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Methadone-induced desensitization of the δ-opioid receptor is mediated by uncoupling of receptor from G protein

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Abstract

Chronic exposure of neuroblastoma × glioma (NG108-15) hybrid cells and rat μ-receptor-transfected Chinese hamster ovary (CHO) cells to 10 μM morphine resulted in a compensatory and antagonist-precipitated increase in cAMP accumulation. However, incubation of these cells with 10 μM methadone during chronic exposure to morphine substantially prevented the actions of morphine. Chronic methadone treatment caused a pronounced reduction in agonist-stimulated binding of [^{35}S]GTPγS to G proteins, but it did not produce significant down-regulation of δ-opioid receptors, whereas chronic morphine treatment failed to induce either uncoupling of δ-opioid receptors from G proteins or down-regulation of δ-opioid receptors. In contrast to chronic treatment with morphine alone, treatment of cells with morphine and methadone simultaneously resulted in a significant decrease in agonist-stimulated binding of [^{35}S]GTPγS to G proteins. The action of methadone-mediated uncoupling of the receptor from the G protein was blocked by the nonselective protein kinase inhibitor [1-(5-isoqinolinesulfony)-2-methylpiprazine](H$_7$), but not by the specific protein kinase C inhibitor, chelerythrine. The data demonstrate that methadone desensitizes the δ-opioid receptor by uncoupling the receptor from the G protein. In this way, methadone antagonizes the morphine-mediated adaptive sensitization and overshoot of adenylate cyclase. The functional desensitization of opioid receptors by methadone may explain why methadone is effective in the treatment of morphine dependence.

Keywords: δ-Opioid receptor; cAMP; Desensitization; Down-regulation; Morphine; Methadone

1. Introduction

Neuroblastoma × glioma (NG108-15) hybrid cells are frequently used to study acute and chronic opioid treatment-mediated transmembrane signaling processes, since they express a high level of δ-opioid receptors (Chang and Cuatrecasas, 1979), G proteins and effectors (Milligan et al., 1990). Activation of δ-opioid receptors in NG108-15 cells results in inhibition of adenylate cyclase (Sharma et al., 1975) and voltage-dependent calcium channels (Hescheler et al., 1987). Chronic treatment of the cells with opioid produces at least three distinct adaptation processes: (1) receptor desensitization as characterized by the loss of receptor-mediated inhibition of adenylate cyclase (Law et al., 1982); (2) receptor down-regulation as characterized by the net loss of δ-opioid receptor protein (Law et al., 1983); (3) sensitization of adenylate cyclase as characterized by the appearance of cAMP overshoot phenomenon upon withdrawal of the drug (Sharma et al., 1975; Greenspan and Musacchio, 1984). This increase in intracellular cAMP level is generally accepted to represent a cellular mechanism of drug dependence (Thomas and Hoffman, 1987).

It is generally accepted that the reaction mediated by G protein-coupled receptors reflects an interaction between the receptor and the G protein, and that the efficacy of agonists is primarily determined by the interaction between the receptor and G protein transducers (Kenakin, 1993). One of the first biochemical events after occupation of the receptor by an agonist is stimulation of the exchange of bound GDP for GTP, which can be measured as an increase in the binding of the stable GTP analog [^{35}S]GTPγS to cell membranes (Traynor and Nahorsri,

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1995; Sim et al., 1995). The interaction between the opioid receptor and the G protein in NG108-15 cells has been directly demonstrated as an agonist-stimulated increase in the binding of hydrolysis-resistant [^{35}S]GTPγS to G protein in a concentration-dependent, antagonist-reversible, and pertussis toxin-sensitive manner (Szekeres and Traynor, 1997; Breivogel et al., 1997). It is now generally accepted that opioid stimulation of [^{35}S]GTPγS binding in membranes provides a useful method to examine the receptor activation of G proteins (Selley et al., 1997), and thus the system also provides a suitable method for investigating mechanisms of tolerance and dependence occurring at the level of the receptor-G protein interaction (Breivogel et al., 1997; Elliott et al., 1997).

