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Acute δ-opioid receptor activation induces CREB phosphorylation in NG108-15 cells

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Abstract

A growing body of evidence supports an important role of the transcription factor cAMP responsive element binding protein (CREB) in mediating opioid-induced changes in the cAMP pathway. Regulation of CREB and subsequent changes in gene expression may underlie some long-term cellular adaptations associated with the administration of opioid drugs. The effect of morphine on the level of the transcription factor CREB, as well as CREB phosphorylation, was investigated in NG108-15 cells. Morphine and the δ-opioid receptor agonist [D-Pen^{2,5}]enkephalin (DPDPE) produced a dose-dependent increase in CREB phosphorylation. The effect was reversed by naloxone and naltrindole, respectively. The calmodulin antagonist *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), the protein kinase inhibitor staurosporine, as well as 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), an inhibitor of protein kinase C and cAMP-dependent protein kinase, but not *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-8), an inhibitor of cAMP- and cGMP-dependent protein kinase, blocked the opioid-induced CREB phosphorylation. The obtained results suggest that in the cells studied opioids affect, via the δ-opioid receptor, stimulatory intracellular mediator systems involving Ca^{2+}/calmodulin and the protein kinase C pathway.

Keywords: CREB (cAMP responsive element binding protein); δ-Opioid receptor; DPDPE ([D-Pen^{2,5}]enkephalin), Morphine; NG108-15 neuroblastoma; Protein kinase

1. Introduction

Considerable interest has focused on molecular alterations which may contribute to pathophysiological states that occur following treatment with opiates and their withdrawal. These processes result from adaptation of the neuronal system to the action of opiates. It is well known that they are usually coupled to G_i/G_o classes of the G proteins and acutely inhibit cAMP formation and Ca^{2+} conductance and activate potassium conductance, leading to hyperpolarization of the cell (Nestler, 1992). The hyperpolarization resolves with time and cAMP levels are normalized and subsequently raised above baseline. The resultant changes in the activation of protein kinase A lead to alterations in the phosphorylation of proteins relevant to opioid signalling and to changes in the expression of the cAMP responsive element binding protein (CREB), which may be important in the development and expression of opioid dependence (Nestler et al., 1993; Widnell et al., 1994; Maldonado et al., 1996).

CREB is a transcription factor that binds to cAMP-responsive elements (CRE) in the promotor region of target genes and modulates their expression. Phosphorylation of Ser^{133} within the kinase-inducible transcriptional activation domain of CREB is required to induce the transcriptional activity of the protein. In turn, CREB can interact with and activate the basal transcriptional machinery (Chrivia et al., 1993). It was found that acute morphine administration decreased, whereas withdrawal increased, the extent of CREB phosphorylation in the locus coeruleus of the rat. In contrast, chronic morphine increased the levels of CREB, but decreased its phosphorylation in that nucleus (Guitart et al., 1992; Widnell et al., 1994, 1996). Chronic morphine decreased CREB protein levels in the nucleus accumbens but was also reported to increase CRE DNA binding and CREB phosphorylation, and to decrease CRE DNA binding in that region (Guitart et al., 1992; Ikemoto et al., 1995; Nibuya et al., 1996). However,

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studies on CREB phosphorylation in the brain require the development of special techniques that do not measure directly CREB phosphorylation on Ser^{133}, the latter being required for a transcriptional activity. Moreover, putative extrinsic mechanisms (such as involvement of projections from other brain areas) seem to be involved in CREB activation in the brain. The direct effect of opioids on the regulation of CREB and its phosphorylation has not yet been studied in cell cultures. To better understand the mechanisms underlying the action of opioids on neurons, we investigated the morphine-produced regulation of the levels of CREB and the phosphorylated form of CREB in NG108-15 neuroblastoma × glioma cells. This cell line is established as a cellular model for studying opioid effects and several phenomena of opioid action in the brain, e.g., explanation of the molecular mechanism of tolerance, dependence and withdrawal on the basis of the action of opioids on cAMP pathway components was first shown in these cells. We report here that acute morphine, via δ-opioid receptors, exerts a stimulatory effect on CREB phosphorylation in NG108-15 cells.

2. Materials and methods

2.1. Materials

Morphine sulphate was purchased from PoCh Gliwice (Poland). Dulbecco's HAT medium (DMEM HAT) and fetal calf serum were purchased from Gibco Laboratories. [D-Pen^{2,5}]enkephalin (DPDPE), staurosporine, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-8), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) and naltrindole were obtained from RBI. All other reagents were purchased from Sigma Chemical (USA).

