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Ubiquitin-specific protease USP2-45 acts as a molecular switch to promote $\alpha_2\delta$-1-induced downregulation of $Ca_v1.2$ channels

Jean-Sebastien Rougier · Maxime Albesa · Ninda Syam · Guillaume Halet · Hugues Abriel · Patricia Viard

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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 $Ca_v1.2$ have been found to be ubiquitylated under basal conditions both in vitro and in vivo. In a previous study, we have shown that $Ca_v1.2$ 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 $Ca_v1.2$ channels together with the accessory subunits β_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 $Ca_v1.2$ 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 $Ca_v1.2$ and $\alpha_2\delta$-1 subunits. Remarkably, $\alpha_2\delta$-1, but not $Ca_v1.2$ nor β_2, co-precipitated with USP2-45. These results suggest that USP2-45 binding to $\alpha_2\delta$-1 promotes the de-ubiquitylation of both $Ca_v1.2$ and $\alpha_2\delta$-1 subunits, in order to regulate the expression of $Ca_v1.2$ 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

Jean-Sebastien Rougier and Maxime Albesa share the first authorship.

J.-S. Rougier · P. Viard
Department of Neurosciences, Physiology, and Pharmacology, University College London, London WC1E 6BT, UK

J.-S. Rougier · M. Albesa · N. Syam · H. Abriel (✉)
Department of Clinical Research, University of Bern, Bern 3010, Switzerland
e-mail: hugues.abriel@dkf.unibe.ch

G. Halet · P. Viard (✉)
CNRS, UMR 6290, Institut de Génétique et Développement de Rennes, 35043 Rennes, France
e-mail: patricia.viard@univ-rennes1.fr

G. Halet · P. Viard
Université Rennes 1, UEB, IFR 140, Faculté de Médecine, 35043 Rennes, France

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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 $Ca_v1.2$ 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 $Ca_v1.2$ 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 $Ca_v1.2$ 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]. $Ca_v1.2$ channels also contribute to neuron excitability and are involved in the control of gene transcription [13]. In addition to the main pore-forming $Ca_v1.2$ subunit, $Ca_v1.2$ channels also contain accessory subunits: β and $\alpha_2\delta$, which regulate both the gating properties and trafficking of the channels [9, 41, 17, 7, 6]. $Ca_v1.2$ channels are predominantly associated with β_2 [10, 43] and $\alpha_2\delta$-1 [5, 28] in the heart. We, and others, have previously shown that these three $Ca_v1.2$ subunits are ubiquitylated in vitro and in vivo [25, 36]. Here, we found that both USP2-45 and USP2-69 regulate cloned cardiac $Ca_v1.2$ 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_v currents obtained with either splice variant suggests that Ca_v channels are the target of both isoforms and that the minimum sequence required for Ca_v current reduction resides within the short variant form of USP. Hence, we focussed our investigation on USP2-45-induced regulation of Ca_v channels. Our study reveals a new role for $\alpha_2\delta$-1 subunits in binding USP2-45 and promoting the de-ubiquitylation of both α1 and $\alpha_2\delta$-1 subunits. USP2-45-induced Ca_v regulation leads to a decrease of $Ca_v1.2$ channels available at the plasma membrane.

Methods

DNA constructs

Rabbit $Ca_v1.2$ (cardiac isoform α1c) (P15381.1), β_{2b} (P54288) and $\alpha_2\delta$-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 $pcDNA_{3.1}$ were gifts from Prof. O. Staub (Department of Pharmacology and Toxicology, Lausanne, Switzerland).

Transfections

For electrophysiological studies, T25-cm^2 flasks of tsA-201 cells were transiently co-transfected using Fugene® 6 mix reagent (Roche Diagnostics, IN, USA) with 0.3 μg of each subunit of voltage-gated calcium channel ($Ca_v1.2$, β_2 and $\alpha_2\delta$-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_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®, Oslo, Norway) were used to identify transfected cells, and only decorated cells were analysed. For biochemistry experiments, T75-cm^2 flasks of HEK-293 cells were transfected using Lipofectamine LTX® (Invitrogen, Basel, Switzerland) according to the manufacturer's instructions. The ratio of cDNAs/Lipofectamine 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_v-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 $MgCl_2$, 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 $BaCl_2$, 1 $MgCl_2$, 10 HEPES and 11 D-glucose, pH 7.4 with NaOH. The osmolarity, assessed using a Löser micro-osmometer (Giessen, Marburger, Germany), was 295 mOsm l^{-1} for both the internal solution and the 5 mM $BaCl_2$ external solution and 325 mOsm l^{-1} for the 20 mM $BaCl_2$ external solution. In experiments performed without β subunits which are known to promote Ca_v targeting, we used the higher 20 mM $BaCl_2$ solution to compensate for the lack of β subunits which greatly reduced Ca_v currents. 5 mM $BaCl_2$ 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 $I=(g(V\mathrm{h}-V_{\mathrm{rev}}))/(1+\exp[(V\mathrm{h}-V_{50})/k])$, in which I is the normalized peak current density (pA/pF) at a given holding potential (Vh), $V_{50,\mathrm{act}}$ is the voltage at which half of the channels are activated, k is the slope factor, V_{rev} is the reversal potential and g is the conductance. The maximal conductance $G_{\max}$ was calculated from the maximal peak current density.

