κ-Opioid receptor-transfected cell lines: modulation of adenylyl cyclase activity following acute and chronic opioid treatments

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Abstract The opioid receptors μ, δ and κ have recently been cloned. Here we show that κ-agonists inhibit adenylyl cyclase activity in Chinese hamster ovary cells stably transfected with rat κ-opioid receptor cDNA. Chronic exposure of the cells to κ-agonists did not lead to significant desensitization of the capacity of the agonists to inhibit adenylyl cyclase. On the other hand, withdrawal of the agonist following the chronic treatment led to the phenomenon of supersensitivity ('overshoot') of adenylyl cyclase activity. Both the inhibition of adenylyl cyclase activity by the acute opioid treatment and the chronic agonist-induced supersensitivity are pertussis toxin sensitive, demonstrating involvement of G_i/G_o proteins in both processes.

Key words: Opioid receptor; Forskolin; Adenylyl cyclase; GTP-binding protein; Addiction; Withdrawal

1. Introduction

Pharmacological studies have defined three types of opioid receptors, termed μ, δ and κ, that differ in their affinity for various opioid ligands and in their distribution in the nervous system [1]. Activation of all three types of opioid receptors leads to inhibition of adenylyl cyclase (AC) activity, and this effect is mediated through pertussis toxin (PTX)-sensitive GTP-binding proteins (G proteins) [2,3].

Chronic activation of opioid receptors in NG108-15 neuroblastoma × glioma cell line (containing δ-receptors), as well as in the human neuroblastoma SH-SY5Y (containing mainly μ-receptors and some δ-receptors), followed by the withdrawal of the opioid agonist, was shown to lead to AC supersensitivity ('overshoot') [4–8]. It has been suggested that this phenomenon represents a possible cellular adaptation mechanism associated with chronic opioid exposure. However, due to the absence of cell lines containing κ-opioid receptors there was no definite information regarding the exact role of acute and chronic activation of κ-receptors and of the effect of agonist withdrawal on AC activity.

The three types of opioid receptors have recently been cloned [9,10]. They are all members of the seven-transmembrane domain G protein-coupled receptor superfamily. The availability of cDNAs for the opioid receptors allows molecular studies of their binding and signal transduction properties. We have stably transfected Chinese hamster ovary (CHO) cells with the rat κ-receptor cDNA and found that κ-opioid agonists markedly inhibit the forskolin-stimulated AC activity. The removal of the opioid agonist following chronic κ-opioid treatment leads to a large increase in the level of the forskolin-induced cAMP accumulation in the cells. These results demonstrate, for the first time, that the 'overshoot' phenomenon can be observed for κ-opioid receptors and can be obtained with non-neuronal cells.

2. Materials and methods

2.1. Materials

[^{3}H-2]Adenine was purchased from Rotem Industries (Be'er Sheba, Israel). [15,16(*n*)-^{3}H]Diprenorphine (36 Ci/mmol) was purchased from Amersham (Bukinghamshire, UK). Opioid ligands were obtained from Research Biochemical International (Natick, MA) and from the National Institute on Drug Abuse, Research Technology Branch (Rockville, MD). 1-Methyl-3-isobutylxanthine (IBMX), forskolin, PTX and cAMP were from Sigma (St. Louis, MO). Geneticin (G418) and tissue culture reagents were from Gibco BRL (Bethesda, MD).

2.2. Cell transfection and culture

CHO-K1 cells were transfected with rat κ-receptor cDNA inserted into the mammalian expression vector pCMV-neo [11], using the calcium phosphate method, as previously described [12]. Clones resistant to G418 (1 mg/ml) were isolated and analyzed for their capacity to bind [^{3}H]diprenorphine. The CHO-κ-opioid receptor cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, non-essential amino acids, 0.2 mg/ml G418, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37°C.

