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Homologous and Heterologous Desensitization of Capsaicin and Mustard Oil Responses Utilize Different Cellular Pathways in Nociceptors

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Abstract

The Transient Receptor Potential channel subtypes V1 (TRPV1) and A1 (TRPA1) play a critical role in the development of hyperalgesia in inflammatory pain models. Although several studies in animals and humans have demonstrated that capsaicin (CAP), a TRPV1-specific agonist, and mustard oil (MO), a TRPA1 agonist, evoke responses that undergo functional cross-desensitization in various models, the mechanisms mediating this phenomenon are largely unknown. In the present study, we evaluated the mechanisms underlying homologous and heterologous desensitization between CAP and MO responses in peripheral nociceptors using an *in vitro* neuropeptide release assay from acutely isolated rat hindpaw skin preparation and *in vivo* behavioral assessments. The pretreatment with CAP or MO significantly inhibited (50–60%) both CAP- and MO-evoked CGRP release indicating homologous and heterologous desensitization using this assay. Further studies evaluating the requirement of calcium in these phenomena revealed that homologous desensitization of CAP responses was calcium-dependent while homologous desensitization of MO responses was calcium-independent. Moreover, heterologous desensitization of both CAP and MO responses was calcium-dependent. Further studies evaluating the role of calcineurin demonstrated that heterologous desensitization of CAP responses was calcineurin-dependent while heterologous desensitization of MO responses was calcineurin-independent. Homologous and heterologous desensitization of CAP and MO was also demonstrated using *in vivo* behavioral nocifensive assays. Taken together, these results indicate that TRPV1 and TRPA1 could be involved in a functional interaction that is regulated via different cellular pathways. The heterologous desensitization of these receptors and corresponding inhibition of nociceptor activity might have potential application as a therapeutic target for developing novel analgesics.

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Keywords

TRPV1; TRPA1; nociceptor; capsaicin; mustard oil; trigeminal; pain

1. Introduction

The transient receptor potential channel subtypes V1 (TRPV1) and A1 (TRPA1) are expressed on sensory neurons and respond to changes in temperature, protons, or local application of certain noxious chemicals such as capsaicin (CAP) or mustard oil (MO) (Moran et al. 2004; Numazaki and Tominaga 2004; Ramsey et al. 2006). Both of these channels also play a critical role in the development and maintenance of hyperalgesia in acute as well as chronic inflammatory pain models (Bautista et al. 2006; Caterina et al. 2000; Obata et al. 2005).

In dorsal root (DRG) and trigeminal (TG) ganglia, the expression of TRPA1 is largely restricted to the TRPV1-positive sub-population of sensory neurons (Obata et al. 2005; Story et al. 2003). Studies in animals and humans demonstrated that MO, like CAP, responses undergo functional homologous desensitization in several physiological and pathophysiological models (Brand and Jacquot 2002; Jacquot et al. 2005; Patacchini et al. 1990; Simons et al. 2003). Moreover, studies in humans have demonstrated cross-desensitization between CAP and MO responses in oral irritation models (Simons et al. 2003). Nevertheless, the molecular mechanisms underlying pharmacological desensitization of MO responses and cross-desensitization between CAP and MO responses in nociceptors are unknown.

Given that desensitization to capsaicin can also lead to a reduction in responsiveness of certain nociceptors to other noxious stimuli (Szallasi and Blumberg 1996; Szallasi and Blumberg 1999), and that mustard oil can evoke the sensation of pain, the goal of the current study was to determine whether capsaicin pretreatment heterologously desensitized mustard oil responses and vice versa as measured by reduced nociceptor activities and behavioral measurements. Further, given the findings that pharmacological desensitization of capsaicin responses is triggered by increased intracellular calcium levels (Bhave et al. 2002; Docherty et al. 1996; Mohapatra and Nau 2005; Tominaga and Tominaga 2005) and that TRPA1 is a Ca^{2+} permeable channel (Bandell et al. 2004; Jordt et al. 2004; Macpherson et al. 2005), we used an *in vitro* model to evaluate the role of Ca^{2+} -dependent pathways in the homologous and heterologous desensitization of CAP and MO responses.