Western blots

T75-cm^2 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-cm^2 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 20,000*g* 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, Glattbrugg, 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 16,000*g* at 4 °C for 15 min. Two milligrams of the supernatant (lysate) was incubated at 4 °C for 24 h with anti-$Ca_v1.2$ 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 transferred 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.

Pull down of ubiquitylated proteins

Expression of GST-S5a fusion proteins in *Escherichia coli* bacteria was induced with 0.2 mM isopropyl β-D-1-thio-galactopyranoside 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 13,000*g* (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 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 $Ca_v1.2$ (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 $\alpha_2\delta$-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). Monoclonal antibody raised against actin was purchased from Sigma-Aldrich (Sigma-Aldrich Chemie, Postfach, Switzerland) and used at a dilution of 1/1,000. Antibody raised against S-tag was purchased from Abcam (ab18588; Abcam, Cambridge, UK) and used at a dilution of 1/200.

Statistical analysis

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

$Ca_v1.2$ currents are downregulated by USP2-45

We expressed $Ca_v1.2$ together with the accessory subunits β_2 and $\alpha_2\delta$-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 significatively 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 ($k=-9.1\pm0.9$ in USP2-69-transfected cells versus $k=-5.9\pm0.3$ in control; $P<0.01$; and $k=-9.0\pm1.1$ in USP2-45-transfected cells versus $k=-5.9\pm0.4$ 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 $Ca_v1.2$ channels. Current densities recorded in USP2-45C67A-transfected cells were similar to control (control 38±5 pA/pF at 0 mV, $n=16$; and USP2C67A 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 isoform only.

USP2-45 decreases the availability of $Ca_v1.2$ 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 $Ca_v1.2$, whereas the surface abundance of $\alpha_2\delta$-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 $Ca_v1.2$ (Fig. 2a). Interestingly, the amount of

