Title: Activation of Human Transient Receptor Potential Melastatin-8 (TRPM8) by Calcium-Rich Particulate Materials and Effects on Human Lung Cells

Authors: John G. Lamb, Erin G. Romero, Zhenyu Lu, Seychelle K. Marcus, Hannah C. Peterson, John M. Veranth, Cassandra E. Deering-Rice, and Christopher A. Reilly

Running Title: Activation of TRPM8 by Particles

*Corresponding author:
Dr. Christopher A. Reilly, Ph.D.
University of Utah
Department of Pharmacology and Toxicology
30 South 2000 East, 201 Skaggs Hall
Salt Lake City, UT 84112
Phone: (801) 581-5236; FAX: (801) 585-5111
Email: Chris.Reilly@pharm.utah.edu

Number of text pages: 41
Number of Tables: 2
Number of Figures: 7
References: 40
Number of words in Abstract: 245
Number of words in Introduction: 704
Number of words in Discussion: 1478

Non-Standard Abbreviations: CFA, coal fly ash; TRPA1, transient receptor potential ankyrin-1; TRPM8, transient receptor potential melastatin-8; TRPV1, transient receptor potential vanilloid-1; TRPV4, transient receptor potential vanilloid-4; IL, interleukin; AITC, allyl isothiocyanate; GSK, GSK1016790A, N-(1-((4-2-(((2,4-dichlorophenyl)sulfonyl)amino)-3-hydroxypropanoyl)-1piperazinyl)carbonyl)-3-methylbutyl)-1-benzothiophene-2-carboxamide; LJO-328, N-(4-tert-butylbenzyl)-N’-(1-[3-fluoro-4-(methylsulfonylamino)phenyl]thiourea; BEAS-2B, human bronchial epithelial cells; NHBE, normal human bronchial epithelial cells; HEK, human embryonic kidney; LHC-9, Lechner and LaVeck media; CFA, coal fly ash; CFA2, laboratory-generated-coal fly ash; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR; β2M, beta-2 microglobulin, GCaMP6s, a fusion of green fluorescent protein, calmodulin, and M13 used as a calcium indicator; ANOVA, analysis of variance; PIP2, phosphatidylinositol 4,5-bisphosphate.
Abstract:

To better understand how adverse health effects are caused by exposure to particulate materials, and to develop preventative measures, it is important to identify the properties of particles and molecular targets that link exposure with specific biological outcomes. Coal fly ash (CFA) is a by-product of coal combustion that can affect human health. We report that human transient receptor potential melastatin-8 (TRPM8) and an N-terminally truncated TRPM8 variant (TRPM8-Δ801) are activated by CFA and calcium-rich nanoparticles and/or soluble salts within CFA. TRPM8 activation by CFA was potentiated by cold temperature involving the phosphatidylinositol 4,5-bisphosphate binding residue (L1008), but was independent of the icilin and menthol binding site residue Y745, and essentially the N-terminal amino acids 1-800. CFA, calcium nanoparticles, and calcium salts also activated TRPV1 and TRPA1, but not TRPV4. CFA treatment induced CXCL1 and IL-8 mRNA in BEAS-2B and primary human bronchial epithelial cells through activation of both TRPM8 and TRPV1. However, neither mouse nor rat TRPM8 were activated by these materials, and Trpm8-knockout had no effect on cytokine induction in the lungs of CFA instilled mice. Amino acids S921 and S927 in mouse Trpm8 were identified as important for the lack of response to CFA. These results imply that TRPM8, in conjunction with TRPV1 and TRPA1, might sense selected forms of inhaled particulate materials in human airways, shaping cellular responses to these materials, and improving our understanding of how and why certain particulate materials elicit different responses in biological systems, affecting human health.
Introduction:

Environmental and occupational-specific particulate materials (PM) often cause adverse effects on human health. PM\textsubscript{2.5-10} derived from various sources has been linked to increased risks for asthma and chronic obstructive pulmonary disease (COPD), lung cancer, acute and chronic cardiovascular diseases, and early mortality (Morakinyo et al., 2016). However, PM can vary in composition depending on the origin, and this variability likely contributes to the observed differences in biological and health effects of these materials. Currently, precise biochemical mechanisms explaining the complex and variable effects of PM in biological systems and human health are not fully understood.

It has been proposed that transient receptor potential (TRP) proteins play roles in both sensing and initiating biological responses to PM in the lung. TRPs detect a variety of environmental stimuli including temperature, pH, constituents of an inflammatory milieu and other endogenous molecules involved in maintaining cellular homeostasis (Venkatachalam and Montell, 2007). TRP channels are also directly activated by environmental particulate pollutants. For example, TRP ankyrin-1 (TRPA1) and vanilloid-1 (TRPV1), which are expressed by airway neurons and epithelial cells, are activated by, and regulate responses to diesel exhaust, cigarette smoke and wood smoke particles, coal fly ash (CFA), and oxidants, as well as the pulmonary irritants allyl isothiocyanate (TRPA1) and capsaicin (TRPV1) (Bessac and Jordt, 2008; Bessac et al., 2008; Deering-Rice et al., 2012; Deering-Rice et al., 2011; Deering-Rice et al., 2015; Deering-Rice et al., 2016; Johansen et al., 2006; Shapiro et al., 2013; Veronesi et al., 1999a; Veronesi et al., 2002; Veronesi et al., 1999b). Activation of C-fiber neurons expressing TRPA1 and V1 triggers the cough reflex, suppresses respiratory drive, and stimulates neurogenic edema, while activation of these channels in epithelial cells stimulates pro-inflammatory cytokine
responses (Bessac and Jordt, 2008; De Logu et al., 2016; Deering-Rice et al., 2015; Deering-Rice et al., 2016). Collectively, these events could negatively impact respiratory health.

In a prior study, TRPV1 was identified as a sensor of CFA particles (Deering-Rice et al., 2012). In human lung epithelial cells TRPV1 activation by CFA triggered calcium-dependent induction of mRNA and protein secretion for interleukin-6 (IL-6) and interleukin-8 (IL-8), while in mouse lungs \textit{Trpv1} partially regulated \textit{Il-6}, \textit{Cxcl1} and \textit{Cxcl2} expression. In this study, CFA was also reported to activate human TRP melatstatin-8 (TRPM8), and to a lesser extent TRPA1, which were proposed as possible alternative mechanisms of CFA induced cytokine gene induction, since neither a TRPV1 antagonist (LJO-328) in human bronchial epithelial cells, nor \textit{Trpv1}-knockout (mice), fully attenuated the responses elicited by CFA.

Here we tested the hypothesis that TRPM8 contributes to the pro-inflammatory effects of CFA. TRPM8, a cold-sensing ion channel, is activated by temperature <16°C, as well as by “cooling” chemicals (e.g., menthol) often found in breath mints, toothpaste, VICK’S vapor rub, and menthol cigarettes (McKemy, 2007). In the lung, TRPM8 is expressed by cold-sensitive neurons that are distinct from TRPV1 and A1 expressing neurons (McKemy, 2007; Memon et al., 2017; Millqvist, 2016; Teichert et al., 2014), and in human lung epithelial cells as an N-terminally truncated variant (TRPM8-Δ801) (Sabnis et al., 2008a; Sabnis et al., 2008b). TRPM8 has been shown to regulate bronchial tone (Kaneko and Szallasi, 2014), suppress the cough reflex (Millqvist, 2016), and reduce irritation and bronchoconstriction caused by cigarette smoke (Ha et al., 2015; Willis et al., 2011). Activation of the TRPM8-Δ801 variant by either cold or menthol in bronchial epithelial cells regulates the expression of multiple cytokines and chemokines (Sabnis et al., 2008b). Similarly, TRPM8 was found to be elevated in the bronchial epithelium of patients with COPD, and to regulate mucin-5AC secretion in human bronchial
epithelial cells (Li et al., 2011). Thus, TRPM8 activation by particles could contribute to their biological effects.

