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Adenylylcyclase Supersensitization in μ-Opioid Receptor-transfected Chinese Hamster Ovary Cells Following Chronic Opioid Treatment*

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Tomer Avidor-Reiss, Michael Bayewitch, Rivka Levy, Noa Matus-Leibovitch, Igal Nevo, and Zvi Vogel‡

From the Department of Neurobiology, The Weizmann Institute of Science, Rehovot 76100, Israel

Using CHO cells stably transfected with rat μ-opioid receptor cDNA, we show that the μ-agonists morphine and [D-Ala^2, *N*-methyl-Phe^4,Gly-ol^5]enkephalin are negatively coupled to adenylylcyclase and inhibit forskolin-stimulated cAMP accumulation. Chronic exposure of cells to morphine leads to the rapid development of tolerance. Withdrawal of morphine or [D-Ala^2, *N*-methyl-Phe^4,Gly-ol^5]enkephalin following chronic treatment (by wash or addition of the antagonist naloxone) leads to an immediate increase in cyclase activity (supersensitization or overshoot), which is gradually reversed upon further incubation with naloxone. Phosphodiesterase inhibitors do not affect the overshoot, indicating that it results from cyclase stimulation rather than phosphodiesterase regulation. Morphine's potency to inhibit cAMP accumulation is the same before and after chronic treatment, suggesting that the apparent tolerance results from cyclase activation, rather than from receptor desensitization. The similar kinetics of induction of tolerance and overshoot support this idea. Both the overshoot and acute opioid-induced cyclase inhibition are blocked by naloxone and are pertussis toxin-sensitive, indicating that both phenomena are mediated by the μ-receptor and G_i/G_o proteins. The supersensitization is cycloheximide-insensitive, indicating that it does not require newly synthesized proteins. This is supported by the rapid development of supersensitization. Taken together, these results show that μ-transfected cells can serve as a model for investigating molecular and cellular mechanisms underlying opiate drug addiction.

Pharmacological studies have defined three types of opioid receptors, μ, δ, and κ, that differ in their affinity for various opioid ligands and in their distribution in the nervous system (Herz, 1993). The three types of opioid receptors have recently been cloned and are all members of the seven-transmembrane domain GTP-binding protein (G protein)-coupled[1] receptor superfamily. Activation of all three types of opioid receptors leads to inhibition of adenylylcyclase (AC) activity, and this effect is mediated through pertussis toxin (PTX)-sensitive G proteins (for review see Reisine and Bell (1993); Uhl *et al.* (1994)). Much less is currently known about the cellular and molecular mechanisms that accompany prolonged opiate exposure leading to opiate tolerance and, upon removal of the agonist, to opiate withdrawal.

Various neuroblastoma cell lines have been used to study the regulation of AC activity by prolonged opioid exposure. Sharma *et al.* (1975, 1977) showed that chronic exposure of NG108–15 neuroblastoma × glioma hybrid cells (a cell line that expresses mainly δ-opioid receptors) to morphine leads to an increase in AC activity (see also Hamprecht (1977)) and suggested that this phenomenon may underlie the tolerant state. Withdrawal of the agonist (*i.e.* by adding the antagonist naloxone or by washing, which relieves the inhibition of AC exerted by the agonist) revealed the phenomenon of AC supersensitization or overshoot. Additional processes have been suggested to underlie tolerance to opioids in this cell line. These include receptor desensitization and down-regulation, as well as uncoupling of the receptor from G proteins (for reviews see Loh *et al.* (1988) and Way (1993)).

The effect of prolonged opioid exposure on μ-opioid receptor signaling has been less well characterized. In the human neuroblastoma SH-SY5Y cell line, which expresses both μ- and δ-opioid receptors (Kazmi and Mishra, 1987), chronic activation of μ-opioid receptors by morphine was shown to lead to partial desensitization (Yu *et al.*, 1990; Yu and Sadée, 1988) and, upon withdrawal of the opiate agonist, to overshoot in the production of cAMP (Ammer and Schulz, 1993a; Wang *et al.*, 1994; Yu *et al.*, 1990). No receptor down-regulation or changes in guanyl nucleotide regulation of agonist affinity were reported in this cell line after chronic morphine treatment (Yu *et al.*, 1990). On the other hand, in the mouse 7315c cell line, which contains a homogeneous population of μ-opioid receptors (Frey and Kebabian, 1984), chronic morphine treatment induced a rapid loss of μ-opioid receptor-mediated inhibition of AC, which was accompanied by a loss of guanyl nucleotide regulation of agonist affinity (Puttfarcken *et al.*, 1988). Reduction in receptor number developed more slowly and required a higher concentration of morphine (Puttfarcken and Cox, 1989; Puttfarcken *et al.*, 1988). In addition, no AC supersensitization was observed in these cells following the chronic opiate exposure (Puttfarcken and Cox, 1989).

The mechanism by which chronic opioid treatment increases AC activity and cellular cAMP levels is not known. According to some authors, AC supersensitization could be the result of a secondary regulatory process during the chronic exposure to opiates, the nature of which is not yet understood (Nestler *et al.*, 1993; Sharma *et al.*, 1975). According to other authors, the increase in cAMP accumulation could be due to the ability of opioid receptors to couple to G_s and to stimulate AC directly, whereas the chronic opioid exposure would attenuate the G_i-mediated AC opioid inhibition (Crain and Shen, 1990; Shen

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‡ To whom correspondence should be addressed. Tel.: 972-8-34-2402; Fax: 972-8-34-4131.

