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Regulator of G protein signaling proteins differentially modulate signaling of μ and δ opioid receptors

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Abstract

Effects of regulator of G protein signaling (RGS) proteins on μ and δ opioid receptors were investigated in HEK293 cells. Co-expression of RGS1, RGS2, RGS4, RGS9, RGS10 or RGS19 (G α -interacting protein (GAIP)) significantly reduced [Tyr-D-Ala-Gly-N-methyl-Phe-Gly-ol]-Enkephalin (DAMGO)-induced inhibition of adenylyl cyclase (AC) mediated by μ opioid receptor, but only RGS9 decreased the effects of [Tyr-D-Pen-Gly-p-Chloro-Phe-D-Pen]-Enkephalin (DPDPE) mediated by δ opioid receptor. When C-tails of the receptors were exchanged (μ/δ C and δ/μ C chimeras), RGS proteins decreased δ/μ C-mediated AC inhibition, but none had significant effects on that via μ/δ C receptor. Thus, the C-terminal domains of the receptors are critical for the differential effects of RGS proteins, which may be due to differences in receptor - G protein - RGS protein interactions in signaling complexes.

Keywords

RGS; opioid receptor; adenylyl cyclase inhibition

1. Introduction

Opioid receptors, including μ , δ , and κ types, mediate physiological actions of endogenous opioid peptides and pharmacological effects of opioid compounds. Opioid receptors are members of G protein coupled receptors (GPCRs) and are mainly coupled with G_{i/o} proteins. Activation of opioid receptors results in inhibition of adenylyl cyclase (AC), increase in potassium conductance, decrease in calcium conductance and activation of mitogen-activated protein kinase pathways [for a review, see (Law et al., 2000)]. The μ opioid receptors mediate analgesia, respiratory depression, immunosuppression, decreased gastrointestinal transit, reward increased locomotor activity and dependence induced by morphine and other μ -preferring compounds (Matthes et al., 1996). The δ opioid receptors have been associated with

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analgesia, morphine tolerance, mood regulation and cardioprotection (Zhu et al., 1999; Filliol et al., 2000; Gross et al., 2004).

G protein-mediated signaling of GPCRs is intrinsically kinetic, mainly including two key processes, i.e., G protein activation (GTP/GDP exchange) and G protein deactivation (GTP hydrolysis). GTP/GDP exchange is accelerated by interaction of receptors with G proteins, and GTP hydrolysis by G_{α} subunits is accelerated by GTPase-activating proteins. Regulator of G protein signaling (RGS) proteins are the most important GTPase-activating proteins for G proteins and thus inhibit G protein functions (Willars, 2006). To date, more than 30 mammalian RGS proteins have been identified. These proteins contain an evolutionarily conserved RGS core domain of ~120 amino acids flanked by highly variable sequences with molecular weights ranging from 17 kDa to 140 kDa. Based on the diversity of amino acid sequence in RGS domain, RGS proteins have been classified into six main subfamilies, Rz, R4, R7, R12, RA and RL (Hollinger and Hepler, 2002). The RGS domain alone is capable of binding G_{α} subunits and accelerating GTP hydrolysis. In addition, RGS proteins also contain other functional sequences such as G-gamma-like, phosphotyrosine-binding and PSD-95/Discs-large/ZO-1 related (PDZ) domains which may contribute to affinity and/or selectivity for G protein targets and involve in regulation of GPCR signaling via other mechanisms (Burchett, 2000; Hollinger and Hepler, 2002). Most RGS proteins act as GTPase-activating proteins for α subunits of $G_{i/o}$ and G_q proteins. In addition to function as GTPase-activating proteins, some RGS proteins compete with effectors for binding to G_{α} proteins and function as effector antagonists. GPCRs, G proteins, effectors, RGS and other regulating proteins have been shown to form complexes (Abramow-Newerly et al., 2006). Direct interactions of GPCRs and RGS proteins have been reported (Abramow-Newerly et al., 2006; Neitzel and Hepler, 2006).

