Pungent General Anesthetics Activate Transient Receptor Potential-A1 to Produce Hyperalgesia and Neurogenic Bronchoconstriction

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ABSTRACT

Background: Volatile anesthetics such as isoflurane and halothane have been in clinical use for many years and represent the group of drugs most commonly used to maintain general anesthesia. However, despite their widespread use, the molecular mechanisms by which these drugs exert their effects are not completely understood. Recently, a seemingly paradoxical effect of general anesthetics has been identified: the activation of peripheral nociceptors by irritant anesthetics. This mechanism may explain the hyperalgesic actions of inhaled anesthetics and their adverse effects in the airways.

Methods: To test the hypothesis that irritant inhaled anesthetics activate the excitatory ion-channel transient receptor potential (TRP)-A1 and thereby contribute to hyperalgesia and irritant airway effects, we used the measurement of intracellular calcium concentration in isolated cells in culture. For our functional experiments, we used models of isolated guinea pig bronchi to measure bronchoconstriction and withdrawal threshold to mechanical stimulation with von Frey filaments in mice.

Results: Irritant inhaled anesthetics activate TRPA1 expressed in human embryonic kidney cells and in nociceptive neurons. Isoflurane induces mechanical hyperalgesia in mice by a TRPA1-dependent mechanism. Isoflurane also induces TRPA1-dependent constriction of isolated bronchi. Nonirritant anesthetics do not activate TRPA1 and fail to produce hyperalgesia and bronchial constriction.

Conclusions: General anesthetics induce a reversible loss of consciousness and render the patient unresponsive to painful stimuli. However, they also produce excitatory effects such as airway irritation and they contribute to postoperative pain. Activation of TRPA1 may contribute to these adverse effects, a hypothesis that remains to be tested in the clinical setting.

What This Article Tells Us That Is New

❖ Volatile anesthetics produce irritant airway effects and can, in low concentrations, increase pain perception
❖ Transient receptor potential (TRP) channels are found in sensory neurons and airway cells and could be activated by volatile agents to cause these effects

What We Already Know about This Topic

❖ Using cells in cultures, bronchial rings in vitro, and behavior tests in rats, general anesthetics activate TRPA1 channels and cause hypersensitivity and bronchial constriction

ANNUALLY, there are approximately 21 million general anesthesia cases in the United States,1 most of which involve administration of volatile anesthetics for maintenance of anesthesia. Although general anesthetics have different effect
profiles, they induce a reversible loss of consciousness and render the patient unresponsive to painful stimuli. Traditionally, the lack of arousal to skin incision has been used to compare different drugs with respect to their anesthetic potency and to test new experimental agents for their anesthetic potential. This latter property is also used in clinical practice as a measure to estimate the depth of anesthesia.2

Nevertheless, despite the lack of response to surgical stimulation, it is unclear whether volatile anesthetics induce analgesia by suppressing the activity of nociceptive neurons. Paradoxically, low concentrations of volatile anesthetics cause hyperalgesia,3–6 which suggests excitation rather than inhibition of nociceptive neurons. The mechanisms by which anesthetics affect the activity of nociceptive neurons are not fully understood.

Volatile anesthetics have distinct odors, and some are irritating to the airway. They produce a pungent sensation when inhaled and induce a strong cough reflex that can precipitate laryngospasm, a potentially life-threatening complication during induction and emergence from anesthesia. Although there is not always agreement with respect to the ranking of the four commonly used volatile anesthetics according to their “irritating potential,” from clinical observation, it is clear that isoflurane and desflurane are irritant and halothane and sevoflurane are nonirritant anesthetics.7 The molecular mechanisms for these irritant effects are not understood, but the nature of the clinical responses suggests that they would involve activation of a chemosensor in the airway.

Transient receptor potential (TRP) ion channels are important receptors in sensory transduction. In particular, two members of the TRP family of ion channels, TRPV1 and TRPA1, are sensors for noxious chemical stimuli and are widely expressed in peripheral nociceptors.8 TRPA1, which is coexpressed with TRPV1, has emerged as a sensor for a wide variety of irritant chemicals, including the pungent ingredients of various plants, such as wasabi, mustard, and garlic.9–11 Environmental irritants, including acrolein and cigarette smoke, also activate TRPA1.12 Given its role as a chemosensor for a wide array of irritants and its expression on peripheral nociceptors, TRPA1 presents a strong candidate for mediating the irritant effects of volatile anesthetics. In fact, in a recent study, Matta et al.13 provided the first evidence for the activation of TRPA1 by irritant anesthetics. Moreover, TRPA1 activation on sensory nerves stimulates the release of substance P and calcitonin gene-related peptide, mediators of neurogenic inflammation and inflammatory pain.14 In this study, we test the hypothesis that pungent volatile anesthetics produce their irritant effects, such as bronchoconstriction of the airway, and hyperalgesia through activation of TRPA1 ion channels on peripheral nociceptors.

