Published in final edited form as:

Neuroscience. 2010 December 29; 171(4): 1109–1119. doi:10.1016/j.neuroscience.2010.09.031.

TRPV1 regulates activation and modulation of TRPA1 by Ca^{2+}

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Abstract

The transient receptor potential A1 (TRPA1) channel contributes to nociceptive signaling in certain pain models. It has been suggested that Ca^{2+} , which activates and modulates TRPA1, could play a critical regulatory role in this process. Since TRPA1 and TRPV1 channels are co-expressed and interact in neurons, we investigated whether activation and modulation of TRPA1 by Ca^{2+} is regulated by TRPV1. Cell-attached recordings showed that TRPA1 is activated by extracellular Ca^{2+} ($[\text{Ca}^{2+}]_e$) in concentration-response fashion. This activation, especially by 2mM $[\text{Ca}^{2+}]_e$ was substantially suppressed by co-expression with TRPV1. Inside-out recordings demonstrated that intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$)-triggered activation of TRPA1 was attenuated by the presence of TRPV1 only at 2 mM $[\text{Ca}^{2+}]_e$, but not in Ca^{2+} -free conditions. Further, depletion of internal Ca^{2+} stores by thapsigargin generated TRPA1-mediated currents, which is affected by TRPV1 in both Chinese hamster ovary cells and sensory neurons.

Since mustard oil current (I_{MO}) is modulated by $[\text{Ca}^{2+}]_e$, we next examined whether alterations in the Ca^{2+} -permeability of TRPV1 by mutating Y671 effect I_{MO} properties. First it was demonstrated that the mutations in TRPV1 did not affect association of the TRPA1 and TRPV1 channels. However, these TRPV1 mutations, particularly Y671K, altered the following characteristics of TRPA1: magnitude of I_{MO} in presence and absence of $[\text{Ca}^{2+}]_e$; the influence of $[\text{Ca}^{2+}]_e$ on the voltage-dependency of I_{MO} , and open probability of single-channel I_{MO} . In summary, activation of TRPA1 by $[\text{Ca}^{2+}]_e$ and $[\text{Ca}^{2+}]_i$ is controlled by the TRPV1 channel, and characteristics of I_{MO} depend on Ca^{2+} permeability of the TRPV1 channel.

Keywords

TRPA1; TRPV1; Pain

Introduction

Physiological studies using TRPA1-null-mutant mice (Bautista et al., 2006, Kwan et al., 2006, Kwan et al., 2009), antisense knock-down (Obata et al., 2005) and *in vivo* effects of

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TRPA1 antagonists (McNamara et al., 2007, Petrus et al., 2007) have demonstrated that TRPA1 controls the processing of nociceptive information in certain inflammatory and nerve injury pain models.

Mechanisms underlying information processing and stimulus integration by the TRPA1 channel in nociceptors have recently been vigorously studied. It was suggested that Ca^{2+} could play important role in these processes (Bautista et al., 2006, Zurborg et al., 2007). TRPA1 and TRPV1 can be activated by extracellular $[\text{Ca}^{2+}]_e$ (Ahern et al., 2005, Cavanaugh et al., 2008) as well as intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) (van der Stelt et al., 2005, Doerner et al., 2007, Zurborg et al., 2007). Activation of these channel by $[\text{Ca}^{2+}]_e$ can result in a baseline supply of Ca^{2+} into cells (i.e. Ca^{2+} leak). This constant supply of Ca^{2+} may maintain a variety of basal Ca^{2+} dependent processes in nociceptors, including transcription regulation and phosphorylation. On the other hand, activation of TRPA1 by $[\text{Ca}^{2+}]_i$ could account for mechanisms of TRPA1 gating by inflammatory mediators (Bandell et al., 2004, Zurborg et al., 2007). Thus, inflammatory mediators can trigger an elevation in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in sensory neurons via two possible pathways: depletion of internal Ca^{2+} stores via $G_{q/11}$ -coupled pathways and/or activation of Ca^{2+} -permeable channels (such as TRPV1, TRPA1 and TRPC3) on the plasma membrane (Bandell et al., 2004, Kim et al., 2004, Suh and Oh, 2005). Such elevation in $[\text{Ca}^{2+}]_i$ can result in activation of a variety of channels, including the TRPA1, by inflammatory mediators (Liu et al., 2010). In addition, since $[\text{Ca}^{2+}]_i$ can activate the TRPA1 channel in expression systems (Doerner et al., 2007, Zurborg et al., 2007), it has been proposed that $[\text{Ca}^{2+}]_i$ could serve as a mediator providing a linkage between the TRPV1 and TRPA1 channels during acute inflammatory hyperalgesia (Bautista et al., 2006, McMahon and Wood, 2006).

Extracellular Ca^{2+} can also modulate TRPA1-mediated responses. Thus, $[\text{Ca}^{2+}]_e$ alters the magnitude (Jordt et al., 2004, Nagata et al., 2005), changes kinetics and regulates single-channel characteristics of mustard oil (MO)-gated responses (Nagata et al., 2005, Kim and Cavanaugh, 2007). Further, extracellular Ca^{2+} -dependent properties of TRPA1-mediated responses are regulated by the TRPV1 channel in sensory neurons (Akopian et al., 2007, Salas et al., 2009, Staruschenko et al., 2010).