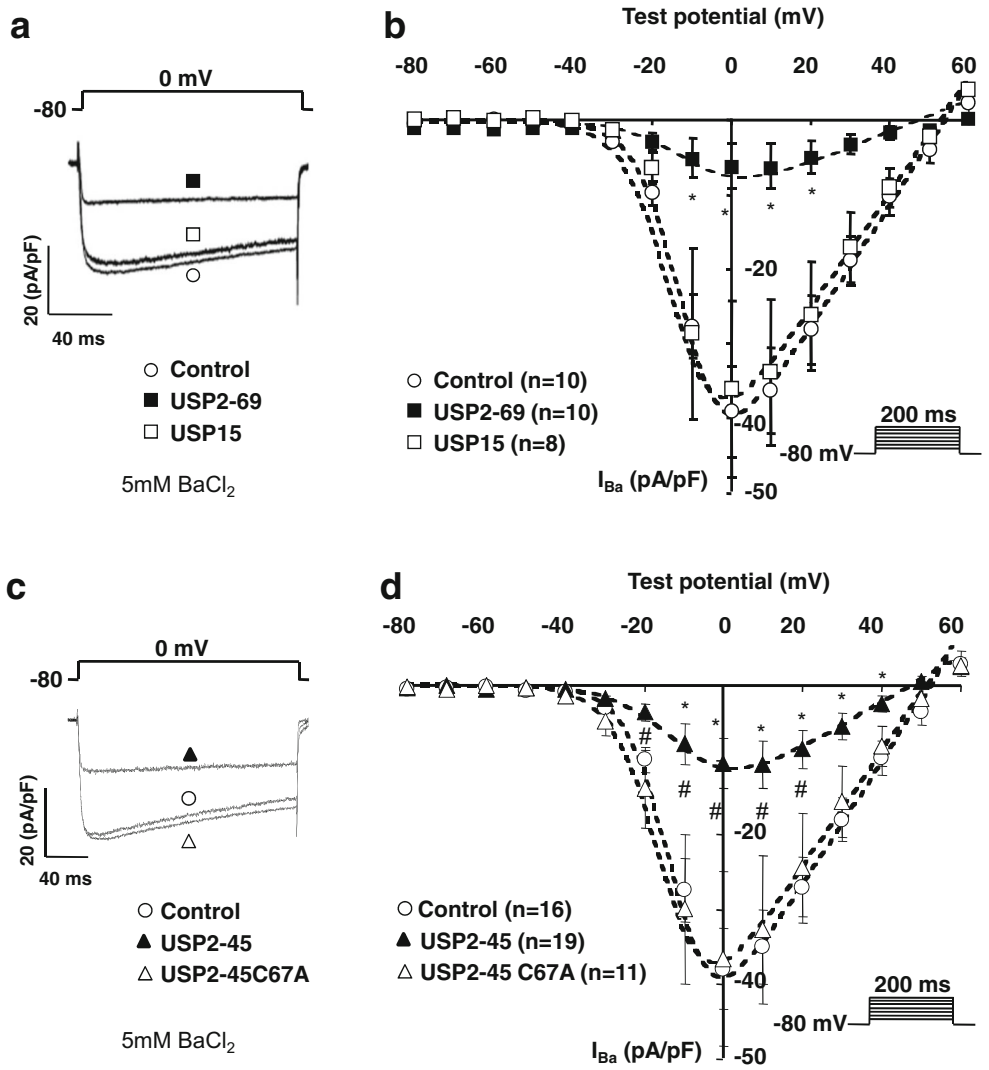


Fig. 1 Effect of USP de-ubiquitylases on $Ca_v1.2$ currents. **a** Representative whole-cell current traces and **b** corresponding current-voltage (I-V) relationships in $Ca_v1.2/\beta_2/\alpha2\delta$-1 channels transfected alone with control (*white circle* (control)), or with USP2-69 (*black square*) 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_{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_{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 $V_{50,act}$ 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 $Ca_v1.2/\beta_2/\alpha2\delta$-1 channels alone (*white circle* (control)), or with USP2-45 (*black triangle*) or the catalytically inactive mutant USP2-45 C67A (*white triangle*). G_{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_{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 $V_{50,act}$ 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

coprecipitated β suggested that the proportion of β 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 β divided by the amount of biotinylated $Ca_v1.2$). The same comparison cannot be made regarding $\alpha_2\delta$-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 $\alpha_2\delta$-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

