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Short communication

Morphine promotes phosphorylation of the human δ-opioid receptor at serine 363

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Abstract

After prolonged stimulation, the δ-opioid receptor becomes desensitized by regulatory mechanisms such as receptor phosphorylation, internalization and down-regulation. In this study, we demonstrate that morphine treatment causes phosphorylation of S^{363} in the C-terminus of the human δ-opioid receptor. Morphine-mediated phosphorylation reached 53±8% of maximum deltorphin II-mediated phosphorylation. Phosphorylation of S^{363} may contribute to δ-opioid receptor desensitization by morphine.

Keywords: δ-opioid receptor; Morphine; Receptor phosphorylation

1. Introduction

Morphine is a commonly used pharmaceutical agent that produces its effects by activating three types of opioid receptors. After repeated or prolonged stimulation, the opioid receptors become less responsive (desensitized)—an adaptation which is thought to contribute to opioid tolerance. Desensitization of most G protein-coupled receptors involves a cascade of regulatory mechanisms, such as receptor phosphorylation, internalization and down-regulation. Phosphorylation of the δ-opioid receptor has been implicated in rapid desensitization (Pei et al., 1995) as well as in β-arrestin- and clathrin-mediated internalization (Law et al., 2000). In contrast to other opioid agonists, morphine elicits minimal δ-opioid receptor phosphorylation and β-arrestin translocation (Zhang et al., 1999), and does not stimulate rapid δ-opioid receptor internalization (Keith et al., 1996). Overexpression of G protein-coupled receptor kinase 2 facilitated morphine-mediated δ-opioid receptor phosphorylation (Zhang et al., 1999), suggesting that the morphine-activated δ-opioid receptor is a sub-optimal substrate for G protein-coupled receptor kinases (Von Zastrow et al., 2003).

The peptide agonist, DADLE ([D-Ala2, D-Leu5]enkephalin) was shown to phosphorylate several residues in the C-terminus of the rodent δ-opioid receptor, with S^{363} as the primary phosphorylation site (Kouhen et al., 2000). It is presently not known whether morphine is able to phosphorylate the same residues as peptide agonists. Therefore, in this study, we investigated phosphorylation of S^{363} in the human δ-opioid receptor upon morphine or deltorphin II treatment. Using an antibody specific for S^{363}-phosphorylated δ-opioid receptor, we demonstrate that in Chinese hamster ovary cells

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(CHO) expressing the human δ-opioid receptor (Malatynska et al., 1996) morphine promotes phosphorylation of S^{363}.

2. Materials and methods

To induce receptor phosphorylation, CHO cells expressing the human δ-opioid receptor were treated with morphine (30 μM) (Sigma) or deltorphin II (100 nM) (Tocris) for 30 min at 37 °C in a serum-free medium. Optimal agonist concentrations and treatment times to achieve maximal receptor phosphorylation were determined in preliminary experiments. After agonist treatment, the cells were rinsed and harvested in homogenization buffer (20 mM Tris, 4 mM EGTA, 2 mM EDTA, pH 7.4) containing 1% protease inhibitor cocktail and 0.1% phosphatase inhibitor cocktails I and II (Sigma). Cell lysates were boiled for 5 min with reducing sample buffer (Invitrogen). Total protein concentration in each sample was determined by the Bradford assay. Sample aliquots containing 5 μg total protein were resolved by 10% polyacrylamide gels (NuPAGE, Invitrogen) and transferred to nitrocellulose membranes. Phosphorylation of the human δ-opioid receptor at S^{363} was measured by Western blots using a rabbit primary antibody specific for phospho-S^{363} of the δ-opioid receptor (1 : 1000, Cell Signaling) and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1 : 100,000, Santa Cruz Biotechnology). Immunoreactive bands were detected using SuperSignal West Dura chemiluminescent kit (Pierce) and quantified using the Image J software (NIH). As an internal control, we also quantified actin, a "housekeeping" protein, in each sample. We normalized the intensities of the phospho-S^{363} bands for actin immunoreactivity. Statistical analyses were performed using GraphPad Prism 4.

3. Results

In samples from agonist-treated CHO cells expressing the human δ-opioid receptor the anti-phospho-S^{363} antibody recognized a diffuse band in the molecular weight range of 50–70 kDa, presumably corresponding to differently glycosylated forms of the S^{363}-phosphorylated human δ-opioid receptor (Fig. 1, lanes 2, 3). Quantitative analyses of the 50–70 kDa bands show that morphine (30 μM)-mediated S^{363} phosphorylation was 53 ± 8% of maximum phosphorylation mediated by deltorphin II (100 nM). Both morphine- and deltorphin II-mediated phosphorylation were completely blocked by concomitant treatment with the selective δ-opioid receptor antagonist, naltrindole (1 μM) (Fig. 1, lanes 4, 5). The anti-phospho-S^{363} antibody did not interact with proteins in untreated cell lysates (Fig. 1, lane 1), indicating that S^{363} phosphorylation is negligible under basal conditions. Finally, no specific immunoreactivity was detected upon morphine or deltorphin II treatment of CHO cells expressing a human δ-opioid receptor in which S^{363} was replaced by alanine (Navratilova et al., 2004), confirming that the antibody recognizes only the S^{363}-phosphorylated human δ-opioid receptor (Fig. 1, lanes 6, 7).

