Pancreatic Polypeptide Is Recognized by Two Hydrophobic Domains of the Human Y₄ Receptor Binding Pocket

Received for publication, July 28, 2013, and in revised form, December 21, 2013 Published, JBC Papers in Press, December 27, 2013 Published, JBC Papers in Press, December 27, 2013 DOI 10.1074/jbc.M113.502021

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Background: The Y₄R is involved in regulation of food intake and gastrointestinal transport.

Results: Mutagenesis studies revealed several residues displaying a significant loss of potency for hPP.

Conclusion: Tops of TM2, TM6, and TM7 interact with the hY₄R native agonist hPP.

Significance: Characterizing the structure of the Y₄R binding pocket is crucial for the development of new anti-obesity drugs.

Structural characterization of the human Y₄ receptor (hY₄R) interaction with human pancreatic polypeptide (hPP) is crucial, not only for understanding its biological function but also for testing treatment strategies for obesity that target this interaction. Here, the interaction of receptor mutants with pancreatic polypeptide analogs was studied through double-cylinder mutagenesis. To guide mutagenesis and interpret results, a three-dimensional comparative model of the hY₄R-hPP complex was constructed based on all available class A G protein-coupled receptor crystal structures and refined using experimental data. Our study reveals that residues of the hPP and the hY₄R form a complex network consisting of ionic interactions, hydrophobic interactions, and hydrogen binding. Residues Tyr²₆₄, Asp²₆₈, Asn⁶₅₅, Asn⁷₃₂, and Phe⁷₃₅ of Y₄R are found to be important in receptor activation by hPP. Specifically, Tyr²₆₄ interacts with Tyr²₇ of hPP through hydrophobic contacts. Asn⁷₃₂ is affected by modifications on position Arg³³ of hPP, suggesting a hydrogen bond between these two residues. Likewise, we find that Phe⁷₃₅ is affected by modifications of hPP at positions 33 and 36, indicating interactions between these three amino acids. Taken together, we demonstrate that the top of transmembrane helix 2 (TM2) and the top of transmembrane helices 6 and 7 (TM6–TM7) form the core of the peptide binding pocket. These findings will contribute to the rational design of ligands that bind the Y₄ receptor more effectively to achieve an enhanced agonistic or antagonistic effect.

G protein-coupled receptors (GPCRs) are the most prominent group of cell surface proteins. They are formed by seven transmembrane helices (TM) that are connected by intracellular and extracellular loops (ECL). GPCRs can be activated by several stimuli such as hormones, light, or odorant molecules. It is estimated that ~30% of all prescribed pharmaceuticals modify the activity of GPCRs (2) indicating that these receptors are fundamental drug targets in modern pharmacology.

The Y₄ receptor (Y₄R) is a member of the NPYR, a class A GPCR family composed of Y₁R, Y₂R, Y₄R, and Y₅R receptors in humans. The NPYR is closely related to other class A GPCR families such as the neuropeptide FF receptor family and the orexin receptor family (3). NPY receptors are physiologically coupled to the G₄ protein; however, other reports show that rabbit Y₂R and rabbit Y₄R are also coupled to the G₅ protein, triggering an increase in inositol phosphate (4). These receptors are activated by the NPY family of peptide hormones, consisting of NPY, peptide YY (PYY), and pancreatic polypeptide (PP). NPY peptides and receptors form a multiligand/multireceptor system that plays a role in several physiological and pathological processes, such as obesity and cancer (5). NPY peptides consist of 36 amino acids, are C-terminally amidated, and share high sequence identity. Shared structural features include a C-terminal helix. Despite high sequence homology and common structural features among NPY receptors and peptide hormones, however, there are significant differences in the affinity of these peptide hormones to the different receptor subtypes as well as differences in how the peptide bind their receptor (6).

The Y₄R, cloned in 1995 (7), has 375 amino acids and was found to be expressed in the colon, small intestine, pancreas, prostate (8), brain, and coronary arteries (7). Physiologically,
the Y4R is involved in the regulation of food intake (9), colonic
anion transport (10), and adipose tissue and bone formation
synergistically with Y3R (11). The Y4R sequence is one of the
least conserved members of the NPYR family among different
species, making it the fastest evolving functional member of the
family (12). This makes it difficult to transfer conclusions from
other Y receptor members to this subtype. Its main agonist,
hPP, is produced by endocrine cells of the Langerhans islets of
the duodenal part of the pancreas. These cells are also found in
the gastrointestinal tract (13), albeit in much lower numbers.

Peptide Synthesis—Peptides were synthesized by automated
solid-phase peptide synthesis on a Syro II peptide synthesizer
(MultiSynTech, Bochum, Germany) and manual coupling steps
following a 9-fluorenylmethoxycarbonyl-4-butyloxycarbonyl-
thioanisole/ethanediolithium (90:7:3 v/v/v). After full cleavage, a
reducing mixture containing TFA/ethanediolithium/trimethylsilyl
bromide was applied to reduce oxidized methionine. Crude pep-
tides were purified using preparative reversed-phase HPLC on a
Phenomenex C18 Jupiter 10u column (Proteo, 250 × 21.20 mm,
90 Å).

Peptide characterization was achieved by matrix-assisted laser
desorption/ionization (Ultraflex III MALDI-ToF/ToF,
Bruker Daltonics, Billerica, MA) and by electrospray ionization
mass spectrometry. Peptide purities were determined on two
analytical reversed-phase HPLC systems using a linear gradient of
0.1% (v/v) TFA in H2O (eluents A) and 0.08% (v/v) TFA in
acetonitrile (eluents B). The gradient used was 20–70% of eluent
B in eluent A in 40 min. The purity of the synthetic peptides was
higher than 92% (data not shown).

Preparation of hY4R Mutants—The single mutations were
inserted by site-directed mutagenesis into the protein
sequence. Pfu Turbo DNA polymerase (Agilent) (2.5 units/μl)
was used as a reaction enzyme in combination with 10×
reaction buffer. The plasmid hY4_EYFP_N1 was used as a template
(50–100 ng), and sense/antisense oligonucleotides were used in
2.5–10 pmol/μl concentrations depending on the reaction
conditions. The deoxyribonucleotide (dNTP) mix was added in 10
mm concentration, and dimethyl sulfoxide (DMSO) was used
occasionally to reduce secondary structures. Dntp (Thermo
Fischer Scientific) was used to eliminate the original dsDNA
template. Constructs were transformed in semi-competent
Escherichia coli DH5α or E. coli JM109 cells, and the plasmid
DNA was isolated using a Wizard plus Mini or Midi DNA purifi-
cation system kit (Promega). The desired mutations were con-
firmed by sequencing of the complete coding sequence. The
mutated positions are named after the system of Ballesteros and
Weinstein (22).

Cell Culture—HEK293 cells (human embryonic kidney) and
COS-7 cells (African green monkey) were cultured as described
previously (21).

