Supplementary Materials and Methods:

**Protein isolation, click chemistry, and fluorescent gel imaging (Fig. 1b):** performed essentially as described previously\(^{19}\). Myc-His-tagged mTRPA1-expressing Human Embryonic Kidney (HEK293T) cells were treated with alkyne-modified TRPA1 activators (100µM, diluted in PBS) for 10 minutes and washed. Total protein was collected, separated into soluble and insoluble fractions, and labeled with rhodamine using click chemistry. After SDS-PAGE, rhodamine fluorescence was visualized in-gel using a Hitachi FMBio IIe flatbed laser-induced fluorescence scanner (MiraiBio, Alameda, CA). Gels were reserved for protein transfer to nitrocellulose membranes and western blotting.

**TRPA1 IP click labeling experiments (Fig. 1c-g):** Similar to above, tagged TRPA1 expressing cells were treated, lysed, immunoprecipitated with anti-myc and Protein G agarose beads, and then click-labeled on bead.

**Click competition labeling experiments (Fig. 1e-g):** Using the IP click labeling method, cells were treated first with unlabeled compound, IA, SC, MO, CA, or icilin (100µM, 5 minutes). Compound was removed, and then cells were treated with IAA (100µM, 5 minutes) and then washed and lysed. Control cells were labeled with IAA (100µM, 5 minutes), and then treated with vehicle (PBS) for 5 minutes, washed and lysed. IP, click labeling, and visualization was performed as described above.

**Mass Spectrometry:**
**Glutathione Adduct Formation:** Equimolar concentrations of TRPA1 agonist and glutathione (~100mM) reacted in water produced glutathione adducts at room temperature as measured by electrospray ionization mass spectrometry (ESI-MS).

**Proteomics of IA labeled TRPA1:** We applied IA (either 100µM for 2 minutes or 400µM for 10 minutes) 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 10mM DTT, alkylated with 55mM IAA (as a substitute for IA in the protocol to differentiate this modification from the IA used in the live-cell treatment), trypsinized, and analyzed by nano-liquid chromatography (LC)-MS/MS on an Agilent ion trap mass spectrometer. 30-40% of TRPA1 residues were identified by spectra analysis using Mascot search engine (Matrix Science, Ltd.).

**Mutagenesis:** Individual cysteine mutants were created using Stratagene Quikchange XL site-directed mutagenesis kit according to manufacturer’s instructions. mTRPA1 IRES-YFP expression vector was used as the template. 31 cysteine to serine mutants were made as well as cysteine to alanine mutants for C415, C422, and C622. Clones were sequenced to confirm mutation, and absence of errors.

**Calcium Imaging/ FLIPR:** performed essentially as described\(^24\). FLIPR (fluorescence imaging plate reader) was used for EC\(_{50}\) determinations. Transiently transfected Chinese Hamster Ovary (CHO) cells were used for calcium imaging experiments, stably expressing mTRPA1 CHO cells were used for FLIPR. In many experiments 100µM
ATP was used as a control at the end of the experiment. Addition of ATP, which causes calcium release from intracellular stores, indicates that the calcium signal is not saturated and that the cells are still alive after the long assays. Concentration of agonists used: IA (400µM), Menthol (300µM), MTSEA (400µM), SC (50µM), DTT (5mM), ATP (100µM).

**Immunofluorescence:** CHO cells were transiently transfected with Myc-His tagged WT mTRPA1, C415S, C422S, C622S, and V5-His tagged TRPM8. 48 hours after transfection, cells were paraformaldehyde fixed, and treated with Wheat Germ Agglutinin (WGA AlexaFluor 488; Invitrogen-Molecular Probes, Carlsbad, CA) according to manufacturer’s instructions. Cells were washed and membranes permeabilized with .4% Triton X-100. After blocking for 1 hour, cells were incubated in primary antibody (anti-myc, or anti-V5) overnight at 4°C. After washing with PBS, secondary antibody (donkey anti-mouse Cy-3) was incubated for 1 hour at RT with shaking. After washing, coverslips were mounted, and images were acquired using an Olympus Fluoview 500 confocal microscope by sequential illumination. Images were analyzed using MetaMorph software (Universal Imaging Corporation, West Chester, PA).

