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Functional differences between $NPFF_1$ and $NPFF_2$ receptor coupling: High intrinsic activities of RFamide-related peptides on stimulation of [^{35}S]GTPγS binding

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Abstract

By using an optimized [^{35}S]GTPγS binding assay, the functional activities (potency and efficacy) of peptides belonging to three members of the RFamide family; Neuropeptide FF (NPFF), prolactin-releasing peptide (PrRP) and 26RFamide, were investigated on $NPFF_1$ and $NPFF_2$ receptors stably expressed in Chinese Hamster Ovary (CHO) cells. Despite their large differences in affinity and selectivity, all analogues tested behaved as agonists toward $NPFF_1$ and $NPFF_2$ receptors. High NaCl concentration in the assay strongly increased the efficacy toward $NPFF_2$ receptors and augmented differences among agonists. In low sodium conditions, whereas the potencies of agonists correlated with their affinities for $NPFF_1$ receptors, $NPFF_2$ receptors exhibited an extraordinary activity since all compounds tested displayed EC_{50} values of GTPγS binding lower than their K_I values. Comparisons of functional values between $NPFF_1$ and $NPFF_2$ receptors revealed unexpected potent selective $NPFF_2$ agonists especially for the PLRFamide and the VGRFamide sequences. By using blocker peptides, we also show that $G\alpha_{i3}$ and $G\alpha_s$ are the main transducers of $NPFF_1$ receptors while $NPFF_2$ are probably coupled with $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$ and $G\alpha_s$ proteins. Our data indicate that $NPPF_1$ and $NPFF_2$ receptors are differently coupled to G proteins in CHO cells.

Keywords: Receptor; NPFF; RFamide; [^{35}S]GTPγS; G protein

1. Introduction

In mammals, four genuine members of the RFamide family (RFamide related peptides) have been so far identified; RFRPs (FMRF-amide related peptides and Neuropeptide FF), PrRP (Prolactin releasing peptide), metastin/kisspeptins and QRFP/P518/26RFa.

Neuropeptide FF (NPFF, FLFQPQRF-NH_2) interacts with two $G_{i/o}$-protein coupled receptors termed $NPFF_1$ and $NPFF_2$ (Bonini et al., 2000; Elshourbagy et al., 2000; Hinuma et al., 2000; Kotani et al., 2001) and acts as modulator of the endogenous opioid functions (Roumy and Zajac, 1998). The study of $NPFF_1$ and $NPFF_2$ receptors localization by using selective labeled radioligands (Gouarderes et al., 2002) revealed that most of the pharmacological effects of NPFF in rodents are mediated through the $NPFF_2$ receptor. Two precursors that could maturate peptides with a C-terminal PQRF-NH_2 sequence have been cloned in mammals (Perry et al., 1997; Hinuma et al., 2000), a $proNPFF_A$ containing especially NPFF and a $proNPFF_B$ generating peptides referred to as RFRP (RFamide-related peptides) with a PQRF-NH_2 or a LPLRF-NH_2 C-terminal sequence.

In isolated neurons, both $NPFF_1$ and $NPFF_2$ receptors have an anti-opioid activity by attenuating the magnitude of the

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inhibitory effect of opioid receptor agonists on the $[Ca^{2+}]_i$ transient induced by depolarization (Roumy et al., 2003). Likewise, in transfected cellular model, $NPFF_1$ and $NPFF_2$ receptors exert a non-reciprocal antagonism on opioid receptors in two different paradigms (Mollereau et al., 2005; Kersanté et al., 2006). $NPFF_1$ and $NPFF_2$ receptors are able to couple with $G_{i/o}$ protein when expressed in Chinese hamster ovary (Hinuma et al., 2000; Kotani et al., 2001), human embryonic kidney (HEK 293) (Elshourbagy et al., 2000) or human SH-SY5Y neuroblastoma cells (Mollereau et al., 2005).