Materials and Methods

Reagents

Isoflurane and desflurane were from Baxter Healthcare Corp. (Deerfield, IL), sevoflurane from Abbott Laboratories (North Chicago, IL) and halothane from Halocarbon Laboratories (River Edge, NJ). The TRPA1 antagonist HC-030031 was purchased from Tocris Bioscience (Ellisville, MO). The neurokinin receptor antagonists SR140333, SR48968, and SR142801 were gifts of X. Emonds-Alt (Sanofi-Aventis, France). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

Animals

All experiments were carried out in accordance with the National Institutes of Health guidelines regarding the use of animals in experimental procedures. All protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the University of California, San Francisco, California, and the University of Florence, Florence, Italy. Dunkin-Hartley guinea pig (250 g) and mice (male, 25 g) were housed in a temperature- and humidity-controlled vivarium (12-h dark/light cycle, with free access to food and water) for at least 5 days before the experiments. TRPA1 heterozygote male mice had been backcrossed with C57BL/6 female mice to obtain the C57BL/6 genetic background. The C57BL/6 genetic background.15 Animals were killed using sodium pentobarbital (200 mg/kg, intraperitoneal) in combination with bilateral thoracotomies.

Cell Lines and Neuronal Cultures

Using the Flp-In T-Rex-293 cell line, derived from human embryonic kidney (HEK) cells, and following the manufacturer’s guidelines (Invitrogen, Carlsbad, CA), stable cell lines were generated expressing TRPA1 (rat TRPA1 complementary DNA in a pcDNA5/FRT/TO expression vector) or the empty vector, both under the control of a tetracycline-inducible promoter. Cells were gifts of X. Emonds-Alt (Sanofi-Aventis, France). All other reagents were purchased from Tocris Bioscience (Ellisville, MO). The neurokinin receptor antagonists SR140333, SR48968, and SR142801 were purchased from X. Emonds-Alt (Sanofi-Aventis, France). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

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5% horse serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine, and 100 ng/ml nerve growth factor. After plating on glass coverslips coated with poly-D-lysine and laminin, cells were cultured for 2–3 days before calcium imaging experiments. Electrophysiology experiments were performed 1 day after dissociation and plating.

**Measurement of Intracellular Calcium Concentration (Ca^{2+})**
HEK cells or DRG neurons were incubated in Hanks’ buffered salt solution, 0.1% bovine serum albumin, 20 mM HEPES, pH 7.4, containing 2.5 mM fura-2-acetoxymethyl ester for 30–45 min at 37°C. Fura-2 fluorescence at 510 nm was measured in response to alternating excitation with 340 nm and 380 nm using a F-2500 spectrophotometer (Hitachi Instruments, San Jose, CA) for HEK cells and a microscope-based calcium imaging system consisting of a Zeiss Axiosvert microscope (Carl Zeiss, Thornwood, NY), a cooled digital video camera (Stanford Photonics, Stanford, CA), and a software system for video acquisition and analysis (Imaging Workbench 6, INDEC Biosystems, Santa Clara, CA) for measurements in individual DRG neurons. Results are reported as fluorescence ratio F_{340}/F_{380} to indicate relative changes in [Ca^{2+}]. Allyl isothiocyanate (AITC), anesthetics, and bradykinin were applied via injection with a calibrated syringe or pipette from concentrated stock solutions. All experiments were performed at room temperature (21–22°C). Experiments with Ca^{2+}-free extracellular solution were used to demonstrate that the activation involves Ca^{2+} influx. For sensitization assays, HEK cells were pretreated with bradykinin (10 mM) for 2 min before the anesthetic was applied.\(^1\)

**Electrophysiology**
Whole cell membrane currents of HEK cells were recorded using a HEKA EPC-10 amplifier (HEKA Instruments,ErrorMsg replaced with <image>) in the voltage clamp mode. Data were recorded as 1-s sweeps with a sampling frequency of 10 kHz and filtered at 3 kHz. The average current for the time interval from 0.2 to 0.8 s was calculated for each sweep and plotted against time. Patch pipettes were pulled from borosilicate glass and had resistances between 2 and 4 MΩ. The composition of the internal solution is KCl 140 mM, MgCl\(_2\) 1 mM, EGTA 5 mM, HEPES 5 mM, and ATPNa\(_2\) 5 mM and the composition of the external solution is NaCl 140 mM, CaCl\(_2\) 2 mM, KCl 4 mM, MgCl\(_2\) 0.6 mM, glucose 11 mM, HEPES 5 mM, and CaCl\(_2\) 3 mM. The recording chamber was at room temperature (20–23°C) and continuously perfused with external solution (2 ml/min). AITC and anesthetics were applied through a capillary positioned 150 μm from the cell. The holding potential was −60 mV.

