Re-evaluation of receptor–ligand interactions of the human neuropeptide Y receptor Y1: a site-directed mutagenesis study

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Interactions of the human NPY (neuropeptide Y) receptor Y1 with the two endogenous agonists NPY and peptide YY and two non-peptide antagonists were investigated using site-directed mutagenesis at 17 positions. The present study was triggered by contradictions among previously published reports and conclusions that seemed inconsistent with sequence comparisons across species and receptor subtypes. Our results show that Asp²⁸⁷, at the border between TM (transmembrane) region 6 and EL3 (extracellular loop 3) influences peptide binding, while two aspartic residues in EL2 do not, in agreement with some previous studies but in disagreement with others. A hydrophobic pocket of the Y1 receptor consisting of Tyr¹⁰⁰ (TM2), Phe²⁸⁶ (TM6) and His²⁹⁸ (EL3) has been proposed to interact with the amidated C-terminus of NPY, a theory that is unsupported by sequence comparisons between Y1, Y2 and Y5. Nevertheless, our results confirm that these amino acid residues are critical for peptide

binding, but probably interact with NPY differently than proposed previously. Studies with the Y1-selective antagonist SR120819A identified a new site of interaction at Asn¹¹⁶ in TM3. Position Phe¹⁷³ in TM4 is also important for binding of this antagonist. In contrast with previous reports, we found that Phe¹⁷³ is not crucial for the binding of BIBP3226, another selective Y1 receptor antagonist. Also, we found that position Thr²¹² (TM5) is important for binding of both antagonists. Our mutagenesis results and our three-dimensional model of the receptor based on the high-resolution structure of bovine rhodopsin suggest new interactions for agonist as well as antagonist binding to the Y1 receptor.

Key words: BIBP3226, G-protein-coupled receptor (GPCR), mutagenesis, neuropeptide Y (NPY), receptor Y1, three-dimensional model.

INTRODUCTION

NPY (neuropeptide Y) and PYY (peptide YY) are highly conserved 36-amino-acid peptides with a C-terminal amide group (Figure 1) [1]. They are widely expressed in the neuronal and gut endocrine systems respectively. In addition to many powerful physiological effects such as appetite stimulation (NPY), appetite reduction [PYY-(3–36)], vasoconstriction and influence on gastrointestinal functions [2–4], there are two features that make the NPY/PYY system particularly interesting for studies of ligand–receptor structures and interactions. First, these neuroendocrine peptides seem to have unusually well-defined three-dimensional structures. X-ray crystallography of PP (pancreatic polypeptide), the third member of this peptide family, identified a proline helix for residues 1–8, a β -turn for residues 9–14, an α -helix for residues 15–32 and a C-terminal flexible structure for residues 33–36 [5]. This characteristic hairpin-like structure has been named the PP fold. Structural modelling has suggested that NPY and PYY share this structure, which has been confirmed by NMR studies for NPY [6], PP [7] and PYY [8], although NPY in solution at low concentrations may not be folded like a hairpin [9,10].

Secondly, the five mammalian receptor subtypes for the NPY-family peptides display unusually divergent primary structures. With only 27–32 % overall identity, the three subtypes Y1, Y2 and Y5 are the most different GPCRs (G-protein-coupled receptors) that bind the same peptide ligand [11]. In fact, two peptide ligands (NPY and PYY) that share 70 % identity bind to these receptors.

Nevertheless, it seems clear that all of the NPY receptors share a single common NPY/PYY receptor ancestor [11]. All three of the receptor subtypes mentioned have interactions with the C-terminal portion of NPY and PYY, but differ in their requirement for the N-terminal part. Y1 requires a complete N-terminus, while Y5 has equal affinity for NPY/PYY and the N-terminally truncated peptide NPY-(2–36) [12,13]. Much shorter peptides, even as small as NPY-(13–36), can bind to the Y2 receptor with only marginal reduction in affinity [14,15]. Such limited amino acid identity among the receptor subtypes and high identity between NPY and PYY might be considered as a complication for structural modelling. However, it may also be turned into an advantage as the relatively few amino acids that are conserved across receptor subtypes are likely to be important for ligand binding and signal transduction to G-proteins and presumably function similarly in the different subtypes.

Although the Y1 receptor was the first to be cloned among the NPY family of receptors [12,16,17], its structure has been challenging to study due to difficulties in achieving functional expression of the receptor in transfected cell lines. A few mutagenesis studies of hY1 (human Y1) have been performed using expression systems less commonly used for this type of analysis, such as a vaccinia virus vector and human HeLa cells [18–20] and even expression in *Escherichia coli* [21]. Only one mutagenesis study of the hY1 receptor in a conventional expression system has been reported using a pcDNA3-based vector in COS-7 cells [22]. Also, the rat Y1 receptor has been studied in this system [23].

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EL, extracellular loop; GPCR, G-protein-coupled receptor; HEK-293 cells, human embryonic kidney 293 cells; hY1 receptor, human Y1 receptor; NPY, neuropeptide Y; pNPY, porcine NPY; PP, pancreatic polypeptide; PYY, peptide YY; pPYY, porcine PYY; TM, transmembrane; WT, wild-type.

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Figure 1 Structures of ligands

(A) Human NPY; (B) human PYY. White circles refer to conserved positions in all known peptide sequences, grey circles refer to those conserved among mammals and black circles refer to variable positions [1]. (C) BIBP3226; (D) SR120819A.