Despite this wealth of research, the potential roles of the TRPV1 channel in regulation of activation and modulation of TRPA1 by Ca^{2+} are poorly understood. To test this possibility, we have examined activation of TRPA1 by extracellular and intracellular Ca^{2+} in the presence and absence of the TRPV1 channel. We also investigated whether mutations in TRPV1 pore affecting Ca^{2+} permeability of the TRPV1 channel modify characteristics of MO-gated responses. These data could provide insight on Ca^{2+} -dependent functional regulation of nociceptive processing by interacting TRPA1 and TRPV1 channels.

Experimental procedures

Animals and primary sensory neuron culture

All experiments on animals conformed to protocols approved by the University Texas Health Science Center at San Antonio (UTHSCSA) Animal Care and Use Committee (ACUC). We followed guidelines issued by the National Institutes of Health and the Society for Neuroscience to minimize the number of animals used and their suffering.

Sprague-Dawley rats, 45-60-days old, were obtained from a commercial breeder (Charles River Laboratories, Inc., Wilmington, MA or Harlan, Indianapolis, IN, USA). B6.129S4 and B6.129S4-trpV1^{tm1/jul} (TRPV1 null-mutant) mice, 40-60-days old, were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). TRPA1 null-mutant mice were generated on the B6129P1/F2J background, and kindly provided by Dr. Kevin Kwan (Kwan et al., 2006).

Mice and rats were deeply anaesthetized with isoflurane (0.3 ml in 1 liter administered for 60-90 sec) and killed by decapitation. The trigeminal ganglia (TG) were quickly removed from the skull and placed in ice-cold Hank's solution (Invitrogen, Carlsbad, California, USA). TG neurons were separated by treatment in a 1mg/ml collagenase-dispase (Roche, Indianapolis, IN) solution. Neurons were plated at low-density on poly-D-lysine/laminin coated coverslips (Clontech, Palo Alto, CA). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 ng/ml NGF-7.02S (Harlan, Indianapolis, IN). The use of dispase instead of trypsin during dissociation of neurons and NGF in the maintenance media was critical to achieve reproducible and consistent responses to mustard oil (MO) application. The experiments were performed 24-36 h after plating.

Constructs and expression in CHO cells

Expression plasmids of green fluorescent protein (GFP) in pEGFP-N1 (Clontech); TRPV1 (accession number - NM031982) in pcDNA3 (Invitrogen, Carlsbad, CA); TRPA1 (NM177781) in pcDNA5/FRT (Invitrogen); D646N TRPV1, Y671A TRPV1, Y671D TRPV1 and Y671K TRPV1 in pcDNA3 were used in the studies. These constructs were delivered into Chinese hamster ovary (CHO) cells using PolyFect (Qiagen, Valencia, CA) according to manufacturers' protocols. Cells were used within 24-48 h after transfection.

Co-immunoprecipitation (Co-IP)

2.5×10^7 CHO cells were transfected with equimolar ratios of TRPV1 (or TRPV1 mutants) and myc-tagged TRPA1. Co-immunoprecipitation (Co-IP) and Western blotting was conducted as previously described (Jeske et al., 2006, Staruschenko et al., 2010) on 2-day transfected CHO cells. Anti-TRPV1 (Oncogene, San Diego, CA) and anti-myc (BD Biosciences) antibodies were utilized (Jeske et al., 2006).

Ca²⁺ imaging

Ca²⁺-imaging was performed as previously described (Akopian et al., 2007). Fluorescence images were analyzed by the MetaFluor Software (MetaMorph, Web Universal Imaging Corporation, Downingtown, PA). Ratiometric data were converted to $[Ca^{2+}]_i$ (in µM) as previously described (Jeske et al., 2006). The net changes in Ca²⁺ influx were calculated by subtracting the basal $[Ca^{2+}]_i$ (collected for 60 s) from the peak $[Ca^{2+}]_i$ value. Increases in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) above 30 nM were considered positive. This minimal threshold criterion was established by application of 0.1% DMSO (capsaicin was dissolved in 100% DMSO to concentration of 100mM) as a vehicle.

Electrophysiology

The whole-cell perforated patch and single-channel voltage-clamp recordings were performed at 22-24°C from the somata of TG neurons (15-45 pF) or CHO cells. Data were acquired and analyzed using an Axopatch200B amplifier and pCLAMP10 software (Axon Instruments, Union City, CA). Currents were filtered with an 8-pole, low pass Bessel filter at 0.1 kHz (cell-attached and inside-out) since dwelling time (τ) of the TRPA1 single-current was >0.5 sec (Cavanaugh et al., 2008); or 0.5 kHz (whole-cell and perforated patch) and sampled at 1 kHz or 2 kHz, respectively. Borosilicate pipettes (Sutter, Novato, CA) were polished to resistances of 5-7 MΩ (perforated patch) or 8-11 MΩ (cell-attached and inside-out) in corresponding pipette solutions. Access resistance (R_s) was compensated to achieve final R_s =12-15 MΩ (perforated-patch). Perforated patch recording were rejected when R_s changed >20% during recording, leak currents were >100pA, or input resistance was <200 MΩ. The amplitudes of the currents were analyzed when they were 5-fold larger than the noise (in root mean square).

Unitary current (i) was determined, as normal, from all-point amplitude histograms fitted with single- or multi-Gaussian curves using the standard 50% threshold criterion to differentiate between events. Events were inspected visually prior to acceptance. Channel activity, defined as NP_o , was calculated using the following equation: $NP_o = (t_1 + 2t_2 + \dots + nt_n)$, where N and P_o are the number of TRP channels in a patch and the mean open probability of these channels, respectively, and t_n is the fractional open time spent at each of the observed current levels. P_o was calculated by dividing NP_o by the number of active channels within a patch as defined by all-point amplitude histograms (Staruschenko et al., 2010). In order to increase accuracy in measurement of P_o , only patches containing 5 channels or fewer were used.