[1] The abbreviations used are: G proteins, GTP-binding proteins; AC, adenylylcyclase; CHO, Chinese hamster ovary; DAMGE, [D-Ala^2, *N*-methyl-Phe^4,Gly-ol^5]enkephalin; FS, forskolin; PTX, pertussis toxin.

and Crain, 1992; Wang and Gintzler, 1994). Ammer and Schulz (1993a, 1993b) suggested that AC supersensitivity may be due to enhanced coupling of the prostaglandin E_1 receptor to G_s in NG108–15 cells and showed that it is correlated with elevated levels of functionally intact G_s in SH-SY5Y cells, whereas Griffin *et al.* (1985) suggested that it may be due to the loss of tonic G_i-mediated inhibition of AC.

Chinese hamster ovary (CHO) cells have been reported to be a suitable system for expressing opioid receptors, with the expressed receptors showing the expected ligand-binding selectivity (Fukuda *et al.*, 1993; Raynor *et al.*, 1994). Moreover, expressed κ- and δ-receptors were shown to inhibit AC activity through a PTX-sensitive G protein (Avidor-Reiss *et al.*, 1995; Law *et al.*, 1994). In this report, we show that this cell system is very useful for the study of the effects of chronic exposure to opioids, including the development of tolerance and dependence. Using CHO cells transfected with the μ-opioid receptor, we show that (i) μ-opioid agonists markedly inhibit forskolin (FS)-stimulated AC activity; (ii) chronic morphine treatment leads to tolerance in the ability of the drug to reduce the level of FS-induced cAMP; and (iii) removal of the agonist following chronic treatment reveals a large overshoot of AC activity. This overshoot does not require protein synthesis and is responsible, at least in part, for the tolerance observed. These opioid receptor-transfected cells are a good model for studying the mechanisms of opiate drug addiction.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H-2]adenine (10.3 Ci/mmol) was purchased from Rotem Industries (Be'er Sheba, Israel). [^{3}H][D-Ala^2,*N*-methyl-Phe^4,Gly-ol^5] enkephalin (DAMGE) (59 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). Unlabeled opioid ligands were obtained from Research Biochemical International (Natick, MA) and from the National Institute of Drug Abuse, Research Technology Branch (Rockville, MD). The phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine and RO-20–1724 were from Calbiochem (La Jolla, CA). FS and cAMP were from Sigma (St. Louis, MO). PTX was from List Biological Laboratories (Campbell, CA). Geneticin (G418) and tissue culture reagents were from Life Technologies, Inc. (Bethesda, MD).

Cell Transfection and Culture—Parental CHO-K1 cells (American Type Tissue Culture Collection, Rockville, MD) were transfected with rat μ-receptor cDNA inserted into the mammalian expression vector pCMV-neo (Thompson *et al.*, 1993) using the calcium phosphate method as described previously (Chen and Okayama, 1987). Clones resistant to G418 (1 mg/ml) were isolated and analyzed for their capacity to bind [^{3}H]diprenorphine. The CHO-μ cell lines isolated were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, nonessential amino acids, 0.2 mg/ml G418, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37 °C.

Opioid Receptor-binding Assays—Aliquots of cell homogenates (50–100 μg protein) were incubated for 1 h at 25 °C with 0.01–8 nM of [^{3}H]DAMGE in a final volume of 200 μl in 50 mM Tris-HCl (pH 7.4). Bound ligand was separated by filtration through Whatman GF/B glass filters using a Brandel cell harvester (Gaithersburg, MD), and the radioactivity was determined by liquid scintillation. K_d and B_{max} values were calculated by Scatchard plot analysis following subtraction of nonspecific binding measured in the presence of 10 μM unlabeled DAMGE.

AC Assay—The assay was performed in triplicate essentially as described (Vogel *et al.*, 1993). In brief, cells cultured in 24-well plates (250,000 cells/well) were incubated for 4 h with 0.25 ml/well of fresh growth medium containing 5 μCi/ml of [^{3}H]adenine. This medium was replaced with 0.5 ml/well of Dulbecco's modified Eagle's medium containing 20 mM Hepes (pH 7.4), 0.1 mg/ml bovine serum albumin, 0.1 mM 1-methyl-3-isobutylxanthine, and 0.5 mM RO-20–1724 (assay medium), after which FS was added to a final concentration of 1 μM. After 10 min at 37 °C, the medium was removed, and the reaction was terminated with 1 ml of 2.5% perchloric acid containing 0.1 mM of unlabeled cAMP. After 15 min with perchloric acid at 4 °C, volumes of 0.9 ml were neutralized with 100 μl of a mixture of 3.8 M KOH and 0.16 M K_2CO_3. Aliquots of 0.9 ml of the supernatants were applied to a two-step column separation procedure as described previously (Salomon, 1991). The [^{3}H]cAMP was eluted into scintillation vials and counted. Opioids were added together with the FS for the 10-min assay period (acute treatment) or incubated with the cells for the times indicated prior to the addition of FS (chronic treatment). Unless otherwise indicated, chronic treatment was for 4 h and was concomitant with the labeling with [^{3}H]adenine. The chronic opioid exposure did not affect the uptake of [^{3}H]adenine by the cells. Withdrawal of the chronically applied opioid agonist was achieved either by its removal (together with the [^{3}H]adenine) and the addition of 1 μM naloxone in assay medium or, where indicated, by four sequential washes with 0.5-ml aliquots of assay medium without phosphodiesterase inhibitors. The two methods of agonist withdrawal resulted in similar effects on cAMP accumulation (see also Avidor-Reiss *et al.*, 1995). In experiments utilizing PTX, it was added, at 100 ng/ml, to the cultures 20 h before the addition of [^{3}H]adenine and was replenished upon the addition of [^{3}H]adenine. The amount of cAMP in the absence of FS was subtracted from the values obtained following FS stimulation. This value ranged between 90–400 cpm and was usually less than 30% of the FS-stimulated value. AC inhibition curves and the EC_{50} values were determined using the equation: $y = (a - d)/[1 + (x/c)^b] + d$, where a is the asymptotic maximum, b is the value of the slope, c is the inflexion point, and d is the asymptotic minimum. All data are expressed as the means ± S.E.