Fluorescence Microscopy Studies—HEK293 cells were seeded
and transfected with cDNA encoding hY4R constructs as earlier
described (23). The nuclei were stained with Hoechst 33342
(0.5 mg/ml) for 10 min after starving the cells for 20 min in
OptiMEM medium. Fluorescence microscopy pictures were
captured using an ApoTome Imaging system with an Axio
Observer microscope (Carl Zeiss, Jena, Germany).

Signal Transduction Assays—Signal transduction assays
were performed on 24- or 48-well plates as described previ-
ously with minor changes (3, 23). As transfection reagents,
Metafectene and Metafectene Pro (Biontex) were used. The
analysis of the data obtained was performed using the
GraphPad Prism 5.03 software (GraphPad Software, San
Diego). For each hypothesis, the data were processed using a
nonlinear regression analysis, obtaining concentration-re-
sponse curves displaying EC50 and Emax values. Furthermore,
EC50 ratios were calculated using the global curve fitting func-
tion from GraphPad Prism 5.03. All the experiments were per-
formed in duplicates of at least two independent experiments.
Iterative Study to Identify the Y₄R Binding Pocket

| Experimental restraints used to guide docking of PP with hY₄R | Proposed interaction | Steps imposed | Experimental evidence |
|---------------------------------------------------------------|----------------------|---------------|-----------------------|
| **hY₄R residue** | **PP residue** | **Low resolution restraint** | **High resolution restraint** | | |
| Tyr²⁶⁴ | Tyr²⁷ | C-β atoms within 8 Å | None | Unknown | hPP helix placement | Table 2 (Tyr²⁶⁴ and Tyr²⁷ single mutants) |
| Asp⁶³⁹ | Arg³⁵ | C-β atoms within 8 Å | Asp⁶³⁹ O-δ and Arg³⁵ NH within 4 Å | Salt bridge | hPP C-terminal folding (low resolution), hY₄R loop building (low resolution), final relaxation (high resolution) | Ref. 20 (Asp⁶³⁹ and Arg³⁵ single mutants) |
| Asn⁷³² | Arg³⁵ | C-β atoms within 8 Å | Asn⁷³² O-δ and Arg³⁵ NH within 4 Å | Hydrogen bond | hPP C-terminal folding (low resolution), hY₄R loop building (low resolution), final relaxation (high resolution) | Table 5, Fig. 5B (Asp⁷³² and Arg³⁵ single mutants) |
| Phe²³⁵ | Arg³⁵ | C-β atoms within 8 Å | None | π-cation stacking | hPP C-terminal folding (low resolution), hY₄R loop building (low resolution), final relaxation (high resolution) | Ref. 50, Table 4 (Phe²³⁵ and Arg³⁵ single mutants) |
| Phe²³⁵ | Tyr²⁰⁶ | None | Phe²³⁵ CZ and Tyr²⁰⁶ CZ within 4 Å | Unknown | Final relaxation (high resolution) | Table 6, Fig. 6 (Phe²³⁵ and Tyr²⁰⁶ single mutants) |

**Fourteen Experimental GPCR Structures Were Considered as Templates for hY₄R Comparative Modeling**—A comparative model of hY₄R was constructed using the protein structure prediction software package Rosetta, version 3.4 (24). Fourteen experimental GPCR structures from the Protein Data Bank (PDB) were considered as possible templates. These structures include the following: rhodopsin (PDB code 1U19) (25); β₂-adrenoceptor (PDB code 2RH1) (26); β₁-adrenoceptor (PDB code 2VT4) (27); A₁₂₆-adenosine receptor (PDB code 3EML) (28); CXC chemokine receptor type 4 (PDB code 3ODU) (29); D₃ dopamine receptor (PDB code 3PBL) (30); H₁ histamine receptor (PDB code 3RZE) (31); M₂ muscarinic receptor (PDB code 3UON) (32); sphingosine 1-phosphate receptor (PDB code 3V2W) (33); M₃ muscarinic receptor (PDB code 4DAJ) (34); α₁-opioid receptor (PDB code 4DJH) (35); μ-opioid receptor (PDB code 4DKL) (36); nocioceptin/orphanin FQ opioid receptor (PDB code 4EA3) (37), and δ-opioid receptor (PDB code 4EJ4) (38).

These structures were aligned with MUSTANG (39), and the resulting multiple sequence alignment was aligned with a multiple sequence alignment of hY₁R, hY₂R, hY₄R, and hY₅R using ClustalW (40). Sequence alignments were adjusted to remove gaps within transmembrane α-helices and ensure that highly conserved residues remain aligned (supplemental Fig. S1). hY₄R residues were threaded onto the three-dimensional coordinates of aligned residues in each of the 14 GPCRs.

**Missing Atom Coordinates Were Constructed Using Rosetta Loop Construction Protocols**—Missing density and loop regions were reconstructed using Monte Carlo Metropolis fragment replacement and cyclic coordinate descent loop closure algorithms in Rosetta (41). All models underwent repacking and gradient minimization with RosettaMembrane (42). An additional constraint was included to account for the expected disulfide bond between hY₄R residues Cys²³⁵ and Cys²⁵².

The final set of models was clustered based on r.m.s.d. using bcl2Cluster (43). The top scoring models from the five largest clusters were used for docking studies.

**Docking of Pancreatic Polypeptide (PP) into the Comparative Model of hY₄R**—A set of NMR structure conformations of bovine pancreatic polypeptide (PDB code 1LVJ) (44) was docked into the hY₄R comparative models. Bovine pancreatic polypeptide differs only on positions 6 and 23 with respect to hPP and has similar affinity for the hY₄R as earlier reported (45, 46). The use of ILJV provided a guide for the structural distinction between the peptide’s helical region and dynamic tail region. The helical region (residues 1⁴⁻PEQMAQYAAELRRY-INML³¹) was first docked into the hY₄R models. Four distinct helix conformations were docked into 37 hY₄R comparative models without ECLs, guided by a predicted interaction between hY₄R Tyr²⁶⁴ and hPP Tyr²⁷.

**C-terminal Residues of hPP Were Added Using de Novo Folding with Experimental Restraints**—The five C-terminal residues of hPP (TRPRY) were constructed using Rosetta’s low resolution de novo folding algorithm where residues are represented as “centroids” (47). Three experimentally derived restraints between hY₄R and PP residues were used to guide this step using an 8-Å distance cutoff between residues Asp⁶³⁹ and Arg³⁵, Phe²³⁵ and Arg³³, and Asn⁷³² and Arg³³ (20, 48). All restraints are detailed in Table 1.

The ECLs were rebuilt as described for the comparative modeling of hY₄R, with the addition of these experimental constraints. Additionally, these models were refined to atomic detail, replacing centroids with side chain rotamers based on a backbone-dependent rotamer library and energy minimization with RosettaMembrane (49–51).

