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Adaptations to chronic agonist exposure of μ-opioid receptor-expressing Chinese hamster ovary cells

Shigehisa Kato, Kazuhiko Fukuda *, Hitoshi Morikawa, Takehiro Shoda, Hiroyuki Mima, Kenjiro Mori

Department of Anesthesia, Kyoto University Hospital, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-01, Japan

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Abstract

To investigate cellular adaptation responses induced by chronic agonist treatment of the μ-opioid receptor, Chinese hamster ovary (CHO) cells were stably transfected with the rat μ-opioid receptor cDNA. Chronic treatment with agonists selective for the μ-opioid receptor, [D-Ala^2, *N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO), morphine and fentanyl, time- and dose-dependently induced down-regulation of the μ-opioid receptor. The down-regulation was not significantly affected by pretreatment with pertussis toxin, but was completely blocked by treatment with hypertonic sucrose, suggesting that receptor internalization mediated by clathrin-coated vesicles is an essential step in the μ-opioid receptor down-regulation. On the other hand, forskolin-stimulated cyclic AMP formation was increased by chronic DAMGO treatment, which was inhibited by pertussis toxin pretreatment. These results indicate that two adaptation responses induced by chronic agonist treatment of the μ-opioid receptor-expressing CHO cells, down-regulation of the μ-opioid receptor and supersensitization of adenylate cyclase, are mediated by distinct mechanisms. © 1998 Elsevier Science B.V.

Keywords: μ-Opioid receptor; Down-regulation; Internalization; cAMP; Supersensitization; CHO (Chinese hamster ovary) cell

1. Introduction

The opioid receptor, pharmacologically classified into at least three types (μ, δ and κ), mediates pharmacological actions of opioid analgesics and physiological effects of endogenous opioid peptides through the action of pertussis toxin-sensitive guanine nucleotide-binding regulatory proteins (G-proteins) (Loh and Smith, 1990). Most of the opioids clinically used, such as morphine and fentanyl, produce a potent analgesic effect by selectively interacting with the μ-opioid receptor (Matthes et al., 1996; Sora et al., 1997). However, the prolonged use of opioids is limited by the development of tolerance, dependence and addiction. A number of studies have indicated that development of opioid tolerance and dependence is based on adaptations in opioid receptor-expressing neurons caused by repeated exposure to opioids (Nestler, 1992). Adaptation responses induced by prolonged opioid exposure on the μ-opioid receptor have been analyzed using human neuroblastoma SH-SY5Y cells and mouse 7315c pituitary tumor cells, which endogenously express the μ-opioid receptor (Puttfarcken et al., 1988; Puttfarcken and Cox, 1989; Yu et al., 1990; Ammer and Schulz, 1993). Furthermore, recent cloning of the μ-opioid receptor cDNA has enabled the study using heterologous expression systems (Arden et al., 1995; Avidor-Reiss et al., 1995a,b, 1996). However, molecular mechanisms underlying opioid tolerance and dependence remain poorly understood.

In the present study, to gain further insight into the molecular mechanisms underlying opioid tolerance and dependence, we have analyzed cellular adaptation responses induced by chronic agonist exposure of the cloned rat μ-opioid receptor expressed in Chinese hamster ovary (CHO) cells. The results obtained indicate that two adaptation responses induced simultaneously by chronic treatment of the μ-opioid receptor-expressing CHO cells, down-regulation of the μ-opioid receptor and supersensitization of adenylate cyclase, are mediated by the pertussis toxin-insensitive and sensitive mechanism, respectively.

* Corresponding author. Fax: +81-75-752-3259.

2. Materials and methods

2.1. Materials

[^{3}H][D-Ala^2, *N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO) (55.0 or 48.9 Ci/mmol) and [^{3}H]naloxone (57.5 Ci/mmol) were purchased from DuPont NEN (Boston, MA, USA). [α-^{32}P]dCTP (~3000 Ci/mmol) was obtained from Amersham (Amersham, UK). The cyclic AMP (cAMP) radioimmunoassay kit was from Yamasa (Tokyo, Japan). Culture medium was purchased from GIBCO (Grand Island, NY, USA), and calf serum was from HyClone (Logan, UT, USA). DAMGO was obtained from Peninsula Laboratories (Belmont, CA, USA). Pertussis toxin was from Funakoshi (Tokyo, Japan). Morphine was from Takeda (Osaka, Japan), and fentanyl was from Sankyo (Tokyo, Japan). All the other reagents were obtained from Wako (Osaka, Japan), Nacalai Tesque (Kyoto, Japan) and Sigma (St. Louis, MO, USA).

