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TRPA1 receptors mediate environmental irritant-induced meningeal vasodilatation

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Abstract

The TRPA1 receptor is a member of the transient receptor potential (TRP) family of ion channels expressed in nociceptive neurons. TRPA1 receptors are targeted by pungent compounds from mustard and garlic and environmental irritants such as formaldehyde and acrolein. Ingestion or inhalation of these chemical agents causes irritation and burning in the nasal and oral mucosa and respiratory lining. Headaches have been widely reported to be induced by inhalation of environmental irritants, but it is unclear how these agents produce headache. Stimulation of trigeminal neurons releases CGRP and substance P and induces neurogenic inflammation associated with the pain of migraine. Here we test the hypothesis that activation of TRPA1 receptors are the mechanistic link between environmental irritants and peptide mediated neurogenic inflammation. Known TRPA1 agonists and environmental irritants stimulate CGRP release from dissociated rat trigeminal ganglia neurons and this release is blocked by a selective TRPA1 antagonist, HC-030031. Further, TRPA1 agonists and environmental irritants increase meningeal blood flow following intranasal administration. Prior dural application of the CGRP antagonist, $CGRP_{8–37}$, or intranasal or dural administration of HC-030031, blocks the increases in blood flow elicited by environmental irritants. Together these results demonstrate that TRPA1 receptor activation by environmental irritants stimulates CGRP release and increases cerebral blood flow. We suggest that these events contribute to headache associated with environmental irritants.

Keywords

migraine; trigeminal ganglia; sensory neurons; blood flow; CGRP

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Introduction

Headache is one of the most common complaints following human exposure to environmental irritants and air pollutants. An increase in air pollutants has been correlated with an increase in the number of emergency room visits for headache symptoms and to their frequency and severity [33,47,46]. Furthermore, exposure to formaldehyde vapors, which has been linked to headache symptomology [40], may be on the rise due to the increased use of formaldehyde in manufacturing building materials [30]. However, how these substances induce headache attacks remains unknown.

The transient receptor potential A1 (TRPA1) receptor is an excitatory nonselective cation channel expressed in a subset of sensory neurons believed to be involved in many forms of acute and chronic hyperalgesia [34,5]. Initially reported to sense noxious cold [45], TRPA1 receptors have subsequently been shown to act as sensors of many environmental irritants, including acrolein [5], formaldehyde [27] and zinc [19], pungent plant ingredients such as mustard oil [4,5], cinnamaldehyde [4], and allicin [6,5], and endogenously produced substances such as hydrogen peroxide [2] and 4-hydroxynonenal [50]. In TRPA1-deficient mice, pain related behavioral responses to TRPA1 agonists are abolished, further supporting a role for TRPA1 receptors in mediating certain painful sensations [5,23,25].

TRPA1 receptors are often colocalized with the inflammatory peptides substance P and calcitonin gene-related peptide (CGRP) in primary sensory neurons [45,6] and neuronal activation leads to release of these peptides [21] producing neurogenic inflammation and vasodilatation in the dura [31,42]. The release of CGRP from trigeminal root ganglia (TRG) neurons innervating the dura and cerebral blood vessels is widely thought to play an important role in migraine headaches [14,15,13], however a role for CGRP release in environmental irritant induced headache remains speculative. Here we tested the hypothesis that environmental irritants activate the trigeminovascular system via TRPA1 receptors. We show that TRPA1 agonists and environmental irritants induce release of CGRP from isolated neurons which is blocked by TRPA1 selective antagonists. We further show that intranasal administration of TRPA1 agonists or environmental irritants increases meningeal blood flow *in vivo*, which is blocked by a CGRP antagonist or TRPA1 receptor antagonist. These results suggest that headaches associated with exposure to environmental irritants may result from activation of TRPA1 receptors in the trigeminovascular system which triggers neurogenic inflammation in the dura.

2. Methods

All animal procedures were approved by the Animal Care and Use Committee at the Indiana University School of Medicine and followed the ethical guidelines of International Association for the Study of Pain [54]. Experiments were performed on 130 male (150–250 g) Sprague-Dawley rats (Harlan Bioproducts, IN).

2.1 TRG cell culture

Trigeminal root ganglia neurons were isolated and grown in culture as previously described [53]. Briefly, rats were rendered unconscious with carbon dioxide before cervical dislocation. For each experiment, trigeminal ganglia from 5–7 rats were dissected from the bony base of the brain, rinsed in ice-cold Puck's saline (Ca^{2+} free, Mg^{2+} free) and minced. The ganglia were pooled and single cells isolated by exposure to Puck's saline containing collagenase (775 units/ml, Worthington) and dispase II (0.24%, Roche, IN) for 50 minutes in a 37°C water bath. After washing, cells were resuspended in F12 medium (Gibco, CA) containing 10% fetal calf serum (Gibco) 2 mM glutamine, 100 μg/ml penicillin and streptomycin and 50 ng/ml NGF (Harlan Bioproducts) and maintained at 37°C and 5% CO_2.