**Synthesis of Super Cinnamaldehyde (SC), Super Cinnamaldehyde Alkyne (SCA), and Iodoacetamide Alkyne (IAA):** See Characterization of Chemical Materials section.

**Electrophysiology:** Cells were plated on 12 mm uncoated glass coverslips and transiently transfected with TRPA1 or cysteine mutant constructs in an IRES vector.
expressing YFP. Cells were maintained at 37 °C, 5% CO₂ in DMEM containing 10% FBS and antibiotic/antimycotic (Invitrogen) for about 24 hour and then switched to 33 °C, 5% CO₂. Low or moderately fluorescent YFP positive cells were chosen 12 hours to 4 days later for electrophysiological experiments. Whole cell electrophysiological methods were as described previously⁹. Excised patch voltage clamp experiments were performed on the equipment used for whole cell studies (i.e., Axon Instruments (Molecular Devices Corporation)-based acquisition system). To repress inactivation of voltage-dependent currents and desensitization of agonist-induced currents, all electrophysiological experiments were performed in the absence of calcium. Calcium-free solution contained (in mM): NaCl 124.5, MgCl₂ 2, EGTA 5, HEPES 10, pH7.4. Cells and excised patches were continuously perfused at 1-2ml/min at a constant temperature (>24 °C) to avoid complications of exposing the channels to cool temperatures. Compounds were usually added from 300-1000-fold stock solutions in ethanol or DMSO. Vehicle at these concentrations had no effect on TRPA1 activity.

**MTSEA-Biotin experiments (Fig 2c):** Recording pipettes contained standard calcium free saline at the tip and were back-filled with calcium free saline containing MTSEA-biotin. Control experiments in which electrodes contained vehicle only (DMSO, 0.1%) revealed no obvious change in conductance over 10 min. Voltage ramp-induced currents were acquired every 5 sec, from -120 mV to +120 mV.

**Whole-cell current responses (Fig. 2d-e):** In whole-cell configuration, voltages were stepped from +215 to -100 mV in increments of 15 mV applied every 10 sec. After 100 msec at the indicated voltage, the cell was stepped to -70 mV to measure inward currents as the channels closed (tail currents).
Normalization of agonist responses to voltage (Fig. 2f-g): Agonist-activated whole cell current densities were calculated from voltage ramp-induced currents elicited at -120 mV (negative) and +120 mV (positive) and normalized to the current elicited by a +180mV voltage step applied before agonist addition in the same cell. This voltage step was chosen to obtain the largest responses without unnecessarily taxing membrane integrity.

Single channel recordings (Fig. 3b): Single-channel activity of an excised inside-out patch (mTRPA1 transfected HEK cell) was initially recorded over a 3.4 min period during which no chemical activator was present (+100mV, bath temperature 28.5 °C) (representative traces are shown in the top left panel). Bath perfusion of 30 µM MO increased the channel activity at +100 mV beginning about 60 sec after addition to the bath and representative traces after exposure for 3 min are shown (bottom left panel). The patch was then challenged with voltage steps to +180 mV, still in the presence of 30 µM MO (top right panel). Activity at +180 mV was further increased by bath perfusion of 100 µM MO (bottom right panel).

Single channel recordings SC, MO, Icilin Responses (Fig. 3c-e): The instantaneous current-voltage relationship was obtained using a voltage ramp protocol, applied every 5 seconds to inside-out excised patches.

