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Peripheral G protein-coupled inwardly rectifying potassium (GIRK) channels are involved in delta opioid receptor-mediated anti-hyperalgesia in rat masseter muscle

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Abstract

Background—Although the efficacy of peripherally administered opioid has been demonstrated in preclinical and clinical studies, the underlying mechanisms of its anti-hyperalgesic effects are poorly understood. G protein-coupled inwardly rectifying potassium (GIRK) channels are linked to opioid receptors in the brain. However, the role of peripheral GIRK channels in analgesia induced by peripherally administered opioid, especially in trigeminal system, is not clear.

Methods—Expression of GIRK subunits in rat trigeminal ganglia (TG) was examined with RT-PCR, western blot and immunohistochemistry. Chemical profiles of GIRK expressing neurons in TG were further characterized. Behavioral and Fos experiments were performed to examine the functional involvement of GIRK channels in delta opioid receptor (DOR)-mediated anti-hyperalgesia under an acute myositis condition.

Results—TG expressed mRNA and proteins for GIRK1 and GIRK2 subunits. Majority of GIRK1- and GIRK2-expressing neurons were non-peptidergic afferents. Inhibition of peripheral GIRK using Tertiapin-Q (TPQ) attenuated anti-nociceptive effects of peripherally administered DOR agonist, DPDPE, on mechanical hypersensitivity in masseter muscle. Furthermore, TPQ

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Young Chul Bae: Participated in the design of the study, provided expert consultation of immunohistochemistry experiments, and approved the final version of this manuscript.

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attenuated the suppressive effects of peripheral DPDPE on neuronal activation in the subnucleus caudalis of the trigeminal nucleus (Vc) following masseteric injection of capsaicin.

Conclusions—Our data indicate that peripheral DOR agonist-induced suppression of mechanical hypersensitivity in the masseter muscle involves the activity of peripheral GIRK channels. These results could provide a rationale for developing a novel therapeutic approach using peripheral GIRK channel openers to mimic or supplement the effects of peripheral opioid agonist.

Introduction

Since activation of K^+ channels is often suggested as the final molecular mechanism of many drugs and natural products K^+ channels have been considered as excellent targets for the development of new anti-nociceptive drugs (Ocaña et al., 2004). Early studies demonstrated that specific agonists for μ opioid receptor (MOR) and δ opioid receptor (DOR) open inwardly rectifying K^+ channels through the activation of Gi/o proteins in neurons (Ikeda et al., 2002). Two types of K^+ channels that are activated by Gi/o proteins, G protein-coupled inwardly rectifying K^+ (GIRK) channel and ATP-dependent K^+ (KATP) channel, have been extensively studied in opioid analgesia.

Functional GIRK channels are homo- and heteromers formed by GIRK1-4 subunits, which are activated by the $\beta\gamma$ subunit of G protein upon activation of G protein-coupled receptors (GPCRs) (Sadjia et al., 2003). Although the functional relevance of subunit composition is not fully explored, GIRK1 and GIRK2 channels have been implicated in mediating anti-nociceptive effects of a variety of GPCRs including opioid receptors (Blednov et al., 2003; Mitrovic et al., 2003). Selective deletion of GIRK1/2 genes produces thermal hyperalgesia and attenuates analgesic responses of spinally administered MOR agonist suggesting GIRK1/2 subunits form functional channels in the spinal cord (Marker et al., 2005). Consistent with these observations GIRK1/2 subunits have been demonstrated in lamina II neurons of the mouse spinal cord (Marker et al., 2006; Marker et al., 2005). These studies also provided evidence that GIRK channels mediate analgesic effects of MOR and DOR, but not κ -opioid receptor (KOR) in the spinal cord (Marker et al., 2004, 2005, 2006).

All three major subtypes of opioid receptors, MOR, DOR, and KOR, are expressed in sensory ganglia and they have been implicated in peripheral analgesia (for review see Stein et al., 2003). Each subtype of opioid receptors in sensory ganglia has been suggested to associate with K^+ channels as a downstream target for the regulation of nociceptor activity (Ortiz et al., 2005; Pacheco and Duarte 2005; Rodrigues and Duarte 2000). Interestingly, transcripts for all 4 subunits of GIRK have been detected in rat dorsal root ganglia (DRG) neurons (Gao et al., 2007). Among them, GIRK2 protein expression was demonstrated in soma and peripheral terminals of DRG neurons and its linkage with MOR was suggested (Khodorova et al., 2003). These studies suggest a potential contribution of GIRK channels in modulating nociception and analgesia at the primary afferent level in the spinal system. Similar data from sensory neurons of trigeminal ganglia (TG) are scarce. It has been shown that KATP channel subunits are expressed in TG and that they are involved in DOR-mediated anti-hyperalgesic effects in the orofacial muscle pain condition (Niu et al., 2011;

Saloman et al., 2011). However, the expression of GIRK channels in TG, and their role in peripheral opioid-mediated analgesia have not been described.

