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Assessing activation of the human neuropeptide FF2 receptor with a non-radioactive GTP binding assay☆

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Abstract

We have evaluated a novel, time-resolved fluorometric GTP binding assay for its suitability for functional screening of neuropeptide FF (NPFF) receptor ligands. Our results suggest that this assay, which relies on the use of a europium-labeled GTP analogue, Eu-GTP, provides a powerful alternative to the [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate binding assay for assessing the functional properties of NPFF analogs. Further, we demonstrate that the tetrapeptide PMRF-NH_2 exhibited high agonist potency at the NPFF2 receptor, and that the efficacies of this peptide and another shortened NPFF analog were greater than that of NPFF.
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Keywords: Neuropeptide FF; Eu-GTP binding assay; [^{35}S]GTPγS binding assay; NPFF2 receptor; Neuropeptides; Structure–activity relationship

1. Introduction

The octapeptide neuropeptide FF (NPFF) acts as a modulator of morphine-induced analgesia, tolerance, and dependence, and influences nociception and several other physiological processes such as neuroendocrine and cardiovascular functions [13,16,19]. NPFF exerts its effects by interacting with specific receptors localized in the central nervous system as well as in the periphery [2,3]. In humans, two G-protein-coupled receptors (GPCRs) for NPFF, termed hNPFF1 and hNPFF2, have been identified [3,4,11]. Because of the scarcity of truly selective NPFF receptor ligands, it has not yet been possible to fully gauge the potential of the two NPFF receptor subtypes as novel drug targets. One possible application of NPFF2 receptor selective agonists might be the treatment of pain [3,20,22].

Although the structure–activity relationships have not been fully explored, a requirement for the C-terminal sequence RF-NH_2 in NPFF for both receptor binding and receptor activation has previously been described [8,15,17]. In addition, Mazarguil et al. [15] have proposed that the amide function of Gln^6 in NPFF represents an essential domain for conferring high affinity and activity of NPFF at its receptor.

Robust and simple screening systems that allow for a functional discrimination between agonists and antagonists are needed to facilitate the testing of compound libraries in a drug discovery process. Such an assay, based on the agonist-stimulated binding of [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate ([^{35}S]GTPγS) to membranes of mammalian cells recombinantly expressing NPFF2 receptor has recently been described [5,6,12]. However, there is an increasing trend within the high-throughput screening field to move to assays that do not rely on the use of radioactive labels. Time-resolved fluorometry is a well-established alternative technology to radioisotopic assays in many high-throughput applications [9]. Very recently, a time-resolved fluorometric method to measure G-protein activation was described [7].

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For this purpose, the human α_{2A}-adrenergic receptor was used as a model GPCR system, and receptor activation was assessed by measuring the binding of a europium-labeled GTP analogue, Eu-GTP, to cell membranes.

The main aim of the current study was to determine whether the Eu-GTP assay represents a suitable method to test the functional properties of novel NPFF ligands at the hNPFF2 receptor. Another aim was to study the structural determinants for binding and functional activity of NPFF analogs at the hNPFF2 receptor.

2. Materials and methods

2.1. Peptides

NPFF and six NPFF analogues, all containing the C-terminal RF-NH_2 domain (see Table 1) were synthesized using a PerSeptive 9050 Plus automated peptide synthesizer employing a Fmoc strategy on a RINK-amide resin and TBTU/ DIPEA as the coupling reagent. The side-chain protecting groups used in the synthesis were trityl for Q and 2,2,4,6,7-pentamethylduhydrobenzofurane-5-sulfonyl for R (Novabiochem, Läufelfingen, Switzerland).

The peptides were purified via HPLC (Shimadzu, Kyoto, Japan) with a C_{18} reversed phase column and acetonitrile as eluent (0.1% TFA in H_2O/0–60% acetonitrile gradient for 60 min). The correctness of the amino acid sequences were verified with MALDI-TOF mass spectrometry (Bruker, Bremen, Germany). Peptide purity was determined via analytical HPLC with a 240 mm × 1.4 mm C_{18} column and acetonitrile as eluent (0.1% TFA in H_2O/0–60% acetonitrile gradient for 30 min).