2. Methods

2.1. Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 250–300 gm were used in these studies. All animal study protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and conformed to the International Association for the Study of Pain and U.S. government guidelines. Animals were housed for 1 week before the experiment with food and water available *ad libitum*.

2.2. Neuropeptide Release Assay

The release of iCGRP from isolated hindpaw skin biopsies was evaluated using a previously described method (Kilo et al. 1997) with the following modifications: the same sized skin biopsies/tissue was dissected from glabrous rat hind paws and simply immersed into wells containing various treatment conditions in a modified Hank's buffer. Capsaicin (Fluka, St. Louis, MO) stock prepared in 100% ethanol, mustard oil (10 M stock solution; Sigma, St Louis,

MO) and calcineurin auto-inhibitory peptide (CAIP) (Calbiochem, San Diego, CA) were diluted with Hanks buffer for the assays. In brief, rats were sacrificed and the hindpaw skin was dissected and pooled in standard modified Hank's buffer containing 10.9 mM HEPES, 4.2 mM sodium bicarbonate, 10 mM dextrose, and 0.1% bovine serum albumin (pH adjusted to 7.4; osmolality 303 mOsm) before subjecting them to various treatments. For the MO concentration-response experiment, 2 biopsies/well, each weighing 20 mg, were immersed into 24 well plates containing 1.2 ml of 1X Hanks buffer (37°C) (described above) for 30 min, and two 20 min baseline samples were collected, followed by 2 min application of a selected concentration of MO (0.001%–10%) or vehicle. The total evoked CGRP release was measured by pooling the 2 min MO exposure sample with an 18 min vehicle post-exposure sample. Biopsies were only used once and only exposed to one treatment condition.

For the desensitization experiments, 2 biopsies/well were placed into 24 well plates containing 1.2 ml of Hanks buffer (37°C), and, following a wash period and collection of two 20 min baseline samples, the skin biopsies were exposed for 20 min to either vehicle, 0.1% mustard oil, or 100µM capsaicin. The skin was then exposed to a 10 min vehicle washout period which was followed by a 2 min application of either 0.1% mustard oil or 100µM capsaicin. As before, the total evoked CGRP release was measured by pooling the 2 min exposure sample with a subsequent 18 min vehicle exposure sample. Biopsies were only used once.

Following the desensitization protocol (described above), the biopsies were evaluated for their responsiveness to a subsequent application of a depolarizing concentration of elevated potassium concentration. In these experiments, the biopsies first underwent the depolarization treatment described above and then exposed to two 20 recovery fractions each containing Hank's buffer; the biopsies were then stimulated by a 20 min application of a modified Hank's buffer containing high potassium that contained 5 mM KCl, 2.5 mM CaCl₂, 1.2mM MgCl₂, 135 mM NaCl, 1 mM Na₂HPO₄, 0.2 M KH₂PO₄, 15 mM HEPES, 25 M sodium bicarbonate, 10 mM dextrose, and 0.1% bovine serum albumin (pH adjusted to 7.4; osmolality 332 mOsm). The amount of CGRP release was measured by collecting the 20 min high K⁺ fraction.

The calcium dependency of MO and CAP desensitization were next evaluated. To test for calcium-dependency, biopsies were first pretreated for 20 min with vehicle, MO or CAP in either regular Hank's buffer or calcium-free Hank's buffer (with 10mM EGTA). This pretreatment was followed by a washout period of 10 min in the same buffer. After the washout period, standard modified Hank's buffer (containing 2mM calcium) was added to all groups. The biopsies were then stimulated for 2 min with either 0.1% MO or 100µM capsaicin and total CGRP release was measured by combining the 2min stimulation fraction with a subsequent 38 min fraction. We measured total CGRP release over a 40 min period in this experiment based on our preliminary studies indicating that pretreatment with calcium-free Hanks buffer resulted in an extended time course of CGRP release induced by subsequent application of CAP and MO in the standard modified Hank's buffer. Accordingly, we evaluated the calcium-dependency of MO and CAP desensitization by measuring total CGRP release over a 40 min period,