I-V relations were obtained as previously described (Salas et al., 2009). Detailed protocols of solutions for electrophysiological recordings are standard and provided in (Akopian et al., 2008, Salas et al., 2009). Briefly, standard external solution (SES) contained 2 mM Ca^{2+} . Ca^{2+} -free solution contains 5 mM EGTA to buffer ambient Ca^{2+} . Low concentrations (up to 1 μM) of Ca^{2+} in solutions were established by mixing software calculated concentrations of EGTA and Ca^{2+} (see <http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm>). The pipette solution for the perforated patch configurations included 250 $\mu\text{g/ml}$ amphotericin B (Sigma). For single-channel recording in cell-attached and inside-out configuration, the bath solution (SES-SCh) consisted of (in mM): 100 K-gluconate, 4 KCl, 10 D-glucose, 10 Hepes (pH 7.3) and the indicated concentration of Ca^{2+} . The pipette solution (SIS-SCh) was (mM): 100 Na-gluconate, 10 NaCl, 10 D-glucose, 10 Hepes (pH 7.3) and the indicated concentration of Ca^{2+} . Drugs were applied using a fast, pressure-driven, computer controlled 8-channel system (ValveLink8; AutoMate Scientific, San Francisco, CA). Application times are stated in the legends to figures.

Data analyses

For detailed statistical analyses, GraphPad Prism 4.0 (GraphPad, San Diego, CA) was used. The data in Figures were given as mean $\pm$ standard error of the mean (SEM), with the value of n referring to the number of analyzed cells for each group. All experiments were performed at least in triplicate. The significant difference between groups was assessed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-hoc test. Two conditions were compared using unpaired t -tests. A difference was accepted as significant when $p < 0.05$, < 0.01 or < 0.001 that were marked *, ** and ***, respectively.

Results

Activation of cells by extracellular Ca^{2+} is mutually regulated by TRPA1 and TRPV1 channel

It has been reported that extracellular Ca^{2+} ($[\text{Ca}^{2+}]_e$; 1-3mM) is able to gate TRPA1 (Cavanaugh et al., 2008) as well as TRPV1 channels (Ahern et al., 2005). We examined whether this activity is influenced by co-expression of TRPA1 with the TRPV1 channel. 2mM $[\text{Ca}^{2+}]_e$ did not activate GFP transfected CHO cells (Fig 1B; the upper left panel). However, $[\text{Ca}^{2+}]_e$ at concentrations ranging from 20 μM to 10mM showed single-channel activity (cell-attached configuration; voltage-clamp $V_h = -60\text{mV}$) in CHO cells expressing the TRPV1 and TRPA1 channels (Fig 1A and Fig 1B). The responses were considered for analysis when TRPV1 and TRPA1 expressing CHO cells were activated by capsaicin (100 nM CAP) and mustard oil (10 μM MO), respectively. Figure 1A and corresponding traces (Fig 1B) also illustrate that co-expression of TRPV1 and TRPA1 (respond for both CAP and MO) affects $[\text{Ca}^{2+}]_e$ -triggered single-channel activity of CHO cells. Thus, at a physiological concentration of $[\text{Ca}^{2+}]_e$ (2 mM), TRPA1 and TRPV1 co-expressing cells exhibit less activity than cells expressing either TRPA1 or TRPV1 alone (Fig 1A). In contrast, at lower

concentrations ($<200 \mu\text{M}$), TRPA1 and TRPV1 co-expressing cells exhibit higher activity (Fig 1A). One of the possible explanations for the biphasic effect in TRPA1-TRPV1 co-expressing cells is that at $2\text{mM } [\text{Ca}^{2+}]_e$, co-expression of TRPV1 with TRPA1 leads to a reduction of TRPA1-mediated activities (Salas et al., 2009, Staruschenko et al., 2010).

It was noted that at physiological concentrations of $[\text{Ca}^{2+}]_e$, TRPA1 exhibits basal activity (Bandell et al., 2004, Akopian et al., 2007, Macpherson et al., 2007). The basal activity of the TRPA1 and the TRPV1 channels could affect input resistance and leak values of whole-cell patched sensory neurons. Indeed, analyses of CAP (TRPV1-expressing), MO (TRPA1-expressing) and CAP and MO (TRPA1+TRPV1-co-expressing) positive neurons from trigeminal ganglia (TG) of rats, wild-type (WT) and TRPV1 null-mutant (TRPV1 KO) mice indicated a correlation (Salas et al., 2009). Thus, TG neurons from rat and WT mice responding for both CAP and MO showed $535 \pm 28 \text{ M}\Omega$ ($n=115$) input resistance and $32 \pm 11 \text{ pA}$ ($n=96$) leak values, while only MO sensitive TG neurons from TRPV1 KO mice revealed lesser input resistance ($247 \pm 54 \text{ M}\Omega$, $n=21$) and higher leak values ($75 \pm 26 \text{ pA}$, $n=19$). These differences are statistically significant (unpaired *t*-test, $P < 0.001$ for leak values and input resistance).

