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Studies of the Structure of the N-Terminal Domain from the Y4 Receptor—a G Protein-Coupled Receptor—and its Interaction with Hormones from the NPY Family

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Binding of peptide hormones to G protein-coupled receptors is believed to be mediated through formation of contacts of the ligands with residues of the extracellular loops of family 1 GPCRs. Here we have investigated whether additional binding sites exist within the N-terminal domain, as studied in the form of binding of peptides from the neuropeptide Y (NPY) family to the N terminus of the Y4 receptor (N-Y4). The N-terminal domain of the Y4 receptor has been expressed in isotopically enriched form and studied by solution NMR spectroscopy. The peptide is unstructured in solution, whereas a micelle-associated helical segment is formed in the presence of dodecylphosphocholine (DPC) or sodium dodecylsulfate (SDS). As measured by surface plasmon resonance (SPR) spectroscopy, N-Y4 binds with approximately 50 μM affinity to the pancreatic polypeptide (PP), a high-affinity ligand to the Y4 receptor, whereas binding to neuropeptide Y (NPY) and peptide YY (PYY) is much weaker. Residues critical for binding in PP and in N-Y4 have been identified by site-directed mutagenesis. The data indicate that electrostatic interactions dominate and that this interaction is mediated by acidic ligand and basic receptor residues. Residues of N-Y4 are likely to contribute to the binding of PP, and in addition might possibly also help to transfer the hormone from the membrane-bound state into the receptor binding pocket.

Introduction

Neuropeptide Y receptors, so-called Y receptors, are members of the rhodopsin-like G protein-coupled receptor (GPCR) family 1b. The neurohormones neuropeptide Y (NPY), peptide YY (PYY), and the pancreatic polypeptide (PP) target a heterologous population of at least five different receptor subtypes: Y1, Y2, Y4, Y5, and y6.[1] Their physiological roles in the regulation of blood pressure, memory retention, food uptake, and seizure have been demonstrated. Y4 receptors have been shown to play pivotal roles, for example, in cardiac function, glucose metabolism in chronic pancreatitis patients, and mediation of intestinal absorption of electrolytes and water.[2] NPY and PYY possess similar pharmacology, displaying nanomolar affinities for all receptor subtypes,[3] whereas PP binds with very high affinity and selectivity to the Y4 receptor.[4]

Little structural information is available for GPCRs. For a long time, in fact, bovine rhodopsin was the only GPCR for which experimental coordinates at atomic resolution had been published,[5] but very recently a structure for the β-adrenergic receptor has appeared.[6,7] The data for rhodopsin confirmed the arrangement of the seven-membered transmembrane (TM) bundle postulated on the basis of lower-resolution cryo-EM data,[8] but also revealed the unanticipated presence of a short antiparallel β-sheet in the N-terminal domain. In contrast, the N-terminal domain of the β-adrenergic receptor was shown to be disordered.[6]

The N-terminal domains of other GPCR (sub)families are known to play important roles in ligand binding. Each of the hormone receptors from GPCR family 2 contains a conserved region, responsible for ligand binding, in the N-terminal domain.[9] The N termini from family 3 GPCRs are the largest among all GPCRs, usually incorporating more than 500 amino acids.[9] Grafting and mutagenesis studies have demonstrated that conserved serine and threonine residues in these domains are directly involved in ligand binding.[10] Surprisingly, the expressed N terminus alone can bind the ligand with affinity similar to that of the full-length receptor.[11]

In contrast, the N-terminal domains of family 1 GPCRs have received little attention, most likely because of their short lengths, usually fewer than 70 amino acids. However, recent studies have suggested pivotal roles of N termini from GPCRs of this class in ligand recognition and binding.[12–14] Furthermore, mutagenesis data highlight the prominent role of charged residues in ligand binding.[15,16] Koller demonstrated that the N terminus of the calcitonin-like receptor is not only essential for binding to the ligands but also presents a determinant for ligand specificity.[17] The 35 amino terminal residues of CCR2, expressed as a membrane-bound fusion protein, bind to its ligand with an affinity similar to that of the intact, wild-type receptor, indicating that in that case the N terminus is sufficient for ligand binding.[18] From the mutagenesis data on the N terminus of the CX3C receptor and previous studies,

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Chen has proposed a two-step binding model involving ligand binding followed by receptor activation. The residues located in the N-terminal domain play distinct roles during the different processes.[19]

Complementarily to the biological work described above, GPCR fragments have also been studied by NMR. Pervushin, for example, investigated the N-terminal domain of bacteriorhodopsin—a protein structurally very closely related to GPCRs—in SDS micelles,[20] and Ulfers studied the extracellular domain of the neurokinin-1 receptor in DPC micelles.[21] Riek presented a high-quality 3D NMR structure of the extracellular domain of CRF-R2β in complexation with the peptide antagonist astressin,[22] Yeagle's group has determined conformational preferences for peptides corresponding to the cytosolic loops,[23] the sixth TM helix,[24] and the N terminus[23] of rhodopsin, and Pellegrini has studied the cytosolic domain[25] and the extracellular loops[26,27] of the PTH1 receptor in the presence of DPC micelles. Furthermore, we have recently determined the conformation of a polypeptide corresponding to the seventh TM helix of the yeast Ste2p receptor extended by 40 residues from the cytosolic tail[28] when integrated into DPC micelles.

