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Identification of amino acids that are selectively involved in Gi/o activation by rat melanin-concentrating hormone receptor 1

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ABSTRACT

Many G-protein-coupled receptors (GPCRs) are known to functionally couple to multiple G-protein subfamily members. Although promiscuous G-protein coupling enables GPCRs to mediate diverse signals, only a few GPCRs have been identified with differential determinants for coupling to distinct Gα proteins. Mammalian melanin-concentrating hormone receptor 1 (MCHR1) couples to dual G-protein subfamilies. However, the selectivity mechanisms between MCHR1 and different subtypes of Gα proteins are unclear. Our previous studies demonstrated that mammalian MCHR1 couples to both Gi/o and Gq, whereas goldfish MCHR1 exclusively couples to Gq. In this study, we analyzed multiple sequence alignments between rat and goldfish MCHR1s, and designed three multisubstituted mutants of rat MCHR1 by replacing corresponding residues with those in goldfish MCHR1, focusing on regions around the cytosolic intracellular loops. By measurement of intracellular Ca^{2+} mobilization, we found that two MCHR1 mutants, i2_6sub and i3_6sub, which contained six simultaneously substituted residues in the second intracellular loop or a combination of substituted residues in the third intracellular loop and fifth transmembrane domain, respectively, significantly reduced Gi/o-sensitive pertussis toxin responsiveness without altering Gq-mediated activity. Analyses of 10 other substitutions revealed that the multiple substitutions in i2_6sub and i3_6sub were necessary for Gi/o-selective responses. As judged by Gi/o-dependent GTPγS binding and cyclic AMP assays, i2_6sub and i3_6sub elicited phenotypes for impaired Gi/o-mediated signaling. We also monitored the dynamic mass redistribution (DMR) in living cells, which reveals receptor activity as an optical trace containing activation of all GPCR coupling classes. Cells transfected with i2_6sub or i3_6sub exhibited reduced Gi/o-mediated DMR responses compared with those transfected with MCHR1. These data suggest that two different regions independently affect the Gi/o-protein preference, and that multiple residues comprise a conformation favoring Gi/o-protein coupling and subsequently result in Gi/o-selective signaling.

1. Introduction

Melanin-concentrating hormone (MCH) is a cyclic neuropeptide composed of 19 amino acids in mammals. MCH-expressing neurons are predominantly localized in the lateral hypothalamus, which is known as the center for feeding behavior and energy expenditure [1–3]. The effects of MCH are mediated through two class A G-protein-coupled receptors (GPCRs), MCHR1 and MCHR2 [4–6], of which MCHR2 is not functionally present in rodents [7]. MCHR1 is expressed at high levels in several brain regions [8], and MCHR1-knockout mice show decreased body weight and increased activity and energy metabolism [9,10]. Rodent behavior studies also indicated that selective MCHR1 antagonists decrease food intake and body weight [11,12]. Furthermore, several antagonists exhibit antidepressant and anxiolytic effects [13,14]. Therefore, the MCH–MCHR1 system could be an important target for the treatment of obesity, anxiety, and depression in mammals. Beside mammals, MCH and MCHRs exist in other vertebrates [15]. MCH not only causes aggregation of melanophores in teleost skin, but regulates feeding behavior via MCHR1 and/or MCHR2 in goldfish [16,17]. Recently, we cloned and characterized four MCHR subtypes from the amphibian *Xenopus tropicalis* and indicated the involvement of two MCHRs in melanin-granule-concentrating and -dispersing activities [18].

