

# Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines

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The nervous system senses peripheral damage through nociceptive neurons that transmit a pain signal<sup>1,2</sup>. TRPA1 is a member of the Transient Receptor Potential (TRP) family of ion channels and is expressed in nociceptive neurons<sup>3–5</sup>. TRPA1 is activated by a variety of noxious stimuli, including cold temperatures, pungent natural compounds, and environmental irritants<sup>6–11</sup>. How such diverse stimuli activate TRPA1 is not known. We observed that most compounds known to activate TRPA1 are able to covalently bind cysteine residues. Here we use click chemistry to show that derivatives of two such compounds, mustard oil and cinnamaldehyde, covalently bind mouse TRPA1. Structurally unrelated cysteine-modifying agents such as iodoacetamide (IA) and (2-aminoethyl)methanethiosulphonate (MTSEA) also bind and activate TRPA1. We identified by mass spectrometry fourteen cytosolic TRPA1 cysteines labelled by IA, three of which are required for normal channel function. In excised patches, reactive compounds activated TRPA1 currents that were maintained at least 10 min after washout of the compound in calcium-free solutions. Finally, activation of TRPA1 by disulphide-bond-forming MTSEA is blocked by the reducing agent dithiothreitol (DTT). Collectively, our data indicate that covalent modification of reactive cysteines within TRPA1 can cause channel activation, rapidly signalling potential tissue damage through the pain pathway.

We noted that many TRPA1-activating compounds are electrophiles able to react with cysteines. For example, the nucleophilic mercapto group of cysteines can attack the  $\alpha,\beta$ -unsaturated bond of cinnamaldehyde (CA) via a Michael addition (Table 1). In support of this mechanism, a more reactive cinnamaldehyde-like Michael acceptor with a carbonyl substitution adjacent to the enone<sup>12</sup> (a substituted oxindole, here referred to as super cinnamaldehyde, SC) is also a more potent activator of TRPA1 (Table 1). Interestingly, chemically inert structural analogues of TRPA1 agonists, such as propionaldehyde, cinnamic alcohol and SC alcohol, do not activate TRPA1<sup>6,9</sup> (Table 1, and not shown). Other activators including isothiocyanates such as mustard oil (MO) could be conjugated with cysteines via an addition to form dithiocarbamates<sup>13</sup> (Table 1).

We predicted that TRPA1 could be activated by covalent binding of electrophiles to cysteines. We tested if structurally unrelated cysteine-modifying agents could also activate TRPA1. We found TRPA1 was activated by both the commonly used cysteine-modifying alkylating agent iodoacetamide (IA; a standard reagent in mass spectrometry used to bind covalently with free cysteines to avoid protein aggregation) and a reagent that forms disulphide bonds with cysteines, (2-aminoethyl)methanethiosulphonate (MTSEA)<sup>14,15</sup> (Table 1, and see later). *N*-hydroxyl succinimide (NHS), a lysine modifying agent<sup>16</sup>, did not activate TRPA1 in calcium imaging experiments at 100  $\mu\text{M}$  (not shown). *In vitro*, MO, CA, SC and IA formed adducts with

the cysteine-containing tripeptide glutathione (Glu-Cys-Gly; Supplementary Fig. 1a–e). These data indicate that solvent-accessible cysteine residues in TRPA1 might be covalently modified by these reactive compounds.

Alkyne groups are rarely found *in vivo* and have been used as a tag to monitor covalent modification of proteins via a copper(I)-catalysed

**Table 1 | Cysteine-modifying properties of TRPA1 agonists.**

TRPA1 agonist	Structure	EC <sub>50</sub>	Inactive analogue structure
Cinnamaldehyde (CA)		19.0 $\mu\text{M}$	
Super cinnamaldehyde (SC)		0.8 $\mu\text{M}$	
SC alkyne (SCA)		0.1 $\mu\text{M}$	
Acrolein		5 $\mu\text{M}^*$	
Pentenal		5 $\mu\text{M}^*$	
Mustard oil (MO)		33.5 $\mu\text{M}$	
Mustard oil alkyne (MOA)		18.4 $\mu\text{M}$	
Iodoacetamide (IA)		357 $\mu\text{M}$	
Iodoacetamide alkyne (IAA)		134 $\mu\text{M}$	
MTSEA		1.58 mM	

\* As reported previously<sup>6</sup>. † As reported previously<sup>9</sup>.

