BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

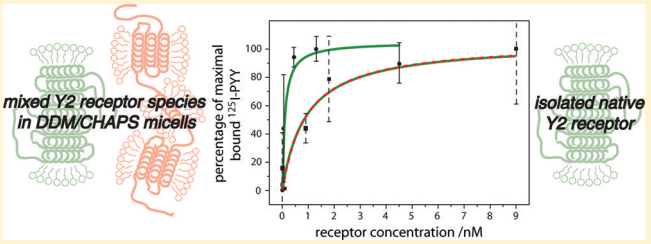
Assessment of a Fully Active Class A G Protein-Coupled Receptor Isolated from *in Vitro* Folding

Mathias Bosse,[†] Lars Thomas,[†] Rayk Hassert,[‡] Annette G. Beck-Sickinger,[‡] Daniel Huster,[†] and Peter Schmidt*[,†]

[†]Institute of Medical Physics and Biophysics, University of Leipzig, Härtelstr. 16-18, D-04107 Leipzig, Germany

[‡]Institute of Biochemistry, University of Leipzig, Brüderstr. 34, D-04103 Leipzig, Germany

ABSTRACT: We provide a protocol for the preparation of fully active Y2 G protein-coupled receptors (GPCRs). Although a valuable target for pharmaceutical research, information about the structure and dynamics of these molecules remains limited due to the difficulty in obtaining sufficient amounts of homogeneous and fully active receptors for *in vitro* studies. Recombinant expression of GPCRs as inclusion bodies provides the highest protein yields at lowest costs. But this strategy can only successfully be applied if the subsequent *in vitro* folding results in a high yield of active receptors and if this fraction can be isolated from the nonactive receptors in a homogeneous form. Here, we followed that strategy to provide large quantities of the human neuropeptide Y receptor type 2 and determined the folding yield before and after ligand affinity chromatography using a radioligand binding assay. Directly after folding, we achieved a proportion of ∼25% active receptor. This value could be increased to ∼96% using ligand affinity chromatography. Thus, a very homogeneous sample of the Y2 receptor could be prepared that exhibited a K_D value of 0.1 ± 0.05 nM for the binding of polypeptide Y, which represents one of the natural ligands of the Y2 receptor.



The G protein-coupled receptors (GPCRs) constitute the largest family of integral membrane proteins and have become of tremendous interest for the research community as well as for pharmaceutical industry over the past one and a half decades. More than 800 different human GPCRs have been identified in the coding sequence of the human genome. Although only 10% of these molecules have been targeted so far, ∼30% of all currently used drugs already act on these proteins. Hence, GPCRs represent a very important and high potential target for pharmaceutical intervention. The most essential function of GPCRs is the transduction of outer signals through the plasma membrane into the interior of a cell. Malfunctions in this process can lead to various diseases while influencing signal transduction provides an enormous potential for therapeutic treatment.[1,2]

Apart from the function of each GPCR and the understanding of the respective signal transduction pathways, the structure and dynamics of the receptor molecules, especially upon their activation, are most important for drug development. The crystal structures of seven different class A GPCRs have been reported to date, some in their activated and some in their nonactivated states, and more will probably come very soon, as reviewed in refs 3−7. Although crystal structures have revealed the largest part of the knowledge about GPCRs, they represent characteristic snapshots in a rather non-native environment and therefore can hardly provide all the essential information about the structure and dynamics of these highly dynamic molecules and the changes of these parameters upon activation. This gap has to be filled by other methods.[8]

By far the most powerful method to provide information about the structure and particularly the molecular dynamics in a more native environment is magnetic resonance spectroscopy.[9−11] For example, EPR measurements using site-directed spin-labeling has revealed the key events during the activation of rhodopsin.[12] It has already been shown that NMR is also a feasible method to investigate membrane proteins of larger size.[13,14] Especially rhodopsin-like proteins have been subjects to intense studies.[15−17] Recently, even the first full structure of an α-helical seven transmembrane receptor was solved using solution NMR methods.[18] Additionally, NMR-based structures of some ligands of GPCRs have been published.[19−22]

The main limitation for the application of NMR techniques to GPCRs is the sample preparation. NMR samples require homogeneous, isotopically labeled, and stable protein molecules in comparatively high concentrations. This sample quality is difficult to achieve for GPCRs, since so far these strongly hydrophobic and very dynamic receptors are challenging to stabilize in a homogeneous fully active state in the required concentrations. A very careful optimization of each step in the preparation appears to be fundamental for successful preparation.[23]

The first challenge is to provide sufficient amounts of isotopically labeled protein. Significant progress has been made in the recombinant expression of GPCRs over the past years.

Received: August 19, 2011
Revised: October 14, 2011
Published: October 14, 2011

The expression of GPCRs as fusion protein in the *Escherichia coli* inner membrane,[24,25] the expression in the yeast strain *Pichia pastoris*,[26,27] or the cell free expression[28] are strategies that can lead to samples that are suited for NMR measurements. But so far, the strategy that provides the highest yields represents the expression of GPCRs in *E. coli* as inclusion bodies. This is not only due to the simplicity, rapidity, safety, scalability, and genetic tractability of the host but also in terms of the quantity and the homogeneity of the recombinantly expressed protein. Further, the solubilization and purification of GPCRs from inclusion bodies are now well established.[29,30]

The difficulty with this strategy is that GPCRs expressed as inclusion bodies have to be subsequently folded *in vitro* in their native states. The folding of membrane proteins has been the subject of intense studies for quite a while now[31] but with growing success.[32] For a few class A[33−37] and also some class B GPCRs[38,39] it has been demonstrated that these receptors can successfully be transferred to their native states by *in vitro* folding, at least in terms of ligand binding. But usage of these molecules in structural studies has remained challenging, since the folding solution always represents a mixture of native protein, non-native intermediates, oligomers, and aggregates,[40] and especially for GPCRs it is not straightforward to discriminate between the different states.

To evaluate the success of the folding strategy, it is fundamental to quantify the proportion of native receptor and even more important to isolate and concentrate this fraction into a homogeneous sample. In the current study, we focus on this question, working with a typical class A GPCR, the human neuropeptide Y receptor type 2 (Y2 receptor).[41] The natural ligands of the Y2 receptor are the neuropeptide Y (NPY) and the polypeptide Y (PYY). Both ligands comprise 36 amino acids and bind to the receptor in a low nanomolar range.[42] The interaction of the Y2 receptor and its ligands plays, among others, an essential role in food intake and in the regulation of the circadian rhythm, which renders this molecule an interesting target for pharmaceutical research.[43]

Here, we describe the quantification of the exact proportion of the native Y2 receptor after *in vitro* folding with ligand affinity chromatography and radioligand binding assays using both natural ligands. Further we investigated the isolation of the native receptor fraction. Using different salts in varying concentrations, the recovery of Y2 receptor from ligand affinity chromatography was determined by fluorescence measurements and radioligand binding assay.