Chemicals: Cinnamaldehyde, mustard oil, iodoacetamide, icilin and dithiothreitol were purchased from Sigma Aldrich (St. Louis, MO). MTSEA, MTSEA-biotin, and MOA (aka propargyl isothiocyanate) are also commercially available (from Toronto Research Chemicals, Inc. North York, Ontario, Canada and Oakwood Products, Inc. West Columbia, SC, respectively).
Characterization of Chemical Materials:

**General Methods for Chemical Synthesis.** All compounds were purchased from commercial sources (Aldrich, Acros, Fisher) and used without further purification, unless otherwise noted. Dry tetrahydrofuran (THF), triethylamine ($\text{NEt}_3$), methylene chloride (DCM), and $N,N'$-dimethylformamide (DMF) were obtained by passing commercially available predried, oxygen-free formulations through activated alumina columns. Unless otherwise noted, reactions were run under an atmosphere of dry argon. Analytical thin layer chromatography (TLC) was carried out on Whatman silica gel plates (catalog no. 4861-820), and developed plates were examined under UV light and stained with cerium molybdate stain or ninhydrin. Flash chromatography was performed with EMD silica gel (catalog no. 11567-1). LC/MS HPLC was performed by using a Hitachi L-7150 pump equipped with a Higgins Analytical C18, 5 mm, 150 × 10-mm reverse phase column. NMR spectra were recorded on a Bruker AMX 400 spectrometer. Chemical shifts are reported in $\delta$ ppm values relative to the residual undeuterated solvent, and coupling constants ($J$) are reported in Hz. Multiplicities are noted using the following abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. Mass spectra were obtained with an Agilent ESI-TOF at the Center for Mass Spectrometry at The Scripps Research Institute. Abbreviations: EtOAc, ethyl acetate; Hex, hexanes; EtOH, ethanol; Burgess reagent, methyl $N$-(triethylammoniumsulphonyl)carbamate.

**Preparation of 1-amino-hex-5-yne:** 1.12 mL of 5-hexynenitrile (10.3 mmol, Aldrich, St. Louis, MO) was dissolved in 100 mL of Et$_2$O and cooled to 0°C under argon. 11.3 mL of LiAlH$_4$ (1.0 M in Et$_2$O) was added dropwise for five minutes at which time the reaction was warmed to room temperature and stirred overnight. Excess LiAlH$_4$ was carefully quenched with water, and the resulting white precipitate was removed by filtration. The organic layer was washed with saturated NaCl (1 x 100 mL), and concentrated to 20 mL, and the product was precipitated from solution by adding HCl (1.0 M in Et$_2$O) dropwise. The resulting yellow solid was filtered and dried to afford 0.82 g (6.2 mmol, 60%) of 1-amino-hex-5-yne as a hydrochloride salt. $^1$H-NMR (400 MHz, CD$_3$OD): $\delta$ 2.56 (m, 2H) 2.05 (m, 2H), 1.82 (t, 1H, $J = 2.6$ Hz), 1.41 (m, 4H).

**Preparation of IA-alkyne:** To a mixture of iodoacetic anhydride (0.1 g, 0.28 mmol), 1-amino-hex-5-yne (0.013 g, 0.10 mmol), and DCM (2.5 mL) was added NEt$_3$ (0.038 mL, 0.28 mmol) dropwise. After stirring at room temperature for 1 hr, the reaction was washed with saturated NaHCO$_3$ (2 x 1 mL) and saturated NaCl (1 x 1 mL). The resulting yellow solid was filtered and dried to afford 0.082 g (6.2 mmol, 60%) of IA-alkyne as a hydrochloride salt. $^1$H-NMR (400 MHz, CD$_3$OD): $\delta$ 6.14 (br, 1H), 3.72 (s, 2H), 3.32 (m, 2H), 2.24 (m, 2H), 1.98 (t, 1H, $J = 2.6$ Hz), 1.67 (m, 2H), 1.58 (m, 2H); HRMS (m/z): [M]$^+$ calcd for C$_8$H$_{12}$INO, 266.0035; found, 266.0035.
Summary of the synthesis of enones 3, 6, and 9: The synthesis of 3 commenced by performing the base-catalyzed aldol addition of acetone to isatin (1; Aldrich, St. Louis, MO) to afford the tertiary alcohol 2 in 75% yield. Acid-catalyzed dehydration of 2 provided the electrophilic enone 3 in suitable yield for further biological investigations. The N-alkylated enone derivatives 6 and 9 were prepared by alkylating isatin with methyl iodide and propargyl bromide to give compounds 4 and 7, respectively. Subsequent aldol addition of acetone to the ketone moieties from 4 and 7 yielded the tertiary alcohols 5 and 8. Dehydration of the resulting tertiary alcohols with the Burgess reagent afforded the α,β-unsaturated enones 6 and 9 in 40% and 14% overall yield, respectively.