Effects of RGS proteins on opioid receptor signaling have been examined in several systems and results are not always consistent [for a review, see (Xie and Palmer, 2005)]. In the present study, we examined regulation of signaling of μ and δ opioid receptor by RGS proteins expressed in HEK293 cells. Six RGS proteins belonging to different subgroups in the RGS family were investigated: RGS19 [G_{α} -interacting protein (GAIP)] of the Rz group, RGS1, RGS2 and RGS4 of the R4 group, RGS9 of the R7 group, and RGS10 of the R12 group. μ or δ opioid receptor and one RGS protein were transfected into HEK293 cells. Agonist-induced inhibition of forskolin-stimulated AC via μ or δ opioid receptor was used as the end point. Unexpectedly, we found that μ and δ opioid receptor signaling was differentially regulated by RGS proteins.

2. Materials and Methods

2.1. Materials

DAMGO (Tyr-D-Ala-Gly-N-Methyl-Phe-Gly-ol), DPDPE (D-Pen²,D-Pen⁵-enkephalin), naloxone, IBMX (3-isobutyl-1-methylxanthine), forskolin and cAMP (Adenosine 3',5'-cyclophosphate) were purchased from Sigma Aldrich (St. Louis, MO). [³H]diprenorphine was purchased from Perkin-Elmer Co. (Boston, MA). MEM (Minimum Essential Medium), heat-inactivated fetal bovine serum, 100× penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). [³H]cAMP was purchased from Perkin-Elmer Co. (Boston, MA) and Institute of Chinese Atomic Physics (Beijing, China). The human RGS constructs (hRGS1, hRGS2, hRGS4, hGAIP plasmids) and the expression vector (pcDNA3) were kindly provided by Dr. John Kehrl in National Institute of Health (Bethesda, MD). hRGS9-pcDNA3 and hRGS10-pcDNA3 constructs were generously provided by Dr. Ernest Peralta in Harvard University (Boston, MA). The rat μ opioid receptor and human δ opioid receptor expression constructs were obtained from Dr. Lei Yu of University of Cincinnati School of Medicine and Dr. Brigitte Kieffer of National Institute of Health and Medical Research, Illkirch, France, respectively (Chen et al., 1993; Simonin et al., 1994).

2.2. Generation of μ/δ C and δ/μ C chimera

Human δ opioid receptor and rat μ opioid receptor have the same amino acid residues 330YAFLDENFK338 at the end of seventh transmembrane domain (TM7) and beginning of C-terminal domain. Sense (TAC GCC TTC CTC GAT GAG AAC TTC AAG C) and antisense oligodeoxynucleotides (GCT TGA AGT TCT CAT CGA GGA AGG CGT A) were synthesized for use as TM7 primers.

μ/δ C chimera—The μ/δ C chimeric receptor is composed of 1-339 amino acids of rat μ opioid receptor and 322-372 amino acids of human δ opioid receptor C-terminal domain with the FLAG epitope at N terminus. The fragment of FLAG- μ opioid receptor (N-terminus-TM7) was generated by polymerase chain reaction (PCR) using the FLAG-ECORI-mutant primer (AGA CCC AAG CTT CAA TTC GAG C) and the antisense of TM7 primer as primers and the FLAG- μ opioid receptor in pcDNA3 as the template. The C-tail of δ opioid receptor was produced by PCR using the sense TM7 primer and a pcDNA3 sequence as primers and the FLAG- δ opioid receptor in pcDNA3 as the template. Overlap PCR was performed using the FLAG- μ opioid receptor (N-terminus-TM7) and C-tail of δ opioid receptor as the templates and the FLAG-ECORI-mutant primer and a pcDNA3 sequence as primers. The resulting PCR product (FLAG- μ opioid receptor with δ opioid receptor C-tail) was treated with Hind III and Xba I and was ligated into HindIII and Xba I sites of the vector pcDNA3 to generate the μ/δ C chimera.

δ/μ C chimera—The same strategy was employed to generate δ/μ C chimera. The δ/μ C chimeric receptor is composed of 1-321 amino acids of human δ opioid receptor and 340-398 amino acids of rat μ opioid receptor C-terminal domain with the FLAG epitope at N terminus.