Activation of the cells by intracellular Ca^{2+} is mutually modulated by TRPA1 and TRPV1

Activation of TRPA1 by intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$; $0.1\text{--}100 \mu\text{M}$) with a Ca^{2+} -free extracellular solution (i.e. pipette solution) has been reported (Doerner et al., 2007, Zurborg et al., 2007). We examined whether the TRPV1 channel alters $[\text{Ca}^{2+}]_i$ -gated activation. Recordings were conducted in inside-out configuration with a Ca^{2+} -free pipette solution. CAP, MO and CAP+MO-sensitive CHO cells were considered TRPV1, TRPA1 and TRPA1-TRPV1 positive, respectively. Figure 2A and corresponding representative traces in Fig 2B demonstrate that with a Ca^{2+} -free extracellular solution, TRPV1 does not significantly influence activation of TRPA1 by $0.1\text{--}100 \mu\text{M } [\text{Ca}^{2+}]_i$. Further, TRPV1 itself is slightly activated by $1\text{--}100 \mu\text{M } [\text{Ca}^{2+}]_i$ (Fig 2A and 2B).

Since TRPA1-mediated responses are regulated by TRPV1 in the presence of $2\text{mM } [\text{Ca}^{2+}]_e$ (Salas et al., 2009) and $2\text{mM } [\text{Ca}^{2+}]_e$ activates both TRPA1 and TRPV1 channels, we investigated the role of the TRPV1 channel in the regulation of TRPA1 activation by $[\text{Ca}^{2+}]_i$ in the presence of physiological concentrations of $[\text{Ca}^{2+}]_e$ (i.e. 2mM). Under these conditions, TRPV1 significantly suppressed TRPA1-mediated responses to 1 and $10 \mu\text{M } [\text{Ca}^{2+}]_i$ (Fig 3A and 3B). Altogether, TRPV1 influences TRPA1-mediated responses to $[\text{Ca}^{2+}]_i$ only at 2 mM pipette (i.e. extracellular) solution. This behavior is similar to those observed for other TRPA1 agonists, such as MO and certain cannabinoids (Salas et al., 2009).

An elevation in $[\text{Ca}^{2+}]_i$ of up to $1 \mu\text{M}$ can be achieved by treating cells with $2 \mu\text{M}$ thapsigargin. Elevation in $[\text{Ca}^{2+}]_i$ can trigger whole-cell currents in TRPA1, TRPV1 and TRPA1-TRPV1 expressing cells (van der Stelt et al., 2005). Accordingly, we evaluated thapsigargin-gated currents (I_{THAP}) in CHO cells sensitive to CAP, MO and both CAP and MO, which are expressing TRPV1, TRPA1 and TRPA1-TRPV1, respectively. To maintain physiological conditions, recordings were carried out in perforated patch configuration with 2 mM extracellular Ca^{2+} . GFP expressing cells were not significantly activated by $2 \mu\text{M}$ thapsigargin ($5.9 \pm 1.4 \text{ pA}$, $n=6$; Fig 4A and 4B). In accordance with results presented in Figure 3, I_{THAP} was substantially smaller in TRPA1-TRPV1 compared to TRPA1 expressing cells (Fig 4A and 4B). In addition, TRPV1 positive cells generated small currents to thapsigargin application (Fig 4A and 4B) in agreement with a previous report (van der Stelt et al., 2005).

Unlike in CHO cells, the absence of the TRPV1 channel in sensory neurons leads to a reduction of TRPA1-currents triggered by MO and WIN55,212-2 (Salas et al., 2009). Therefore, we also measured I_{THAP} in TG sensory neurons from WT, TRPA1 KO and TRPV1 KO mice. Thapsigargin activated TG sensory neurons from these animals, and often displayed biphasic current (Fig 4D). Figure 4C and representative traces (Fig 4D) demonstrate that I_{THAP} is significantly reduced in sensory neurons lacking either TRPV1 or TRPA1. The presence of TRPV1 and TRPA1 channels in sensory neurons were confirmed by application of CAP and MO, respectively. Altogether, these data indicate that both TRPA1 and TRPV1 contribute to I_{THAP} in sensory neurons. However, other channels such as TRPC3, TRPC6 and Ca^{2+} -dependent Cl^{-} -channels expressed in nociceptors may influence the magnitude of I_{THAP} (Chen et al., 2006, Liu et al., 2010). Thus, small I_{THAP} (12.1 ± 4.2 pA, $n=10$) was recorded from capsaicin and mustard oil insensitive sensory neurons. This current is still statistically significantly smaller than I_{THAP} recorded from capsaicin-sensitive (TRPA1-KO animals) and mustard oil-sensitive (TRPV1-KO) sensory neurons.

The Magnitude of MO-activated responses depend on TRPV1 Ca^{2+} permeability

One of the major differences in the properties of TRPA1-mediated currents when comparing TRPA1- versus TRPA1-TRPV1-expressing systems is a voltage-dependent blockade of channel activity that is influenced by extracellular Ca^{2+} (Salas et al., 2009). Removal of $[Ca^{2+}]_i$ for inside-out, single-channel recording, and the chelation of intracellular Ca^{2+} with EGTA or BAPTA, did not reverse this effect (Salas et al., 2009, Staruschenko et al., 2010). Therefore, we sought to examine whether mutations in the pore of the TRPV1 channel affecting Ca^{2+} permeability could alter features of I_{MO} in TRPA1-TRPV1 co-expressing CHO cells. In this regard, there are several appropriate candidate mutations of TRPV1. The first is the D646N mutation (Garcia-Martinez et al., 2000). This mutation corresponds to the D682N mutation in the TRPV4 channel which is strongly linked to voltage-dependent pore blockage of TRPV4 by Ca^{2+} (Voets et al., 2002). Secondly, the Y671K mutation was reported to drastically reduce Ca^{2+} -permeability of the TRPV1 channel (Mohapatra et al., 2003). In contrast, the Y671D mutation significantly increases Ca^{2+} permeability of the TRPV1 channel, whereas the Y671A mutation has no reported effect on Ca^{2+} permeability (Mohapatra et al., 2003). Accordingly, we evaluated whether the D646N, Y671K, Y671D and Y671A mutations of TRPV1 altered the magnitudes of MO responses in TRPA1-TRPV1 co-expressing CHO cells.