In this work, we focus on structural studies of the isolated 41-residue N terminus of the Y4 receptor, a family 1b GPCR that is targeted by members of the NPY family. The location of this segment in the context of the entire human Y4 receptor is shown in snake plot in Figure S1 in the Supporting Information. In addition, we have investigated possible interactions with the hormones both qualitatively and quantitatively. By limiting the system of the study to just the N-terminal domain, and with the help of various biophysical methods, we were able to develop a rather detailed picture that would at present be difficult to achieve with the entire receptor. Moreover, we also report on the synthesis of the difficult-to-express N-terminal domain, suggesting a generally useful method to produce such polypeptides in isotopically labeled form. The structure of N-Y4 and its topology in the presence of DPC or SDS micelles was elucidated by high-resolution NMR techniques. While it is unstructured in solution, in the presence of micelles a hydrophobic segment associates with the micelle and folds into an α-helix. Chemical shift mapping revealed potential interaction sites between PP and N-Y4. SPR techniques quantified the strength of this interaction. Mutagenesis studies identified residues of PP that are likely to be important for binding N-Y4. The data indicate that the isolated N-Y4 is capable of weakly binding to PP, and that much of the binding affinity is due to electrostatic interactions. To simulate the receptor milieu the carboxyl terminus of N-Y4 was additionally conjugated to a C12 fatty amino alcohol (dodecylphosphoethanolamine) chain, thereby mimicking its conjugation to the first TM helix in the whole receptor. In this lipopeptide the structure of the N-Y4 moiety was not significantly affected. The study shows that PP associates with the flexible, central segment of N-Y4, and we speculate that transient binding to the N-terminal domain might facilitate transfer of PP from the membrane-bound state into the receptor binding pocket.

Results

Recombinant production of N-Y4

The N terminus of the Y4 receptor comprises 41 residues and is highly water-soluble. However, attempts to express it in the form of a soluble ubiquitin fusion in *E. coli* resulted in unspecific fragmentation. To circumvent this problem, the N-Y4 was expressed as a fusion product with the highly insoluble protein ketosteroidisomerase (KSI), which resulted in accumulation of the fusion protein in inclusion bodies. A TEV protease cleavage site was introduced to facilitate removal of the fusion partner.[29,30] The sequence recognized by the TEV protease is ENLYFQ, with Q as the P1′ residue. To achieve the natural peptide sequence after cleavage, the P1′ residue was replaced with the first residue from the target sequence (here it is Met),[31] and an additional GSGSGS linker was inserted to prevent steric hindrance during cleavage.

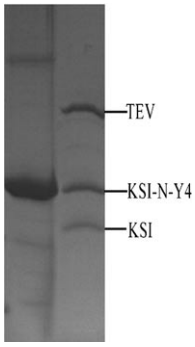

Figure 1. SDS-PAGE of the cleavage product of the ketosteroidisomerase-N-Y4 fusion after cleavage with the TEV protease. A size marker is shown on the left. Note that N-Y4 cannot be detected on the gel, due to its small size.

A problem of the chosen strategy was that the fusion protein must be solubilized in detergent that is compatible with the active protease. After extensive detergent screening, we observed that the ionic detergent sarcosyl solubilizes the fusion protein while preserving TEV protease activity to some extent. As shown in Figure 1, cleavage efficiency is around 40%, allowing recovery of about 2 mg of ^{15}N-labeled N-Y4 from 1 L of culture.

The structure of N-Y4

Although the N-terminal domain is rather small, its analysis was complicated by reduced chemical shift dispersion due to the fact that the peptide in water is largely unstructured. Nevertheless, with the aid of 3D ^{15}N-resolved NOESY and TOCSY spectra it was possible to assign the ^{15}N,^{1}H-correlation map. Furthermore, no NOE cross-peaks between amide protons could be detected. Recording of a second set of 2D and 3D spectra in the presence of DPC micelles resulted in large chemical shift changes in some parts of the sequence (see Figure 2). Moreover, sequential NOEs between amide protons were seen, as well as Hα,Hβ (i, i+3) contacts usually only observed in helices (see Figure S2). A structure calculation using restraints derived from the NOESY spectra revealed the presence of a helical stretch encompassing residues 5 to 10 (Figure 3). To verify the formation of a stable secondary structure, ^{15}N{^{1}H} NOE spectra were recorded both in the absence and in the presence of DPC. The heteronuclear NOE allows highly sensitive measurement of the rigidity of the backbone at the corresponding residue, with negative values characteristic of flexible

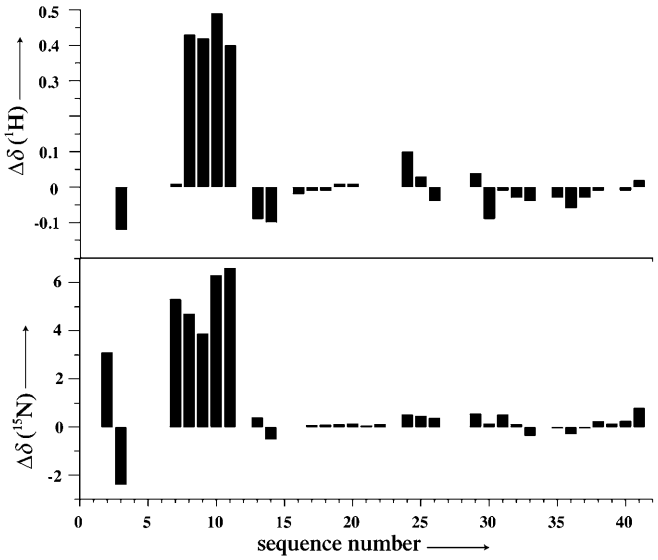

Figure 2. Differences in backbone amide ^{1}H (top) and ^{15}N (bottom) chemical shifts of N-Y4 in the presence and absence of DPC micelles.

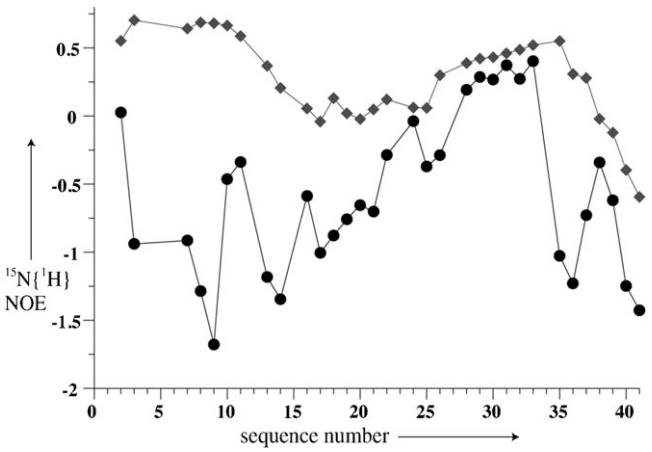

Figure 4. $^{15}N\{^{1}H\}$ NOE values for N-Y4 in plain buffer (spheres) and in the presence of DPC micelles (diamonds). Data were recorded on 1 mM samples at pH 5.6, 310 K at 700 MHz proton frequency.