It is reported here that human, but not mouse or rat TRPM8, and the TRPM8-Δ801 variant, were activated by CFA, calcium nanoparticles, and calcium salts. Additionally, TRPM8 activation contributed to increased pro-inflammatory cytokine gene expression by human lung epithelial cells treated with CFA, but not in mouse lungs. Specific amino acid residues in the pore loop region of TRPM8 were identified as the basis for the lack of activation of mouse Trpm8 by CFA. The potential significance to these findings in regard to human health effects of selected forms of particulate materials is also discussed.

Materials and Methods:

Chemicals and Reagents: Icilin, (-) menthol, allyl isothiocyanate (AITC), capsaicin, N-(1-((4-(2,4-dichlorophenyl)sulfonyl)amino-)-3-hydroxypropanoyl)-1-piperazinyl)carbonyl)-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A), N-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)-benzamide hydrochloride (AMTB), calcium chloride-2H2O, calcium sulfate (anhydrous), and ionomycin, were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture supplements including fetal bovine serum (FBS), geneticin (G418), trypsin, and penicillin/streptomycin (pen/strep) were purchased from Life Technologies (Carlsbad, CA).

Particulate Materials: Aluminum oxide (Al2O3; 8-14 nm), silicon dioxide (SiO2; 10 nm), titanium dioxide (TiO2, 5 nm) were purchased from Nanostructured and Amorphous Materials (Los Alamos, NM). Iron oxide (Fe2O3; 100 nm, spherical and <5 μm), cerium oxide (CeO2; 9-15 nm), and zinc oxide (ZnO; 20-30 nm) were purchased from Alfa-Aesar (Ward Hill, MA).
Nickel oxide (NiO) nanopowder (<50 nm) and 10 µm powder, silicon dioxide (0.5-10 µm), calcium oxide (CaO; <160 nm), calcium phosphate (Ca₂P₂O₇·xH₂O; <150 nm; abbreviated as CalPhos. for simplicity), and hydroxyapatite (<200 nm) were purchased from Sigma-Aldrich.

**Coal Fly Ash:** CFA (referred to as CFA1 in our prior studies) (Deering-Rice et al., 2012; Deering-Rice et al., 2011) was collected from the Hunter power plant in Utah. CFA2 was generated in a laboratory furnace under low oxygen conditions producing a more soot rich particle, as described elsewhere (Deering-Rice et al., 2012; Deering-Rice et al., 2011). CFA was size fractioned for some experiments, using a rotary tumbler and an Anderson Cascade Impactor, as well as by centrifugation at 1000 xg for 5 minutes to separate larger and smaller materials. The supernatant was further separated by filtering through a 0.22 µm syringe filter to yield what is referred to herein as filtered CFA or CFA <0.22 µm. To remove soluble calcium salts from the CFA (or other) particles, ~10 g or material was suspended in water containing 1 mM EGTA and injected into a 2000 MW cutoff Slide-A-Lyzer cassette. The material was then dialyzed against 1 mM EGTA and water (2 L) for 24 h, then deionized nanopure water (2 L) for 24h x 2. Particles were recovered, dried gently under air, and re-suspended at the desired concentrations prior to experiments.

**Cells and Cell Culture:** Immortalized human bronchial epithelial (BEAS-2B) cells (ATCC; Rockville, MD), Normal Human Bronchial Epithelial (NHBE) cells (Tissue Acquisition number: 13057 / Lonza; Walkersville, MD) and HEK-293 cells (ATCC; Rockville, MD) were maintained in a humidified cell culture incubator at 37°C with a 95% air: 5% CO₂ atmosphere. BEAS-2B cells were cultured in LHC-9 medium (Life Technologies; Carlsbad, CA). NHBE cells were cultured in BEGM media (Lonza; Walkersville, MD). HEK-293 cells were cultured in DMEM/F12 (Life Technologies; Carlsbad, CA) media containing 5% FBS and 1x penicillin-
streptomycin. HEK-293 cells stably overexpressing human TRPM8, TRPV1, TRPV4, and TRPA1 were created as described previously (Deering-Rice et al., 2011), and were cultured as above with 0.3 mg/ml G418 added to the media. HEK-293 cells stably over-expressing the ultra-sensitive fluorescent protein calcium sensor GCaMP6s (Chen et al., 2013), mouse, and rat TRPM8, were also generated using protocols described previously (Deering-Rice et al., 2011). The GCaMP6s (Addgene, Cambridge, MA) gene was sub-cloned into pcDNA3.1 and transfected into HEK-293 cells as below. Clones exhibiting the greatest change in fluorescence intensity following ionomycin (1 μM) in calcium flux assays were selected and expanded. The stability of the GCaMP6s over-expressing cell line response used herein has been verified for >30 passages. Mouse and rat TRPM8 overexpressing cells were generated in the same way using icilin 20 μM as the agonist. The stability of TRPM8 overexpression in these cells has been verified for >10 passages. BEAS-2B TRPM8 “knock-out” (TRPM8-KO) cells were generated by overexpression of TRPM8 shRNA or a scrambled (non-specific) shRNA, as previously described (Sabnis et al., 2008a; Sabnis et al., 2008b). All cell types were sub-cultured using trypsin. Plates (96-well) were pre-coated with 1% gelatin for HEK-293 experiments.

**TRP Channel Cloning, TRP Channel Overexpression and Site Directed Mutagenesis:** All TRP genes including human TRPM8, and the N-terminal truncated TRPM8 variant (TRPM8-Δ801) were cloned into pcDNA3.1 (Life Technologies; Carlsbad, CA). Mouse and rat TRPM8 expression plasmids were generously provided by Dr. David Julius (UCSF), and the genes were sub-cloned into pcDNA3.1/V5-His. Transient transfection of expression plasmids into HEK-293 GCaMP6s over-expressing cells (HEK-GCaMP6s) was performed using Lipofectamine 2000 (Life Technologies; Carlsbad, CA) at a 2:1 lipid/DNA ratio in 50 μl of Opti-MEM media (Life Technologies; Carlsbad, CA) with sequence verified plasmid DNA (175 ng/well), as previously
described for HEK-293 cells (Deering-Rice et al., 2012). Site-directed mutagenesis of TRPM8 was performed using the QuickChange II XL kit (Stratagene, La Jolla, CA). Sequences were verified, and mutants were assayed in comparison to wild-type human TRPM8 after transient-transfection in HEK-293-GcAMP6s cells. Results were normalized to icilin (50μM for transfected cells, 20μM for TRPM8-OE cells).

