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Peripheral Group I metabotropic glutamate receptor activation leads to muscle mechanical hyperalgesia through TRPV1 phosphorylation in the rat

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Abstract

Elevated glutamate levels within injured muscle play important roles in muscle pain and hyperalgesia. In this study, we hypothesized that PKC-dependent TRPV1 phosphorylation contributes to the muscle mechanical hyperalgesia following activation of Group I metabotropic glutamate receptors (mGlu1/5). Mechanical hyperalgesia induced by dihydroxyphenylglycine (DHPG), an mGlu1/5 agonist, in the masseter muscle was attenuated by AMG9810, a specific TRPV1 antagonist. AMG9810 also suppressed mechanical hyperalgesia evoked by pharmacological activation of PKC. DHPG-induced mechanical hyperalgesia was suppressed by pretreatment with a decoy peptide that disrupted interactions between TRPV1 and A-kinase anchoring protein (AKAP), which facilitates phosphorylation of TRPV1. In dissociated trigeminal ganglia (TG), DHPG upregulated serine phosphorylation of TRPV1 (S800) during which DHPG-induced mechanical hyperalgesia was prominent. The TRPV1 phosphorylation at S800 was suppressed by a PKC inhibitor. Electrophysiological measurements in TG neurons demonstrated that TRPV1 sensitivity was enhanced by pretreatment with DHPG, and this was prevented by a PKC, but not by a PKA, inhibitor. These results suggest that mGlu1/5 activation in masseter afferents invoke phosphorylation of TRPV1 serine residues including S800, and that phosphorylation-induced sensitization of TRPV1 is involved in masseter mechanical hyperalgesia. These data support a role of TRPV1 as an integrator of glutamate receptor signaling in muscle nociceptors.

PERSPECTIVE—This article demonstrates that activation of mGlu1/5 leads to phosphorylation of a specific TRPV1 residue via PKC and AKAP150 in trigeminal sensory neurons, and that functional interactions between glutamate receptors and TRPV1 mediate mechanical hyperalgesia in the muscle tissue.

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DISCLOSURES

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Keywords

Peripheral; trigeminal; sensory neurons; muscle pain

1. INTRODUCTION

TRPV1 is a non-selective cationic channel that can be activated by capsaicin, proton, and noxious heat. TRPV1 mediates cutaneous thermal hyperalgesia during inflammation and tissue injury^{7, 12}. Under pathophysiological conditions, multiple inflammatory mediators invoke the activation of multiple kinases that phosphorylate TRPV1 enhancing its functionality. Increased heat sensitivity of TRPV1 following phosphorylation underpins mechanisms of thermal hyperalgesia^{12, 32}. Recently, the causative association between phosphorylation of TRPV1 and cutaneous thermal hyperalgesia has been demonstrated in studies that broke the association of TRPV1 with A-kinase anchoring protein (AKAP), which facilitates TRPV1 phosphorylation⁹. TRPV1 is expressed not only in skin but also is expressed in nociceptors projecting to deep tissues such as gut, joint, and muscle⁷. In contrast to its contribution to thermal hyperalgesia in skin, accruing evidence suggests that TRPV1 in deep tissue afferents contributes to sensitization mediated by mechanical stimuli. Inflammation or injury to deep tissues induces enhanced responses to mechanical stimuli, and these enhanced responses in deep tissues are attenuated by pharmacological or genetic inhibition of TRPV1^{1, 15, 25, 29, 35}. Therefore, TRPV1 represents a candidate target not only for treating cutaneous thermal hyperalgesia but also for treating mechanical hyperalgesia in deep tissues.

Glutamate is one of the substances released upon muscle injury and is a major contributor to muscle hypersensitivity^{4, 33}. Activation of ionotropic or metabotropic glutamate receptors (mGluR) induces mechanical hyperalgesia in masseter muscles^{4, 20, 21}. Interestingly, mechanical hyperalgesia induced by masseteric injection of N-methyl-D-aspartate (NMDA) depends not only on peripheral NMDA receptors but also on TRPV1²⁰, demonstrated by the fact that an antagonist of TRPV1 prevents the development of NMDA-induced masseter hypersensitivity. NMDA enhances capsaicin-evoked responses in a subset of primary afferents and increases phosphorylation of TRPV1 in primary afferents, which further supports the functional involvement of TRPV1 in NMDA-induced masseter hypersensitivity¹⁹. We previously demonstrated that masseteric injection of R,S-3,5-dihydroxyphenylglycine (DHPG), an agonist of Group I mGluR (mGlu1/5), induced masseter hypersensitivity [21]. It is unclear, however, whether masseter hypersensitivity induced by the activation of mGlu1/5 also involves TRPV1.