The reference peptide DYL(NMe)FQPQRF-NH_2 ([1DMe]Y8Fa) was purchased from Bachem (Bubendorf, Switzerland).

2.2. Cell culture and membrane preparation

Recombinant CHO-K1 cells expressing the hNPFF2 receptor (CHO-hNPFF2 cells) (Euroscreen, Brussels, Belgium) were grown in Ham's F12 medium (Nutrient Mixture Ham's F12, Life Technologies, Glasgow, UK) supplemented with 10% fetal bovine serum (Euroclone, UK) and 400 μg/ml of the neomycin analogue G418 (Calbiochem, San Diego, CA). Confluent cells were harvested in phosphate-buffered saline containing 0.6 mM EDTA, pH 7.4 and frozen at −70 °C. Membranes were prepared from thawed cell pellets as previously described [5].

2.3. Radioligand binding assay

Competition binding assays with [^{125}I]-(1DMe)Y8Fa (custom iodinated by Amersham, Buckinghamshire, UK; specific activity: 2000 Ci/mmol) were carried out as described previously [5]. Briefly, membranes of CHO-hNPFF2 cells (2.5 μg of total protein per sample) were incubated in assay buffer (50 mM Tris–HCl, 60 mM NaCl, 1 mM $MgCl_2$, 3 μg/ml aprotinin, 7.5 g/l bovine serum albumin, and 30 μM bestatin, pH 7.5 at room temperature) with 20–70 pM final concentration of [^{125}I]-(1DMe)Y8Fa, and the desired concentrations of test peptides. Each concentration was tested in duplicate. Nonspecific binding was defined with 1 μM (1DMe)Y8Fa and corresponded to about 15% of total binding. After 45 min at room temperature, incubations were stopped by rapid filtration and the radioactivity retained on the filter was determined by scintillation counting.

2.4. Guanine nucleotide binding assays

The agonist activities of test compounds were determined as their ability to stimulate the receptor-mediated binding of Eu-GTP or [^{35}S]GTPγS to G-proteins in membranes from CHO-hNPFF2 cells.

The Eu-GTP binding assay was performed in Acro-Well filter plates, essentially as previously described for membranes of CHO cells stably expressing the human α_{2A}-adrenergic receptor [7]. The reaction was started by adding membranes (2–5 μg protein/sample) to the assay solution (in dose–response experiments the assay buffer consisted of 50 mM Tris–HCl, 1 mM EDTA, 1 mM dithiothreitol, 1 μM GDP, 20 mM NaCl, and 5 mM $MgCl_2$, pH 7.4 at room temperature) containing the desired concentration of test peptide. A 30-min preincubation period without label, was followed by a 30-min stimulation period after the addition of 10 nM Eu-GTP (Perkin-Elmer Life Sciences, Wallac, Turku, Finland; product code: AD0260). The reaction was terminated by vacuum filtration (MultiScreen Vacuum Manifold, Millipore), and the filter plate was washed five times with 200 μl of ice-cold wash buffer (20 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4 at room temperature) per well. Eu-GTP retained on the filter was then measured with a VICTOR^{2TM} V Multilabel Counter (Perkin-Elmer Life Sciences, Wallac, Turku, Finland) using the factory-set protocol for europium measurements (340 nm excitation/615 nm emission, 0.4 ms delay, 0.4 ms window).

The [^{35}S]GTPγS binding assay was based on the filtration of samples incubated in 96-well plates with the help of a Tomtec Harvester96 (Tomtec, Inc., Hamden, CT, USA). The assay and the subsequent scintillation counting was carried out essentially as described earlier [5]. In order to be able to compare the two guanine nucleotide binding assays, the [^{35}S]GTPγS binding assay was carried out using the same conditions as in the Eu-GTP binding assay.