PrRP (Prolactin releasing peptide) is a RFamide peptide identified by the reverse pharmacological approach to identify ligands for orphan GPCRs. Prolactin releasing peptides, PrRP20 and PrRP31, have been identified by Hinuma et al. (1998), as ligands of the orphan receptor GPR10/hGR3. Expression and distribution studies indicated the presence of PrRP in the central and peripheral nervous system. The physiological function of PrRP is not yet completely understood (Hinuma et al., 2000; Samson and Taylor, 2006) but it has been shown that it could interact with $NPFF_2$ receptors (Engstrom et al., 2003). Originally, its role was associated with its prolactin releasing property, but it seems now that it is involved as a regulator of the CNS or that it regulates the hypothalamic secretion of CRF and LH/FSH or oxytocin. Some evidence also suggests that PrRP is involved in the regulation of food intake (Gu et al., 2004).

The most recent mammalian RFamide peptides discovered are QRFP/P518/26RFa (Jiang et al., 2003; Chartrel et al., 2005). Intracerebroventricular injection of 26RFa in mice induces a dose-dependent increase in food consumption (Thuau et al., 2005).

Several G-protein-coupled receptors have now been identified as targets for the mammalian -RFamide peptides but the cellular mechanisms by which NPFF, PrRP and 26RFa exert their functions are poorly understood. Receptor-mediated G-protein activation can be directly investigated by determination of agonist-induced guanine nucleotide exchange. This can be achieved by measurement of binding of the non-hydrolysable GTP analogue $[^{35}S]GTP\gamma S$ to membrane preparations. This quantitative technique measures the primary response in the signaling pathway following receptor activation (Harrison and Traynor, 2003). By modifying the cellular environment with different sodium concentrations, it is possible to detect inverse agonists under situation of basal constitutive activity.

Results obtained from structure–activity relationship studies of NPFF-related peptides suggest that the C-terminal -RFamide is essential for NPFF receptor activation (Mazarguil et al., 2001) and/or occupation but the N-terminus is responsible for binding (Gicquel et al., 1994). Based on the message/address model, C-terminal -RFamide may be considered to be a ''message domain'' of NPFF. However, little is actually known concerning the role of this message domain in receptor signaling. The effects of RFamide-related peptides such as FMRFamide, h26RFa, fRRFa, aLPLRFa, fPP36 and other $NPFF_A$ and $NPFF_B$-derived peptides have thus to be evaluated.

In the present work, the potencies and efficacies of diverse NPFF and other RFamide-related peptides have been characterized and compared at human $NPFF_1$ and $NPFF_2$ receptors stably expressed in CHO cells by using the $[^{35}S]GTP\gamma S$ binding assay.

2. Materials and methods

Analogues and peptides derived from the C-terminal regions of the alpha subunits of G proteins $\alpha_{i1,2}$ 345–354: [C]KNNLKDCGLF, α_{i3} 345–354: [C]KNNLKECGLY, α_o 345–354: [C]ANNLRGCGLY, and α_s 385–394: [C]RMHLRQYELL were synthesized using an automated peptide synthesizer (Applied Biosystems model 433A) as described previously (Mazarguil et al., 2001). Peptides related to the h26RFa, their derivatives and the fRRFa were synthesized by the solid phase methodology as previously described (Chartrel et al., 2003). FMRF-NH_2 and hPrRP31 were from NeoMPS (Strasbourg, France). The integrity of peptides was confirmed by mass spectrometry analysis.

Peptides were initially dissolved in 20% methanol-distilled water. HEPES, saponin, bovine serum albumin (BSA), bestatin, GDP and $GTP\gamma S$ were purchased from Sigma (France). All other reagents were from Euromedex (France).

$[^{35}S]GTP\gamma S$ (1000–1178 Ci/mmol) and $[^{125}I]Na$ (80.5 TBq = 2175 Ci/mmol) were from Amersham (France). $[^{125}I]EYF$ and $[^{125}I]YVP$ were obtained by iodination of EYWSLAAPQRFa (EYF-NPSF) and YVPNLPQRFa (YNPVF), respectively, by electrophilic substitution as previously described (Gouarderes et al., 2002). Radio-iodinated peptides were stored at 4 °C in the presence of 0.1% BSA.

