Mechanisms of Transient Receptor Potential Vanilloid 1 Activation and Sensitization by Allyl Isothiocyanate

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ABSTRACT

Allyl isothiocyanate (AITC; aka, mustard oil) is a powerful irritant produced by Brassica plants as a defensive trait against herbivores and confers pungency to mustard and wasabi. AITC is widely used experimentally as an inducer of acute pain and neurogenic inflammation, which are largely mediated by the activation of nociceptive cation channels transient receptor potential ankyrin 1 and transient receptor potential vanilloid 1 (TRPV1). Although it is generally accepted that electrophilic agents activate these channels through covalent modification of cytosolic cysteine residues, the mechanism underlying TRPV1 activation by AITC remains unknown. Here we show that, surprisingly, AITC-induced activation of TRPV1 does not require interaction with cysteine residues, but is largely dependent on SS13, a residue that is involved in capsaicin binding. Furthermore, AITC acts in a membrane-delimited manner and induces a shift of the voltage dependence of activation toward negative voltages, which is reminiscent of capsaicin effects. These data indicate that AITC acts through reversible interactions with the capsaicin binding site. In addition, we show that TRPV1 is a locus for cross-sensitization between AITC and acidosis in nociceptive neurons. Furthermore, we show that residue F660, which is known to determine the stimulation by low pH in human TRPV1, is also essential for the cross-sensitization of the effects of AITC and low pH. Taken together, these findings demonstrate that not all reactive electrophiles stimulate TRPV1 via cysteine modification and help understanding the molecular bases underlying the surprisingly large role of this channel as mediator of the aalgic properties of AITC.

Introduction

Transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1), two members of the transient receptor potential superfamily of cation channels, are expressed in sensory nerve endings, where they act as molecular sensors of noxious thermal and chemical stimuli (Caterina et al., 1997, 2000; Jordt et al., 2004; Bautista et al., 2006; Kwan et al., 2006). TRPV1 is activated by heat, low pH, and by a variety of endogenous and exogenous compounds, including vanilloids such as the hot pepper compound capsaicin (Caterina et al., 1997; Tominaga et al., 1998). TRPA1 is directly activated by cold (Story et al., 2003; Sawada et al., 2007; Karashima et al., 2009) and by a wide variety of pungent chemicals, including menthol (Karashima et al., 2007), nicotine (Talavera et al., 2009), and isoflurane (Matta et al., 2008), among many others (Nilius et al., 2012). TRPA1 is also activated by highly reactive electrophilic compounds such as cinnamaldehyde (Bandell et al., 2004), allyl isothiocyanate (AITC; aka, mustard oil) (Jordt et al., 2004), acrolein (Bautista et al., 2006), endogenous aldehydes (Macpherson et al., 2007; Trevisani et al., 2007; Andersson et al., 2008), and aldehydes contained in cigarette smoke (Andrè et al., 2008).

Capsaicin and AITC have been extensively used to probe the in vivo function of TRPV1 and TRPA1, respectively (see for example Caterina et al., 2000; Bautista et al., 2006; Kwan et al., 2006). However, although the specificity of capsaicin for TRPV1 remains unchallenged, recent studies demonstrated that TRPA1 is not the sole molecular target of AITC. For instance, Kwan et al. (2006) showed that Trpa1 knockout (KO) mice have significant aversion toward AITC and residual nociceptive response to intraplantar injection of this compound. Furthermore, Ohta et al. (2007) reported that AITC activates porcine TRPV1 and suggested that inflammatory and nociceptive responses to this compound in vivo could be partly mediated by TRPV1 activation. Finally, it was

ABBREVIATIONS: AITC, allyl isothiocyanate; [Ca²⁺], intracellular Ca²⁺ concentration; DRG, dorsal root ganglion; GFP, green fluorescent protein; HEK, human embryonic kidney; hTRPV1, human transient receptor potential vanilloid 1; KO, knockout; mTRPV1, mouse transient receptor potential vanilloid 1; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; rTRPV1, rat transient receptor potential vanilloid 1; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1; WT, wild-type.
recently shown that AITC activates mouse and human TRPV1, especially in recording conditions that better resembled experimental settings in vivo, such as prolonged application of pharmacologically relevant concentrations at physiologic temperatures (Everaerts et al., 2011). Consistent with these data, it was also found that TRPV1 has a large relative role in several effects of AITC in vivo and ex vivo, including bladder irritation, calcitonin gene-related peptide release, and acute pain. In contrast, TRPA1 was shown to be more important for aversion responses in a drinking test and for AITC-induced acute inflammation (Everaerts et al., 2011).

