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C-terminal motif of human neuropeptide Y_4 receptor determines internalization and arrestin recruitment

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ABSTRACT

The human neuropeptide Y_4 receptor is a rhodopsin-like G protein-coupled receptor (GPCR), which contributes to anorexigenic signals. Thus, this receptor is a highly interesting target for metabolic diseases. As GPCR internalization and trafficking affect receptor signaling and *vice versa*, we aimed to investigate the molecular mechanism of hY_4R desensitization and endocytosis. The role of distinct segments of the hY_4R carboxyl terminus was investigated by fluorescence microscopy, binding assays, inositol turnover experiments and bioluminescence resonance energy transfer assays to examine the internalization behavior of hY_4R and its interaction with arrestin-3. Based on results of C-terminal deletion mutants and substitution of single amino acids, the motif $^{7.78}$**EESE**HLPL**ST**VH**T**EV**S**KG**S**$^{7.96}$ was identified, with glutamate, threonine and serine residues playing key roles, based on site-directed mutagenesis. Thus, we identified the internalization motif for the human neuropeptide Y_4 receptor, which regulates arrestin-3 recruitment and receptor endocytosis.

1. Introduction

The human neuropeptide Y_4 receptor (hY_4R) is a 375-amino acid transmembrane protein that belongs to class A of G protein-coupled receptors (GPCRs). The hY_4R mediates its signaling through pertussis-toxin sensitive $G_{i/o}$ proteins like the three other members of the human Y receptor family (hY_1R, hY_2R and hY_5R) [1]. Several studies reveal that the hY_4R is predominately located in the gastrointestinal tract [2,3]. Additionally, receptor expression was detected in distinct brain regions that are either involved in the communication of the brain-gut axis or accessible by circulating factors such as the area postrema [4–7]. The most prominent role of the hY_4R is its involvement in food intake, as it mediates satiety signals after activation by its endogenous carboxyl(C)-terminally amidated 36-amino acid peptide ligand, the human pancreatic polypeptide (hPP) [8,9]. This and additional contribution of the hY_4R to anti-secretory effects [3], as well as its possible participation in colon cancer progression [10], and anxiety- and depression-like behaviors [5] suggest this GPCR as a valuable target in several diagnostic and therapeutic approaches. Indeed, several PP-based anti-obesity drugs have already been tested in clinical trials [11–13].

Over the past decades, it became clear that several GPCRs desensitize and undergo a process that is referred to as internalization. This process regulates the propagation and duration of the intracellular signal and can alter the output of a receptor and thus the overall cellular response [14]. Alternatively, G protein-independent signaling pathways might be promoted by proteins of the endocytic machinery. Frequently, internalization of activated GPCRs is induced by phosphorylation of [S/T] rich sequences within the receptor C-terminus or the third intracellular loop. GPCR endocytosis is often triggered by receptor phosphorylation by G protein-coupled receptor kinases (GRKs), although other kinases were also found to be involved in receptor phosphorylation. Nonvisual arrestin-2 (arr-2) and arr-3 can then bind to the active phosphorylated receptor, mediate internalization and transduce further downstream signaling. After passage through different stages of endosomes, finally the GPCR is transported to lysosomes (degradation) or back to the cell surface (recycling).

The hY_4R was reported to undergo such an agonist-induced internalization process in an arr-3-dependent manner and recycles back passing the perinuclear compartment (indirect recycling route) [3,15,16]. Here we aim to investigate the molecular mechanism of hY_4R internalization to shed more light on its activation and regulation profile. To this end, hY_4R deletion mutants were generated lacking parts of a suggested internalization motif ϕ-H-[S/T]-[D/E]-V-S (with ϕ representing hydrophobic residues) located in the receptor C-tail. Further single amino

Abbreviations: ANOVA, analysis of variance; arr, arrestin; BRET, bioluminescence resonance energy transfer; EYFP, enhanced yellow fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; hPP, human pancreatic polypeptide; hY_1R, human neuropeptide Y_1 receptor; hY_2R, human neuropeptide Y_2 receptor; hY_4R, human neuropeptide Y_4 receptor; hY_5R, human neuropeptide Y_5 receptor; *R*Luc8, *Renilla* Luciferase 8; TAMRA, (5,6-)carboxytetramethylrhodamine.

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acid substitutions revealed that additional [S/T] and acidic residues of the medial C-terminus are responsible for receptor internalization. Hence, distinct amino acids playing a role in the internalization process and being responsible for arr-3 binding have been identified and the internalization motif was characterized on a molecular level.

2. Experimental procedures

2.1. Plasmid construction

The cDNA of the N-terminally hemagglutinin (HA)-tagged hY_4R enhanced yellow fluorescent protein (EYFP) fusion protein was cloned into the pVitro2-hygro-mcs vector (Cayla-Invivogen, Toulouse, France) as described [17]. The hY_4R was cloned into the pcDNA3 vector to result in a C-terminal fusion protein with the *Renilla* Luciferase 8 variant (*R*Luc8) using *Asi*SI and *Sbf*I restrictions sites [18]. Bovine arr-3 was cloned into the mCherry-NE/S vector for live cell imaging or N-terminally fused to Venus and cloned into the pcDNA3 vector for bioluminescence resonance energy transfer (BRET) experiments [19]. $G\alpha_{\Delta 6qi4myr}$ was kindly provided by E. Kostenis (Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany) [20]. All deletions and point mutants of hY_4R were obtained from QuikChange site-directed mutagenesis (Stratagene) using appropriate primer pairs.