2.5. Data analysis

Experimental results were analyzed by non-linear least squares fitting with each experiment repeated at least three times. The K_D used in the analysis of the competition binding experiments with [^{125}I]-(1DMe)Y8Fa was 0.1 nM, as determined in a pilot study. The statistical significance of

Table 1
Agonist activities (EC_{50}'s and efficacies relative to (1DMe)Y8Fa) were assessed as the ability of the ligands to stimulate Eu-GTP or [^{35}S]GTPγS binding to membranes of CHO cells stably expressing the human NPFF2 receptor

Peptide	Eu-GTP		[^{35}S]GTPγS	
	EC_{50} (nM)	Efficacy [% of (1DMe)Y8Fa]	EC_{50} (nM)	Efficacy [% of (1DMe)Y8Fa]
Reference (1DMe)Y8Fa	6 ± 3	100	17 ± 2	100
FLFQPQRF-NH_2 (NPFF)	1.6 ± 0.3	94 ± 6	13 ± 4	98 ± 9
FLFQGQRF-NH_2	120 ± 50	97 ± 5	290 ± 150	102 ± 2
FLFQPMRF-NH_2	3.8 ± 1.8	85 ± 10	15 ± 9	87 ± 8
PQRF-NH_2	51 ± 27	107 ± 10	300 ± 80	125 ± 5
PMRF-NH_2	11 ± 3	135 ± 5**	11 ± 4	126 ± 6
FLLQPQRF-NH_2	15 ± 4	113 ± 12	110 ± 50	119 ± 7
FRF-NH_2	90 ± 25	140 ± 4**	190 ± 30	139 ± 8*

Efficacy significantly different from NPFF (Student's unpaired *t* test). The data are given as the mean ± S.E.M. of at least three experiments. Representative curves for (1DMe)Y8Fa, PMRF-NH_2 and FRF-NH_2 as tested in the Eu-GTP assay are shown in Fig. 3.
* $p < 0.05$.
** $p < 0.01$.

differences in the results was evaluated using Student's two-tailed unpaired *t* test.

3. Results

3.1. Eu-GTP binding assay with membranes from CHO-hNPFF2 cells

A recently developed Eu-GTP binding assay for GPCRs [7] was evaluated for its suitability as a method to determine the functional properties of NPFF2 receptor ligands. In this assay, cell membrane-based binding of a GTP analog labeled with an Eu-chelate (Fig. 1) is used to measure the extent of G-protein activation [10]. We first performed pilot optimization experiments with CHO cell membranes containing hNPFF2 receptors in order to establish optimal concentrations of Na^+, Mg^{2+}, and GDP for the (1DMe)Y8Fa-mediated activation of hNPFF2 receptors (Fig. 2A and B). In these optimization experiments, 4 μg of CHO-hNPFF2 cell membrane protein per sample was used. In line with our previous findings [6], high Na^+ concentrations (100 and 150 mM) provided for a greater relative response in terms of stimulation over basal compared to low (50 mM or less) Na^+ concentrations (Fig. 2B). However, high Na^+ concentrations also lead to reduced agonist potencies of the tested compounds on the hNPFF2 receptor [6]. At 20 mM NaCl, 1 μM GDP and 5 mM $MgCl_2$, a stimulation of about 220% over basal was obtained (Fig. 2B), and these buffer conditions were chosen for the generation of concentration–response curves of the NPFF analogs.

A concentration-dependent europium signal was observed for agonists activating the hNPFF2 receptor (Fig. 3). The increase in Eu-GTP binding caused by (1DMe)Y8Fa, PQRF-NH_2, PMRF-NH_2, and FRF-NH_2 was clearly mediated through hNPFF2 receptors, since control membranes from CHO cells not expressing this receptor failed to give rise to

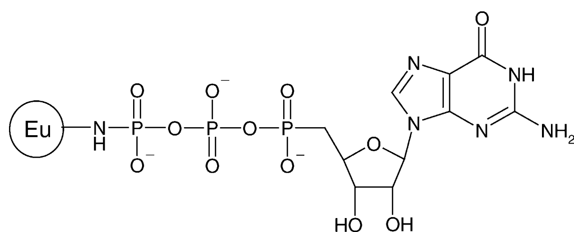

Fig. 1. Structure of the GTP analog labeled with an Eu-chelate. Adapted from [10].

