Cellular/Molecular

Molecular Determinants of Species-Specific Activation or Blockade of TRPA1 Channels

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TRPA1 is an excitatory, nonselective cation channel implicated in somatosensory function, pain, and neurogenic inflammation. Through covalent modification of cysteine and lysine residues, TRPA1 can be activated by electrophilic compounds, including active ingredients of pungent natural products (e.g., allyl isothiocyanate), environmental irritants (e.g., acrolein), and endogenous ligands (4-hydroxynonenal). However, how covalent modification leads to channel opening is not understood. Here, we report that electrophilic, thioaminal-containing compounds [e.g., CMP1 (4-methyl-*N*-[2,2,2-trichloro-1-(4-nitro-phenylsulfanyl)-ethyl]-benzamide)] covalently modify cysteine residues but produce striking species-specific effects [i.e., activation of rat TRPA1 (rTRPA1) and blockade of human TRPA1 (hTRPA1) activation by reactive and nonreactive agonists]. Through characterizing rTRPA1 and hTRPA1 chimeric channels and point mutations, we identified several residues in the upper portion of the S6 transmembrane domains as critical determinants of the opposite channel gating: Ala-946 and Met-949 of rTRPA1 determine channel activation, whereas equivalent residues of hTRPA1 (Ser-943 and Ile-946) determine channel block. Furthermore, side-chain replacements at these critical residues profoundly affect channel function. Therefore, our findings reveal a molecular basis of species-specific channel gating and provide novel insights into how TRPA1 respond to stimuli.

Key words: **TRPA1; species specific; covalent modification; channel gating; activation; block**

Introduction

TRPA1 is a nonselective cation channel that belongs to the superfamily of transient receptor potential (TRP) ion channels. It is expressed in sensory neurons and colocalizes with pain markers such as TRPV1, calcitonin gene-related peptide, and bradykinin receptors (Story et al., 2003; Jordt et al., 2004; Nagata et al., 2005; Obata et al., 2005). In animal studies, activation of TRPA1 by noxious compounds caused pain and neurogenic inflammation, which could be blocked by treatments with TRPA1 antagonists or gene-specific antisense oligonucleotides (Obata et al., 2005; McNamara et al., 2007; Petrus et al., 2007; Trevisani et al., 2007). In addition, TRPA1 gene knock-out resulted in impaired sensory functions and deficits in bradykinin-evoked pain hypersensitivity (Bautista et al., 2006; Kwan et al., 2006). Collectively, these studies suggest that TRPA1 plays an important role in sensory functions and represents an important target for development of analgesic and anti-inflammatory drugs.

Like other TRP channels, a functional TRPA1 channel is formed by coassembly of four subunits, each containing six transmembrane domains, intracellular N, C termini, and an ion conduction pore formed between transmembrane domain 5 (S5) and 6 (S6) (Clapham, 2003; Voets et al., 2005). It can be activated by a variety of stimuli, including intracellular Ca^{2+}, noxious cold, hypertonic solutions, amphipathic molecules [e.g., trinitrophenol (TNP)], and pharmacological agents [e.g., 3′-carbamoylbiphenyl-3-yl cyclohexylcarbamate (URB597)] (Story et al., 2003; Bandell et al., 2004; Hill and Schaefer, 2007; Niforatos et al., 2007; Zurborg et al., 2007; Zhang et al., 2008). Most notably, TRPA1 can be activated by electrophilic compounds, such as allyl isothiocyanate (AITC), acrolein, and 4-hydroxynonenal (Jordt et al., 2004; Bautista et al., 2005; Hinman et al., 2006; Macpherson et al., 2007; Trevisani et al., 2007). These electrophilic agents covalently modify cysteine and lysine residues localized in the N terminus of channel proteins, and the elimination of several critical cysteine/lysine residues dramatically reduced channel sensitivity (Hinman et al., 2006; Macpherson et al., 2007; Trevisani et al., 2007). Presumably covalent modification by electrophilic compounds induces conformational changes of channel proteins. However, how covalent modification leads to channel opening and whether other electrophilic compounds exhibit the same functional effects on TRPA1 remain to be determined.

From a high-throughput screen, we identified electrophilic, thioaminal-containing modulators of human and rat TRPA1 (hTRPA1 and rTRPA1, respectively) channels [e.g., 4-methyl-*N*-[2,2,2-trichloro-1-(4-nitro-phenylsulfanyl)-ethyl]-benzamide (CMP1)]. Similar to previously identified electrophilic activators, these thioaminal compounds likely covalently modify cysteine resi-

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dues in the N terminus of channel proteins; however, they exhibit striking species-specific effects: activation of rTRPA1 and block of hTRPA1 activation by reactive and nonreactive agonists. These effects are not caused by the expression in particular host cells, or particular assay methodologies, but rather intrinsic to respective channel proteins. By characterizing rTRPA1-hTRPA1 chimeras and point mutations, we identified single residues in the S6 domains as the molecular determinants for the species-specific activation and block of TRPA1.

Materials and Methods

Molecular biology and transient expression. Human TRPA1, TRPV1, TRPM8, TRPV4, and rat TRPA1 full-length cDNAs were cloned in the pcDNA3.1/V5-His Topo vector (Invitrogen, Carlsbad, CA). rTRPA1/hTRPA1 chimeras were generated by PCR, and single mutations were introduced using QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). For transient expression in human embryonic kidney 293F (HEK293F) cells, FreeStyle 293 Expression System (Invitrogen) was used as reported previously (Chen et al., 2007). For expression in ND7/23 and PC12 cells, adherent cells were transfected by using 293fectin (Invitrogen).

ALARM nuclear magnetic resonance spectroscopy and ALARM mass spectrometry to assess compound reactivity. Compound reactivity was assessed as reported previously (Huth et al., 2005). In ALARM nuclear magnetic resonance (NMR) experiments, compounds were incubated with ^{13}C-labeled La antigen, and two-dimensional (2D) [^{13}C-^{1}H] heteronuclear single quantum coherence (HSQC) spectra of the labeled proteins were detected in the presence or absence of 20 mM DTT. Elimination of the protein spectral changes by DTT indicated compound reactivity with surface-exposed cysteines. In ALARM mass spectrometry (MS) experiments, La antigen was incubated with compounds overnight, diluted, and analyzed by liquid chromatography/mass spectrometry.

