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The role of TRPA1 in muscle pain and mechanical hypersensitivity under inflammatory conditions in rats

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Abstract

TRPA1 is expressed in muscle afferents and direct activation of these receptors induces acute mechanical hypersensitivity. However, the functional role of TRPA1 under pathological muscle pain conditions and mechanisms by which TRPA1 mediate muscle pain and hyperalgesia are not clearly understood. Two rodent behavioral models validated to assess craniofacial muscle pain conditions were used to study ATP- and NMDA-induced acute mechanical hypersensitivity and complete Freund's adjuvant (CFA)-induced persistent mechanical hypersensitivity. The rat grimace scale was utilized to assess inflammation-induced spontaneous muscle pain. Behavioral pharmacology experiments were performed to assess the effects of AP18, a selective TRPA1 antagonist under these conditions. TRPA1 expression levels in trigeminal ganglia were examined before and after CFA treatment in the rat masseter muscle. Pre-treatment of the muscle with AP18 dose-dependently blocked the development of acute mechanical hypersensitivity induced by NMDA and $\alpha\beta\text{meATP}$, a specific agonist for NMDA and P2X₃ receptor, respectively. CFA-induced mechanical hypersensitivity and spontaneous muscle pain responses were significantly reversed by post-treatment of the muscle with AP18 when CFA effects were most prominent. CFA-induced myositis was accompanied by significant up-regulation of TRPA1 expression in TG. Our findings showed that TRPA1 in muscle afferents plays an important role in the development of acute mechanical hypersensitivity and in the maintenance of persistent muscle pain and hypersensitivity. Our data suggested that TRPA1 may serve as a downstream target of pro-nociceptive ion channels, such as P2X₃ and NMDA receptors in masseter afferents, and that increased TRPA1 expression under inflammatory conditions may contribute to the maintenance of persistent muscle pain and mechanical hyperalgesia. Mechanistic studies elucidating transcriptional or post-translational regulation of TRPA1 expression under pathological pain

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CONFLICT OF INTEREST

There is no conflict of interest to declare.

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conditions should provide important basic information to further advance the treatment of craniofacial muscle pain conditions.

Keywords

Myositis; trigeminal ganglia; craniofacial; muscle afferents; AP18

INTRODUCTION

Transient receptor potential cation channel, subfamily A, member 1 (TRPA1) was initially described as a nociceptive channel responsive to noxious cold (Story et al., 2003, Bandell et al., 2004), as well as a putative mechanical transducer in sensory neurons (Kwan et al., 2006). It is now well established that TRPA1 is activated by a variety of environmental irritants and endogenous mediators generated during injury or inflammation (Bautista et al., 2013). The role of TRPA1 in pain and hyperalgesia has been repeatedly demonstrated in a variety of tissue types including skin and visceral organs (Chung et al., 2011). In muscle tissue, direct injections with a TRPA1 agonist, mustard oil evoke reliable nocifensive responses and mechanical hyperalgesia that is mediated by TRPA1 in muscle afferents (Ro et al., 2003, Ro et al., 2009). However, it is not known whether TRPA1 plays a functional role under pathological muscle pain conditions.

Among many algogenic substances that are released during muscle injury, ATP and glutamate are most extensively studied. When muscle cells are lysed, a high concentration of ATP is released into extracellular space, activating approximately 70% of group IV muscle afferents (Reinohl et al., 2003, Hoheisel et al., 2004). In the masseter muscle, direct P2X₃ activation, via the selective agonist alpha,beta-methylene ATP ($\alpha\beta\text{meATP}$), induces a dose-dependent acute mechanical hyperalgesia that lasts for hours (Shinoda et al., 2008, Saloman et al., 2013). Similarly, tissue injury or inflammation leads to elevated extracellular excitatory amino acid concentrations (Omote et al., 1998, Lawand et al., 2000). Direct injections with N-Methyl-D-aspartate (NMDA), a selective agonist for NMDA receptors, into the masseter muscle activate muscle nociceptors (Dong et al., 2007) and induce acute mechanical hypersensitivity (Lee et al., 2012b). Importantly, both ATP- and NMDA-induced mechanical hypersensitivity involves TRPV1 sensitization (Lee et al., 2012b, Saloman et al., 2013). It is not known whether mechanical hypersensitization induced by ATP and NMDA also involves TRPA1.

Selective TRPA1 antagonists, such as AP18 or HC-030031, attenuate persistent inflammatory mechanical hyperalgesia in the skin (Petrus et al., 2007, Eid et al., 2008). Recent studies provided compelling evidence that TRPA1 is also involved in the maintenance of persistent mechanical hyperalgesia arising from deep tissues, such as knee joint and the bladder (DeBerry et al., 2014, Garrison and Stucky, 2014). Early intervention with TRPA1 antagonists attenuates the transition of acute to chronic pain in a pancreatitis model (Schwartz et al., 2013). Moreover, the level of TRPA1 expression closely correlates with mechanical hypersensitivity in several inflammatory pain models (Chen et al., 2013, DeBerry et al., 2014, Malsch et al., 2014). These results are consistent with the role of

TRPA1 as an important signal integrator during inflammation (Levine and Alessandri-Haber, 2007). However, the role of TRPA1 in an inflammatory muscle pain condition has never been demonstrated.

