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Title: The Pore Loop Domain of TRPV1 is Required for its Activation by the Volatile Anesthetics Chloroform and Isoflurane.

Corinna Kimball, Jialie Luo, Shijin Yin, Hongzhen Hu, Ajay Dhaka

Department of Biological Structure, University of Washington, Seattle, WA 98195, USA (C.K., A.D.); and The Center for the Study of Itch, Washington University Pain Center, Department of Anesthesiology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110, USA (J.L., S.Y., H.H.).

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Running title: VGAs activate TRPV1 via pore loop domain

Correspondence should be addressed to Ajay Dhaka, Department of Biological Structure, The University of Washington School of Medicine,1959 NE Pacific Street, Box 357420, Seattle, WA 98195. dhaka@uw.edu.

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Abstract

The environmental irritant chloroform, a naturally occurring small volatile organohalogen, briefly became the world's most popular volatile general anesthetic (VGA) before being abandoned due to it low therapeutic index. When chloroform comes in contact with skin or is ingested, it causes a painful burning sensation. The molecular basis for the pain associated with chloroform remains unknown. In this study we assess the role of transient receptor potential (TRP) channel family members in mediating chloroform activation and the molecular determinants of VGA activation of TRPV1. We identify the subpopulation of DRG neurons that are activated by chloroform. Additionally, we transiently expressed wildtype or specifically mutated TRP channels in human embryonic kidney cells and used calcium imaging or whole cell patch clamp electrophysiology to asses the effects of chloroform or the VGA isoflurane on TRP channel activation. The results revealed that chloroform activates DRG neurons via TRPV1 activation. Furthermore while chloroform activates TRPV1, it also activates TRPM8 and functions as a potent inhibitor of the noxious chemical receptor TRPA1. The results also indicate that residues in the outer pore region of TRPV1 previously thought to be required for either proton or heat activation of the channel are also required for activation by chloroform and isoflurane. In addition to identifying the molecular basis of DRG neuron activation by chloroform and the opposing effects chloroform has on different TRP channel family members, the findings of this study provide novel insights into the structural basis for the activation of TRPV1 by VGAs.

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Introduction

The transient receptor potential vanilloid type 1(TRPV1) cation channel is promiscuously activated or potentiated by a wide range of noxious and inflammatory stimuli, including temperatures above ~42°C, acids, bases, capsaicin, and inflammatory molecules like bradykinin (Julius, 2013). Similarly, TRPA1 is a sensor of noxious chemical stimuli such as isothiocyanates, the pungent agents in mustard oil, volatile air pollutants such as acrolein and formalin, endogenous products of oxidative and nitrative stress and inflammatory factors such as bradykinin and ATP (Julius, 2013). Both receptors are expressed in a population of small diameter nociceptive somatosensory neurons (C-fibers) with TRPA1 being expressed in a subset of TRPV1+ neurons (Dhaka et al., 2006). Null mutation studies have confirmed essential roles for TRPV1 and TRPA1 in acute and inflammatory nociception (Bautista et al., 2006; Caterina et al., 2000).

Volatile general anesthetics (VGAs) are comprised of a diverse group of chemicals that are able to reversibly inhibit CNS activity, rendering patients unresponsive to stimuli, though the molecular mechanisms by which they act is still somewhat open to debate. Most reports indicate that VGAs bind directly to ligand gated ion channels/receptors while others suggest that VGAs may act directly on the lipid bilayer to influence neuronal activity (Franks, 2008; Lynch, 2008). VGAs have enabled hundreds of million surgeries annually worldwide saving countless lives. Perhaps paradoxically, VGAs have been reported to activate nociceptive A-delta and C-fibers neurons in a number of animal models (Campbell et al., 1984; MacIver and Tanelian, 1990; Mutoh et al., 1998).

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Moreover VGAs appear to activate exclusively capsaicin-sensitive or TRPV1+ sensory neurons (Mutoh et al., 1998). Indeed volatile anesthetics such as isoflurane and desflurane are perceived as pungent and cause airway irritation when inhaled, which have limited their use as induction agents (Eger, 1995; TerRiet et al., 2000). Following up on these findings it has been shown that these VGAs directly activate TRPA1 and sensitize TRPV1 (Cornett et al., 2008; Eilers et al., 2010; Matta et al., 2008). It has therefore been hypothesized that VGAs can elevate postsurgical pain through direct action on these TRP channels.

Chloroform ($CHCl_3$), a small volatile organohalogen, is a naturally occurring environmental irritant that is produced by many seaweeds and algae. It is produced synthetically at an industrial scale (93-350 million pounds/year) and is a commonly used solvent, bonding agent, a precursor to Teflon and refrigerants, and was once found in many consumer products (Li et al., 1993; Winslow and Gerstner, 1978). It is however best known as one of the earliest used VGAs. Discovered in the latter 19th century, it quickly became the most popular general anesthetic. It was abandoned in the early 20th century because of its low therapeutic index, as the effective anesthetic dose did not differ markedly from doses that would induce cardiac arrest. Prolonged exposure to chloroform has also been shown to be toxic to organs, tissues and cells (Winslow and Gerstner, 1978).

Here, we report that chloroform directly activates TRPV1 while also being a reversible inhibitor of TRPA1, and that TRPV1 mediates the majority of responses of DRG

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neurons to chloroform. Chloroform acts synergistically with heat and protons to potentiate TRPV1 activity. Furthermore, we found that residues in the outer pore domain of TRPV1 required for heat- or proton-induced channel activation are also required for activation of the channel by chloroform and isoflurane.

Material and Methods

Ratiometric calcium imaging.

DRGs were isolated from 6-12 week old C57Bl/6J mice. All experiments were performed in compliance with institutional animal care and use committee standards. Dissociation and culturing of mouse dorsal root ganglia (DRG) neurons was performed as described with the following modifications (Story et al., 2003). Dissected DRGs were dissociated by incubation for 1 h at 37°C in a solution of culture medium (Ham's F12/DMEM with 10% Horse Serum, 1% penicillin-streptomycin (Life Technologies)) containing 0.125% collagenase (Worthington Biochemicals) followed by a 30 min incubation in 10 ml of culture media plus 1.25 units papain. Ca^{2+} imaging was performed essentially as described previously (Story et al., 2003). Growth media was supplemented with 100ng/ml nerve growth factor. For experiments involving heterologous expression, human embryonic kidney (HEK)293T cells were transiently transfected co-transfected with *piresegfp* and one of the following: *rTrpv1, rTrpv1 H378Q, rTrp1 N628K, N652T, Y653T, rTrpv1 N628K, rTrpv1 N652T, rTrpv1 Y653T, rTrpv1 E600V, mTrpv1 E649Q* or *hTrpa1*. The threshold for activation was defined as 20% above baseline for chloroform and 30% above baseline for capsaicin/ allyl

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isothiocyanate (AITC) for DRG experiments and heterologous expression experiments. Student's *t* test was used for all statistical calculations. All averaged traces represent mean ± SEM. The buffer solution for all experiments was 1× Hanks' balanced salt solution (HBSS) (Invitrogen), 10 mM HEPES (Invitrogen), except for studies involving acidic or basic solutions where 1× HBSS was buffered with 10 mM Citric acid or 10 mM TRIS (Sigma), respectively. To determine chloroform responses of TRPV1-transfected HEK cells, background chloroform responses from untransfected HEK cells were subtracted. All dose response curves and mutant channel experiments were normalized except when noted to either peak capsaicin (1μM) for TRPV1, AITC (100μM) for TRPA1 or menthol (250μM) for TRPM8 based experiments to account for experiment/transfection variability. Though channel desensitization could potentially skewer results causing an overestimation of chloroform activity, we did not observe changes in peak channel responses that corresponded with increases in chloroform concentration and the variability of peak responses across all experiments was minor (data not shown).

Electrophysiology

Whole-cell patch-clamp recordings in HEK 293 cells expressing TRPA1 or TRPV1. HEK293T cells were grown as a monolayer using passage numbers less than 30 and maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies), supplemented with 10% fetal bovine serum (Life Technologies), 100 units/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies) in a humidified

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incubator at 37°C with 5% CO_2. The cells were transiently transfected with 100ng cDNA of mouse Trpv1 (m*Trpv1*) or 100ng cDNA of mouse *Trpa1* (m*Trpa1*) and 100ng cDNA of C1-GFP, using Lipofectamine 2000 (Invitrogen). Following transfection, the cells were maintained in DMEM at 37 °C for 24 hrs before use.

