Published in final edited form as:

Eur J Neurosci. 2009 April ; 29(8): 1568–1578. doi:10.1111/j.1460-9568.2009.06702.x.

TRPA1-mediated responses in trigeminal sensory neurons: interaction between TRPA1 and TRPV1

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Abstract

The Transient Receptor Potential A1 channel (TRPA1) is involved in the transduction of inflammation-induced noxious stimuli from the periphery. Prior studies have characterized the properties of TRPA1 in heterologous expression systems. However, there is little information on the properties of TRPA1-mediated currents in sensory neurons. A capsaicin-sensitive subset of trigeminal ganglion (TG) sensory neurons was activated with TRPA1-specific agonists, mustard oil and the cannabinoid, WIN55,212. Mustard oil- and WIN55,212-gated currents exhibited marked variability in their kinetics of activation and acute desensitization. TRPA1-mediated responses in neurons also possess a characteristic voltage-dependency with profound outward rectification that is influenced by extracellular Ca^{2+} and the type and concentration of TRPA1-specific agonists. Examination of TRPA1-mediated responses in a TRPA1-containing cells indicated that the features of neuronal TRPA1 are not duplicated in cells expressing only TRPA1, and instead, can be restored only when TRPA1 and TRPV1 channels are co-expressed. In summary, our results suggest that TRPA1-mediated responses in sensory neurons have distinct characteristics that can be accounted for by the co-expression of the TRPV1 and TRPA1 channels.

Introduction

A wide range of transient receptor potential (TRP) channels, including TRPA1, control the processing of information by nociceptors, a specialized class of damage sensing afferent neurons (Tominaga and Caterina, 2004; Wang and Woolf, 2005; Tai et al., 2008). Physiological studies using either TRPA1 null-mutant mice, TRPA1 antisense knock-down or TRPA1-specific antagonists have demonstrated that this channel plays important roles in the development of thermal and/or mechanical hyperalgesia in certain inflammatory (Obata et al., 2005; Bautista et al., 2006; Kwan et al., 2006; Klionsky et al., 2007; Petrus et al., 2007) and nerve injury pain models (Katsura et al., 2006).

TRPA1 was originally identified as a cold-gated (Story et al., 2003) and a putative mechanical transduction channel (Corey et al., 2004). It is predominantly expressed in the TRPV1-positive subset of sensory neurons and regulated by nerve growth factor (NGF) (Story et al., 2003; Kobayashi et al., 2005; Diogenes et al., 2007). TRPA1 can be activated

by a variety of exogenous and endogenous ligands such as mustard oil (MO), formalin (McNamara et al., 2007), 4-hydroxynonenal (Macpherson et al., 2007b; Trevisani et al., 2007), certain cannabinoids (Jordt et al., 2004; Akopian et al., 2008) and allicin (Bautista et al., 2005; Macpherson et al., 2005). Studies of the TRPA1 channel in heterologous expression systems show that it has unique characteristics in terms of activation modes and biophysical properties. Thus, the channel has mild Ca^{2+} permeability and almost linear current-voltage characteristics under physiological conditions (Story et al., 2003; Jordt et al., 2004; Karashima et al., 2007; Kim and Cavanaugh, 2007). It was also demonstrated that Ca^{2+} inhibits MO-gated currents (Nagata et al., 2005), and is capable of activating TRPA1 in a heterologous expression system (Doerner et al., 2007; Zurborg et al., 2007).

Despite prior studies describing certain TRPA1 channel characteristics in sensory systems, no study has systematically evaluated the pharmacological and biophysical properties of TRPA1-mediated responses in sensory neurons. Therefore, we have characterized TRPA1-mediated currents in adult rat trigeminal ganglion (TG) sensory neurons. Since TRPA1 is primarily co-expressed with TRPV1 in a subset of sensory neurons, TRPA1 has also been studied in TRPA1 as well as TRPA1/TRPV1-coexpressing cells.

Material and Methods

Cell cultures

Expression plasmids of green fluorescent protein (GFP; pEGFP-N1 (Clontech), TRPV1 (accession number - NM031982) in pcDNA3 (Invitrogen, Carlsbad, CA) and TRPA1 (NM177781) in pcDNA5/FRT (Invitrogen) were delivered into Chinese hamster ovary (CHO) cells using PolyFect (Qiagen, Valencia, CA) according to manufacturers' protocols. CHO cells were used within 36-72 h after transfection.

Adult Sprague-Dawley rat TG neurons were cultured as previously described (Akopian et al., 2007), and were plated at low-density on poly-D-lysine/laminin coated coverslips (Clontech, Palo Alto, CA). TG cultures were maintained in the presence of 100 ng/ml 7.02S NGF (Harlan, Indianapolis, IN). The experiments were carried out 24-48 h after preparation. Note that trypsin was not used in sensory neuron culture preparation, because it strongly affected the functional expression of TRPA1 that is not fully recovered within 24-48 h of culturing.

