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Up-regulation of RGS4 mRNA by opioid receptor agonists in PC12 cells expressing cloned μ- or κ-opioid receptors

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Abstract

The regulators of G-protein signaling (RGS) proteins have been shown to modulate the function of some heterotrimeric G-proteins by stimulating the GTPase activity of G-protein α subunits. In this study, by northern blotting analysis, we investigated the regulation of RGS4 mRNA by opioid receptor agonists in PC12 cells stably expressing either cloned μ- or κ-opioid receptors. Treatment with respective opioid receptor agonists (μ: morphine) and [D-Ala^2, MePhe^4, Gly(ol)^5] enkephalin (DAMGO), κ: (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro-(4,5)dec-8-y1]benzeneacetamide (U69,593)) for 0.5–24 h significantly and transiently increased the expression of RGS4 mRNA by 140–170% of the control level in a concentration-dependent manner which peaked when treated for 2 h, while treatment of non-transfected PC12 cells with opioid receptor agonists did not. The up-regulation of RGS4 mRNA was significantly blocked by co-treatment with respective opioid antagonists (μ: naloxone, κ: norbinaltorphimine) or pretreatment with pertussis toxin. These results suggest that the activation of μ- or κ-opioid receptors increases RGS4 mRNA level, which might contribute to opioid desentilization.

Keywords: RGS4 (regulator of G-protein signaling); Opioid receptor; PC12 cell; Opioid receptor agonist; Desensitization

1. Introduction

Desensitization of opioid receptors by continuous agonist stimulation is a possible mechanism underlying the development of opioid tolerance, which is a characteristic problem of opiate drugs in clinical usage, although its mechanisms remain relatively poorly understood (Nestler, 1992; Nestler and Aghajanian, 1997). Opioid receptors have been pharmacologically classified into at least three types, designed as μ, δ and κ, each with distinct binding properties for various opioid ligands and with distinct distributions in the nervous system (Pasternak, 1988; Minami and Satoh, 1995; Satoh and Minami, 1995). All of them belong to the superfamily of seven-transmembrane domain GTP-binding protein (G-protein)-coupled receptors, and couple via the pertussis toxin-sensitive $G_{i/o}$ family of G proteins to various effectors, including adenylyl cyclase, Ca^{2+} channels and K^+ channels. The cloning of cDNAs encoding opioid receptors has allowed us to study receptor structures, receptor localization, ligand binding, signal transduction properties and receptor desensitization (Mansour et al., 1995; Minami and Satoh, 1995; Satoh and Minami, 1995). Research on mechanisms of desensitization has focused on receptor modulation, either by changes in receptor expression or by G protein-coupled receptor kinase (GRK)-mediated receptor phosphorylation, β-arrestin binding, and subsequent receptor internalization (Hausdorff et al., 1990; Kobilka, 1992; Krupnick and Benovic, 1998).

The family of regulators of G protein signaling (RGS) proteins were originally identified by genetic complementation of a yeast homologue (Sst2p) and identification of a closely related homologue in *Caenorhabditis elegans* (EGL10), which negatively regulated G protein signaling (Druey et al., 1996; Koelle and Horvitz, 1996; De Vries et al., 1995). To date, more than 20 mammalian RGS proteins have been isolated and shown to down-regulate signal transduction via G protein-coupled receptors by acting as GTPase-activating proteins (GAPs) for α subunits of heterotrimeric G_i, G_o, G_z, G_t and G_q, but not G_s proteins (Berman et al., 1996; Watson et al., 1996; Hunt et al., 1996; Dohlman and Thorner, 1997; Hepler, 1999; De Vries et al., 2000). By facilitating the ability of α subunits to hydrolyze bound GTP, RGS proteins increase the rate at which the α subunits reassociate with their corresponding βγ dimers. In this way,

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RGS proteins promote the return of G proteins to their inactive GDP-bound states as negative regulators of G protein signaling, suggesting that the RGS proteins might be involved in receptor desensitization. Indeed, it has been shown that overexpression of RGS proteins inhibited the signal transduction via opioid receptors and other G protein-coupled receptors (Hepler at al., 1997; Huang et al., 1997; Potenza et al., 1999). More recently, knock down of some RGS proteins using antisense oligodeoxynucleotides has been reported to enhance the antinociceptive effects of morphine and β-endorphine, and to attenuate morphine tolerance in mice (Garzón et al., 2001).

Rat pheochromocytoma (PC12) cells, which display a number of neuronal characteristics and accordingly have been extensively utilized as neuronal models, were reported to endogenously have some RGS proteins, particularly RGS4 (Pepperl et al., 1998). Therefore, PC12 cells transfected with the cDNAs for opioid receptors are considered to be a suitable system for studying the involvement of RGS proteins in alterations of the signal transduction system via opioid receptors by continuous receptor stimulation. In this study, we investigated the effects of continuous treatment with opioid receptor agonists for 0.5–24 h on the expression of RGS4 mRNA in PC12 cells stably expressing either cloned μ- or κ- opioid receptors.