2.3. Opioid receptor-binding assays

Cell homogenates (75–200 μg protein) were incubated for 1 h at 25°C with 0.01–3 nM [^{3}H]diprenorphine or [^{3}H]U69593, in a final volume of 200 μl of 50 mM Tris-HCl (pH 7.4). Bound ligand was separated by filtration through Whatman GF/B glass filters using the Brandel cell harvester (Gaithersburg, MD) and the radioactivity determined by liquid scintillation. K_d and B_{max} values were calculated by Scatchard analysis following subtraction of non-specific binding, measured in the presence of 10 μM U69593.

2.4. AC assay

This assay was performed essentially as described [13]. 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 DMEM containing 20 mM HEPES (pH 7.4), 0.1 mM IBMX and 1 μM forskolin. After 10 min at 37°C, the medium was removed and the reaction terminated with 1 ml of 2.5% perchloric acid containing 0.1 mM of unlabeled cAMP. Opioids were added either together with the forskolin

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Abbreviations: AC, adenylyl cyclase; CHO, Chinese hamster ovary; DAMGE, [D-Ala^2,*N*-Me-phe^4,Gly-ol^5]enkephalin; DMEM, Dulbecco's modified Eagle's medium; G proteins, GTP-binding proteins; IBMX, 1-methyl-3-isobutylxanthine; nor-BNI, nor-binaltrophimine; PTX, pertussis toxin.

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for the 10-min incubation period (acute exposure) or together with the [^{3}H]adenine for a 4-h incubation (chronic treatment). The chronic opioid exposure did not affect the uptake of [^{3}H]adenine by the cells. 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 previously described [14]. The [^{3}H]cAMP was eluted into scintillation vials and counted. In experiments utilizing PTX, it was added to the cultures 20 h before adding [^{3}H]adenine and was replenished upon the addition of [^{3}H]adenine.

2.5. Statistical analysis

Data were analyzed using Student's *t*-test. Binding parameters (K_d and B_{max} values) were determined by Scatchard plot analysis. AC inhibition curves were generated with the Sigma Plot 4.11 computer program (Jandel Scientific, Corta Madera, CA) and the EC_{50} were determined, using an equation from the ALLFIT program [15]; *n* represents the number of experiments.

3. Results

3.1. Characterization of CHO cells expressing κ-opioid receptors

We have transfected CHO-K1 cells and isolated clones expressing κ-receptors. Two of the clones, CHO-κ/5 and CHO-κ/8, were used for further studies. The K_d value for binding of the κ-agonist [^{3}H]U69593 to the receptors in these cell lines was 1.05 ± 0.09 nM (n = 3). The K_d values for [^{3}H]diprenorphine binding to CHO-κ/5 and CHO-κ/8 were 0.35 ± 0.06 nM (n = 4) and 0.61 ± 0.03 nM (n = 5), and the B_{max} values were 252 ± 52 and 388 ± 66 fmol/mg protein, respectively. The application of κ-opioid agonists to these cells led to a marked inhibition of cAMP accumulation. The effect of various opioid ligands on the forskolin-stimulated cAMP accumulation in CHO-κ/8 cell line is illustrated in Fig. 1. Similar results were obtained with CHO-κ/5. The non-selective opioid agonist etorphine and the κ-selective opioid agonists (−)U50488, U69593 and dynorphin-

