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Molecular Brain Research 136 (2005) 54–64
MOLECULAR BRAIN RESEARCH
www.elsevier.com/locate/molbrainres

Research report

Purification and mass spectrometric analysis of the δ opioid receptor

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Accepted 8 January 2005
Available online 2 March 2005

Abstract

A mouse δ opioid receptor was engineered to contain a FLAG epitope at the amino-terminus and a hexahistidine tag at the carboxyl terminus to facilitate purification. Selection of transfected human embryonic kidney (HEK) 293 cells yielded a cell line that expressed the receptor with a B_{max} of 10.5 pmol/mg protein. [^{3}H]Bremazocine exhibited high affinity binding to the epitope-tagged δ opioid receptor with a K_D of 1.4 nM. The agonists DADL, morphine, and DAMGO competitively inhibited bremazocine binding to the tagged δ receptor with K_I's of 0.9, 370, and 620 nM, respectively. Chronic treatment of cells expressing the epitope-tagged δ receptor with DADL resulted in downregulation of the receptor, indicating that the tagged receptor retained the capacity to mediate signal transduction. The δ receptor was solubilized from HEK 293 cell membranes with *n*-dodecyl-β-D-maltoside in an active form that maintained high affinity bremazocine binding. Sequential use of Sephacryl S300 gel filtration chromatography, wheat germ agglutinin (WGA)-agarose chromatography, immobilized metal affinity chromatography, immunoaffinity chromatography, and SDS/PAGE permitted purification of the receptor. The purified δ opioid receptor was a glycoprotein that migrated on SDS/PAGE with an apparent molecular mass of 65 kDa. MALDI-TOF mass spectrometry was used to identify and characterize peptides derived from the δ opioid receptor following in-gel digestion with trypsin, and precursor-derived ms/ms confirmed the identity of peptides derived from enzymatic digestion of the δ opioid receptor.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Opioid receptors

Keywords: Opioid; Purification; Mass spectrometric analysis; Morphine; Enkephalin; Mass spectrometry; Affinity chromatography

1. Introduction

Opioid receptors mediate the effects of endogenous opioid peptides in the central, peripheral, and enteric nervous systems and are also the molecular targets of opioid drugs, such as heroin, morphine, fentanyl, and methadone [5]. The three types of opioid receptors, μ, δ, and κ, are members of the G protein-coupled receptor superfamily [10,28]. Like other G protein-coupled receptors, opioid receptors contain seven transmembrane domains, have extracellular amino termini that contain sites for N-linked glycosylation (Asn-X-Ser/Thr), and have carboxyl termini located intracellularly. Opioid ligands approach and bind the receptor from the extracellular side, and agonist-induced opioid receptor activation results in the coupling to G proteins on the intracellular aspect of the plasma membrane. Opioid-induced signal transduction is mediated through a variety of effectors, including adenylyl cyclase, K^+ channels, Ca^{2+} channels, MAP kinase, and phosphatidylinositol 3-kinase [3,4,16,24,25].

Abbreviations: DADL, [D-Ala^2, D-Leu^5]enkephalin; DAMGO, [D-Ala^2, *N*-Me-Phe^4,Gly^5-ol]enkephalin; HEK, human embryonic kidney; MALDI, matrix-assisted laser desorption ionization; ms/ms, tandem mass spectrometry; Ni-NTA, nickel–nitrilotriacetic acid; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; TOF, time of flight; WGA, wheat germ agglutinin

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doi:10.1016/j.molbrainres.2005.01.016

Chronic use of opioid drugs leads inevitably to physical dependence, which becomes evident when prolonged opioid drug use is terminated, resulting in a withdrawal syndrome lasting from several days to weeks [13]. Chronic opioid use also induces a state of tolerance, in which the pharmacological efficacies of agonists are attenuated. Intensive research efforts over the last several decades have indicated that opioid addiction is complex, and probably involves neural plasticity associated with alterations in the strength of neural circuits, changes in gene expression, and alterations in opioid-regulated signaling pathways [15,20].

