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Ankyrin-rich membrane spanning protein as a novel modulator of transient receptor potential vanilloid 1-function in nociceptive neurons

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Abstract

Background—The ion channel TRPV1 is mainly expressed in small diameter dorsal root ganglion (DRG) neurons, which are involved in the sensation of acute noxious thermal and chemical stimuli. Direct modifications of the channel by diverse signaling events have been intensively investigated, but little is known about the composition of modulating macromolecular TRPV1 signaling complexes. Here, we hypothesize that the novel adaptor protein ankyrin-rich

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membrane spanning protein/kinase D interacting substrate (ARMS) interacts with TRPV1 and modulates its function in rodent DRG neurons.

Methods—We used immunohistochemistry, electrophysiology, microfluorimetry and immunoprecipitation experiments to investigate TRPV1 and ARMS interactions in DRG neurons and transfected cells.

Results—We found that TRPV1 and ARMS are co-expressed in a subpopulation of DRG neurons. ARMS sensitizes TRPV1 towards capsaicin in transfected HEK 293 cells and in mouse DRG neurons in a PKA-dependent manner. Using a combination of functional imaging and immunocytochemistry, we show that the magnitude of the capsaicin response in DRG neurons depends not only on TRPV1 expression, but on the co-expression of ARMS alongside TRPV1.

Conclusion—These data indicate that ARMS is an important component of the signaling complex regulating the sensitivity of TRPV1.

Introduction

The transient receptor potential vanilloid 1 (TRPV1) is predominantly expressed in nociceptive dorsal root ganglion (DRG) neurons. It is a key player in the sensation of painful stimuli, in the generation of thermal hyperalgesia during inflammation, and it became a major target in the preclinical development of pain therapeutics (Caterina et al., 2000; Caterina et al., 1997). TRPV1 is modulated by opioid receptor/cAMP interactions, phospholipases, phospholipids, kinases and phosphatases (Endres-Becker et al., 2007; Spahn et al., 2013; Tominaga and Tominaga, 2005).

Little is known about the intracellular TRPV1 signaling complexes in sensory neurons. A first discovery was the A-kinase anchoring protein 150 (AKAP150 in rodents, AKAP79 in humans) which functions as a scaffold for protein kinase A (PKA), facilitating the association of the enzyme with its substrates at distinct subcellular loci (Beene and Scott, 2007). This complex is involved in PKA- and PKC ϵ -induced TRPV1 sensitization in DRG neurons (Distler et al., 2003; Jeske et al., 2008; Jeske et al., 2009; Rathee et al., 2002; Schnizler et al., 2008). Recently, it was shown that the RII β subunit of the PKA II isoform is predominantly expressed in nociceptors and can be used as a marker of these neurons (Isensee et al., 2014a). Additionally, a direct non-canonical interaction of TRPV1 and the GABAB1 receptor was identified (Hanack et al., 2015).

Another potential interaction partner of TRPV1 is the adaptor protein ankyrin-rich membrane spanning protein/kinase D interacting substrate (ARMS). ARMS was previously identified as a large adaptor protein featuring numerous protein-protein interaction motifs, specifically expressed in the nervous system (Iglesias et al., 2000; Kong et al., 2001). So far, ARMS has been shown to be involved in neurotrophic signaling, neuronal development, synaptic transmission, and in the control of neurotransmitter release (reviewed in (Neubrand et al., 2012)). It was also identified as an essential interaction partner of the nerve growth factor receptor TrkA (Kong et al., 2001). Recently we showed ARMS mRNA to be co-expressed in TRPV1-positive nociceptive neurons (Isensee et al., 2014b). Although ARMS was initially identified in DRG neurons, most functional studies on ARMS focused on central neurons or used heterologous expression systems (Kong et al., 2001). Here, we

explore the functional role of ARMS in the context of TRPV1 signaling in DRG neurons and transfected cells.

Materials and Methods

Mice and preparation of tissues

Experimental procedures were approved by the state animal care and use committee ("Landesamt für Arbeitsschutz, Gesundheit und Technische Sicherheit Berlin"). Male C57BL/6 mice (*Mus musculus*) were obtained from the animal housing facility of the Charité (FEM; Forschungseinrichtung für experimentelle Medizin). For cryosectioning mice were sacrificed using an overdose of isoflurane (Piramal Critical Care, Bethlehem, USA) and perfused with 0.1 M phosphate buffer (PB) followed by fixation with 4 % paraformaldehyde (PFA) in PB. Spinal cord (SC) and DRG were removed and postfixed for 20 min in 4 % PFA/PB. The tissue was cryoprotected in 30 % sucrose in PB overnight at 4 °C and mounted with Neg50 (Microm, Germany). Twelve µm thick sections were cut using a Microm HM560 cryostat (Microm, Germany). Sections were transferred to poly-L-lysine coated glass slides (Thermo Fisher) and stored at -20 °C until staining procedures.

Primary cell culture

DRG neurons were prepared as described previously (Bessac et al., 2009; Endres-Becker et al., 2007). A brief description is provided in the supporting information section (methodsS1).

Cell culture and heterologous expression

Human embryonic kidney (HEK) 293 cells (DSMZ) were maintained at 37 °C, 5 % CO₂ in DMEM (Biochrom, Germany) containing 10 % fetal bovine serum and 1 % penicillin/streptomycin. They were passaged 1:2 – 1:5 every second to third day depending on the confluence. The plating and transfection procedure for the different experiments is described in the supporting information (methodsS2).

Patch clamp recordings

Whole-cell voltage clamp recordings were performed in TRPV1- or TRPV1 and ARMS-expressing HEK 293 cells with or without pretreatment of 10 µM myristoylated PKA inhibitor fragment 14-22 (myr-PKI, Sigma Aldrich, Germany) for 30 min at -60 mV holding potential with an EPC-10 patch clamp amplifier and PULSE software (HEKA Elektronik, Lambrecht, Germany) as described previously (Endres-Becker et al., 2007; Spahn et al., 2013; Spahn et al., 2014). Experiments were performed 16 - 24 h after transfection and the TRPV1 channel was activated by the addition of 50 nM capsaicin.