2. Materials and methods

2.1. Materials

The cDNAs for rat μ- and κ-opioid receptor encoded within pcDNA3 (Invitrogen, San Diego, USA) have been described previously (Minami et al., 1993, 1994). The κ-opioid receptor-selective antagonist norbinaltorphimine was a gift from Dr. H. Nagase (Toray Industries, Kamakura Japan). The κ-opioid receptor-selective agonist (+)-(5α, 7α, 8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro-(4,5) dec-8-yl]benzeneacetamide (U69,593) was a gift from the Upjohn (Kalamazoo, USA). Morphine hydrochloride was purchased from Takeda Chemical Industries (Osaka, Japan). [D-Ala^2, MePhe^4, Gly(ol)^5]Enkephalin (DAMGO), a μ-opioid receptor-selective agonist, and [D-Pen^2, D-Pen^5]enkephalin (DPDPE), a δ-opioid receptor-selective agonist, were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). Naloxone hydrochloride and salmon sperm DNA were from Sigma (St. Louis, USA). All other reagents were of the best available quality from commercial sources.

2.2. Cell culture and development of cell lines stably expressing μ- or κ-opioid receptor

PC12 cells were kindly provided by Dr. M. Negishi (Laboratory of Molecular Neurobiology, Graduate School of Biostudies, Kyoto University). PC12 cells were cultured in Dulbecco's Modified Eagle's Medium containing 5% fetal bovine serum, 10% horse serum, 50 U/ml penicillin and 50 μg/ml streptomycin under a 5% CO^2 atmosphere at 37 °C. Though it has been reported that the expression of RGS4 mRNA was not altered between undifferentiated and nerve growth factor-induced differentiated PC12 cells (Pepperl et al., 1998), we used undifferentiated PC12 cells in the subsequent studies. The cells were transfected with plasmids containing μ- or κ-opioid receptor cDNAs for eukaryotic expression using LipofectAMINE reagent (GibcoBRL, Gaithersburg, USA). A single clone expressing each type of opioid receptor was selected by cultivation in the presence of 500 μg/ml G418 (GibcoBRL) followed by binding assay with [^{3}H]bremazocine, a non-selective opioid ligand. The stable expression of μ- or κ-opioid receptors in PC12 cells was confirmed by binding assay with [^{3}H]DAMGO or [^{3}H]U69,593, respectively, and by the ability of subtype-specific opioid receptor agonists to inhibit forskolin-stimulated intracellular cAMP accumulation.

2.3. Preparation of antisense RNA probe for RGS4

The rat RGS4 cDNA was cloned from the cerebral cortex of Sprague–Dawley rats by a reverse transcription-polymerase chain reaction (RT-PCR)-based method. PCR primers were designed on the basis of the sequence of rat RGS4 cDNA (GeneBank U32327). The sequences of forward and reverse primers were 5′-ATCAGCTGTGAGGAGTACAA-3′ and 5′-CAGGTTGAAAATCTTCTTCT-3′, respectively. The DNA fragment obtained by RT-PCR was cloned into pBluescript II (Stratagene, San Diego, CA) and confirmed to be a partial cDNA for RGS4 by sequence analysis. The plasmid containing RGS4 cDNA was linearized at the *Spe* I site for preparation of an antisense RNA probe. ^{32}P-Labeled antisense RNA probe for RGS4 was synthesized in the presence of [α-^{32}P] uridine triphosphate (15 TBq/mmol, Amersham, Buckinghamshire, UK) using T7 RNA polymerase (Promega, Madison, USA).

2.4. Northern blotting analysis of RGS4 mRNA

For northern blotting analysis, total RNA was extracted from cultured PC12 cells by a single-step acid phenol/chloroform method with Isogen (Nippon Gene, Tokyo, Japan). Aliquots of 10 μg of total RNA samples were fractionated by electrophoresis in 1.2% agarose gels containing 6% formaldehyde, transferred onto nylon membranes (Biodyne, Pall, Glen Cove, USA) and baked at 80 °C for 2 h. The membranes were prehybridized for 2 h and then hybridized with the ^{32}P-labeled antisense RNA probe for RGS4 at 68 °C. The membranes were washed twice in 2 × saline sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) for 5 min at 68 °C, and then washed twice in 0.1 × SSC/0.1% SDS for 30 min at 68 °C. The membranes were exposed to X-ray film at − 80 °C with an intensifying screen. Quantitative analysis of film

autoradiograms was conducted with a BAS2000 Bioimaging analyzer (Fuji Photo Film, Japan). The obtained value of non-treated cells served as a control (100%) and the results are presented as the mean percentage of the control ± S.E.M. Statistical analysis was performed using Student's *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Effects of opioid receptor agonists on the expression of RGS4 mRNA in non-transfected PC12 cells

In preliminary experiments, non-transfected undifferentiated PC12 cells highly expressed RGS4 mRNA, but the levels of expression of RGS2, 7, 8 or 10 mRNA were very low or below the level of detection by northern blotting analyses (data not shown). In subsequent studies, we investigated the expression of RGS4 mRNA in undifferentiated PC12 cells.

We examined the effects of various opioid receptor agonists on the expression of RGS4 mRNA in non-transfected undifferentiated PC12 cells. However, treatment with morphine (10 μM), DAMGO (1 μM), DPDPE (1 μM) or U69,593 (1 μM) for 2 h had no significant effects on the expression of RGS4 mRNA (Fig. 1A).

3.2. Effects of opioid receptor agonists on the expression of RGS4 mRNA in PC12 cells expressing μ- or κ-opioid receptor

In PC12 cells stably expressing the cloned μ-opioid receptor, treatment with morphine and DAMGO for 2 h significantly increased the expression of RGS4 mRNA by 151 ± 13% and 153 ± 9.8% of the control level, respectively, while DPDPE and U69,593 had no effect (Fig. 1B). The up-regulation of RGS4 mRNA by morphine and DAMGO was concentration-dependent and their EC_{50} values were 74 ± 34 and 15 ± 9.7 nM, respectively (Fig. 2A). The increasing effect of morphine (10 μM) was relatively rapid and transient, peaking when treated for 2 h, and declined finally reaching control level when treated for 12 h (Fig. 3A).