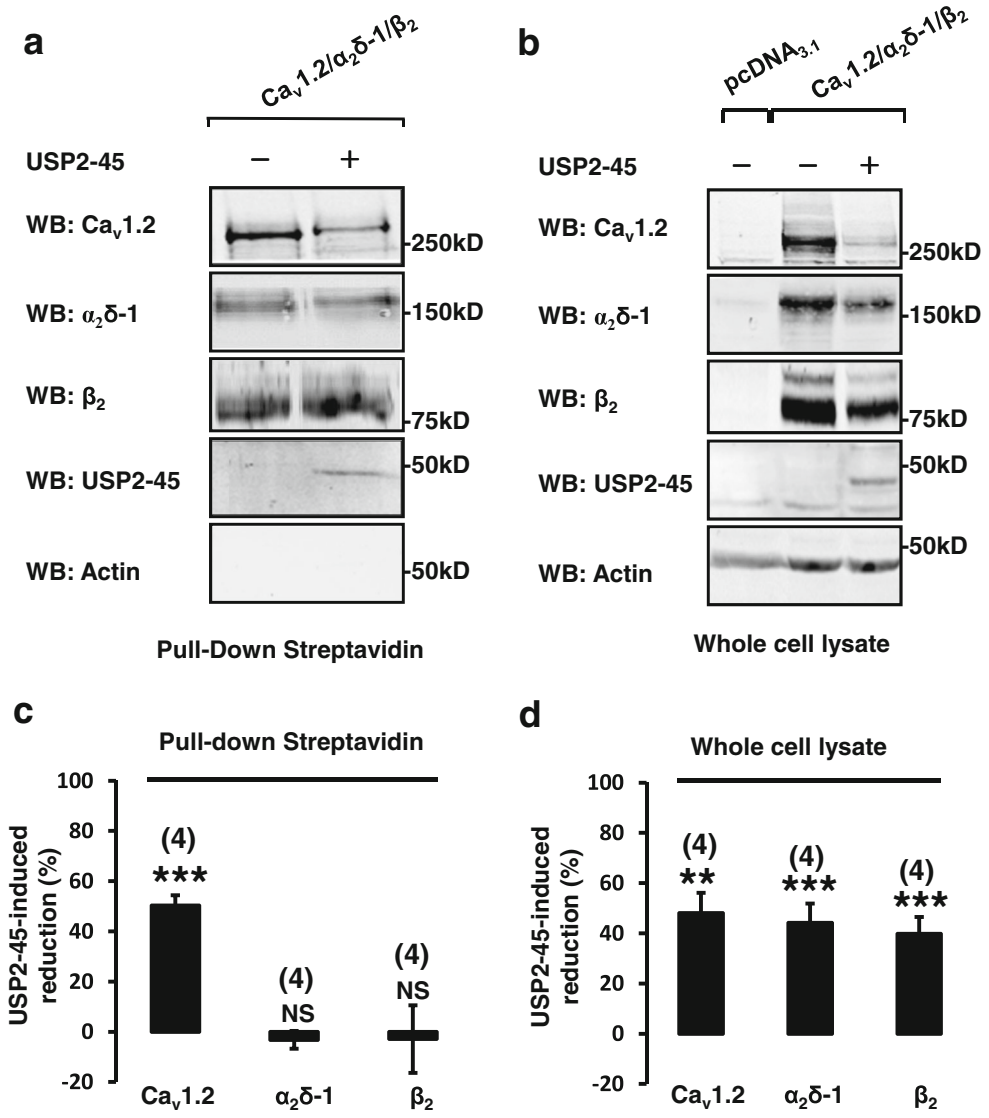


Fig. 2 USP2-45 reduces the expression $Ca_v1.2$ channels at the plasma membrane. **a**, **b** Surface biotinylation assays were performed in control and USP2-45-transfected HEK-293 cells. Steptavidin-covered beads were used to pull down the biotinylated proteins and their binding partners. Western blots (*WB*) show Ca_v channels and USP2-45 detected in the whole-cell lysates (**a**) and recovered by pull down with streptavidin (**b**) from a same experiment (n=4). Note that the cytosolic USP2-45 was co-precipitated with the biotinylated fraction in (**a**), suggesting that this de-ubiquitylase binds to proteins expressed at the plasma membrane. As expected, the cytosolic β2 was co-purified with the biotinylated channels, because of their known interaction with $Ca_v1.2$. As a control, another cytosolic protein (actin) was not recovered in the biotinylated fraction, indicating that the membrane integrity of the cells was preserved in our experimental conditions. **c** *Bar graphs* showing how USP2-45 alters the amount of Ca_v subunits recovered in pull-down experiments. The effect of USP2-45 was expressed as a percentage of reduction in the intensity of Ca_v protein bands relative to control. USP2-45 specifically decreased the membrane insertion of $Ca_v1.2$, whereas the amount of biotinylated $\alpha_2\delta$-1 and the proportion of the cytosolic β_2 that co-purified with the biotinylated $Ca_v1.2$ channels were unchanged. The number of independent experiments is indicated in *parentheses*. *NS* non-significant. ***$p<0.001$ when compared with control. **d** *Bar graphs* showing the USP2-45-induced decrease in the amount of total Ca_v proteins (both biotinylated and non-biotinylated) recovered in the corresponding whole-cell lysates of cells exposed to biotin. The effect of USP2-45 was expressed as a percentage of reduction in the intensity of Ca_v protein bands relative to control. The decreased amount of Ca_v proteins detected in whole-cell lysates indicates that USP2-45 downregulated all three $Ca_v1.2$, β_2 and $\alpha_2\delta$-1 subunits. **$p<0.01$ and ***$p<0.001$ when compared with control

antibody, which recognizes both mono- and poly-ubiquitinated proteins. We found that USP2-45 reduced the ubiquitylation status of $Ca_v1.2$ (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 $Ca_v1.2$, suggesting that USP2-45 de-ubiquitylates $Ca_v1.2$ subunits. By contrast, USP2-45 did not significantly alter the ubiquitylation status of immunoprecipitated β_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