The objectives of this study are (1) to demonstrate the expression of GIRK1 and GIRK2 subunits in TG, (2) to define neurochemical properties of GIRK-expressing TG neurons, and (3) to investigate whether the pharmacological inhibition of peripheral GIRK channels modulate anti-nociceptive effects of peripherally administered DOR agonist on mechanical hypersensitivity in masseter muscle and activation of trigeminal brainstem neurons.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (150 to 350 g; Harlan, IN, USA) were used. Rats 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.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from TG tissue by using Trizol (Invitrogen, CA) and further purified with RNeasy kit (Qiagen, MD). Reverse transcription was carried out using Superscript II First strand synthesis kit (Invitrogen) with a oligo-dT. For negative control, first strand synthesis was performed without Superscript II enzyme. PCR program consisted of a 5 min initial denaturation at 94°C followed by 50 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72 °C for 45 s and a final extension at 72°C for 5 min. To detect the expression of GIRK mRNAs, we used specific primers to amplify unique regions of each GIRK mRNA based upon sequences provided in Kawano et al. (2004). The PCR primers for GIRK1 (Gene accession #L25264) and GIRK2 (Gene accession #X83583) were as follows: GIRK1 (247 bp) 5'-CAGCAGCTTGTACCCAAGAAG-3' (forward) and 5'-ACATGGGCTTTGTTTCAGGTC-3' (reverse); GIRK2 (253 bp) 5'-GTGAGGAAGGATGGGAAATG-3' (forward) and 5'-AGACAAACCGTTGAGGTTG-3' (reverse). PCR products were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide.

Immunohistochemistry

The rats were transcardially perfused with cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS (250 mL; pH 7.3 to 7.4; Sigma). TG were extracted and postfixed for 90 minutes, placed in 30% sucrose solution at 4 °C overnight, and sectioned coronally at 12 µm. Every eighth section was collected and mounted on gelatin-coated slides for double-labeling immunohistochemistry. After blocking, the sections were incubated overnight with primary antibody against GIRK1 (1:400), GIRK2 (1:400), substance P (1:3000), and calcitonin gene-related peptide (CGRP, 1:3000). Details of the primary antibodies are provided in Table 1. Specificity of these primary antibodies was verified previously (Kim et al., 2008; Kim et al., 2010; Marker et al., 2004; Yang et al., 2008). In our

hands, the specificity of GIRK1 and GIRK2 antibodies was further validated by preabsorption with antigenic peptides of GIRK1 (Santa Cruz, 1.5 µg/ml) or GIRK2 (Alomone labs, 7.2 µg/ml). The sections were incubated with appropriate secondary antibodies conjugated with fluorophore. To detect IB4-binding neurons, the sections were incubated with biotin-labeled IB4 (5 µl/1ml; Sigma L3759) followed by avidin conjugated with alexa 488. Image acquisition was performed using Exi digital camera (Q-imaging Inc., Surrey, CA) attached to Zeiss Axioplan 2 microscope or Image analysis: Size analysis (cross-sectional area, µm²) and colocalization (%) from 3 rats, using I-solution software (IMT i-solution inc., Korea).

Western blot analysis

TG tissue was lysed in lysis buffer including protease inhibitor cocktail. A concentration in lysates was determined using a Bio-rad protein assay kit. Protein sample containing loading buffer was denatured at 100 °C for 5 min and separated by 4-12 % NuPAGE gel. Fractionated protein was blotted onto a polyvinylidene fluoride (PVDF) membrane in a semi-dry system. The membrane was blocked with 5 % skim milk for 1 h at room temperature. Each membrane was incubated with GIRK1 antibody (1:200; rabbit polyclonal; Alomone labs), GIRK2 antibody (1:200; rabbit polyclonal; Santa Cruz), respectively. Specificity of these antibodies was validated by preabsorption with antigenic peptides (18 µg/ml for GIRK1 and 10 µg/ml for GIRK2). The bound primary antibodies were detected with a horseradish peroxidase-conjugated anti-rabbit IgG antibody. The immunocomplex was visualized with ECL reagents (Amersham).