2.2. Cell culture and cDNA expression

CHO cells, maintained in minimum essential medium alpha (MEMα) containing deoxyribonucleosides and ribonucleosides supplemented with 6% calf serum, streptomycin (50 μg/ml) and penicillin (50 u/ml), were transfected with the expression plasmid pRORS15-1 (Fukuda et al., 1993), which contains the entire protein-coding region of the rat μ-opioid receptor cDNA and the neomycin-resistant marker gene, by the calcium phosphate method (Sambrook et al., 1989). Clones stably expressing the μ-opioid receptor were isolated by screening G418-resistant clones by RNA blot hybridization analysis.

2.3. Preparation of crude membrane and intact cells for binding assay

Cells were seeded in 10-cm culture dishes at $3–5 \times 10^6$ cells/dish. After 36–48-h incubation at 37°C, when growth reached 80–100% confluence, cells were treated with opioid ligands for indicated times at 37°C.

After treatment with ligands, crude membrane was prepared as follows. Cells were washed three times with phosphate-buffered saline (PBS), harvested and stored at −80°C until membrane preparation. Frozen cells were thawed, homogenized with a Dounce homogenizer in 50 mM Tris–HCl, pH 7.5, and centrifuged at $1000 \times g$ for 10 min. The supernatant was 6-fold diluted with the same buffer, incubated at room temperature for 30 min, and centrifuged at $20\,000 \times g$ for 30 min. The pellet was washed twice and suspended in the same buffer for binding assay. Protein concentration of the membrane preparation was determined by the method of Bradford (Bradford, 1976), using bovine serum albumin as the standard. Ligand binding activity of the membrane from cells treated with 1 μM DAMGO or morphine for 1 min before harvesting showed no significant difference compared with that of the membrane from non-treated cells (data not shown), indicating that our procedure for membrane preparation is sufficient to remove residual ligands.

For binding assay using intact cells, agonist-treated cells were washed three times with PBS, harvested and suspended in 150 mM Tris–HCl, pH 7.5. To prevent intracellular redistribution of the receptor protein, all procedures were carried out on ice. The viability of the cells suspended in 150 mM Tris–HCl, pH 7.5, assessed by trypan blue exclusion, was higher than 90%, which was affected little by binding reaction on ice for 1 h.

2.4. Radioligand binding assay

Crude membrane (~10 μg of protein) in 0.2 ml of 50 mM Tris–HCl, pH 7.5 was incubated at 37°C for 1 h in the presence of [^{3}H]DAMGO or [^{3}H]naloxone. Intact cells ($3–5 \times 10^5$) suspended in 0.2 ml of 150 mM Tris–HCl, pH 7.5 were incubated on ice for 1 h in the presence of 10 nM [^{3}H]DAMGO. After incubation, the samples were collected on Whatman GF/B filters and washed twice with 10 ml of ice-cold 50 mM Tris–HCl, pH 7.5. Radioactivity on the filters was determined by liquid scintillation counting after soaking in scintillation cocktail for more than 12 h. The nonspecific binding was determined as [^{3}H]DAMGO or [^{3}H]naloxone binding in the presence of 500 μM naloxone. All determinations were performed in duplicate.

2.5. RNA blot hybridization analysis

Total cellular RNAs prepared from the cells by the acid guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987) were analyzed as described previously (Fukuda et al., 1993). The hybridization probe used was the 1.4-kb *Eco*RI/*Hin*dIII fragment from the plasmid pROR15 (Fukuda et al., 1993). The probe was labelled with [α-^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983). Autoradiography was performed at −80°C with an intensifying screen for 24 h.

2.6. cAMP accumulation assay

Cells were washed three times with PBS, harvested and suspended in MEMα. Cells (1×10^6) in 0.5 ml of MEMα were pretreated with 1 mM isobutylmethylxanthine for 10 min at room temperature, and then stimulated with 10 μM forskolin in the presence or absence of DAMGO. After incubation at 37°C for 40 min, 0.25 ml of 30% trichloroacetic acid was added and the cells were centrifuged at $20\,000 \times g$ for 5 min. The supernatants were extracted with diethylether and subjected to measurement of cAMP concentration by radioimmunoassay.