**Fluorometric Calcium Flux Assays:** Responses to agonist treatments at ~16 and 37°C were assayed using an Olympus 1x50 inverted microscope, as previously described (Johansen et al., 2006), with the addition of a heat plate to maintain 37°C temperature of the HEK and TRPM8-OE cells. We found that maintaining cells at 37°C versus room temperature was essential to prevent TRPM8 inactivation, as reported by Fujita et al. (Fujita et al., 2013). An electronic probe was placed in a representative well to measure and continually adjust the temperature of the heated plate for the duration of the assay. Fluo-4 calcium imaging was performed using the Fluo-4 direct kit (Life Technologies; Carlsbad, CA) as previously described (Deering-Rice et al., 2012; Deering-Rice et al., 2011). Images were collected at 30 s intervals after addition of soluble or particle agonists. The fluorescence of the pre-treatment baseline image was subtracted from all images, prior to normalizing to the maximum fluorescence elicited from ionomycin (10 μM) treatment.

All other calcium assays were performed using an EVOS FL Auto Imaging System (Life Technologies; Carlsbad, CA) and either stably over-expressing HEK-293 cells or HEK-293 GCaMP6s cells transiently transfected with the desired constructs. The integrated environmental chamber maintained ideal experimental conditions at 5% CO₂ and 37°C with humidification. Images were taken prior to agonist treatment, and at 3 s intervals for 45 s following application of ice cold agonists at 3x concentration, diluted in LHC-9 media/buffer. Cells were either loaded
with Fluo-4 AM, or in the case of the transfected HEK-293-GcAMP6s cells, were prepared for imaging by replacing the media with LHC-9 containing 0.75 mM trypan red, and maintained at 37°C for up to thirty minutes prior to imaging. Pretreatment with the antagonist AMTB (20 μM in LHC-9 containing 0.75 mM trypan red at 37°C) was performed 5-10 min prior to imaging. Ionomycin (10 μM) at room temperature was added prior to final image and used to elicit the maximum fluorescent response in each well. Baseline responses elicited by LHC-9 are subtracted from each treatment and then further normalized to the wild-type response or as a percent of the icilin response. Quantification of calcium flux was performed using the maximum change in fluorescence observed at any point during the duration of the assay. This typically occurred within 20-30s of application of the particles versus almost immediately (<10 s) for soluble agonists.

**Calcium Content of Particles:** Free calcium in particle suspensions (CFA, CaO, CalPhos., and hydroxyapatite) in ddH₂O were determined at 0.3, 0.6, 1.15 and 2.3 (CFA only) mg/mL using the Calcium Colorimetric Assay Kit (Sigma; St. Louis, MO), as per manufacturer’s recommendations.

**SEM-EDS:** The physical and elemental characteristics of CFA and filtered CFA (<0.22 μm) were defined using an FEI Quanta 600 FEG Scanning Electron Microscope with Energy Dispersive X-ray Spectroscopy (EDAX X-ray detector). Electron microscopy was performed at the University of Utah Electron Microscopy Core Laboratory.

**PCR analysis of Gene Expression in Human Lung Cells:** NHBE cells or BEAS-2B (TRPM8-KO and scrambled) were sub-cultured into 12-well cell culture plates, grown to 90% density and treated for 24 hours at 37°C. Total RNA was extracted from cells using GeneElute Mammalian Total RNA Miniprep kit (Sigma; St. Louis, MO) and 2.5 μg of the total RNA was
converted to cDNA using iScript Reverse Transcriptase Supermix (BioRad; Hercules, CA). The resulting cDNA was diluted 1:20 for analysis by quantitative real-time PCR (qPCR). qPCR was performed using predesigned Taqman probes for β2-microglobulin (B2M; Hs00984230_m1), IL-8/CXCL8 (Hs00174103_m1); and CXCL1 (Hs00236937_m1) on a QuantStudio 6 (Life Technologies; Carlsbad, CA) using Taqman Fast Advanced Mastermix (Life Technologies; Carlsbad, CA), as per manufacturer protocols.

**CFA Treatment of Mice:** All procedures were approved by the University of Utah IACUC committee. C57BL/6 and *Trpm8* -/- (Jackson Laboratories, Bar Harbor, ME) were generously provided by Dr. Baldomero Olivera (University of Utah). Mice were anesthetized using ketamine (50 mg/kg) + xylazine (10 mg/kg i.p.) and suspended vertically at a ~30° angle on an immobilizer. The larynx was visualized by gently grasping tongue with tweezers and inserting an otoscope into the mouth; treatment solutions (25 μL ice-cold sterile saline or CFA suspended in sterile saline) were slowly dispensed at the tracheal opening. Mice remained in the recumbent position for ~1 min after aspiration was completed and returned to their cages for 4h, upon which they were terminally anesthetized by i.p. injection of ketamine and xylazine (300 mg/kg + 30 mg/kg) and exsanguinated via the abdominal aorta. The lungs were inflated with cold RNAlater, excised, and subsequently processed for gene expression by qPCR.

**Gene Expression Analysis in Mouse Lung Tissue:** Total RNA was isolated using Trizol (Life Technologies; Carlsbad, CA) followed by on column purification with DNaseI treatment using the RNeasy mini kit (Qiagen, Valencia, CA). RNA (2.5 μg) was converted to cDNA using iScript Reverse Transcriptase Supermix (BioRad; Hercules, CA). The resulting cDNA was diluted 1:20 for analysis by quantitative real-time PCR (qPCR). qPCR was performed using predesigned Taqman probes for *mGAPDH* (Mm99999915_g1), *mIL-6* (Mm00446190_m1),
mCxcl1 (Mm04207460_m1), and mCxcl2 (Mm00436450_m1) on a QuantStudio 6 (Life Technologies; Carlsbad, CA) using Taqman Fast Advanced Mastermix (Life Technologies; Carlsbad, CA), as per manufacturer protocols.

Statistical methods: Statistical analyses were performed in GraphPad Prism 7 software. Statistically significant responses were determined as described in the figure legends and typically involved regular, non-repeated measures 1- and 2-way ANOVA and post-testing using the Bonferroni or Tukey’s multiple comparisons tests.

Results:

Activation of TRPM8 by CFA: Cold solutions, more than warm solutions, of icilin, menthol, and suspensions of CFA and CFA2, activated full-length human TRPM8 in HEK-293 cells over-expressing TRPM8 (Figure 1A). For cold samples, the final temperature of the treatment well was measured at ~15-16°C which slowly increased over the duration of the assay. No significant responses were observed using cold LHC-9 buffer (vehicle) alone. However, 5 minute pre-application of warm CFA followed by cold buffer/LHC-9 also elicited a response ~90% that of cold CFA. The kinetics of TRPM8-mediated calcium flux exhibited an initial rapid rise in intracellular calcium followed by a reduction in fluorescence intensity/intracellular calcium content over time (data not shown). Further, when assays were performed without maintaining the cells at 37°C, the cells quickly became desensitized to all stimuli, as previously reported (Fujita et al., 2013). Accordingly, all subsequent calcium flux experiments were performed at 37°C using cold treatment solutions.