**Models Were Relaxed Using Atomic Resolution Experimental Restraints**—Models were again clustered based on r.m.s.d. Top scoring models from the largest clusters were visually inspected for binding poses that preserved the experimental restraints. Selected models underwent an additional relaxation step with constraints adjusted to reflect atomic level interactions between residues Asp⁶³⁹ and Arg³⁵ (3 Å distance between the two δ-oxygens on Asp⁶³⁹ and the side chain nitrogen atoms on Arg³⁵), and residues Asn⁷³² and Arg³³ (4 Å distance between the δ-oxygens on Asn⁷³² and the two side chain nitrogen atoms on Arg³³). These constraint distances allow for possible hydrogen bonding and salt bridge interactions. An additional restraint between hY₄R Phe²³⁵ and PP Tyr²⁰⁶ was introduced. Final models were clustered and visually inspected, and nine representative models were selected. The overall workflow for receptor modeling and peptide docking is summarized in Fig. 1.

**RESULTS**

The comparative models presented here reflect an iterative process where multiple rounds of modeling were performed in parallel with in vitro experiments. Early models were generated based on comparative modeling with only seven GPCR templates and limited experimental restraints. The number of tem-

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**TABLE 1**

| Experimental restraints used to guide docking of PP with hY₄R | Proposed interaction | Steps imposed | Experimental evidence |
|---------------------------------------------------------------|----------------------|---------------|-----------------------|
| **hY₄R residue** | **PP residue** | **Low resolution restraint** | **High resolution restraint** | | |
| Tyr²⁶⁴ | Tyr²⁷ | C-β atoms within 8 Å | None | Unknown | hPP helix placement | Table 2 (Tyr²⁶⁴ and Tyr²⁷ single mutants) |
| Asp⁶³⁹ | Arg³⁵ | C-β atoms within 8 Å | Asp⁶³⁹ O-δ and Arg³⁵ NH within 4 Å | Salt bridge | hPP C-terminal folding (low resolution), hY₄R loop building (low resolution), final relaxation (high resolution) | Ref. 20 (Asp⁶³⁹ and Arg³⁵ single mutants) |
| Asn⁷³² | Arg³⁵ | C-β atoms within 8 Å | Asn⁷³² O-δ and Arg³⁵ NH within 4 Å | Hydrogen bond | hPP C-terminal folding (low resolution), hY₄R loop building (low resolution), final relaxation (high resolution) | Table 5, Fig. 5B (Asp⁷³² and Arg³⁵ single mutants) |
| Phe²³⁵ | Arg³⁵ | C-β atoms within 8 Å | None | π-cation stacking | hPP C-terminal folding (low resolution), hY₄R loop building (low resolution), final relaxation (high resolution) | Ref. 50, Table 4 (Phe²³⁵ and Arg³⁵ single mutants) |
| Phe²³⁵ | Tyr²⁰⁶ | None | Phe²³⁵ CZ and Tyr²⁰⁶ CZ within 4 Å | Unknown | Final relaxation (high resolution) | Table 6, Fig. 6 (Phe²³⁵ and Tyr²⁰⁶ single mutants) |
It is hypothesized that this amino acid would be important in hY1R (16). This amino acid is conserved in all receptor subtypes except for hY5R (Fig. 2B), which contains Gly at this position. Asp2.68 was found to be important in all receptor subtypes, was described in some GPCR to belong to the motif WFG and to be important for receptor activation (6). In hY4R, the exchange of Asp2.68 to Ala led to a 94-fold loss in activity and decreased the efficacy dramatically to 39% of the wild type receptor response (Fig. 3B and Table 3). The loss in efficacy fits with the high intracellular accumulation of receptors demonstrated by fluorescence microscopy (Fig. 4). In contrast, the exchange to Glu or Asn regained the efficacy (93 to 87%, respectively) and displayed a loss of potency for hPP of only 9- and 16-fold, respectively.

Additionally, position Trp2.70, which is conserved in all receptor subtypes, was described in some GPCR to belong to the motif WXFG and to be important for receptor activation (53). To prove its relevance, Trp was mutated to Ala, leading to a 107-fold loss in potency. Mutation to Tyr displayed only a 2-fold loss of potency (Table 3). These exchanges reveal that an aromatic or bulky side chain is necessary at this position to keep wild type-like activity.

Because alanine-scan studies of pNPY (52) revealed Tyr27 as an important position for pNPY binding on hY2R, this led to the hypothesis this residue might be the interaction partner of Tyr2.64. To investigate the role of Tyr27, the analogs [Ala27]hPP, [Leu27]hPP, [Cha27]hPP, and [Phe27]hPP were synthesized. The substitution of Tyr27 to Leu pursued the aim of introducing a longer aliphatic amino acid, whereas the introduction of Cha was constructed to investigate the effects of a more bulky hydrophobic amino acid. Additionally, the substitution of Tyr27 to Phe was made to investigate the relevance of the hydroxyl group and to discard a possible hydrogen bond. [Ala27]hPP displayed an 8-fold loss of activity (EC$_{50}$ 11.78 nM), whereas the residue and Cha variants showed wild type like potency on hY4R (Table 2). To investigate the effect of modifications on position Tyr27 with a hypothetical interaction to Tyr2.64, the mutants Y2.64A and Y2.64L were also tested with the peptide analogs in a double-cycle mutagenesis approach. [Ala27]hPP revealed a dramatic activity shift when tested on Y2.64A (424-fold), whereas [Leu27]hPP displayed no further loss on Y2.64A (Fig. 3A). In contrast, [Cha27]hPP activated Y2.64A with higher potency (EC$_{50}$ 22.34 nM) compared with hPP. However, testing [Phe27]hPP on Y2.64L displayed a great activity loss of 138-fold (EC$_{50}$ 205.20 nM), whereas [Leu27]hPP and [Cha27]hPP activated Y2.64L with a moderate loss of potency of 21–23-fold compared with hY4R with hPP (Table 2). Besides these findings, [Phe27]hPP was tested with Y2.64F to further investigate the interaction type. [Phe27]hPP displayed only a 9-fold loss of potency on Y2.64F. Taken together, this confirms that the presence of a bulky hydrophobic amino acid is favorable for this interaction site of the binding pocket and suggests that Tyr2.64 might interact with a second amino acid. This is supported by the comparative models, because seven models have Tyr2.64 within 8 Å of Tyr27 and eight models have Tyr2.64 within 8 Å of Leu31, which might be the second interaction point. Nevertheless, experimental data are needed to confirm this second interaction point.

Other Positions Highlight the Importance of ECL1 — Another amino acid investigated was Asp2.68. This residue is conserved in all receptor subtypes except in hY3R (Fig. 2A), which contains Gly at this position. Asp2.68 was found to be important in mutagenesis studies on the hY1R (17), and it is one of the interaction points between receptor and NPY on the hY3R system (6). In hY4R, the exchange of Asp2.68 to Ala led to a 94-fold loss in activity and decreased the efficacy dramatically to 39% of the wild type receptor response (Fig. 3B and Table 3). The loss in efficacy fits with the high intracellular accumulation of receptors demonstrated by fluorescence microscopy (Fig. 4). In contrast, the exchange to Glu or Asn regained the efficacy (93 to 87%, respectively) and displayed a loss of potency for hPP of only 9- and 16-fold, respectively.