2.7. Data analysis

Data are presented as means ± S.E.M. Statistical difference of the means was analyzed by analysis of variance (ANOVA) followed by Fisher's protected least significance difference test or Student's *t*-test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Stable expression of the μ-opioid receptor in CHO cells

From the clones of CHO cells expressing the rat μ-opioid receptor, a clone, CROR-B22, was selected for further experiments. Scatchard analysis using crude membrane preparation with [^{3}H]DAMGO, a peptide agonist selective for the μ-opioid receptor, demonstrated that the μ-opioid receptor is expressed in CROR-B22 cells at the level of 8.0 ± 0.8 pmol/mg protein ($n = 4$) with the K_d value of 1.5 ± 0.2 nM ($n = 4$), which is comparable with the reported value for the μ-opioid receptor expressed in brain (Leslie, 1987). In CROR-B22 cells, DAMGO dose-dependently induced inhibition of cAMP accumulation elicited by 10 μM forskolin for 40 min at 37°C; the maximal inhibition and the EC_{50} value were 66 ± 7% ($n = 4$) and 15 ± 6 nM ($n = 4$), respectively. Pretreatment with 100 ng/ml pertussis toxin for 20 h completely suppressed the DAMGO-induced inhibition of cAMP accumulation, indicating that the μ-opioid receptor expressed in CHO cells is negatively coupled with adenylate cyclase through the action of the pertussis toxin-sensitive G-protein (G_i and/or G_o), similarly as the μ-opioid receptor expressed in nervous tissues (Loh and Smith, 1990).

3.2. Down-regulation of the μ-opioid receptor induced by chronic treatment with opioid agonists

Agonist-induced change in the ligand binding activity of the μ-opioid receptor was analyzed by the [^{3}H]DAMGO binding assay using crude membrane preparations from CROR-B22 cells treated with opioid agonists. Fig. 1A shows that treatment with 1 μM DAMGO time-dependently reduced the specific [^{3}H]DAMGO binding activity in membrane preparation, with a maximal decrease [58 ± 3% ($n = 4$) of the value from non-treated control cells] reached within 4 h of the treatment. As shown in Fig. 1B, the decrease in binding activity was dependent on DAMGO concentration with the EC_{50} value of ~100 nM. Treatment of CROR-B22 cells with morphine and fentanyl, non-peptide agonists selective for the μ-opioid receptor, also dose-dependently induced reduction in [^{3}H]DAMGO