Methadone and morphine are similar opioid receptor agonists. However, their clinical actions are distinctly different. Methadone has been demonstrated to have a lower dependence potential than morphine (Blake et al., 1997) and is effectively used in the treatment of opioid addiction, whereas morphine induces dependence. Despite the clinical importance of methadone in the treatment of opioid addiction, the mechanisms underlying the efficacy of methadone are poorly understood (Blake et al., 1997). Several studies have shown that δ-opioid receptors may be critical in the development of morphine-induced tolerance and dependence (Abdelhamid et al., 1991; Suzuki et al., 1994; Fundytus et al., 1995). Although morphine and methadone are generally accepted as μ-opioid receptor-preferring agonists, they also interact with δ-opioid receptors (Takemori and Portoghese, 1987; Kristensen et al., 1995; Bot et al., 1997). In a μ-opioid receptor-transfected cell line, methadone has been demonstrated to be distinct from morphine in inducing spontaneous cAMP overshoot and desensitization of opioid receptors (Blake et al., 1997; Yu et al., 1997). We have also found that there is a significant difference between methadone and morphine in the regulation of δ-opioid receptors in NG 108-15 cells although both of them are non-specific δ-opioid receptor agonists (unpublished data). Methadone substantially desensitizes δ-opioid receptors, while morphine fails to have a similar action. Incubation of the cells with methadone during exposure to morphine significantly inhibits the morphine-mediated cellular reaction. It is known that the cellular effects of hormones, neurotransmitters and drugs are regulated at several levels, most notably at the level of the receptor. Even in the continued presence of hormone, neurotransmitter or drug, the cellular response can be attenuated by modulation of agonist affinity, G protein-coupling efficiency, or receptor density (Pak et al., 1996). To investigate the mechanisms of methadone desensitization of δ-opioid receptors and to elucidate the cellular and molecular basis of methadone antagonism of the effect of morphine, we investigated the effects of chronic methadone and morphine treatment on δ-opioid receptors down-regulation and functional uncoupling of δ-opioid receptors from G proteins.

2. Materials and methods

2.1. Materials

Morphine was purchased from Qing-Hai Pharmaceutical Factory (Xi Ning, China); methadone was purchased from Tian-Jing Central Pharmaceutical Factory. [D-Ala^2, D-Leu^5]enkephalin (DADLE), [1-(5-isoqinolinesulfony)-2-methylpiperazine], chelerythrine chloride, and naloxone were purchased from Sigma. ^{3}H-naloxone (1.48 TBq/mmol), and guanosine-5′-*O*-(3-[^{35}S]-thio) triphosphate (GTPγS) (46.3 TBq/mmol) were purchased from Dupont New England Nuclear Research Products (Boston, MA).

2.2. Cell culture and membrane preparation

NG108-15 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal calf serum, antibiotics and 100 μM hypoxanthine, 1 μM aminopterin and 17 μM thymidine (HAT), as described previously (Vachon et al., 1987). Briefly, the cells grew in monolayers to confluence at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The mouse μ-opioid receptor-transfected CHO cell line was a generous gift of Dr. G. Pei (Shanghai Institute of Cell Biology, Shanghai, China). Stably transfected Chinese Hamster ovary (CHO) cells were cultured in the same medium as NG108-15 cells but without HAT, supplemented with 0.25 mg/ml Geneticin (Sigma). Membranes for receptor binding assays were prepared as described previously (Vachon et al., 1987) with some modifications. Briefly, confluent monolayers of NG108-15 cells were rinsed 2–3 times with cold phosphate buffer solution and harvested by gentle agitation in DMEM, followed by centrifugation at $345 \times g$ for 10 min. The pellet was resuspended in 50 volumes of 50 mM Tris–HCl, pH 7.4, with 1 mM EGTA, and was homogenized with a Polytron homogenizer. The homogenate was centrifuged at $29{,}600 \times g$ for 15 min; the pellet was resuspended in the same volume of Tris–HCl buffer and centrifuged a second time. The pellet was finally resuspended in fresh buffer to give a protein concentration of 2.5 mg protein per ml (Bradford, 1976) and immediately used for the binding assay. For the [^{35}S]GTPγS binding assay, membranes were prepared as described previously (Szekeres and Traynor, 1997), with some modifications. Briefly, the cells were homogenized in a buffer of 20 mM HEPES (*N*-2-hydroxyethyl) piperazine-*N*-[2-ethanesulphonic acid], pH 7.4, 100 mM NaCl, 4 mM $MgCl_2$ (buffer A) and homogenized using a Polytron homogenizer. The resultant homogenate was centrifuged at 48,000 $\times g$ for 20 min and washed with buffer A and recentrifuged as before. The pellet was finally resuspended in buffer A to give a protein concentration of 1.25–2.5 mg/ml (Bradford, 1976). All procedures were performed at 0°C to 4°C. To examine the effect of chronic opioid exposure, 5 days after passage morphine (10 μM),

methadone (10 μM), 10 μM morphine and 10 μM methadone (Musacchio and Greenspan, 1986; Liu et al., 1999), or sterile vehicle (water) was added to the medium. Two or 3 days later, the cells were harvested and cell membranes were prepared as above.

