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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.

Keywords

G protein-coupled receptors; Neuropeptide Y receptors; Internalization; Arrestin

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 pertussistoxin sensitive $G_{i/o}$ proteins like the three other members of the

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Author contribution

LW, SB and KB generated mutants and performed microscopic internalization studies as well as inositol turnover experiments. LW and SB performed binding studies and microscopic studies for arrestin recruitment and ligand uptake. LW performed BRET experiments. LW, SB, KM, VVG and AGB-S designed the study and wrote the manuscript.

Conflict of interests

The authors declare no conflict of interest.

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 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 *AsiS*I 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 fused arr-3 was clearly visible after stimulation of WT, Δ7.93 and Δ7.97 with 100 nM hPP (Fig. 2A). The deletion mutant Δ7.78 was not able to induce arr-3 recruitment to the cell membrane after agonist stimulation, although it was

expressed at the cell surface and was active with respect to G protein signaling. Quantitative investigations by BRET assay, using *R*Luc8-fused receptor constructs supported the results obtained in the cell imaging studies (Fig. 2B). Again, Δ7.78 showed the weakest recruitment (1 ± 1%), whereas Δ7.93 (174 ± 6%) and Δ7.97 (186 ± 27%) displayed significantly increased arr-3 recruitment compared to WT receptor (set to 100%). Thus, arr-3 interaction of Δ7.93 and Δ7.97 are strongly pronounced even when the slightly enhanced cell surface expression is considered (Table 1), which might be due to a better orientation of the BRET-donor-acceptor pair. Regarding these results based on the deletion mutants, we concluded that sequences of the intermediate hY_4R C-tail, between residues 7.78 and 7.96, are essential for receptor endocytosis as well as arr-3 recruitment.

3.3. Distinct serine, threonine and glutamic acid residues within the hY_4RC-terminus promote internalization

Based on the results of the deletion mutants, point mutations were introduced by replacing either glutamic acid, serine or threonine residues within the intermediate C-tail by alanine (Fig. 3A), suggesting that those amino acids might serve as an anchor for GRK interaction and phosphorylation sites, respectively. All point mutants displayed good signal transduction activity (Table 1) and showed good cell surface expression of > 78% compared to WT (set to 100%) as determined by cell surface binding of [^{125}I]-hPP (Fig. 3C). Interestingly, only one mutant, $S^{7.100/103}A$, appeared to be still able to undergo agonist-induced endocytosis (Fig. 3B and C), which was not statistically significant. The substitution of threonine and glutamic acid within the intermediate part of the hY_4R C-tail as well as the replacement of serine residues at positions 7.80/86 and 7.93/96 resulted in abolished receptor internalization. Surprisingly, each point mutant was at least capable to mediate some TAMRA-labeled ligand uptake (Fig. 3D). However, uptake quantities were significantly reduced when threonine (31 ± 4%) and glutamic acid residues (29 ± 7%) or serine residues at the position 7.80/86 (42 ± 3%) and 7.93/96 (21 ± 5%) were replaced. This confirms that those residues are crucial for the internalization of the receptor-ligand complex. The substitution of serine residues at positions 7.100/103 was well tolerated regarding internalization and ligand uptake (78 ± 11%), indicating that these two serine residues do not play a role in the internalization process and are not part of the internalization motif.

3.4. Confirmation of the internalization motif by Arr-3 recruitment studies

Assessing the influence of the point mutations on the arr-3 recruitment, the receptor-arr-3 interaction was qualitatively analyzed by fluorescence microscopy (Fig. 4A). Neither $E^{7.78/79/81}A$ nor $T^{7.87/90}A$ nor the serine point mutants $S^{7.80/86}A$ and $S^{7.93/96}A$ induced arr-3 recruitment to the cell membrane after agonist stimulation. The effect of the point mutant $S^{7.93/96}A$ as an example was compared to the hY_4RWT and was tested at lower hPP concentration up to 1 nM (Supplementary Fig. S2). All hPP concentrations clearly induced arr-3 recruitment.

