Transient receptor potential ankyrin 1 antagonists block the noxious effects of toxic industrial isocyanates and tear gases

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ABSTRACT **The release of methyl isocyanate in Bhopal, India, caused the worst industrial accident in history. Exposures to industrial isocyanates induce lacrimation, pain, airway irritation, and edema. Similar responses are elicited by chemicals used as tear gases. Despite frequent exposures, the biological targets of isocyanates and tear gases *in vivo* have not been identified, precluding the development of effective countermeasures. We use Ca^{2+} imaging and electrophysiology to show that the noxious effects of isocyanates and those of all major tear gas agents are caused by activation of Ca^{2+} influx and membrane currents in mustard oil-sensitive sensory neurons. These responses are mediated by transient receptor potential ankyrin 1 (TRPA1), an ion channel serving as a detector for reactive chemicals. In mice, genetic ablation or pharmacological inhibition of TRPA1 dramatically reduces isocyanate- and tear gas-induced nocifensive behavior after both ocular and cutaneous exposures. We conclude that isocyanates and tear gas agents target the same neuronal receptor, TRPA1. Treatment with TRPA1 antagonists may prevent and alleviate chemical irritation of the eyes, skin, and airways and reduce the adverse health effects of exposures to a wide range of toxic noxious chemicals.—Bessac, B. F., Sivula, M., von Hehn, C. A., Caceres, A. I., Escalera, J., Jordt, S.-E. Transient receptor potential ankyrin 1 antagonists block the noxious effects of toxic industrial isocyanates and tear gases. *FASEB J.* 23, 1102–1114 (2009)**

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Isocyanates are reactive organic chemicals widely used in the industrial production of polyurethane polymers, pesticides, fungicides, and other materials. Methyl isocyanate (MIC), a precursor in pesticide production, was the major causative agent of the environmental disaster in Bhopal, India, responsible for more than 3000 immediate deaths and several thousand additional casualties in the years after the accident (1). In the United States, MIC exposures have occurred after spills of the pesticide metam sodium (sodium *N*-methyl-dithiocarbamate) in railroad and agricultural accidents (2, 3). In these accidents, metam sodium reacted with soil components and water to produce MIC and other reactive agents (3–5). MIC exposure caused immediate unbearable irritation of eyes, nose, and throat (6). The airways are especially sensitive to MIC and other isocyanates. Dependent on exposure levels and duration, MIC-exposed individuals present with airway hyperresponsiveness, inflammation, reactive airway dysfunction syndrome (RADS), and airway edema and injuries (1). Bifunctional isocyanates such as 2,4-toluene-diisocyanate, diphenylmethane-4,4′-diisocyanate (MDI), and hexamethylene-diisocyanate (HDI), used in the production of polyurethane products, are equally strong irritants and cause asthma-related symptoms on repeated exposures (7).

The severe irritation after exposures to isocyanates is surprisingly similar to the incapacitating effects of tear gas agents (8, 9). The development of tear gas agents dates back to World War I, when almost all factions used airway irritants and chemical lacrimatory agents (tear gases), such as acrolein (Papite), chloropicrin (PS), bromoacetone, benzyl bromide, and others (9–11). CN tear gas, a riot control agent, was developed in the 1920s and was widely used by law enforcement until the 1960s (12). The active lacrimatory agent in CN is 2-chloroacetophenone. Because of its toxicity, CN was supplanted by CS tear gas, containing 2-chlorobenzylidene malononitrile as its active ingredient. CS is currently the most widely used riot control agent worldwide. CR is another modern riot control agent, containing dibenzo[*b,f*][1,4]oxazepine as its lacrimatory principle (**Fig. 1*B***) (12).

Despite the infamy of isocyanate exposures in occupational and environmental medicine and the widespread and frequent use of tear gas agents for more than 90 yr, with possibly millions of exposures, little is known about the molecular and cellular actions of these agents. Current medical treatment of exposures includes the removal of the toxicants by dilution, washing, and chemical neutralization, treatment of

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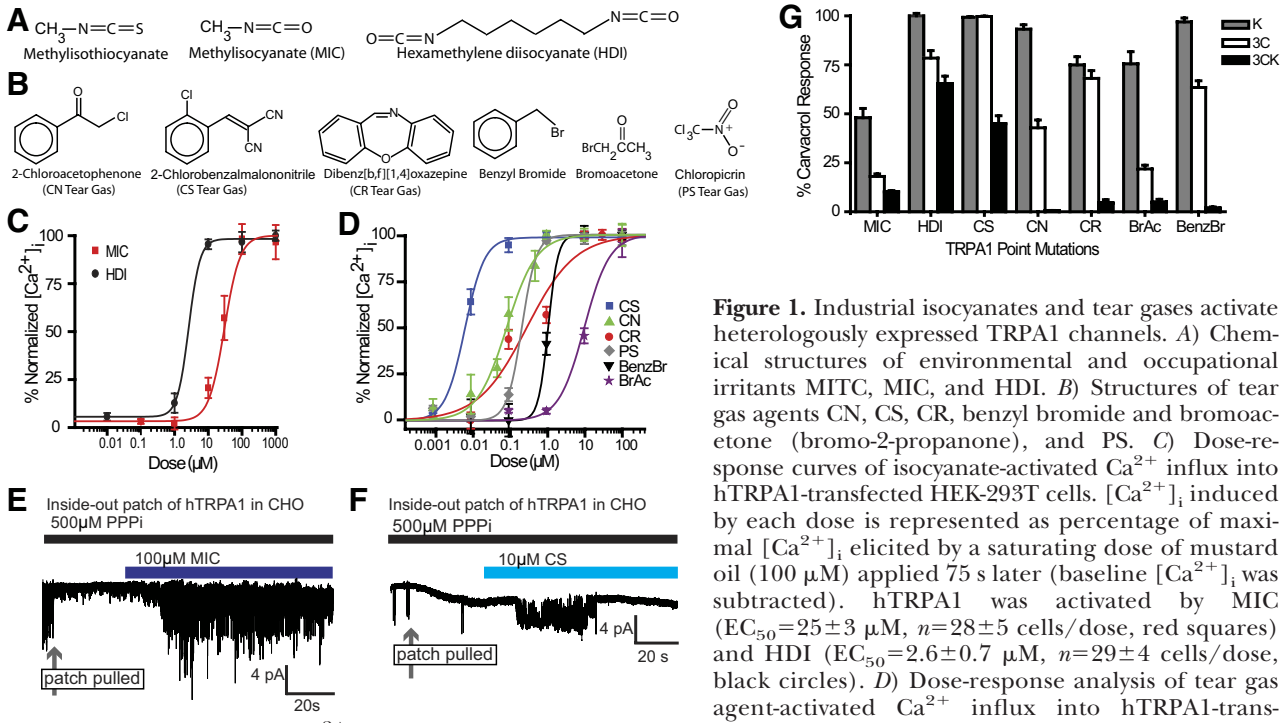

Figure 1. Industrial isocyanates and tear gases activate heterologously expressed TRPA1 channels. *A*) Chemical structures of environmental and occupational irritants MITC, MIC, and HDI. *B*) Structures of tear gas agents CN, CS, CR, benzyl bromide and bromoacetone (bromo-2-propanone), and PS. *C*) Dose-response curves of isocyanate-activated Ca^{2+} influx into hTRPA1-transfected HEK-293T cells. $[Ca^{2+}]_i$ induced by each dose is represented as percentage of maximal $[Ca^{2+}]_i$ elicited by a saturating dose of mustard oil (100 μM) applied 75 s later (baseline $[Ca^{2+}]_i$ was subtracted). hTRPA1 was activated by MIC (EC_{50}=25±3 μM, n=28±5 cells/dose, red squares) and HDI (EC_{50}=2.6±0.7 μM, n=29±4 cells/dose, black circles). *D*) Dose-response analysis of tear gas agent-activated Ca^{2+} influx into hTRPA1-transfected HEK-293T cells. $[Ca^{2+}]_i$ represented as in *A*. hTRPA1 was activated by CN (EC_{50}=91±12 nM, n=49±1 cells/dose, green triangles), CS (EC_{50}=7±1 nM, n=52±5, blue squares), CR (EC_{50}=308±150 nM, n=70±9, red circles), PS (EC_{50}=215±62 nM, n=32±4, gray diamonds), benzyl bromide (BenzBr, EC_{50}=12±1 μM, n=66±17, black triangles) and bromoacetone (BrAc, EC_{50}=1.1±0.3 μM, n=78±14 cells/dose, purple stars). *E*) Application of MIC (100 μM, purple bar) induces an increase of single-channel openings of an excised patch in the inside-out configuration from hTRPA1-overexpressing CHO cells. Voltage was held at −40 mV; bath solution contained 0.5 mM PPP_i and was devoid of Ca^{2+} (10 mM EGTA). *F*) Application of CS (10 μM, blue bar) induces an increase of single-channel openings in an excised patch in the inside-out configuration from hTRPA1-overexpressing CHO cells. Conditions were same as for *E*. *G*) Responses of hTRPA1 mutant-expressing HEK-293T cells to 100 μM MIC, 100 μM HDI, 100 μM CS, 100 μM CN, 300 μM CR, 100 μM bromoacetone (BrAc), and 100 μM benzyl bromide (BenzBr). Increase in $[Ca^{2+}]_i$ is displayed as percentage of $[Ca^{2+}]_i$ activated by a saturating dose of carvacrol (300 μM). Mutants tested are coded as follows: K (gray) = K708 (MIC, n=84; HDI, n=53; CS, n=157; CN, n=108; CR, n=154; BrAc, n=44; BenzBr n=64 cells); 3C (white) = C619, C639, and C663 combined (MIC, n=34; HDI, n=47; CS, n=96; CN, n=56; CR, n=90; BrAc, n=25; BenzBr, n=40 cells); 3CK (black) = C619, C639, and C663; and K708 combined (MIC, n=30; HDI, n=123; CS, n=129; CN, n=18; CR, n=17; BrAc, n=38; BenzBr, n=41).