Similarly, in PC12 cells stably expressing the cloned κ-opioid receptor, treatment with U69,593 for 2 h significantly increased the expression of RGS4 mRNA by 167 ± 5.6% of the control level, while morphine, DAMGO and DPDPE

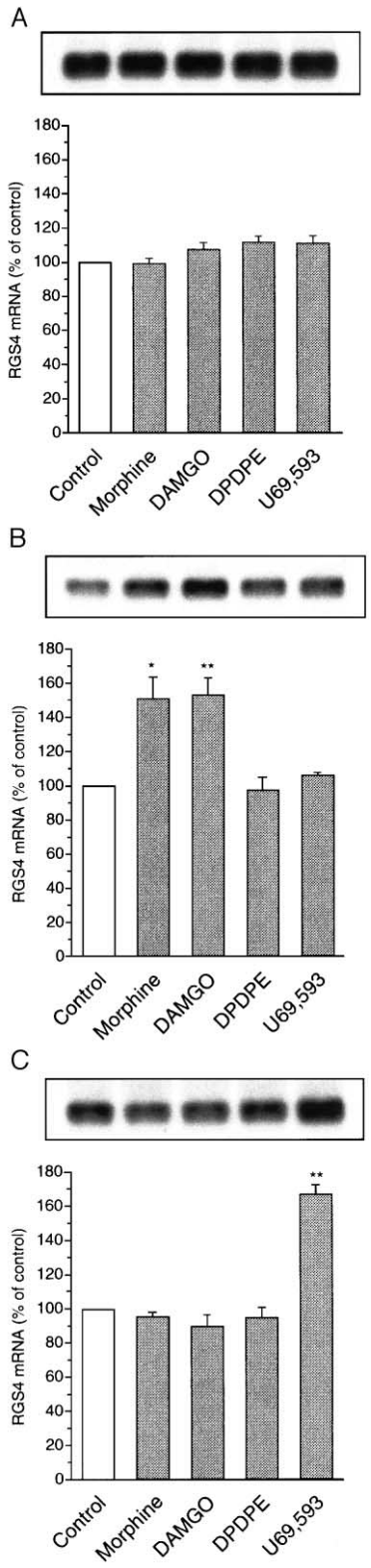

Fig. 1. Effects of various opioid receptor agonists on the expression of RGS4 mRNA in non-transfected PC12 cells and PC12 cells stably expressing cloned μ- or κ-opioid receptors. Non-transfected PC12 cells (A), PC12 cells expressing the cloned μ-opioid receptors (B) or κ-opioid receptors (C) were treated with morphine (10 μM), DAMGO (1 μM), DPDPE (1 μM) or U69,593 (1 μM) for 2 h. Total RNA was extracted, and northern blotting analysis was carried out with 10 μg of total RNA per lane using a ^{32}P-labeled antisense RNA probe for RGS4. The upper panels show representative blots. In the lower panels, the expression of RGS4 mRNA in non-treated cells (control) was assigned a value of 100% and data are presented as means of the percentage of control ± S.E.M. of four to five separate experiments. * $P < 0.05$, ** $P < 0.01$ versus control.

had no effect (Fig. 1C). The up-regulation by U69,593 was dose-dependent with an EC_{50} value of 12 ± 4.2 nM (Fig. 2B). The increasing effect of U69,593 (1 μM) was also