Activation of TRPM8-Δ801 by CFA and Inhibition of Calcium Flux with the TRPM8 Antagonist AMTB: Full-length wild-type TRPM8, TRPM8-Δ801, and a control vector (CV;
pcDNA3.1/V5-His/lacZ) were transiently transfected into HEK-293-GcAMP6s cells. An increase in calcium flux was observed with full-length TRPM8 (wild-type; WT) with all treatments, as in Figure 1A (Figure 1B). For the TRPM8-Δ801 variant, reduced but significant calcium flux was observed with menthol (~30% full-length) and CFA (~60% wild type; Figure 1B); icilin does not activate TRPM8-Δ801 because the variant lacks the full icilin (Y745/N799/D802/G805) binding site (Bandell et al., 2006; Brauchi et al., 2007; Liu and Qin, 2005; Malkia et al., 2009; Sabnis et al., 2008b; Voets et al., 2007; Zakharian et al., 2010). All responses were inhibited by co-treatment with the TRPM8 antagonist AMTB (Figure 1B, pattern filled bars).

Role of the Menthol/Icilin- and Phosphatidylinositol 4,5-Bisphosphate (PIP<sub>2</sub>)-Binding Site Residues in TRPM8 Activation by CFA: Previous studies have identified Y745, R1008, and L1009 as residues critical for menthol, icilin, and cold activation of TRPM8 (Bandell et al., 2006; Brauchi et al., 2007; Dragoni et al., 2006; Liu and Qin, 2005; Malkia et al., 2009; Voets et al., 2007; Zakharian et al., 2010). Y745 is critical for high affinity menthol and icilin binding/activation, while L1008 and L1009 alters activation efficiency by affecting PIP<sub>2</sub> binding. Y745H, R1008Q, and L1009R mutations were introduced into full-length human TRPM8 by site-directed mutagenesis. These constructs were transiently-transfected into HEK-GCaMP6s cells and subsequently assayed for agonist-induced calcium flux. As expected, mutation of Y745 abolished calcium flux caused by icilin, and substantially reduced activation by menthol (Figure 2A). Both the R1008Q and L1009R mutations reduced activation by menthol ~30-50%, also as expected (Figure 2B). However, the Y745A mutation had no effect on CFA-induced calcium flux (Figure 2A), while the R1008Q and L1009R mutants exhibited essentially 100% and ~60% reduced response, respectively (Figure 2B). These data imply that CFA activates TRPM8
through interactions that do not involve the traditional soluble ligand binding site, but via a mechanism that is influenced by PIP$_2$ binding. The effects of these and other point mutations are summarized in Table 2.

**Chemical Composition of CFA:** Size and chemical composition of PM often influence the biological effects of particulate materials. SEM-EDS analysis of CFA and filtered CFA (<0.22 μm) was performed (Figure 3). CFA was a mixture of mineral-rich, mostly spherical particles ranging in size (~50 nm -150 μm), with occasional needle- and prism-shaped materials. CFA particles were composed primarily of Si (45.6%), Al (22.0%), Ca (12.1%), and Fe (7.5) oxides and/or salts (Table 1). The filtered CFA (<0.22 μm) contained mostly aggregated spherical, nanoparticles approximately 70 nm, composed almost exclusively of Ca oxides and/or salts (97.8%) (Table 1). For most of the elements detected by SEM-EDS, homogenous mineral oxide particles of micron and nano sizes were available. These materials were tested for their ability to activate TRPM8 (Table 1). Only CaO nanoparticles activated TRPM8. This agrees with the result that the calcium-rich filtered CFA (<0.22 μm) material was a more potent TRPM8 agonist relative to the unfractionated CFA (46% icilin response vs. 15% for CFA; Table 1).

**Soluble Calcium and TRPM8 Activation by CFA and Related PM:** Dialysis of CFA essentially eliminated the response of TRPM8 to CFA (Figure 4A). As such, activation of hTRPM8 by calcium associated with CFA and other calcium nanoparticles was further explored. The free calcium content of commercial CaO nanoparticles (<160 nm), CalPhos. nanoparticles (<150 nm), hydroxyapatite particles (<200 nm), and CFA was determined (Figure 4B). CaO particles contained the highest concentration of free calcium on a per mass basis (~38 μg/mL in a 1.15 mg/mL suspension or 0.95 mM), followed by CalPhos., CFA (~18 μg/mL in a 2.3 mg/mL suspension or ~0.450 mM), and hydroxyapatite particles.
To assess the importance of free calcium in TRPM8 activation, TRPM8-OE cells were treated with CaO, CalPhos., CFA, and hydroxyapatite, normalizing the particle treatments by adjusting the mass dose of particles to yield equivalent concentrations of free calcium at 0.25 and 0.5 mM (Figure 4C). However, treatment of cells with equivalent concentrations of free calcium, delivered as particle suspensions did not elicit equivalent calcium flux responses; the rank order was CaO>CFA>CalPhos.>hydroxyapatite. Further, responses for CalPhos. and hydroxyapatite particles were not dose-dependent. As such, TRPM8-OE cells were also treated with solutions prepared using CaCl$_2$·2H$_2$O and anhydrous CaSO$_4$. CaCl$_2$ and CaSO$_4$ also activated TRPM8 comparably to CFA on a per mass basis (Figure 4D), even though CaCl$_2$ should yield the highest concentration of free calcium ions (solubility = 0.595 g/mL versus 0.0021 g/mL for CaSO$_4$). Accordingly, the CaSO$_4$ treatment was turbid.

**Selectivity of TRPM8 Activation by CFA and Related Calcium Particles:** HEK-293 cells over-expressing human TRPM8, TRPA1, TRPV1, or TRPV4 were also treated with CFA as well as CaCl$_2$, and CaSO$_4$ (Figure 4D). The rank order for sensitivity to CFA and other calcium-rich materials was TRPM8≥TRPV1>TRPA1>TRPV4, with no responses observed for TRPV4. Interestingly, CaCl$_2$ produced the lowest response for TRPA1, which has previously been suggested to be activated by CFA by cell surface interactions and a mechanosensitive mechanism involving N-terminal residues (Deering-Rice et al., 2015). As with TRPM8, dialysis of CFA also eliminated the activation of TRPV1 and A1.

**Induction of Pro-Inflammatory Genes in Human Lung Epithelial Cells by CFA:** BEAS-2B cells stably over-expressing shRNA to attenuate TRPM8-Δ801 expression were used to evaluate the role of TRPM8 in regulating the pro-inflammatory effects of CFA on human bronchial epithelial cells. The TRPM8-KO cells lack response to both menthol and cold
treatment (Sabnis et al., 2008a; Sabnis et al., 2008b). CFA treatment of normal BEAS-2B cells or BEAS-2B cells over-expressing a scrambled shRNA resulted in concentration-dependent IL-8 mRNA induction (Figure 5A). Note, the treatment of lung cells with CFA was performed at 37°C for 24h, indicating that TRPM8 activation by CFA does not require cold temperature, and furthermore that the acute sensitivity of TRPM8 to CFA and calcium-rich materials in the short-term calcium assays was due to potentiation by cold temperature primarily involving PIP2 binding. Regardless, IL-8 induction was substantially reduced (~70-80%) in the TRPM8-Δ801 shRNA over-expressing “knock-out” cells (Figure 5A).

To compare the relative contributions of TRPM8-Δ801 and TRPV1 in primary human lung epithelial cell responses to CFA, NHBE cells were also treated with CFA in the presence or absence of either the TRPM8 antagonist AMTB or the TRPV1 antagonist LJO-328 (Figure 5B and C). Both CXCL1 and IL-8 mRNA were induced in NHBE cells by treatment with CFA. The increase in mRNA for CXCL1 was inhibited ~80% by LJO-328, but not AMTB, while the increase in IL-8 mRNA was attenuated roughly equally (~40-50%) by AMTB and LJO-328.