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The contribution of non-neuronal cells was reduced by adding 50 µM 5-fluoro-2’-deoxyuridine (Sigma, MO) and 150 µM uridine (Sigma) to the media. Cells were plated onto 12 well plates coated with polylysine and laminin (Sigma). The F12 growth medium was changed every other day and the cells used for release studies 7–10 days after plating. We utilized trypan blue exclusion to ensure that similar numbers of viable cells were present in all wells within an experiment [53]. Cultures contained 1800–2500 viable cells/well at the time the release studies were performed.

2.2 CGRP release studies

The protocol for release studies followed published reports [52,53] and were performed at 37°C. Prior to the release experiments, cells were washed three times in freshly prepared HEPES buffer containing 25 mM HEPES, 140 mM NaCl, 3.5 mM KCL, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 3.3 mM D-glucose, and 1% bovine serum albumin (pH 7.4). Cells were then exposed to three successive 10 minute incubations in the absence or presence of test compounds to assay their effect on CGRP release. After the third incubation, cells were exposed to HEPES buffer alone to re-establish basal release. The supernatants (400 µl) were collected following each 10 minute interval and CGRP content was measured by radioimmunoassay. Some TRG cultures were pretreated with the TRPV1 antagonist capsazepine (10 µM, Sigma) or the TRPA1 selective antagonist, HC-030031 (50 µM, Tocris Bioscience) prior to stimulation with capsaicin, formaldehyde, cinnamaldehyde, acrolein, or mustard oil (Sigma) dissolved in HEPES buffer.

2.3 CGRP radioimmunoassay

Total immunoreactive CGRP released into the buffer was determined by radioimmunoassay as previously described [52,53]. Briefly, 25 µl of rabbit polyclonal CGRP antibody (1:130,000 dilution, kindly provided by Dr. Michael Iadorola, NIH, Bethesda, MD) and 25 µl of [^{125}I] [Tyr^0] (28–37) containing 3000–5000 cpm were added to each standard and sample and incubated overnight at 4°C. Tracer not bound to antibody was removed from tracer bound to antibody by adding 0.5 ml of a 0.1 M phosphate buffer (pH 7.4) containing 1% Norite charcoal, 50 mM NaCl, and 0.1% bovine serum albumin. Following centrifugation at 1000 × G for 20 minute at 4°C, the supernatant was decanted and radioactivity measured by gamma scintillation spectrometry. The amount of CGRP in unknown samples was estimated by comparing the percent bound radioactivity in unknowns to a standard curve using a four-point nonlinear least squares regression analysis. Using this method, the minimal detectable amount of CGRP was 0.5 fmol.

2.4 Laser Doppler flowmetry

Male rats were anesthetized with ketamine/xylazine (80 and 10 mg/kg body weight, respectively), followed by additional doses of ketamine/xylazine (40 and 5 mg/kg body weight) as needed. The animal’s body temperature was maintained at 37°C with a homeothermic blanket. In some cases, the trachea was cannulated and the animal allowed to breathe room air spontaneously. For the measurement of meningeal blood flow, the animals head was fixed in a stereotaxic frame and a cranial window prepared [22] with the dura left intact. Dural blood flow was measured with a laser Doppler flowmeter (TSI, MN). A needle type probe was placed over a large branch of the middle meningeal artery (MMA), distant from visible cortical blood vessels and the cranial window kept moist with synthetic interstitial solution (SIF) consisting of: 135 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose (pH 7.3). Blood flow was recorded on-line at a frequency of 1Hz using Axoscope software (Axon Instruments, CA).

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2.5 Blood flow drug administration

The CGRP antagonist h-α$CGRP_{8-37}$ (Sigma) was topically administered to the dural surface (50 μl) at a concentration of 1 μM in SIF. HC-030031 was dissolved in SIF to 50 μM for nasal or dural administration. To stimulate the nasal mucosa, 50 μl of test compound or vehicle solution was applied over a 30 sec period at a site 2 mm into the right nostril using a Pipetman pipette [18]. Solutions of acrolein (30 μM), mustard oil (100 μM), and capsaicin (100 nM) were diluted in SIF and prepared fresh daily. A thirty minute stabilization period preceded all experimental procedures to ensure stable blood flow measurements. SIF was applied to the dura or nasal mucosa as a control in all experiments 15 minutes prior to drug application and had no effect on meningeal blood flow.

