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Antinociception depends on the presence of G protein γ_2-subunits in brain

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Abstract

We have shown previously [Hosohata, K., Logan, J.K., Varga, E., Burkey, T.H., Vanderah, T.W., Porreca, F., Hruby, V.J., Roeske, W.R., Yamamura, H.I., 2000. The role of the G protein γ2 subunit in opioid antinociception in mice. Eur. J. Pharmacol. 392, R9–R11] that intracerebroventricular (i.c.v.) treatment of mice with a phosphorothioate oligodeoxynucleotide antisense to the γ_2 subunit ($G\gamma_2$) of the heterotrimeric G proteins (antisense ODN) significantly attenuates antinociception by a δ-opioid receptor agonist. In the present study, we examined the involvement of $G\gamma_2$ in antinociception mediated by other (μ- or κ-opioid, cannabinoid, α_2-adrenoreceptor) analgesic agents in a warm (55 °C) water tail-flick test in mice. Interestingly, i.c.v. treatment with the antisense ODN attenuated antinociception by each analgesic agent. Missense phosphorothioate oligodeoxynucleotide treatment, on the other hand, had no effect on antinociception mediated by these agonists. The antinociceptive response recovered in 6 days after the last antisense ODN injection, indicating a lack of nonspecific tissue damage in the animals. These results suggest a pervasive role for the G protein γ_2 subunits in supraspinal antinociception.

Keywords: Guanine nucleotide binding protein; G protein γ_2 subunit; Opioid receptor agonist; Cannabinoid receptor agonist; Clonidine; Antinociception

1. Introduction

Intracerebroventricular (i.c.v.) injection of μ-, δ-, or κ-opioid (Lohmann and Welch, 1999), cannabinoid (Edsall et al., 1996), or α_2-adrenoceptor (Raffa et al., 1996) agonists was shown to mediate antinociception against noxious thermal stimuli. The antinociceptive action of these drugs is mediated by activation of inhibitory $G_{i/o}$ proteins, as indicated by pertussis toxin sensitivity of the analgesic effect (Sanchez-Blazquez and Garzon, 1991; Lichtman et al., 1996). Agonist binding to inhibitory $G_{i/o}$ protein-coupled receptors (GPCR) promotes nucleotide exchange in the α-subunits of the heterotrimeric $G_{i/o}$ proteins, leading to activation of both the α- and the βγ-subunits. Both the activated $\alpha_{i/o}$-subunits and the liberated βγ-subunits regulate the activity of numerous cellular effectors (Neer, 1994).

The exact nature of the cellular effectors that mediate analgesia in response to $G_{i/o}$ protein-coupled receptor stimulation is presently not entirely clear. It is also not clear whether the antinociceptive actions of different analgesic agents are mediated through the same cellular effector(s). It is generally agreed, however, that regulation of cation channel permeability at pre- and postsynaptic locations plays crucial role in $G_{i/o}$ protein-coupled receptor agonist-mediated antinociception (Ikeda et al., 2002). $G_{i/o}$ protein-coupled receptors regulate cell membrane cation channels using the free βγ-subunits, released upon the dissociation of the G protein heterotrimer (Gautam et al., 1998). However, it is not clear whether the regulation of the membrane ion channels by antinociceptive agents is mediated selectively by only certain βγ-subunit combinations.

Interestingly, we found earlier that a phosphorothioate oligodeoxynucleotide antisense to the γ_2 subunit of the rodent heterotrimeric G proteins (antisense ODN) attenuates

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cyclic-D-Pen^2-D-Pen^5-enkephalin (DPDPE)-mediated antinociception in a tail-flick test in mice (Hosohata et al., 2000). In the present study, we investigated the role of the $G\gamma_2$ subunit in antinociception produced by analgesic drugs acting at other (μ- and κ-opioid, cannabinoid, and α_2) $G_{i/o}$ protein-coupled receptors.

2. Materials and methods

2.1. Synthesis of the phosphorothioate antisense and missense oligodeoxynucleotides

The phosphorothioate-modified antisense ODN corresponding to nucleotides 1–24 in the open reading frame of the mouse $G\gamma_2$ subunit cDNA (5′-GCT-GGC-GGT-GTT-GTT-GCT-GGC-CAT-3′) and the phosphorothioate oligodeoxynucleotide missense ODN (5′-CGG-TCG-TTG-TGG-TGC-TTG-TCA-CGG-3′) were described previously (Hosohata et al., 2000). Neither the antisense nor the missense oligodeoxynucleotides produced significant alignment with any cloned mammalian nucleotide sequences in the BLAST Search program (NCBI, Bethesda, MD). The oligodeoxynucleotides were dissolved in distilled water and injected through the i.c.v. route into the brain of mice in the treated groups.

