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The difference between methadone and morphine in regulation of δ-opioid receptors underlies the antagonistic effect of methadone on morphine-mediated cellular actions

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Abstract

To investigate the cellular and molecular basis for using methadone in substitution therapy for morphine addiction, the difference between methadone and morphine in causing desensitization of δ-opioid receptors was examined, and the effects of methadone pretreatment on opiate-induced inhibition of forskolin-stimulated cAMP accumulation was studied. Methadone substantially attenuated the ability of [D-Ala^2,D-Leu^5]enkephalin (DADLE), morphine and methadone to inhibit forskolin-stimulated cAMP accumulation. Methadone was able to block the morphine-induced compensatory increase in intracellular cAMP levels and naloxone-precipitated cAMP overshoot after chronic exposure to morphine. The protein kinase inhibitor (1-5-isoquinolinesulfony)-2-methylpiperazine) (H_7) could significantly block the chronic methadone treatment-induced loss of the ability of DADLE to inhibit adenylate cyclase. The protein kinase inhibitor chelerythrine was able to block the acute methadone treatment-induced loss of the ability of DADLE to inhibit adenylate cyclase. In contrast, morphine did not cause a substantial desensitization of the δ-opioid receptor. These results indicate that methadone is different from morphine in its regulation of the δ-opioid receptor. In addition, these results also indicate that the mechanisms of δ-opioid receptor desensitization induced by acute and chronic methadone treatment are different.

Keywords: δ-Opioid receptor; Methadone; Morphine; Desensitization; Phosphorylation; Protein kinase inhibitor

1. Introduction

Methadone has been used for the maintenance and detoxification of opioid addicts since 1965 (Dole and Nyswander, 1965). As a substitute for opiate in illicit use, methadone is highly effective in preventing of withdrawal symptoms and in helping people to reduce their craving for opioids (Greenstein et al., 1984; Strain et al., 1993). Methadone, like morphine, is a μ-opioid receptor-preferring agonist (Reisine and Pasternak, 1996). Whereas morphine induces dependence, methadone is used in the treatment of opioid addiction. Despite the clinical importance of methadone in the treatment of opioid addiction, relatively little is known about the molecular and cellular events induced by methadone. Also the cellular events that accompany the therapeutic action of methadone in the treatment of addiction are poorly understood (Blake et al., 1997). Study of these problems may offer potential molecular insights into the cellular basis of the substitution therapy for opioid addiction.

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). Several studies have suggested 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). Considerable evidence has accumulated that opioid treatment of NG108-15 cells that endogenously express the mouse δ-opioid receptor activates at least two separate cellular adaptation processes. One is opioid receptor desensitization, which is characterized by the loss of opiate agonist ability to regulate cyclic AMP levels. The other is receptor down-regulation, which is characterized

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by a decrease in the number of binding sites without apparent alteration in the dissociation constant (Law et al., 1982, 1983; Vachon et al., 1987b; Louie et al., 1990; Cai et al., 1996). Receptor desensitization is one of the cellular mechanisms that may play a significant role in neuroadaptive processes (Zhang et al., 1996). The actions of opioid agonists on opioid receptor desensitization may be different and related to the ability to cause the development of tolerance and dependence.

In this study, we examined the difference between methadone and morphine in causing δ-opioid receptor desensitization, studied the alteration of the ability of morphine to inhibit forskolin-stimulated cAMP accumulation induced by methadone pretreatment, and explored the possible mechanism of methadone-induced δ-opioid receptor desensitization.

2. Materials and methods

2.1. Materials

Morphine hydrochloride 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), naloxone hydrochloride, forskolin, (1-(5-isoquinolinesulfonyl)-2-methylpiperazine), chelerythrine chloride were purchased from Sigma. cAMP assay kit was purchased from the Chinese Academy of Atomic Energy Sciences.

2.2. Cell culture

The mouse neuroblastoma × rat glioma hybrid cell line, NG108-15, was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum, 0.1 mM hypoxanthine, 10 μM aminopterin, 17 μM thymidine, 2 mM glutamine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a humidified atmosphere of 5% CO_2 and 95% air. Experiments were routinely carried out with confluent cultures, passage numbers 20–30, 3–5 days after seeding in 17-mm plates.