2.6 Data collection and statistics

For blood flow experiments, data was collected at 1 Hz and binned by averaging 60 samples (1 minute intervals) for statistical analysis or 10 samples (10 sec intervals) for graphical representation. Basal blood flow was determined as the mean flow rate measured during a 3 minute period prior to drug application and the effects of test compounds were calculated by comparing the peak response within three minutes of administration to the average blood flow in the three minutes preceding administration. Changes in blood flow for each animal were calculated, averaged within treatment groups and expressed as percentage changes relative to the basal blood flow. Comparison of blood flow changes was performed using an unpaired Student's t-test. For CGRP release studies data from three to five independent experiments were pooled and analysis of variance and Bonferroni posthoc test were used to determine significance. Graphical presentation and statistical analysis was performed using GraphPad Prism 4 software (GraphPad, CA). Data values are presented as means ± SEM. The significance level for all tests was set at $p < 0.05$.

3. Results

3.1. TRP agonists induce CGRP release from trigeminal neurons

CGRP released from trigeminal neuron terminals in the dura is believed to be involved in vascular responses leading to headache. Thus we first examined the effects of TRPA1 and TRPV1 agonists on release of CGRP from rat trigeminal neurons grown in culture. As seen in Fig 1A, exposing trigeminal ganglion neurons to the TRPA1 agonist cinnamaldehyde (300 μM) or the environmental irritant, acrolein (30 μM) induced a robust increase in CGRP release. Formaldehyde (500 μM) and mustard oil (100 μM) also induced large increases in CGRP release respectively (Fig 1B), suggesting that both prototypic TRPA1 agonists and known environmental irritants are capable of eliciting CGRP release from trigeminal neurons in culture. As both TRPA1 and TRPV1 receptors are expressed in trigeminal neurons [6] we confirmed that capsaicin (100 nM), the prototypic TRPV1 agonist induced an increase in CGRP release as had been previously shown [37] (Fig 1C). The capsaicin induced release of CGRP was significantly reduced in the presence of capsazepine (Fig 1C). In contrast, capsazepine did not affect the release of CGRP by mustard oil (Fig 1D). Finally, stimulated release of CGRP by mustard oil was completely abolished in Ca^{2+}-free release buffer. These results suggest that TRPA1 agonists stimulate release of CGRP which is calcium dependent, but not through TRPV1 receptors.

The results of the CGRP release studies are summarized in Fig 2. Stimulated release of CGRP was normalized to the basal release in the first 10 minute incubation interval for each sample and averaged by treatment. Bars represent stimulated CGRP release and are expressed as mean ± SEM. In some cases, multiple concentrations of agonists were tested in separate experiments. All agonists induced a significant increase in CGRP release when compared to basal release ($p < 0.05$). These results demonstrate that TRPA1 agonists and

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environmental irritants are capable of inducing CGRP release from trigeminal neurons to levels comparable to the TRPV1 agonist, capsaicin.

To confirm that the stimulated release of CGRP by TRPA1 agonists and environmental irritants was mediated through TRPA1 receptors, we next tested the ability of the selective TRPA1 antagonist, HC-030031 [27] to block the agonist effects (Fig 3). Pretreatment with HC-030031 (50 μM), significantly reduced the mustard oil (Fig 3A) and acrolein (Fig 3B) induced release of CGRP, while having no effect on basal release. In contrast, HC-030031 did not alter capsaicin induced release of CGRP (Fig 3C). These results indicate that the release of CGRP *in vitro* due to mustard oil and the environmental irritant, acrolein is mediated through TRPA1 receptors.

3.2. Nasal administration of TRPV1 and TRPA1 agonists induce increased meningeal blood flow

Representative traces of meningeal blood flow changes in response to nasal application of TRP agonists are shown in Fig 4. Capsaicin (100 nM), mustard oil (100 μM) and acrolein (30 μM) each induced over a two-fold increase in peak blood flow (Fig 4A, C, E respectively). The blood flow changes were rapid and generally of short duration peaking within the first 2–3 minutes before returning toward basal values within 10 – 15 min.

To determine whether CGRP release is involved in TRP agonist induced blood flow changes, the CGRP antagonist $CGRP_{8–37}$ (1 μM) was applied to the dura 3 minutes prior to nasal agonist administration. To eliminate the effects of prior agonist administration on blood flow responses (i.e., desensitization of receptor function or neurotransmitter depletion) separate animals were used for this set of experiments. As show in Fig 4B, D and F, $CGRP_{8–37}$ significantly blunted the blood flow increases induced by all 3 TRP agonists. Dural administration of $CGRP_{8–37}$ alone had no effect on recorded blood flow (−5 ± 4%; n = 10), compared to SIF only application (−1 ± 2%; n = 14) suggesting that CGRP has minimal tonic influence on blood flow. Meningeal blood flow was likewise not altered by nasal administration of SIF (2 ± 3%; n = 16) or SIF containing 0.1% ethanol (0.3 ± 7%; n = 4).