2.1. Cell lines

CHO-$hNPFF_1$ and CHO-$hNPFF_2$ cell lines stably expressing the human recombinant Neuropeptide FF receptors were produced as described (Mollereau et al., 2002). Cells (from the clone $hNPFF_1C3$ and the clone $hNPFF_2S\#2$, obtained by limit dilution) were grown in Ham-F12 medium supplemented with 7% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate and 400 μg/ml G418 (Gibco-BRL, France), in an atmosphere of 5% CO_2 at 37 °C. Cells were passaged every 2–3 days. CHO-$hNPFF_1$ and CHO-$hNPFF_2$ expressed approximately 1.3 and 4.8 pmol of receptors/mg of proteins, as determined by binding of 0.1 nM $[^{125}I]YVP$ on $hNPFF_1$ and 0.05 nM $[^{125}I]EYF$ on $hNPFF_2$, respectively.

2.2. Preparation of cell membranes

Cells grown to confluence were harvested in phosphate buffered saline (PBS), frozen at least for 1 h at −80 °C, and then homogenized in 50 mM Tris–HCl, pH 7.4 in a Potter Elvehjem tissue grinder. The nuclear pellet was discarded by centrifugation at 1000 × *g* for 15 min at 4 °C, and the membrane fraction was collected upon centrifugation of the supernatant at 100,000 × *g* for 30 min at 4 °C. The pellets were re-suspended in Tris–HCl 50 mM, pH 7.4, homogenized, and aliquots (0.6–1.3 mg protein) were stored at −80 °. Protein concentration was determined by the Lowry method with BSA as standard.

2.3. $[^{125}I]EYF$ and $[^{125}I]YVP$ binding assays

Binding of $[^{125}I]YVP$ ($[^{125}I]YVPNLPQRFa$, 0.1 nM) and $[^{125}I]EYF$ ($[^{125}I]EYWSLAAPQRFa$, 0.05 nM) to $hNPFF_1$ and $hNPFF_2$ receptors, respectively, was measured by rapid filtration. Membranes (1–2 μg protein) were incubated in polypropylene tubes in a final volume of 500 μl containing 50 mM Tris–HCl, pH 7.4, 60 mM NaCl, 25 μM bestatin, 0.1% BSA and the radioligand. Non-specific binding was determined in the presence of 1 μM $YVPNLPQRFa$ ($NPFF_1$) or 1 μM $EYWSLAAPQRFa$ ($NPFF_2$). After 1 h incubation at 25 °C, samples were rapidly filtered on Whatman GF/B filters preincubated for at least 1 h in 50 mM Tris–HCl, pH 7.4, 0.1% BSA. Filters were rinsed three times with 4 ml of ice-cold Tris–HCl containing 0.1% BSA, and the bound radioactivity was quantified using a Packard γ-counter.

Table 1
Apparent affinities (K_I, nM) of RFamide-related peptides on human $NPFF_1$ and $NPFF_2$ receptors expressed in CHO cells