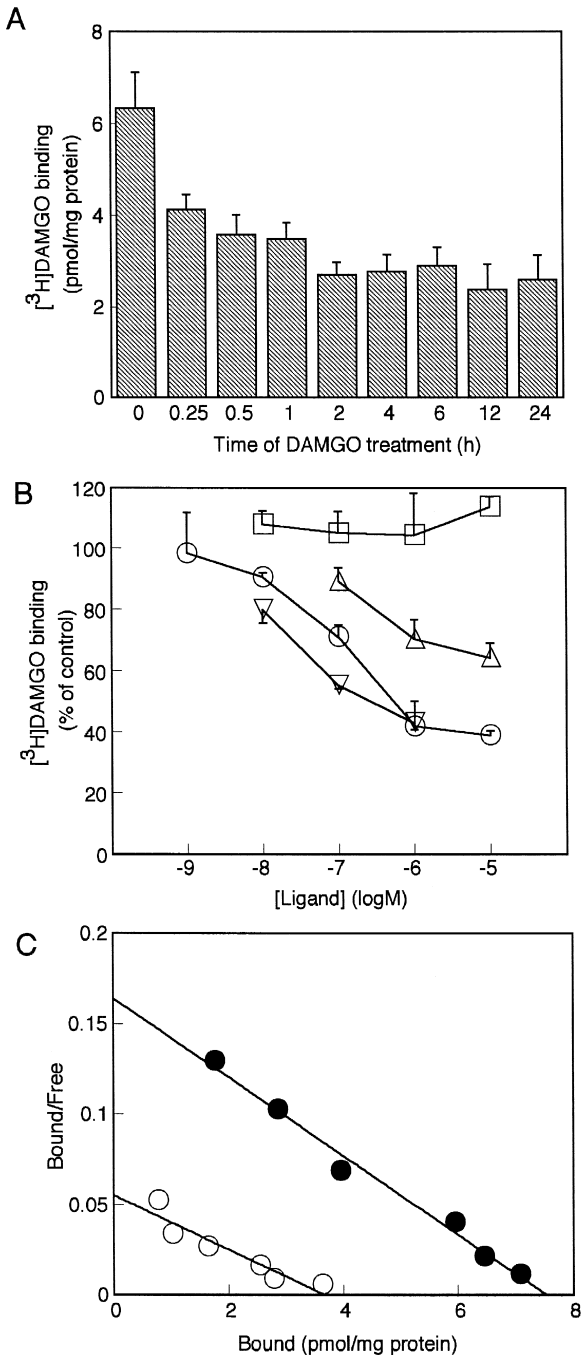

Fig. 1. Effect of chronic treatment with opioid agonists on [^{3}H]DAMGO binding activity in membrane preparation from CROR-B22 cells. (A) Time course of the decrease in [^{3}H]DAMGO binding activity after exposure to 1 μM DAMGO. The values represent mean ± S.E.M. of four separate experiments. (B) Dose-dependent change in [^{3}H]DAMGO binding activity induced by chronic treatment with opioid ligands. CROR-B22 cells were treated with DAMGO (○), morphine (△), fentanyl (▽) and naloxone (□) for 6 h at 37°C. The values are shown as percentage of the value from the non-treated control cells, and represent mean ± S.E.M. of three separate experiments. (C) Scatchard analysis of [^{3}H]DAMGO binding to membrane preparations from non-treated CROR-B22 cells (●) and CROR-B22 cells treated for 6 h at 37°C with 1 μM DAMGO (○). Nonspecific binding of [^{3}H]DAMGO was assessed in the presence of 500 μM naloxone. Representative results from four separate experiments are shown. K_d and B_{max} values from each experiment were obtained by linear regression analysis of Scatchard plots, assuming a single site model, and averaged to produce mean values.

binding activity. Naloxone, a nonselective opioid antagonist, at 1 μM completely blocked the decrease in ligand binding activity induced by 1 μM DAMGO (data not shown), indicating that the decrease in [^{3}H]DAMGO binding activity is caused by interaction of DAMGO with the μ-opioid receptor. Treatment with naloxone alone induced a slight increase in [^{3}H]DAMGO binding activity (Fig. 1B), which may agree with a previous report that treatment with naloxone induces up-regulation of the μ-opioid receptor in SH-SY5Y cells (Zadina et al., 1993).

Scatchard analysis (Fig. 1C) showed that the B_{max} values for [^{3}H]DAMGO binding of membrane preparations from non-treated control cells and cells treated with 1 μM DAMGO for 6 h were 8.0 ± 0.8 pmol/mg protein ($n = 4$) and 3.3 ± 0.1 pmol/mg protein ($n = 4$), respectively, whereas the K_d values were 1.5 ± 0.2 nM ($n = 4$) and 2.0 ± 0.1 nM ($n = 4$), respectively. These results indicate that the agonist-induced decrease in [^{3}H]DAMGO binding activity of the μ-opioid receptor is mainly caused by the decrease in the receptor number rather than the change in binding affinity. Scatchard analysis using [^{3}H]naloxone also revealed similar decrease in the B_{max} value induced by DAMGO (data not shown). Thus, our results show that chronic agonist treatment induces down-regulation of the μ-opioid receptor expressed in CHO cells.

RNA blot hybridization analysis showed that the amount of RNA species hybridizable with the μ-opioid receptor cDNA probe in total cellular RNA was not significantly changed by treatment with 1 μM DAMGO up to 24 h (Fig. 2). This result suggests that the agonist-induced reduction in [^{3}H]DAMGO binding activity of the μ-opioid receptor in CHO cells is not due to the decrease in the transcription rate of the transfected μ-opioid receptor cDNA. On the other hand, treatment of CROR-B22 cells with 100 μg/ml cycloheximide for 1 h, which inhibits translation and suppresses cellular protein synthesis by more than 90% (Schmidt et al., 1995), did not significantly affect the [^{3}H]DAMGO binding activity in the following 6 h (data not shown). Therefore, it is unlikely that the down-regulation of the μ-opioid receptor in CHO cells is due to the decrease in the translation rate, because the agonist-induced reduction in [^{3}H]DAMGO binding activity was detectable within 15 min of agonist treatment (see Fig. 1). Taken together, it is conceivable that the agonist-induced μ-opioid receptor down-regulation in CHO cells is caused by degradation, rather than decrease in the rate of synthesis of the receptor molecules.

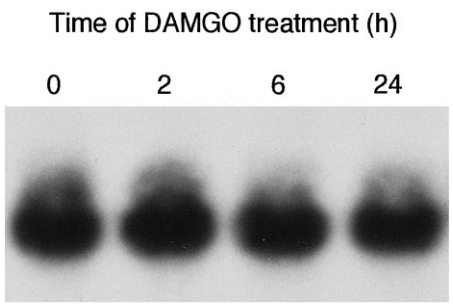

Fig. 2. Autoradiogram showing the effect of chronic DAMGO treatment on the expression level of the μ-opioid receptor messenger RNA in CROR-B22 cells. CROR-B22 cells were treated with 1 μM DAMGO for the indicated times at 37°C. After DAMGO treatment, total cellular RNA was prepared from the cells and subjected to RNA blot hybridization analysis, using the protein-coding region of the rat μ-opioid receptor cDNA as the probe. Representative result from three separate experiments is shown.