The summarized data comparing agonist induced blood flow changes in the presence or absence of $CGRP_{8–37}$ are shown in Fig. 5. Mustard oil, acrolein and capsaicin all increased blood flow that was blocked by the CGRP receptor antagonist, $CGRP_{8–37}$. These results demonstrate that both TRPA1 and TRPV1 agonists increase meningeal blood flow and may do so through a common mechanism of inducing CGRP release.

To confirm that the *in vivo* blood flow changes were mediated through TRPA1 receptors, we tested the effects of HC-030031 on the ability of acrolein to stimulate blood flow (Fig 6). Intranasal administration of HC-030031 (50 μM) 3 minutes prior to the combined intranasal administration of acrolein (30 μM) and HC-030031 significantly reduced the TRPA1 agonist induced blood flow increase previously observed (Fig 6B). The large blood flow changes induced by acrolein (103 ± 25%, n = 7) were dramatically attenuated in the presence of HC-030031 (7 ± 8%; n = 5). Similar results were observed when HC-030031 was placed on the dura (Fig 6C). Dural application of HC-030031 prior to intranasal administration of acrolein significantly attenuated the TRPA1 agonist affects on blood flow (22 ± 8%, n = 8), though less so than intranasal HC-030031 application. Basal meningeal blood flow was not altered by nasal administration of HC-030031 (8 ± 3%, n = 6), but was slightly reduced by dural application (−7 ± 1%, n = 8). Together these results suggest that TRPA1 receptors mediate the increased blood flow *in vivo* in response to TRPA1 agonists.

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4.0 Discussion

Headache is the most common complaint related to indoor and outdoor air pollution. Despite this association, the mechanism by which environmental irritants induce headache pain has not been elucidated. Previous studies on the health risks associated with pollution have focused primarily on respiratory ailments, such as asthma or allergy. Asthma and allergic rhinitis symptoms exhibited after environmental irritant exposure are the consequence of neurogenic inflammation in the respiratory tract [28]. Neurogenic inflammation in the dura is also considered an important mediator of migraine pain. Recent evidence suggests a connection between TRP receptors and the neuronal and inflammatory actions of chemical irritants in asthma, rhinitis and other respiratory ailments [36]. For example, TRPV1 receptors have been described as hyper-sensitive in patients suffering from Multiple Chemical Sensitivity [49], a disorder linked to chemical exposure with multi-organ symptoms including respiratory symptoms and headache [10]. TRPA1 receptors are activated by environmental irritants including acrolein [5], formaldehyde [27], toluene [48] and tear gas [7] and neurogenic inflammation of the lungs resulting from cigarette smoke has been linked to activation of TRPA1 receptors [3]. TRPA1 receptors have also been proposed to be “key integrators” of the immune and neuronal actions in asthma [9]. Based on these links between TRPA1 receptors, environmental irritants and neurogenic inflammation we hypothesize that environmental irritants induce headache by activation of TRPA1 receptors on trigeminal neurons, triggering neuronal excitation and subsequent neurogenic inflammation.

Neurogenic inflammation of the meninges has been widely used as a marker for activation of the trigeminovascular system in animal models of migraine. The particular role of CGRP in migraine has recently been highlighted by clinical trials demonstrating the efficacy of CGRP antagonists in migraine [35]. Therefore we have examined two components of neurogenic inflammation (CGRP release and blood vessel dilatation) to determine whether TRPA1 agonists and environmental irritants activate the trigeminovascular system. Earlier studies had demonstrated that TRPA1 receptors are present in a subset of sensory trigeminal neurons which also express TRPV1, CGRP or Substance P [20,45]. In addition, TRPA1 agonists stimulate CGRP release from spinal cord, esophageal sensory neurons [50], rat hindpaw skin [41] and dorsal root ganglion neurons [38], but TRPA1 related CGRP release has not previously been demonstrated from trigeminal neurons, the cells important in headache. Our data fills this gap by demonstrating that environmental irritants elicit CGRP release from trigeminal neurons and that this release is mediated by TRPA1 receptors as a TRPA1 antagonist attenuates the response.

We have also demonstrated that dilatation of meningeal blood vessels occurs after intranasal administration of TRPA1 agonists and environmental irritants. This dilatation is blocked by an antagonist of TRPA1. Furthermore our data indicate that the vasodilation is induced by CGRP release from perivascular sensory nerve terminals since it can be blocked by topical administration of the CGRP antagonist, $CGRP_{8-37}$. Thus our data supports the notion that TRPA1 receptors mediate increases in meningeal blood flow by environmental irritants. Previous studies support this hypothesis as neurogenic inflammation occurs in the nasal cavity after acrolein [32] and asphalt fume exposure [43].