Peptides	Sequences	$NPFF_1$	$NPFF_2$	$S_{1/2}$
NPFF	FLFQ**PQRF**-NH_2	2.82 ± 0.06	0.21 ± 0.03	13.4
SQA-NPFF	SQAFLFQPQRF-NH_2	4.16 ± 0.31	0.16 ± 0.02	26.0
SPA-NPFF	SPAFLFQPQRF-NH_2	2.62 ± 0.51	0.047 ± 0.003	55.7
NPA-NPFF	NPAFLFQPQRF-NH_2	3.4 ± 0.2	0.044 ± 0.006	77.3
dNPA-NPFF	D.NP(N-Me)AFLFQPQRF-NH_2	2.9 ± 0.5	0.027 ± 0.001	107.4
1DMe	D.YL(N-Me)FQPQRF-NH_2	1.09 ± 0.03	0.18 ± 0.04	6.1
NPSF	SLAAPQRF-NH_2	32.0 ± 6.0	20.0 ± 2.0	1.6
hNPAF	AGEGLNSQFWSLAAPQRF-NH_2	13.0 ± 2.0	0.14 ± 0.01	92.9
bNPAF	AGEGLSSPFWSLAAPQRF-NH_2	10.1 ± 1.4	0.16 ± 0.02	63.1
QFW-NPSF	QFWSLAAPQRF-NH_2	8.8 ± 1.4	0.19 ± 0.01	46.3
EFW-NPSF	EFWSLAAPQRF-NH_2	20.8 ± 0.8	0.21 ± 0.01	99.0
EYF (EYW-NPSF)	EYWSLAAPQRF-NH_2	18.0 ± 3.0	0.24 ± 0.03	75.0
NPVF	VPNLPQRF-NH_2	0.59 ± 0.07	23.0 ± 2.1	0.026
YVP (YNPVF)	YVPNLPQRF-NH_2	0.69 ± 0.09	8.9 ± 1.5	0.078
FMRFa	**FMRF-NH_2**	1.95 ± 0.25	7.4 ± 0.7	0.26
fPP36	APSEPHHPGDQATQDQLAQYYS DLYQYITFVT**RPRF-NH_2**	>3000	5.15 ± 0.51	> 583
hPrRP31	SRTHRHSMEIRTPDINPAWYAS RGIRP**VGRF-NH_2**	44.6 ± 8.2	3.4 ± 0.3	13.1
h9RFa	KKGGF**SFRF-NH_2**	181 ± 30	14.4 ± 1.1	12.6
h26RFa	TSGPLGNLAEELNGYSRKK GGFSFRF-NH_2	38.5 ± 4.7	10.1 ± 1.0	3.8
h43RFa	———— TSGPLGNLAEELNGYSR KKGGFSFRF-NH_2	331 ± 45	53.0 ± 3.1	6.2
h26RFa (19–26)	KGGFSFRF-NH_2	88.6 ± 14.3	25.1 ± 1.4	3.5
h26RFa (20–26)	GGFSFRF-NH_2	190 ± 22	76.3 ± 3.8	2.5
h[Ala5]26RFa (20–26)	GGFSARF-NH_2	968 ± 104	530 ± 33	1.8
h[Ala6]26RFa (20–26)	GGFSFAF-NH_2	>5000	>10000	
h[Ala7]26RFa (20–26)	GGFSFRA-NH_2	>5000	>10000	
LPLRFa	L**PLRF-NH_2**	1.7 ± 0.1	10.6 ± 0.5	0.16
hRFRP-1	MPHSFANLPLRF-NH_2	0.38 ± 0.05	2.8 ± 0.2	0.14
fRRFa	SLKPAANLPLRF-NH_2	2.5 ± 0.3	24.2 ± 2.3	0.10

Data represent the mean K_I values ± S.E.M. (nM) of 3–9 independent experiments, each performed in duplicate samples. K_I values of PQRFa-related peptides are from our recent reports (Gouarderes et al., 2002; Mollereau et al., 2002). $S_{1/2} = K_I$ $NPFF_1/K_I$ $NPFF_2$ for the selectivity index. b, bovine; f, frog; h, human.

2.4. [^{35}S]GTPγS binding assay

The assay buffer consisted of 20 mM HEPES, pH 7.4, 20 or 150 mM NaCl, 3 mM $MgCl_2$ and 0.1% BSA. $hNPFF_1$ or $hNPFF_2$ CHO-transfected-membranes were thawed slowly on ice and diluted with buffer. Aliquots (50 µl, equivalent to 1.5–3 µg and to 0.7–1.4 µg protein, for $NPFF_1$ and $NPFF_2$, respectively), were incubated in polypropylene tubes at 30 °C for 60 min in 500 µl of buffer with 5 µg saponin and 0.066 nM [^{35}S]GTPγS, 0.1 µM ($NPFF_1$) or 1 µM ($NPFF_2$) GDP. Non-specific binding, measured with 10 µM GTPγS, only represented 2.2% and 3.8% of total binding in 20 or 150 mM NaCl, respectively, in both transfected cells. Reaction was stopped by rapid vacuum filtration through GF/B Whatman glass fiber filters, preincubated in the buffer at room temperature for 1 h, and washed three times with 4 ml ice-cold buffer. Membrane-bound radioactivity retained on the filters was determined by liquid scintillation spectrophotometry (94% efficiency) (Packard counter) after overnight extraction of the filters in 4 ml Ready Protein scintillation fluid (Beckman).