Electrophysiology

The whole-cell voltage-clamp ($V_h = -60$ mV) 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 pCLAMP9.0 software (Axon Instruments, Union City, CA). Currents were filtered with an 8-pole, low pass Bessel filter at 0.5 kHz (whole-cell) and sampled at 2 kHz. Borosilicate pipettes (Sutter, Novato, CA) were polished to resistances of 2-4 M Ω (whole-cell) in standard pipette solution. Access resistance (R_s) was compensated when appropriate to achieve $R_s < 10$ M Ω . Cell diameters were calculated using $d = \sqrt{100 \cdot C_m / g}$, where d (μm) is cell diameter and C_m (pF) is membrane capacitance. Concentration-response curves were fitted to the Hill equation $I/I_{\max} = 1/[1 + (EC_{50}/C)^h]$, where EC_{50} is the half maximal effective concentration, C is the drug concentration, h is the Hill coefficient and $I_{\max}$ is the maximum current. I-V relations were obtained as previously described (Liu et al., 1997) using voltage-ramp protocols applied at a frequency of 1 Hz to TG neurons and 0.33 Hz to CHO cells. The currents were ascertained by subtraction of control traces as previously described (Liu et al., 1997). I-V relations were established only at peak currents. To identify the E_{rev} of shallow I-V curves (i.e. small inward currents), fitting with standard exponential functions were used. Liquid junction

potentials (E_{LJ}) were subtracted from measured E_{rev} to calculate corrected reverse potentials, which are presented in this study. Relative ion permeability ratios were calculated using the following constant-field equations for bi-ionic solutions:

$$P_X/P_Y = [Y]_i/[X]_o \cdot \exp(E_{rev} \cdot F/RT) \text{ for } P_{Na}/P_{Cs} \text{ and } P_{NMDG}/P_{Cs},$$

$$P_{Ca}/P_Y = \{1 + \exp(E_{rev} \cdot F/RT)\} \cdot \{[Y]_i/4[Ca]_o \cdot \exp(E_{rev} F/RT)\} \text{ for } P_{Ca}/P_{Cs},$$

where $RT/F=25.5$ at $T=20^\circ\text{C}$, and E_{rev} are reverse potentials (in mV) at given ionic conditions: $[Na]_o=143$ mM, $[Cs]_i=145$ mM, $[Ca]_o=5$ mM and $[NMDG]_o=140$ mM.

Solutions

Standard external solution (SES) contained (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 D-glucose and 10 HEPES, pH 7.4. In the Ca²⁺-free external solution (0 Ca-ES), 5 mM EGTA was added to buffer ambient Ca²⁺. In a separate set of experiments, 1 mM MgCl₂ was omitted from 0Ca-ES (0 mM Ca+Mg). To determine ion permeability, solutions containing (in mM) 10 HEPES, 10 D-glucose, 0.0003 TTX (Tocris, Ellisville, MO) and 0.1 verapamil (Tocris), in addition to the stated concentration of Na⁺, Ca²⁺ and Mg²⁺. N-methyl-D-glucamine (NMDG) was used as a Na⁺ substitute. The standard pipette solution (SIS) consisted of (in mM): 140 KCl, 1 MgCl₂, 1 CaCl₂, 10 EGTA, 10 D-glucose, 10 HEPES, 0.2 Na-GTP and 2.5 Mg-ATP, pH 7.3. To suppress voltage-activated K⁺ currents, the pipette solution (Cs-IS) was modified as following (in mM): 140 CsCl, 2 EGTA, 10 HEPES, 0.2 Na-GTP and 2.5 Mg-ATP, pH 7.3. Drugs were applied using a fast, pressure-driven, computer controlled 8-channel system (ValveLink8; AutoMate Scientific, San Francisco, CA). Application of cannabinoids required siliconization of the perfusion system with Sigma Coat (Sigma).

Data analysis

For detailed statistical analysis, GraphPad Prism 4.0 (GraphPad, San Diego, CA) was used. The data in Figs are presented as the mean $\pm$ standard error of the mean (SEM), with the value of n referring to the number of analyzed cells. 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 paired or unpaired t -test. A difference was accepted as significant when $p<0.05$, <0.01 or <0.001 and are indicated as *, ** and ***, respectively.

Results

Ca²⁺-dependency of mustard oil and WIN-gated currents in sensory neurons

Mustard oil (MO) and an aminoalkylindole cannabinoid, WIN 55,212-2 (WIN), were used in this study, because (1) in sensory neurons cultured with NGF, the application of WIN (5-100 μM) and MO (5-500 μM) activate currents and generate Ca²⁺-influx specifically and exclusively via the TRPA1 channel (Bautista et al., 2006; Akopian et al., 2008); (2) they are structurally distinct agonists for TRPA1 that possibly activate TRPA1 via different mechanisms (Hinman et al., 2006; Macpherson et al., 2007a); and (3) different TRPA1-specific agonists display distinct current properties in the heterologous expression system (Hill and Schaefer, 2007).

The application of MO and WIN generated whole-cell inward currents (I_{MO} and I_{WIN}) in a subset of small-to-medium-sized neurons (Figs 1A and 1B). In some experiments, recordings were carried out with a K-gluconate pipette solution which demonstrated that TRPA1 is selective for cations in sensory neurons (data not shown). In accordance with

previously published results (Story et al., 2003; Jordt et al., 2004; Diogenes et al., 2007), I_{MO} and I_{WIN} was almost exclusively detected in capsaicin-sensitive sensory neurons. Neuronal cell-size distributions of I_{MO} and I_{WIN} and co-expression with capsaicin-gated currents (I_{CAP}) are presented in the Table 1. I_{MO} and I_{WIN} exhibited concentration-dependent relations that can be fitted with the Hill equation with the following parameters: EC_{50} (in μM) and h for I_{MO} (18; 1.4) and I_{WIN} (18; 1.7); and the I_{max} for WIN and MO were -271.5 ± 67.49 pA ($n=15$) and -481 ± 90.93 pA ($n=12$), respectively (Fig 1C).