The original model of the hY1 receptor suggested that the endogenous peptide ligands, which have several basic amino acid side chains, interact with four aspartic residues, namely Asp¹⁰⁴ in EL1 (extracellular loop 1), Asp¹⁹⁴ and Asp²⁰⁰ in EL2 and position Asp²⁸⁷ on the border between TM (transmembrane) region 6 and EL3 [18]. However, contradictory findings in later studies suggested that only Asp¹⁰⁴ in EL1 and Asp²⁸⁷ in TM6 are important [20–22]. Moreover, residues Tyr¹⁰⁰ in TM2, Phe²⁸⁶ in TM6 and His²⁹⁸ in EL3 of the receptor were proposed to form a hydrophobic pocket important for binding of the amidated C-terminus of the peptide ligand [19]. However, the amino acids at the corresponding positions in other NPY receptor subtypes differ. The equivalent of position Tyr¹⁰⁰ in the Y5 receptor is a serine residue, Phe²⁸⁶ has differing amino acids in the Y2, Y4 and Y5 subtypes and the position His²⁹⁸ has amino acids with variable characteristics in the Y2 and Y4 subtypes [11]. As all NPY-family receptors recognize the amidated C-terminal part of the endogenous peptide ligands, it seems unlikely that two of the three proposed Tyr³⁶ amide-interacting positions would differ in Y2 and Y4, and all three in Y5. Indeed, Du et al. [24] reported that PYY exhibited unaltered binding when Phe²⁸⁶ and His²⁹⁸ were mutated to alanine and glycine residues respectively. Taken together, these contradictory observations and the improved high-resolution crystal structure for bovine rhodopsin [25] demand new modelling and mutagenesis studies of the Y1 receptor and a re-evaluation of previous results. This will give a better understanding of other NPY-receptor subtypes as well as improving NPY receptor-targeted drug development.

In the present study, we report an extensive site-directed mutagenesis study of 17 positions of the hY1 receptor, followed by pharmacological characterization of the receptors by binding studies using the two endogenous peptides NPY and PYY (Figures 1A and 1B) as well as two selective non-peptide antagonists, BIBP3226 [26] and SR120819A [27] (Figures 1C and 1D). Our results together with our three-dimensional model confirm the importance of some of the previously proposed residues, question

others and identify new important positions not previously known to influence the receptor–ligand interactions.

EXPERIMENTAL

Construction of expression vectors carrying mutant hY1 receptors

The positions for mutagenesis were selected after sequence comparisons of Y1 sequences from different species as well as comparisons with other NPY receptor subtypes [11] (Figure 2; see Supplementary Figures 1 and 2 at <http://www.BiochemJ.org/bj/393/bj3930161add.htm>).

Some mutant hY1 receptors were constructed in the pUC18 vector, according to previously described procedures [28,29]. Due to difficulties of expressing the receptor in mammalian cell cultures, no binding data were obtained for these mutants at the time. We introduced a FLAG epitope by PCR and transferred the mutated hY1 receptors to a modified pCEP4 vector using the Gateway cloning method (see below). Other mutations were introduced by four-primer PCR, using a WT (wild-type) hY1 receptor with a FLAG epitope as a template. The PCR products were cloned into the modified pCEP4 vector using the Gateway method (see below).

Introduction of FLAG epitope into the WT hY1 receptor

The WT hY1 receptor was subcloned into a modified pCEP4 vector, previously shown to enhance protein expression [30]. Prior to mutagenesis, a C-terminal FLAG epitope was introduced by PCR and binding assays were performed to ensure that the epitope did not affect the binding properties of the receptor for pPYY (porcine PYY) (results not shown).

Gateway cloning

A PCR was run with the proofreading enzyme PLATINUM[®] Pfx DNA polymerase (Life Technologies, Stockholm, Sweden) and

Figure 2 Schematic serpent model of the hY1 receptor, highlighting residues mutated in the present study

Shaded regions show the seven-TM regions and the short C-terminal cytoplasmic α -helix.

the PCR product was gel-purified with a QIAquick Gel Extraction kit (Qiagen, Stockholm, Sweden). The PCR product was cloned using the GatewayTM system according to the manufacturer's instructions (Life Technologies). Briefly, the PCR product was cloned into an expression vector via a donor vector (GatewayTM pDONRTM201 Vector; Life Technologies) using modified phage lambda enzymes (Life Technologies). The expression vector was a modified pCEP4 plasmid (Invitrogen, Groningen, the Netherlands), with an intron from pCI-neo (Promega, Falkenberg, Sweden) located 5' to the cloning site. This construct has previously been shown to enhance gene expression from the cytomegalovirus promoter [30]. Also, the vector contained a GatewayTM cloning cassette, obtained by using the GatewayTM Vector Conversion Reagent System (Life Technologies), with a *ccdB* gene (confers cell death when expressed in *E. coli*) and recognition sites for modified lambda recombinases. The cloned receptor in the expression vector was fully sequenced from both directions.