For experiments using a cell permeable form of the calcineurin auto-inhibitory peptide (CAIP), the skin biopsies were pretreated for 20 min with 100µM CAIP (Patwardhan et al. 2006) or vehicle (standard modified Hank's buffer as described above) followed by a co-treatment of 100µM CAIP with MO or CAP for 20 min. The skin biopsies were then washed for 10 min, and stimulated with MO or CAP for 2 min. The total evoked CGRP release was measured by pooling the 2 min MO exposure sample with an 18 min vehicle post-exposure sample.

2.3. iCGRP RIA

The CGRP RIA was conducted essentially as described (Kilo et al. 1997; Patwardhan et al. 2005). In brief, 100 µl of primary antibody against CGRP (final dilution, 1:1,000,000; kindly donated by Dr. M. J. Iadarola, NIH, Bethesda, MD) was added to 1 ml aliquots and incubated at 4°C for 48 hours. After this incubation, 100 µl of [¹²⁵I]-Tyr⁰-CGRP_{28–37} (~20,000 cpm) and 50 µl of goat anti-rabbit antisera coupled to ferric beads (PerSeptive Diagnostics, Cambridge, MA) were added to the tubes and incubated at 4°C for an additional 24 hours. The reaction was stopped using immunomagnetic separation of bound from the free tracer. The minimum detectable levels for CGRP for this assay are ~3 fmol/ml and the 50% displacement at 28 fmol. All test compounds were evaluated for potential interference in the RIA.

2.4. Behavioral Assays

On the day of the experiment, capsaicin was dissolved in 5% Tween and 5% DMSO, mustard oil was dissolved in mineral oil and calcineurin auto-inhibitory peptide was dissolved in saline. Vehicle controls contained the appropriate vehicle for each compound. For the mustard oil dose-response experiments, the right hindpaw of the animals received an intraplantar (ipl) injection (25 µl) of a selected concentration of mustard oil (0.001%–20%) and the duration (s) that the animal spent grooming and flinching the injected hind paw over a 5 min period was collected by an observer blinded to treatment allocation.

For the desensitization experiments, the animals were first injected with 50 µl of either the appropriate vehicle, 0.1% MO or 10 µg CAP, followed 15 min later with a 25 µl second ipl injection of either MO or CAP (same concentrations) and the duration (s) spent grooming and flinching the injected hindpaw over a 5 min period for CAP and MO-induced behavior was collected.

For the *in vivo* studies using a cell permeable form of calcineurin auto-inhibitory peptide (CAIP), the animals were first injected with 50 µl of either vehicle or 400 µg of CAIP (Patwardhan et al. 2006) followed 10 min later with a 50 µl second injection of either vehicle, CAP or MO which was then followed 15 min later with a 25 µl stimulus injection of either CAP or MO. As mentioned above, the duration spent by the animal grooming and flinching the injected hindpaw over a 5 min period for CAP or MO-induced nocifensive behavior was collected.

2.6. Data Analysis

All iCGRP release desensitization experiments were conducted with n=4–6 wells/group and repeated at least three times for statistical analysis. Overall, a sample size (n) of 12–18 was used for statistical analysis for these experiments. The data are presented as percentage above basal (mean ± SEM) and actual basal CGRP levels are provided in the figure legends. The behavioral experiments were performed with n=6–8 rats/group and the data are presented as time (s) spent displaying nocifensive behavior (mean ± SEM). For experiments involving only two groups, an unpaired Student's t-test was performed; all other experiments were analyzed by a one-way ANOVA with Dunnett's multiple comparison test. Data were analyzed using GraphPad (San Diego, CA) Prism software version 4.