Although these data strongly suggest that both TRPV1 and TRPA1 are required for normal nociceptive responses to AITC in mice, they also indicate that more studies are needed to clarify the precise role of these channels in different experimental paradigms over a wide range of pharmacologically relevant concentrations. In addition, two other questions remain, the first regarding the mechanisms underlying AITC-induced activation of TRPV1 and the second concerning the possibility that AITC sensitizes TRPV1 to low extracellular pH. The latter question is particularly important because it relates to the mechanisms by which AITC induces neurogenic inflammation (Jancso et al., 1968; Inoue et al., 1997; Grant et al., 2005), where extracellular acidosis is a key feature (Julius and Basbaum, 2001; Holzer, 2009). We therefore set out to investigate the mechanism by which AITC activates TRPV1, and whether AITC interacts with low pH. We provide compelling evidence that AITC activates TRPV1 in a membrane-delimited manner by interacting with the binding site for capsaicin and demonstrate that TRPV1 is a locus for cross-sensitization between AITC and acidosis in sensory neurons.

Materials and Methods

Culture and Transfection of Human Embryonic Kidney 293 Cells. Human embryonic kidney (HEK)-293 cells were seeded on 18-mm glass coverslips coated with poly-L-lysine (0.1 mg/ml) and grown in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum, 2 mM L-glutamine, 2 mM penicillin, and 10 mM streptomycin at 37°C in a humidity controlled incubator with 10% CO2. HEK-293 cells were transiently transfected with either human (ENSG00000196869) or mouse (ENSMUSG00000005952) TRPV1 cDNAs cloned into the pCAGGS-M2-IRES-green fluorescent protein (GFP) vector (Meseguer et al., 2008), using Trans-IT-293 reagents (Mirus, Madison, WI). All human TRPV1 and the F660A and F660S mutants. In all experiments, TRPA1 mutants were obtained using the reaction overlap extension technique (Ho et al., 1989) and verified by sequencing. For rat TRPV1 experiments, HEK-293 cells were cotransfected with pcDNA3.1 vector expressing corresponding rat TRPV1 cDNA (ENSRNOG00000019486) (Caterina et al., 1997) or the C18 rat TRPV1 mutant (Salazar et al., 2008) and the pEGFP-C1 vector. We used the vectors constructed by Aneiros et al. (2011) for the human TRPV1 and the F660A and F660S mutants. In all experiments, transfected cells were identified by GFP expression and sensitivity to the TRPV1 agonist capsaicin or low extracellular pH.

Patch-Clamp Experiments. For current recordings, coverslips with cells were placed in the stage of an inverted microscope (Olympus IX70; Olympus, Tokyo, Japan) and rinsed for a few minutes with Krebs (stabilization) solution containing (in mM) 150 NaCl, 6 KCl, 1 MgCl2, 1.5 CaCl2, 10 glucose, and 10 HEPES and titrated to pH 7.4 with NaOH. TRPV1 currents were recorded in the whole-cell and inside-out configurations, using an extracellular solution containing (in mM) 150 NaCl, 2 CaCl2, 1 MgCl2, and 10 HEPES and titrated to 7.4 with NaOH. The intracellular solution contained (in mM) 150 NaCl, 10 HEPES, 5 EGTA, and 1 MgCl2 and was titrated to pH 7.2 with NaOH. Currents were recorded using the patch-clamp technique using an EPC-7 (List Electronics, Darmstadt, Germany) amplifier and filtered with an eight-pole Bessel-filter (Kemo, Bekenham, UK). For control of voltage-clamp protocols and data acquisition, we used an IBM-compatible PC with a TL-1 DMA interface (Axon Instruments, Foster City, CA) and pCLAMP software (version 9.0; Axon Instruments). Bath solutions were perfused by gravity via a multibarreled pipette. Patch pipettes were pulled from Vircapillary tubes (Modulohm, Herlev, Denmark) using a DMZ Universal puller (Zeitz-Instruments, Augsburg, Germany). An Ag-AgCl wire was used as reference electrode. Adequate voltage control was achieved by using low pipette resistances (1–2.5 MΩ) and series resistance compensation to the maximum extent possible (40–50%). Membrane capacitive transients were electronically compensated. Current traces were filtered at 2.5–5 kHz and digitized at 5–10 kHz. The temperature of the bath solution was controlled as previously described (Voets et al., 2004).