\[ \text{Burgess reagent} \]

\[ \text{HCl (aq)} \]

\[ \text{HOAc} \]


Figure caption:
Synthesis of enones. Isatin and N-alkylated isatin were converted in similar two-step sequences. Burgess reagent = methyl N-(triethylammoniumsulphonyl)carbamate,

3-Hydroxy-3-(2-oxo-propyl)-1,3-dihydro-indol-2-one (2) and 3-(2-Oxo-propylidene)-1,3-dihydro-indol-2-one (3) were synthesized starting from 3 g isatin according to literature procedures. The aldol 2 was purified by silica chromatography (EtOAc:hexanes, 1:1 – 80:20 v/v) and the enone 3 was purified by preparative HPLC. TLC (EtOAc): $R_F = 0.39$; $^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 8.52 (1H, d, $J = 7.92$ Hz), 7.70 (br, 1H), 7.33 (dt, 1H, $J = 1.16, 7.64$ Hz), 7.16 (s, 1H), 7.04 (dt, 1H, $J = 1.16, 7.64$ Hz), 6.84 (d, 1H, $J = 7.6$ Hz); HRMS (m/z): [M]+ calcd for C$_{11}$H$_9$NO$_2$, 188.0706; found, 188.0699.

![Chemical structure of 3-Hydroxy-3-(2-oxo-propyl)-1,3-dihydro-indol-2-one (2)](image)

1-Methyl-1H-indole-2,3-dione (4): A published procedure$^5$ for the synthesis of the dione 4 was slightly modified: 1.5 g (10 mmol) isatin was dissolved in 5 mL DMF. The mixture was cooled to 0 °C and 0.52 g of sodium hydride (13 mmol, 1.3 eq., 60% in mineral oil) was added directly to the reaction mixture in several small portions. Methyl iodide (0.93 mL, 15 mmol, 1.5 eq.) was added and the reaction mixture was subsequently stirred at room temperature. After 3 hr, glacial acetic acid was added to quench the reaction. The resulting mixture was filtered to remove precipitates, washed with EtOAc (1 x 50 mL), and the filtrate was concentrated under reduced pressure. The crude residue was coated on silica gel with EtOH, and purified by silica chromatography (20-50% EtOAc in hexanes) to yield 1.60 g of 4 (9.93 mmol, 99%) as a bright orange solid. TLC (EtOAc:hexanes, 1:1 v/v): $R_F = 0.36$. $^1$H NMR (400 MHz, CD$_3$OD) d 7.44 (m, 1H), 7.37 (m, 1H), 7.18 (m, 1H), 7.12 (m, 1H) 3.18 (s, 3H); HRMS (m/z): [M]+ calcd for C$_9$H$_7$NO$_2$, 162.055; found, 162.0550.

![Chemical structure of 1-Methyl-1H-indole-2,3-dione (4)](image)

3-Hydroxy-1-methyl-3-(2-oxo-propyl)-1,3-dihydro-indol-2-one (5)$^1$: Potassium carbonate (0.071 g, 0.51 mmol, 0.19 eq.) was added directly to a solution of N-methyl isatin (4, 0.443 g, 2.75 mmol) in acetone (15 mL). The reaction mixture was stirred

overnight, and reaction progress was monitored by LC/MS. The reaction mixture was filtered to remove salts, evaporated to dryness, and the resulting crude residue was purified by silica chromatography (EtOAc:hexanes, 1:1 - 66:34 v/v) to afford 0.575 g of 5 (1.62 mmol, 95%) as a colorless solid. TLC (EtOAc:hexanes, 66:34 v/v): \( R_f = 0.19 \). 1H NMR (400 MHz, CD3OD) \( \delta 7.35 \text{ (m, 2H)}, 7.19 \text{ (m, 2H)}, 7.10 \text{ (d, 2H, J=7.64 Hz), 3.39 (m, 2H), 3.18 (s, 3H), 2.66 (s, 3H)} \); HRMS (m/z): [M]Na+ calcd for C12H13NO3, 242.0788; found, 242.0788.