Anecdotally, the use of garlic and other plants from the Allium family has been recommended as a natural treatment for hypertension for many years. Indeed, a vasorelaxant effect in mesenteric blood vessels in response to TRPA1 agonists has been reported [6]. It was further shown that the vasorelaxant effect was attenuated in the presence of $CGRP_{8-37}$ or ruthenium red but not capsazepine. In addition, it has been reported that the odorants

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propionic acid, cyclohexanone and amyl acetate increase cortical blood flow [26] via TRPV1 or other as yet unidentified receptors [44].

Our results demonstrating meningeal vasodilatation with capsaicin are in agreement with other reports of vasodilatation in response to dural or i.v. administration of capsaicin which is attenuated by $CGRP_{8-37}$ [16,1]. Gottselig and Messlinger [18] suggested a role for parasympathetic pathways in vasodilatation after nasal administration of capsaicin since the effect could be blocked by a vasoactive intestinal polypeptide antagonist. We cannot rule out a small role for parasympathetic mediators in capsaicin stimulated blood flow as 1 μM $CGRP_{8-37}$ attenuates but does not completely abolish the effects of 100 nM capsaicin. Differences in experimental protocol and drug concentrations between this study and ours make it difficult to draw direct comparisons. Although parasympathetic pathways may be triggered by capsaicin and environmental irritants as symptoms include lacrimation and rhinorrhea, trigeminal sensory pathways appear to play the biggest role in environmental irritant-induced vasodilatation in this series of experiments.

We presume that the site of action of air-borne environmental irritants is TRPA1 receptors on trigeminal nerve terminals in the epithelium of nasal mucosa. The expression of TRPV1 receptors in trigeminal neurons innervating the nasal mucosa has been demonstrated by receptor binding [39] and immunocytochemical studies [12,11]. It is likely that TRPA1 receptors are also present in trigeminal neurons innervating the nasal mucosa because TRPA1 receptors are usually expressed in a large subset of TRPV1 expressing sensory neurons, but this remains to be explicitly demonstrated in our system. Environmental irritants such as acrolein are highly reactive compounds and usually present in low concentrations when inhaled. It seems unlikely that they can accumulate in their original chemical structure at sufficient concentrations to activate TRPA1 receptors at a distance from the original site of exposure, further suggesting a nasal site of action.

Although the initial exposure to air-borne environmental pollutants is the nasal and respiratory epithelium, it is not certain how this leads to meningeal blood vessel dilatation. As topical (dural) administration of CGRP antagonist blocks the effects of nasally administered TRPA1 agonists on blood flow we presume that the vasodilatation is mediated by CGRP release from perivascular nerve terminals within the meninges. Activation of trigeminal neurons innervating the nasal mucosa may propagate the signal to the meninges via trigeminal nerve collaterals as trigeminal neurons innervating the nasal cavity have collaterals projecting back into the cranium [17]. Intraganglionic transmission [51] may transmit the signal from nasal innervating neurons to nearby trigeminal neurons which innervate the meninges. Alternatively, a hypothesis of ‘neurogenic switching’ has been proposed to explain the neurogenic inflammation at a peripheral site distant from the initial site of exposure [29] via activation of a pathway ascending to the central nervous system and then back down to produce inflammation. These are questions that remain to be resolved.

In summary, our results demonstrate that TRPA1 receptor activation by environmental irritants stimulates CGRP release from trigeminal neurons and increases cerebral blood flow and may contribute to headache associated with environmental irritants. However interpretation of our data in the clinical context is not straightforward as human exposure to airborne irritants is generally to lower concentrations and of longer duration before the appearance of symptoms. Headache and other symptoms associated with air pollution are a significant and increasing health burden, so understanding the mechanism of environmental-irritant induced headache may have a significant impact on public health. Although migraine triggers are not well-understood [24] in some cases migraine may be initiated or aggravated by odors or irritants [8]. Thus understanding how environmental irritants provoke headache may shed some light on migraine triggers as well.