Behavioral Studies for 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 (Lee et al., 2012; Ro et al., 2009). Initially, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). 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 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) 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 nocifensive responses was determined 15 min prior to drug injection using the electronic von Frey (VF) anesthesiometer (IITC Life Science, Inc, Woodland Hills, CA). A rigid tip (diameter = 2 mm) 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 min following drug treatments. In order to maintain the consistency of assessing behavioral responses all behavioral observations were made by one experimenter blinded to the experimental conditions.

The VF thresholds before and after the drug treatment were plotted against time. The time-dependent changes in mechanical thresholds were analyzed with a two-way ANOVA with repeated measures. In order to assess the overall magnitude of drug-induced changes in masseter sensitivity over time, time-dependent mean percent changes in mechanical thresholds normalized to the baseline threshold were obtained, and the area under the curve (AUC) was calculated for the normalized data for each rat using the trapezoid rule. 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 (Dunnett's). The significance of all statistical analyses presented in this report was set to $p < 0.05$. Each group consisted of 6-8 rats.

To activate DOR, we used a DOR agonist, [D-Pen², D-Pen⁶]-enkephalin (DPDPE) at a dose of 100 µg. DPDPE at this dose was demonstrated to effectively suppress masseter hypersensitivity in this behavioral model (Saloman et al., 2011). To inhibit GIRK channel activity, we administered Tertiapin-Q (TPQ; 0.1 and 1 nmol), a peptide from honey bee venom blocking inwardly rectifying potassium channels including GIRKs (Kanjhan 1995). DPDPE and TPQ were dissolved in PBS. All drugs were injected directly into the mid region of the masseter muscle in 20 µl volume via 27-gauge needle.

Assessment of Fos protein expression in the brainstem

In order to further examine the functional contribution of GIRK in DOR-mediated responses, we complemented the behavioral studies with Fos-like immunoreactivity (Fos-LI) assessment in the subnucleus caudalis of the trigeminal nucleus (Vc) as an index of neuronal activation. All animals received intramuscular capsaicin in the masseter muscle in the same manner as described for the behavioral studies. One group of animals was pre-treated with DPDPE (100 µg/20 µl) in the masseter muscle prior to the injection of capsaicin, and control group was pretreated with vehicle. Additional two groups of animals received TPQ (0.1 nmol/20 µl) or vehicle followed by DPDPE prior to intramuscular capsaicin treatment. No additional post-injection experimental manipulation was performed on these animals to ensure that Fos data was not influenced by any procedural variables.

After 2 h following the last injection, these animals received a lethal dose of sodium pentobarbital (100 mg/kg, i.v.) followed by transcardiac perfusion with 4% paraformaldehyde in PBS (pH 7.3). The lower brainstem (from the obex to 5 mm caudal to the obex) was blocked and post-fixed overnight. Blocks were serially sectioned (30 µm) and every fourth section was processed to evaluate Fos-LI. Free floating sections were incubated successively in 5% normal donkey serum (30 min), affinity-purified rabbit polyclonal anti-Fos antibody (Oncogene Science; 1:20,000; overnight), biotinylated donkey anti-rabbit antibody (Chemicon International; 1:300; 1 h), and avidin-biotin-peroxidase complex (Vector Laboratory; 1 h). Diaminobenzidine was used for visualization of Fos-LI. Primary

antibody was omitted from processing of selected sections to serve as a control for nonspecific staining.

The Vc was referenced to the obex according to coordinates provided by Paxinos and Watson (Paxinos and Watson 2007). For the purpose of this study, we analyzed Fos-LI in the caudal Vc by counting Fos-LI positive cells in 10 representative tissue sections taken from 3 to 5 mm caudal from the obex. The average number of Fos-LI positive cells per section was obtained for each rat and used for statistical comparisons between the groups with Student's t-test. All counts were made by one experimenter to maintain the consistency in application of criteria used to select profiles as Fos-LI positive cells and reduce the likelihood for subject variability. The experimenter was blinded to experimental conditions. All data are expressed as mean $\pm$ sd.