Transfection method and membrane harvesting

For transient transfections, HEK-293 (human embryonic kidney 293) EBNA-1 cells were transfected with FuGENETM6 Transfection reagent (Boehringer Mannheim, Biberach an der Riss, Germany), diluted in OptiMEM medium (Gibco BRL, Stockholm, Sweden) according to the manufacturer's instructions. After transfection, cells were grown in DMEM (Dulbecco's modified Eagle's medium)/Nut Mix F-12 without L-glutamine (Gibco BRL) containing 10% (v/v) fetal calf serum (Biotech Line AS), 2.4 mM L-glutamine (Gibco BRL), 0.25 mg/ml G418 (Gibco BRL) and 100 units of penicillin/100 μ g of streptomycin/ml (Gibco BRL) until harvesting after 48 h. Cells with semi-stable expression were chosen by growth in the presence of 200 μ g/ml hygromycin, starting 1 day after transfection, in the same growth medium as described above. Cell membranes were washed, collected by centrifugation and frozen in aliquots at -80°C . Cell membranes used for saturation assays were concentrated (by homogenization and centrifugation) to obtain

about the same receptor density needed to carry out reliable binding assays.

Peptides and non-peptide ligands

pNPY (porcine NPY) was purchased from Bachem (King of Prussia, PA, U.S.A.) and [^{125}I]pPYY was purchased from Amersham Biosciences. The non-peptide Y1 antagonist BIBP3226 [26] was provided by Boehringer Ingelheim (Biberach an der Riss, Germany). The non-peptide Y1 antagonist SR120819A [27] was provided by Sanofi-Synthelabo Recherche (Toulouse, France).

Binding assays

On the day of the binding assay, membrane aliquots were thawed and resuspended in 25 mM Hepes buffer (pH 7.4) containing 2.5 mM CaCl_2 and 1.0 mM MgCl_2 , with 2.0 g/l bacitracin, and homogenized using an Ultra-Turrax homogenizer. Saturation experiments were performed in a final volume of 100 μ l with 10–25 μ g of protein and [^{125}I]pPYY for 2 h at room temperature (23°C). This radioligand had iodinated tyrosine residues at positions 21 and 27 and had a specific radioactivity of 4000 Ci/mmol. Saturation experiments were carried out with serial dilutions of radioligand. Non-specific binding was defined as the amount of radioactivity remaining bound to the cell membranes after incubation in the presence of 100 nM unlabelled pNPY. Competition experiments were performed in a final volume of 100 μ l. Various concentrations of the competitor, i.e. pNPY, SR120819A or BIBP3226, were included in the incubation mixture along with [^{125}I]pPYY. Incubations were terminated by filtration through GF/C filters (Filtermat A from Wallac Oy, Turku, Finland), which had been presoaked in 0.3% poly(ethyleneimine), using a Tomtec (Orange, CT, U.S.A.) cell harvester. The filters were washed with 50 mM Tris (pH 7.4) at 4°C and dried at 60°C . The dried filters were treated with MeltiLex A (Wallac Oy) melton scintillator sheets and the radioactivity retained on the filters was counted using the Wallac 1450 Microbeta counter.

The results were analysed with nonlinear regression curve fitting using the Prism 2.0 software package (GraphPad, San Diego, CA, U.S.A.). Saturation experiments were also analysed with linear regression using Scatchard transformation. Hill coefficients were calculated for each individual competition experiment. One- and two-site curve fitting was tested in all experiments. The two-site model was accepted when it significantly improved the curve fit ($P < 0.05$; F test) and when each site accounted for $> 20\%$ of the receptors. Before statistical analysis, dissociation constant (K_d) and inhibition constant (K_i) values obtained in each binding assay were converted into pK_d and pK_i values by the formula $-\log K_d$ or $-\log K_i$ respectively. One-way ANOVA followed by Dunnett's multiple comparison test for comparisons with the WT group was used on the pK_d and pK_i values to determine which mutants differed in binding characteristics from the WT.

Protein concentrations were measured by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad, Solna, Sweden) with BSA as the standard.

Construction of the hY1 receptor molecular structure model

The model was based on the $2.8 \text{ \AA}$ ($1 \text{ \AA} = 0.1 \text{ nm}$) resolution X-ray crystallographic structure of bovine rhodopsin [25]. Residues conserved among the GPCR family were tethered by constraints to each other in the rhodopsin template. The amino acids of the rhodopsin protein sequence were replaced with the corresponding amino acids of the hY1 receptor. Regions that could not be aligned, e.g. the extracellular and intracellular regions, were excluded. Modelling was performed with Sybyl 6.9.1 software (Tripos, Munich, Germany) running on workstation Fedora core 2 linux kernel 2.4 using the subroutine Powell to energy-refine the receptor model. The whole model was minimized with the Kollmann United Force field (maximum iteration 10.000), which gave a receptor model with energy of -3321 kcal/mol ($1 \text{ kcal} = 4.184 \text{ kJ}$). The WHAT IF V4.99 program was used to evaluate the validity of the obtained structural model by analysing different conformational interaction factors such as bond angles, bond length, interatomic distances and packing quality [31]. Furthermore, the RAMPAGE program was used to create a Ramachandran plot to evaluate the Φ and ψ angles of the C- α amino acids of the helices and compare them with the rhodopsin template [32].