Ratiometric [Ca²⁺]_i measurements

18-24 h post transfection HEK 293 cells were loaded with the fluorescent calcium indicator dye Fura 2-AM (2 µM) supplied with 0.02 % pluronic F-127 (Invitrogen, USA) for 20 min. TRPV1 was activated by capsaicin (1 pM, 100 pM, 500 pM, 1 nM, 20 nM, 100 nM, 200 nM, 1 µM, 3 µM) contained in calcium imaging buffer (140 mM NaCl, 4 mM KCl, 2 mM CaCl₂,

1 mM MgCl₂, 4.55 mM NaOH, 5 mM Glucose and 10 mM HEPES, adjusted to pH 7.4 with NaOH) in the presence or absence of the unspecific PKA inhibitor H89 (°N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride, Sigma-Aldrich, Germany), the specific PKA inhibitor myr-PKI (Rathee et al., 2002) (Sigma-Aldrich, Germany) or 1 µM of the PKC inhibitor GF 109203X (Sigma Aldrich, Germany). The cells were treated with H89 or GF 109203X 20 min and with myr-PKI 30 min prior to the experiment. After 20 s baseline determination, fluorescent signals were recorded for 100 s. Images were acquired as described previously (Spahn et al., 2013). Viability was tested using a 3 µM capsaicin stimulus at the end of each measurement. Cells were included in the analyses, if the Fura ratio at baseline did not fluctuate more than 10 % and if the cells responded to 3 µM capsaicin with a Fura ratio (F340/380) greater than 1.

For the combined calcium imaging/immunocytochemistry experiments (see methodsS3), DRG neurons were loaded with 0.25 µg Fura 2-AM to 80 µl cell culture wells and incubated at 37 °C and 5 % CO₂. After 40 min, the wells were washed twice for 10 min with warmed media, supplemented with Neurobasal A for neurons. Image capturing was performed after alternating excitations (340 and 380 nm) at 510 nm in the Cellomics ArrayScan High Content Screening platform. Stimulation of cultures was handled by an integrated pipetting robot. A maximum of 8 wells was measured in a row to prevent Fura intoxication.

Immunohistochemistry

DRG sections of mice were thawed, rehydrated in PBS, permeabilized in PBS with 0.2 % Triton X-100 (PBT) for 10 min and blocked for 1 h in blocking buffer (PBT, 1 % BSA, 5 % serum of the host animal of the second antibody). For double or triple staining, the Zenon antibody labeling technology (Invitrogen, USA) was used, according to the manufacturer's instructions. This technique avoids cross-reactivity, if the primary antibodies were raised in the same host. The stained sections were analyzed using confocal laser scanning microscopy (LSM 510meta, Carl Zeiss, Germany) and quantified using ImageJ. Further information is provided in methodsS4.

Immunoprecipitation and western blot analysis

Samples of transfected HEK 293 cells, DRG neurons or SC were lysed in a buffer containing 150 mM NaCl, 50 mM TRIS, 10 mM HEPES, 1 % Triton X-100, 1× Protease Inhibitor Mix (Complete protease inhibitor cocktail, Roche Diagnostics, Switzerland) at pH 7.8. Nuclei and debris were removed by centrifugation at 1000 g for 5 min and protein concentrations were measured by the BCA assay (Thermo Fisher Scientific, USA). Equal protein concentrations (in µg protein ml⁻¹ buffer) of the resulting cell homogenates were subjected to immunoprecipitation (methodsS5). Western blot analysis was performed as described previously (Busch-Dienstfertig et al., 2012) (methodsS5).

For the interaction analysis experiments using cAMP-analog-agarose (Hanke et al., 2011), cells were treated as described in methodsS5.

Radioligand binding assay

48 h post transfection, TRPV1- and TRPV1/ARMS-expressing HEK 293 were prepared using either the plasma membrane protein extraction kit (Abcam, ab65400, Great Britain) to separate total (including plasma and organelle membrane proteins, TMF) and plasma membrane fractions (PMF) according to the manufacturer's instructions or membrane preparations according to a modified protocol for transfected HEK 293 cells (Szallasi et al., 1999; Szallasi et al., 1992).

The TRPV1 expression was determined using the radioactively labeled TRPV1 agonist [³H] resiniferatoxin (RTX). The experiment was carried out according to a modified protocol (Szallasi et al., 1999; Szallasi et al., 1992). Briefly, appropriate concentrations of membranes (100-200 µg) were prepared and incubated in assay buffer (50 mM Trizma, 0.25 mg/ml bovine serum albumin, pH 7.4) with 1.1 nM [³H] RTX (39.2 Ci/mmol) (Perkin Elmer, Waltham, USA) in the absence or presence of 10 µM unlabeled RTX to assess affinity by displacement (Spahn et al., 2013).

Statistics

Data are presented as means ± SEM. Statistical significance is denoted as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Statistical analysis was performed by using 1-way ANOVA with Bonferroni's post test, Kruskal Wallis test with Dunn's post test, or Mann Whitney test in co-expression studies. For patch clamp and calcium imaging experiments, unpaired t-test, Mann Whitney test and 2-way ANOVA with Bonferroni's post test were used. Dose response curves were fitted with the Hill equation. All tests were performed using Prism 5 (GraphPad, USA).

Results

Co-expression of TRPV1 and ARMS in DRG neurons

Immunohistochemistry revealed 4 different subpopulations in mouse DRG neurons, namely cells expressing TRPV1 but not ARMS (TRPV1+/ARMS-) (5.16 % of the investigated cells; $340.2 \pm 9.4 \mu\text{m}^2$, $n = 706$; Fig. 1A, cells indicated by arrows), cells expressing ARMS but not TRPV1 (48.92 % of all investigated cells; $675.5 \pm 10 \mu\text{m}^2$, $n = 6579$; Fig. 1B, cells indicated by arrows), cells expressing both proteins (30.32 % of all investigated cells; $405.7 \pm 4.7 \mu\text{m}^2$, $n = 4121$; Fig. 1C, cells indicated by asterisks), and cells expressing none of the proteins. The cell sizes of the subpopulations differed significantly. The TRPV1+/ARMS- subpopulation displayed the smallest size (Kruskal Wallis test with Dunn's post test, ***, $p < 0.001$). The average size of all investigated neurons ($n = 13349$ of 6 mice) was $530.9 \pm 3.8 \mu\text{m}^2$. Of these cells, 35.49 % were TRPV1-positive with a mean cell size of $396.1 \pm 4.27 \mu\text{m}^2$. 79.24 % of the cells were ARMS-positive. The size of this subpopulation was $546.6 \pm 4.33 \mu\text{m}^2$ (Fig. 1D). Fig. 1E shows the size distribution of all TRPV1-positive cells, cells positive for TRPV1 but negative for ARMS, and positive for both TRPV1 and ARMS. Cells expressing only TRPV1 (blue bars) were smaller compared to those expressing both proteins (red bars). In Fig. 1F the ratio of subpopulations is visualized as Venn-diagram.