Additionally, position Trp2.70, which is conserved in all receptor subtypes, was described in some GPCR to belong to the motif WXFG and to be important for receptor activation (53). To prove its relevance, Trp was mutated to Ala, leading to a 107-fold loss in potency. Mutation to Tyr displayed only a 2-fold loss of potency (Table 3). These exchanges reveal that an aromatic or bulky side chain is necessary at this position to keep wild type-like activity.
Tested Residues in TM3, ECL2, and TM5 Do Not Play a Relevant Role in the Binding Pocket—The single residue tested in TM3 was Gln\textsuperscript{3.32}. This position has been shown to participate in the binding pocket of nearly all crystallized class A GPCRs (2). In the hY\textsubscript{4}R, the exchange of Gln\textsuperscript{3.32} to Ala displayed a wild type-like potency when tested with hPP.

The ECL2 is the least conserved region between receptor subtypes. To elucidate the role of this ECL, several amino acids were mutated to Ala (Fig. 2B). To investigate polar interactions with the positively charged residues of the peptide Arg\textsuperscript{33} and Arg\textsuperscript{35}, amino acids with negatively charged side chains Glu\textsuperscript{4.67}, Glu\textsuperscript{4.79}, and Asp\textsuperscript{4.83} were mutated to Ala. These three Ala mutants revealed wild type-like activity with EC\textsubscript{50} values from 1- to 2-fold over hY\textsubscript{4}R. Furthermore, preliminary comparative models suggested that the residues Lys\textsuperscript{4.72}, Phe\textsuperscript{4.80}, Trp\textsuperscript{5.29}, His\textsuperscript{5.34}, Tyr\textsuperscript{5.38}, and Phe\textsuperscript{5.41} located on ECL2 and the top of TM5 might be involved in the receptor binding pocket. The exchange of Lys\textsuperscript{4.72}, Trp\textsuperscript{5.29}, and Phe\textsuperscript{5.41} to Ala displayed only a 3–4-fold loss of potency when tested with hPP. Phe\textsuperscript{4.80} and His\textsuperscript{5.34} revealed wild type-like potency for hPP when exchanged to Ala. Only Tyr\textsuperscript{5.38} showed a reduced efficacy for hPP (43%, Table 4) but wild type-like potency. A further mutation to Ser at this position led to a partial regain of efficacy and displayed a 3-fold loss of potency for hPP, whereas an additional exchange to Phe at this position, to prove the relevance of the hydroxyl moiety, led to wild type-like potency for hPP.

TM6 Plays a Crucial Role in Building the Binding Pocket—Asp\textsuperscript{6.59} on top of TM6 has already been shown to form a direct contact to the ligand (20). To further characterize the role of this part of the receptor, residues in close proximity to Asp\textsuperscript{6.59} were chosen for further mutagenesis studies. Phe\textsuperscript{6.54} was mutated to Ala because of its importance in other GPCRs closely related to the hY\textsubscript{4}R, its aromatic character, and its close proximity to Asp\textsuperscript{6.59}. F6.54A displayed a 4-fold loss of hPP potency with reduced efficacy (73%, Table 4) that corresponds to poor membrane localization observed by fluorescence microscopy (Fig. 4). Asn\textsuperscript{6.55} was found to be involved in the hY\textsubscript{4}R binding pocket (18), and because initial comparative models oriented the side chain of this amino acid toward the interior of the hY\textsubscript{4}R binding pocket, this indicated its possible involvement in ligand-receptor interactions. Stimulation of N6.55A with hPP resulted in an 8-fold loss in activity and 79% of efficacy (Fig. 5A and Table 5) suggesting that this position is important in the receptor pocket.

Additionally, His\textsuperscript{6.62} was investigated because of its proximity to Asp\textsuperscript{6.59} at the beginning of ECL3. H6.62A revealed an EC\textsubscript{50} of 0.4 nM, which is moderately better than wild type.

TM7 Is a Contact Point of hPP in the hY\textsubscript{4}R and Asn\textsuperscript{7.32} Interacts with Arg\textsuperscript{33} of hPP—The first position investigated in TM7 was Asn\textsuperscript{7.32}. This residue was found to be relevant for PYY binding on the Y\textsubscript{5}R (54), and initial comparative models indicated the possible importance of this position as well.
**Iterative Study to Identify the Y₄R Binding Pocket**

was mutated to Ala, Arg, and Asp. The effect of these substitutions increased in the following manner: Arg > Ala > Asp (Table 5 and Fig. 5B). This suggested that the introduction of a positive charge might cause a repulsion that was eliminated with the introduction of the negative charge Asp. Hence, we supposed that one of the C-terminal positively charged arginines might be the interaction partner. Because Arg to hPP was already identified to interact with hY₄R Asp.⁶·⁵⁹, Arg was suggested as a possible interaction partner of Asn7.32. To clarify the binding hypothesis, the hPP analogs [ADMA₃³]hPP, [SDMA₃³]hPP, and [Lys₃³]hPP were synthesized. Position 33 of hPP was modified to SDMA and ADMA to maintain the positive charge and simultaneously reduce the possibility of hydrogen bond formation. Whereas [Lys₃³]hPP revealed wild type-like activity, the exchange of Arg to ADMA or SDMA displayed a 7- to 6-fold loss of activity (Table 5). Next, the hPP variants were tested on the receptor mutants N7.32A and N7.32D. [Lys₃³]hPP displayed a great loss of activity on N7.32A (60-fold, EC₅₀ 87.78 nM), and [ADMA₃³]hPP and [SDMA₃³]hPP displayed a dramatic activity loss compared with the wild type peptide with EC₅₀ values of 1416 and >2000 nM, respectively (Table 5 and Fig. 5B). These experiments demonstrated that the introduction of a shorter amino acid such as Lys was better tolerated than the double methylation of Arg, which was not tolerated at all. Unlike N7.32A, N7.32D showed only a 2–3-fold loss of potency when tested with [Lys₃³]hPP and [ADMA₃³]hPP, revealing that the introduction of the double methylation or the reduction of the side chain length did not affect the binding pocket.