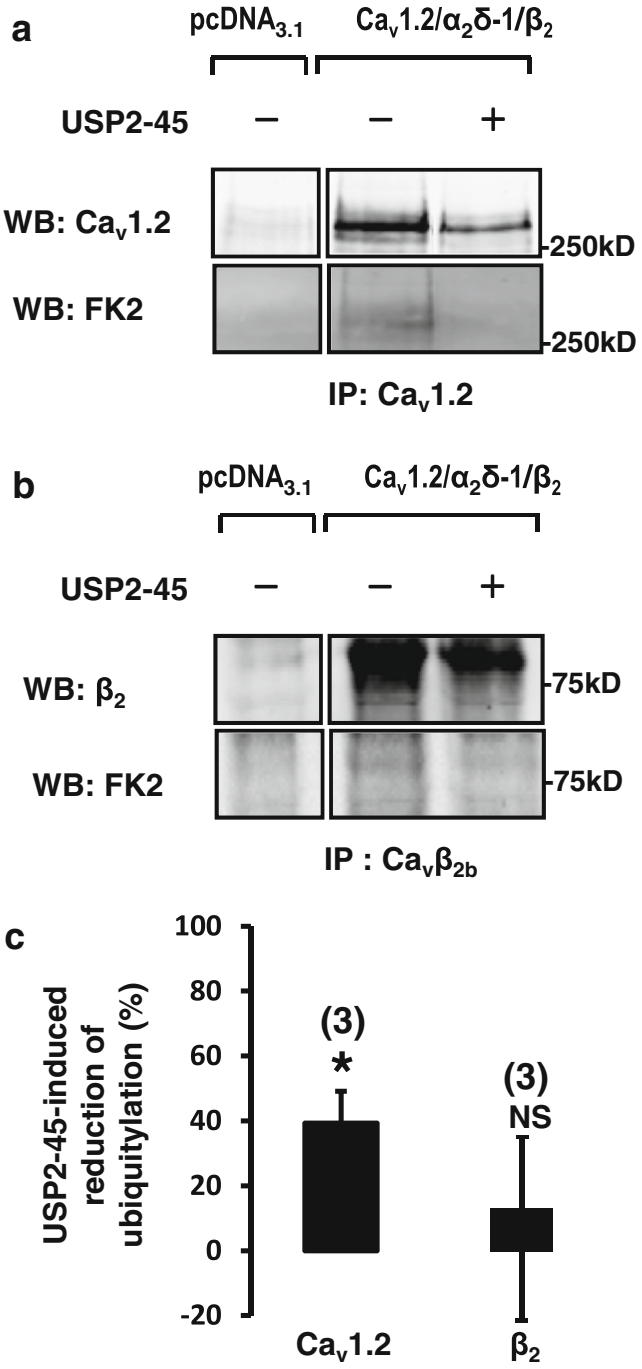
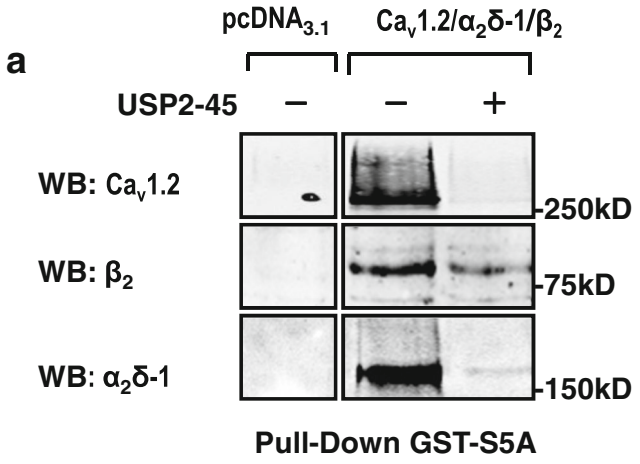


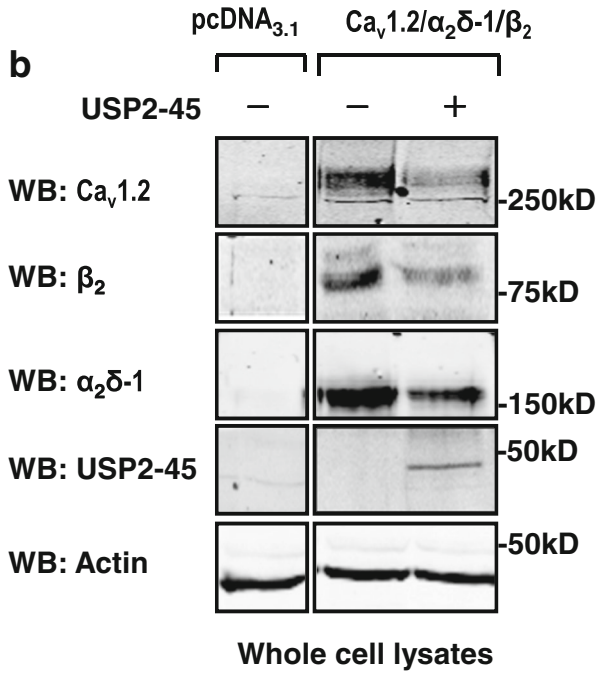
Fig. 3 De-ubiquitylation of immunoprecipitated $Ca_v1.2$ by USP2-45. **a** Immunoprecipitation of $Ca_v1.2$ and **b** β_2 subunits. Western blots were performed to confirm the purification of Ca_v subunits from HEK-293 cells, whilst anti-ubiquitin FK2 antibodies were used to detect the ubiquitylated channels. Control experiments show that $Ca_v1.2$ and β_2 are tonically ubiquitylated. **c** *Bar graph* showing that USP2-45 decreased the ubiquitylation level of $Ca_v1.2$, but not β_2 subunits. The intensity of the FK2 signal was normalized to take in account the reduction Ca_v protein (i.e. dividing by the intensity of the signal obtained with the corresponding Ca_v antibody), and the effect of USP2-45 was expressed as a percentage of change from the control value