The fragment of FLAG- δ opioid receptor (N-terminus-TM7) was generated by PCR using the FLAG-EcoRI-mutant primer and the antisense TM7 primer and the FLAG- δ opioid receptor in pcDNA3 as the template. The μ opioid receptor (C-tail) was produced by PCR using the sense TM7 primer and the RMXbaI(-) primer (GTC TAG ACC CAG TTA GGG CAA T) and the FLAG- μ opioid receptor in pcDNA3 as the template. Overlap PCR was performed using the FLAG- δ opioid receptor (N-terminus-TM7) and μ opioid receptor (C-tail) as the templates and the FLAG-ECORI-mutant primer and RMXbaI(-) primer as primers. The resulting PCR product (FLAG- δ opioid receptor with μ opioid receptor C-tail) was treated with Hind III and Xba I and was ligated into HindIII and Xba I sites of the vector pcDNA3 to generate the δ/μ C chimera.

The DNA sequences of chimeric cDNA clones were determined to ensure correct generation and no unwanted mutations.

2.3. Cell culture and transfection

HEK293 cells with the similar passage numbers (5-25) were used for all the experiments after we obtained cells from ATCC (Manassas, VA); however, the absolute passage numbers were unknown. HEK293 cells were grown in 100mm cell culture dishes in MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 μ g/ml streptomycin sulfate at 37°C in a humidified atmosphere containing 5% CO₂. Cells were grown to ~70% confluence before DNA transfection using calcium phosphate reagents [250 mM of calcium chloride and 2× HBS buffer (50 mM HEPES, 1.5 mM Na₂HPO₄, 280mM NaCl, pH 7.1)]. The amount of each plasmid DNA used for transfection was 1.0 μ g/ml medium. For co-transfection, the total amount of DNA transfected was held constant using empty pcDNA3 plasmid. The cells were incubated with the transfection solution for 24 h at 37°C, and then grown in the medium for another 24 h before harvest.

2.4. [³H]diprenorphine binding assay

Transfected HEK293 cells were collected and suspended in serum-free MEM at a density of 1×10^6 cells/ml. Cell suspension (100 μ l) was incubated with [³H]diprenorphine (1nM) in binding assay buffer (10mM Tris-HCl, 50mM sucrose, 20mM MgCl₂, pH 7.4) with a final volume of 300 μ l. Naloxone (10 μ M) was employed to define nonspecific binding. The cells were incubated at 25°C for 1 h and filtered rapidly through Brandel 48-well harvester (Brandel, Gaithersburg, MD) using Whatman GF/C glass fiber filter paper (Whatman, Florham Park, NJ), presoaked with 0.25% polyethyleneimine in water. The filters were rinsed three times with ice-cold 50mM Tris-HCl, pH 7.4 and the radioactivity in filters was determined by liquid scintillation counting in LS3801 Beckman counter (Beckman Coulter, Fullerton, CA).

2.5. Drug treatment and cAMP measurement

Cells were harvested and suspended in reaction buffer (10mM Tris-HCl, 50mM sucrose, 20mM MgCl₂, pH 7.4) to a density of 1×10^6 cells/ml. Cells were incubated with DAMGO or DPDPE at various concentrations (1 nM ~10 μ M), forskolin (10 μ M) and IBMX (1 mM) for 10 min at 37 ° C. The reaction was terminated by boiling in the water bath for 5 min. The cells were homogenized by brief sonication and centrifuged at $2,000 \times g$ for 5 min. cAMP level in the supernatant was measured with cAMP binding protein method as described previously by Huang et al. (2001). Briefly, the supernatant or standards with known amounts of cAMP was incubated with [³H]cAMP (~30,000 dpm) and cAMP binding protein for 2h at 4 ° C to yield 10,000 to 20,000 dpm of binding in the absence of cAMP. Then 5% (w/v) ice-cold charcoal suspension pre-saturated with bovine serum albumin was added into the solution. The bound [³H]cAMP was separated from the free by centrifugation at $10,000 \times g$ for 5 min. The radioactivity of supernatants was determined by liquid scintillation counting. cAMP level was determined based on the standard curve.