Results

In DRG, GIRK 1-4 subunits are known to be expressed (Gao et al., 2007). Among these four, we focused our analysis on GIRK1 and GIRK2 since these two subunits are suggested to be involved in nociceptive processing (Blednov et al., 2003; Marker et al., 2005; Mitrovic et al., 2003). To investigate whether GIRK 1 and 2 subunits are expressed in TG, we initially performed the conventional RT-PCR analysis in the mRNA isolated from TG using primer sets designed for specifically detecting GIRK1 and GIRK2 subunits. As shown in Fig. 1A, each set of reaction amplified a band of expected size. In contrast, there was no band amplified in samples without reverse transcription. These results demonstrate that transcripts of GIRK1 and GIRK2 subunits are expressed in TG.

To examine whether GIRK1 and GIRK2 subunits are expressed at protein level, we performed western blot analysis using antibodies raised against GIRK1 or GIRK2 subunit in the protein lysates obtained from rat TG. Fig. 1B shows that antibodies against GIRK1 and GIRK2 subunits detected band sized that are consistent with a previous finding (Jelacic et al., 2000). These bands were not detected in negative control groups, in which primary antibodies were preadsorbed with antigen peptides.

We further investigated the pattern of expression of GIRK1 and GIRK2 subunits in TG neurons. Immunohistochemical staining of TG sections revealed that GIRK1 and GIRK2 subunits were expressed in subsets of TG neurons (Fig. 1D, E). Specific labeling with the GIRK1 or GIRK2 antibody was completely abolished by preabsorption with antigen peptides or omission of primary antibodies. The proportion of TG neurons expressing GIRK1 and GIRK2 subunit was approximately 3% and 7% of total TG neurons, respectively. The GIRK subunits-expressing neurons were mostly small to medium in size distribution (Fig. 1F).

We then examined neurochemical properties of GIRK-expressing primary afferents by performing double labeling of GIRK1 or GIRK2 and neurochemical markers. We investigated whether GIRK channels are expressed in peptidergic or non-peptidergic subpopulation nociceptors by co-labeling with substance P, CGRP or IB4. Vast majority of GIRK1-positive neurons coexpressed neuropeptides Substance P ($17.6\pm3.8\%$; $n=3$ rats; Fig.

2A) or CGRP ($98.9 \pm 1.2\%$, $n=3$ rats; Fig. 2B). Interestingly, most GIRK1-positive neurons were also bound to IB4, a marker of non-peptidergic nociceptors ($96.7 \pm 4.8\%$ in GIRK1; $n=3$ rats). The expression of GIRK1 in peptidergic IB4-positive neurons was confirmed by a triple labeling of GIRK1, CGRP and IB4 (Fig. 2D).

Similar to GIRK1 expression, GIRK2-positive neurons were mostly positive to IB4 (Fig. 3; $80.1 \pm 6.0\%$; $n=3$ rats). In contrast to GIRK1, however, no GIRK2-positive neurons express neuropeptides such as CGRP (0/590 neurons) or Substance P (0/610 neurons).

Given the neurochemical properties of GIRK-expressing TG neurons, we hypothesized that GIRK channels are preferentially linked with DOR, which is expressed in IB4-positive nociceptors (Scherrer et al., 2009; Wang et al., 2010; Zhang and Bao 2012). To examine the involvement of GIRK in DOR agonist-induced analgesia in masseter muscle, we adopted a rat model of masseter mechanical hyperalgesia. As shown in Fig. 4A, injection of 1 μmol capsaicin induced a robust mechanical hypersensitivity in rats. Pretreatment of masseter muscle with 100 μg of DPDPE completely prevented the development of mechanical hyperalgesia, which is consistent with our previous findings (Saloman et al., 2011). When a small dose of TPQ (0.1 nmol) was co-administered with DPDPE, it partially reversed the anti-nociceptive effect of DPDPE. TPQ at 1 nmol completely reversed the DPDPE-induced anti-nociception. TPQ (1 nmol) alone, without capsaicin, did not produce any changes in masseter sensitivity. The same dose of TPQ administered in the contralateral masseter did not significantly alter the DPDPE effect (data not shown), suggesting that TPQ at this dose is blocking local GIRKs. In order to examine the overall magnitude of drug effect irrespective of time, AUC was calculated for the normalized data for each rat. Both doses of TPQ effectively reversed the DPDPE effect (Fig. 4B).