In this study, we examined whether the blockade of TRPA1 in muscle attenuates ATP- and NMDA-induced acute mechanical hypersensitivity as well as complete Freund's adjuvant (CFA)-induced spontaneous muscle pain and persistent mechanical hypersensitivity, and whether CFA-induced myositis alters TRPA1 expression in trigeminal ganglia (TG).

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague Dawley rats (250–350 g; Harlan, Indianapolis, IN) were used. All animals were housed in a temperature-controlled room under a 12:12 light-dark cycle with access to food and water *ad libitum*. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Publication No. 80–23) and under a University of Maryland approved Institutional Animal Care and Use Committee protocol.

Drugs

For behavioral experiments, NMDA (10 μ mol/40 μ l), a specific agonist for the NMDAR, and α , β -methylene adenosine triphosphate ($\alpha\beta$ meATP) (750 μ g/20 μ l, pH adjusted to 7.0 using NaOH), a specific agonist for P2X₃, were dissolved in phosphate buffered saline (PBS). AP18 (10 nmol to 2 μ mol), a TRPA1 antagonist, was dissolved in 1% DMSO, 10% Tween-80, and 89% PBS. HC-030031 (50 nmol), another TRPA1 antagonist, was dissolved in 10% DMSO. NMDA, $\alpha\beta$ meATP and HC-030031 were purchased from Sigma-Aldrich (St. Louis, MO). AP18 was purchased from Enzo Life Sciences (Farmingdale, NY). Doses of NMDA, $\alpha\beta$ meATP, and AP18 were adapted from our previous studies (Ro et al., 2009, Lee et al., 2012b, Saloman et al., 2013). All drugs were administered directly into the masseter muscle.

Masseter Inflammation

Inflammation was induced by injecting 50 μ l of 50 % CFA in isotonic saline (Sigma-Aldrich, St. Louis, MO) into the mid-region of the masseter muscle via a 27 gauge needle. Rats were briefly anesthetized with 3 % isoflurane for the injection procedure. The characteristics of inflammation following CFA injections in the rat masseter have been described previously (Imbe et al., 1999, Ambalavanar et al., 2006).

Behavioral Studies

Assessment of acute mechanical hypersensitivity—Noxious chemical or mechanical stimulation of the masseter muscle evokes characteristic wiping-like movement of the ipsilateral hindpaw in lightly anesthetized rats. We have previously described the use of this behavior for testing mechanical sensitivity of the masseter muscle (Ro et al., 2007, Ro et al., 2009, Chung et al., 2015). This lightly anesthetized rodent paradigm allows the delivery of calibrated and reliable mechanical stimuli on the masseter muscle before and after pharmacological manipulations, which is not possible in awake animals. Initially, rats

were anesthetized with an intraperitoneal injection of sodium pentobarbital (40mg/kg). As the rats recover from the anesthesia, a level of 'light' anesthesia was determined by providing a noxious pinch to the tail or the hindpaw with a serrated forceps. Animals typically responded to the noxious pinch on the tail with an abdominal contraction and with a withdrawal reflex to the noxious pinch of a hindpaw within 30 min after the initial anesthesia. Once the animal reached this level a metal clip calibrated to produce 600 gm of force was applied 5 consecutive times, and experiments were continued only after the animals showed reliable reflex responses to every clip application. A tail vein was connected to an infusion pump (Harvard Apparatus, Pump11) for continuous infusion of pentobarbital. The rate of infusion was adjusted to maintain a relatively light level of anesthesia throughout the duration of the experiment (3–5 mg/h).

A baseline mechanical threshold for evoking the hindpaw responses was determined 15 min prior to NMDA or ATP injection using the electronic von Frey (VF) anesthesiometer (IITC Life Science, Inc, Woodland Hills, CA). A rigid tip attached to the VF meter was applied to the masseter muscle until the animals responded with hindpaw shaking. The animal's head was rested flat against the surface of the table when pressing the anesthesiometer on the masseter in order to provide stability. The threshold was defined as the lowest force needed to evoke a hindpaw response. Changes in masseter sensitivity were then assessed at 15, 30, 45, 60 and 90 minutes following NMDA or ATP treatments. AP18 (10 nmol to 1 μ mol in 50 μ l) or vehicle was administered in the masseter muscle 15 minutes prior to NMDA or ATP treatment in the same muscle. The percent changes in VF thresholds following drug treatment were calculated with respect to the baseline threshold and plotted against time. In order to assess the overall magnitude of drug-induced changes in masseter sensitivity over time, the area under the curve (AUC) was calculated for the normalized data for each rat using the trapezoid rule.

All animals were kept warm throughout the experiments with thermal blankets. In order to maintain the consistency of assessing behavioral responses all behavioral observations were made by one experimenter blinded to the experimental conditions. The time-dependent mean percent changes in mechanical thresholds were normalized to the baseline threshold and analyzed with a two-way ANOVA with repeated measures. One-way ANOVA was used to evaluate the overall magnitude of mechanical hyperalgesia assessed as AUC. All multiple group comparisons were followed by a post hoc test (Duncan's). Data are presented as mean $\pm$ S.E.M. and a $p < 0.05$ was considered significant for all statistical analyses presented in this report.

