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Title page

Modulation of Transient Receptor Vanilloid 1 Activity by Transient Receptor Potential Ankyrin 1

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TRPA1 modulation of TRPV1

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Abbreviations: AC, adenylyl cyclase; CA, cinnamaldehyde; cAMP, cyclic adenosine monophosphate; Caps, capsaicin; CIB, calcium imaging buffer; Ctrl, control group; DRG, dorsal root ganglion; ECS, extracellular solution; FBS, fetal bovine serum; H89, N-[2-(p-Bromocinnamylamino)ethyl]- 5-isoquinolinesulfonamide·2HCl; HC-030031**,** 1,2,3,6-Tetrahydro-1,3-dimethyl-N-[4-(1-methylethyl)phenyl]-2,6-dioxo-7H-purine-7-acetamide, 2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide; HEK 293 cells, human embryonic kidney 293 cells; MEM, Minimum Essential Media; MO, mustard oil; PKA, protein kinase A; RTX, resiniferatoxin; SQ 22.536**,** 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine; TRPA, transient receptor potential ankyrin; TRPV1, transient receptor potential vanilloid 1; wt, wild type; YFP, yellow fluorescent protein

Abstract

Transient Receptor Potential Vanilloid 1 (TRPV1) is a nonselective ligand-gated cation channel responding to noxious heat, protons, and chemicals like capsaicin. TRPV1 is expressed in sensory neurons and plays a critical role in pain associated with tissue injury, inflammation or nerve lesions. Transient Receptor Potential Ankyrin 1 (TRPA1) is co-expressed with TRPV1. It is activated by compounds that cause a burning sensation (e.g. mustard oil) and, indirectly, by components of the inflammatory milieu eliciting nociceptor excitation and pain hypersensitivity. Previous studies indicate an interaction of TRPV1 and TRPA1 signaling pathways. Here we sought to examine the molecular mechanisms underlying such interactions in nociceptive neurons. We first excluded physical interactions of both channels using radioligand binding studies. By microfluorimetry, electrophysiological experiments, cAMP measurements, and site-directed mutagenesis we found a sensitization of TRPV1 after TRPA1 stimulation with mustard oil in a calcium- and cAMP/PKA-dependent manner. TRPA1 stimulation enhanced TRPV1 phosphorylation *via* the putative PKA phosphorylation site serine 116. We also detected calcium-sensitive increased TRPV1 activity after TRPA1 activation in dorsal root ganglion (DRG) neurons. The inhibition of TRPA1 by HC-030031 after its initial stimulation and the calcium-insensitive TRPA1 mutant D477A still showed increased capsaicin-induced TRPV1 activity excluding an additive TRPA1 current after TRPV1 stimulation. Our study shows sensitization of TRPV1 *via* activation of TRPA1, which involves adenylyl cyclase, increased cAMP, subsequent translocation and activation of PKA, and phosphorylation of TRPV1 at PKA phosphorylation residues. This suggests that cross-sensitization of TRP channels contributes to enhanced pain sensitivity in inflamed tissues.

Introduction

The transduction and transmission of painful stimuli is initiated by activation of nociceptive dorsal root ganglion (DRG) neurons, which convey information to the central nervous system, resulting in the sensation of pain (Tominaga, 2007). The transient receptor potential (TRP) ion channel family plays essential roles in such responses (Venkatachalam and Montell, 2007). Important and extensively investigated are TRP vanilloid 1 (TRPV1) and TRP ankyrin 1 (TRPA1). Both are nonselective cation channels sharing the common feature of six transmembrane domains and intracellular N- and C-termini. One functional channel is formed by four monomers (Venkatachalam and Montell, 2007). Both channels play crucial roles in neurogenic inflammation (reviewed in (Geppetti et al., 2008)) and in the development of hyperalgesia (Bautista et al., 2006; Davis et al., 2000; Obata et al., 2005; Petrus et al., 2007; Spahn et al., 2013; Tominaga et al., 1998). Additionally, it was shown that 97 % of TRPA1-positive sensory neurons also express TRPV1, and that 30 % of TRPV1-positive neurons co-express TRPA1 (Story et al., 2003). Since previous studies indicated modulating effects of TRPA1 stimulation on TRPV1 activity (Akopian et al., 2008; Anand et al., 2008; Jeske et al., 2006), we sought to investigate the underlying mechanisms at the molecular level.

TRPV1 expressing DRG neurons respond to temperatures above 43°C, protons and chemical stimuli such as capsaicin (Caterina et al., 2000; Caterina et al., 1997). This channel features several putative phosphorylation sites that can be modulated by the cyclic adenosine monophosphate (cAMP)/protein kinase A pathway (reviewed in (Tominaga and Tominaga, 2005)). Important sites are serine 6, serine 116, threonine 144, threonine 370, serine 502, serine 774 and serine 820 (Bhave et al., 2002; Mohapatra and Nau, 2003).

TRPA1 is the sole vertebrate member of the TRPA family (Tai et al., 2008; Venkatachalam and Montell, 2007). Initially TRPA1 was proposed to be the sensor of noxious cold, albeit TRPA1 knock-out mice yielded controversial results (Bandell et al., 2004; Bautista et al., 2006; Kwan et al., 2006; Story et al., 2003). This channel is also activated by the chemicals

allyl isothiocyanate (an ingredient of mustard, horseradish and wasabi), allicin, diallyldisulfide (in garlic), cinnamaldehyde (in cinnamon), and acrolein (in tear gas) through covalent modifications of cysteine residues. *In vivo*, the topical application of mustard oil (MO) to the skin produces pain, inflammation and hypersensitivity to thermal and mechanical stimulation (Jordt et al., 2004). Additionally, TRPA1 can be activated by cannabinoids and intracellular calcium, which modulates the channel via a partial EF-hand domain at the N-terminal (Doerner et al., 2007; Jordt et al., 2004; Zurborg et al., 2007).

Similar to the TRPV subfamily, TRPA1 features a tetrameric arrangement with a dense transmembrane region and a basket-like cytoplasmic domain structure. However, the intracellular termini differ from other TRP channels in that the N-terminus consists of numerous ankyrin repeats (Bessac and Jordt, 2008; Cvetkov et al., 2011; Premkumar and Abooj, 2012).

In the current study we sought to investigate the molecular mechanisms underlying the effects of TRPA1 activation on TRPV1 since this interaction is of great interest for the development of novel pain therapies in the context of inflammation. To this end, we used microfluorimetry, electrophysiology, radioligand binding studies and site-directed mutagenesis. We hypothesized that MO-induced calcium influx through TRPA1 results in the activation of calcium sensitive adenylyl cyclases (ACs) which produce intracellular cAMP. The latter might stimulate PKA to phosphorylate and sensitize TRPV1.

Materials and Methods

Cell culture

Experiments were performed in HEK 293 Tet-On cells because they do not constitutively express TRPV1 or TRPA1. The HEK 293 Tet-On® Advanced cell line stably expresses a reverse tetracycline-controlled transactivator protein, providing the ability of high expression levels after addition of doxycycline to the culture medium. This way, the known cell toxicity due to constitutive expression of TRPA1 is prevented (Story et al., 2003).

HEK 293 Tet-On cells were maintained in MEM alpha medium supplemended with 10 % Tet system approved FBS, 1 % penicillin/streptomycin, 4 mM L-glutamine and 200 µg/ ml geneticin (G418) at 37° C and 5 % CO_2 in a cell incubator. They were passaged 1:3 - 1:10 every second to third day depending on the confluence.

DRG were prepared as described previously (Bolyard et al., 2000). Briefly, thoracic and lumbar DRG of male Wistar rats were removed and placed in sterile MEM at 4°C. DRG were digested with collagenase type 2 in MEM at 37°C for 50 min and 0.025% trypsin for 10 min at 37°C. After digestion, DRG were carefully dissociated by mechanical agitation, centrifuged at 500 g for 5 min and then at 300 g for 5 min. The cells were maintained in MEM (Biochrom AG, Berlin, Germany) growth media supplemented with 10% horse serum, 50 µg/ml penicillin and streptomycin, and plated in 35-mm polylysine coated culture dishes at 37°C in an atmosphere of 5% CO_2 (Spahn et al., 2013).

Site directed mutagenesis and heterologous expression

Mutagenesis of several PKA phosphorylation sites of TRPV1 cDNA was realized with TRPV1-pcDNA3.1 using a site-directed mutagenesis kit (Stratagene, La Jolla, USA) as described previously (Mohapatra et al., 2003). All constructs were confirmed by DNA sequencing by a commercial provider (LCG Genomics, Berlin, Germany) (Spahn et al., 2013). The TRPA1 mutant D477A was kindly provided by S. Zurborg and P. Heppenstall.

Heterologous expression of wild type (wt) or mutant plasmids of TRPV1 (Rattus norvegicus) (0.5 μg) and wild type or mutant TRPA1 (Homo sapiens) (0.5 μg) was achieved using Fugene 6 or X-tremeGENE DNA transfection reagent (Roche Diagnostics, Indianapolis, IN 46250). Gene expression was induced after addition of 1μg/ml doxycycline and experiments were performed 24 h after transfection. Transfection-positive cells were identified by YFP tagged TRPV1 receptors. All cells responding to MO also responded to capsaicin, indicating co-expression of TRPA1 and TRPV1.

