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Short communication

Chronic morphine-mediated adenylyl cyclase superactivation is attenuated by the Raf-1 inhibitor, GW5074

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Abstract

The utility of morphine for the treatment of chronic pain is limited by the development of analgesic tolerance. Adenylyl cyclase (AC) superactivation, induced by chronic opioid agonist administration, is regarded as one of the molecular mechanisms leading to tolerance. In the present work, we tested the role of Raf-1 in morphine-mediated AC superactivation in CHO cells stably expressing the human μ-opioid receptor. We found that pretreatment of CHO cells stably expressing the human μ-opioid receptor with the selective Raf-1 inhibitor, 3-(3,5-dibromo-4-hydroxybenzylidene)-5-iodo-1,3-dihydroindol-2-one (GW5074, 10 μM, 60 min) completely abolished chronic morphine-mediated AC superactivation ($P<0.01$). This finding indicates that Raf-1 may have a crucial role in compensatory feedback regulation of cellular cAMP levels by clinically important opioid analgesics.

Keywords: Adenylyl cyclase superactivation; Human μ-opioid receptor; Raf-1

1. Introduction

The μ-opioid receptor agonist, morphine, is one of the most important analgesics in clinical practice for the alleviation of severe pain. Prolonged morphine administration, however, attenuates the responsiveness of the cellular μ-opioid receptors. Consequently, higher morphine doses become necessary to achieve pain relief (analgesic tolerance). The molecular and cellular mechanisms of morphine tolerance are not entirely clear. Previous studies suggest that a compensatory increase in the activity of adenylyl cyclase (AC) isoenzymes (AC superactivation) in response to chronic opioid receptor stimulation may contribute to cellular opioid tolerance (Williams et al., 2001).

Phosphorylation of Ser/Thr residues in the vicinity of the catalytic domain of AC isoenzymes is an important molecular mechanism to regulate their catalytic activity. Thus, recently, it was found that the protein kinase Raf-1 directly phosphorylates several AC isoenzymes, such as AC II, V and VI (Ding et al., 2004). Importantly, it was also shown (Tan et al., 2001) that Raf-1-mediated phosphorylation of AC VI leads to the sensitization of this isoenzyme to stimulators, such as forskolin (i.e. AC superactivation). Since AC VI is a major AC isoenzyme in Chinese hamster ovary (CHO) cells (Varga et al., 2002), we previously investigated the role of Raf-1 in chronic δ-opioid agonist-mediated AC superactivation in CHO cells stably expressing the human δ-opioid receptor. Interestingly, we found that a selective Raf-1 inhibitor, 3-(3,5-dibromo-4-hydroxybenzylidene)-5-iodo-1,3-dihydroindol-2-

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one (GW5074), significantly attenuated chronic deltorphin II-mediated cAMP overshoot in CHO cells stably expressing the human δ-opioid receptor (Varga et al., 2002). In the present work, since most clinically used opioid analgesics activate predominantly the μ-opioid receptor type, we tested the role of Raf-1 in chronic morphine-mediated AC superactivation in CHO cells stably expressing human μ-opioid receptor.

2. Materials and methods

The cDNA encoding the human μ-opioid receptor (GENBANK accession no.: L25119; a generous gift from Dr. J.B. Wang) was inserted into a pHβApr-1-neo mammalian expression vector, and stably transfected into CHO cells using the Superfect transfection reagent (Qiagen, CA). Saturation binding assays indicated that the selected neomycin-resistant clonal cell line exhibits high affinity (K_d=1.88 nM) for the μ-selective opioid receptor agonist, [^{3}H][D-Ala^2,N-Me-Phe^4, Gly^5-ol]-enkephalin ([^{3}H]DAMGO), with a B_{max} value of 0.16 pmol/mg protein (data not shown).

Two days before the assay, recombinant CHO cells were plated in 24-well plates at a density of 30,000 cells/well and were grown in a humidified incubator at 37 °C (5% CO_2) in Ham's F-12 medium (Invitrogen, CA) containing 10% fetal calf serum. On the day of the experiment, recombinant CHO cells were pre-incubated (60 min, 37 °C, 5% CO_2) in the presence or absence (control) of the Raf-1 inhibitor, GW5074 (10 μM) in Iscove's modified Dulbecco's medium (IMDM). After inhibitor treatment, the cells were co-incubated (4 h, 37 °C, 5% CO_2) with saturating concentrations of morphine (500 nM) in continuous presence of GW5074. Control cells were incubated in IMDM in the presence or absence of GW5074. Morphine treatment was also performed in the presence or absence of the opioid receptor antagonist, naloxone (10 μM). Forskolin-stimulated cAMP formation was determined as previously described (Varga et al., 2003). Unpaired *t*-tests were employed to determine statistical significance, using the Prism 4.0 software.

3. Results

Pretreatment (4 h) of the CHO cells stably expressing human μ-opioid receptor with morphine (500 nM) augmented forskolin (100 μM)-stimulated cAMP formation to 209 ±17.8% of the IMDM-treated control (n=9, P<0.01) (Fig. 1). The opioid receptor antagonist, naloxone (10 μM), completely prevented morphine-mediated cAMP overshoot (n=3, P<0.01) (Fig. 1) indicating that opioid receptor stimulation is necessary for the development of AC superactivation. Pretreatment (60 min, 37 °C, 5% CO_2) of CHO cells stably expressing human μ-opioid receptor with the selective Raf-1 inhibitor, GW5074 (10 μM), completely attenuated chronic morphine-mediated increase in forskolin-stimulated cAMP formation (n=7, P<0.01) (Fig. 1). Basal cAMP formation, on the other hand, was not significantly different between IMDM-treated and GW5074-treated recombinant CHO cells (P>0.05) (Fig. 1). Finally, radioligand-binding