The serine point mutant $S^{7.100/103}A$ was still able to redistribute the mCherry-tagged arr-3 to the cell membrane after stimulation with 100 nM of the endogenous ligand hPP.

These results were confirmed by quantitative investigations using the BRET-assay (Fig. 4B). The point mutants fused to the *R*Luc8 showed a significant decrease in arr-3 recruitment ($E^{7.78/79/81}$A: 25 ± 6%; $T^{7.87/90}$A: 51 ± 6%; $S^{7.80/86}$A: 33 ± 10%; $S^{7.93/96}$A: 49 ± 10%), except of $S^{7.100/103}$A (95 ± 18%) which was able to interact with arr-3 similar to the WT receptor (set to 100%). These experiments clearly demonstrate that glutamic acid and threonine residues and also distinct serine residues within the hY_4R C-tail dictate receptor internalization and arr-3 recruitment.

4. Discussion

Desensitization and internalization of cell surface receptors is known to be a fundamental process to maintain cellular homeostasis and responsiveness. Besides being simply a termination mechanism in terms of receptor signaling, endocytosis and receptor trafficking were also found to determine the quality of the receptor response due to cell-area-specific signaling events. Detailed knowledge of internalization and trafficking is therefore essential to estimate the druggability of a receptor. Like many GPCRs the hY_1R, hY_2R, hY_4R, but not the hY_5R, were reported to undergo rapid internalization in an arr-3-dependent manner [3,22–24]. However, specific sequences that drive endocytosis of the hY_4R have not yet been reported. Therefore, deletion mutants lacking different parts of the hY_4R C-terminus were generated to identify the residues responsible for internalization and arr-3 recruitment. It is known that the very proximal part of the C-terminus of GPCRs can be involved in the anterograde transport of the receptor. Conserved motifs, playing a role in this process, were found in the α_{2B}-adrenergic receptor, angiotensin II type 1A receptor as well as in the hY_2 receptor [25,26]. Likewise, the hY_4R bears a motif ($^{7.60}F(x)_3I(X)_3V^{7.68}$) within the proximal C-tail, which was found to be responsible for the transport of the receptor from the Golgi apparatus to the cell surface [26]. Correspondingly, deletion mutants which were lacking only the medial and distal part of the hY_4R C-terminus were generated, to ensure the correct transport of the receptor to the cell surface. Based on these mutants, it was shown that the medial part of the C-tail, residues 7.78 to 7.96, was responsible for internalization of the hY_4R. We identified distinct serine, threonine and glutamic acid residues within this section ($^{7.78}$**EESE**HLPL**ST**VH**T**EV**S**KG**S**$^{7.96}$) that promoted arr-3 interaction and receptor internalization. Moreover, we demonstrated that the substitution of these specific amino acids had no influence on cell surface expression or activation of G proteins.

Sequence alignments are helpful tools to identify relevant positions as they are conserved in evolution [27]. The sequence alignment of the Y_4 receptor from different species [27] demonstrates that the identified motif contains well conserved residues such as the threonine residues at positions 7.87 and 7.90 and the serine residues at positions 7.86, 7.93 and 7.96, which indicates the relevance of this motif (Fig. 5).