Treatments

TRPV1 and TRPA1 co-expressing HEK 293 Tet-On cells or DRG neurons were preincubated with MO (20 μM) or cinnamaldehyde (CA; 100 μM) for 2 min followed by a 1 min washout with buffer (MO; CA in Fig. 1) unless otherwise noted. Thereafter, cells were stimulated with capsaicin. Control cells were treated with the corresponding buffer for 3 min, followed by capsaicin (control group, Ctrl). To examine whether the capsaicin-induced current is a result of an additive TRPA1 current, cells were perfused with the TRPA1 inhibitor HC-030031 (25 μM) (Sigma-Aldrich, Steinheim, Germany) for 1 min immediately after the MO-induced TRPA1 current. Thereafter, they were stimulated with capsaicin in the presence of HC-030031. In these experiments control cells were pretreated with buffer for 2 min, afterwards with buffer containing HC-030031 (25 μM) for 1 min.

To completely block TRPA1 activity *a priori*, HC-030031 was applied simultaneously with MO for 2 min followed by a 1 min wash out and capsaicin stimulation. Control cells were treated with buffer for 3 min followed by capsaicin application.

To simulate calcium-free conditions in patch clamp experiments, cells were washed and incubated with Ca^{2+}-free extracellular solution (ECS) complemented with EGTA (100 μM) (ECS Ca^{2+}-free) before the recording of currents. To inhibit PKA, cells were incubated with H89 (Sigma-Aldrich, Steinheim, Germany) (10 μM) for at least 6 h prior to the experiment.

The activity of AC was inhibited by the addition of 100 µM SQ 22,536 (Sigma-Aldrich, Steinheim, Germany) for 10 min.

Patch clamp recordings

Whole-cell voltage clamp recordings were performed in HEK 293 Tet-On cells and dissociated small-sized DRG neurons at –60 mV holding potential with an EPC-10 patch clamp amplifier and PULSE software (HEKA Elektronik, Lambrecht, Germany). Borosilicate glass electrodes (Hilgenberg, Malsfeld, Germany), pulled on a horizontal puller (Sutter Instrument Company, Novato, USA), had resistances of 2-5 MΩ after filling with 140 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES and 5 mM EGTA, with the pH adjusted to 7.4 with KOH according to (Mohapatra and Nau, 2003; Mohapatra and Nau, 2005; Spahn et al., 2013). The ECS consisted of 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 2 mM $CaCl_2$ and 10 mM HEPES at pH 7.4 adjusted by NaOH. In experiments using DRG neurons 10 µM forskolin and 2 mM isobutymethylxanthin were added to the solution. Recordings were performed at room temperature. Only small-sized sensory neurons (cell diameter ≤ 26µm) sensitive to capsaicin (1µM or 100 nM) and MO (20 µM) were included in the study. These cells were considered responsive if the inward current was at least 100 pA. Solutions were applied with a polytetrafluoroethylene glass multiple-barrel perfusion system. The PULSE software (HEKA Elektronik, Lambrecht, Germany) was used for data acquisition and offline analysis.

Ratiometric $[Ca^{2+}]_i$ measurements

Transiently transfected cells were loaded with 3µM of the fluorescent calcium indicator dye Fura-2-AM supplied with 0.02% pluronic F-127 (Invitrogen, Carlsbad, USA) for 20 min. Afterwards, cells were washed with calcium imaging buffer (CIB) (140 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 10 mM glucose, 20 mM HEPES; adjusted to pH 7.4) to remove

extracellular Fura-2-AM. 20 µM MO was added to the culture plates with cover slips. After 2 min the cover slips were transferred to the measurement chamber and cells were covered with CIB (wash out). 1 min later the recording started and capsaicin was added. Every 2 s images were acquired using a CCD camera with a monochromator (Till Photonics) coupled to an Eclipse TE 2000-s microscope (Nikon, Düsseldorf, Germany). Fura-2-AM was excited at 340 nm and 380 nm with 100 ms exposure time at 2 Hz. Fluorescence at more than 420 nm was collected. Background for both wavelength intensities was continuously recorded and subtracted. Results were reported as a ratio. Cells were continuously superfused with CIB. Experiments were performed at room temperature and data were stored for offline analysis as described (Spahn et al., 2013).

TRPV1 expression

Binding of the labeled TRPV1 agonist [^{3}H] resiniferatoxin (RTX) was examined according to Szallasi et. al. (Szallasi et al., 1999). Briefly, appropriate concentrations of membranes from cells transfected with TRPV1 and TRPA1, or with TRPV1 and the empty vector Ptre2 (100 µg) were prepared and incubated in assay buffer (50 mM Trizma, 0.25 mg/ml bovine serum albumin, pH 7.4) containing increasing concentrations of [^{3}H] RTX (100-2400 pM) (39.2 Ci/mmol) (Perkin Elmer, Waltham, USA) in the absence or presence of 10 µM unlabeled RTX to assess affinity by displacement.

cAMP accumulation

The cAMP levels of transiently transfected HEK 293 Tet-On cells were measured using enzyme linked immunosorbant assays (GE Healthcare) according to the manufacturer's instructions. Cells were pretreated for 20 min with the phosphodiesterase inhibitor isobuthylmethylxanthine (2mM) and 10 µM forskolin to avoid cAMP degradation and induce AC activity, respectively (Xia et al., 2011). TRPA1 was activated by 20 µM MO 5 min

previous to the membrane preparation. Calcium was removed in the respective cells by the addition of 100 µM EGTA and 10 µM BAPTA-AM 12 h prior to the experiment.

Statistics

Data are represented as means ± S.E.M. Gaussian distribution was examined by the Shapiro-Wilk normality test. We used the unpaired t-test and 1-way ANOVA with Dunnett's multiple comparison test for parametric data, and the Mann-Whitney test and Kruskal-Wallis with Dunn's multiple comparison test for non-parametric data. For grouped analyses we used 2-way ANOVA with Bonferroni's multiple comparison test. Binding data are reported as means ± S.E.M. of at least four independent experiments performed in duplicate. Differences were considered significant if $p<0.05$. All tests were performed using Prism 5 (GraphPad, San Diego, USA) statistical software (Spahn et al., 2013).

Results

Increased TRPV1 activity after TRPA1 stimulation

We first measured capsaicin (1µM)-induced TRPV1 currents in transiently transfected HEK 293 Tet-On cells co-expressing wild type (wt) TRPV1 and TRPA1. Pretreatment with 20 µM MO for 2 min followed by 1 min washout resulted in 3.3 fold increase of capsaicin-induced TRPV1 currents compared to control cells without MO pretreatment (Kruskal-Wallis test, $p<0.01$). In the absence of TRPA1 or after inhibition by simultaneous application of the TRPA1-specific inhibitor HC-030031 (25 µM) this effect was not detected (Fig. 1A and Fig. 2C and D).

Representative current traces of control and MO-pretreated cells are shown in Fig. 1B. Calcium imaging confirmed these data in that the capsaicin (100 nM)-induced change of the fura ratio over time, as well as the maximum change of the fura ratio of MO-pretreated cells was significantly higher compared to control cells (Fig. 2A; 2-way ANOVA, $p<0.001$; Fig. 2B Mann-Whitney test, $p<0.05$). In cells only expressing TRPV1 (Fig 2E; n=53) or TRPA1 (Fig 2F; n=16), 20 µM MO or 1µM capsaicin, respectively, had no effect.

To further exclude a direct effect of MO at TRPV1, we investigated another TRPA1 agonist, CA (100 µM). Pretreatment of TRPV1 and TRPA1 co-expressing HEK 293 cells with CA for 2 min followed by a 1 min washout also resulted in a significantly increased capsaicin (100 nM)-induced TRPV1 current (Fig. 1E; Mann-Whitney test; $p<0.01$). Representative current traces are shown in Fig 1F.

To study sensory neurons natively expressing TRPV1 and TRPA1, we performed whole cell patch clamp experiments in small to medium-sized DRG neurons. Again, MO pretreatment resulted in a significantly elevated capsaicin (100 nM)-induced TRPV1 current (Fig. 1C; Mann-Whitney test, $p<0.05$). Representative current traces of control and MO-pretreated cells are shown in Fig. 1D. 69.2% of capsaicin-responsive neurons also responded to MO with an

average MO (20 µM)-induced current of -0.44 ± 0.1 nA, indicating TRPA1 expression. Only neurons responding to MO were analyzed.

To exclude that the increased capsaicin-induced current is based on an additive TRPA1 current, TRPA1 was inhibited with HC-030031 (25 µM) immediately after MO (or vehicle) pretreatment. Capsaicin was applied in the presence of HC-030031. This treatment still resulted in significantly increased capsaicin-induced TRPV1 currents in HEK 293 Tet-On cells (Figs. 3A, B; Mann-Whitney test, $p<0.05$). In DRG neurons this effect was similar but not statistically significant (not shown; Mann-Whitney test, $p>0.05$). We further studied HEK 293 Tet-On cells co-expressing wt TRPV1 and D477A TRPA1. This mutant lacks calcium sensitivity (Zurborg et al., 2007) and therefore, increased intracellular calcium (due e.g. to TRPV1 activation) cannot activate TRPA1 and account for the increased current. These experiments also showed significantly increased capsaicin-induced currents in the MO-pretreated group (Fig. 3C, Mann-Whitney test, $p<0.001$).

Unaltered TRPV1 expression in the presence of TRPA1

Next, we examined whether TRPV1 and TRPA1 co-transfection may alter the TRPV1 expression level or [^{3}H] RTX binding affinity. The co-transfection of TRPV1 and TRPA1 did not influence total [^{3}H] RTX binding compared to cells transfected with TRPV1 alone (2-way ANOVA with Bonferroni post test, $p>0.05$) (Fig. 4A). The maximum number of [^{3}H] RTX binding sites on cells expressing only TRPV1 was 427.2 ± 244.1 fmol/mg compared to 445.6 ± 236.1 fmol/mg on TRPV1/TRPA1 co-expressing cells. The dissociation constants were 6.9 ± 4.8 nM and 4.7 ± 3.4 nM, respectively, indicating similar binding affinities. Binding experiments with 0.4 nM [^{3}H] RTX in TRPV1/TRPA1 co-expressing cells revealed that MO pretreatment for 1 h did not change the number TRPV1 binding sites (Mann-Whitney test, $p>0.05$) (Fig. 4B).