Intracellular Ca^{2+} assay, membrane potential assay, and electrophysiological recordings. Intracellular Ca^{2+} and membrane potential assays were performed using the fluorescent imaging plate reader (FLIPR), calcium dye R8033, or membrane potential dye R8034 (Molecular Devices, Sunnyvale, CA), as reported previously (Bianchi et al., 2007; Chen et al., 2007). Electrophysiological recordings were performed as described previously (Niforatos et al., 2007). External and internal solutions contained the following (in mM): 140 NaCl, 2 $MgCl_2$, 5 EGTA, and 10 HEPES (300 mOsm/L and pH 7.4).

Reagents. CMP1, 4-methyl-*N*-[2,2,2-trichloro-1-(4-chlorophenylsulfanyl) ethyl]benzamide (CMP2), *N*-[2,2,2-trichloro-1-(4-chlorophenylsulfanyl) ethyl] acetamide (CMP3), and 4-bromo-*N*-[2,2,2-trichloro-1-*m*-tolyamino-ethyl]benzamide (CMP4) (see Fig. 1*A*) were prepared at Abbott Laboratories (Abbott Park, IL), each with purity >98% as determined by ^{1}H-NMR and mass spectrometry. Other compounds were obtained from Sigma-Aldrich (St. Louis, MO) or Calbiochem (La Jolla, CA).

Results

Species-specific activation or blockade of TRPA1 channels expressed in HEK293F cell

Species-specific pharmacological properties have been exploited to elucidate structure–function relationships of TRP channels, including TRPV1 and TRPM8 (Jordt and Julius, 2002; Chuang et al., 2004). Based on the relatively low homology between rTRPA1 and hTRPA1 (79.0% identify at amino acid level), we hypothesized that differences in pharmacological properties may exist between the two channels. Indeed, from a high-throughput screen of 700,000 compounds (Chen et al., 2007), we identified thioaminal-containing molecules (CMP1, CMP2, and CMP3) (Fig. 1*A*) that activated rTRPA1 but inhibited hTRPA1. The effects of CMP1 were characterized in greater detail.

rTRPA1, hTRPA1, channels with single mutations, and rTRPA1-hTRPA1 chimeras were transiently expressed in HEK293F cells unless otherwise noted. In cells expressing rTRPA1 or hTRPA1, the nonreactive agonist URB597 (100 μM) and electrophilic agonist AITC (30 μM) activated channels and induced a rapid increase in intracellular Ca^{2+}, as reflected by increases of relative fluorescence units (RFU) (Fig. 1*B*,*C*,*E*,*F*). The URB597 concentration required to induce 50% of maximal Ca^{2+} signals (EC_{50}) was 46.4 ± 4.2 μM on rTRPA1 and 24.3 ± 3.9 μM on hTRPA1, respectively ($n = 4$). The EC_{50} value of AITC was 7.5 ± 1.0 μM on rTRPA1 and 11.3 ± 2.3 μM on hTRPA1, respectively ($n = 4$). Likewise, 32 of 33 mutant channels from rTRPA1-hTRPA1 chimeras or point mutations (except for rM949P) responded robustly to URB597, AITC, and TNP, indicative of Ca^{2+} permeability and channel functions. EC_{50} values of AITC on mutant channels are summarized in supplemental Table 1 (available at www.jneurosci.org as supplemental material).

Similar to URB597 and AITC, CMP1 activated rTRPA1 in a concentration-dependent manner (EC_{50}, 4.0 ± 0.4 μM; $n = 12$) (Fig. 1*B–D*). CMP1 was slightly less efficacious, with E_{max} of 0.91 ± 0.02 compared with URB597 and 0.84 ± 0.02 compared with AITC. In contrast to its effect on rTRPA1, CMP1 (at concentration up to 300 μM) did not activate hTRPA1; instead, CMP1 blocked hTRPA1 responses to URB597 and AITC (Fig. 1*E*,*F*). The inhibitory effect of CMP1 on hTRPA1 was determined using 100 μM URB597 or 30 μM AITC (equivalent to EC_{80} concentration) to activate the channels. The IC_{50} value of CMP1 was 0.85 ± 0.04 μM on URB597 responses (Fig. 1*G*) ($n = 4$) and 2.0 ± 0.4 μM on AITC responses (Fig. 1*G*) ($n = 12$). Moreover, CMP1 inhibited hTRPA1 activation by a variety of other stimuli, including 300 μM 4-hydroxynonenal (IC_{50}, 1.7 ± 0.6 μM; $n = 4$), 30 μM cinnamaldehyde (IC_{50}, 2.5 ± 0.3 μM; $n = 4$), 300 μM TNP (IC_{50}, 0.36 ± 0.00 μM; $n = 8$), and hypertonic solution of 400 mOsm (IC_{50}, 0.56 ± 0.2 μM; $n = 4$). Therefore, CMP1 blocked hTRPA1 activation independent of the nature of agonists (reactive, nonreactive, or hypertonicity).

The effects of CMP1 on rTRPA1 and hTRPA1 were further confirmed using whole-cell and cell-attached single-channel recordings. Cells were transiently transfected with rTRPA1/green fluorescent protein (GFP) or hTRPA1/GFP. To prevent pronounced Ca^{2+}-mediated desensitization (Nagata et al., 2005; Hill and Schaefer, 2007), a Ca^{2+}-free external/internal solution was used. In rTRPA1-expressing cells, slow inward currents (at −60 mV) were induced by CMP1 (100 μM) and subsequently decayed after its removal (Fig. 2*A*). A follow-up application of AITC (100 μM) also evoked large inward currents. In hTRPA1-expressing cells, large inward currents were evoked by AITC, whereas currents were not evoked by CMP1 (Fig. 2*B*). Instead, coapplication of CMP1 and AITC completely abolished the AITC-elicited currents. At the single-channel level, CMP1 and AITC evoked similar activities in rTRPA1 (Fig. 2*C*) without a change in conductance. For example, at −60 mV, the unitary conductance was 65.8 ± 6.5 pS for CMP1 and 64.2 ± 5.9 pS for AITC ($n = 4$). In hTRPA1-expressing cells, AITC elicited single-channel events (Fig. 2*D*). As observed in the whole-cell recordings, CMP1 failed to activate hTRPA1 and blocked AITC-induced single-channel activity.

Activation of TRPA1 mediates changes in the membrane potential; hence, membrane potential dyes can be used to assess TRPA1 function (Bianchi et al., 2007). In rTRPA1-expressing cells, CMP1 induced membrane potential changes, indicative of activation of rTRPA1 (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material). In contrast, CMP1 did not evoke membrane potential changes through hTRPA1 but rather blocked its activation by AITC (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material).