I_{MO} and I_{WIN} displayed complicated kinetics that neither fitted with exponential functions, nor demonstrated uniform activation curves (Fig 1A and 1B). In addition, a wide cell-to-cell variation in kinetics of the currents was observed. Overall, I_{MO} and I_{WIN} displayed relatively slow activation kinetics (Fig 2A; see also (Patwardhan et al., 2006; Akopian et al., 2007)). Both currents exhibited a delayed rise in some neurons and little-to-no desensitization during a 2min drug application in the majority of analyzed cells. Nevertheless, fast activating and desensitizing I_{MO} and I_{WIN} were recorded from a small subset ($\approx 22\%$ of MO and WIN-responsive cells) of sensory neurons (Fig 1A and 1B; second traces from the right).

To determine the effect of Ca^{2+} on I_{MO} and I_{WIN} in sensory neurons, we recorded from separate groups of neurons bathed either in extracellular solution with 2 mM Ca^{2+} or in a Ca^{2+} -free solution. Figure 2B and representative traces (Fig 1A and 1B) show that, unlike results from TRPA1-expressing cell lines (Nagata et al., 2005), Ca^{2+} strongly and significantly attenuates peak I_{MO} and I_{WIN} values in TG neurons. Thus, for I_{MO} , removal of Ca^{2+} from the extracellular solution increased the magnitude of the current >2 -fold (for 0Ca-ES -634.9 ± 120.0 pA, $n=12$ vs SES -292.3 ± 39.57 pA, $n=34$, $p > 0.001$). This effect of Ca^{2+} is even more pronounced for I_{WIN} than for I_{MO} (Fig 2B). The removal of both Mg^{2+} and Ca^{2+} did not further increase the magnitude of I_{MO} and I_{WIN} (Fig 2B). An analysis of the activation kinetics of I_{MO} and I_{WIN} revealed that this parameter was not altered by extracellular Ca^{2+} levels (Fig 2A; see also (Akopian et al., 2007)). In addition, a majority of cells exhibited little-to-no desensitization of I_{MO} or I_{WIN} in Ca^{2+} -free solutions (Fig 1A and 1B).

Current-voltage characteristics of I_{MO} and I_{WIN} in sensory neurons

The evaluation of current-voltage (I-V) relationships under different experimental conditions defines important functional parameters of ligand-gated channels. In physiological solution (SES), the I-V relationships for I_{WIN} (Fig 3A) and I_{MO} (Fig 3B) exhibited a reversal potential close to 0 mV (for I_{MO} , 1.9 ± 1.18 mV, $n=13$ and for I_{WIN} , -0.45 ± 0.92 mV, $n=16$) and pronounced outward rectification with a ratio (I_{+60}/I_{-60}) of 9 ± 1.6 ($n=13$) for I_{MO} and 12 ± 2.3 ($n=15$) for I_{WIN} . Removal of Ca^{2+} from the extracellular solution drastically reduced the rectification ratio for I_{MO} (in SES, 9 ± 1.6 , $n=13$ vs in 0Ca-ES, 2.45 ± 0.58 , $n=17$; t -test; $p < 0.001$) as well as I_{WIN} (in SES, 12 ± 2.3 , $n=15$ vs in 0Ca-ES, 2.6 ± 0.47 , $n=20$; t -test; $p < 0.001$). Thus, the influential factor controlling the voltage dependency of I_{MO} and I_{WIN} was the presence of extracellular Ca^{2+} (Fig 3A and 3B). The mild outward rectification detected in the absence of Ca^{2+} still persisted in a symmetrical extra-intracellular solution (for $I_{WIN} \approx 2.1$, $n=7$, Fig 3C; and for $I_{MO} \approx 1.77$, $n=4$). This voltage-dependent blocking effect of Ca^{2+} could also explain the observation that the amplitude of I_{MO} and I_{WIN} recorded at $V_h = -60$ mV is 2.5-3-fold large when neurons were bathed in a Ca^{2+} -free solution (Fig 2B).

To define the contribution of various cations to I_{MO} and I_{WIN} , I-V traces were recorded from neurons bathed in solutions of different cationic composition. I_{MO} and I_{WIN} showed a similar behavior in this respect. Thus, I_{WIN} did not discriminate between Na^+ and Cs^+ (Fig 3C), but showed a clear preference to Ca^{2+} with relative permeability ratios

$P_{Ca}:P_{Na}:P_{Cs}:P_{NMDG} = 5.5:1:0.88:0.07$. For I_{MO} , the Ca^{2+} permeability relative to Na^{+} (i.e. $P_{Ca}:P_{Na}$) was approximately 4.3.