Increased TRPV1 activity after TRPA1 stimulation is dependent on calcium, AC and PKA

In transfected HEK cells the removal of extracellular calcium *per se* induced significantly increased TRPV1 currents under control conditions (with calcium: I = - 0.73 ± 0.1 nA, without calcium: I = -2.72 ± 0.35 nA, unpaired t-test, $p<0.001$) and reversed the TRPV1 sensitization after TRPA1 stimulation with MO (Fig. 5A; unpaired t-test, $p>0.05$).

The unspecific AC inhibitor SQ 22.536 (100µM) abolished the increased capsaicin-induced TRPV1 current after TRPA1 stimulation (Fig. 5B; control: I = -1.42 ± 0.31 nA; MO: I = - 2.0 ± 0.54 nA; unpaired t-test, $p>0.05$). Enzyme-linked immunosorbant assays showed significantly elevated cAMP levels in TRPV1/TRPA1 expressing cells pretreated with MO compared to controls (1-way ANOVA, Bonferroni posthoc test, $p<0.05$). This was not detectable in the absence of calcium or in the absence of TRPA1 (Fig. 5C). In patch clamp experiments the PKA inhibitor H89 (10µM) prevented TRPV1 sensitization after TRPA1 stimulation (Fig. 5D; control: I = -1.34 ± 0.2 nA; MO: I = -2.06 ± 0.55 nA; Mann-Whitney test, $p>0.05$).

In DRG neurons the removal of extracellular calcium as well as the inhibition of PKA prevented TRPV1 sensitization after TRPA1 stimulation, i.e. capsaicin currents did not differ significantly between control and MO-pretreated cells (Figs. 5E, F; calcium free controls: I = - 1.12 ± 0.27 nA; MO: I = - 0.58 ± 0.07 nA, unpaired t-test, $p>0.05$; H89 controls: I = - 0.39 ± 0.15 nA; MO: I = - 0.25 ± 0.1 nA; Mann-Whitney test, $p>0.05$).

Increased TRPV1 activity after TRPA1 stimulation is absent in TRPV1 mutant S 116A

We investigated PKA phosphorylation sites of TRPV1 by replacing serine 116 (S116), threonine 144 (T144), and serine 774 (S774) by alanine, respectively. In HEK 293 Tet-On cells co-expressing mutant TRPV1 and wt TRPA1 the increased capsaicin-induced TRPV1 current after TRPA1 stimulation was absent in mutants S116A and S774A (Figs. 6A and C; unpaired t-test, $p>0.05$) but was still present in cells co-expressing T144A and wt TRPA1

(Fig. 6B, unpaired t-test, $p<0.001$). In microfluorimetry experiments the increased capsaicin-induced calcium mobilization after MO pretreatment was reversed in mutant S116A (Mann-Whitney test, $p>0.05$, Fig. 7A) but was still present in mutant T144A (unpaired- t-test, $p<0.001$; Fig. 7B) and in mutant S774A (unpaired t-test, $p<0.01$; Fig. 7C).

Discussion

In the present study we showed increased TRPV1 activity after pre-stimulation of TRPA1 both in HEK cells and DRG neurons. This was dependent on calcium, AC and PKA. Mutation of the putative phosphorylation site serine 116 in TRPV1 also abolished increased TRPV1 activity after TRPA1 stimulation. Together, our findings suggest that TRPA1 activation causes an influx of calcium, increases calcium-sensitive AC activity, cAMP accumulation and subsequent PKA activation. This results in phosphorylation and sensitization of TRPV1.

Although some studies showed direct activation of TRPV1 by MO at high concentrations (Everaerts et al., 2011; Gees et al., 2013; Ohta et al., 2007), our control experiments and other studies showed that MO at a concentration of 20 µM did not directly activate TRPV1 (Fig. 2D and (Everaerts et al., 2011; Jordt et al., 2004)).

Approximately 30-50 % of TRPV1 expressing small to medium-sized peripheral sensory neurons co-express TRPA1 and almost all TRPA1 positive neurons co-express TRPV1 (Hjerling-Leffler et al., 2007; Kobayashi et al., 2005; Linte et al., 2007; Story et al., 2003). Furthermore, currents induced by the TRPA1 agonists MO and WIN55,212 were almost exclusively detected in TRPV1 positive cells (Diogenes et al., 2007; Jordt et al., 2004; Story et al., 2003) and both channels are activated by compounds which cause a pungent burning sensation. Thus, we hypothesized that TRPV1 sensitization can result from functional interactions of both channels.

While most TRP channel complexes are made up of monomers from members of the same subfamily, numerous studies identified physical interactions between members of different TRP channel subfamilies through formation of heteromultimeric complexes (Bai et al., 2008; Dietrich et al., 2005; Hellwig et al., 2005; Park et al., 2008; Schilling and Goel, 2004). Although it was shown that TRPV1 and TRPA1 can form such complexes resulting in different pharmacological properties of the individual channels (Ruparel et al., 2011; Salas et al., 2009; Staruschenko et al., 2010), we did not find any alterations in the amount of TRPV1

protein expression or binding in the presence of TRPA1 with the methods used in the current study. Previously, a stabilizing effect of TRPV1 on the membrane expression of TRPA1 was suggested (Akopian et al., 2007). Internalization of membrane-bound channels can be suppressed by interactions with other proteins including subunits of the channels (Bernstein and Jones, 2007), and functional TRPV1 tetramers can be modulated by cAMP-dependent translocation of TRPV1 monomers from intracellular pools to the cell membrane (Vetter et al., 2008). Again, since neither the affinity nor the number of TRPV1 binding sites changed during TRPA1 activation, our experiments indicate that TRPV1 was not internalized or recruited to the cell membrane from intracellular pools.

Functional interactions between TRPV1 and TRPA1 via signaling pathways were suggested before, however with some inconsistent results. One group showed inhibition of TRPV1 activity by TRPA1 via calcineurin-mediated desensitization of TRPV1 in CHO cells and trigeminal ganglion neurons (Akopian et al., 2007; Jeske et al., 2006). In these studies TRPA1 activation resulted in a recruitment of the calcium-dependent calcineurin pathway, whereby calcineurin dephosphorylated threonine 144 and 370 and subsequently desensitized TRPV1. In line with our results, CAMKII and PKC activation can increase TRPV1 activity after TRPA1 stimulation in a calcium dependent manner (Jung et al., 2004; Mandadi et al., 2004). Phosphorylation and dephosphorylation events might be dependent on calcium, TRPA1 agonist concentration or incubation time.

Additionally, activation of calcium sensitive ACs with subsequent PKA activation can induce TRP channel phosphorylation (Distler et al., 2003; Ferguson and Storm, 2004; Wang et al., 2008). A recent study measured enhanced capsaicin responses of DRG neurons pre-incubated with low concentrations of CA (225 µM) in calcium imaging experiments, which indicated enhanced TRPV1 activity after pretreatment with a TRPA1 agonist (Anand et al., 2008). Another group showed that the TRPV1 response to mild acidification was significantly

enhanced in the presence of 100 µM MO (Gees et al., 2013). These findings are in line with our results showing that pretreatment with MO increased TRPV1 activity.

The activation of TRPA1 leads to an increase of intracellular calcium since TRPA1 is a non-selective cation channel. Previous studies showed that calcium directly activates TRPA1 through an intracellular EF-hand domain (Zurborg et al., 2007), possibly resulting in an (auto-) amplification of TRPA1 activity. This might induce an additive TRPA1 current due to calcium entering TRPV1 after capsaicin application. We excluded this possibility using the TRPA1 inhibitor HC-030031 or the calcium-insensitive TRPA1 mutant D447A. Therefore, we conclude that the increased TRPV1 activity after TRPA1 stimulation is only carried by TRPV1. In the present study TRPA1 activation failed to sensitize TRPV1 in the absence of calcium, suggesting an important role for calcium in mediating TRPV1 sensitization. In sensory neurons an increase of intracellular calcium can lead to elevated cAMP, elevated translocation of the catalytic PKA subunit via AC 1 and AC 8, and subsequently to increased TRPV1 phosphorylation (Distler et al., 2003). In line with these findings, we measured increased cAMP levels after MO pre-stimulation, which were abolished in the absence of calcium or of TRPA1. Importantly, in native DRG neurons we also found a significant increase of capsaicin-induced TRPV1 activity after TRPA1 activation. This supports our results in transfected cells, suggesting that this mechanism also plays an important role in cells endogenously expressing TRPV1 and TRPA1.

Finally, we used TRPV1 mutants S116A, T144A and S774A to identify potential PKA phosphorylation sites at TRPV1. TRPV1 sensitization after TRPA1 stimulation was absent in mutant S116A, however not in T144A or S774A. This supports our hypothesis that TRPA1 pre-stimulation increases PKA activity and subsequently increases TRPV1 sensitivity at S116. This is in accordance with previous studies that identified S116 as a candidate PKA phosphorylation site for PKA-dependent TRPV1 sensitization (Bhave et al., 2002; Mohapatra and Nau, 2005).

In summary, our data show that TRPA1 activation sensitizes TRPV1 in a calcium- and cAMP/PKA-dependent manner. The activation of TRPA1 resulted in an enhanced cAMP-accumulation, likely by the stimulation of calcium-dependent ACs. The increased cAMP may stimulate the release of the catalytic PKA subunit which in turn phosphorylates TRPV1 at the putative phosphorylation site serine 116. Concurrently, we detected calcium-sensitive increased TRPV1 activity after TRPA1 activation in DRG neurons. Future studies might identify new analgesics based on the prevention of TRPV1 sensitization.