Functional and physical interaction of TRPV1, ARMS and PKA in HEK 293 cells and DRG neurons

To investigate possible functional interaction of TRPV1 and ARMS, we performed calcium imaging experiments. TRPV1/ARMS-expressing HEK 293 cells showed stronger calcium increase upon stimulation with a very low capsaicin concentration (1 nM) than cells expressing TRPV1 alone (Fig. 2A, C and F). At the end of each measurement, all cells were stimulated with 3 μ M capsaicin and showed no significant differences between the groups (Fig. 2A, D and G). Fig. 2 B and E present HEK 293 cells expressing only TRPV1 (B) and TRPV1/ARMS (E) prior to capsaicin stimulation. Dose-response relationships upon capsaicin stimulation showed a leftward shift in cells co-expressing TRPV1 and ARMS compared to cells only expressing TRPV1. The calculated EC₅₀ values differed significantly (Fig. 2H and I, TRPV1: n = 51; TRPV1/ARMS: n = 68; unpaired t-test, **, p<0.01). Pretreatment of the TRPV1 and TRPV1/ARMS expressing cells with 10 μ M of the (unspecific) PKA blocker H89 (20 min) not only abolished the significantly lower EC₅₀-value for capsaicin obtained in TRPV1/ARMS expressing cells compared to cells only expressing TRPV1, but resulted in significantly higher EC₅₀-values in this group (Fig 2I; TRPV1+H89: n = 127; TRPV1/ARMS+H89: n = 42; unpaired t-test, **, p<0.01).

Whole cell patch clamp experiments demonstrated functional interaction of both proteins in HEK 293 cells. Application of 50 nM capsaicin resulted in significantly increased currents in HEK 293 cells co-expressing TRPV1 and ARMS compared to cells expressing TRPV1 alone (Fig. 3A and B, unpaired t-test, *, p<0.05). Repetitive capsaicin application to cells co-expressing TRPV1 and ARMS or TRPV1 alone did not show different desensitization patterns (Fig. 3C-E, 2-way ANOVA with Bonferroni's post test, p>0.05).

We further investigated the role of kinases in mediating increased capsaicin-induced TRPV1 activity in cells expressing TRPV1 and ARMS compared to cells only expressing TRPV1. Fig. 4A shows 1 nM capsaicin-induced maximum changes of F340/380. The significantly stronger calcium influx was abolished by pretreating the cells with H89 and the PKC inhibitor GF 109203X. Additionally, inhibition of PKC resulted in significantly weaker calcium influx in both expression groups compared to untreated cells, possibly due to more general effects of PKC inhibition in this context. Since H89 is an unspecific PKA inhibitor we used myr-PKI, which was shown to be specific for PKA. In calcium imaging and patch clamp experiments, pretreatment with 10 μ M myr-PKI for 30 min reversed the increased capsaicin-induced TRPV1 activity in TRPV1/ARMS co-expressing cells compared to cells only expressing TRPV1 (Fig. 4A and B).

To investigate whether the increased TRPV1 activity is due to a physical interaction between the proteins, TRPV1 and ARMS plasmids were co-transfected into HEK293 cells. Co-immunoprecipitation experiments were performed with an antibody directed against ARMS (Fig. 5A, left panel) or TRPV1 (Fig. 5B, left panel) bound to agarose beads. The precipitate was further analyzed by immunoblotting with antibodies against TRPV1 or ARMS, respectively. ARMS co-precipitated with TRPV1 in this heterologous system. A clear band in the western blot marked the precipitated TRPV1 at about 120 kDa (Fig. 5A, left panel, lane TRPV1/ARMS), suggesting an interaction between tagged full length TRPV1 and full length ARMS. In Fig. 5B the co-immunoprecipitation was performed using anti-TRPV1

with or without control peptide for TRPV1 and the western blot using anti-ARMS antibody. A band for ARMS (at ~ 200 kDa) was only detectable in samples without the control peptide (Fig. 5B, lane 6 and 3, respectively). A similar co-immunoprecipitation experiment was conducted with tissue of DRG neurons suggesting that TRPV1 and ARMS interact in native tissue as well (Fig. 5A and B, right panel).

The calcium imaging experiments revealed that components of PKA might be involved in these processes. For that reason physical interaction analysis of ARMS, TRPV1 and the PKA holoenzyme were carried out. Transfected HEK293 cells expressing ARMS, TRPV1 or ARMS/TRPV1 were lysed and endogenous PKA holoenzyme was co-precipitated with the cAMP derivative, Rp-8-AEA-cAMPS, coupled to agarose beads. Rp-8-AEA-cAMPS is a cAMP-analog that couples regulatory subunits of PKA (de Wit et al., 1984). However, in contrast to cAMP, it prevents the dissociation of the PKA holoenzyme leading to a stable and inactive PKA heterotetramer. This way PKA-holoenzyme associated protein complexes can be precipitated. Elution of precipitated PKA holoenzymes and their associated proteins was carried out by boiling of Rp-cAMPS-agarose beads. SDS-PAGE was applied and proteins were transferred to a nitrocellulose membrane. Afterwards, western blot analysis with antibodies against proteins of interest (ARMS, TRPV1, C – subunit of PKA, pan RI-subunit and RII β of PKA) were used to identify the respective protein-protein interactions. Therefore, two antibodies from different species were used simultaneously.

ARMS (~ 190 kDa, green) and TRPV1 (~ 90 kDa, red) of cells expressing ARMS, TRPV1 and TRPV1/ARMS, respectively, could be detected in the PKA precipitated samples. Therefore, ARMS, TRPV1 and ARMS/TRPV1 were able to bind the PKA holoenzyme, giving support for the co-immunoprecipitation experiments shown in Fig. 5 A and B. Untransfected (nt) cells yielded no fluorescence signal after incubation with anti-ARMS and anti-TRPV1 antibodies, demonstrating the specificity of these antibodies. Input sample of ARMS/TRPV1 co-expressing cells served as expression control for TRPV1 and ARMS. ARMS could not be detected, probably due to a too low concentration in total cell lysate. Purified recombinant proteins of PKA isoforms (RI α , RI β , RII α , RII β /C α) were loaded to the SDS-gel as control for immunodetection of PKA subunits. They were not detected by anti-ARMS and anti-TRPV1 antibodies.