Whole-cell patch-clamp recordings were performed using an EPC 10 amplifier (HEKA Elektronik) at room temperature (22-24 °C) on a stage of an inverted phase-contrast microscope equipped with a filter set for green fluorescence protein visualization. Pipettes pulled from borosilicate glass (BF 150-86-10; Sutter Instrument) with a Sutter P-97 pipette puller had resistances of 2-4 MΩ for whole-cell recordings, when filled with pipette solution containing 140 mM CsCl, 2 mM EGTA, and 10 mM HEPES with pH 7.3 and 315 mOsm/L in osmolarity. A Ca^{2+}-free extracellular solution was used for whole-cell recording to avoid Ca^{2+}-dependent desensitization of TRP channels, containing (in mM): 140 NaCl, 5 KCl, 0.5 EGTA, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted to ≈340 mOsm/L with sucrose). The whole-cell currents were recorded using voltage ramps from -100 to +100 mV during 500 ms at holding potential of 0 mV. Data were acquired using Patchmaster software (HEKA Elektronik). Currents were filtered at 2 kHz and digitized at 10 kHz. Data were analyzed and plotted using Clampfit 10 (Molecular Devices).

Reagents

Chloroform, Isoflurane, AITC, capsaicin, menthol, AMG9810, 2-aminoethoxy diphenyl borate (2-APB) and GSK101 and all other reagents were obtained from Sigma-Aldrich

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unless otherwise noted.

Statistics

All statistics reported utilized the student's t-test except when noted. Statistical effects of chloroform on temperature, pH or capsaicin on TRPV1 activity were measured by ANOVA. All error reported as S.E.M.

Results

Chloroform activates capsaicin-sensitive DRG neurons

Nociceptive DRG neurons are specialized to sense noxious chemical and physical stimuli in tissues such as the skin, muscles and internal organs (Dhaka et al., 2006). Using Ca^{2+} imaging we observed that 40% of DRG neurons responded to chloroform (0.1% or 12.5mM) and the vast majority of these neurons (78%) also responded to the TRPV1 agonist capsaicin (Fig. 1A,B). It is therefore possible the pain and irritation associated with chloroform ingestion, as well as eye and skin contact, could be caused by the direct activation of nociceptive sensory neurons. Since the vast majority of chloroform responsive neurons were also activated by capsaicin, we investigated whether or not TRPV1 was required for the neuronal responses elicited by chloroform. Pharmacological blockade of TRPV1 with the specific TRPV1 antagonist AMG9810 inhibited responses to chloroform within the capsaicin sensitive population as well as neurons that did not respond to capsaicin suggesting that many non-capsaicin responding DRG neurons that responded to chloroform were TRPV1 expressing neurons (360/380 fluorescence ratio to chloroform (9.4mM); Capsaicin(+), 0.58 ± 0.06

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vs Capsaicin(+) + AMG9810 (1μM), 0.09 ± 0.009, p< 0.001; Capsaicin(-), 0.21 ± 0.02 vs Capsaicin(-) + AMG9810 (1μM), 0.091 ± 0.01, p<0.001) (Fig. 1C,D) (Gavva et al., 2005).

Previous studies had suggested that VGAs do not directly activate TRPV1 but rather sensitize the channel to activation by capsaicin, protons and heat (Cornett et al., 2008). We found that application of chloroform elicited a strong influx of calcium in TRPV1-expressing HEK cells that can be blocked by TRPV1 antagonist AMG9810 (Fig. 2A,B). Furthermore, application of chloroform produced inward currents in voltage-clamped TRPV1-expressing cells that were also blocked by AMG9810 (TRPV1 basal: 86±31 pA/pF, TRPV1+Chloroform (9.4mM): 215±42 pA/pF, TRPV1+Chloroform (9.4mM) + AMG9810 (1μM): 85±19 pA/pF; measured at +100mV, n=7 cells/condition) (Fig. 2C). Using Ca^{2+}-imaging, we found that chloroform activated TRPV1-expressing cells in a dose-dependent manner with an EC50 of 9.18mM (Fig. 2D). This data argues that TRPV1 is directly activated by chloroform.

Since other VGAs have been shown to activate the noxious chemosensor TRPA1, we investigated whether chloroform also activated this pain-initiating channel (Matta et al., 2008). Interestingly, isoflurane, which activated TRPA1 with an EC_{50}~0.18mM, was shown to have pore-blocking effects at concentrations above 2.7mM (Matta et al., 2008). We found that chloroform at concentrations above 0.075% (9.4mM) did not activate TRPA1 as observed via Ca^{2+} imaging and whole-cell patch-clamp experiments (Fig. 3A,B) (Matta et al., 2008). Chloroform did seem to reduce intracellular calcium

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levels in TRPA1-expressing HEK cells, suggesting that it might be inhibiting baseline TRPA1-activity (Fig. 3A,B). Indeed using Ca^{2+}-imaging, we found that preincubation with chloroform blocked AITC-mediated activation of TRPA1 in a dose-dependent manner (IC_{50}=0.046mM) with inhibition seen at concentrations as low as 50μM and, like the TRPA1 antagonist HC-030031, chloroform potently and reversibly inhibited TRPA1 activity (Fig. 3C,D) (McNamara et al., 2007). Unlike isoflurane, chloroform at concentrations as low as 10μM did not appear to activate TRPA1, implying that these VGAs interact with the channel differentially.

We next investigated the effects of chloroform on other thermoTRPs (Dhaka et al., 2006). None of the heat-activated channels TRPV2, TRPV3 nor TRPV4 were activated by chloroform at a concentration that elicited a robust activation of TRPV1. However, they did respond robustly to their known agonists, indicating that the channels were functional in our experiments (Fig. 4A-C). However, the cold-activated channel TRPM8 was found to be activated by chloroform (9.4mM), in line with previous reports that TRPM8 activity is modulated by VGAs (Fig. 4D) (Abeele et al., 2013; McKemy et al., 2002; Peier et al., 2002). Furthermore TRPM8 was activated in a dose-dependent manner by chloroform with an EC_{50} of 7.95mM (Supplemental Figure 1A/data not shown). To determine if TRPM8 could be contributing to chloroform evoked DRG responses, we used Ca^{2+} imaging to measure the response of isolated enhanced EGFPf-positive DRG neurons from a previously characterized TRPM8-EGFPf knockin mouse line where EGFPF expressing neurons from heterozygous animals were shown to express TRPM8 (Dhaka et al., 2008). EGFPf-expressing DRG neurons from

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heterozygous TRPM8$^{Egfpf/+}$ mice were found to be responsive to chloroform. Furthermore capsaicin-insensitive, menthol-sensitive EGFPf-expressing neurons were found to be significantly more responsive to chloroform (9.4mM) versus EGFPf-negative/Capsaicin-insensitive neurons, indicating that TRPV1 was not contributing to chloroform responsiveness of these neurons (360/380 fluorescence ratio to chloroform (9.4mM): EGFPf(+)/Capsaicin(+), 1.4 ± 0.29 (n=16 neurons), EGFPf(+)/Capsaicin(-), 0.90 ± 0.28 (n=9 neurons) or EGFPf(-)/Capsaicin(+), 0.89 ± 0.11 (n=152 neurons) vs EGFPf(-)/Capsaicin(-), 0.22 ± 0.02 (n=215), $p<0.001$, $p<0.05$, $p<0.001$, respectively) (Supplemental Figure 1B). This data suggests that in addition to TRPV1, TRPM8 could also contribute to DRG neuronal responses to chloroform.

Chloroform potentiates heat- and pH-induced activation of TRPV1 but not activation by capsaicin

Many TRPV1 agonists act cooperatively to potentiate the activity of the channel. Protons, for example, potentiate the responses of the channel to heat or capsaicin. Isoflurane has also been shown to potentiate activation of TRPV1 by both heat and capsaicin (Cornett et al., 2008). Using Ca^{2+}-imaging, we investigated the ability of chloroform to act cooperatively with other TRPV1 agonists. We found that 6.3mM chloroform, a concentration which shows minimal or no activation of TRPV1, potently shifted thermal activation of TRPV1 towards cooler temperatures (Fig 5A), i.e. TRPV1 has a thermal threshold near 42°C, but in the presence of 6.3mM chloroform activation of the channel was seen at temperatures greater than or equal to 29°C. This activation