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Authorship contributions

Participated in research design: Spahn, Zöllner, and Stein

Conducted experiments: Spahn

Contributed new reagents or analytic tools:

Performed data analysis: Spahn, Stein

Wrote or contributed to the writing of the manuscript: Spahn, Stein, and Zöllner

References

Akopian AN, Ruparel NB, Jeske NA and Hargreaves KM (2007) Transient receptor potential TRPA1 channel desensitization in sensory neurons is agonist dependent and regulated by TRPV1-directed internalization. *J Physiol* **583**(Pt 1):175-193.

Akopian AN, Ruparel NB, Patwardhan A and Hargreaves KM (2008) Cannabinoids desensitize capsaicin and mustard oil responses in sensory neurons via TRPA1 activation. *J Neurosci* **28**(5):1064-1075.

Anand U, Otto WR, Facer P, Zebda N, Selmer I, Gunthorpe MJ, Chessell IP, Sinisi M, Birch R and Anand P (2008) TRPA1 receptor localisation in the human peripheral nervous system and functional studies in cultured human and rat sensory neurons. *Neurosci Lett* **438**(2):221-227.

Bai CX, Giamarchi A, Rodat-Despoix L, Padilla F, Downs T, Tsiokas L and Delmas P (2008) Formation of a new receptor-operated channel by heteromeric assembly of TRPP2 and TRPC1 subunits. *EMBO Rep* **9**(5):472-479.

Bandell M, Story GM, Hwang SW, Viswanath V, Eid SR, Petrus MJ, Earley TJ and Patapoutian A (2004) Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron* **41**(6):849-857.

Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, Yamoah EN, Basbaum AI and Julius D (2006) TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* **124**(6):1269-1282.

Bernstein GM and Jones OT (2007) Kinetics of internalization and degradation of N-type voltage-gated calcium channels: role of the alpha2/delta subunit. *Cell Calcium* **41**(1):27-40.

Bessac BF and Jordt SE (2008) Breathtaking TRP channels: TRPA1 and TRPV1 in airway chemosensation and reflex control. *Physiology (Bethesda)* **23**:360-370.

Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS and Gereau RWt (2002) cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron* **35**(4):721-731.

Bolyard LA, Van Looy JW and Vasko MR (2000) Sensitization of rat sensory neurons by chronic exposure to forskolin or 'inflammatory cocktail' does not downregulate and requires continuous exposure. *Pain* **88**(3):277-285.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI and Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **288**(5464):306-313.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD and Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**(6653):816-824.

Cvetkov TL, Huynh KW, Cohen MR and Moiseenkova-Bell VY (2011) Molecular architecture and subunit organization of TRPA1 ion channel revealed by electron microscopy. *J Biol Chem* **286**(44):38168-38176.

Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A and Sheardown SA (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* **405**(6783):183-187.

Dietrich A, Mederos y Schnitzler M, Kalwa H, Storch U and Gudermann T (2005) Functional characterization and physiological relevance of the TRPC3/6/7 subfamily of cation channels. *Naunyn Schmiedebergs Arch Pharmacol* **371**(4):257-265.

Diogenes A, Akopian AN and Hargreaves KM (2007) NGF up-regulates TRPA1: implications for orofacial pain. *J Dent Res* **86**(6):550-555.

Distler C, Rathee PK, Lips KS, Obreja O, Neuhuber W and Kress M (2003) Fast Ca2+-induced potentiation of heat-activated ionic currents requires cAMP/PKA signaling and functional AKAP anchoring. *J Neurophysiol* **89**(5):2499-2505.

Doerner JF, Gisselmann G, Hatt H and Wetzel CH (2007) Transient receptor potential channel A1 is directly gated by calcium ions. *J Biol Chem* **282**(18):13180-13189.

Everaerts W, Gees M, Alpizar YA, Farre R, Leten C, Apetrei A, Dewachter I, van Leuven F, Vennekens R, De Ridder D, Nilius B, Voets T and Talavera K (2011) The capsaicin receptor TRPV1 is a crucial mediator of the noxious effects of mustard oil. *Curr Biol* **21**(4):316-321.

Ferguson GD and Storm DR (2004) Why calcium-stimulated adenylyl cyclases? *Physiology (Bethesda)* **19**:271-276.

Gees M, Alpizar YA, Boonen B, Sanchez A, Everaerts W, Segal A, Xue F, Janssens A, Owsianik G, Nilius B, Voets T and Talavera K (2013) Mechanisms of transient receptor potential vanilloid 1 activation and sensitization by allyl isothiocyanate. *Mol Pharmacol* **84**(3):325-334.

Geppetti P, Nassini R, Materazzi S and Benemei S (2008) The concept of neurogenic inflammation. *BJU Int* **101 Suppl 3**:2-6.

Hellwig N, Albrecht N, Harteneck C, Schultz G and Schaefer M (2005) Homo- and heteromeric assembly of TRPV channel subunits. *J Cell Sci* **118**(Pt 5):917-928.

Hjerling-Leffler J, Alqatari M, Ernfors P and Koltzenburg M (2007) Emergence of functional sensory subtypes as defined by transient receptor potential channel expression. *J Neurosci* **27**(10):2435-2443.

Jeske NA, Patwardhan AM, Gamper N, Price TJ, Akopian AN and Hargreaves KM (2006) Cannabinoid WIN 55,212-2 regulates TRPV1 phosphorylation in sensory neurons. *J Biol Chem* **281**(43):32879-32890.

Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Hogestatt ED, Meng ID and Julius D (2004) Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature* **427**(6971):260-265.

Jung J, Shin JS, Lee SY, Hwang SW, Koo J, Cho H and Oh U (2004) Phosphorylation of vanilloid receptor 1 by Ca2+/calmodulin-dependent kinase II regulates its vanilloid binding. *J Biol Chem* **279**(8):7048-7054.

Kobayashi K, Fukuoka T, Obata K, Yamanaka H, Dai Y, Tokunaga A and Noguchi K (2005) Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with adelta/c-fibers and colocalization with trk receptors. *J Comp Neurol* **493**(4):596-606.

Kwan KY, Allchorne AJ, Vollrath MA, Christensen AP, Zhang DS, Woolf CJ and Corey DP (2006) TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron* **50**(2):277-289.

Linte RM, Ciobanu C, Reid G and Babes A (2007) Desensitization of cold- and menthol-sensitive rat dorsal root ganglion neurones by inflammatory mediators. *Exp Brain Res* **178**(1):89-98.

Mandadi S, Numazaki M, Tominaga M, Bhat MB, Armati PJ and Roufogalis BD (2004) Activation of protein kinase C reverses capsaicin-induced calcium-dependent desensitization of TRPV1 ion channels. *Cell Calcium* **35**(5):471-478.

Mohapatra DP and Nau C (2003) Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. *J Biol Chem* **278**(50):50080-50090.

Mohapatra DP and Nau C (2005) Regulation of Ca2+-dependent desensitization in the vanilloid receptor TRPV1 by calcineurin and cAMP-dependent protein kinase. *J Biol Chem* **280**(14):13424-13432.

Mohapatra DP, Wang SY, Wang GK and Nau C (2003) A tyrosine residue in TM6 of the Vanilloid Receptor TRPV1 involved in desensitization and calcium permeability of capsaicin-activated currents. *Mol Cell Neurosci* **23**(2):314-324.

Obata K, Katsura H, Mizushima T, Yamanaka H, Kobayashi K, Dai Y, Fukuoka T, Tokunaga A, Tominaga M and Noguchi K (2005) TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. *J Clin Invest* **115**(9):2393-2401.

Ohta T, Imagawa T and Ito S (2007) Novel agonistic action of mustard oil on recombinant and endogenous porcine transient receptor potential V1 (pTRPV1) channels. *Biochem Pharmacol* **73**(10):1646-1656.

Park JY, Hwang EM, Yarishkin O, Seo JH, Kim E, Yoo J, Yi GS, Kim DG, Park N, Ha CM, La JH, Kang D, Han J, Oh U and Hong SG (2008) TRPM4b channel suppresses store-operated Ca2+ entry by a novel protein-protein interaction with the TRPC3 channel. *Biochem Biophys Res Commun* **368**(3):677-683.

Petrus M, Peier AM, Bandell M, Hwang SW, Huynh T, Olney N, Jegla T and Patapoutian A (2007) A role of TRPA1 in mechanical hyperalgesia is revealed by pharmacological inhibition. *Mol Pain* **3**:40.

Premkumar LS and Abooj M (2012) TRP channels and analgesia. *Life Sci* **92**(8-9):415-424.

Ruparel NB, Patwardhan AM, Akopian AN and Hargreaves KM (2011) Desensitization of transient receptor potential ankyrin 1 (TRPA1) by the TRP vanilloid 1-selective cannabinoid arachidonoyl-2 chloroethanolamine. *Mol Pharmacol* **80**(1):117-123.

Salas MM, Hargreaves KM and Akopian AN (2009) TRPA1-mediated responses in trigeminal sensory neurons: interaction between TRPA1 and TRPV1. *Eur J Neurosci* **29**(8):1568-1578.

Schilling WP and Goel M (2004) Mammalian TRPC channel subunit assembly. *Novartis Found Symp* **258**:18-30; discussion 30-43, 98-102, 263-106.

Spahn V, Fischer O, Endres-Becker J, Schafer M, Stein C and Zollner C (2013) Opioid withdrawal increases transient receptor potential vanilloid 1 activity in a protein kinase A-dependent manner. *Pain* **154**(4):598-608.