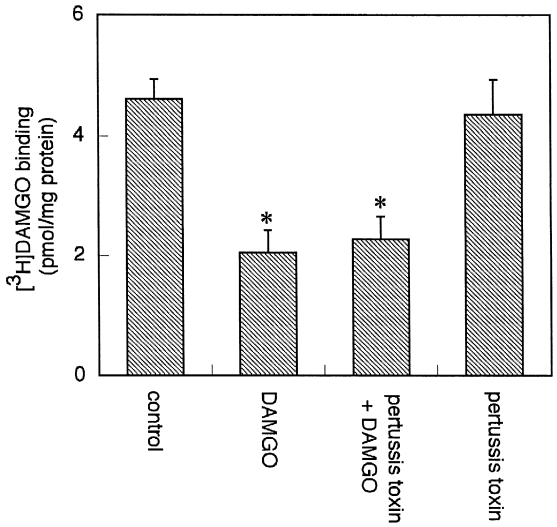

Fig. 3. Effect of pertussis toxin on the DAMGO-induced down-regulation of the μ-opioid receptor expressed in CHO cells. With or without pretreatment with 100 ng/ml pertussis toxin for 20 h at 37°C, CROR-B22 cells were treated with 1 μM DAMGO for 6 h. Crude membrane was prepared and subjected to the binding assay using 10 nM [^{3}H]DAMGO. The values represent mean ± S.E.M. of three separate experiments. $* P < 0.05$ compared with the control value (ANOVA, Fisher's protected least significance difference test).

As shown in Fig. 3, pretreatment with pertussis toxin (100 ng/ml, 20 h) did not significantly affect the μ-opioid receptor down-regulation induced by treatment with 1 μM DAMGO for 6 h, although the same pretreatment completely suppressed the DAMGO-induced adenylate cyclase inhibition (see above). This result indicates that activation of pertussis toxin-sensitive G-proteins is not necessary for the agonist-induced down-regulation of the μ-opioid receptor in CHO cells.

[^{3}H]DAMGO binding activity on the cell surface was time-dependently reduced by treatment with 1 μM DAMGO, which suggests that agonist treatment induces μ-opioid receptor internalization (Fig. 4A). Fig. 4A also demonstrates that the μ-opioid receptor internalization was inhibited by hypertonic sucrose. This suggests that the agonist-induced μ-opioid receptor internalization is mediated by clathrin-coated vesicles, because treatment with hypertonic sucrose was demonstrated by electron microscope to inhibit formation of clathrin-coated vesicles in fibroblasts (Heuser and Anderson, 1989), and agonist-induced internalization of the G-protein-coupled receptors