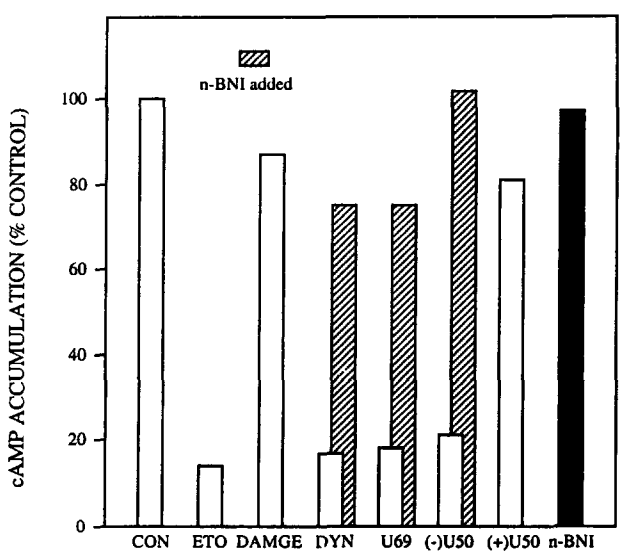

Fig. 1. Effect of opioids on forskolin-stimulated cAMP accumulation in CHO cells transfected with κ-opioid receptors. Agonists were used at 10 nM, and nor-BNI was used at 100 nM. Control (CON); etorphine (ETO); dynorphin (DYN); U69593 (U69); U50488 (U50). The background level (210 cpm) was subtracted from all values. The value of forskolin-stimulated AC in the absence of opioids was defined as 100% (750 cpm). The data show a representative experiment performed with the CHO-κ/8 cell line. Similar results were obtained with CHO-κ/5.

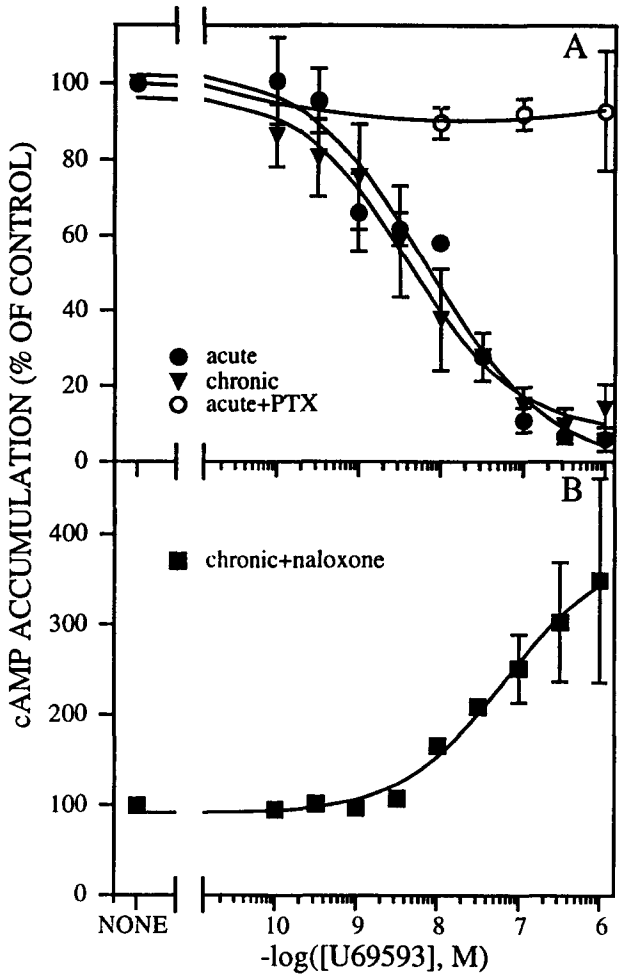

Fig. 2. Effects of acute and chronic κ-agonist exposure on cAMP accumulation in CHO-κ/8 cells. (A) Forskolin-stimulated cAMP accumulation in cells incubated for either 10 min (●) or 4 h (▼) with the indicated concentrations of U69593. The EC_{50} for cAMP inhibition did not significantly differ for the acute (7.8 ± 1.8 nM) and chronic (4.0 ± 2.1 nM) U69593-treated cells. The incubation for 10 min was repeated in cells (○) pretreated with PTX (100 ng/ml, 24 h). (B) Forskolin-stimulated cAMP accumulation in cells treated chronically (4 h) with the indicated concentration of U69593, to which 1 μM naloxone was added 10 min prior to the assay (■). The EC_{50} for cAMP stimulation was 203 ± 37 nM. Data are the mean ± S.E.M. of three experiments.

A_{1-13}, were very effective in inhibiting cAMP accumulation in the κ-transfected CHO cells, reaching levels of 75–85% inhibition with 10 nM of added opioid agonists. In agreement with the known pharmacology of the κ-receptors, the inactive stereoisomer (+)U50488 and the μ-selective opioid agonist DAMGE were not active. Moreover, the κ-opioid antagonist nor-binaltrophimine (nor-BNI) had no effect by itself and blocked the inhibitory effects of κ-agonists on cAMP accumulation in the cells.