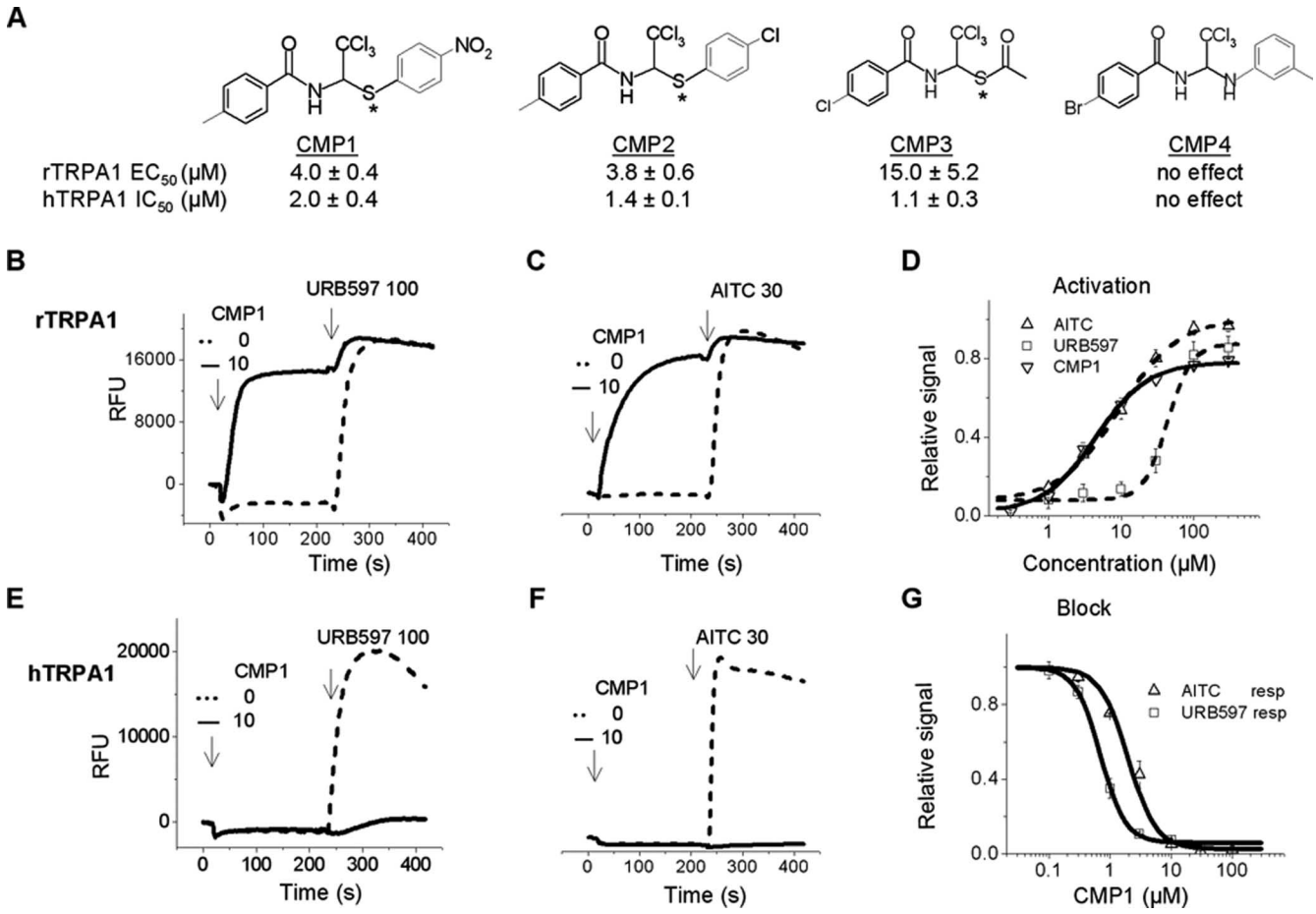

Figure 1. Thioaminal compounds and their species-specific effects on rTRPA1 and hTRPA1 expressed in HEK293F cells. ***A***, Chemical structures, activities, and potencies on rTRPA1 (EC_{50}) and hTRPA1 (IC_{50}). CMP1–3 contain thioaminal groups with a reactive sulfur atom labeled by an asterisk. The sulfur atom is replaced by a nitrogen in CMP4. Compound nomenclature is described in Materials and Methods. To obtain the IC_{50} value for CMP1–3, 30 μM AITC ($\sim EC_{80}$) was used to activate hTRPA1 channel. ***B***, ***C***, In cells expressing rTRPA1, URB597, AITC, and CMP1 evoked increases of intracellular Ca^{2+} as represented by the changes of fluorescence signals (RFU) in the FLIPR-based Ca^{2+} assay. ***D***, Concentration– effect relationship for rTRPA1 activation by AITC, URB597, and CMP1. ***E***, ***F***, In cells expressing hTRPA1, CMP1 did not induce Ca^{2+} increases but blocked URB597 and AITC-evoked responses. ***G***, Concentration– effect relationship of CMP1 inhibition of AITC (30 μM) or URB597 (100 μM) evoked Ca^{2+} responses in hTRPA1 cells.

Species-specific activation or blockade of TRPA1 channels expressed in ND7/23 and PC12 cells

In the above studies, rTRPA1 and hTRPA1 were expressed in HEK293F, a cell line originated from human embryonic kidney. We wondered whether the opposite gating responses were related to expression in this particular host. Hence, two other cell lines, the mouse neuroblastoma-rat neuron hybrid ND7/23 and the rat pheochromocytoma cell line PC12, were used to transiently express rTRPA1 or hTRPA1. In ND7/23 and PC12 cells, CMP1 activated rTRPA1 and inhibited hTRPA1 (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Therefore, the species-specific activation and block by CMP1 are not attributable to expression in particular host cells (HEK293F, ND7/23, or PC12) or resulted from a particular methodology (Ca^{2+} flux, membrane potential assay, whole-cell recordings, and single-channel recordings). Instead, the opposite pharmacological properties are intrinsic to respective channel proteins.

Lack of effects on TRPV1, TRPV4, and TRPM8 channels

Many TRPA1 modulators affect functions of other TRP channels, including gingerol, eugenol (TRPV1); icilin, menthol, URB597 (TRPM8); and arachidonic acid (TRPV4) (Watanabe et al., 2002; Bandell et al., 2004; Macpherson et al., 2006; Karashima et al., 2007; Niforatos et al., 2007). As shown in supplemental Figure 3 (available at www.jneurosci.org as supplemental material), CMP1 did not activate TRPV1, TRPV4, or TRPM8. Moreover, CMP1 did not inhibit TRPV1 activation by capsaicin, TRPV4 activation by hypotonicity, or TRPM8 activation by menthol. Therefore, CMP1 appears to be relatively specific for TRPA1.