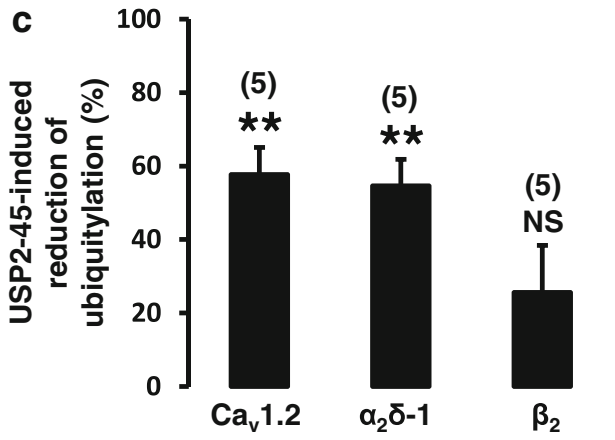
ubiquitin on $\alpha_2\delta$-1 was too weak to be reliably analysed using the FK2 antibody (not shown). Hence, we used an alternative approach and performed pull-down experiments using GST-S5a fusion proteins, which recognize poly-ubiquitylated proteins [12]. The specificity of the GST-S5a was demonstrated in our previous works [36]. In cells transfected with the channels only, GST-S5A pulled down all three Ca_v 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 $Ca_v1.2$ and $\alpha_2\delta$-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_v 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 $Ca_v1.2$ and $\alpha_2\delta$-1 but not β_2 subunits. Altogether, these results indicate that both $Ca_v1.2$ and $\alpha_2\delta$-1, but not β_2, are regulated by the USP2-45 de-ubiquitylase.

The $\alpha_2\delta$-1 subunit is essential for the regulation of Ca_v channels by USP-45

In support of a direct effect of USP2 on the channels, we found that USP2-45 co-immunoprecipitated with $\alpha_2\delta$-1 but not with $Ca_v1.2$ subunits (Fig. 5). As expected, we found no evidence for interaction of USP2-45 with β_2 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 $\alpha_2\delta$-1 and that this interaction is sufficient to promote the de-ubiquitylation of both $\alpha_2\delta$-1 and $Ca_v1.2$. To confirm the involvement of $\alpha_2\delta$-1, we assessed the effect of USP2-45 on channels expressed without $\alpha_2\delta$-1 or β subunits. We show that, in the absence of $\alpha_2\delta$-1, USP2-45 did not alter $Ca_v1.2/\beta_2$ current densities (reduced by 9±16 % at +10 mV, n=19; NS; Fig. 6a), nor the current-voltage relationship: $V_{50,act}$ for $Ca_v1.2/\beta_2$ 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 $Ca_v1.2/\alpha_2\delta$-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 $\alpha_2\delta$-1, β subunits are not required for regulation of Ca_v channels by USP2-45. Altogether, these data demonstrate







the paramount role of $\alpha_2\delta$-1 in USP2-45-induced downregulation of Ca_v channels.

◀ **Fig. 4** USP2-45-induced de-ubiquitylation of $Ca_v1.2$ and $\alpha_2\delta$-1 subunits. **a** Western blots showing the reduction of total $Ca_v1.2$, β_2 and $Ca_v\alpha_2\delta$-1 proteins in HEK-293 whole-cell lysates used for **b** pull down of ubiquitylated channels using ubiquitin binding GST-S5A (n=5). Note that USP2-45 appears to reduce further the amount of $Ca_v1.2$ and $\alpha_2\delta$-1 subunits recovered in the pull-down assay. **c** *Bar graph* comparing the effect of USP2-45 on the ubiquitylation of the three different Ca_v subunits. To illustrate that the decrease in ubiquitylation of $Ca_v1.2$ and $\alpha_2\delta$-1 cannot be solely explained by the concomitant reduction in total Ca_v proteins, the intensity of each protein bands recovered by pull-down assays (shown in **b**) was divided by the intensity of the corresponding band in whole-cell lysates (ubiquitylated and non-ubiquitylated proteins shown in **a**). The data was expressed as a percentage change of this ratio relative to control. USP2-45 significantly decreased the ubiquitylation of $Ca_v1.2$ and $\alpha_2\delta$-1 but not β_2 subunits. The number of experiments is indicated in *parentheses*. *NS* non-significant. ***p<0.001 when compared with control