Many GPCRs can activate more than one G-protein subfamily member. In mammalian cell expression systems, rat MCHR1 promiscuously couples to both Gi/o and Gq, resulting in activation of multiple signaling pathways including Ca^{2+} mobilization, phosphorylation of extracellular signal-regulated kinase, and inhibition of cyclic AMP (cAMP) generation [4,5,19]. Extensive mutagenesis analyses have identified several amino

Abbreviations: cAMP, cyclic AMP; DMR, dynamic mass redistribution; GPCR, G-protein-coupled receptor; GTPγS, guanosine 5′-O-[gamma-thio]triphosphate; i1, first intracellular; i2, second intracellular; i3, third intracellular; MCH, melanin-concentrating hormone; MCHR, melanin-concentrating hormone receptor; PTX, pertussis toxin; TM, transmembrane domain.

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acid residues that are essential for mammalian MCHR1 activation. The highly conserved DRY motif in MCHR1 has a role in governing receptor conformation and dual G-protein coupling/recognition [20]. Another study indicated the importance of complete glycosylation of MCHR1 (Asn13, Asn16, and Asn23) for efficient trafficking [21]. Molecular modeling of MCHR1 with MCH demonstrated that Asp123 in the third transmembrane domain (TM3) of MCHR1 is crucial for ligand binding [22]. In the second intracellular (i2) loop, Arg155 was found to be a key residue for receptor signaling via dual G-protein-mediated pathways [23]. In addition, Thr255, which is located at the junction of the third intracellular (i3) loop and sixth transmembrane domain (TM6), is important for receptor folding and correct trafficking to the cell surface [24]. In the intracellular C-terminus, two dibasic amino acids (Arg319 and Lys320) in helix 8, a common short amphiphilic helical domain, are essential for Gi/o- and Gq-mediated signaling [25]. Another mutant in the C-terminus, T317A/S325A/T342A, has no effects on the signal transduction for Ca^{2+} mobilization, but significantly prevents MCH-induced receptor internalization through protein kinase C and β-arrestin 2-dependent processes [26,27]. To date, there remain fundamental questions about how the G-protein selectivity of GPCRs is determined. We previously reported that F318K in the highly conserved NPxxY$(x)_{5,6}$F motif in MCHR1 provides an efficient signaling property that selectively increases the Gq-mediated pathway [28]. However, the distinct amino acid residues required for optimal Gi/o-protein responses in mammalian MCHR1 have not been identified.

Most GPCR studies have emphasized that membrane-proximal regions in the i2 and i3 loops and/or the C-terminal tail of the receptor play prominent roles in coupling to G-proteins [29–31]. However, only a few GPCRs belonging to class A have been demonstrated to contain molecular determinants for coupling to distinct G-proteins [32–34]. Using mammalian cell-based assays, we found that the signaling properties of MCHR1 are quite different between mammals and fish. Thus, diverse transduction assays revealed that rat MCHR1 promiscuously couples to both Gi/o and Gq, while goldfish MCHR1 exclusively couples to Gq [5,35,36]. Based on these different signaling features, we wanted to identify the regions of rat MCHR1 responsible for Gi/o coupling. First, multiple sequence alignments were made between rat and goldfish MCHR1s, and then three mutants of rat MCHR1 containing fish type-substituted helix and/or intracellular cytoplasmic loop residues were constructed. Each mutant was tested for its capacity of Gi/o coupling by measurement of intracellular Ca^{2+} mobilization. Next, we examined the phenotypes induced using Gi/o-mediated guanosine 5′-O-[gamma-thio]triphosphate (GTPγS) binding and cAMP assays. Finally, the MCH-induced responses in living cells were measured by dynamic mass redistribution (DMR) assays, which involve multiple G-protein-mediated pathways. We identified two mutants, in which multiple residues in the i2 loop or i3 loop/TM5 were simultaneously substituted, that caused decreases in Gi/o-mediated signaling without changing the MCHR1 activity via Gq protein.