Activation of NMDA receptors induces masseter hypersensitivity in a protein kinase C (PKC)- and TRPV1-dependent manner²⁰. Pharmacological inhibition of PKC or knock down of AKAP150 decreases NMDA-induced phosphorylation of TRPV1 in primary afferents¹⁹. Since masseter hypersensitivity induced by mGlu1/5 activation is primarily dependent on PKC²¹, it is possible that mGlu1/5 is also linked to TRPV1 through PKC. However, PKC-dependent interactions between mGlu1/5 and TRPV1 in primary afferents have not been reported. Activation of mGlu1/5 was reported to attenuate capsaicin-induced desensitization of TRPV1 through protein kinase A (PKA) via *de novo* synthesis of PGE₂¹¹.

Activation of mGluR was also suggested to generate diacylglycerol, which directly activates TRPV1 through a PKC-independent mechanism¹⁷. Therefore, in this project, we tested the hypothesis that PKC-dependent phosphorylation of TRPV1 contributes to masseter hypersensitivity following the activation of mGlu1/5. This hypothesis was tested by a combination of biochemical and electrophysiological analyses and behavioral assays in a rodent model of masseter hypersensitivity.

2. MATERIALS AND METHODS

2.1. Animals

Adult male Sprague-Dawley rats (150 to 350 g; Harlan, IN, USA) 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 National Institutes of Health 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.

2.2. Drugs and peptide preparation for behavioral experiments

DHPG was dissolved in PBS (1 μ mol in 100 μ l). AMG9810, a specific antagonist of TRPV1, was dissolved in a vehicle containing 5% dimethylsulfoxide (DMSO), 10% Tween-80, and 85% PBS (1 or 100 nmol in 10 μ l). Phorbol 12-myristate 13-acetate (PMA), an activator of PKC, was dissolved in PBS (300 nmol in 30 μ l) and forskolin (FSK), an activator for protein kinase A (PKA), was dissolved in PBS (50 nmol in 20 μ l). A Peptide spanning residues 736–745 of A-kinase anchoring protein 150 (AKAP150) fused to the TAT sequence (transactivator of transcription of HIV) (735–757-TAT: KDDYRWCFRVYGRKKRRQRRR; TAT sequence is underlined) that interferes with the interaction of TRPV1 with AKAP150 was synthesized from the published sequences⁹. As a control, a scramble-TAT peptide (RFVCWDKYRDYGRKKRRQRRR) was also synthesized. Both peptides contained the TAT sequence to mediate transmembrane transport. Purity of the peptides were >95% in mass spectroscopic analysis (Genscript). Both 736–745-TAT (10 and 30 μ M) and scramble-TAT (30 μ M) were dissolved in PBS.

2.3. Behavioral assays – assessment of mechanical sensitivity in masseter muscle

Noxious chemical or mechanical stimulation of the masseter muscle evokes characteristic shaking 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^{20, 21, 29}. This lightly anesthetized rodent model allows the delivery of a calibrated and reliable mechanical stimulus to the masseter muscle or temporomandibular joint before and after pharmacological manipulations, which is difficult to accomplish in awake animals. Initially, rats (250 to 350 g) were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg, i.p.). 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 minutes after the initial anesthesia. Once the animal reached this level, a metal clip calibrated to produce 600 g of

force was applied 5 consecutive times to the tail, 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 Holliston, Massachusetts) 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 to 5 mg/h). A baseline mechanical threshold for evoking the nocifensive response was determined 15 minutes before drug injection using the electronic von Frey (VF) anesthesiometer (IITC Life Science, Inc, Woodland Hills, CA, USA). A rigid tip (2 mm diameter) 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 assessed at 15, 30, 45, 60, and 90 minutes after drug treatments. To maintain consistency of assessing behavioral responses, all behavioral observations were made by a single experimenter blinded to the experimental conditions. The percent changes in VF thresholds after drug treatment were calculated with respect to the baseline threshold and plotted against time. The time-dependent mean percent changes in mechanical thresholds normalized to the baseline threshold were analyzed by two-way analysis of variance (ANOVA) with repeated measures. All multiple-group comparisons were followed by a post-hoc test (Bonferroni). The significance of all statistical analyses presented in this report was set to $P < 0.05$.