EXPERIMENTAL PROCEDURES

Preparation of the Y2 Receptor Samples. Recombinant expression in *E. coli*, solubilization of the expressed inclusion bodies, and purification of the Y2 receptor in sodium dodecyl sulfate (SDS) micelles were performed as previously described.[44] Receptor folding in detergent micelles composed of *n*-dodecyl-*β*-D-maltoside (DDM) provided by GLYCON Biochemical, Luckenwalde, Germany, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) provided by Sigma-Aldrich, Deisenhofen, Germany, was carried out by rapid dilution as described in ref 33, but with a final receptor concentration of ∼900 nM without any further concentration. The folding buffer also contained 20 mM *β*-cyclodextrin for the removal of SDS as described before.[33]

Preparation of the N-Terminal Biotinylated NPY. All peptides were synthesized by automated multiple-solid-phase peptide synthesis on a Syro II peptide synthesizer (MultiSynTech, Bochum, Germany) by Fmoc/tBu-strategy on a 15 μM scale using Rink amide resin (resin loading = 0.045 mmol/g) to yield C-terminally amidated peptides. Side-chain protecting groups included tBu (Ser, Tyr, Asp, Glu), Trt (Asn, Gln, and His), Pbf (Arg), and Boc (Lys). For automated amino acid coupling, a 10-fold excess of the N_{α}-Fmoc-protected amino acid was activated *in situ* with equimolar amounts of DIC and HOBt and was performed in a double coupling procedure (2 × 40 min). Cleavage of the Fmoc group was carried out using 40% (v/v) piperidine in DMF for 3 min and 20% (v/v) piperidine in DMF for 10 min. The N-terminal biotin group was introduced into the peptide by manual coupling, using a 3-fold excess of biotin and *in situ* DIC/HOBt (0.3 M in DMF) activation for 16 h. Final cleavage of the peptide was achieved with a mixture of TFA/thioanisole/thiocresol (90:5:5 v/v/v) in 3 h while shaking at room temperature. The peptides were precipitated in ice-cold Et_2O and washed six times with Et_2O. The precipitate was then dissolved in tBu/H_2O (1:3 v/v) and finally lyophilized from the mixture. Peptides were purified by preparative RP-HPLC (Phenomenex Jupiter Proteo C-18 column, 22 mm × 250 mm, 4 μm/90 Å) using 0.1% (v/v) TFA in H_2O (eluent A) and 0.08% (v/v) TFA in ACN (eluent B) to yield homogeneous peptides (>95%). The identity of the peptides was verified by MALDI-ToF mass spectrometry (Ultraflex III MALDI-ToF/ToF, Bruker Daltonics), and the purity was determined by analytical RP-HPLC (Phenomenex Jupiter Proteo C-18 column, 4.6 mm × 250 mm, 4 μm/90 Å) using a linear gradient system of 0.1% (v/v) TFA in H_2O (eluent A) and 0.08% (v/v) TFA in ACN (eluent B).

Ligand Affinity Chromatography. The matrix for ligand affinity chromatography was prepared using a modified protocol, which was successfully applied for the neurotensin receptor type 1 before.[45] Intensively washed avidin resin from egg white (Sigma-Aldrich, Deisenhofen, Germany) was incubated with 230 nmol of biotinylated NPY per mL of resin for 1 h and then transferred into an empty column. Unbound ligand was removed by washing the matrix with 20 column volumes (cv) of a buffer containing 1 M NaCl. To avoid nonspecific protein binding to avidin, the resin was succinylated.

Ligand affinity chromatography was used analytically to determine the folding yield of the native receptor and for purification to isolate the receptor in its native form from the folding solution. For quantification of the folding yield, different volumes of folding solution with a constant receptor concentration of ∼900 nM were mixed with 1 mL matrix by gentle stirring. After incubation overnight at room temperature, this solution was transferred into a column and washed with 10 cv of a buffer containing 30% (v/v) glycerol, 0.3% (w/v) DDM, 0.6% (w/v) CHAPS, and 0.1 M Tris/HCl at pH 8. Elution of the bound receptor was performed with 2 cv of the same buffer, but additionally 1 M NaCl. The protein concentrations of the elution fractions were measured by using the intrinsic tryptophan fluorescence of the receptor as described below.

To determine the optimal salt species and its concentration for elution, 4.5 nmol of Y2 receptor in the folding solution were incubated again with 1 mL of matrix and washed as stated above. The elutions were performed in a step gradient of increasing salt concentration with 2 cv for each step. For calculation the measured receptor amounts in the elution fractions were added to the previously measured amounts in lower salt concentrations.

Fluorescence Measurement. Fluorescence emission was used to determine receptor concentrations in the nanomolar range. The spectra were recorded on a FluoroMax-2 (JOBIN YVON) in a 10 mm quartz cuvette at 20 °C using slit widths of 1 nm. The excitation wavelength was set to 295 nm, and the emission was recorded from 300 to 400 nm. All spectra were corrected for buffer contributions.

The receptor concentration was determined by comparing the fluorescence signal at 335 nm with a calibration curve (Figure 1, inset). For the calibration curve, the Y2 receptor in

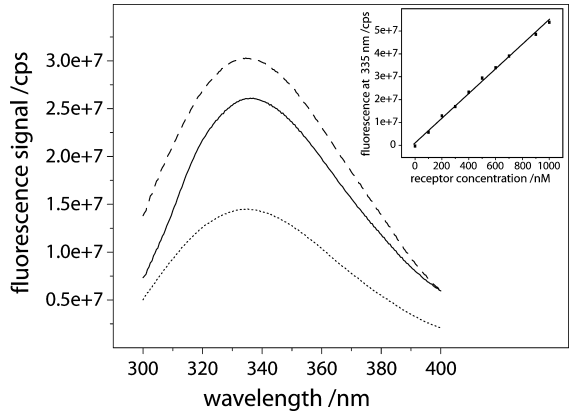


Figure 1. Tryptophan fluorescence spectra of different receptor samples. The solid and the dotted lines represent the spectra of the Y2 receptor recovered from ligand affinity chromatography by elution with 40 mM $CaCl_2$ and 400 mM NaCl, respectively, while the dashed line represents the spectra of the Y2 receptor before folding in SDS. A set of these receptor spectra solubilized in SDS was used for a calibration curve of fluorescence emission at 335 nm in linear dependence on the receptor concentration as shown in the inset. A slight shift in the maxima from 333 to 335 nm could be observed in the sample eluted with 40 mM $CaCl_2$ compared to the other samples, what might be a hint to structural changes related to successful refolding.

SDS micelles with a spectrophotometrically determined concentration was diluted to different nanomolar concentrations and measured as stated above.