2.5. Identification of G proteins associated with $NPFF_1$ and $NPFF_2$ receptors in CHO cells

The $G\alpha_{i1,2}$, $G\alpha_{i3}$, $G\alpha_o$ and $G\alpha_s$ synthetic peptides were tested for their ability to modulate the [^{35}S]GTPγS binding in CHO-$hNPFF_1$ and CHO-$hNPFF_2$ cell membranes in 20 mM NaCl. The effects of increasing concentrations of each synthetic peptide (0 to 100 µM) were determined at basal and maximal 1DMe-stimulated [^{35}S]GTPγS binding. The basal and maximal agonist-stimulated [^{35}S]GTPγS binding using 10 µM ($NPFF_1$) or 0.1 µM ($NPFF_2$) of various RFa-related peptides were determined in the absence (control binding) or presence of 100 µM of each synthetic peptide.

2.6. Analysis of the data

Non-linear regression analysis was performed with GraphPad Prism software (San Diego, USA). [^{125}I]EYF or [^{125}I]YVP competition binding data curves were analyzed to generate IC_{50} values that were converted to K_I values with the Cheng–Prusoff equation. [^{35}S]GTPγS binding data were fitted to a sigmoidal curve with variable slope. Maximum response (E_{max}, %) elicited by a peptide was defined as the maximum increase in [^{35}S]GTPγS binding over basal unstimulated binding. In isotopic dilution experiments, binding inhibited by cold GTPγS was fitted as an homologous competitive binding curve with one or two-classes of binding sites to determine the parameters (K_D and B_{max}) on membranes of transfected $hNPFF_1$ and $hNPFF_2$ cells.

The mean effects of Gα peptides on basal and maximal agonist-stimulated [^{35}S]GTPγS binding were expressed as a percentage of respective control value (absence of Gα peptides) and compared to a theoretical value of zero (*t*-test); when this test revealed significant differences, statistical comparisons of samples were carried out with one-way or two-way ANOVA followed by post hoc appropriate Bonferroni multiple tests. The level of significance was chosen as 0.05.

3. Results

3.1. Affinity and selectivity of RFamide peptides

The binding characteristics of NPFF peptides derived from pro-$NPFF_A$ and pro-$NPFF_B$ precursors, and of RFamide-derived peptides such as h26RFa, fRRFa, LPLRFa, FMRFa, fPP36 and hPrRP31 were determined on $hNPFF_1$ and $hNPFF_2$ receptors by using [^{125}I]YVP (0.1 nM) and [^{125}I]EYF (0.05 nM), respectively. The results are summarized in Table 1. Six groups of RFamide peptides have been tested presenting different affinities and selectivities towards $NPFF_1$ and $NPFF_2$ receptors: peptides exhibiting PQRFamide, FMRFamide, RPRFamide, VGRFamide, SFRFamide and PLRFamide C-terminal sequences. Peptides derived from $NPFF_A$ and $NPFF_B$ precursors, except NPSF and NPVF, exhibited the highest affinity for $NPFF_2$ receptors. However, hRFRP-1 (PLRFamide) or hPrRP31 (VGRFamide) displayed high affinity. In contrast, only peptides from $NPFF_B$ precursor (NPVF and RFRP-1 displayed a sub-nanomolar affinity toward $NPFF_1$ receptors and analogues possessing the C-terminal PLRFamide or FMRFamide sequence exhibited a K_I value of about 2 nM. The selectivity (K_I $NPFF_1$/K_I $NPFF_2$, $S_{1/2}$) varied from 0.026 to 100, dNPA-NPFF and NPVF behaved as the most $NPFF_2$ and $NPFF_1$ selective agonist, respectively. Alanine scanning showed that h26RFa peptides displayed low affinity and selectivity. GGFSFAFamide and GGFSFRAamide have completely lost their ability to interact with NPFF receptors, demonstrating that both NPFF receptors selectively recognized the RFamide C-terminal segment.