The hY_1R and hY_4R share the highest sequence identity among the human Y receptor family, including the C-terminal sequence S-T-ϕ-H-T-[D/E]-V-S-K-x-S. For the hY_1R, all serine and threonine residues within this sequence were reported to be phosphorylated with the final consequence of arrestin recruitment and internalization [15,28]. However, the number of phosphorylation sites rather than their actual position seems to be relevant for hY_1R desensitization [15]. Correspondingly, we showed that the same serine and threonine

residues of this sequence are relevant for arr-3 recruitment and hY_4R endocytosis. Furthermore, additional glutamic acid and serine residues upstream of this motif seem to be crucial for arr-3 recruitment and hY_4R internalization. Acidic residues were found to favor GRK association to several GPCRs which is fundamental for receptor phosphorylation and arrestin recruitment. However, other kinases, such as the casein kinases and arrestin itself are guided by acidic residues to recognize particular serine and threonine residues within the receptor sequence [29]. Clearly, further experiments will be necessary to prove which kinases participate in hY_4R phosphorylation. Nevertheless, the phosphorylation of serine and threonine residues within the internalization motif seems to be essential for arrestin binding to the hY_4R, comparable to the rhodopsin or the hY_1 receptor [28,30]. The phosphate sensor of the arrestin molecule binds to these phosphates, which have to be in close proximity independent of the surrounding sequence. Next, the arrestin is able to recognize the active receptor conformation and can act as adaptor protein to connect the receptor with members of the endocytotic machinery such as clathrin and the adaptor protein 2 [31–33]. The fluorescence microscopic studies of arr-3 recruitment to the activated hY_4R displayed a spot-like arrestin pattern at the cell surface, indicating that the hY_4R-arrestin complex is guided to clathrin-coated pits. We showed that substitution of distinct serine and threonine residues decreased the arr-3 binding, but only the lack of all phosphorylation sites and glutamic acid residues leads to a complete loss of arr-3 recruitment to the hY_4R. This indicates an essential interplay between all phosphorylation sites and acidic residues within the C-terminal motif. It is noteworthy, that for the hY_4R no arrestin-independent internalization was observed. In contrast, the hY_2R switches to an arrestin-independent internalization mechanism after truncation of the C-terminal phosphorylation motif; the ghrelin receptor was also found to internalize in an arrestin-independent manner [19,34]. After internalization of the hY_4R, it is supposed that the receptor passes endosomal vesicles and is recycled back to the cell surface, but details of the hY_4R recycling process are still a matter of investigation [3]. Interestingly, although the hY_1R and hY_4R share the same internalization motif and arr-3 binding site, these two subtypes of the same receptor family mediate opposing functions. Together with the hY_5R, the hY1R induces food intake and is involved in the regulation of energy homeostasis, whereas the hY_4R mediates anorexigenic effects [8,9,35]. Since, the hY_1R is predominantly expressed in brain tissues, whereas the hY_4R can be found in the gastrointestinal tract [36], these two receptors mediate different functions, using the same molecular determinants for internalization and arr-3 interaction depending on tissue specific expression.

5. Conclusion

In conclusion, we identified distinct serine, threonine and glutamic acid residues within the medial C-tail of the hY_4R between residues 7.78 to 7.96, which are responsible for arr-3 recruitment and for agonist-induced receptor internalization and thus, this study sheds more light on the regulation of the hY_4 receptor signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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$_1$R	human neuropeptide Y$_1$ receptor
hY$_2$R	human neuropeptide Y$_2$ receptor
hY$_4$R	human neuropeptide Y$_4$ receptor
hY$_5$R	human neuropeptide Y$_5$ receptor
***R*Luc8**	*Renilla* Luciferase 8
TAMRA	(5,6-)carboxytetramethylrhodamine