3 Results

3.1. Cross-desensitization between TRPV1 and TRPA1 in *vitro* skin preparations

A major assumption in any study evaluating heterologous desensitization is that the agonists exhibit receptor selectivity. While the specificity of CAP for TRPV1 has been demonstrated in multiple reports (Caterina et al. 2000; Davis et al. 2000), the specificity of MO for TRPA1 has been questioned (Kwan et al. 2006). Moreover, a recent report demonstrated that at least

some agonists (i.e., menthol and cinnamaldehyde) are non-selective in activating certain thermoTRPs including TRPV1, TRPV3, TRPM8 and TRPA1 (Macpherson et al. 2006). However, our previous studies and that of other investigators have demonstrated that, at least in rats, MO (5–500 μ M) responses in sensory neurons is specifically and exclusively mediated by TRPA1 (Bautista et al. 2006; Jeske et al. 2006; Jordt et al. 2004).

We initially tested our hypothesis using a functional *in vitro* assay consisting of acutely isolated and superfused hindpaw skin biopsies containing peripheral terminals of sensory neurons (Kilo et al. 1997). In order to select a desensitizing concentration of MO, we first determined the lowest concentration of MO that is required to evoke maximum CGRP release from these peripheral terminals. As presented in Fig 1A, MO evoked maximum neuropeptide release at 0.1% MO (i.e., 10 mM), with no further increase observed at concentrations as high as 20% (Fig. 1A). The concentration-response relation between CAP stimulation and CGRP release from the hindpaw skin preparations was determined previously (Kilo et al. 1997). For desensitization experiments, hindpaw skin biopsies were first pretreated with either CAP or MO and then CAP- (Fig 1B) or MO-evoked (Fig 1C) CGRP release was measured. CAP pretreatment attenuated CAP-evoked CGRP release by 66%, as compared to the control group and MO pretreatment attenuated CAP-evoked CGRP release by approximately 58% (Fig. 1B). Similarly, MO pretreatment inhibited MO-evoked CGRP release by ~54% and CAP pretreatment inhibited MO-evoked CGRP release by 64% (Fig. 1C). Altogether, our data suggest that homologous and heterologous desensitization of CAP and MO responses occur in peripheral terminals of acutely prepared hindpaw skins.

3.2. Cross desensitization between TRPV1 and TRPA1 does not affect generalized exocytosis of the cell

It is possible that a CAP- or MO-induced depletion of the releasable pool of CGRP might mimic heterologous desensitization because both conditions would lead to reduced rates of peptide release. In order to exclude this potential confound, skin biopsies were first exposed to CAP or MO (using the same design as in Fig 1B/C) and then subjected to a 40 min recovery after which they were exposed to a depolarizing concentration of potassium. Neither CAP/CAP nor MO/CAP significantly altered K^+ -evoked CGRP release compared to the control group (Fig. 2A). Similarly, neither MO/MO nor CAP/MO significantly altered K^+ -evoked CGRP release compared to its respective control group (Fig. 2B). Collectively, these data indicate that CAP and MO heterologously and selectively modulate CGRP release without generalized inhibition of the overall exocytotic functions of these peripheral fibers.

