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Neuropeptide Y and the Nonpeptide Antagonist BIBP 3226 Share an Overlapping Binding Site at the Human Y_1 Receptor

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SUMMARY

Neuropeptide Y (NPY) is a 36-amino acid peptide that exhibits actions on the cardiovascular system and the central nervous system. NPY can regulate blood pressure, psychomotor function, anxiety, food intake, and endocrine secretions. BIBP 3226, the first potent and selective nonpeptide antagonist at the NPY Y_1 receptor, was designed by mimicking the carboxyl-terminal structure of NPY. We investigated the interaction of NPY and BIBP 3226 with the human Y_1 receptor at the molecular level. Alanine mutants at positions Y100, D104, W288, and H298 of the human Y_1 receptor showed no or significantly reduced binding for NPY but were not affected in their ability to bind BIBP 3226. Receptors with alanine mutations at positions W163, F173, Q219, N283, F286, and D287 showed reduced binding for both NPY and BIBP 3226. Mutations at other positions were tested (H105, S170, L174, V178, D200, D205, S206, H207, S210, T212, T280, T284, N289, H290, and Q291) and did not affect the binding of NPY or BIBP 3226. The human Y_1 receptor mutant Y211A showed no affinity for BIBP 3226 but retained wild-type affinity for NPY. Based on these experimental results, a detailed model for the interaction of BIBP 3226 with the human Y_1 receptor was developed using a Y_1 receptor model and a three-dimensional model of BIBP 3226. The experimental results, supported by modeling studies, clearly suggest that the native ligand (NPY) and the antagonist (BIBP 3226) share an overlapping binding site.

NPY is a 36-amino acid peptide amide that exhibits a high homology with the pancreatic polypeptide and peptide YY in both sequence and three-dimensional structure (1) (Fig. 1). NPY, isolated from pig brain and sequenced in 1982 (2), is one of the most abundant neurohormones in the mammalian peripheral and central nervous system. It is a sympathetic cotransmitter that mediates vasoconstriction through direct effects or through potentiation of other vasoconstrictors. Centrally, NPY is involved in the regulation of food intake, memory retention, and anxiolysis (for recent reviews, see Refs. 3–7). Receptor competition binding studies using analogs or segments of NPY revealed that at least two NPY receptor subtypes (termed Y_1 and Y_2) exist (8–10). Unequivocal pharmacological characterization of NPY receptor subtypes and investigations of the true (patho)physiological importance of NPY have been severely hampered by the lack of potent and selective NPY receptor antagonists. Recently, however, the nonpeptide compound BIBP 3226 (Fig. 1) was designed by mimicking the carboxyl-terminal part of NPY and shown to behave as a competitive, specific, and selective Y_1 receptor antagonist (11, 12). The cDNA of the rat and the human Y_1 receptor has been cloned (13–16). Sequence analysis showed that the Y_1 receptor belongs to the G protein-coupled hormone receptor family. Like the other members of this family of receptors, the Y_1 receptor is supposed to have seven transmembrane domains connected by three extracellular and three intracytoplasmic loops. Using site-directed mutagenesis, we recently demonstrated that certain acidic residues present in extracellular loops of the human Y_1 receptor are important for ligand binding (17). In addition, an L-alanine scan of the 36-amino acid peptide NPY revealed that positively charged arginine residues contribute considerably to the receptor binding activity of the ligand (18). Based on these experimental results, a three-dimensional model for the interaction of NPY with the human Y_1 receptor was designed (19). In this model, the carboxyl-terminal tyrosine amide moiety of NPY binds to a hydrophobic pocket formed by the residues Y100, F286, and H298. We describe our efforts to locate possible contact points between the human Y_1 receptor and the nonpeptide NPY antagonist BIBP 3226.

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ABBREVIATION: NPY, neuropeptide Y.