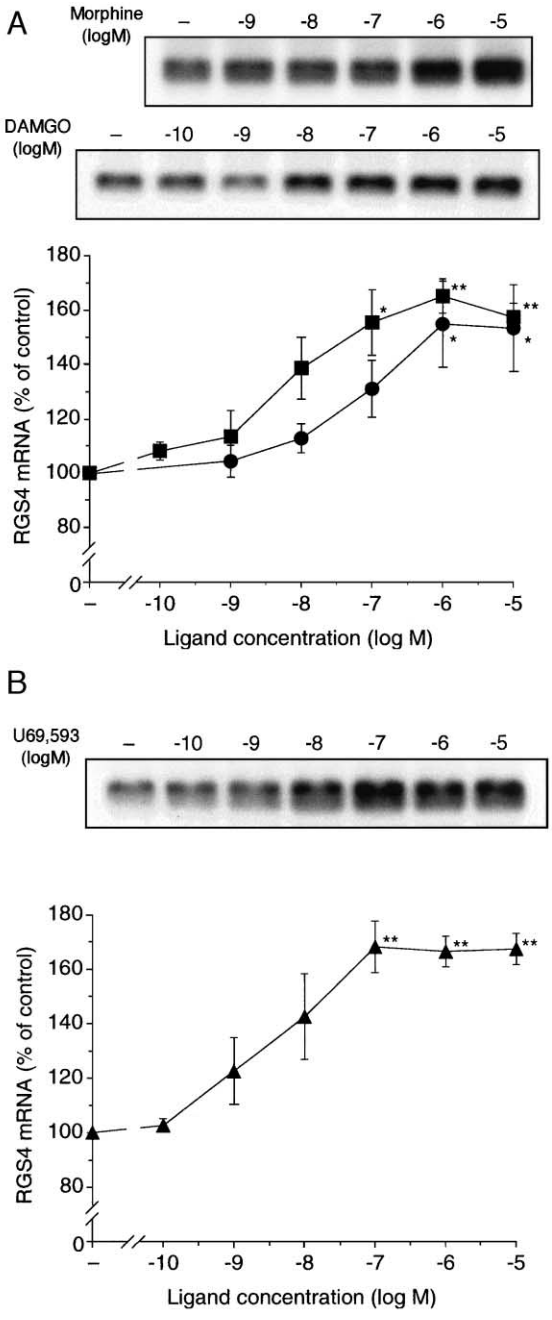

Fig. 2. Dose-dependent up-regulation of RGS4 mRNA induced by opioid receptor agonists. PC12 cells stably expressing the cloned μ-opioid receptors were treated with morphine (●) or DAMGO (■) at the indicated doses for 2 h (A). PC12 cells stably expressing the cloned κ-opioid receptors were treated with U69,593 (▲) at the indicated doses for 2 h (B). The upper panels show representative blots. In the lower panels, the expression of RGS4 mRNA in non-treated cells (control) was assigned a value of 100% and data are presented as means of the percentage of control ± S.E.M. of three to four separate experiments. $*P<0.05$, $**P<0.01$ versus control.

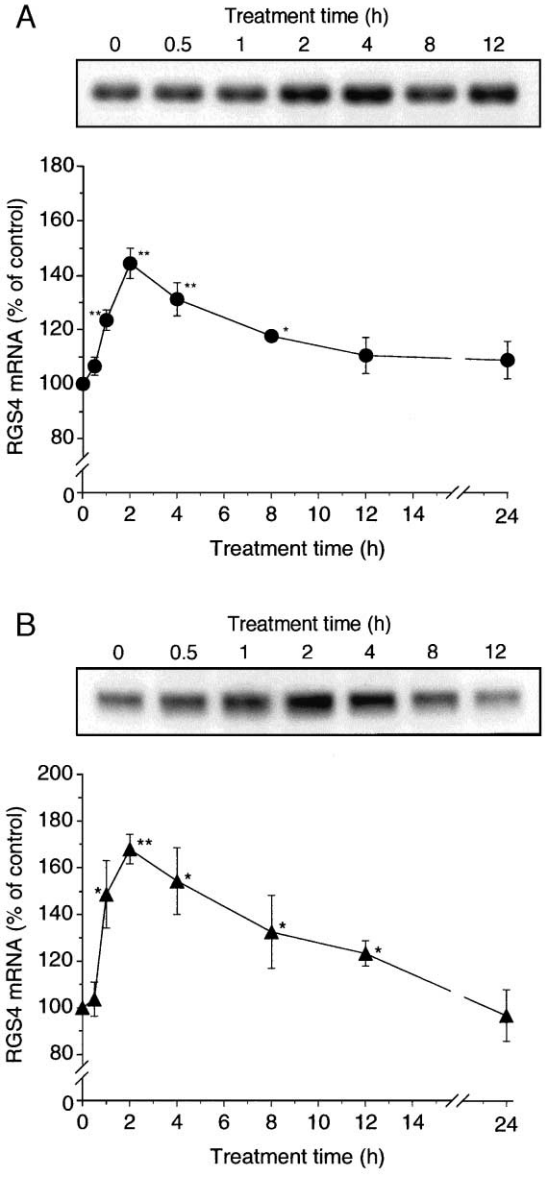

Fig. 3. Time course of up-regulation of RGS4 mRNA induced by opioid receptor agonists. PC12 cells stably expressing the cloned μ-opioid receptors (A) or κ-opioid receptors (B) were treated with morphine (10 μM) or U69,593 (1 μM), respectively, for 0.5–24 h. The upper panels show representative blots. In the lower panels, the expression of RGS4 mRNA in non-treated cells (control) was assigned a value of 100% and data are presented as means of the percentage of control ± S.E.M. of three to five separate experiments. $*P<0.05$, $**P<0.01$ versus control.

relatively rapid and transient peaking when treated for 2 h, and declined finally reaching control level when treated for 24 h (Fig. 3B).

3.3. Effects of opioid antagonists on the up-regulation of RGS4 mRNA by opioid receptor agonists

In PC12 cells expressing the cloned μ-opioid receptor, the up-regulation of RGS4 mRNA by morphine (10 μM)

was significantly antagonized by co-treatment with naloxone (1 μM), although treatment with naloxone alone had no effect on the expression of RGS4 mRNA (Fig. 4A). Similarly, in PC12 cells expressing the cloned κ-opioid receptor, the up-regulation by U69,593 (1 μM) was significantly antagonized by co-treatment with norbinaltorphimine (1 μM), although treatment with norbinaltorphimine alone had no effect (Fig. 4B).