Staruschenko A, Jeske NA and Akopian AN (2010) Contribution of TRPV1-TRPA1 interaction to the single channel properties of the TRPA1 channel. *J Biol Chem* **285**(20):15167-15177.

Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S and Patapoutian A (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* **112**(6):819-829.

Szallasi A, Blumberg PM, Annicelli LL, Krause JE and Cortright DN (1999) The cloned rat vanilloid receptor VR1 mediates both R-type binding and C-type calcium response in dorsal root ganglion neurons. *MolPharmacol* **56**(3):581-587.

Tai C, Zhu S and Zhou N (2008) TRPA1: the central molecule for chemical sensing in pain pathway? *J Neurosci* **28**(5):1019-1021.

Tominaga M (2007) Nociception and TRP channels. *Handb Exp Pharmacol*(179):489-505.

Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI and Julius D (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* **21**(3):531-543.

Tominaga M and Tominaga T (2005) Structure and function of TRPV1. *Pflugers Arch* **451**(1):143-150.

Venkatachalam K and Montell C (2007) TRP channels. *Annu Rev Biochem* **76**:387-417.

Vetter I, Cheng W, Peiris M, Wyse BD, Roberts-Thomson SJ, Zheng J, Monteith GR and Cabot PJ (2008) Rapid, opioid-sensitive mechanisms involved in transient receptor potential vanilloid 1 sensitization. *J Biol Chem* **283**(28):19540-19550.

Wang S, Dai Y, Fukuoka T, Yamanaka H, Kobayashi K, Obata K, Cui X, Tominaga M and Noguchi K (2008) Phospholipase C and protein kinase A mediate bradykinin sensitization of TRPA1: a molecular mechanism of inflammatory pain. *Brain* **131**(Pt 5):1241-1251.

Xia M, Guo V, Huang R, Shahane SA, Austin CP, Nirenberg M and Sharma SK (2011) Inhibition of morphine-induced cAMP overshoot: a cell-based assay model in a high-throughput format. *Cell Mol Neurobiol* **31**(6):901-907.

Zurborg S, Yurgionas B, Jira JA, Caspani O and Heppenstall PA (2007) Direct activation of the ion channel TRPA1 by Ca2+. *Nat Neurosci* **10**(3):277-279.

Footnotes

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b) Parts of this work have been published previously in thesis form [Spahn V. Interaction of Transient Receptor Potential Vanilloid 1 (TRPV1) with G-protein coupled receptors and TRP ion channels. Freie Universität Berlin; 2011; Berlin; Germany] and in abstract form [Spahn V, Endres-Becker J, Fischer O, Schäfer M, Stein C, Zöllner C. Interaction of Transient Receptor Potential Vanilloid 1 (TRPV1) with G-protein coupled receptors and TRP ion channels. 13th World Congress On Pain; August 29th – September 2nd 2010; Montreal, Canada].

c) For reprint request please contact: Viola Spahn, Charité-Universitätsmedizin Berlin, Klinik für Anästhesiologie und operative Intensivmedizin, Campus Benjamin Franklin, Hindenburgdamm 30, 12203 Berlin, Germany; viola.spahn@charite.de

Figure legends:

Figure 1: *Sensitization of TRPV1 after TRPA1 activation.* A) Preincubation with the TRPA1 agonist mustard oil (MO) significantly increased capsaicin (Caps)-induced TRPV1 currents in HEK 293 Tet-On cells co-transfected with TRPV1/TRPA1, but not with TRPV1/Ptre2 (empty control vector) (Kruskal-Wallis test, **, $p<0.01$). B) Representative current traces of TRPV1/TRPA1 co-expressing HEK 293 Tet-On cells without (lower trace, Ctrl) and with MO pretreatment (upper trace). For the MO pretreated cell, the MO-induced TRPA1 current is also shown followed by washout and Caps-induced TRPV1 current. C) Capsaicin-induced TRPV1 currents of DRG neurons with (MO, black bar) and without (Ctrl, white bar) MO pretreatment differed significantly (Mann-Whitney test, *, $p<0.05$). D) Representative capsaicin-induced current traces of neurons without (Ctrl, left) and with MO pretreatment (right). E) Preincubation with the TRPA1 agonist cinnamaldehyde (CA) (100 µM) significantly increased capsaicin-induced TRPV1 currents in HEK 293 Tet-On cells co-transfected with TRPV1/TRPA1 (Mann-Whitney test, **, $p<0.01$). F) Representative current traces of TRPV1/TRPA1 co-expressing HEK 293 Tet-On cells without (lower trace, Ctrl) and with CA-pretreatment (upper trace). For the CA-pretreated cell, the CA-induced TRPA1 current is also shown followed by washout and capsaicin-induced TRPV1 current. Numbers (n) of cells are given in brackets.

Figure 2: *Sensitization of TRPV1 is dependent on TRPA1*. A) Capsaicin-induced calcium influx in TRPV1/TRPA1 co-expressing HEK 293 Tet-On cells without (Ctrl, open circle) and with MO pretreatment (solid squares) differed significantly (2-way ANOVA, ***, $p<0.001$; Ctrl: n=73; MO: n=36, 7 independent experiments). B) Maximum change of fura ratio in MO-pretreated cells (black bar) was significantly increased compared to Ctrl cells (white bar) (Mann-Whitney test, **, $p<0.05$). C) Co-application of MO and the TRPA1 inhibitor HC-

030031 reversed capsaicin-induced TRPV1 sensitization in calcium imaging experiments (2-way ANOVA, ***, $p<0.001$; Ctrl: n=67; MO/HC-030031: n=77; 8 independent experiments). D) Simultaneous application of MO with the TRPA1 inhibitor HC-030031 reversed capsaicin-induced TRPV1 sensitization in patch clamp experiments (Mann-Whitney test, n.s., $p>0.05$). Numbers (n) of cells are given in brackets. E) HEK 293 Tet-On cells only expressing TRPV1 did not respond to MO (indicated by arrow), but to capsaicin (indicated by arrow) (n=53, 4 independent experiments). F) HEK 293 Tet-On cells only expressing TRPA1 strongly respond to MO (n=16, 2 independent experiments).

Figure 3: *An additional TRPA1 current is not involved in the increased capsaicin-induced currents.* Capsaicin-induced TRPV1 currents were significantly higher in the MO group (black bar) compared to the Ctrl group (white bar) (Mann-Whitney test, *, $p<0.05$), both if TRPA1 was blocked by HC-030031 after the initial MO activation (A) and in cells co-transfected with wt TRPV1 and the TRPA1 mutant D447A (Mann-Whitney, ***, $p<0.001$) (C). B) Representative capsaicin-induced current traces of a Ctrl and MO-pretreated cell in the presence of HC-030031. Numbers (n) of cells are given in brackets.

Figure 4: *[^{3}H] RTX-binding in transfected HEK 293 Tet-On cells.* A) [^{3}H] RTX binding was not different between cells co-transfected with TRPV1 and TRPA1 (n=7) and those transfected with TRPV1 alone (n=4) (2-way ANOVA Bonferroni post test, $p>0.05$). B) Binding of 0.4 nM [^{3}H] RTX did not differ between TRPV1/TRPA1 co-transfected cells with (MO, black bar) and without (Ctrl, white bar) MO pre-treatment (Mann-Whitney test, $p>0.05$). Numbers of independent experiments are given in brackets.

Figure 5: *Involvement of calcium, AC and PKA in TRPA1-mediated TRPV1 sensitization.* Capsaicin-induced TRPV1 currents (in % of Ctrl) in TRPV1/TRPA1 co-transfected HEK 293

Tet-On cells with (black bar) and without (white bar) MO pretreatment were not significantly different following removal of extracellular calcium (A; unpaired t-test, $p>0.05$), inhibition of AC by SQ 22.536 (B; unpaired t-test, $p>0.05$) or inhibition of PKA by H89 (D; Mann-Whitney test, $p>0.05$). C) cAMP-accumulation in HEK 293 Tet-On cells co-transfected with TRPV1/TRPA1 (left three bars) without (Ctrl, white bar) and with MO pre-treatment (black bar), in the absence of calcium (third bar) or of TRPA1 (fourth bar) (Kruskal-Wallis test with Dunn's multiple comparison test, *, $p<0.05$). Capsaicin-induced TRPV1 currents (in % of Ctrl) in DRG neurons with (black bar) and without (white bar) MO pretreatment were not significantly different in the absence of calcium (E; unpaired t-test, $p>0.05$) or in the presence of PKA inhibitor H89 (F; Mann-Whitney test, $p>0.05$). Numbers of cells and independent experiments are given in brackets.

Figure 6: *Cells co-expressing mutant TRPV1 and wild type TRPA1 in patch clamp experiments.* Capsaicin-induced TRPV1 currents of mutants *S116A* (A) and *S774A* (C) did not differ between Ctrl and MO-pretreated cells (unpaired t-test, $p>0.05$) but were still increased in mutant *T144A* (B) after MO pretreatment (unpaired t-test, ***, $p<0.001$). Numbers of cells are given in brackets.

Figure 7: *Cells co-expressing mutant TRPV1 and wild type TRPA1 in calcium imaging experiments.* A) Maximum capsaicin-induced fura ratios were not different after MO pre-treatment in TRPV1 mutant *S116A* (Ctrl: n=28; MO: n=24; Mann-Whitney test, $p>0.05$) but were significantly increased in mutants *T144A* (B; Ctrl: n=120; MO: n=70; unpaired t-test, ***, $p<0.001$) and *S774A* (C; Ctrl: n=71; MO: n=54; unpaired t-test; **, $p<0.01$).