2.4. Dissociation of rat TG neurons

Male Sprague Dawley rats (150–250 g) were used for primary trigeminal ganglia (TG) neuron cultures as described previously¹⁹. TG were minced in cold Hanks's Balanced Salt Solution (HBSS) and incubated in 5 ml of Dulbecco's Modified Eagle Medium (DMEM)/F-12 containing collagenase and trypsin in a shaking incubator at 37°C for 30 min. TG extracts were mechanically dissociated and resuspended in the culture medium before plating in 12 well plates coated with laminin. Dissociated TG neurons were maintained with DMEM/F-12 media containing 10% FBS, 1% penicillin/streptomycin at 37°C in a 5% CO₂ incubator. Cultures were used in immunoprecipitation experiments and biotinylation assays three to four days after plating. For electrophysiological assays, the neurons were plated onto glass coverslips coated with poly-L-ornithine and laminin, and were maintained in DMEM/F-12 media containing 10% FBS, 1% penicillin/streptomycin. In experiments testing PKC and PKA inhibitors, 100 ng/ml nerve growth factor was added to the medium. Electrophysiological recordings were performed between 18–48 hours.

2.5. Immunoprecipitation

TG cultures were treated with lysis buffer containing protease inhibitor cocktail. To extract protein, the lysate was centrifuged at 12,000 rpm at 4°C for 20 min. The protein concentration of the cell lysate was measured using a Bio-Rad protein assay reagent kit. Proteins were immunoprecipitated from lysates with TRPV1 antibody (1 µg, polyclonal, anti-rabbit, Calbiochem) overnight at 4°C, and then with protein A/G-Sepharose beads (Santa Cruz) for 2 hr. LDS loading dye including sodium dodecyl sulfate was added and samples were treated at 100°C for 5 min to elute proteins from the bead complex. The

denatured protein was then fractionated on a 4–12% gradient NuPAGE electrophoresis gel and blotted onto a polyvinylidene difluoride or nitrocellulose membrane. The membrane was blocked and incubated overnight at 4°C with a monoclonal phosphoserine antibody (1:500, monoclonal, anti-mouse, Santa Cruz). The bound primary antibody was detected with a horseradish peroxidase conjugated anti-mouse IgG secondary antibody. As a loading control, the membranes were stripped and re-probed with anti-TRPV1 (1:1000, polyclonal, anti-rabbit, Calbiochem). Immune complexes were visualized using an enhanced chemiluminescence reagent (Amersham) and recorded on X-ray film. Films were scanned, and bands were quantified using Image J software. The re-probed TRPV1 on the same membrane was used to normalize p-Ser concentrations.

2.6. Biotinylation of cell surface proteins and western blot analysis

To examine changes in proteins localized to the plasma membrane, we performed biotinylation as described previously^{16, 19}. Briefly, dissociated TG cells were washed three times in cold PBS. For membrane protein biotinylation, TG cells were incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in PBS at 4°C for 30 min. To quench the reaction, cells were washed three times with cold PBS containing 100 mM glycine. TG neurons were then lysed in lysis buffer containing protease inhibitor cocktail followed by centrifugation at 1000 *g* for 5 min. The 100–150 µg of collected lysate was incubated with streptavidin cross-linked to agarose beads (Pierce) for 2 hours at 4°C. The beads were then washed twice with lysis buffer, and eluted with LDS loading buffer by heating at 100°C for 5 min. The membranes were incubated with antibody against p-S800 TRPV1 antibody (1:500, polyclonal, anti-rabbit, Cosmo) for three days at 4°C. The specificity of this antibody was previously verified²³. In order to normalize the amount of protein loaded and to examine contamination of cytosolic components in the biotinylation assay, the stripped membranes were incubated with GAPDH antibody (1:5000, monoclonal, anti mouse, Sigma). For the relative quantification of p-S800 TRPV1, the GAPDH level of the corresponding sample was used as the normalization control.