2.2. Cell culture and treatments

NG108-15 cells were maintained at 37°C in a humidified CO_2 incubator in Dulbecco's modified Eagle's HAT medium containing 10% fetal calf serum and L-glutamine. After being left for 1–2 days in growth medium, cells were placed in serum-free medium and cultured overnight. Experiments were carried out after an adaptation period, and reagents were added directly to the medium.

2.3. Western blotting

Cells were immediately lysed with warm sodium dodecylsulfate (SDS) sample buffer. Aliquots of crude extracts (containing 5–50 μg of protein) were then subjected to electrophoresis through a 12% SDS–polyacrylamide gel, and proteins were electroblotted on microporous polyvinylidene difluoride (PVDF) membranes (Boehringer-Mannheim, Germany). The membranes were blocked for 1 h, washed, and incubated overnight with primary antibodies at 4°C. After a wash step, immunocomplexes were detected using a Chemiluminescence Western Blotting Kit (mouse/rabbit Boehringer-Mannheim). The following rabbit polyclonal antibodies were used: anti-rat CREB (Upstate Biotechnology, USA), which recognizes both phosphorylated and unphosphorylated forms of CREB, and anti-rat phosphorylated CREB (Upstate Biotechnology) which recognizes the CREB protein only when the Ser^{133} amino acid residue is phosphorylated (Ginty et al., 1993).

In our immunoblots of cell extracts from NG108-15 cells, the phosphorylated CREB antibody reacted with two bands of a similar molecular weight. An additional band was probably due to phosphorylation of sites other than the Ser^{133} site, which resulted in a shift visible on some blots. The specificity of each of the antibodies was demonstrated by the recognition of bands of an expected molecular weight of 43 kDa. After each transfer SDS–polyacrylamide gels were stained with Coomassie Brilliant Blue G250 in order to control the relative level of proteins in samples. Levels of immunoreactivity were quantified with a Microcomputer Imaging Device (MCID, Canada) system and M4 software.

2.4. Data analysis

Data are given as means ± S.D. for all the experiments. We performed at least three separate experiments, each of them conducted in triplicate. Differences between treatment groups were analysed by a one-way analysis of variance (ANOVA). When the ANOVA indicated a significant difference between treatments, Bonferroni's post-test was applied.

3. Results

3.1. CREB Ser-133 phosphorylation following morphine treatment

We attempted to find out whether morphine triggered CREB Ser^{133} phosphorylation in NG108-15 cells, an event necessary for the transcription-activating function of this protein (Gonzalez and Montminy, 1989; Lee et al., 1990). When NG108-15 cells were incubated with increasing concentrations of morphine for 30 min, significant stimulation of CREB phosphorylation was observed. The effect produced by morphine was dose-dependent, the most effective doses of morphine being higher than 0.5 μM (Fig. 1). Morphine elicited a rapid and transient increase in the phosphorylated CREB level, which peaked at 30 min after receptor activation, remained constant for at least 90 min, and decreased after 5 h (Fig. 2). The δ-opioid receptor agonist DPDPE as well as morphine triggered sustained CREB phosphorylation (Fig. 3A, B). In contrast, an im-

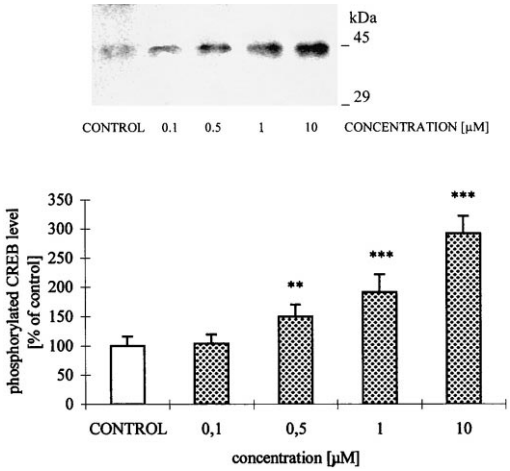

Fig. 1. The effect of morphine treatment on CREB phosphorylation in NG108-15 cells. The cells were incubated with increasing concentrations of morphine for 30 min. Data are expressed as means ± S.D. from three experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with the control.

munoblot analysis with an Anti-CREB antiserum showed no change in the total CREB protein level after incubation with morphine (data not shown). The opioid receptor antagonist naloxone (Fig. 3A), or the highly selective δ-opioid receptor antagonist naltrindole (Fig. 3B), reversed the effects of morphine and DPDPE on CREB phosphorylation. Summing up, the above results indicate that the transcription factor CREB in NG108-15 cells is a target for a signalling pathway that is initiated by activation of δ-opioid receptors.