2.2. Antinociceptive tests

Antinociception was measured 24 h after the last oligodeoxynucleotide administration using a hot (55 °C) water tail-flick test in male ICR mice (4 mice/group, n=2–3). Mice not responding within an initial baseline tail-flick latency time (5 s) were excluded from further experiments. The distal 1/3 of the mouse tail was dipped into the hot water and the time that it took for the animal to withdraw his tail was recorded in seconds. All compounds were injected into the right lateral ventricle of the mice under light ether anesthesia. Mice in the treated groups received 10 μg of antisense or missense phosphorothioate oligodeoxynucleotide in 5 μl volume once daily, for 2 or 3 days. Control mice were injected with 5 μl of distilled water at the same times.

Twenty-four hours after the last treatment, both control and ODN-treated mice received a single i.c.v. injection of the indicated analgesic drug in 5 μl final volume. The minimal doses necessary to produce the maximal possible analgesic effect (MPE) were determined by measuring dose–response curves for each drug in naïve untreated mice. The doses of the analgesic compounds were as follows: 6 nmol of morphine, 20 nmol of deltorphin II, 40 nmol of (5α7α,8β)-(−)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide (U69,593), 40 nmol of (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (WIN55212-2), and 4 nmol of clonidine.

Before drug administration, the basal latency of the nociceptive response was determined again for each mouse and animals with basal latencies longer than 5 s were excluded from further experiments. After drug administration, the latency of tail withdrawal from a hot (55 °C) water bath was measured in 10 min intervals up to 60 min, as previously described (Hosohata et al., 2000). The animals were assigned 100% antinociception when not responding within 15 s (to avoid tissue damage, 15 s is used as a maximal cutoff in the hot water tail-flick tests). Antinociception was calculated as the percent of the maximal possible effect by the following formula: %MPE=100×(test latency−baseline latency)/(15 s (cutoff)−baseline latency).

2.3. Statistical analysis

The percent MPE versus time plots were used to calculate the area under the curve ($AUC_{0–60}$) values for each mouse over a period 0–60 min after antinociceptive drug administration. The $AUC_{0–60}$ values were determined by trapezoidal approximation, using the Prism 4.0 software. One-way ANOVA followed by Bonferroni's and Neuman–Keuls post-hoc tests were used to evaluate statistical significance between treatment groups. The reported P values were obtained using the Bonferroni tests. P values of less than 0.05 were considered significant.

3. Results

Confirming our previous results with DPDPE, i.c.v. pretreatment with the phosphorothioate oligodeoxynucleotide antisense to $G\gamma_2$ (antisense ODN) attenuated antinociception by an additional selective δ-opioid receptor agonist, deltorphin II (20 nmol). Deltorphin II produced maximal (68±14%) antinociception 20 min after i.c.v. administration in vehicle-treated control mice. Conversely, after antisense ODN pretreatment, the same dose of deltorphin II elicited only 20±4% of the maximal possible antinociceptive effect. The $AUC_{0–60}$ values were significantly different (P<0.05) between control and antisense ODN-treated mice. The $AUC_{0–60}$ values for control and missense ODN-treated mice, on the other hand, were not significantly different (P>0.05), indicating the lack of nonspecific neurotoxicity from our phosphorothioate-modified ODNs.