3.3. Requirement of calcium in the heterologous desensitization between TRPV1 and TRPA1

Several studies have demonstrated that in sensory neurons the repeated application of CAP leads to an accumulation of intracellular calcium levels and subsequent calcium- and calcineurin-dependent desensitization of TRPV1 via dephosphorylation (Docherty et al. 1996; Koplas et al. 1997; Mohapatra and Nau 2005; Tominaga and Tominaga 2005). In addition, since TRPA1 is a Ca^{2+} -permeable channel, MO is capable of accumulating intracellular Ca^{2+} (Jordt et al. 2004). Therefore, we evaluated whether the heterologous desensitization of MO and CAP responses was mediated by Ca^{2+} -dependent pathways. To evaluate this hypothesis, skin biopsies were pretreated with 100 μ M CAP or 0.1% MO under calcium-free conditions followed by subsequent stimulation with either CAP or MO in Hanks buffer containing 2mM Ca^{2+} . As expected, the application of CAP or MO in the absence of calcium blocked evoked release of iCGRP (data not shown). As presented in Fig 3A, pretreatment with CAP or MO under calcium-free conditions failed to inhibit CAP-evoked CGRP release, demonstrating the requirement of calcium in homologous and heterologous desensitization of CAP responses. Analogous studies were performed to evaluate the calcium dependency of TRPA1 mediated CGRP release. Interestingly, as shown in Fig 3B, pretreatment

with MO under calcium-free conditions did attenuate MO-evoked CGRP release demonstrating a calcium-independent mechanism mediating homologous desensitization of MO responses. On the other hand, pretreatment with CAP under calcium-free conditions failed to inhibit MO-evoked CGRP release, indicating an essential role of a calcium-dependent pathway mediating heterologous desensitization of MO responses. Several conclusions were evident from this study. TRPV1 undergoes homologous (CAP) and heterologous (MO) desensitization by calcium-dependent pathways. In contrast, TRPA1 undergoes homologous (MO) desensitization via a calcium-independent pathway, but heterologous (CAP-induced) desensitization via a calcium-dependent pathway.

3.4. Differential requirement of calcineurin in the heterologous desensitization between TRPV1 and TRPA1 *in vitro*

It is well documented that CAP-induced Ca^{2+} -dependent desensitization of TRPV1 occurs via a protein phosphatase 2B (calcineurin) pathway. Since the results from previous experiments demonstrated that heterologous desensitization between MO and CAP responses requires extracellular Ca^{2+} , we evaluated the role of calcineurin in mediating this effect. Skin biopsies were pretreated with either vehicle or a cell-permeable analog of calcineurin autoinhibitory peptide (CAIP), followed by application of either CAP or MO. The tissue was then stimulated with either 100 μM CAP or 0.1% MO. As shown in Fig 4A, pretreatment with CAIP prevented MO-induced desensitization of CAP-evoked CGRP release by ~72%. However, pretreatment with CAIP did not alter CAP induced desensitization of MO-evoked CGRP release (Fig. 4B). Collectively, these results suggest that heterologous desensitization of TRPV1 by MO occurs via calcineurin pathways. In contrast, the heterologous desensitization of TRPA1 by CAP does not involve calcineurin.

3.5. Homologous and heterologous desensitization between TRPV1 and TRPA1 *in vivo*

The cross-desensitization of evoked CGRP release from the hindpaw skin biopsies implies that heterologous desensitization might occur in the whole animal. To test this hypothesis, we carried out a set of behavioral assays with observers blinded to treatment allocation. The hindpaw grooming and flinching model was selected for a nocifensive assay because doses of CAP and MO can be selected that produce a similar magnitude and duration of effects. We first determined the lowest concentration of MO required to produce a maximal nocifensive effect (Fig 5A). The injection of MO induced a dose-dependent nocifensive behavioral response with a maximum effect observed at a concentration of 0.1% (10 mM; Fig 5A); interestingly, this concentration was the same as that producing maximal CGRP release from hindpaw skin preparations (Fig 1A). For the desensitization experiments, rats were first injected with either vehicle, CAP or MO, followed 15 min later by an injection of CAP (Fig. 5B). Based on previous reports, we employed 10 μg CAP for the desensitization experiments (Gilchrist et al. 1996). Pretreatment with either CAP or MO significantly inhibited capsaicin-induced nocifensive behavior by approximately 69% and 60%, respectively when compared with the controls (Fig. 5B). Analogous studies were performed to determine desensitization effects of MO-evoked behavior (Fig. 5C). As compared with the vehicle controls, pretreatment with either MO or CAP significantly inhibited MO-induced nocifensive behavior by approximately 76% and 71%, respectively (Fig. 5C). Our results indicate that homologous and heterologous desensitization between MO and CAP responses could be observed in both *in vitro* and *in vivo* models.