The inhibition of the forskolin-stimulated cAMP accumulation by U69593 was dose dependent (Fig. 2A) with an EC_{50} of 7.8 ± 1.8 nM. The maximal level of inhibition (i.e. 90–95% of the forskolin-stimulated cAMP accumulation) was obtained with 0.1–1 μM of U69593. Pretreatment of the cells with PTX completely blocked the inhibitory effects of opioids on AC,

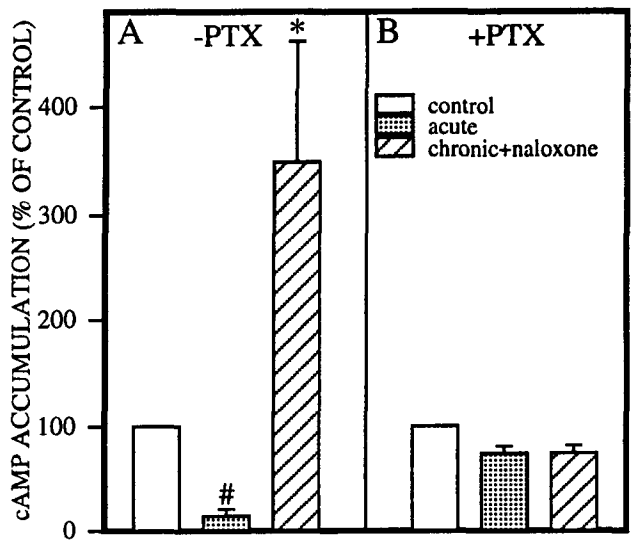

Fig. 3. PTX sensitivity of the forskolin-stimulated cAMP accumulation in CHO-κ/8 cells following acute or chronic treatments with 1 μM U69593. Forskolin-stimulated cAMP accumulation was determined in control cells (A), or in PTX-pretreated (100 ng/ml, 24 h) cells (B), following acute (10 min) or chronic (4 h) treatment with 1 μM U69593. Data are the mean ± S.E.M. of three experiments. Significantly different from control cells, $^{*}P<0.05$ and $^{\#}P<0.01$, respectively.

indicating the involvement of G_i or G_o protein in mediating this process.

3.2. Effect of chronic agonist exposure on AC activity

CHO-κ/8 cells were pretreated for 4 h with the indicated concentrations of U69593 and the forskolin-induced AC activity was determined. The results show (Fig. 2A) that exposure of CHO-κ/8 cells to U69593 for 4 h did not affect the capacity of U69593 to inhibit the forskolin-stimulated cAMP accumulation. Both the EC_{50} and the maximal inhibition of AC were not markedly affected, suggesting that in this transfected CHO cell line the opioid receptors did not undergo desensitization following treatment with the indicated concentrations of U69593. In agreement with this result, no reduction in the maximal inhibition of the forskolin-stimulated cAMP accumulation was observed when the cells were pretreated with 1 μM U69593 for 24 h (data not shown).

A compensatory increase in PGE_1- or forskolin-stimulated AC activity has been reported following long-term exposure of NG108-15 or SH-SY5Y to opioid agonists [4–7]. We examined whether a similar effect could also be detected in the CHO cell lines stably transfected with κ-receptors. For this purpose, cells were treated for 4 h with the indicated concentrations of U69593, and the antagonist naloxone was applied 10 min prior to the assay to remove the agonist inhibitory effect on AC activity. The results (Fig. 2B) clearly indicate that the withdrawal of U69593 following the 4-h chronic treatment induced an 'overshoot' in forskolin-stimulated cAMP accumulation, as compared with control forskolin-stimulated cells (not treated with U69593). Treatment with naloxone alone (without previous exposure to opioid agonists) did not influence the forskolin-stimulated cAMP accumulation (96 ± 4% of control, $n = 9$) (see also Fig. 3). The increase in cAMP accumulation under the 'withdrawal' conditions proved to be dependent on the agonist concentration used in the chronic exposure step, and reached, in this experiment, levels as high as 250% above control levels (Fig. 2B). The level of overshoot in various experiments, using 1 μM U69593 at the chronic step, ranged between 100 and 440% above control levels. It should be stated that treatment with low concentrations of U69593 (e.g. 0.1–3 nM), which were sufficient to inhibit accumulation of cAMP (by up to 40%), did not induce the overshoot effect. The EC_{50} of the U69593-triggered naloxone-induced overshoot in cAMP accumulation was found to be equivalent to 203 ± 37 nM of U69593.