After removal of the anti-TRPV1 and anti-ARMS antibodies, the membrane was probed with antibodies against PKA isoforms (Fig. 5D). Using a pan-RI antibody, RI α (MW of ~50 kDa, green) could be detected in cell lysates including that of untransfected cells. RI β subunits could not be detected using the pan-RI antibody probably due to low expression levels in transfected HEK293 cells. In the input sample, R-subunits could not be detected by pan-RI antibody probably because of the antibody's detection limit. Purified RI α and RI β subunits were detected as well (~ 50 kDa, green) serving as positive control for the specificity of the pan-RI antibody. To demonstrate the functionality of Rp-8-AEA-cAMPS-agarose on PKA holoenzyme, an anti-C-subunit antibody was applied simultaneously. At a molecular weight of a 40 kDa a signal of catalytic subunits in all elutions as well as purified C α could be detected (red). Catalytic subunits were detected in the input sample of ARMS/TRPV1 transfected cells (Fig 5D). After the removal of pan-RI and C-subunit antibodies, membrane was probed with a specific anti-RII β antibody (Fig.S2). RII β was detected in all

elutions (~ 50 kDa, green) and in the positive control. Weak fluorescence signals (green) in some elutions (narrow below 50 kDa) and in RI α and RI β samples most likely result from incomplete removal of anti-pan-RI antibody since anti-pan-RI and anti-RII β were raised in the same species.

To demonstrate the specificity of ARMS, TRPV1 and components of the PKA holoenzyme to the agarose beads, a control hydrolysed ethanolamine agarose was used. Figure 5E shows no detection of ARMS and TRPV1 in the boiled control-agarose. TRPV1 gave fluorescence signal in the input sample (Input-TRPV1, 90 kDa, red), serving as expression control of single transfected cells. In the input-ARMS sample ARMS was below the detection limit of the anti-ARMS-antibody and could not be detected. Its expression was proven in the elution of ARMS transfected cells (Figure 5C, 190 kDa, green). Components of PKA did not stick to the control- agarose beads (Fig. 5F). Therefore, only positive controls of RI β , RI α and Ca could be detected in this experiment. Weak fluorescence signals were present for Ca present in the input samples. RII β also did not stick to control-agarose beads (FigS2 B). Only recombinant RII β serving as specificity control was detected (50 kDa, green).

To relate the expression pattern of TRPV1 and ARMS to neuronal functionality, we combined calcium imaging and immunocytochemistry. After loading cultured mouse DRG neurons with Fura 2-AM, imaged cells were fixed and stained for TRPV1 and ARMS (Fig 6A-H). In neurons expressing TRPV1 and ARMS separately (Fig 6E-G), the change of the Fura ratios after 50 nM capsaicin of the neurons only expressing TRPV1 (Fig. 6H) was lower compared to the neurons co-expressing TRPV1 and ARMS (Fig. 6D). The comparison of the maximum change of the Fura ratio after 50 nM capsaicin between groups showed a strong tendency towards higher intensities in neurons co-expressing TRPV1 and ARMS. However, this difference was not statistically significant (Fig. 6I, Mann Whitney test, $p>0.05$). Fig. 6J presents the change of the Fura ratio over time of all investigated neurons. At 60 s medium was added, at 80 s capsaicin and at 130 s 30 mM KCl was administered to confirm cell viability. Of 458 investigated DRG neurons, less than 10 % expressed only TRPV1, confirming the immunocytochemistry results.

TRPV1 expression in transfected HEK 293 cells

To address the question whether ARMS facilitates the expression of membrane-bound TRPV1 channels, we performed single point radioligand binding experiments in HEK 293 cells expressing TRPV1 alone or TRPV1 and ARMS. To separate plasma membrane-bound TRPV1 channels from TRPV1 expressed in total cell lysates (containing protein from both plasma membranes and organelle membranes), we used a plasma membrane protein extraction kit. The TMF of HEK 293 cells expressing TRPV1 and ARMS showed significantly increased [3 H]-resiniferatoxin (RTX) (1.1 nM) binding compared to cells only expressing TRPV1 (Fig. 7A, Mann Whitney test, *, $p<0.05$, $n=7$). The percentage of PMF-bound TRPV1 relative to the TMF was not different between TRPV1 and TRPV1/ARMS expressing cells (Fig. 7B, Mann Whitney test, $p>0.05$, $n=6$). Repetition of binding experiments without membrane separation showed that TRPV1 expression was significantly increased in the presence of ARMS compared to cells only expressing TRPV1. This effect was reversed by pretreating the cells with myr-PKI, indicating the involvement of PKA (Fig.

7C). However, this experiment does not distinguish between total TRPV1 and plasma membrane TRPV1 expression. We assume that the increased total TRPV1 expression in TRPV1/ARMS co-expressing cells is based on a higher amount of TRPV1 stored in intracellular vesicles.

Co-expression of TRPV1 and ARMS with CGRP, IB4, NF200 and AKAP in DRG neurons

To classify the different subpopulations of TRPV1+/ARMS+ and TRPV+/ARMS-expressing neurons, we co-stained the neurons for different markers. The results are presented in the supporting information section (resultsS1, figureS1 and tableS2).

Discussion

In this study we provide evidence for ARMS expression in mouse DRG neurons at the protein level. Previous studies demonstrated prominent ARMS mRNA expression in the brain and other cells of neuronal origin, while heart tissue displayed lower expression levels (Iglesias et al., 2000). Kong et al. showed ARMS DNA and mRNA expression in adult rat DRG neurons using Northern Blot and *in situ* hybridization (Kong et al., 2001). Other studies showed ARMS mRNA and protein expression in spleen and peripheral blood after airway allergen challenge (Li et al., 2013). A functional role of ARMS was suggested by the investigation of Kidins220 (ARMS) knock-out mice. DRG subpopulations of these animals showed cell loss, proposing ARMS as a general survival factor during sensory neuron development (Cesca et al., 2011). To our knowledge, our study is the first demonstrating ARMS expression in DRG neurons at the protein level, supporting those previous findings at the mRNA level.

Furthermore, our data suggest that ARMS represents a novel interaction partner of TRPV1 and PKA. Our pull down experiments indicate that all three proteins interact physically in a signaling complex regulating TRPV1 sensitization. We detected functional interaction of ARMS and TRPV1 in mouse DRG neurons and in TRPV1/ARMS-expressing HEK 293 cells using immunohistochemistry, electrophysiology, microfluorimetry and a combination of microfluorimetry and immunocytochemistry. Cells expressing both proteins displayed enhanced response amplitudes to capsaicin in patch clamp and calcium imaging experiments compared to cells only expressing TRPV1. Using pull down experiments in transfected HEK 293 cells with Rp-8-AEA-cAMPS-agarose, we found that PKA holoenzyme forms a complex with both TRPV1 and ARMS. Physical interaction of TRPV1 and PKA has already been demonstrated by others (Bhave et al., 2002). We now show that ARMS and PKA form complexes too. To our knowledge this was shown for the first time. While PKA phosphorylation sites of TRPV1 are well known, further studies will have to be performed to identify PKA – ARMS and TRPV1 – ARMS interaction sites.