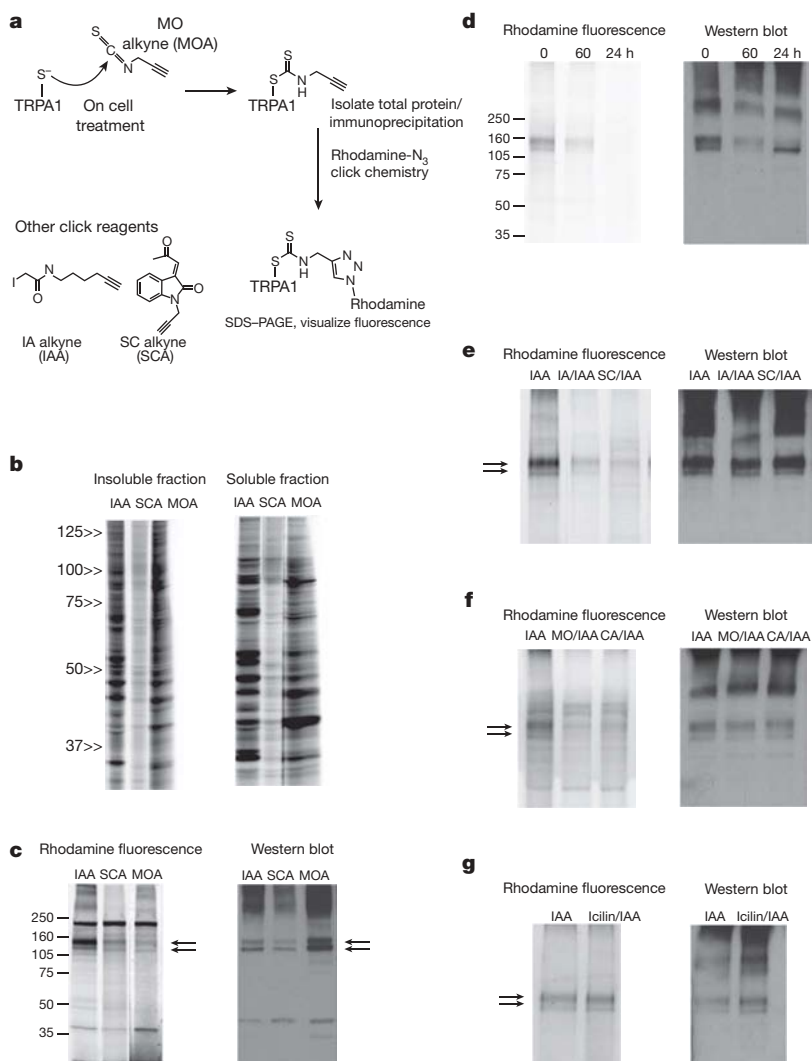
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[3 + 2] cycloaddition reaction between alkyne and azide groups ('click chemistry') (Fig. 1a)<sup>17–19</sup>. We tested whether alkyne-tagged IA, MO and SC (all three of which retained TRPA1 activity, Table 1) covalently bind to TRPA1 using click chemistry. As expected, a large number of proteins are labelled by the alkyne derivative of IA (IAA; Fig. 1b). Interestingly, alkyne-modified MO (MOA) and SC (SCA) also covalently bound many proteins (Fig. 1b). We next used pull-down experiments and click chemistry to monitor TRPA1 modification directly. All three compounds covalently labelled immunoprecipitated TRPA1 (Fig. 1c). In pulse-chase experiments, covalent modification of TRPA1 by IA or SC derivatives was present 1 h post-wash but returned to background levels by 24 h, possibly owing to protein turnover (Fig. 1d, not shown). These experiments suggest that SC and MO, similar to IA, covalently modify free cysteines within proteins, including TRPA1.

To determine whether TRPA1 agonists label similar sites on the channel, we used the pull-down click labelling method to measure the amount of labelling by IAA after TRPA1-expressing cells were first exposed to IA, SC, MO, CA and icilin. Pre-incubation of cells with untagged IA, SC, CA or MO with subsequent treatment with IAA reduced the rhodamine fluorescence of immunoprecipitated TRPA1

compared with cells treated with IAA alone (Fig. 1e, f). These results indicate that SC, CA and MO covalently label many of the same cysteines that are labelled by IAA. Interestingly, pre-incubation of TRPA1 cells with 100  $\mu\text{M}$  icilin did not inhibit labelling of the channel by IAA (Fig. 1g). Icilin, an activator of TRPA1 with no obvious reactivity to cysteines, did not react *in vitro* with glutathione as measured by electrospray ionization–mass spectrometry (ESI–MS) (Supplementary Fig. 1f). Therefore, it seems likely that icilin activates TRPA1 through an independent mechanism.