Currents were routinely recorded every 2 seconds during the application of 500 milliseconds-lasting voltage ramps from −100 to +160 mV with a holding potential of 0 mV. The voltage dependence of channel activation was studied using a voltage-step protocol consisting of increasing depolarizations in the range from −100 to +200 mV from a holding potential of 0 mV followed by an invariant step to +60 mV (or −60 mV for experiments with capsaicin). Activation curves were obtained from instantaneous currents at the beginning of the pulse to +60 mV (−60 mV) determined using monoeponential fits (Talavera and Nilius, 2011). These curves were fitted with Boltzmann function of the form:

\[
I_{\text{act}}(+60) = \frac{I_{\text{max}}}{1 + \exp(-(V - V_{\text{act}})/s_{\text{act}})}
\]

where \(I_{\text{max}}\) is the current at very positive potentials, \(V_{\text{act}}\) is the voltage for half-maximal activation, and \(s_{\text{act}}\) is the slope factor. The lack of saturation of the open probability at positive potentials in control conditions made it impossible to estimate the fitting parameters independently. To overcome this limitation, the value of \(I_{\text{max}}\) which is proportional to the maximal whole-cell conductance for TRPV1, and \(s_{\text{act}}\) were assumed not to be affected by AITC. In practice, data in control and AITC were simultaneously fit with values for \(I_{\text{max}}\) and \(s_{\text{act}}\) being shared in both conditions. Data were analyzed using WinAASC (G. Droogmans, KU Leuven, Leuven, Belgium).

Animals. All animal experiments were carried out in accordance with the European Community Council guidelines and were approved by the local ethics committee of the KU Leuven. Wild-type (WT) C57Bl/6 mice aged 8–14 weeks were obtained from Janvier Labs (Saint Berthevin, France). Trpa1 (Karashima et al., 2009) and Trpv1 KO mice were backcrossed at least 10 generations in C57Bl/6J background. Double Trpa1/Trpv1 KO mice were generated by crossing these single Trpa1 and Trpv1 KO mice. The mice were maintained under standard conditions in a 12-hour light/dark cycle with free access to food pellets and tap water.

Isolation and Culture of Dorsal Root Ganglion Neurons. Mouse dorsal root ganglion (DRG) neurons were cultured using a variant of a previously described method (Desesco et al., 2011). In brief, for each series of experiments, 3–4 mice were killed by cervical dislocation and DRGs were bilaterally excised under a dissection microscope. The ganglia were washed in 10% fetal calf serum (Gibco, Grand Island, NY) and 2.5 mg/ml dispase (Gibco) for 3 minutes, and the ganglia were then transferred to a depuration bath containing (in mM) 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 glucose, 10 HEPES and titrated to pH 7.4 with NaOH, and then incubated at 37°C in a mix of 1 mg/ml collagenase (Gibco, Grand Island, NY) and 2.5 mg/ml dispase (Gibco) for 45 minutes. Digested ganglia were gently washed twice with basal medium and mechanically dissociated by mixing with syringes fitted with increasing needle gauges. Neurons were seeded on poly-L-ornithine/laminin-coated glass bottom chambers (FluoroBath; World Precision Instruments, Hitchin, Hertfordshire, UK) and cultured for 12–18 hours at 37°C in B27 supplemented Neurobasal A medium (Invitrogen) containing 2 ng/ml glial cell line-derived neurotrophic factor (GDNF).
factor (Invitrogen) and 10 ng/ml neurotrophic factor 4 (PeproTech, Rocky Hill, NJ).