**Phe₇.₃₅ Interacts with Arg₃₃ as Well as Tyr₇.₃₆**—Phe₇.₃₅ on the top of TM7 was also investigated, because this conserved position might be a suitable interaction point because of its aromatic characteristics and location. Furthermore, in the hY₄R, Tyr₇.₃₆ was found to be relevant for the receptor and was suggested to belong to the receptor binding pocket (6). The initial comparative models also suggested its orientation to the interior of the proposed binding pocket. The amino acid was mutated to Ala and Ile, displaying a moderate loss in potency (7-fold) for the Ala mutant, whereas the Ile mutant revealed a 41-fold loss of potency compared with wild type (Table 6). The higher potency loss caused by the Ile variant might indicate that the distance or the space available between Phe₇.₃₅ and the ligand or other positions in the receptor is important. F7.35I also showed reduced efficacy that corresponds to single intracellular receptor localization as demonstrated by fluorescence microscopy (Fig. 4). Preliminary comparative hY₄R models suggested that this residue was in close proximity to Arg₃₃ and Tyr₇.₃₆. Furthermore, Arg₃₃ and Tyr₇.₃₆ revealed to be critical residues for pNPY binding on the hY₄R (52). Taken all these facts together, we hypothesized that Phe₇.₃₅ might interact with one or both amino acids, Arg₃₃ or Tyr₇.₃₆, of the peptide. As described above, the exchange of Phe₇.₃₅ to Ile displayed higher impact on receptor activity than the Ala substitution (Table 6). [Lys₃³]hPP and [ADMA₃³]hPP were used to investigate the relationship between Phe₇.₃₅ and Arg₃₃. [Lys₃³]hPP displayed a dramatic loss of 451-fold in activation (EC₅₀ 640.4 nM) on F7.35A. Following tests on F7.35A with [ADMA₃³]hPP revealed a 107-fold loss of activity compared with hPP on hY₄R. This corresponds to the potency loss produced by the mutant

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**Table 2**

| Peptides        | EC₅₀ (nM) ± S.E. | EC₅₀ ratio a | EC₅₀ (nM) ± S.E. | EC₅₀ ratio a |
|-----------------|-----------------|--------------|-----------------|--------------|
| Peptides        | (mutant/wildtype) | (mutant/wildtype) |
| MP hPP          | 1.04 (0.84 ± 0.03) | 1.00 | 1.04 (0.84 ± 0.03) | 1.00 |
| T7.35M hPP      | 1.19 (0.82 ± 0.17) | 1.14 | 1.19 (0.82 ± 0.17) | 1.14 |
| L7.35M hPP      | 2.81 (1.55 ± 0.26) | 2.62 | 2.81 (1.55 ± 0.26) | 2.62 |
| Phe₇.₃₅ hPP     | 0.85 (0.97 ± 0.12) | 0.60 | 0.85 (0.97 ± 0.12) | 0.60 |

a EC₅₀ and EC₅₀ values were determined from the mean ± S.E. of n independent experiments, each performed in duplicate.
plus the loss produced by the analog. Finally, F.7.35I leads to a dramatic loss in activity when tested with both analogs.

Preliminary models suggested Tyr2.64 as a second interaction partner to Phe7.35. To characterize this hypothetical interaction, several analogs with modifications on Tyr36 were synthesized (Table 6). The introduction of Phe36 was well tolerated on the wild type receptor (123-fold). Shortening the length of aromatic amino acid such Ile brought a dramatic loss in potency (Table 6 and Fig. 6). Tyr was not playing a relevant role. The introduction of a non-aromatic amino acid such as Cha and Nle was better tolerated (EC50 2000-fold). Surprisingly, the introduction of unnatural amino acid such Ile brought a dramatic loss in potency between the inactive and active states. Previous modeling studies explored with inactive templates. We compared the r.m.s.d. of our templates with the latest agonist-bound GPCR crystal structures. Pairwise alignments using the structure-based alignment tool MAMMOTH revealed that the average r.m.s.d. value of our inactive structures (2.9 ± 0.6) is not significantly different from that of the active structures (2.8 ± 0.6). Importantly, the average r.m.s.d. value is unchanged when combining the two groups (3.1 ± 0.5).

In addition to our analysis, Tautermann and Pautsch (55) examined the binding sites of active and inactive β2-adrenergic receptors. They show that the binding site is very similar between the inactive and active states. Previous modeling studies with the inactive structure predicted the binding mode of an agonist that overlapped well with that seen in the agonist-bound crystal structure (55).

The final comparative models support these results because within the nine best models obtained, 8 out of 9 showed Asn7.32 within 8 Å of distance to Arg13, and 9 out of 9 models showed Phe7.35 within 8 Å of proximity to Arg33 (Fig. 8).

In addition to the residues mentioned above, the conserved residue His7.39, which was one helix turn deeper in TM7, was also investigated. The Ala mutant did not reveal any detectable activity, and fluorescence microscopy pictures confirmed intracellular localization of the receptor. No further studies have been performed as this receptor variant is stuck in trafficking.

Docking of PP to the hY4R Comparative Model—Pancreatic polypeptide was docked into the comparative model of hY4R to assist interpretation of experimental results. Because inactive GPCR structures were used for our templates, it was important to consider the effects this may have on docking an agonist to this model.

Rosetta’s comparative modeling protocol is insensitive to the state of GPCR templates. Templates are used only in the initial transmembrane helix positioning. Several relaxation steps allow for energy-based adjustments to these placements. Additionally, all extracellular loops are rebuilt in accordance with the helical conformation of an active template may be altered enough to fall outside of the conformational area explored with inactive templates. We compared the r.m.s.d. of our templates with the latest agonist-bound GPCR crystal structures. Pairwise alignments using the structure-based alignment tool MAMMOTH revealed that the average r.m.s.d. value of our inactive structures (2.9 ± 0.6) is not significantly different from that of the active structures (2.8 ± 0.6). Importantly, the average r.m.s.d. value is unchanged when combining the two groups (3.1 ± 0.5).

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The initial placement of the PP helix was guided specifically by the altered activity of hY4R Tyr2.64 and hPP Tyr27 mutants (Table 2). This placement provided a starting position from which the dynamic ECLs and C-terminal tail of hPP might be

### TABLE 3

| Y4R mutants | EC50 (nM) | EC50 ratio | Emax (nM) | n |
|-------------|----------|------------|-----------|---|
| Wild type   | 1.4 (8.84 ± 0.04) | 1 | 100 | 39 |
| D2.68A      | 135 (6.87 ± 0.24) | 94 | 39 ± 2 | 3 |
| D2.68E      | 13.66 (7.87 ± 0.18) | 9 | 93 ± 4 | 2 |
| D2.68N      | 22.88 (7.64 ± 0.13) | 16 | 87 ± 3 | 2 |
| W2.70A      | 157.7 (6.80 ± 0.36) | 107 | 95 ± 9.5 | 6 |
| W2.70Y      | 3.06 (8.52 ± 0.45) | 2 | 108 ± 7 | 2 |
| Q3.32A      | 1.25 (8.90 ± 0.22) | 0.87 | 95 ± 4 | 3 |