Activation of Rodent TRPM8 by CFA: Activation of rodent (mouse and rat) TRPM8 was also tested using HEK-293 cells stably over-expressing these channels. Similar to human TRPM8, both rodent channels forms of TRPM8 were activated by menthol and icilin (Figure 6A). However, neither mouse nor rat TRPM8 were activated by CFA, CaO or CaCl2 (Figure 6A).

Role of TRPM8 in CFA-Induced Cytokine Gene Induction in Mouse Lungs: Wild-type and Trpm8/- mice were instilled with cold CFA (10 mg/kg) via the oropharyngeal route. After 4h, the lungs were removed and processed for analysis of Il-6, Cxcl1 and Cxcl2 mRNA induction as previously described for Trpv1/- mice (Deering-Rice et al., 2012). CFA treatment produced
variable, but significant increases in mRNA abundance for all three genes, as anticipated (Figure 6B-D). However, neither the basal levels of mRNA expression nor the level of induction of these genes differed between the wild-type and Trpm8-/− mice, in agreement with Figure 6A showing that rodent TRPM8 is not activated by CFA.

Specific amino acids in the pore loop region of TRPM8 are important for species specific response to CFA: The amino acid sequences of human, mouse, and rat TRPM8 were compared (Figure 7A). Residues differing between human and rodent TRPM8 within the extracellular pore-loop region were specifically tested based on prior results demonstrating a role for pore-loop residues in the activation of TRPV1 by CFA (Deering-Rice et al., 2012). Chimeric constructs of human and mouse TRPM8 were generated by site directed mutagenesis by substituting residues S921, S927, and S932 of mouse Trpm8 to those of human TRPM8 (G921, A927, and T932). These constructs were transiently transfected into HEK-GCaMP6s cells and assayed for calcium flux (Figure 7B). It was found that “humanizing” mouse Trpm8 at all three positions increased the response of mouse Trpm8 to CFA, with a rank order of S927A (p=0.012), S921G (p=0.158), and S932T (p=0.767); the p-value comparing mouse to human wild-type responses was 0.033 in this experiment. All three mutations also slightly increased responses of mouse Trpm8 to menthol (~25-50%), indicating that the pore-loop region of TRPM8 is also critical for activation by CFA. The effects of these and other point mutations are also summarized in Table 2.

Discussion:

Inflammation and irritation of the respiratory tract are common effects of inhaled particulates. CFA is a model particulate that, based on in vitro and animal models, may stimulate inflammation and irritation through interactions with TRPV1, TRPA1 and possibly
TRPM8 (Deering-Rice et al., 2012; Deering-Rice et al., 2015; Deering-Rice et al., 2016; Sabnis et al., 2008a; Veronesi et al., 2002; Veronesi et al., 1999b). This study sought to determine: 1) How CFA activates TRPM8; and 2) to what extent TRPM8 and TRPM8-Δ801 contribute to the pro-inflammatory effects of CFA in human lung cells and mouse lungs.

The discovery of hTRPM8 activation by CFA and related materials (Figures 1 and 4, Table 1) was a novel finding for which biochemical mechanisms were unknown. Examination of specific amino acid residues involved in TRPM8 activation by prototypical soluble agonists revealed that activation by CFA and related calcium-rich particles and salts occurred independent of the menthol and icilin binding site residue Y745 (Figure 2A) (Bandell et al., 2006; Brauchi et al., 2007; Liu and Qin, 2005; Malkia et al., 2009; Voets et al., 2007; Zakharian et al., 2010). This conclusion was further supported by results showing that the TRPM8-Δ801 variant, which lacks Y745 due to truncation, was also activated by CFA, albeit to a lesser (~60%) degree (Figure 1B). However, similar to menthol and icilin, TRPM8 activation by CFA was significantly attenuated by mutation of R1008 (Figure 2B), which is critical for PIP₂ binding and TRPM8 activity. Similarly, TRPM8 activation by CFA was inhibited by mutation of L1009 (Figure 2B), which also reduces sensitivity to menthol and interfering with PIP₂ binding (Bandell et al., 2006; Brauchi et al., 2007; Liu and Qin, 2005; Voets et al., 2007; Zakharian et al., 2010). Collectively, these data show that CFA activates hTRPM8 through a mechanism unique from that of traditional soluble agonists such as menthol and icilin, presumably involving specific interactions with structural elements on the extracellular pore-loop domain which differ between human and rodent TRPM8 (Figures 6 and 7), and through an inherently fundamental mechanism that overlaps with the well-established thermal- and voltage-dependent activation mechanisms of TRPM8. However, it should be noted that the potentiation effect of cold and PIP₂ on TRPM8
activity in short-term calcium assays was found to be irrelevant in cellular assays, since TRPM8 significantly contributed to the regulation of cellular responses to CFA at 37°C (Figure 5A-C).

How particles such as CFA ultimately interact with biological systems to elicit specific responses is both complex and not fully understood. The importance of particle, size, shape, surface charge, and chemical/mineral composition in determining the biological effects of particles is an area of intense research, with evidence that all of these criteria can modify the effects of different materials. Activation of TRP ion channels by particles is a relatively new and an incompletely understood concept. As such, the relationship between the physico-chemical properties of CFA and TRPM8 were further explored. CFA was characterized using SEM-EDS (Figure 3 and Table 1). CFA is a mixture of physically and chemically distinct materials ranging in size, shape, and chemical make-up. In functional assays, smaller particles (Filtered CFA <0.22 μm), specifically calcium-rich, calcium oxide nanoparticles, and/or leachable calcium salts, promoted hTRPM8 activation. A survey of TRPM8 activation by various commercially available micron- and nano-sized mineral oxide particles, representative of the major minerals identified in CFA, also demonstrated selectivity for CaO (Table 1). However, activation of an ion channel by a particle, even a nanoparticle, was difficult to comprehend, warranting further investigation.

Dialysis of CFA was used to remove soluble calcium from the CFA in an effort to differentiate particle-bound versus soluble calcium in TRPM8 activation. Dialysis of CFA ameliorated TRPM8 activation (Figure 4A) which suggested that calcium ions liberated from CFA may be responsible for driving TRPM8 activation. To further explore this hypothesis, TRPM8 activation was evaluated by testing CFA, CaO, CalPhos, and hydroxyapatite suspensions at different mg/mL concentrations to yield equimolar concentrations of free calcium.
at 0.25 and 0.5 mM (Figure 4B and C). However, despite normalizing the treatments for free calcium content, TRPM8 activation was not equivalent.