We next examined possible parameters and reasons underlining the rectification of I_{WIN} . Neither the slope of the voltage ramp (i.e. dV/dt) nor application of hyperpolarized voltage ramps changed the I_{WIN} I-V relations derived from the delivery of depolarized ramps to whole-cell patched neurons (Fig 4A; mean of rectification ratios were 12, 13.5 and 13.1 ($n=6-15$) for depolarized ramp, hyperpolarized ramp and steep depolarized ramp, respectively). Acute and pharmacological desensitization of the channels can also affect the shape of the I-V relationship and its rectification ratio. Thus, it was reported that an increase in outward rectification ratios occurred after tachyphylaxis of I_{CAP} in sensory neurons (Piper et al., 1999). Consistent with this finding, the rectification ratio of I_{CAP} (300 nM) was substantially increased in sensory neurons after tachyphylaxis (3.4 vs. 7.1; $p<0.001$; Fig 4B). In contrast, the I-V relation of I_{WIN} was not significantly changed (15.3 vs 13.7; $p>0.05$; Fig 4B).

We next evaluated whether the rectification ratios of I_{MO} and I_{WIN} were affected by the concentration of agonists. Threshold (5 μM) and near maximally effective (50 or 100 μM) concentrations of MO and WIN were selected to record I-V relations from sensory neurons. Figures 4C and 4D demonstrate that the magnitude of rectification ratios for both currents significantly increased when the agonist concentration are reduced from 100 μM to 5 μM for MO (4.3 vs. 16.8; $p<0.001$; Fig 4C) and from 50 μM to 5 μM for WIN (10.9 vs. 30.3; $p<0.001$; Fig 4D). Interestingly, this concentration-dependent alteration in rectification ratios was not noted when recordings were carried out in a Ca^{2+} -free solution (Fig 4C and 4D). Altogether, the I_{MO} and I_{WIN} voltage-dependency in sensory neurons is regulated mainly by the presence of extracellular Ca^{2+} , and the types and/or concentrations of TRPA1-specific agonists.

Characterization of I_{MO} and I_{WIN} in TRPA1- and TRPA1/TRPV1-expressing CHO cells

Several properties of I_{MO} , including voltage- and Ca^{2+} -dependency, in sensory neurons differ from previously reported properties of I_{MO} in TRPA1-expressing cell lines (Bandell et al., 2004; Jordt et al., 2004; Nagata et al., 2005; Zurborg et al., 2007). One possible reason is that the TRPA1/TRPV1 co-expression that occurs in a majority of TRPA1-containing sensory neurons could alter TRPA1-mediated responses. To test this hypothesis, we next compared the properties of I_{MO} and I_{WIN} acquired from TRPA1 expressing (A1- I_{MO} or A1- I_{WIN}) and TRPA1/TRPV1 co-expressing CHO cells (A1/V1- I_{MO} or A1/V1- I_{WIN}).

MO- (10 μM) and WIN- (25 μM) gated currents exhibited faster activation kinetics in TRPA1-expressing than in the TRPA1/TRPV1-co-expressing cells (Fig 5A; the 5-95% rise time for A1- I_{WIN} ; 17.8 ± 3.5 sec vs for A1/V1- I_{WIN} 29.22 ± 7.1 sec; $p<0.01$). The activation kinetics of A1- I_{MO} and A1- I_{WIN} were slightly slower in Ca^{2+} -free solution (for A1- I_{WIN} , in SES, 17.8 ± 3.5 sec vs. in 0Ca-ES, 22.8 ± 6.6 sec). However, removal of extracellular Ca^{2+} did not affect the activation kinetics of A1/V1- I_{MO} and A1/V1- I_{WIN} in the TRPA1/TRPV1-coexpressing CHO cells.

A1- I_{WIN} and A1/V1- I_{WIN} reached the half-time for acute desensitization in approximately 70% ($n=42$) and 40% ($n=53$) cells, respectively, within 2 min of drug application (Fig 5A). A1- I_{MO} and A1/V1- I_{MO} exhibited similar behaviors in this respect. Removal of extracellular Ca^{2+} significantly reduced the development of acute desensitization of A1- I_{MO} and A1- I_{WIN} (data not shown, (Akopian et al., 2007). However, this effect was not apparent for either A1/V1- I_{MO} or A1/V1- I_{WIN} . One possible explanation for this effect is that the co-expression of TRPV1 with TRPA1 reduces desensitization of I_{MO} and I_{WIN} (Akopian et al., 2007). Together, these findings replicate prior observations of the role of Ca^{2+} in regulating I_{MO} and I_{WIN} in expression systems (Jordt et al., 2004; Nagata et al., 2005), and extend them by

demonstrating that the TRPV1 channel also regulates TRPA1 function. Further, the kinetic characteristics of I_{MO} and I_{WIN} in TRPA1-expressing cells differ from kinetic parameters of I_{MO} and I_{WIN} recorded from TRPA1/TRPV1-expressing cells or TG neurons.

We next evaluated the influence of extracellular Ca^{2+} on the magnitude of I_{MO} and I_{WIN} in TRPA1- and TRPA1/TRPV1-expressing cells. The removal of external calcium produced insignificant changes (5-15%) in the density of $A1-I_{MO}$ and $A1-I_{WIN}$ at $V_h = -60$ mV (Fig 5B). However, the co-expression of TRPV1 with TRPA1 alters the Ca^{2+} -dependency of I_{MO} and I_{WIN} in CHO cells. Thus, Figure 5B illustrates that I_{WIN} and I_{MO} in these co-expressing cells are significantly larger in Ca^{2+} -free solution.