The functional effects of CMP1 are correlated with its cysteine reactivity

Electrophilic compounds such as AITC activate TRPA1 through covalent modification of key cysteine and lysine residues localized in the N terminus of channel proteins (Hinman et al., 2006; Macpherson et al., 2007). We reasoned that the thioaminal functionality in CMP1 acted on TRPA1 through a similar mechanism. The electron withdrawing nitrophenyl substituent in CMP1 activates the thioaminal for elimination of the aryl sulfide, generating a reactive electrophilic intermediate that is prone to covalent attachment to nucleophilic cysteine (Fig. 3*A*). A similar modification can also occur at lysine residues (data not shown). The reactivity of CMP1 was confirmed in La antigen-based ALARM NMR and ALARM MS, which use 2D nuclear magnetic resonance spectroscopy and mass spectrometry, respectively, to detect interactions of electrophilic compounds with surface-exposed cysteine and lysine residues (Huth et al., 2005). In ALARM NMR, CMP1 caused significant spectral perturbation,

which was completely prevented by DTT (20 mM), suggesting the modification of cysteine residues (Fig. 3*B*). In ALARM MS, CMP1 generated covalent La antigen adducts, with spectral shifts consistent with modification of one to several cysteine/lysine sites (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Similar to CMP1, its structural analogs with electron withdrawing groups attached to the sulfur atom (e.g., CMP2 and CMP3) demonstrated reactivity (data not shown) and effects on TRPA1 (Fig. 1*A*). In contrast, many analogs with the reactive sulfur atom replaced by nitrogen (e.g., CMP4) were not reactive (Fig. 3*B*) and had no effects on rTRPA1 or hTRPA1 in terms of activation and block (Fig. 1*A*). Therefore, CMP1 activities on TRPA1 rely on its chemical reactivity.

AITC, cinnamaldehyde, diallyl disulfide, acrolein, *N*-methyl maleimide, iodoacetamide, and 4-hydroxynonenal activate TRPA1 by covalently modifying cysteine and lysine residues within channel proteins (Hinman et al., 2006; Macpherson et al., 2007; Trevisani et al., 2007). At least 17 residues within TRPA1 can be modified chemically, and functional consequences were observed for several consensus residues. In hTRPA1, Cys-621 (as in ^{618}Gly-Asn-Lys-Cys-Pro-Ile-Thr^{624}) is one of the major sites of modification. The equivalent residue in mouse TRPA1 was also the most critical (Macpherson et al., 2007). We mutated Cys-621 in hTRPA1 and its counterpart in rTRPA1 to serine (hC621S and rC622S) and determined channel sensitivities to reactive (AITC and CMP1) and nonreactive [TNP and ruthenium red (RR)] modulators. As shown in the concentration–effect relationships (Fig. 3*C–E*), neither rC622S nor hC621S altered sensitivity to TNP or RR. In contrast, rC622S reduced the sensitivity to AITC (5.4-fold) and CMP1 (2.7-fold), whereas hC621S reduced the sensitivity to AITC (5.6-fold) and CMP1 (7.2-fold). To a lesser extent, the hC641S mutation (as in ^{638}Leu-Asp-Phe-Cys-Met-Leu-His^{644} (Hinman et al., 2006) also reduced sensitivity to AITC (2.1-fold) and CMP1 (1.8-fold). Therefore, it is likely that CMP1 exhibits its effects on TRPA1 through covalently modifying cysteine residues.

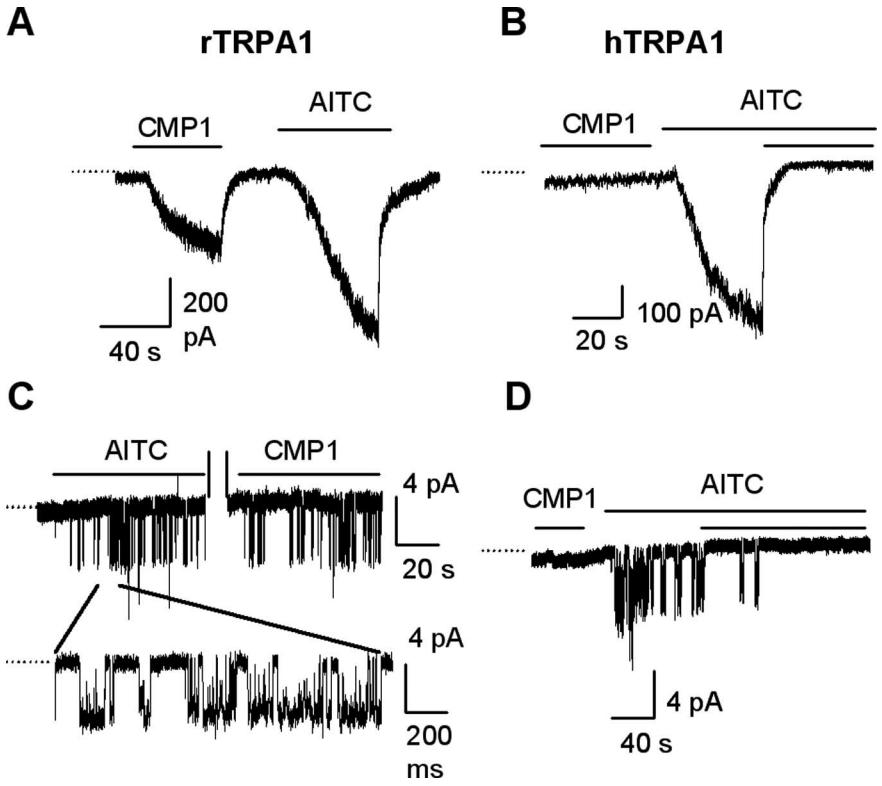

Figure 2. CMP1 activated rTRPA1 and blocked hTRPA1, as assessed in whole-cell and cell-attached single-channel recordings. ***A***, In a representative cell expressing rTRPA1 (held at −60 mV), CMP1 evoked whole-cell currents in a reversible manner. A follow-up application of AITC also evoked inward currents. The dotted line indicates zero current level. ***B***, CMP1 blocked AITC-induced hTRPA1 currents. ***C***, CMP1 evoked rTRPA1 single-channel activities (−60 mV). ***D***, CMP1 blocked AITC-induced hTRPA1 single-channel activities. The concentrations for CMP1 and AITC were 100 μM.