Discussion

In this study, we identify the de-ubiquitylase USP2-45 as a novel regulator of Ca_v channels. We show that USP2-45 binds to $\alpha_2\delta$-1, but not $Ca_v1.2$ subunits, and that $\alpha_2\delta$-1 is required for USP2-45-induced $Ca_v1.2$ downregulation. This result suggests that the first critical event is the binding of USP2-45 to the auxiliary $\alpha_2\delta$-1 subunit which may act as an anchor allowing for USP2-45 to de-ubiquitylate $Ca_v1.2$ channels. USP2-45 promotes the de-ubiquitylation of both $Ca_v1.2$ and $\alpha_2\delta$-1 subunits, reducing the availability of $Ca_v1.2$ 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

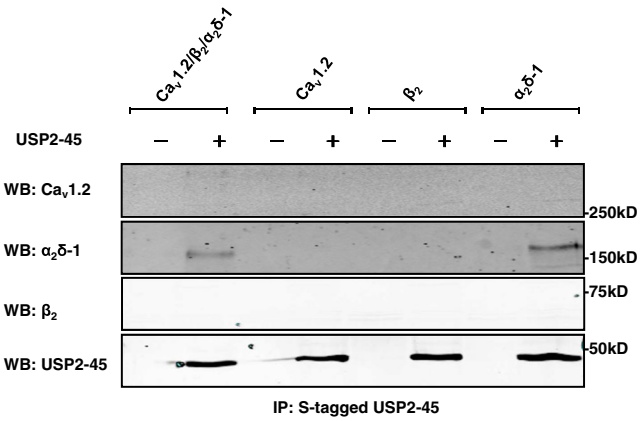


Fig. 5 USP2-45 binds to $\alpha_2\delta$-1 subunits. Pull-down experiments were performed on HEK-293 cells using biotinylated S-protein which recognizes an S-tag epitope inserted into USP2-45. Western blots show that USP2-45 co-precipitated with $\alpha_2\delta$-1 but not $Ca_v1.2$ nor β_2 subunits (n=3)