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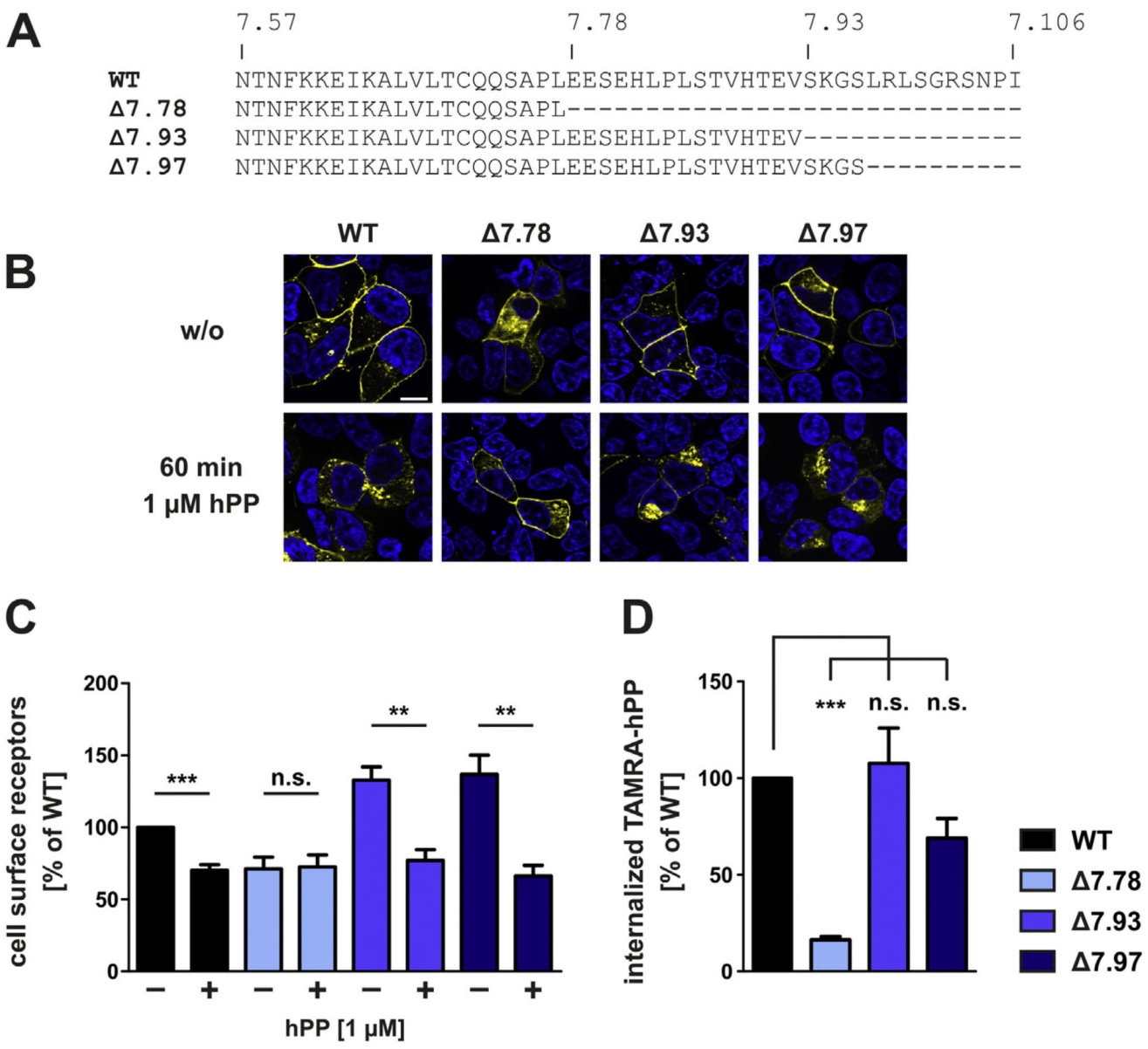

Fig. 1.

Internalization properties and uptake of TAMRA-labeled hPP of hY_4R deletion mutants. (A) C-terminal sequence of hY_4RWT and different deletion mutants in single-letter amino acid code. (B) Receptor localization of WT and deletion mutants was determined in transiently transfected HEK293 cells by fluorescence microscopy prior to (w/o) and after stimulation with the endogenous ligand hPP (1 μM for 60 min) (scale bar: 10 μm). (C) Relative cell surface receptors were quantified by [^{125}I]-hPP binding before (−) and after stimulation with 1 μM hPP for 60 min (+) (Mean ± S.E.M. of n ≥ 3, unpaired *t*-test, $^{**}P \leq 0.0099$; $^{***}P \leq 0.0001$; n.s. – not significant). (D) HEK293 cells were transiently transfected with hY_4RWT and mutant receptors, respectively, and treated with 100 nM TAMRA-labeled hPP for 60 min. Intracellular TAMRA fluorescence was quantified from n ≥ 4 experiments (Mean ± S.E.M.; Dunnett's multiple comparison test; $^{***}P < 0.001$; n.s. – not significant).