Radioligand Binding Assay. Radioligand binding studies were performed for Y2 receptors in DDM/CHAPS micelles before and after ligand affinity chromatography. Ligand binding was determined using ^{125}I-PYY (PerkinElmer, Rodgau, Germany) with a final ligand concentration of 0.3 nM. The Y2 receptors in micelles were diluted to a certain concentration in 30% (v/v) glycerol, 0.3% (w/v) DDM, 0.6% (w/v) CHAPS, and 0.1 M Tris/HCl, pH 8, and incubated with the ligand in a reaction volume of 60 μL. After incubation at room temperature for 60 min, the samples were loaded on Micro Bio-Spin Chromatography columns (Bio-Rad Lab., Munich, Germany) filled with Ni-NTA-agarose (Qiagen, Hilden, Germany) to separate the PYY bound to the C-terminal His-tagged Y2 receptor from unbound ligand. The columns enclosed a bed volume of 300 μL resin and were equilibrated with 3 cv of the dilution buffer stated above. To minimize nonspecific binding, the loaded columns were washed with 3 cv dilution buffer containing 50 mM imidazol. The elution of the Y2 receptor–ligand complex was performed again with 3 cv dilution buffer containing this time 500 mM imidazol to eliminate all metal–histidine interactions. The radioactivity of the eluted samples was measured by liquid scintillation counting[46] with a counting time of 2 min. All measurements were performed with at least two samples either in duplicate or triplicate. The general equation for a sigmoidal dose–response curve was fitted to the data points in all experiments.[47]

RESULTS

Quantification of Native Receptor by Ligand Affinity Chromatography. To quantify the yield of native receptor after *in vitro* folding, ligand affinity chromatography with a bed volume of 1 mL was used. According to the distributor (Sigma-Aldrich), the capacity of the matrix is specified with 60–160 nmol/mL resin. Since protein concentrations in this nanomolar range can hardly be measured spectrophotometrically, the tryptophan signal of the receptors at 335 nm was used and compared with a calibration curve to determine the respective protein concentration (Figure 1).

Different amounts of the receptor were loaded onto the ligand affinity column directly after *in vitro* folding, washed intensively, and then eluted from the matrix using high salt concentrations. The receptor concentrations in the respective elution fractions were measured using the intrinsic fluorescence of the receptor as stated above. Figure 2 illustrates that the

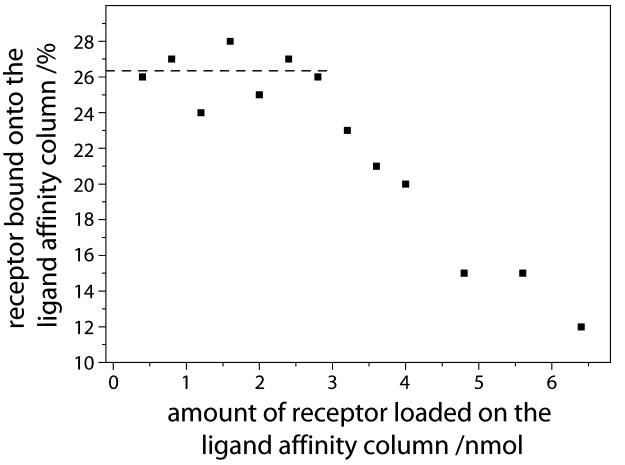


Figure 2. Yield of receptor recovered from ligand affinity chromatography. Different amounts of Y2 receptor in folding solution were loaded onto 1 mL of matrix. The bound fractions were eluted with 1 M NaCl. The maximum yield was determined at around 26% by using the mean value of the data points from the amounts of loaded receptor below 3 nmol (dashed line), representing the folding yield of native Y2 receptor. The capacity of the affinity column is ~730 pmol of native receptor per mL of matrix, calculated from the data point at 2.8 nmol. All receptor amounts were determined by the fluorescence assay.

relative amount of receptor eluted from the column remains almost constant up to a certain amount of receptor loaded onto the column. For amounts above 2.8 nmol the column capacity is reached as indicated by a drastic decrease in the relative amount of receptor bound to the column. From the fraction of receptor bound to the column at low concentration an approximate value of 26 ± 2% active receptor can be concluded. Also, the maximum capacity of the matrix could be calculated to be around 730 pmol bound Y2 receptor per mL of matrix.

As a control experiment we loaded Y2 receptor in SDS micelles before folding onto the ligand affinity column. In this case, no receptor could be detected in the elution fraction.

Isolation of Native Receptor. Besides the quantification of the folding yield, the isolation of the native receptor from the *in vitro* folding solution in its active form was the main focus of this study. The interaction between the Y2 receptor and NPY is dominated by electrostatic attraction.[48] Thus, for the elution of the native receptor from the ligand affinity column the monovalant ion Na^+ and the bivalent ion Ca^{2+} were tested in different concentrations to break off the receptor ligand interactions.

As illustrated in Figure 3, the Y2 receptor could be eluted from the affinity column, using NaCl as well as $CaCl_2$ with a similar yield of around 730 pmol of Y2 receptor. While a concentration of at least 800 mM NaCl was required to elute most of the receptor from the ligand affinity column, a concentration of only 40 mM $CaCl_2$ was sufficient to elute more than 90% of the active receptor. These results are in agreement with the reported tolerated salt concentration for the interaction between NPY and the Y5 receptor.[42] Also, the efficiency of the receptor elution as a function of salt concentration varies significantly between the two salt species. While a rather gradual increase in the receptor amount that can be eluted from the column is observed for NaCl, the elution profile for $CaCl_2$ shows a sudden increase at ~40 mM. The elution with $CaCl_2$ is approximately an all or nothing effect with a critical concentration of ~40 mM.

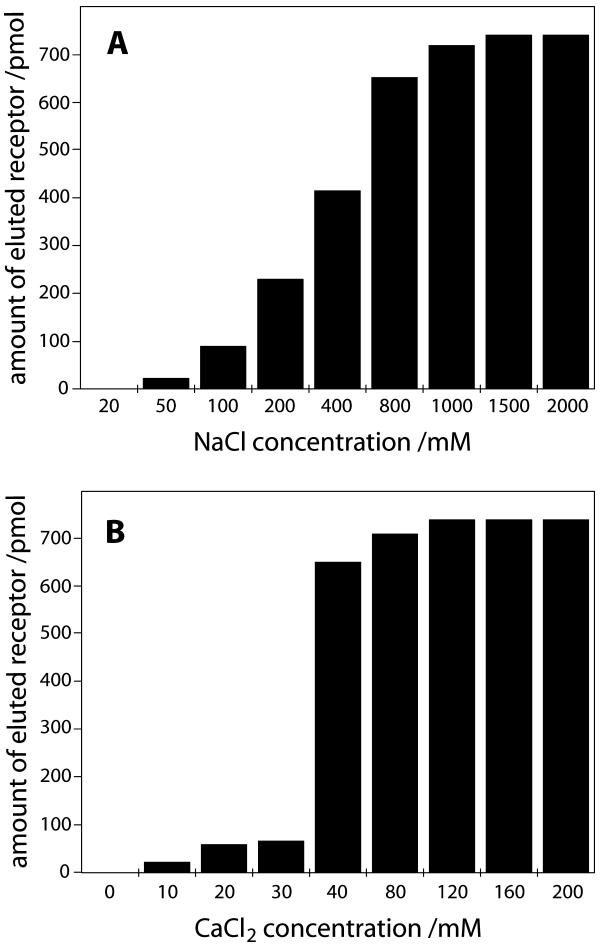


Figure 3. Fraction of eluted Y2 receptor from the ligand affinity column at varying salt concentrations. Different concentrations of the monovalent Na^+ (A) and the bivalent Ca^{2+} (B) cations were tested. While 800 mM NaCl was required to elute ~90% of the bound receptor, already 40 mM $CaCl_2$ was sufficient, resulting in a 20 times higher effectiveness. The data are from one representative experiment for each salt species. All receptor amounts were determined by the fluorescence assay.