The voltage-current (I-V) analysis showed that $A1-I_{MO}$ and $A1-I_{WIN}$ as well as $A1/V1-I_{MO}$ and $A1/V1-I_{WIN}$ exhibited close to 0 mV reversal potentials (for 25 μ M WIN, 2.8 ± 1.4 mV, $n=14$; and for 10 μ M MO 4.2 ± 3.1 mV, $n=12$). However, there were ~6-fold differences in the outward rectification ratios for I_{MO} and I_{WIN} recorded from TRPA1- versus TRPA1/TRPV1-expressing CHO cells (Figs 6A and 6C; in TRPA1-expressing cells for MO, 1.3 ± 0.24 ; $n=8$; for WIN, 1.7 ± 0.16 ; $n=17$ and in TRPA1/TRPV1-expressing cells for MO, 6 ± 1.3 , $n=12$; for WIN, 8 ± 2.8 , $n=13$). As a further distinction, the rectification ratios of $A1-I_{MO}$ and $A1-I_{WIN}$ were not substantially dependent on the presence of extracellular Ca^{2+} , while there was a voltage-dependent alteration in $A1/V1-I_{MO}$ and $A1/V1-I_{WIN}$ that was influenced by Ca^{2+} (Figs 6A and 6C).

We next evaluated I-V relationships in TRPA1-expressing CHO cells treated with two different concentrations of agonists (i.e. WIN and MO). Figure 6B illustrates that $A1-I_{MO}$ and $A1-I_{WIN}$ I-V relationships were not affected by either types or concentrations of antagonists. In contrast, the outward rectification ratios of TRPA1-mediated currents in TRPA1/TRPV1-expressing CHO cells were strongly influenced by the types and concentrations of the specific agonists (Fig 6B). Thus, the $A1/V1-I_{MO}$ activated by 50 μ M MO produced an outward rectification with a ratio of 3.7 ± 2.2 , $n=7$ (Fig 6B), while the generation of 500 μ M MO-gated current revealed an almost negligent rectification (1.65 ± 0.54 , $n=6$). This implies that the voltage-dependency of TRPA1-mediated currents in TRPA1/TRPV1-expressing cells is influenced by the magnitude of the currents (at $V_h = -60$ mV) which, in turn, is specified by the types (i.e. agonist intrinsic activity) and concentrations of agonists, and expression systems (i.e. heterologous expression systems versus sensory neurons).

I_{MO} in wild-type and TRPV1 null-mutant mouse TG sensory neurons

The evaluation of I_{MO} and I_{WIN} in TRPA1- and TRPA1/TRPV1-expressing CHO cells (Figs 5 and 6) indicate that the characteristic properties of neuronal TRPA1-mediated currents are not observed in TRPA1-expressing CHO cells, but can be reproduced when TRPA1 and TRPV1 are co-expressed in the CHO cells. Since the biochemical machinery in CHO cells and sensory neurons could be very different and over-expression of TRPA1 and TRPV1 in CHO cells could lead to quantitatively (or even qualitatively) distinct results, we next evaluated the modulation of TRPA1 by TRPV1 in sensory neurons by comparing I_{MO} features between neurons derived from TRPV1 null-mutant (KO) mice and wild type controls (WT). I_{MO} measured from TRPA1-expressing CHO cells was on average larger than I_{MO} generated in TRPA1/TRPV1-expressing CHO cells (Fig 5B). However, recordings of I_{MO} in TG neurons isolated from WT and TRPV1 KO mice demonstrated that the magnitude of I_{MO} in sensory neurons from TRPV1 KO mice is significantly smaller compared to I_{MO} recorded from TG neurons of WT mice (Fig 7A and 7C). In addition, I_{MO} was recorded in 35.2% (50/142) of TRPV1 KO mouse small-to-medium sized TG neurons, while 52.3% (45/86) of small-to-medium sized TG neurons of WT mice were MO responsive. Another notable difference was that unlike TRPA1-expressing CHO cells, no

difference in the activation kinetics of I_{MO} was detected in TG neurons from WT versus TRPV1 KO mice (Fig 7D).

We next examined the voltage- and Ca^{2+} -dependency of I_{MO} in these mouse lines. The I-V relationships for I_{MO} (25 μ M) exhibited a reversal potential close to 0 mV (Fig 7B). Figure 7A (representative traces) and 7C illustrate that the removal of extracellular Ca^{2+} leads to an increase in I_{MO} magnitude in WT, but not TRPV1 KO mice. The voltage-dependency of I_{MO} in WT mouse neurons (Fig 7B and 7E) showed similar characteristics to that obtained from rat TG neurons, with both exhibiting strong Ca^{2+} -dependent outward rectification. In contrast, the I_{MO} I-V curve from TRPV1 KO neurons was similar to I-V currents recorded from TRPA1-expressing CHO cells (Fig 7B and 7E). Moreover, the concentration of MO also affected the rectification ratio of I-V I_{MO} only in WT mouse, but not in the TRPV1 KO TG neurons (Fig 7G). Thus, in neurons from WT mice when recordings were carried out in SES, I_{MO} stimulated by 5 μ M MO produced a I-V curve with outward rectification of 14 ± 2 , $n=12$, while increasing the MO concentration to 25 μ M or 100 μ M reduced the rectification ratio to 7.3 ± 1.1 , $n=12$ and 2.8 ± 0.94 , $n=9$, respectively. However, this effect was not observed in TG neurons from TRPV1 KO mice, in which both 25 μ M MO and 100 μ M MO generated I_{MO} voltage-dependent relations with quite similar rectification ratios. In summary, studies of I_{MO} in WT and TRPV1 KO mouse TG neurons confirmed that several important properties of TRPA1-mediated currents are modulated by the presence of TRPV1.