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was greater than the additive activation of the channel by 6.3mM chloroform at baseline temperature (24.5°C) or temperature alone, suggesting a synergistic interaction between temperature and chloroform (Effect of chloroform (6.3mM) on heat activation of TRPV1, $p<0.001$; 24.5°C, 1.1 ± 0.49 a.u. (n=221 cells) vs 24.5°C + Chloroform, 10.0 ± 1.36 a.u. (n=667 cells); 29°C, 1.7 ± 0.1 a.u. (n=128 cells) vs 29°C + Chloroform, 33.0 ± 3.15 a.u. (n=175 cells); 34°C, 4.9 ± 0.83 a.u. (n=242 cells) vs 34°C + Chloroform, 24.9 ± 6.82 a.u. (n=79 cells); 38°C, 6.3 ± 2.00 a.u. (n=127 cells) vs 38°C + Chloroform, 44.4 ± 7.92 a.u. (n=129 cells); 39°C 16.7 ± 4.35 a.u. (n=234 cells) vs 38°C + Chloroform, 42.3 ± 4.98 a.u. (n=403 cells); 44°C 36.8 ± 7.89 a.u. (n=153 cells) vs 44°C + Chloroform, 53.1 ± 7.72 a.u. (n=211 cells)). Chloroform (6.3mM) also potentiated TRPV1 responses to protons with activation of the channel at pH 5.5 greater than additive of each stimulus alone and there was a significant effect of chloroform on acidic pH activation of TRPV1 ($p<0.001$; pH5, 81.9 ± 7.89 (n=145 cells) vs pH5 + chloroform, 94.6 ± 10.19 (n=125 cells); pH5.5, 45.9 ± 4.36 (n=130 cells) vs pH5.5 + chloroform, 63.0 ± 0.78 (n=150 cells); pH6, 4.3 ± 1.21 (n=223 cells) vs pH6 + chloroform, 9.4 ± 0.94 (n=273 cells); pH6.2, 2.8 ± 0.84 (n=60 cells) vs pH6.2 + chloroform, 5.7 ± 2.46 (n=97 cells; pH6.5, 1.8 ± 0.71 (n=222 cells) vs pH5 + chloroform, 6.4 ± 0.90 (n=247 cells)) (Fig. 5B). Surprisingly, capsaicin-mediated activation of TRPV1 was not synergistically potentiated by 6.3mM chloroform. Instead we observed that 6.3mM chloroform had a merely additive effect on channel activation by this agent at concentrations less than or equal to 5nM capsaicin. Concentrations above 10nM capsaicin yielded indistinguishable activations in the presence or absence of chloroform (effect of chloroform (6.3mM) on capsaicin-mediated activation of TRPV1, $p>0.05$; capsaicin (1nM), 0.23 ± 0.11 (n=260

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cells) vs capsaicin (1nM) + chloroform, 0.51 ± 0.06 (n=108 cells); capsaicin (5nM), 0.20 ± 0.11 (n=115 cells) vs capsaicin (5nM) + chloroform, 0.75 ± 0.15 (n=79 cells); capsaicin (10nM), 2.39 ± 0.62 (n=304 cells) vs capsaicin (10nM) + chloroform, 2.32 ± 0.96 (n=95 cells); capsaicin (20nM), 3.74 ± 0.38 (n=167 cells) vs capsaicin (20nM) + chloroform, 4.24 ± 0.17 (n=221 cells); capsaicin (50nM), 4.81 ± 0.33 (n=260 cells) vs capsaicin (1nM) + chloroform, 5.64 ± 1.01 (n=108 cells); all units: peak Δ of 360/380 ratio to stimulus) (Fig. 5C).

Isoflurane activates TRPV1 at high concentrations

Previous studies had found that VGAs such as isoflurane sensitized TRPV1 activity in the presence of other known TRPV1 agonists (Cornett et al., 2008). However, these studies were biased towards concentrations typically used in clinical settings. Since we found that chloroform activated TRPV1 at millimolar concentrations, we speculated that perhaps isoflurane might also activate TRPV1 at similar concentrations. Indeed, using ratiometric Ca^{2+} imaging, we found that isoflurane activated TRPV1 in a dose-dependent manner with an EC_{50} of 7.47mM (Fig. 6A,B). These data suggest that chloroform and isoflurane might utilize similar mechanisms to activate TRPV1.

Mutations of residues in the outer pore domain required for heat or acid activation impair chloroform activation of TRPV1.

To investigate what residues/domains of TRPV1 are required for chloroform-mediated

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activation of the channel, we characterized the response properties of previously described mutations that affect the ability of the channel to respond to specific stimuli using Ca^{2+}-imaging. A concentration of 9.4mM chloroform was used for these experiments since this concentration was near the top of the dose response curve for TRPV1 activation. An intracellular amino-terminal mutation, H378Q, that specifically abrogates the ability of the channel to respond to basic pH, without affecting sensitivity to heat, acid and capsaicin, had no effect on the ability of the channel to be activated by chloroform (WT, 60.9 ± 4.6 a.u. vs H378Q, 66.7 ± 11.2 a.u.; $p>0.05$. $n\geq3$ trials/concentration, ~100 cells/trial with each trial weighted to account for number of cells) (Fig. 6C) (Dhaka et al., 2009). However, when we tested a triple mutation of residues in the outer pore domain of TRPV1 (N628K, N652T, Y653T) that specifically impairs activation to heat by ablating temperature-dependent long channel openings without affecting responses to acid, or capsaicin, we observed a dramatically reduced sensitivity to chloroform (WT, 60.9 ± 4.6 a.u. vs N628K, N652T, Y653T, 17.4 ± 4.0 a.u.; $p<0.001$. $n\geq3$ trials/concentration, ~100 cells/trial with each trial weighted to account for number of cells) (Fig. 6C) (Grandl et al., 2008). Furthermore each individual mutation N628K, N652T and Y653T demonstrated impaired responses to chloroform (WT, 60.9 ± 4.6 a.u. vs N628K, 7.0 ± 1.1 a.u., N625K, 22.1 ± 3.7 a.u. or Y653K 16.7, ± 4.6 a.u.; $p<0.0.001$, $p<0.001$, $p<0.001$, respectively. $n\geq3$ trials/concentration, ~100 cells/trial with each trial weighted to account for number of cells) (Fig. 6C). This finding indicates that residues within the outer pore domain linked to heat activation of TRPV1 are required for normal sensitivity to chloroform. We next investigated the response to chloroform of a mutation to amino acid E600Q, which inhibits acid sensitivity while potentiating heat

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and capsaicin responsiveness (Jordt et al., 2000). It has been postulated that upon protonation of this residue, hydrogen bonds are disrupted which in turn facilitates gating associated movements of the outer pore and a widening of the selectivity filter (Cao et al., 2013a). As with the triple heat mutant, chloroform sensitivity was reduced by this mutation (WT 60.9 ± 4.6 a.u. vs E600Q 7.6 ± 1.1 a.u., $p<0.001$. $n\geq3$ trials/concentration, ~100 cells/trial with each trial weighted to account for number of cells) (Fig. 6C). We also tested the triple heat mutant TRPV1 (N628K, N652T, Y653T) for chloroform responsiveness at concentrations up to 12.5mM (the maximum concentration we could use before we observed significant changes in intracellular calcium in HEK cells alone) and did not see any increase in activation compared to 9.4 mM chloroform (9.4mM chloroform 17.5 ± 3.8 a.u. vs 12.5 mM chloroform 13.9 ± 4.5 a.u., $p>0.05$) (Supplemental Figure 2). These data argue that the outer pore domain of TRPV1 is required for sensitivity to chloroform. A mutation of another residue (E649Q) that selectively abrogates proton sensitivity while maintaining normal heat and capsaicin responsiveness also located in the pore loop domain had no effect on chloroform mediated activation, suggesting that indiscriminate mutation of residues in the pore loop domain would not lead to selective reduction in TRPV1 sensitivity to chloroform (WT 60.9 ± 4.6 a.u. vs E649Q 51.0 ± 10.9 a.u.; $p<0.05$) (Jordt et al., 2000).

We next assessed the effect these mutations had on TRPV1 sensitivity to isoflurane. Similar to chloroform we chose a concentration of isoflurane (8.1mM) that was near the top of the dose response curve for isoflurane activation of TRPV1. In alignment with the findings from chloroform, the H378Q and E649Q mutations had no effect on the

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sensitivity to isoflurane, while the triple (N628K, N652T, Y653T), individual heat mutants (N628K, N652T and Y653T) and E600V mutant were all deficient in their ability to respond to isoflurane (WT 41.7 ± 7.0 a.u. vs H378Q 41.8 ± 0.5 a.u, E649Q 41.9 ± 8.3 a.u., N628K N652T Y653T 2.5 ± 1.6 a.u., N628K 9.7 ± 3.8 a.u., N652T 9.5 ± 3.1 a.u., Y653T 9.5 ± 5.3 a.u. or E600V 7.8 ± 0.7 a.u.; $p>0.05$, $p>0.05$, $p<0.001$, $p<0.01$, $p<0.01$, $p<0.001$, or $p<0.01$, respectively. $n\geq3$ trials/concentration, ~100 cells/trial with each trial weighted to account for number of cells)(Fig. 6D). This would argue that isoflurane and chloroform interact with the outer pore domain of TRPV1 utilizing overlapping determinants. Furthermore our findings indicate that VGAs utilize structural determinants previously thought to be specific to protons or heat to activate the channel.

Discussion

TRPV1 and TRPA1 have a remarkable ability to detect a wide range of noxious stimuli (Julius et al, 2013). Here we have provided conclusive evidence that the environmental irritant and VGA chloroform activates TRPV1 while also functioning as a reversible antagonist of TRPA1 activity. We have shown that the responses of DRG neurons to chloroform are mainly mediated by TRPV1 as almost all neurons responding to chloroform also responded to the TRPV1 agonist capsaicin and pretreatment with the TRPV1 antagonist AMG9810 abrogated responses to chloroform. TRPM8, which is expressed a small percentage of DRG neurons (<10%), may also contribute to the response of DRG neurons to chloroform (Dhaka et al., 2008). Neurons expressing TRPM8 that were insensitive to capsaicin, thus not likely expressing TRPV1, also responded to chloroform.

