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Full paper

Neuropeptide oxytocin enhances μ opioid receptor signaling as a positive allosteric modulator

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ABSTRACT

Oxytocin (OT) is a 9-amine neuropeptide that plays an essential role in mammalian labor, lactation, maternal bonding, and social affiliation. OT has been reported to exert an analgesic effect in both humans and animals, and the results of certain animal experiments have shown that the analgesic effect of OT is partially blocked by opioid receptor antagonists. To investigate the relationship between OT and μ opioid receptor (MOR), we evaluated how OT affects MOR in vitro by performing an electrical impedance-based receptor biosensor assay (CellKey™ assay), an intracellular cAMP assay, and a competitive receptor-binding analysis by using cells stably expressing human MOR and OT receptor. In both the CellKey™ assay and the intracellular cAMP assay, OT alone exerted no direct agonistic effect on human MOR, but treatment with 10^{-6} M OT markedly enhanced the MOR signaling induced by 10^{-6} M endomorphin-1, β-endorphin, morphine, fentanyl, and DAMGO. Moreover, in the competitive receptor-binding assay, 10^{-6} M OT did not alter the affinity of endomorphin-1 or morphine for MOR. These results suggest that OT could function as a positive allosteric modulator that regulates the efficacy of MOR signaling, and thus OT might represent a previously unrecognized candidate analgesic agent.

1. Introduction

Oxytocin (OT) is a 9-amine neuropeptide synthesized in the paraventricular nucleus (PVN) and the supraoptic nucleus of the hypothalamus. OT is secreted from the posterior pituitary into the systemic circulation, where it plays an essential role in mammalian

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labor and lactation through its peripheral actions,[1] and suggested to modulate numerous functions including maternal bonding and social affiliation through its central actions.[2,3]

Recently, the effect of OT on pain sensitivity in both humans and animals has been increasingly investigated by several research groups including ours.[4–11] The first crucial mechanism linking OT and pain involves hypothalamo-spinal oxytocinergic pathways. Stimulation of the PVN or administration of OT activates presynaptic OT receptors (OTRs) located superficially in the dorsal horn (Laminae I and II) and subsequently excites inhibitory GABAergic interneurons. Activation of GABAergic interneurons, in turn, presynaptically inhibits A δ-fiber and C-fiber signals at nociceptive-specific and wide-dynamic-range neurons in Laminae I and II.[7,12–14] These effects can be reversed by selective OTR antagonists.[13] The second potential mechanism linking OT and pain involves an indirect pathway through the endogenous opioid system. Injection of opioid receptor (OR) antagonists has been reported to partially block the analgesic effects of OT.[4–6] In terms of the relationship between OT and endogenous opiates, at least two potential mechanisms exist: OT can stimulate the release of endogenous opioids in the brain[15,16]; and OT can bind to ORs and act as an orthosteric agonist or an allosteric modulator.

μORs (MORs) as key targets in pain management are among the most studied G protein-coupled receptors (GPCRs). MORs are grouped in the Class A family of GPCRs and signal through the Gi/o family of heterotrimeric G proteins, which results in the inhibition of adenylate cyclase to cause cAMP level reduction, modulation of ion channel activity (through G protein βγ subunits), and transcriptional changes in cells.[17] Although widely recognized for their therapeutic values, opioid drugs frequently cause serious side effects, including respiratory suppression, constipation, allodynia, tolerance, dependence, and withdrawal symptoms, as well as produce rewarding effects and hold abuse potential.[18] Recently, research attention has been focused on MOR positive allosteric modulators (μ-PAMs), which were first described by Burford et al., in 2013[19] and followed by us.[20] GPCR allosteric ligands bind to a site on the receptor distinct from the site that binds the orthosteric agonist,[21] and an allosteric modulator can change a range of activities at the target protein. PAMs might present no intrinsic efficacy in terms of receptor activation, whereas enhances the binding affinity and/or efficacy of orthosteric agonists when bound to a receptor. PAMs thus offer the specific advantage of acting as modulators of receptor activity only when orthosteric agonists occupy the targeted receptors. This advantageous feature of PAMs could emerge as a novel target in the discovery of drugs that present improved side-effect profiles or fewer tolerance and dependence problems as compared with orthosteric OR agonists.[21]

In this study, we evaluated the effect of OT on MOR by performing an electrical impedance-based receptor biosensor assay (CellKey™, MDS Sciex, Ontario, Canada),[22] an intracellular cAMP assay, and a competitive receptor-binding analysis by using cells stably expressing human MOR (hMOR).