Figure 3. Comparison of the structures calculated for N-Y4 in the presence of DPC (left) or SDS (right) micelles (only bonds from backbone atoms are depicted). For purposes of clarity, bonds from disordered residues 16–41 are not shown.

parts and values larger than 0.5 usually observed in elements of secondary structure. The $^{15}N\{^{1}H\}$ NOE data show dramatic differences in aqueous medium and DPC. Residues 1–27 have values <0 for N-Y4 in water, whereas all of these residues have $^{15}N\{^{1}H\}$ NOE values >0 in the DPC bound state (Figure 4). Strikingly, residues 5–10 have $^{15}N\{^{1}H\}$ NOE values >0.5. Interestingly, a segment encompassing residues 26 to 33 is rather rigid in both environments. We observed sequential amide proton contacts in that region for almost all residues, but the corresponding Hα,Hβ (i, i+3) contacts were generally missing. When chemical shifts of amide protons in the two environments were compared, the largest differences were observed in that segment that obviously becomes structured in the presence of the micelles, indicating the presence of a nascent helix in that part. To conclude, the N terminus is largely unstructured in the absence of a membrane, whereas a short helical stretch comprising a hydrophobic segment in the N terminus of the sequence is formed in the presence of DPC micelles.

Considering the importance of electrostatic interactions for ligand binding and to investigate whether (stabilizing) interactions of the N-terminal domain with the membrane head groups might be formed, we further initiated structural studies of N-Y4 in the presence of SDS micelles, a negatively charged membrane mimetic. $^{15}N\{^{1}H\}$ NOE values rapidly revealed that N-Y4 was not significantly better structured in this environment. Moreover, a structure calculation again revealed the presence of an α-helix spanning the region between residues 3 to 10 (Figure 3). NOEs between sequential amide protons were seen at the C-terminal end from residue 36 on, but the corresponding Hα,Hβ (i, i+3) contacts were missing, indicating that a transient helix is formed towards the C terminus. Interestingly, this region in the full-length receptor is connected to the first TM. In general, sequential amide proton contacts in the more flexible regions were stronger in relation to the data recorded in the presence of DPC; this suggests that the negatively charged surface promotes the formation of transient helical structures to a slightly greater extent. This is particularly well documented in the heteronuclear NOEs for residues of the segment encompassing residues 19–25, which is less flexible in the presence of SDS micelles (see Figure S9). In general, though, the structural features of the peptide in DPC and in SDS were similar (for more data on the SDS-recorded sample see the Supporting Information).

Topology of membrane association

The proximity of protons of the N-terminal domain to the micelle surface was probed with the aid of micelle-integrating

spin-labels. The paramagnetic moiety of 5-doxyl stearic acid has been shown to reside in the headgroup region.[32] Consistently with the assumption that structuring of the N-terminal segment is induced by binding to the micelle, signals from the amide moieties within that segment experienced the largest signal reduction (see Figure S10). The spin-label data indicate that the N-terminal helix is tightly associated with the micelle, whereas the central segment makes more transient contacts. Motions in that region are likely limited at both ends by the adjacent hydrophobic residues 24–30 and the membrane-anchored N-terminal helix. It has previously been demonstrated that attenuations in helical regions of surface-associated peptides follow periodic patterns.[33,34] The data here indicate that the helical region is not bound in a parallel fashion to the micelle surface. Moreover, from the lack of a clear pattern in the attenuation we conclude that this part is also not anchored in a precisely defined mode.

We also tested whether binding of bPP to N-Y4 could possibly trigger dissociation of the N terminus from the micelle. However, no decrease in signal reduction from the spin-label could be detected upon addition of bPP to micelle-bound N-Y4, indicating that N-Y4–micelle contacts are largely unchanged, even in the presence of a large excess of bPP (concentration ratio of N-Y4 to bPP 1:30; data not shown). This indicates that bPP cannot initiate detachment of N-Y4 from the micelle surface, supporting the view that the contact site between bPP and NY-4 is not located in the helical segment of NY-4 and hence does not interfere with micelle association.

Immobilizing the N terminus on the membrane

In the native Y4 receptor, the segment that has been studied in this work is connected to the first TM helix. In order to address whether anchoring of N-Y4 to the membrane at its C-terminal end influences the structure or the binding properties of the N-terminal domain, a lipopeptide in which receptor residues 1–41 were covalently linked at the C terminus to dodecylethanolamine—to provide stable anchoring of the lipopeptide in the micelles—was chemically synthesized. The lipopeptide was prepared by standard amino acid coupling chemistry, purified, and could be tightly integrated into the DPC micelles. A superposition of the NOESY spectra of N-Y4 and the lipopeptide in the presence of DPC micelles revealed that chemical shift differences are exclusively observed in the vicinity of the lipid attachment site. Moreover, cross-peaks between amide protons occur at identical positions, indicating that the secondary structures of the two peptides are highly similar. To conclude, anchoring of N-Y4 onto the micelle does not influence its secondary structure, which is more likely to be determined by partitioning of residues of the hydrophobic Leu-rich segment into the membrane. As is evident from Figure 4, the carboxy terminal segment of N-Y4 possesses high flexibility both in the presence and in the absence of DPC micelles. Whether this will also be true when the C terminus is linked to the first TM helix is presently under investigation.

Interaction between N-Y4 and neuropeptides from the NPY family

Possible interactions between peptides from the NPY family and N-Y4 were probed both by chemical shift mapping and by surface plasmon resonance (SPR). PP represents a natural ligand for the Y4 receptor, and accordingly the binding affinity between N-Y4 and PP was measured under physiological conditions (10 mM HEPES, pH 7.4, 150 mM NaCl) both in the absence and in the presence of DPC micelles. The data for chemical-shift mapping were acquired with ^{15}N-labeled NPY, PP, or PYY and with unlabeled N-Y4, as well as by using ^{15}N-labeled NY-4 and unlabeled neuropeptides. The shift mapping experiments revealed significant shift changes in the PP/N-Y4 interaction studies (see Figure 5). Large changes in the PP/N-Y4 system occurred close to positions that were later on shown to be sensitive to replacement by Ala residues (vide infra). In addition, the shift changes involving PYY and NPY are generally much smaller than those seen with PP (data not shown).