**1-Methyl-3-(2-oxo-propylidene)-1,3-dihydro-indol-2-one (6)**: A solution of 5 (0.051 g, 0.235 mmol) in THF (0.6 mL) was added to a solution of the Burgess reagent (0.059 g, 0.251 mmol, 1.1 eq.) in THF (0.3 mL). The reaction mixture was stirred at room temperature and monitored by LC/MS at periodic intervals over 36 hr. The reaction was quenched with water, and the mixture was washed with EtOAc (2 x 1 mL). The combined organic fractions were dried over MgSO₄, concentrated under reduced pressure, and purified with silica chromatography (EtOAc:hexanes 25:75 v/v) to afford 0.02 g of 6 (0.099 mmol, 42%) as a red solid. TLC (EtOAc:hexanes, 33:67 v/v): \( R_f = 0.27 \). 1H NMR (400 MHz, CD3OD) \( \delta 8.42 \text{ (d, 1H, J=7.6 Hz)}, 7.41 \text{ (m, 1H)}, 7.17 \text{ (s, 1H)}, 7.03 \text{ (m, 1H)}, 6.96 \text{ (d, 1H, J=7.92 Hz), 3.21 (s, 3H), 2.45 (s, 3H)} \); HRMS (m/z): [M]+ calcd for C12H11NO2, 202.0863; found, 202.0862.

**1-Prop-2-ynyl-1H-indole-2,3-dione (7)**: A solution of isatin (1.5 g, 10 mmol) in DMF (5 mL) was cooled to 0 °C, and sodium hydride (0.52 g, 13 mmol, 1.3 eq, 60% in mineral oil) was periodically added to the solution in small portions. Propargyl bromide (0.17 mL, 1.50 mmol, 1.5 eq.; 80% in toluene) was added to the slurry via syringe, and the reaction mixture was slowly warmed to room temperature. The reaction was quenched with water after 10 hr, and the resulting solid was removed via filtration. The filtrate was concentrated under reduced pressure and the crude product was purified via recrystallization (toluene:EtOAc, 1:1 v/v). Successive recrystallizations yielded 1.24 g of 7 (6.7 mmol, 67%) as an orange solid. TLC (EtOAc:hexanes, 1:1 v/v): \( R_f = 0.52 \). 1H MR

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(400 MHz, CD3OD) d 7.43 (m, 1H), 7.26 (m, 1H), 7.17 (m, 2H), 4.52 (m, 2H), 2.68 (t, 1H, J=2.3 Hz); HRMS (m/z): [M]+ calcd for C11H7NO2, 186.055; found, 186.0550.

3-Hydroxy-3-(2-oxo-propyl)-1-prop-2-ynyl-1,3-dihydro-indol-2-one (8): Potassium carbonate (0.38 g, 2.7 mmol, 0.83 eq.) was added directly to a stirring mixture of N-propargyl isatin (7, 0.61g, 3.3 mmol, 1 eq.) in acetone (50 mL). The reaction continued stirring at room temperature and reaction progress was monitored by LC/MS. After complete conversion of 7, the reaction mixture was filtered to remove salts, and concentrated under reduced pressure. The crude residue was purified via silica column chromatography (EtOAc:hexanes, 66:34 v/v) to afford 0.465 g of 8 (58%) as a white solid. TLC (EtOAc:hexanes, 66:34 v/v): R_F = 0.34. 1H NMR (400 MHz, CD3OD) d 7.36 (m, 2H), 7.11 (m, 2H), 4.48 (m, 2H), 3.23 (m, 2H), 2.68 (t, 1H, J=2.3 Hz), 2.66 (s, 3H); HRMS (m/z): [M]Na+ calcd for C14H13NO3, 266.0788; found, 266.0784.