2.3. Measurement of cyclic AMP accumulation

Confluent monolayers were treated with vehicle, different opiates or opiates plus protein kinase inhibitor for different periods as described in the legends of the respective figures. After pretreatment, the cells were washed 2–3 times with 3 ml DMEM. cAMP accumulation was measured after a 10-min incubation period with 10 μM forskolin in the presence or absence of different concentrations of DADLE or morphine. 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 washing media contained the relevant opiate. 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 well, transferred to 1.5-ml Eppendorff tubes and centrifuged ($700 \times g$, 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 a competitive protein binding assay, and the protein concentration was determined with the method of Bradford (1976).

2.4. Preparation of cell membranes for adenylate cyclase assay

The naive or opiate-treated cells were harvested and homogenized in Tris–HCl (50 mM, pH 7.4) containing 1 mM EGTA with a polytron. The homogenate was centrifuged at $500 \times g$, 0°C for 10 min. The pellet was resuspended in Tris–EGTA and centrifuged at $500 \times g$ for 10 min again. The two supernatants were combined and centrifuged at $22\,500 \times g$, 0°C for 30 min. The pellet was resuspended in the original volume of the buffer and centrifuged at $22\,500 \times g$, 0°C for 30 min again. The pellet was finally resuspended in Tris–EGTA at a final concentration of 1 mg of protein/ml and aliquots were stored at −70°C.

2.5. Adenylate cyclase assay

NG108-15 cell membranes were incubated with or without various drugs at 32–35 for 10 min as described by Vachon et al. (1987a). The assay system contained 50 mM Tris–HCl, pH 7.4, 0.2 mM EGTA, 0.2 mM D,L-dithiotheitol, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM ATP, 5 mM phosphocreatine (Sigma), 10 units of creatine phosphokinase (Sigma) in a final volume of 100 μl. The reaction was terminated by boiling in a water bath for 2 min. The amount of cAMP generated was measured by the method described above. Adenylate cyclase activity is expressed as pmol of cAMP per mg of protein per min.

3. Results

3.1. Differential effects of methadone and morphine on DADLE-mediated inhibition of forskolin-stimulated cAMP accumulation in intact cells

To examine whether there is difference between methadone and morphine in causing desensitization of δ-opioid receptors, we observed the effect of methadone and morphine on the inhibition induced by DADLE, a δ-opioid receptor-selective agonist, of forskolin-stimulated cAMP accumulation. As shown in Fig. 1, DADLE caused a concentration-dependent inhibition of forskolin-stimulated intracellular cAMP accumulation in untreated cells,

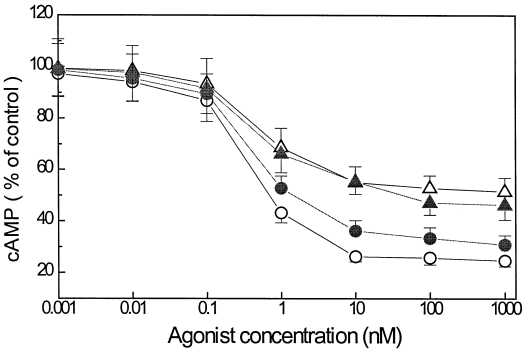

Fig. 1. Differential effect of morphine and methadone on DADLE inhibition of forskolin-stimulated cAMP accumulation. Cells were either untreated (○) or treated with 10 μM morphine (●) or 10 μM methadone (△) or with 10 μM morphine in combination with methadone (▲) for 48 h and then exposed to 100 nM DADLE in fresh medium for 10 min after removal of the pretreatment drugs by aspiration and thorough washing. cAMP accumulation, stimulated by 10 μM forskolin, was determined by competitive protein binding assay. The values represent means ± S.D. of triplicate determinations from four experiments.

whereas pretreatment of cells with 10 μM methadone substantially decreased the ability of DADLE to inhibit forskolin-stimulated cAMP accumulation, with a marked increase in the EC_{50} for the DADLE-induced inhibition of forskolin-stimulated cAMP accumulation (EC_{50}: untreated, 5.8 ± 0.6 nM; treated, 114 ± 18 nM) and a reduction in the maximal level of inhibition (from 75.4 ± 8.6% in untreated cells to 48.5 ± 5.3% in treated cells). These results suggest that the δ-opioid receptor underwent significant desensitization. A 48-h pretreatment of cells with 10 μM morphine failed to produce a pronounced decrease in the ability of DADLE to inhibit forskolin-stimulated cAMP accumulation, suggesting that morphine did not induce a substantial desensitization of δ-opioid receptors. This is in agreement with studies previously reported (Benalal and Barch, 1985; Vachon et al., 1987b; Bot et al., 1997). However, a 48-h pretreatment with 10 μM morphine and 10 μM methadone produced a substantial desensitization similar to that induced by methadone alone, with an increase in the EC_{50} value from 5.8 ± 0.6 nM in morphine-treated cells to 74 ± 23 nM in morphine and methadone-treated cells. These results further indicate that methadone can effectively decrease the morphine-induced inhibition of forskolin-stimulated cAMP accumulation by desensitizing δ-opioid receptors to morphine.