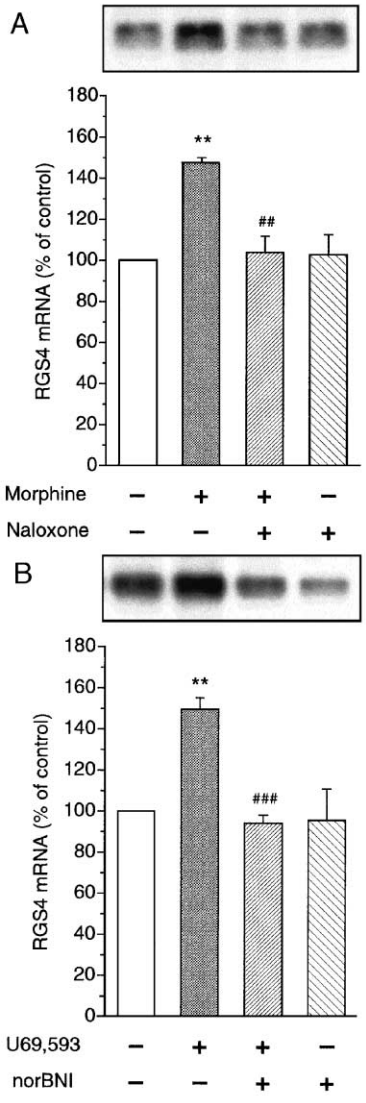

Fig. 4. Effects of opioid antagonists on the up-regulation of RGS4 mRNA induced by opioid receptor agonists. PC12 cells stably expressing the cloned μ-opioid receptors were treated with morphine (10 μM) for 2 h in the presence or absence of naloxone (1 μM) (A). PC12 cells stably expressing the cloned κ-opioid receptors were treated with U69,593 (1 μM) for 2 h in the presence or absence of norbinaltorphimine (1 μM) (B). The upper panels show representative blots. In the lower panels, the expression of RGS4 mRNA in non-treated cells (control) was assigned a value of 100% and data are presented as means of the percentage of control ± S.E.M. of three to four separate experiments. $^{**}P<0.01$ versus control. $^{\#\#}P<0.01$ versus treatment with morphine alone. $^{\#\#\#}P<0.001$ versus treatment with U69,593 alone.

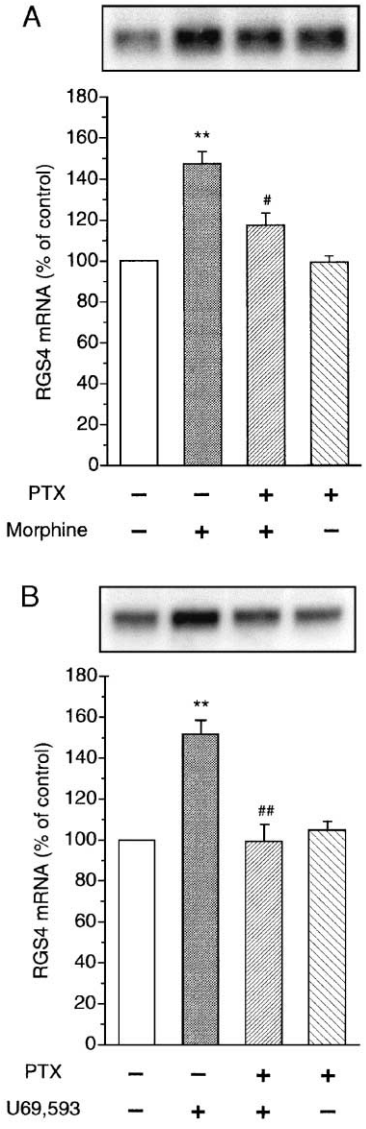

Fig. 5. Effects of pertussis toxin on the up-regulation of RGS4 mRNA induced by opioid receptor agonists. PC12 cells stably expressing the cloned μ-opioid receptors (A) or κ-opioid receptors (B) were treated with morphine (10 μM) or U69,593 (1 μM) for 2h. Pertussis toxin (20 ng/ml) was added to the growth medium 14 h previously and was present during the 2 h of agonist treatment. The upper panels show representative blots. In the lower panels, the expression of RGS4 mRNA in non-treated cells (control) was assigned a value of 100% and data are presented as means of the percentage of control ± S.E.M. of three to four separate experiments. $^{**}P<0.01$ versus control. $^{\#}P<0.05$ versus treatment with morphine alone. $^{\#\#}P<0.01$ versus treatment with U69,593 alone.

3.4. Effects of pertussis toxin on the up-regulation of RGS4 mRNA by opioid receptor agonists

Pretreatment of the PC12 cells expressing the cloned μ-opioid receptor with pertussis toxin (20 ng/ml for 16 h) significantly blocked the up-regulation of RGS4 mRNA by morphine (10 μM), although pretreatment with pertussis toxin alone had no effect on the expression of RGS4 mRNA

(Fig. 5A). Similarly, the up-regulation of RGS4 mRNA by U69,593 (1 μM) in PC12 cells expressing the cloned κ-opioid receptor was significantly blocked by pretreatment with pertussis toxin (Fig. 5B).

4. Discussion

To examine the involvement of RGS proteins in alterations of the opioid receptor mediated-signal transduction system by continuous agonist stimulation, in this study, we established PC12 cells stably expressing either cloned μ- or κ-opioid receptors. In preliminary experiments, we found that undifferentiated PC12 cells dominantly expressed RGS4, rather than RGS2, 7, 8 or 10. It has been shown that RGS4 is predominantly expressed in the brain (Gold et al., 1997) and is one of the best characterized RGS proteins (Tesmer et al., 1997; Yan et al., 1997; Srinivasa et al., 1998; Druey et al., 1998). In this study, therefore, we focused on endogenous RGS4 expressed in PC12 cells as a representative RGS protein.