TRPV1 can be modulated by phosphorylation, transcriptional/translational regulation, interaction with phospholipids and protein-protein interactions. The latter are increasingly recognized, since TRPV1 has been shown to interact with modulatory proteins forming multi-protein signaling complexes with tubulins, the plasma membrane-associated protein Pirt, the scaffolding protein AKAP79/150, the Fas-associated factor, TRPV2, snapin, synaptotagmin IX, and GABAA receptor associated protein. These interactions influence the

expression and activity of functional TRPV1 heteromers at the plasma membrane, the trafficking and insertion of TRPV1, the sensitivity to various stimuli and desensitization (Bhave et al., 2002; Kim et al., 2014; Lukacs et al., 2007; Morenilla-Palao et al., 2004; Spahn et al., 2013; Spahn et al., 2014; Zhang et al., 2005; Zhang et al., 2008). For PKA it was shown that the complex of AKAP/PKA and TRPV1 is necessary for the insertion of functional tetramers into the plasma membrane (Vetter et al., 2008). Expression studies showed that the majority of TRPV1 is cytosolic and needs to be inserted into the plasma membrane (Brandao et al., 2012). A recent study identified that nearly 95 % of TRPV1-positive DRG neurons co-express the regulatory subunit of the PKA, RII β , another member of the TRPV1/AKAP signalosome (Isensee et al., 2014a). The authors suggest that RII β is a response predictor for capsaicin sensitivity and that the classical separation of DRG neurons into subpopulations based on morphological neuronal markers is insufficient. By demonstrating physical and functional interactions of TRPV1, ARMS and PKA, our data suggest that all three proteins are part of a signaling complex sensitizing TRPV1 towards capsaicin stimulation. Thus, future classifications of nociceptive neurons should take into account that excitatory ion channels such as TRPV1 are modulated by signalosomes consisting of proteins such as PKA and/or ARMS.

Our calcium imaging and patch clamp experiments using the specific PKA inhibitor myr-PKI and the pull down experiments with Rp-8-AEA-cAMPS-agarose indicate that the cAMP/PKA pathway may play a role in the ARMS-mediated TRPV1 sensitization. Furthermore, in radioligand binding studies ARMS co-expression with TRPV1 increased the percentage of ^3H -RTX-bound TRPV1 compared to cells only expressing TRPV1. This effect was abolished using the PKA inhibitor myr-PKI. Although the plasma membrane fraction of TRPV1/ARMS expressing cells was not increased compared cells only expressing TRPV1, we assume that the higher total TRPV1 expression is based on enhanced TRPV1 in intracellular vesicles. These stores can be rapidly inserted into the membrane leading to increased TRPV1 activity in calcium imaging and patch clamp experiments. This notion is supported by findings that PKA mediates the insertion of functional TRPV1 into the plasma membrane. Moreover, it was shown that phosphorylation events at TRPV1 sensitized TRPV1 to capsaicin (Jeske et al., 2008; Vetter et al., 2008). Therefore, it is necessary to investigate phosphorylation events on TRPV1, but also on ARMS in future studies.

To our knowledge, a scaffolding effect of a protein regulating the activity of TRPV1 was only shown for AKAP79/150 and β -arrestin-2 so far. It was shown that AKAP 150 can arrange TRPV1, adenylyl cyclases, PKA and PKC to form a signalosome important for TRPV1 sensitization (Btesh et al., 2013; Efendiev et al., 2013; Jeske et al., 2008; Jeske et al., 2009; Rathee et al., 2002; Schnizler et al., 2008). On the other hand, TRPV1 and β -arrestin-2 association was shown to regulate TRPV1 desensitization (Por et al., 2012; Por et al., 2013; Rowan et al., 2014).

Interestingly, we found that about 20 % of TRPV1/ARMS-expressing DRG neurons co-express AKAP 150 (resultsS1 and tableS2). In line with a recent study, we found co-expression of TRPV1 and AKAP 150 predominantly in small sized neurons (Brandao et al., 2012). However, the co-expression of TRPV1 and ARMS is not restricted to one population

of nociceptors (either peptidergic, non-peptidergic or myelinated), since our co-expression studies using the neuronal markers CGRP, IB4 and NF200 showed expression of both proteins in all types of neurons (Fig. S1, table S2).

Numerous interaction partners were identified for ARMS in the last decade. A role of ARMS in the formation of receptor complexes was shown for the Trk receptor, and functional interactions with glutamate, Eph, VEGF receptors (VEGFRs), P-loop nucleotide phosphatases and ATP were identified. Due to the multiple binding domains of ARMS, this protein is a suitable candidate as a signaling platform (reviewed in (Neubrand et al., 2012)). ARMS regulates cytoskeletal remodelling, neuronal differentiation, neurotrophin signaling and interacts with cytoskeletal components such as tubulin (Arevalo et al., 2004; Higuero et al., 2010). A very recent study identified ARMS as an interaction partner of brain voltage-gated sodium channels, in which ARMS was able to modulate the activity of sodium channels. This study, in addition to ours, also underlines the ability of ARMS to act as signaling platform resulting in the modulation of ion channel activity (Cesca et al., 2015).

In conclusion, our study showed co-expression of ARMS and TRPV1 protein in mouse DRG neurons. Furthermore, we identified a functional interaction of TRPV1 and ARMS, resulting in a PKA-dependent sensitization of TRPV1 to capsaicin. This emphasizes the role of signaling complex formation in the context of pain sensitization. With regard to the development of new analgesics, it might be necessary to investigate not only single proteins important for pain generation, transmission and sensitization, but also whole signaling complexes including several interaction partners.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Arevalo JC, Yano H, Teng KK, Chao MV. A unique pathway for sustained neurotrophin signaling through an ankyrin-rich membrane-spanning protein. *EMBO J.* 2004; 23:2358–2368. [PubMed: 15167895]
- Beene DL, Scott JD. A-kinase anchoring proteins take shape. *Curr Opin Cell Biol.* 2007; 19:192–198. [PubMed: 17317140]
- Bessac BF, Sivula M, von Hehn CA, Caceres AI, Escalera J, Jordt SE. Transient receptor potential ankyrin 1 antagonists block the noxious effects of toxic industrial isocyanates and tear gases. *FASEB J.* 2009; 23:1102–1114. [PubMed: 19036859]
- Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RWt. cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron.* 2002; 35:721–731. [PubMed: 12194871]