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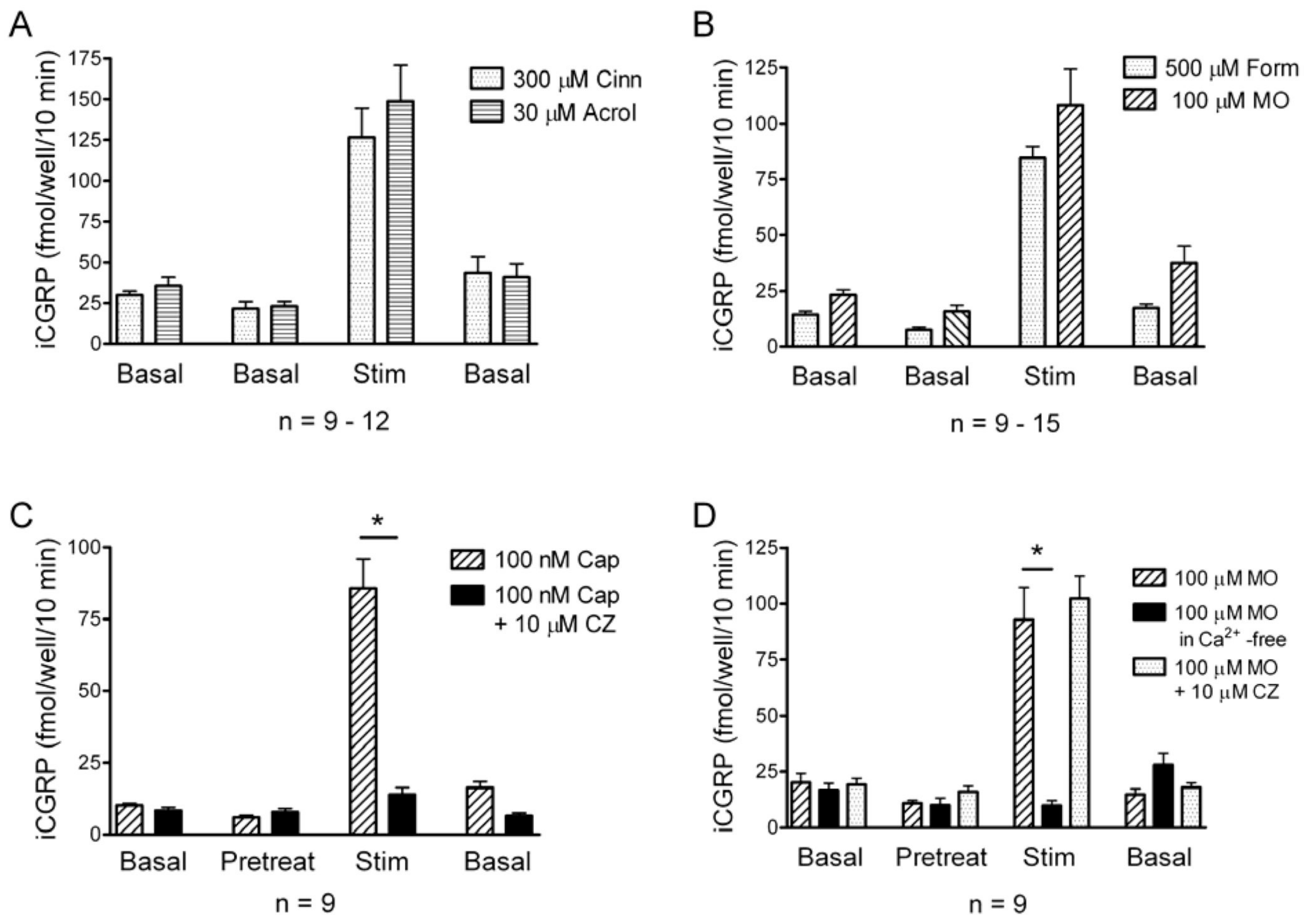

Fig 1.

Representative release experiments showing TRP channel agonists stimulate release of CGRP from adult rat trigeminal neurons in culture. Bars indicate immunoreactive CGRP (iCGRP) in the buffer measured after successive 10 minute incubations in the absence or presence of drugs and antagonist. Agonists were present during stimulus (Stim) incubations. The TRPV1 antagonist, capsazepine (CZ) was also present during pretreatment (Pretreat) in (black bar; C), but not during basal incubations. Similarly, TRG neurons were incubated in normal release buffer, Ca^{2+}-free release buffer or capsazepine respectively during pretreatment in (D). (A) Cinnamaldehyde (Cinn) and acrolein (Acrol) stimulate release of CGRP. (B) Formaldehyde (Form) and mustard oil (MO) also stimulate CGRP release. (C) Capsaicin (Cap) stimulates release of CGRP that is significantly blocked by capsazepine. (D) Mustard oil stimulates release of CGRP which is calcium dependent, but not through TRPV1 receptors. Data is presented as mean ± SEM and the number of samples per condition is indicated. *$p < 0.05$ compared to release in the presence of antagonist or in the presence of Ca^{2+}-free buffer.

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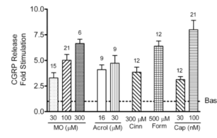

Fig 2.