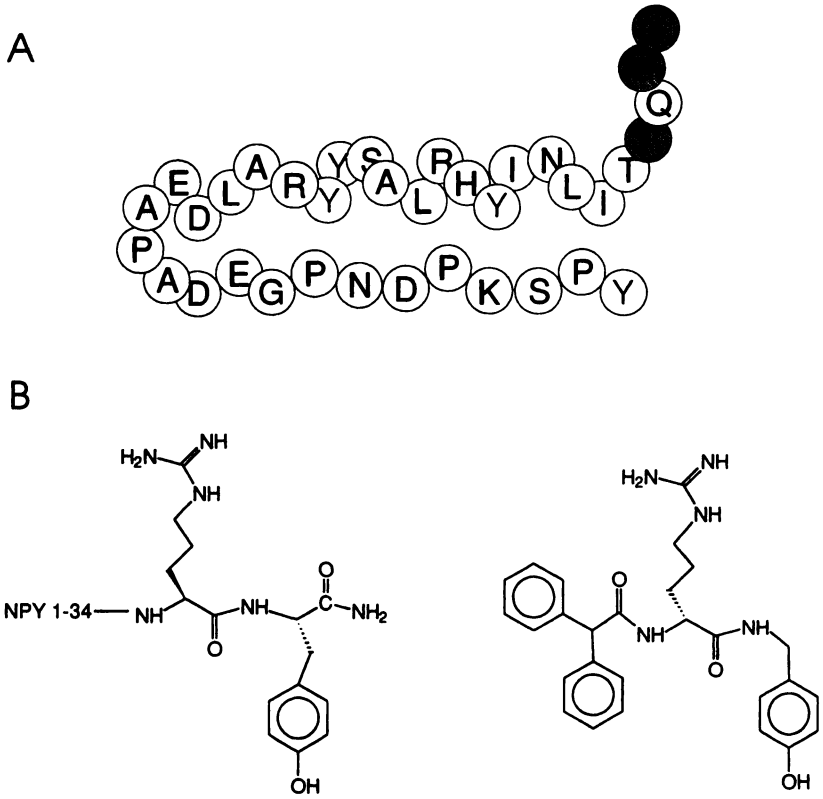


Fig. 1. A, Sequence of NPY. Gray, residues necessary for Y_1 receptor binding (18). B, Structures of the NPY carboxyl terminus and of BIBP 3226.

Materials and Methods

Chemicals. [^{125}I]NPY and [^{35}S]dATP were obtained from Amersham (Buckinghamshire, UK). BIBP 3226 was tritium-labeled (New England Nuclear, Boston, MA) and purified by high performance liquid chromatography (Inertsil ODS, 5 μm) using a 0.1% KH_2PO_4 (pH 7.4) methanol gradient of 20–95% to give a radiochemical purity of >93% (specific activity, 1.73 TBq/mmol). Restriction enzymes and T4 DNA ligase were from Boehringer-Mannheim (Mannheim, Germany) or Clontech (Palo Alto, CA). Cell culture supplies were from GIBCO (Grand Island, NY) except Dulbecco's modified Eagle's medium and fetal calf serum, which were from Seromed (Berlin, Germany). *N*-(1-(2,3-Dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium methylsulfate was from Boehringer-Mannheim.

Site-directed mutagenesis and transient expression of human Y_1 receptor. The construction of pNPYr-FLAG has been described previously (20). The mutations were introduced into pNPYr-FLAG by site-directed mutagenesis using a primer extension procedure (20, 21). For transient expression of receptor constructs, HeLa cells were grown to confluency in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamycin. Cells were infected with wild-type vaccinia (strain WR) as described previously (17, 19). Five micrograms of plasmid DNA containing cDNA for the receptor (wild-type or mutant) was then transfected using liposome-aided technology as described by the supplier (transfection reagent: DOTAP; Boehringer Mannheim Biochemicals, Mannheim, Germany). The transfected cells were subsequently incubated for 16–18 hr at 37°. Expression rate was ~10% of confluent cells (i.e., 10^5 cells/well). All mutants that lost binding were tested with the FLAG assay to ensure that the receptor has been correctly expressed as described previously (20).

Binding assay on whole cells. NPY and BIBP 3226 binding to whole cells (10^6 cells/well) was performed as described previously using radiolabeled [^{125}I]NPY (50 pM; 200,000 dpm/well) or [^{3}H]BIBP 3226 (1.73 TBq/mmol, 1 nM; 100,000 dpm/well) either alone or mixed with unlabeled compound (i.e., NPY or BIBP, respectively) in a total volume of 1 ml (19). Confluent cells were used in each assay. To assess the capacity of each mutant to bind to NPY or BIBP 3226, the total specific binding was measured and normalized via the expression of the ratio mutant/wild-type receptor. Nonspecific binding was defined as the total binding for cells transfected with the same plasmid but devoid of the coding sequence for the receptor. Nonspecific binding represented 20% of total binding. The affinity of each mutant for NPY and BIBP 3226 was estimated by competition binding experiment in two to four different experiments. For mutants with very low specific binding (ratio of total specific binding to the wild-type value <30%), a saturation curve was performed using tritiated BIBP of low specific activity, to estimate binding affinities of > 200 nM. All of the data were analyzed by nonlinear curve fitting using EBDA software (22).

General computational procedures. All energy minimizations of the human Y_1 receptor model were performed with the DISCOVER simulation package (BIOSYM software package, BIOSYM Technologies, San Diego, CA) using the CVFF force field parameters included in the program. The amino acids did not contain a formal charge. The interactive modeling program INSIGHT II V.2.3.0 (BIOSYM software package, BIOSYM Technologies) was used for visualization of the results and for protein modeling. Conformational analysis of the low-molecular-weight Y_1 receptor antagonists was carried out with the software package SYBYL (SYBYL software package, Tripos Associates, St. Louis, MO) using the standard TRIPOS force field without atomic charges.

Modeling of BIBP 3226 binding conformation. To obtain an independent reference point by which the validity of the human Y_1 receptor model could be tested, we tried to define the receptor-bound conformation of our Y_1 receptor antagonists without restraint induced by the receptor. Here, we used the active analog approach, as defined by Marshall (23). Fifteen selective, nonpeptide human Y_1 antagonists were compared. Due to the high flexibility of the compounds, it was not possible to derive a single model. After a thorough comparison of all structures, eight possible models remained. Each of these was used in our docking study.