Assessment of persistent mechanical hypersensitivity—Persistent mechanical hypersensitivity in the masseter muscle was assessed under CFA-induced inflammatory condition utilizing a behavioral model specifically developed for testing masseter sensitivity in awake rats (Ren, 1999). In this model, a series of calibrated von Frey filaments (1–125 gm) were applied to the region over the masseter muscle. An active withdrawal of the head from the filament application was defined as a positive response. Each von Frey filament was applied five times and the response frequencies [(number of responses/number of stimuli) $\times$ 100 %] to a range of filament forces were determined. After a non-linear regression analysis, an EF50 value, the filament force (g) necessary to produce a 50 %

response frequency, was determined. The EF50 value was used as a measure of mechanical threshold. A reduction of EF50 after inflammation suggested the presence of mechanical hypersensitivity.

Mechanical sensitivity of the masseter muscle was determined before and 1, 3, 7, 10, 14, 21 and 28 days after the CFA injection in the masseter muscle. The effect of a TRPA1 antagonist, AP18 on mechanical sensitivity was examined on 1 and 3 days following CFA injection, time point during which mechanical hypersensitivity is most pronounced. On test day, AP18 (2 μ mol in 50 μ l) or the same volume of vehicle was administered directly in the masseter muscle under anesthesia using isoflurane. The post AP18 or vehicle effect was measured 1, 2 and 24 hrs after the drug injection. In order to maintain consistency in assessing behavioral responses an experimenter who was blinded to treatment conditions conducted all behavioral experiments. The time-dependent changes in mechanical thresholds (EF50) before and after CFA were analyzed with a Two-Way ANOVA with repeated measures. Drug effects were compared before and after the drug treatment and analyzed with one-way ANOVA. All multiple group comparisons were followed by Duncan's post hoc test.

Evaluation of spontaneous muscle pain—In order to evaluate spontaneous pain in rats following CFA-induced masseter inflammation, we adapted the Rat Grimace Scale (RGS) as a method for quantifying pain by assessing facial expression patterns (Sotocinal et al., 2011). First, we established the temporal profile of CFA-induced muscle pain, and then examined whether AP18 treatment would attenuate the maintenance of CFA-induced pain.

Video Imaging: The rats were acclimated to the testing environment for 2 to 3 days prior to behavioral assessment. The rats were placed in a cubicle (21.0 $\times$ 10.5 $\times$ 9.0 cm), with four 9 transparent Plexiglas walls, a ventilated metal shelf bottom and an opaque middle wall that separate two cubicles, which allows the recording of two rats at a time. Two digital video cameras (Sony HDR-CX230/B High Definition Handycam Camcorder) were placed at a fixed distance from the cubicle, with one on each side of the cubicle to maximize the probability of capturing the faces of the rats. In the first part of the experiment, free behaviors of rats were videotaped for 30-min blocks before and 6 hr, and 1, 3, and 7 days after CFA or vehicle treatment in the masseter muscle. In the second part of the experiment, CFA- or vehicle-treated rats were videotaped one day after the CFA administration in 30-min blocks before and 1, 2, and 24 hrs after either AP18 or vehicle treatment in the same muscle.

Scoring System for RGS: To capture face image of rats in unbiased manners, we used Rodent Face Finder (Sotocinal et al., 2011). This software automatically extracts images of rat facial expression from video images. In every 10 min video segment, ten clear and high quality images were captured when the rat was found to be directly facing the camera. Randomized and unlabeled photos were presented on a large, high-resolution computer monitor one at a time. One experimenter, who was blinded to the condition of the rat and experienced in the use of the scoring system, scored the image. Four facial action units (AUs) were scored: orbital tightening, nose and cheek flattening, ear position and whisker change (Langford et al., 2010, Sotocinal et al., 2011). The observer scored 0, 1, or 2 for each

AU based on the criteria used by Sotocinal and colleagues (2011). Briefly, for each photo, the scorer assigned a value of 0, 1 or 2 for each of the four RGS action units. A score of “0” represented absence of the action unit, a score of “2” indicated obvious detection of the action unit. A score of “1” was assigned when the scorer was not highly confident that the action unit is present or absent, or a moderate appearance of the action unit. An initial RGS score of each photograph was calculated by averaging the scores of the four AUs. Then, a mean RGS score for each video was obtained from 10 photographs, which was presumed to reflect the level of spontaneous pain. The mean RGS score of each rat before CFA treatment was used as the baseline value. The time-dependent changes in RGS scores before and after CFA or vehicle were analyzed with a Two-Way ANOVA with repeated measures. The effects of AP18 and vehicle were also analyzed with a Two-Way ANOVA with repeated measures. All multiple group comparisons were followed by Bonferroni post hoc test.

