC, Smith SJ, Luo ZD, Rosenthal A, Mosher DF, Barres BA (2009) Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell 139(2):380–392. doi:10.1016/j.cell.2009.09.025