For a detailed analysis of CREB activation, we used 1 μM morphine (because of strong stimulation) and DPDPE (in order to compare the action of the two opioids). The concentration of opioids used in our study is consistent

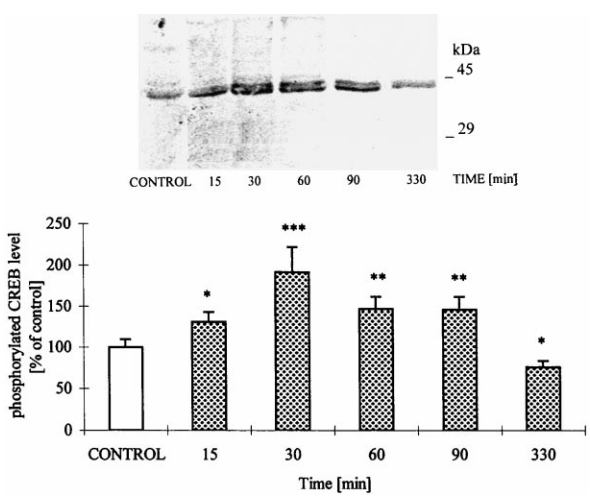

Fig. 2. Time course of morphine-induced CREB phosphorylation. The cells were incubated with 1 μM morphine for the indicated periods of time. Data are expressed as means ± S.D. from three experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with the control.

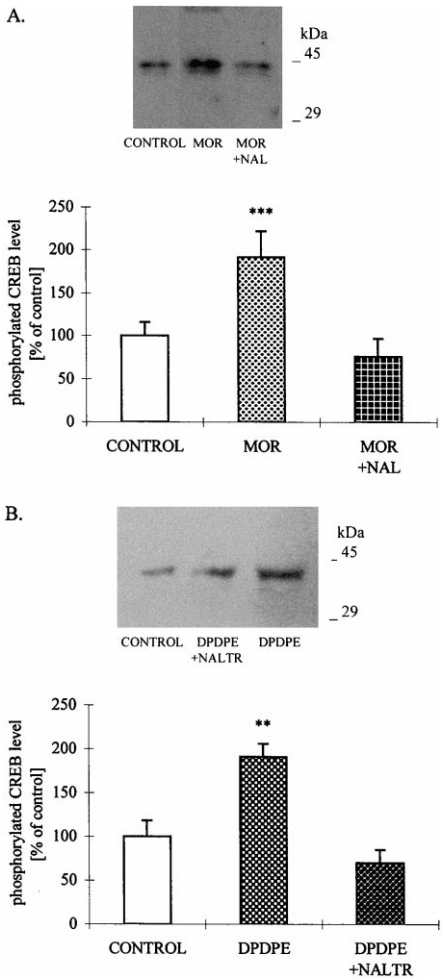

Fig. 3. The effect of opioid antagonists on morphine- or DPDPE-induced CREB phosphorylation in NG108-15 cells. Data are expressed as means ± S.D. from three experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control. (A) CREB phosphorylation after morphine (MOR, 1 μM; 30 min of incubation) treatment. Naloxone (NAL) was used simultaneously with the given opioid at 1 μM concentration. (B) CREB phosphorylation after DPDPE (0.5 μM; 30 min of incubation) treatment. Naltrindole (NALTR) was used simultaneously with the given opioid (1 μM).

with that used to evoke stimulation of Ins(1,4,5)P_3 formation by DPDPE in NG108-15 cells (Smart and Lambert, 1996), as well as with the dose producing an increase in $[Ca^{2+}]_i$ after [D-Ala^2, D-Leu^5]-Enkephalin (DADLE)/DPDPE application in these cells (Jin et al., 1992). Micromolar concentrations of opioid agonists usually exert a maximal effect on NG108-15; however, opioid concentrations as high as 100 μM have also been used in these cells (Yoon et al., 1998).

3.2. Effect of the protein synthesis and protein kinase inhibitors on CREB phosphorylation

The first step taken to investigate the mechanism underlying the opioid-induced increase in CREB phosphoryla-

tion was to treat NG108-15 cells with the protein synthesis inhibitor cycloheximide (20 μg/ml, 60 min before morphine). As shown in Fig. 4A, cycloheximide did not block the opioid-induced increase in CREB phosphorylation. A similar lack of effect was observed after anisomycin administration (data not shown). When given alone, cycloheximide did not affect the phosphorylated CREB level (Fig. 4B). These findings prove that the above-described stimulation did not depend on newly synthesized proteins.