2. Materials and methods

2.1. Construction of plasmids and generation of stable cell lines

The hMOR cDNA (NM_000914) with or without a stop codon was amplified from a Flexi ORF clone (Promega, Madison, WI, USA), and the amplified MOR fragment was introduced into pcDNA3.1 (+) vector (Life Technologies, Carlsbad, CA, USA). Human Embryonic Kidney 293 (HEK293) cells were obtained from American Type Culture Collection (ATCC®, Manassas, VA, USA), and HEK293 cells stably expressing hMOR were generated through transfection of the constructed plasmids by using Lipofectamine reagent (Life Technologies) and selection based on MOR activity measured using the CellKey™ assay. OTR-expressing HEK293 cells were also generated using human OTR (hOTR) cDNAs (Promega) as in the case of hMOR. Moreover, pGloSensor™-22F plasmid (Promega) was transfected into cells that already stably expressed hMOR, and stable cell lines expressing both hMOR and pGloSensor™-22F were generated for monitoring intracellular cAMP levels.

2.2. Cell culture

HEK293 cells (stably expressing hMOR, both hMOR and GloSensor™, or hOTR) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 mg/mL), and genistein (700 μg/mL) in a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C (in a cell-culture incubator).

2.3. Chemicals

The following reagents were purchased from the listed sources: human OT, Peptide Institute Inc. (Osaka, Japan); endomorphin-1, Tocris Bioscience (Bristol, UK); DAMGO (D-Ala(2)-N-Me-Phe(4)-Gly-ol(5)-enkephalin), β-endorphin, forskolin, 3-isobutyl-1-methylxanthine (IBMX), and Ro 20-1724, Sigma–Aldrich (St. Louis, MO, USA); morphine hydrochloride, Takeda Pharmaceutical Co., Ltd. (Tokyo, Japan); and fentanyl, Janssen Pharmaceutical K.K. (Tokyo, Japan). Forskolin, IBMX, and Ro 20-1724 were diluted with dimethyl sulfoxide (DMSO), whereas other reagents were diluted with H_2O.

2.4. Functional analysis of human ORs by using the CellKey™ system

The CellKey™ assay has been described previously.[22–24] Briefly, cells stably expressing hMOR and hOTR were seeded at densities of 6.0×10^4 and 5.0×10^4 cells/well, respectively, in CellKey™ 96-well microplates containing an electrode at the bottom of each well, and then incubated for 21–24 h according to the method of Miyano et al.[22] The wells were washed with CellKey™ buffer composed of Hanks' balanced salt solution (1.3 mM $CaCl_2$nbsp;$2H_2O$, 0.81 mM $MgSO_4$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 136.9 mM NaCl, 0.34 mM Na_2HPO_4, and 5.6 mM D-glucose) containing 20 mM HEPES and 0.1% bovine serum albumin, and then the cells were allowed to equilibrate in the CellKey™ buffer for 30 min at 29 °C before assays. Changes in the impedance of an induced extracellular current (dZiec) in each well were monitored, using the CellKey™ system, once every 10 s for up to 30 min; a 5-min baseline was recorded, after which drugs were added and then the dZiec was measured for 25 min. The extent of changes in dZiec values was expressed as the ΔZiec (ΔZiec = dZiec maximum - dZiec minimum) after drug application. The ΔZiec value of each sample was normalized by that of the control sample.

2.5. Intracellular cAMP assay

The GloSensor™ cAMP biosensor (Promega) includes a modified form of firefly luciferase harboring a cAMP-binding motif.[25] cAMP accumulation was analyzed using HEK293 cells stably expressing both GloSensor™-22F protein and hMOR. The cells were seeded (at 3×10^4/well) into poly-D-lysine-coated 96-well, white, clear-bottomed plates (Corning, Corning, NY, USA), and intracellular cAMP accumulation after 24 h was assayed using the GloSensor™ reagent. Cells were incubated with diluted GloSensor™ reagent at room temperature for 2 h, and then treated with a test compound diluted with CellKey™ buffer containing 250 μM IBMX and 50 μM

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