2. Materials and methods

2.1. cDNA constructs for rat MCHR1 and mutant receptors

The generation of a cDNA encoding a Flag epitope tag before the first methionine of rat MCHR1 (NM_031758/GenBank/EMBL) was described previously [21]. Wild-type MCHR1 and Flag-tagged MCHR1 (Flag-MCHR1) have similar EC50 values for MCH, indicating that the addition of the Flag-tag did not affect the receptor function. Genetyx5 software (Genetyx Corporation, Tokyo, Japan) was used to process the nucleotide and amino acid sequences of rat and goldfish, and perform the amino acid sequence alignments. Substitution mutants around the intracellular regions were produced by oligonucleotide-mediated site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). All mutations in the MCHR1 cDNA sequence were confirmed by sequencing analysis. Mutated MCHR1 cDNAs were excised by digestion with *Eco*RI and *Xho*I and inserted into the pcDNA3.1/Zeo(+) expression vector.

2.2. Cell culture and transfection

Human embryonic kidney HEK293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). The plasmid DNA was mixed with FuGENE HD transfection reagent (Promega, Madison, WI), diluted with Opti-MEM (Life technologies, Carlsbad, CA), and added to 70–80% confluent cells [21]. The transfected cells were replated onto LAB-TEK 8-well plates (Nunc, Rochester, NY) for immunocytochemical analyses, 96-well plates (BIOCOAT; Becton Dickinson, Belford, MA) for Ca^{2+} mobilization assays, and 100-mm cell culture dishes for GTPγS-binding assays. For FACScan flow cytometric analyses and cAMP assays, the cells were reseeded onto 24-well plates (Becton Dickinson). The transfected cells were cultured for a further 18–24 h. For stable transfection, the transfected cells were selected in the presence of zeocin at a final concentration of 0.4 mg/ml for 3 weeks and used for measurements of the cAMP levels.

2.3. Western blotting

Western blotting analyses were performed as described previously [23]. To generate whole-cell extracts, transiently transfected HEK293T cells or CHO-K1 cells were washed with phosphate-buffered saline (PBS), and lysed with ice-cold sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 50 mM β-mercaptoethanol, 10% glycerol). The lysates were homogenized at 4 °C by sonication (SONICAOR Ultrasonic processor W-225; Wakenyaku Ltd., Kyoto, Japan) using five 30-s bursts at 20% power. The proteins were separated by SDS-PAGE and electrotransferred to Hybond-P PVDF membranes (GE Healthcare UK Ltd., Little Chalfont, UK). After blocking with 5% skim milk, Flag-MCHR1 on the membranes was detected by incubation with 1 μg/ml anti-DYKDDDDK primary antibody (Wako, Osaka, Japan), followed by a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (GE Healthcare UK Ltd.). The reactive bands were visualized with an enhanced chemiluminescence (ECL) reagent (GE Healthcare UK Ltd.) and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD).

2.4. Immunofluorescence microscopy

Transfected HEK293T cells were fixed in 3.7% paraformaldehyde-PBS solution for 15 min. After two washes with PBS, cells with or without permeabilization (0.05% Triton X-100 in PBS for 5 min) were transferred into a blocking solution (20% goat serum in PBS) for 30 min, and then incubated with 0.5 μg/ml anti-DYKDDDDK antibody at 4 °C overnight. The bound antibodies were detected using an Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR). Fluorescence imaging was performed using a FLUOVIEW FV1000 confocal microscope (Olympus, Tokyo, Japan). Confocal images were opened in ImageJ software and relative intensity was quantified. The relative intensity represents the ratio pixel density/cell, and statistical analysis of staining intensity was performed with a Student's *t*-test.

2.5. FACScan flow cytometric analysis of cell surface receptors

FACScan flow cytometric analyses were performed as described [21]. Transfected HEK293T cells in 24-well plates were fixed in 1.5% paraformaldehyde-PBS solution for 10 min at room temperature, and then incubated with 0.67 μg/ml anti-DYKDDDDK antibody in PBS containing 20% FBS for 1 h. After three washes with PBS, the cells were incubated with the Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody for 1 h. The cells were washed, collected from the wells with 5 mM EDTA, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems Inc., Franklin