The strength of the interaction of PP with N-Y4 was quantified by SPR in the absence of detergent, the N-terminally biotinylated neuropeptides being immobilized on a streptavidin-

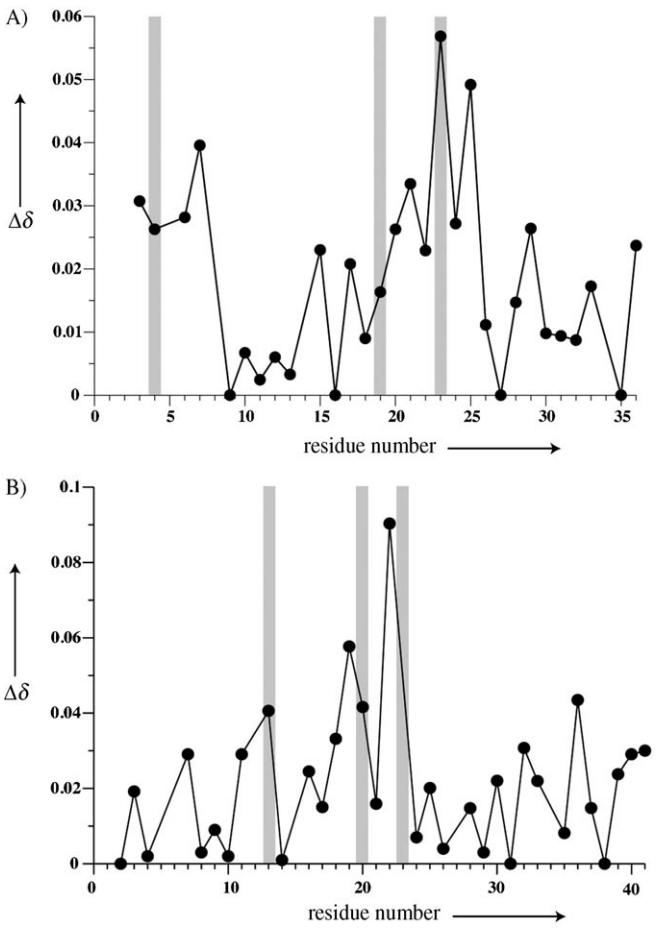

Figure 5. Differences in chemical shifts of amide proton and nitrogen frequencies of backbone resonances of bPP in the presence and in the absence of N-Y4 (A) and of N-Y4 upon addition of bPP (B). Values are computed from $\Delta\delta(^1H,^{15}N) = SQR[(\Delta\delta^1H)^2 + 0.2 \times (\Delta\delta^{15}N)^2]$. Positions at which mutations were performed (E4K, Q19R, and E23A in PP and K13A, R20A, and K23A in N-Y4) are indicated by gray bars.

coated chip and the cells being flushed with solutions of N-Y4 (see Figure 6). The K_D value derived from both kinetic and steady-state analysis was 50 μM for bPP, whereas binding affinity for NPY and PYY was too low to be measured with this technique (> 1 mM).

Measuring the binding of membrane-immobilized peptides with N-Y4 by SPR methods is technically very challenging, and so K_D values in the presence of micelles were measured with the aid of NMR data, by fitting changes in chemical shifts as derived from peak positions of the neuropeptides in [^{15}N,^{1}H] HSQC spectra in the presence of varying amounts of N-Y4. For micelle-bound bPP the K_D to N-Y4 is approximately 600 μM, and experiments in which varying amounts of PP were added to N-Y4 resulted in a very similar value. Apparently, the K_D value in the presence of micelles is much higher than in their absence. This is not really surprising, because it reflects the affinity of the ligand towards the N-terminal domain in the presence of competing membrane binding, and hence accounts for the difference in binding affinity between the two sites.

NPY and PYY possess 80% sequence identity between one another,[35] while PP only shares about 50% homology to either of them. All these neuropeptides display a remarkable separation of charges along the sequence: the positively charged residues occur in the C-terminal half of PP from almost all organisms sequenced so far (see Table 1). In order to identify residues that could contribute significantly to the different pharmacological profiles of NPY/PYY and PP at the Y4 receptor we have aligned the sequences. Particular attention was paid to charged or aromatic residues that are known to be generally involved in GPCR–ligand interactions. The N termini of all Y receptor subtypes are generally negatively charged, with the exception of N-Y4, which contains a net positive charge (see Table 1). In view of the high number of positive charges in N-Y4 and negative charges in the N-terminal half of bPP, electrostatic interactions are likely to be responsible for binding, and such forces would also be expected to result in the observed rather weak binding affinities.

Table 1. Sequence alignment of the principal members of the NPY family and of the N-terminal domains from the various Y receptor subtypes. Positions in bPP and hN-Y4 replaced by other amino acids in this work have been underlined.

pNPY:	YPSKPDNPGE DAPAEDMARY YSALRHYINL ITRQRY-NH_2
pPYY:	YPAKPEAPGE DASPEELSRY YASLRHYLNL VTRQRY-NH_2
bPP:	APLEPEYPGD NATPEQMAQY AAELRRYINM LTRPRY-NH_2
	* * * * * * ** * * ** **
hN-Y1:	MNSTLFSQVE NHSVHSNFSE KNAQLLAFEN DDCHLPLAMI
hN-Y2:	MGPIGAEADE NQTVEEMKVE QYGPQTTPRG ELVPDPEPEL IDSTKLIEVQ
hN-Y4:	MNTSHLLALL LPKSPQGENR SKPLGTPYNF SEHCQDSVDV M
hN-Y5:	MSFYSKQDYN MDLELDEYYN KTLATENNTA ATRNSDFPVW DDYKSSVDDL Q

As depicted in Table 1, common acidic residues in PP, NPY, and PYY are located at positions 6, 10, and 15. PP mutants E4K, Q19R, and E23A were produced by site-directed mutagenesis in order to probe for the importance of differently charged residues between PP and NPY/PYY at these positions. The dissociation constant for Q19R–bPP was only marginally reduced to 89 μm, whereas binding of E4K–bPP and E23A–bPP to N-Y4 was too weak to be detected by SPR. The data indicate that it is the additional negative charges in PP and their distribution along the sequence that might be important for its different binding affinities at N-Y4.