Morphine (6 nmol), a μ-opioid receptor-preferring alkaloid analgesic, elicited 93±4% of the maximal possible analgesic effect in vehicle-treated mice 20 min after i.c.v. administration (Fig. 1B). The $AUC_{0–60}$ values were not significantly different between the missense ODN- and vehicle-treated groups of mice (P>0.05). On the other hand, morphine antinociception was significantly (P<0.01) attenuated after pretreatment with the antisense ODN. Importantly, we found that 6 days after cessation of the antisense ODN treatment, morphine-mediated analgesia completely recovered (81±19% of MPE). As shown in Fig. 2, the

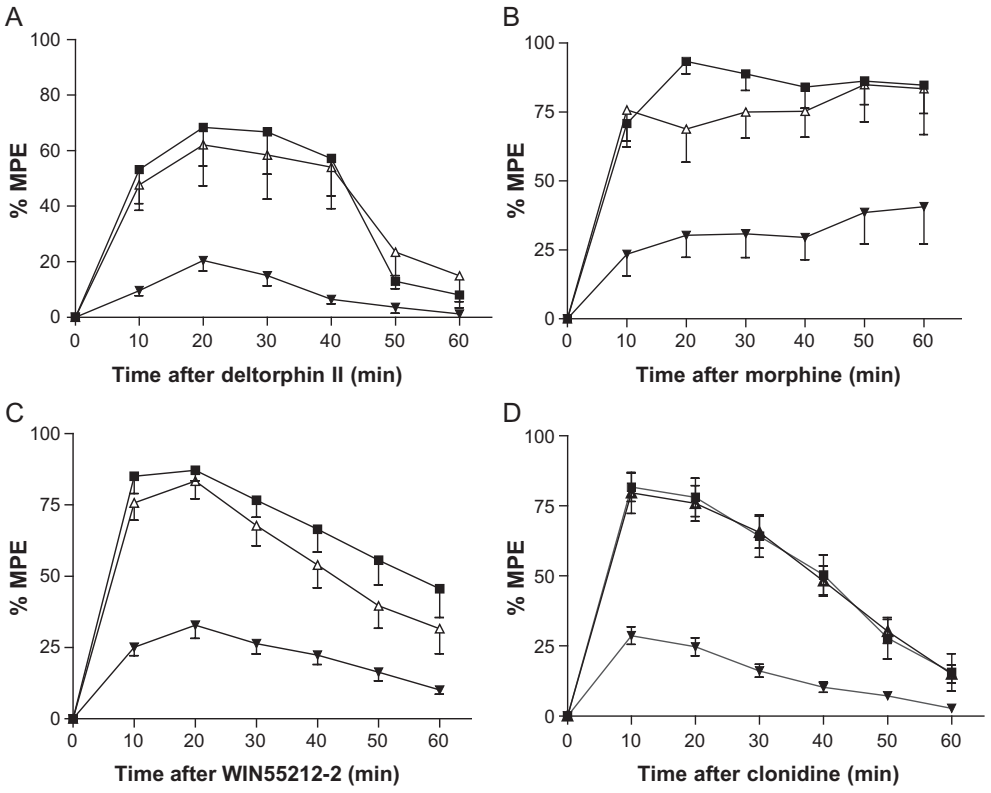

Fig. 1. The time course of (A) deltorphin II-mediated, (B) morphine-mediated, (C) WIN55212-2-mediated, and (D) clonidine-mediated antinociception in mice after antisense or missense ODN treatment. Antinociception was measured 24 h after the last oligodeoxynucleotide administration using a 55 °C warm water tail-flick test in male ICR mice (4 mice/group, n=2–3). Mice in the treated groups received 10 μg of antisense (▼) or missense (△) phosphorothioate oligodeoxynucleotide in 5 μl volume once daily, for 2 days. Control mice (■) were injected with 5 μl of distilled water. After pretreatment, the mice received a single i.c.v. injection of (A) morphine (6 nmol), (B) deltorphin II (20 nmol), (C) WIN5212-2 (40 nmol), or (D) clonidine (4 nmol), in 5 μl final volume. The latency of tail withdrawal from a warm (55 °C) water bath was measured, as previously described (Hosohata et al., 2000). The animals were assigned 100% antinociception when not responding within 15 s. Antinociception was calculated by the following formula: MPE%=(test latency−basal latency)×100/(15 s−basal latency). The graph represents the mean±standard error. The %MPE–time curves were used to calculate area under the curve values ($AUC_{0–60}$) for each mouse. Statistical significance was tested by one-way ANOVA of the $AUC_{0–60}$ values, followed by Bonferroni's post-hoc tests.

$AUC_{0–60}$ values (mean±S.E.M.) for morphine were significantly (P<0.01) different from control 1 day after antisense (but not missense) ODN treatment. Conversely, 6 days after the ODN treatment, the $AUC_{0–60}$ value was not significantly different from control in either the antisense or missense ODN-treated group (P>0.05), indicating that the phosphorothioate ODNs did not cause tissue damage in the brain.