Immunocytochemistry

To monitor the expression of the hY1 receptor protein and some of the mutants, EBNA cells semi-stably expressing these constructs were grown in DMEM/F-12 medium on coverslips in 6-well plates. On the day of the experiment, the cells were washed once with PBS and fixed for 30 min in 2% (w/v) paraformaldehyde/PBS at room temperature and washed three times with PBS. Thereafter, the cells were permeabilized with 0.1% Triton X-100/PBS (Sigma–Aldrich Sweden AB, Stockholm, Sweden) for 2 h at room temperature and washed three times with PBS. Non-specific binding was blocked with 5% fetal calf serum/PBS for 1 h at room temperature. The cells were washed once with PBS and then the primary antibody, anti-FLAG BioM2 (Sigma–Aldrich) diluted 1:1000 in BSA/PBS, was added for 1 h at room temperature. The cells were washed three times with 0.1% Tween 20/PBS with each wash lasting for 10 min while shaking. Mouse anti-biotin Cy3-conjugated secondary antibody (Sigma–Aldrich) was added at a dilution of 1:1000 in BSA/PBS for 1 h at room temperature. The cells were then washed three times with 0.1% Tween 20/PBS as above. Finally, the cells were mounted on slides in Vectashield™ (Vectashield Laboratories, Burlingame, CA, U.S.A.) containing $1.5 \mu\text{g/ml}$ DAPI (4,6-dia-

Figure 3 Binding characteristics of the WT hY1 receptor

(A) Saturation and Scatchard analyses (inset) of $[^{125}\text{I}]\text{pPYY}$ binding to cloned WT hY1 receptors expressed in HEK-293 EBNA-1 cells. Results shown are from one typical experiment. $K_d = 63.7 \text{ pM}$ and $B_{\text{max}} = 80.0 \text{ fmol/mg}$ of protein. Scatchard analysis gave $K_d = 61.3 \text{ pM}$ and $B_{\text{max}} = 79.3 \text{ fmol/mg}$ of protein. (B) Inhibition of $[^{125}\text{I}]\text{pPYY}$ binding to cloned hY1 receptors expressed in HEK-293 EBNA-1 cells by competitors pNPY, BIBP3226 or SR120819A. Results shown are from one typical experiment. $K_i = 0.19 \text{ nM}$ for pNPY, 6.80 nM for BIBP3226 and 0.42 nM for SR120819A.

midino-2-phenylindole). Fluorescently stained cells were viewed with a Zeiss Axioplan 2 microscope. Pictures were taken using an AxioCam HRm camera and the Openlab software of Improvision.

RESULTS

A total of 17 positions of the hY1 receptor were mutated (two of them in two different ways) and changes in ligand interactions and expression level were analysed. The positions for mutagenesis were selected after sequence comparisons of Y1 sequences from different species as well as comparisons with other NPY receptor subtypes [11] (Figure 2, and Supplementary Figures 1 and 2). The peptides used in the binding assays were pNPY and pPYY, which differ by only one and two positions respectively from the human peptides. The porcine peptides have been used extensively in previous studies and their properties appear to be functionally indistinguishable from those of the human peptides, which is why they can be considered equivalent to the truly endogenous human peptides. The binding affinities of the agonists observed in our study are 2–14-fold higher than those reported by some other groups [19–22]. This is probably mainly due to methodological differences.

Binding characteristics of $[^{125}\text{I}]\text{pPYY}$ to the WT and mutant hY1 receptors

Figure 3(A) shows a representative saturation curve of $[^{125}\text{I}]\text{pPYY}$ binding to the cloned WT hY1 receptor. $[^{125}\text{I}]\text{pPYY}$ bound with high affinity, saturability and low non-specific binding to the Y1

Table 1 K_d and B_{max} values of [125 I]pPYY binding to WT and mutated hY1 receptors

The results are means $\pm$ S.E.M. for n independent experiments performed in duplicate. Non-specific binding was defined in the presence of 100 nM pNPY. The data were analysed using nonlinear regression (GraphPad Prism 2.0 software). Abbreviations: nb, no binding; nm, not measurable; ne, not expressed; na, not analysed in the saturation assay. ** $P < 0.01$ as determined by one-way ANOVA followed by Dunnett's test, indicating a significant difference in pK_d value compared with the WT. K_d values were converted into pK_d values by the formula $-\log K_d$. K_d/K_d WT stands for K_d mutant/ K_d WT.

Mutant	[125 I]pPYY			n
	K_d (nM)	K_d/K_d WT	B_{max} (fmol/mg of protein)	
WT	0.065 ± 0.001	1.0	68 ± 9.8	3
A90T	na	—	—	—
Y100A	nb	—	—	2
N116A	$0.15 \pm 0.01^{**}$	2.3	63 ± 0.6	3
Q120A	ne	—	—	2
R138K	0.095 ± 0.005 (0.10, 0.09)	1.5	47 ± 3.4	2
V167T	na	—	—	—
F173A	$0.14 \pm 0.03^{**}$	2.2	28 ± 5.5	3
D194A	na	—	—	—
D200A	$0.21 \pm 0.03^{**}$	3.2	95 ± 28	3
T212A	$0.28 \pm 0.02^{**}$	4.3	193 ± 6.1	3
T213A	na	—	—	—
L214T	na	—	—	—
Q219A	nb	—	—	2
W276A	$0.17 \pm 0.01^{**}$	2.6	86 ± 11	3
F286E	0.29 ± 0.04 (0.25, 0.33)	4.4	278 ± 73	2
F286A	nb	—	—	2
D287A	nb	—	—	2
H298G	nb	—	—	2
H298A	nm	—	—	2

receptor. Scatchard analysis of the specific [125 I]pPYY binding resulted in a linear plot consistent with a non-co-operative, apparently single class of binding sites (Figure 3A). As shown in Table 1, the K_d value of [125 I]pPYY for the WT hY1 receptor was 0.065 ± 0.001 nM and the maximum binding value (B_{max}) was 68 ± 9.8 fmol/mg of protein (mean $\pm$ S.E.M., $n = 3$).