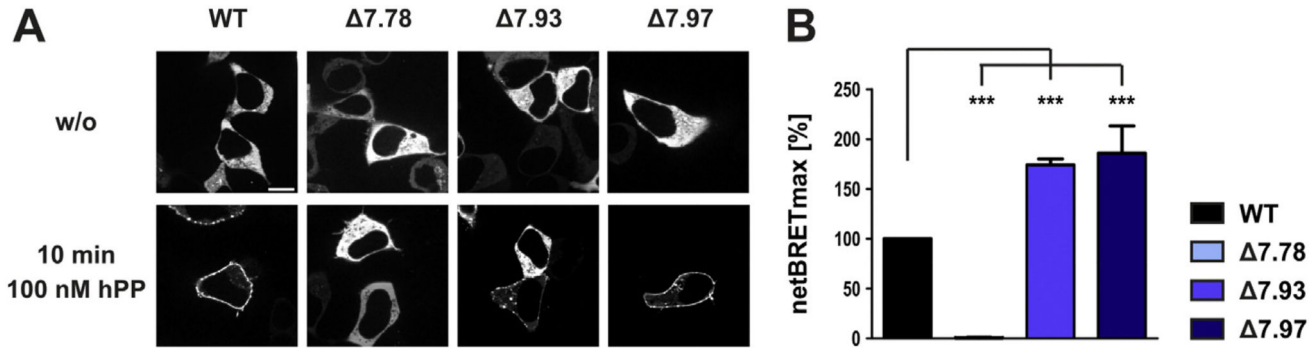

Fig. 2.

Qualitative and quantitative arr-3 recruitment of hY_4R deletion mutants. (A) Arr-3 redistribution in HEK293 cells, co-expressing arr-3-mCherry (shown in white) and hY_4RWT or mutants fused to EYFP (not shown), was documented prior to (w/o) and after stimulation with 100 nM hPP for 10 min (scale bar: 10 μm). (B) Arr-3 receptor interaction was quantified by BRET assay in HEK293 cells. Maximum net BRET values were determined from saturation curves, measuring ligand-induced arr-3 recruitment after 10 min of stimulation (100 nM hPP). (Mean ± S.E.M. of n ≥ 3, Dunnett's multiple comparison test; *** $P < 0.001$).