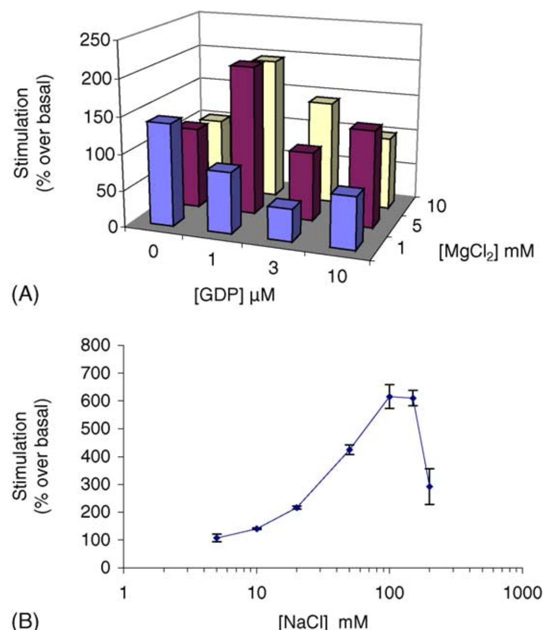

Fig. 2. Optimization of the assay buffer composition for the Eu-GTP binding assay with membranes of CHO-hNPFF2 cells. Effects of $MgCl_2$ and GDP (A) or NaCl (B) on the (1DMe)Y8Fa-stimulated binding of Eu-GTP. Membranes of transfected cells (4 μg protein/sample) were incubated with Eu-GTP (10 nM) in the presence (stimulation) or absence (basal) of 10 μM (1DMe)Y8Fa. Results in (A) were obtained in buffer containing 20 mM NaCl, and results in (B) were obtained in the presence of 5 $MgCl_2$ and 1 μM GDP. The results are expressed as percentage stimulation over basal and one representative example of three independent determinations performed in triplicate is shown.

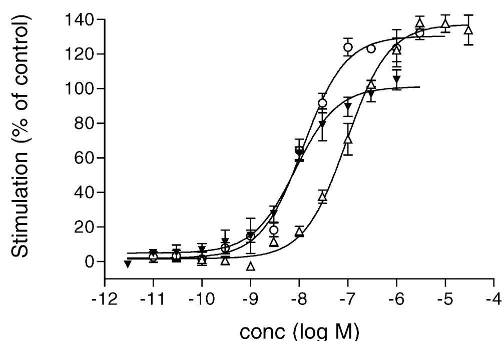

Fig. 3. Stimulation of Eu-GTP binding to membranes of CHO cells expressing the hNPFF2 receptor. Eu-GTP (10 nM) was added to membranes of CHO-hNPFF2 cells (5 μg protein/sample) that had been pre-incubated with the indicated concentration of (1DMe)Y8Fa (▼), PMRF-NH_2 (◯), or FRF-NH_2 (△). The extent of Eu-GTP binding was normalized against the maximal effect of the reference compound (1DMe)Y8Fa, which was set to 100%. The combined results of three different experiments performed in duplicate are shown. The sequence of the peptides as well as the EC_{50} and E_{max} values are shown in Table 1.