2.3. Measurement of cyclic AMP accumulation

Confluent monolayers were treated with vehicle, 10 μM morphine, 10 μM methadone, or 10 μM morphine and 10 μM methadone for 48 h. Following the pretreatment, cells were washed 2–3 times with 3 ml of DMEM. cAMP accumulation was measured over a 10-min incubation period with 10 μM forskolin. The rebound response of cAMP was elicited by the addition of 10 μM naloxone. To prevent the rebound response of cAMP prior to the addition of naloxone, the relevant opiate was added to the washing media. The reaction was terminated by adding 500 μl of ice-cold 20% (wt/vol) trichloroacetic acid directly to the cell culture medium. The cells were then scraped from the wells, transferred to 1.5-ml Eppendorff tubes and centrifuged (700 × *g*, 15 min, 0°C). The supernatants were extracted three times with 5 ml of water-saturated diethyl ether. After evaporation of the residual ether, the cyclic AMP concentration was determined by competitive protein binding assay (Brown et al., 1971), and the protein concentration was determined with Bradford's method (Bradford, 1976).

2.4. Receptor binding assays

Membrane protein (≈ 250 μg) was incubated in Tris–HCl buffer containing 0.01% bovine serum albumin, pH 7.4, with [^{3}H]naloxone (0.5–16 nM) in a final volume of 1 ml for 90 min at 25°C (Selley et al., 1993). The reaction was terminated by rapid filtration through Whatman GF/B glass fiber filters, and the filters were rinsed three times with 3 ml of ice-cold buffer. Bound radioactivity was determined by liquid scintillation counting (37% efficiency). Nonspecific binding was defined as the difference between total binding and binding in the presence of 10 μM naloxone. The B_{max} and K_d values were calculated using the computer program described previously (Zhang et al., 1988).

2.5. [^{35}S]GTPγS binding assay

Cell membranes (≈ 250 μg of protein) were incubated in buffer A containing [^{35}S]GTPγS (0.1 nM) and GDP (100 μM) for 1 h at 30°C in a total volume of 1 ml and in the presence of various concentrations of opioids as described previously (Szekeres and Traynor, 1997). Nonspecific binding was defined with unlabelled [^{35}S]GTPγS (10 μM). Bound and free [^{35}S]GTPγS were separated by vacuum filtration through GF/B filters, which were washed three times with 3 ml of ice-cold buffer A. Radioactivity was quantified by liquid scintillation counting. In data analysis, percent maximal stimulation is defined as (net stimulated binding by agonist/by 10 μM DADLE) × 100%. This parameter was determined for each individual experiment with 10 μM DADLE, and the normalized data were subjected to nonlinear regression analysis to determine EC_{50}.

3. Results

3.1. Antagonism of methadone against morphine adaptive sensitization or overshoot of adenylate cyclase activity

The overshoot of adenylate cyclase activity has been implicated in the cellular mechanism of opioid tolerance and dependence (Thomas and Hoffman, 1987; Avidor-Reiss et al., 1995). The naloxone-precipitated cAMP overshoot in vitro has been proposed to be linked to abstinence in animals (Sharma et al., 1975). Several studies have demonstrated that chronic morphine treatment results in spontaneous and naloxone-precipitated cAMP overshoot in NG108-15 cells (Musacchio and Greenspan, 1986), SH-SY5Y cells (Wang et al., 1994) and μ-opioid receptor-transfected human embryonic kidney 293(HEK 293) cells (Blake et al., 1997). In a previous study, we observed that methadone could antagonize the effect of morphine by desensitization of δ-opioid receptors. To further confirm the results and to investigate the mechanism underlying methadone antagonism against the action of morphine, we examined the effect of methadone on chronic morphine treatment-induced spontaneous and naloxone-precipitated cAMP overshoot in NG108-15 and μ-opioid receptor-transfected CHO cells. As shown in Table 1, pretreatment with morphine for 48 h led to significant spontaneous and naloxone-precipitated cAMP overshoot as compared with the response of untreated cells. No marked overshoot of adenylate cyclase was observed in the cells pretreated with methadone for 48 h. However, incubation of NG108-15 cells and μ-opioid receptor-transfected CHO cells with methadone (10 μM, 48 h), during exposure to morphine (10 μM, 48 h), substantially prevented the morphine-induced spontaneous and naloxone-precipitated cAMP overshoot. To exclude the possibility that the prevention of morphine-induced cAMP overshoot by methadone was caused by its long occupation of opioid receptors due to its high lipophilicity, cells treated with methadone for 48 h were continuously treated with 10 μM morphine for 24 h after removal of methadone by thorough washing, and then cAMP accumulation was measured. Under this condition, morphine also did not cause marked spontaneous and naloxone precipitated cAMP overshoot (Liu et al., 1999). These results suggest that methadone can antagonize the morphine-induced adaptive sensitization or overshoot of