Table 1 summarizes the results from saturation studies with [125 I]pPYY. [125 I]pPYY lost binding completely to seven mutants: Y100A, Q120A, Q219A, F286A, D287A, H298A and H298G. Five mutants, i.e. N116A, F173A, D200A, T212A and W276A, displayed a decrease in affinity ($P < 0.01$).

Competition studies with pNPY and the antagonists BIBP3226 and SR120819A

Figure 3(B) shows representative competition curves of [125 I]pPYY binding to cloned WT hY1 by pNPY and the two selective Y1 antagonists BIBP3226 and SR120819A, designed to mimic the C-terminal structure of NPY. All three ligands displayed high affinity for the hY1 receptor with K_i values (nM) of 0.22 ± 0.03 for pNPY, 7.6 ± 0.98 for BIBP3226 and 4.2 ± 0.2 for SR120819A (means $\pm$ S.E.M., $n = 3$). The K_i values of pNPY and the antagonists are summarized in Table 2(a). The Hill coefficients of all competition curves were close to 1 (results not shown). The K_d value of [125 I]pPYY was not determined for some of the mutants because of the unchanged IC_{50} values of NPY and the antagonists compared with the WT. Therefore, in Table 2(b), we present the IC_{50} and not the K_i values for these mutants.

In competition with [125 I]pPYY, pNPY displayed a 2–3-fold reduced affinity for the mutants F173A, T212A and W276A ($P < 0.01$), while the affinity decreased by 7- and 11-fold for mutants D200A and F286E respectively ($P < 0.01$; Table 2a). Notable changes in binding were also observed for the antagonists. BIBP3226 showed a 3-fold decrease in affinity for T213A and

Table 2 Inhibition of [125 I]pPYY binding to WT and mutated hY1 receptors by various ligands

The results are means $\pm$ S.E.M. for three independent experiments performed in duplicate. Non-specific binding was defined in the presence of 100 nM pNPY. The data were analysed using nonlinear regression (GraphPad Prism 2.0 software). Abbreviations: nd, not displaced up to 10μ M. * $P < 0.05$ and ** $P < 0.01$ as determined by one-way ANOVA followed by Dunnett's test, indicating a significant difference in pK_i and pIC_{50} values compared with the WT. K_i and IC_{50} values were converted into pK_i and pIC_{50} values by the formula $-\log K_i$ or $-\log IC_{50}$ respectively. †Exhibits two-site binding. The IC_{50} value refers to high-affinity site. K_i/K_i WT stands for K_i mutant/ K_i WT; likewise for IC_{50}/IC_{50} WT.

(a) K_i values

Mutant	pNPY		BIBP3226		SR120819A	
	K_i (nM)	K_i/K_i WT	K_i (nM)	K_i/K_i WT	K_i (nM)	K_i/K_i WT
WT	0.22 ± 0.03	1.0	7.6 ± 0.98	1.0	4.2 ± 0.2	1.0
N116A	$0.07 \pm 0.01^{**}$	0.3	7.7 ± 0.5	1.0	$149 \pm 13^{**}$	35
R138K	0.31 ± 0.03	1.4	$1.8 \pm 0.1^{**}$	0.2	3.5 ± 0.2	0.8
F173A	$0.57 \pm 0.04^{**}$	2.6	$23 \pm 1.0^{**}$	3.0	nd	—
D200A	$1.5 \pm 0.2^{**}$	6.8	$2.1 \pm 0.3^{**}$	0.3	3.9 ± 0.3	0.9
T212A	$0.46 \pm 0.02^{**}$	2.1	$133 \pm 12^{**}$	18	$459 \pm 14^{**}$	109
W276A	$0.60 \pm 0.13^{**}$	2.7	$153 \pm 18^{**}$	20	$65 \pm 3.6^{**}$	15
F286E	$2.4 \pm 0.4^{**}$	11	$318 \pm 61^{**}$	42	nd	—

(b) IC_{50} values

Mutant	pNPY		BIBP3226		SR120819A	
	IC_{50} (nM)	IC_{50}/IC_{50} WT	IC_{50} (nM)	IC_{50}/IC_{50} WT	IC_{50} (nM)	IC_{50}/IC_{50} WT
WT	0.57 ± 0.08	1.0	20 ± 3.5	1.0	10 ± 0.6	1.0
A90T	$1.2 \pm 0.2^{*†}$	2.1	$4.8 \pm 0.45^{**}$	0.2	$5.0 \pm 0.2^{**}$	0.5
V167T	$0.22 \pm 0.01^{**}$	0.4	$7.8 \pm 0.4^{**}$	0.4	14 ± 2.4	1.4
D194A	0.98 ± 0.15	1.7	31 ± 2.6	1.6	9.7 ± 0.7	1.0
T213A	0.87 ± 0.12	1.5	$61 \pm 12^{**}$	3.1	16 ± 2.2	1.6
L214T	0.71 ± 0.04	1.2	17 ± 2.8	0.9	8.4 ± 1.0	0.8

Figure 4 Immunocytochemistry studies of the expression of NPY receptor Y1

WT and mutant Y1 receptors semi-stably expressed in EBNA cells were visualized using an anti-biotin Cy3-conjugated secondary antibody towards an anti-FLAG BioM2 primary antibody. Untransfected HEK-293 EBNA-1 cells were used as the negative control. Receptor expression was visible on the cell membranes in cells expressing WT hY1 and the mutants Y100A, F173A, H298G, H298A, Q219A and D287A. Cells expressing Q120A and negative control cells did not fluoresce. The results shown are representative of three independent experiments performed in duplicate.