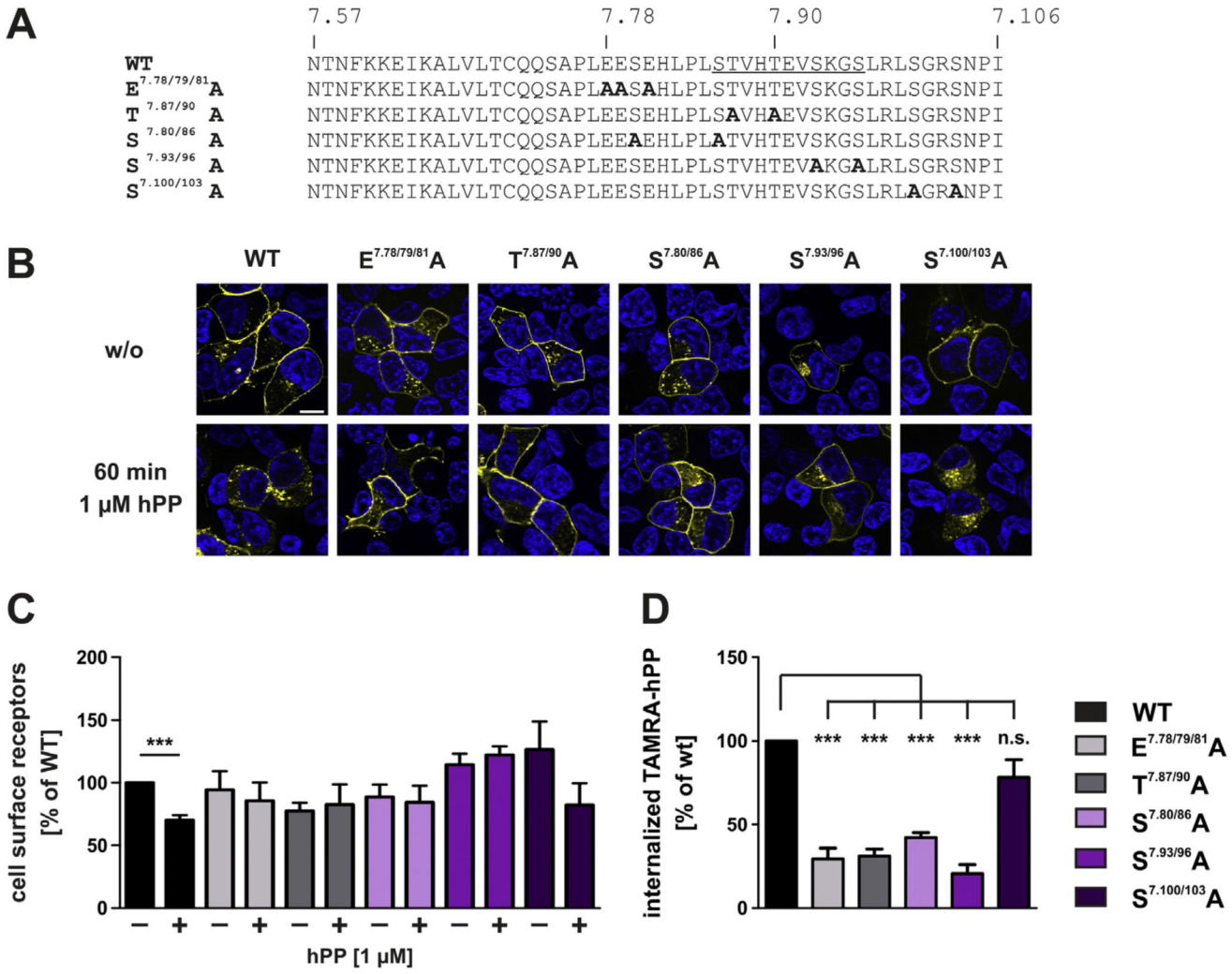

Fig. 3.

Internalization properties and uptake of TAMRA-labeled hPP of hY_4R point mutants. (A) C-terminal sequence of hY_4R WT and different point mutants in single-letter amino acid code. Potential internalization motif is underlined. Introduced Ala substitutions are shown in bold. (B) Transiently transfected HEK293 cells were used to determine receptor localization of WT and point mutants by fluorescence microscopy prior to (w/o) and after stimulation with the endogenous ligand hPP (1 µM for 60 min) (scale bar: 10 µm). (C) Relative cell surface receptors were quantified by [^{125}I]-hPP binding before (−) and after stimulation with 1 µM hPP for 60 min (+) (Mean ± S.E.M. of n ≥ 3, unpaired *t*-test, ***$P \leq 0.0001$). (D) HEK293 cells were transiently transfected with hY_4R WT and mutant receptors, respectively, and treated with 100 nM TAMRA-labeled hPP for 60 min. Intracellular TAMRA fluorescence was quantified from n ≥ 4 experiments (Mean ± S.E.M.; Dunnett's multiple comparison test; ***$P < 0.001$; n.s. – not significant).