Table 1
Effects of methadone on chronic morphine treatment-induced compensatory and naloxone-precipitated increase in forskolin-stimulated cAMP accumulation in NG108-15 cells and μ-opioid receptor-transfected CHO cells. Cells were treated for 48 h with 10 μM morphine, 10 μM methadone alone or with 10 μM morphine and 10 μM methadone, and the drugs were then removed by thorough washing. cAMP accumulation, stimulated by 10 μM forskolin, was determined by competitive protein binding assay in the absence or presence of 10 μM naloxone. Data are $\bar{x} \pm$ S.D. of triplicate determination from five experiments $^{*}P < 0.05$ vs. control. Statistical significance was determined by paired, or unpaired Student's *t*-test. The values in parentheses reflect the compensatory increase and naloxone-precipitated increase in forskolin-stimulated cAMP accumulation

Treatment (48 h)	NG108-15 cells		CHO cells	
	Naloxone −	Naloxone +	Naloxone −	Naloxone +
cAMP (pmol / min / mg protein)				
Control	172 ± 36	169 ± 21	161 ± 28	149 ± 19
Morphine	225 ± 29* (30.8%)	356 ± 52* (58.2%)	225 ± 46* (33.5%)	367 ± 71* (70.7%)
Methadone	189 ± 41 (9.9%)	224 ± 33 (18.5%)	172 ± 33 (6.8%)	195 ± 29 (13.4%)
Morphine + methadone	181 ± 32 (5.2%)	206 ± 47 (13.8%)	165 ± 32 (2.5%)	184 ± 43 (11.5%)

adenylate cyclase activity by desensitization of δ- and μ-opioid receptor function.

3.2. [^{3}H]naloxone binding to membranes prepared from intact cells exposed chronically to morphine or methadone

We previously demonstrated that there was a difference between morphine and methadone in inducing functional desensitization of δ-opioid receptors in NG108-15 cells. It has been shown that receptor down-regulation is one of the mechanisms responsible for the desensitization of opioid receptors (Law et al., 1983; Vachon et al., 1987; Pak et al., 1996). In order to investigate whether the difference between morphine and methadone in causing desensitization of δ-opioid receptors is due to their different ability to induce down-regulation of δ-opioid receptors, the properties of δ-opioid receptor binding were studied using [^{3}H]naloxone in membranes derived from cells pretreated for 48 h with 10 μM morphine or 10 μM methadone. Significant alteration in the binding of [^{3}H]naloxone to NG108-15 cell membranes was not observed during chronic exposure to morphine, methadone alone or the two drugs in combination (Fig. 1). Chronic morphine or methadone treatment did not result in a substantial reduction in B_{max} and change in K_d. Analysis of saturation experiments showed that [^{3}H]naloxone bound to the δ-opioid receptor in membranes derived from naive cells with a K_d value of 2.9 ± 0.6 nM and a B_{max} value of 82 ± 12 fmol/mg protein. The specific [^{3}H]naloxone binding to δ-opioid receptors in membranes derived from cells treated chronically with morphine, methadone alone or the combination of morphine and methadone was $95.1 \pm 6.7\%$, $89.0 \pm 5.4\%$ and $91.5 \pm 3.7\%$ of that in control cells, respectively. The affinity of [^{3}H]naloxone bound to δ-opioid receptors in membranes prepared from cells treated chronically with morphine, methadone or the combination of morphine and methadone also did not change significantly compared with that in control cells. We conclude that there is no significant difference between morphine and methadone in their ability to induce down-regulation of δ-opioid receptors, and that the methadone-induced functional desensitization of the δ-opioid receptor in NG108-15 cells is not mediated by down-regulation of δ-opioid receptors.