To further explore the relationship between soluble calcium ions and particle-bound calcium, CaCl$_2$ and CaSO$_4$ were compared as agonists of TRPM8 (Figure 4D). Both salts activated TRPM8 in a dose dependent manner with CaSO$_4$ being slightly more potent than both CFA and CaCl$_2$ on a per mass basis (Figure 4D). However, CaCl$_2$ is significantly more soluble than either CFA or CaSO$_4$ and should have provided the highest levels of free calcium in the treatments solutions; the CaSO$_4$ was visibly turbid. A ~10-15% greater response to CaSO$_4$ (versus CaCl$_2$) was observed at 18 µg/cm$^2$ (i.e., 1.7 mM; 1/10th that used in Figure 4D), and a ~25% positive control response was observed for CaSO$_4$ at 1.8 µg/cm$^2$ or 0.17 mM; CaCl$_2$ was inactive at this concentration. Thus, it seems unlikely that soluble calcium ions are driving the activation of TRPM8, and that simply increasing the bioavailability of free calcium in the media upon stimulation with cold treatment solutions may not fully explain how TRPM8 activation occurs with particle agonists. Of note, it is possible that the relatively high levels of free calcium in the CaCl$_2$ solution partially inhibits calcium-dependent TRPM8 activation based on a report by Mahieu et al. (Mahieu et al., 2010) who demonstrated that extracellular calcium ions (>2 mM) inhibited TRPM8 activation by menthol and icilin via surface charge screening and shifting the voltage dependence of TRPM8 to higher values. However, we did not see evidence for this in our studies.

Unfortunately, the experiments described above did not fully discern whether TRPM8 activation was the result of direct activation by soluble calcium leached from the particles, or if very small insoluble aggregates activated TRPM8. It may be possible that both are important, but our current hypothesis is that hTRPM8 activation by CFA and related calcium-containing...
particles and salts may be the result of the formation of very small insoluble aggregates of calcium salts in solution and at the cell surface, which interact with hTRPM8 at a unique site (or sites) which has not yet been fully elucidated. Formation of colloidal aggregates has been shown to be an important determinant of the biochemical activity of many “soluble” molecules at their biological targets (Duan et al., 2015), which may be relevant to TRPM8, as well as TRPV1 and TRPA1 activation by particles. In support of TRPM8 activation by soluble calcium, TRPM2, M4, and M5 are activated by intracellular calcium ion binding at specific residues (Du et al., 2009; Liman, 2007; Yamaguchi et al., 2014). However, this has not been shown for TRPM8 thus far, and as described above, high (>2 mM) extracellular calcium may inhibit TRPM8.

Regardless of the precise mechanism of by which TRPM8, TRPV1, and TRPA1 are activated by CFA and other calcium-rich materials and salts, the ability of these materials to stimulate specific TRPs and associated subcellular signaling cascades is intriguing with potential pathophysiologica implicaisions. Specifically, results in Figures 5A-C show that both TRPM8 and TRPV1 contribute to cytokine gene induction in human lung epithelial cells, which is unique in that Trpm8 does not appear to be a sensor and/or mediator of similar responses in mouse lungs (Figure 6).

Exactly how the results of this study translate to human health effects of PM will require further investigation, but it may be possible that human TRPM8, alone or in combination with TRPA1 and/or TRPV1, may play direct roles in regulating some of the documented adverse effects caused by CFA and similar inhaled calcium-rich particles/materials. For example, CFA, CaO, and CaSO₄ can be more prevalent in ambient air near coal-fired power plants because flue-gas desulfurization utilizes CaO to trap SO₂ emissions, producing calcium sulfite (CaSO₃) and eventually CaSO₄ (Srivastava and Jozewicz, 2001). Living in close proximity to coal-burning
power plants has been associated with increased risks for adverse respiratory and general health effects (Buchanan et al., 2014). Additionally, according to the American Coal Ash Association, approximately 100 million tons of CFA is produced annually in the United States, and substantial quantities of this material is “recycled” as components of concrete and gypsum-containing products including wall board/sheetrock and plasters. According to the Centers for Disease Control and Prevention, occupational exposure to gypsum dusts adversely affects the respiratory system, causing inflammation and irritation, among other effects. Perhaps these are regulated in part by TRPM8 and/or TRPV1 and A1? Finally, through inhibition of acute airway irritation and bronchoconstriction, TRPM8 activation could paradoxically increase inhalation delivery of a variety of other inhaled and potentially toxic co-pollutants, as reported for nicotine and cigarette smoke (Ha et al., 2015; Willis et al., 2011). As such, in conjunction with TRPV1 and/or TRPA1, TRPM8 may be an important particle sensor to consider as a mediator of adverse effects of particles, particularly when performing naturalistic exposure/health effect studies in certain locations and in relation to specific occupational exposure sources.
Acknowledgments:

None

Author Contributions:

Participated in research design: Romero, Deering-Rice, Lu, Veranth, Reilly
Conducted experiments: Romero, Marcus, Lu, Lamb, Peterson, Deering-Rice, Reilly
Performed data analysis: Romero, Marcus, Lu, Lamb, Peterson, Deering-Rice, Reilly
Wrote or contributed to writing of the manuscript: Lamb, Romero, Deering-Rice, Veranth, Reilly
References:


Footnotes:

This work was supported the National Institute of Environmental Health Sciences [Grant ES017431].
Figure legends:

Figure 1: (A) Calcium flux in TRPM8-OE HEK-293 cells elicited by CFA and CFA2 (2.3 mg/mL/180 µg/cm²; n=8 and 8, respectively), icilin (20 µM n=10), and menthol (1 mM; n=3) at 4°C and 37°C. Data are represented as baseline and HEK-293 subtracted calcium flux, normalized to the maximum fluorescence change elicited by ionomycin (10µM). Values are mean ± SEM. Statistically significant responses relative to vehicle and warm treatments using 2-way ANOVA and post-testing using the Bonferroni multiple comparisons test are represented as ** p<0.01, ***p< 0.001. (B) Calcium flux in GCaMP6s over-expressing cells transfected with human TRPM8-WT (n=16, 3, and 16 for icilin, menthol, and CFA, respectively), TRPM8-Δ801 (n=3, 3, and 8 for icilin, menthol, and CFA, respectively), or the control vector (CV; pcDNA3.1/V5-His/lacZ; n=13, 3, and 14 for icilin, menthol, and CFA, respectively) in response to icilin (20 µM), menthol (1 mM) and CFA (2.3 mg/mL/180 µg/cm²) treatments. Inhibition of calcium flux by the TRPM8 antagonist with AMTB at 20 µM (n=16, 3 and 16 for full-length TRPM8, TRPM8-Δ801, and CV using icilin, menthol and CFA, respectively) is shown for each treatment as pattern filled bars. Values shown represent the mean ± SEM. Statistically significant responses using 2-way ANOVA and post-testing using the Bonferroni multiple comparisons test relative to CV are represented as ***p< 0.001, relative to AMTB represented as † p<0.05, ††† p< 0.001, and relative TRPM8-WT represented as ‡‡ p< 0.001.

Figure 2: (A) Calcium flux elicited by CFA (2.3 mg/mL/180 µg/cm²), icilin (50 µM), and menthol (500 µM) on GCaMP6-overexpressing cells transiently transfected with either human TRPM8-WT (n=47, 16, and 47 for icilin, menthol, and CFA, respectively) or the TRPM8 icilin and menthol binding site mutant Y745H (n=3, 3 and 3 for icilin, menthol and CFA,
respectively). (B) Calcium flux elicited by CFA (2.3 mg/mL/180 µg/cm²), icilin (50 µM), and menthol (500 µM) on GCaMP6-overexpressing cells transiently transfected with either human TRPM8-WT (n=47, 16, and 48 for icilin, menthol, and CFA, respectively), the PIP₂ binding site mutant R1008Q (n=3, 3, and 3 for icilin, menthol, and CFA, respectively), or adjacent residue L1009R (n=3, 3, and 3 for icilin, menthol, and CFA, respectively). Calcium flux responses were first subtracted for control vector (CV) responses and then normalized to responses of the TRPM8-WT cells. Values represent the mean ± SEM. Statistically significant responses using 2-way ANOVA and post-testing using the Bonferroni multiple comparisons test relative to TRPM8-WT are represented as *p< 0.05 and ***p< 0.001.