S6 domains determine CMP1-mediated channel opening and block

How does the modification by CMP1 lead to activation of rTRPA1 but blockade of hTRPA1? To address this question, we constructed rTRPA1-hTRPA1 chimeras, focusing on the pore modules, including S5, the linker between S5 and S6 (L56), and S6 domains (Fig. 4*A*) (for sequence alignment, see supplemental Fig. 4*A*, available at www.jneurosci.org as supplemental material). Chimera h-rS5L56S6 was constructed by introducing S5, L56, and S6 of rTRPA1 into hTRPA1. Strikingly, h-rS5L56S6 was activated by CMP1, whereas the reverse chimera r-hS5L56S6 (constructed by introducing S5, L56, and S6 of hTRPA1 into rTRPA1) was not activated by CMP1 (up to 300 μM); instead, it was blocked by CMP1 when either AITC or URB597 was used as agonist. Therefore, determinants of channel gating are located within the ion conduction pore.

To further narrow down critical domains within the pore regions, S5+L56 or S6 domains were individually swapped (Fig. 4*A*). In the hTRPA1 background, the introduction of the S5+L56 from rTRPA1 (h-rS5L56) maintained the sensitivity to blockade by CMP1, whereas introducing S6 of rTRPA1 (h-rS6) yielded a channel that could be neither blocked nor activated by CMP1 (Fig. 4*B*). In the rTRPA1 background, introducing S5+L56 of hTRPA1 (r-hS5L56) resulted in clear, albeit diminished, activation by CMP1. For example, the Ca^{2+} response evoked by 300 μM CMP1 was 54.0 ± 4.9% of those evoked by 30 μM AITC ($n = 4$). In contrast, introducing the S6 domain of hTRPA1 (r-hS6) produced a channel that mimicked the property of hTRPA1, viz., blockade by CMP1 (Fig. 4*C*). These data suggest that the S6 domain of hTRPA1 is necessary and sufficient to confer channel block by CMP1, whereas the S6 domain of rTRPA1 is necessary, but not sufficient, for channel activation.

Single residues in the S6 domains determine channel gating

The sequence alignment around S6 domains revealed six amino acid differences between rTRPA1 and hTRPA1 (Fig. 5*A*). Substitutions of each of the four residues near the N terminus of the rTRPA1 S6 domain with equivalent hTRPA1 residues retained activation by CMP1 (Fig. 5*B*). Consistently, the four reverse mutations in hTRPA1 retained blockade by CMP1. In contrast, substitutions in the two other positions produced profound changes in gating responses to CMP1. rA946S and rM949I abolished