**Microfluorometric Intracellular Ca\textsuperscript{2+} Imaging Experiments.** Before the measurements, cells were incubated with 2 μM Fura-2 AM ester for 30 minutes at 37°C. Intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) was measured on an Olympus Cell M system. Fluorescence was measured during excitation at 340 and 380 nm, and after correction for the individual background fluorescence signals, the ratio of the fluorescence at both excitation wavelengths (F\textsubscript{340}/F\textsubscript{380}) was monitored. Experiments were performed using the standard Krebs solution (see above). The data were classified semiautomatically using a function programmed in MATLAB (MathWorks, Natick, MA) and analyzed with Origin 7.0 software (Origin-Lab Corporation, Northampton, MA).

**Drugs.** AITC was kept as a 10 M stock solution (4°C) and fresh dilutions were prepared daily. Solutions containing capsaicin were prepared from an ethanol-based 1 mM stock solution that was kept for not longer than 1 month at −20°C. Both AITC and capsaicin were purchased from Sigma-Aldrich (Bornem, Belgium). Allicin was purchased from LKT Laboratories (St. Paul, MN) and kept as a 60 mM stock solution at −80°C and fresh dilutions were prepared every 4 hours.

**Statistical Analysis.** Statistical analyses were performed with Origin 7.0 software. Data are presented as mean ± S.E.M. Paired and unpaired t tests were performed whenever appropriate and P < 0.05 was considered for statistical significance.

**Results**

We first investigated whether activation of TRPV1 by AITC requires an intracellular signal transduction pathway, by

![Fig. 1.](image-url) AITC activates TRPV1 in cell-free inside-out patches. (A) Current traces of mTRPV1 in inside-out patches. Currents were evoked by a voltage ramp from −100 to +150 mV (holding potential of 0 mV) in the control condition and in the presence of 3 mM AITC or 1 μM capsaicin in the bath solution. Traces are labeled to indicate the correspondence with the filled data points shown in (B). The inset shows magnification of the currents at negative potentials. (B) Example of the time course of the effects of application of 3 mM AITC or 1 μM capsaicin on the amplitude of mTRPV1 currents at +75 and −75 mV. (C) Average increase of mTRPV1 inside-out currents during AITC (3 mM) and capsaicin (1 μM) application at −75 and +75 mV (n = 5). Caps, capsaicin; NMDG, N-methyl-D-glucamine.
testing whether this effect is preserved in cell-free inside-out patches. Application of 3 mM AITC to the cytosolic side of membrane patches of TRPV1-expressing HEK-293 cells resulted in robust and reversible stimulation of outwardly rectifying currents (Fig. 1, A and B). As previously observed for whole-cell currents (Everaerts et al., 2011), the amplitudes of AITC-activated inward and outward currents were several-fold smaller than those upon application of 1 μM capsaicin (Fig. 1). These data indicate that AITC activates TRPV1 in a membrane-delimited manner.

It was reported previously that the activation of TRPA1 by AITC and various other electrophiles occurs via covalent binding to cysteine residues (Hinman et al., 2006; Macpherson et al., 2007). Similarly, the activation of rat TRPV1 by allicin, a pungent electrophilic component of raw garlic, or by 2-aminoethyl methanethiosulfonate hydrobromide (MTSEA), a cysteine-reactive reagent, requires a single N-terminal cysteine residue (C157) (Salazar et al., 2008). Thus, we tested whether AITC activates TRPV1 via covalent modification of cysteine residues on the channel.

We found that, similar to the rat ortholog, WT mouse TRPV1 was activated by allicin (6.5-fold ± 1.0-fold current increase at −75 mV; n = 3; P = 0.017) (Fig. 2, A and C), whereas the corresponding C158A mutant was not (n = 4) (Fig. 2, B and C). However, the C158A mutation did not affect AITC-induced stimulation [5.4-fold ± 0.6-fold increase at −75 mV compared with 5.8 ± 1.2 for WT mouse TRPV1 (mTRPV1); n = 5–6; P = 0.74] (Fig. 2, B–D). Since TRPV1 contains multiple cysteine residues, these data do not exclude that AITC activation of TRPV1 is mediated via covalent modification of other cysteine residues on the channel. We therefore analyzed the effect of AITC on C18− rat TRPV1

