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Design and functional evaluation of an optically active μ-opioid receptor

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ABSTRACT

The use of opioids, which achieve therapeutic analgesia through activation of μ-opioid receptors, are limited in the management of chronic pain by adverse effects including tolerance and addiction. Optogenetics is an emerging approach of designing molecular targets that can produce cell-specific receptor-mediated analgesia with minimal side effects. Here we report the design and functional characterization of a chimeric μ-opioid receptor that could be photoactivated to trigger intracellular signaling. A prototype optoactive μ-opioid receptor (optoMOR) was designed by replacing the intracellular domains from rhodopsin with those of the native μ-opioid receptor and was transiently expressed in human embryonic kidney (HEK293) cells. Expression and distribution of the protein were confirmed by immunocytochemistry. The signal-transduction mechanisms induced by photoactivation of the optoMOR were evaluated and compared with the native μ-opioid receptor stimulation by an agonist, D-Ala^2, N-MePhe^4, Gly-ol-enkephalin (DAMGO). Cells were depolarized by extracellular potassium and the depolarization-induced calcium (Ca^{2+}) influx was quantified by using Fura-2 imaging. The forskolin-stimulated adenylate cyclase/cAMP cascade was evaluated by ELISA or western blotting of brain-derived neurotrophic factor (BDNF) and the phosphorylation of cAMP response element binding protein (CREB). The optoMOR protein distribution was observed intracellularly and on the plasma membrane similar to the native μ-opioid receptor in HEK293 cells. Photoactivation of optoMOR decreased the Ca^{2+} influx and inhibited the forskolin-induced cAMP generation, activation of CREB, and BDNF levels in optoMOR-expressing cells similar to the activation of native μ-opioid receptor by DAMGO. Thus the current study has accomplished the design of a prototype optoMOR and characterized the cellular signaling mechanisms activated by light stimulation of this receptor.

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1. Introduction

Chronic pain is a debilitating disease that has significant impact on the quality of life of individuals suffering from pain (Gureje et al., 2001; Verhaak et al., 1998). The most effective currently available therapies for the treatment of chronic pain are opioids, however, the long-term use of opioids is associated with adverse effects including tolerance and addiction (Ballantyne and Mao, 2003). A safer therapy for chronic pain could be achieved by focal interruption of nociceptive signaling in a targeted and cell type specific manner. Despite tremendous efforts to minimize nonspecific receptor activation, no comprehensive solution has been accomplished to reduce tolerance and addiction associated with opioid therapy of chronic pain (Ahlbeck, 2011; Lesniak and Lipkowski, 2011).

Optogenetics is an emerging technology with potential application in the therapy of chronic diseases (Boyden et al., 2005a; Deisseroth et al., 2006). Optogenetic approaches involve selective and precise control of physiological function(s) by photostimulation of cell-surface proteins. This technology requires genetic modification of a group of cells and enables the remote control of intracellular signaling mechanisms. Optical G-protein-coupled receptor (GPCR) approaches have been used successfully in experimental models in vivo for remote regulation of neuronal firing (Airan et al., 2009) or to succesfully compensate for loss of signaling in slice cultures from knockout mice (Oh et al., 2010).

Opioids produce analgesia by activating cell surface receptors belonging to the GPCR family of receptors (Chen et al., 1993). GPCRs share a common activation mechanism based on their conserved structure that enables the design of functional chimeric receptors (Kim et al., 2005; Kobilka et al., 1988). The optically active XR (optoXR) family of chimeric receptors have been engineered based on mammalian rhodopsin that can activate

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intracellular signaling pathways associated with the β_2-adrenoceptor (G_q), the α_1-adrenoceptor (G_s), or the 5-HT_{1A} receptor ($G_{i/o}$), when exposed to light (Airan et al., 2009; Oh et al., 2010). These receptors can be expressed in a cell-type specific manner using different promoter elements or by targeted viral delivery and can be activated/deactivated with temporal precision comparable to the corresponding native receptors (Oh et al., 2010). Coupled with the finding that there is adequate endogenous retinal for receptor function in mammalian brain tissue, these chimeric GPCRs could be potentially useful to achieve precise optical control over intracellular signaling cascades (Boyden et al., 2005b; Zhang et al., 2006).

In this study we describe the design and functional characterization of an optically active μ-opioid receptor (optoMOR), an important step towards the development of an optogenetic approach for the treatment of chronic pain. The design of the chimeric receptor conserved critical regions involved in light responsiveness of rhodopsin and the regions that mediate intracellular signaling to the native μ-opioid receptor. The engineered receptor was cloned and expressed in human embryonic kidney (HEK293) cells. The cellular distribution of the receptor was delineated and the functional coupling to intracellular signaling pathways was characterized (Piros et al., 1995, 1996; Rhim and Miller, 1994).