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It is well established that multiple agonists of TRPV1 can sensitize the activation of the channel by other agonists and that this cooperation is often synergistic as opposed to additive, arguing that the known different activator sites of these ligands are coupled within TRPV1 (Julius, 2013; Tominaga et al., 1998). We found that chloroform appears to sensitize the activation of TRPV1 by heat and protons but under the testing conditions utilized in this study chloroform only appeared to have additive effects on activation by capsaicin. Why this may be warrants further investigation.

While we have not determined the mechanisms by which chloroform activates TRPV1, it is more likely than not that chloroform activates TRPV1 via a ligand gating mechanism, which would align with previous findings on VGA activation of TRP channels and the action of other membrane partitioning compounds such as camphor on TRPV1 activation (Marsakova et al., 2012; Matta et al., 2008). This also corresponds with many studies which suggest that chloroform and other VGAs act on transmembrane receptors and ion channels such as the GABA receptors, NMDA receptors and two-pore domain potassium channels via ligand receptor binding dynamic (Franks, 2008; Franks and Lieb, 1994). However, others point to the lipophilic properties of chloroform and have shown that chloroform readily inserts itself into the plasma membrane causing a loosening and rearrangement of the plasma membrane (Reigada, 2011; Turkyilmaz et al., 2009). These studies argue that this could lead to relaxed and dysfunctional transmembrane proteins, which in turn would account for the anesthetic effects of chloroform and other VGAs. Like chloroform, high temperatures are known to fluidize

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and increase disorder in the plasma membrane whereas cooling temperatures lead to increased rigidity and stiffen the plasma membrane (Digel et al., 2008). It has been proposed that thermoTRP channel activity in response to changes in temperature may be mediated by temperature-dependent alterations in their interactions with the plasma membrane(Clapham, 2003). However, it has recently been shown that artificial liposomes containing purified TRPV1 display intrinsic heat sensitivity that is not altered by changes in lipid bilayer tension (Cao et al., 2013b). Nevertheless, since heat-activated TRPV1 was activated by chloroform and cold-activated TRPA1 was inhibited, we considered that alterations in membrane fluidity by chloroform could mimic the effects of temperature on these channels such that increasing membrane fluidity would lead to TRPV1 activation and perhaps inhibition of TRPA1 activation. At the time we reasoned that if this was true, other structurally similar heat -activated TRPV channels such as the warm-activated channels TRPV3 and TRPV4 as well as the high heat threshold channel TRPV2 might be activated by lower to higher concentrations of chloroform, respectively, and perhaps cool-activated TRPM8 might be inhibited by chloroform (Dhaka et al., 2006). This was not the case; TRPV2, TRPV3, and TRPV4 were not activated by chloroform. Additionally, cool-activated TRPM8 was activated by chloroform, which would not be expected if decreasing membrane fluidity by cooling temperatures were responsible for activation of this channel. These data argue that chloroform does not activate TRPV1 or inhibit TRPA1 by mimicking the effects of temperature on plasma membrane fluidity nor does it provide any evidence that temperature might activate thermoTRPS by modulating membrane fluidity.

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We have shown that residues in the outer pore region previously thought to be required for specific activation by either heat or protons are critical for activation by the VGAs chloroform and isoflurane (Grandl et al., 2010; Jordt et al., 2000). Intriguingly, structural analysis of TRPV1 suggests that residue E600 required for proton and VGA activation and residue Y653 required for heat and VGA activation of TRPV1 could potentially form a hydrogen bond that upon disruption could lead to the facilitation of channel activation. Indeed it has been postulated that protonation of E600 disrupts this bond leading to channel activation (Cao et al., 2013a). One possibility is that chloroform and isoflurane interaction with TRPV1 could lead to the disruption of this hydrogen bond, potentiating channel activation. In the future it will be interesting to determine if specific residues in the outer pore region are required for chloroform activation of TRPM8.

In summary our results show that chloroform excites somatosensory neurons by activating TRPV1. However chloroform has divergent effects on TRP family members *in vitro.* It has no effect on warm -sensitive TRPV3 or TRPV4 channels or high heat-activated TRPV2. In addition to TRPV1, chloroform activates TRPM8 and unlike other VGAs such as isoflurane, which activate TRPA1, chloroform is a potent inhibitor of TRPA1. Finally, we show that chloroform and isoflurane use overlapping determinants in the outer pore loop region to activate TRPV1.

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Authorship Contributions

Participated in research design: Dhaka, Hu and Kimball.
Conducted experiments: Kimball, Luo and Yin.
Performed data analysis: Kimball, Luo and Yin.
Wrote or contributed to the writing of the manuscript: Dhaka and Kimball

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Footnotes

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Figure Legends

Figure 1. DRG neuron response to chloroform is primarily mediated by TRPV1. Responses of dissociated DRG neurons were assessed with ratiometric calcium imaging. **A.** Chloroform-sensitive neurons are primarily localized within the capsaicin-sensitive population. DRG neurons from C57Bl/6J were challenged with a pulse of chloroform (12.5mM) followed by a pulse of capsaicin (1μM). **B.** Average peak response over baseline to chloroform of capsaicin-sensitive and capsaicin-insensitive DRG neurons (n=4 trials, 30-100 neurons/trial with each trial weighted to account for number of neurons). **C.** The TRPV1 antagonist AMG9810 (1μM) blocks chloroform (9.4mM) responses in capsaicin-sensitive and capsaicin-insensitive neurons. **D.** Average peak response over baseline to chloroform (9.4mM) of DRG neurons preincubated with AMG9810 (1μM) or vehicle only of capsaicin-sensitive and capsaicin-insensitive neurons (n=4 trials, 30-100 neurons/trial with each trial weighted to account for number of neurons). ** $p<0.01$, *** $p<0.001$.

Figure 2. TRPV1 is activated by chloroform. **A.** Utilizing ratiometric calcium imaging, TRPV1-expressing HEK cells (red) respond to chloroform (9.4mM) and capsaicin (1μM). Untransfected HEK cells (black). **B.** Preincubation with AMG9810 blocks responses of TRPV1-expressing HEK cells (red) to chloroform. Untransfected HEK cells (black). **C.** Chloroform (9.4mM, red) increases TRPV1 channel activity recorded using whole-cell configuration with TRPV1-expressing HEK cells in an AMG9810 (1μM, blue) dependent manner. Whole cell patches were challenged with a voltage ramp protocol. **D.** Dose-

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response curve of TRPV1-expressing HEK cells to chloroform normalized to peak capsaicin response (1μM), utilizing ratiometric calcium imaging (n≥3 trials/concentration, ~100 cells/trial with each trial weighted to account for number of cells).

Figure 3. TRPA1 is inhibited by chloroform. **A.** Chloroform (9.4mM) appears to suppress baseline activity in TRPA1-expressing HEK cells (red), which are potently activated by the TRPA1 agonist AITC (100μM) whereas no change is seen in baseline activity of untransfected HEK cells (black). **B.** Whole cell recording utilizing a voltage ramp protocol of TRPA1 expressing HEK cell shows no increase in current to chloroform (9.4mM, red) while showing potent increases in current to AITC (100μM). At +100mV; TRPA1 basal: 89±27 pA/pF; TRPA1+chloroform (9.4mM): 35±6 pA/pF; TRPA1+AITC (100μM): 442±77 pA/pF; n=5 cells/condition. **C.** Preincubation with chloroform inhibits activation of TRPA1-expressing HEKs by AITC (5μM) in a dose dependent manner as measured by ratiometric calcium imaging (n≥3 trials/concentration, ~100 cells/trial with each trial weighted to account for number of cells). **D.** Chloroform is a reversible antagonist of TRPA1. Chloroform (9.4mM) reversibly suppresses AITC (5μM) mediated activation of TRPA1-expressing HEK cells (black).

Figure 4. Chloroform does not activate TRPV2, TRPV3 or TRPV4 but does activate TRPM8. Responses of TRP channel expressing HEK cells were assessed with ratiometric calcium imaging. **A.** Chloroform (9.4mM) does not activate TRPV2 which is activated by known agonist 2-APB (100μM)(Hu et al., 2004). **B.** TRPV3 does not

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respond to chloroform (9.4mM) but is activated by known agonist 2-APB (100μM)(Hu et al., 2004). **C.** TRPV4 which is activated by known agonist GSK101 (10nM) is not activated by chloroform (9.4mM) but is activated by known agonist (Thorneloe et al., 2008). **D.** TRPM8 is activated by chloroform (9.4mM) and its known agonist menthol (Peier et al., 2002).