3.6. Differential requirement of calcineurin in the heterologous desensitization between TRPV1 and TRPA1 *in vivo*

Our *in vitro* studies using the calcineurin auto-inhibitory peptide demonstrate that heterologous desensitization of TRPV1 by MO is mediated via a calcium activated calcineurin-dependent

pathway (Fig 3A and 4A). In contrast, heterologous desensitization of TRPA1 by CAP occurs via a calcium-dependent but calcineurin-independent pathway (Fig 3B and 4B). In order to confirm that our *in vitro* findings operate under *in vivo* conditions, we performed *in vivo* behavioral assays measuring CAP or MO-induced nocifensive behavior using the calcineurin auto-inhibitory peptide (CAIP). Pretreatment with 400 μ g CAIP fully reversed MO inhibition of CAP-induced nocifensive behavior indicating calcineurin-dependent heterologous TRPV1 desensitization. Collectively, heterologous desensitization of TRPV1 is mediated via a Ca^{2+} -activated calcineurin pathway. However, pretreatment with 400 μ g CAIP did not have an effect on CAP inhibition of MO-induced nocifensive behavior consistent with our *in vitro* findings demonstrating calcineurin-independent heterologous TRPA1 desensitization. These data verify our *in vitro* CGRP results and demonstrate different heterologous desensitization pathways for TRPV1 and TRPA1.

4. Discussion

The heterologous modulation between membrane receptors contributes to the dynamic plasticity of the nervous system. Here, we present a study on homologous and heterologous desensitization between CAP and MO responses which constitutes a type of functional interaction between TRPV1 and TRPA1. A critical aspect of this study is the prior demonstration that both CAP and MO, at least in rats, are selective for TRPV1 and TRPA1 respectively (Jeske et al. 2006; Jordt et al. 2004), indicating that the effects cannot be attributed to the lack of agonist specificity. CAP has long been known to be able to desensitize and influence nociceptor activities to a variety of noxious stimuli including CAP itself (Santicioli et al. 1987; Simons et al. 2003; Szallasi and Blumberg 1996). Stimulation of TRPV1 by CAP can lead to heterologous regulation of nicotinic channels (Dessirier et al. 2000) which, in turn, could control the actions of other ion channels (Liu et al. 2001). CAP treatment can also heterologously desensitize voltage-gated calcium channels as well as ATP-gated channels in sensory neurons (Liu et al. 2001; Piper and Docherty 2000; Wu et al. 2005). In addition, it was demonstrated that repeated application of MO produces diminished perceptual responses (Brand and Jacquot 2002; Simons et al. 2003). However, despite the accumulation of a large amount of information on the homologous desensitization of CAP and MO responses, no studies have examined heterologous desensitization between capsaicin and mustard oil in nociceptors; and to our knowledge, no study has evaluated potential mechanisms for this heterologous desensitization.

We found that MO, a TRPA1-selective agonist, was able to significantly reduce CAP-evoked neuropeptide release from skin terminals. Similarly, CAP pretreatment led to suppression of MO-evoked CGRP release. In addition, cross-desensitization was observed in a whole-animal, nocifensive behavioral test. Several conclusions are evident from these experiments. TRPV1 undergoes homologous (CAP) and heterologous (MO) desensitization by calcium-dependent pathways. The present studies also demonstrated that MO induced desensitization of TRPV1 occurs via a calcium-calcineurin dependent pathway. A possible explanation for the partial blockade of CAIP on MO-inhibition of CAP responses in the *in vitro* versus the complete blockade of CAIP on MO observed in the *in vivo* assay could be due to the concentration of CAIP used in the CGRP release experiments (100 μ M). A full concentration response curve might have revealed full inhibitory efficacy at higher concentrations of CAIP. In contrast, CAP induced desensitization of TRPA1 employs a Ca^{2+} -dependent, but calcineurin-independent pathway. Moreover, TRPA1 desensitization by MO is independent of any signaling pathway triggered by an influx of extracellular calcium. A possible explanation for this latter finding is that activation of TRPA1 by MO depends on covalent modification of several cysteine residues (Hinman et al. 2006; Macpherson et al. 2007). These modifications could occur in Ca^{2+} -free extracellular media (Macpherson et al. 2007) and thus constitute one possible calcium-independent mechanism. If these modifications are not totally and fully reversed under the