3-(2-Oxo-propylidene)-1-prop-2-ynyl-1,3-dihydro-indol-2-one (9): A solution of 8 (0.047 g, 0.196 mmol) in THF (0.3 mL) was added to a solution of the Burgess reagent (0.052 g, 0.220 mmol, 1.1 eq.) in THF (0.3 mL). The reaction was stirred at room temperature for two hours, at which time the reaction was quenched with saturated aqueous NaCl (1 mL). The mixture was extracted with EtOAc (2 x 1 mL), and the organic fraction was dried (MgSO4). The solution was concentrated under reduced pressure, and the crude residue was purified via silica chromatography (EtOAc:hexanes, 20:80 v/v) to afford 0.016 g of 9 (36%) as an orange solid. TLC (EtOAc:hexanes, 20:80 v/v): R_F = 0.24. 1H NMR (400 MHz, CD3OD) ; 8.46 (d, 1H, J=7.0 Hz), 7.44 (m, 1H), 7.21 (s, 1H), 7.09 (m, 2H), 4.57 (d, 2H, J=2.6 Hz), 2.70 (t, 1H, J=2.3 Hz), 2.46 (s, 3H); HRMS (m/z): [M]+ calcd for C14H10NO2, 266.0863; found, 226.0863.
Supplementary FIG. S1. Characterization of cysteine reactive compound glutathione adducts and controls for click labeling experiments (Supplementary for Main Figure 1).

(a-f) Electrospray ionization mass spectrometry (ESI-MS) spectra of glutathione alone or reacted with TRPA1 agonists. Equimolar concentrations of GSH + agonist (~100mM each in water) were combined and allowed to react at room temperature. Samples were diluted in MeOH for ESI-MS analysis. GSH alone in water/methanol exists as monomer and dimer in solution, MH+, m/z (336, 669) (a). When reacted with MO, IA, CA, and SC (b-e), glutathione adducts of predicted mass are observed. Glutathione + icilin shows no adduct, only monomer and dimer of GSH and unreacted icilin (312) (f).

(g) Control experiments for click chemistry method reveal no rhodamine labeled TRPA1 in the absence of exposure to alkyne reagents (left panels), and no rhodamine fluorescence at the position where TRPA1 runs in control untransfected cells treated with SCA (right panels). Rhodamine fluorescence (Rhod) and western blot (Wstrn) images of protein gels from the indicated experimental treatment. Left: TRPA1 immunoprecipitated from untreated TRPA1-HEK cells shows no labeling at TRPA1 molecular weight; a non-specific band of high molecular weight is visible in many rhodamine fluorescence images. Right: Rhodamine and western blot of negative control untransfected HEK cells immunoprecipitated with anti-myc.

Supplementary FIG. S2. Identification of reactive cysteines by mass spectrometry (Supplementary for Main Figure 2).
(a) Table of cysteine residues modified by IA (live cell) or IAA (subsequent in vitro treatment as it is required for residue identification in MS) identified by MS analysis using two experimental conditions: on cell application of 100µM IA for 2 minutes and 400µM IA for 10 minutes. Cysteine residues modified by IA and IAA are indicated by a check mark in the corresponding box. ND = peptide containing the individual cysteine was not detected by MS. Note that many cysteines are modified by both compounds, suggestive of incomplete reaction by the live-cell IA treatment.

(b) Representative spectra of an IA modified cysteine-containing peptide. Table below shows masses of all predicted fragmentation ions; those in red were identified within the above spectra. IA modification (+57 amu) of C415 and C422 were identified in this spectra.

Supplementary FIG. S3. Characterization of cysteine mutants (Supplementary for Main Figure 2).