Figure 5. Chloroform potentiates TRPV1 activation by heat and protons but not capsaicin. Responses of TRPV1-expressing HEK cells were assessed with ratiometric calcium imaging. **A.** Temperature response curve in the presence or absence of chloroform (6.3mM). **B.** Proton response curve in the presence or absence of chloroform (6.3mM). **C.** Capsaicin response curve in the presence or absence of chloroform (6.3mM). All data points for **A** and **B** correspond to peak activation to stimulus normalized to peak response to a subsequent pulse of capsaicin (1μM). Data points for capsaicin response curves (**C**) were not normalized to a second pulse capsaicin (1μM), since first pulse capsaicin concentrations greater than 10nM produced tachyphylaxis in the subsequent capsaicin (1μM) response. For all experiments n≥3 trials/condition, ~100 cells/trial with each trial weighted to account for number of cells.

Figure 6. Residues in the outer pore domain of TRPV1 necessary for activation by protons or heat are required for activation by chloroform and isoflurane. Responses of cells were assessed with ratiometric calcium imaging. **A.** TRPV1-expressing HEK cells respond to isoflurane (12.2mM) and capsaicin (1μM). **B.** Dose response curve of TRPV1-expressing HEK cells to isoflurane. Peak isoflurane responses over baseline

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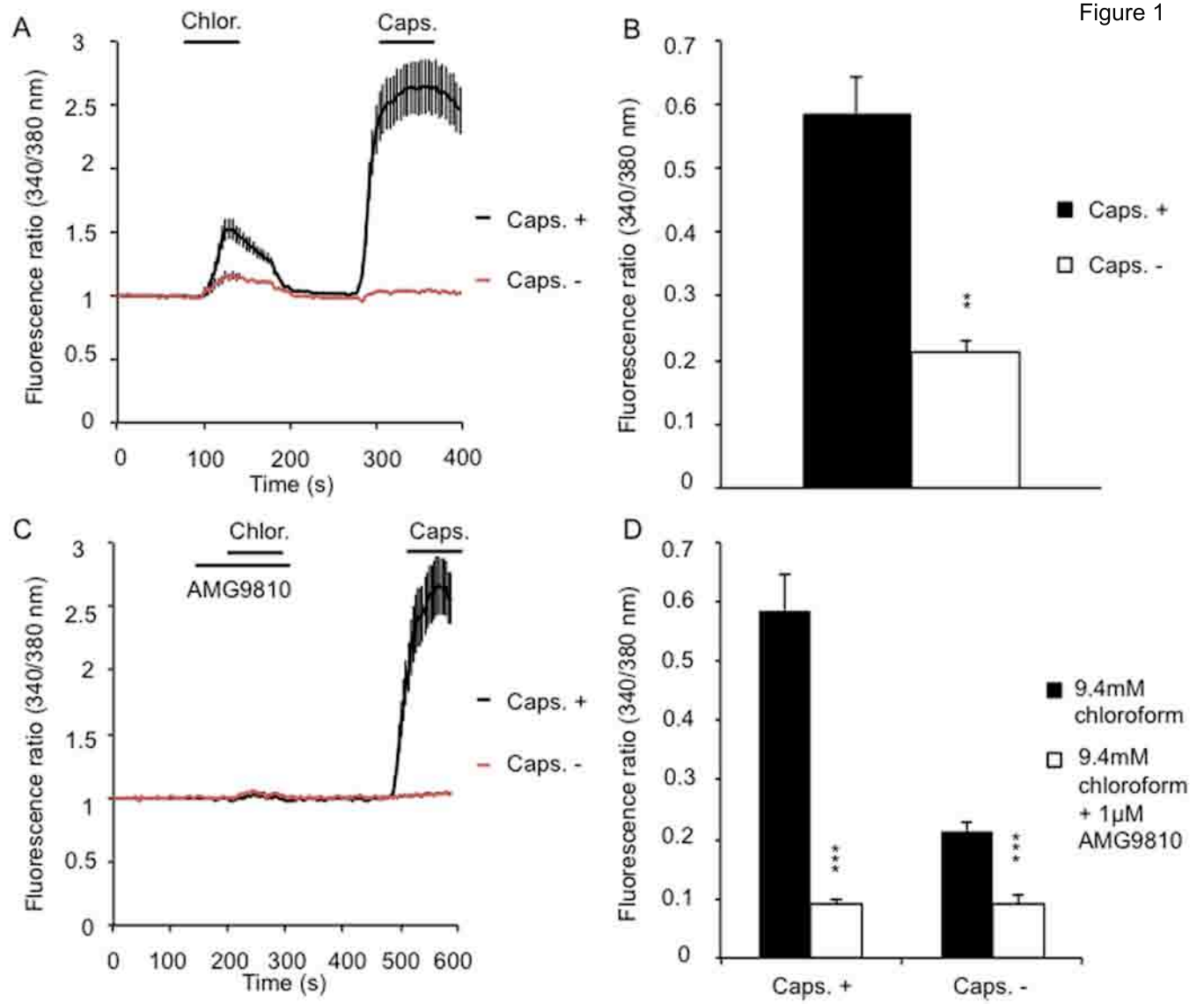
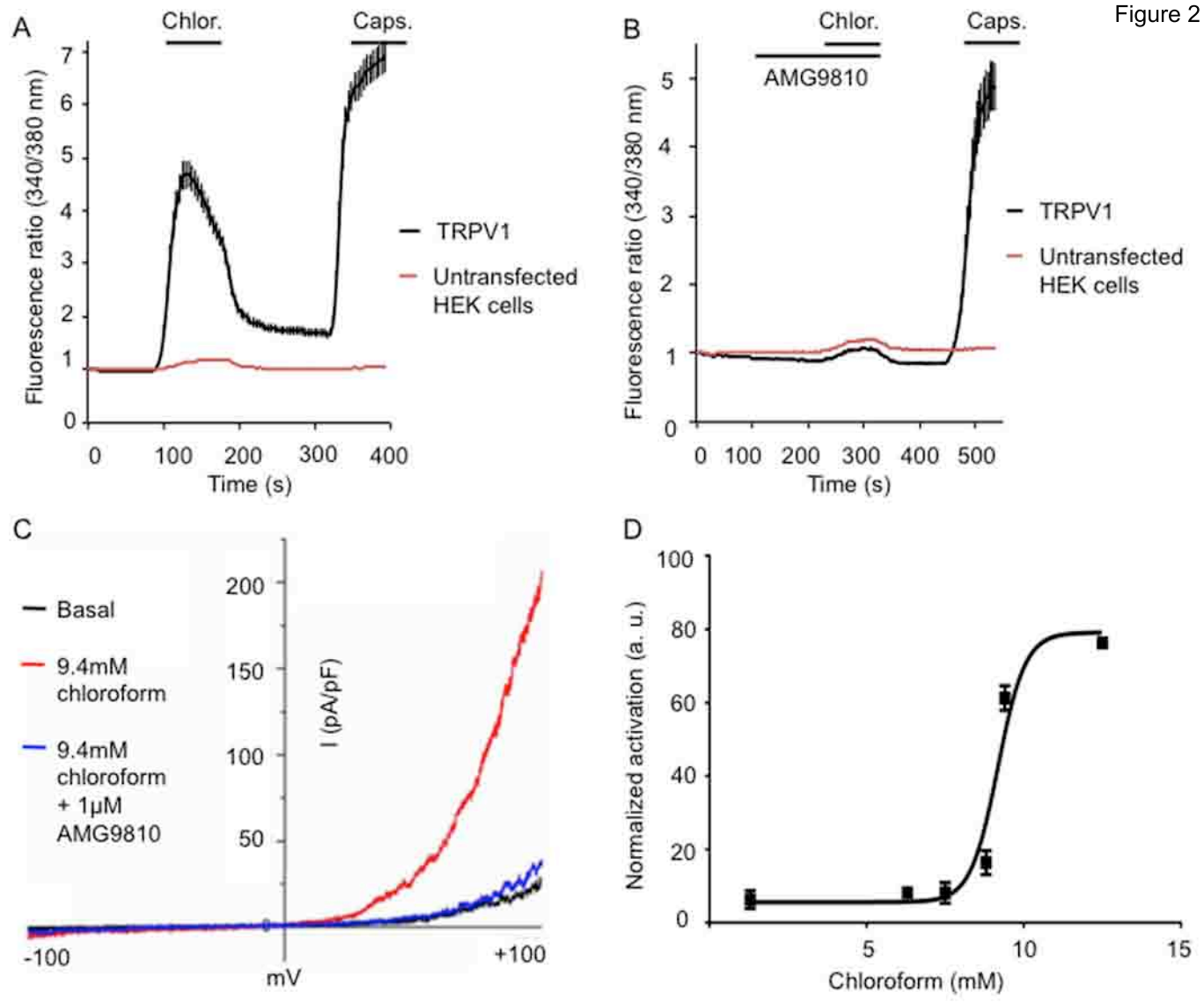
normalized to peak capsaicin (1μM) responses (n≥3 trials/concentration, ~100 cells/trial with each trial weighted to account for number of cells). **C.** TRPV1 mutants E600V, N628K, N652T, Y653T and Triple heat mutant (N628K,N652T,Y653T) showed inhibited responses to chloroform (9.4mM) while H378Q and E649Q did not differ from wildtype TRPV1. Peak chloroform over baseline responses normalized to peak capsaicin (1μM) responses. Each bar represents an n≥4 trial with ~100 cells/trial. **D.** TRPV1 mutants E600V, N628K, N652T, Y653T and Triple heat mutant (N628K,N652T,Y653T) showed inhibited responses to isoflurane (8.1mM) while H378Q and E649Q did not differ from wildtype TRPV1. Peak isoflurane over baseline responses normalized to peak capsaicin (1μM) responses. Each bar represents an n≥4 trial with ~100 cells/trial. **p<0.01, ***p<0.001.