RESULTS

Characterization of CHO Cells Expressing μ-Opioid Receptors—We have transfected CHO-K1 cells and isolated several clones expressing μ-receptors. As expected, these cells bind the μ-specific ligand [^{3}H]DAMGE, whereas the parental CHO-K1 cells exhibit no specific binding of the agonist. The K_d and B_{max} values for binding of [^{3}H]DAMGE to these receptors in CHO-μ/1 cells (one of the clones isolated and the one used in this study) were 1.94 ± 0.57 nM and 1.7 ± 0.5 pmol/mg protein, respectively (n = 4).

The application of μ-opioid agonists to CHO-μ/1 cells led to a marked inhibition of FS-stimulated cAMP accumulation in these cells. The μ-selective opioid agonists DAMGE and morphine, as well as the nonselective opioid agonist etorphine, were very effective in inhibiting FS-stimulated cAMP accumulation, reaching levels of ~85% inhibition with 100 nM of added opioid agonist. The κ- and δ-selective opioid agonists U69593 and [D-penicillamine^2, D-penicillamine^5]enkephalin, respectively, were not active (Fig. 1*a*). The nonselective opioid antagonist naloxone (1 μM) did not inhibit FS-stimulated cAMP accumulation and blocked the inhibitory effects of the μ-agonists (Fig. 1*b*). In agreement with the lack of specific opioid binding in CHO-K1 parental cells, morphine (1 μM) did not lead to any inhibition of cAMP accumulation in these cells (data not shown). Altogether, these results demonstrate that μ-agonists activate the μ-receptor in the transfected cells, leading to inhibition of FS-stimulated cAMP accumulation.

As can be seen in Fig. 2, both morphine and DAMGE inhibit FS-stimulated cAMP accumulation in a dose-dependent manner. However, their ability to inhibit AC is different. Morphine is much less potent, with an EC_{50} of 41 ± 7 nM, whereas DAMGE has an EC_{50} of 1.7 ± 0.8 nM. The maximal level of inhibition (90–98% inhibition of FS-stimulated cAMP accumulation) was obtained with 0.032–1 μM of DAMGE and with 1 μM of morphine. Pretreatment of the cells with PTX blocked the inhibitory effects of DAMGE on AC activity, indicating the involvement of the G_i/G_o type of G proteins in mediating this process.

Effect of Chronic Agonist Exposure on AC Activity—A compensatory increase in prostaglandin E_1- or FS-stimulated AC activity has been reported following long term exposure of neuroblastoma × glioma hybrid NG108–15 cells or of human neuroblastoma SH-SY5Y cells to opiate agonists (Ammer and Schulz, 1993a, 1993b; Sharma *et al.*, 1975, 1977; Traber *et al.*, 1975; Wang *et al.*, 1994). We examined whether a similar effect could be detected in CHO cells stably transfected with μ-receptors. For this purpose, cells were treated for 4 h with the