These results strongly suggest the involvement of GIRK channels in DOR-mediated anti-hyperalgesia in orofacial muscle pain.

In order to further investigate the functional contribution of GIRK in DOR-mediated anti-hyperalgesia, we examined Fos-LI in Vc as a marker of neuronal activation. The injection of capsaicin into masseter muscle (0.1 %/100 μl) induced a robust increase in Fos-LI in Vc. Pretreatment of masseter muscle with DPDPE (100 μg /20 μl) prior to the injection of capsaicin significantly attenuated the extent of Fos-LI (Fig. 5A, B and E). We then performed an additional experiment to examine whether a GIRK inhibitor, TPQ can reverse the DPDPE effect. This experimental procedure, which involved the triple injection (i.e., TPQ, DPDPE and Capsaicin), evoked a greater level of FOS-LI compared to that from the double injection (i.e. DPDPE or PBS and capsaicin). Nevertheless, when the masseter muscle was pretreated with TPQ (0.1 nmol/20 μl), the suppressive effects of DPDPE on capsaicin-induced Fos-LI was significantly reduced (Fig. 5C, D and F). These data suggest that peripheral GIRK channels functionally contribute to DOR-mediated reduction of neuronal activation in the trigeminal system.

Discussion

The evidence supporting the role of peripheral opioid receptors in suppressing pain and hyperalgesia has been accumulated for decades (Stein et al., 2003). Not only in preclinical studies (Garlicki et al., 2006; Guan et al., 2008; Nunez et al., 2007; Obara et al., 2009), but clinical studies in patients also demonstrated the effects of peripheral opioid in multiple pain conditions (Dionne et al., 2001; Duckett et al., 1997; Eisenach et al., 2003; Keskinbora and Aydinli 2009; Likar et al., 2001; Rorarius et al., 1999). We previously demonstrated the role of peripheral opioid receptors in attenuating mechanical hypersensitivity (Auh and Ro 2012; Nunez et al., 2007; Saloman et al., 2011). However, the downstream signaling mechanisms following the activation of these receptors in trigeminal nociceptors have not been clear.

In this study, we demonstrated that 1) rat TG neurons express GIRK1 and GIRK2 subunits, 2) the majority of GIRK1-positive afferents are colocalized with CGRP and IB4, 3) the majority of GIRK2-positive afferents are colocalized with IB4, but not with neuropeptides, 4) intramuscular injection of TPQ, a GIRK inhibitor, dose-dependently attenuates the anti-hyperalgesic effects of peripherally administered DPDPE, a DOR agonist, and blocks the DPDPE effect on the activation of Vc neurons. Taken together, these results support our hypothesis that peripheral GIRK channels are involved in peripheral DOR agonist-induced suppression of pain in the masseter muscle.

The role of GIRK channels in opioid-induced analgesia has been well-established in the central nervous system. However, the contribution of GIRK channels in primary afferents has been questioned due to the early studies reporting the absence of GIRK2 in sensory ganglia (Mitrovic et al., 2003) and the absence of opioid-induced hyperpolarization of membrane potential or activation of K⁺ conductance in primary afferent neurons (Akins and McCleskey 1993). However, growing evidence indicates the role of GIRK channels in analgesia mediated by peripherally applied opioids. Transcripts for all 4 subunits of GIRK have been detected in rat DRG neurons (Gao et al., 2007). Among them, GIRK2 protein expression was demonstrated in soma and peripheral terminals of DRG neurons and its linkage with MOR was suggested (Khodorova et al., 2003). Consistent with these reports, we identified transcripts and proteins of GIRK1 and GIRK2 subunit in the rat TG. Moreover, a peripherally injected GIRK inhibitor, TPQ reversed the suppressive effects of a peripheral DOR agonist on masseter hypersensitivity and neuronal activation in the brainstem. These studies suggest that GIRK channels are downstream effectors of DOR in trigeminal nociceptors and that they contribute to peripheral opioid-induced analgesia in craniofacial regions.