- Brandao KE, Dell'Acqua ML, Levinson SR. A-kinase anchoring protein 150 expression in a specific subset of TRPV1- and CaV 1.2-positive nociceptive rat dorsal root ganglion neurons. *J Comp Neurol.* 2012; 520:81–99. [PubMed: 21674494]
- Btesh J, Fischer MJ, Stott K, McNaughton PA. Mapping the binding site of TRPV1 on AKAP79: implications for inflammatory hyperalgesia. *J Neurosci.* 2013; 33:9184–9193. [PubMed: 23699529]
- Busch-Dienstfertig M, Labuz D, Wolfram T, Vogel NN, Stein C. JAK-STAT1/3-induced expression of signal sequence-encoding proopiomelanocortin mRNA in lymphocytes reduces inflammatory pain in rats. *Mol Pain.* 2012; 8:83. [PubMed: 23146666]
- Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeit KR, Koltzenburg M, Basbaum AI, Julius D. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science.* 2000; 288:306–313. [PubMed: 10764638]
- Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature.* 1997; 389:816–824. [PubMed: 9349813]
- Cesca F, Satapathy A, Ferrea E, Nieus T, Benfenati F, Scholz-Starke J. Functional Interaction between the Scaffold Protein Kidins220/ARMS and Neuronal Voltage-Gated Na⁺ Channels. *J Biol Chem.* 2015; 290:18045–18055. [PubMed: 26037926]
- Cesca F, Yabe A, Spencer-Dene B, Arrigoni A, Al-Qatari M, Henderson D, Phillips H, Koltzenburg M, Benfenati F, Schiavo G. Kidins220/ARMS is an essential modulator of cardiovascular and nervous system development. *Cell Death Dis.* 2011; 2:e226. [PubMed: 22048169]
- de Wit RJ, Hekstra D, Jastorff B, Stec WJ, Baraniak J, Van Driel R, Van Haastert PJ. Inhibitory action of certain cyclophosphate derivatives of cAMP on cAMP-dependent protein kinases. *Eur J Biochem.* 1984; 142:255–260. [PubMed: 6086345]
- Distler C, Rathee PK, Lips KS, Obreja O, Neuhuber W, Kress M. Fast Ca²⁺-induced potentiation of heat-activated ionic currents requires cAMP/PKA signaling and functional AKAP anchoring. *J Neurophysiol.* 2003; 89:2499–2505. [PubMed: 12740405]
- Efendiev R, Bavencoffe A, Hu H, Zhu MX, Dessauer CW. Scaffolding by A-kinase anchoring protein enhances functional coupling between adenylyl cyclase and TRPV1 channel. *J Biol Chem.* 2013; 288:3929–3937. [PubMed: 23264624]
- Endres-Becker J, Heppenstall PA, Mousa SA, Labuz D, Oksche A, Schafer M, Stein C, Zollner C. Mu-opioid receptor activation modulates transient receptor potential vanilloid 1 (TRPV1) currents in sensory neurons in a model of inflammatory pain. *Mol Pharmacol.* 2007; 71:12–18. [PubMed: 17005903]
- Hanack C, Moroni M, Lima WC, Wende H, Kirchner M, Adelfinger L, Schrenk-Siemens K, Tappe-Theodor A, Wetzel C, Kuich PH, et al. GABA blocks pathological but not acute TRPV1 pain signals. *Cell.* 2015; 160:759–770. [PubMed: 25679765]
- Hanke SE, Bertinetti D, Badel A, Schweinsberg S, Genieser HG, Herberg FW. Cyclic nucleotides as affinity tools: phosphorothioate cAMP analogues address specific PKA subproteomes. *N Biotechnol.* 2011; 28:294–301. [PubMed: 21147280]
- Higuero AM, Sanchez-Ruiloba L, Doglio LE, Portillo F, Abad-Rodriguez J, Dotti CG, Iglesias T. Kidins220/ARMS modulates the activity of microtubule-regulating proteins and controls neuronal polarity and development. *J Biol Chem.* 2010; 285:1343–1357. [PubMed: 19903810]
- Iglesias T, Cabrera-Poch N, Mitchell MP, Naven TJ, Rozengurt E, Schiavo G. Identification and cloning of Kidins220, a novel neuronal substrate of protein kinase D. *J Biol Chem.* 2000; 275:40048–40056. [PubMed: 10998417]
- Isensee J, Diskar M, Waldherr S, Buschow R, Hasenauer J, Prinz A, Allgower F, Herberg FW, Hucho T. Pain modulators regulate the dynamics of PKA-RII phosphorylation in subgroups of sensory neurons. *J Cell Sci.* 2014a; 127:216–229. [PubMed: 24190886]
- Isensee J, Wenzel C, Buschow R, Weissmann R, Kuss AW, Hucho T. Subgroup-elimination transcriptomics identifies signaling proteins that define subclasses of TRPV1-positive neurons and a novel paracrine circuit. *PLoS One.* 2014b; 9:e115731. [PubMed: 25551770]
- Jeske NA, Diogenes A, Ruparel NB, Fehrenbacher JC, Henry M, Akopian AN, Hargreaves KM. A-kinase anchoring protein mediates TRPV1 thermal hyperalgesia through PKA phosphorylation of TRPV1. *Pain.* 2008; 138:604–616. [PubMed: 18381233]