Real-Time RT-PCR

Total RNAs were extracted from TG of CFA injected rats 1, 3, 7, and 14 days following the injection in the masseter muscle. TG were extracted from additional rats treated with vehicle 3 and 14 days following the injection in order to assess the effects of injection procedure. All samples were processed using Trizol (Invitrogen, Carlsbad, CA) and purified according to the RNeasy kit (Qiagen, Germantown, MD) that included a DNase treatment to remove genomic DNA. Reverse transcription was carried out using the SuperScript First strand synthesis kit (Invitrogen). SuperScript II (Invitrogen) was used to generate cDNA from 500 ng of RNA along with 2.5 ng of random primer per reaction. Real-time PCR analysis of cDNA equal to 15 ng of RNA was then performed using Maxima SYBR Green/ROX qPCR Master Mix in an Eppendorf Mastercycler Ep Realplex 2.0 (Fermentas, Forest City, CA). The following primer pairs were used to detect TRPA1 mRNA: forward 5'-TCCTATACTGGAAGCAGCGA-3', reverse 5'-CTCCTGATTGCCATCGACT-3', and GAPDH mRNA: forward 5'-TCACCACCATGGAGAAGGCG-3', reverse 5'-GCTAAGCAGTTGGTGGTGCA-3'. We obtained the ratios between TRPA1 and GAPDH to calculate the relative abundance of mRNA levels in each sample. Relative quantification of the TRPA1 mRNA was calculated by the comparative CT method (2^{-CT} method) between control and experimental groups and fold changes were analyzed with two-way ANOVA.

Western blotting

Total proteins were extracted from TG of naive rats, and from inflamed rats 3 days following CFA injection in the masseter muscle. The protein samples were dissolved in RIPA buffer containing protease inhibitor cocktail. The protein concentration of lysates was determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Fifty micrograms of protein for each sample were separated on 4–12 % NuPAGE gel with MOPS SDS running buffer and transferred to a PVDF membrane (Bio-rad, Hercules, CA). After blocking 1 h in 5 % milk PBST at room temperature, membranes were probed with primary antibodies for TRPA1 (1:5000, Millipore #ABN-1009) and GAPDH (1:5000, Calbiochem), used as an internal control protein, diluted in blocking solution. The TRPA1 antibody was raised against the N-terminus of rat TRPA1 and detects a 90–98 kDa protein, which disappears in

TG lysates probed with TRPA1 antibody pre-incubated with a commercially available peptide used to generate the antibody.

Membranes from TG samples were incubated with primary antibodies overnight at 4° C and washed four times with PBST. HRP conjugated secondary antibodies (anti-rabbit secondary antibody (Cell Signaling) and anti-mouse secondary antibody (Millipore)] were diluted to 1:5000 in PBST and incubated with membranes for 1 h at room temperature. Bands were visualized using ECL (Western Lightning, PerkinElmer Inc.) or ECL plus Western blotting detection reagent (Lumigen PS-3, GE Healthcare). Protein level for TRPA1 was normalized to that of GAPDH in the same sample. Data from CFA-inflamed rats were normalized to that of naive rats and analyzed with Mann-Whitney U test.

In order to further validate the TRPA1 antibody in our system, we transfected human embryonic kidney (HEK) 293 cells with either an expression vector (pFrog3) containing cDNA encoding rat TRPA1 (generously gifted by Dr. David Julius) or empty vector. HEK 293 cells were maintained and transfected as previously described (Wang et al., 2012). Briefly, cells were cultured in a 6-well plate in Dulbecco's modified Eagle's medium (DMEM) with 10 % FBS at 37 °C, 5 % CO₂ incubator. Transfection procedures were performed using Lipofectamine 2000 following manufacturer's protocol (Invitrogen). Forty-eight hours after transfection, the cells were lysed and processed for immunoblotting with the anti-TRPA1 antibody.

RESULTS

TRPA1 is involved in P2X₃-mediated acute mechanical hypersensitivity

The baseline mechanical threshold that evoked the nocifensive response ranged from 500 to 600 g in lightly anesthetized rats, which was similar to those we previously reported (Ro et al., 2009, Lee et al., 2012b, Saloman et al., 2013). We have previously shown that intramuscular injection of $\alpha\beta$ meATP induces a dose- and time-dependent mechanical hypersensitivity, and that A-317491, a selective antagonist for P2X₃ receptor, dose-dependently blocked the effects of $\alpha\beta$ meATP, confirming the specificity of $\alpha\beta$ meATP on P2X₃ receptors (Saloman et al., 2013). Here we confirmed that 750 μ g of $\alpha\beta$ meATP injected directly in to the masseter muscle induced significant mechanical hypersensitivity that was observed at 15, 30 and 45 min post injection, with mechanical thresholds returning to baseline by 60 min (Fig. 1A). To determine if TRPA1 is involved in P2X₃-induced masseter hypersensitivity, animals were treated with a TRPA1 antagonist, AP18, prior to $\alpha\beta$ meATP in the same muscle. AP18 dose-dependently prevented P2X₃-induced masseter hypersensitivity ($F=22.69$, $P < 0.001$, Fig. 1A). In order to assess the overall magnitude of drug effect irrespective of time a one-way ANOVA was performed on the area under the curve (AUC) (Fig. 1B). AP18 significantly prevented $\alpha\beta$ meATP-induced mechanical hypersensitivity compared to vehicle ($F = 22.0$, $P < 0.001$, Fig. 1B). When the higher dose of AP18 (200 nmol) or DMSO was given alone there was no significant drug effect ($F = 2.619$, $P = 0.137$). While there was a slight variation in the mechanical sensitivity over time neither AP18 nor DMSO altered mechanical thresholds at earlier time points during which $\alpha\beta$ meATP effects were prominent. In order to confirm the involvement of TRPA1 in $\alpha\beta$ meATP-induced mechanical hypersensitivity, another selective TRPA1 antagonist,