Similar results were obtained with analgesics acting at other nonopioid $G_{i/o}$-coupled receptors. Thus, pretreatment with the antisense ODN significantly attenuated antinociception by the CB_1-cannabinoid receptor agonist, WIN 55212-2 (Fig. 1C) and the α_2-adrenoceptor agonist, clonidine (Fig. 1D). In vehicle-treated mice, WIN 55212-2 and clonidine produced 85±6% and 78±6% maximal antinociceptive effect, respectively. WIN 55212-2 and clonidine caused 87±4% and 78±8% maximal possible analgesic effect in missense-treated mice, respectively. Conversely, in the antisense ODN-treated group, the same doses elicited antinociception of 18±3% and 25±3% of MPE, 20 min after drug administration. For both agonists, the $AUC_{0–60}$ values were significantly different from the

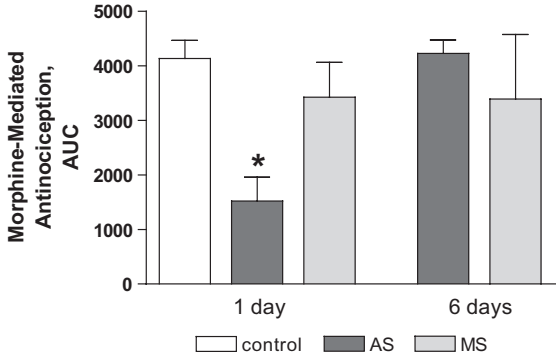

Fig. 2. Recovery of morphine-mediated analgesia after cessation of the phosphorothioate ODN treatment. Morphine (6 nmol)-mediated antinociception was measured 1 and 6 days after phosphorothioate ODN administration in hot (55 °C) water tail-flick tests in male ICR mice. The graph represents the mean±standard error of the $AUC_{0–60}$ values calculated from the antinociception (%MPE)–time curves for each mouse. Statistical significance was determined by one-way ANOVA, followed by Bonferroni's multiple comparison tests, using the PRISM 4 software. Morphine-mediated antinociception was significantly different from control (P<0.01) 24 h, but not 6 days (P>0.05), after the last antisense ODN treatment.

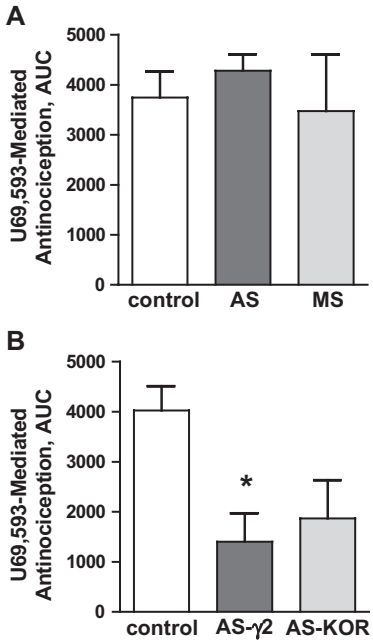

Fig. 3. Three days of antisense ODN treatment is necessary to attenuate κ-opioid receptor agonist-mediated antinociception. Male ICR mice were treated for (A) 2 days with 10 μg of phosphorothioate ODN antisense to $G\gamma_2$ (as-γ_2) or the corresponding missense ODN, or (B) 3 days with as-γ_2 or a phosphorothioate ODN antisense to the rodent κ-opioid receptor (as-KOR), in 5 μl volume, once daily. U69,593 (40 nmol)-mediated antinociception was measured 24 h after the last oligodeoxynucleotide administration using the tail-flick test. The graph represents the mean± standard error of the AUC_{0-60} values calculated from the antinociception (%MPE)–time curves for each mouse. U69,593-mediated antinociception was significantly different from control ($P<0.05$) after 3 days of treatment with as-γ_2.

vehicle-treated group in the antisense ($P<0.001$) (but not in the missense) ODN-treated groups of mice.