A
Chlor.
Caps.
Fluorescence ratio (340/380 nm)
Time (s)
Caps. +
Caps. -
B
Fluorescence ratio (340/380 nm)
Caps. +
Caps. -
C
Chlor.
AMG9810
Caps.
Fluorescence ratio (340/380 nm)
Time (s)
Caps. +
Caps. -
D
Fluorescence ratio (340/380 nm)
9.4mM chloroform
9.4mM chloroform + 1μM AMG9810
Caps. +
Caps. -


A
Chlor.
Caps.
Fluorescence ratio (340/380 nm)
Time (s)
TRPV1
Untransfected HEK cells
B
Chlor.
Caps.
AMG9810
Fluorescence ratio (340/380 nm)
Time (s)
TRPV1
Untransfected HEK cells
C
Basal
9.4mM chloroform
9.4mM chloroform + 1μM AMG9810
I (pA/pF)
-100
+100
mV
D
Normalized activation (a. u.)
Chloroform (mM)

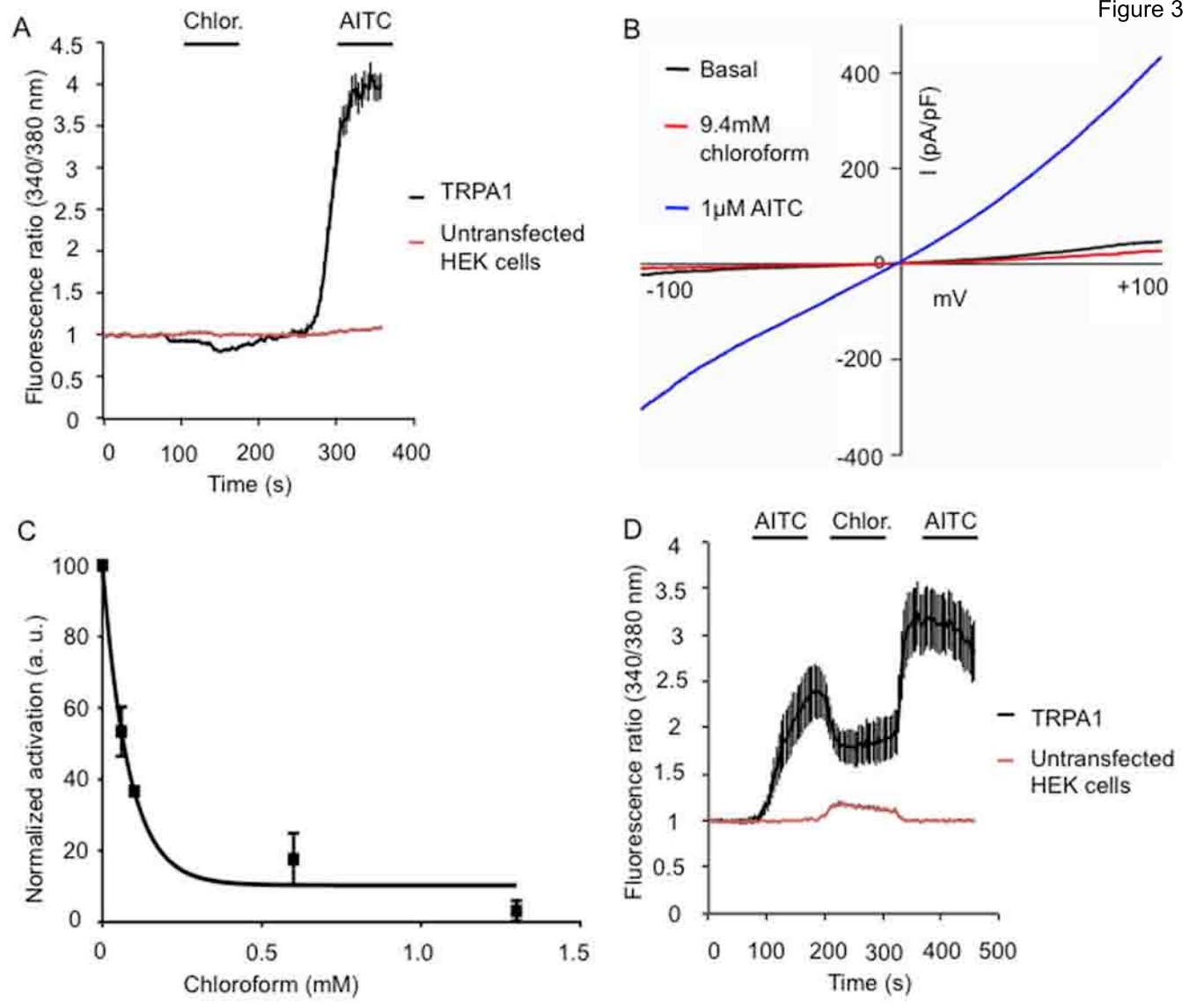

A
Chlor.
AITC
Fluorescence ratio (340/380 nm)
Time (s)
TRPA1
Untransfected HEK cells
B
Basal
9.4mM chloroform
1μM AITC
I (pA/pF)
mV
-100
+100
C
Normalized activation (a. u.)
Chloroform (mM)
D
AITC
Chlor.
AITC
Fluorescence ratio (340/380 nm)
Time (s)
TRPA1
Untransfected HEK cells