In order to verify that electrostatic interactions between acidic residues of PP and basic residues in the N-Y4 contribute to binding, the K13A, R20A, and K22A mutants of the N-terminal domain of the Y4 receptor were synthesized and investigated by SPR. In all of these mutants, binding to bPP was significantly reduced. The measured values of K_D were 249 μM (R20A) and 281 μM (K22A), whereas for K13A binding was too weak to be detected by SPR. The combination of the mutagenesis studies performed on acidic residues of PP and basic residues of N-Y4 suggests that the binding affinity between the two is to a large extent determined by electrostatic interactions. In this work we have abstained from experiments in which residues in PP and N-Y4 were charged-reversed simultaneously, because in those mutants electrostatics are likely to be perturbed in

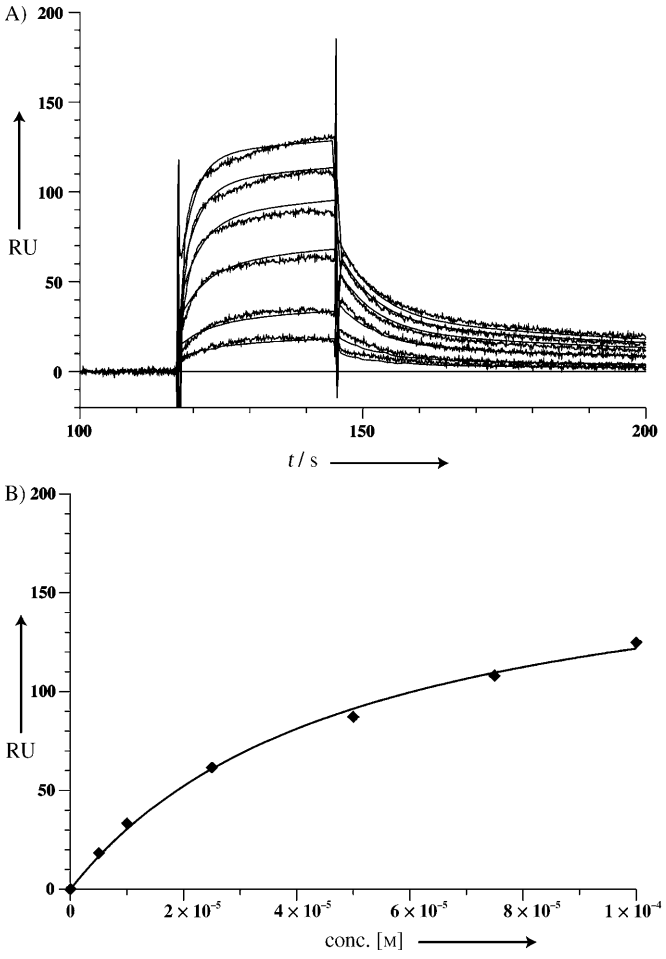

Figure 6. A) SPR sensogram of the interactions of N-Y4 with bPP at various concentrations of N-Y4 (in the 5 to 100 μM range). B) Plot of the steady-state value of the sensograms versus the concentration of N-Y4, used for extraction of the dissociation constant K_D.

both molecules, and hence it is questionable whether activity could have been rescued.

Discussion

The mechanism for recognition of ligands by their receptors is of prime biological and pharmaceutical interest. Because of the enormous problems involved in expression, purification, and reconstitution of sufficient amounts of GPCRs, little progress in structural studies has been made over the last decade, and so far bovine rhodopsin and the β-adrenergic receptors are the only GPCRs for which high-resolution X-ray data have been published. In this work we have attempted to investigate the structure of the isolated N-terminal extracellular domain of the Y4 receptor, a GPCR targeted by hormones of the NPY family that binds to PP with very high affinity. Moreover, we have determined the interaction with PP and the other members of the NPY family and have investigated the role of specific residues for binding.

Structural studies of GPCR fragments could possibly suffer from the fact that interactions with the remainder of the receptor—that might be structurally relevant—are missing. With regard to the present analysis, the N-terminal domain of the published crystal structure of the β-adrenergic receptor was largely unstructured, and did not display interactions with other parts of this GPCR, and in particular not with the extracellular loops. This supports our contention that the conformations of the N-terminal domains of a GPCR are not significantly determined by interactions with the remainder of the receptor. Such a study also allows us to define contributions of residues from the N terminus of the Y4 receptor to ligand binding directly.

While the N-terminal domain of the Y4 receptor is largely unfolded in solution, upon binding to zwitterionic (DPC) or negatively charged (SDS) micelles, a hydrophobic segment comprising residues 5 to 10 forms a rather stable α-helix, and the nascent helix encompassing residues 26–35 is slightly rigidified. The central region and the C-terminal hexapeptide remain largely unstructured. The helical segment comprising residues 5 to 10 is entirely made up of hydrophobic residues. The structural data and the internal backbone dynamics of N-Y4 in the presence of zwitterionic (DPC) and anionic (SDS) headgroups display only minor differences, indicating that the conformation does not depend on specific features of the surrounding lipids. Both the formation of secondary structure and the association with the membrane seem to be controlled by the hydrophobicity of the residues and their partitioning into the membrane.[36] Strongly favorable values for the latter are encountered only in the α-helical stretch and in the segment between residues 24 to 30, exactly those regions for which the spin-label data indicate proximity to the water–membrane interface. Spin-label, dynamics, and structural data of N-Y4 reveal the central segment to be rather flexible. The segregation of N-Y4 into structured and flexible regions is very similar in the presence of zwitterionic or negatively charged lipid headgroups. As a consequence of these features it appears likely that this domain can experience larger movements on the membrane surface, and hence could possibly undergo various structural or translational transitions in order to interact with the extracellular loops or with the membrane-bound ligands. At this point we would like to mention that the N-terminal domain of the β2-adrenergic receptor was also disordered in Kobilka's crystal structure[6,7] and that the N-terminal domains from many other class-1 GPCRs are predicted to be largely unfolded. This indicates that the fact that N-Y4 is mainly flexible is likely not an artifact due to the usage of a receptor fragment but rather reflects a commonly encountered feature of these receptors.