pain with antiinflammatory drugs and general and local anesthetics, and stabilization of the airways with bronchodilators (13). Although these procedures are helpful, the additional use of pharmacological agents to block the specific targets of isocyanates and tear gases would allow a more efficient treatment to alleviate acute irritation and pain and to prevent the development of chronic health effects.

Although immunological pathways are thought to mediate the allergic sensitization to isocyanates in the airways, studies in animal models point to a role of peripheral sensory C-fibers in their acute noxious effects and in exposure-induced airway hyperreactivity (14–19). In guinea pigs, isocyanates stimulate the release of neuropeptides from capsaicin-sensitive (C-fiber) airway nerve endings, leading to constriction of isolated bronchial segments (20, 21). Similar to the airways, the cornea of the eye is densely innervated by peripheral sensory nerve fibers. A majority of these fibers are trigeminal chemosensory C-fibers that trigger the lacrimation reflex after exposure to a noxious chemical stimulus (22). In addition to lacrimation, activation of corneal C-fibers induces ocular pain and blepharospasm, both of which are symptoms associated with tear gas exposures (23). Ocular pretreatment with local anesthetics abolishes the tear gas-induced lacrimation reflex, suggesting that these agents target corneal chemosensory nerve endings (22).

Peripheral sensory neurons express a large number of excitatory or sensitizing chemosensory receptors, including members of the transient receptor potential (TRP) ion channel family (24–26). Natural products activating the sensory neuronal TRP channels, transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1), induce effects similar to those of industrial isocyanates and tear gases. For example, the key ingredient of pepper spray, capsaicin, is a specific agonist of TRPV1 (27, 28). TRPA1 is the receptor for mustard oil (allyl isothiocyanate), the pungent ingredient in mustard, for allicin and diallyl disulfide, the lacrimatory principles in garlic and onions, and pungent natural dialdehyde sesquiterpenes (29–33). In addition to natural products, TRPA1 is also activated by industrial and environmental elec-

trophilic and oxidizing chemicals (34–36). For example, TRPA1 is activated by hypochlorite, the reactive mediator of the potent irritant gas chlorine, and is crucial for oxidant-induced respiratory depression and nocifensive behavior in mice (36–38). The role of TRPA1 as a major chemical irritant sensor in airway sensory neurons was further corroborated by experiments showing its essential requirement for cigarette smoke extract-induced neurogenic inflammation in mice and guinea pigs and by findings describing its interaction with endogenous reactive mediators enriched during airway inflammation (39–43).

Recent studies by us and others have shown that TRPA1 is activated by chemical tear gas agents *in vitro*, including acrolein, CN, CS, and CR (34, 44). Because these chemicals are highly reactive and may induce nonspecific tissue damage, it is questionable whether all of them selectively and potently target TRPA1 *in vivo*. Reactive agents may be inactivated before reaching sensory neuronal targets or activate neurons indirectly through factors released from damaged tissue. For example, adenosine or ATP released from airway tissue damaged by inhalation of organic chemical or acidic fumes has been shown to activate sensory neurons through interaction with purinergic receptors (45). Thus, detailed whole animal physiological, pharmacological, and behavioral studies are required for validation of TRPA1 as a specific target for any given chemical *in vivo*.

The molecular targets for industrial isocyanates in sensory neurons are unknown. Isocyanates are highly electrophilic compounds chemically related to isothiocyanates such as mustard oil. Methylisothiocyanate (MITC), the isothiocyanate analog of MIC, is a widely used soil fumigant that frequently causes irritation and occupational injuries in agricultural workers (Fig. 1*A*) (3, 4). In comparison with mustard oil, MITC is only a weak agonist of TRPA1 *in vitro* (29). Evidence suggests that activation of TRPA1 by reactive chemicals such as isocyanates and isothiocyanates occurs through covalent modification of cytosolic amino acid residues in the N terminus of the ion channel protein (46, 47). Intriguingly, ruthenium red, a blocker of TRPA1 and other TRP channels, inhibits isocyanate-induced contraction of isolated guinea pig bronchi (21). Thus, activation of sensory neuronal TRP ion channels may contribute to the immediate noxious effects of isocyanate exposures *in vivo*.

The purpose of our present study was to identify and characterize potential targets for industrial isocyanates and for tear gas agents in peripheral sensory neurons and to examine their roles in ocular and facial irritant sensation *in vivo*. Our findings suggest that TRPA1 is the major mediator of sensory neuronal activation by isocyanates and tear gas agents, both *in vitro* and *in vivo*. Newly developed TRPA1 antagonists selectively block neuronal activation by these agents, providing a promising lead for future therapies of chemical exposures.

MATERIALS AND METHODS

Animals

Mice were housed at an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility in standard environmental conditions (12-h light/dark cycle and ~23°C). All animal procedures were approved by the Yale Institutional Animal Care and Use Committee. Animals were identically matched for age (12–22 wk) and sex, and the experimenter was masked to the genotype. *Trpa1*$^{-/-}$ mice were a gift from David Julius (University of California, San Francisco, CA, USA) and were genotyped as described (33). C57 mice were purchased commercially (Charles River Breeding Laboratories, Inc., Wilmington, MA, USA). In certain experiments, 200-μl i.p. injections of 0, 1, 2, or 6 mg of HC-030031 dissolved in 0.5% methylcellulose (Methocel; Fluka AG, Buchs, Switzerland) were administered to mice.

Cell culture

Adult mouse dorsal root ganglia and trigeminal ganglia were dissected and dissociated by a 1-h incubation in 0.28 Wünsch units/ml Liberase Blendzyme 1 (Roche Diagnostics, Mannheim, Germany), followed by washes with Hanks' buffered saline, trituration, and straining (70 μM; Falcon; BD Biosciences Discovery Labware, Bedford, MA, USA). Trigeminal ganglia were further purified using centrifugation over a Percoll gradient (GE Healthcare, Chalfont St. Giles, UK). Neurons were cultured in Neurobasal-A medium (Invitrogen, Carlsbad, CA, USA) with B-27 supplement, 0.5 mM glutamine, and 50 ng/ml nerve growth factor (Merck Biosciences, Darmstadt, Germany) on 8-well chambered coverglass or 35-mm dishes (Nunc, Roskilde, Denmark) coated with polylysine (Sigma-Aldrich Corp., St. Louis, MO, USA) and laminin (Invitrogen). Human embryonic kidney (HEK-293T) and Chinese hamster ovary (CHO) cells for Ca^{2+} imaging and electrophysiology were cultured and transfected with human and mouse TRPA1, mutant TRPA1, rat TRPV1, or empty vector (pcDNA3) cDNAs as described previously (29, 33).