Construction of a three-dimensional model for the human Y_1 receptor. The coordinates of the C-α atoms of the human Y_1 receptor [obtained from the TM7 server of EMBL, Heidelberg, Germany (24)] were used as a starting structure to construct a three-dimensional model of the human Y_1 receptor. The seven antiparallel helices are arranged counter-clockwise as viewed from outside the cell membrane. The following steps were taken to build up the final model of the helical regions of human Y_1 receptor: amino acid replacement, local geometry optimization, and side-chain rotation to minimize overlaps between residues. The extracellular and intracellular loop regions were constructed from entries of the Brookhaven Protein Data Bank library following the geometric loop search algorithm implemented in INSIGHT II. The loop regions were energy minimized with tethered backbone atoms of the helical segments.

Method of docking. The docking of BIBP 3226 into the human Y_1 receptor was guided by the results of the various point mutations, which indicated the possible location of the binding site of BIBP 3226. Initially, eight possible binding conformations were fitted into the binding site. During this fitting procedure, the geometry of the inhibitor as well as that of the receptor was not adjusted. The studies clearly showed that only one conformation of BIBP 3226 did not show strong repulsive interactions with the receptor binding site and was therefore chosen as the "active conformation." BIBP 3226 was docked at the human Y_1 receptor model to form a salt bridge between its guanidium group and residue D287 located in helix 6 of the Y_1 receptor. The carboxyl-terminal hydroxybenzyl group of BIBP 3226 binds to the hydrophobic pocket formed by Q219 (helix 5) and W163 (helix 4). The diphenylmethane group points in the direction of F173 (helix 4), Y211 (helix 5), and F286 (extracellular region 4). Subsequently, the total ligand receptor complex was energy minimized to optimize the possible interactions.

Results

The human Y_1 receptor mutants D104A and D287A, which were reported to reduce NPY binding activity (17), were tested for their ability to bind tritiated BIBP 3226. Aspartate residues were chosen in previous studies to identify the binding partner of the arginine residues of NPY that were found to be involved in receptor interaction (18). D287A was found to abolish the binding of [^{3}H]BIBP 3226 (Table 1). This is consistent with the respective structures in that BIBP 3226 possesses one arginine residue and NPY possesses two arginine residues that are important for receptor binding at positions 33 and 35 (Fig. 1). In a previous study (17), a loss of binding was also reported for the receptor mutant D200A, a result that could not be confirmed after recent improvement in the expression system that resulted in a higher detection limit in the NPY binding experiment.

Previous results have suggested that the amino acids Y100, F286, and H298 of the human Y_1 receptor interact with the carboxyl-terminal tyrosine amide of NPY (19). It has been suggested that Y100 interacts with the carboxyl-terminal amide group. Because the denoted amide group is absent in BIBP 3226, it is not surprising that the binding of this antagonist is not affected by the Y100A mutation. H298 of the human Y_1 receptor was hypothesized to interact with the phenolic OH of ^{36}Tyr of NPY by forming a hydrogen bridge, whereas the hydroxy group of BIBP 3226 cannot reach H298, because its spatial orientation is different from that of the carboxyl-terminal Y36 of NPY due to the loss of one CH_2 group. Correspondingly, the human Y_1 receptor mutant H298A showed no binding for NPY and was found to bind BIBP 3226 as effectively as the wild-type receptor; i.e., H298 is important for the binding of NPY only, not for that of BIBP 3226. With these initial experimental data, the first three-dimensional model of the interaction of BIBP 3226 with the

TABLE 1

Binding of NPY and BIBP 3226 to the mutants of the human Y_1 receptor

Binding sites marked with FLAG+ were identified with FLAG antibodies because no ligand bound to them.