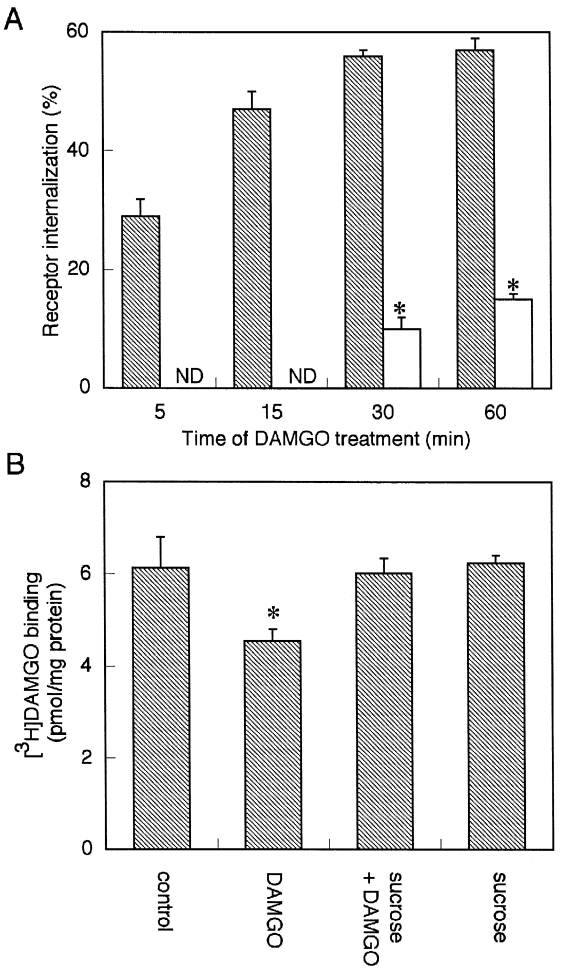

Fig. 4. Agonist-induced internalization and down-regulation of the μ-opioid receptor expressed in CHO cells are suppressed by hypertonic sucrose. (A) Effect of hypertonic sucrose on receptor internalization. CROR-B22 cells were treated with 1 μM DAMGO in the presence (open bars) or absence (hatched bars) of 0.5 M sucrose at 37°C for the indicated times. After agonist treatment, cell surface [^{3}H]DAMGO binding activity was determined using 10 nM [^{3}H]DAMGO. Receptor internalization is defined as agonist-induced decrease in cell surface [^{3}H]DAMGO binding activity, and expressed as percentage of surface [^{3}H]DAMGO binding activity in non-treated control cells. The values represent mean ± S.E.M. of three separate experiments. ND, not determined. $* P < 0.05$ compared with the value obtained in the absence of sucrose (Student's *t*-test). (B) Effect of hypertonic sucrose on receptor down-regulation. CROR-B22 cells were treated with 1 μM DAMGO in the presence or absence of 0.5 M sucrose for 1 h at 37°C. After treatment, crude membrane was prepared and subjected to the binding assay using 10 nM [^{3}H]DAMGO. The values represent mean ± S.E.M. of four separate experiments. $* P <$ 0.05 compared with the control value (ANOVA, Fisher's protected least significance difference test).

via clathrin-dependent mechanism was inhibited by this treatment (Slowiejko et al., 1996; Petrou et al., 1997). As shown in Fig. 4B, the μ-opioid receptor down-regulation induced by treatment with DAMGO for 1 h was completely abolished by hypertonic sucrose. These results together suggest that internalization mediated by clathrin-coated vesicles is an essential step in the agonist-induced down-regulation of the μ-opioid receptor expressed in CHO cells.

3.3. Effects of chronic DAMGO treatment on forskolin-stimulated cAMP accumulation

As described above, acute activation of the μ-opioid receptor induces inhibition of adenylate cyclase via pertussis toxin-sensitive G-proteins, leading to reduction in forskolin-stimulated cAMP accumulation. In order to analyze the effects of chronic μ-opioid receptor-agonist treatment on the adenylate cyclase activity, we next measured forskolin-stimulated cAMP accumulation in the cells

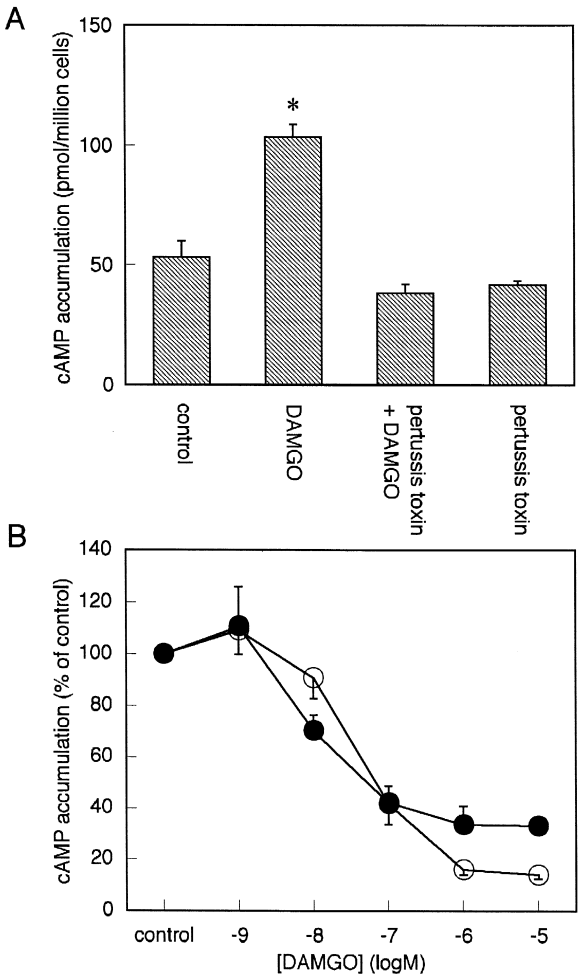

Fig. 5. Effect of DAMGO treatment on forskolin-stimulated cAMP accumulation in CROR-B22 cells. (A) Induction of adenylate cyclase supersensitization following exposure of the cells to DAMGO. With or without pertussis toxin pretreatment (100 ng/ml, 20 h), CROR-B22 cells were treated with 1 μM DAMGO for 6 h, washed three times with PBS and subjected to cAMP accumulation assay using 10 μM forskolin. The values represent mean ± S.E.M. of three separate experiments. $* P < 0.05$ compared with the control (ANOVA, Fisher's protected least significance difference test). (B) Inhibition of forskolin-stimulated cAMP accumulation by various concentration of DAMGO in non-treated control cells (●) and cells treated with 1 μM DAMGO for 6 h (○). The values represent mean ± S.E.M. of four separate experiments.