Chemicals and solutions

If not otherwise indicated, chemicals were purchased from Sigma-Aldrich Corp. Whole-cell electrophysiological and Ca^{2+}-imaging experiments were performed in modified standard Ringer's bath solution: 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES-NaOH, and 5 mM glucose (pH 7.3, 315–320 mosM). Pipette and chip solutions for whole-cell intracellular application contained 75 mM CsCl, 70 mM CsF, 2 mM $MgCl_2$, 10 mM EGTA, and 10 mM HEPES-CsOH (pH 7.3, 315–320 mosM). Pipette and bath solutions for single-channel electrophysiological recordings contained solutions identical to the standard Ringer's bath solution with the exception of being Ca^{2+}-free and containing 10 mM EGTA. Solutions for recordings in the inside-out configuration contained 0.5 mM sodium tripolyphosphate (PPPi) (Acros Organics, Fairlawn, NJ, USA). In certain cell-attached recordings, solutions contained 2 mM $CaCl_2$ and did not contain EGTA and PPPi. Isocyanate solutions of MDI (Chem Service Inc., West Chester, PA, USA) and HDI and tear gas solutions of CN (Scientific Exchange, Inc., Center Ossipee, NH, USA) and CR (Key Organics Ltd., Camelford, UK) were initially dissolved in dimethyl sulfoxide (DMSO) at 40 mM. Ionomycin (4 mM; MP Biomedicals, Solon, OH, USA), capsaicin (100 mM), and 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine (ET-18-OCH3) (20 mM) were dissolved in ethanol, and ruthenium red (100 mM;

Latoxan, Valence, France) was dissolved in water. Stock solutions were diluted to their final concentration in appropriate solution for applications. For eye applications, HDI, CN, and CS were dissolved in 75% DMSO/PBS to 100 mM. A freezing point osmometer (Advanced Instruments, Norwood, MA, USA) was used to measure the osmolarity of all solutions. The TRPA1 antagonists 4-(4-chlorophenyl)-3-methylbut-3-en-2-oxime (AP-18) (10 mM; Maybridge, Trevillett, UK) and 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)-*N*-(4-isopropylphenyl)acetamide (HC-030031) (20 mM; Hydra Bioscience, Cambridge, MA, USA) were dissolved in DMSO. For i.p. injections, 5, 10, and 30 mg/ml HC-030031 was suspended in 0.5% methylcellulose (Methocel).

Ca^{2+} imaging and electrophysiology

Cultured neurons and HEK-293T cells were loaded in modified Ringer's solution with 10 μM Fura-2-AM (Calbiochem, San Diego, CA, USA) and 0.02% Pluronic F127 (BASF, Mount Olive, NJ, USA) for 1 h and subsequently washed and imaged in glucose-free modified Ringer's solution. Fura-2-AM emission ratios were obtained with alternating 0.100-ms exposures at 340 and 380 nm from a Polychrome V monochromator (Till Photonics, Gräfelfing, Germany) on a microscope (IX51; Olympus, Center Valley, PA, USA), captured with a PCO camera (Sensicam QE; Cooke, Auburn Hills, MI, USA), and analyzed with Imaging Workbench 6 software (Indec; Santa Clara, CA, USA). Intracellular calcium ($[Ca^{2+}]_i$) concentrations were derived from the F_{340}/F_{380} ratio adjusted by the K_d of Fura-2-AM (238 nM) and the F_{380} and ratiometric data at minimum and maximum $[Ca^{2+}]_i$ (48–50). The latter was determined by incubation in 10 μM ionomycin Ringer's solution with 0 Ca^{2+} 10 mM EGTA or 25 mM Ca^{2+} (90 mM NaCl to compensate for a final osmolarity of 350 mosM). Ratiometric images were generated using ImageJ software (http://rsbweb.nih.gov/ij/).

Whole-cell configuration patch-clamp experiments were performed at ~25°C with borosilicate glass pipettes (World Precision Instruments, Sarasota, FL, USA) for neurons and a planar patch-clamp system for HEK-293T cells (NPC-1; Nanion, Munich, Germany). High-resolution currents were filtered at 2.3 kHz and digitized at 100-μs intervals using an EPC-10 amplifier (HEKA, Lambrecht, Germany) and Pulsemaster acquisition software (HEKA). Voltage ramps of −100 to +100 mV or −80 to +80 mV were applied over 100 ms at every 0.5 Hz from a holding potential of 0 mV as described previously (51). A liquid junction potential of 6.7 mV (JPCalc software; Axon Instruments, MA, USA) and capacitance were compensated for by the amplifier system.

Single-channel patch-clamp experiments were performed in the cell-attached or inside-out configurations on CHO cells at ~25°C with wax-coated borosilicate glass pipettes (World Precision Instruments). High-resolution currents were filtered at 3 kHz and digitized at 20-μs intervals using an EPC-10 amplifier and Pulsemaster acquisition software (HEKA).

Analysis of nocifensive responses

The nocifensive responses to intraocular instillation of 10 μl of 100 or 200 mM HDI, 100 mM CN, or 100 mM CS into the right eye or vehicle control (70% DMSO/PBS) into the left eye of *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice were video recorded (DCR-SR80; Sony, New York, NY, USA) in a clear Plexiglass cylinder (5-inch inside diameter) for 2 or 3 min. At the conclusion of every test, the treated eye was irrigated with PBS. Mice responded to HDI or tear gas agent application by lowering and subsequent pushing or rubbing of the facial area on the floor of the behavioral recording chamber; responses were individually counted. Nearly identical experiments were conducted on C57 mice after a 200-μl i.p. injection of 0.5% vehicle control (100 mM HDI, 100 mM CN, or 100 mM CS intraocular instillation into the right eye) and ~1 h after a 200-μl i.p. injection of the TRPA1 antagonist HC-030031 (1 or 6 mg) (100 mM HDI, 100 mM CN, or 100 mM CS into the right eye).

Nocifensive responses in the paw were also examined by 25-μl intraplantar injections using a 30-gauge needle. For experiments in *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice, vehicle was injected into the left paw and then ~1 h later either CN (2 mM in 5% DMSO/PBS) or bromoacetone (4 mM in PBS) was injected in the right paw. For experiments using HC-030031 on C57 mice, HDI (6 mM in 5% DMSO/PBS) or CN (4 mM in 5% DMSO/PBS) was injected into the right paw after a 200-μl i.p. injection of 0.5% vehicle control, and ~1 h after mice received a 2-mg i.p. injection of HC-030031 (200 μl), 6 mM HDI or 4 mM CN was injected into the left paw. Videorecorded responses (licking, lifting, and flicking of the injected paw) in a Plexiglas cylinder for 3–5 min were visualized and quantified by slowing the video frame speed using Microsoft Windows Media Player software (Microsoft, Redmond, WA, USA). The more hydrophobic agents were not used, because they were insoluble in 5% DMSO.

Statistics

Statistical analysis was performed, and graphical displays of both electrophysiological and Ca^{2+}-imaging data were made using IGOR Pro (Wavemetrics, Lake Oswego, OR, USA) or ORIGIN (OriginLab, Northampton, MA, USA). Statistical errors are SE unless indicated otherwise.

An independent two-sample Student's *t* test was performed between mice lacking a functional *Trpa1* gene (*Trpa1*$^{-/-}$) and wild-type littermates (*Trpa1*$^{+/+}$) on the total quantity of paw licking, lifting, and flicking (paw pain response, n=8/group for CN and n=6/group for bromoacetone) and the "facial pain" of stroking the orbitofacial area in response to isocyanate or tear gases (CS and HDI n=6 *Trpa1*$^{+/+}$, n=7 *Trpa1*$^{-/-}$; n=6/group for CN). Differences were seen in the paw response to CN (P=0.023) and bromoacetone (P=0.045). Differences were seen in the facial pain response to CN (P=0.001), CS (P=0.001), and HDI (P=0.008).

Dependent (repeated-measure) Student's *t* tests were performed on the mouse facial pain and paw pain responses to isocyanate or tear gases after vehicle control injection compared with the responses ~1 h after the mice were injected with 6 mg of HC-030031 (n=6/group for CS and HDI; n=9 for CN), 1 mg of HC-030031 (n=6/group), or 2 mg of HC-030031 (n=6/group). Differences were seen in facial pain with the 1-mg HC-030031 treatment to applications of CN (P=0.004), CS (P=0.04), and HDI (P=0.01) and with the 6-mg HC-030031 treatment to applications of CN (P=0.0), CS (P=0.005), and HDI (P=0.029). Differences in paw pain were observed after the 2-mg HC-030031 treatment to intraplantar injections of CN (P=0.005) and HDI (P=0.015).