Figure 1

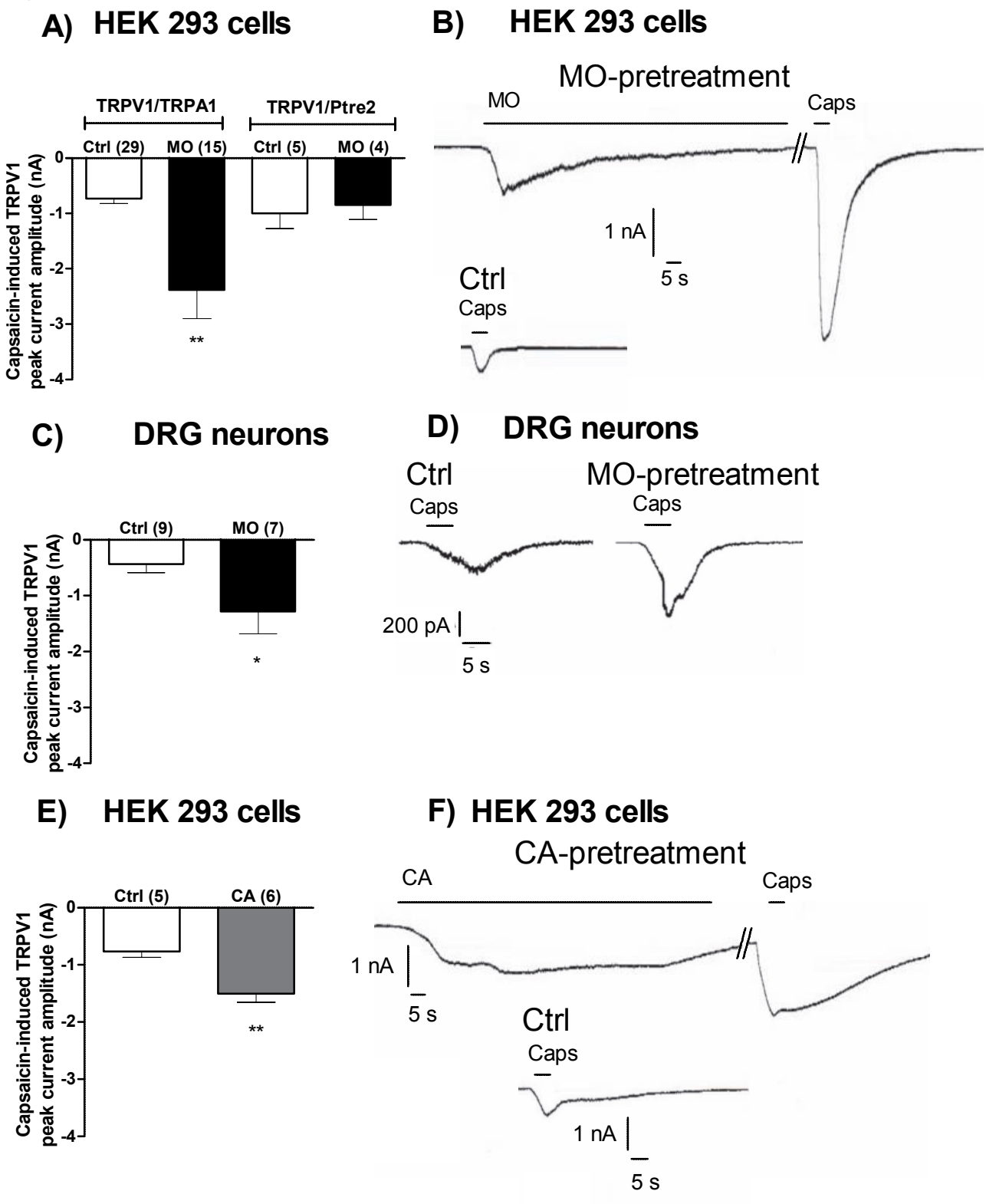

A) HEK 293 cells
TRPV1/TRPA1
TRPV1/Ptre2
Ctrl (29)
MO (15)
Ctrl (5)
MO (4)
Capsaicin-induced TRPV1 peak current amplitude (nA)
0
-1
-2
-3
-4
**
B) HEK 293 cells
MO-pretreatment
MO
Caps
1 nA
5 s
Ctrl
Caps
C) DRG neurons
Ctrl (9)
MO (7)
Capsaicin-induced TRPV1 peak current amplitude (nA)
*
D) DRG neurons
Ctrl
MO-pretreatment
Caps
Caps
200 pA
5 s
E) HEK 293 cells
Ctrl (5)
CA (6)
Capsaicin-induced TRPV1 peak current amplitude (nA)
**
F) HEK 293 cells
CA-pretreatment
CA
Caps
1 nA
5 s
Ctrl
Caps
1 nA
5 s

Figure 2

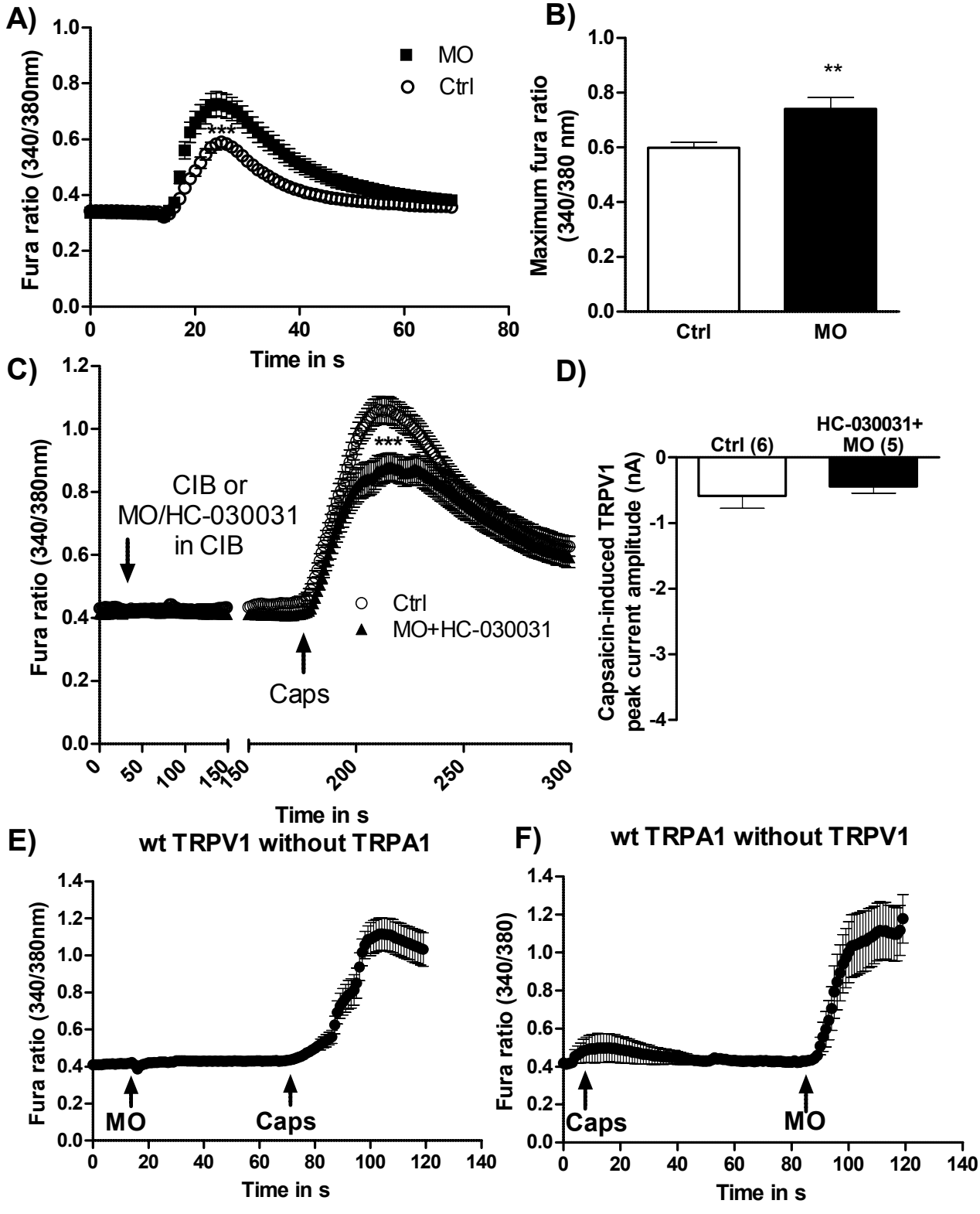
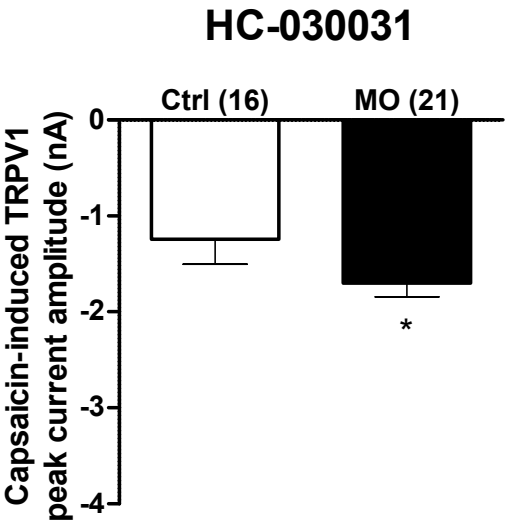
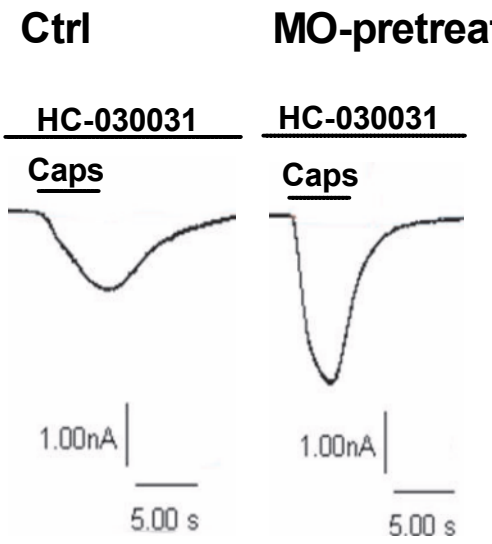