**Organ Bath Studies**
Guinea pigs were killed by cervical dislocation, the lungs were removed, and rings from the main bronchi (~2 mm width) were isolated and suspended with a resting tension of 1.5 g in a 5-ml organ bath. The contractile forces were measured using isometric transducers (Ugo Basile, Comerio, Italy). The tissues were bathed with Krebs’ solution (composition: NaCl 119.0 mM; KCl 4.7 mM; MgSO\(_4\) 1.5 mM; CaCl\(_2\) 2.5 mM; NaHCO\(_3\) 25.0 mM; KHPO\(_4\) 1.2 mM, and glucose 11.0 mM; pH 7.4) containing phosphoramidon (1 μM) to minimize peptide degradation and indomethacin (5 μM) to prevent generation of prostanooids, aerated with carbogen (95% O\(_2\) and 5% CO\(_2\)) and maintained at 37°C. Tissues were equilibrated for 60 min before the beginning of the experiments (washed every 5 min). In all experiments, the tissues were first contracted with carbachol (1 μM) to record the maximal contractile response of each preparation, as previously reported.\(^1\)

After carbachol wash-out, atropine (1 μM) was added to the buffer and maintained for the remainder of the experiment. Contractile responses for isoflurane (150 μM) were measured in the absence or presence of the TRPA1 antagonist HC-030031 (50 μM),\(^1\)\(^2\)\(^,\)\(^1\)\(^7\)\(^,\)\(^1\)\(^8\) or the TRPV1 antagonist capsazepine (10 μM), or a combination of NK\(_1\),2,3 receptor antagonists (SR140333, SR 48968, and SR142801, respectively; all 1 μM) and their respective vehicles, or in preparations previously desensitized by capsaicin (10 μM for 20 min).\(^1\)\(^2\) All antagonists were left in contact with the tissue for at least 15 min before isoflurane challenge (150 μM). In another set of experiments, the contractile response to halothane (150 μM) was measured. The results were expressed as percent of the maximal carbachol-induced response (1 μM).

**Mechanical Hyperalgesia**
Mechanical nociception was measured in trpa1\(^+/+\) and trpa1\(^{-/-}\) mice using the up–down paradigm of Chaplan et al.\(^1\)\(^8\) Calibrated von Frey monofilaments (0.008–4.0 g; North Coast Medical, Morgan Hill, CA) were applied to the plantar aspect of the left hind paw of the animal. If a paw withdrawal response was not observed on stimulation with the 0.4 g monofilament, a stronger stimulation was applied, and if the withdrawal response was detected, a weaker stimulus was used. The responses were tabulated, and the 50% response threshold was determined. Behavioral testing occurred between 9:00 AM and 1:00 PM. Starting 2 days before behavioral testing, trpa1\(^+/+\) and trpa1\(^{-/-}\) mice were habituated (at least 1 h per day) in plastic cylinders with wire mesh bottoms. On day 3, mice received intraplantar injections into the left hind paw of a saturated solution of either isoflurane (10 μl; ~15 mM) or halothane (10 μl; ~17 mM) in saline or the same volume of normal saline (control group). One group of trpa1\(^+/+\) mice was pretreated with the TRPA1 antagonist HC-030031 (100 mg/kg; intraperitoneal).\(^1\)\(^9\) The paw injections were given without anesthesia using restraint only to not confound the results. The mechanical nociceptive thresholds were determined before the injections (baseline, time 0) followed by measurements at 15, 30, 45, and 60 min after the injection. Data are expressed in grams and represent the mean threshold values.
Measurement of Anesthetic Concentrations and Preparation of Anesthetic Solutions

Buffers saturated with anesthetics were prepared by adding excess liquid anesthetic to the experimental buffer (bath solution for Ca\(^{2+}\) measurements, electrophysiology, and bronchoconstriction assay or normal saline for the behavioral assay) in glass vials sealed with glass or Teflon stoppers. Solutions were equilibrated by stirring overnight. Anesthetic concentrations in liquid samples were measured using gas chromatography after extraction into a known gas volume using established methods. These gas samples were then analyzed using a gas chromatograph (Gow-Mac, Bethlehem, PA), and concentrations in the liquid phase were determined using the gas equations and published data for the solubility of the volatile anesthetics.