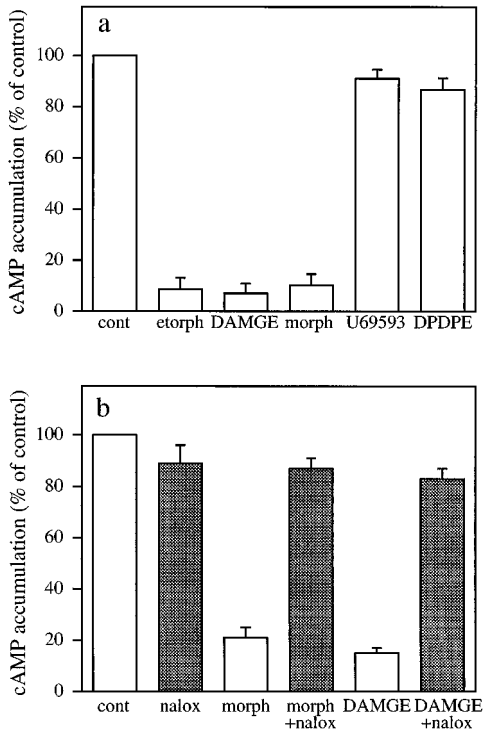

FIG. 1. **Effect of various opioid ligands on FS-stimulated cAMP accumulation in CHO-μ cells.** *a*, inhibition of FS-stimulated cAMP accumulation by various opioid agonists. The agonists, etorphine (*etorph*), morphine (*morph*), U69593, DAMGE, and [D-penicillamine^2, D-penicillamine^5]enkephalin (*DPDPE*), were used at a concentration of 100 nM. The data are expressed as the means ± S.E. of four experiments. 100% defines FS-stimulated cAMP accumulation in cells that have not been treated with opioids. *b*, blockade by naloxone of the opioid agonist inhibition of FS stimulation. Morphine and DAMGE were used at concentrations of 100 and 10 nM, respectively; naloxone (*nalox*) was used at 1 μM. The data show the means ± S.E. of a representative experiment. 100% defines FS-stimulated cAMP accumulation in cells that have not been treated with opioids and is equivalent to 1561 ± 42 cpm. *cont*, control.

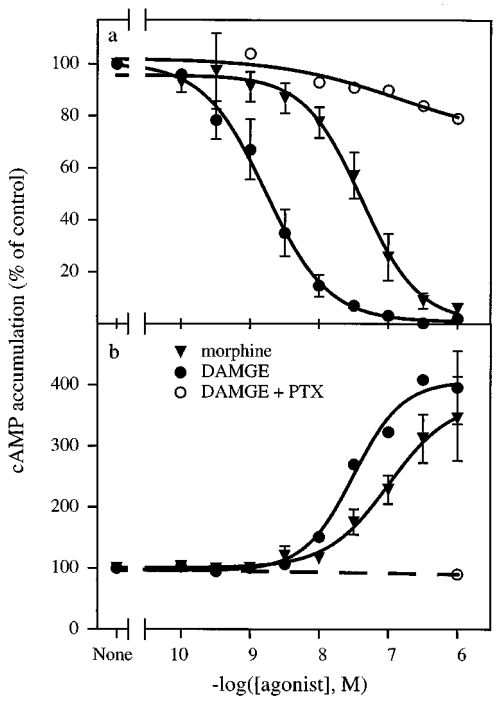

FIG. 2. **Effects of acute and chronic exposures to DAMGE and morphine on cAMP accumulation in CHO-μ cells.** *a*, inhibition of FS-stimulated cAMP accumulation by various concentrations of morphine (▼) and DAMGE (●). *b*, induction of AC supersensitization following chronic (4 h) exposure to DAMGE or morphine and their withdrawal by the addition of naloxone prior to the AC assay (see "Experimental Procedures"). Where indicated (○), cells were pretreated with PTX (100 ng/ml) for 24 h, and the effect of DAMGE was studied. The data are expressed as the means ± S.E. of three or four experiments.

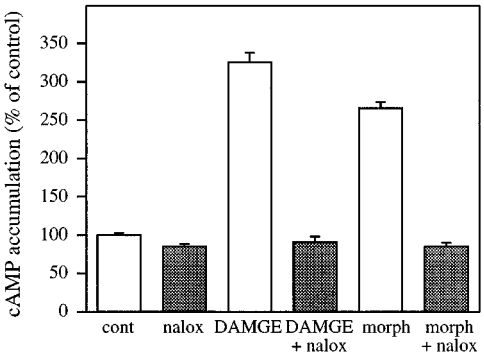

FIG. 3. **Effect of chronic treatment with naloxone on agonist-induced AC supersensitization.** CHO-μ cells were treated for 4 h with 1 μM of DAMGE or morphine (*morph*) in the presence (*shaded bars*) or absence (*white bars*) of 1 μM naloxone (*nalox*). After this time, all cultures were washed once, and 1 μM naloxone was added just prior to the addition of FS. The data show the means ± S.E. of a representative experiment. Control (*cont*) represents cells that have not been pretreated with opioids.

indicated concentrations of DAMGE or morphine, and the antagonist naloxone was applied (to remove the inhibitory agonist effect on AC activity) prior to the AC assay. The results (Fig. 2*b*) clearly demonstrate that withdrawal of the agonists (morphine or DAMGE) following the 4-h chronic treatment induced an overshoot in FS-stimulated cAMP accumulation, as compared with control FS-stimulated cells (*i.e.* cells not treated with the agonist). This supersensitization of AC activity was blocked when the antagonist, naloxone, was present during the chronic agonist treatment (Fig. 3). Moreover, this supersensitization could not be observed in CHO-K1 parental cells, which do not express the μ-opioid receptor (data not shown), demonstrating that the increase in cAMP accumulation under the withdrawal conditions is dependent on the activation of the μ-receptor by the agonist during the chronic exposure step. The overshoot could also be observed following a rapid wash of the agonist (see Fig. 6), indicating that this effect is due to the removal of the agonist *per se* rather than to a specific activity of the antagonist.

Fig. 2*b* shows that both morphine and DAMGE evoke AC supersensitization in a dose-dependent manner. The maximal level of overshoot was obtained when the cells were exposed to 0.32–1 μM of DAMGE or to 1 μM of morphine. However, whereas DAMGE induced AC supersensitization with an EC_{50} of 34 ± 6 nM, morphine induced AC supersensitization with an EC_{50} of 102 ± 21 nM. Thus, as with the acute inhibition of AC, DAMGE is more potent than morphine. Moreover, for both ligands, the induction of AC supersensitization requires chronic incubation with higher concentrations of agonists than those needed to induce the acute opioid inhibition of FS-stimulated cAMP accumulation. However, although DAMGE was 24-fold more potent than morphine in inhibiting AC activity, it was only 3-fold more potent in evoking AC supersensitization.