Several studies have indicated that PC12 cells also endogenously express μ-, κ- or δ-opioid receptors and that opioid receptor agonists affected PC12 cell function, although the results were controversial. For example, Margioris et al. (1995) reported that κ-opioid receptor agonists inhibited the secretion of catecholamines, proliferation and differentiation in their PC12 cell lines. In one PC12 subclone, the levels of δ-opioid receptors markedly increased in response to nerve growth factor (Inoue and Hatanaka, 1982; Abood and Tao, 1995). On the other hand, another PC12 subclone showed low levels of μ-opioid receptor mRNA, although δ- and κ-opioid receptor mRNA expression were not detected by RT-PCR (Yoshikawa et al., 2000). Thus, the expression levels and characteristics of opioid receptors in PC12 cells seem to vary with the PC12 cell lines used (Yoshikawa et al., 2000). In this study, however, treatment of our undifferentiated PC12 cells with various opioid receptor agonists had no effect on the expression of RGS4 mRNA, suggesting that stimulation of endogenous opioid receptors that might be expressed in non-transfected PC12 cells is insufficient to regulate the expression of RGS4 mRNA. Indeed, we could not detect the high affinity binding of [^{3}H]DAMGO or [^{3}H]U69,593 in the membrane fraction from non-transfected PC12 cells (our unpublished data).

In the present study, we found that continuous treatment with μ- or κ-opioid receptor agonists for 0.5–2.4 h increased the expression of RGS4 mRNA in PC12 cells stably expressing either cloned μ- or κ-opioid receptors, respectively. The up-regulation of RGS4 mRNA by opioid receptor agonists occurred in a concentration-dependent manner, and was antagonist- and pertussis toxin-sensitive. These results suggest that the up-regulation was mediated via activation of μ- or κ-opioid receptors and subsequent activation of pertussis toxin-sensitive $G_{i/o}$-like G proteins. The time course of up-regulation of RGS4 mRNA was relatively rapid and transient, peaking when treated for 2 h and disappearing when treated for 12–24 h. It has been shown that desensitization, phosphorylation and internalization of opioid receptors by continuous agonist treatment occurred within several minutes and reached a maximum within several hours (approximately 2 h) (Blake et al., 1997; Hasbi et al., 1998; Koch et al., 1998). In addition, a similar time course has been reported in the supersensitization of the adenylyl cyclase system (cAMP overshoot) induced by continuous opioid receptor agonist treatment (Avidor-Reiss et al., 1995, 1996; Nakagawa et al., 1999). Thus, the time courses of these phenomena seem to be correlated with that of up-regulation of RGS4 mRNA.

It has been reported that the expression of RGS mRNAs can be regulated by a variety of factors (De Vries et al., 2000). For example, the electroconvulsive seizure, which was used as a stimulus to evoke neuronal plasticity, rapidly and transiently up-regulated RGS2 mRNA in the rat hippocampus, cortex, and striatum (Ingi et al., 1998), while it down-regulated RGS10 mRNA in the rat parietal neocortex and dentate gyrus granule cell layer (Gold et al., 1997). A single injection of amphetamine induced RGS2, 3, 5 and 8, but not RGS4 mRNAs in the caudate putamen (Burchett et al., 1998). In addition, the expression of RGS2 mRNA, but not RGS3, 10 or 12 or G alpha interacting protein (GAIP) mRNAs, was rapidly increased by angiotensin II and a protein kinase C activator in cultured vascular smooth muscle cells (Grant et al., 2000). RGS2 mRNA was up-regulated in response to a calcium ionophore, ionomycin, in human lymphocytes (Siderovski et al., 1990) and human blood mononuclear cells (Heximer et al., 1997), but not in PC12 cells (Pepperl et al., 1998). These findings suggest that the RGS mRNAs are regulated by a variety of factors, although the regulation seems to be different depending on RGS subtype, stimuli used and cells examined. On the other hand, in undifferentiated PC12 cells, treatment with forskolin, cAMP analogues, or a G_s-coupled adenosine receptor agonist for 3 h has been reported to down-regulate RGS4 mRNA by nearly 50% (Pepperl et al., 1998), which strongly suggests that RGS4 expression is modulated by the cAMP signaling pathway. Stimulation of opioid receptors leads to inhibition of adenylyl cyclase activity via $G_{i/o}$-family G proteins. Indeed, we observed that treatment of opioid receptor agonists markedly inhibited forskolin-stimulated intracellular cAMP accumulation in PC12 cells expressing cloned μ- or κ-opioid receptors (our unpublished data). Taken together, these findings suggest that the up-regulation of RGS4 by opioid receptor agonists might be due to inhibition of the cAMP signaling pathway.

The RGS proteins are a family of proteins that have been shown to be negative regulators of G protein α subunit signaling GTPase activity. RGS4 has also been shown to increase GTPase activity of α_i, α_o and α_q, and to inhibit signal transduction via $G_{i/o}$- and G_q-coupled receptors including opioid receptors (Watson et al., 1996; Huang et al., 1997; Hepler et al., 1997; Potenza et al., 1999). We also

observed that overexpression of RGS4 slightly but significantly attenuated the inhibitory effects of morphine on forskolin-stimulated intracellular cAMP accumulation in HEK293 cells stably expressing the cloned μ-opioid receptors (our unpublished data). Taken together, these findings suggest that up-regulation of RGS4 by opioid receptor agonists might play a role in the desensitization of opioid receptors. More recently, Garzón et al. (2001) investigated the involvement of RGS proteins in morphine antinociception and tolerance in vivo. They found that knock down of RGS7, 9, 12, 14, 16 and RGS4 by respective antisense oligodeoxynucleotides brought about increases in the potency and, in particular, duration of morphine antinociception, while that of RGS2 and 3 attenuated them in mice. Furthermore, in mice impaired in RGS9, but not RGS3, morphine tolerance was markedly diminished. These findings suggest that RGS proteins including RGS4 might modulate morphine antinociception and tolerance in vivo, as well as receptor desensitization in vitro.