HC-030031, or its vehicle was pre-administered in the masseter prior to $\alpha\beta$ meATP injection (Fig. 1C). Consistent with AP18 data, HC-030031, but not vehicle, significantly blocked the mechanical hypersensitivity ($t=4.85$, $p < 0.05$).

TRPA1 is involved in NMDAR-mediated acute mechanical hypersensitivity

We first confirmed that direct activation of NMDARs in the masseter muscle evokes a time-dependent increase in mechanical sensitivity (Fig. 2A). Masseter injection of NMDA (10 μ mol/40 μ L) significantly decreased the mechanical threshold reaching the peak effect at 15 minutes and gradually returning to the baseline level within 90 minutes after the injection. We have previously shown that a pretreatment of the muscle with a systemically low dose of AP5 (1 μ mol), a competitive NMDAR antagonist, completely prevented the development of NMDA-induced masseter hypersensitivity, indicating that the responses are produced specifically by the activation of local NMDARs (Lee et al., 2012b).

We then examined whether the NMDA-induced mechanical hypersensitivity was altered by pretreatment of the muscle with AP18. There was a significant group effect ($F = 11.2$, $P < .01$) and a significant time effect ($F = 99.1$, $P < .001$) (Fig. 2A). The NMDA-induced mechanical hypersensitivity was almost completely blocked when 1 μ mol of AP18 was pre-administered in the masseter. A lower dose of AP18 (10 nmol) did not significantly block the NMDA-induced responses indicating a dose-dependent effect of the antagonist. The vehicle did not alter the NMDA-induced mechanical hypersensitivity. In order to assess the overall magnitude of drug effect irrespective of time a one-way ANOVA was performed on the AUC. AP18 significantly prevented NDMA-induced mechanical hypersensitivity compared to vehicle ($F = 11.7$, $P < 0.01$, Fig. 2B). In order to further strengthen our data on the involvement of TRPA1 in NDMA-induced mechanical hypersensitivity, we examined whether NMDA-induced mechanical hypersensitivity is also blocked by HC-030031 (Fig 2C). Consistent with AP18 data, HC-030031, but not vehicle, significantly blocked the mechanical hypersensitivity ($t=9.99$, $p < 0.05$).

TRPA1 is involved in CFA-induced mechanical hypersensitivity

Masseter injection of CFA in the rat induces a time-dependent and significant decrease in mechanical thresholds that lasts over 14 days (Ambalavanar et al., 2006, Shimizu et al., 2009, Niu et al., 2012). We have confirmed the development of mechanical hypersensitivity following CFA injection in the masseter with a peak decrease in EF₅₀ during the first 3 days. A significant decrease in mechanical threshold was observed until day 21, which gradually returned toward baseline level by 28 days post CFA treatment ($F=27.06$, $P < 0.001$, Fig. 3A). The vehicle treatment in the same manner did not alter mechanical thresholds for 28 days, at which point we stopped the further assessment of mechanical thresholds in this group.

In order to examine the involvement of TRPA1 in CFA-induced masseter mechanical hypersensitivity, we tested the effects of AP18 on days 1 and 3 after CFA treatment. We chose these time points since the mechanical hypersensitivity was most prominent during these time points. Based on our preliminary experiments and previous studies, we determined that AP18 at 2 μ mol/50 μ L can be locally administered without producing

systemic effects. The masseter muscle was treated with either AP18 or vehicle and changes in mechanical thresholds were assessed at 1, 2 and 24 hrs following the drug treatment. On day 1, AP18, but not the vehicle, significantly attenuated the CFA-induced mechanical hypersensitivity ($F=25.8$, $P<0.001$, Fig. 3B). The AP18 effect was significant only during the first hour and completely abated by 24 hrs. AP18 also significantly reversed the CFA-induced mechanical hypersensitivity on day 3 ($F=31.4$, $P<0.001$, Fig. 3C). Interestingly, AP18 effect was time-dependent in that the relative attenuation of mechanical hypersensitivity by the same dose of AP18 was greater on day 3 compared to day 1 following the CFA treatment.