Interestingly, however, in mice treated with the antisense ODN for 2 days, the κ-selective opioid receptor agonist, U69,593 was still able to produce 100% antinociception at the time of the peak response (20 min after administration). We reasoned that the insensitivity of the κ-opioid receptor agonist-mediated analgesia to antisense ODN treatment can be due to a difference in the molecular mechanism of κ- versus μ- and δ-opioid receptor agonist-mediated antinociception. Alternatively, it can be caused by differential localization of the κ-opioid receptors around the cerebral ventricle. In order to differentiate between these possibilities, we performed control experiments using an antisense ODN that was previously shown to knockdown κ-opioid receptors selectively in mouse brain, upon longer (>2 days) i.c.v. treatment (Lohmann and Welch, 1999). Groups of mice were treated with the antisense ODNs against either the G protein γ_2 subunit or the rodent κ-opioid receptor (as-KOR) for two or three consecutive days, respectively, and U69,593-mediated antinociception was evaluated. κ-Opioid agonist-mediated antinociception was unaffected after a 2-day treatment with antisense ODNs against either the κ-opioid receptor ($P>0.05$, not shown) or $G\gamma_2$ ($P>0.05$; Fig. 3A). Interestingly, however, 3-day treatment with either antisense ODN attenuated antinociception (Fig. 3B). Maximal U69,593 mediated antinociception after 3 days of treatment with ODN against $G\gamma_2$ or against KOR was 40±20% and 46±21% of the MPE, respectively. The AUC_{0-60} values between control and $G\gamma_2$-antisense ODN-treated groups of mice were also significantly different after 3 days of ODN treatment ($P<0.05$). These findings suggest that the κ-opioid receptors are localized in brain areas that are less easily accessible from the ventricle; but the molecular mechanism of κ-agonist-mediated antinociception is still dependent on the presence of G protein γ_2-subunits.

4. Discussion

In the present work, we examined the role of the heterotrimeric G protein γ_2-subunits ($G\gamma_2$) in $G_{i/o}$ protein-coupled receptor agonist-mediated antinociception. We found that i.c.v. pretreatment of mice with a phosphorothioate oligodeoxynucleotide antisense to the $G\gamma_2$-subunits (as ODN) attenuates antinociception by opioid, cannabinoid receptor, and α_2-adrenoceptor agonists. The results suggest that the G protein γ_2 subunits have a central role in supraspinal antinociception mediated by $G_{i/o}$ protein-coupled receptor agonists.

The role of the various pertussis toxin-sensitive G protein types in antinociception was previously investigated by down-regulation of $G_{i/o}$ protein α-subunits using antisense oligodeoxynucleotide treatment (Sanchez-Blazquez et al., 1995; Raffa et al., 1996; Standifer et al., 1996) as well as by affinity labeling of the G protein α-subunits activated by the antinociceptive agonist (Prather et al., 2000). However, since nucleotide exchange at the α-subunit is a condition for both α- and βγ-subunit-mediated signal transduction, these data do not necessarily establish that α-subunit-regulated effectors are involved in analgesia.

The exact nature of the intracellular second messenger pathways that mediate the in vivo analgesic response is presently not entirely clear. It is generally agreed, however, that regulation of cation channel permeability by the βγ-subunits of the $G_{i/o}$ proteins plays a crucial role in $G_{i/o}$ protein-coupled receptor agonist-mediated antinociception (Ikeda et al., 2002).

The β and γ subunits of the heterotrimeric G proteins remain associated after dissociation from the activated α-subunits, and thus work as functional monomers inside the cell. Five β-subunits (plus a splice variant) and 11 γ-subunits have been identified by molecular cloning. The β-subunits are highly homologous, while the sequences of γ-subunits are more diverse (Gautam et al., 1998). G protein βγ-subunits were initially thought to serve only as membrane-anchoring components in the heterotrimeric G proteins. After examination of individual G protein βγ-subunit complexes, however, it is becoming clear that βγ

subunits play a more active and specific role in signal transduction. Thus, antisense oligonucleotide- or ribozyme-mediated selective genetic knockdown of individual β- and/or γ-subunits demonstrated that individual βγ-subunit combinations exhibit selectivity toward both G protein-coupled receptors and their effectors (Robillard et al., 2000). For instance, a prenylated peptide corresponding to the C-terminus of the G protein γ_5 subunit specifically inhibited the regulation of N-type calcium channels by muscarinic receptor agonists in superior cervical ganglion neurons, while peptides corresponding to the C-termini of $G\gamma_7$ or $G\gamma_{12}$ subunits had no effect (Azpiazu et al., 1999). Similarly, a ribozyme that selectively reduced the level of $G\gamma_7$ mRNA in human embryonic kidney cells selectively inhibited β-adrenoceptor, but not prostaglandin E1 receptor-mediated stimulation of adenylate cyclase (Wang et al., 1997).