We next employed mass spectrometry to identify the individual modified cysteines within TRPA1 (Fig. 2a)<sup>14,20,21</sup>. IA application at 100  $\mu\text{M}$  covalently labelled six cysteines, whereas 400  $\mu\text{M}$  labelled an additional eight for a total of 14 cysteines (Fig. 2b; Supplementary Fig. 2a, b). These experiments establish that at least 14 residues are reactive and covalently bind IA during a live-cell pulse. It is likely that some of the undetected cysteines could also have been modified by the initial IA pulse. Because the majority of TRPA1 cysteines reside in the intracellular portion of the channel, we used the membrane-impermeable cysteine-disulphide-forming reagent MTSEA-biotin to determine the site of TRPA1 activation. In whole-cell patch clamp electrophysiology experiments, MTSEA-biotin (400  $\mu\text{M}$ ) applied to



**Figure 1 | TRPA1 is covalently modified by reactive compounds.**

**a**, Schematic of the proposed reactions of alkyne reagents with TRPA1, and the click chemistry labelling with rhodamine- $\text{N}_3$  to visualize covalently bound activators on the protein. **b**, **c**, Many cellular proteins (**b**) and immunoprecipitated TRPA1 (**c**) are labelled by rhodamine using the click chemistry method after a 10 min incubation with 100  $\mu\text{M}$  IAA (left lanes), SCA (middle lanes) or MOA (right lanes). Molecular weight markers are

indicated (kDa). Arrows indicate immunoprecipitated TRPA1 (runs as a doublet). **d**, IAA labelling of immunoprecipitated TRPA1 at 0 min, 60 min and 24 h after initial treatment with 100  $\mu\text{M}$  IAA. **e–g**, Pre-incubation of TRPA1-expressing cells with untagged compounds followed by labelling of immunoprecipitated TRPA1 with IAA. Left panels, rhodamine fluorescence gel images; right panels, corresponding western blots.



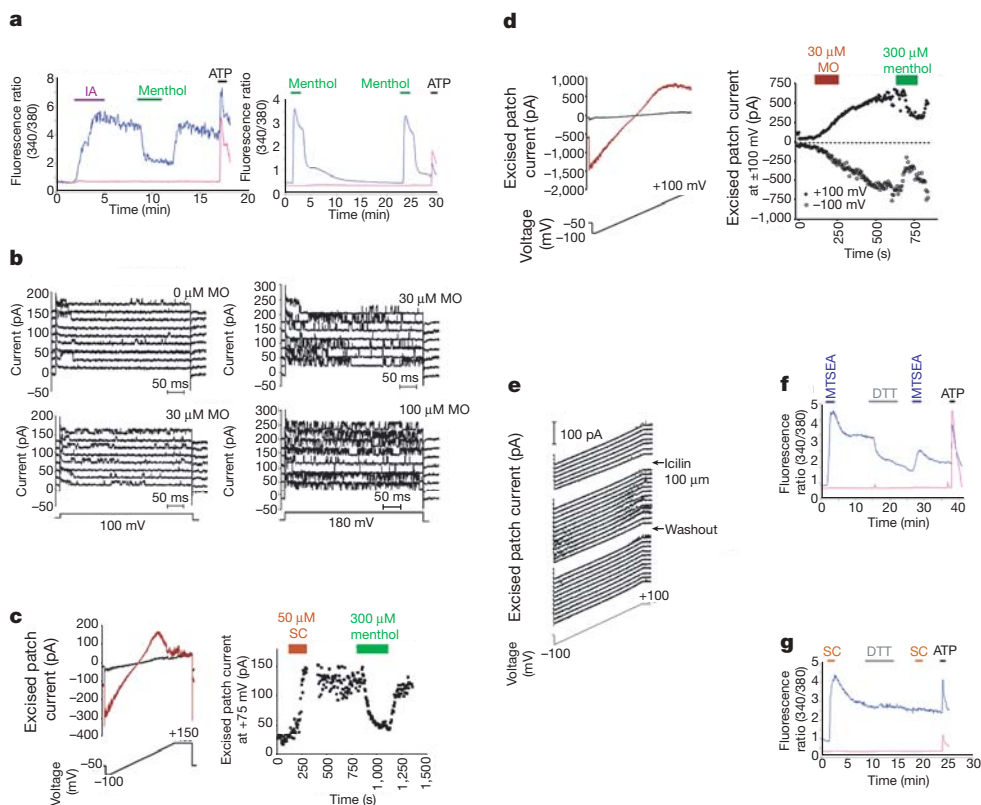
transduction of reactive compound labelling to channel activation, such that modifications (covalent modification or amino acid substitutions) at these locations can profoundly affect channel gating. However, we cannot rule out the possibility that the three cysteines are also required for appropriate channel conformation. Furthermore, it is likely that other reactive cysteines involved in TRPA1 activation were not highlighted by individual mutation (Fig. 2b).