3.3. Effects of morphine and methadone on [^{35}S]GTPγS binding

The δ-opioid receptor belongs to the superfamily of G-protein-coupled receptor that inhibits adenylate cyclase through the activation of G_i/G_o-type G proteins (Childers, 1991; Evans et al., 1992; Reisine and Bell, 1993). G protein-coupled receptors have been shown to adapt to the presence of chronic agonist exposure by means of two adaptive mechanisms that involve the functional uncoupling of the receptors from G proteins and the net loss of receptor protein (Law et al., 1983; Nestler, 1993; Raynor et al., 1994). We demonstrated that chronic treatment of cells with morphine or methadone did not cause obvious down-regulation of δ-opioid receptors. Therefore, we speculated that the uncoupling of δ-opioid receptors from G proteins might be an important mechanism responsible

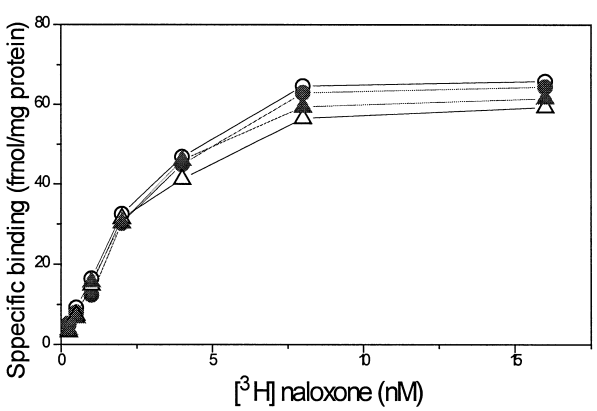

Fig. 1. Effects of chronic morphine or methadone treatment on saturation binding of [^{3}H] naloxone to NG108-15 cell membranes. Membranes from naive cells (-○-) and cells treated for 48 h with 10 μM morphine (-●-), 10 μM methadone (-△-) alone or with 10 μM morphine and 10 μM methadone (-▲-) were incubated in Tris–HCl buffer (pH 7.4, 50 mM) with [^{3}H] naloxone at various concentrations in a final volume of 1 ml for 90 min at 25°C. Data are $\bar{x} \pm$S.D. of three separate experiments performed in duplicate.

for the functional desensitization of δ-opioid receptors. To confirm this speculation, we examined the effects of acute and chronic methadone or morphine treatment on the opioid activation of G proteins. In naive cells, morphine, methadone and DADLE, a selective agonist of the δ-opioid receptor, stimulated the binding of [^{35}S]GTPγS (0.1 nM) to membranes in a concentration-dependent manner (Fig. 2A, B and C). Because DADLE was the most efficacious agonist examined, the efficacy of all agonists was expressed as percentage of the maximal stimulation produced by DADLE. Pretreatment of cells for 48 h with 10 μM morphine did not result in an obvious rightward shift of the curve for morphine, methadone or DADLE stimulation of the binding of [^{35}S]GTPγS to membranes (Fig. 2A, B and C). No significant changes in the value of EC_{50} and percent maximal stimulation were observed (Table 2). In contrast to chronic treatment with morphine, pretreatment of cells for 48 h with 10 μM methadone resulted in a marked rightward shift of the curve for morphine, methadone or DADLE stimulation of the binding of [^{35}S]GTPγS to membranes (Fig. 2A, B and C), with there being a significant increase in the value of EC_{50} and an obvious reduction in the percent maximal stimulation (Table 2; Fig. 2A, B and C). A similar increase in EC_{50} value and decrease in percent maximal stimulation for agonist-stimulated [^{35}S]GTPγS bound to membranes was also observed in membranes isolated from cells co-pretreated chronically with morphine and methadone (Table 2; Fig. 2A, B and C).

3.4. Inhibition of methadone treatment-induced uncoupling of δ-opioid receptors from G proteins by H_7 but not by chelerythrine