Further, using a radioligand binding assay, we tested whether the recovered receptor eluted by NaCl or $CaCl_2$ was still in the native state (Figure 4). To measure the ligand binding ability of the receptors, all samples were diluted at least 10 times in a buffer without the respective salt. This means that the maximum salt concentrations were ≤80 and ≤16 mM for NaCl and $CaCl_2$, respectively. Almost no ^{125}I-PYY binding could be detected for the receptor samples eluted by either 400 or 800 mM NaCl. In contrast, the fractions eluted with either 160 or 40 mM $CaCl_2$ showed a clear concentration-dependent binding behavior. From the fit of the data points the maximal radioactivities of bound ^{125}I-PYY could be determined to 16 250 ± 950 cpm for the sample eluted with 160 mM $CaCl_2$ and 14 050 ± 1200 cpm for 40 mM $CaCl_2$, with similar inflection points at a receptor concentration of 5 ± 2 pM. The basal radioactivity was measured in all experiments at around 5800 ± 200 cpm.

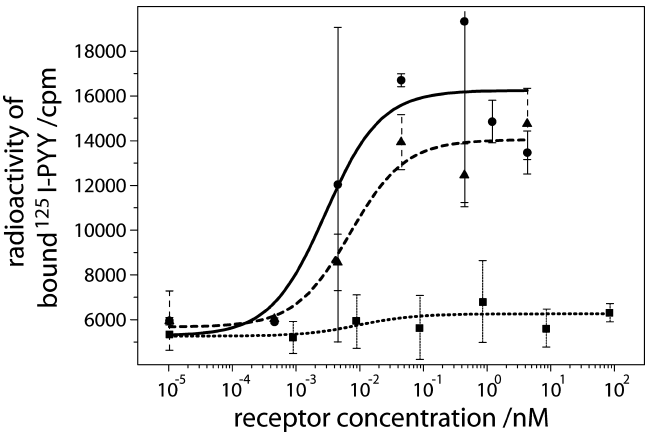


Figure 4. Radioactivity of Y2 receptor bound ^{125}I-PYY for different samples recovered from ligand affinity chromatography. The samples eluted with 160 mM $CaCl_2$ (solid line) and 40 mM $CaCl_2$ (dashed line) show a clear increase of bound ligand in a receptor concentration dependent manner. The maximal amounts of bound ligand have slightly different values, but the inflection points of the curves from the fitted model are very similar, indicating very equal samples with similar amounts of native receptor. In contrast, the ligand shows almost no binding to the receptor fraction eluted with 800 mM NaCl (dotted line). The data shown are from several merged experiments.

Quantification of Native Receptor by Radioligand Binding Assays. To assess the functionality of the Y2 receptor preparations before and after ligand affinity chromatography, we measured the radioactivity of bound ^{125}I-PYY as a function of increasing receptor concentrations. Representative binding curves are shown in Figure 5A. While the fitted maximal and minimal radioactivities in both curves are very similar, the determined inflection points strongly vary with values of 800 ± 50 pM in the sample before and 5 ± 4 pM in the sample after separation of native Y2 receptors by ligand affinity chromatography. Considering the fact that before ligand affinity chromatography the sample contains native receptor and non-native intermediates, this difference was expected.

To quantify the proportion of native Y2 receptor, the data points from the experiments above were normalized to the

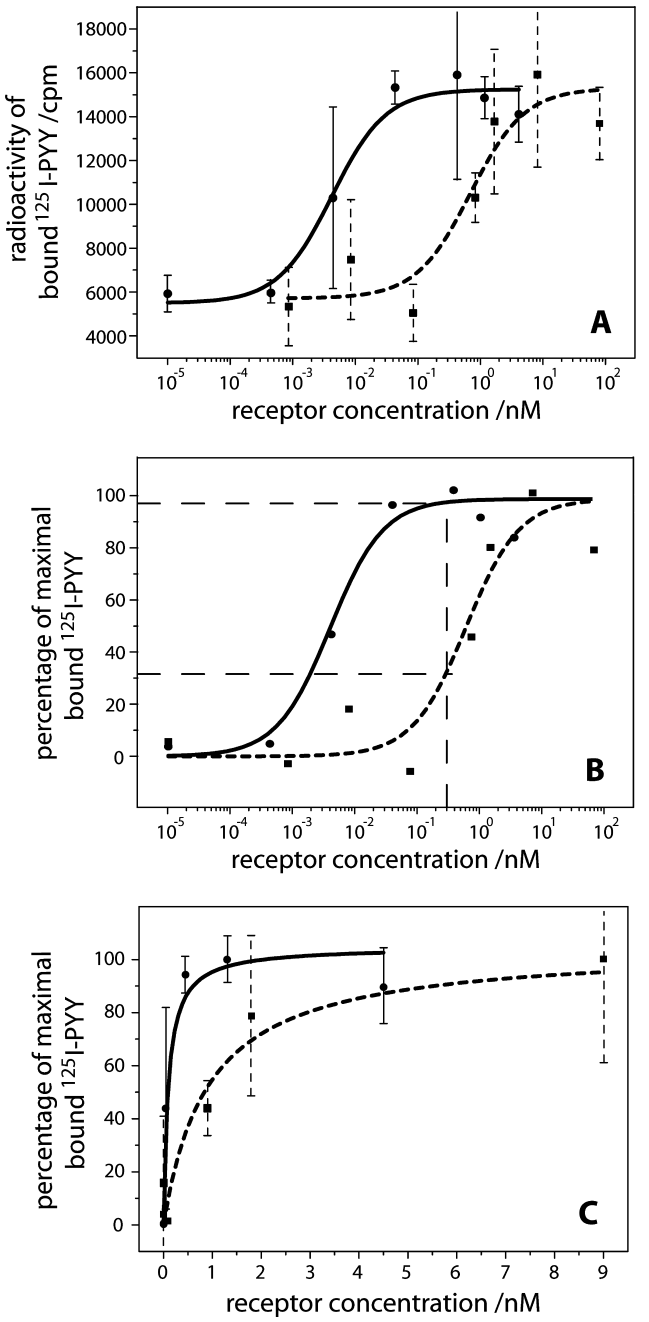


Figure 5. Radioligand binding assay of Y2 receptor samples before (dashed line) and after (solid line) ligand affinity chromatography, eluted by using $CaCl_2$. The radioactivities of bound ligand from several merged experiments in a receptor concentration dependent manner are presented (A). The applied receptor concentrations are based on fluorescence measurements. To determine the amount of native receptor the data points were normalized to the percentage of maximal bound ligand (B, error bars are omitted for clarity). In the assay, a constant ligand concentration of 0.3 nM was used in all samples. Assuming a 1:1 mol/mol stoichiometry in the receptor–ligand complex, the proportion of native receptor could be calculated at 32 ± 9% before and 96 ± 4% after ligand affinity chromatography. In (C), the calculated binding affinity curves are shown providing K_D values of 0.7 ± 0.3 and 0.1 ± 0.05 nM for the respective samples as determined from the fit to the data points. The different K_D values result from the different proportions of native receptor in the samples, since the total receptor concentration (native and non-native) is used for the fit.

percentage of minimal and maximal bound ^{125}I-PYY (Figure 5B). In the assay, constant amounts of 0.3 nM ligand were used. Assuming that one ligand binds to one natively folded receptor, the percentage of native receptors could be determined at values of 32 ± 9% in the sample before and 96 ± 4% in the sample after ligand affinity chromatography, indicating the high efficiency of the separation method.