HEK 293 cells
A)
MO
Ctrl
***
Fura ratio (340/380nm)
Time in s
B)
**
Maximum fura ratio (340/380 nm)
Ctrl
MO
C)
CIB or MO/HC-030031 in CIB
***
Ctrl
MO+HC-030031
Caps
Fura ratio (340/380nm)
Time in s
D)
Ctrl (6)
HC-030031+ MO (5)
Capsaicin-induced TRPV1 peak current amplitude (nA)
E)
wt TRPV1 without TRPA1
MO
Caps
Fura ratio (340/380nm)
Time in s
F)
wt TRPA1 without TRPV1
Caps
MO
Fura ratio (340/380)
Time in s

Figure 3

A) HEK 293 cells



B) HEK 293 cells HC-030031



C) HEK 293 cells

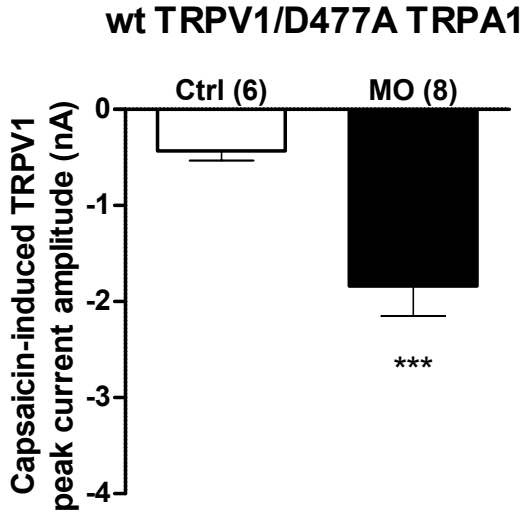
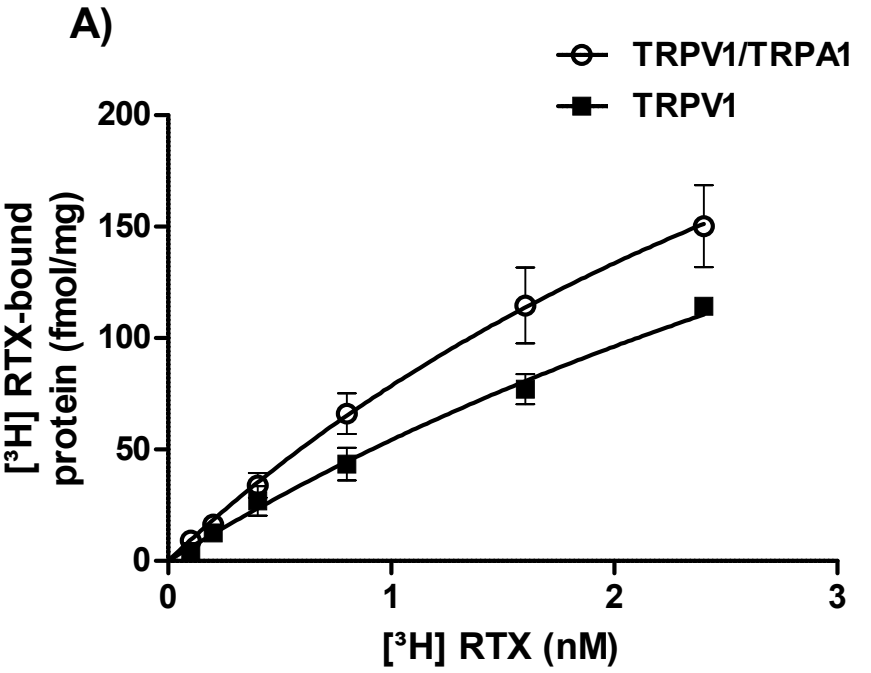


Figure 4


A)
TRPV1/TRPA1
TRPV1
200
150
100
50
0
0
1
2
3
[³H] RTX-bound protein (fmol/mg)
[³H] RTX (nM)

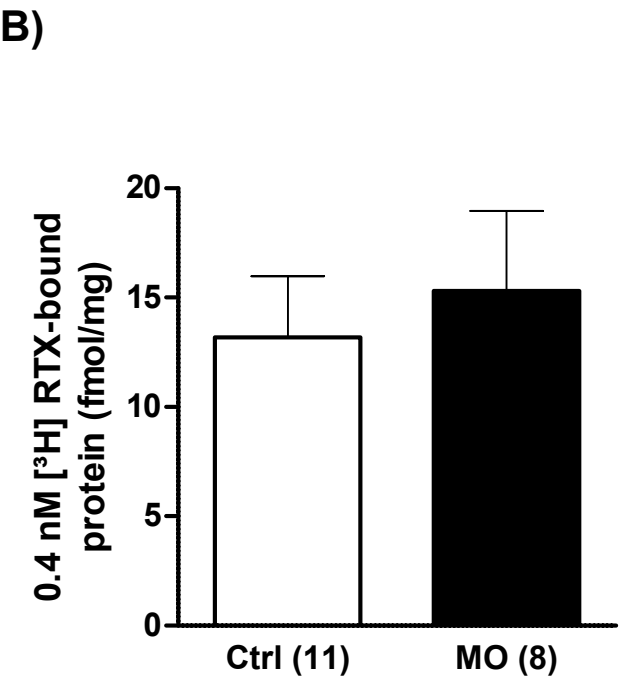

B)
20
15
10
5
0
0.4 nM [³H] RTX-bound protein (fmol/mg)
Ctrl (11)
MO (8)

Figure 5

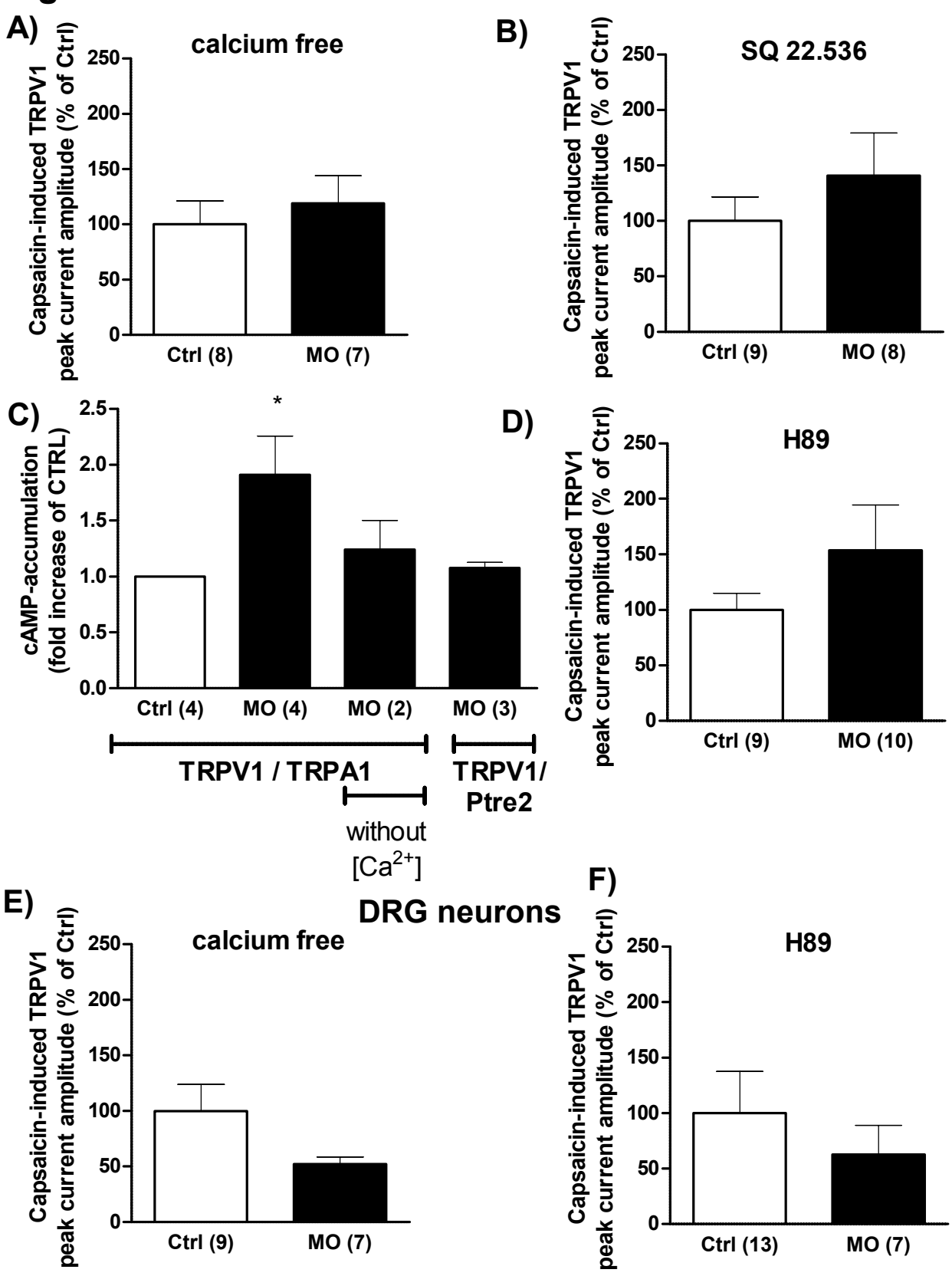

HEK293 cells
A)
calcium free
Capsaicin-induced TRPV1 peak current amplitude (% of Ctrl)
250
200
150
100
50
0
Ctrl (8)
MO (7)
B)
SQ 22.536
Capsaicin-induced TRPV1 peak current amplitude (% of Ctrl)
Ctrl (9)
MO (8)
C)
cAMP-accumulation (fold increase of CTRL)
2.5
2.0
1.5
1.0
0.5
0.0
*
Ctrl (4)
MO (4)
MO (2)
MO (3)
TRPV1 / TRPA1
without [Ca2+]
TRPV1/ Ptre2
D)
H89
Capsaicin-induced TRPV1 peak current amplitude (% of Ctrl)
Ctrl (9)
MO (10)
DRG neurons
E)
calcium free
Capsaicin-induced TRPV1 peak current amplitude (% of Ctrl)
Ctrl (9)
MO (7)
F)
H89
Capsaicin-induced TRPV1 peak current amplitude (% of Ctrl)
Ctrl (13)
MO (7)