Discussion

Behavioral studies indicated that the TRPA1 channel plays a key role in pain transduction, especially during pathological conditions triggered by tissue damage and inflammation (Obata et al., 2005; Bautista et al., 2006; Kwan et al., 2006; Petrus et al., 2007). TRPA1 is thoroughly characterized in heterologous expression systems. However, there is a lack of information on TRPA1 properties in sensory neurons. We evaluated the TRPA1 channel activated by two distinct agonists, MO and WIN 55, 212, in adult rat TG sensory neurons. A main conclusion of this study is that MO and WIN-gated responses in sensory neurons are distinct and can be accounted for by co-expression of TRPA1 and TRPV1 in both sensory neurons and a heterologous expression system. Further, substantial differences are observed in the functional properties of TRPA1-mediated currents in TRPA1- versus TRPA1/TRPV1-expressing sensory neurons.

Our results demonstrate that MO responses are significantly diminished in cultured sensory neurons from TRPV1 KO mice (Fig 7C). This observation was reported for *in vitro* (Akopian et al., 2007) and *in vivo* behavioral responses in TRPV1 KO mice (unpublished observation; see also (Bautista et al., 2006)). Further, our unpublished data indicate that WIN and bradykinin responses are also smaller in TG neurons from TRPV1 KO mice. The mechanisms controlling the reduction of TRPA1 expression by TRPV1 are unknown. It could be either on transcriptional, translational or post-translational levels. Thus, this difference may be explained either by a possible down regulation of TRPA1 expression in TRPV1 KO mouse sensory neurons, by a requirement of TRPV1 for functional stability of TRPA1 channels on the plasma membranes of sensory neurons, or by regulation of TRPA1 activity by the TRPV1 channel.

In TRPA1-expressing cells, the activation of TRPA1 by specific agonists generates relatively fast currents (Fig 5A; (Jordt et al., 2004; Nagata et al., 2005; Akopian et al., 2007)). Previous reports also demonstrated that in the absence of extracellular Ca^{2+} , TRPA1-mediated currents were faster activating (Nagata et al., 2005). However, our data indicate that Ca^{2+} only slightly influences the activation kinetics of MO currents in sensory neurons from WT or TRPV1 KO mice (see also (Akopian et al., 2007)).

Our results are in agreement with previously published reports that demonstrated a slight reduction in the magnitudes of I_{MO} and cold-activated currents after removal of extracellular Ca^{2+} in TRPA1-expressing cell lines (Story et al., 2003; Bandell et al., 2004). However, other reports indicated that removal of extracellular Ca^{2+} substantially decreases I_{MO} in TRPA1-expressing cells (Jordt et al., 2004; Nagata et al., 2005). Importantly, the co-expression of TRPV1 with TRPA1 in sensory neurons and CHO cells dramatically enhances peaks of I_{MO} and I_{WIN} after removal of extracellular Ca^{2+} .

A distinct feature of I_{MO} and I_{WIN} in TRPA1/TRPV1-expressing cells is a voltage-dependent change of the channel activity which is influenced by extracellular Ca^{2+} and the types and concentrations of TRPA1 agonists. Specifically, it is apparent that the absence of extracellular Ca^{2+} and the presence of highly efficacious agonists reduce outward rectification of TRPA1-mediated currents in TRPA1/TRPV1 expressing cells. These voltage-dependent properties clearly distinguish the behavior of the currents recorded from TRPA1/TRPV1 expressing cells, including sensory neurons, versus TRPA1 containing cells (Bandell et al., 2004; Jordt et al., 2004; Nagata et al., 2005; Zurborg et al., 2007). The mechanisms underlying voltage-dependent blockage of TRPA1-mediated responses by Ca^{2+} in TRPA1/TRPV1 co-expressing cells are unclear. However, two possibilities could be proposed. First, Ca^{2+} could directly block TRPA1 activities in a voltage-dependent fashion. A similar mechanism was proposed for the TRPV4, TRPV5 and TRPV6 channels (Voets et al., 2002; Owsianik et al., 2006). Thus, mutation analysis has demonstrated that neutralization of D⁶⁸² in the TRPV4 channel strongly decreases voltage-dependent pore blockage by Ca^{2+} (Voets et al., 2002). It is possible that the lack of the positively charged residues in the fourth transmembrane domain of the TRPA1 (836-854; Corey et al., 2004) may explain the Ca^{2+} - and voltage-independent behavior of the TRPA1 channel that was expressed alone in cell lines. Second, the influx of Ca^{2+} after activation of TRPA1 could indirectly influence the voltage-dependency of TRPA1-mediated responses in the co-expressing cells. However, we routinely used high (10 mM) concentration of EGTA in pipette solution to chelate free intracellular Ca^{2+} .