Effects of TRP agonists on CGRP release from trigeminal neurons grown in culture. Stimulated CGRP release was normalized to the first basal release incubation sample for each sample and averaged across treatments in 3–7 independent experiments. Data is presented as the mean ± SEM and tested for significant differences ($p < 0.05$) using a one sample Student's t-test. In all conditions tested, CGRP release was significantly increased during stimulus incubation compared to the respective basal release values. The number of samples tested for each condition is indicated.

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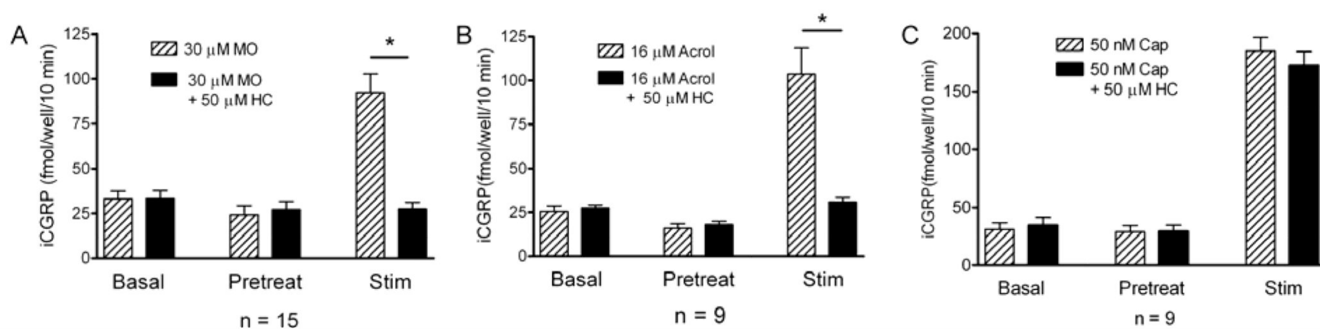

Fig. 3.

Representative experiments showing TRPA1 receptor antagonists blocks release of CGRP stimulated by TRPA1 agonists but not TRPV1 agonists from adult rat trigeminal neurons in culture. Pretreatment (Pretreat) with the selective TRPA1 antagonist HC-030031 (HC) significantly blocks the CGRP release induced by mustard oil (MO)(A) and acrolein (Acrol) (B) but not by capsaicin (Cap) (C). Data is presented as mean ± SEM. The number of samples per condition is indicated. *$p < 0.05$ compared to release in the presence of antagonist.

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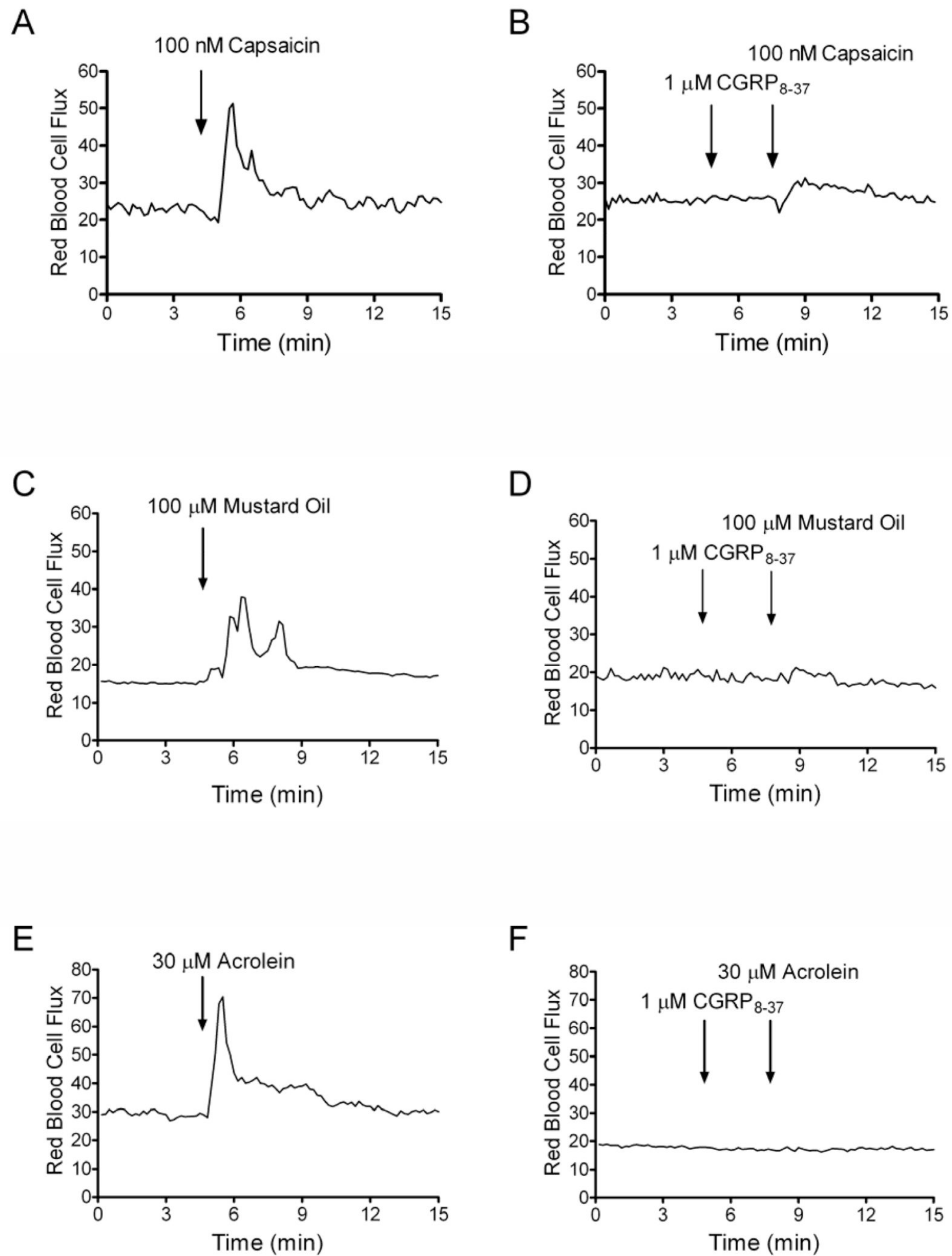