### FIGURE 3

Concentration-response curves of hY4R receptor mutants and hPP analogs determined with an immunoprecipitation accumulation assay. A functional characterization of Y4R mutant Y2.64A with the modified ligands [Ala27]hPP and [Cha27]hPP to study the influence between Tyr2.64 of hY4R and Tyr7.35 of hPP. B, functional investigation of hY4R mutant D2.68A.
folded to simulate additional interactions suggested by the mutational data. These interactions specifically include a predicted salt bridge between hY4R Asp6.59 and PP Arg35, a predicted hydrogen bond between hY4R Asn7.32 and PP Arg33, and an interaction between hY4R Phe7.35 and PP Tyr36. The residues imposed by these experimental results were included initially as low resolution restraints based on residue proximity. To complete the model, several restraints were adjusted to higher resolution atom level restraints in an attempt to capture the proposed interactions on an atomic level. The specific restraints imposed and their corresponding steps are described in Table 1. When PP was docked using the low resolution restraints, 81% of the generated models did not significantly violate any of the restraints. In the final step, when the high resolution restraints were imposed, 29.8% of the generated models were able to fit these restraints with no significant violations. This was encouraging in that a significant portion of our models were capable of fitting proposed atom-level interactions. A subset of nine top-scoring models that showed no significant violation of high resolution restraints was selected as the final ensemble for discussion. These models fit well with the majority of the experimental results, accurately portraying residues found to affect activity as well as those residues that failed to show any effect on activity. Specifically, the predicted salt bridge between Asp6.59 and Arg35 is well represented in eight of the nine models. All models show less than a 4.0 Å distance between both inter-residue oxygen-nitrogen pairs, providing possible salt bridge interactions or hydrogen bonding. Six of the nine models demonstrate a distance of less than 3.2 Å between the oxygen in hY4R Asn7.32 and amine group in PP Arg33, providing for the possibility of a hydrogen bond between these residues. hY4R Phe7.35 and PP Arg35 point toward each other in all nine models, which is conducive to the proposed cation-π interaction. Additionally, hY4R Phe7.35 and hPP Tyr36 were oriented toward each other in four models. Finally, hY4R Asp2.68 is within 8 Å and points toward the PP helix in five models, suggesting an interaction between the hPP helix and hY4R Asp2.68.

The importance of hY4R Trp2.70 for hPP binding is the only experimental finding not well reflected in the models. In all but one of the nine models, it is pointing away and/or not in close proximity to hPP. Possible explanations include inaccuracy of the model in this region, increased dynamics of this region as displayed in our models, or an indirect effect that involves a second site on the receptor that interacts with both hPP and Trp2.70. It is interesting that the length of TM2 varies in the models, thereby changing the length of the first intracellular loop dramatically from three residues in two of the models, 9–11 residues in five models, and 12–13 residues in two models. Because the models did not converge on a consistent length of ECL1, and precision is a prerequisite for accuracy, we expect that accuracy in this region might be low. This discrepancy in loop length is shown in Fig. 7C. The residues that failed to show a significant effect on activity in the mutational assays are generally not contacting PP in the model. The majority of these residues are located in ECL2, which is consistently localized on the edge of the receptor away from hPP. Specifically, Lys4.72, Glu4.79, Phe4.80, Asp4.83, His5.34, and Phe5.35 are further than 8 Å away from any PP residue. Gln3.32, Glu4.67, Trp5.29, His6.62, and His7.39 are within 8 Å of an hPP residue in only three of the nine models, and Tyr5.38 is within 8 Å of an hPP residue in only two of the nine models. ECL2 and the residues not involved in PP binding are shown in Fig. 7D. The ensemble of nine models was analyzed for ligand-receptor interactions. These predictions can serve as hypotheses to direct future mutational assays. Residue pairs between PP and hY4R with a distance of less than 8 Å were collected across all nine models. The total counts are shown in Fig. 8. This map can

### TABLE 4

| Y4R mutants | hPP | EC_{50} (nM) | EC_{50} ratio | E_{max} ± S.E. | n |
|-------------|-----|-------------|--------------|---------------|---|
| Wild type   |     | 1.44 (8.84 ± 0.04) | 100 ± 0 S.E. | 39            |   |
| E4.67A      |     | 2.74 (8.56 ± 0.30) | 88 ± 6 2     |              |   |
| K4.72A      |     | 5.07 (8.30 ± 0.17) | 105 ± 5 2    |              |   |
| E4.79A      |     | 1.77 (8.75 ± 0.20) | 114 ± 6 2    |              |   |
| F4.80A      |     | 1.58 (8.8 ± 0.24) | 100 ± 6 2    |              |   |
| D4.83A      |     | 2.58 (8.59 ± 0.15) | 88 ± 3 3     |              |   |
| W5.29A      |     | 4.03 (8.40 ± 0.17) | 96 ± 4 3     |              |   |
| H5.34A      |     | 1.26 (8.89 ± 0.33) | 86 ± 7 3     |              |   |
| Y5.38A      |     | 1.36 (8.87 ± 0.11) | 44 ± 3 5     |              |   |
| Y5.38E      |     | 4.19 (8.38 ± 0.18) | 73 ± 3 5     |              |   |
| Y5.38F      |     | 1.63 (8.79 ± 0.29) | 109 ± 6 5    |              |   |
| F5.41A      |     | 5.02 (8.30 ± 0.24) | 92 ± 6 3     |              |   |

* EC_{50} and EC_{50} ratio values were determined from the mean ± S.E. of n independent experiments, each performed in duplicate.
* The ratio was calculated using the global fitting function for EC_{50} shift determination of Prism 5.03.
* The E_{max} was determined at the highest peptide concentration.
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**Figure 5. Concentration-response curves obtained with an immunoprecipitation accumulation assay using increasing concentrations of the ligands.**

**A,** functional characterization of hY₁₄R mutants N6.55A. B, investigation of the relationship between position 33 of hPP and the amino acids Asn²² and Tyr²⁷ of hY₁₄R using the ligands hPP, [ADMA³³]hPP, and [Lys³³]hPP.

serve as a foundation from which to identify the residues that line the binding pocket. For example, five of nine models show that hY₄R Ser²⁵ and PP Thr³² are within 8 Å of each other, suggesting a possible interaction between these two residues.

**DISCUSSION**

In the NPY receptor family, the ECL1 and TM6 were regions described to form the binding pocket and interact with the peptide (6, 20). Additionally, hY₁₄R, which shares high sequence homology with hY₄R, has been extensively characterized in the past. Many amino acids located on ECL1, TM6, and TM7 are crucial for the interaction (16, 19, 54). Taking all these data into consideration, we expected the hY₄R binding pocket to be composed of amino acids located in these areas of the receptor. Furthermore, it was also expected that hY₄R has a second interaction site with the peptide on top of TM2 or beginning of ECL1, as suggested for hY₅R (6).

We have now identified a binding pocket for the hY₄R system that is composed of several residues located on TM2, TM6, and TM7. The first identified position in the pocket, Tyr²⁶⁴, is conserved in hY₁₄R, hY₂₄R, and hY₄R. It is also present in the prolatin-releasing peptide receptor from several species, including human, rat, and mouse. Tyr²⁶⁴ was found to be involved in ligand binding on the hY₁₄R and was suggested to belong to a hydrophobic pocket (19). In the hY₄R, Tyr²⁶⁴ demonstrated that bulkiness and not aromaticity is critical for the interaction with hPP.