Pretreatment of the cells with PTX, which prevents the opioid inhibition of AC (see Fig. 2*a*), also blocked the naloxone-induced overshoot of cAMP accumulation in the chronically opioid-treated cells (Fig. 2*b*). This finding demonstrates that the overshoot phenomenon is linked to the chronic activation of the opioid receptors, as well as to the activation of the PTX-

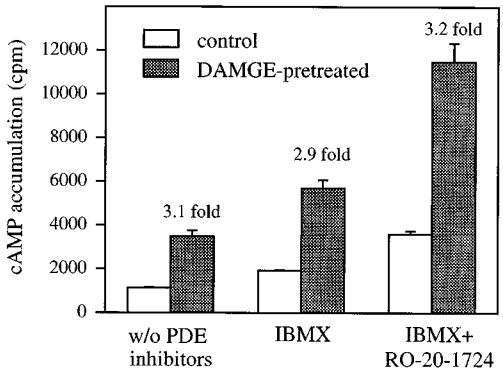

FIG. 4. **Effects of phosphodiesterase inhibitors on FS-stimulated cAMP accumulation.** FS-stimulated cAMP accumulation was determined in control and in chronically (4 h, 1 μM DAMGE) pretreated CHO-μ cells under withdrawal conditions. The phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine and RO-20–1724 were present during the assay only where indicated. Withdrawal was achieved by a rapid wash and the addition of naloxone to all cultures. The data show the average values (in cpm of [^{3}H]cAMP) of a representative experiment. The ratio between the levels of cAMP in FS-stimulated chronic DAMGE-treated and control cells is provided. *PDE*, phosphodiesterase; *IBMX*, 1-methyl-3-isobutylxanthine.

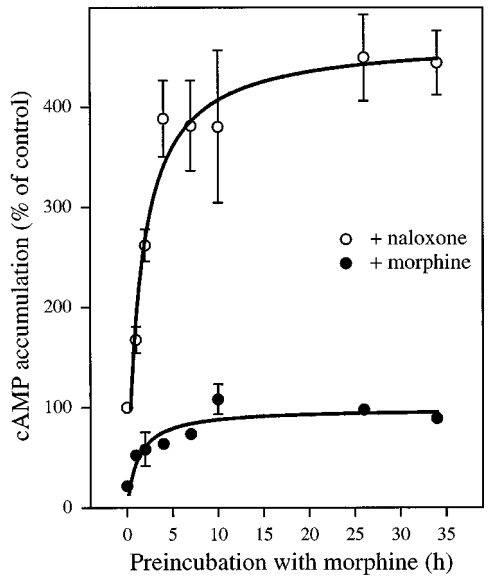

FIG. 5. **Time course of chronic morphine pretreatment on FS-stimulated cAMP accumulation.** CHO-μ cells were incubated with 0.32 μM morphine for the periods indicated. At the end of the preincubations, the morphine was removed (together with the [^{3}H]adenine), and morphine (0.32 μM; ●) or naloxone (1 μM; ○) was added to the cultures in assay medium, followed by the addition of FS for the 10-min assay period. The data are expressed as the means ± S.E. of a representative experiment. 100% (1283 ± 55 cpm) represents the FS-stimulated cAMP value in cells not pretreated with morphine.

sensitive G_i/G_o proteins during the chronic opioid exposure.

To exclude the possibility of involvement of phosphodiesterase regulation in the cAMP overshoot phenomenon, we routinely included phosphodiesterase inhibitors in the assay. In Fig. 4, we show that the presence of phosphodiesterase inhibitors (1-methyl-3-isobutylxanthine alone or together with RO-20–1724) during the 10-min assay period increased the level of cAMP accumulation both in control and in chronic DAMGE-treated cells. However, the extent of the overshoot, in comparison with cells that were not pretreated with opioid agonist, was not affected by the phosphodiesterase inhibitors. This suggests that the AC supersensitization involves changes in AC activity rather than in phosphodiesterase activity.

Fig. 5 depicts the kinetics of the effect of morphine on the activity of AC in the presence of the drug and after its withdrawal. It shows that the cells have to be exposed to morphine (0.32 μM) for at least 4 h in order to reach the maximal level of AC supersensitization. This level is maintained even after 35 h of exposure to the drug. The time needed to achieve half-maximal supersensitization was equivalent to ~2 h. Chronic treatment with morphine also leads to the development of tolerance in the μ-receptor-transfected CHO cells. As can be seen from Fig. 5, the kinetics of the appearance of tolerance are very similar to those of the overshoot. Namely, acute morphine (0.32 μM) inhibited FS-stimulated cAMP accumulation by 80%. However, despite the continued presence of morphine, FS-stimulated cAMP accumulation slowly recovered (after 4–10 h with morphine) to approximately the level of untreated control cells. This tolerance to morphine is not due to a loss of the capacity of morphine to inhibit AC, because removal of morphine by adding naloxone (withdrawal conditions) markedly increased the level of FS-stimulated cAMP accumulation. Thus, morphine was still able to inhibit FS-stimulated cAMP accumulation and to a similar extent (around 80%) as before the chronic treatment.