Mutant	Binding of [^{125}I]NPY K_i	K_i (mutant)/K_i (wild-type)	B_{max}	No. of binding sites/cell	Binding of [^{3}H]BIBP 3226 K_i	K_i (mutant)/K_i (wild-type)	B_{max}	No. of binding sites/cell
	nM		M		nM		M	
Wild-type	1.5 ± 0.01	1	1.40E − 10	8.40E + 05	4.3 ± 0.5	1	2.90E − 10	1.74E + 06
Y100A	>200	>130		FLAG+	6.6 ± 4.1	1.5	1.10E − 09	6.60E + 06
D104A	>200	>130		FLAG+	4.1 ± 4.0	0.9	1.11E − 09	6.66E + 06
H105A	8.5 ± 2.3	5.7	3.87E − 10	2.32E + 06	4.8 ± 3.1	1.1	1.15E − 10	6.90E + 05
W163A	>200	>130		FLAG+	>200	>40		FLAG+
S170A	9.3 ± 2.7	6.2	3.25E − 10	1.95E + 06	2.3 ± 1.4	0.5	1.74E − 10	1.04E + 06
F173A	>200	>130		FLAG+	>200	>40		FLAG+
L174A	2.6 ± 0.1	1.7	3.20E − 10	1.92E + 06	2.9 ± 0.8	0.7	6.77E − 10	4.06E + 06
V178A	3.4 ± 0.3	2.3	5.80E − 10	3.48E + 06	3.6 ± 1.1	0.8	6.10E − 10	3.66E + 06
D200A	2.5 ± 0.5	1.7	1.70E − 10	1.02E + 06	2.2 ± 1.6	0.5	2.29E − 10	1.37E + 06
D205A	12.8 ± 1.2	8.5	2.96E − 10	1.78E + 06	5.7 ± 4.5	1.3	2.99E − 10	1.79E + 06
S206A	0.3 ± 0.1	0.2	4.70E − 11	2.82E + 05	1.7 ± 1.1	0.4	3.40E − 10	2.04E + 06
H207A	1.1 ± 0.1	0.7	7.32E − 11	4.39E + 05	2.9 ± 1.3	0.7	6.60E − 10	3.96E + 06
S210A	2.7 ± 0.5	1.8	2.33E − 10	1.40E + 06	2.7 ± 1.9	0.6	3.93E − 10	2.36E + 06
Y211A	2.1 ± 0.2	1.4	1.10E − 10	6.60E + 05	>200	>40		FLAG+
T212A	2.3 ± 0.8	1.5	1.80E − 10	1.08E + 06	7.5 ± 2.4	1.7	4.11E − 10	2.47E + 06
Q219A	>200	>130		FLAG+	>200	>40		FLAG+
T280A	1.3 ± 0.6	0.9	3.24E − 10	1.94E + 06	1.3 ± 0.9	0.3	1.10E − 10	6.60E + 05
N283A	>200	>130		FLAG+	>200	>40		FLAG+
T284A	8.4 ± 5.1	5.6	1.56E − 09	9.38E + 06	3.3 ± 0.3	0.8	9.41E − 10	5.65E + 06
F286A	>200	>130	2.70E − 08	1.62E + 08	21.7 ± 4.1	5.1	7.20E − 10	4.32E + 06
D287A	>200	>130		FLAG+	>200	>40		FLAG+
W288A	>200	>130		FLAG+	7.5 ± 5.2	1.7	1.50E − 10	9.00E + 05
N289A	0.6 ± 0.1	0.4	1.93E − 10	1.16E + 06	2.5 ± 1.3	0.6	5.10E − 10	3.06E + 06
H290A	0.3 ± 0.2	0.2	5.00E − 11	3.00E + 05	4.2 ± 2.2	1.0	8.74E − 10	5.24E + 06
Q291A	0.9 ± 0.2	0.6	2.61E − 11	1.57E + 05	3.2 ± 1.5	0.7	9.10E − 10	5.46E + 06
H298A	>200	>130		FLAG+	5.7 ± 3.4	1.3	5.10E − 10	3.06E + 06

human Y_1 receptor was built using a human Y_1 receptor model according to Walker *et al.* (17).

The aim of the next step was to identify the interaction points of BIBP 3226 with the human Y_1 receptor and to compare them with those of NPY. Guided by the preliminary model of the binding of BIBP 3226 at the human Y_1 receptor, further alanine substitutions at distinct positions in the transmembrane segments 4–6 were generated. The corresponding mutant receptors were expressed and tested for their ability to bind radiolabeled NPY and BIBP 3226. K_i and B_{max} values for [^{125}I] NPY and [^{3}H] BIBP 3226 (Table 1) were determined and compared with the corresponding values obtained with the wild-type receptor. All mutants showed ~10^6 binding sites/cell, with exception of S206A (2.82×10^5 binding sites for NPY/cell). For BIBP 3226, however, B_{max} of S206A is comparable to that of the wild-type. For wild-type and all other mutants, B_{max} values range from 7×10^{-11} to 3×10^{-10} for NPY and from 2×10^{-10} to 1×10^{-9} for BIBP 3226. Binding sites per cell could not be determined for mutants, which bound neither NPY nor BIBP 3226. However, to ensure correct expression, those cells were investigated with fluorescent labeled anti-FLAG antibodies because the FLAG epitope was expressed as part of the receptor protein. Accordingly, all mutants were correctly expressed.

Four different types of mutants could be distinguished: mutants that show K_i values similar to those of wild-type for both NPY and BIBP 3226; mutants that show a significant loss of affinity for either NPY or BIBP 3226; and mutants that show high K_i values for both NPY and BIBP 3226 (Fig. 2). A large number of Y_1 mutations belong to the first type and did not reduce the binding of NPY or BIBP 3226 (H105A, S170A, L174A, V178A, D200A, D205A, S206A, H207A, S210A, T212A, T280A, T284A, N289A, H290A, and Q291A). An increase in K_i values for both NPY and BIBP 3226 was found for the receptor mutants W163A, F173A, Q219A, N283A, F286A, and D287A, suggesting that these residues are involved in ligand binding. A loss of affinity for NPY but not for BIBP 3226 was found for Y100A, D104A, W288A, and H298A. On the other hand, Y211A was the only mutant that did not bind BIBP 3226 (K_i = >200 nM) but showed wild-type binding for NPY (K_i = 2.1 nM; wild-type K_i = 1.5 nM).