2.6. Data analysis

The AC activity inhibition was expressed as the percentage of forskolin-stimulated cAMP accumulation in agonist treated cells to vehicle treated cells. Statistical significance was determined by Student's *t* test.

3. Results

3.1. Inhibition of forskolin-stimulated AC activity by activation of μ and δ opioid receptor

As an initial step, we examined the effects of opioid agonists on forskolin-stimulated AC activity in HEK293 cells transiently transfected with μ or δ opioid receptor. Expression of either receptor was confirmed by [³H] diprenorphine binding and the expression level was approximately 20 fmole/ 10^5 cells for both receptors. Transfection of the vector pcDNA3 did not result in any specific [³H] diprenorphine binding. In cells transfected with μ opioid receptor, the selective μ agonist DAMGO inhibited forskolin-stimulated AC activity in a dose-dependent manner and 10 μ M caused ~ 45% inhibition (Fig. 1A). In cells transfected with δ opioid receptor, the selective δ agonist DPDPE dose-dependently inhibited AC activity and 10 μ M induced ~ 42% inhibition (Fig. 1B). Since there were no apparent plateaus at low and high doses, no IC₅₀ or I_{max} values were calculated. In cells transfected with the vector, neither agonist caused any inhibition.

3.2. Expression of RGS proteins on μ opioid receptor -mediated inhibition of forskolin-stimulated AC activity

HEK293 cells were transiently co-transfected with μ opioid receptor and one of six RGS proteins [RGS1, RGS2, RGS4, RGS19 (GAIP), RGS9, RGS10] or the vector pcDNA3. [³H] diprenorphine specific binding was not significantly different between the pcDNA3- and RGS-

transfected groups. Expression of each of RGS1, RGS2, RGS4, RGS19 (GAIP), RGS9 and RGS10 significantly attenuated DAMGO-induced inhibition of forskolin-stimulated AC activity (Fig. 2). RGS9 reduced the DAMGO inhibition of AC activity to a less extent than other RGS proteins (Fig. 2E). The RGS proteins attenuated DAMGO response at almost every concentration. Since all six RGS proteins tested attenuated DAMGO effect, there is little specificity in RGS proteins on μ opioid receptor signaling.

3.3. Expression of RGS proteins on δ opioid receptor -mediated inhibition of forskolin-stimulated AC activity

Similar experiments were performed on HEK293 cells co-transfected with δ opioid receptor and one of the six RGS proteins or the vector pcDNA3. RGS protein expression did not significantly change the expression of δ opioid receptor as determined by [3 H]diprenorphine binding, compared with pcDNA3-transfected control. DPDPE caused dose-dependent decreases in forskolin-stimulated AC activity in RGS- and pcDNA3-transfected cells. Surprisingly, RGS1, RGS2, RGS4, RGS19(GAIP), and RGS10 had no significant effect on DPDPE-induced inhibition of AC activity (Fig. 3), which is different from their effects on μ opioid receptor -mediated AC inhibition. Interestingly, RGS9 reduced DPDPE-induced inhibition significantly, albeit weakly, similar to what it did in cells transfected with μ opioid receptor.