Statistics

Data were analyzed using one-way ANOVA with Student-Newman–Keuls post test as well as t test (Prism 5, GraphPad Software, La Jolla, CA) as indicated in the Results section and in the figure legends of the respective experiments. The results of the behavioral experiments were analyzed with two-way ANOVA followed by a Bonferroni post test to compare each group with the saline control. A value of P < 0.05 was considered statistically significant for all statistical comparisons. The data are presented as mean ± SEM unless otherwise noted. For the calculation of the nonlinear regression of the concentration response data, we used a sigmoid dose–response curve model with a standard slope (Hill equation with Hill coefficient of 1). The equation used is

\[ E_x = E_0 + \frac{(E_{\text{max}} - E_0)}{1 + 10^{(\log EC_{50} - X)}} \]

where \( E_0 \) = predicted effect for concentration X, \( E_0 \) = minimal effect, bottom plateau of the curve, \( E_{\text{max}} \) = maximal effect, top plateau of the curve, \( EC_{50} \) = concentration that produces the half maximal effect (Prism 5, GraphPad Software).

Results

Irritant Volatile Anesthetics Activate TRPA1 Heterologously Expressed in HEK293 Cells

To test the hypothesis that the irritant effects of certain volatile anesthetics are mediated through activation of TRPA1, we first examined whether isoflurane produces an increase in the intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) in HEK293 cells expressing rat TRPA1 under control of a tetracycline-inducible promoter. Functional expression of TRPA1 was confirmed by the [Ca\(^{2+}\)]\(_i\) increase in response to AITC, a known TRPA1 agonist (fig. 1A). When HEK-TRPA1 cells were challenged with isoflurane at clinically relevant concentrations (300 μM, approximately equivalent to 1 minimal alveolar concentration [MAC]), we observed a similar increase in [Ca\(^{2+}\)]\(_i\) that was dependent on induction of TRPA1 expression by tetracycline and also on extracellular Ca\(^{2+}\) (fig. 1B). The increase in [Ca\(^{2+}\)]\(_i\), induced by isoflurane, was concentration-dependent over the techni-}

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Fig. 1. Irritant volatile anesthetics activate heterologously expressed transient receptor potential (TRP)-A1 in a dose-dependent manner. (A) Representative traces of fura-2-fluorescence ratios indicating increases in intracellular calcium concentration [Ca$^{2+}$]$_{i}$ induced by allylisothiocyanate (AITC, 20 $\mu$M) in human embryonic kidney (HEK) cells after tetracycline-induced (tet) TRPA1 expression (TRPA1 + tet). Control traces show cells without tetracycline induction of TRPA1 expression (TRPA1 – tet) and TRPA1 expressing cells in the absence of extracellular calcium. The horizontal line indicates application of AITC. (B) Typical traces of isoflurane-induced increase in [Ca$^{2+}$]$_{i}$ in TRPA1-expressing HEK cells. Same controls as in (A). The horizontal line indicates application of isoflurane (300 $\mu$M). (C) Dose–response curve for the changes in intracellular calcium (change in fura-2-fluorescence ratio) induced by isoflurane in TRPA1-expressing HEK cells. N = 3–5 coverslips per data point. The calculated EC$_{50}$ is 534 $\mu$M ($\approx$1.6 MAC, $E_{0}$ = 0.52, $E_{\text{max}}$ = 2.58). (D) Comparison of the change in [Ca$^{2+}$]$_{i}$ induced by equianesthetic concentrations of the four most commonly used volatile anesthetics. Bars represent mean ± SEM, numbers in parentheses indicate the number of observations per group. All intergroup comparisons are statistically significant* (one-way ANOVA followed by Student–Newman–Keuls post hoc test for all intergroup comparisons). (E) Comparison of the concentration–response relationships for the four most commonly used volatile anesthetics. Parameters calculated for the concentration–response curve of desflurane: EC$_{50}$ = 1150 mM, $E_{0}$ = 0.38, $E_{\text{max}}$ = 3.45. (F) Inward current responses induced by AITC (50 $\mu$M) and isoflurane (300 $\mu$M) in TRPA1-expressing HEK cells. Responses were measured by whole cell patch clamp in a voltage clamp configuration with a holding potential of −60 mV.
tile anesthetics to induce \([\text{Ca}^{2+}]_{i}\) influx, we cultured neurons derived from knockout mice (\(\text{trpa}1^{-/-}\)). A total of 35.5% of neurons from \(\text{trpa}1^{+/+}\) mice responded to the anesthetic with an increase in calcium influx. In contrast, only 3% of cells derived from \(\text{trpa}1^{-/-}\) mice responded to desflurane exposure (figs. 2B and C). Thus, the activation of peripheral sensory neurons by the pungent anesthetics desflurane (fig. 2A) and isoflurane (fig. 2D) is dependent on TRPA1 expression and is largely absent in mice lacking TRPA1.