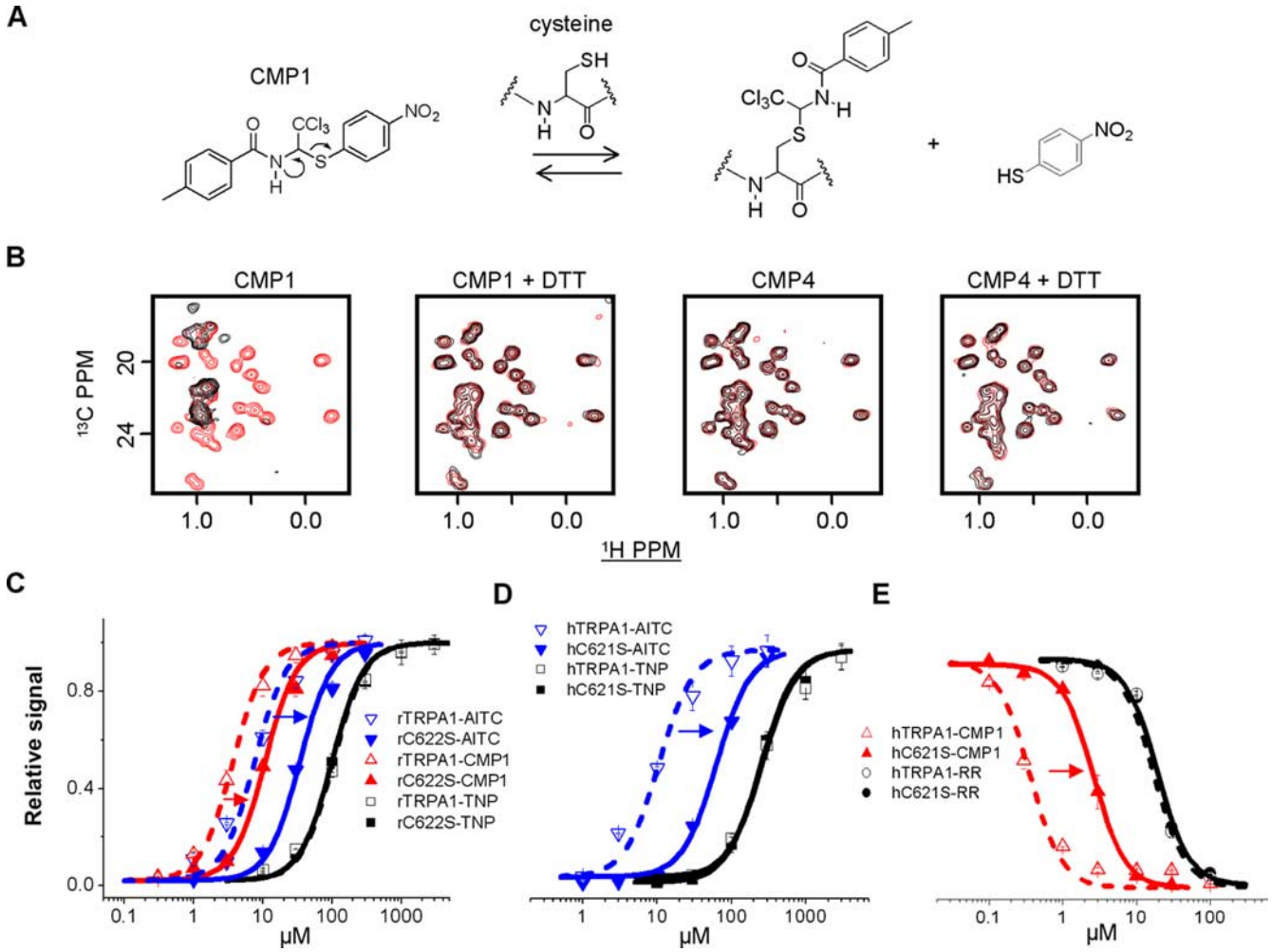

Figure 3. CMP1 activities on TRPA1 depend on its chemical reactivity. ***A***, Schematic of the chemical reaction between CMP1 and a cysteine residue with a free SH group. ***B***, Reactivity in CMP1 but not in CMP4, as assessed by La antigen-based ALARM NMR. Each panel contains an overlay of the [^{13}C- ^{1}H] HSQC with the leucine and valine methyl proton chemical shifts (protein alone, red contours; protein with compound, black contours). Compounds were tested with and without 20 mM DTT. ***C***, Concentration-effect relationships of rC622S and rTRPA1 activation by AITC, CMP1, and TNP, as determined in the Ca^{2+} assay. AITC EC_{50}, 7.5 ± 1.0 μM (rTRPA1), 40.3 ± 3.6 μM (rC622S); CMP1 EC_{50}, 4.0 ± 0.4 μM (rTRPA1), 10.7 ± 0.9 μM (rC622S); TNP EC_{50}, 108.0 ± 6.6 μM (rTRPA1), 103.5 ± 5.7 μM (rC622S). Note reductions in sensitivity to AITC and CMP1 (arrows) but not to TNP. ***D***, hC621S reduced the activation sensitivity to AITC but not TNP. AITC EC_{50}, 11.3 ± 2.3 μM (hTRPA1), 62.9 ± 3.1 μM (hC611S); TNP EC_{50}, 259 ± 19 μM (hTRPA1), 245 ± 16 μM (hC611S). ***E***, hC621S reduced inhibition potency of CMP1 but not RR. CMP1 IC_{50}, 0.36 ± 0.00 μM (hTRPA1), 2.6 ± 0.1 μM (hC611S); IC_{50} RR, 18.9 ± 0.4 μM (hTRPA1), 19.9 ± 0.1 μM (hC611S). TNP (300 μM) was used to activate channels. $n = \sim 4–12$.

channel activation and introduced block, whereas the reverse mutations hS943A and hI946M resulted in loss of CMP1 sensitivity (no activation or blockade). Therefore, AL-946 and Met-949 in rTRPA1 are critical for channel activation by CMP1, whereas the equivalent residues in hTRPA1 (Ser-943 and Ile-946) determine channel block.

As structural analogs of CMP1, the thioaminal-containing CMP2 and CMP3 covalently modify cysteine residues, producing identical or similar protein adducts. CMP2 and CMP3 activated rTRPA1 and blocked hTRPA1 (Fig. 1*A*). In addition to its effect on CMP1 responses, the rM949I mutation abolished activation and introduced block by CMP2 and CMP3 (Fig. 5*C*). Therefore, Met-949 of rTRPA1 is critical for channel activation by three thioaminal compounds.

Side-chain substitutions at residues 946 and 949 profoundly affect gating of rTRPA1

To further understand its role in CMP1-mediated channel gating, Met-949 in rTRPA1 was mutated to Leu, Val, Tyr, Thr, Gly, Ile, or Pro, each possessing different combinations of physicochemical properties (supplemental Table 2, available at www.jneurosci.org as supplemental material). rM949P did not respond to AITC, 4-hydroxynonenal, URB597, TNP, or CMP1, suggesting a loss of channel function and/or surface expression. All other mutant channels exhibited relatively normal responses to AITC and URB597, and they were blocked by CMP1. Therefore, Met-949 is specifically required for CMP1-mediated channel activation.

Likewise, AL-946 of rTRPA1 was mutated to Gly, Asp, Pro, Ser, Thr, Tyr, and Lys, When tested against CMP1, three distinctive gating responses were observed (supplemental Table 3, available at www.jneurosci.org as supplemental material): rA946D and rA946P could neither be activated nor blocked by CMP1; rA946S, rA946T, rA946Y, and rA946K could only be blocked by CMP1; only rA946G retained activation by CMP1. Because Ala and Gly are the smallest amino acid residues, as reflected by their total accessible surface areas, side-chain accessible surface areas, and molecular weights, these results suggest that a small side chain at residue 946 is required for CMP1 activation of rTRPA1.

Discussion

In this study, we identified thioaminal-containing compounds that activated rTRPA1, but blocked hTRPA1 activation by a variety of stimuli, including AITC, cinnamaldehyde, 4-Hydroxynonenal, URB597, TNP, and hypertonicity. The opposite effects were not caused by usage of a particular expression system (HEK293F, ND7/23, PC12) or methodologies (Ca^{2+} assay, membrane potential assay, whole-cell and single-channel recordings) but rather intrinsic to channel proteins. These results are consistent with a recent report that several structural analogs of CMP1 have similar species-specific effects on TRPA1 (Klionsky et al., 2007). Several lines of evidence indicate that thioaminal compounds act on rTRPA1 and hTRPA1 through covalent modification. First, these compounds are predicted to be electrophilic and reactive to nucleophilic cysteine or lysine residues. Second, the chemical reactivity was confirmed by La antigen-based ALARM NMR and ALARM MS, in which surface-exposed cysteine residues were modified and predicted adducts were formed. Third, structural analogs with the reactive sulfur atom (CMP2 and CMP3) exhibited reactivity and effects on channel functions. Fourth, close analogs without the reactive sulfur atom (e.g., CMP4) (Fig. 1*A*) did not affect rTRPA1 or hTRPA1, suggesting that effects on channel functions depend on the chemical reactivity. Finally, Cys-621 of hTRPA1 and Cys-622 of rTRPA1, the residues critical for modification by AITC, iodoactamide, and 4-hydroxynonennal (Hinman et al., 2006; Macpherson et al., 2007; Trevisani et al., 2007), are also important for CMP1 mediated effects. Specifically, the elimination of these residues (rC622S and hC621S) reduced sensitivities to the reactive AITC, CMP1, but not to the nonreactive TNP and RR (Fig. 3*C–E*). Together, the correlation between cysteine reactivity and functional activities and the reduced sensitivities of cysteine mutants are consistent with the idea that thioaminal compounds exert their effects on TRPA1 through covalent modification.