Figure 3: Scanning electron microscopy (SEM) images of CFA particles at 1,000X (top left image), 2,828X (bottom left image) and CFA after filtration through a 0.22 µm syringe filter at 500X (top right image) and 17,830X (bottom right image).

Figure 4: (A) Calcium flux in TRPM8-OE HEK-293 cells exposed to icilin (20 µM; n=3), CFA (2.3 mg/mL/180 µg/cm²; n=3), and dialyzed CFA (2.3 mg/mL/180 µg/cm²; n=3). Data were subtracted from HEK-293 cell responses, and normalized to icilin responses. Data are represented as the mean ± SEM. Statistically significant responses were determined using a 1-way ANOVA and post-testing with Tukey’s multiple comparisons test relative to icilin ***p< 0.001 or to CFA †††p< 0.001. (B) Soluble calcium content of suspensions of CFA 2.3 mg/mL/180 µg/cm² or 1.15 mg/mL/90 µg/cm² (n=3, 3, 3), 0.6/45 µg/cm² (n=3, 3, 3), and 0.3 mg/mL/23 µg/cm²; (n=3, 3, 3) CaO, CalPhos., and hydroxyapatite, respectively. Values were determined from a standard curve and are represented as the mean ± SEM. (C) TRPM8-OE cells
treated with CaO (n=3), CalPhos. (n=3), hydroxyapatite (n=3), and CFA (n=3), normalizing treatments to achieve 0.5 and 0.25 mM free calcium by application of different mass doses of PM. HEK-293 cell responses were subtracted from particle responses and data are represented as % icilin (20 µM). N.D.=none detected. Values shown represent the mean ± SEM. Prior to HEK-293 cell response subtraction, statistical difference from the HEK-293 treated control was performed using a paired t-test (***p<0.001). All values were statistically different from HEK-293 cells except hydroxyapatite. (D) Calcium flux in human TRPM8 (n=8, 4, 6 for CFA, CaCl₂, and CaSO₄, respectively), TRPV1 (n=5, 3, 4 for CFA, CaCl₂, and CaSO₄, respectively), TRPV4 (n=4, 3, 3 for CFA, CaCl₂, and CaSO₄, respectively), and TRPA1 over-expressing HEK-293 cells (n=3, 3, 3 for CFA, CaCl₂, and CaSO₄, respectively) were treated with CFA, CaCl₂, or CaSO₄ at 2.3 mg/mL/180 µg/cm². Values are HEK-293 (control) subtracted and expressed as the percent of each channel’s response to the appropriate positive control (TRPM8: 50µM icilin; n=5, TRPV1: 5µM capsaicin; n=4, TRPV4: 25 nM GSK; n=3, TRPA1: 150µM AITC; n=3). Values shown are mean ± SEM. Prior to HEK-293 response subtraction, statistical difference from the HEK-293 treated controls was performed using 2-way ANOVA and post-testing with Tukey’s multiple comparisons (***p<0.001).

Figure 5: (A) IL-8 mRNA quantification in BEAS-2B cells, TRPM8 shRNA (TRPM8-KO), and scramble shRNA over-expressing cells treated with CFA (0.34 and 0.68 mg/mL/90 and 180 µg/cm²) for 24 hours at 37°C. Data are represented as the mean ± SEM (n=3). Statistically significant responses using 2-way ANOVA and post-testing with the Bonferroni multiple comparisons test relative to vehicle are represented as ***p< 0.001 or relative to BEAS-2B-WT and scramble cells †††p< 0.001. (B) CXCL1 and (C) IL-8 mRNA quantification in NHBE cells
treated with CFA (0.68 mg/mL/180 µg/cm²) and CFA+AMTB (2 µM) or CFA+LJO-328 (20 µM) for 24h at 37°C. CXCL1 and IL-8 mRNA expression in both cell types was normalized to the reference gene β2M and quantified using ΔΔCT relative to the vehicle control. Data are represented as mean ± SEM (n=3). Statistically significant responses using 2-way ANOVA and post-testing with the Bonferroni multiple comparisons test relative to vehicle are represented as ***p< 0.001 or relative to CFA †††p< 0.001.

**Figure 6:** (A) Calcium flux in HEK-293 cells stably over-expressing either human TRPM8, mouse *Trpm8*, or rat *Trpm8* and treated with icilin (20 µM), menthol (0.5 mM), CFA (2.3 mg/mL/180 µg/cm²), CaO (2.3 mg/mL/180 µg/cm²), or CaCl₂ (2.3 mg/mL/180 µg/cm²). Data are normalized to hTRPM8-OE responses and represented as the mean ± SEM (n=6). Statistically significant responses were determined using a 1-way ANOVA and post-testing with Bonferroni multiple comparisons test relative to hTRPM8-OE ***p< 0.001 or to CFA †††p< 0.001. Quantification of (B) *Il-6*, (C) *Cxcl1* and (D) *Cxcl2* mRNA in lugs of wild-type C57/Bl6 and *Trpm8*/- mice treated via oropharyngeal aspiration (25 µL) with either ice-cold saline or CFA (10 mg/kg in saline) for 4h. Data are represented as mean ± SEM (n=6). Statistically significant responses using 2-way ANOVA and post-testing with the Bonferroni multiple comparisons test relative to vehicle are represented as *p<0.05 and **p< 0.01. No statistically significant differences were observed for comparisons between the saline and CFA groups as a function of genotype, indicated as p>0.05.

**Figure 7:** (A) Amino acid sequence alignment of human (NM_024080), mouse (NM_134252) and rat TRPM8 (NM_134371). The alignment and text graphics were generated using Clustal
Omega software. (B) Calcium flux elicited by CFA (2.3 mg/mL/180 µg/cm²), and menthol (500 µM) on GCaMP6-overexpressing cells transiently transfected with either wild-type human (menthol n=35, CFA n=32) or mouse (menthol n=6, CFA n=6) or the mouse-to-human mutants S921G (menthol n=14, CFA n=16), S927A (menthol n=17, CFA n=11) and S932T (menthol n=14, CFA n=10). Data are represented as mean ± SEM. Statistically significant responses using 2-way ANOVA and post-testing with the Dunnett’s multiple comparisons test relative to mouse TrpM8-WT (*p<0.05).
Table 1. Elemental analysis of CFA and filtered CFA and TRPM8-OE activation by micron and nano mineral oxide particles.