TRPA1 is involved in CFA-induced spontaneous muscle pain responses

To assess the contribution of TRPA1 to ongoing spontaneous pain under muscle inflammation, we evaluated RGS scores. As shown in Figure 4A, the RGS score was significantly increased from the baseline at 6hr, day 1 and day 3 and returned to the baseline level by day 7 following CFA treatment in the masseter muscle ($F=10.97$, $P<0.001$). The vehicle treatment also evoked consistently higher RGS scores than the baseline scores at the same time points, but CFA treatment induced significantly higher RGS scores compared to those of vehicle treated rat. The RGS score was highest on day 1 and the difference in RGS scores between vehicle and CFA groups was also greatest on day 1. Subsequently, we examined whether AP18 treatment in the masseter muscle one day after CFA treatment can reverse CFA-induced muscle pain responses. AP18 (2 μmol), but not vehicle, effectively attenuated the CFA-induced RGS score ($F=8.81$, $P<0.001$). The AP18 effect lasted for 1 hr and was ineffective after 2 hrs (Fig 4B). Since vehicle for CFA also evoked a slightly higher RGS scores it is possible that the AP18 reduced the vehicle-evoked responses. However, our data showed that AP18 did not significantly alter the vehicle-evoked responses, confirming that CFA-induced pain responses are mediated by TRPA1.

Masseter inflammation leads to TRPA1 up-regulation in TG

In order to assess the impact of inflammation on TRPA1 expression, we measured the changes in the TRPA1 mRNA content in TG following intramuscular injection with CFA. CFA-induced masseter inflammation resulted in a time-dependent increase in the level of TRPA1 mRNA in TG (Fig. 5A). The level of TRPA1 mRNA was significantly greater in CFA-treated groups on days 1, 3 and 7 compared with the naïve group ($F=6.67$, $P<0.01$). The CFA treatment did not alter TRPA1 expression in TG contralateral to the injected side (data not shown). In order to rule out the possibility that the intervention altered TRPA1 expression in TG, we analyzed the changes in TRPA1 mRNA following vehicle injection in the masseter muscle in the same manner. We chose 3 days and 14 days following the injection as representative time points and showed that vehicle injection itself did not significantly alter TRPA1 mRNA expression levels in either time point (Fig 5B).

To determine whether CFA induced changes in TRPA1 protein levels, we performed western blot analysis in TG from naïve and CFA-inflamed rats. We first validated the specificity of the antibody in HEK293 cells. The band size consistent with TRPA1 protein was reliably detected in only the cells transfected with TRPA1 cDNA, but not in empty vector transfected cells (Fig 5C). Using the same antibody we assessed whether TRPA1

protein is upregulated in TG 3 days following CFA treatment. We chose a 3 day post CFA time point since TRPA1 mRNA up-regulation was most prominent and AP18 effectively reversed mechanical hyperalgesia at this time point. Consistent with the RNA data, there was a significant up-regulation of TRPA1 protein level in TG of CFA inflamed rats compared to that of naive TG by approximately 43% (Fig 5D, $t=3.93$, $P<0.05$).

DISCUSSION

The present study demonstrated that 1) inhibition of TRPA1 suppressed mechanical hypersensitivity following masseteric injection of NMDA or ATP, 2) inhibition of TRPA1 attenuated mechanical hypersensitivity and facial grimace scores following CFA-induced myositis, 3) masseter inflammation up-regulated TRPA1 transcript and protein in TG. These results suggest that TRPA1 up-regulation contributes to mechanical hyperalgesia and spontaneous ongoing pain under masseter inflammation in rats.

While the literature on TRPA1 is not as extensive as TRPV1, TRPA1 is certainly emerging as another ‘integrator’ of various inflammatory signals in sensory neurons (Story and Gereau, 2006). Our results suggest that, like TRPV1, TRPA1 may also form ‘functional units’ with not only G-protein coupled receptors (Bandell et al., 2004, Bautista et al., 2013), but also with other ion channels, such as P2X₃ and NMDAR in sensory neurons. We have previously shown that ATP- and NMDA-induced acute mechanical hypersensitivity arising from masseter muscle involves TRPV1 sensitization (Lee et al., 2012b, Saloman et al., 2013). These results led us to conclude that excess ATP or glutamate in muscle tissue leads to the development of mechanical hypersensitivity through both TRPV1 and TRPA1. ATP or glutamate-induced mechanical hypersensitivity is prevented when either PKC or CaMKII is inhibited, and the inhibition of TRPV1 similarly suppressed the muscular hypersensitivity (Lee et al., 2012b, Saloman et al., 2013). Both P2X₃ and NMDAR activation induces phosphorylation of TRPV1, including serine 800 site, which is the major underlying mechanism for TRPV1 sensitization (Lee et al., 2012a). However, intracellular signaling mechanisms by which P2X₃ and NMDAR interact with TRPA1 are not known. Unlike TRPV1, phosphorylation of TRPA1 has never been demonstrated. While the same kinases could potentially mediate the interaction, it is unclear at this time in what ways they might be modifying TRPA1. Potentiation of TRPA1 function by PLC and cAMP-PKA pathway, but not PKC, has been reported in dorsal root ganglia (DRG) neurons (Dai et al., 2007, Wang et al., 2008). Thus, it is possible that NMDA or ATP injected in the muscle may invoke signaling cascades that are unique to TRPA1, which should be further explored in the future.