Based on this new information, the initial model for the interaction of [^{3}H]BIBP 3226 with the human Y_1 receptor could be further refined (Figs. 3 and 4). In addition to the ionic interaction of the guanidino group of BIBP 3226 with D287, a cation/π interaction (25) of the guanidino group of BIBP 3226 with F286 and π/π interaction of the phenyl rings of BIBP 3226 with F173 (diphenylmethyl group), Y211 (diphenylmethyl group), and W163 (benzyl group) can be postulated. Two hydrogen bridges are suggested in the carboxyl-terminal segment of BIBP 3226: Q219 (NH_2) could interact with the 4-OH of the carboxyl-terminal 4-hydroxybenzylamine moiety of BIBP 3226 (187 pm) and N283 (NH_2) could interact with the backbone carbonyl group of BIBP 3226 (218 pm). Interestingly, Y211A, the only mutant that shows no affinity for BIBP 3226 but high affinity for NPY, was suggested to bind to the diphenylmethyl segment of BIBP 3226, a segment that is present only in the nonpeptide antagonist ligand.

Discussion

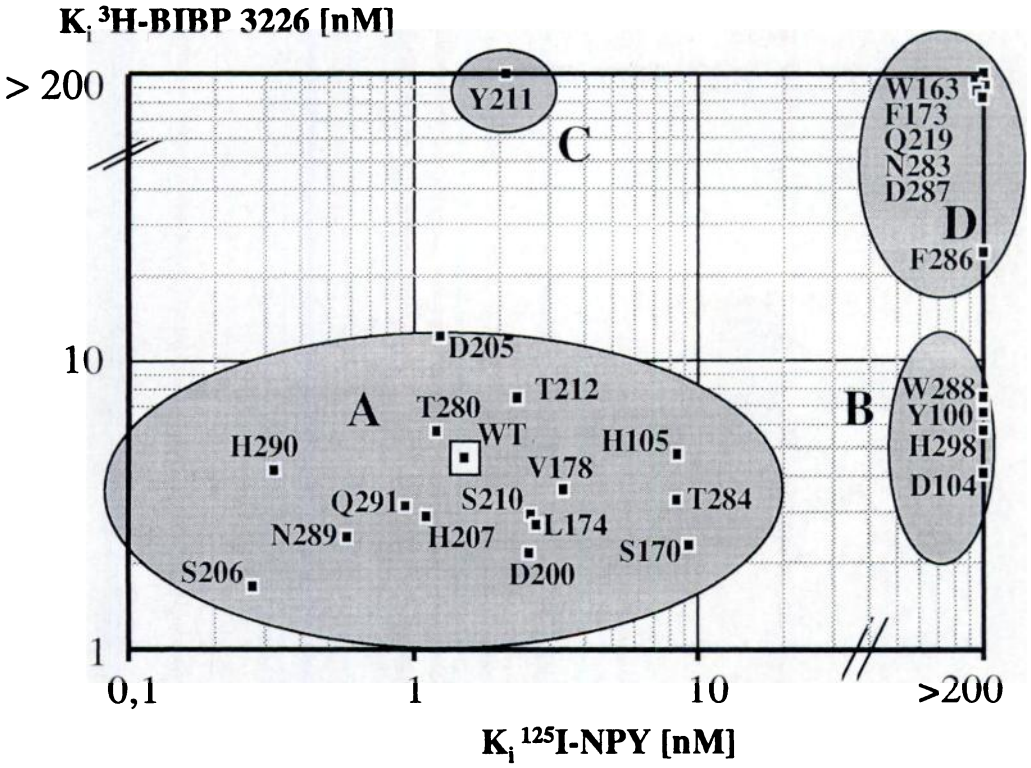
The localization of ligand binding sites in seven-transmembrane receptors is a prerequisite for a deeper understanding of the interaction between G protein-coupled receptors and their cognate ligands and has been the focus of considerable efforts (26). Because high resolution structures for a G protein-coupled receptor do not exist, electron-density maps of bacteriorhodopsin (27) and bovine visual rhodopsin (28) obtained by cryoelectron microscopy analysis are used as a scaffold for molecular modeling (17, 29).

Through construction of chimeric receptors and the performance of site-directed mutagenesis experiments, the ligand binding sites of substance P (30), bradykinin (31), cholecystokinin/gastrin (32), and endothelin (33) have been investigated. Nonpeptide antagonists with high affinity for tachykinin receptors (34, 35), angiotensin receptors (36), cholecystokinin/gastrin (32), or κ-opioid receptors (37) were shown to bind to different domains compared with the native ligands. An overlapping binding site of agonist and antagonist has very recently been suggested for the endothelin-1 receptor based on a single mutation finding (33).