Determination of the K_D Value. From the binding data as a function of receptor concentration, the K_D value for receptor–ligand interaction can also be calculated for the samples before and after ligand affinity chromatography. As shown in eq 1, the ligand–receptor interaction is described by the mass action law with an equal molar stoichiometry between ligand (L) and receptor (R) according to L + R ⇔ LR.

$$K_D = \frac{[L][R]}{[LR]} \quad (1)$$

The free ligand concentration [L] is always represented by the total ligand concentration [L_{tot}], which was kept constant, reduced by the receptor bound ligand concentration [LR].

$$[L] = [L_{tot}] - [LR] \quad (2)$$

Through rearranging the law of mass action, the K_D value for ligand binding of the receptor can be determined as a function of the total receptor concentration [R] and the concentration of the ligand receptor complex (eq 3) measured by the radioactivity of the receptor bound ligand

$$K_D = \frac{([L_{tot}] - [LR])[R]}{[LR]} \quad (3)$$

yielding an equation for the concentration of the ligand receptor complex:

$$[LR] = \frac{[L_{tot}][R]}{K_D + [R]} \quad (4)$$

Figure 5C shows the measured radioactivity of bound ^{125}I-PYY for increasing receptor concentrations on a linear scale. From the fit of the data points to eq 4 K_D values of 0.7 ± 0.3 and 0.1 ± 0.05 nM for the receptor samples before and after ligand affinity chromatography, respectively, could be calculated. These K_D values strongly depend on the proportion of native receptor in the respective sample, since the total receptor concentrations (native and non-native) are used for the fit, based on fluorescence measurements. The resulting ratio of the K_D values also offers the opportunity for a conclusion of the ratio of active to inactive receptors in the receptor samples before ligand affinity chromatography, calculated with 0.15 ± 0.20, although this only represents a rough estimate due to the error margin of the fit.

DISCUSSION

Despite the large efforts that have been undertaken and the tremendous progress that has been achieved over the past few years, there is still limited information on the structure and dynamics of GPCRs and their ligands. Such data can be provided by solution NMR[49] and especially solid-state NMR spectroscopy,[50,51] which both require highly concentrated homogeneous samples. To date, the most effective strategy to provide the milligram amounts of isotopically labeled GPCRs required for NMR measurements is the recombinant expression of these molecules in *E. coli* as inclusion bodies, as used in this

study. In this approach, the expression level exceeds those achieved for most of the reported expression systems by orders of magnitude,[52] and the labeling is comparatively simple. But this strategy can only be successfully applied if (i) the subsequent *in vitro* folding yields a native receptor proportion of a two-digit percentage[30] and (ii) the native receptor can be isolated in a homogeneous form. To address these two fundamental points for the Y2 receptor, we implemented ligand affinity chromatography using biotin-NPY and studied the activity of the Y2 receptor before and after the column by a radioligand binding assay using ^{125}I-PYY.

Biotin binds to avidin with an extremely high specificity and an affinity of 10^{-15} M and hence presents one of the strongest noncovalent interactions. Very harsh conditions like molar concentration of chaotropic agents at very low pH are required to break this interaction, rendering it long-term stable.[53] Therefore, the use of biotinylated ligand bound to an avidin matrix is exploited in many areas of biotechnology to separate native proteins, even for GPCRs.[54] One consideration that has to be made is that the ligand is fixed to the avidin matrix on one end and hence is not fully accessible for the receptor. Fortunately, this does not represent a problem for the Y2 receptor, since NPY is biotinylated at the N-terminus, which is not involved in the binding to the Y2 receptor. Common truncated analogues like NPY (3−36) or NPY(13−36) and even shorter fragments (18−36, 22−36) can bind to the Y2 receptor with the same nanomolar affinity as the full length ligand.[55]

Using ligand affinity chromatography, we were able to estimate a folding yield of the Y2 receptor of 26 ± 2%, which represents the exact proportion of natively folded molecules in the receptor preparation. This value could be confirmed in the radioligand binding assay, where a value of 32 ± 9% was determined. Despite the fact that the micellar system does not present an optimal environment for membrane proteins,[56] it allows the detection of the precise amount of native receptor molecules, since the receptors are supposed to be monomeric in micelles[57] and all ligand binding sides are accessible.[58] Estimating the exact proportion of native receptors is a prerequisite for evaluating folding conditions. Just to assume that all receptors in the soluble fraction after folding are in their native state, as it is often presented, might not be sufficient,[30] even if general ligand binding could be shown.

In this study, we also used ligand affinity chromatography to separate native Y2 receptors from the folding solution. The capacity of the column was calculated to be 730 pmol receptor per mL of matrix. This value is below the maximal capacity of the matrix indicated by the manufacture, which can be explained simply by the size of the involved proteins. With its 43.8 kDa, which is at least doubled in the protein-detergent complex,[59] the Y2 receptor occupies more space than the avidin (16.8 kDa) or the NPY (4.3 kDa), and hence not all ligands can be saturated by receptors.

It was found that the use of $CaCl_2$ for the elution of the Y2 receptor in ligand affinity chromatography is about 20 times more effective than using NaCl. This is somewhat surprising since the ion radii of the hydrated ions are nearly identical, and only the charge states of the monovalent ion Na^+ and the bivalent ion Ca^{2+} are different.[60] At first sight, one would expect that the required elution concentration for Na^+ is only twice as high as for Ca^{2+}. In detail, the calculation of electrostatic forces in (dilute) electrolyte solutions is given by the Gouy−Chapman and Debye−Hückel theories.[60] The Debye length in 40 mM $CaCl_2$ electrolyte is about 0.96 nm, which almost equals the distance (0.85 nm) for the one identified salt bridge between NPY and the Y2 receptor Arg^{33}-Asp$^{2.68}$.[61] In contrast, the Debye length for 800 mM NaCl, which is required to elute a similar amount of receptor from the column, is 0.34 nm. This means that the higher NaCl concentration screens the electrostatic potentials more efficiently than the much lower $CaCl_2$ concentration, which suggests that pure electrostatic effects cannot explain the differences in the elution properties of the two salts.