3.4. Effect of activation of μ/δ C and δ/μ C chimeras on forskolin-stimulated AC activity

Since both μ and δ opioid receptor are coupled to the same G proteins, the differences in effects of RGS proteins on AC inhibition mediated by the two receptors are likely due to differences in their interactions with RGS proteins and/or associated interacting proteins. Comparison of the amino acid sequences of the intracellular domains of μ and δ opioid receptor shows that only the C-terminal domains are significantly different. Two chimeras, μ/δ C and δ/μ C, were constructed, in which the C-terminal domains of μ and δ opioid receptor were swapped, and employed to investigate the involvement of C-terminal domains in the RGS regulation of the receptor function. Expression of μ/δ C and δ/μ C receptors was detected with [3 H] diprenorphine binding. The K_d value of [3 H] diprenorphine and K_i value of DAMGO for the μ/δ C receptor were not significantly different from those for the μ opioid receptor (data not shown). In addition, the K_d value of [3 H] diprenorphine and K_i value of DPDPE for the δ/μ C receptor were not significantly different from those for the δ opioid receptor (data not shown). In cells transfected with the μ/δ C or δ/μ C receptor, DAMGO or DPDPE, respectively, inhibited forskolin-stimulated AC activity in a dose-dependent manner, and 10 μ M caused ~ 40% inhibition (Fig. 4). DAMGO and DPDPE were active at the μ/δ C and δ/μ C chimeric receptors in similar concentration ranges as at μ and δ opioid receptor, respectively.

3.5. Expression of RGS proteins on inhibition of forskolin-stimulated AC activity caused by the μ/δ C chimera activation

In cells transfected with the μ/δ C chimera and one of the RGS proteins or the vector, the dose-response curves of DAMGO in inhibiting forskolin-stimulated AC activity were almost superimposable with or without RGS protein expression (Fig. 5). Thus, replacement of the μ opioid receptor C-terminal domain with that of δ opioid receptor resulted in loss of sensitivity to RGS proteins. In addition, RGS9-transfected cells did not show appreciable attenuation of the inhibition in response to varying concentrations of DAMGO, which is different from the RGS9 effect on responses of μ or δ opioid receptor.

3.6. Effects of RGS proteins on inhibition of AC activity caused by δ/μ C chimera receptor activation

Co-expression of RGS1, RGS2, RGS4, RGS19(GAIP), RGS9 or RGS10 with the δ/μ C chimera significantly attenuated DPDPE-induced inhibition of forskolin-stimulated AC activity, compared with the vector-transfected control (Fig. 6). Thus, substitution of the C-tail of δ opioid receptor with that of μ opioid receptor leads to acquisition of sensitivity to RGS proteins, demonstrating that the C-tail of the μ opioid receptor confers the sensitivity.

4. Discussion

Our study demonstrated that RGS1, RGS2, RGS4, RGS19 (GAIP), RGS9 and RGS10 significantly attenuated μ opioid receptor-mediated inhibition of AC activity. However, only RGS9 had a similar effect on δ opioid receptor-mediated actions. These results indicate that RGS proteins differentially regulate signaling of opioid receptors. Replacement of the μ opioid receptor C-tail with that of the δ opioid receptor resulted in loss of sensitivity to RGS proteins, whereas C-tail substitution of the δ opioid receptor by the μ opioid receptor C-tail led to acquisition of response to RGS proteins, demonstrating the critical role of the C-tail in RGS sensitivity.

Most RGS proteins tested affect signaling of μ opioid receptor, but not that of δ opioid receptor

The differential RGS sensitivity of μ - vs. δ - opioid receptor -mediated AC inhibition is consistent with the findings from the group of Drs. Sanchez-Blazquez and Garzon (Sanchez-Blazquez et al., 2003; Garzon et al., 2004). These researchers found that reductions in RGS9-2, RGS19(GAIP), or RGS20 levels enhanced antinociceptive effects of morphine and DAMGO, but did not affect those of DPDPE.

Although it is possible that differential effects on μ - or δ - opioid receptor signaling may be caused by different transfection efficiency of RGS proteins, it is very unlikely in our study. It is generally accepted that when co-transfected with two different cDNAs, cells taking up one cDNA will take up the other. The same amount of cDNAs of RGS proteins was co-transfected with μ - or δ - opioid receptor (adjusted to the same amount of plasmid DNAs with the vector) into the cells of the same type using the same transfection reagent. μ - or δ - opioid receptor was expressed at similar levels as shown by binding results. The successful expression of RGS proteins was demonstrated by their effects on μ opioid receptor signaling. Since the same system and procedure were employed, it is highly unlikely that RGS proteins were expressed only in cells transfected with μ opioid receptor, but not with δ opioid receptor, while the co-transfected δ opioid receptor was expressed as expected.