Our model of the human Y_1 receptor is based on the knowledge of human Y_1 receptor residues important for binding of the native agonist ligand NPY. The binding site of the nonpeptide antagonist BIBP 3226 was localized initially by investigating the binding of [^{3}H]BIBP 3226 to a series of point-mutated human Y_1 receptors. A preliminary receptor binding model consistent with all experimental results could be established. This model was challenged by the forecast of additional sensitive mutants and subsequently refined in a second set of experiments with further receptor variants. Of course, we cannot exclude that some mutants lost binding due to an overall change in conformation. This hypothesis could explain why the mutation W163A induced an almost complete loss of receptor affinity, even if the residue W163 is located deep in the transmembrane region and at least the peptide ligand is suggested to bind preferentially at the top of the transmembrane segments (19). However, all mutants were correctly expressed at the cell surface according to FLAG epitope binding studies. Point-mutated receptors with exchanges next to very sensitive residues showed wild-type binding, indicating that we did not hit any receptor segments of general sensitivity.

Our data provide evidence that the nonpeptide antagonist BIBP 3226 binds to a site on the top of transmembrane segments 4–6 of the human Y_1 receptor. The mutant Y211A, the only one that does not bind BIBP 3226 yet retains wild-type NPY binding, is located at the top of transmembrane segment 5. Y211 is suggested to interact with the benzene rings of the diphenylmethyl moiety through π/π interaction. Further experiments with analogs of BIBP 3226 or further receptor mutants are, however, required to confirm this model. Preliminary structure-affinity investigations with retro/inverso compounds and backbone methylated compounds suggest that, within the carboxyl-terminal amide structure of BIBP 3226, the carbonyl is more important than the NH group for the interaction with the human Y_1 receptor because this NH group can be alkylated without a significant loss of affinity.[1] This is in agreement with our model, which

[1] K. Rudolf, W. Engel, and W. Eberlein, unpublished observations.



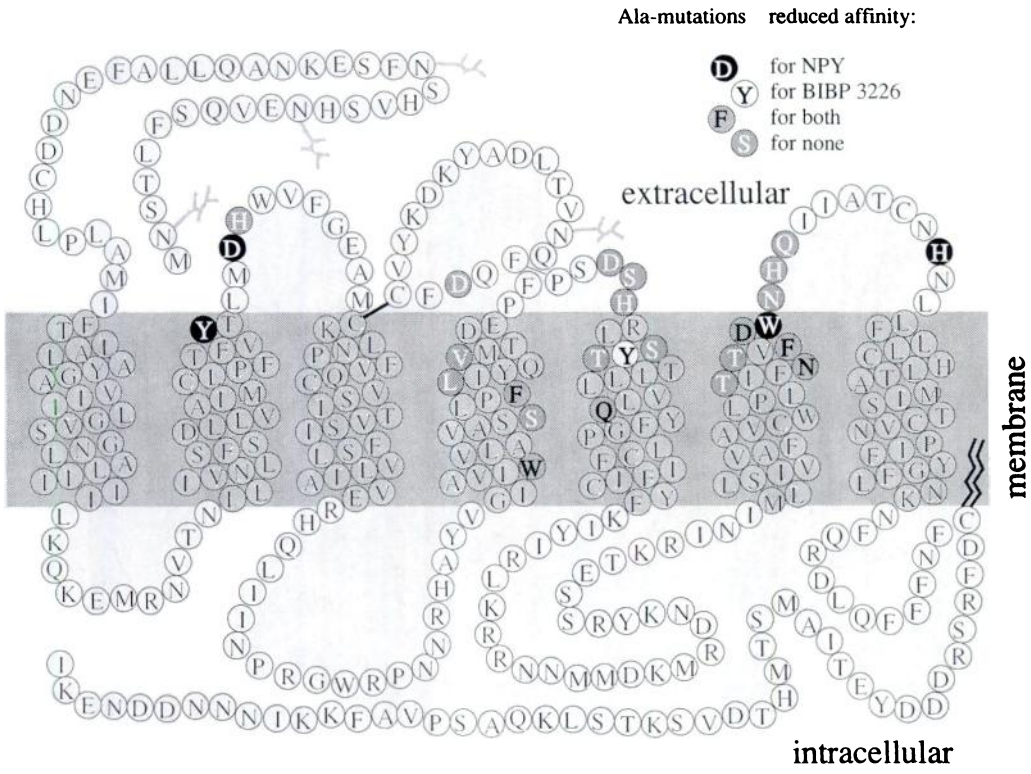


Fig. 2. *Top*, correlation between K_i values of [^{125}I]NPY and [^{3}H]BIBP 3226. Four different types of mutants are distinguished: mutants that show K_i values similar to those of wild-type for both NPY and BIBP 3226 (*A*), mutants that show a significant loss of affinity for either NPY (*B*) or BIBP 3226 (*C*), and mutants that show high K_i values for both (*D*). *Enlarged box*, wild-type (*WT*) indicator. *Bottom*, sequence of the human Y_1 receptor with marking of mutated residues. *Shaded region*, transmembrane segments.

suggests the formation of a hydrogen bridge between the carboxyl-terminal carbonyl group of BIBP 3226 and the NH of N283. This region matches the binding site of nonpeptide antagonists to the neurokinin receptors (34, 35).