RESULTS

TRPA1 is activated by industrial isocyanates and all major tear gas agents *in vitro*

We used fluorescent $[Ca^{2+}]$ imaging to examine the effects of 2 major industrial isocyanates (Fig. 1*A*) and 6 different tear gas agents (Fig. 1*B*) on 2 members of the TRP ion channel family, TRPV1, the capsaicin receptor,

and TRPA1, the mustard oil receptor, expressed in HEK-293T cells. TRPA1 was strongly activated by MIC, HDI, and all of the tear gas agents tested (CN, CS, CR, PS, bromoacetone, and benzyl bromide). Dose-response analysis revealed that the isocyanates MIC (EC_{50}=25±3 μM, n=28±5 cells/dose) and HDI (EC_{50}=2.6±0.7 μM, n=29±4 cells/dose) activated TRPA1 with a potency comparable to that of the chemically similar mustard oil (allyl isothiocyanate) (Fig. 1*C*). In our hands, CS was the most potent activator of human TRPA1 channels, with half-maximal activation occurring at EC_{50} CS = 7 ± 1 nM (n=52±5 cells/dose) and was 3 orders of magnitude more potent than mustard oil. CN, CR, and PS were also highly potent, with half-maximal activation of human TRPA1 (hTRPA1) at EC_{50} CN = 91 ± 12 nM (n=49±1 cells/dose), EC_{50} CR = 308 ± 150 nM (n=70±9 cells/dose), and EC_{50} PS = 308 ± 150 nM (n=32±4 cells/dose) (Fig. 1*D*). The tear gas agents benzyl bromide (EC_{50}=12.0±0.6 μM, n=66±17 cells/dose) and bromoacetone (EC_{50}=1.1±1.1 μM, n=78±14 cells/dose) also activated hTRPA1. At saturating doses of the noxious chemical activation of hTRPA1, neither rat TRPV1- nor empty vector (pcDNA3)-transfected HEK-293T cells responded (Supplemental Fig. 1*A, B*). Only HDI and benzyl bromide induced minor TRPV1 activity after significant delays following irritant application (Supplemental Fig. 1*B*).

Recent studies support the idea that reactive irritants activate TRPA1 through covalent modification of cysteine and lysine residues within the cytosolic N terminus of the channel protein (46, 47). Whereas isocyanates and some tear gas agents can undergo electrophilic chemical reactions, CN, CS, and CR also share structural similarities with nonreactive TRPA1 agonists, including terpenes such as carvacrol or thymol (44, 52, 53). Chemical agents may also activate TRPA1 indirectly, through stimulation of phospholipase C (PLC)-coupled receptor pathways and subsequent release of Ca^{2+} from intracellular stores or through other Ca^{2+}-mobilizing pathways (29, 30, 54–56). To examine the requirement for Ca^{2+} or other cytosolic factors, we performed inside-out patch-clamp recordings of hTRPA1 channels expressed in CHO-K1 cells in the absence of Ca^{2+} on both sides of the membrane. In this configuration, PLC- and any other second messenger-dependent pathways are disrupted. Sodium triphosphate (0.5 μM), an essential intracellular cofactor for TRPA1 activation, was included in the bath solution (57). Application of 100 μM MIC or 10 μM CS specifically induced a large increase in single-channel openings (124±3 pS for MIC and 120±3 pS for CS at −40 mV; 3 patches/agent), similar to TRPA1 single conductances recorded by others in the absence of Ca^{2+} (Fig. 1*E, F*; Supplemental Fig. 2*A, B*) (58). These results suggest that isocyanates and tear gas agents activate TRPA1 in a membrane-delimited fashion that does not require increases in cytosolic Ca^{2+} or activation of second messenger pathways. hTRPA1 single channels were also activated in the cell-attached configuration, indicating that the chemical activator needs to traverse the plasma membrane to activate the ion channels positioned under the patch electrode (Supplemental Fig. 2*D, E*). The open channel current-voltage relationship of HDI-activated channels in the cell-attached configuration was linear in the absence of Ca^{2+} (single channel conductance: 127±4 pS at −40 mV) but outwardly rectifying in the presence 2 mM Ca^{2+} (51±2 pS at −40 mV) (Supplemental Fig. 2*D, E*).

We next examined whether isocyanates and tear gas agents would require putative covalent acceptor sites in hTRPA1 for channel activation (46, 47). We examined three different mutant channels in which critical reactive sites (C619, C639, C663, and K708) were replaced by inert residues. In the first mutant (K) K708 was replaced. A second mutant (3C) had mutations in all three cysteine residues, and a third mutant (3CK) had mutations in all four sites. In previous studies these mutations dramatically reduced the potencies and efficacies of electrophiles and oxidants to activate TRPA1 (33, 36, 46, 47). As a positive control, we used the TRPA1 agonist carvacrol, a pungent nonreactive terpene, which does not activate TRPA1 by covalent binding. Whereas the lysine mutant was activated by all agents (MIC, n=84; HDI, n=53; CS, n=157; CN, n=108; CR, n=154; bromoacetone, n=44; and benzyl bromide, n=64), mutant 3C showed significantly reduced responses to MIC (n=34), CN (n=56) and CR (n=90), and bromoacetone (n=25) but did not greatly affect the efficacy of HDI (n=47), CS (n=96), or benzyl bromide (n=40). CS, the most potent tear gas agent also showed significant activity on the 3CK mutant (n=129), as did the isocyanate HDI (n=123), indicating that these agents may require additional reactive sites for their activity or activate TRPA1 through a different mechanism. In contrast, the activities of the other tested chemicals were dramatically reduced or eliminated (MIC, n=30; CN, n=18; CR, n=17; bromoacetone, n=38; and benzyl bromide, n=41) (Fig. 1*G*).

Cellular responses of native sensory neurons to industrial isocyanates have not been reported. We therefore used fluorescent Ca^{2+} imaging to investigate the effects of MIC and HDI on dissociated murine trigeminal ganglion (TG) and dorsal root ganglion (DRG) neurons. Fibers derived from the TG innervate the eyes, facial skin, and upper airways that were the initial contact sites of exposure in patients during the Bhopal incident. DRG neurons innervate parts of the lower airways affected after inhalation of the toxicant. We observed that MIC (100 μM) and HDI (100 μM) induced a rapid increase in $[Ca^{2+}]_i$ in a subset of capsaicin-sensitive TG and DRG neurons, overlapping with the mustard oil-sensitive neuronal population (**Fig. 2*A, B*;** Supplemental Fig. 1*E*).

Responsiveness of native sensory neurons to the two most widely used tear gas agents, CS and CN, has not been described. CR was recently reported to activate Ca^{2+} influx into cultured DRG neurons (44). However, although implying that TRPA1 was a neuronal target for CR, this study did not use any specific pharmaco-