In summary, the expression of RGS4 mRNA was up-regulated by opioid receptor agonists in PC12 cells expressing either cloned μ- or κ-opioid receptors. These results suggest that the up-regulation of RGS4 might be involved in alterations of signal transduction occurring after activation of opioid receptors, such as desensitization of opioid receptors.

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References

Abood, M.E., Tao, Q., 1995. Characterization of a delta opioid receptor in rat pheochromocytoma cells. J. Pharmacol. Exp. Ther. 274, 1566–1573.

Avidor-Reiss, T., Bayewitch, M., Levy, R., Matus-Leibovitch, N., Nevo, I., Vogel, Z., 1995. Adenyclyclase supersensitization in μ-opioid receptor-transfected Chinese hamster ovary cells following chronic opioid treatment. J. Biol. Chem. 270, 29732–29738.

Avidor-Reiss, T., Nevo, I., Levy, R., Pfeuffer, T., Vogel, Z., 1996. Chronic opioid treatment induces adenylyl cyclase V supersensitization: involvement of Gβγ. J. Biol. Chem. 271, 21309–21315.

Berman, D.M., Wilkie, T.M., Gilman, A.G., 1996. GAIP and RGS4 are GTPase-activating proteins for the G_i subfamily of G protein α subunits. Cell 86, 445–452.

Blake, A.D., Bot, G., Freeman, J.C., Reisine, T., 1997. Differential opioid receptor agonist regulation of the mouse μ opioid receptor. J. Biol. Chem. 272, 782–790.

Burchett, S.A., Volk, M.L., Bannon, M.J., Granneman, J.G., 1998. Regulators of G protein signaling: Rapid changes in mRNA abundance in response to amphetamine. J. Neurochem. 70, 2216–2219.

De Vries, L., Mousli, M., Wurmser, A., Farquhar, M.G., 1995. GAIP, a protein that specifically interacts with the trimeric G protein $G\alpha_{i3}$, is a member of a protein family with a highly conserved core domain. Proc. Natl. Acad. Sci. U.S.A. 92, 11916–11920.

De Vries, L., Zheng, B., Fischer, T., Elenko, E., Farquhar, M.G., 2000. The regulator of G protein signaling family. Annu. Rev. Pharmacol. Toxicol. 40, 235–271.

Dohlman, H.G., Thorner, J., 1997. RGS proteins and signaling by heterotrimeric G proteins. J. Biol. Chem. 272, 3871–3874.

Druey, K.M., Blumer, K.J., Kang, V.H., Kehrl, J.H., 1996. Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family. Nature 379, 742–746.

Druey, K.M., Sullivan, B.M., Brown, D., Fischer, E.R., Watson, N., Blumer, K.J., Gerfen, C.R., Scheschonka, A., Kehrl, J.H., 1998. Expression of GTPase-deficient $G_{i\alpha 2}$ results in translocation of cytoplasmic RGS4 to the plasma membrane. J. Biol. Chem. 273, 18405–18410.

Garzón, J., Rodriguez-Diaz, M., López-Fando, A., Sánchez-Blázquez, P., 2001. RGS9 proteins facilitate acute tolerance to mu-opioid effects. Eur. J. Neurosci. 13, 801–811.

Gold, S.J., Ni, Y.G., Dohlman, H.G., Nestler, E.J., 1997. Regulation of G-protein signaling (RGS) proteins: Region-specific expression of nine subtypes in rat brain. J. Neurosci. 17, 2024–2037.

Grant, S.L., Lasségue, B., Griendling, K.K., Ushio-Fukai, M., Lyons, P.R., Alexander, R.W., 2000. Specific regulation of RGS2 messenger RNA by angiotensin II in cultured vascular smooth muscle cells. Mol. Pharmacol. 57, 460–467.

Hasbi, A., Polastron, J., Allochue, S., Stanasila, L., Massotte, D., Jauzac, P., 1998. Desensitization of the d-opioid receptor correlates with its phosphorylation in SK-N-BE cell: involvement of a G protein-coupled receptor kinase. J. Neurochem. 70, 2129–2138.

Hausdorff, W.P., Caron, M.G., Lefkowitz, R.J., 1990. Turning off the signal: desensitization of β-adrenergic receptor function. FASEB J. 4, 2881–2889.

Hepler, J.R., 1999. Emerging roles for RGS proteins in cell signaling. Trends Pharmacol. Sci. 20, 376–382.

Hepler, J.R., Berman, D.M., Gilman, A.G., Kozasa, T., 1997. RGS4 and GAIP are GTPase-activating proteins for $G_{q\alpha}$ and block activation of phospholipase Cβ by γ-thio-GTP-$G_{q\alpha}$. Proc. Natl. Acad. Sci. U. S. A. 94, 428–432.

Heximer, S.P., Cristillo, A.D., Forsdyke, D.R., 1997. Comparison of mRNA expression of two regulators of G-protein signaling, RGS1/BL34/1R20 and RGS2/G0S8, in cultured human blood mononuclear cells. DNA Cell Biol. 16, 589–598.

Huang, C., Hepler, J.R., Gilman, A.G., Mumby, S.M., 1997. Attenuation of G_i- and G_q-mediated signaling by expression of RGS4 or GAIP in mammalian cells. Proc. Natl. Acad. Sci. 94, 6159–6163.

Hunt, T.W., Fields, T.A., Casey, P.J., Peralta, E.G., 1996. RGS10 is a selective activator of $G\alpha_i$ GTPase activity. Nature 383, 175–177.