Our results strongly suggest that the receptor binding site of the nonpeptide antagonist BIBP 3226 and of the native ligand NPY do overlap, as indicated by the large number of mutants that were found to be either sensitive or insensitive for both ligands. Our four types of mutants could be clearly distinguished by the significant change in K_i values. The positions of exchange of those sensitive mutants are located in transmembrane segments 4–6 (Fig. 2, *bottom*). Whether the corresponding side chains of NPY in the positions R35 and Y36 are interacting with the receptor in a similar way as the antagonist cannot be determined. However, it is most likely that the guanidino groups of both R35 in NPY and of

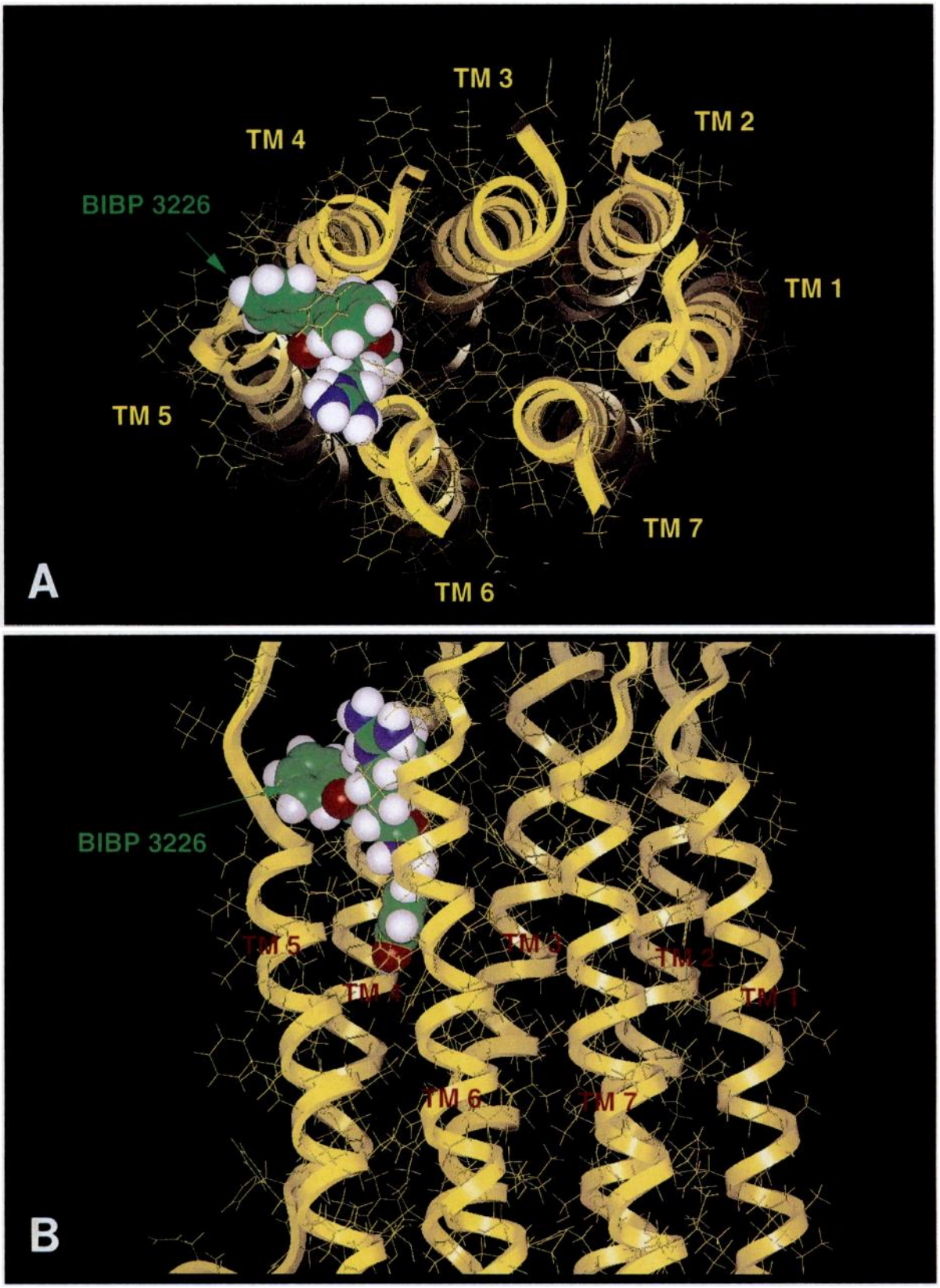


Fig. 3. View of the human Y_1 receptor model with the antagonist BIBP 3226 docked at the binding site. *Solid yellow ribbon*, transmembrane (*TM*) helices. *Space-filling representation*, inhibitor. The loop regions of the receptor model are clipped. *Top*, view down the helix axis. *Bottom*, view in the plane of the helices. *Top portion*, extracellular space.