2.7. Whole-cell voltage clamp recordings

Whole-cell voltage clamp techniques were performed as described previously^{8, 34}. The recording pipettes (2–3 MΩ) were pulled from borosilicate glass using a P-97 (Sutter Instrument). The pipettes were filled with an internal solution containing 140 mM KCl, 5 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 2.5 mM Mg-ATP, 10 mM EGTA, 10 mM HEPES pH 7.4 (adjusted with KOH). Unless otherwise indicated, the recording bath contained an external solution containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES pH 7.4 (adjusted with NaOH). Osmolarity of each solution was measured on a vapor pressure osmometer (Wescor Inc) and was adjusted with mannitol to 290 to 310 mOsm as necessary.

3. RESULTS

3.1. TRPV1 contributes to masseter mechanical hyperalgesia induced by the activation of mGlu1/5 and PKC

Previously, we demonstrated that injection of DHPG into masseter muscle induces mechanical hyperalgesia in a PKC-dependent manner ²¹. Here, we tested whether DHPG-induced muscle mechanical hyperalgesia depends on TRPV1 by using AMG9810, a TRPV1 antagonist. Intramuscular injection of DHPG (i.m.) significantly decreased the mechanical threshold of masseter muscle, with peak effects at 15 minutes that gradually declined toward baseline levels over 90 minutes following the injection (Fig. 1A). Pretreatment of masseter muscle with AMG9810 (i.m.) significantly attenuated DHPG-induced masseter hyperalgesia (Fig. 1A, B). In contrast, pretreatment with vehicle did not alter the DHPG-induced responses. Injection of AMG9810 (i.m.) alone did not alter muscle mechanical sensitivity ²⁰. These results suggest that TRPV1 is involved in DHPG-induced masseter mechanical hyperalgesia.

Pharmacological activation of PKC by intramasseteric injection of PMA or forskolin (i.m.), respectively, induced robust masseter mechanical hyperalgesia (Fig. 2A). Pretreatment with AMG9810 (i.m.) significantly suppressed PMA-induced mechanical hyperalgesia (Fig. 2A, B). In contrast, AMG9810 did not significantly attenuate forskolin-induced mechanical hyperalgesia (Fig. 2C, D). Although direct injections of PMA and forskolin can induce peripheral inflammation due to non-specific activation of various cell types, only PMA-induced mechanical analgesia was attenuated with the TRPV1 antagonist. These results suggest that TRPV1-mediated masseter mechanical hyperalgesia involves PKC but not PKA.

3.2. Breaking the interactions between TRPV1 and AKAP150 attenuates DHPG-induced muscle mechanical hyperalgesia

AKAP79/150 is a scaffolding protein that harbors PKA and PKC in the proximity of TRPV1 channels, which facilitates phosphorylation efficiency ^{13, 14, 31, 37}. We previously showed that knockdown of AKAP150 consistently attenuates NMDA-induced phosphorylation at the Ser800 residue as well as total serine of TRPV1 in sensory neurons ¹⁹. Recently, it was demonstrated that interfering with interactions between AKAP and TRPV1 using a membrane-permeable decoy peptide (736–745-TAT) attenuated thermal hyperalgesia in skin ⁹. Here, we tested whether disrupting the association of AKAP and TRPV1 would also alter muscle mechanical hyperalgesia. We found that pretreatment of the masseter muscle with 736–745-TAT peptide (i.m.) caused a dose-dependent reduction in DHPG-induced mechanical hyperalgesia (Fig.3). However, pretreatment with scramble-TAT peptide did not affect masseter hyperalgesia. These results indicate that DHPG-induced masseter hyperalgesia involves the interactions of TRPV1 and AKAP, and hence, suggest a causal relationship between DHPG-induced masseter hyperalgesia and phosphorylation of TRPV1.