Therefore, it further has to be considered that the binding of monovalent Na^+ ions to the protein and/or ligand is likely to be weaker than the binding of the bivalent Ca^{2+} ions to the negatively charged binding sites at the protein. For instance, the intrinsic binding constant of Ca^{2+} ions to phospholipid membranes is about 40 times higher than that of Na^+ ions,[62] which is about the same difference as seen for the elution concentrations of Ca^{2+} and Na^+. Therefore, the increased binding affinity of Ca^{2+} to the protein and/or ligand should be the main contribution for the lower elution concentration observed for Ca^{2+}.

In contrast to the receptors eluted using $CaCl_2$, the fractions eluted by NaCl show no ligand binding at all and therefore no activity. Under these conditions, the receptors bound to the NPY on the column are most likely eluted by denaturing and not by weakening of the electrostatic NPY−receptor interactions, which might explain the shallower slope in the plot of the percentage of eluted receptor at increasing salt concentrations (see Figure 3). Alternatively, the receptor molecules could be denatured directly after dissociating from their ligands. It is known that GPCRs generally show a higher stability when bound to their ligands.[63]

Whether the receptors eluted from the affinity column by 800 mM NaCl were denatured or aggregated was not determined in detail. It has been shown before that the Y receptors overexpressed in COS-7 cells can tolerate quite high salt concentrations.[42] However, at a certain salt concentration the tolerance level of the receptors is exceeded; this appears to be reached at 800 mM NaCl, but not at 160 mM $CaCl_2$. We did not investigate in detail what happens to the Y2 receptors in the presence of 800 mM NaCl; it is also conceivable that the micelles change their properties, which would lead to receptor aggregation or, if the ions interfere with the electrostatic forces within the soluble loops of the receptor, to denaturation. As we were most interested in finding conditions that allow elution of functional receptor from the column, we did not further investigate the precise cause of the loss of activity of the Y2 receptors at high NaCl.

The receptors eluted by using $CaCl_2$ seem to be fully active after elution, as confirmed by radioligand binding assays with ^{125}I-PYY. Also, the K_D value is in the same low nanomolar range as is obtained for Y2 receptors endogenously expressed in mammalian cells (0.2 ± 0.1 nM) for both ligands NPY and PYY.[48] Obviously, the 40 mM $CaCl_2$ had no critical influence on the receptor structure. The higher K_D value for the receptors before ligand affinity chromatography can be explained by the fact that the preparation directly after *in vitro* folding represents a mixture of active and nonactive receptors. The model used to describe the binding data as a function of receptor concentration always assumes that the entire receptor population is active. The fact that only a fraction of the receptor is active before ligand affinity chromatography leads to a shift in the K_D to higher values. Therefore, the ratio of the K_D

values before and after ligand affinity chromatography represents another measure of the ratio of active and nonactive receptors in the preparation. Adjusting the receptor concentrations to the actual amount of active receptors (~25%) in the model leads to very similar K_D values for all preparations. In our study, the binding assays were performed with increasing receptor concentrations instead of using increasing ligand concentrations as in the usual performance of this assay. Because of the high availability of receptors from preparations from *E. coli* inclusion bodies, this experiment is much more cost efficient than performing an assay with increasing concentrations of the valuable radioactive ligand.

We reported earlier that during *in vitro* folding at 1.5 μM receptor concentration and a subsequent concentration increase to 32 μM up to 80% of the Y2 receptors stay in solution.[33] With a maximal protein concentration of 900 nM without any further concentration steps, as it is performed in this recent study, almost 100% of the receptors remain soluble, which has been determined by the fluorescence assay. After expression and purification we obtained around 13 mg of pure and labeled Y2 receptor from 1 L of cultivation medium.[44] With the folding yield determined here (of above 25%), the achieved overall yield is more than 3 mg of isolated and fully active Y2 receptor per liter of expression medium, which represents the required amount for NMR studies. Within 48 h no significant change in the yields or K_D values of the receptor preparations could be determined by the radioligand binding assay. We are aware that all experiments in this study were performed in fairly low concentrations, and it has to be proven that the results can be transferred to the Y2 receptor in concentrations required for NMR measurements. But following the philosophy of a carefully optimized step by step strategy, this work presents a significant achievement toward the preparation of functional GPCR samples useful for NMR. The next logical steps will be an upscaling of the affinity chromatography and the development of a concentration strategy, where the methods established here can be used for quality control.

AUTHOR INFORMATION

Corresponding Author

*Tel: +49 (0) 341 97-15742. Fax: +49 (0) 341 97-15709. E-mail: peter.schmidt@medizin.uni-leipzig.de.

Funding

This study was supported by the Deutsche Forschungsgemeinschaft (SFB 610, Projects A1 and A14) and by the "Nachwuchsförderung" of the Medical Department, University of Leipzig.

ACKNOWLEDGMENTS

We thank Dr. M. Köck from the service laboratory at the Biozentrum of the Martin Luther University Halle-Wittenberg, Germany for supporting the radioassays. We are very grateful to Prof. Dr. Scott E. Feller for a critical review of this manuscript.

ABBREVIATIONS

ACN, acetonitrile; Boc, *tert*-butoxycarbonyl; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; cv, column volume; DDM, *n*-dodecyl-β-D-maltoside; DIC, *N,N'*-diisopropylcarbodiimide; Et_2O, diethyl ether; Fmoc, 9-fluorenylmethoxycarbonyl; GPCR, G protein-coupled receptor; HOBt, hydroxybenzotriazole; NPY, neuropeptide Y; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PYY, polypeptide Y; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; tBu, *tert*-butyl; TFA, trifluoroacetic acid; Trt, trityl.

REFERENCES

(1) Watts, A. (2005) Solid-state NMR in drug design and discovery for membrane-embedded targets. *Nat. Rev. Drug Discovery 4*, 555−568.

(2) Ulloa-Aguirre, A., and Conn, P. M. (2009) Targeting of G protein-coupled receptors to the plasma membrane in health and disease. *Front Biosci. 14*, 973−994.

(3) Hanson, M. A., and Stevens, R. C. (2009) Discovery of new GPCR biology: one receptor structure at a time. *Structure 17*, 8−14.

(4) Goncalves, J. A., Ahuja, S., Erfani, S., Eilers, M., and Smith, S. O. (2010) Structure and function of G protein-coupled receptors using NMR spectroscopy. *Prog. Nucl. Magn. Reson. Spectrosc. 57*, 159−180.

(5) Shimamura, T., Shiroishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., Abagyan, R., Cherezov, V., Liu, W., Han, G. W., Kobayashi, T., Stevens, R. C., and Iwata, S. (2011) Structure of the human histamine H1 receptor complex with doxepin. *Nature 475*, 65−70.

(6) Rosenbaum, D. M., Rasmussen, S. G., and Kobilka, B. K. (2009) The structure and function of G-protein-coupled receptors. *Nature 459*, 356−363.

(7) Rasmussen, S. G., Devree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, S. T., Lyons, J. A., Caffrey, M., Gellman, S. H., Steyaert, J., Skiniotis, G., Weis, W. I., Sunahara, R. K., and Kobilka, B. K. (2011) Crystal structure of the beta(2) adrenergic receptor-Gs protein complex. *Nature 477*, 611−615.