We have recently proposed that binding of hormones from the NPY family to their receptors is preceded by association of the ligands at the membrane. According to ideas originally proposed by Kezdy and Kaiser[37,38] and later developed by Schwyzer into the membrane-compartment model,[39,40] binding to the membrane reduces the search for the receptor to two dimensions, increases the concentration in the vicinity of the receptor, and possibly induces conformations that facilitate receptor binding. Structural studies of porcine (p) NPY[33] and PYY[41] and of bovine (b) PP[42] bound to membrane-mimicking dodecylphosphocholine (DPC) micelles revealed large structural changes occurring during membrane association.[43] From this picture the important question that arises is how the hormones enter the binding pocket once the membrane-bound species has laterally diffused along the membrane into the vicinity of the receptor. The seven-helix bundle provides a rather rigid scaffold that most likely does not allow the necessary rearrangements required for direct diffusion of the membrane-bound ligand into the binding pocket. Therefore, the hormones need to detach from the membrane. Our SPR data for binding affinities of the hormones towards phospholipid membranes indicate that membrane binding is only moderate.[41] Any part of the receptor that possesses higher affinity towards the peptides than the membrane does, and could be accessed by a ligand that is in proximity to the membrane surface, might help to guide the ligand into the binding pocket. The N-terminal domains of the Y receptors are polypeptide segments of 40–50 amino acid residues in length located in the extracellular space,[44] and hence present potential interaction sites for the ligands. This work now indicates that, at least for PP, transient association with the N-Y4 might be part of the cascade of events leading to receptor activation. It should be emphasized here that transient binding to the N-terminal domain does not exclude structural changes in the conformations of loop residues later on, which might occur when the ligands have diffused into the genuine receptor binding pockets. Such changes or rotations of the TM helices are believed to be important for receptor activation, and the events described above merely serve to guide the ligand from the membrane-bound state into the binding pocket.

Binding of PP to the N-terminal domain of the Y4 receptor—often referred to as the PP-preferring receptor—is moderate, with a dissociation constant of about 50 μM. NPY and PYY, two hormones from the NPY family with very similar pharmacology and high sequence similarity with respect to one another, do not bind to this domain. Sequence alignments reveal that PP

overall is more negatively charged than NPY or PYY, particularly in the N-terminal region, and our studies show that replacement of E4 or E23 in PP largely abolished binding to N-Y4. Furthermore, introduction of Arg into position 19 led to only marginal changes in binding affinity. The N-Y4 domain, unlike the N-terminal domains from all other receptor subtypes, contains a comparably large number of positively charged residues (K13, R20, and K22), which are also relatively close to each other in sequence. Their replacement by Ala as described above leads to significant losses in binding affinity. To conclude, taking the importance of acidic PP and basic N-Y4 residues into account, we speculate that electrostatic interactions between PP and N-Y4 are crucial for this interaction. However, it must be emphasized that a priori it is not clear in our case whether residues from the N terminus are interacting with residues from the extracellular loops, thereby modulating the effective charge experienced by the peptides. This question can only be addressed experimentally with confidence when structural studies of the full-length receptor in a functional state become available.

Unfortunately, few pharmacological data for the entire Y4 receptor are available. In the case of the human Y1 receptor it has been proposed that an Asp residue at the interface between TM helix 6 and the third extracellular loop might contribute strongly to binding of NPY[45] in the full-length Y1 receptor. Because Asp at this position is conserved amongst all Y receptor subtypes it was speculated that this residue generally contributes to binding in all subtypes. Nicole et al. investigated the role of this $Asp^{6.59}$ in more detail[46] and verified the proposed interaction of Arg33 or Arg35 with acidic third extracellular loop (EL3) residues in the other Y receptor subtypes. Our data now indicate that in addition to the interaction described above, additional contacts between acidic residues of PP and basic residues of the N-terminal domain of the Y4 receptor might contribute to binding. Association of the N-Y4 with PP might therefore not only be of a transient nature, helping the ligand to be transferred from the membrane-bound state into the receptor binding pocket, but might also exist in the ligand-bound state, contributing to the high binding affinity and selectivity of PP at the Y4 receptor.

Conclusions

From the data described above, we speculate that the N-terminal domain of the Y4 receptor might help in transferring PP from the membrane-bound state into the receptor binding pocket. As proposed by us in the case of ligands of the Y receptors,[43] PP initially associates with the membrane. Through binding to the membrane, its effective concentration in the vicinity of the receptor is increased, the search is reduced from three to two dimensions, and conformations closer to those of the bound state could be induced, according to the membrane-compartment model.[39,40] BIACore data for PP binding to phospholipid surfaces indicated that binding to membranes is moderate.[47] Accordingly, an equilibrium is formed, in which PP rapidly diffuses on and off the membrane, but mostly remains in the vicinity of the membrane. When PP has diffused into proximity to the receptor, where interactions with the latter can occur, it may transiently bind to N-Y4 from solution. Whether the complex of PP and N-Y4 itself will move into the vicinity of the extracellular loops, or whether the position of N-Y4 is fixed by interactions with the membrane or the remaining portion of the receptor is presently unclear.