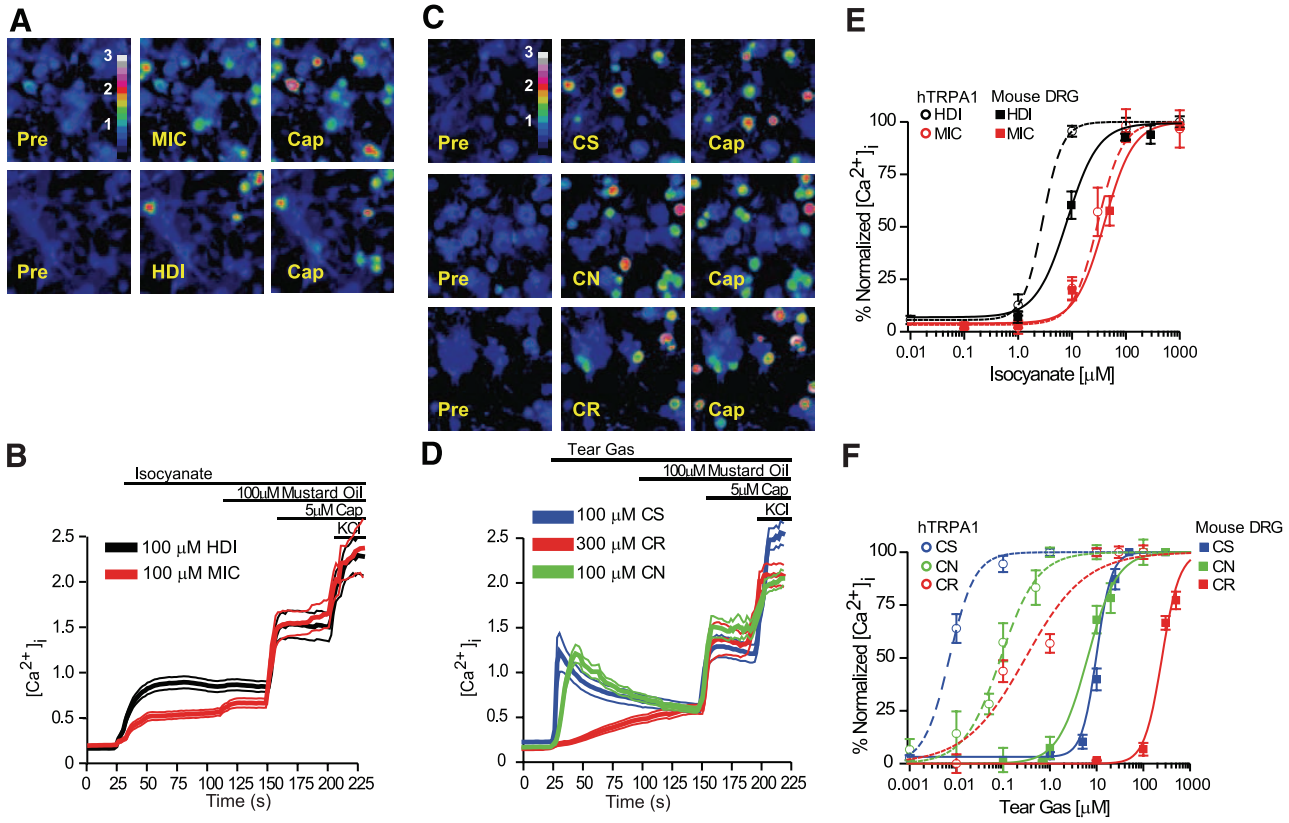

Figure 2. Industrial isocyanates and tear gas agents activate native TRPA1 channels in cultured sensory neurons. *A*) Industrial isocyanates induced Ca^{2+} influx into cultured mouse DRG neurons, as measured by fluorescent Fura-2 imaging. Neurons are shown before activation (Pre, left column), 70 s after challenge (middle column) with MIC (100 μM, top row) or HDI (100 μM, bottom row), and after application of 5 μM capsaicin (Cap, right column) after 50 s. Pseudocolors denote 0–3 μM $[Ca^{2+}]_i$. Original view, ×10. *B*) Average $[Ca^{2+}]_i$ of mouse DRG neurons (thick lines) with an application of MIC (100 μM, n=168 neurons from 2 mice, black line) or HDI (100 μM, n=270 from 2 mice, red line), followed by 100 μM mustard oil, 5 μM capsaicin (Cap), and 65 mM KCl. Thin lines represent SE. *C*) Tear gas agent-induced Ca^{2+} influx into cultured murine DRG neurons, as measured by fluorescent Fura-2 imaging. Neurons are shown before activation (Pre, left column), 70 s after challenge (middle column) with CS (100 μM, top row) or CN (100 μM, middle row) or CR (300 μM, bottom row) and after application of 5 μM capsaicin (Cap, right column) after 50 s. Pseudocolors denote 0–3 μM $[Ca^{2+}]_i$. Original view, ×10. *D*) Average $[Ca^{2+}]_i$ of mouse DRG neurons (thick lines) with an application of CS (100 μM, blue line, n=161 neurons from 2 mice), CN (100 μM, green line, n=335 from 5 mice), or CR (300 μM, red line, n=137 from 2 mice), followed by 100 μM mustard oil, 5 μM capsaicin (Cap), and 65 mM KCl. Thin lines represent SE. *E*) Dose-response curves of isocyanate-activated Ca^{2+}-influx into mouse DRG neurons are similar to hTRPA1-transfected HEK-293T cells. $[Ca^{2+}]_i$ induced by each dose is represented as percentage of maximal $[Ca^{2+}]_i$ elicited by a saturating dose of mustard oil (100 μM) applied 75 s later (baseline $[Ca^{2+}]_i$ was subtracted). Mustard oil-sensitive mouse DRG neurons were activated by MIC (EC_{50}=36±7 μM, n=30±6 neurons/dose, solid black squares) and HDI (EC_{50}=8.4±1.4 μM, n=39±12 /dose, solid red squares). Dashed lines and open circles represent hTRPA1-transfected HEK-293T cells, as shown in Fig. 1*C*. *F*) Dose-response curves of tear gas agent-activated Ca^{2+}-influx into mouse DRG neurons are right-shifted compared with responses in hTRPA1-transfected HEK293T cells. $[Ca^{2+}]_i$ represented as in *E*. Mustard oil-sensitive mouse DRG neurons were activated by CS (EC_{50}=12.1±0.3 μM, n=41±9 neurons/dose, solid blue squares), CN (EC_{50} =6±1 μM, n=23±5 /dose, solid green squares), and CR (EC_{50}= 246±27 μM, n=37±16 /dose, solid red squares). Dashed lines with open circles represent dose-response curves of Ca^{2+}-influx into hTRPA1-transfected HEK-293T cells shown in Fig. 1*D*.

logical, genetic, or *in vivo* approaches to substantiate this point. We found that CS, CN, bromoacetone, and benzyl bromide (100 μM each) rapidly induced Ca^{2+} influx into a subset of DRG neurons (Fig. 2*C, D, F*; Supplemental Fig. 1*C*). Exposure to CS, CN, bromoacetone, and benzyl bromide eliminated the neuronal sensitivity to subsequent application of mustard oil. CR (300 μM) only slowly induced neuronal activity and did not completely prohibit further neuronal activation by mustard oil (Fig. 2*D*). CS and CN also induced Ca^{2+} influx into TG neurons (Supplemental Fig. 1*F*).

Similar to previously characterized TRPA1 agonists such as mustard oil or acrolein, the isocyanates have very similar potencies in mustard oil-sensitive DRG neurons (EC_{50} MIC=36±7 μM, n=30±6 neurons/dose) and (EC_{50} HDI=8.4±1.4 μM, n=39±12 neurons/dose) and in hTRPA1-transfected cells (EC_{50} MIC=25±3 μM) and HDI (EC_{50} HDI=2.6±0.7 μM) (Fig. 2*E*). Most surprisingly, we found the tear gas agents CS and CR to be ~1000-fold less potent and CN to be 100-fold less potent for activating Ca^{2+} influx into native neurons (EC_{50} CS=12.1±0.3 μM, n=41±9 neu-

rons/dose; EC_{50} CN=6±1 μM, n=23±5 neurons/dose; and EC_{50} CR=246±27 μM, n=37±16 neurons/dose) compared with heterologous cells expressing hTRPA1 (EC_{50} CS=7±1 nM, EC_{50} CN=91±12 nM, and EC_{50} CR=308±150 nM) (Fig. 2*F*) or mouse TRPA1 (EC_{50} CN=66±14 nM) (Supplemental Fig. 1*F*).

The large divergence of tear gas agent potencies between heterologous cells expressing TRPA1 and primary neurons suggests that either native TRPA1 channels have different pharmacological properties or that alternative targets may be involved in neuronal responses to these agents. To further examine the neuronal response to tear gas agents we performed patch-clamp electrophysiological recordings of primary neurons in the whole-cell configuration. CN (100 μM) induced sizable, slightly outwardly rectifying membrane currents in 4 of 16 recorded neurons, which were efficiently blocked by ruthenium red, a pore blocker of TRPA1 and other TRP ion channels (**Fig. 3*A*, *B***). The percentage of responsive neurons, the size, and the current-voltage (I-V) relationship of the CN-induced currents were similar to neuronal TRPA1 currents we recorded in previous studies using the TRPA1 agonists sodium hypochlorite and isovelleral (33, 36). Furthermore, the CN-induced neuronal currents were remarkably similar in their relationship to voltage to CN-induced currents in hTRPA1-expressing HEK-293T cells (Fig. 3*C*). Compared with neuronal currents, TRPA1 currents in the heterologous HEK-293T cells were larger and desensitized rapidly, as characterized by us and others with a variety of agonists (n=4) (Fig. 3*D*) (36).

Genetic deletion of TRPA1 or pharmacological blockade with TRPA1 antagonists renders sensory neurons insensitive to isocyanates and tear gas agents

Our results gathered from cultured primary neurons and heterologous cells suggest that industrial isocyanates and tear gas agents excite sensory neurons through activation of TRPA1. However, concentrations of tear gas agents required to induce Ca^{2+} influx into cultured sensory neurons were >100-fold higher than required for activation of cloned mouse and human TRPA1 channels expressed in heterologous cells. It remained a possibility that isocyanates and tear gas agents activated alternative targets in sensory neurons, through direct interactions with other Ca^{2+}-permeable ion channels with relatively similar electrophysiological profiles, or indirectly, through activation of signal transduction cascades involving PLC. PLC pathways have been shown to activate or sensitize TRPA1 and many other Ca^{2+}-permeable TRP ion channels (26, 59). To investigate the potential involvement of PLC pathways in the neuronal response to isocyanates and tear gases, we performed Ca^{2+}-imaging experiments in the presence of ET-18-OCH_3, a PLC inhibitor used in a previous study to inhibit activation of TRPA1 through PLC-coupled protease-activated receptors in sensory neurons (56). ET-18-OCH_3 (4 μM) did not diminish neuronal Ca^{2+} influx activated by any of the noxious agents applied (Supplemental Fig. 1*D*).