To investigate the mechanism of the uncoupling of δ-opioid receptors from G proteins induced by methadone, we examined the ability of H_7, a mixed of inhibitor of cAMP-dependent protein kinase and protein kinase C (chelerythrine), a specific inhibitor of protein kinase C, to block methadone-induced uncoupling of δ-opioid receptors from G proteins. As shown in Fig. 3, incubation of NG108-15 cells with H_7 (50 μM) (Elliott et al., 1997), a mixed of inhibitor of cAMP-dependent protein kinase and protein kinase C, during chronic exposure to either methadone alone (10 μM, 48 h) or the combination of methadone and morphine (10 μM of each, 48 h), largely prevented the reduction in agonist modulation of [^{35}S]GTPγS binding. H_7 treatment alone did not alter the maximum stimulation of [^{35}S]GTPγS binding by morphine and methadone, nor did acute H_7 treatment have any effect on the response of the [^{35}S]GTPγS binding to membranes stimulated by morphine or methadone. The results are in agreement with those observed for SH-SY5Y cells. (Elliott et al., 1997). However, incubation of NG108-15 cells with chelerythrine (10 μM), a specific inhibitor of protein kinase C, during exposure of the cells to either methadone alone (10 μM, 48 h) or the combination of methadone and morphine (10 μM of each, 48 h), did not significantly

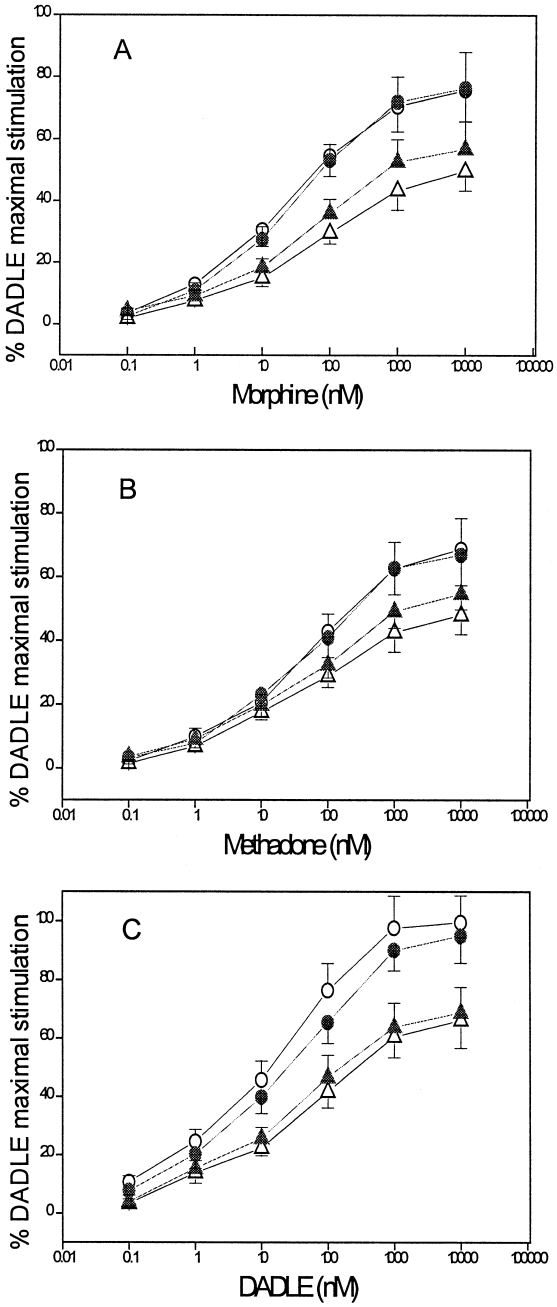

Fig. 2. Concentration–effect curves for the stimulation of [^{35}S]GTPγS binding to membranes prepared from different opioid-treated NG108-15 cells by morphine (A), methadone (B) and DADLE (C). Membranes were incubated with 0.1 nM [^{35}S]GTPγS, 100 μM GDP and opioids at various concentrations for 1 h at 30°C in a total volume of 1 ml. Basal binding of [^{35}S]GTPγS was 56.5±7.7 fmol/mg protein and the binding of [^{35}S]GTPγS stimulated by DADLE (10 μM) was 132.8±12.4 fmol/mg protein. Data are $\bar{x}$±S.D. of percent stimulation produced by 10 μM DADLE. (-○-): membranes from untreated NG108-15 cells. (-●-): membranes from cells treated with morphine for 48 h, (-△-): membranes from cells treated with methadone for 48 h, (-▲-): membranes from cells treated with combination of 10 μM morphine and 10 μM methadone.