2.2. Peptide synthesis

Peptides were synthesized by solid-phase peptide synthesis according to 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/*t*Bu) strategy as reported recently [18].

2.3. Cell culture

Cells were grown in a humidified atmosphere at 37 °C and 5% CO_2. Human embryonic kidney cells (HEK293) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g l^{-1} glucose and L-glutamine and Ham's F12 (1:1, *v*/*v*; Lonza) supplied with 15% (*v*/*v*) heat-inactivated fetal calf serum (FCS; Lonza). African green monkey kidney (COS-7 from ATCC, CRL-1651) cells were maintained in DMEM with 4.5 g l^{-1} glucose and L-glutamine supplied with 10% (*v*/*v*) FCS, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Invitrogen).

2.4. Fluorescence microscopy

HEK293 cells were grown on μ-slide 8 wells (ibidi) to 70–80% confluence and subsequently transfected with 1 μg total DNA using Lipofectamine® 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. For single transfection, 1 μg plasmid encoding the receptor construct was used. For co-transfection, 0.9 μg receptor plasmid and 0.1 μg of plasmid encoding arr-3-mCherry fusion protein was applied. One day post transfection, cells were starved with Opti-MEM® reduced serum medium (Gibco®) optionally containing Hoechst33342 (Sigma) for 30 min at 37 °C. Cells were then stimulated with 10^{-7} M (5,6-)carboxytetramethylrhodamine (TAMRA)-hPP for peptide uptake experiments, 10^{-7} M unlabeled hPP to visualize arr-3 redistribution or 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M hPP to study receptor internalization and trafficking. For visualization of receptor internalization, medium was additionally supplied with 100 μg ml^{-1} cycloheximide (CHX; Calbiochem) and 6 μg ml^{-1} brefeldin A (BFA; Santa Cruz). For ligand uptake experiments, cells were washed twice with acidic wash buffer (50 m M glycine, 100 m M NaCl, pH 3.0) and once with Hank's balanced salt solution (HBSS; PAA). The peptide uptake was documented immediately after washing.

2.5. Binding assay

To determine the relative amount of cell surface receptors, HEK293 cells were grown in a 6-well plate and transfected with 7 μg receptor DNA using 10.5 μl Lipofectamine® 2000 transfection reagent. One day post-transfection, cells were re-seeded into poly-D-lysine coated 48-well plates and grown to confluence. Cells were prestimulated with 10^{-6} M hPP in presence of 100 μg ml^{-1} CHX and 6 μg ml^{-1} BFA for 60 min. Non-stimulated cells were treated with CHX and BFA only. Washing and [^{125}I]-hPP binding experiments were performed as described recently [18].

2.6. BRET experiments

Arr-3 interaction with individual hY_4R constructs was determined by BRET assay as reported earlier [18]. Cells were stimulated with 10^{-9}, 10^{-8} and 10^{-7} M hPP for 10 min. All assays were performed at least in triplicate.

2.7. Inositol turnover assay

Inositol turnover experiments were conducted in duplicate from transiently transfected COS-7 cells as reported recently [21].

2.8. Statistical analysis

Nonlinear regression and calculation of means, S.E.M. and statistical analysis were determined using PRISM 5.0 (GraphPad Software). Significances were calculated by one-way ANOVA and Dunnett's multiple comparison test or unpaired *t*-test.

3. Results

3.1. Localization of C-terminal parts responsible for internalization

To estimate the role of the hY_4R C-tail for internalization, a series of deletion mutants was generated (Fig. 1A). These mutants displayed good cell surface expression (Fig. 1B) and also good activation properties at the G protein as determined by inositol turnover experiments, which is reflected in wild type(WT)-like potency and efficacy (Table 1). In live cell imaging, as well as in radioligand binding studies, internalization was observed for the Δ7.93 and Δ7.97 mutants, while the shortest Δ7.78 form did not internalize in response to 1 μM agonist (Fig. 1B and C). Furthermore, we demonstrated for the hY_4RWT that internalization occurred after stimulation with 1 nM hPP (Supplementary Fig. S1).

The hY_4R is known to co-internalize with its ligand hPP [18] which raised the question whether distinct internalization behavior of hY_4R mutants is reflected by hPP uptake. With the help of a hPP-derivative that was N-terminally modified with a TAMRA fluorophore, ligand uptake was estimated by fluorescence microscopy (Fig. 1D). The intensity of fluorescence was measured per cell, to account for different cell numbers within an image section. The TAMRA-hPP uptake of at least ten different cells expressing the receptors was documented per experiment. Ligand uptake corresponded very well with the internalization properties of the mutants, with Δ7.78 showing the lowest intracellular fluorescence (16 ± 2%) and Δ7.93 the strongest uptake (108 ± 18%). The latter was not statistically significantly different from WT (set to 100%). Although Δ7.97 showed an increased internalization in the radioligand binding assay, no increase in ligand uptake was observable (69 ± 10%).

3.2. Arrestin-3 recruitment to hY_4R deletion mutants

To get an insight into the molecular mechanism of hY_4R receptor internalization, arr-3 recruitment was estimated in transiently transfected HEK293 cells. In fluorescence microscopy, redistribution of mCherry