To examine the requirement for TRPA1 in sensory neuronal responses to isocyanates and tear gas agents, we studied the responses of sensory neurons dissociated from TRPA1-deficient mice. When superfused with MIC (n=217 neurons from 2 mice), HDI (n=204 neurons from 2 mice), CS (n=229 neurons from 2 mice), CN (n=270 neurons from 5 mice), or CR (n=108 neurons), TRPA1-deficient neurons failed to respond with an increase in $[Ca^{2+}]_i$. These neurons responded normally to capsaicin, used as a control stimulus (Fig. 3*A–D*).

Recently, the structures and efficacies of two newly developed TRPA1 antagonists were reported (35, 60). These antagonists, HC-030031 and AP-18, blocked the activation of TRPA1 by mustard oil and other reactive

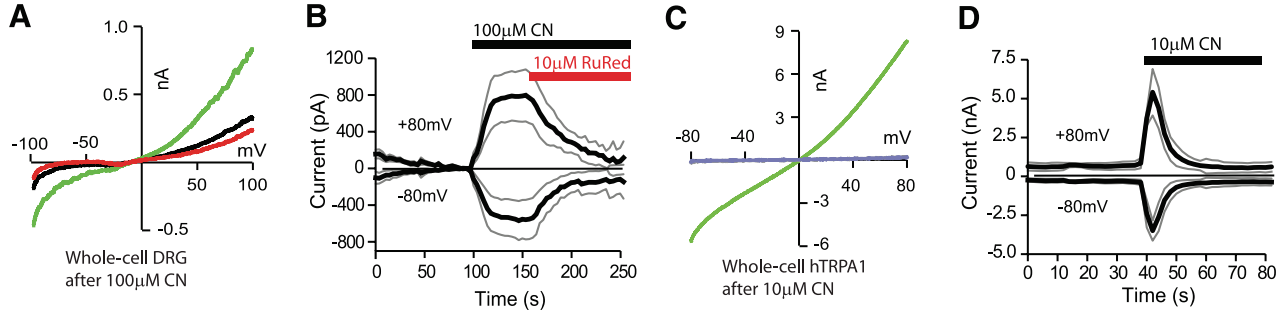

Figure 3. CN induces TRPA1-like currents in mouse DRG neurons. *A)* TRPA1-like current-voltage curves of a representative mouse DRG neuron before activation (black trace), activation by 100 μM CN (green trace), and inhibition by ruthenium red (10 μM, red trace) in whole-cell configuration. $V_{holding}$ = 0 mV to minimize voltage-gated channels. Currents were measured with a voltage ramp from −100 to +100 mV over 100 ms at 0.5-Hz intervals. Intracellular Cs-based solution with 10 mM EGTA was used. *B*) Average native TRPA1-like currents at −80 and +80 mV in mouse DRG neurons superfused with 100 μM CN (black bar), followed by ruthenium red (RuRed; 10 μM) as described for Fig. 1*B* (n=4 of 16 neurons). Baseline current was subtracted for each trace. *C*) hTRPA1 current-voltage curves before activation (black trace), at maximal activation by 10 μM CN (green trace), and after inactivation phase (blue trace) in whole-cell configuration. Currents were measured with a voltage ramp from −80 to +80 mV over 100 ms at 0.5 Hz intervals, $V_{holding}$= 0 mV. Intracellular Cs-based solution with 10 mM EGTA was used. *D*) Averaged TRPA1 currents at −80 and +80 mV in hTRPA1-transfected HEK-293T cells superfused with 10 μM CN (black bar) as described for Fig. 1*C* (n=4).

chemical stimuli *in vitro*. We examined the effects of these antagonists on CS-, CN- and CR-induced activation of hTRPA1 expressed in HEK-293T cells. Both HC-030031 and AP-18, used at a concentration of 25 μM, efficiently blocked the activation of hTRPA1 by all three tear gas agents (Supplemental Fig. 3*A*). The antagonist HC-030031 effectively blocked native TRPA1 responses to 10 μM HDI (IC_{50}=74±3 μM, n=31±4), CN (IC_{50}=884±23 nM, n=25±5), and CS (IC_{50}=4.5±0.4 μM, n=26±6) in cultured sensory neurons dissociated from wild-type mice (**Fig. 4*D***; Supplemental Fig. 3*B, C*). These neurons responded normally to a saturating dose of capsaicin, used as a control stimulus.

Taken together, our results show that TRPA1 is the sole target of industrial isocyanates and tear gas agents in sensory neurons, allowing influx of Ca^{2+} and neuronal excitation. Furthermore, we show that TRPA1 antagonists completely block neuronal activity in response to isocyanates or tear gas agents. This finding suggests that TRPA1 antagonists may prevent and alleviate the noxious effects of isocyanates and tear gas agents *in vivo*.

TRPA1 antagonists effectively block the noxious effects of isocyanates and tear gas agents in vivo

Human exposure to airborne industrial isocyanates and tear gases results in immediate extreme ocular and facial pain, as well as in airway irritation, mucus secretion, and obstruction. Our data suggest that these effects are triggered by activation of TRPA1 channels in trigeminal sensory neurons. However, it is unclear whether isocyanates and tear gas agents interact specifically with TRPA1 *in vivo* or whether these highly reactive chemicals activate sensory neurons indirectly through factors released during tissue damage. We