Our immunohistochemical results demonstrated that GIRK1 and GIRK2 subunits are expressed primarily in small to medium-sized nociceptive neurons. Interestingly, GIRK1 and GIRK2 subunits are enriched in IB4-positive afferents. Ablation of a large proportion of IB4-positive nociceptors reduces mechanical pain in mice suggesting the involvement of these afferents in cutaneous mechanical pain (Cavanaugh et al., 2009). Recently, it was demonstrated that IB4-positive muscle afferents play a critical role in mechanical pain and hyperalgesia in rat muscles (Alvarez et al., 2012a; Alvarez et al., 2012b; Hendrich et al., 2012). Furthermore, DOR is expressed in non-peptidergic afferents as well as peptidergic

nociceptors whereas MOR is primarily expressed in peptidergic afferents (Scherrer et al., 2009; Wang et al., 2010; Zhang and Bao 2012). Therefore, our data suggest that GIRK1 and GIRK2 subunits may be substantially colocalized and functionally linked to DOR in muscle afferents that are limited to IB4-positive, and that the activation of DOR opens GIRK channels in this subset of afferents to attenuate mechanical hyperalgesia in the masseter muscle.

Neurochemical properties of subpopulation of TG neurons expressing GIRK1 are particularly interesting. Although GIRK1-expressing neurons compose only a small proportion of TG neurons (~3%), these neurons are mostly expressed in IB4-positive neurons containing CGRP. Although CGRP and IB4 are rarely colocalized in mouse DRG and IB4 has been often used as a marker for labeling non-peptidergic primary afferents (Ruscheweyh et al., 2007), it is known that IB4 binds to a subpopulation of peptidergic neurons in rat TG (Ambalavanar and Morris 1992; Price and Flores 2007) and DRG (Hwang et al., 2005; Kashiba et al., 2001). Since GIRK1 is exclusively expressed in the peptidergic IB4-positive neurons, this molecule may be a novel marker and target for studying unique functions of the rare subpopulation of primary afferents.

Along with GIRK, KATP channels are also known to mediate anti-nociception by peripherally administered opioids (Rodrigues and Duarte 2000). Activation of peripheral DOR in the cutaneous tissue or the masseter muscle involves the opening of KATP channels (Pacheco and Duarte 2005; Saloman et al., 2011). Although the interaction between opioid receptors and GIRK or KATP channels in nociceptors is evident, we speculate that physiological consequences of opioid receptor-mediated activation of the two potassium channels could be different. Mechanisms of activation of KATP and GIRK channels following the activation of opioid receptors are apparently different since GIRK channels are coupled with opioid receptors through G proteins while KATP channels are downstream of nitric oxide and cGMP pathway (Luscher and Slesinger 2010; Sachs et al., 2004). Unlike GIRK channels that are enriched in small diameter non-peptidergic afferents, KATP channels-expressing neurons include myelinated or CGRP-positive subpopulations (Zoga et al., 2010). Therefore, it is possible that the activation of peripheral opioid receptors may invoke different signaling cascades through either GIRK or KATP channels in different subset of primary afferents. Detailed investigation of GIRK and KATP channels is important since these two channels may function as common downstream effectors of multiple inhibitory signaling mechanisms in nociceptors.

Besides DOR, peripheral GIRK channels are also suggested to be linked with multiple GPCRs that regulate excitability of nociceptors such as endothelin(B) receptors, MOR, and GABA(B) receptors (Gao et al., 2007; Khodorova et al., 2003). KATP channels are also a downstream of peripheral A1 adenosine receptors and α_2c adrenergic receptors in primary afferents (Lima et al., 2010; Romero and Duarte 2009). Therefore, as a novel therapeutic approach, peripheral administration of specific GIRK or KATP openers could mimic or supplement the effects of activation of multiple inhibitory receptors.

In conclusion, our results support the hypothesis that peripheral opioid receptor agonist-induced suppression of mechanical hypersensitivity in the masseter muscle involves the

activity of peripheral GIRK channels. These results provide a rationale for developing a novel anti-hyperalgesic therapeutic approach to manipulate peripherally localized GIRK channels.

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What's already known about this topic?

- Activation of peripheral delta opioid receptors in sensory ganglia leads to anti-hyperalgesic effects in various preclinical pain models.
- G protein-coupled inwardly rectifying potassium (GIRK) channels function as a downstream effector of opioid receptors.

What does this study add?

- GIRK channels are expressed in specific subsets of trigeminal sensory neurons.
- Anti-hyperalgesic effects of peripheral delta opioid receptors under muscle pain conditions are mediated by GIRK channels in trigeminal sensory neurons.