Fig 4.

Representative traces of middle meningeal blood flow changes in response to nasally administered capsaicin (A, B) mustard oil (C, D) or acrolein (E, F) in the absence (A, C, E) or presence (B, D, F) of the CGRP antagonist, $CGRP_{8-37}$ applied to the dura. Laser Doppler flowmetry measurements were collected at 1 Hz and binned by averaging every 10 samples for graphical representation. Nasal application of capsaicin, mustard oil or acrolein induced a rapid and robust increase in meningeal blood flow which returned toward baseline values within minutes. Dural application of $CGRP_{8-37}$ prior to nasal drug administration blocked the blood flow changes. Arrows indicate administration of agonist or antagonist.

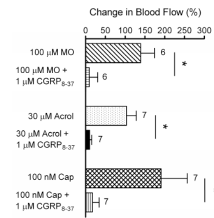

Fig 5.

Blood flow changes in the middle meningeal artery following nasal administration of mustard oil (MO), acrolein (Acrol) or capsaicin (Cap) in the absence or presence of previous administration of $CGRP_{8-37}$ to the dura. Values are mean ± SEM. *$p < 0.05$ compared to blood flow changes in the presence of antagonist (unpaired Student's t-test). TRP agonist induced blood flow changes are significantly reduced in the presence of the CGRP antagonist. The number of animals tested is indicated.

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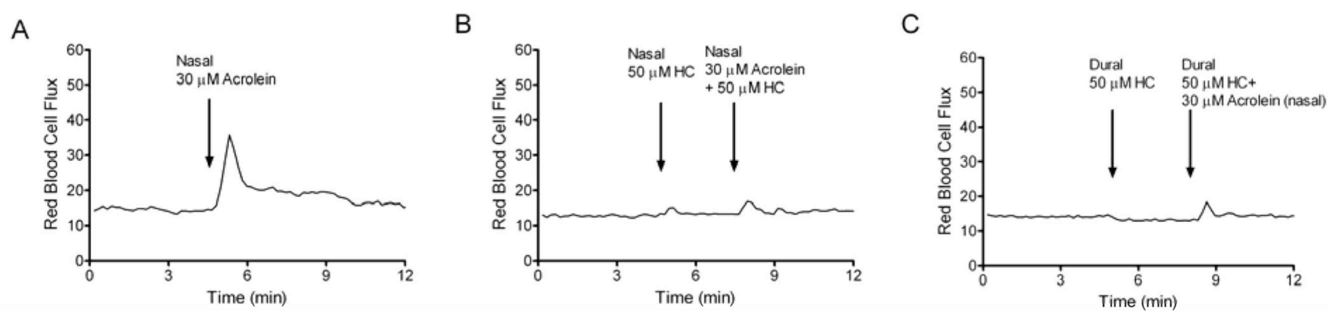

Fig 6.

Representative experiments showing TRPA1 channel antagonist, HC-030031 (HC) block of blood flow changes *in vivo* (A–C). Acrolein significantly increases meningeal blood flow (A) while nasal administration of HC-030031 prior to nasal administration of acrolein blocks changes in middle meningeal blood flow (B). A similar result was observed when HC-030031 was applied to the dura prior to intranasal acrolein exposure (C).

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