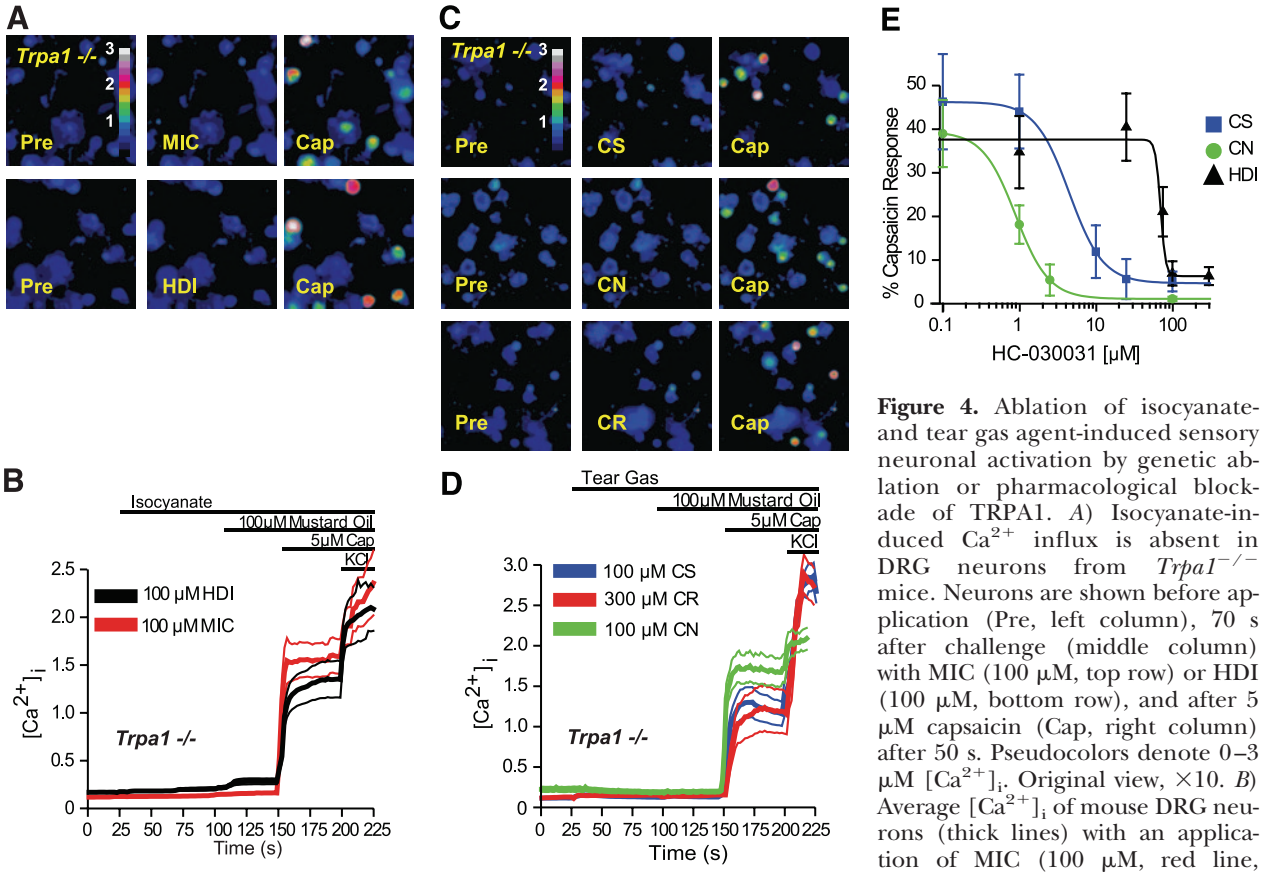

Figure 4. Ablation of isocyanate- and tear gas agent-induced sensory neuronal activation by genetic ablation or pharmacological blockade of TRPA1. *A*) Isocyanate-induced Ca^{2+} influx is absent in DRG neurons from *Trpa1*$^{-/-}$ mice. Neurons are shown before application (Pre, left column), 70 s after challenge (middle column) with MIC (100 μM, top row) or HDI (100 μM, bottom row), and after 5 μM capsaicin (Cap, right column) after 50 s. Pseudocolors denote 0–3 μM $[Ca^{2+}]_i$. Original view, ×10. *B*) Average $[Ca^{2+}]_i$ of mouse DRG neurons (thick lines) with an application of MIC (100 μM, red line, n=217 from 2 mice) and HDI (100 μM, black line, n=204 neurons from 2 mice), followed by 100 μM mustard oil, 5 μM capsaicin (Cap), and 65 mM KCl. Thin lines represent SE. *C*) Tear gas agent-induced Ca^{2+} influx is absent in DRG neurons from *Trpa1*$^{-/-}$ mice, shown before activation (Pre, left column), 70 s after challenge (middle column) with CS (100 μM, top row), CN (100 μM, middle row), or CR (300 μM, bottom row), followed by 5 μM capsaicin (Cap, right column) after 50 s. Pseudocolors denote 0–3 μM $[Ca^{2+}]_i$. Original view, ×10. *D*) Average $[Ca^{2+}]_i$ of mouse DRG neurons (thick lines) with an application of CS (10 μM, blue line, n=229 neurons from 2 mice), CN (100 μM, green line, n=270 neurons from 5 mice), or CR (300 μM, red line, n=108 neurons), followed by 100 μM mustard oil, 5 μM capsaicin (Cap), and 65 mM KCl. Thin lines represent SE. *E*) Dose-response curves of inhibition of industrial isocyanate or tear gas agent-activated Ca^{2+}-influx into mouse DRG neurons by the TRPA1 antagonist HC-030031. $[Ca^{2+}]_i$ induced by each dose is represented as percentage of $[Ca^{2+}]_i$ elicited by a saturating dose of capsaicin (5 μM, Cap) applied 125 s later (baseline $[Ca^{2+}]_i$ was subtracted). HC-030031 inhibited the $[Ca^{2+}]_i$ induced by 10 μM HDI (IC_{50}=74±3 μM, n=31±4 Cap-sensitive neurons/dose, black triangles), 10 μM CS (IC_{50}=4.5±0.4 μM, n=26±6/ dose, blue squares), and 10 μM CN (IC_{50}=884±23 nM, n=25±5/dose, green circles) in mouse DRG neurons.

therefore examined the effects of pharmacological inhibition and genetic ablation of TRPA1 on the behavioral responses to isocyanates and tear gas agents in mice. HDI, CN, and CS (100 mM each) caused immediate nocifensive responses on application to the mouse eye (MIC was too volatile and dangerous to test). The mice initially wiped their eyes and facial area and then continued with characteristic nocifensive behavior by vigorously stroking their heads and facial area against the bottom of the observation chamber (33). This behavior was completely absent when just vehicle was applied. We then injected the mice with the TRPA1 antagonist HC-030031 (300 or 50 mg/kg body weight i.p.) and applied the same dose of noxious chemical to the opposite eye 1 h later (300 mg/kg HC-030031 (n=6/group for CS and HDI; n=9 for CN) and 50 mg/kg HC-030031 (n=6/group). Remarkably, HC-030031 dramatically reduced the frequency of nocifensive responses to all three agents (**Fig. 5*A***). We then used a more conventional method of examining TRPA1-associated nocifensive responses, comparing nocifensive responses after intraplantar injections of HDI (6 mM) or the tear gas agent CN (4 mM) into the mouse hindpaw before and after treatment with 100 mg/kg body weight HC-030031. After the initial intraplantar injections, mice responded with immediate nocifensive behavior, including flinching, lifting, and licking of the paw (Fig. 5*B*). This behavior was greatly reduced in the same mice approximately 1 h after treatment with HC-030031 (Fig. 5*B*).

Because HC-030031 may inhibit the effects of isocyanates and tear gases in a nonspecific manner, we also compared isocyanate- and tear gas agent-induced behavior between TRPA1-deficient mice after eye application. Strikingly, nocifensive responses to tear gas agents (CN and CS) were completely absent in $Trpa1^{-/-}$ mice in this test (Fig. 5*C*). These results suggest that $Trpa1^{-/-}$ mice failed to detect tear gas agents as noxious stimuli. Responses to the isocyanate HDI were significantly abated (Fig. 5*C*). In addition to facial exposures, we compared responses of $Trpa1^{-/-}$ and $Trpa1^{+/+}$ mice after injections of the relatively soluble tear gas agents CN (n=8/group) and bromoacetone (n=6/group) into the hindpaw. After injections, wild-type mice responded with immediate nocifensive behavior, which was greatly reduced in $Trpa1^{-/-}$ mice (Fig. 5*D*).

In summary, our behavioral tests support an essential role for TRPA1 in the sensory detection of industrial isocyanates and tear gas agents (CN, CS, and bromoacetone) *in vivo*. Furthermore, exposure-related pain and irritation by these agonists can be prevented by TRPA1 antagonists.

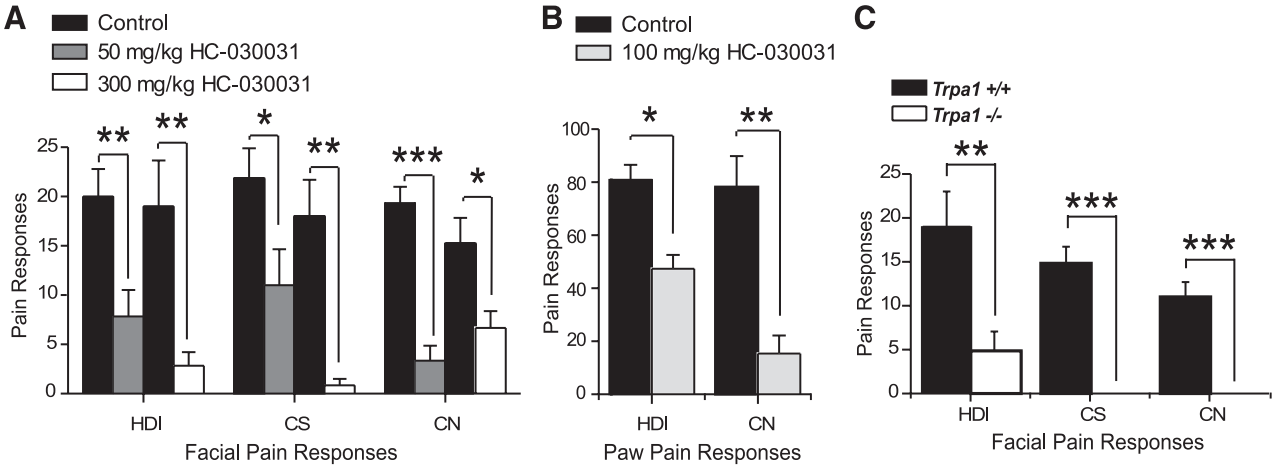

Figure 5. Ablation of isocyanate- and tear gas agent-induced nocifensive responses in mice by genetic deletion or pharmacological blockade of TRPA1. *A*) Nocifensive responses after application of 10 μl of 100 mM HDI (n=6), 100 mM CS (n=6), or 100 mM CN (n=9) to the eye of untreated C57/BL6 wild-type mice (black bars) and the same mice after an injection of 1 mg i.p.(50 mg/kg, gray bar) or 6 mg i.p.(300 mg/kg, white bar) of TRPA1 antagonist HC-030031. Nocifensive responses were quantified by counting strokes of the orbitofacial area on the observation chamber floor over 2 min for CS and CN for 300 mg/kg experiments and over 3 min for other treatments. Significance: $^{**}P < 0.01$; $^{*}P < 0.05$. *B*) Nocifensive responses (licks, lifts, and flicks) over 3 min after 25-μl subplantar injections of 4 mM CN or 6 mM HDI (n=6/group) into hindpaws of untreated C57/BL6 wild-type mice (black bars) and the same mice after an injection of 2 mg i.p.(100 mg/kg, gray bars). Significance: $^{**}P < 0.01$; $^{*}P < 0.05$. *C*) Comparison of nocifensive responses of wild-type (black bars) and $Trpa1^{-/-}$ mice (white bars) after application of 10 μl of 200 mM HDI, 100 mM CS, or 100 mM CN to right eye. Strokes of the orbitofacial area against the observation chamber floor were counted over 2 min for CS and CN and for 3 min for HDI (n=6 wild-type and n=6 $Trpa1^{-/-}$ mice were tested with CN, n=6 wild-type and n=7 $Trpa1^{-/-}$ mice tested with CS, and n=6 wild-type and n=7 $Trpa1^{-/-}$ mice were tested with HDI). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. *D*) Nocifensive responses (licks, lifts, and flicks) over 5 min after 25-μl subplantar injections of 2 mM CN (n=8/group) or 4 mM bromoacetone (Br acetone, n=6/group) into hindpaws of $Trpa1^{+/+}$ and $Trpa1^{-/-}$ mice. $^{*}P < 0.05$.