We initially examined CAP responses in cells co-expressing TRPA1 and one of the TRPV1 previously mentioned TRPV1 mutants. In agreement with the previous report (Mohapatra et al., 2003), CAP (50 nM)-induced Ca^{2+} influx detected by Ca^{2+} -imaging was significantly increased or decreased in cells co-expressing TRPA1 and Y671D or Y671K mutations, respectively (Fig 5A). We next characterized MO (10 μ M)-induced Ca^{2+} influx in TRPA1/mutant TRPV1 co-expressing cells. Similar to CAP responses, the co-expression of TRPA1 with Y671D or Y671K TRPV1, but not with D646N and Y671A TRPV1, led to significantly increased or reduced MO-triggered Ca^{2+} influx, respectively (Fig 5A). It is possible that the increased Ca^{2+} -permeability of the Y671D TRPV1 channel could contribute to activation of TRPA1 by $[Ca^{2+}]_i$ accumulation in transfected CHO cells (Fig 3; Doerner et al., 2007, Zurborg et al., 2007). In addition, an increase in $[Ca^{2+}]_i$ could stimulate anandamide production that activates TRPV1 in concentration-response manner (van der Stelt et al., 2005). To evaluate the possibility that mutations in TRPV1 could affect association with TRPA1, co-immunoprecipitation (Co-IP) of *myc* tagged TRPA1 with wild-type (wt) and TRPV1 mutants was carried out. Figure 5B illustrates that mutations to TRPV1 did not ablate TRPA1-TRPV1 interactions. Altogether, mutations in TRPV1

affecting Ca^{2+} permeability influenced magnitudes of MO-evoked Ca^{2+} -influx, but did not disrupt TRPA1-TRPV1 interaction

MO-gated current (I_{MO}) properties are influenced by TRPV1 Ca^{2+} permeability

We next examined whether mutations affecting TRPV1 Ca^{2+} permeability alter properties of I_{MO} in TRPA1-TRPV1 expressing CHO cells. The magnitude of I_{MO} depends on extracellular Ca^{2+} in TRPA1-TRPV1, but not cells expressing TRPA1 alone (Salas et al., 2009). Therefore, we first investigated the effects of the TRPV1 mutations on I_{MO} peaks. In the presence of Ca^{2+} , I_{MO} ($V_h = -60$ mV) in TRPA1-TRPV1 expressing cells was substantially smaller than in cells expressing TRPA1 and either Y671D or Y671K TRPV1, but not TRPA1 with Y671A or D646N TRPV1 (Fig 6A and 6B). Further, I_{MO} was not affected by removal of external Ca^{2+} in TRPA1-Y671D TRPV1 or TRPA1-Y671K TRPV1 cells (Fig 6A and 6B). In contrast, I_{MO} recorded from TRPA1-WT, TRPA1-D646N or TRPA1-Y671A TRPV1 cells were significantly larger in Ca^{2+} -free solution and comparable with I_{MO} from TRPA1-Y671D or TRPA1-Y671K TRPV1 cells (Fig 6B). Altogether, the alteration of TRPV1 Ca^{2+} -permeability by mutations in position Y671 leads to I_{MO} characteristics in TRPA1-TRPV1 co-expressing cells similar to those displayed in cells expressing TRPA1 alone (Salas et al., 2009).

Since Ca^{2+} influences the voltage-dependency of I_{MO} (Salas et al., 2009), we sought to determine whether the TRPV1 mutations altered the rectification ratios of the I_{MO} current-voltage (I-V) curves. In this respect, D646N and Y671A mutations were not different from WT TRPV1 (Fig 6C and 6D). Removal of external Ca^{2+} also resulted in a dramatic reduction of pronounced outward rectification in I-V I_{MO} recorded from TRPA1-Y671A TRPV1 and TRPA1-TRPV1 expressing cells (Fig 6C and 6D). In contrast, in TRPA1-Y671D and TRPA1-Y671K TRPV1 cells, the I_{MO} I-V curves exhibited no substantial outward rectification. Moreover, the presence of external Ca^{2+} has no effect on I_{MO} I-V rectification in these cells (Fig 6C and 6D). Altogether, alterations in TRPV1 Ca^{2+} permeability abolishes the influence of extracellular Ca^{2+} on the voltage-dependency of I_{MO} , resulting in I_{MO} properties similar to those displayed in TRPA1-expressing cells (Salas et al., 2009).

Single-channel I_{MO} is regulated by TRPV1 Ca^{2+} permeability—To further evaluate properties of I_{MO} in TRPA1-Y671K TRPV1 co-expressing cells, we examined single-channel I_{MO} in cells co-expressing TRPA1 with Y671K TRPV1 channels. In the presence of extracellular Ca^{2+} , I_{MO} in TRPA1-Y671K TRPV1 cells responded similarly to TRPA1-expressing cells (Fig 7; (Staruschenko et al., 2010)). Thus, single-channel conductance did not undergo substantial rectification (Fig 7; $G_{60} = 66.3$ vs $G_{-60} = 44.9$), as for TRPA1 expressing CHO cells (Staruschenko et al., 2010). In TRPA1-TRPV1 co-expressing cells, the open probability (P_o) was significantly larger at positive holding potentials (Staruschenko et al., 2010). In contrast, P_o of I_{MO} in TRPA1-Y671K TRPV1 CHO cells displayed similar values at -60 mV and 60 mV (Fig 7; $P_{o60} = 0.67$ vs $P_{o-60} = 0.63$). In summary, alteration of Ca^{2+} permeability of the TRPV1 channels leads not only to whole-cell properties of I_{MO} in TRPA1-TRPV1 containing cells similar to those recorded from TRPA1-expressing cells (Salas et al., 2009), but also to single-channel characteristics observed in TRPA1-expressing cells (Staruschenko et al., 2010).