3.3. Activation of mGlu1/5 leads to PKC-dependent phosphorylation of TRPV1 in primary afferents

Our recognition of the involvement of PKC, TRPV1, and AKAP in DHPG-induced mechanical hyperalgesia led us to examine whether activation of mGlu1/5 would lead to PKC-mediated phosphorylation of TRPV1. Since it is not feasible to biochemically quantify the extent of phosphorylated TRPV1 at the muscle afferent terminals, we used dissociated TG neurons as an alternative model. Dissociated neurons were exposed to DHPG for 15, 30, and 45 minutes, and the extent of TRPV1 phosphorylation was assessed using phospho-specific antibodies. To assess the extent of phosphorylation of global serine residues of TRPV1, we performed immunoprecipitation using an antibody against TRPV1, and probed blots with an anti-phospho-serine antibody. The application of DHPG enhanced phosphorylation of serine residues compared to that of the baseline condition without DHPG. The increase was significant at 15 and 30 minutes, time points during which NMDA-induced mechanical hyperalgesia is prominent (Fig. 4A). The phosphorylation level declined to the baseline level by 45 minutes. The total amount of TRPV1 did not change during this time course. To evaluate phosphorylation of the S800 residue of TRPV1, we used an antibody specific for the phosphorylated S800 residue. For quantifying phosphorylation of TRPV1 located at the plasma membrane, we performed a biotinylation assay. Using these methods, we assayed p-S800 levels at 15, 30, and 45 minutes following the application of DHPG. The DHPG application resulted in a significant increase in plasma membrane levels of p-S800 at 15 and 30 minutes, which was consistent with the time course of phospho-serine increase and DHPG-induced muscle mechanical hyperalgesia (Fig. 4B). In these assays, GAPDH was only detected in cytosolic samples and not in biotinylation samples, which suggests minimal contamination of the cytosolic component in our preparation (data not shown). The increase in p-S800 was prevented when DHPG was co-applied with GF10920X, a PKC inhibitor, but was not prevented by co-application with vehicle (Fig. 4C, D). These results showed that activation of mGlu1/5 leads to the phosphorylation of S800 in membrane-delimited TRPV1 via PKC in rat TG neurons.

3.4. Activation of mGlu1/5 induces PKC-dependent sensitization of TRPV1 in primary afferents

Since treatment of primary afferents with DHPG upregulated phosphorylation of TRPV1 through PKC, we examined whether TRPV1 sensitization by DHPG is PKC-dependent. To test this, we performed electrophysiological recordings in dissociated primary afferent neurons. A brief application of capsaicin (200 nM) induced modest activation of currents. Under our recording conditions, two consecutive applications of capsaicin evoked responses of almost identical size without obvious desensitization. When DHPG was applied for two minutes prior to the second application of capsaicin, the ratios of 2nd/1st responses were 1.2 ± 0.2 (n=4), which was not significantly different from vehicle (1.9 ± 0.4 ; n=7; $p > 0.6$). However, prolonged DHPG treatment (10 min) greatly enhanced the 2nd response, and this enhancement was significantly different from the vehicle treated control (Fig. 5A–B). The effects of DHPG were abrogated by the co-application with the PKC inhibitor GF109203X. However, co-administration of DHPG with KT5720, an inhibitor of PKA, did not significantly alter the extent of DHPG-induced sensitization. These results provide evidence

that DHPG induces sensitization of TRPV1 through the activation of PKC rather than through activation of PKA (Fig. 5C–D).

4. DISCUSSION

In this study, we demonstrated that 1) DHPG-induced masseter mechanical hyperalgesia depends on TRPV1; 2) TRPV1 is involved in masseter mechanical hyperalgesia evoked by the activation of PKC but not PKA; 3) DHPG-induced masseter mechanical hyperalgesia is suppressed by disrupting interactions between TRPV1 and AKAP150; 4) DHPG upregulates phosphorylation of TRPV1 serine residues including S800; 5) DHPG enhances capsaicin-evoked responses in TG neurons in a PKC-dependent manner. Since our previous study identified mGlu5 and PKC ϵ as major contributors to DHPG-induced masseter hypersensitivity²¹, these results collectively suggest that mGlu5 activation invokes the activation of PKC, which phosphorylates TRPV1 serine residues including S800, and that such phosphorylation-induced sensitization of TRPV1 enhances masseter mechanical hyperalgesia in an AKAP150-dependent manner.

AKAP150 is the product of the rat AKAP5 gene (the human homolog is AKAP79). AKAP150 is a scaffolding protein that harbors PKA and PKC in the proximity of TRPV1 channels, which facilitates phosphorylation efficiency^{13, 14, 31, 37}. Genetic knockout or siRNA-mediated knockdown of AKAP150 attenuates PKA or PKC-mediated sensitization of TRPV1. Using phospho-specific antibodies, we previously reported direct evidence that knockdown of AKAP150 resulted in attenuation of PKC-induced phosphorylation at pan-serine residues as well as S800 of TRPV1¹⁹. In the hind paw, interference with interactions between AKAP and TRPV1 using a membrane-permeable decoy peptide reduces thermal hyperalgesia⁹, suggesting that the suppression of phosphorylation of TRPV1 alters hyperalgesia *in vivo*. In the current study, we demonstrated that DHPG-induced masseter mechanical hyperalgesia was also suppressed by disrupting associations between AKAP and TRPV1. These data suggest causal relationships between TRPV1 phosphorylation in muscle afferents and mechanical hyperalgesia *in vivo*.