DISCUSSION

Even though the release of methyl isocyanate in Bhopal caused the largest chemical industrial accident in history, the molecular basis of injury and irritation elicited by isocyanate exposures has remained unknown. In our present study, we have demonstrated that industrial isocyanates target the same sensory neuronal receptor as tear gas agents, TRPA1, to rapidly activate pain and sensory irritation. We find that TRPA1 channels expressed in primary sensory neurons and heterologous cells are robustly activated by both classes of agents. Isocyanate- and tear gas-induced nocifensive behavior is greatly reduced in TRPA1-deficient mice. Moreover, treatment of mice with newly developed TRPA1 antagonists leads to a dramatic reduction in sensitivity to isocyanates and tear gas agents.

Activation of TRPA1 by industrial isocyanates may have contributed to the acute and chronic health effects experienced by victims of the Bhopal incident and by agricultural and industrial laborers (1, 6). We show that industrial isocyanates strongly activate human TRPA1 channels and, in mice, have effects very similar to those of tear gases, activating trigeminal nerve endings in the eyes and facial area to elicit nocifensive responses. Trigeminal nerve fibers innervating the facial skin, mucous membranes, and eyes are the first line of defense against chemical exposures threatening tissue integrity and function (22). By acting similarly to tear gas agents, isocyanates induce ocular pain, lacrimation, and blepharospasm through trigeminal-autonomic and trigeminal-motor reflexes in exposed individuals. In addition to ocular and facial cutaneous nerve endings, isocyanates may also target TRPA1 channels in nerve endings lining the airways. In humans, activation of airway nerve endings by chemical irritants triggers cough, sneezing, airway mucus secretion, edema, and obstruction through activation of sensory nerves. In mice, these effects result in respiratory depression, significantly lowering respiratory rates (61). Recently, we showed that TRPA1 is essential for the activation of murine sensory neurons by the irritant chlorine and for chlorine-induced respiratory depression (36). Similar to chlorine, isocyanates and other TRPA1 agonists such as acrolein induce respiratory depression in rodents and other mammalian species, suggesting a crucial role of TRPA1 in this physiological response to chemical sensory irritation (14, 62, 63).

Our study is the first to provide a clear mechanistic basis for the biological actions of tear gases *in vivo*, supporting a central role of TRPA1 in the neuronal sensation of all major tear gas agents and subsequent activation of involuntary nocifensive reflex responses, including lacrimation, mucus secretion, and muscle contraction. We identify CS and CN as the most highly potent activators of heterologously expressed human TRPA1 channels. In our hands, CR agent is less potent than CS and CN, a finding that is in contrast to a recent study reporting a higher potency of CR on hTRPA1 *in vitro* (44). The reason for this discrepancy may lie in the differing purity of the agents used or in differences in experimental conditions.

We observed large differences in potencies of tear gas agents in heterologous cells and native sensory neurons. Although divergence of potencies have been observed for TRPA1 agonists before, we found that some tear gas agents have >100-fold higher potencies in human or mouse TRPA1-expressing HEK-293T cells than in mouse sensory neurons (36). In contrast, isocyanates show largely equal potencies in heterologous cells and native neurons. Our results indicate that *in vitro* studies alone are insufficient to evaluate specific TRPA1 agonist activity for a given chemical. We also found that previously identified covalent acceptor sites in TRPA1 are essential for activation by some agonists (CN and CR) but not by others (MIC, HDI, and CS). These results suggest that, in addition to electrophilic reactivity, other factors affect the ability of chemical agents to activate TRPA1. Some chemical agonists may bind to additional, as yet unidentified, covalent acceptor sites. Other agents may have different membrane permeabilities in heterologous cells or neurons, or their actions may be affected by intracellular reducing agents. Finally, responses by native TRPA1 channels may be affected by additional protein subunits, post-translational modifications, or differences in regulation of the local Ca^{2+} microenvironment (64).

The essential role of TRPA1 as the sole mediator of tear gas-related irritation *in vivo* is supported by our observation that TRPA1-deficient mice are largely impervious to the noxious effects of tear gases. In contrast to isocyanates, exposure to tear gas agents causes less tissue damage and long-term health effects. CS and CN are much less volatile than MIC and are usually dispersed as aerosols together with organic solvents or burned to reach irritating airborne concentrations (12). Nevertheless, adverse health effects and even deaths have been reported after tear gas exposures, especially when exposures occurred in closed environments. Responses include acute bronchospasm, pulmonary edema, asthma-like symptoms, and severe contact dermatitis (65–70). Individuals affected by preexisting allergic conditions seem to be especially prone to hypersensitivity reactions after tear gas exposures. In addition to the two major tear gas agents, TRPA1 is also activated by CR, benzyl bromide, bromoacetone, and PS. Presently, PS is widely used as a soil fumigant in agriculture, causing frequent occupational and environmental exposures (71, 72). TRPA1 activation is likely to contribute to the health effects caused by PS, including eye and respiratory tract irritation.

Irritant-induced sensory reflexes and pain are thought to be essential for the protection of eyes, skin, and airways from further chemical exposures. However, in the cases of isocyanates and tear gases, sensory responses usually occur rapidly and with very

high intensity, leading to partial or complete incapacitation. During the Bhopal incident, the TRPA1-mediated acute noxious effects of methyl isocyanate may thus have prevented many victims from escaping further exposure, leading to aggravated tissue damage due to the nonspecific corrosive effects of the toxicant. Individuals having airway infections or chronic inflammatory airway conditions, both highly prevalent in developing countries, may have responded more violently to MIC exposure. Activation of inflammatory signaling pathways in asthma, rhinitis, or airway infections could explain hypersensitivity responses to isocyanates and tear gases, because these pathways dramatically increase the sensitivity of TRPA1 to its agonists (9, 29, 30, 34, 56).

Individuals exposed to high levels of TRPA1 agonists, including chlorine and isocyanates, often present with RADS (73–75). RADS is characterized by highly increased sensitivity to chemical and physical stimuli, in addition to the initial sensitizing stimulus, resulting in asthma-like symptoms such as cough, wheezing, chest tightness and dyspnea (73). For example, agricultural workers exposed to MIC during a spill of the pesticide, metam sodium, subsequently became highly sensitive to diesel exhaust (5). Diesel exhaust contains high levels of the TRPA1 agonist, acrolein, and induced lacrimation, strong nasal irritation, and cough in the MIC-preexposed individuals (5). The multiple chemical sensitivity of TRPA1 readily explains the symptoms observed in patients with RADS. After an initial sensory challenge and tissue injury by a high-level chemical exposure, sensory TRPA1 channels become sensitized through inflammatory signaling pathways, establishing prolonged hypersensitivity to multiple reactive chemicals (29, 30, 34, 56). The role of TRPA1 in chemical hypersensitivity may extend to other, less clearly defined, conditions, including sensory hyperreactivity and multiple chemical sensitivity (76, 77).

RADS and related conditions are only partially responsive to the therapeutic interventions developed for the treatment of asthma. Our data suggest that TRPA1 antagonists may be effective in blocking the exaggerated chemosensory responses accompanying these conditions. Moreover, we show that TRPA1 antagonists prevent the acute sensory irritation elicited by exposures to isocyanates and tear gasses. TRPA1 antagonists may also be useful for postexposure treatment, reducing sensory irritation and, potentially, preventing adverse long-term health effects elicited by neurogenic inflammatory mechanisms. FJ

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