F173A ($P < 0.01$), whereas binding to T212A, W276A and F286E decreased considerably by 18–42-fold ($P < 0.01$; Table 2). The affinity of the antagonist SR120819A decreased by 15-fold for W276A, 35-fold for N116A and 109-fold for T212A ($P < 0.01$; Table 2a). Mutants F173A and F286E completely lost their ability to interact with SR120819A. Without the access to additional radiolabelled ligands other than [125 I]pPYY, it was not possible to test the affinity of the antagonists or pNPY for mutants that lost [125 I]pPYY binding.

The residues Ala⁹⁰ in TM2, Val¹⁶⁷ in TM4 and Leu²¹⁴ in TM5 (Figure 2, and Supplementary Figure 1) are conserved in all known mammalian Y1 receptors but differ in chicken. Mutagenesis of these positions to threonine changed a non-polar residue (Ala) or a hydrophobic residue (Val or Leu) to a polar residue. As can be seen, the IC₅₀ values of pNPY for A90T and V167T were barely changed (Table 2b). The affinities of the antagonists for A90T were slightly increased by 2–4-fold, while remaining practically unaffected for V167T and L214T. These three mutants bound [125 I]pPYY with largely unaltered affinity.

Detection of receptor expression using immunological techniques

For all mutants that failed to bind the radioligand, we investigated the expression of the receptor at the cell surface using immunological detection techniques. The results showed that all mutants, except Q120A, were expressed on the cell surface (Figure 4). The receptor mutants were detected mainly in the cell-surface membrane. Occasionally, these receptors could be detected intracellularly as well, probably corresponding to receptors being transported to the cell membrane or being internalized from the cell membrane (however, no ligand had been added to these cells).

Modelling of the human NPY receptor Y1

A homology-based three-dimensional model of the seven-TM α -helices of the hY1 receptor was constructed using as the template the structure of high-resolution bovine rhodopsin [25]. Superimposition of the obtained Y1 model on the rhodopsin model showed high structural similarity. The WHAT IF V4.99 program [31] gave high support to the reliability of the obtained structural model. The Ramachandran plot evaluation [32] showed that the amino acids were located in the region of the plot supporting a right-handed α -helix; 96.7% were in the favoured region and 2.8% were in the allowed region, whereas 0.6%, e.g. Cys³¹⁴ in TM7, were in the outlier region. The most striking conformational difference from earlier models of the Y1 receptor is the longer, diagonal and more centrally located TM3 region (Figure 5; see Supplementary Figure 3 at <http://www.BiochemJ.org/bj/393/bj3930161add.htm>). The computer model agrees well with the mutagenesis results of the present study.

DISCUSSION

The prevailing model of NPY binding to the hY1 receptor was based on two types of interactions as deduced from mutagenesis experiments. First, a series of negatively charged aspartic residue side chains in the ELs were proposed to interact with basic side chains in the peptide ligands. Secondly, a hydrophobic pocket consisting of three amino acid side chains was suggested to interact with the amidated C-terminus of NPY. However, contradictory findings for the pharmacological characteristics of the mutated receptors as well as comparisons of Y1 receptor sequences across species and with other NPY receptor subtypes

Figure 5 Three-dimensional model of the hY1 receptor based on the rhodopsin high-resolution structure

The extracellular and intracellular loops have been excluded since their three-dimensional structures are unknown. Highlighted residues are positions critical for peptide binding: Tyr¹⁰⁰ in TM2, Gln²¹⁹ in TM5, Phe²⁸⁶ in TM6, Asp²⁸⁷ in TM6 and His²⁹⁸ in EL3. The latter residue resides in a loop, one amino acid before the start of TM7 (Figure 2), which is why it is unconnected to TM7 in the Figure, located above the curved TM6. (A) Side view of the receptor and (B) extracellular view of the receptor.

(Supplementary Figure 1) have given reasons to question this model.

In the present study, we describe the pharmacological characterization of a series of mutants using binding studies with the peptides pNPY and pPYY as well as two non-peptide antagonists, BIBP3226 and SR120819A. In the evaluation of the results, we take into consideration our three-dimensional model of Y1, based on the high-resolution structure of bovine rhodopsin, and information from other mutagenesis studies. It is currently unknown how similar the rhodopsin structure is to other GPCRs and this model only represents the inactive form of rhodopsin. However, it is the best available crystal model and has served as the template for several studies of other receptors whose results have confirmed their similarities to rhodopsin [33].