Discussion

Behavioral tests in TRPA1 and TRPV1 null-mutant mouse lines and pharmacological studies indicate that these channels play a critical role in pain transduction during pathological conditions triggered by tissue damage and inflammation (Caterina et al., 2000,

Davis et al., 2000, Bolcskei et al., 2005, Bautista et al., 2006, Kwan et al., 2006, Petrus et al., 2007). It has been suggested that activation and regulation of TRPA1 and TRPV1 by inflammatory mediators can underlie mechanisms for inflammatory hyperalgesia (Caterina et al., 2000, Bandell et al., 2004, Jordt et al., 2004, Bautista et al., 2006, Kwan et al., 2006). An elevation in $[Ca^{2+}]_i$ could play critical roles in both activation (Zurborg et al., 2007) and regulation (Schmidt et al., 2009) of the TRPA1 channel, as well as functional interaction between TRPA1 and TRPV1 (Bautista et al., 2006, McMahon and Wood, 2006). In addition, it was shown that $[Ca^{2+}]_e$ can modulate activity of the TRPA1 channel (Salas et al., 2009, Staruschenko et al., 2010).

Since TRPA1 and TRPV1 are extensively co-expressed in sensory ganglia (Story et al., 2003, Bandell et al., 2004, Jordt et al., 2004, Obata et al., 2005, Diogenes et al., 2007) and undergo functional interaction, we investigated how TRPV1 influences TRPA1 activation and modulation by Ca^{2+} . Our data demonstrate that: co-expression of TRPA1 and TRPV1 leads to significant suppression of $[Ca^{2+}]_e$ -gated activity at a physiological concentration of extracellular Ca^{2+} (2 mM); TRPV1 attenuates TRPA1-mediated $[Ca^{2+}]_i$ -gated activity at 2 mM of extracellular Ca^{2+} ; both TRPV1 and TRPA1 contributes to thapsigargin-gated currents in the expression system and sensory neurons; Ca^{2+} permeability of TRPV1 underlies the mechanism accounting for Ca^{2+} -dependent properties of TRPA1-mediated responses in TRPA1-TRPV1 co-expressing cells. It is also worth noting that with accordance with previous report (Ahern et al., 2005), TRPV1 can be activated by extracellular Ca^{2+} , and small currents are also detected at elevated levels of $[Ca^{2+}]_i$. Altogether, responses of Ca^{2+} -gated currents in TRPA1 and TRPA1-TRPV1-expressing cells are similar to currents generated by other TRPA1 agonists such as MO and WIN55,212-2 (Salas et al., 2009, Staruschenko et al., 2010). Most feasible explanation for these events is direct functional interaction between the TRPA1 and TRPV1 channels. However, some aspects of TRPA1-TRPV1 interaction could still remain indirect.

Responses to $[Ca^{2+}]_e$ are mutually modulated by the TRPV1 and TRPA1 channels. Interestingly, this modulation is biphasic (Fig 1A). Thus, at low concentrations of $[Ca^{2+}]_e$ (< 1 mM), it appears that activity in TRPA1-TRPV1 co-expressing cells is a sum of activities in TRPA1 and TRPV1 homomer expressing cells. However, at physiological concentrations of $[Ca^{2+}]_e$ (> 2mM), $[Ca^{2+}]_e$ -gated activity is suppressed in TRPA1-TRPV1 co-expressing cells (Fig 1A). One possible explanation for such a biphasic effect is that TRPV1 inhibits TRPA1-mediated responses only at presence of 2 mM $[Ca^{2+}]_e$ (Akopian et al., 2007, Salas et al., 2009, Staruschenko et al., 2010). This inhibition is noted only at negative potentials, because 2 mM of $[Ca^{2+}]_e$ generates strong voltage-dependence in cells co-expressing TRPA1 and TRPV1 (Salas et al., 2009).

What could be the physiological relevance in regulation of the activation of TRPA1-containing cells by 2mM $[Ca^{2+}]_e$? Such activation could result in a basal Ca^{2+} leak into TRPA1-expressing cells. The basal Ca^{2+} leak may be necessary to maintain the required Ca^{2+} -dependent cell processes such as transcription, translation and/or protein trafficking. However, high levels of Ca^{2+} -influx could lead to chemo-taxis and eventual death for sensory neurons. Thus, almost no sensory neurons from TRPV1 KO TG displayed large (>300 pA) MO-gated currents (Salas et al., 2009). One possible explanation for this finding is that TRPV1 suppresses the Ca^{2+} leak into sensory neurons and protects them from cell-death. In the absence of TRPV1, highly expressing TRPA1-positive cells (~8% of MO-sensitive sensory neurons; (Salas et al., 2009) either do not survive during development or suffer down-regulation in TRPA1 expression. Further, it was observed that many TRPA1-expressing CHO cells do not appear healthy in the absence of the TRPV1 channel (Bandell et al., 2004, Akopian et al., 2007, Salas et al., 2009). Altogether, TRPV1 may play certain protective roles for sensory neurons with high expression levels of TRPA1.