present experimental conditions (i.e., before the second application of MO), then TRPA1 activation could be reduced. This hypothesis would predict the observed pharmacological desensitization of TRPA1, although this mechanism may not be solely responsible for the calcium-independent pharmacological desensitization of TRPA1 by MO. One possible approach to address this mechanism is to study TRPA1 desensitization with compounds which do not modify cysteine residues, such as cannabinoids (Hinman et al. 2006). Addressing this possibility, we have recent preliminary data that indicate calcium-independent TRPA1 desensitization by the cannabinoid WIN 55,212, a specific TRPA1 agonist in neurons (unpublished results). Hence, an incomplete reversal of TRPA1 covalent modifications by MO could contribute to the observed calcium-independent homologous desensitization of TRPA1 by MO under the present experimental conditions and yet still be one of several mechanisms of TRPA1 desensitization.

Collectively, our data demonstrate that TRPA1 desensitization recruits different cellular pathways from those required for TRPV1 desensitization. Furthermore, TRPA1 desensitization, unlike TRPV1, is agonist-dependent. This implies that activation of at least two independent cellular pathways could lead to TRPA1 desensitization. Hence, a logical area of future research should focus on identifying detailed molecular mechanisms of the pharmacological desensitization of TRPA1.

Nevertheless, drawing an analogy between mechanisms for TRPA1 desensitization and mechanisms for desensitization of other TRP channels, we can speculate on several possible models. One model involved in the desensitization of TRPs, like TRPM8 in an expression system, predicts a role for calcium-dependent forms of phospholipase C that can deplete PIP₂ and thereby reduce activities of TRP channels (Liu and Qin 2005; Rohacs et al. 2005). Another model could involve activation of certain calcium-dependent kinases that could lead to inactivation of the TRP channels (Zhu et al. 2005).

In conclusion, this study is an important step towards understanding the function of polymodal nociceptors that are capable of integrating various mechanical, chemical and thermal stimuli and regulating their responses through the inhibition of channels such as TRPV1 and TRPA1. Hence the heterologous modulation of these channels expressed by these neurons could serve as a novel strategy to inhibit nociceptor activation in response to a variety of stimuli that form a major component of the inflammatory pain pathway.

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Fig. 1. Effect of capsaicin and mustard oil on CGRP release from isolated hindpaw skin biopsies (A) Evaluation of the concentration-dependent effect of mustard oil (MO) over the range of 0.001–10% and the amount of iCGRP released from isolated and superfused rat hindpaw skin biopsies over a 20 min period. (B) Evaluation of the effect of pretreatment with vehicle, capsaicin (CAP; 100 μ M for 20 min) or mustard oil (MO; 0.1% for 20 min) to inhibit CAP-evoked CGRP release from hindpaw skin biopsies. (C) Evaluation of the effect of pretreatment with vehicle, mustard oil (MO; 0.1% for 20 min) or capsaicin (CAP; 100 μ M for 20 min) to inhibit MO-evoked iCGRP release from superfused skin. Basal levels of CGRP release are 6–7 fmol/ml. N = 12–18; error bars = SEM. * p <0.05, **<0.01.