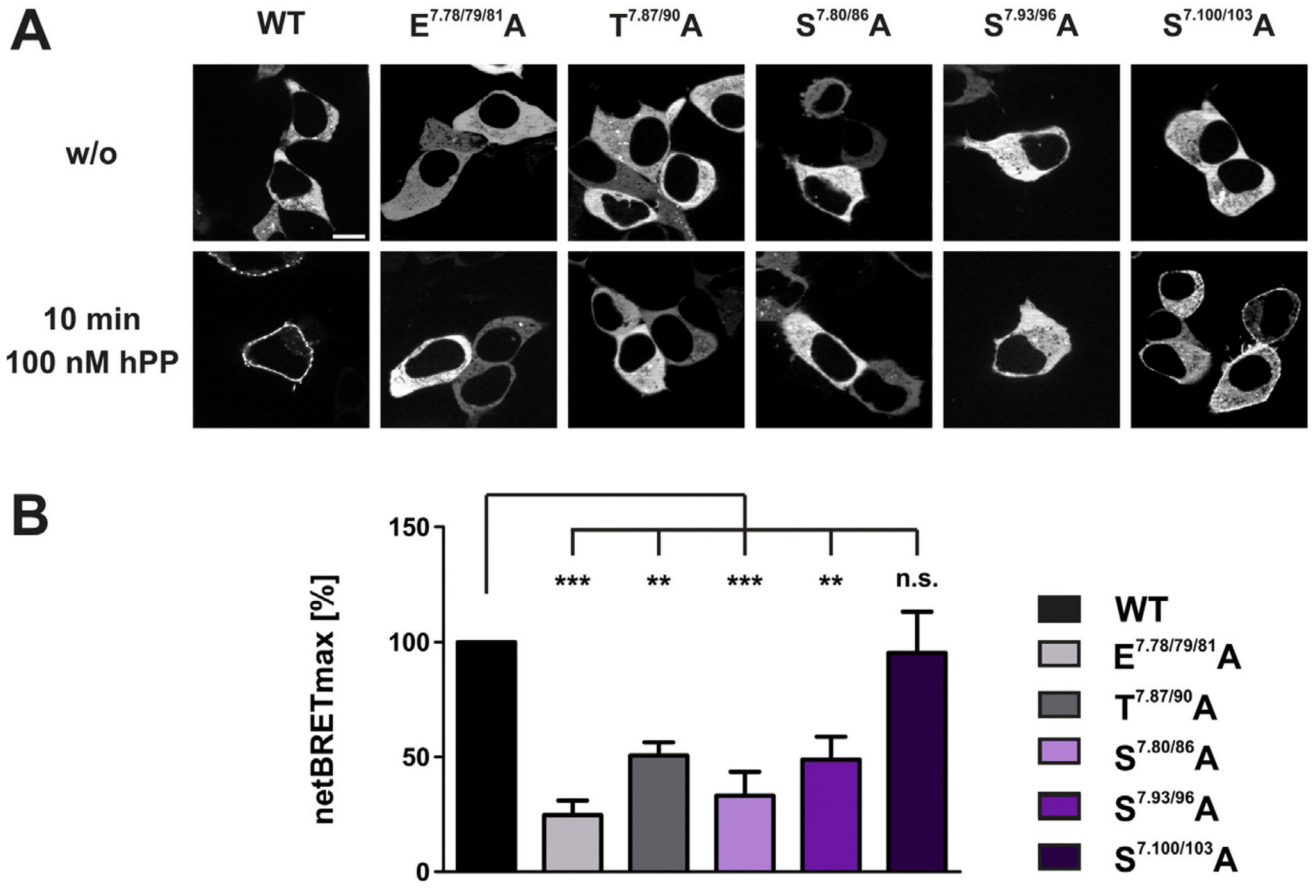

Fig. 4.