chronically stimulated with DAMGO. CROR-B22 cells were treated for 6 h with 1 μM DAMGO, and then, after withdrawal of the agonist, stimulated by 10 μM forskolin for 40 min. As shown in Fig. 5A, the chronic DAMGO treatment induced 2.0 ± 0.3-fold ($n = 3$) increase in forskolin-stimulated cAMP accumulation, as compared with control cells not treated with DAMGO, indicating that supersensitization of adenylate cyclase was elicited by chronic agonist treatment of the μ-opioid receptor. The increase in forskolin-stimulated cAMP accumulation was completely blocked by pretreatment of the cells with 100 ng/ml pertussis toxin for 20 h. This suggests that activation of the pertussis toxin-sensitive G-protein (G_i and/or G_o) by the μ-opioid receptor is essential for the adenylate cyclase supersensitization.

It has been reported that chronic agonist exposure on the μ-opioid receptor in 7315c pituitary tumor cells induces a loss of μ-opioid receptor-mediated adenylate cyclase inhibition (desensitization) (Puttfarcken et al., 1988; Puttfarcken and Cox, 1989). To examine whether chronic agonist treatment induces μ-opioid receptor desensitization in CHO cells, we compared the dose-response relationships for DAMGO-induced inhibition of forskolin-stimulated cAMP accumulation, before and after chronic DAMGO treatment. As shown in Fig. 5B, chronic DAMGO treatment for 6 h induced slight rightward shift of the dose-response curve: the EC_{50} values before and after DAMGO treatment were 15 ± 6 nM ($n = 4$) and 47 ± 13 nM ($n = 4$), respectively.

4. Discussion

In the present study, to gain information on the changes of the μ-opioid receptor induced in the opioid addictive processes, we stably expressed the cloned rat μ-opioid receptor in CHO cells, and analyzed cellular adaptation responses induced by chronic agonist treatment of the μ-opioid receptor. The results obtained indicate that chronic agonist treatment of μ-opioid receptor-expressing CHO cells induces down-regulation of the μ-opioid receptor and supersensitization of adenylate cyclase, and further suggest that these two phenomena are mediated by distinct mechanisms.

Acute cellular responses induced by activation of the μ-opioid receptor, including inhibition of adenylate cyclase, activation of mitogen-activated protein kinase, inhibition of voltage-dependent Ca^{2+} channel activity and activation of the inwardly rectifying K^+ channel, are mediated by the pertussis toxin-sensitive G-protein (G_i and/or G_o) (Loh and Smith, 1990; Fukuda et al., 1996). In contrast, our data demonstrate that the μ-opioid receptor down-regulation induced by chronic agonist exposure is not affected by pertussis toxin pretreatment. This suggests that receptor activation and G-protein coupling are not required for the μ-opioid receptor down-regulation, although the possibility cannot be completely excluded that the μ-opioid receptor activates pertussis toxin-insensitive G-proteins, which mediate down-regulation of the opioid receptor. In agreement with our result, it has been reported that agonist-induced down-regulation of the δ-opioid receptor endogenously expressed in NG108-15 cells is not affected by pertussis toxin pretreatment (Law et al., 1985). In contrast, Chakrabarti et al. (1997) has recently reported that the ability of DAMGO to internalize and down-regulate the μ-opioid receptor expressed in $Neuro_{2A}$ cells was completely abolished by pertussis toxin pretreatment. Therefore, mechanism for the opioid receptor down-regulation may be different in different cell types.

Our results indicate that receptor internalization mediated by clathrin-coated vesicles is an essential step of the agonist-induced μ-opioid receptor down-regulation. In agreement with this finding, chronic agonist treatment of NG108-15 cells results in internalization of the endogenously expressed δ-opioid receptor followed by degradation in lysosomes (Law et al., 1984). The pertussis toxin-insensitivity of the agonist-induced μ-opioid receptor down-regulation suggests that the agonist-induced μ-opioid receptor internalization also does not require G-protein coupling. In line with this, it has been reported that internalization of the cholecystokinin receptor expressed in CHO cells is induced not only by agonists but also by antagonists, suggesting that activation of the G-protein is not involved in the internalization process (Roettger et al., 1997). Therefore, it is likely that ligand binding with the G-protein-coupled receptor induces conformational change in the domain of the receptor molecule distinct from the domain necessary for G-protein coupling, leading to interaction of the receptor with molecules involved in receptor internalization. Recently, it has been reported that internalization of the β_2-adrenoreceptor via clathrin-coated vesicles is mediated by β-arrestin and dynamin (Ferguson et al., 1996; Zhang et al., 1996). It is possible that similar proteins are involved in the opioid receptor internalization.