<table>
<thead>
<tr>
<th>Element</th>
<th>% Abundance in CFA</th>
<th>hTRPM8 Activity (% 50µM icilin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFA &lt;150 µm</td>
<td>Filtered &lt;0.22 µm</td>
</tr>
<tr>
<td>Na</td>
<td>4.01</td>
<td>2.18</td>
</tr>
<tr>
<td>Mg</td>
<td>1.96</td>
<td>0.00</td>
</tr>
<tr>
<td>Al</td>
<td>22.0</td>
<td>0.00</td>
</tr>
<tr>
<td>Si</td>
<td>45.6</td>
<td>0.00</td>
</tr>
<tr>
<td>S</td>
<td>1.61</td>
<td>0.00</td>
</tr>
<tr>
<td>K</td>
<td>3.28</td>
<td>0.00</td>
</tr>
<tr>
<td>Ca</td>
<td>12.1</td>
<td>97.8</td>
</tr>
<tr>
<td>Ti</td>
<td>2.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Fe</td>
<td>7.47</td>
<td>0.00</td>
</tr>
<tr>
<td>Ce</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Ni</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Zn</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.T. = not tested; N.D. = not detected; *Statistically different from vehicle controls (p<0.05) using 2-way ANOVA, and post-testing using the Bonferroni multiple comparisons test.
Table 2: Summary of TRPM8 mutant responses to chemical agonists and CFA, compared to human TRPM8-WT or mouse-WT

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Icilin</th>
<th>Menthol</th>
<th>CFA</th>
<th>Description/Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>hΔ801</td>
<td>↓</td>
<td>↓</td>
<td>=</td>
<td>Lung epithelial variant</td>
</tr>
<tr>
<td>hY745D</td>
<td>↓</td>
<td>↓</td>
<td>=</td>
<td>Menthol/icilin binding site</td>
</tr>
<tr>
<td>hR1008Q</td>
<td>N.T.</td>
<td>↓</td>
<td>↓</td>
<td>PIP₂ binding, cold sensitivity</td>
</tr>
<tr>
<td>hL1009R</td>
<td>N.T.</td>
<td>↓</td>
<td>↓</td>
<td>PIP₂ binding, cold sensitivity</td>
</tr>
<tr>
<td>mS921G</td>
<td>N.T.</td>
<td>=</td>
<td>↑</td>
<td>Mouse to human substitution</td>
</tr>
<tr>
<td>mS927S</td>
<td>N.T.</td>
<td>=</td>
<td>↑</td>
<td>Mouse to human substitution</td>
</tr>
<tr>
<td>mS932S</td>
<td>N.T.</td>
<td>=</td>
<td>=</td>
<td>Mouse to human substitution</td>
</tr>
</tbody>
</table>

N.T. = not tested
Figure 1:

A.

```
<table>
<thead>
<tr>
<th></th>
<th>C FA</th>
<th>C FA2</th>
<th>Icilin</th>
<th>Menthol</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COLD (4.0°C)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WARM (37.0°C)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

B.

```
<table>
<thead>
<tr>
<th></th>
<th>TRPM8-WT</th>
<th>TRPM8-WT+AMTB</th>
<th>TRPM8-Δ801</th>
<th>TRPM8-Δ801+AMTB</th>
<th>CV</th>
<th>CV+AMTB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcium Flux</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 2

A.

![Graph showing calcium flux as % of hTRPM8 WT for three treatments: CFA, Icilin, and Menthol. The graph compares hTRPM8 and Y745H proteins.]

B.

![Graph showing calcium flux as % of hM8 WT for four treatments: Menthol, CFA, R1008Q, L1009R, and CV proteins. The graph includes the hM8 WT, R1008Q, and L1009R treatments.]

Figure 3:

CFA

Filtered CFA (0.22 µm)
Figure 4:

A. 

![Bar chart showing calcium flux (% ilulin) for icillin, CFA, and CFA dialyzed.]

B. 

![Bar chart showing calcium concentration (µg/mL) for CaO, CalPhos, and hydroxyapatite at different particle concentrations.]

C. 

![Bar chart showing calcium flux (% ilulin) for icillin, CaO, CalPhos, CFA, and hydroxyapatite with 0.5 mM and 0.25 mM concentrations.]

D. 

![Bar chart showing calcium flux (% positive control) for TRPM8, TRPV1, TRPV4, and TRPA1 with positive controls, CaCl₂, and CaSO₄.]
Figure 5:

A.

**BEAS-2B**

- IL-8 mRNA Relative Expression
- Vehicle, CFA 90 μg/cm², CFA 180 μg/cm²
- BEAS-2B, Scramble, TRPM8-KO

B.

**NHBE**

- CXCL1 mRNA Relative Expression
- Vehicle, CFA
- CFA, CFA + AMTB, CFA + LJO

C.

**NHBE**

- IL-8 mRNA Relative Expression
- Vehicle, CFA
- CFA, CFA + AMTB, CFA + LJO

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 6:

A.

B.

C.

D.

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 7:

A.

<table>
<thead>
<tr>
<th></th>
<th>S4</th>
<th>S5</th>
<th>900</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPM8_HUMAN</td>
<td>LRLIHIFTSRNLGPKIIMLQRMLIDVFFFLLFLFAVWMVAFCVARQGILRQNEQRWRWIF</td>
<td>900</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>TRPM8_MOUSE</td>
<td>LRLIHIFTSRNLGPKIIMLQRMLIDVFFFLLFLFAVWMVAFCVARQGILRQNEQRWRWIF</td>
<td>900</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>TRPM8_RAT</td>
<td>LRLIHIFTSRNLGPKIIMLQRMLIDVFFFLLFLFAVWMVAFCVARQGILRQNEQRWRWIF</td>
<td>900</td>
<td>----------------------------------------------------------------------</td>
</tr>
</tbody>
</table>

|                | RSVIYEPYLAMFGQVPSDVDTHCTFGNESKPLCVELDEHNLPREFEWITIPLV               | 960                                                                 |----------------------------------------------------------------------|
| TRPM8_HUMAN    | RSVIYEPYLAMFGQVPSDVDTHCTFGNESKPLCVELDEHNLPREFEWITIPLV               | 960                                                                 |----------------------------------------------------------------------|
| TRPM8_MOUSE    | RSVIYEPYLAMFGQVPSDVDTHCTFGNESKPLCVELDEHNLPREFEWITIPLV               | 960                                                                 |----------------------------------------------------------------------|
| TRPM8_RAT      | RSVIYEPYLAMFGQVPSDVDTHCTFGNESKPLCVELDEYNLPREFEWITIPLV               | 960                                                                 |----------------------------------------------------------------------|

|                | CIYMLSTNILLVNLVAMFGYTVGTVCENNQVVKFQRYFLVQECRNLIPEFIVFAY             | 1020                                                                |----------------------------------------------------------------------|
| TRPM8_HUMAN    | CIYMLSTNILLVNLVAMFGYTVGTVCENNQVVKFQRYFLVQECRNLIPEFIVFAY             | 1020                                                                |----------------------------------------------------------------------|
| TRPM8_MOUSE    | CIYMLSTNILLVNLVAMFGYTVGTVCENNQVVKFQRYFLVQECRNLIPEFIVFAY             | 1020                                                                |----------------------------------------------------------------------|
| TRPM8_RAT      | CIYMLSTNILLVNLVAMFGYTVGTVCENNQVVKFQRYFLVQECRNLIPEFIVFAY             | 1020                                                                |----------------------------------------------------------------------|

B.

The figure shows the calcium flux in human and mouse TRPM8 WT and mutant cells, comparing menthol and CFA treatments. The graph displays % calcium flux across different cell lines and treatments, with bars indicating statistical significance (p < 0.05).