- Jeske NA, Patwardhan AM, Ruparel NB, Akopian AN, Shapiro MS, Henry MA. A-kinase anchoring protein 150 controls protein kinase C-mediated phosphorylation and sensitization of TRPV1. *Pain*. 2009; 146:301–307. [PubMed: 19767149]
- Kim YS, Chu Y, Han L, Li M, Li Z, Lavinka PC, Sun S, Tang Z, Park K, Caterina MJ, et al. Central terminal sensitization of TRPV1 by descending serotonergic facilitation modulates chronic pain. *Neuron*. 2014; 81:873–887. [PubMed: 24462040]
- Kong H, Boulter J, Weber JL, Lai C, Chao MV. An evolutionarily conserved transmembrane protein that is a novel downstream target of neurotrophin and ephrin receptors. *J Neurosci*. 2001; 21:176–185. [PubMed: 11150334]
- Li N, Dong X, Yang C, Liu Y, Ni X. Expression of neuronal protein Kidins220/ARMS in the spleen and peripheral blood of mice following airway allergen challenge. *Mol Med Rep*. 2013; 8:1871–1875. [PubMed: 24126542]
- Lukacs V, Thyagarajan B, Varnai P, Balla A, Balla T, Rohacs T. Dual regulation of TRPV1 by phosphoinositides. *J Neurosci*. 2007; 27:7070–7080. [PubMed: 17596456]
- Morenilla-Palao C, Planells-Cases R, Garcia-Sanz N, Ferrer-Montiel A. Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. *J Biol Chem*. 2004; 279:25665–25672. [PubMed: 15066994]
- Neubrand VE, Cesca F, Benfenati F, Schiavo G. Kidins220/ARMS as a functional mediator of multiple receptor signalling pathways. *J Cell Sci*. 2012; 125:1845–1854. [PubMed: 22562556]
- Por ED, Bierbower SM, Berg KA, Gomez R, Akopian AN, Wetsel WC, Jeske NA. beta-Arrestin-2 desensitizes the transient receptor potential vanilloid 1 (TRPV1) channel. *J Biol Chem*. 2012; 287:37552–37563. [PubMed: 22952227]
- Por ED, Gomez R, Akopian AN, Jeske NA. Phosphorylation regulates TRPV1 association with beta-arrestin-2. *Biochem J*. 2013; 451:101–109. [PubMed: 23360390]
- Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang SY, Nau C, Kress M. PKA/AKAP/VR-1 module: A common link of Gs-mediated signaling to thermal hyperalgesia. *J Neurosci*. 2002; 22:4740–4745. [PubMed: 12040081]
- Rowan MP, Bierbower SM, Eskander MA, Szteyn K, Por ED, Gomez R, Veldhuis N, Bunnett NW, Jeske NA. Activation of mu opioid receptors sensitizes transient receptor potential vanilloid type 1 (TRPV1) via beta-arrestin-2-mediated cross-talk. *PLoS One*. 2014; 9:e93688. [PubMed: 24695785]
- Schnizler K, Shutov LP, Van Kanegan MJ, Merrill MA, Nichols B, McKnight GS, Strack S, Hell JW, Usachev YM. Protein kinase A anchoring via AKAP150 is essential for TRPV1 modulation by forskolin and prostaglandin E2 in mouse sensory neurons. *J Neurosci*. 2008; 28:4904–4917. [PubMed: 18463244]
- Spahn V, Fischer O, Endres-Becker J, Schafer M, Stein C, Zollner C. Opioid withdrawal increases transient receptor potential vanilloid 1 activity in a protein kinase A-dependent manner. *Pain*. 2013; 154:598–608. [PubMed: 23398938]
- Spahn V, Stein C, Zollner C. Modulation of transient receptor vanilloid 1 activity by transient receptor potential ankyrin 1. *Mol Pharmacol*. 2014; 85:335–344. [PubMed: 24275229]
- Szallasi A, Blumberg PM, Annicelli LL, Krause JE, Cortright DN. The cloned rat vanilloid receptor VR1 mediates both R-type binding and C-type calcium response in dorsal root ganglion neurons. *Mol Pharmacol*. 1999; 56:581–587.
- Szallasi A, Lewin NE, Blumberg PM. Identification of alpha-1-acid glycoprotein (orosomucoid) as a major vanilloid binding protein in serum. *J Pharmacol Exp Ther*. 1992; 262:883–888. [PubMed: 1527730]
- Tominaga M, Tominaga T. Structure and function of TRPV1. *Pflügers Arch*. 2005; 451:143–150. [PubMed: 15971082]
- Vetter I, Cheng W, Peiris M, Wyse BD, Roberts-Thomson SJ, Zheng J, Monteith GR, Cabot PJ. Rapid, opioid-sensitive mechanisms involved in transient receptor potential vanilloid 1 sensitization. *J Biol Chem*. 2008; 283:19540–19550. [PubMed: 18482991]
- Zhang X, Huang J, McNaughton PA. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO J*. 2005; 24:4211–4223. [PubMed: 16319926]

Zhang X, Li L, McNaughton PA. Proinflammatory mediators modulate the heat-activated ion channel TRPV1 via the scaffolding protein AKAP79/150. *Neuron*. 2008; 59:450–461. [PubMed: 18701070]

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What does the study add?

The study identifies ARMS as an important component of the signaling complex regulating the sensitivity of excitatory ion channels (TRPV1) in peripheral sensory neurons (DRG neurons) and transfected cells.

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Figure 1. Expression patterns of TRPV1 and ARMS in mouse DRG neurons

A) Representative immunohistochemical staining of TRPV1 prepared for confocal microscopy. B) Representative immunohistochemical staining of ARMS prepared for confocal microscopy. C) Merged images of TRPV1 (blue) and ARMS (red) immunoreactivity. Asterisks indicate co-expression of TRPV1 and ARMS. Arrows label exemplary TRPV1-positive and ARMS-negative neurons. Arrowheads indicate exemplary neurons positive for ARMS and negative for TRPV1. D) Size distribution histogram of ARMS-positive, TRPV1-positive and all DRG neurons. E) Size distribution histogram of all TRPV1-positive neurons versus neurons co-expressing TRPV1 and ARMS and neurons only positive for TRPV1. F) Venn-diagram of TRPV1 positive/ARMS negative, TRPV1 and ARMS positive, TRPV1 negative/ARMS positive and TRPV1 and ARMS negative neurons.

Figure 2. Capsaicin-induced calcium influx in TRPV1- and TRPV1/ARMS-expressing HEK 293 cells

A) Mean change of the fura ratio ($\pm$ SEM, dashed lines) after 1 nM (20 s) and 3 μ M (80 s) capsaicin of TRPV1 (black line) and TRPV1/ARMS (red line) expressing cells. B-D) Representative images of the capsaicin-induced (0 nM (B), 1 nM (C) and 3 μ M (D)) change of the fluorescence intensity of cells only expressing TRPV1. E-F) Representative images of the capsaicin-induced (0 nM (E), 1 nM (F) and 3 μ M (G)) change of the fluorescence intensity of cells co-expressing TRPV1 and ARMS. H) Concentration-response curves of capsaicin-induced calcium influx normalized to the maximum induced response at 3 μ M capsaicin of TRPV1- (black line) and TRPV1/ARMS- (red line) expressing cells. Curves are fitted according to the Hill equation. Data are shown as means + S.E.M. (TRPV1 represented by the black curve; n = 74 (1 pM), n = 34 (100 pM), n = 26 (500 pM), n = 41 (1 nM), n = 46 (20 nM), n = 45 (100 nM), n = 62 (200 nM), n = 85 (1 μ M), n = 44 (3 μ M) or TRPV1/ARMS represented by the red curve; n = 38 (1 pM), n = 59 (100 pM), n = 69 (500 pM), n = 57 (1 nM), n = 141 (20 nM), n = 64 (100 nM), n = 80 (200 nM), n = 46 (1 μ M), n = 54 (3 μ M)). I) EC₅₀ values ($\pm$ 95% CI) generated from capsaicin-induced dose response curves of HEK 293 cells expressing TRPV1 alone (black bar) or TRPV1 and ARMS (empty bar) without pre-treatment or with 20 min H89 pre-treatment (unpaired t-test, **, p<0.01).