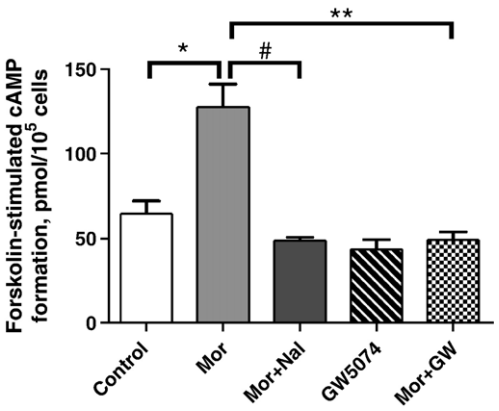

Fig. 1. The selective Raf-1 inhibitor, GW5074 attenuates chronic morphine-mediated AC superactivation in CHO cells expressing the human μ-opioid receptor. Chronic treatment (4 h) of CHO cells expressing the human μ-opioid receptor with morphine (Mor, 500 nM) augmented forskolin (100 μM)-stimulated cAMP formation (*P<0.01, n=9). Morphine-mediated cAMP overshoot was completely prevented by co-incubation with the opioid antagonist, naloxone (Mor+Nal, $^{\#}P$<0.01, n=3). Pre-treatment (1 h) of the cells with 10 μM GW5074 followed by 4 h co-incubation with morphine (500 nM, Mor+GW) completely attenuated chronic morphine-mediated adenylyl cyclase superactivation (**P<0.01, n=7). The results (mean±S.E.M.) are expressed as cAMP formation (pmol) per 100,000 cells.

assays have confirmed that GW5074 does not compete for opioid agonist binding sites in CHO cells stably expressing human μ-opioid receptor (data not shown).

4. Discussion

In the present study, we demonstrate that, similar to δ-opioid agonist-mediated AC superactivation in δ-opioid receptor-expressing CHO cells (Varga et al., 2002), a Raf-1 inhibitor (GW5074) attenuates long-term morphine-mediated AC superactivation in recombinant CHO cells stably expressing the human μ-opioid receptor, indicating that Raf-1 may have a crucial role in the development of opioid tolerance. Interestingly, while our present data indicates that GW5074 completely attenuates morphine-mediated AC superactivation in CHO cells expressing the human μ-opioid receptor, this inhibitor was less efficient to prevent AC superactivation by deltorphin II in CHO cells expressing the human δ-opioid receptor (Varga et al., 2002). The discrepancy may be due to at least three factors: (a) subtle differences in μ- and δ-opioid receptor-mediated cellular signaling pathways; (b) differences in the efficacies of the two agonists (deltorphin II is a full opioid receptor agonist, while morphine is a partial agonist); and (c) differences in opioid receptor expression levels between the two recombinant cell lines (0.16 pmol/mg protein in CHO cells stably expressing the human μ-opioid receptor vs. 1 pmol/mg protein in CHO cells stably expressing the human δ-opioid receptor).

Previously, it was thought that the major physiological function of Raf-1 is the regulation of the ERK1/2 mitogen activated protein kinase pathway. Recent data however demonstrate that Raf-1 may also have other important cellular effects. Thus, it was shown that Raf-1 directly binds and

phosphorylates several AC isoenzymes, leading to their sensitization towards stimulators, such as forskolin or activated Gs proteins (Tan et al., 2001; Ding et al., 2004).

The intracellular pathway(s) leading to the activation of Raf-1 upon long-term μ-opioid receptor stimulation are at present not fully characterized. However, we have found earlier that multiple parallel signal transduction pathways (chelerythrine sensitive isoforms of protein kinase C as well as calmodulin- and tyrosine kinase-regulated pathways) are involved in chronic δ-opioid receptor agonist-mediated phosphorylation and super-activation of AC VI (Varga et al., 2003). Further investigations are in progress to test the role of these pathways in morphine-mediated AC superactivation. Interestingly, recently, Beazely et al. (2005) have investigated the role of Raf-1, PKC and tyrosine kinases in the regulation of the activity of AC VI in human embryonic kidney (HEK) cells overexpressing AC VI. Similar to our data, these authors have found that activation of both receptor tyrosine kinases and chelerythrine sensitive PKC isoenzymes enhanced forskolin-stimulated activity of AC VI in a GW5074 sensitive manner. These data lend further support to the hypothesis that multiple signaling pathways may merge at Raf-1 to potentiate forskolin-stimulated cyclic AMP accumulation in mammalian cells expressing AC VI. Furthermore, since Raf-1-dependent phosphorylation represents a common regulatory mechanism for several AC isoenzymes (such as ACVI, ACV and ACII) (Ding et al., 2004), Raf-1-mediated sensitization of AC activity may be a common feature for a number of mammalian cells. Finally, current data indicate that Raf-1-mediated phosphorylation and sensitization of AC isoenzymes may be a common mechanism for feedback-regulation of cellular cAMP concentrations upon chronic stimulation of Gi/o-coupled receptors. Thus, Beazely and Watts (2005) have found that, in HEK 293 cells expressing the D2 dopamine receptor, GW5074 inhibited long-term D2-dopamine receptor agonist-mediated PKC-dependent augmentation of forskolin-stimulated cAMP formation.

In conclusion, our data demonstrate that Raf-1-mediated sensitization of one or more AC isoenzymes has a crucial role in the compensatory increase in cellular cAMP concentration after chronic morphine treatment. Since it is well established that cAMP-regulated protein kinases play a central role in the regulation of nociceptor responsiveness to pain stimuli (Rathee et al., 2002), identification of the cellular effectors responsible for cellular cAMP overshoot upon chronic morphine treatment should provide novel pharmacological targets to prevent analgesic tolerance.

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