What could be the physiological relevance in the regulation of activation of TRPA1-containing cells by $[Ca^{2+}]_i$? In a TRPA1-TRPV1 expression system, TRPV1 attenuates $[Ca^{2+}]_i$ -gated and thapsigargin-gated activity. However, in sensory neurons, the absence of TRPV1 produces significantly smaller thapsigargin-gated currents. This behavior resembles responses generated by other TRPA1 agonists such as MO and WIN 55,212-2 (Salas et al., 2009). Single-channel analysis of I_{MO} in CHO cells and sensory neurons showed that intrinsic properties of I_{MO} are independent on the type of cells (i.e. CHO or sensory neurons) co-expressing TRPA1-TRPV1 (Staruschenko et al., 2010). This implies that the density of functional TRPA1 on the plasma membrane of sensory neurons may be reduced in cells lacking TRPV1 (Staruschenko et al., 2010). Considering these findings, it could be proposed that TRPA1-TRPV1 co-expression on the plasma membrane of sensory neurons is necessary to generate large responses to the elevation in $[Ca^{2+}]_i$ that can be triggered by inflammatory mediators such as bradykinin, histamine and 5-HT. Thus, in sensory neuron culture, it appear that particular conditions (i.e. NGF, absence of trypsin treatment) likely promote TRPA1 expression on the plasma membrane (Salas et al., 2009). In summary, in sensory neurons, TRPV1 could promote expression of functional TRPA1 on the plasma membrane; this in turn may be necessary to generate $[Ca^{2+}]_i$ -dependent responses to certain inflammatory mediators.

TRPA1 agonist such as MO, certain cannabinoids and $[Ca^{2+}]_i$ activate TRPA1 through different pathways (Kim and Cavanaugh, 2007, Macpherson et al., 2007, Zurborg et al., 2007, Cavanaugh et al., 2008). However, TRPV1 regulation of TRPA1 activities is independent of agonist nature (Salas et al., 2009). Regulation of TRPA1 activities by the TRPV1 channel happens only in the presence of 2 mM Ca^{2+} (Staruschenko et al., 2010). It appears that extracellular Ca^{2+} inhibits TRPA1-mediated responses in TRPA1-TRPV1, but not in TRPA1-expressing cells. Our data imply that the Y671 amino acid located in the trans-membrane domain 6 of the TRPV1 channel and responsible for Ca^{2+} permeability of TRPV1 (Mohapatra et al., 2003), is responsible for the blocking effects of external Ca^{2+} in TRPA1-TRPV1 expressing cells. Interestingly, mutation of TRPV1 in the Y671 position does not alter co-IP of the channels. This finding provides additional evidence on functional interaction of TRPA1 and TRPV1, and regulation of TRPA1 activities by the TRPV1 channel. This suggests that the main modification to TRPA1 in TRPA1-TRPV1 co-expressing cells happens in or near pore. Although, it still remains a possibility that TRPV1 presence will alter the interaction of certain agonists with the TRPA1 channel. Thus, it is possible that a binding position for drugs could modulate TRPA1-responses by controlling Ca^{2+} permeability of TRPV1. Altogether, the co-expression of TRPA1 and TRPV1 channels that influence the activation of cells by Ca^{2+} may sub-serve regulatory control of peripheral mechanisms of inflammatory hyperalgesia.

Acknowledgments

We would like to thank Dr. David Julius (UCSF, San Francisco, CA) for kindly gifting rTRPV1 cDNA, Dr. Ardem Patapoutian (The Scripps Research Institute, San Diego, CA) for kindly gifting TRPA1 cDNA and Dr. Carla Nau (University Erlangen-Nuremberg, Erlangen, Germany) for providing Y671 TRPV1 cDNA. Research was supported by grants NS061884 (to NAJ) and DE019311 and DE017696 (to ANA).

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Figure 1. Activation of TRPA1, TRPV1 and TRPA1-TRPV1 expressing CHO cells by extracellular Ca²⁺

(A) Activation of TRPV1, TRPA1 and TRPA1-TRPV1 expressing CHO cells by a variety concentrations of extracellular Ca²⁺ ([Ca²⁺]_e). Recordings were carried out in cell-attached configuration in voltage-clamp (V_h=-60mV) mode. Indicated concentrations of Ca²⁺ (X-axis) were included in the pipette solution. The bath solution contained 100nM Ca²⁺, which approximately corresponds to basal intracellular concentration of Ca²⁺. Recordings were made for 1 min. Open probability (P_o) was measured for recordings between time points of 10 sec and 50 sec. Data analysis was by unpaired *t*-test. n=9-18. (B) Representative traces show single-channel activity during ≈10 sec stretch. Expression constructs and concentrations of Ca²⁺ in the pipette solution are indicated above the traces.

Figure 2. Activation of TRPA1, TRPV1 and TRPA1-TRPV1 expressing CHO cells by intracellular Ca²⁺ in Ca²⁺-free extracellular solution

(A) Activation of TRPV1, TRPA1 and TRPA1-TRPV1 expressing CHO cells by a variety concentrations of intracellular Ca²⁺ ([Ca²⁺]_i). Recordings were carried out in inside-out configuration in voltage-clamp (V_h=-60mV) mode. Indicated concentrations of Ca²⁺ (X-axis) were applied to the patches for 1 min. The pipette solution is Ca²⁺-free. Open probability (P_o) was measured for recordings between time points of 10 sec and 50 sec. Data analysis was by unpaired *t*-test. n=10-18. (B) Representative traces show single-channel activity during ≈10 sec stretch. Expression constructs and concentrations of applied Ca²⁺ (i.e. [Ca²⁺]_i) are indicated above the traces.