To confirm the observation that morphine does not lead to receptor desensitization, we checked the ability of increasing concentrations of morphine to inhibit FS-stimulated cAMP accumulation both before and after chronic treatment with morphine (Fig. 6*a*). Four sequential washes were found to be effective in removing the bound agonist, as was deduced from the inability of added naloxone to induce a further increase in FS-stimulated cAMP accumulation (data not shown). Chronic

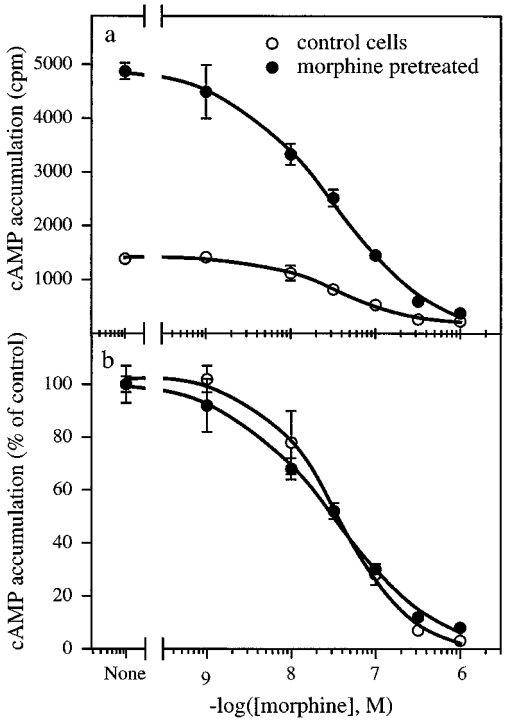

FIG. 6. **Effect of morphine pretreatment on morphine inhibition of FS-stimulated cAMP accumulation.** CHO-μ cells were either pretreated with morphine (0.1 μM; ●) for 4 h or served as controls (○). Withdrawal was achieved by four washes as described under "Experimental Procedures." Morphine was then readded at the indicated concentrations and cAMP accumulation (*a*) and percent of inhibition (*b*) were determined. The data are expressed as the means ± S.E. of a representative experiment.

treatment with morphine (0.1 μM) followed by these four sequential washes led to a 3.5-fold increase in the level of FS-stimulated cAMP accumulation. The readdition of morphine for

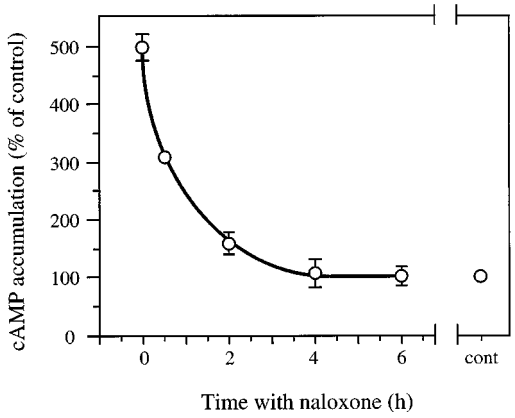

FIG. 7. **Reversibility of AC supersensitization following long term treatment with naloxone.** CHO-μ cells were pretreated for 18 h with 1 μM DAMGE. Naloxone (1 μM) was added for the indicated times prior to (and remained present during) the 10-min AC assay. Control (*cont*) represents cells that were not pretreated with DAMGE or naloxone and is defined as 100% (586 ± 32 cpm). The data are expressed as the means ± S.E. of a representative experiment.

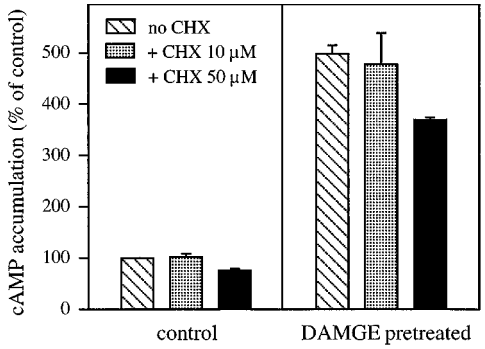

FIG. 8. **Effect of cycloheximide on AC supersensitization.** 10 or 50 μM cycloheximide (*CHX*) was added to the cultures 1 h before and was present during a 4-h chronic pretreatment with 1 μM DAMGE. FS-stimulated cAMP accumulation was determined in control and in DAMGE-pretreated CHO-μ cells under withdrawal conditions. The data are expressed as the means ± S.E. of a representative experiment. 100% is equivalent to the level of cAMP in FS-stimulated control cells not treated with cycloheximide.

the 10-min assay period inhibited FS-stimulated cAMP accumulation in both the control and the chronic morphine-treated cells in a dose-dependent manner. Moreover, as shown in Fig. 6*b* (by normalizing the data obtained in the experiment shown in Fig. 6*a*), it is clear that morphine is able to inhibit FS-stimulated cAMP accumulation following chronic morphine treatment to a similar extent as in control cells.