To examine the role of TRPA1 in mechanical hypersensitivity under a myositis condition, we evaluated the effect of a selective TRPA1 antagonist, AP18, in the CFA model of inflammatory pain. When injected into craniofacial muscles, CFA induces a profound mechanical hypersensitivity that peaks within the first 3 days and gradually returns to baseline in 2–3 weeks (Ambalavanar et al., 2006, Shimizu et al., 2009, Niu et al., 2012). In the present study intramuscular administration of AP18 was found to reverse CFA-induced mechanical hypersensitivity at 24 hours and 72 hours post CFA treatment when mechanical hypersensitivity was most prominent. The anti-hyperalgesic effect of AP18 lasted

approximately one hour in our hands, which is shorter than the previously reported duration of its effects in the hindpaw (Petrus et al., 2007, Bonet et al., 2013). The hyperalgesia returned as the effect of AP18 dissipated, data suggesting that a sustained TRPA1 activation is required for the maintenance of mechanical hyperalgesia. These results are consistent with a number of previous reports that have supported a role for TRPA1 receptors in inflammatory pain. Intra-paw injection of AP18 partially reverses CFA-induced mechanical hyperalgesia (Petrus et al., 2007). In a separate study, oral administration of another TRPA1 antagonist, HC-030031, inhibited CFA-induced mechanical hypersensitivity (Eid et al., 2008). Additionally, a recent study demonstrated that intra-articular CFA-induced mechanical hyperalgesia in CD1 mice was attenuated by AP18, which was administered 22 hours after the CFA treatment in the same knee joint (Fernandes et al., 2011). In their study, intra-articular CFA-induced mechanical hyperalgesia was maintained for 3 weeks in TRPA1 wild type mice, whereas TRPA1 knock out mice exhibited mechanical hyperalgesia for only 24 hours. TRPA1 was also found to be a central factor in the mechanical hypersensitivity displayed during the first 2 weeks in young mice, and that TRPA1 plays an even more critical role in maintaining chronic joint hypersensitivity in aged mice (Garrison and Stucky, 2014). These data suggest that endogenous activation of TRPA1 plays a critical role in sustaining the mechanical hyperalgesia observed after knee joint inflammation. Our data provided novel findings that TRPA1 also plays a role in the maintenance of mechanical hypersensitivity in the presence of muscle inflammation.

In addition to mechanical hypersensitivity, we demonstrated that TRPA1 is involved in maintaining spontaneous ongoing pain arising from craniofacial muscle tissue. In order to capture ongoing muscle pain responses, we have implemented the RGS assay in our model. This model has been used to characterize inflammation- as well as nerve injury-induced spontaneous pain by assessing facial expression patterns (Sotocinal et al., 2011). Interestingly, the duration and magnitude of RGS scores following CFA treatment in the masseter muscle were similar to those documented after CFA treatment in the hindpaw. Recently, the RGS assay was validated for assessing orofacial pain following tooth movements in a rat model of orthodontic treatment (Liao et al., 2014). Our data provide additional support that the RGS assay can serve as an effective tool to study ongoing pain in orofacial regions, an important aspect of pathological pain responses under myositis. Our data showed that the temporal profiles of the development, maintenance and resolution of spontaneous pain and mechanical hypersensitivity are clearly different. The underlying mechanisms for these pain-related responses from craniofacial deep tissues can be more rigorously explored with the various behavioral tools that are now available. Nevertheless, our data indicate the involvement of TRPA1 in the maintenance of both spontaneous pain and mechanical hypersensitivity, especially when TRPA1 expression is upregulated.

The contribution of TRPA1 under inflammatory conditions could be sustained or enhanced through various mechanisms. As discussed above, inflammatory pain has been suggested to occur as a consequence of receptor sensitization and/or activation by a variety of pro-inflammatory mediators (Dai et al., 2007, Materazzi et al., 2008, Taylor-Clark et al., 2008, Wang et al., 2008). Alternatively, reactive oxygen species (ROS) released in response to tissue damage during inflammation can either directly activate TRPA1 (Macpherson et al., 2007) or could lead to the production of endogenous agonists by lipid peroxidation

(Trevisani et al., 2007). Recently, we showed that ROS is released within TG following masseter inflammation, which could lead to direct or indirect activation of TRPA1 within TG (Chung, 2015). Therefore, a sustained elevation of ROS in the damage tissue could potentially maintain TRPA1 activation. One study has revealed that activation of PKA and PLC increases TRPA1 surface expression suggesting a possible mechanism of sensitization related to channel trafficking (Schmidt et al., 2009).