Only Asp¹⁰⁴ and Asp²⁸⁷ are important for assumed electrostatic peptide interactions

The initial straightforward electrostatic hypothesis suggested interaction of basic NPY side chains with four aspartic residues in the Y1 receptor, namely Asp¹⁰⁴ in EL1, Asp¹⁹⁴ and Asp²⁰⁰ in EL2 and Asp²⁸⁷ in TM6 [18]. However, later studies by the same investigators described virtually unaltered binding to D194A [21] and D200A [20,21] mutants, whereas a more recent report observed loss of binding to D200A [22]. We mutated three of these residues, namely Asp¹⁹⁴, Asp²⁰⁰ and Asp²⁸⁷. Our results confirmed loss of [¹²⁵I]pPYY binding to D287A, whereas both peptide ligands retained high affinity for D194A and D200A (Tables 1 and 2). A study by Kanno et al. [22] in 2001 also confirmed virtually intact peptide binding to D194A. This leaves only two aspartic residues for assumed electrostatic peptide interaction, namely Asp¹⁰⁴ in EL1 and Asp²⁸⁷ in TM6.

The three positions proposed to form a hydrophobic pocket are essential for peptide binding

The hydrophobic pocket that was suggested to interact with the amidated C-terminus of NPY or PYY involves Tyr¹⁰⁰ in TM2, Phe²⁸⁶ in TM6 and His²⁹⁸ in EL3 [19] (Figure 5). Since all NPY-family receptors interact with the C-terminal part of the peptides and share a common ancestor [11,34], it would be expected that the receptors recognize the highly conserved amidated Tyr³⁶ in a similar way and hence have identical or similar amino acid residues at the relevant positions. However, the three positions mentioned above are not well conserved between receptor subtypes. We replaced each of the three residues independently with alanine and/or the amino acid at the corresponding position of mammalian Y4 (the PP receptor), to which NPY and PYY bind with lower affinity [35] and for which the Y1-selective antagonists show no detectable affinity [36].

We found that substitution of the tyrosine residue at position 100 by an alanine resulted in loss of peptide binding, as seen in earlier studies [19–21,24]. The F286A mutant in the outer part of TM6 (Figure 2) also lost the ability to bind [¹²⁵I]pPYY, in agreement with a more recent report [22] and in line with earlier studies by Sautel et al. [19,20]. However, another publication reported unaltered NPY binding (no numerical data were presented) [24]. In addition, we exchanged the phenylalanine with a glutamic residue (present in the Y4 receptor), and unsurprisingly observed reduction of pNPY and BIBP3226 affinity for the receptor, while SR120819A binding was completely lost. Thus Phe²⁸⁶ is indeed involved in both agonist and antagonist interactions.

The third position of interest, His²⁹⁸, was converted into an alanine or a glycine residue. [¹²⁵I]pPYY lost its affinity for the glycine mutant, in disagreement with the results reported by

Du et al. [24], who observed no effect on binding, albeit without presenting any numerical data. Similarly to our observations, various groups have reported radical decrease [19,20] or loss [21,22] of peptide binding to the H298A mutant (Table 1). Only one earlier study reported unaltered binding of BIBP3226 to this mutant [20].

Taken together, our results for the proposed hydrophobic pocket show loss of [¹²⁵I]pPYY binding to the mutants Y100A, H298A and F286A, suggesting that these positions are vital for peptide binding. Of the three positions, Tyr¹⁰⁰ is the most conserved position across receptor subtypes and, thus, is likely to be the most essential for peptide binding. Positions in the Y2 and Y5 receptors that correspond to the Y1 position Tyr¹⁰⁰ possess a tyrosine and a serine residue respectively. Since both serine and tyrosine residues contain a hydroxy group, they may display a hydroxy interaction with the peptide as suggested by Sautel et al. [19]. The latter study hypothesized that the hydroxy group of Tyr¹⁰⁰ binds the amide group on Tyr³⁶, while Phe²⁸⁶ and His²⁹⁸ may display aromatic and hydroxy interactions with Tyr³⁶ respectively. Judging from the three-dimensional model that we have calculated (Figure 5), it is not possible for Tyr¹⁰⁰ to interact with the amidated Tyr³⁶ together with Phe²⁸⁶ and His²⁹⁸, mainly because of the large distance between Tyr¹⁰⁰ and the other two residues. Instead, two other possible interactions may be that Tyr¹⁰⁰, Phe²⁸⁶ and His²⁹⁸ sequentially interact with Tyr³⁶ during different conformational changes during receptor binding and activation, or that they actually interact with different amino acid residues of the peptide. Regardless, it is highly questionable that these three variable residues would form a hydrophobic pocket that interacts with the invariant C-terminus of the peptide ligands.

Arg¹³⁸ and Trp²⁷⁶ are important for antagonist binding

Two highly conserved residues in rhodopsin-like receptors have been shown to be involved in the conformational changes leading to the active conformation of the receptor and the subsequent activation of G-proteins, namely those corresponding to hY1 positions Arg¹³⁸ on the inner side of TM3 and Trp²⁷⁶ in the middle of TM6 (Figure 2) [37]. The arginine residue at position 138 is the most highly conserved position among rhodopsin-like GPCRs and was mutated to see whether indirect effects on the receptor structure might influence peptide binding. We chose to introduce a lysine residue in order to maintain a positive charge at this position in case this is important for the folding and membrane insertion of the receptor. We observed no significant changes in the affinities of the peptides for R138K, indicating the absence of direct peptide interactions, as expected. Compared with an earlier report of unaltered binding [24], we observed a slight decrease in affinity of [¹²⁵I]pPYY for W276A. Signal transduction experiments will be necessary to show whether these mutations render the receptor unable to switch between inactive and active conformations. In contrast, the binding affinity of the antagonist BIBP3226 for W276A was reduced by 20-fold (Table 2a). Also, SR120819A lost affinity for this mutant (Table 2a). An effect of Trp²⁷⁶ on BIBP3226 binding was also reported in previous mutagenesis experiments [24]. It is likely that the two antagonists interact directly with the tryptophan residue, thereby perhaps stabilizing the receptor in its inactive conformation.