Although the addition of an antagonist during the assay after the chronic treatment reveals the phenomenon of AC supersensitization (due to blockade of the inhibition induced by the agonist), prolonged exposure to the antagonist, after the chronic treatment, results in a gradual reduction of FS-stimulated cAMP accumulation (Fig. 7). The half-life of the disappearance of the AC supersensitization was ~30 min, and after 4 h, the levels of cAMP in the cells had returned to their original values (*i.e.* those obtained under normal, nonopioid-treated conditions). It is therefore apparent that the occurrence of AC supersensitization depends upon sustained activation of the receptor and that the cells maintain the ability to rapidly return to the original levels of cyclase activity and cAMP concentration following the withdrawal of the opioid agonist.

In order to clarify whether protein synthesis is involved in the induction of AC supersensitization, we checked the effect of the protein synthesis inhibitor cycloheximide on this phenomenon. We found that incubation with 10 or 50 μM cycloheximide did not affect the ability of the cells to induce AC supersensitization (Fig. 8). Under these conditions, cycloheximide was able to inhibit >85% of the incorporation of [^{3}H]-labeled amino acids (which were added to the cells in parallel to the opioid agonist). Some inhibition of FS-stimulated AC activity (~24%) was observed in chronic opioid-treated cells exposed to 50 μM cycloheximide, but the same level of inhibition was also observed in cells that had not been treated with opioids. This result suggests that the opioid-induced AC supersensitization does not require newly synthesized proteins.

DISCUSSION

In this study, we have used CHO cells expressing the rat μ-opioid receptor to gain information on μ-receptor signal transduction and its role in opiate addictive processes. We have demonstrated that the μ-transfected cells are able to interact with μ-agonists and that this interaction leads to several processes of AC regulation, including the inhibition of FS-stimulated cAMP accumulation, apparent tolerance, and the development of AC supersensitization.

Long term exposure of the transfected cells to opioids can offer a model system to study the molecular mechanisms of drug tolerance and withdrawal. In their review, Nestler *et al.* (1993) defined tolerance as "a reduced effect upon repeated exposure to a constant drug dose, or the need for an increased dose to maintain the same effect." Using the μ-opioid receptor-transfected CHO cell line, we have observed that chronic morphine treatment led to an increase in the level of FS-stimulated cAMP accumulation, which could be overcome by higher levels of the drug. This result indicates the development of tolerance to the effect of morphine.

Withdrawal of opioid agonists after chronic treatment (either by wash or by the addition of an antagonist) markedly enhances FS-stimulated cAMP accumulation, indicating that AC was under inhibition during the chronic treatment and that this treatment led to AC supersensitization. Moreover, we have observed that the μ-opioid receptors did not desensitize during the chronic morphine treatment and that the ability of morphine to inhibit the FS-stimulated AC remained the same both before and after the chronic exposure. These results demonstrate that the tolerance observed is a result of the capacity of the cells to undergo opioid-induced AC supersensitization and is not due to changes in the receptor capacity to transduce the signal. These results are consistent with the model suggested by Sharma *et al.* (1975) for the role of AC regulation in the development of morphine tolerance and dependence in NG108–15 cells. On the other hand, these results differ from the observations reported by Frey and Kebabian (1984), who detected full desensitization by morphine in mouse 7315c cells, and from those of Yu and Sadée (1988) and Yu *et al.* (1990), who detected partial desensitization (4-fold increase in the EC_{50} in SH-SY5Y cells).

The degrees of both AC inhibition and supersensitization by DAMGE or morphine are concentration-dependent. Although application of low concentrations of agonist was sufficient to inhibit FS-stimulated cAMP production, the supersensitization required pretreatment with higher doses of the drugs. A similar observation was also obtained for the κ-agonist U69593 applied to CHO cells transfected with the κ-receptor (Avidor-Reiss *et al.*, 1995). This difference in concentrations needed for the two phenomena suggests that activation of the overshoot requires the recruitment of threshold amounts of signaling components, the nature of which remains to be clarified. It may involve spare receptors, as suggested by Law *et al.* (1994), or other mechanisms (see below). Nevertheless, this difference in agonist concentration needed for the two phenomena is in

agreement with the clinical observation that the development of physical dependence requires prolonged exposure to relatively high doses of opiates (Bhargava, 1994).

It has been reported that various μ-ligands could differentially activate the μ-opioid receptor, leading to different effects on the AC signaling system. For example, although both morphine and DAMGE inhibited AC activity in SH-SY5Y cells (through the μ-receptors), it was shown that morphine but not DAMGE was able to induce AC supersensitization (Ammer and Schulz, 1993a). In contrast, in our μ-transfected CHO cells, both DAMGE and morphine were able to induce AC supersensitization. However, although DAMGE was 24-fold more potent than morphine in inhibiting AC activity, it was only 3-fold more potent in evoking AC supersensitization. This difference could in part be due to the ability of DAMGE but not of morphine to induce receptor desensitization (Carter and Medzihradsky, 1993). In this regard, it is worthwhile to note that Zimprich *et al.* (1995) reported a difference in the ability of DAMGE to induce desensitization of the inhibition of FS-stimulated cAMP accumulation with two isoforms of the rat μ-opioid receptor. The longer of the two forms of μ-opioid receptor, which is equivalent to the one used in our study, was more prone to desensitization than the shorter form. No information was provided to account for the mechanism of this desensitization phenomenon.