Figure 4

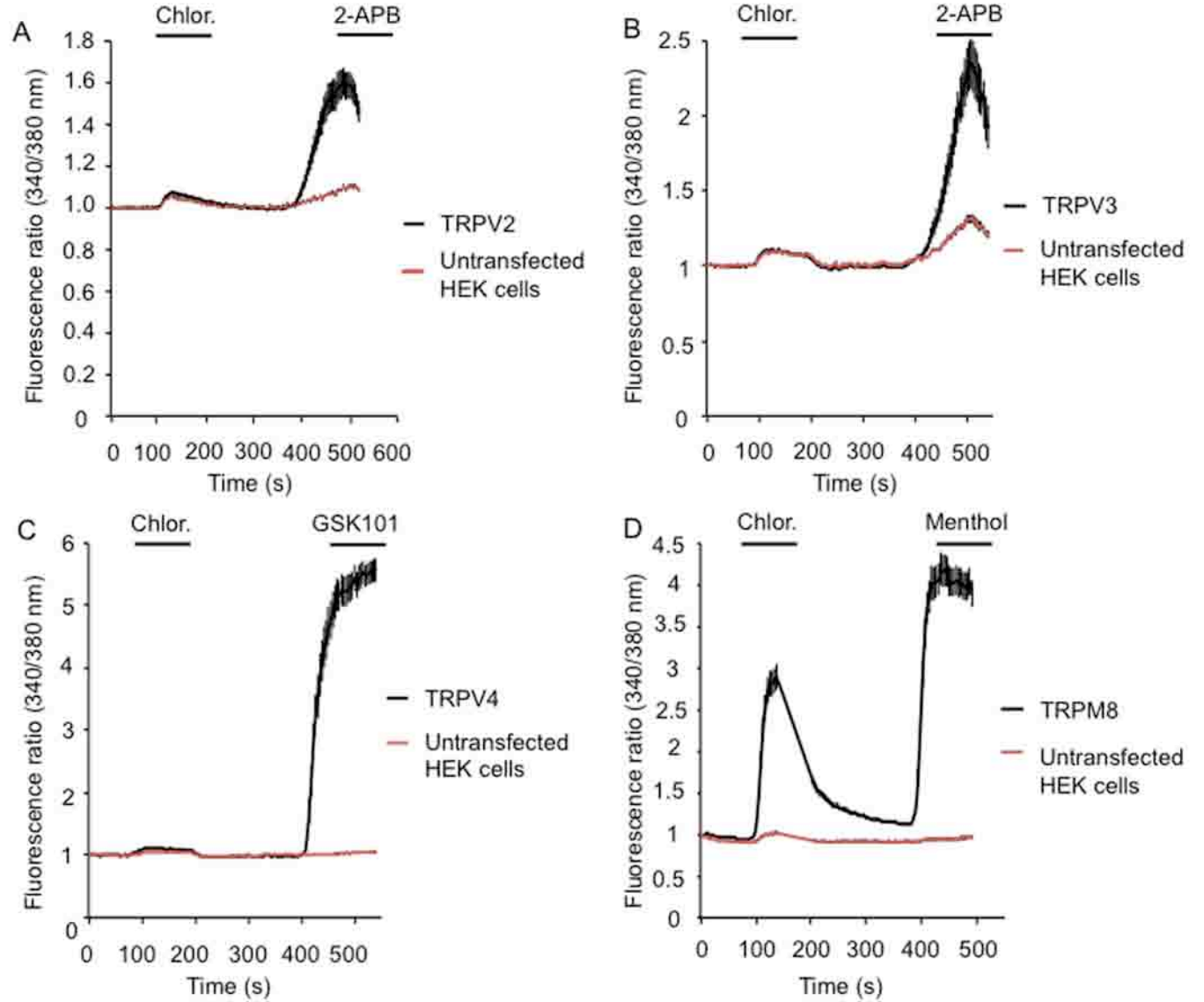
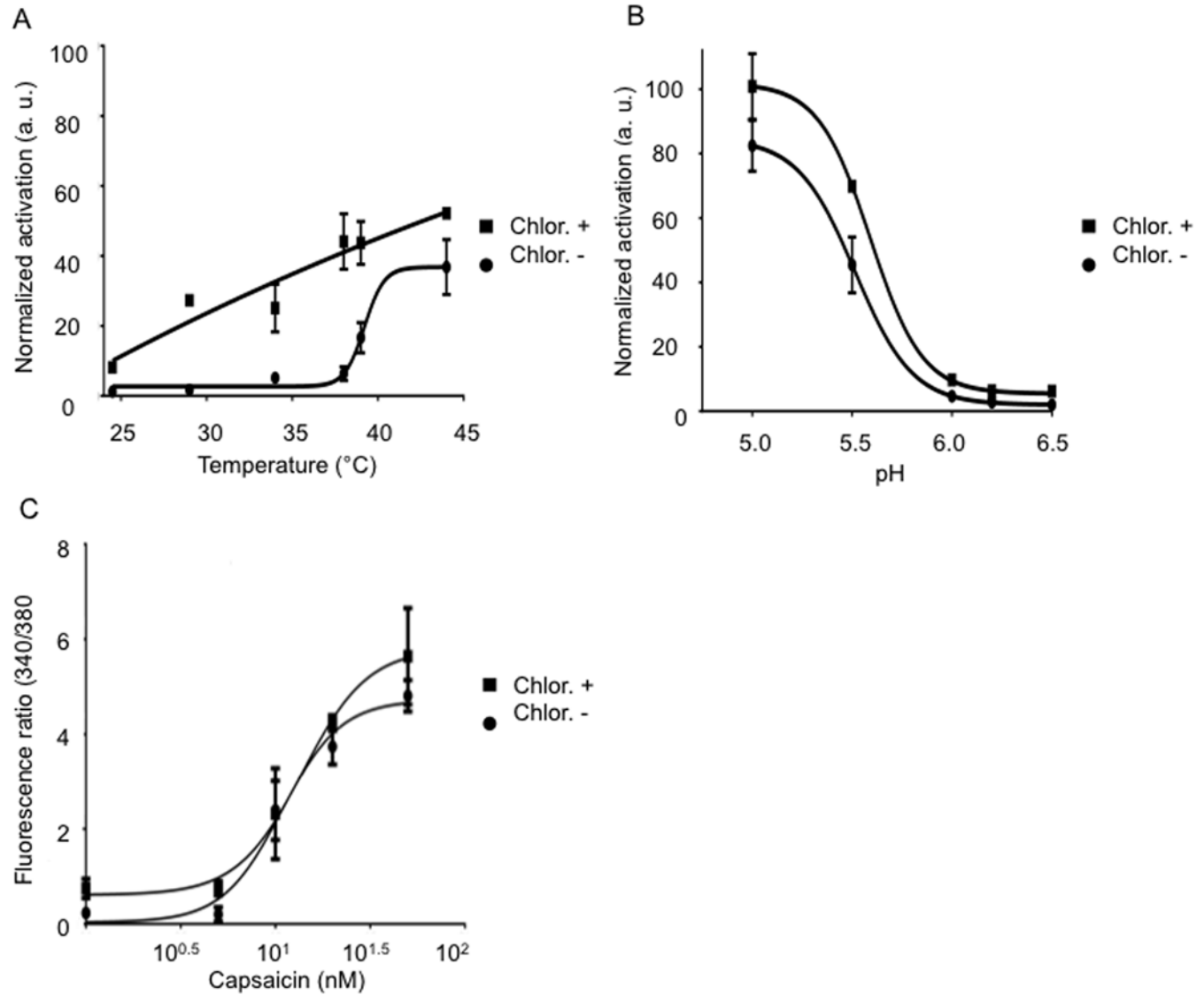
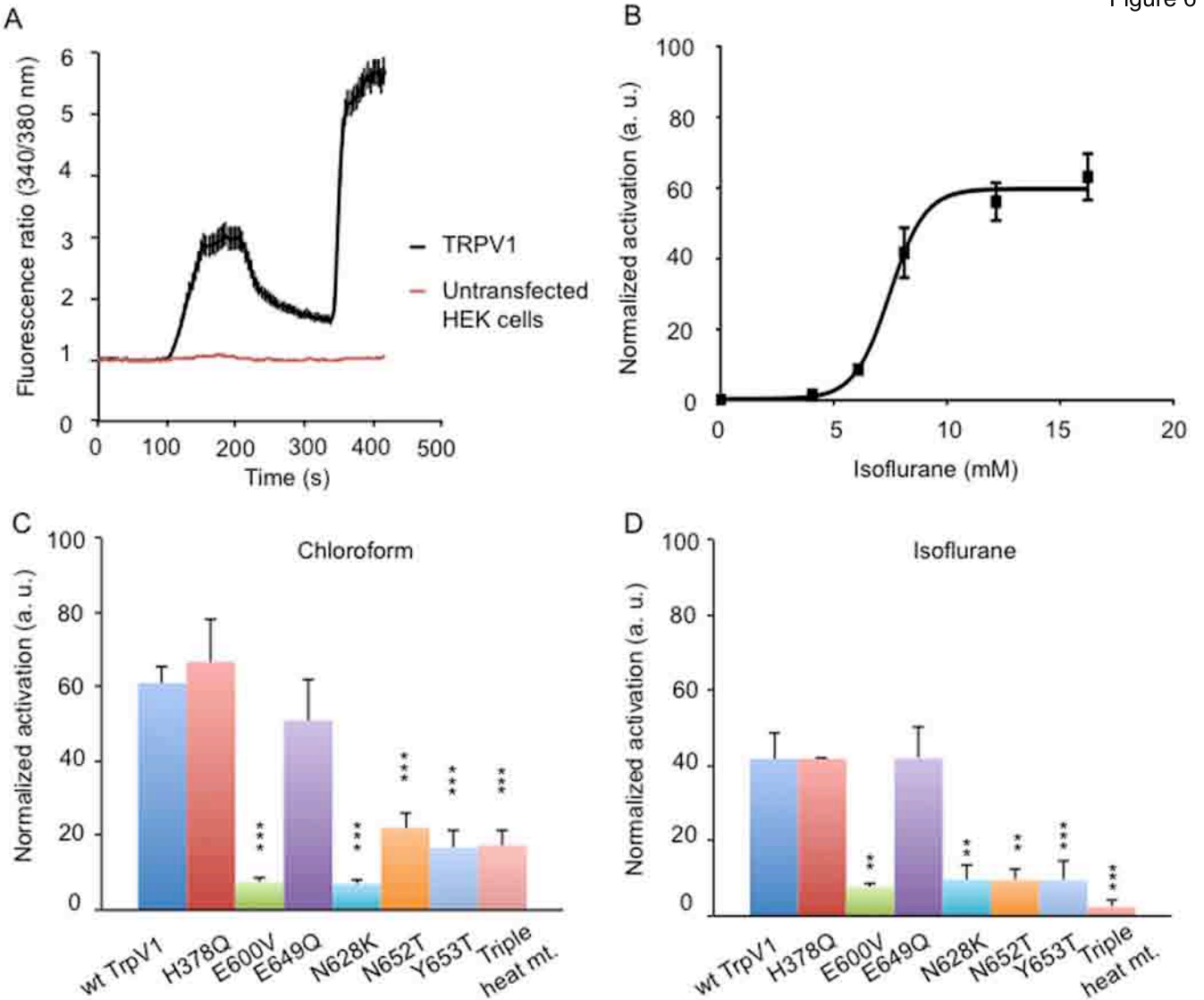