![Fig. 2. The mTRPV1 C158A mutant is activated by AITC. (A and B) Examples of the time course of the amplitude of WT mTRPV1 and mTRPV1 C158A mutant currents at +75 and −75 mV in control or in the presence of extracellular allicin (200 μM) or AITC (3 mM). The letters next to the filled symbols indicate the time points when the traces shown on the right were recorded. (C) Increase of amplitude for WT mTRPV1 and C158A mutant mTRPV1 currents during application of AITC (3 mM) and allicin (200 μM) (n = 3–6). *P < 0.05 for the comparison of current amplitudes measured before and during stimulation. (D) Effect of extracellular application of 3 mM AITC on [Ca2+]i in HEK-293 cells expressing the C158A mutant mTRPV1. Transfected cells were identified by the expression of GFP and their responsiveness to 1 μM capsaicin (upper traces, n = 68). Nontransfected cells did not respond to either AITC or capsaicin (lower traces, n = 26). Thick traces represent the means and dashed traces represent the means ± the corresponding S.E.M. Caps, capsaicin.]
(rTRPV1), a cysteine-less rTRPV1 mutant that was previously shown to be insensitive to allicin and MTSEA (Salazar et al., 2008). We found that the C18^rTRPV1 channel was activated by AITC (3.6-fold ± 0.4-fold current increase at −75 mV; n = 8; P = 0.0025) (Fig. 3), similar to WT rTRPV1 (4.3-fold ± 0.6-fold current increase at −75 mV; n = 7) (Fig. 3B). We therefore conclude that, in contrast to allicin and MTSEA, AITC activates TRPV1 via a mechanism independent of covalent linkage of AITC to cysteine residues on the channel.

Next, we explored the possibility that residues important for the stimulatory effects of capsaicin are also involved in the response to AITC. Mutation S512Y in rat TRPV1 results in a selective ablation of capsaicin-induced activation, whereas activation by low pH or heat was largely preserved (Jordt and Julius, 2002). We studied the corresponding mutant in mouse TRPV1 (S513Y) using Ca^{2+} imaging and whole-cell patch-clamp recordings and confirmed that it had a strongly reduced sensitivity to capsaicin, whereas responses to low pH were conserved (Fig. 4, A and B). Interestingly, mutations in other positions, Y512A and T551I, which correspond to mutants previously reported to significantly impair the response to capsaicin in rat (Jordt and Julius, 2002; Gavva et al., 2004) and human TRPV1 (Gavva et al., 2004), did not have significant effect on the responses to AITC or capsaicin in the mouse isoform (Fig. 4A). Surprisingly, cells expressing the S513Y mutant mTRPV1 also showed strongly reduced responses to 3 mM AITC (Fig. 4, A and B).

Activation of TRPV1 by capsaicin, heat, or low pH involves a shift of the voltage dependence of activation toward more negative potentials (Voets et al., 2004; Aneiros et al., 2011). Thus, we tested whether the mutation S513Y interferes with this mechanism by recording mTRPV1 currents during the application of a voltage-step protocol that allows estimating the steady-state TRPV1 open probability at different membrane potentials (see Materials and Methods). Capsaicin induced a leftward shift of the voltage dependence of activation in the S513Y mutant (55 ± 10 mV) (Fig. 4, C and D), but in a much smaller extent that in mTRPV1 WT (225 ± 18 mV).

Application of 3 mM AITC caused a substantial leftward shift of the activation curve by an estimated 210 ± 13 mV (n = 5; Fig. 5, A and C), resulting in significant inward currents in the physiologic voltage range (Fig. 5D). These data demonstrate that AITC acts on TRPV1 in a similar way to capsaicin (Voets et al., 2004) and raised the possibility that both compounds have common interaction sites on the channel. Consistently with this contention, AITC caused a much smaller leftward shift of the voltage-dependent activation curve in the S513Y mutant than in the WT (Fig. 5, E and F). This explains the limited effects of AITC and capsaicin on calcium influx and inward currents at physiologic potentials (Figs. 4A and 5D).