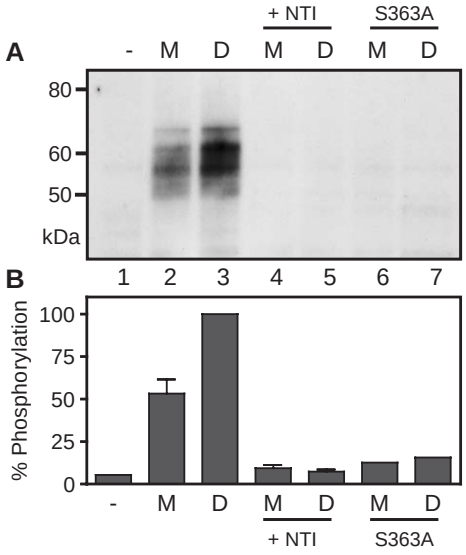

Fig. 1. Agonist-mediated phosphorylation of the human δ-opioid receptor at S^{363}: recombinant CHO cells expressing the human δ-opioid receptor or the S363A mutant receptor were treated in serum-free medium with opioid ligands for 30 min at 37 °C and samples prepared as described. Aliquots from each sample, corresponding to 5 μg total protein, were loaded onto 10% polyacrylamide gels, resolved and transferred to nitrocellulose membranes. Western blot analyses were performed using an anti-phospho-S^{363} antibody (Cell Signaling). Treatment of cells for 30 min with morphine (M, 30 μM) or deltorphin II (D, 100 nM) led to phosphorylation of the human δ-opioid receptor at S^{363} (lanes 2, 3). This phosphorylation was completely blocked by concomitant incubation of cells with 1 μM naltrindole (+NTI) (lanes 4, 5). No immunoreactivity was detected in cells expressing the S363A mutant receptor after identical treatment with morphine or deltorphin II (S363A) (lanes 6, 7). A representative immunoblot is shown in (A). Intensities of the 50–70 kDa protein bands were quantified using the Image J software. For each sample the intensity of phospho-S^{363} bands was normalized to the intensity of actin immunoreactivity. The results were analyzed using GraphPad Prizm 4 and plotted as percent of the maximal phosphorylation produced by deltorphin II (B). In four independent experiments, morphine produced 53 ± 8% (mean ± S.E.M.) of the maximal deltorphin II-mediated phosphorylation, which was significantly greater than basal phosphorylation (5.4 ± 1.2%, $P < 0.05$).

4. Discussion

Our findings indicate that, like a full δ-selective opioid agonist deltorphin II, morphine is also able to promote phosphorylation of S^{363} in the C-terminus of the human δ-opioid receptor. Phosphorylation of S^{363} is the initial step in δ-opioid receptor regulation and is a prerequisite for phosphorylation of other residues in the C-terminus including T^{358}, T^{361} (Kouhen et al., 2000). Additional sites may also be phosphorylated in other intracellular domains. Since total morphine-mediated phosphorylation of the δ-opioid receptor is only 6% of total phosphorylation by ethorphine (Zhang et al., 1999), we hypothesize that morphine fails to phosphorylate residues other than S^{363}. Phosphorylation of at least two to three serine/threonine residues is necessary for binding of arrestin to rhodopsin (Gurevich and Gurevich, 2004). Therefore, phosphorylation of S^{363} alone may not be sufficient for recruitment of β-arrestin, receptor internalization and down-regulation. Recently, phosphorylation of a homologous residue in the μ-opioid receptor, S^{375}, upon morphine treatment was demonstrated (Schultz et al., 2004). Phosphorylation of

these homologous residues may therefore be involved in responses mediated by morphine, including desensitization, while phosphorylation of additional residues may be necessary for recruitment of β-arrestin, receptor internalization and down-regulation.

In conclusion, we were able to demonstrate for the first time that activation of the human δ-opioid receptor by morphine leads to phosphorylation of S^{363} in the C-terminus of the receptor. Since receptor phosphorylation is a crucial step in G protein-coupled receptor desensitization, we suggest that phosphorylation of S^{363} may have an important role in the regulation of human δ-opioid receptor signaling in response to morphine treatment.

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