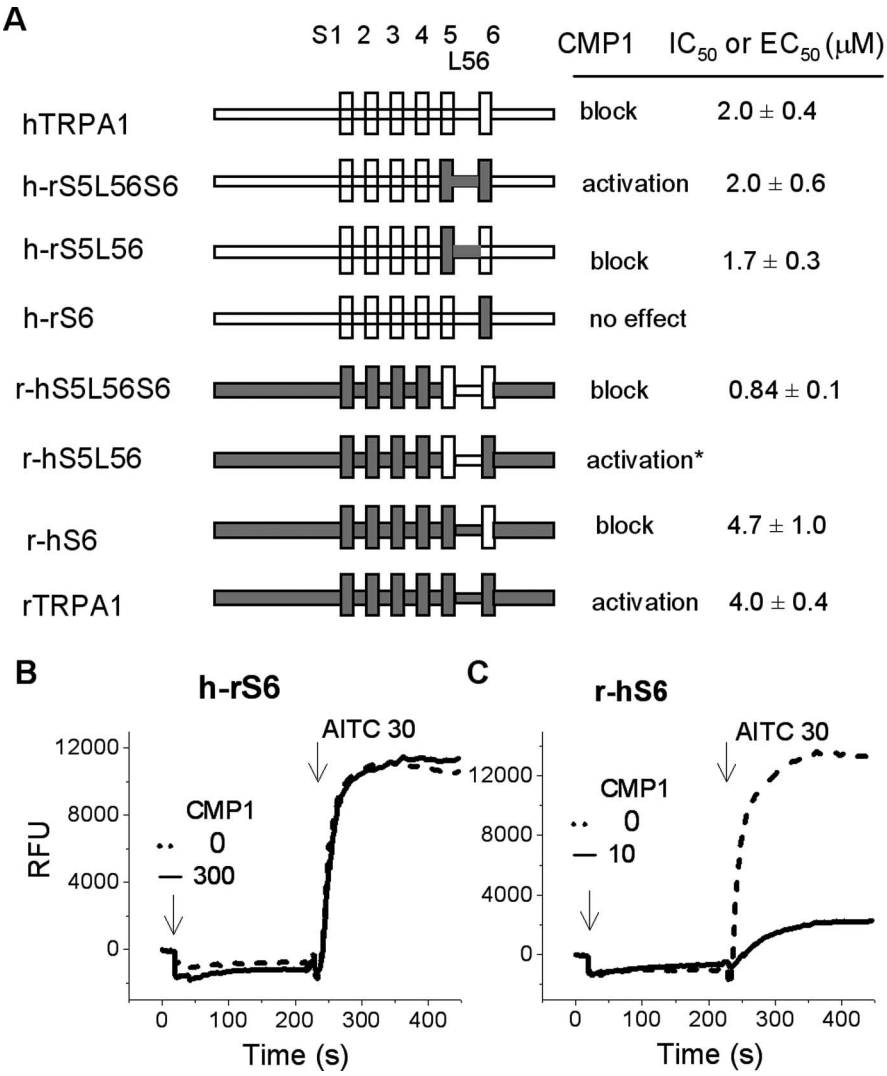

Figure 4. S6 domains of rTRPA1 and hTRPA1 dictate CMP1-mediated channel gating. ***A***, Schematic representations of chimeras and their responses to CMP1, as measured in the Ca^{2+} assays. The amino acid compositions of chimeras are included in the supplemental material (available at www.jneurosci.org) and also indicated by arrows in supplemental Figure 5*A* (available at www.jneurosci.org as supplemental material). ***B***, h-rS6 was insensitive to block or activation by CMP1 (in μM). ***C***, r-hS6 was blocked by CMP1. Asterisk, In r-hS5L56, 300 μM CMP1 evoked 54.0 ± 4.9% of Ca^{2+} signals compared with AITC.

Given the specific chemical modification by thioaminals, we expect that covalent attachment of CMP1 to cysteine residues would be reversible (Fig. 3*A*). In fact, previous exposure of CMP1 followed by a wash-off period did not abrogate subsequent hTRPA1 activation by AITC, consistent with reversibility of the CMP1 modification, whereas the coapplication of CMP1 rapidly blocked channels already activated by AITC (Fig. 2*B*). Many other electrophiles have been reported to act on TRPA1 in a reversible manner, including AITC, benzyl isothiocyanate, allicin, and cinnamaldehyde (Jordt et al., 2004; Bautista et al., 2005; Macpherson et al., 2005; Hinman et al., 2006). Electrophilic compounds such as AITC and CMP1 likely modify many cysteine residues (Hinman et al., 2006; Macpherson et al., 2007), although it is unclear whether all sites are identical between rTRPA1 and hTRPA1, between different channel conformations, and between AITC and CMP1 modifications. For example, in the simultaneous presence of CMP1 and AITC, it is possible that CMP1 modifies additional sites to block AITC-conjugated hTRPA1. Alternatively, CMP1 may compete with AITC for modification at the same site to confer blockade of hTRPA1. It is also unclear whether the chemical modification and channel functions are affected by cellular oxidative states and endogenous nucleophiles.

Remarkably, the opposite functional responses to thioaminals can be contributed to single residue differences in the S6 domains, implicating the essential role of these residues in channel gating. Specifically, AL-946 and Met-949 are critical for rTRPA1 activation, and the equivalent residues in hTRPA1 are critical for channel block. In addition, side-chain substitutions at these key residues have profound functional consequences. For example, substitutions at Ala-946 can result in channel activation (rA946G), blockade (e.g., rA946T), and no responses (e.g., rA946D), suggesting conformational changes at this residue are