3.2. Different effects of methadone and morphine pretreatment on DADLE-mediated inhibition of adenylate cyclase

In order to further confirm the difference between methadone and morphine in their desensitization of the δ-opioid receptor, we further observed the action of acute and chronic methadone or morphine treatment on DADLE-mediated inhibition of adenylate cyclase. Membranes derived from NG108-15 cells pretreated with methadone or morphine for 1 h (acute treatment) or 48 h (chronic treatment)were used to assay adenylate cyclase activity. Fig. 2 shows that pretreatment for 1 or 48 h with 10 μM methadone resulted in a similar loss of responsiveness of adenylate cyclase to δ-opioid receptor stimulation. In contrast, pretreatment for 1 or 48 h with 10 μM morphine could not desensitize the δ-opioid receptor-adenylate cyclase system. These results agree well with those obtained from intact cells. Chelerythrine, a specific protein kinase C inhibitor, significantly inhibited the desensitization of δ-opioid receptors induced by acute methadone treatment, but it was unable to block the desensitization induced by chronic methadone treatment. In contrast, H_7, a mixed inhibitor of cyclic AMP-dependent protein kinase and protein kinase C, could fully block the desensitization produced by chronic methadone pretreatment, but it only partially blocked the desensitization produced by acute pretreatment with methadone. These results suggest that the mechanisms underlying the devel-

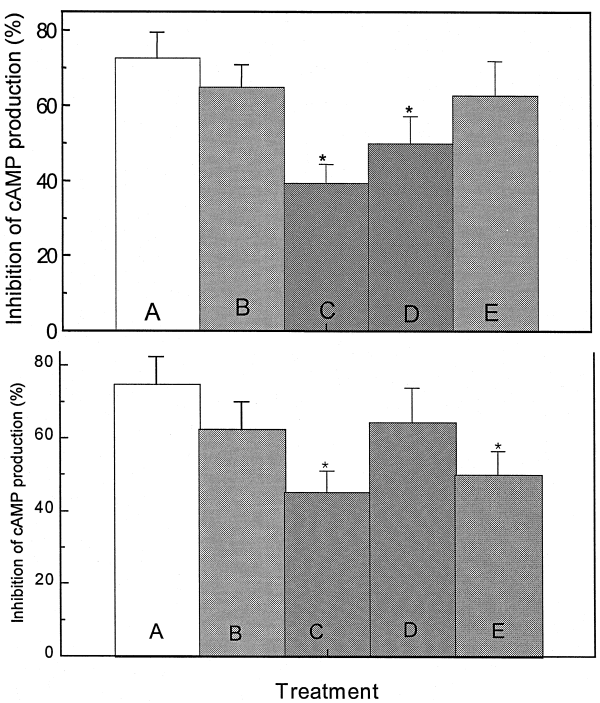

Fig. 2. Differential effects of protein kinase inhibitor H_7 and chelerythrine on methadone-induced acute and chronic desensitization of δ-opioid receptors in NG108-15 cells. Intact cells were either untreated (A) or treated with 10 μM morphine (B) or 10 μM methadone alone (C), or 10 μM methadone and 10 μM H_7 (D), 10 μM methadone or/and 5 μM chelerythrine (E) for 1 h (upper panel) or 48 h (lower panel). Adenylate cyclase activity in membranes was assayed in the presence of 100 nM DADLE and 10 μM forskolin. Reactions were carried out at 32–35°C for 10 min, and terminated by boiling in a water bath for 2 min. The amount of cAMP generated was measured by competitive protein binding assay. The values represent means ± S.D. of quadruplicate determinations from four experiments. *: $P < 0.05$ compares with the untreated cell values by Student's *t*-test.

opment of acute and chronic desensitization of δ-opioid receptors are different.