In addition to regulation of functions and localization, inflammation could also enhance TRPA1-mediated nociception by up-regulating TRPA1 expression. An early study showed that inflammation increases TRPA1 expression in DRG neurons and the blockade of TRPA1 up-regulation suppresses inflammation-induced enhancement of TRPA1 functionality (Obata et al., 2005). More recent studies in the visceral pain also correlate TRPA1 expression and hyperalgesia. For example, when the up-regulation of TRPA1 in colonic afferents in DRG is prevented, so is the colitis-induced hyperalgesia (Yang et al., 2008). Under a chronic stress condition, increased expression of TRPA1 was found to be critical for visceral hypersensitivity as well as spinal neuronal hyper-excitability (Chen et al., 2013). Cyclophosphamide (CYP)-induced bladder hyperalgesia is also associated with significant increase in TRPA1 expression, and blockade of TRPA1 alleviated CYP-induced bladder hyperalgesia (DeBerry et al., 2014). Notably, mice with a null mutation of glycoprotein 130, a signal transducing subunits for interleukin-6 and an upstream to TRPA1 expression, show reduced mechanosensitivity and TRPA1 mRNA expression in DRG (Malsch et al., 2014). These reports strongly support a correlation between TRPA1 expression and hyperalgesia. Our results also suggested that inflammation-induced increases in TRPA1 expression in TG play a critical role in masseter hypersensitivity. While TRPA1 expressed in neurons is likely a key contributor to the inflammation-induced upregulation, it is also possible that TRPA1 expressed in non-neuronal cells may also contribute to the change in total TRPA1 in TG. Detailed mechanisms involving transcription regulation of TRPA1 under inflammatory conditions is currently lacking. Therefore, future studies determining factors that regulate TRPA1 expression and the context in which TRPA1 up-regulation is sustained could provide important new insights into the contribution of TRPA1 in pathological pain conditions. Taken together, our study suggest that TRPA1 plays a key role in both acute and persistent types of mechanical hyperalgesia and spontaneous ongoing pain in the craniofacial muscle, and that targeting TRPA1 in muscle afferents is a promising strategy for alleviating inflammatory muscle pain and hyperalgesia.

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Abbreviations

$\alpha\beta$meATP	α,β -methylene adenosine triphosphate
CFA	complete Freund's adjuvant
NMDA	N-Methyl-D-aspartate

TG	trigeminal ganglia
TRPA1	Transient receptor potential cation channel, subfamily A, member 1

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HIGHLIGHTS

- TRPA1 mediates acute muscle mechanical hypersensitivity induced by ATP and glutamate.
- TRPA1 contributes to the maintenance of mechanical hypersensitivity under a myositis condition.
- TRPA1 contributes to the maintenance of spontaneous muscle pain under a myositis condition.
- Inflammation in the masseter muscle significantly up-regulates TRPA1 expression in trigeminal ganglia.

FIGURE 1.

$\alpha\beta$ meATP-induced acute masseter hypersensitivity involves TRPA1. (A) Line graph shows changes in masseter mechanical thresholds induced by $\alpha\beta$ meATP in the presence of the TRPA1 antagonist, AP18 (n=6 per group). (B) Bar graph shows the overall magnitude of AP18 effect as measured by area under curve. (C) Overall effects of HC-030031 during the same time course are shown as area under curve (n=4 per group). BL: baseline 15 min prior to $\alpha\beta$ meATP injection. Arrow: vehicle or antagonist injection 5 min prior to $\alpha\beta$ meATP injection. +, * indicates significant ($p<0.05$) group and time effects, respectively.

FIGURE 2.

NMDA-induced acute masseter hypersensitivity involves TRPA1. (A) Line graph shows changes in masseter mechanical thresholds induced by NMDA in the presence of the TRPA1 antagonist, AP18 (n=6 per group). (B) Bar graph shows the overall magnitude of drug effect as measured by area under curve. (C) Overall effects of HC-030031 during the same time course are shown as area under curve (n=4 per group). BL: baseline 15 min prior to NMDA injection. Arrow: vehicle or antagonist injection 5 min prior to $\alpha\beta$ meATP injection. +, * indicates significant ($p<0.05$) group and time effects, respectively.

FIGURE 3.

CFA-induced persistent masseter hyperalgesia involves TRPA1. (A) Line graphs show changes in mechanical hyperalgesia in the rat masseter muscle following CFA and vehicle. Mechanical force (g) that produced the head withdrawal responses 50% of the trials is plotted for pre- and 1, 3, 7, 14, 21 and 28 days post injection. * denotes significant time effects at $p < 0.05$ compared to the pre-injection values. (B, C) Effects of intramuscular AP18 or vehicle on mechanical sensitivity 1 and 3 days after CFA treatment. Bar graphs show mechanical thresholds for pre- and 1, 2 and 24 hrs post-treatment. * denotes significant differences at $p < 0.05$ compared to the pre drug treatment values.

FIGURE 4.

Mean RGS scores following CFA treatment in the masseter muscle. (A) RGS scores are presented for rats treated with either intramuscular CFA (50 μ l) or vehicle. ** denotes $p < 0.01$, *** $p < 0.001$ and # $p < 0.0001$ between CFA and vehicle treated groups. N=8 per each group. (B) RGS scores were compared between AP18 and vehicle treatment in CFA- and vehicle-treated rats at day 1. # denotes $p < 0.0001$ between AP18 and vehicle treated groups. The arrow indicates the time of AP18 or vehicle injection. N=6 per group.

FIGURE 5.

Masseter inflammation increases TRPA1 expression in TG. Real time RT-PCR data showing (A) CFA- and (B) vehicle-induced changes in TRPA1 mRNA levels in TG compared to that of naive untreated TG. N=4. (C) HEK293 cells transfected with either TRPA1 cDNA or empty vector were used for antibody validation. (D) Up: Immunoblots of TRPA1 in naive and CFA treated TG. Bottom: Quantification of TRPA1 protein level relative to naive untreated TG samples. * denotes $p < 0.05$. Each group consisted of 4 animals and data are shown as mean $\pm$ S.E.M.