A
Chlor.
2-APB
Fluorescence ratio (340/380 nm)
Time (s)
TRPV2
Untransfected HEK cells
B
Chlor.
2-APB
Fluorescence ratio (340/380 nm)
Time (s)
TRPV3
Untransfected HEK cells
C
Chlor.
GSK101
Fluorescence ratio (340/380 nm)
Time (s)
TRPV4
Untransfected HEK cells
D
Chlor.
Menthol
Fluorescence ratio (340/380 nm)
Time (s)
TRPM8
Untransfected HEK cells

Figure 5


A
Normalized activation (a. u.)
100
80
60
40
20
0
25
30
35
40
45
Temperature (°C)
Chlor. +
Chlor. -
B
Normalized activation (a. u.)
100
80
60
40
20
0
5.0
5.5
6.0
6.5
pH
Chlor. +
Chlor. -
C
Fluorescence ratio (340/380
8
6
4
2
0
$10^{0.5}$
10^{1}
$10^{1.5}$
10^{2}
Capsaicin (nM)
Chlor. +
Chlor. -


A
Fluorescence ratio (340/380 nm)
Time (s)
TRPV1
Untransfected HEK cells
B
Normalized activation (a. u.)
Isoflurane (mM)
C
Chloroform
Normalized activation (a. u.)
wt TrpV1
H378Q
E600V
E649Q
N628K
N652T
Y653T
Triple heat mt.
D
Isoflurane
Normalized activation (a. u.)
wt TrpV1
H378Q
E600V
E649Q
N628K
N652T
Y653T
Triple heat mt.

Molecular Pharmacology

The Pore Loop Domain of TRPV1 is Required for its Activation by the Volatile Anesthetics Chloroform and Isoflurane.

Corinna Kimball, Jialie Luo, Shijin Yin, Hongzhen Hu, Ajay Dhaka

Supplementary Data

Fig. S1

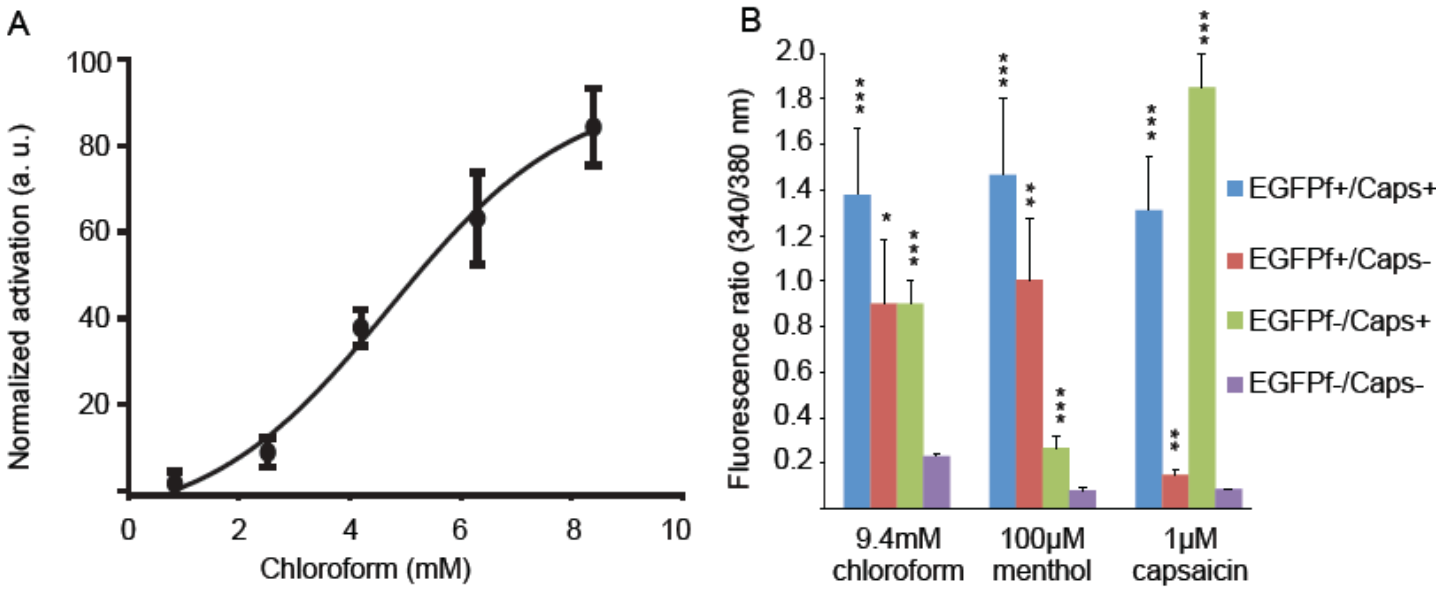


Figure S1. Chloroform activates TRPM8 and TRPM8-expressing DRG neurons. **A**. Dose-response curve of TRPM8-expressing HEK cells to chloroform normalized to peak menthol (250μM) response, utilizing ratiometric calcium imaging (n≥3 trials/concentration, ~100 cells/trial with each trial weighted to account for number of cells). **B.** Average peak over baseline responses of *TRPM8*$^{EGFPF/+}$ DRG neurons to chloroform (9.4mM), menthol (100μM) and capsaicin (1μM). * comparison of indicated condition versus the EGFPf-/Caps- condition. *p<0.05, **p<0.01, ***p<0.001. While capsaicin response of EGFPf+/Caps- is significantly different that EGFPf-/Caps- neither groupings met the 30% above baseline set for capsaicin responsiveness.

Molecular Pharmacology

The Pore Loop Domain of TRPV1 is Required for its Activation by the Volatile Anesthetics Chloroform and Isoflurane.

Corinna Kimball, Jialie Luo, Shijin Yin, Hongzhen Hu, Ajay Dhaka

Fig. S2.

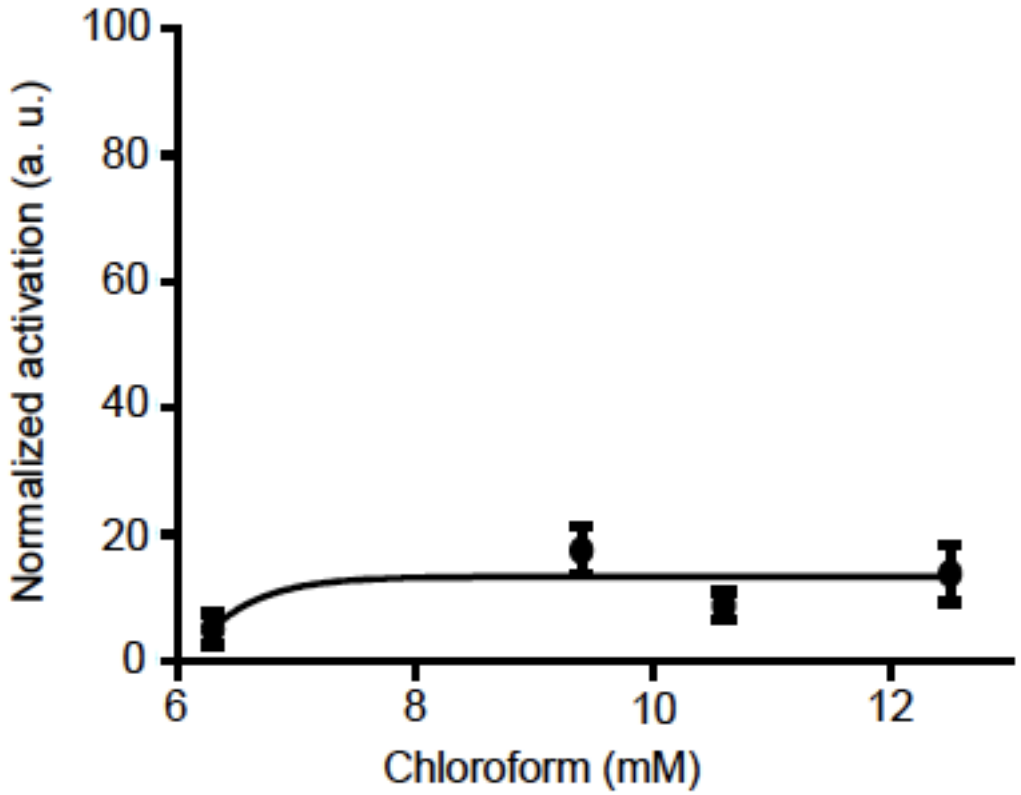


Figure S2. The outer pore domain TRPV1 triple heat mutant (N628K, N652T, Y653T) shows impaired responsiveness to chloroform across a range of dosages. Dose response curve of TRPV1 (N628K, N652T, Y653T)-expressing HEK cells. Peak chloroform over baseline response normalized to peak capsaicin (1μM) responses (n≥3 trials/concentration, ~100 cells/trial with each trial weighted to account for number of cells).