Isoflurane-induced \([\text{Ca}^{2+}]_{i}\) Increase Is Augmented by Pretreatment with Bradykinin

Surgical procedures under general anesthesia cause tissue injury and inflammation. One mechanism by which inflammatory agents cause pain involves the sensitization of nociceptive neurons. Bradykinin, a prominent inflammatory peptide, sensitizes ion channels including TRPA1, which magnifies neuronal responses to TRPA1 agonists, and TRPA1 is also required for bradykinin-induced pain. We evaluated whether bradykinin also sensitizes anesthetic-induced activation of TRPA1. We examined sensitization in HEK-TRPA1 cells that naturally express the bradykinin B2 receptor and compared the anesthetic-induced increase in \([\text{Ca}^{2+}]_{i}\) without (fig. 3A) and with preincubation with bradykinin (fig. 3B). HEK-TRPA1 cells were exposed to bradykinin (10 nM) or vehicle (control) for 2 min and were then challenged with isoflurane (100 \(\mu\text{M} \sim 0.3 \text{ MAC}\)). Pretreatment with bradykinin augmented the isoflurane-induced \([\text{Ca}^{2+}]_{i}\) increase by 70% (fig. 3C). Thus, the inflammatory mediator bradykinin sensitizes the activation of TRPA1 by the pungent anesthetic isoflurane.

Irritant Volatile Anesthetics Cause Neurogenic Contractions of the Airway

The best known and most commonly accepted adverse effect of irritant volatile anesthetics is their irritating effect on the airway, which is clinically apparent as an unpleasant sensation, cough, or laryngospasm. The effect of anesthetics on the bronchomotor tone is controversial: the general notion that anesthetics are bronchodilators is in contrast with airway

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**Fig. 2.** Irritant volatile anesthetics activate transient receptor potential (TRP)-A1 expressed in peripheral nociceptors. (A) Typical traces of fura-2 fluorescence ratios representing changes in intracellular calcium concentration \([\text{Ca}^{2+}]_{i}\) induced by desflurane (des, 1.15 mm–2 MAC), allyl-isothiocyanate (aitc, 100 \(\mu\text{M}\)), and capsaicin (cap, 1 \(\mu\text{M}\)) in dorsal root ganglion (DRG) neurons isolated and cultured from TRPA1 wild-type mice. Application of desflurane, mustard oil, and capsaicin is indicated by horizontal lines. Two cells respond to all the three substances, one cell only to capsaicin (bold black trace), and two cells do not respond to any of the stimuli (dashed traces). (B) Representative traces of changes in \([\text{Ca}^{2+}]_{i}\) in response to desflurane, mustard oil, and capsaicin in DRG neurons isolated and cultured from TRPA1 knock-out mice. Labeling of the traces is similar to that described for (A). (C) Quantitative analysis of responses to desflurane in DRG neurons from TRPA1 wild-type (\(\text{trpa}1^{+/+}\)) and knock-out (\(\text{trpa}1^{-/-}\)) mice. A total of 35.5% of wild-type cells responded to desflurane but only 3% of cells isolated from knock-out mice responded (n = 28 [\(\text{trpa}1^{+/+}\)] and n = 42 [\(\text{trpa}1^{-/-}\)]). (D) Representative traces of changes in \([\text{Ca}^{2+}]_{i}\) in response to isoflurane, mustard oil, and capsaicin in DRG neurons from TRPA1 wild-type mice. Experimental setup and labeling of traces is similar to (A) but isoflurane (iso) was used instead of desflurane.
irritation and reports of bronchoconstriction. To examine the effects of anesthetics on the musculature of the airway, we measured constriction of isolated bronchial rings from guinea pigs. Exposure to isoflurane (150 μM) produced a significant contractile response in the bronchial ring that was 49% of the effect induced by carbachol (1 mM). The response to isoflurane was abolished by desensitization of sensory nerves with a high concentration of capsaicin (10 μM for 20 min) and by pretreatment with the TRPA1 selective antagonist HC-030031 (50 μM; fig. 4) but not by the TRPV1 selective antagonist capsazepine (10 μM; fig. 4). To determine whether neuropeptides released from primary sensory neurons mediate isoflurane-induced contraction, we repeated the experiment after preincubation of tissues with a combination of SR140333, SR48968, and SR142801 antagonists of neurokinin receptor.

Fig. 3. Bradykinin sensitizes transient receptor potential (TRP)-A1 activation by irritant volatile anesthetics. Representative traces of fura-2-fluorescence ratios showing changes in intracellular calcium concentration [Ca^{2+}] in human embryonic kidney (HEK) cells expressing TRPA1. (A) A trace in response to isoflurane (100 μM, application indicated by horizontal line) and (B) a trace in response to isoflurane (100 μM, application indicated by gray line) after pretreatment with bradykinin (10 nM, application indicated by black line). (C) Average responses to isoflurane (100 μM) in cells without (iso) and with (bk + iso) bradykinin pretreatment. The average response to 100 μM isoflurane is increased by 70% after bradykinin. The asterisks symbol indicates significant difference (t test, n = 4 coverslips per group).