Differences in RGS sensitivity do not reflect coupling of the μ and δ opioid receptors to different G_α subunits of the $G_{i/o}$ class. Law's group (Prather et al., 1994a; Prather et al., 1994b; Chakrabarti et al., 1995) has reported that μ and δ opioid receptor are coupled simultaneously to several G_α subunits including $\alpha i2$, $\alpha i3$ and two αo subunits, regardless of receptor density and agonist potency.

The RGS proteins examined in this study belong to different subfamilies: RGS19 (GAIP) of the Rz group, RGS1, RGS2 and RGS4 of the R4 group, RGS9 of the R7 group, and RGS10 of the R12 group. Except RGS9, the other RGS proteins have short sequences outside of the RGS domains. RGSs 1, 2 and 4 and RGS19(GAIP) have short amphipathic helices in the N-terminal regions. RGS19(GAIP), in addition, has a cysteine string in the N-terminal region and a PDZ domain in the C-terminal region. RGS9 contains distinct DEP (disheveled, EGL-10, pleckstrin), R7H (R7 homology) and G-gamma-like domains in the N-terminus to the RGS

domain (Hollinger and Hepler, 2002). It is unlikely that all the RGS proteins examined bind directly to the μ opioid receptor C-tail, but not the δ opioid receptor C-tail.

The differential sensitivity may be due to differences in signaling complex formation, in which the C-tail of the receptors plays a role. There is increasing evidence that RGS proteins are part of signaling complexes which include, in addition to RGS proteins, GPCRs, $G\alpha$ proteins, and downstream effectors, such as adenylyl cyclase, GIRK channels, phosphodiesterase, PLC β and Ca^{2+} channels (Abramow-Newerly et al., 2006). Georgoussi et al. (2006) reported that both the δ opioid receptor C-tail and the i3 loop bound $G\alpha t\beta 1\gamma 1$, inactive $G\alpha t$ (GDP) and active $G\alpha t$ (GTP γ S), but the μ opioid receptor C-tail did not. In addition, the δ opioid receptor was noted for being coupled tightly to $G\alpha$ proteins. Law et al. (1991) demonstrated that chronic treatment of NG108-15 cells with D-Ala², D-Leu⁵-enkephalin (DADLE) resulted in homologous desensitization of the δ opioid receptor; however, about 40% of the total binding sites remained in high agonist affinity states. Similar results were found following pertussis toxin treatment. The δ opioid receptors in high-affinity states after DADLE or pertussis toxin treatment represent receptors still coupled to G-proteins. In addition, the δ opioid receptor has been shown to have high levels of agonist-independent activities, indicative of tight coupling (Costa and Herz, 1989). In contrast, RGS4 directly interacted with the μ opioid receptor C-tail, the δ opioid receptor C-tail and i3 loop (Georgoussi et al., 2006). Thus, in signaling complexes, μ and δ opioid receptor may have different interaction with G proteins. The low sensitivity of δ opioid receptor response to RGS proteins may be also attributed to the presence of spare δ opioid receptors for agonist-induced AC inhibition. Costa et al. (1988) reported that following treatment of NG108-15 cells with the alkylating antagonist beta-chlornaltrexamine (CNA), the reduction of GTPase responsiveness corresponded to the loss of binding sites and occurred at CNA concentrations lower than those necessary to reduce AC responsiveness. The loss of responsiveness of GTPase occurred as reduction of maximal response, whereas that of AC involved an initial reduction of apparent agonist potency that was followed by a decrease in maximal effect. These results indicate the presence of spare δ opioid receptors for agonist-induced AC inhibition.