Fig. 2. Effect of capsaicin and mustard oil pretreatment on K^+ -evoked CGRP release from isolated hindpaw skin biopsies

(A) Evaluation of the effect of pretreatment with vehicle or capsaicin (CAP; 100 μ M for 20 min and washed for 40 min) to desensitize K^+ -evoked iCGRP release from hindpaw skin. (B) Evaluation of the effect of pretreatment with vehicle or mustard oil (MO; 0.1% for 20 min and washed for 40 min) to inhibit K^+ -evoked iCGRP release from superfused skin. Basal levels of CGRP release are 4–6 fmol/ml. N = 12–18; error bars = SEM. * p <0.05, **<0.01.

Fig. 3. Effect of capsaicin and mustard oil on CGRP release under calcium-free conditions from isolated hindpaw skin

(A) Evaluation of the effect of pretreatment with vehicle, capsaicin (CAP; 100 μ M for 20 min in calcium-free buffer) or mustard oil (MO; 0.1% for 20 min in calcium-free buffer) to desensitize CAP-evoked iCGRP release from hindpaw skin. (B) Evaluation of the effect of pretreatment with vehicle, mustard oil (MO; 0.1% for 20 min in calcium-free buffer) or capsaicin (CAP; 100 μ M for 20 min in calcium-free buffer) to desensitize MO-evoked iCGRP release from hindpaw skin. Basal levels of CGRP release are 4–5 fmol/ml. N = 12–18; error bars = SEM. * p <0.05 and ** p <0.01 vs vehicle pretreatment. ## p <0.01 and ### p <0.001 vs respective treatment (eg., MO with vs MO without calcium).

Fig. 4. Effect of calcineurin-auto inhibitory peptide on capsaicin and mustard oil-induced inhibition of CGRP release from isolated hindpaw skin

(A). Evaluation of the effect of vehicle or calcineurin-auto inhibitory peptide (CAIP; 100 μ M for 20 min) on the efficacy of pretreatment of vehicle, capsaicin (CAP; 100 μ M for 20 min) or mustard oil (MO; 0.1% for 20 min) to desensitize CAP-evoked CGRP release. (B) Evaluation of the effect of vehicle or calcineurin-auto inhibitory peptide (CAIP; 100 μ M for 20 min) on the efficacy of pretreatment of vehicle, mustard oil (MO; 0.1% for 20 min) or capsaicin (CAP; 100 μ M for 20 min) to desensitize MO-evoked CGRP release. Basal levels of CGRP release are 5–6 fmol/ml. N = 12–18; error bars = SEM. * p <0.05, **<0.01 vs vehicle. ## p <0.01 vs respective treatment.

Fig. 5. Effect of capsaicin and mustard oil on nocifensive behavior in rats

A: Determination of the mustard oil dose response curve by injecting 0.001–20% mustard oil into the intraplantar surface of the rat right hind paw and measuring flinching by blinded observers. **B:** Evaluation of the effect of pre-injection of vehicle, capsaicin (CAP; 10 μ g) or mustard oil (MO; 0.1%) to desensitize CAP-induced nocifensive behavior after 15 min. **C:** Evaluation of the effect of vehicle, mustard oil (MO; 0.1%) or capsaicin (CAP; 100 μ M) to desensitize MO-induced nocifensive behavior after 15 min. N = 6–8; error bars = SEM.

* $p < 0.05$, ** $p < 0.01$.

Fig. 6. Effect of calcineurin-auto inhibitory peptide on capsaicin and mustard oil-induced inhibition of nocifensive behavior *in vivo*

A: Evaluation of the effect of pre-injection of vehicle or calcineurin auto-inhibitory peptide (CAIP; 400 μ g) on the efficacy of vehicle or mustard oil (MO; 0.1%) to desensitize CAP-induced nocifensive behavior after 15 min. **C:** Evaluation of the effect of pre-injection of vehicle or calcineurin auto-inhibitory peptide (CAIP; 400 μ g) on the efficacy of vehicle or capsaicin (CAP; 10 μ g) to desensitize MO-induced nocifensive behavior after 15 min. N = 6–8; error bars = SEM. * $p < 0.05$, ** $p < 0.01$.