Figure 3. Activation of TRPA1 and TRPA1-TRPV1 expressing CHO cells by intracellular Ca²⁺ in 2 mM Ca²⁺ extracellular solution

(A) Activation of TRPA1 and TRPA1-TRPV1 expressing CHO cells by 1 μM and 10 μM of [Ca²⁺]_i. Recordings were carried out in inside-out configuration in voltage-clamp (V_h = -60 mV) mode. Indicated concentrations of Ca²⁺ (under X-axis) were applied to the patches for 1 min. Pipette solutions contain 2 mM Ca²⁺. Open probability (P_o) was measured for recordings between time points of 10 sec and 50 sec. Data analysis was by unpaired *t*-test. n=10-15. (B) Representative traces show single-channel activity during ≈10 sec stretch. Expression constructs and concentrations of applied Ca²⁺ (i.e. [Ca²⁺]_i) are indicated above the traces.

Figure 4. Activation of TRPA1, TRPV1 and TRPA1-TRPV1 expressing CHO cells and sensory neurons by thapsigargin

(A) Activation of TRPV1, TRPA1 and TRPA1-TRPV1 expressing CHO cells by 2 μ M thapsigargin. Recordings were carried out in perforated patch whole-cell configuration in voltage-clamp mode. Magnitude of I_{THAP} at $V_h = -60$ mV is presented. Thapsigargin was applied for 1 min. Expression plasmids are indicated below X-axis. Numbers of analyzed cells are noted. (B) Representative current-voltage (I-V) traces at maximal responses to 2 μ M thapsigargin are presented for CHO cells expressing TRPV1, TRPA1 and TRPA1-TRPV1. I-V was generated by voltage ramp (from -100 mV to +60 mV applied for 450 msec). The ramp was applied to cells every 3 sec. (C) Activation of sensory neurons from trigeminal ganglia (TG) of wild-type (WT), TRPV1 (TRPV1 KO) and TRPA1 (TRPA1 KO) null-mutant mice by 2 μ M thapsigargin. Recordings were carried out in perforated patch whole-cell configuration in voltage-clamp mode ($V_h = -60$ mV). Thapsigargin was applied for 1 min. Mouse lines are indicated below the X-axis. Numbers of analyzed cells are noted. (D) Representative whole-cell voltage clamp I_{THAP} traces recorded from sensory neurons of WT and TRPV1 KO TG. Application times of 2 μ M thapsigargin are indicated by horizontal bars.

Figure 5. TRPV1 mutations affect magnitude of MO responses in TRPA1-TRPV1 cells, but do not ablate TRPA1-TRPV1 co-immunoprecipitation (Co-IP)
(A) MO- or CAP-induced accumulation of $[Ca^{2+}]_i$ in CHO cells co-transfected with TRPA1 and wild-type (WT) TRPV1 or indicated TRPV1 mutants. Numbers of responding CHO cells are indicated. **(B)** N-terminally *myc*-tagged TRPA1 was co-expressed with TRPV1 (wt) or TRPV1 mutants (D646N, Y671A, Y671D and Y671K; marked DN, YA, YD and YK respectively) in CHO cells. Anti-TRPV1 and anti-*myc* immunoreactivity was detected by WB which are noted at the right side of the panels. WBs were performed after co-IP with either TRPV1 or *myc* antibodies (noted above panels). *Myc*-TRPA1 (upper panel) and TRPV1 (lower panel) corresponding bands are denoted by arrows.

Figure 6. The Y671 TRPV1 mutation effects on properties of I_{MO} in TRPA1/TRPV1 cells
 Recordings were carried out in perforated patch voltage clamp ($V_h = -60$ mV) configuration. **(A)** Representative traces of I_{MO} in TRPA1-TRPV1 (marked WT) and TRPA1-Y671K TRPV1 (marked Y671K) CHO cells bathed in extracellular solution containing 2 mM or 0 mM Ca^{2+} . Durations of drug applications are denoted. **(B)** Magnitude of I_{MO} depends on mutations in TRPV1 and extracellular Ca^{2+} . Cells were bathed in SES (2mM Ca^{2+}) or 0Ca-ES (Ca^{2+} -free). External solutions and mutations of TRPV1 are indicated. Numbers of recorded cells are marked within bars. **(C)** Representative I_{MO} current-voltage (I-V) traces in TRPA1-TRPV1 (marked WT), TRPA1-Y671K TRPV1 (marked Y671K) TRPA1-Y671D TRPV1 (marked Y671D) and TRPA1-Y671A TRPV1 (marked Y671A) CHO cells bathed in extracellular solution containing 2 mM Ca^{2+} or Ca^{2+} -free solution. **(D)** Rectification ratios of I-V I_{MO} are differentially affected by mutations in TRPV1. $n=6-14$.

Figure 7. Single-channel I_{MO} in cells expressing TRPA1/Y671K TRPV1

Recordings were performed in cell-attached configuration in voltage-clamp mode from TRPA1/Y671K TRPV1 co-transfected CHO cells. **(A)** Representative traces of single-channel I_{MO} (25 μ M) were recorded at different indicated holding potentials. *c* indicates the closed state of the channel **(B)** Averaged current-voltage relationship for single-channel I_{MO} (25 μ M), $n=8-10$. **(C)** Summary graphs of open probability (P_o) for I_{MO} at +60 and -60 mV, $n=8-10$.