The increase in cAMP that occurs in intact NG108–15 and in several other cells following withdrawal after chronic treatment with substances that stimulate receptors negatively coupled to AC has been suggested to be due to the increased AC activity detected in membrane preparations from these cells (Sabol and Nirenberg, 1979; Sharma *et al.*, 1975; Thomas and Hoffman, 1987). Moreover, in our experiments, the relative increase in FS-stimulated cAMP accumulation was the same in both the presence and absence of phosphodiesterase inhibitors. Thus, the increase in cAMP appears to result from an up-regulation of AC activity rather than from opioid-induced inhibition of phosphodiesterase. Nevertheless, it is worth noting that in intact NG108–15 cells but not in membrane preparations chronic treatments with carbachol or opiate agonists were shown to result in an attenuated degradation rate constant for cAMP, suggesting a contribution of phosphodiesterase regulation to the cAMP overshoot observed in these cells (Law and Loh, 1993; Thomas *et al.*, 1990).

Several reports suggest that activation of opioid receptors could either inhibit or stimulate AC activity, depending on opioid agonist concentration. Opioid agonists at low concentrations were shown to activate the G_s pathway and stimulate AC activity, whereas high concentrations of opioid agonists activate G_i and inhibit AC activity in myenteric plexus (Wang and Gintzler, 1994) and in dorsal root ganglion cells (Crain and Shen, 1990). Using μ-opioid receptor-transfected CHO cells, we found that low concentrations of DAMGE or morphine (*e.g.* as low as 0.1 nM) did not stimulate AC activity. Moreover, PTX pretreatment abolished the inhibitory opioid effect without revealing any opioid activation (see Fig. 2*a*), suggesting that the acute opioid effects in the CHO cells are mediated through G_i/G_o and not through G_s proteins.

The PTX sensitivity clearly demonstrates that chronic opioid activation, like acute opioid inhibition, has to be mediated through both opioid receptors and PTX-sensitive G proteins in order to produce the elevated cyclase activity observed following removal of the agonist. The PTX sensitivity is in agreement with the observation that PTX prevents the development of opiate dependence when administered to rats (Parolaro *et al.*, 1990). Because μ-receptors expressed in CHO-K1 cells were found to activate $G_{i\alpha 2}$, $G_{i\alpha 3}$ and $G_{o\alpha 2}$ (Chakrabarti *et al.*, 1995), this PTX sensitivity could be attributable to these G_i and/or G_o protein α subunits. Similarly, in NG108–15 cells, where the δ-receptor is coupled to G_{i2}, G_{i3}, and G_o proteins (McKenzie and Milligan, 1990; Roerig *et al.*, 1992), PTX abolished the AC supersensitivity (Griffin *et al.*, 1985).

Regarding the mechanism of the AC supersensitization, it has been suggested, based on results obtained using S49 mouse lymphoma cells chronically treated with a somatostatin analog, that the mechanism involved in AC supersensitization could be attributed to a decrease in the concentration of intracellular cAMP, due to the inhibition of AC by G_i, which would affect PKA activity in the cells (Thomas and Hoffman, 1988). However, this possibility does not fit with our finding that elevated intracellular cAMP levels, as obtained using cholera toxin or dibutyryl cAMP, were unable to block the development of AC supersensitivity (data not shown). It has recently been suggested that chronic opiate exposure could up-regulate AC expression. For example, Matsuoka *et al.* (1994) demonstrated increased mRNA of AC type VIII in the amygdala and locus coeruleus of chronic morphine-treated rats. However, we show here that cycloheximide at concentrations that inhibit protein synthesis does not inhibit AC supersensitivity. Moreover, the development of the overshoot is relatively rapid, with half-maximal supersensitivity achieved within 2 h, kinetics that do not seem to be consistent with induction of new protein synthesis. Ammer and Schulz (1993b) suggested that AC supersensitivity in morphine-treated NG108–15 cells may be due to enhanced coupling of prostaglandin E_1 receptors to G_s. However, as described here for CHO cells and by Ammer and Schulz (1993a) for SH-SY5Y cells, AC supersensitization is also being detected when AC is activated directly by FS in a receptor-independent manner.

Another possible mechanism that could be related to AC supersensitivity is the activation of AC by the $\beta\gamma$ dimers released from G_i/G_o proteins upon opioid receptor activation. $\beta\gamma$ dimers have been shown to activate a variety of signal transduction pathways (for review see Clapham and Neer (1993)), including AC of type II (Federman *et al.*, 1992) and of type IV (Gao and Gilman, 1991). Additional experiments will have to be performed to determine the involvement of the $\beta\gamma$ dimers in AC supersensitization. Nevertheless, the finding of a supersensitized state up to 2 h after withdrawal from chronic treatment is consistent with a model requiring a secondary regulatory process that could be mediated by the $\beta\gamma$ dimers (*i.e.* phosphorylation) rather than a direct action on the AC of the various types of G protein subunits upon receptor activation.

In summary, using CHO cells transfected with rat μ-opioid receptor, we have shown that acute μ-agonist exposure leads to inhibition of AC, whereas chronic exposure leads to the development of supersensitization of AC activity. This activation of AC is reversible and is gradually lost following removal of the agonist. Our results suggest that the primary mechanism for morphine tolerance in chronic morphine-treated CHO cells is the underlying increase in AC activity, resulting in the necessity to use higher concentrations of agonist to reach the same low levels of cAMP in the cell as before. These observations are in line with the phenomena of opiate action, tolerance, and withdrawal.

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