As shown in Fig. 3, the overshoot effect could also be obtained by a rapid wash of the agonist, as well as by the addition of other antagonists besides naloxone (e.g. 1 μM nor-BNI or diprenorphine). Moreover, the combination of a wash with the subsequent application of an antagonist was as efficient as either wash or antagonist alone. These results demonstrate that the overshoot effect is due to the removal of the agonist per se rather than to a specific activity of the antagonist.

Pretreatment of the cells with PTX, a treatment which blocks the opioid inhibition of AC (see Fig. 2), also blocked the naloxone-induced overshoot of cAMP accumulation (Fig. 4) in the chronically opioid-treated cells. These results indicate that the overshoot phenomenon is tightly linked to the chronic activation of the opioid receptors and to the subsequent activation of the PTX-sensitive G_i/G_o proteins during the chronic opioid exposure.

4. Discussion

In this study, we have used CHO cell lines expressing the κ-opioid receptor to gain information on the κ-receptor signal transduction and its role in the opioid addictive processes. We have demonstrated that the κ-transfected cells are able to interact with κ-agonists and that this interaction leads to inhibition

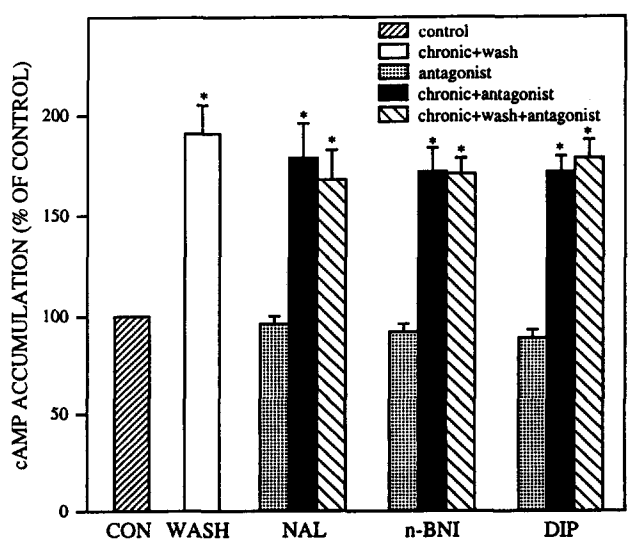

Fig. 4. Effect of wash or antagonists on the forskolin-stimulated AC activity in CHO-κ/8 cells chronically treated with U69593. Cells were treated for 4 h with 1 μM U69593. The cells were either rapidly washed, treated for 10 min with 1 μM of the indicated antagonists (naloxone (Nal), n-BNI, or diprenorphine (DIP)) or washed and subsequently treated with the antagonists. Data are the mean ± S.E.M. of three experiments. Significantly different from control or antagonist-treated cultures, $^{*}P < 0.05$.

of the forskolin-stimulated cAMP accumulation. Several reports suggest that activation of opioid receptors could both inhibit and stimulate AC activity, depending on opioid agonist concentration. Opioid agonists at low concentrations activate G_s proteins and stimulate AC activity, while high concentrations of opioid agonists activate G_i and inhibit AC activity in myenteric plexus and dorsal root ganglion cells [16,17]. Using κ-opioid receptor-transfected CHO cells, we found that low concentrations of U69593 (e.g. as low as 0.1 nM) did not stimulate AC activity. Moreover, PTX pretreatment abolished the inhibitory opioid effect without unmasking any opioid activation, suggesting that the acute opioid effects on the CHO cells are mediated through G_i/G_o and not through G_s proteins.