Mutations of positions highly conserved in the Y1 receptor of various species

The position Gln¹²⁰ in TM3 is an invariant glutamine across NPY receptor subtypes in all vertebrates investigated. Du et al. [24] showed that mutation of this residue to tyrosine resulted in a drastic decrease in [¹²⁵I]pPYY binding (results not shown), but the

effects on receptor expression were not investigated. We found no expression of Q120A at the cell membranes (Figure 4). This may be due to incorrect membrane insertion of the mutant receptor or abnormal post-transcriptional modification in the endoplasmic reticulum, leading to degradation.

The asparagine residue at position 116 in TM3 (Figure 2) is conserved in all Y1 sequences known but differs in all other subtypes (Supplementary Figure 1). This position has not previously been investigated by mutagenesis in the Y1 receptor. The 35-fold reduced affinity of SR120819A may indicate a direct interaction with Asn¹¹⁶. Our three-dimensional model of the hY1 receptor, with TM3 located diagonally across the inner side of TM4, would be compatible with a direct interaction.

Another position conserved in Y1 of various species is Phe¹⁷³ in TM4 (Figure 2). Both NPY and BIBP3226 were originally reported to lose detectable affinity for this mutant [20], but in two later studies only 2.6-fold or no loss of affinity of PYY was reported [22,24]. Indeed, it seems less likely that this phenylalanine is crucial for binding of endogenous ligands as the corresponding position in Y2 and Y5 is a leucine residue. We found a slight decrease in pNPY and BIBP3226 binding to F173A (Table 2a), while SR120819A was unable to compete with the radioligand. Thus Phe¹⁷³ probably does not interact directly with NPY and BIBP3226, but rather stabilizes the receptor conformation. Phe¹⁷³ is more important for binding of SR120819A than of BIBP3226, confirming that these two antagonists have partially different binding sites (Supplementary Figure 3).

A series of polar residues in the outer part of TM5 seemed to be likely candidates for interaction with ligands, namely Thr²¹², Thr²¹³ and Gln²¹⁹. Of these, Thr²¹² is conserved in all Y1 receptors and is a threonine or a serine residue in other subtypes. In disagreement with an earlier study [20], we found that Thr²¹² is important for both BIBP3226 and SR120819A binding (Table 2a). The amino acid residue Thr²¹³ is conserved in the Y1 subfamily (which includes also Y4 and Y6) but differs in Y2 and Y5 (Supplementary Figure 1). It has not previously been investigated. We found that the antagonist BIBP3226 displayed a slightly reduced affinity for this mutant (Table 2b).

Position 219 deeper in the TM5 helix is a conserved glutamine in Y1, Y4 and Y5 but is a leucine residue in Y2 (Supplementary Figure 1). We found that [¹²⁵I]pPYY lost binding to Q219A (Table 1), in accordance with the drastically reduced or lost affinity reported previously [20,22,24]. Thus PYY probably binds directly to Gln²¹⁹.

Antagonist binding to the hY1 receptor

A comparison of the two antagonists thus reveals several differences (Supplementary Figure 3). Although both of the antagonists were based on modelling of the C-terminus of NPY, this result is not unexpected because they differ extensively from each other in some parts of the molecules (Figures 1C and 1D). The binding of BIBP3226 involves TM4–TM6, whereas the binding interactions for SR120819A span over a larger area, involving TM2 and TM3 as well, notably Asn¹¹⁶ in TM3. Both of the antagonists probably interact directly with Thr²¹² in TM5 and Trp²⁷⁶ in TM6.

Conclusions

The results presented here lead to extensive revision of the Y1 structural model. Only two, at most, of the original four aspartic residues may be involved in peptide binding. The three positions previously proposed to form a hydrophobic pocket interacting with the amidated C-terminus of NPY do seem to interact with the peptide, but appear to be too far apart to form a hydrophobic

pocket. Their divergence across receptor subtypes makes it unlikely that they would interact with the invariant C-terminus of NPY and PYY. Our results also suggest new sites of interaction with the antagonists BIBP3226 and SR120819A. Thus we here propose a new structural model for the hY1 receptor with implications for the other NPY receptor subtypes, which may facilitate development of subtype-selective drugs for this important receptor family.

We are grateful to Dr R. Fredriksson and Henry Elisell (Department of Neuroscience, Uppsala University, Uppsala, Sweden) for help with structural modelling. We thank Dr K. Rudolf and Dr H. Wieland (Boehringer Ingelheim) for kindly providing BIBP3226 and Dr C. Serradeil-Le Gal (Sanofi-Synthelabo Recherche) for kindly providing SR120819A. This work was supported by a grant from the Swedish Research Council for Natural and Engineering Sciences.

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Received 29 April 2005/4 August 2005; accepted 12 August 2005

Published as BJ Immediate Publication 12 August 2005, doi:10.1042/BJ20050708