(8) Kobilka, B., and Schertler, G. F. (2008) New G-protein-coupled receptor crystal structures: insights and limitations. *Trends Pharmacol. Sci. 29*, 79−83.

(9) Huster, D. (2005) Investigations of the structure and dynamics of membrane-associated peptides by magic angle spinning NMR. *Prog. Nucl. Magn. Reson. Spectrosc. 46*, 79−107.

(10) Torres, J., Stevens, T. J., and Samso, M. (2003) Membrane proteins: the 'Wild West' of structural biology. *Trends Biochem. Sci. 28*, 137−144.

(11) Farrens, D. L. (2010) What site-directed labeling studies tell us about the mechanism of rhodopsin activation and G-protein binding. *Photochem. Photobiol. Sci. 9*, 1466−1474.

(12) Altenbach, C., Kusnetzow, A. K., Ernst, O. P., Hofmann, K. P., and Hubbell, W. L. (2008) High-resolution distance mapping in rhodopsin reveals the pattern of helix movement due to activation. *Proc. Natl. Acad. Sci. U. S. A. 105*, 7439−7444.

(13) Hiller, S., Garces, R. G., Malia, T. J., Orekhov, V. Y., Colombini, M., and Wagner, G. (2008) Solution structure of the integral human membrane protein VDAC-1 in detergent micelles. *Science 321*, 1206−1210.

(14) Chill, J. H., Louis, J. M., Miller, C., and Bax, A. (2006) NMR study of the tetrameric KcsA potassium channel in detergent micelles. *Protein Sci. 15*, 684−698.

(15) Zheng, L., and Herzfeld, J. (1992) NMR studies of retinal proteins. *J. Bioenerg. Biomembr. 24*, 139−146.

(16) Etzkorn, M., Martell, S., Andronesi, O. C., Seidel, K., Engelhard, M., and Baldus, M. (2007) Secondary structure, dynamics, and topology of a seven-helix receptor in native membranes, studied by solid-state NMR spectroscopy. *Angew. Chem., Int. Ed. 46*, 459−462.

(17) Pfleger, N., Worner, A. C., Yang, J., Shastri, S., Hellmich, U. A., Aslimovska, L., Maier, M. S., and Glaubitz, C. (2009) Solid-state NMR and functional studies on proteorhodopsin. *Biochim. Biophys. Acta 1787*, 697−705.

(18) Gautier, A., Mott, H. R., Bostock, M. J., Kirkpatrick, J. P., and Nietlispach, D. (2010) Structure determination of the seven-helix transmembrane receptor sensory rhodopsin II by solution NMR spectroscopy. *Nat. Struct. Mol. Biol. 17*, 768−774.

(19) Lopez, J. J., Shukla, A. K., Reinhart, C., Schwalbe, H., Michel, H., and Glaubitz, C. (2008) The structure of the neuropeptide