3.3. Inhibition of chronic methadone treatment-induced opioid receptor desensitization by protein kinases inhibitor

Studies from several laboratories have demonstrated that agonist-induced opioid receptor desensitization may be correlated with receptor phosphorylation (Chen and Yu, 1994; Fukushima et al., 1994; Terwilliger et al., 1994). In order to investigate the mechanism of methadone desensitization of δ-opioid receptors, we examined the ability of H_7 and chelerythrine, to block methadone-induced δ-opioid receptor desensitization. As observed above, methadone desensitized the δ-opioid receptor (Fig. 3). A 48-h pretreatment of cells with 10 μM methadone resulted in a clear increase in the EC_{50} for the methadone-mediated inhibition of forskolin-stimulated cAMP accumulation (from 192.5 ± 29 nM in control cells to 2867.4 ± 347 nM in treated cells), as well as a marked reduction in the level of maximal inhibition (from 67.1 ± 5.6% in control cells to 46.2 ± 3.4% in treated cells). Pretreatment of the cells for 48-h with 10 μM methadone and 10 μM H_7 significantly inhibited the methadone-induced desensitization (EC_{50} from 2867.4 ± 347 nM in cells treated with methadone alone to 388.5 ± 45 nM in cells treated with methadone and H_7). However, pretreatment of cells with 5 μM chelerythrine and 10 μM methadone for 48 h did not result in substantial blockade of the δ-opioid receptor desensitization (EC_{50}: cells treated with methadone and chelerythrine, 1602 ± 291 nM; cells treated with methadone alone, 2867.4 ± 347 nM). The results were consistent with those obtained with membranes derived from chronic methadone-treated cells. These results clearly demonstrated the involvement of cyclic AMP-dependent protein kinase in the chronic desensitization of δ-opioid receptors.

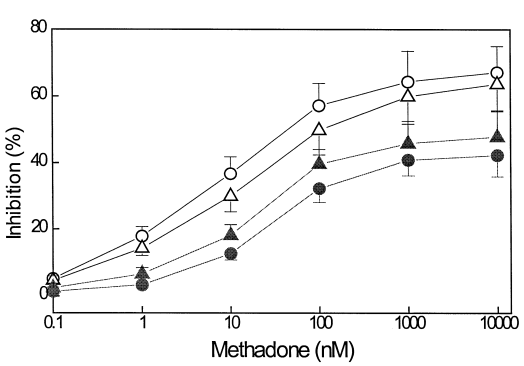

Fig. 3. Effects of protein kinase inhibitor H_7 or chelerythrine on the chronic methadone pretreatment-induced decrease in inhibition of forskolin-stimulated cAMP accumulation. Cells were either untreated (○) or pretreated with 10 μM methadone alone (●) or with 10 μM methadone and 10 μM H_7 (△) or with 10 μM methadone and 5 μM chelerythrine for 48 h (▲). The drugs were then removed by thorough washing and cAMP accumulation was determined as previously described in the presence of 10 μM forskolin. The values represent means ± S.D. of triplicate determinations from three experiments.

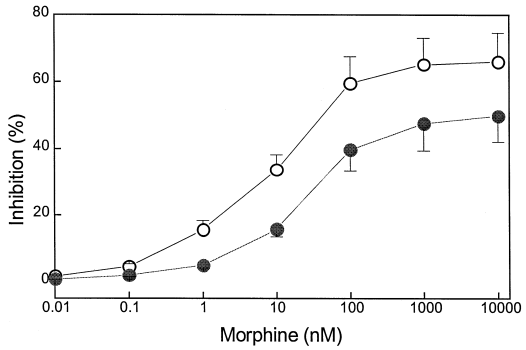

Fig. 4. Effect of chronic pretreatment with methadone on morphine-induced inhibition of forskolin-stimulated cAMP accumulation. Cells were either untreated (○) or pretreated with 10 μM methadone for 48 h (●). The drug was then removed by thorough washing, and cells were exposed to different concentrations of morphine in the medium for 10 min. cAMP accumulation, stimulated by 10 μM forskolin, was then determined by competitive protein binding assay. The values represent means ± S.D. of triplicate determinations from five experiments.