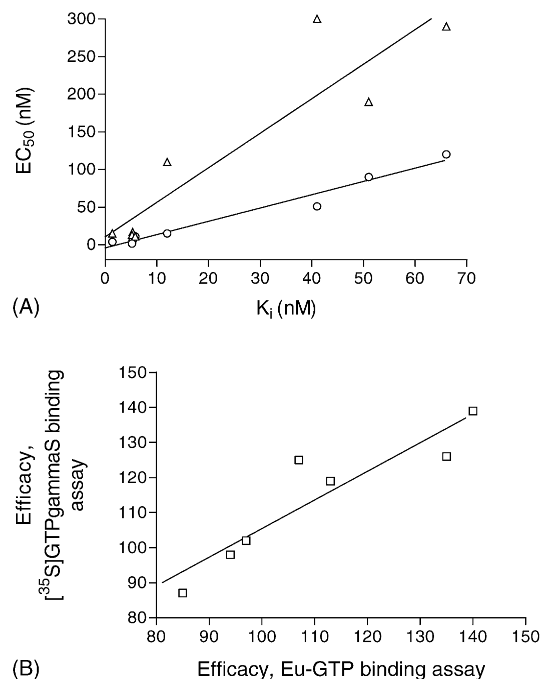

Fig. 4. Correlation of binding affinities and agonist activities at the hNPFF2 receptor. (A) For each peptide, the binding affinity (K_i) as determined in competition binding assays with [^{125}I](1DMe)Y8Fa is shown on the *x*-axis, whereas the agonist potency (EC_{50}) as determined in the Eu-GTP binding assay (◯) or in the [^{35}S]GTPγS binding assay (△) is plotted on the *y*-axis. The comparison of EC_{50}' s as determined in the Eu-GTP binding assay vs. the K_i values gave a regression line with $r^2 = 0.9692$ and a slope = 1.8 ± 0.1. The comparison of EC_{50} values as determined in the [^{35}S]GTPγS binding assay vs. the K_i values gave a regression line with $r^2 = 0.8451$ and a slope = 4.6 ± 0.8. (B) The agonist efficacies as determined in the Eu-GTP binding assay are shown on the *x*-axis, whereas the agonist efficacies as determined in the [^{35}S]GTPγS binding assay are plotted on the *y*-axis. The comparison of efficacies as determined in the two assays gave a regression line with $r^2 = 0.8477$ and a slope = 0.8 ± 0.2.

any significant increase in Eu-GTP binding, when challenged with micromolar concentrations of these peptides (data not shown). Table 1 shows the EC_{50} values and agonist efficacies of the NPFF analogs as determined in the Eu-GTP and the [^{35}S]GTPγS binding assays. Both types of assays were performed under the same conditions.

For reference purposes, the binding affinities (K_i values) of the NPFF analogs at the hNPFF2 receptor were determined in competition binding assays with [^{125}I](1DMe)Y8Fa as the labeled ligand (Table 2). The agonist potencies (EC_{50}'s) from the two functional assays were plotted against the binding affinities (K_i's) determined in the competition binding assays (Fig. 4A). The agonist EC_{50} values as determined in the Eu-GTP binding assay were generally lower than the corresponding values from the [^{35}S]GTPγS binding assay and showed a better correlation with the affinity values (Table 1 and Fig. 4A). However, the K_i values and the EC_{50} values from both functional assays showed high degrees of correlation (the r^2 values for the comparison between K_i values and Eu-GTP EC_{50} values or K_i values and [^{35}S]GTPγS EC_{50} values were 0.9692 and 0.8451, respectively). There was also a high degree of correlation when the agonist properties as determined in the Eu-GTP binding assay versus the [^{35}S]GTPγS binding assay were compared. The r^2 values were 0.7175 for the comparison of agonist potencies (data not shown) and 0.8477 for the comparison of agonist efficacies (Fig. 4B).

Table 2
Binding affinities (K_i's) of NPFF analogs at the human NPFF2 receptor

Peptide	K_i (nM)
Reference DYL'e)FQPQRF-NH2 ([1DMe]Y8Fa)	5.3 ± 1.0
FLFQPQRF-NH_2 (NPFF)	5.2 ± 2.4
FLFQGQRF-NH_2	66 ± 21
FLFQPMRF-NH_2	1.4 ± 0.5
PQRF-NH_2	41 ± 7
PMRF-NH_2	5.8 ± 3.0
FLLQPQRF-NH_2	12 ± 6
FRF-NH_2	51 ± 23

The affinities were determined in competition binding assays with [^{125}I](1DMe)Y8Fa in membranes of CHO cells stably expressing the human NPFF2 receptor. The data are given as the mean ± S.E.M. of at least three experiments.