Considering that local extracellular acidosis is a hallmark of neurogenic inflammation (Julius and Basbaum, 2001; Holzer, 2009) and a well known TRPV1 sensitizer (Tominaga et al., 1998; Aneiros et al., 2011), we determined whether there is cross-sensitization between AITC and low pH. We found that mild acidification to pH 6.8 caused a modest but significant stimulation of human TRPV1 (hTRPV1) (1.7-fold ± 0.2-fold current increase at −75 mV; n = 6; P = 0.038) (Fig. 6) and that this response was significantly larger in the presence of 100 μM AITC (3.3-fold ± 0.4-fold current increase at −75 mV; n = 8; P = 0.008).

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Fig. 3. The cysteine-less rTRPV1 mutant C18^rTRPV1 is activated by AITC. (A) Example of the effects of extracellular application of 3 mM AITC and 1 μM capsaicin on the amplitude of currents through the C18^rTRPV1 mutant expressed in HEK-293 cells. The letters next to the filled symbols indicate the time points when the traces shown on the right were recorded. (B) Average current increase for rTRPV1 and C18^rTRPV1 induced by application of 3 mM AITC and 1 μM capsaicin (n = 7). Caps, capsaicin.
at $-75 \text{ mV; } n = 8; P = 0.002$ (Fig. 6C). Conversely, $\text{Ca}^{2+}$ imaging experiments revealed that application of 100 $\mu$M AITC evoked responses in TRPV1-expressing HEK-293 cells at extracellular pH 6.8 but not at pH 7.4 (unpublished data).

To investigate the molecular basis for the cross-sensitization of the effects of AITC and low pH on TRPV1, we tested the effects of mutations at position F660, which are known to ablate stimulation by low pH in human TRPV1 (Aneiros et al., 2011). We found that mutants F660A and F660S responded to high concentrations of AITC in a similar way to WT hTRPV1, but fully lacked the cross-sensitization between AITC and pH 6.8 (Fig. 6C).

Finally, we tested whether the TRPV1-mediated cross-sensitization between AITC and low extracellular pH is also present in sensory neurons. For this we performed microfluorometric intracellular $\text{Ca}^{2+}$ imaging in primary cultures of DRG neurons isolated from Trpa1 KO mice, to avoid interference with the AITC responses mediated by this channel. In line with previous results (Everaerts et al., 2011), we found that extracellular application of 300 $\mu$M AITC at pH 7.4 induced responses in 3.9% (21 of 537) of DRG neurons with a mean amplitude of 330 $\pm$ 50 nM (Fig. 7, A and C). These responses were strongly potentiated when AITC was applied at extracellular pH 6.8 (16.2%, 117
of 721; mean amplitude 490 ± 30 nM; Fig. 7, B and C), indicating that acidosis sensitizes TRPV1 for the AITC effect.

Conversely, preapplication of AITC strongly enhanced the responses of Trpa1 KO mouse neurons to acidification (Fig. 7, D–F). Indeed, lowering the extracellular pH from 7.4 to 6.8 induced responses in 3.9% (17 of 432), with a mean amplitude of 270 ± 40 nM, whereas when acidosis was applied in the presence of 100 μM AITC the responses were observed in 24.4% of the cells (144 of 589), with a mean amplitude of 350 ± 20 nM. Notably, the amplitude of the responses to acidification in the presence of AITC largely correlated with the amplitudes of the responses to capsaicin (R = 0.72; P < 0.0001). The responses were strongly reduced by application of 20 μM capsazepine, a TRPV1 inhibitor (10.5%, 38 of 361; mean amplitude 280 ± 20 nM) (Fig. 7F), and were totally absent in neurons isolated from double Trpa1/Trpv1 KO mice (0 of 240).