Fig. 1. Expression of GIRK subunits in rat trigeminal ganglia (TG)

A. Conventional RT-PCR analysis of GIRK1 and 2 subunits in male TG. RT +, with reverse transcription; RT -, without reverse transcription.

B-C. Western blot analysis of rat TG samples using antibodies against GIRK1 (in panel B) and GIRK2 (in panel C) subunit (upper panels) followed by GAPDH (lower panels). In negative controls, primary antibodies against GIRK subunits were preadsorbed by antigen peptide. Same TG samples were duplicated in each group.

D-E. Immunohistochemical staining of TG sections using specific antibodies against GIRK1 (in panel E) and GIRK2 (in panel E) subunit (left panels). In negative controls, primary antibodies were preabsorbed with antigen peptides (middle panels) or omitted (right panels). Scale bar 100 μ M.

F. Size distribution of the neurons expressing GIRK1 and GIRK2 subunits.

Fig. 2. GIRK1 expression in IB4-binding peptidergic TG neurons

A-C. Double-labeling of GIRK1 (left panels in A to C) and substance P (middle panel in A), CGRP (middle panel in B) or IB4 (middle panel in C). Right panels show merged images. Arrowheads indicate the colocalized neurons. Scale bar 50 μM.

D. Triple-labeling of GIRK1, IB4 and CGRP as indicated. A merged image is also shown. Scale bar 50 μM.

Fig. 3. GIRK2 expression in IB4-binding non-peptidergic TG neurons

Double-labeling of GIRK2 (left panels in A to C) and substance P (middle panel in A),

CGRP (middle panel in B) or IB4 (middle panel in C). Right panels show merged images.

Arrowheads indicate the colocalized neurons. An arrow in C indicates the neuron expressing only GIRK2. Scale bar 50 μ M.

Fig. 4. Peripheral GIRK channels mediate the anti-nociceptive effects of peripherally injected DOR agonist

A. Mechanical sensitivity of masseter muscle in lightly-anesthetized rats before and after the intra-masseteric injection of Cap (1 μ mol). Indicated drugs, 100 μ g DPDPE and TPQ at 0.1 or 1 nmol, were pre-injected into masseter muscle 15 minutes before the injection of capsaicin. N=6-8 rats in each group.

B. Area under the curve calculated from the plot shown in A. Data represent mean $\pm$ sd. $P < 0.05$ in one-way ANOVA; *, $p < 0.05$ in Dunnet's post-test.

Fig. 5. Peripheral GIRK channels are involved in the inhibitory effects of peripherally injected DOR agonist on neuronal activation in Vc

A-D. Representative brainstem sections showing FOS-LI-positive neurons following masseteric injection of Cap (0.1 %/100 μ l). The animals were pretreated by vehicle in A or DPDPE (100 μ g/20 μ l) in panel B. In separate groups of animals, vehicle (panel C) or TPQ (0.1 nmol, panel D) was injected prior to the sequential injection of DPDPE and capsaicin. E-F. Mean number of Fos-LI-positive neurons in Vc per section. E is the quantification of

experiments exemplified in A-B. F is the quantification of the experiments exemplified in C-D. Data represent mean $\pm$ sd. *, $p<0.05$ in Student's t-test. N=5 rats/group.

Table 1

Information of primary antibodies

Protein	company	Catalogue number	Host species	Antigen epitope	Cross reactivity
GIRK1 (IHC)	Santa Cruz	sc-22926	goat	residues near c-terminus of human GIRK1	Human, rodents, canine, bovine
GIRK1 (WB)	Alomone labs	APC-005	rabbit	64 residues near c-terminus of mouse GIRK1	rat
GIRK2 (IHC)	Alomone labs	APC-006	rabbit	41 residues near c-terminus of mouse GIRK2	rat
GIRK2 (WB)	Santa Cruz	Sc-16135	goat	residues near c-terminus of human GIRK2	Mouse, rat, human
Substance P (IHC)	Immunostar	20064	rabbit	synthetic substance P	Rodents, human, guinea pig, canine
	Chemicon	AB5892	guinea pig	aa 1-11 of rat substance P	Human, rodents
CGRP (IHC)	Immunostar	24112	rabbit	synthetic rat α -CGRP	human, pig, rat
	Peninsula lab	T-5027	guinea pig	Synthetic human α -CGRP	Human, rat, chicken

HC, immunohistochemistry; WB, western blot