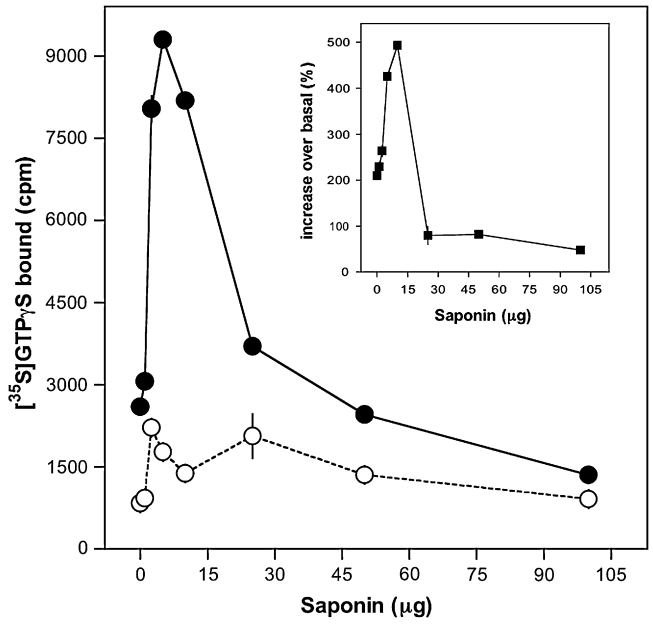

Fig. 1. Effect of saponin on 1DMe-stimulated [^{35}S]GTPγS binding in CHO-$hNPFF_2$ cell membranes. [^{35}S]GTPγS binding in the absence (open circles) and presence (closed circles) of 10 μM 1DMe was determined in a standardized assay. Results are expressed as mean ± S.E.M. values of three independent experiments, each performed in triplicate. Inset: Increase in [^{35}S]GTPγS binding by 10 μM 1DMe expressed as percent over the respective basal binding at various saponin amounts.

3.2. Optimization of [^{35}S]GTPγS binding assay

The addition of graded concentrations of NaCl induced a concentration-dependent reduction of [^{35}S]GTPγS both in the absence and presence of 10 μM 1DMe in both cell membranes. But the presence of NaCl improved the agonist stimulated binding relative to basal conditions from 78% (absence of Na^+) to 264% (150 mM NaCl).

As shown in Fig. 1, saponin, used to permeabilize vesicles present in membrane preparations, from 1 to 5 μg per assay increased by 3-fold 1DMe-stimulated [^{35}S]GTPγS binding without noticeable effects on the basal binding.