The co-expression of TRPA1 with TRPV1 also leads to agonist-regulated voltage-dependency of TRPA1 activities. In contrast, the expression of TRPA1 alone does not produce this effect. Interestingly, a voltage-dependent activity of TRPV1 has been reported. Thus, although relatively high concentrations of the potent TRPV1 agonist CAP (>0.1 μ M) do not significantly alter voltage-dependency of the TRPV1 channel (Piper et al., 1999; Gunthorpe et al., 2000), lower concentrations of CAP does alter voltage-dependent TRPV1 activity (Voets et al., 2004). Based on this finding, Voets and colleagues (Voets et al., 2004) proposed that CAP could function (with $EC_{50} = 28$ nM) as a gating modulator of the TRPV1 channel. Extending this hypothesis to the TRPA1/TRPV1 co-expression system could imply that agonist-induced conformational changes could lead to alteration of the channel gating properties that occur at physiological concentrations of Ca^{2+} . Considering this hypothesis, the co-expression of TRPV1 with TRPA1 may allow TRPV1 agonists to regulate or heterologously modulate activities of the TRPA1 channel. Such regulation could have substantially important physiological significance for integration of multiple stimuli by sensory neurons and the somatosensory system in general. Thus, TRPA1 and TRPV1 could mediate an accumulation of intracellular Ca^{2+} in response to stimulation of sensory neurons with inflammatory mediators coupled to the phospholipase C β pathway such as bradykinin (Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2006). These data strongly suggest that certain inflammatory mediators could activate sensory neurons via TRPV1 and/or TRPA1 which, in this case, can play an integrative role in regulating nociceptor function (McMahon and Wood, 2006).

Acknowledgments

We would like to thank Mayur Patil for technical assistance, Dr. David Julius (UCSF, San Francisco, CA) for kindly gifting rTRPV1 cDNA and Dr. Ardem Patapoutian (The Scripps Research Institute, San Diego, CA) for providing TRPA1 cDNA. Research was supported by NIH grants DA19585 and DE014928.

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Figure 1. MO and WIN-activated whole-cell currents in adult rat TG neurons

Recordings were carried out in whole-cell voltage clamp ($V_h = -60$ mV) configuration.

Details of current acquisition parameters and compositions of solutions are noted in “Material and Methods”. (A) and (B) Typical slow and fast inward currents in TG sensory neurons generated by application of 25 μ M MO (A) and 25 μ M WIN (B). The currents were acquired from separate neurons bathed in SES (standard extracellular solution; marked Ca+) or 0 Ca-ES (Ca^{2+} -free; marked Ca-). Standard intracellular solution was SIS. (C)

Concentration-response curves for I_{WIN} and I_{MO} recorded from TG neurons. Data for each point were generated from separate neurons by application of drugs for 2 min. $n=6-18$. Error bars are SEM.

Figure 2. Activation kinetic and modulation of I_{MO} and I_{WIN} by extracellular Ca^{2+}
 Recordings were carried out in whole-cell voltage clamp ($V_h = -60$ mV) configuration. Details of current acquisition parameters and compositions of solutions are noted in “Material and Methods”. **(A)** Activation kinetics measured for I_{WIN} and I_{MO} generated in TG neurons by WIN and MO (25 μ M each). Activation kinetics were calculated as the 5-95% of current rise time. The external solutions were SES (Ca+) or 0Ca-ES (Ca-) and the internal (pipette) solution was SIS. Numbers of recorded cells are indicated within the bars. “Extern” is external solution. **(B)** The peak magnitude of I_{WIN} and I_{MO} (25 μ M each) were measured after recording in indicated extracellular solutions. Pipette solution was SIS. Numbers of recorded cells for each experiment are denoted. Error bars are SEM.
 *** $p < 0.001$

Figure 3. Voltage-dependency of I_{MO} and I_{WIN} in rat TG neurons

All data were collected from recording in whole-cell voltage clamp configuration. In all presented recordings, the electrode pipettes were filled with Cs-IS ("intracellular solution"). Depolarizing ramps (-100 to $+60$ mV in 400 ms) as indicated on Figure 4A were used to generate traces depicted on the *panels (A-C)*. I-V curves were recorded from separate neurons. I-V traces are averaged from $n = 2-4$. **(A)** and **(B)** The effect of Ca^{2+} removal on I_{WIN} **(A)** and I_{MO} **(B)** I-V relationships. I-V traces were recorded from neurons maintained in indicated extracellular solution with 2 mM Ca^{2+} (SES) or without Ca^{2+} (0Ca-ES). **(C)** I_{WIN} ($25 \mu\text{M}$) was carried by monovalent cations and Ca^{2+} . Mean I-V relations for I_{WIN} were constructed from recordings of 6-12 neurons in bath solutions with the indicated cationic compositions (external solutions). 0 Ca - 140 Cs represents symmetrical solution to Cs-IS (pipette solution).

Figure 4. Factors influencing I_{MO} and I_{WIN} I-V in TG neurons

All data were collected from recording in whole-cell voltage clamp configuration. In all presented recordings, the electrode pipettes were filled with Cs-IS (“intracellular solution”). Depolarizing ramps (−100 to +60 mV in 400 ms) as indicated *in panel A* were used to generate traces depicted in *panels (B–D)*. **(A)** Rectification ratios of I-V for I_{WIN} (25 μ M) in TG neurons are independent on speed and type (i.e. depolarizing or hyperpolarizing) of ramps. Shapes of the ramps are depicted. **(B)** Pharmacological desensitization of I_{WIN} does not alter the I_{WIN} I-V relationship. Second application of WIN (25 μ M) does not alter I_{WIN} I-V rectification ratios, while rectification ratios for I_{CAP} (0.1 μ M) increase after the second application of the drug resulting in tachyphylaxis of I_{CAP} . **(C)** and **(D)** Rectification ratios of I_{MO} **(C)** and I_{WIN} **(D)** depend on WIN and MO concentrations (5 μ M and 50 μ M or 100 μ M) when standard external solution contains 2 mM Ca^{2+} (SES), but not in Ca^{2+} -free solutions (0Ca-ES).