3.4. Effect of methadone pretreatment on morphine-mediated inhibition of forskolin-stimulated cAMP accumulation

To further confirm the observation that methadone was able to block the effects initiated by morphine, we compared the ability of increasing concentrations of morphine to inhibit forskolin-stimulated cAMP accumulation in naive and chronic methadone-treated cells. As shown in Fig. 4, morphine inhibited forskolin-stimulated cAMP accumulation in naive cells in a dose-dependent manner. However, the ability of morphine to inhibit cAMP accumulation was markedly attenuated in the cells treated with 10 μM methadone for 48 h, as indicated by a rightward shift of the dose–response curve of morphine against forskolin-stimulated cAMP accumulation. The EC_{50} value of morphine for inhibition of forskolin-stimulated cAMP accumulation increased from 212 ± 33 nM in naive cells to 1767 ± 217 nM in methadone-treated cells.

3.5. Effects of methadone on time course of the spontaneous and naloxone-precipitated cAMP overshoot induced by morphine

Chronic exposure of NG108-15 cells to morphine causes a marked rebound of cAMP levels upon withdrawal of morphine or challenge with naloxone, a response which is proposed to be linked to abstinence in animals (Sharma et al., 1975). To investigate the cellular mechanisms by which methadone is effective in the treatment of morphine addiction, we examined the effect of methadone on the time course of the spontaneous and naloxone-precipitated cAMP overshoot induced by morphine. Pretreatment of

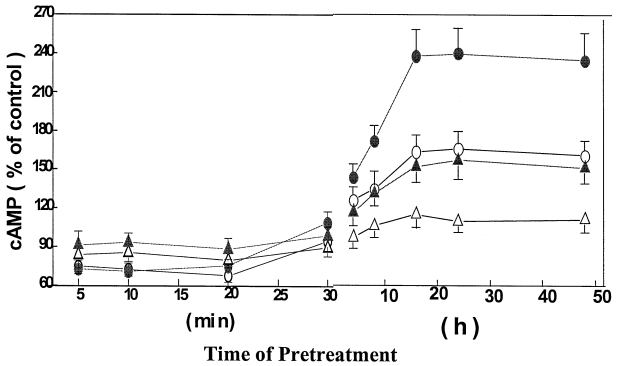

Fig. 5. Effect of methadone on the time course of the spontaneous and naloxone-precipitated cAMP overshoot induced by morphine. NG108-15 cells were pretreated for up to 48 h with 10 μM morphine alone (○,●) or with 10 μM morphine and 10 μM methadone (△,▲) and the drug was then removed at the indicated time by thorough washing. cAMP accumulation, stimulated by 10 μM forskolin, was determined by competitive protein binding assay in the absence (open symbols) or presence of 10 μM naloxone(solid symbols). The values represent means ± S.D. of quadruplicate determinations from four experiments.

cells with 10 μM morphine for 20 min or less caused a slight decrease in forskolin-stimulated cAMP accumulation, which was not reversed by the addition of naloxone (Fig. 5). A significant increase in cAMP accumulation was observed after pretreatment of the cells with 10 μM morphine for more than 4 h. Addition of 10 μM naloxone led to a further increase in the level of forskolin-stimulated cAMP accumulation. However, when cells were treated with 10 μM morphine in combination with 10 μM methadone, the forskolin-stimulated spontaneous increase in intracellular cAMP level and the naloxone-precipitated further increase in the level of intracellular cAMP were reversed. These results imply that methadone is capable of blocking the compensatory increase in intracellular cAMP and naloxone-precipitated cAMP overshoot observed after chronic treatment with morphine.