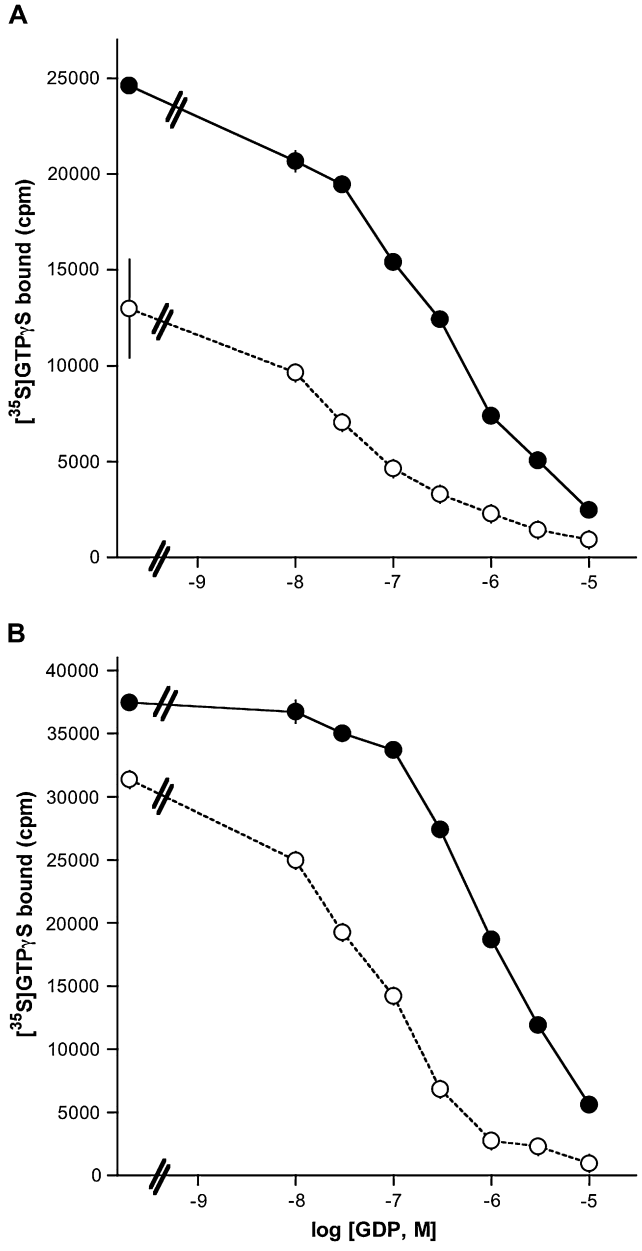

Fig. 2. Effects of GDP on 1DMe-stimulated [^{35}S]GTPγS binding in CHO-$hNPFF_1$ (A) and in CHO-$hNPFF_2$ (B) cell membranes. [^{35}S]GTPγS binding in the absence (open circles) and presence (closed circles) of 10 μM 1DMe was determined in a standardized assay. Results are expressed as mean ± S.E.M. values of three independent experiments, each performed in triplicate.

Basal and 1DMe-stimulated [^{35}S]GTPγS binding decreased with increasing (0.01 to 10 μM) GDP concentrations (Fig. 2). The intensity of 1DMe-induced [^{35}S]GTPγS binding expressed as percentage over the basal binding, peaked around 0.1−1 μM GDP depending on the receptor, indicating a difference in the optimal signal-to-noise ratio. Thus, the sensitivity of each NPFF receptors to GDP was dissimilar and a tenfold difference in optimal concentration was observed; 0.1 μM GDP was used for $NPFF_1$ and 1 μM for $NPFF_2$.

3.3. Affinity of [^{35}S]GTPγS to G protein in CHO-hNPFF$_1$ and CHO-hNPFF$_2$ membranes

Inhibition of [^{35}S]GTPγS binding by increasing concentrations of unlabeled GTPγS was performed in the absence or presence of 1DMe (10 μM) in low and high sodium concentrations in both transfected cells. Results are summarized in Table 2. The basal [^{35}S]GTPγS binding curves were monophasic, similar for both receptors and comparable to that observed in CHO-K1 untransfected cells (not shown). In CHO-hNPFF$_2$ cell membranes, saturation isotherms of [^{35}S]GTPγS binding in the presence of 1DMe were biphasic in low sodium and always monophasic in CHO-hNPFF$_1$ (Table 2). A high sodium concentration caused a 4.25-fold decrease in GTPγS basal binding. 1DMe, by stimulating $NPFF_1$ receptors whatever the sodium concentration, directed the G-protein binding in a high-affinity state since it decreased the K_D value of GTPγS (1.7- and 2.7-fold in 20 mM and 150 mM NaCl, respectively), without a clear change in the maximal amount of G-protein accessible (Table 2). CHO-hNPFF$_2$ receptors displayed a similar behavior since 1DMe increased the amount of high-affinity G-protein; 6.4- and 8.6-fold in 20 mM and 150 mM NaCl, respectively.