Fig. 4. Isoflurane induces a contractile response in guinea pig bronchus. (A) Typical traces and (B) pooled data of the motor responses of guinea pig isolated bronchus to halothane (Hal; 150 μM; open triangle) and isoflurane (Iso; 150 μM; filled circles) or their vehicle (Veh) either alone or after pretreatment with capsaicin (CAP; 10 μM for 20 min), the combination of neurokinin receptor antagonists SR140333, SR48968, and SR142801 (NK, 1 μM; HC, 50 μM; CPZ, 10 μM). Each column in (B) represents the mean ± SEM, and the number of observations for each group is listed in parentheses. * P < 0.05 vs. Veh (one-way analysis of variance, followed by Student–Newman–Keuls post hoc test).
Injection of the nonirritant volatile anesthetic halothane (10 μl, 15 mM in saline) or saline (10 μl) into the hind paw of trpa1+/+ and trpa1−/− mice. Only the wild-type animals injected with isoflurane develop a transient hyperalgesia. *P < 0.05 vs. trpa1+/+ saline, trpa1−/− iso, and trpa1−/− saline. (B) Mechanical nociceptive thresholds after halothane injection (hal, 10 μl, 15 mM in saline). No change in nociceptive threshold develops after halothane injection. (C) Development of hyperalgesia after isoflurane injection is blocked by systemic pretreatment with HC-030031 (100 mg/kg intraperitoneal). Mice injected with isoflurane and pretreated with vehicle only (iso-veh[HC]) develop hyperalgesia. *P < 0.05 vs. trpa1+/− iso-HC, trpa1+/− saline-HC, and trpa1+/− saline-veh[HC]. The number of observations per data point is listed in parentheses. Data were analyzed using a two-way analysis of variance followed by Bonferroni post hoc test.

**Isoflurane Causes Mechanical Hyperalgesia**

The transmission of nociceptive signals is associated with the release of substance P and calcitonin gene-related peptide from the central endings of peptidergic nociceptors. Given that volatile anesthetics are capable of exciting such a subset of peptidergic primary sensory neurons, we sought to determine their effects on nociception. To assess mechanical hyperalgesia, we measured hind paw withdrawal threshold to mechanical stimulation with Von Frey filaments. Intraplantar injection of the irritant volatile anesthetic isoflurane (10 μl, 15 mM in 0.9% NaCl) significantly decreased the nociceptive threshold in trpa1+/+ mice 15–30 min after injection, indicative of mechanical hyperalgesia (fig. 5A, trpa1+/+ iso and trpa1+/+ iso-veh[HC]). In contrast, intraplantar injection of the nonirritant volatile anesthetic halothane (10 μl, 17 mM in 0.9% NaCl) did not affect the nociceptive threshold (fig. 5B, trpa1+/+ hal). To further confirm that the hyperalgesic effect of isoflurane was mediated by a TRPA1-dependent mechanism, we examined isoflurane-induced mechanical hyperalgesia in both mice lacking the gene that encodes for TRPA1 (trpa1−/−) and in wild-type mice pretreated with the specific TRPA1 antagonist HC-030031 (100 mg/kg; intraperitoneal). As predicted, trpa1−/− mice (fig. 5A, trpa1−/− iso) as well as trpa1+/+ mice pretreated with systemic administration of HC-030031 (fig. 5C, trpa1+/+ iso-HC) failed to develop mechanical hyperalgesia after intraplantar injection of isoflurane, suggesting that the mechanism of isoflurane-induced hyperalgesia involves activation of TRPA1 ion channels. Thus, the pungent anesthetic isoflurane, when injected locally into the mouse hind paw, produces mechanical hyperalgesia by a mechanism requiring the expression of functional TRPA1.

**Discussion**

We report, for the first time, that the activation of TRPA1 by pungent volatile anesthetics plays a pivotal role in mediating their irritant effects in the airways and their proposed hyperalgesic effects. Specifically, our studies suggest a mechanism by which irritant volatile anesthetics produce their characteristic pungent sensation and cause neurogenic bronchoconstriction through activation of TRPA1 expressed in nocicep-
Irritant Volatile Anesthetics and TRPA1 Channels

Sensitive A

TRPA1 detects a wide variety of chemical irritants, including endogenous alimentary and environmental substances. Given the proposed role of TRPA1 as a mediator of protective airway reflexes in response to chemical stimulation, it seems likely that the irritant effects of certain volatile anesthetics are also mediated through the same pathway.