Michael addition, conjugation, alkylation and disulphide formation of cysteines are relatively stable modifications, and a pulse of an activator could potentially cause prolonged channel activity. Indeed, 2-min pulses of SC, MO, MTSEA and IA caused sustained elevation of intracellular calcium levels over a period of 15–60 min in CHO cells expressing TRPA1 (Fig. 3a; Supplementary Fig. 4a–d). The addition of menthol during the extended period of activation reversibly reduced the elevated calcium levels, arguing that the sustained calcium signal is due to ongoing TRPA1 activity (Fig. 3a, left panel; Supplementary Fig. 4c, d). In contrast, TRPM8 activation by pulses of maximally activating concentrations of menthol shows fast recovery from each pulse, suggesting that prolonged activation is not a general mechanism of all TRP channels (Fig. 3a, right panel). We also investigated the reversibility of TRPA1 activity in isolated patches from TRPA1-expressing HEK cells, in a calcium-free environment to reduce desensitization. TRPA1 single-channel activity (92 pS) was both voltage- and agonist-concentration-dependent (Fig. 3b)<sup>25</sup>. Similar to results obtained from calcium imaging experiments, a 2-min application of SC (50  $\mu$ M) or MO (30  $\mu$ M) to an inside-out patch showed continued activity for more than 10 min, which could

be reversibly reduced by menthol, demonstrating that irreversible activation can occur even at moderate concentrations of agonist (Fig. 3c, d). Interestingly, application of 100  $\mu$ M icilin to excised inside-out patches enhanced channel activity in a rapidly reversible manner (Fig. 3e), again demonstrating the qualitative differences in mechanism of action of icilin on TRPA1 compared with cysteine-reactive agonists.

Dithiothreitol (DTT) is a cell-permeable reducing agent that can reverse the disulphide modification of cysteines, but not that of the Michael addition or cysteine conjugation reactions (for example, see refs 15, 26). Therefore, DTT should reverse MTSEA-induced, but not SC- or MO-induced activation. This is indeed what we observed in calcium imaging experiments in CHO cells and cultured dorsal root ganglia neurons (Fig. 3f, g; Supplementary Fig. 4i–k, and not shown).

Redox-sensitive cysteine residues have been implicated in modulation of ion channel function, including activation of ryanodine receptors through S-nitrosylation<sup>27,28</sup>. S-nitrosylation of cysteine residues next to the pore of TRPC5 has also recently been shown to activate the channel<sup>29</sup>. However, the covalent modification of TRPA1 by pungent compounds is apparently irreversible. How, then, is the channel inactivated? Activated channels still display gating; for instance, inactivation at high voltage (Fig. 3c, left panel; Supplementary Fig. 4h). At a single-channel level, compound-activated channels showed open/closed transitions reflecting open and desensitized states (Fig. 3b). Furthermore, TRPA1 activity can be blocked by intracellular calcium<sup>25</sup>. Thus, mechanisms exist for inactivating the covalently modified channel.