Differential regulation of GPCRs coupled to the same $G\alpha$ protein by RGS proteins has been reported previously (Neitzel and Hepler, 2006). In rat pancreatic cells, phospholipase C activation and subsequent Ca^{2+} release following activation of Gq-coupled receptors is differentially regulated by several RGS proteins (Zeng et al., 1998; Xu et al., 1999). Muscarinic receptor is 3 $\times$ and 10 $\times$ more sensitive to RGS4 than bombesin and cholecystokinin receptors, respectively. Both the N- and C-terminal flanking regions and the RGS domain of RGS4 are required for the differential sensitivities (Zeng et al., 1998; Xu et al., 1999). RGS1 was $\sim$ 1000-fold more potent in inhibiting muscarinic than CCK signaling, whereas RGS2 was equi-potent. RGS16 was as effective as RGS1 in attenuated muscarinic signaling but only partially inhibited the cholecystokinin response (Xu et al., 1999). Ghavami et al. (2004) reported that RGS4 and RGS10 significantly attenuated inhibition of cAMP accumulation mediated by 5-HT1A receptor, but not that by dopamine D2 receptor.

δ opioid receptor - or μ/δ C chimera-mediated AC inhibition was not affected by co-expression of RGS4 in our study. However, purified RGS4 was shown to bind directly to C-tail and the i3 loop of the δ opioid receptor in pull-down assay (Georgoussi et al., 2006). It is not known whether RGS4 can interact with δ opioid receptor and μ/δ C chimera in the cellular milieu where proteins may exist in different conformations compared with purified forms. It is also possible that in cells, RGS4 and the δ opioid receptor or μ/δ C chimera are parts of a complex in which the access of RGS4 to the δ opioid receptor C-tail and i3 loop may be hindered by other proteins.

RGS9 is different from other RGS proteins in modulating μ - and δ - opioid receptor -mediated signaling

RGS9 is different from other RGS proteins in that it attenuated signaling of the δ opioid receptor, indicating that RGS proteins play different roles in the same GPCR signaling. Its different function is probably due to the involvement of its DEP domain in membrane attachment (Martemyanov et al., 2003) and the binding of its G-gamma-like domain to G β 5 protein (Sondek and Siderovski, 2001). Interestingly, RGS9 had no attenuation effect on the DAMGO-induced AC inhibition mediated by the μ/δ C chimera, indicating more complex interactions other than the C-tail.

Differential effects of RGS proteins on opioid receptor signaling have been reported. Potenza et al. (1999) showed that RGS2, but not RGS1, RGS3, or RGS4, attenuated morphine-induced responses in a melanophore cell line stably expressing the μ opioid receptor. Reductions in RGS2 or RGS3 levels decreased μ opioid receptor -mediated antinociception, whereas those of RGS6, RGS7, RGS9-2, RGS11 or RGS12 increased this activity (Garzon et al., 2001; Garzon et al., 2003). The potency and the duration of opioid antinociception were enhanced in RGS9-null mice and RGS11-null mice, but not in RGS2-null mice (Garzon et al., 2001; Garzon et al., 2003; Sanchez-Blazquez et al., 2003).

Regulation of μ and δ opioid receptor signaling by RGS proteins

Our finding that RGS proteins attenuated μ opioid receptor -mediated AC inhibition is similar to those of Garnier et al. (2003) and Georgoussi et al. (2006). In addition, Ippolito et al. (2002) reported that RGS4 accelerated Kir3 potassium channel deactivation following termination of μ opioid receptor signaling in *Xenopus* oocytes. Moreover, Clark et al. (2003) reported that in cells expressing RGS-insensitive G α o, DAMGO and morphine were much more potent and/or had an increased maximal effect in inhibiting AC and in activating p42/p44 MAPK. Intracellular application of wild-type RGS4 into locus coeruleus neurons diminished electrophysiological responses to morphine (Gold et al., 2003). RGS9 $^{-/-}$ mice showed a dramatic increase in morphine reward and increased morphine analgesia with delayed tolerance (Zachariou et al., 2003).

However, our results are different from those of Hepler et al. (1997). They reported that addition of exogenous RGS4 to NG108-15 cell membranes attenuated δ opioid receptor -mediated inhibition of prostaglandin E1-stimulated AC activity and that RGS4 was more potent than RGS19 in this regard. Perhaps different signaling complexes are formed in different cells (see above).

In summary, our study showed that μ opioid receptor -mediated AC inhibition is more sensitive to RGS protein regulation than that of δ opioid receptor and the C-tails of the receptors play an important role in the differences.