Y2.64L displayed a small decrease in efficacy for hPP. This might be caused by a small portion of receptors being trapped intracellularly (Fig. 4). The substantial amount of intracellular accumulation could be due to high expression levels of the mutant receptor, although it is not very likely because all constructs share the same promoter. It is more probable that this intracellular increase is due to an impaired folding of the mutant. However, the signal intensities suggest that enough active receptors are present in the cell membrane.

To elucidate a candidate position on the peptide side to interact with position Tyr²⁶⁴ of hY₄R, earlier Ala-scanning mutagenesis studies on the NPY peptide family were considered (56). Among others, Tyr²⁷ of NPY and PYY is relevant for binding in all NPY receptor subtypes (52, 56). This conserved residue in the three peptide ligands of the NPY family was thought to be a likely candidate to interact with Tyr²⁶⁴ of hY₄R. Because Leu and Cha at position 27 of hPP have a nonplanar configuration compared with the wild type Tyr, in the presence of Leu or Cha on position 2.64 of hY₄R the interaction might be slightly impeded, and a lack of space between Tyr²⁶⁴ and Tyr²⁷ seems to be a limiting factor for the interaction to take place. This fact would support the close distance between these two positions suggested by the comparative models (Fig. 7A).

Additionally, a hydrogen bonding interaction could be discarded between hY₄R Tyr²⁶⁴ and hPP Tyr²⁷. Besides this, we could not explain the relevance of the hydroxyl moiety of Tyr²⁶⁴ because an aromatic amino acid lacking the hydroxyl moiety like Phe is not as well tolerated as a hydrophobic amino acid like Leu. One hypothesis could be that Phe would adopt a slightly different orientation than Tyr or Leu, and therefore the interaction with the ligand could be slightly impeded. Overall, our data are most consistent with a hydrophobic interaction between hY₄R Tyr²⁶⁴ and hPP Tyr²⁷. Furthermore, the fact that the activity shift obtained for Y2.64A with hPP was larger than the shift obtained for [Ala²⁷]hPP on hY₄R would indicate that Tyr²⁶⁴ might interact with another position in the peptide or within the receptor.

Confirming the importance of ECL1, the nearby residues Asp²⁶⁶ and Trp²⁷⁰ proved critical for the hY₁₄R-hPP interaction. D2.68A displayed high loss of potency and efficacy for hPP. Further mutations on Asp²⁶⁶ suggest that perhaps a polar or negatively charged amino acid is needed for correct export to or stability in the membrane. The relevance of this position is supported by the fact that on the hY₅₄R Asp²⁶⁶ has been proven to interact with Arg²⁵ of pNPY (6). Additionally this residue was hypothesized to form electrostatic interactions with NPY in the hY₅₄R (17, 19). On hY₄R, Asp²⁶⁶ may form hydrogen bonds with the peptide. Also, a polar effect on the structure that stabilizes the receptor binding pocket might be a feasible function for this position. The nearby residue Trp²⁷⁰ needs a bulky hydrophobic amino acid. Although the models show this residue not directly pointing to the peptide, further data are needed to elucidate the role of this residue on the hY₄R binding pocket. This position could participate on direct ligand binding or have a more structural role affecting the nearby important positions when mutated.

On TM6, Asn⁶.⁵⁵ participates in the binding pocket of the hY₄R. Our results are supported by the loss in NPY binding displayed by N6.55A on studies with the hY₁₄R (18). The com-
TABLE 5
Signal transduction of hY4 receptor mutants, mutated residues located on the TM6, ECL3, and TM7

The immunoprecipitation accumulation assays were performed using increasing concentrations of hPP, [Lys33]hPP, [ADMA33]hPP, or [SDMA33]hPP. The incubation time lasted for 1 h. EC50 values from dose-response curves were determined, each performed in duplicate. n represents the number of independent experiments.

| Y4R mutants | hPP | [Lys33]hPP | [ADMA33]hPP | [SDMA33]hPP |
|-------------|-----|-----------|-------------|-------------|
|             | EC50 (nM) | EC50 ratio | Emax ± S.E. | EC50 (nM) | EC50 ratio | Emax ± S.E. | EC50 (nM) | EC50 ratio | Emax ± S.E. | EC50 (nM) | EC50 ratio | Emax ± S.E. |
| Wild type   | 1.44 (8.84 ± 0.04) | 1 | 100 | 39 | 1.68 (8.78 ± 0.07) | 1 | 96.67 (8.02 ± 0.07) | 7 | 9.13 (8.04 ± 0.08) | 6 | 4 |
| F6.54A      | 4.46 (8.35 ± 0.34) | 4 | 73 ± 6 | 3 | NT | 107 ± 3 | 4 | NT | 3 |
| H6.62A      | 0.42 (9.37 ± 0.24) | 0.33 | 98 ± 5 | 2 | NT | 97 ± 4 | 3 | 3.44 (8.48 ± 0.21) | 2 | 3 |
| N6.55A      | 11.39 (7.94 ± 0.10) | 8 | 79 ± 2 | 2 | NT | NT | NT | NT | NT |
| N7.32A      | 6.92 (8.16 ± 0.26) | 5 | 84 ± 5 | 7 | 87.78 (7.06 ± 0.17) | 60 | NT | NT | NT |
| N7.32R      | 24.54 (7.61 ± 0.12) | 18 | 107 ± 3 | 4 | NT | NT | NT | NT | NT |
|               |               |           |           |             |           |           |           |           |           |           |           |           |
| Peptide     | EC50 (nM) | EC50 ratio | Emax ± S.E. | EC50 (nM) | EC50 ratio | Emax ± S.E. | EC50 (nM) | EC50 ratio | Emax ± S.E. | EC50 (nM) | EC50 ratio | Emax ± S.E. |
| hPP         | 1.44 (8.84 ± 0.04) | 1 | 100 | 39 | 11.63 (7.93 ± 0.11) | 8 | 104 (7.28 ± 0.11) | 3 | 11.63 (7.93 ± 0.11) | 8 | 104 (7.28 ± 0.11) | 3 |
| [ADMA33]hPP | 9.67 (8.02 ± 0.07) | 7 | 97 ± 2 | 9 | 160.5 (6.79 ± 0.15) | 107 | 78 ± 4 | 2 | 451 |
| [Lys33]hPP  | 1.68 (8.78 ± 0.07) | 1 | 98 ± 2 | 9 | 640.4 (6.19 ± 0.14) | 451 | 93 ± 4 | 3 | 451 |
| [Ala36]hPP  | >2000 | ND | ND | ND | ND | ND | ND | ND | ND |
| [Leu36]hPP  | 191 (6.72 ± 0.15) | 123 | 99 ± 4 | 3 | NT | NT | NT | NT | NT |
| [Phe36]hPP  | 2.07 (8.68 ± 0.23) | 1 | 102 ± 4 | 4 | 25.29 (7.60 ± 0.43) | 17 | 138 ± 15 | 3 | NT |
| [Cha36]hPP  | 0.76 (9.14 ± 0.18) | 0.6 | 107 ± 4 | 3 | 211.2 (6.67 ± 0.09) | 138 | 93 ± 3 | 3 | NT |
| [Nle36]hPP  | 14.40 (7.84 ± 0.16) | 10 | 100 ± 4 | 5 | 1030.5 (5.99 ± 0.14) | 679 | 81 ± 4 | 4 | NT |

* EC50 and pEC50 values were determined from the mean ± S.E. of n independent experiments, each performed in duplicate.