Treatment of cells with H-8, an inhibitor of cAMP- and cGMP-dependent protein kinases, resulted in an increase in CREB phosphorylation. In contrast, staurosporine, a potent inhibitor of protein kinases, with some selectivity for protein kinase C, caused an almost complete blockade of CREB phosphorylation in NG108-15 cells. The effect of opioids was also markedly attenuated in the presence of H-7, a potent inhibitor of protein kinase C- and cAMP-dependent protein kinase (Fig. 4A).When given alone, staurosporine and H-8 tended to decrease, whereas H-7 slightly increased the phosphorylated CREB level. Nevertheless, these changes did not exceed 10% of the basal CREB phosphorylation (Fig. 4B). These data indicate that stimulation of δ-opioid receptors in NG108-15 cells results in protein kinase C activation.

3.3. Calmodulin is essential for CREB phosphorylation

Calmodulin is a small, ubiquitous Ca^{2+}-binding protein that acts as a Ca^{2+} sensor in neurons and binds to several enzymes, thus modulating their activity. These include calmodulin-dependent kinases, phosphatases and adenylyl cyclases. Of calmodulin-dependent kinases, isoforms II and IV are particularly involved in the stimulation of CREB phosphorylation, and the presence of both these isoforms has been demonstrated in NG108-15 cells (Enslen et al., 1996). We found that W-7, a calmodulin antagonist, almost completely blocked the morphine-induced CREB phosphorylation in the cells studied (Fig. 4A). When given alone, W-7 caused a slight decrease in the phosphorylated CREB level (Fig. 4B).

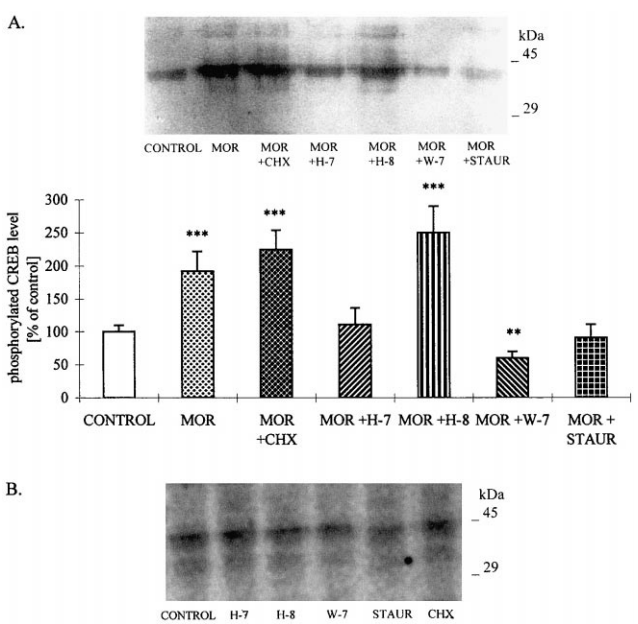

Fig. 4. The effect of protein synthesis and protein kinases inhibitors on CREB phosphorylation in NG108-15 cells. (A) The cells were incubated with one of the following inhibitors: cycloheximide (CHX, 20 μg/ml), H-7 (40 μM), H-8 (40 μM), W-7 (100 μM) or staurosporine (STAUR, 1 μM) and treated with 1 μM morphine (MOR) for 30 min. (B) The cells were incubated with the above inhibitors alone. Data are expressed as means ± S.D. from three experiments. $^{**}P < 0.01$; $^{***}P < 0.001$, compared with the control.

4. Discussion

In our study we investigated the action of opioids (morphine and DPDPE) on NG108-15 cells expressing delta opioid receptors. Despite the fact that morphine analgesia and dependence are connected predominantly with μ-opioid receptors, morphine given in vivo acts through δ receptors as well. We studied the effect of opioids on CREB phosphorylation in several cell lines expressing μ- or δ-opioid receptors. The stimulatory effect of opioids on CREB phosphorylation was most pronounced in NG108-15 cells. This cell line has been established as a cellular model for studying opioid effects, and several phenomena of opioid actions in the brain (e.g., explanation of molecular mechanism of tolerance, dependence and withdrawal explained on the basis of opioid action on components of the cAMP pathway) were first shown in these cells.