A

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                          S6
rTRPA1 (934)LAYPVLTFGQLIAFTMFVPIVLMNLLIGLAVGDI
hTRPA1 (931)LAHPVLSFAQLVSFTIFVPIVLMNLLIGLAVGDI
                      * *
```

B

rTRPA1	CMP1	IC50/EC50	hTRPA1	CMP1	IC50/EC50
rTRPA1	act	4.0±0.4	hTRPA1	block	2.0±0.4
rY936H	act	13.7±4.0	hH933Y	block	5.0±0.2
rT940S	act	4.8±0.5	hS937T	block	2.9±0.5
rG942A	act	11.7±2.2	hA939G	block	3.0±0.4
rI945V	act	10.7±4.1	hV942I	block	4.0±0.7
rA946S	block	9.1±2.5	hS943A	no effect	
rM949I	block	2.3±0.2	hI946M	no effect	

C

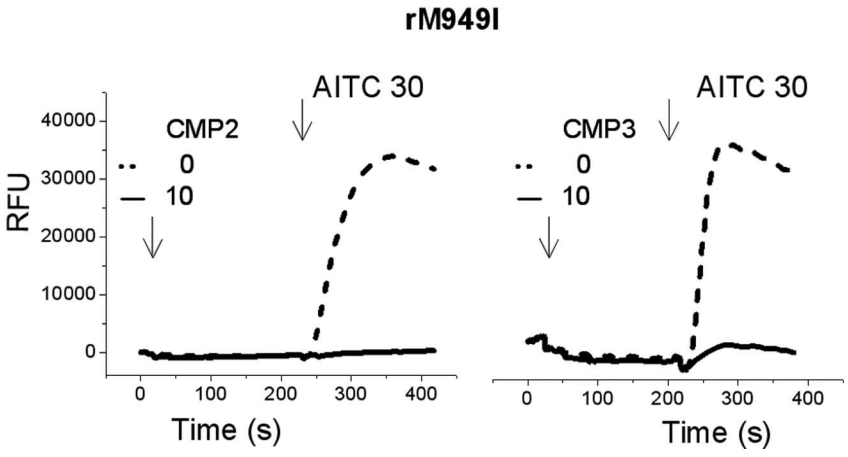

Figure 5. S6 residues critical for CMP1-mediated channel gating. ***A***, A sequence alignment around the S6 domains of rTRPA1 and hTRPA1. Arrows indicate junction sites for S6 swaps. Positions with residue differences are marked in bold, and critical residues are marked by an asterisk. ***B***, Single-residue substitutions and their responses to CMP1. act, Activation; no effect, no activation or block at concentration up to 300 μM. IC_{50} and EC_{50} values are in μM ($n = \sim 4-12$). ***C***, rM949I was blocked by CMP2 and CMP3 (the thioaminal containing analogs of CMP1), as accessed in the Ca^{2+} assay. Representative traces were taken from four trials.

critical to channel gating (supplemental Table 3, available at www.jneurosci.org as supplemental material). TRPA1 is predicted to have the same architecture as voltage-gated K^+ channels and cyclic nucleotide-gated channels. In these channels, opening and closing of the ion conduction pore are controlled by two distinct molecular gates: an intracellular gate formed by bundle crossing of the four S6 domains and a selectivity filter gate localized near the extracellular side of the membrane (Berneche and Roux, 2005; Long et al., 2005). Similar gating mechanisms may occur in TRP channels, but the physical nature of the gate and mechanisms underlying its opening/closing are not understood (Clapham, 2003; Voets et al., 2005). Given their location in the upper portion of S6 domains (e.g., AL-946 and Met-949 in rTRPA1), it is possible that conformational changes in these residues may lead to movements of cytoplasmic ends of S6, where an intracellular gate is formed as in K^+ channels (Doyle et al., 1998). Alternatively, conformational changes in Ala-946 may affect a gate localized in the selectivity filter. To explain our experimental data, we generated a homology model for the S5-S6 region of rTRPA1 (supplemental Fig. 5, available at www.jneurosci.org as supplemental material) by using the Kv1.2 channel structure as a scaffold (Long et al., 2005). This model predicts that Ala-946 is in close proximity with the selectivity filter, and that slight conformational changes of Ala-946, either as results of channel gating or substitution by a larger residue, change selectivity filter conformation. Indeed, substitutions with larger side chains at position 946 (Asp, Pro, Ser, Tyr, and Lys) all abolish channel activation, whereas the substitution by the smaller glycine retains channel activation (supplemental Table 3, available at www.jneurosci.org as supplemental material). Of note, the selectivity filters of K^+ channel and cyclic nucleotide-gated channels play an important role in channel gating (Liu et al., 1997; Berneche and Roux, 2005). Recent evidence suggests TRPV1 and TRPV5 gating may also involve the pore-helix and selectivity filter (Yeh et al., 2005; Ryu et al., 2007). Our data, corroborated by the homology model, suggest that a similar channel gating may occur at the selectivity filter of TRPA1. Nonetheless, this hypothesis still awaits confirmation by future experiments.

Given the seemingly disparate locations between covalent modification (e.g., Cys-622 in the N terminus) and key S6 residues (e.g., AL-946), a coupling between cysteine modification and Ala-946 movement may involve other parts of the channel protein. Transmembrane domains, S4-S5 linker, and C terminus of other TRP channels have been shown to affect channel functions (Jordt and Julius, 2002; Bandell et al., 2006; Kuhn et al., 2007; Voets et al., 2007). Whether these or other structural domains are involved in TRPA1 function remains to be determined. Nonetheless, our data unequivocally demonstrate that S6 residues determine the species-specific responses and constitute a critical link between covalent modification and channel gating.

The finding of hTRPA1 blockade by thioaminal-containing compounds extends the current understanding of TRPA1 channel function. Previous studies have suggested that the chemical reactivity of electrophilic compounds (e.g., AITC), not the structure per se, determines activation of TRPA1 (Macpherson et al., 2007). Our data demonstrate, in fact, the electrophilic, thioaminal-containing molecules may modify cysteine residues within hTRPA1, yet the covalent modification results in channel closing instead of channel opening. Therefore, it is clear that covalent modification can lead to different functional consequences. Whether channels open or close after covalent modification depends not only on their respective proteins (e.g., rTRPA1 and hTRPA1) but also on the nature of chemical adducts donated by electrophilic compounds. AITC modification leads to similar conformational changes and channel activation in rTRPA1, hTRPA1, whereas modification by thioaminal compounds leads to different conformational changes and opposite functional consequences between the two channels. 4-Hydroxynonenal, abundantly produced during tissue injury, inflammation, and oxidative stress, mediates pain and inflammation by endogenously modifying and activating TRPA1 (Trevisani et al., 2007). It will be interesting to determine whether

nature also uses endogenous, electrophilic blockers of TRPA1 to counter-balance excessive hyperalgesia, tissue injury, and inflammation.

Our current findings also have important implications for drug discovery efforts targeting TRPA1. The susceptibility of TRPA1 to modification by reactive compounds represents a special challenge. Despite rigorous compound selection and filtering, it is estimated that ~10% of compounds from chemical libraries and medicinal chemistry efforts are reactive, resulting from parent compounds, impurities, or breakdown products (Huth et al., 2005). Specifically, CMP1–3 are potent, selective TRPA1 antagonists, but the chemical reactivity significantly limits their preclinical or clinical utilities. In addition to TRPA1, these agents likely modify cysteine or lysine residues randomly in other cellular proteins, leading to toxicity or other unwanted side effects. Therefore, chemical reactivity assessments should be rigorously performed during the drug discovery process, and only nonreactive compounds should be advanced for clinical development. Another serious challenge is the significant pharmacological differences between hTRPA1 and its rat counterpart. Currently, rodents, particularly rats, are most widely used in animal pain models to assess compounds in preclinical studies. Our data point to the necessity of evaluating compounds against both hTRPA1 and rTRPA1 before clinical development. Of note, the structurally related thioaminal compounds (CMP1–3) exhibit similar inhibition on hTRPA1 but more divergent activation on rTRPA1 (Fig. 1*A*). It will be important to determine whether, and to what extent, the species-specific effects can be addressed through medicinal chemistry efforts.

In summary, we demonstrated that electrophilic, thioaminal compounds likely covalently modify TRPA1 channels but lead to activation of rTRPA1 and block of hTRPA1. The opposite gating effects can be attributed to residue differences in the S6 domains. Although more work will be required, the results described above provide a hint of how TRP channels respond to chemical stimuli.

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