Several observations support the hypothesis that irritant volatile anesthetics activate TRPA1. First, isoflurane and desflurane activated TRPA1 expressed in HEK cells. Isoflurane-induced calcium influx was entirely dependent on TRPA1 expression because no calcium influx was observed in control cells without induced TRPA1 expression. Irritant anesthetics induced a robust, concentration-dependent effect that is comparable with that of AITC, a specific TRPA1 agonist. Our results confirm a recent report that irritant anesthetics directly activate TRPA1. The EC_{50} reported for isoflurane in this study (180 μM, ~0.6 MAC) is lower than the value we determined (534 μM, ~1.6 MAC), possibly related to the use of electrophysiology instead of measurement of [Ca^{2+}]. However, both reported potencies are within the clinically useful concentration range. Sevoflurane and halothane, both nonirritant volatile anesthetics, did not produce increases in the influx of calcium at any tested dose, leading us to conclude that they do not activate TRPA1.

Second, irritant anesthetics caused calcium influx in DRG neurons from trpa1^+/+ but not trpa1^-/- mice, which confirms that these agents activate TRPA1 in nociceptive neurons. The activation induced by irritant volatile anesthetics is restricted to a neuronal subpopulation sensitive to AITC and capsaicin, indicating TRPA1 and TRPV1 coexpression. This coexpression of TRPA1 and TRPV1 in a subpopulation of peripheral nociceptors has been previously reported.

The activation of sensory neurons by anesthetics has been previously reported by others. Halothane, isoflurane, and enfurane increase the discharge of C-fibers innervating the rabbit cornea. The effects of these anesthetic mechanosensitive Aδ-fibers are more complex: halothane increases and isoflurane and enfurane decrease spike latency, and all three anesthetics slightly decrease spike amplitude. These findings confirm and extend previous reports of sensorineuronal activation by volatile anesthetics in a variety of models. However, the underlying molecular mechanisms for any of these findings have not been elucidated.

TRPA1 and TRPV1 are likely candidates given their sensitivity to irritant chemicals. In preliminary studies, we did not observe irritant anesthetics activating TRPV1 in HEK cells (unpublished observations, July and August 2007, Helge Eilers, M.D., San Francisco, CA, experiments). However, a recent report suggests that volatile anesthetics sensitize TRPV1 to capsaicin, protons, and heat. Inflammatory mediators such as bradykinin and downstream targets such as protein kinase C enhanced these effects of volatile anesthetics on TRPV1.

We report that the inflammatory mediator bradykinin sensitizes isoflurane-induced calcium signals in TRPA1 expressing HEK cells. Because isoflurane mobilizes calcium in these cells by a TRPA1-dependent mechanism, these results suggest that bradykinin sensitizes TRPA1. Surgery induces tissue injury, resulting in the formation of multiple inflammatory mediators, including bradykinin, which can sensitize TRPA1. This sensitization would act in synergy with the effects of irritant anesthetics, resulting in amplified neurogenic inflammation, hyperalgesia, and pain.

Although anesthetics are widely considered to be bronchodilators and are even suggested as therapeutics in the setting of status asthmaticus refractory to other interventions, this concept is at odds with their well-described pungency and ability to cause airway irritation. To address this paradox and further elucidate the effects of TRPA1 activation by irritant volatile anesthetics, we examined bronchoconstriction of the guinea pig bronchi. Irritant anesthetics caused constriction of the bronchi that was abolished by antagonism of neurokinin receptors and is thus mediated by the release of tachykinins from nociceptive neurons. This effect is entirely dependent on C- or Aδ-fiber neurons expressing TRPV1 because selective desensitization of these neurons by high concentrations of capsaicin abolished bronchoconstriction.

These neurogenic bronchoconstrictor effects of isoflurane are in contrast to its widely conceived bronchodilator effect and suggest two distinct cellular mechanisms, one that mediates the neurogenic bronchoconstriction by TRPA1-dependent activation of sensory neurons and another that mediates bronchodilation by direct action on calcium homeostasis in smooth muscle. One possible explanation for the contradictory findings from functional studies may be that one or the other mechanism predominates depending on the experimental model and the anesthetic concentrations used. Alternatively, the duration and timing of the anesthetic exposure may determine the effect. The sensorineuronal transduction mechanism may desensitize during prolonged exposure, leading to a dominance of the irritant effects during the initial exposure. Because airway resistance is measured at steady state, the initial bronchoconstriction may be missed in many experimental models.

Another proposed adverse effect of pungent volatile anesthetics is their pronociceptive action. Although the hyperalgesic effect of volatile anesthetics has been established in animal experiments at low anesthetic concentrations, these findings are not confirmed in human subjects. Low concentrations of volatile anesthetics cause a decrease in hind paw withdrawal latency to painful heat in rats and mice, indicating hyperalgesia. Several mechanisms have been proposed, including nicotinic and α-adrenergic activation. However, a consensus has not been reached, and it is difficult to compare directly the results of these studies because the models used differ substantially. In human...
subjects, nitrous oxide and methoxyflurane have analgesic properties at subanesthetic concentrations, whereas halothane, isoflurane, enflurane, and sevoflurane have no effect on thermal pain. Interestingly, the observed effect of the latter four drugs trended toward increased pain sensitivity but was not statistically significant. Extensive studies designed to assess the analgesic effect of subanesthetic concentrations of isoflurane in human volunteers using a wide variety of experimental pain models failed to demonstrate a clear analgesic effect. However, hyperalgesia was also not observed.