The major finding of this study is that morphine and DPDPE induce CREB phosphorylation in neuroblastoma × glioma NG108-15 cells. This is a striking observation, since as a rule opioids exert an inhibitory action on neurons. NG108-15 cells contain a homogenous population of δ-opioid receptors (Evans et al., 1992; Morikawa et al., 1998) and on exposure to δ-opioid receptor agonists there is inhibition of adenylate cyclase activity, Ca^{2+} currents and opioid receptor downregulation in these cells (Sharma et al., 1975; Vachon et al., 1987; Gucker and Bidlack, 1992; Morikawa et al., 1998). Therefore we did not expect these changes to result in CREB phosphorylation. The induction of CREB phosphorylation in NG108-15 cells following morphine and DPDPE treatment was time-dependent and was blocked by naloxone or naltrindole, which indicates that the effect under study was mediated by activation of δ-opioid receptors. After prolonged exposure to morphine, a decrease with time in the ability of the opioid agonist to stimulate CREB phosphorylation occurred. This might be due to a general desensitization/downregulation mechanism of agonist-occupied receptors. The CREB protein is generally thought to be constitutively expressed, hence no change in its total amount in response to some stimuli is to be expected. This finding is supported by the observations of the current study in which no significant changes in the CREB protein level were found

after opioid administration. It was shown previously that opioids can stimulate neurotransmission (Crain and Shen, 1990) during which both the neural firing rate (Lin and Carpenter, 1994) and the action potential duration are increased (Shen and Crain, 1994). The stimulatory effect of opioids involves not only disinhibition of interneurons but also direct stimulation of the target neurons themselves (Crain and Shen, 1990). The concentration required for stimulation varies considerably (up to 1000-fold) in different cell types (Bourgoin et al., 1994) and opioid receptor subtypes (Devine et al., 1993). It was also shown previously that morphine administration caused induction of the immediate early gene c-*fos* in localized areas of the brain (Gutstein et al., 1998). This phenomenon may also relate to the known role of opioids as inhibitors of inhibitory circuits. Furthermore, opioids may directly cause c-*fos* activation in some target cells (Gutstein et al., 1998). The δ-specific opioids have also been shown to produce rapid transient increases in the $[Ca^{2+}]i$ level in NG108-15 cells. Moreover, removal of extracellular Ca^{2+} did not block the response, which suggests that Ca^{2+} is mobilized from intracellular Ca^{2+} stores (Jin et al., 1992). The stores involved in this process were thapsigargin-sensitive, and inhibition of phospholipase C prevented the δ-opioid-induced increase in $[Ca^{2+}]i$ which suggests that Ca^{2+} was mobilized from inositol 1,4,5-triphosphate-sensitive stores (Jin et al., 1994). Opioids have also been shown to activate protein kinase C, an effect reported to cause desensitization of opioid-induced K^+- and Ca^{2+}-activated Cl^- currents (Mestek et al., 1995; Ueda et al., 1995). Concomitantly, opioid-induced activation of protein kinase C has been reported to exert an autoinhibitory feedback effect on the activation of phospholipase C in NG108-15 cells (Smart and Lambert, 1996). It is noteworthy that an increased protein kinase C activity has been linked to the development of tolerance to opioids in rats (Narita et al., 1994).

Ca^{2+} directly influences the activity of many key regulatory enzymes, such as Ca^{2+}-calmodulin-dependent kinases, protein kinase C, and Ca^{2+}-calmodulin-dependent adenylate cyclase, which, in turn, may activate protein kinase A. All these kinases phosphorylate CREB Ser^{133} in vitro (Yamamoto et al., 1988; Sheng et al., 1991). Our results with NG108-15 cells indicate that calmodulin-dependent kinases may be Ca^{2+}-activated enzymes which phosphorylate CREB in response to an opioid signal. Furthermore, protein kinase C is also activated since inhibition of protein kinase C reduced the level of phosphorylated CREB. In contrast, inhibition of cAMP- or cGMP-dependent protein kinases caused a moderate increase in phosphorylated CREB upon opioid stimulation. CREB phosphorylation at Ser^{133} is usually followed by transcriptional activation of CRE-dependent genes (Gonzalez and Montminy, 1989; Sheng et al., 1990; Ginty et al., 1994) thus, the stimulation of CREB phosphorylation is likely to contribute to the long-lasting consequences of exposure to opioids.

In the present study we obtained evidence that morphine, via δ-opioid receptors, exerts a stimulatory effect on CREB phosphorylation in NG108-15 cells. This stimulation neither depends on newly synthesized proteins nor involves the cAMP pathway, but requires Ca^{2+}/calmodulin and activation of protein kinase C. The obtained results indicate that in the studied cells opioids affect two different intracellular mediator systems: inhibitory, involving the cAMP pathway, and stimulatory, involving Ca^{2+} and the protein kinase C pathway.

Acknowledgements

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