2. Materials and methods

2.1. Cloning and expression of optoMOR

Rhodopsin and optoMOR clones were tagged C-terminally with mCherry immediately following the last coding codon and a short 5′-GCGGCCGCC-3′ linker sequence coding for a NotI restriction site. Fusion clones were constructed in the pSP72 cloning vector (Promega, Madison, WI, USA) between the BamHI and EcoRI sites. These fragments were then inserted immediately following the CamKIIa promoter in the pLenti viral expression vector (Zhang et al., 2007) using the BamHI and EcoRI sites. For expression in HEK293 cells, the CamKIIa promoter was replaced with the cytomegalovirus immediate early promoter (CMV) promoter from the pCMV-Tag1 plasmid (Agilent, Sant Clara, CA, USA) using the primers 5′-GGATAACCGTACGATCGCCATGCAT-TAGTTA-3′ and 5′-GTAATCGCTAGCGGATCCGACGGTTCAC-3′ and PvuI and BamHI restriction enzymes. All constructs were sequence verified. Wild type μ_1-opioid receptor was a gift from Luda Diatchenko.

2.2. Cell culture

HEK293 cells were obtained from ATCC (Manassas, VA, USA). Cells were grown to 90% confluence and transfected with optoMOR, rhodopsin, μ-opioid receptor, or mCherry expression constructs using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) and incubated for 18–24 h before testing or fixing for immunohistochemistry.

Twenty-four hours after transfection, cells were lysed using the QIAshredder (Qiagen, Valencia, CA, USA) spin column. mRNA was isolated from these lysates using the RNeasy Mini Kit (Qiagen) and samples were treated with DNase. Using the mRNA as a template, cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). PCR reactions were amplified using cDNA, gene specific primers, and iQ Supermix (Bio-Rad). For optoMOR the primers 5′-ACC TGA GGA GGA TCA CCA GGA TGG-3′ and 5′-CCT GAA GCA CCT CTT GAA CTG CTT G-3′ were used to generate a 218 bp product. Rhodopsin primers, 5′-GCC CAG CAA CAG GAG TCG GCT A-3′ and 5′-CGG TCT TAG CAA AGA AAG CGG GAA G-3′ generate a 190 bp product. μ-opioid receptor primers, 5′-TGG GCG GGA GAG ACA GCC TG-3′ and 5′-AGG GCA GGG TAC TGG TGG CT-3′ generate a 219 bp product. Amplification was performed with a MJ Mini thermocycler (Bio-Rad) with 30 amplification cycles and an annealing temperature of 59 °C. Samples were run on a 1.5% agarose gel and imaged using UV illumination.

2.3. Confocal microscopy

HEK293 cells were fixed with 4% paraformaldehyde for 9 min and subsequently permeabilized with 0.3% Triton X-100 in TBS 24 h post-transfection. Anti-RFP (Alexa Fluor 647 conjugate) was used to label mCherry tagged receptors, and Anti-MYC (Alexa Fluor 647 conjugate) was used to label μ-opioid receptor. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain and mounted under diazobicyclooctane (DABCO; 2.5% DABCO, 10% polyvinyl alcohol and 20% glycerol in TBS; Sigma Aldrich). Images were acquired with a Zeiss LSM 710 confocal microscope.

2.4. Fluorescence imaging of real-time changes in the intracellular calcium (Ca^{2+}) by Fura-2

Cells were loaded for 20 min with 1 μM all-*trans*-retinal (Toronto Research Chemicals, North York, Ontario, Canada), followed by 30 min with 5 μM Fura-2-AM (Biotium, Hayward, CA, USA) and 0.02% Pluronic-F127 (Invitrogen). Cells were washed and incubated for 15 min in DPBS supplemented with 1 mg/ml glucose.

Fura-2-loaded dishes were placed on the stage of Axiovert inverted microscope (S1000 TV, Zeiss,) with 10X fluar objective for high-speed digital fluorescence imaging. Fura-2 ratio images were obtained using a computer controlled monochromator with xenon excitation light source (TILL Polychrome II, TILL-Photonics, Martinsried, Germany) and a cooled CCD camera with exposure control (SensiCam, Till-photonics). During excitation at 340 or 380 nm, emission was collected at 510 nm by the camera at 2×2 binning and images were acquired by Till Vision software (Till Photonics). Ratio (340/380) images were generated with background subtraction and quantified in terms of arbitrary fluorescence units (AFUs). Data was further analyzed of-line using Microsoft Excel and GraphPad Prism.

Fura-2 imaging was carried out before and immediately after the addition of an agonist or following 60 s illumination with light at 500 nm wavelength. Cells were depolarized by increasing the extracellular concentration of potassium (K^+) by adding 100 mM KCl and the resultant Ca^{2+} influx was recorded for 1 min. At the conclusion of each experiment the presence of transfected cells was confirmed by imaging the expressed GFP or mCherry using 488 nm or 560 nm excitation, respectively.

2.5. Quantification of cAMP

Transfected cells were loaded for 20 min with 1 μM all-*trans*-retinal followed by 15 min incubation in DPBS supplemented with 1 mg/ml glucose and 100 μM 3-Isobutyl-1-methylxanthine (IBMX). Receptors were stimulated by exposure to room lighting or 1 μM D-Ala^2, N-$MePhe^4$, Gly-ol-enkephalin (DAMGO) and immediately incubated for 10 min with 20 μM of forskolin, an activator of adenylyl cyclase (Sigma). Cells were lysed with 0.1 M HCl (Thermo Fisher, Waltham, MA USA) with 0.5% Triton-X 100 (MP Biomedicals, Santa Ana, CA, USA). Protein concentration was determined by BSA method and samples were diluted to 0.1 mg/ml. Lysates were analyzed (100 μl/well) using cAMP, EIA kit (Enzo Life