A scenario in which N-Y4-bound PP would be transferred into the binding pocket by a translational movement of parts of the N-terminal domain is at least compatible with the experimental data. These indicate that the binding region for PP is located in its central segment, which at the same time is the only part of N-Y4 that is not making significant contacts with the membrane surface, and which also possesses sufficient internal flexibility to allow the necessary movements. We presently favor a view that describes the N-terminal domain as a large flexible loop, anchored onto the membrane at the amino terminus through the membrane-associated helix and at the C terminus through the first TM. This view is also supported by the recent crystal structures of the β2-adrenergic receptor, in which the N-terminal domain is so flexible that electron density in this part could not be traced.[6,7] We have now initiated work on constructs that include parts of the TM bundle to see whether conformational preferences of N-Y4 are influenced by the remainder of the receptor.

Experimental Section

Expression of the N-Y4 sequence as a soluble fusion to ubiquitin resulted in heterogeneous fragmentation. In order to prevent in vivo processing, the N-terminal domain was fused to the highly insoluble protein ketosteroidisomerase that is encoded in the commercial plasmid pET31b, from which it was liberated by cleavage with the TEV protease in mild detergent.

Plasmid construction, expression, and purification of N-Y4: The cDNA of the Y4 receptor was obtained from the University of Missouri–Rolla (UMR) cDNA Resource Center. The following two primers were used to amplify the cDNA corresponding to N-Y4 by PCR. Forward primer: GCG*CTCGAG***GGTTCCGGTTCCGGTTCC**GAAAACCTGTACTTCCAGATGAACACCTCTCACCTGCTGGC, in which italic letters denote a XhoI cleavage site, bold letters denote a Gly-Ser linker sequence, and underlined letters identify a TEV cleavage sequence. Reverse primer: CTG*GCTGAGC*TCACATCACGTCCACGGAATCCT, with italic letters denoting an EspI cleavage site. The amplified PCR product and the target vector, pET 31b (Novagen), were simultaneously digested with XhoI and EspI, and ligated together with T4 ligase. The construct was confirmed by DNA sequencing (Synergene Biotech, Switzerland). All mutants were constructed by site-directed mutagenesis by use of the QuikChange Kit (Stratagene, USA).

The fusion protein was expressed in inclusion bodies by use of the BL21(DE3) *E. coli* strain. Protein expression was performed by growing cells at 37 °C in minimal media containing ^{15}N-NH_4Cl as the sole nitrogen source for ^{15}N-labeled peptide. IPTG (1 mM) was added to induce protein expression when the OD_{600} reached 0.8, and cells were harvested after 5–6 h. The fusion protein was purified from inclusion bodies in guanidinium hydrochloride (6 M) by Ni-NTA affinity chromatography. After removal of GdnHCl by dialysis, the precipitated fusion protein was solubilized in Tris (pH 8.0, 50 mM) in the presence of *N*-lauryl sarcosine (2%) upon sonication

to give a final concentration of 2 mg mL^{-1}. The resulting solution was dialyzed four to six times against a 20-fold excess of Tris (pH 8.0, 50 mM). The solution was diluted 10 times with Tris (pH 8.0, 50 mM), and EDTA and DTT were added to give final concentrations of 0.5 mM and 1 mM, respectively. TEV protease was added to give a final concentration of 100 mM, and the cleavage mixture was kept at 4 °C overnight. The target peptide was purified by C18-RP-HPLC (Vydac, USA) and the correctness of the peptide was verified by MALDI-TOF MS: ^{15}N-labeled N-Y4: 4614 Da [theoretical mass (for 100% labeling): 4611.1 Da].

Synthesis and purification of the neuropeptides and of unlabeled N-terminal fragments: ^{15}N-labeled peptides from the NPY family were expressed as soluble fusions to ubiquitin. Ubiquitin was liberated from the neuropeptide by use of yeast ubiquitin hydrolase, and C-terminal amidation was performed with the α-amidating peptidyl glycine amidase (PAM). We have used the protocols for expression, ubiquitin cleavage, and C-terminal amidation many times before and have described them in great detail elsewhere (for example, in Bader et al.[33]).

Wild-type and mutant N-Y4 peptides and peptides from the NPY family containing ^{15}N nuclei at natural abundance were prepared by solid-phase peptide synthesis with use of a robot system (ABI433A, Applied Biosystems). 2-Chlorotrityl chloride resin preloaded with Fmoc-Met-OH was used to assemble the linear peptide by standard Fmoc chemistry [piperidine in DMF (20%) for Fmoc deprotection, HOBt/HBTU (4 equiv) for activation, diisopropylethylamine as base, and *N*-methylpyrrolidone as solvent]. The peptides were cleaved from the resin and deprotected with TFA/water/ethane-1,2-dithiol/triisopropylsilane 95:2.5:2.5:2.5. The product was lyophilized and purified by C18 RP-HPLC, and correctness was confirmed by ESI-MS: wild-type N-Y4: 4556.8 Da (theoretical mass: 4556.1 Da); K13A N-Y4: 4501 Da (theoretical mass: 4499 Da); R20A N-Y4: 4473 Da (theoretical mass: 4471 Da); K22A N-Y4: 4501 Da (theoretical mass: 4499 Da).

In order to synthesize the N-terminally biotinylated forms, the peptides were mixed with biotin-$(PEO)_4$-NHS-propionate (Molecular Biosciences, USA) in a 1:2 ratio in phosphate buffer (pH 7.0, 100 mM), incubated for 2 h at RT, purified by C18 RP-HPLC, and confirmed by ESI-MS. To confirm that in the case of E4K-bPP the biotin was coupled to the N terminus instead of the side chain of lysine, the biotinylated peptide was first digested with pepsin and the fragment containing residues 1–16 was subsequently analyzed by MALDI-TOF MS-MS. The result from this analysis demonstrated that the biotin was exclusively coupled to the N terminus.

Dodecylphosphoethanolamine coupling to the carboxy terminus of N-Y4: The peptide from solid-phase peptide synthesis was cleaved off the resin with TFA (0.8 vol%) in DCM with all the protecting groups remaining intact. After removal of solvents, the protected peptide was precipitated in cold water, lyophilized, and redissolved in DMF. The solution was stirred at RT for 5 h with dodecylphosphoethanolamine (3 equiv) in the presence of HATU (1 equiv), HOAt (1 equiv), and DIEA (1.5 equiv). After extraction with an ethyl acetate/water mixture (1:1, *v/v*) the lipopeptide was deprotected under the same conditions as described above. Finally, the lipopeptide was purified by C4 RP-HPLC (Vydac, USA) and lyophilized, and purity higher than 95% was confirmed by MALDI-TOF-MS [4848 Da (theoretical mass: 4847.1 Da)] and LC-MS.