Figure 5. I_{MO} and I_{WIN} in TRPA1 or TRPA1/TRPV1-coexpressing CHO cells combination
 All data were collected from recordings in whole-cell voltage clamp ($V_h = -60$ mV) configuration. In all presented recordings, the electrode pipettes were filled with Cs-IS. **(A)** Representative traces show I_{MO} (10 μ M) and I_{WIN} (25 μ M) in TRPA1- and TRPA1/TRPV1-expressing CHO cells. Horizontal bars denote durations of agonist applications. **(B)** I_{MO} and I_{WIN} density in CHO cells expressing TRPA1 (A1) or TRPA1/TRPV1 (A1+V1). $n=10-38$ External solutions (Extern solut), expression constructs and agonist types and concentrations are denoted below X-axis. SES and 0Ca-ES represent Ca^{2+} -containing and Ca^{2+} free external solutions, respectively. Significant and non-significant (NS) differences between groups are indicated. Error bars are SEM. *** $p < 0.001$

Figure 6. Current-voltage relationship of I_{MO} and I_{WIN} in TRPA1 or TRPA1/TRPV1-coexpressing CHO cells

All data were collected from recordings in whole-cell voltage clamp ($V_h = -60$ mV) configuration. In all presented recordings, the electrode pipettes were filled with Cs-IS. (A) Representative I_{WIN} (25 μ M) I-V relations were recorded from TRPV1/TRPA1 (A1+V1) and TRPA1 (A1) expressing CHO cells. Recordings were performed in the presence (SES; left panel) and absence (0Ca-ES; right panel) of Ca^{2+} in extracellular solution. Ramp protocol is presented in Figure 4A. Recordings were performed on separate cells. Each I-V curve is averaged from 3-6 recordings. (B) I-V curves of I_{MO} and I_{WIN} at different concentrations (5-50 μ M) of agonists in TRPA1/TRPV1- and TRPA1-expressing CHO cells (marked above panels). The types and concentrations of TRPA1 and TRPV1 agonists are denoted. (C) Summary of experiments that are illustrated on panel (A) and similarly designed experiments in which CHO cells were treated with 10 μ M MO and I-V relations recorded. $n=8-17$. External solutions (Ext solut), expression constructs (A1 or A1+V1) and agonist types and concentrations are denoted below X-axis. Significant differences between groups are indicated. Error bars are SEM. *** $p < 0.001$

Figure 7. Analysis of I_{MO} in wild-type and TRPV1 null-mutant mouse TG neurons

All data were collected from recording in whole-cell voltage clamp ($V_h = -60$ mV) configuration. The pipette solution was Cs-IS for recording of I-V curves. The depolarizing ramp for generation of I-V curves is shown in Fig 4A (upper panel). External solutions, mouse lines and concentrations of drugs are noted. I-V curves were recorded from separate neurons. I-V traces are averaged from $n = 2-4$. (A) Representative traces of I_{MO} in TG neurons isolated from wild-type (WT) and TRPV1 null-mutant (TRPV1 KO) mice. Neurons were bathed in SES (Ca^{2+}) or 0Ca-ES (Ca^{2+}). (B) Representative I_{MO} I-V traces recorded from WT and TRPV1 KO mouse TG neurons. (C) Magnitude of I_{MO} (at $V_h = -60$ mV) is affected by extracellular Ca^{2+} in WT, but not TRPV1 KO, mouse TG neurons. $n = 13-24$ (D) Activation kinetics for I_{MO} is independent of the presence of extracellular Ca^{2+} or mouse line. $n = 11-21$. (E) The effect of Ca^{2+} removal on I_{MO} rectification ratio in WT and TRPV1 KO TG neurons. $n = 6-12$ (F) and (G) Rectification ratios of I_{MO} are differentially affected by concentration of MO (indicated) in WT and TRPV1 KO TG neurons. $n = 7-12$. Representative traces are presented on the panel G. Error bars are SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 1

Expression patterns of MO- and WIN-gated responses in TG neurons.

Drugs ¹	Cell-size distribution of MO- and WIN-gated currents ²				Co-expression of I _{WIN} and I _{MO} with I _{CAP} ³	
	15-25µm	26-31µm	32-36µm	>37µm	WIN-MO/ ⁴ CAP	CAP ⁺ -WIN-MO ⁵
MO	22/65	77/111	23/40	2/25	142/185 (76.7%)	5/152 (3.2%)
WIN	16/75	93/159	17/51	3/18	123/173 (72%)	9/144 (6.2%)

¹ The responses were stimulated with 5-100µM WIN or 10-500µM MO;

² Data was generated using both whole-cell and perforated-patch configurations;

³ Data was accumulated using electrophysiological recordings as well as Ca²⁺ imaging;

⁴ Percentage of MO or WIN-responsive neurons in CAP-positive neurons;

⁵ Percentage of CAP-negative neurons in MO or WIN-responsive neurons