**Discussion**

AITC is a natural compound largely responsible for the pungent effect of mustard and wasabi and has been widely used for research purposes to induce experimental irritation and local inflammation (see for example Reeh et al., 1986; McMahon and Abel, 1987; Caterina et al., 2000; García-Martínez et al., 2002; Simons et al., 2004; Carstens and Mitsuyo, 2005; Bautista et al., 2006; Kwan et al., 2006; Albin et al., 2008; Merrill et al., 2008; Sawyer et al., 2009; Dunham et al., 2010). Until recently, these responses were attributed to the nociceptor channel TRPA1 (Bautista et al.,
However, it was recently shown that AITC activates another key nociceptor cation channel, TRPV1 (Ohta et al., 2007; Everaerts et al., 2011). Moreover, it was shown that the genetic ablation of TRPV1 reduced some of the responses to AITC in vivo (Everaerts et al., 2011). This surprising result was partly explained in terms of the differences between the effects of AITC on these channels. Although AITC interacts covalently with cysteine residues of TRPA1 (Hinman et al., 2006; Macpherson et al., 2007), leading to a strong and long-lasting desensitization of this channel (Macpherson et al., 2007), this compound induces a sustained but quickly reversible activation of TRPV1. These fundamental differences prompted us to investigate the mechanism whereby AITC activates TRPV1 in more detail.

We found that activation of TRPV1 by AITC is conserved in cell-free inside-out patches, which implies that the underlying mechanism does not involve soluble cytosolic component and suggests that AITC acts directly on the channel. The installation of effects of AITC in this recording configuration was faster (approximately 30 seconds) than previously reported for the extracellular application in the whole-cell.
configuration (>1 minute; Everaerts et al., 2011), which suggests for an intracellular access to the active site(s). Yet, AITC effects in the inside-out patches were rather slow, which in turn indicates a restricted access to the site(s) and fits with the relatively low apparent affinity of AITC for the channel (300 μM at 35°C; Everaerts et al., 2011).

Next, we found that the sensitivity to AITC is not altered by mutation of C158, a residue that is necessary and sufficient to confer sensitivity to cysteine-modifying agents such as MTSEA and allicin (Salazar et al., 2008). Even more strikingly, sensitivity to AITC was conserved in the completely cysteine-less C18 rTRPV1 mutant. These data demonstrate that AITC-induced activation of TRPV1 does not critically depend on covalent cysteine modification.

On the other hand, we found that, surprisingly, AITC-induced activation of TRPV1 is in many aspects similar to activation by capsaicin. First, AITC induces a shift of the voltage dependence of channel activation toward negative voltages, therefore acting as some other transient receptor potential channel agonists (Nilius et al., 2005; Voets et al., 2005; Talavera et al., 2008). Second, TRPV1 sensitivity to both AITC and capsaicin critically depends on the residue SS13, which has been suggested to form part of the capsaicin binding site (Jordt and Julius, 2002). It should be noted, however, that the apparent affinity of TRPV1 for AITC is several orders of magnitude lower than for capsaicin (Everaerts et al., 2011), and that maximal AITC response amplitudes are significantly lower. Finally, as for capsaicin, the effect of AITC cross-sensitizes with that of low extracellular pH.

The latter aspect is of particular importance, because it helps explain the surprisingly large relevance of TRPV1 (relative to that of TRPA1) as a AITC receptor in vivo (Everaerts et al., 2011). Indeed, this potentiating interaction renders TRPV1 even more sensitive to AITC in real life and experimental scenarios, since this compound is typically present in acid food (mustard, vinaigrettes) and acidosis is a typical feature of local inflammation (Julius and Basbaum, 2001; Holzer, 2009).

Our analysis of the effects of AITC on the gating properties of TRPV1 indicates that the mechanism of cross-sensitization between acidosis and AITC is the same as the one between heat and capsaicin, i.e., the induction of additive shifts of the voltage dependence of channel activation (Voets et al., 2004). Indeed, the sensitizing effect of extracellular acidosis on the response to AITC was fully ablated by the same mutations that determine the shift of the activation curve of TRPV1 by low pH (Aneiros et al., 2011).

In conclusion, we have provided evidence that AITC activates TRPV1 in a membrane-delimited manner via an interaction with the capsaicin binding site. Hence, TRPV1 can be activated by highly reactive electrophilic compounds either through cysteine-dependent (allicin and MTSEA) and cysteine-independent (AITC) mechanisms. In addition, our results unveil molecular mechanisms that help to understand the complex effects of AITC as an inflammatory and sensitizing agent.
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