4. Discussion

In the current study, the functional activity of morphine and methadone was examined at mouse δ-opioid receptors which are endogenously expressed in NG108-15 cells. A major finding of this study was the demonstration of a difference between methadone and morphine in the regulation of the mouse δ-opioid receptor. Methadone, which is currently used in the treatment of addiction, substantially desensitized the δ-opioid receptor, whereas morphine, a highly addictive opiate, barely caused desensitization of the δ-opioid receptor. The observation that morphine fails to desensitize the δ-opioid receptor expressed in NG108-15 cells is supported by other findings (Benalal and Barch, 1985; Vachon et al., 1987b). The resistance of the opioid receptor to morphine desensitization has also been observed with the mouse δ-and μ-opioid receptors expressed in human embryonic kidney 239 cells (Blake et al., 1997; Bot et al., 1997). The inability of morphine to desensitize the opioid receptor suggests that morphine can cause long-term activation of the opioid receptor. Morphine dependence may be a consequence of continued activation of the opioid receptor, with subsequent long-term cellular changes in the nervous system. Chronic exposure to opiates has been shown to elicit adaptation in some of the intracellular signal transduction pathways. An up-regulation of the cAMP pathway is known to be an important adaptive changes induced by chronic exposure to opiate and is related to tolerance and dependence, which have been demonstrated at the level of individual neurons (Self and Nestler, 1995; Nestler, 1996). Chronic exposure of NG108-15 cells and SH-SY5Y cells, which express abundant μ-opioid receptors, to morphine (Wang et al., 1994) leads to spontaneous cAMP overshoot and naloxone-precipitated cAMP overshoot. The spontaneous and naloxone-precipitated cAMP overshoot is markedly blocked by the addition of methadone. Buprenorphine, a promising anti-addiction agent for substitution therapy, has also been demonstrated to desensitize μ-opioid receptors and to block the compensatory increase in cAMP level (Blake et al., 1997) and naloxone-precipitated cAMP overshoot (Liu et al., 1999). The ability of methadone and buprenorphine to desensitize δ and μ-opioid receptors functionally may be critical for the efficacy of these opioids in the treatment of addiction. The obvious desensitization of opioid receptors can allow methadone to interfere with the effects initiated by morphine, even in its continued presence, thereby interrupting a cascade of cellular events and avoiding the prolonged neurochemical alteration induced by chronic exposure to morphine.

The mechanism of opioid receptor desensitization induced by opioid has been reported to involve receptor phosphorylation (Yu et al., 1997) for example, agonist-induced desensitization of any receptor coincides with phosphorylation of the receptor itself (Pei et al., 1995). Recently, protein kinase C and some G protein-coupled receptor kinases and β-adrenoceptor kinase-1 have been demonstrated to be involved in acute opioid desensitization (Louie et al., 1990; Fukushima et al., 1994; Cai et al., 1996). However, up to now little is known about the mechanism of chronic opioid desensitization. This study provides the first direct evidence that the chronic desensitization induced by methadone may involve cyclic AMP-dependent protein kinase, which has been demonstrated not to contribute to the acute desensitization induced by opioid agonist (Pei et al., 1995; Hasbi et al., 1998). The desensitization induced by chronic methadone treatment was significantly blocked by H_7, an inhibitor of cyclic AMP-dependent protein kinase and protein kinase C, but not by chelerythrine, a specific protein kinase C inhibitor. In contrast, chelerythrine could markedly block the desensitization induced by acute methadone treatment, whereas H_7 could not substantially block the acute desensitization in-

duced by methadone. These results suggest that the mechanisms underlying the acute and chronic opioid receptor desensitization may be different.

Perhaps the difference between methadone and morphine in δ-opioid receptor desensitization observed in the present study reflects a difference in δ-opioid receptor phosphorylation. Studies of the interaction of ligands with opioid receptors suggest that receptor occupancy by opiates of different classes may alter opioid receptor conformation in a distinct fashion. The ligand-to-ligand difference in μ-opioid receptor phosphorylation can reflect ligand-to-ligand differences in receptor/ligand conformation that can improve or worsen the receptor's ability to serve as substrate for activated kinases (Yu et al., 1997). Because the chemical structure of methadone is different from that of opium derivatives, it is possible that the interaction of methadone with δ-opioid receptors may alter the δ-opioid receptor conformation, which provides an improved substrate for kinases and leads to an increase in δ-opioid receptor phosphorylation and desensitization. It has recently been demonstrated that methadone treatment increases μ-opioid receptor phosphorylation to a greater extent than that caused by morphine treatment (Yu et al., 1997). Further experiments are necessary to obtain more detailed knowledge of the molecular events occurring during the phosphorylation and desensitization of receptors.

In summary, the results of the present study show that methadone and morphine, which are chemically distinct, can produce different cellular consequences by activation of δ-opioid receptors. The study reveals that there is a difference between methadone and morphine in their ability to cause δ-opioid receptor desensitization. The ability of methadone to desensitize δ-opioid receptors and to block the adaptive alterations induced by the continued activation of the δ-opioid receptor by morphine may be the underlying mechanisms of its effectiveness in the treatment of morphine addiction. The findings also provide an understanding of the molecular and cellular basis of substitution therapy for opiate addiction, and also provide insight into the mechanisms of dependence and tolerance, thereby facilitating further studies on the design and development of highly effective but poorly addictive substitute drugs for the therapy of opioid addiction.

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