Table 2
Effects of chronic methadone or morphine alone treatment or methadone and morphine co-treatment on efficacy and potency of opioids for stimulating [^{35}S]GTPγS binding to NG108-15 cell membranes. Membranes derived from naive cells and cells treated for 48 h with 10 μM morphine, 10 μM methadone alone or with 10 μM morphine and 10 μM methadone were incubated with opioid receptor agonist at various concentrations in the presence of 0.1 nM [^{35}S]GTPγS and 100 μM GDP. Percent maximal stimulation was measured as the stimulation of binding in response to DADLE (10 μM). Data are $\bar{x} \pm$ S.D. from three or more separate experiments performed in duplicate

Agonist treatment (48 h)	DADLE		Morphine		Methadone	
	EC_{50} (nM)	Maximum stimulation (%)	EC_{50} (nM)	Maximum stimulation (%)	EC_{50} (nM)	Maximum stimulation (%)
Control	5.7 ± 0.8	100 ± 9.1	162.8 ± 28	75.6 ± 9.9	447 ± 56	68.8 ± 9.6
Morphine (Mor)	19.1 ± 3.4	94.9 ± 9.2	185.5 ± 25	76.3 ± 11.7	500 ± 74	66.9 ± 9.5
Methadone (Met)	494 ± 68	66.1 ± 9.6	3165.5 ± 386	49.6 ± 6.3	3174.2 ± 496	47.9 ± 5.9
Mor + Met	319 ± 48	68.7 ± 8.9	1427.1 ± 189	56.8 ± 8.8	1911.1 ± 217	54.6 ± 4.7

prevent the reduction in agonist modulation of [^{35}S]GTPγS binding. These results suggest that cAMP-dependent protein kinase but not protein kinase C is involved in the methadone-induced uncoupling of δ-opioid receptors from G proteins.

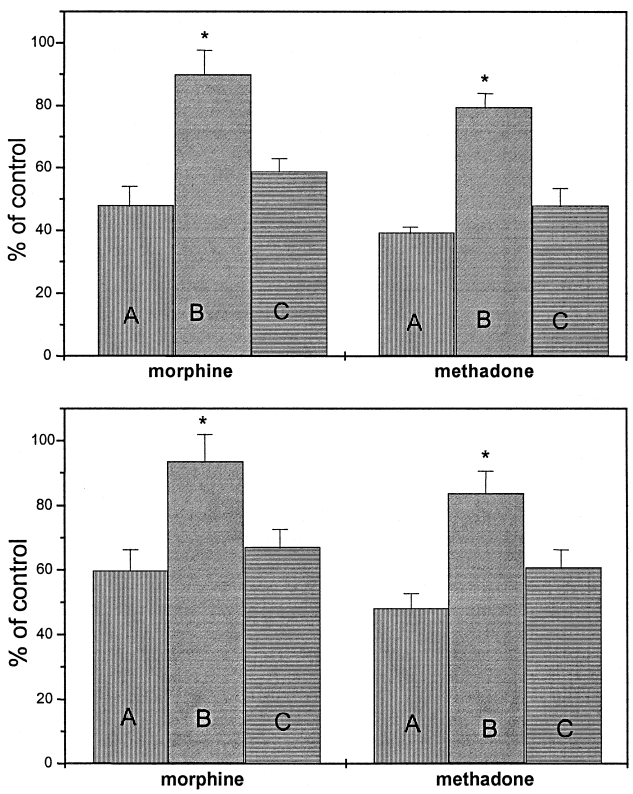

Fig. 3. Effects of protein kinase inhibitor H_7 and chelerythrine on methadone chronic treatment-induced decrease in stimulation of [^{35}S]GTPγS binding by morphine and methadone itself. Membranes from NG108-15 cells treated for 48 h with methadone alone or with the combination of methadone and morphine were incubated with 0.1 nM [^{35}S]GTPγS and 100 μM GDP for 1 h at 30°C. Upper panel: intact cells treated with 10 μM methadone alone (A) or in the presence of 50 μM H_7 (B) or 5 μM chelerythrine (C). Lower panel: intact cells treated with 10 μM methadone and 10 μM morphine (A), or in the presence of 50 μM H_7 (B) or 5 μM chelerythrine (C). Values are given as percentages of the control (maximal response to each agonist in naive cells) and represent $\bar{x} \pm$S.D. from four separate experiments performed in duplicate.