bradykinin bound to the human G-protein coupled receptor bradykinin B2 as determined by solid-state NMR spectroscopy. *Angew. Chem., Int. Ed. 47*, 1668−1671.
(20) Luca, S., White, J. F., Sohal, A. K., Filippov, D. V., van Boom, J. H., Grisshammer, R., and Baldus, M. (2003) The conformation of neurotensin bound to its G protein-coupled receptor. *Proc. Natl. Acad. Sci. U. S. A. 100*, 10706−10711.
(21) Inooka, H., Ohtaki, T., Kitahara, O., Ikegami, T., Endo, S., Kitada, C., Ogi, K., Onda, H., Fujino, M., and Shirakawa, M. (2001) Conformation of a peptide ligand bound to its G-protein coupled receptor. *Nat. Struct. Biol. 8*, 161−165.
(22) Catoire, L. J., Damian, M., Giusti, F., Martin, A., van, H. C., Popot, J. L., Guittet, E., and Baneres, J. L. (2010) Structure of a GPCR ligand in its receptor-bound state: leukotriene B4 adopts a highly constrained conformation when associated to human BLT2. *J. Am. Chem. Soc. 132*, 9049−9057.
(23) Tapaneeyakorn, S., Goddard, A. D., Oates, J., Willis, C. L., and Watts, A. (2011) Solution- and solid-state NMR studies of GPCRs and their ligands. *Biochim. Biophys. Acta 1808*, 1462−1475.
(24) Harding, P. J., Attrill, H., Ross, S., Koeppe, J. R., Kapanidis, A. N., and Watts, A. (2007) Neurotensin receptor type 1: Escherichia coli expression, purification, characterization and biophysical study. *Biochem. Soc. Trans. 35*, 760−763.
(25) Krepkiy, D., Gawrisch, K., and Yeliseev, A. (2007) Expression and purification of CB2 for NMR studies in micellar solution. *Protein Pept. Lett. 14*, 1031−1037.
(26) O'Malley, M. A., Lazarova, T., Britton, Z. T., and Robinson, A. S. (2007) High-level expression in Saccharomyces cerevisiae enables isolation and spectroscopic characterization of functional human adenosine A2a receptor. *J. Struct. Biol. 159*, 166−178.
(27) Fan, Y., Shi, L., Ladizhansky, V., and Brown, L. S. (2011) Uniform isotope labeling of a eukaryotic seven-transmembrane helical protein in yeast enables high-resolution solid-state NMR studies in the lipid environment. *J. Biomol. NMR 49*, 151−161.
(28) Klammt, C., Srivastava, A., Eifler, N., Junge, F., Beyermann, M., Schwarz, D., Michel, H., Doetsch, V., and Bernhard, F. (2007) Functional analysis of cell-free-produced human endothelin B receptor reveals transmembrane segment 1 as an essential area for ET-1 binding and homodimer formation. *FEBS J. 274*, 3257−3269.
(29) Sarramegn, V., Muller, I., Milon, A., and Talmont, F. (2006) Recombinant G protein-coupled receptors from expression to renaturation: a challenge towards structure. *Cell. Mol. Life Sci. 63*, 1149−1164.
(30) Baneres, J. L., Popot, J. L., and Mouillac, B. (2011) New advances in production and functional folding of G-protein-coupled receptors. *Trends Biotechnol. 29*, 314−322.
(31) Booth, P. J., and Curnow, P. (2009) Folding scene investigation: membrane proteins. *Curr. Opin. Struct. Biol. 19*, 8−13.
(32) Malia, T. J., and Wagner, G. (2007) NMR structural investigation of the mitochondrial outer membrane protein VDAC and its interaction with antiapoptotic Bcl-xL. *Biochemistry 46*, 514−525.
(33) Schmidt, P., Lindner, D., Montag, C., Berndt, S., Beck-Sickinger, A. G., Rudolph, R., and Huster, D. (2009) Prokaryotic expression, in vitro folding, and molecular pharmacological characterization of the neuropeptide Y receptor type 2. *Biotechnol. Prog. 25*, 1732−1739.
(34) Kiefer, H., Krieger, J., Olszewski, J. D., Von, H. G., Prestwich, G. D., and Breer, H. (1996) Expression of an olfactory receptor in Escherichia coli: purification, reconstitution, and ligand binding. *Biochemistry 35*, 16077−16084.
(35) Dahmane, T., Damian, M., Mary, S., Popot, J. L., and Baneres, J. L. (2009) Amphipol-assisted in vitro folding of G protein-coupled receptors. *Biochemistry 48*, 6516−6521.
(36) Schimmer, S., Lindner, D., Schmidt, P., Beck-Sickinger, A. G., Huster, D., and Rudolph, R. (2010) Functional characterization of the in vitro folded human Y1 receptor in lipid environment. *Protein Pept. Lett. 17*, 605−609.
(37) Kiefer, H., Maier, K., and Vogel, R. (1999) Refolding of G-protein-coupled receptors from inclusion bodies produced in Escherichia coli. *Biochem. Soc. Trans. 27*, 908−912.
(38) Michalke, K., Huyghe, C., Lichiere, J., Graviere, M. E., Siponen, M., Sciara, G., Lepaul, I., Wagner, R., Magg, C., Rudolph, R., Cambillau, C., and Desmyter, A. (2010) Mammalian G protein-coupled receptor expression in Escherichia coli: II. Refolding and biophysical characterization of mouse cannabinoid receptor 1 and human parathyroid hormone receptor 1. *Anal. Biochem. 401*, 74−80.
(39) Schroder-Tittmann, K., Bosse-Doenecke, E., Reedtz-Runge, S., Ihling, C., Sinz, A., Tittmann, K., and Rudolph, R. (2010) Recombinant expression, in vitro refolding, and biophysical characterization of the human glucagon-like peptide-1 receptor. *Biochemistry 49*, 7956−7965.
(40) Rudolph, R., and Lilie, H. (1996) In vitro folding of inclusion body proteins. *FASEB J. 10*, 49−56.
(41) Michel, M. C., Beck-Sickinger, A., Cox, H., Doods, H. N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., and Westfall, T. (1998) XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol. Rev. 50*, 143−150.
(42) Lindner, D., van Dieck, J., Merten, N., Morl, K., Gunther, R., Hofmann, H. J., and Beck-Sickinger, A. G. (2008) GPC receptors and not ligands decide the binding mode in neuropeptide Y multireceptor/multiligand system. *Biochemistry 47*, 5905−5914.
(43) Parker, S. L., and Balasubramaniam, A. (2008) Neuropeptide Y Y2 receptor in health and disease. *Br. J. Pharmacol. 153*, 420−431.
(44) Schmidt, P., Berger, C., Scheidt, H. A., Berndt, S., Bunge, A., Beck-Sickinger, A. G., and Huster, D. (2010) A reconstitution protocol for the in vitro folded human G protein-coupled Y2 receptor into lipid environment. *Biophys. Chem. 150*, 29−36.
(45) White, J. F., and Grisshammer, R. (2007) Automated large-scale purification of a recombinant g-protein-coupled neurotensin receptor, *Curr. Protoc. Protein Sci.* Chapter 6: Unit 6.8.
(46) Belcher, E. H. (1950) Measurement of gamma-ray energies with the scintillation counter. *Nature 166*, 826.
(47) Klinkhardt, U., and Harder, S. (2000) Dose-response relationship: relevance for medical practice. *Med. Klin. (Munich) 95*, 9−14.
(48) Merten, N., Lindner, D., Rabe, N., Rompler, H., Morl, K., Schoneberg, T., and Beck-Sickinger, A. G. (2007) Receptor subtype-specific docking of Asp6.59 with C-terminal arginine residues in Y receptor ligands. *J. Biol. Chem. 282*, 7543−7551.
(49) Walser, R., Kleinschmidt, J. H., and Zerbe, O. (2011) A Chimeric GPCR Model Mimicking the Ligand Binding Site of the Human Y1 Receptor Studied by NMR Spectroscopy. *ChemBioChem*, 10.
(50) Scheidt, H. A., and Huster, D. (2008) The interaction of small molecules with phospholipid membranes studied by ^{1}H NOESY NMR under magic-angle spinning. *Acta Pharmacol. Sin. 29*, 35−49.
(51) McDermott, A. (2009) Structure and dynamics of membrane proteins by magic angle spinning solid-state NMR. *Annu. Rev. Biophys. 38* (385−403), 385−403.
(52) Michalke, K., Graviere, M. E., Huyghe, C., Vincentelli, R., Wagner, R., Pattus, F., Schroeder, K., Oschmann, J., Rudolph, R., Cambillau, C., and Desmyter, A. (2009) Mammalian G-protein-coupled receptor expression in Escherichia coli: I. High-throughput large-scale production as inclusion bodies. *Anal. Biochem. 386*, 147−155.
(53) Wilchek, M., and Bayer, E. A. (1988) The avidin-biotin complex in bioanalytical applications. *Anal. Biochem. 171*, 1−32.
(54) White, J. F., and Grisshammer, R. (2010) Stability of the neurotensin receptor NTS1 free in detergent solution and immobilized to affinity resin. *PLoS One 5*, e12579.
(55) Cabrele, C., and Beck-Sickinger, A. G. (2000) Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *J. Pept. Sci. 6*, 97−122.

(56) Raschle, T., Hiller, S., Etzkorn, M., and Wagner, G. (2010) Nonmicellar systems for solution NMR spectroscopy of membrane proteins. *Curr. Opin. Struct. Biol. 20*, 471−479.
(57) Harding, P. J., Attrill, H., Boehringer, J., Ross, S., Wadhams, G. H., Smith, E., Armitage, J. P., and Watts, A. (2009) Constitutive dimerization of the G-protein coupled receptor, neurotensin receptor 1, reconstituted into phospholipid bilayers. *Biophys. J. 96*, 964−973.
(58) O'Malley, M. A., Helgeson, M. E., Wagner, N. J., and Robinson, A. S. (2011) The morphology and composition of cholesterol-rich micellar nanostructures determine transmembrane protein (GPCR) activity. *Biophys. J. 100*, L11−L13.
(59) Warschawski, D. E., Arnold, A. A., Beaugrand, M., Gravel, A., Chartrand, E., and Marcotte, I. (2011) Choosing membrane mimetics for NMR structural studies of transmembrane proteins. *Biochim. Biophys. Acta 1808*, 1957−1974.
(60) Israelachvili, J. N. (1991) Electrostatic Forces Between Surfaces in Liquids, in *Intermolecular and Surface Forces* 2nd ed., pp 213−259, Academic Press, New York.
(61) Lindner, D., Stichel, J., and Beck-Sickinger, A. G. (2008) Molecular recognition of the NPY hormone family by their receptors. *Nutrition 24*, 907−917.
(62) Huster, D., Arnold, K., and Gawrisch, K. (2000) Strength of Ca^{2+} binding to retinal lipid membranes: consequences for lipid organization. *Biophys. J. 78*, 3011−3018.
(63) O'Malley, M. A., Naranjo, A. N., Lazarova, T., and Robinson, A. S. (2010) Analysis of adenosine Aa receptor stability: effects of ligands and disulfide bonds. *Biochemistry 49*, 9181−9189.