Qualitative and quantitative arr-3 recruitment to hY_4R point mutants. (A) Arr-3 redistribution in HEK293 cells, co-expressing arr-3-mCherry (shown in white) and hY_4RWT or mutants fused to EYFP (not shown), was documented prior to (w/o) and after stimulation with 100 nM hPP for 10 min (scale bar: 10 μm). (B) Arr-3 receptor interaction was quantified by BRET assay in HEK293 cells. Maximum net BRET values were determined from saturation curves, measuring ligand-induced arr-3 recruitment after 10 min of stimulation (100 nM hPP). (Mean ± S.E.M. of $n \geq 4$, Dunnett's multiple comparison test; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. – not significant).

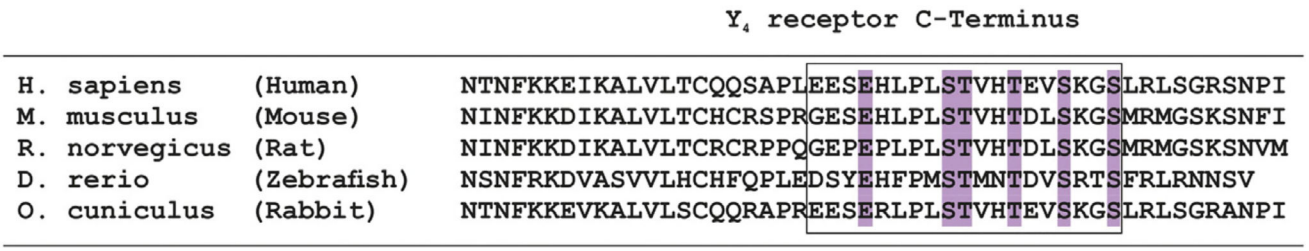

Fig. 5.

Sequence alignment of Y_4 receptor C-terminus. The identified internalization motif is shown within the box. Conserved residues are color-coded

Table 1

Functional characterization of hY_4R wild type and mutants C-terminally fused to EYFP or *R*Luc8. G protein activation was confirmed by inositol turnover experiments in COS-7 cells. Specific binding of EYFP-fused mutants was obtained from transiently transfected HEK293 cells.

Receptor mutants	**EYFP**			***R*Luc8**	
	pEC_{50} ± S.E.M.[a]	**E_{max} [%]**[a]	**Specific binding [% ± S.E.M.]**[b]	**pEC_{50} ± S.E.M.**[a]	**E_{max} [%]**[a]
WT	9.22 ± 0.06	99 ± 2	100	8.92 ± 0.08	98 ± 3
Δ7.78	8.78 ± 0.20	132 ± 7	71 ± 8	9.24 ± 0.11	105 ± 5
Δ7.93	9.05 ± 0.09	100 ± 3	133 ± 9	8.67 ± 0.15	103 ± 8
Δ7.97	9.48 ± 0.18	91 ± 6	137 ± 13	9.26 ± 0.17	89 ± 6
$E^{7.78/79/81}A$	9.38 ± 0.24	84 ± 5	94 ± 15	9.11 ± 0.07	94 ± 3
$T^{7.87/90}A$	9.47 ± 0.24	116 ± 7	78 ± 7	9.44 ± 0.15	104 ± 6
$S^{7.80/86}A$	9.08 ± 0.20	127 ± 6	89 ± 10	9.28 ± 0.12	99 ± 5
$S^{7.93/96}A$	9.12 ± 0.18	161 ± 7	114 ± 9	9.55 ± 0.26	81 ± 8
$S^{7.100/103}A$	9.49 ± 0.16	118 ± 5	126 ± 22	9.51 ± 0.17	118 ± 6

[a] Mean of pEC_{50} and E_{max} values were obtained from concentration-response curves of at least two independent experiments.

[b] Mean of specific [^{125}I]-hPP binding of each mutant was compared to the specific binding of hY_4RWT.