4. Discussion

Opioid dependence is characterized by an enhanced neuronal excitability toward stimulatory input (Collier, 1980). The underlying cellular mechanism involves up-regulation of the cAMP second messenger system (Nestler, 1993), which results from sensitization of adenylate cyclase activity (Johnson and Fleming, 1989; Reisine and Bell, 1993). A recent study of the cloned rat μ-opioid receptor suggested that the physiological consequence of receptor sensitization, rather than receptor desensitization, may be critical to tolerance development (Avidor-Reiss et al., 1995). Chronic morphine treatment-induced adaptive sensitization or overshoot of adenylate cyclase activity has been observed in NG108-15 cells (Musacchio and Greenspan, 1986), SK-N-SH cells (Wang et al., 1994) and μ-opioid receptor-transfected CHO (Avidor-Reiss et al., 1995), HEK293 cells (Blake et al., 1997), as well as δ-opioid receptor-transfected HEK 293 cells (Bot et al., 1997). The results of the present study confirm these findings. Chronic morphine treatment was demonstrated to result in a compensatory increase in forskolin-stimulated cAMP accumulation (spontaneous cAMP overshoot) and the addition of naloxone led to a further increase in cAMP accumulation (naloxone-precipitated cAMP overshoot), but chronic methadone treatment failed to cause substantial spontaneous and naloxone precipitated cAMP overshoot. Furthermore, incubation with methadone during chronic exposure to morphine substantially prevented morphine-induced spontaneous and naloxone-precipitated cAMP overshoot. Buprenorphine has been reported to have a similar effect (Blake et al., 1997). These results suggest that methadone can prevent chronic morphine treatment-induced adaptive sensitization or overshoot of adenylate cyclase activity. We have previously demonstrated that methadone desensitizes the opioid receptor. In this study, desensitization was examined at the level of the activation

of G proteins. The development of agonist-stimulated [^{35}S]GTPγS binding assay with membranes offers the opportunity to study the direct coupling of opioid to the activation of G proteins. We used this technique to investigate the mechanism of methadone-induced δ-opioid receptor desensitization. In untreated cell membranes, DADLE, methadone and morphine were able to stimulate the binding of [^{35}S]GTPγS to membranes in a concentration-dependent manner. Incubation of the cells with methadone for 48 h led to a decrease in the ability of subsequently added morphine, methadone and DADLE to stimulate the binding of [^{35}S]GTPγS to G proteins, but no reduction in agonist-stimulated binding of [^{35}S]GTPγS to G proteins was observed in the membranes from chronic morphine-treated cells. However, a significant decrease in agonist-stimulated binding of [^{35}S]GTPγS to G protein was also observed in the membranes from methadone and morphine-cotreated cells, suggesting that methadone can antagonize the action of morphine by uncoupling δ-opioid receptors from G proteins. The loss of agonist activity following chronic exposure of the cells to methadone, or the combination of methadone and morphine, was not accompanied by a substantial reduction in the number of δ-opioid receptor binding sites, suggesting that receptor down-regulation is not the mechanism of desensitization, and that desensitization results from a decrease in the number of G proteins being activated. We have previously shown that H_7 but not chelerythrine significantly inhibits chronic methadone treatment-induced desensitization of δ-opioid receptors. The present study showed that H_7 but not chelerythrine prevented the decrease in the binding of [^{35}S]GTPγS to G proteins induced by chronic methadone treatment, suggesting that cAMP-dependent protein kinase may be involved in the uncoupling of δ-opioid receptors from G proteins, and that the desensitization of δ-opioid receptors by methadone is caused by uncoupling of δ-opioid receptors from G proteins. These results support the evidence that receptor phosphorylation is a key step in agonist-induced receptor desensitization (Childers, 1991; Nestler et al., 1993; Pei et al., 1995; Zhang et al., 1996; Hasbi et al., 1998).

In the light of our present and previous studies, we conclude that there is a significant difference between morphine and methadone in the regulation of opioid receptor function. Chronic methadone treatment results in substantial desensitization of opioid receptors, whereas chronic morphine treatment fails to desensitize opioid receptors. The desensitization of δ-opioid receptors by methadone is caused by uncoupling of δ-opioid receptors from G proteins but not by down-regulation of δ-opioid receptors. The cAMP-dependent protein kinase-catalyzed receptor phosphorylation may be an important mechanism of methadone-induced desensitization of δ-opioid receptors. The functional desensitization of opioid receptors by methadone is the critical cellular event responsible for the efficacy of methadone in the treatment of opioid addiction. By desensitization of δ-opioid receptor function, methadone can interfere with the effects initiated by morphine, even in its continued presence, thereby interrupting the cascade of cellular events, avoiding the opioid receptor sensitization induced by chronic exposure to opiates. In this way methadone can prevent the chronic morphine treatment-induced spontaneous and naloxone-precipitated cAMP overshoot.

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