** The ratio was calculated using the global fitting function for EC50 shift determination of Prism 5.03.

*** The Emax value was determined at the highest peptide concentration.

** NT represents not tested.
Comparative models demonstrate this amino acid pointing to the inner side of the proposed binding pocket close to Phe7.35 (Fig. 7, A and B). Asn6.55 is a candidate for interaction with Arg7.35 of hPP as in six models these residues are within a distance of 8 Å (Fig. 8). Moreover, as reported recently, this position is involved in ligand receptor interactions of many crystallized class A GPCRs (2). This fact strongly supports our data and confirms the role that Asn6.55 of hY4R has in the hPP binding pocket.

The results obtained on position Asn7.32 suggest that this residue is a key player in the binding pocket of hY4R. The mutation to Ala displays a small loss in potency for hPP. Prior studies on the hY1R, PYY and 1229U91 (GR231118), a Y4R agonist and a Y1R antagonist, displayed a loss in binding for N7.32A (54). It could be shown that Asn7.32 might be in close proximity with a positively charged residue, probably one of the two Arg of the C-terminal segment of hPP. To characterize the relationship between hY4R Asn7.32 and Arg33 of hPP, position 33 was modified to Lys to investigate the influence of the side chain length. Also the asymmetric and symmetric side chain dimethylations were tested at this position. Side chain methylations block

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FIGURE 6. Concentration-response curves were determined with an immunoprecipitation accumulation assay with increasing concentrations of the analogs. Functional investigations are shown for position 36 of hPP and Phe7.35 of hY4R. COS-7 cells were transiently co-transfected with hY4R constructs and the chimeric G protein Gα12q4myr

FIGURE 7. Characterization of the binding pocket of PP docked in the hY4R comparative model. A, side view of PP (purple) docked to hY4R (cyan). Residues found to be important in the activation of hY4R by hPP are labeled. Predicted interactions are indicated by dotted red lines (salt bridge between Asp6.59 and Arg7.35 and hydrogen bond between Arg33 and Asn7.32), B, top-down view of the same docked model. C, two docked models show the variability in ECL1. The model shown in gray has a significantly longer ECL1 than that shown in cyan. Trp2.70, which was experimentally shown to be important in hY4R activation by PP, is shown to be in different proximity to PP depending on the size of ECL1. D, side view of the same docked model shown in A and B. Residues experimentally shown to be inactive in the binding of hPP to hY4R are indicated in black. The disulfide bond in ECL2 is also shown in yellow. a = His6.26; b = Glu5.39; c = Phe6.54; d = His6.62; e = Tyr5.38; f = His3.34; g = Trp3.29; h = Phe4.80; i = Glu4.67; j = Glu3.79; k = Lys4.72; and l = Asp4.83.
hydrogen bond donor positions and increase hydrophobicity and bulkiness of the residue (57). Furthermore, the ability to form polar interactions such as dipole-dipole interactions might be impeded by double side chain methylation. The asymmetric and symmetric double methylation on position Arg33 of hPP produced a potency loss (6–7-fold) on hY4R, probably by blocking potential hydrogen bonding positions, potential dipoles, or due to steric hindrance. On N7.32A, the double side chain methylation at position Arg33 of hPP had a more dramatic effect. This might cause conformational changes in doubly methyated Arg33 of hPP and impeding interactions with close by residues such as Phe7.35. These data are in agreement with the shortening of the side chain in [Lys33]hPP that resulted in a smaller potency loss on N7.32A, suggesting that Asn7.32 and Arg33 of hPP are in very close proximity. The fact that these three peptide analogs displayed potencies similar to wild type on N7.32D also supports an interaction with Arg33, because Asp maintains the hydrogen bonding capability and incorporates a negative charge able to form an ionic bond with position Arg33. Accordingly, we were able to demonstrate that Asn7.32 interacts with Arg33 possibly by hydrogen bonding or polar interactions. This hypothesis is supported by the great relevance of Arg33 as already demonstrated in the Ala scan (52).

The last residue of the proposed binding pocket is Phe7.35. The exchange of Phe7.35 to Ile led to a higher potency and efficacy loss than the exchange to Ala, possibly due to steric hindrance. This position has been found to belong to the binding pocket of several class A GPCRs, among them the peptide receptors human CXC chemokine receptor type 4 and the rat neurotensin receptor 1 (2). Furthermore, this position might highlight the singularity of the Y4R binding pocket with respect to the Y1R. To investigate the role of hydrophobicity and size of Arg33 of hPP toward Phe7.35 of hY4R, [ADMA33]hPP was tested on F7.35A. The obtained results fit with the higher potency of [ADMA33]hPP compared with [Lys33]hPP on F7.35A, because the methyl groups can reduce the distance between both positions. A second interaction point of Phe7.35 was suggested by preliminary models to be Tyr36 as hypothesized in previous studies on the Y1R (58). The fact that an aliphatic amino acid such as Ile with a branched β-carbon is not tolerated in contrast to Cha or Phe could suggest a need for space close to the peptide backbone. Moreover, the effect of these ligands on F7.35A indicates that in the absence of Phe at position 7.35 an aromatic amino acid must be present at position 36 of hPP. This may arise for conformational reasons as only an aromatic amino acid with a planar structure might be able to contact position 7.35 in the absence of Phe. So, in the presence of both aromatic groups a π-π interaction might be established between Phe7.35 and Tyr36. In the absence of the aromatic group at position 36 of hPP, this residue might form hydrophobic interactions instead.

Our data provide the first insights into the complex binding pocket of the hY4R system derived from a combination of modeling and mutagenesis. As it may not be possible to solve the structure of all GPCRs, we demonstrate that this iterative method of study is very promising for understanding structurally uncharacterized receptors. As the model is in agreement with experimental data, it can be used to generate further testable hypotheses regarding the receptor-peptide interaction contributing to the development of ligands with enhanced hY4R activity.

Acknowledgments—We thank Regina Reppich-Sacher, Kristin Löbner, and Janet Schwesinger for technical support. We also thank Daniel Rathmann and René Meier for research discussions, Stephanie Deluca for assistance in developing the peptide docking protocols, and Eric Dawson for developing preliminary comparative models.
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