(a) Response of WT and three cysteine mutant TRPA1 clones to CA in ratiometric calcium imaging studies. WT TRPA1-expressing CHO cells reveal a concentration dependent increase in intracellular calcium during application of 100 and 250 µM CA. No response is observed from C415S and C622S, although ATP responses could be elicited subsequently (not shown). While no response is observed in C422S transfected cells when challenged with 100 µM CA, a higher concentration was effective. C422S also responds to high concentrations of allicin and MO (50µM and 200µM, not shown). Alanine substitutions of these three cysteines also resulted in similarly inactive clones (not shown).

(b-c) We monitored the sub-cellular (b) and whole cell (c) distribution of the transfected TRPA1 WT, TRPA1 mutant clones and TRPM8 WT via a C-terminal tagged
TRPA1 and TRPM8 (the WT tagged clones had normal activity, not shown). All three mutants had a similar distribution pattern as TRPA1 WT, with the majority of expression present in intracellular compartments. Similar subcellular distribution was observed for TRPM8 WT. We quantified fluorescence levels in whole cells, and at or near the plasma membrane (via co-localization with alexa-488 wheat germ agglutinin). The fluorescent counts were similar across clones, with the exception of C622S which showed reduced signal both at the membrane and whole-cell levels.

(d) Representative confocal fluorescence microscopy images of surface bound WGA (red) overlayed with anti-myc (or anti-V5 for TRPM8) immunofluorescence (green) of CHO cells transfected with WT TRPA1, TRPA1 cysteine mutants (C415S, C422S, and C622S), and WT TRPM8. Untransfected CHO is also shown.

(e) Whole cell currents elicited by voltage steps to either +215 mV (WT, C622S, Vector) or +200 mV (C422S, C415S) and in decreasing increments of 15 or 20 mV, respectively, at 10 sec intervals from a holding potential of -50 mV. After 100 msec at the indicated voltage, the cell was stepped to -70 mV to measure inward currents as the channels closed (tail currents). The amplitudes of the tail currents after each prepulse voltage for each example shown are indicated to the right of each current family.

(f) Representative whole cell currents elicited by a voltage ramp protocol (shown below) are shown from a single cell before (black), and after near steady state activation during exposure to 30 µM (light orange) and 100 µM (amber) MO under calcium free conditions to avoid desensitization. Note the different amplitude scales.
Supplementary FIG. S4: Reactive compounds can cause sustained activation of TRPA1, and DTT can block activation by MTSEA. (Supplementary for Main Figure 3).

(a-b) SC and MO produce prolonged activation of TRPA1. Calcium imaging of TRPA1-expressing CHO cells (blue traces: TRPA1-YFP cells, pink traces: untransfected cells). Short term (2 minute) application of agonist (50µM SC, 400µM MO) followed by extensive washout revealed stable activation of TRPA1 for at least 20 minutes (60 minutes for SC). The application of a second pulse of TRPA1 activator 20-60 minutes after the first did not cause any further appreciable increase in calcium levels, consistent with full occupancy of TRPA1 throughout the experiment. 100µM ATP is applied at the end of each experiment as a control for cell viability.

(c-d) Stable activation of TRPA1 by MTSEA (c) and SC (d) is reversed by the TRPA1 antagonist menthol. Application of 300µM menthol reversibly reduces activation of TRPA1 expressing CHO cells by MTSEA and SC (400µM, and 50µM, respectively).

(e-h) Currents observed in excised inside out patches from cells expressing TRPA1 were determined to be mediated by TRPA1 channels based on both electrophysiological and pharmacological criteria. Large conductance non-selective channel activity has similar voltage dependence as that observed in WT but not vector-transfected cells, is activated by agonists in a concentration-dependent manner (see also Fig. 3b) and is blocked by 300 µM (-)-menthol (see also Fig. 3c, right panel), and reveals similar voltage-dependent inactivation at positive voltages in the presence of agonist as observed in whole cell recordings (Supplementatry Fig. S4i).

(e) TRPA1 WT single channel activity is elicited by voltage steps above ~ +60 mV from an excised inside-out membrane patch in calcium free conditions, similar to that
observed from whole cell recordings (see Fig. 2c). The traces are nudged by 10 or 20 pA for clarity. The voltage step is shown to the right.