Since AKAP harbors both PKC and PKA, we cannot exclude the possibility of a role for PKA. However, we conclude that PKC is the major contributor to DHPG-induced TRPV1-dependent masseter hypersensitivity because: 1) Inhibition of PKC substantially prevents DHPG-induced masseter hyperalgesia²¹; 2) TRPV1 antagonist suppressed masseter hyperalgesia induced by pharmacological activation of PKC but not PKA; 3) DHPG upregulated phosphorylation of the S800 residue of TRPV1, which is known to be specifically affected by PKC, but not PKA; 4) DHPG-induced phosphorylation of the S800 residue of neuronal TRPV1 was prevented by inhibition of PKC; 5) Functional sensitization of capsaicin responses of TRPV1 by DHPG in primary afferents was dependent on PKC but not on PKA. Our results on PKC-dependent DHPG-induced regulation of TRPV1 in primary afferents are novel. It was suggested that DHPG directly activate TRPV1 through *de novo* synthesis of diacylglycerol without involvement of PKC¹⁷. Under our conditions, however, instantaneous increases in currents upon DHPG treatment were not readily observed. Our analysis focused on evaluating the effects of prolonged treatment of DHPG on the capsaicin sensitivity of TRPV1 rather than on the instantaneous direct activation by DHPG. It has also

been shown that DHPG attenuates capsaicin-induced desensitization of TRPV1 through PKA via *de novo* synthesis of PGE₂ but not through PKC¹¹. In contrast, our electrophysiological recordings showed that DHPG-induced sensitization of TRPV1 responses to capsaicin were prevented by the PKC inhibitor but not by the PKA inhibitor. The sources of this apparent discrepancy are not clear. We presume that different experimental conditions might contribute to the difference. In Hu et al.¹¹, the effects of DHPG were tested under conditions in which consecutive application of capsaicin induces a moderate amount of desensitization. A relatively brief DHPG application (3.5 min) reversed desensitization by the second application of capsaicin. In our recordings, low concentration of capsaicin did not induce obvious desensitization. More importantly, a short (2 min duration) DHPG application did not significantly enhance capsaicin sensitivity under our conditions. However, a prolonged (10 min) application increased the capsaicin response, and this increased response was prevented by the PKC inhibitor. Although it is known that phosphorylation of TRPV1 commonly induces sensitization of TRPV1 and reverses capsaicin-induced desensitization²³, complicated yet unknown mechanisms of TRPV1 desensitization make it difficult to directly compare our data with previous reports. Nonetheless, the DHPG-induced PKC-mediated sensitization of capsaicin-evoked responses in primary afferents is consistent with the effects of DHPG on the PKC-dependent TRPV1 phosphorylation *in vitro* and masseter mechanical hyperalgesia *in vivo*.

Our previous and current studies indicate that the activation of NMDA receptors and mGlu5 in trigeminal primary afferents leads to increases in PKC-mediated phosphorylation of serine residues of TRPV1. PKC functionally alters TRPV1 through phosphorylation of at least three residues, S502, T704, and S800^{3, 26}. Using a specific antibody and pharmacology, we demonstrated that application of NMDA or DHPG caused elevated levels of phosphorylation of the neuronal TRPV1 S800 residue by PKC^{19, 23}. Although the apparently direct causal relationship between the phosphorylation of TRPV1 S800 and masseter hyperalgesia *in vivo* needs to be verified, these results suggest a major role of the TRPV1 S800 residue in masseter hyperalgesia. In addition to glutamate, muscle injury or inflammation causes increases in multiple inflammatory mediators, such as bradykinin and ATP, which also promote PKC-mediated phosphorylation of TRPV1^{7, 24}. Therefore, TRPV1 may function to integrate signals from multiple receptors located on nociceptor membranes²². Phosphorylation of TRPV1 enhances functionality of TRPV1 such that substantial channel activity is maintained even at body temperature. Although muscle may not be directly exposed to ambient temperature changes, exercise can increase the intramuscular temperature up to 39 °C³⁶, which may allow TRPV1 to maintain constitutively elevated activity following PKC-mediated phosphorylation^{26, 32}. Moreover, intramuscular acidosis following exercise, injury or ischemia of skeletal muscle^{2, 10, 27} should further enhance the activity of TRPV1 following phosphorylation in muscle nociceptors.