Ingi, T., Krumins, A.M., Chidiac, P., Brother, G.M., Chung, S., Snow, B.E., Barnes, C.A., Lanahan, A.A., Siderovski, D.P., Ross, E.M., Gilman, A.G., Worley, P.F., 1998. Dynamic regulation of RGS2 suggests a novel mechanism in G-protein signaling and neuronal plasticity. J. Neurosci. 18, 7178–7188.

Inoue, N., Hatanaka, H., 1982. Nerve growth factor induces specific enkephalin binding sites in a nerve cell lines. J. Biol. Chem. 257, 9238–9241.

Kobilka, B., 1992. Adrenergic receptors as model for G protein-coupled receptors. Annu. Rev. Neurosci. 15, 87–114.

Koch, T., Schulz, S., Schröder, H., Wolf, R., Raulf, E., Höllt, V., 1998. Carboxyl-terminal splicing of the rat μ opioid receptor modulates agonist-mediated internalization and receptor resensitization. J. Biol. Chem. 273, 13652–13657.

Koelle, M.R., Horvitz, H.R., 1996. EGL-10 regulates G protein signaling in the C. elegans nervous system and shares a conserved domain with many mammalian proteins. Cell 84, 115–125.

Krupnick, J.G., Benovic, J.L., 1998. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu. Rev. Pharmacol. Toxicol. 38, 289–319.

Mansour, A., Fox, C.A., Akil, H., Watson, S.J., 1995. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci. 18, 22–29.

Margioris, A.N., Venihaki, M., Stournaras, C., Gravanis, A., 1995. PC12 cells as a model to study the effects of opioids on normal and tumoral adrenal chromaffin cells. Ann. N.Y. Acad. Sci. 771, 166–172.

Minami, M., Satoh, M., 1995. Molecular biology of the opioid receptors: structures, functions and distributions. Neurosci. Res. 23, 121–145.

Minami, M., Toya, T., Katao, Y., Maekawa, K., Nakamura, S., Onogi, T., Kaneko, S., Satoh, M., 1993. Cloning and expression of a cDNA for the rat kappa-opioid receptor. FEBS Lett. 329, 291–295.

Minami, M., Onogi, T., Toya, T., Katao, Y., Hosoi, Y., Maekawa, K., Katsumata, S., Yabuuchi, K., Satoh, M., 1994. Molecular cloning and in situ hybridization histochemistry for rat μ-opioid receptor. Neurosci. Res. 18, 315–322.

Nakagawa, T., Ozawa, T., Watanabe, T., Minami, M., Satoh, M., 1999. Sensitization of the adenylyl cyclase system in cloned κ-opioid receptor-transfected cells following sustained agonist treatment: a chimeric study using G protein α_{i2}/α_q subunits. Jpn. J. Pharmacol. 81, 353–361.

Nestler, E.J., 1992. Molecular mechanism of drug addiction. J. Neurosci. 12, 2439–2450.

Nestler, E.J., Aghajanian, G.K., 1997. Molecular and cellular basis of addiction. Science 278, 58–63.

Pasternak, G.W., 1988. Multiple morphine and enkephalin receptors and the relief of pain. JAMA 259, 1362–1367.

Pepperl, D.J., Shah-Basu, S., VanLeeuwen, D., Granneman, J.G., MacKenzie, R.G., 1998. Regulation of RGS mRNAs by cAMP in PC12 cells. Biochem. Biophys. Res. Commun. 243, 52–55.

Potenza, M.N., Gold, S.J., Roby-Shemkowitz, A., Lerner, M.R., Nestler, E.J., 1999. Effects of regulators of G protein-signaling proteins on the fuctional response of the μ-opioid receptor in a melanophore-based assay. J. Pharmacol. Exp. Ther. 291, 482–491.

Satoh, M., Minami, M., 1995. Molecular pharmacology of the opioid receptors. Pharmac. Ther. 68, 343–364.

Siderovski, D.P., Blum, S., Forsdyke, R.E., Forsdyke, D.R., 1990. A set of human putative lymphocyte G0/G1 switch genes includes genes homologous to rodent cytokine and zinc finger protein-encoding genes. DNA Cell Biol. 9, 579–587.

Srinivasa, S.P., Watson, N., Overton, M.C., Blumer, K.J., 1998. Mechanism of RGS4, a GTPase-activating protein for G protein α subunits. J. Biol. Chem. 273, 1529–1533.

Tesmer, J.J.G., Berman, D.M., Gilman, A.G., Sprang, S.R., 1997. Structure of RGS4 bound to AlF_4^- activated $G_{i\alpha 1}$: stabilization of the transition state for GTP hydrolysis. Cell 89, 251–261.

Watson, N., Linder, M.E., Druey, K.M., Kehrl, J.H., Blumer, K.J., 1996. RGS family members. GTPase-activating proteins for heterotrimeric G-protein α-subunits. Nature 383, 172–175.

Yan, Y., Chi, P.P., Bourne, H.R., 1997. RGS4 inhibits G_q-mediated activation of mitogen-activated protein kinase and phosphoinositide synthesis. J. Biol. Chem. 272, 11924–11927.

Yoshikawa, M., Nakayama, H., Ueno, S., Hirano, M., Hatanaka, H., Furuya, H., 2000. Chronic fentanyl treatments induce the up-regulation of μ-opioid receptor mRNA in rat pheochromocytoma cells. Brain Res. 59, 217–223.