(f) (top) Current histogram of single channel amplitudes determined from patches containing single channels from TRPA1 WT at +100 (blue) and +180 mV (dark blue), and endogenous activity observed from a vector transfected cell (grey). Data were analyzed using Clampfit. (bottom) Single channel currents as a function of voltage (determined from single channel activity elicited by voltage steps to +90 to +200 mV from 4 separate experiments). Single channel conductance ($\gamma$) was calculated using linear regression ($r=0.84$) as 92 pS [95% confidence interval: 77 to 107 pS]. The extrapolated reversal potential was +3 mV [95% CI: -25 to +23 mV].

(g) TRPA1 activity in excised patch can be observed for up to 15 min after exposure to 50 µM SC. Shown are voltage ramp-induced currents 4 min after activation by 50 µM SC (amber), during block by 300 µM (-)-menthol (green), and after washout (blue). Raw data is from the experiment shown in Fig. 3c.

(h) Voltage-dependent inactivation at positive potentials in the presence of the chemical activator MO is similar in excised and whole cell recordings. Currents elicited by a step to +180 mV from excised patches (left) and from whole cell recording (right) before (black traces) and after (amber traces) activation by MO (100 µM).

(i and j) DTT (5mM) can block TRPA1 activation by 400µM MTSEA but not by 50µM SC. SC (not predicted to form disulfide bonds) applied during the DTT exposure elicits a large response (i). DTT blocks the response to MTSEA, and subsequent exposure to SC effectively activates TRPA1 (j). See also Fig. 3h and Supplementary Fig. S2c.
(k) Increasing concentrations of MO occlude the DTT reversal of the MTSEA/MO induced response. We took advantage of the differential ability of DTT to reverse MTSEA- but not MO-induced activation of TRPA1 to determine whether MO can compete with MTSEA’s labelling of cysteine residues. TRPA1-expressing cells were challenged with 400μM MTSEA in the presence of a range of MO concentrations which produced a large stable calcium signal, and subsequently exposed to DTT (5mM). We reasoned that the extent of reduced signal would depend (at least in part) on the percentage of channels labelled with MTSEA in the initial channel activation (the greater the MTSEA-labeling, the larger the effect of DTT to close channels). Increasing the MO concentration indeed decreased the effectiveness of DTT to reduce the TRPA1-dependent intracellular calcium signal. After maximally activating TRPA1-expressing cells by application of 400μM MTSEA in combination with 0 (blue), 50 (green), or 400μM (red) MO, subsequent reduction of covalently bound MTSEA with 5mM DTT reveals a concentration-dependent maintenance of the intracellular calcium signal consistent with the interaction of MO and MTSEA at a population of identical sites. MTSEA and MO are applied together at 60-180s (not shown), and washed out from 180-420s. 5mM DTT (black bar) is applied from 425-550s. Remaining activity after DTT application is normalized to the control stimulus of 400μM MTSEA with 0μM MO to show the percent signal remaining.
Macpherson et al. Supplementary Figure 1

(a) GSH Alone
- GSH monomer (336)
- GSH dimer (669)

(b) GSH + MO
- MO Adduct (+99)
- GSH monomer

(c) GSH + IA
- IA Adduct (+57)
- GSH monomer

(d) GSH + CA
- CA Adduct (+132)
- GSH monomer

(e) GSH + SC
- SC Adduct (+187)
- GSH monomer

(f) GSH + Icilin
- Icilin (312)
- GSH monomer

(g) Untreated TRPA1
- Rhod
- Wstrn

SCA Treated HEK
- Rhod
- Wstrn

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(a) Fluorescence ratio (pF/pC380) vs. Time (s) for various CA and TRPA1 conditions.

(b) Membrane Region Average Fluorescence.

(c) Whole Cell Fluorescence for different conditions.

(d) Fluorescence images of different cell types and conditions.

(e) Whole cell current (pA) vs. Voltage (mV) for different conditions.

(f) Tail current (pA) vs. Voltage (mV) for different conditions.