TRPV1 functions as a heat sensor and mediator of thermal hyperalgesia in cutaneous tissues⁷. Although TRPV1 alone does not fully explain heat sensitivity *in vivo*, TRPV1 labels the majority of heat-sensitive cutaneous afferents. Ablation of the TRPV1-expressing subpopulation of primary afferents in experimental animals attenuates thermal sensitivity

without altering mechanical sensitivity of skin ^{6, 28}. In contrast, TRPV1-expressing afferents projecting to deep tissues apparently label primary afferents that transduce mechanical pain. Lack of TRPV1 reduces sensitivity to colorectal distension ¹⁵, and pharmacological inhibition of TRPV1 attenuates visceral mechanical hyperalgesia following experimental colitis ²⁵. Knocking out TRPV1 inhibits cystitis-induced mechanical hyperalgesia ³⁵. Mechanical hyperalgesia following knee joint inflammation is reduced in TRPV1 knockout mice ¹, and intra-articular injection of resiniferatoxin enhances weight-bearing behavior in an experimental arthritis model ¹⁸. These results suggest that TRPV1 is expressed in primary afferents responsible for mechanical pain and hyperalgesia in deep tissues. Our results from current and previous studies ^{19, 20} provide further evidence that enhanced activity of TRPV1 following PKC-induced phosphorylation leads to mechanical hyperalgesia in muscle tissue. However, the mechanistic link between enhanced activity of TRPV1 and mechanical hyperalgesia remains unclear. Apparently, TRPV1 does not function as a transducer of mechanical stimuli *per se* in muscle afferents. We presume that enhanced activity of TRPV1 might alter the excitability of mechano-nociceptors in muscle afferents increasing sensitivity of muscle afferents to increases in mechanical stimuli. These putative roles of TRPV1 in mechano-nociceptors need to be further investigated.

Peripheral glutamate is important for masseter muscle hyperalgesia. The level of endogenous glutamate is elevated in the masseter muscle of temporomandibular disorder patients relative to healthy subjects, which suggests that peripheral glutamate receptors are involved in persistent muscle pain ⁵. The peripheral glutamate receptors have been implicated in acute pain and mechanical hyperalgesia arising from orofacial muscle tissue in humans and experimental animals ^{4, 20, 21, 30, 33}. Previously, we demonstrated that masseter hyperalgesia following the activation of peripheral NMDA receptors involves PKC-mediated phosphorylation of TRPV1 ^{19, 20}. Together with data from the current study, we suggest that PKC and TRPV1 phosphorylation-mediated signaling is a common mechanism underlying masseter hyperalgesia evoked by the activation of peripheral NMDA and mGlu5. Therefore, selective attenuation of TRPV1 phosphorylation in masseter afferents may lead to development of a new strategy for suppressing mechanical hyperalgesia in muscle tissues.

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HIGHLIGHTS

- Activation of mGlu1/5 leads to phosphorylation of serine-800 of TRPV1 in primary afferents.
- Peripheral mGlu1/5 activation leads to TRPV1 sensitization and muscle hyperalgesia via PKC.
- AKAP150 is involved in mGlu1/5-mediated mechanical hyperalgesia in the muscle.

Figure 1. TRPV1 is involved in masseter mechanical hyperalgesia induced by dihydroxyphenylglycine (DHPG), a mGlu1/5 agonist

A. Mechanical threshold of rat masseter muscle following intramasseteric injection of 100 μ l DHPG (1 μ mol) under lightly anesthetized conditions. Five minutes before injection of DHPG, 10 μ l of Vehicle (Veh, 5% DMSO in PBS), or 10 μ l of 1 nmol or 100 nmol AMG9810 (a TRPV1 antagonist) was injected into the masseter muscle. Mechanical threshold values were normalized to the baseline (BL) in each animal. † $p < 0.05$ in Two-way ANOVA with repeated measures; N=4 per group.