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Fig. 1. Effects of activation of the μ and δ opioid receptors on forskolin-stimulated AC activity HEK293 cells were transfected with the μ or δ opioid receptor or the vector pcDNA3 as described in **Materials and Methods**. Approximately 48 h later, cells were harvested and suspended at 10^5 cells/ml and incubated with 10 μ M forskolin and 1 mM IBMX with and without different concentrations of DAMGO or DPDPE. cAMP levels were determined and normalized to % of forskolin-stimulated cAMP level. Each value represents mean $\pm$ S.E.M. of three independent experiments performed in duplicate.

α opioid receptor

Fig. 2. Effects of RGS proteins on the μ opioid receptor-mediated inhibition of forskolin-stimulated AC activity

HEK293 cells were transfected with the μ opioid receptor and one of the RGS proteins or the vector pcDNA3 as described in **Materials and Methods**. Approximately 48 h later, cells were harvested and experiments were conducted as described in Fig. 1. Each value represents mean $\pm$ S.E.M. of three independent experiments performed in duplicate. Data were fitted with linear regression. RGS- and vector-transfected lines were compared.

- □- RGS + μ opioid receptor, -○- pcDNA3 + μ opioid receptor.

$P < 0.01$ for all RGS groups compared with compared with the vector-transfected control.

δ opioid receptor

Fig. 3. Effects of RGS proteins on δ opioid receptor-mediated inhibition of forskolin-stimulated AC activity

HEK293 cells were transfected with the δ opioid receptor and one of the RGS proteins as indicated or the vector pcDNA3 as described in **Methods and Materials**. Approximately 48 h later, cells were harvested and experiments were conducted as described in Fig. 1. cAMP levels were determined and normalized to % of forskolin-stimulated cAMP level, and each value represents mean $\pm$ S.E.M. of three independent experiments performed in duplicate. -□- RGS + δ opioid receptor, -○- pcDNA3 + δ opioid receptor.

$P < 0.01$ for the RGS9 group compared with the control group. Other RGS protein groups did not differ significantly from the control group.

Fig. 4. μ/δ C or δ/μ C chimera-mediated inhibition of forskolin-stimulated AC activity
HEK293 cells were transfected with μ/δ C or δ/μ C opioid receptor or the vector pcDNA3. Approximately 48 h later, cells were harvested and experiments were conducted as described in Fig. 1. cAMP levels were determined and normalized to % of forskolin-stimulated cAMP level, and each value represents mean $\pm$ S.E.M. of three independent experiments performed in duplicate.

Fig. 5. Effects of RGS proteins on μ/δ C chimera-mediated inhibition of forskolin-stimulated AC activity

HEK293 cells were transfected with the μ/δ C opioid receptor and one of the RGS proteins or the vector pcDNA3 as described in **Materials and Methods**. Approximately 48 h later, cells were harvested and experiments were conducted as described in Fig. 1 using DAMGO as agonist. cAMP levels were determined and normalized to % of forskolin-stimulated cAMP level. Each value represents mean $\pm$ S.E.M. of three independent experiments performed in duplicate.

□- RGS + μ/δ C chimeric receptor, ○- pcDNA3 + μ/δ C chimeric receptor.

None of the RGS protein groups differ significantly from the respective control group.

Fig. 6. Effects of RGS proteins on δ/μ C chimera-mediated inhibition of forskolin-stimulated AC activity

HEK293 cells were transfected with the δ/μ C chimeric receptor and one of the RGS proteins or the vector pcDNA3. Approximately 48 h later, cells were harvested and experiments were conducted as described in Fig. 1 using DPDPE as the agonist. cAMP levels were determined and normalized to % of the baseline which represents forskolin-stimulated cAMP level. Each value represents mean $\pm$ S.E.M. of three independent experiments performed in duplicate.

□- RGS + δ/μ C chimeric receptor, ○- pcDNA3 + δ/μ C chimeric receptor.

* $P < 0.01$ for all RGS groups, compared with the control group.