**Figure 3 | Reactive compounds can cause sustained activation of TRPA1, and DTT reverses activation of TRPA1 by MTSEA.** **a**, Calcium imaging experiments of CHO cells transiently transfected with TRPA1 (left panel) and TRPM8 (right panel). Blue trace indicates the transfected cell average; pink trace, untransfected; average of 15–30 cells. **b**, Single-channel activity evoked by voltage steps to either +100 or +180 mV (from –50 mV) in a single, excised, inside-out wild-type TRPA1 patch, challenged with increasing concentrations of MO. **c**, **d**, SC and MO (50  $\mu$ M and 30  $\mu$ M, respectively; applied as indicated by the red bars, right panels) activated a non-selective cation conductance in an excised patch from wild-type

TRPA1-expressing HEK cells (left panels). The currents were measured over the entire experiment and plotted versus time (right panels). The currents were reversibly reduced by 300  $\mu$ M menthol application (green bars). **e**, Single-channel wild-type TRPA1 activity evoked by voltage ramps recorded every 15 s before, during and after washout of 100  $\mu$ M icilin in the bath (arrows). **f**, **g**, DTT (5 mM) reverses TRPA1 activation induced by the disulphide-forming activator MTSEA (400  $\mu$ M; **f**), but not the Michael addition activator SC (50  $\mu$ M; **g**). TRPA1-expressing cells (blue traces) and untransfected cells (pink traces) were imaged for intracellular calcium levels.

TRPA1 is not unique among proteins to be modified by cysteine reactive agents, but we show it is uniquely activated in response to such stimuli. Interestingly, cytosolic Kelch-like ECH-associated protein 1 (KEAP1) is activated by many of the same compounds that activate TRPA1<sup>14,15,21,30</sup>. Apparently, reactive compounds can activate at least two pathways through cysteine modification: KEAP1-regulated transcriptional activation of antioxidant enzymes and TRPA1-mediated rapid activation of nociceptors as a warning signal against cell damage. By tuning TRPA1 to respond to covalent modification by reactive compounds, the nervous system can directly assess the noxious environment of sensory neurons.

*Note added in proof:* Following review of this manuscript, another study reported some of the findings reported here<sup>31</sup>.

## METHODS

**Protein isolation, click chemistry and fluorescent gel imaging.** These were performed essentially as described previously<sup>19</sup>. The protocol was modified for TRPA1 immunoprecipitation experiments where cells were treated, lysed and immunoprecipitated with anti-Myc and Protein G agarose beads, and then click-labelled on bead. After SDS-polyacrylamide gel electrophoresis (PAGE), rhodamine fluorescence was visualized in-gel using a Hitachi FMBio Iie flatbed-laser-induced fluorescence scanner (MiraiBio, Alameda, California). Gels were probed for protein transfer to nitrocellulose membranes and western blotting. **Proteomics of IA-labelled TRPA1.** We applied IA (either 100  $\mu$ M for 2 min or 400  $\mu$ M for 10 min) to living TRPA1-expressing HEK cells. Cells were subsequently lysed, TRPA1 protein immunoprecipitated, and proteins run on SDS-PAGE. Coomassie-stained bands of appropriate TRPA1 mass were excised from the gel, reduced with 10 mM DTT, alkylated with 55 mM IAA (as a substitute for IA in the protocol to differentiate this modification from the IA used in the live-cell treatment), trypsinized and analysed by nano-liquid-chromatography-mass spectrometry/mass spectrometry (LC)-MS/MS on an Agilent ion trap mass spectrometer. A proportion (30–40%) of TRPA1 residues were identified by spectra analysis using the Mascot search engine (Matrix Science).

**Electrophysiology.** Whole-cell electrophysiological methods were as described previously<sup>9</sup>. Excised patch voltage clamp experiments were performed on the equipment used for whole-cell studies (that is, the Axon Instruments (Molecular Devices Corporation)-based acquisition system). Calcium-free solution contained (in mM): NaCl 124.5, MgCl<sub>2</sub> 2, EGTA 5, HEPES 10, pH 7.4. Cells and excised patches were continuously perfused at 1–2 ml min<sup>-1</sup> at a constant temperature (>24 °C) to avoid complications of exposing the channels to cool temperatures. Compounds were usually added from 300–1,000-fold stock solutions in ethanol or DMSO. Vehicle control at these concentrations had no effect on TRPA1 activity.

**Calcium imaging/ FLIPR.** This was performed essentially as described<sup>22</sup>.

**Chemicals.** Cinnamaldehyde, mustard oil, iodoacetamide and DTT were purchased from Sigma Aldrich (St Louis, Missouri). MTSEA and MOA (also known as propargyl isothiocyanate) are also commercially available (from Toronto Research Chemicals, North York, Ontario and Oakwood Products, West Columbia, South Carolina, respectively). For synthesis of SC, SCA and IAA see the 'Characterization of Chemical Materials' section in the Supplementary Information.

See Supplementary methods for more detailed information on chemicals.

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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