Figure 6

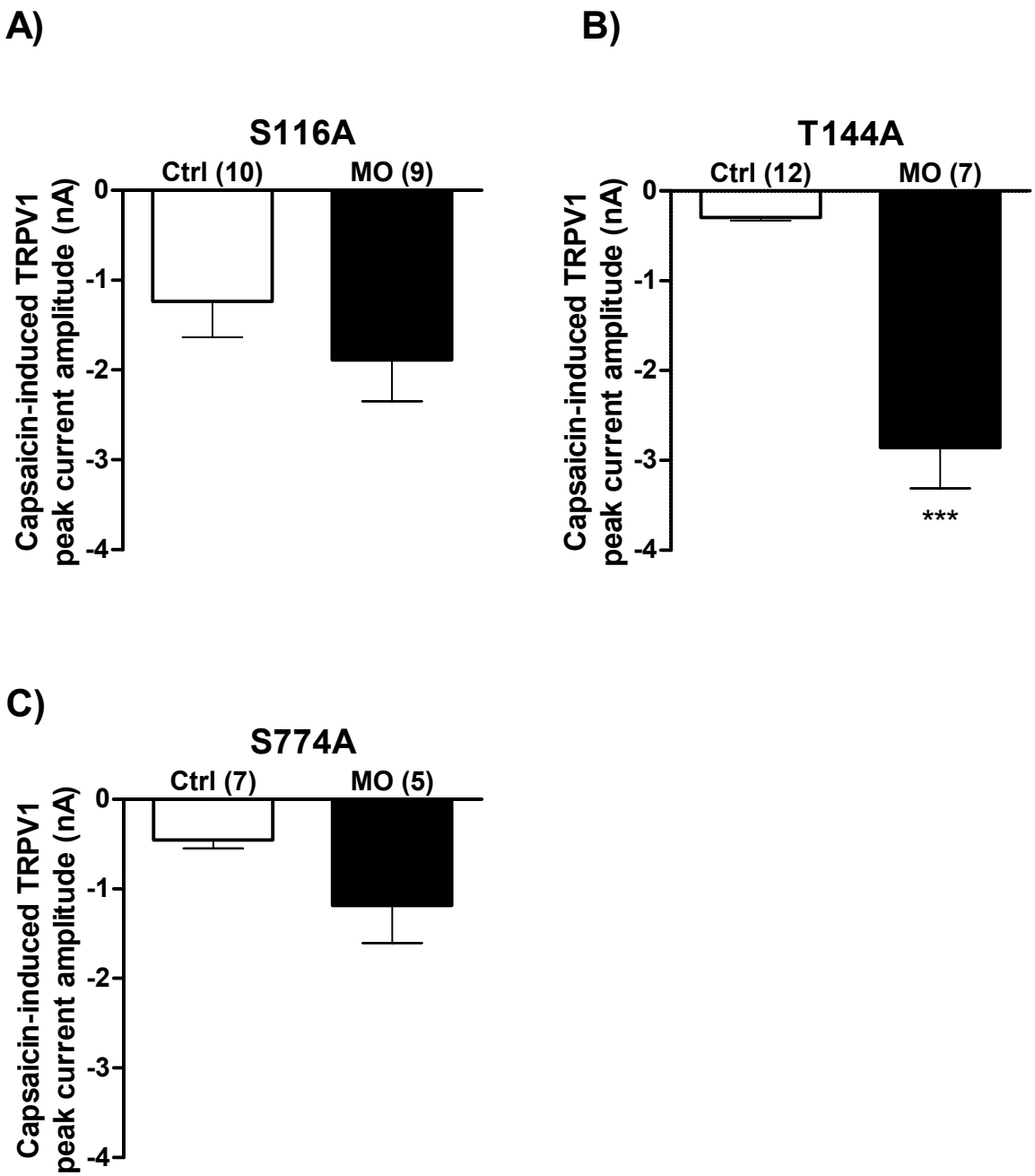
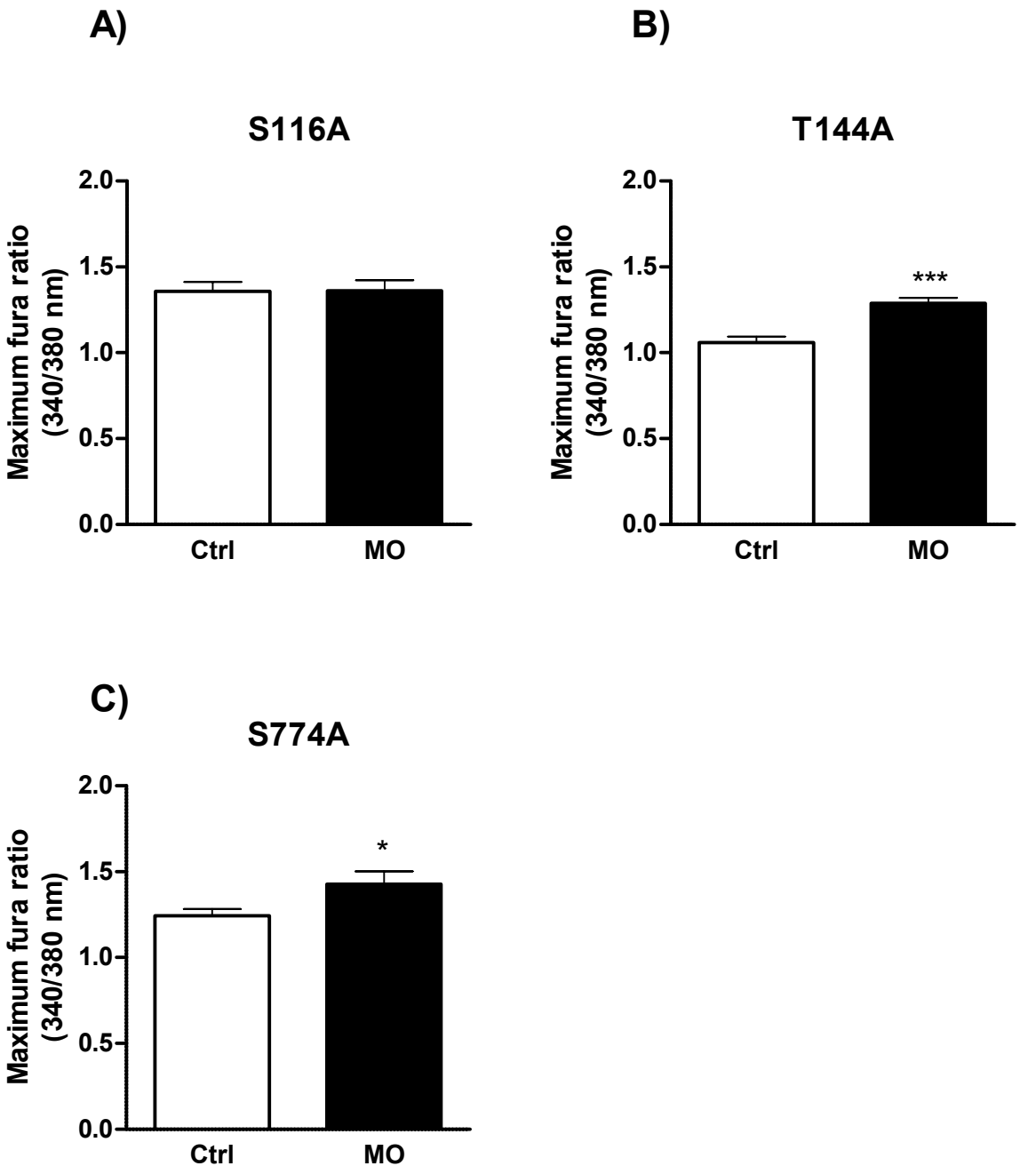

A)
S116A
Ctrl (10)
MO (9)
Capsaicin-induced TRPV1 peak current amplitude (nA)
0
-1
-2
-3
-4
B)
T144A
Ctrl (12)
MO (7)
Capsaicin-induced TRPV1 peak current amplitude (nA)
0
-1
-2
-3
-4
***
C)
S774A
Ctrl (7)
MO (5)
Capsaicin-induced TRPV1 peak current amplitude (nA)
0
-1
-2
-3
-4

Figure 7


A)
S116A
Maximum fura ratio (340/380 nm)
2.0
1.5
1.0
0.5
0.0
Ctrl
MO
B)
T144A
***
C)
S774A
*