NMR experiments: All samples of N-Y4 for structural studies were measured at 1 mM concentration, $[D_{13}]$MES (40 mM) at pH 5.6. For measurements mimicking membrane environments, $[D_{38}]$DPC (300 mM) or $[D_{25}]$SDS (300 mM) were added. All experiments were performed at 700 MHz, 310 K with a triple-resonance cryoprobe. Resonance assignments were initially performed in the absence of DPC or SDS by [^{15}N,^{1}H]-HSQC, 3D [^{15}N,^{1}H]-HSQC-TOCSY (80 ms mixing time), and 300 ms 3D [^{15}N,^{1}H]-HSQC NOESY experiments. Details of the spectroscopy were similar to those described by us earlier.[33] Spectra were analyzed with the aid of the programs CARA[48] and XEASY.[49] After nearly complete resonance assignments in water had been obtained, 3D [^{15}N,^{1}H]-HSQC NOESY (200 ms) was recorded in the presence of DPC, and the assignments in water were adjusted to the DPC spectra. Upper distance restraints in DPC or SDS were then derived from 50 ms 2D NOESY spectra. Internal backbone dynamics were studied by means of a ^{1}H-detected version of a ^{15}N{^{1}H} NOE experiment. Structures were computed on the basis of upper-distance restraints derived from the NOESY spectra with the program CYANA[50,51] by the standard simulated annealing protocol. ^{15}N{^{1}H} NOEs were computed from the ratio of integrals from signals in the presence of amide proton irradiation to those in its absence.[52] Chemical shifts of the ^{15}N,^{1}H-correlation map in the absence of DPC and SDS and full assignments in their presence can be found in the Supporting Information. Proton chemical shifts were referenced to the water line, taken at 4.63 ppm at 310 K, from which the nitrogen scale was derived indirectly through multiplication with the factor $\gamma(^{15}N)/\gamma(^{1}H)$.

The coordinates, chemical shift values, and heteronuclear NOEs of N-Y4 in the presence of SDS and DPC have been deposited in the BMRB database under the accession number 15708.

Membrane-association topology by use of spin-labels: In the spin-label studies [^{15}N,^{1}H]-HSQC spectra of solutions of ^{15}N N-Y4 (0.5 mM) containing DPC (300 mM) were measured in the absence and in the presence of 5-doxyl and 16-doxyl stearic acid (7 mM and 8.8 mM, respectively). Signal attenuation was computed from the ratio of integrals from peaks in the corresponding spectra. The signal attenuation in the presence of the spin-label is related to proximity of protons to the label. In another set of experiments, ^{15}N-labeled N-Y4 (0.1 mM) was mixed with various concentrations of bPP in order to test whether N-Y4 is released from the micelle upon interaction with PP.

Surface plasmon resonance (SPR) studies: HBS buffer [HEPES (pH 7.4, 10 mM), NaCl (150 mM), EDTA (3.4 mM), P20 (0.005%)] was used as the running buffer to achieve physiological pH. N-terminally biotinylated neuropeptides were immobilized onto the sensor chip SA (BIACore, Sweden), which contains a streptavidin-coated surface, resulting in about 200 response units (RUs) on a BIAcore 1000 instrument (BIAcore, Sweden). Different concentrations of N-Y4 spanning a range of 5 to 100 μM were applied to the surface for 30 seconds at a flow rate of 20 μL min^{-1} at 25 °C. After each injection of analytes, the flow cell was flushed with regeneration buffer [NaCl (1 M), NaOH (50 mM)] for 30 seconds. Because unspecific binding occurred at concentrations higher than 100 μM, K_D values larger than 100 μM could not be determined precisely. Nevertheless, trends in reduction of binding could still be computed from a limited set of data points, in which values at high concentrations were excluded from the analysis. All sensograms were analyzed with the BIA evaluation software with use of a two-state binding model.

Abbreviations

AM: adrenomedullin. CCR2: chemokine C-C motif receptor 2. CCR5: chemokine C-C motif receptor 5. CX3C rec.: chemokine C-X3-C motif receptor. CGRP: calcitonin gene-related peptide. DCM: dichloromethane. DIEA: diisopropylethylamine. DMF: dimethylforma-

mide. DPC: dodecylphosphocholine. Doxyl: (4,4-dimethyl-3-oxazolidin-*N*-oxyl). DTT: 1,4-dithiothreitol. EDTA: ethylenediaminetetraacetic acid. ESI-MS: electrospray ionization mass spectroscopy. Fmoc: 9-fluorenylmethoxycarbonyl. GdnHCL: guanidinium hydrochloride. GPCR: G protein-coupled receptor. HATU: 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. HBTU: 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate). HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid. HOBT: *N*-hydroxybenzotriazole. HSQC: heteronuclear single-quantum correlation. IPTG: isopropyl β-D-1-thiogaloctopyranoside. K_D: dissociation constant. LC: liquid chromatography. KSI: ketosteroidisomerase. MCP-1: monocyte chemoattractant protein 1. MES: 2-(*N*-morpholino)ethanesulfonic acid. $^{15}N\{^1H\}$ NOE: nuclear Overhauser enhancement of ^{15}N following saturation of ^1H (heteronuclear NOE). NPY: neuropeptide (h, human; p, porcine). N-Y4: N-terminal domain of Y4 receptor. Ni-NTA: nickel-nitrilotriacetic acid. PAM: peptidylglycine α-amidating enzyme. PP: pancreatic polypeptide (a, avian; b, bovine; h, human). PYY: peptide YY (h, human; p, porcine). RU: response unit; arbitrary unit in SPR. SPR: surface plasmon resonance. TEV protease: tobacco etch virus protease. TFA: trifluoroacetic acid. TM: transmembrane.

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