Assay performance was monitored in form of Z' values as described by Zhang et al. [23]. The Z' value for the Eu-GTP assay was 0.7 and the corresponding value for the [^{35}S]GTPγS binding assay was 0.6. This indicates that the performance of the Eu-GTP binding assay was equal to or slightly better than the performance of the [^{35}S]GTPγS binding assay.

3.2. Structure–activity relationships at the hNPFF2 receptor

The substitution of Gln^6 with Met appeared to lead to an increase in affinity as detectable in the pair-wise comparisons of PMRF-NH_2 versus PQRF-NH_2 and FLFQPMRF-

NH_2 versus FLFQPQRF-NH_2 (Table 2). However, this did only translate into an increased potency in the case of PMRF-NH_2 versus PQRF-NH_2 (Table 1). Cutting off four or five of the N-terminal amino acids (FRF-NH_2; Gln^6 replaced by Phe and PQRF-NH_2) as well as substituting Pro^5 with Gly (FLFQGQRF-NH_2) resulted in a considerable loss in both affinity and potency (Tables 1 and 2). The substitution of Phe^3 in the native NPFF with Leu (to yield FLLQPQRF-NH_2) resulted in a slight loss of affinity and a 8–10 fold lower agonist potency compared to NPFF.

The replacement of a single amino acid in positions 3, 5, or 6 of the NPFF sequence had little effect on agonist efficacy, since FLLQPQRF-NH_2, FLFQGQRF-NH_2, and FLFQPMRF-NH_2 acted as strong or full agonists in both the Eu-GTP and the $[^{35}S]GTP\gamma S$ binding assay. Furthermore, the C-terminal half of NPFF appears to be sufficient for agonist activity at the NPFF2 receptor; as a matter of fact, the efficacies of two shortened peptides (PMRF-NH_2 and FRF-NH_2) were higher compared to NPFF (for PMRF-NH_2 this was significant only in the Eu-GTP binding assay).

According to our preliminary NMR studies, the NPFF parent peptide is too flexible to assume a discernable preferred conformation, thus preventing the possibility to deduce any three-dimensional structure (unpublished observation).

4. Discussion

We have evaluated a novel, time-resolved fluorometric GTP binding assay for its suitability for functional screening of NPFF2 receptor ligands, and in particular, for the purpose of quantitative structure–activity relationship determinations. G-protein activation has traditionally been assessed with GTPase or $[^{35}S]GTP\gamma S$ binding assays. As a reflection of receptor activation, the amount of radioactivity released from $[\gamma\text{-}^{32}P]GTP$ as $[^{32}P]$, or associated with cell membranes in form of $[^{35}S]GTP\gamma S$ has been determined. Due to a centrifugation step in the GTPase assay that is not amenable to automation, $[^{35}S]GTP\gamma S$ binding assays have largely displaced GTPase assays. Problems associated with environmental and occupational safety, waste disposal, and shelf-life have, however, given rise to a growing trend away from the use of radioactivity in screening applications. Very recently, a non-radioactive G-protein activation assay based on Eu-labeled GTP and time-resolved fluorescence has been described [7].

We have compared the Eu-GTP binding assay with the $[^{35}S]GTP\gamma S$ binding assay. Both types of assays were conducted with membranes of a CHO cell line stably expressing the hNPFF2 receptors. There was a high degree of correlation between the receptor affinities, as determined in a $[^{125}I]$(1DMe)Y8Fa competition binding assay, and the agonist potencies, as determined in the Eu-GTP binding assay or the $[^{35}S]GTP\gamma S$ binding assay. Also, the agonist efficacies determined in the two functional assays showed a very high degree of correlation. A comparison of the Z' values from the two functional assays, makes us conclude that the Eu-GTP binding assay performed as well or even slightly better than the traditional $[^{35}S]GTP\gamma S$ binding assay.

However, in terms of agonist potencies there was a noticeable difference between the two functional assays, with the EC_{50} values in the Eu-GTP binding assay being systematically lower than those of the $[^{35}S]GTP\gamma S$ binding assay. The reason for these potency differences seems to be due to the label, since the conditions in the two functional assays, other than the label, were the same.