Short-term agonist activation of opioid receptors leads to an adaptive response to acute effects that is referred to as receptor desensitization. It has been shown that μ and δ opioid receptors are phosphorylated by G protein-coupled receptor kinases following agonist treatment [21,31] and are internalized in a dynamin-dependent process via clathrin-coated pits [8]. Overexpression of arrestin or G protein-coupled receptor kinase increases agonist-induced opioid receptor internalization [29,31]. Morphine analgesia is enhanced in knockout mice lacking β-arrestin 2, pointing to a role for β-arrestin 2 in desensitization of the opioid receptor [1]. In mice lacking β-arrestin 2, chronic morphine treatment does not cause desensitization of the μ opioid receptor, and the knockout mice do not develop tolerance to the analgesic effects of morphine [2]. Endosome-associated receptors can be resensitized via dephosphorylation by protein phosphatases and recycled back to the plasma membrane, or be proteolytically degraded within the cell [11].

Chronic opioid administration leads to receptor down-regulation, as a result of proteolytic degradation of the receptors [17,23]. It is probable that agonist-induced down-regulation of opioid receptors contributes to opioid tolerance. The mechanism for G protein-coupled receptor proteolysis has been generally assumed to involve internalization and trafficking from endosomes to lysosomes; however, evidence that the ubiquitin/proteasome pathway plays a prominent role in agonist-induced opioid receptor downregulation has been reported [7]. In that study, proteasome inhibitors blocked agonist-induced downregulation of μ and δ opioid receptors, while inhibitors of calpain, caspases, and lysosomal cathepsins had no effect. It was also shown that μ and δ opioid receptors are ubiquitinated, which serves to target protein substrates to the proteasome complex. Downregulation of the human κ opioid receptor was also partially attenuated by proteasome inhibitors [18]. The ubiquitin/proteasome pathway also mediates degradation of improperly folded δ opioid receptors, as part of the quality control system for newly synthesized proteins in the endoplasmic reticulum [22]. Observations on the involvement of the ubiquitin/proteasome system in opioid receptor turnover have recently been extended to other G protein-coupled receptors, including the β2-adrenergic receptor [26], the CXCR4 chemokine receptor [9,19], and the CCR5 chemokine receptor [9].

In this study, we report the solubilization and purification of the δ opioid receptor expressed in HEK 293 cells, and the identification and characterization of peptides derived from proteolytic digestion of the purified receptor using MALDI-TOF and TOF-TOF ms/ms mass spectrometry.

2. Materials and methods

2.1. Construction of hexahistidine-tagged opioid receptors

A plasmid encoding the δ opioid receptor with a FLAG epitope at the N-terminus (kindly provided by Dr. Mark von Zastrow, UCSF) was used as a PCR template to generate a receptor that also contained a hexahistidine tag at its C-terminus. Oligonucleotide primers employed for the PCR were 5′-GCC ATG AAG ACG ATC ATC GCC, which was the sense primer used to amplify the 5′-end of the Flag-tagged δ opioid receptor and 5′-GGC GGC AGC GCC ACC GCC CGG, an antisense primer that deletes the termination codon from the 3′-end of the δ opioid receptor open reading frame. The PCR product was inserted into the eukaryotic expression vector, pcDNA3.1/V5-His-TOPO (Invitrogen). Deletion of the stop codon at the 3′-end of the δ receptor open reading frame allowed it to be placed in frame with a plasmid open reading frame encoding the V5-epitope and hexahistidine tag. The recombinant plasmid encoding the FLAG- and His-tagged δ opioid receptor was used to transform TOP10 *Escherichia coli*, and positive colonies were screened by PCR. Plasmids were purified on Qiagen columns and then submitted to the Molecular Resource Facility at UMDNJ-NJMS for DNA sequence verification of the entire receptor open reading frame.

2.2. Cell culture and transfection

HEK 293 cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 100 U/ml penicillin and 100 μg/ml streptomycin sulfate. HEK 293 cells were transfected with the receptor expression plasmid using Lipofectamine (Invitrogen). Cells expressing the epitope-tagged δ opioid receptor were selected in media containing 1 mg/ml G418 (Life Technologies, Gaithersburg, MD).

2.3. Membrane preparation and radioligand binding assays

HEK 293 cells expressing the epitope-tagged δ opioid receptor were grown to near confluence in 150 mm diameter dishes. For membrane preparations, the culture medium was aspirated and cells were harvested in 50 mM Tris–HCl buffer, pH 7.5. The cell suspension was homogenized with a Tekmar tissuemizer (Cincinnati, OH), then centrifuged at 100,000 × *g* for 30 min. The membrane pellet was washed three times in Tris buffer and then