Figure 3. Capsaicin-induced TRPV1 currents in HEK 293 cells expressing TRPV1 and ARMS or only TRPV1

A) Averaged capsaicin-induced (50 nM for 10 s) TRPV1 current traces ($\pm$ SEM, dashed lines) of cells only expressing TRPV1 (black line) and cell co-expressing TRPV1 and ARMS (red line). B) Mean amplitudes of the capsaicin-induced current of TRPV1- (black bar) and TRPV1/ARMS- (red bar) expressing cells (unpaired t-test, *, $p < 0.05$). C) Repetitive capsaicin-induced current traces (0 s, 60 s, 120 s, 210 s) of TRPV1 expressing cells. D) Repetitive capsaicin-induced current traces (0 s, 60 s, 120 s, 210 s) of TRPV1 and ARMS expressing cells. E) Capsaicin-induced TRPV1 currents normalized to the first pulse (in % $\pm$ SEM) of TRPV1- (black bars, $n=25$) and TRPV1/ARMS- (red bars, $n=20$) expressing cells (2way ANOVA with Bonferroni's post test, $p > 0.05$).

Figure 4. Capsaicin-induced calcium influx and TRPV1 currents in TRPV1- and TRPV1/ARMS-expressing HEK 293 cells pretreated with protein kinase inhibitors

A) Maximum change of the fluorescence intensity induced by 1 nM capsaicin in HEK cells expressing TRPV1 or TRPV1/ARMS without pretreatment, with 10 μ M H89-, 1 μ M GF 109203X- or 10 μ M myr-PKI-pretreatment (2way ANOVA with Bonferroni's post test, **, $p < 0.01$; untreated: TRPV1 $n = 39$, TRPV1/ARMS $n = 56$; H89: TRPV1 $n = 127$, TRPV1/ARMS $n = 42$; GF 109203X: TRPV1 $n = 58$, TRPV1/ARMS $n = 114$; myr-PKI: TRPV1 $n = 81$, TRPV1/ARMS $n = 106$). B) Average capsaicin-induced (50 nM for 10 s) TRPV1 currents of HEK 293 cells expressing TRPV1 and TRPV1/ARMS either untreated or pretreated with 10 μ M myr-PKI (1way ANOVA with Bonferroni's post test, *, $p < 0.05$; untreated: TRPV1 $n = 12$, TRPV1/ARMS $n = 12$; myr-PKI: TRPV1 $n = 6$, TRPV1/ARMS $n = 7$).

Figure 5. Pull down experiments of TRPV1 and ARMS in transfected HEK 293 cells and native neuronal tissue

A) Co-immunoprecipitation studies were performed in a heterologous (HEK293 cells, left panel) and native (DRG neurons, right panel) expression system. ARMS was precipitated using an antibody directed against ARMS followed by a subsequent detection of TRPV1 using a TRPV1 antibody (solid arrows). BSA-coated agarose beads served as a control. B) Co-immunoprecipitation studies were performed in a heterologous (HEK293 cells, left panel) and native (DRG neurons, right panel) expression system. TRPV1 was precipitated using an antibody directed against TRPV1 followed by a subsequent detection of ARMS using an ARMS antibody (open arrows). BSA-coated agarose beads and control peptide against TRPV1 served as a control. C) Pull-down experiments with Rp-8-AEA-cAMPS-agarose to pull down PKA holoenzyme of TRPV1 and ARMS expressing HEK 293 cells and control proteins of PKA isoforms. Afterwards SDS-PAGE analysis was applied with antibodies directed against TRPV1 (red at 90 kDa) and ARMS (green at 190 kDa). D) Membrane of C was stripped to remove the first set of antibodies and incubated with antibodies against PKA subunits RI (green at 50 kDa) and C (red at 40 kDa). Both samples of transfected cells and control protein samples showed positive fluorescent signals RI and C-subunit of PKA. E) Pull down experiments with control agarose (PKA cannot bind to the coupled beads) of transfected HEK 293 cells and control proteins for PKA isoforms.

Afterwards SDS-PAGE was applied and the membrane was treated with antibodies against TRPV1 (red at 90 kDa) and ARMS (green at 190 kDa). F) Membrane of E was stripped to remove the first set of antibodies and incubated with antibodies against PKA subunits RI (green at 50 kDa) and C (red at 40 kDa). Only control protein samples showed fluorescent signals. (B = beads, FT = flow through, I = input IP = immunoprecipitation, nt = not transfected)

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Figure 6. Results of combined calcium imaging and immunocytochemistry experiments of DRG neurons

A-D). A neuron immunoreactive to TRPV1 (A) and ARMS (B). Merge (purple) of A) and B). D) Capsaicin-induced (50 nM) change of the fluorescence intensity of the same neuron. E-H). Neurons immunoreactive either to TRPV1 (E) or ARMS (F). Merged image (blue=TRPV1, red=ARMS) of E) and F). Capsaicin-induced (50 nM) change of the fluorescence intensity of the same neurons. I) Maximum change of the fura ratio after 50 nM capsaicin of neurons only positive for TRPV1 (black bar) and neurons positive for TRPV1 and ARMS (white bar) (Mann Whitney test, $p>0.05$). J) Change of the fura ratio over time after perfusion with medium (60 s), 50 nM capsaicin (80 s) and 30 mM potassium chloride (KCl) (130 s) of TRPV1+/ARMS- (black) and TRPV1+/ARMS+ (red) neurons.

Figure 7. TRPV1 expression in total membrane (TMF) and plasma membrane fraction (PMF) of transfected HEK 293 cells

A) [³H]-RTX- (1.1 nM) bound protein (in % total TRPV1) in total membrane fraction (TMF) of TRPV1- and TRPV1/ARMS-expressing HEK 293 cells (paired t-test, *, $p < 0.05$, $n = 7$). B) [³H]-RTX- (1.1 nM) bound protein (in % of TMF) in the plasma membrane fraction (PMF) of cells expressing TRPV1 and TRPV1/ARMS (unpaired t-test, $p > 0.05$, $n = 6$). [³H]-RTX- (1.1 nM) bound protein (in cpm) of transfected HEK 293 cells untreated and pretreated with myr-PKI (1way ANOVA with Bonferroni's post test, *, $p < 0.05$). (cpm = counts per minute)