Current thinking of receptor function ascribes to activated receptor the role of an exchange catalyst, whose task is to accelerate the exchange of GDP for GTP in the alpha subunits of receptor-bound G-proteins. During the exchange process, an activated receptor is assumed to bind a GDP-loaded G-protein, in which it then induces the release of the GDP. Agonist-activated receptor and guanine nucleotide-free G-proteins are considered to form rather stable ternary complexes, which only dissociate upon the binding of a guanine nucleotide to the G-protein alpha subunit. In principle, the ternary complex can bind either GTP or GDP. Which guanine nucleotide the complex will bind depends on the concentrations of the guanine nucleotides and their affinities to the ternary complex. The binding of GDP leads to a non-productive outcome, because it pushes the exchange process in reverse direction, while the binding of GTP drives the process forward. This competition between GDP and GTP (or analogs thereof) for binding to the ternary complex most likely represents the bases for the well-known effects of increasing concentrations of GDP to reduce agonist potencies in G-protein-based functional assays.

While the GDP concentrations in the Eu-GTP and the $[^{35}S]GTP\gamma S$ assay were the same, the concentrations of the labeled GTP analogs were significantly different, with the Eu-GTP being used at 10 nM and the $[^{35}S]GTP\gamma S$ at 0.08 nM. Thus, concentration-wise the Eu-GTP had a clear advantage over $[^{35}S]GTP\gamma S$. This imbalance in terms of concentrations was presumably mitigated to a certain degree by the fact that Eu-GTP has a somewhat lower affinity for the ternary complex than $[^{35}S]GTP\gamma S$ [7]. However, the overall outcome most likely still was that the Eu-GTP was at an advantage compared to the $[^{35}S]GTP\gamma S$ in terms of competing with the GDP for binding to the ternary complex. This would explain the systematically higher agonist potencies in the Eu-GTP versus the $[^{35}S]GTP\gamma S$ binding assays within the framework of known GDP effects on agonist potencies.

By measuring receptor-mediated G-protein activation, we were able to obtain information on how structural modifications to the sequence of NPFF affect agonist efficacies and potencies. In second messenger-based assays, such as adenylyl cyclase assays, especially the efficacy of partial agonists is often masked due to high receptor densities in recombinant expression systems [1,18]. Our results indicate that the C-terminal half of NPFF is sufficient to activate the hNPFF2 receptor. Indeed, we show for the first time that the agonist

efficacies of shortened peptides appear to be higher than those of full length NPFF or of (1DMe)Y8Fa, a stable analog of NPFF. While the removal of four of the N-terminal amino acids leads to a loss of affinity and potency on the NPFF2 receptor, the substitution of Gln with Met in the C-terminal tetrapeptide sequence of NPFF recovers the affinity and agonist potency almost back to that of the full length NPFF. The same tetrapeptide analog, i.e. PMRF-NH_2, has been shown to bind with high affinity to the mixed population of NPFF receptor subtypes in the rat spinal cord [17]. Our results therefore suggest that at the NPFF2 receptor the N-terminal half of NPFF and the presence of Gln^6 are not absolute requirements for high affinity and agonist activity, as has been proposed earlier [8,15]. However, in the context of investigating RFamide-related peptides (RFRPs) [11,14], it was suggested that the three amino acids (i.e. PNL in hRFRP-3) added to the N-terminus of PQRF-NH_2 play a role in directing the specificity towards the NPFF1 receptor subtype [14,21]. In line with this notion, it was also previously shown that the Asn residue in the N-terminus of RFRP-3 slightly hinders the binding of RFRP-3 to the hNPFF2 receptor and that the agonist potencies of the RFRPs are moderate or low at the hNPFF2 receptor subtype [5,14,21].

We conclude that the Eu-GTP binding assay is a powerful alternative to the $[^{35}S]GTP\gamma S$ binding assay and has the potential to offer a high throughput and non-radioactive alternative to the traditional $[^{35}S]GTP\gamma S$ binding assay. Our results in terms of the structure–activity relationship on the NPFF2 receptor will be of use in further drug discovery efforts, e.g. in the discovery of novel, small molecule peptidomimetics.

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