Chronic agonist exposure of the G_i-coupled receptor, including the α_2-adrenoreceptor and the muscarinic M_2 acetylcholine receptor, induces supersensitization of adenylate cyclase activity, leading to an increase in forskolin-stimulated cAMP accumulation (Thomas and Hoffman, 1987). A recent report has demonstrated that adenylate cyclase supersensitization induced by chronic agonist exposure of the μ-opioid receptor expressed in COS-7 cells can be inhibited by coexpression of the carboxy terminus of β-adrenoreceptor kinase or α-transducin (scavengers of the βγ subunits of the G-protein), suggesting the involvement of the βγ subunits of the G-protein in adenylate cyclase supersensitization (Avidor-Reiss et al., 1996). In agreement with this report, our data demonstrate that μ-opioid receptor-agonist-induced adenylate cyclase supersensitization in CHO cells was abolished by pertussis toxin pretreatment, suggesting that receptor activation and coupling with the G-protein are necessary for adenylate cy-

clase supersensitization, in contrast to agonist-induced receptor down-regulation.

Down-regulation of receptors has been known as a cellular adaptation process in response to chronic exposure to agonists in many neurotransmitter systems (Lohse, 1993). However, there have been equivocal reports whether down-regulation of the opioid receptor occurs during development of opioid tolerance in vivo. Chronic infusion of morphine to guinea pigs was reported to induce a decrease in the high-affinity [^{3}H]DAMGO binding sites (Werling et al., 1989), whereas an increase in [^{3}H]DAMGO binding sites was demonstrated in brain membranes from rats chronically exposed to morphine (Rothman et al., 1989). Therefore, further study will be required to elucidate the relationship between opioid tolerance and down-regulation of the opioid receptor. On the other hand, up-regulation of the cAMP pathway, including adenylate cyclase and protein kinase A, is an established adaptation mechanism to chronic drug exposure. It has been demonstrated that chronic opioid exposure induces up-regulation of the cAMP system in the locus coeruleus, leading to an increase in the intrinsic firing rate of the neurons, which has been related to opiate withdrawal syndrome (Nestler, 1992).

Receptor desensitization, uncoupling of the receptor from the heterotrimeric G-protein, is another adaptation response induced by chronic activation of the G-protein-coupled receptor (Lohse, 1993). Chronic agonist treatment induced complete desensitization of the μ-opioid receptor expressed in 7315c pituitary tumor cells (Puttfarcken et al., 1988; Puttfarcken and Cox, 1989) and the κ-opioid receptor expressed in COS-7 cells (Raynor et al., 1994). In contrast, it has been recently reported that chronic agonist treatment induces no significant desensitization of the μ- and κ-opioid receptors in CHO cells (Avidor-Reiss et al., 1995a,b). Furthermore, in slices of rat striatum (De Vries et al., 1991) and cultured striatal neurons (Van Vliet et al., 1991), chronic agonist treatment did not induce μ-opioid receptor desensitization, but caused adenylate cyclase supersensitization. Our data also demonstrate that DAMGO treatment induced only a small rightward shift of the dose-response curve for DAMGO-induced inhibition of forskolin-stimulated cAMP accumulation, whereas the μ-opioid receptor was down-regulated and adenylate cyclase was supersensitized. This may suggest that the down-regulated population of the μ-opioid receptors are not coupled with adenylate cyclase and/or that the cells have a very large reserve of the μ-opioid receptor. Meanwhile, evidence has been accumulated that the opioid receptor desensitization involves phosphorylation of the receptor molecule by one or more G-protein-coupled receptor kinases (Pei et al., 1995). Therefore, it is also possible that CHO cells do not possess G-protein-coupled receptor kinases sufficient for the opioid receptor desensitization.

In conclusion, chronic agonist treatment of the μ-opioid receptor expressed in CHO cells simultaneously induces two adaptation responses, receptor down-regulation and adenylate cyclase supersensitization, via pertussis toxin-insensitive and sensitive mechanism, respectively, although the acute cellular responses mediated by the μ-opioid receptor are elicited via pertussis toxin-sensitive G-proteins (G_i and/or G_o).

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