Long-term exposure of the transfected cells to opioids can offer a model system to study the molecular mechanisms of drug addiction and withdrawal. Using the transfected CHO cell lines, we have observed that the κ-opioid receptors did not significantly desensitize following chronic opioid treatment. These results differ from the observations reported by Raynor et al. [18], who detected κ-agonist-induced desensitization in COS-7 cells transiently transfected with the clonal mouse κ-receptor, and from our previous results demonstrating desensitization of κ-inhibition of AC in co-cultures of spinal cord and dorsal root ganglion cells [2]. Moreover, these results demonstrate that the desensitization (or tolerance) phenomenon and the capacity of the cells to undergo the opioid-induced withdrawal or overshoot, following removal of the agonist, can be dissociated from each other, thus offering a cell system to study the overshoot phenomenon individually.

Withdrawal of opioid agonists after chronic treatment (either by wash or the addition of an antagonist) markedly enhances the forskolin-stimulated cAMP accumulation. Both the forskolin-induced AC activity (without opioid treatment) and the overshoot activity (following chronic exposure and addition of naloxone) were increased as a function of forskolin concentration (0.1–100 μM). The ratio between the two activities was not affected by forskolin concentration (data not shown), suggesting that the overshoot phenomenon is not due to increased affinity of AC to forskolin. Since the overshoot was observed in the presence of IBMX (which inhibits phosphodiesterase activity) and was much weaker in the absence of this inhibitor (data not shown), it appears to result from up-regulation of AC activity rather than from opioid-induced inhibition of phosphodiesterase.

The degree of AC inhibition and supersensitization by U69593 are concentration-dependent. While application of low concentrations of agonist were sufficient to inhibit the forskolin-stimulated cAMP production, the overshoot phenomenon required pretreatment with higher doses of the drug. This discrepancy suggests that activation of the overshoot phenomenon requires threshold amounts of signaling components to be recruited. The nature of these signaling components remains to be clarified. Nevertheless, this difference in concentration is in agreement with the clinical observations that development of physical dependence requires a prolonged and high-dose opiate treatment [19].

Several cellular mechanisms could account for the overshoot phenomenon. The reduction in cAMP levels during the agonist opiate exposure could initiate a cascade of cellular changes, which could lead to up-regulation of the amount and/or activity of AC [7,20]. Enhanced coupling of the stimulatory PGE_1 receptor to G_s or elevated levels of functionally intact G_s proteins were proposed by Ammer and Schulz [4,5] for opioid-treated NG108-15 and SH-SY5Y cells. Alternatively, we would like to suggest that continuous release of βγ-dimers from G_i or G_o proteins upon prolonged activation of opioid receptors could eventually lead to direct or indirect activation of AC [21,22].

It has been claimed that in SH-SY5Y cells, naloxone blocks the μ-receptor activity by acting as an antagonist with negative intrinsic activity [23]. Our results show that the supersensitivity of AC is induced by the chronic agonist activation and that naloxone only serves as a means to remove the opioid agonism. Agonist wash was as effective as the application of naloxone or other antagonists. In spite of that, chronic agonist occupation of the receptor per se is not sufficient to induce the withdrawal activation of AC. The PTX sensitivity clearly demonstrates that the chronic opioid activation has to be mediated through both opioid receptors and PTX-sensitive G proteins, in order to encode for the elevated cyclase activity observed following the agonist removal.

In summary, using CHO cells transfected with rat κ-opioid receptor, we have shown that acute κ-agonist exposure leads to inhibition of AC, while chronic exposure leads to sensitization of AC activity. These observations are in line with the phenomenon of opiate action, addiction and withdrawal. Moreover, the results generalize the findings for the three different types of opioid receptors and show that the overshoot phenomenon could be observed not only with neuronal cells that express the opioid receptors normally, but also with non-neuronal cells transfected with a defined type of opioid receptor.

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