B. Area under the curve (AUC) calculated from experiments in panel A. * $p < 0.05$ in one-way ANOVA.

Figure 2. TRPV1 is involved in masseter hypersensitivity induced by phorbol myristate acetate (PMA), an activator of PKC but not by forskolin, an activator of PKA

A. Mechanical threshold of rat masseter muscle following masseteric injection of 30 μ l PMA (300 nM in PBS) under lightly anesthetized condition. Five min before injection of PMA, 10 μ l Vehicle (Veh, 5% DMSO in PBS) or 10 μ l of 1 nmol or 100 nmol AMG9810 was injected into masseter muscle. + $p < 0.05$ in Two-way ANOVA with repeated measures; N=6 in each group.

B. Area under the curve (AUC) calculated from experiments in panel A. * $p < 0.05$ in one-way ANOVA.

C. Mechanical threshold of rat masseter muscle following masseteric injection of 20 μ l forskolin (FSK; 20 μ g in PBS) under lightly anesthetized condition. Five minutes before injection of FSK, 10 μ l Vehicle (Veh, 5 % DMSO in PBS) or 10 μ l of 100 nmol AMG9810 was injected into masseter muscle.

D. Area under the curve (AUC) calculated from experiments in panel C.

Figure 3. Disruption of interactions between TRPV1 and AKAP150 attenuates DHPG-induced masseter hypersensitivity

A. Mechanical threshold of rat masseter muscle following intramasseteric injection of 100 μ l DHPG (1 μ mol in PBS) under lightly anesthetized conditions. Five minutes before injection of DHPG, 20 μ l Vehicle (Veh, PBS), or 20 μ l scramble peptide (30 μ M), or 20 μ l 736-745 peptide (10 μ M or 30 μ M) was injected into masseter muscle. Mechanical threshold values were normalized to the baseline (BL) in each animal. N=5 per group.

B. Area under the curve (AUC) calculated from experiments in panel A. * $p < 0.05$ in one-way ANOVA

Figure 4. DHPG enhances TRPV1 phosphorylation in a PKC-dependent manner in neurons dissociated from rat trigeminal ganglia (TG)

A–B. Effects of DHPG on phosphorylation of serine residues (panel A) or S800 (panel B) of TRPV1 in samples from rat TG culture. (Upper panels) Representative immunoblot of phospho-serine (panel A) or phospho-S800 of TRPV1 in biotinylated (surface) or GAPDH in corresponding total lysates (TL) (panel B) after indicated time following treatment of DHPG (200 μ M). (Lower panels) Percent change in intensity ratio between phospho-serine (panel A) or phospho-S800 TRPV1 (panel B) and GAPDH in the same total lysate. DHPG-treated groups were normalized to non-treated group. * $p < 0.05$ in one-way ANOVA; $N = 5$ for each group.

C–D. Inhibition by GF109203X of DHPG-induced increase of phospho-S800 TRPV1 expression. (Upper panels) Representative immunoblot of p-S800 of TRPV1 from TG neurons treated for 15 min with DHPG and 10 μ M GF109203X, a PKC inhibitor (panel C) or vehicle (DMSO; panel D). (Lower panels) Percent change in intensity ratio between phospho-S800 and GAPDH in the same total lysate. The intensity of the DHPG-treated

group was normalized to that of the non-treated group in the same blot. * $p < 0.05$ in one-way ANOVA. N=5 for each group.

Figure 5. DHPG sensitizes TRPV1 in a PKC-dependent manner in TG neurons

A. Representative current traces evoked by consecutive application of capsaicin (200 nM) in rat TG neurons. Vehicle (Veh, PBS; upper) or DHPG (50 μ M; lower) was applied for 10 minutes as indicated. Holding potential = -70 mV. Scale bars, 20 pA/pF - 2 min.

B. Averaged normalized responses. Current density of the 1st and 2nd response from each neuron was normalized to the average of 1st response in the given group. * $p < 0.05$, Student's t-test. Numbers in parentheses represent the number of observations.

C. Representative current traces evoked by consecutive application of capsaicin (300 nM) in rat TG neurons. DHPG (50 μ M) was co-applied with vehicle (Veh, DMSO; upper), GF109203X (GF, 1 μ M; middle) or KT5720 (KT, 1 μ M; lower) for 10 minutes as indicated. Holding potential = -70 mV. Scale bars, 20 pA/pF - 2 min.

D. Averaged normalized responses. NS, not significant; * $p < 0.05$, Student's t-test. Numbers in parentheses represent the number of observations.