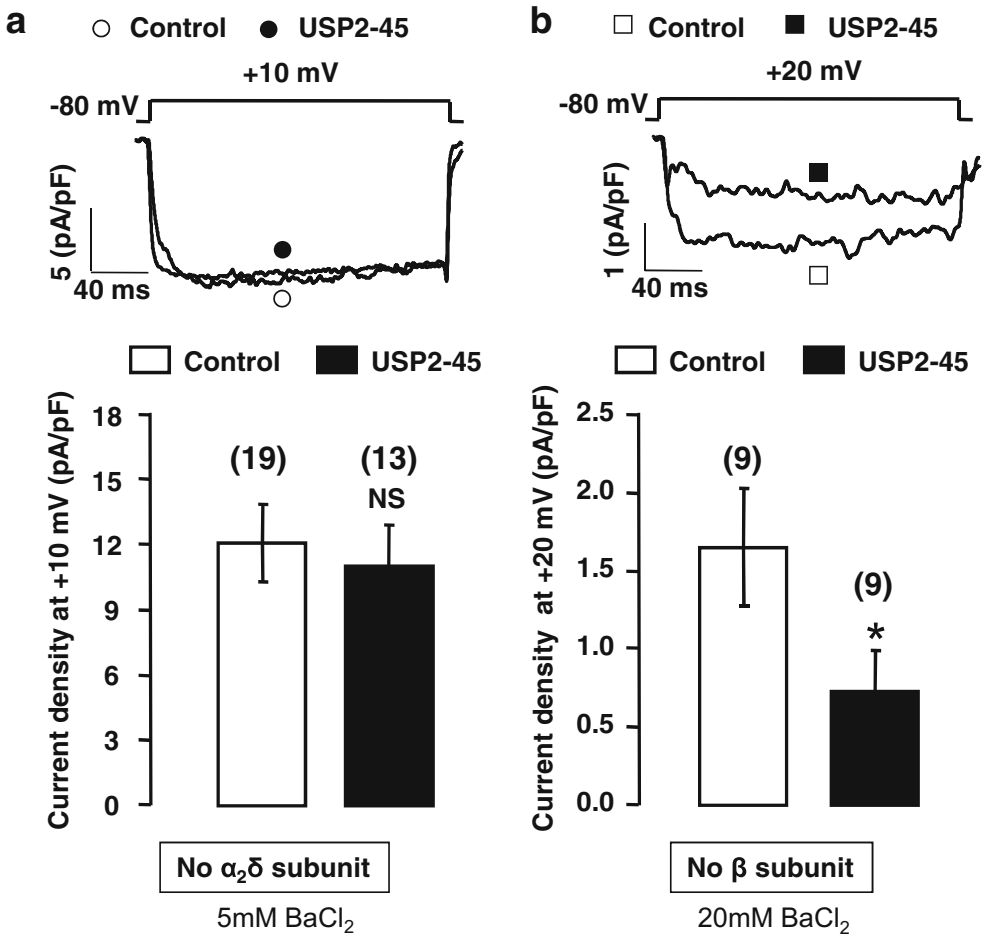


Fig. 6 $\alpha_2\delta$-1 is necessary for the USP2-45-induced decrease of $Ca_v1.2$ currents. **a**, **b** Representative whole-cell current traces and corresponding bar graphs showing that USP2-45 failed to regulate $Ca_v1.2$ channels in the absence of $\alpha_2\delta$-1 subunits (**a**), whereas USP2-45 still decreases $Ca_v1.2$ currents in the absence of β_2 (**b**). tsA-201 cells were transfected with $Ca_v1.2/\beta_2$ alone (*white circle*, (control)) or together with USP2-45 (*black circle*) in **a** and $Ca_v1.2/\alpha_2\delta$-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 β 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 $Ca_v1.2/\alpha_2\delta$-1 channels. The data show the amplitude of the current densities recorded at 20 mV which generates the maximal current in the absence of β. As for prior experiments, 5 mM $BaCl_2$ was used to record $Ca_v1.2/\beta_2$ 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

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 $Ca_v1.2$, leading to the reduction of $Ca_v1.2$ surface expression. The conserved amount of membrane-associated $\alpha_2\delta$-1 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 α1 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 $Ca_v1.2$. Noteworthingly, $\alpha_2\delta$-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 α1 (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

synthase complex (ATP5b) [19]. The $\alpha_2\delta$-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_v 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 $Ca_v1.2$ and $\alpha_2\delta$-1. One possible explanation is that USP2-45 preferentially regulates $Ca_v1.2$ when associated with both accessory subunits β and $\alpha_2\delta$-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 $Ca_v1.2$. In favour of this hypothesis, a recent report showed that β subunits which fail to associate with $Ca_v2.2$ are degraded by the proteasome [42]. Importantly, both β and $\alpha_2\delta$-1 can independently promote $Ca_v1.2$ insertion [9, 17], but only $\alpha_2\delta$-1-associated channels are sensitive to USP2-45. Hence, the fraction of Ca_v channels insensitive to USP2-45 and able to reach the plasma membrane would be mostly composed of $Ca_v1.2/\beta$ channels. In favour of this model, we found that the fraction of Ca_v channels expressed at the plasma membrane of USP2-45-transfected cells is slightly enriched in $Ca_v\beta$.

In our study, we exogeneously expressed cloned cardiac Ca_v channels and USP in mammalian cell lines but the extent of such regulation and the role of $\alpha_2\delta$-1 in the de-ubiquitylation of Ca_v channels in cardiac myocytes remain to be investigated. The $\alpha_2\delta$-1 subunits are also associated with Ca_v2 channels which are predominantly expressed in neurons [8]. $\alpha_2\delta$-1 was reported to control synaptic release probability by regulating presynaptic Ca_v2 channel availability [23]. Ca_v2 is also ubiquitylated [2]. Hence, it would be worth assessing whether $\alpha_2\delta$-1 enables USP2-45, which is known to be expressed in the brain [22], to also de-ubiquitylate neuronal Ca_v2 channels.

Overall, our results argue for a dual role of $\alpha_2\delta_1$ on Ca_v trafficking: increasing (as previously shown [16, 7, 17]) or decreasing Ca_v expression at the plasma membrane. We propose that USP2-45 acts as a switch to redirect the action of $\alpha_2\delta_1$ towards a decrease in Ca_v channels availability, thus revealing a new role both for $\alpha_2\delta_1$ 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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