The contradictory effects of irritant anesthetics on pain in experimental animals (algesia) and human subjects (analgesia) may be attributable to the route of administration. Systemic administration by inhalation causes hypnotic effects in the central nervous system as a confounding factor. Loss of the righting reflex in combination with the tail-flick latency has been used to separate hypnotic from analgesic effects. To avoid the contribution of central effects, we chose local application of anesthetics by intraplantar injection to test for anesthetic-induced hyperalgesia. Intraplantar injection is routinely applied to measure hyperalgesia induced by other irritants such as capsaicin and AITC. Our results demonstrate that intraplantar injection of the irritant volatile anesthetic isoflurane, but not the nonirritant halothane, caused mechanical hyperalgesia in trpa1+/+ but not trpa1−/− mice. Moreover, to further support the specificity of the detected effect, we showed that pretreatment of trpa1+/+ mice with the TRPA1 antagonist HC-030031 abolished isoflurane-induced mechanical hyperalgesia. The isoflurane concentration was at saturation (~15 mM) and, therefore, considerably higher than clinically useful concentrations. However, the absolute amount of anesthetic delivered was small (10 µl = 150 nmol, in the unlikely event that the injection process can be accomplished without loss) and even if equilibration of the anesthetic would be restricted to the paw, the maximal achievable concentration would be equivalent to 3 MAC, assuming an average paw volume of 150 µl. Furthermore, the observation that these effects were absent when TRPA1 was deleted or antagonized argues against a nonspecific effect produced by a high concentration. Furthermore, a saturated solution of halothane failed to produce hyperalgesia. Together, we interpret this as evidence for isoflurane-induced mechanical hyperalgesia by activating TRPA1.

Locally administered volatile anesthetics have been reported to have analgesic actions, which contradict our findings of hyperalgesia after injection into the hind paw. However, these studies used extremely high concentrations such as pure sevoflurane applied to the spinal cord or lipid preparations of anesthetics at concentrations exceeding 0.5 M. Although in our experiments the lack of effect in knockout animals and the lack of effect induced by an equimolar concentration of the nonirritant anesthetic halothane provide convincing evidence for a specific, TRPA1-mediated effect, other studies may lack adequate controls to assure specificity.

Our observations provide strong evidence for a mechanism by which pungent volatile anesthetics may not only produce their well-described airway irritation but also contribute to the severity of postoperative pain. Unequivocal clinical evidence for anesthetic-induced postoperative pain is currently lacking, although more severe postoperative pain has been observed in patients anesthetized with isoflurane (an irritant volatile anesthetic) than in patients who received propofol, as determined by morphine requirement and verbal pain scores. Although a hyperalgesic effect of isoflurane is only one possible explanation for their findings, this study emphasizes the possibility that the hyperalgesic effects become only apparent under conditions of inflammation after surgery.

The hypothesis that irritant volatile anesthetics, such as isoflurane and desflurane, activate TRPA1 ion channels on peripheral sensory neurons is supported by multiple lines of evidence presented here as well as by recent reports of other investigators. For the first time, we demonstrate a TRPA1-specific hyperalgesic effect of irritant volatile anesthetics and provide a plausible mechanism not only for the airway irritation produced by anesthetics but also for anesthetic-induced hyperalgesia.

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**ANESTHESIOLOGY REFLECTIONS**

**The Morton House I by Vandam**

In August of 1819, William Thomas Green Morton (1819–1868) was born in Charlton, Massachusetts. His original birthplace burned down to its foundations, so the house depicted above is “a successor to the original edifice.” So remarked artist-anesthesiologist Leroy D. Vandam (1914–2004), a retired Editor of Anesthesiology, who painted this watercolor (see above). Championing Morton as the first to publicly demonstrate ether for surgical anesthesia, Dr. Vandam delighted in painting this Morton House at various seasons of the year. Only a few copies of the 100-print run autographed by the late Dr. Vandam remain for sale as a benefit for the Wood Library-Museum. (Copyright © the American Society of Anesthesiologists, Inc. This image appears in color in the Anesthesiology Reflections online collection available at www.anesthesiology.org.)

George S. Bause, M.D., M.P.H., Honorary Curator, ASA’s Wood Library-Museum of Anesthesiology, Park Ridge, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. \(\text{UJC}@\text{aol.com}\).