The diversity of the Gγ-subunit isoforms led to the suggestion that the γ-subunits may play an important role in determining the specificity βγ-subunit-mediated signal transduction. However, it is presently not clear whether selectivity is determined at the receptor/βγ-subunit interaction surface, or at the effector/βγ-subunit interaction surface. Evidence indicates that the γ-subunits of the βγ-complex directly interact with a hydrophobic pocket in the GPCRs (Kisselev et al., 1995). On the other hand, initial experiments suggested that it is the β-subunit of the complex that interacts with the effectors (Yan and Gautam, 1997).

In the present study, we demonstrate that i.c.v. treatment of mice with a phosphorothioate ODN antisense to $G\gamma_2$ significantly attenuates $G_{i/o}$ protein-coupled receptor-mediated analgesia, indicating that $G\gamma_2$ may have a pervasive role in supraspinal antinociception in mice. Further investigation will be necessary to identify the final common effector(s) regulated by $G\gamma_2$-containing βγ-subunits to produce antinociception in vivo.

Voltage-dependent calcium channels (VDCC) are important candidates for effectors selectively regulated by $\beta\gamma_2$ complexes to produce analgesia. Thus, inhibitors of VDCCs or genetic knockout of VDCC subunits were shown to regulate nociception in mice (Muth et al., 2001). G protein βγ-subunits directly interact with the cytoplasmic region of the α_1 subunit of VDCCs, resulting in current inhibition (Herlitze et al., 1997). The extent of the inhibition was shown to be dependent on the βγ-subunit isoform combination (Zamponi, 2001). Inhibition of VDCCs is thought to play an important role in antinociception by inhibiting the release of pain neurotransmitters from presynaptic cells (Quock et al., 1999).

On the other hand, electrophysiological studies reveal that opioids hyperpolarize neurons in the descending antinociceptive pathway by activating postsynaptic G protein-regulated inwardly rectifying potassium (GIRK) channels (Vaughan et al., 2003). Interestingly, recent data have indicated that postsynaptic GIRK channels may play a pervasive role in GPCR-mediated analgesia. Thus, the '*weaver*' mice, carrying a spontaneous mutation in the GIRK2 subunits, display low level of analgesia upon opioid receptor agonist administration (Ikeda et al., 2000). Similarly, targeted mutation of the GIRK2 subunit markedly reduced (or completely eliminated) antinociceptive responses to opioid (Mitrovic et al., 2003), cannabinoid, $GABA_B$, and M_2 muscarinic receptor and α_2-adrenoceptor (Blednov et al., 2003) agonists in transgenic mice. These findings indicate that a large group of analgesic agents mediates their antinociceptive effects by activating a single signal transduction pathway, involving the GIRK2 subunits.

The GIRK channels were the first effectors shown to be regulated by $G_{i/o}$ protein βγ-subunits (Reuveny et al., 1994). Initial research indicated that it is the β-subunit of the complex that interacts with the GIRK channels. Interestingly, however, later it was found that although separate β_1 subunits are able to bind to the GIRK channel, they fail to enhance channel activity in the absence of the $G\gamma_2$-subunits, suggesting an important role of the $G\gamma_2$ subunits in channel activation. A recent study, using chimeric constructs between the mammalian $G\gamma_2$-subunit and the yeast Gγ-subunit, also clearly demonstrates that GIRK activation depends on a definite region of $G\gamma_2$ (Peng et al., 2003). At present, however, it is not clear which βγ-subunit combinations take part in the regulation of GIRK channels, and whether specific βγ-subunit combinations are selectively involved in GIRK2 regulation, and thus, in the analgesic response.

The present study demonstrates that i.c.v. treatment of mice with a phosphorothioate oligodeoxynucleotide antisense to $G\gamma_2$ significantly attenuates GPCR agonist-mediated antinociception in a hot (55 °C) water tail-flick test in mice. These data indicate that the G protein $G\gamma_2$-subunits may have a pervasive role in supraspinal antinociception in mice, presumably by selectively regulating the effectors that mediate analgesia.

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