BIBP 3226 bind to D287 of the Y_1 receptor. On the other hand, the orientation of Y36 might be slightly different (38). Mutants that lost their affinity for NPY but not for BIBP 3226 are located either at the top of transmembrane segment 2 or in the first or third extracellular loop (Fig. 2, *bottom*). Therefore, the binding site of the native ligand is thought to

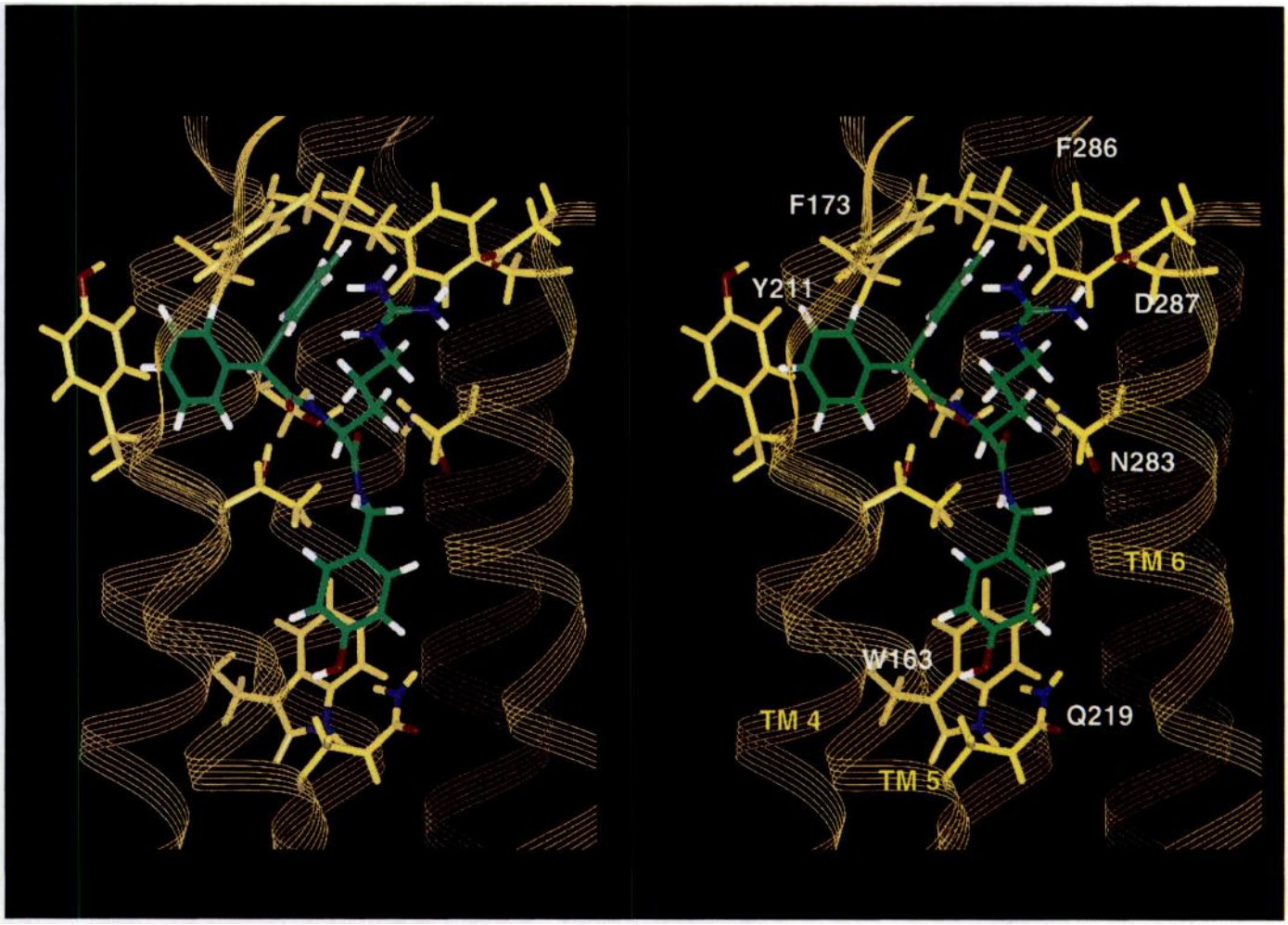


Fig. 4. Stereo view of the BIBP 3226 binding site. *Yellow line ribbons*, helices of the human Y_1 receptor. *Yellow*, side chains of the binding site. BIBP 3226 is colored by atom type (*green*, carbon; *red*, oxygen; *blue*, nitrogen; *white*, hydrogen).

be larger, and in addition to the transmembrane segments 4–6 (binding site of BIBP 3226), the transmembrane segment 2 and the extracellular loops 1 and 3 may be involved in NPY recognition. A different conformation of the receptor for the binding of agonist or antagonist might be required and account for the differences in the binding modes of agonist and antagonist. Further experiments, including covalent cross-linking, are in progress. However, because the nonpeptide antagonist BIBP 3226 was derived from the natural peptide ligand (NPY) by a rational approach mimicking the carboxyl-terminal part of NPY, we suggest, in agreement with the conclusion of Ohlstein *et al.* (39), that the rational design of nonpeptide antagonists for G protein-coupled peptide receptors is indeed possible.

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