3.4. Comparison of RFa-related peptide activity on optimized [^{35}S]GTPγS binding on NPFF$_1$ and NPFF$_2$ receptors

The concentration−response curves were measured for several representative NPFF and RFamide-related peptides under low (20 mM) and high (150 mM) sodium conditions. We used Na^+ as an agent to unmask constitutive activity of NPFF receptors. Results are shown in Fig. 3 and Tables 3 and 4. At both NaCl concentrations, peptides stimulated [^{35}S]GTPγS binding in a concentration-dependent manner. Under high sodium, the concentration−response curves were shifted rightward with higher EC_{50}, and this effect was clearly seen in CHO-hNPFF$_2$ since the EC_{50} ratios ranged from 230 (NPFF) to 235,000 (hRFRP-1) (Table 4). The E_{max} increased by a factor 1.5 in CHO-hNPFF$_1$ and 6 to 8 in CHO-hNPFF$_2$, except for hNPAF and h26RFa (E_{max} ratio around 3), when sodium concentration rose from 20 to 150 mM.

Despite a great variability in potency, all compounds exhibited similar E_{max} values ranging from 143% (dNPA-NPFF) to 226% (bNPAF) in 20 mM sodium for $NPFF_1$ receptors (Table 3). The highest efficacies (>200%) corresponded to PQRFamide C-terminal sequence in 20 mM sodium. In contrast, in CHO-hNPFF$_2$ membranes, the highest efficacies, approximately 180% in 20 mM sodium, corresponded not only to PQRFa peptides but also to h26RFa, hRFRF-1 and fRRFa. Generally speaking, all peptides exhibiting a PQRFamide C-terminal sequence displayed a high activity in low and high sodium conditions.

The effects of peptides containing a LPLRFamide C-terminal sequence such as fRRFamide, RFRP-1 and LPLRFamide must be pointed out. Whereas their affinities to $NPFF_1$ receptor were close to that of NPVF (the most selective $NPFF_1$ agonist exhibiting a LPQRFa sequence), their EC_{50} values in 20 mM NaCl were 2−10 times larger but with greater E_{max} (Table 3). In contrast, these peptides had lower affinity to $NPFF_2$ receptor (Table 1) whereas they were very potent agonists in low sodium (Table 4). Their activities were similar to those of NPA-NPFF, 1DMe or hNPAF, which have 100 times higher affinity to $NPFF_2$ (Table 1).

Potencies for stimulation of [^{35}S]GTPγS binding were compared with abilities to compete for [^{125}I]YVP ($NPFF_1$) or [^{125}I]EYF ($NPFF_2$) binding. In high sodium, agonists stimulated [^{35}S]GTPγS binding and competed for iodinated NPFF binding with identical rank orders of potency in both transfected cells; and there was a statistically significant ($P < 0.001$) correlation between potencies (EC_{50}) and affinities (K_I) of $r = 0.934$ (for $NPFF_1$) and 0.831 (for $NPFF_2$). However, agonists were 100−600 ($NPFF_1$) or 25−500 ($NPFF_2$)-fold less active in stimulating [^{35}S]GTPγS binding than in competing for NPFF binding sites. In low sodium, a significant ($r = 0.507$, $P < 0.05$) correlation was found only in CHO-hNPFF$_1$ cells in which agonists were 2- to 76-fold less active in stimulating [^{35}S]GTPγS binding than

Table 2
Inhibition by GTPγS of basal and 1DMe-stimulated [^{35}S]GTPγS binding at CHO-hNPFF$_1$ and CHO-hNPFF$_2$ cell membranes

		Basal 20 mM NaCl	1DMe 20 mM NaCl	Basal 150 mM NaCl	1DMe 150 mM NaCl
$NPFF_1$	K_D (nM)	1.16 ± 0.06	0.68 ± 0.02	4.93 ± 0.56	1.84 ± 0.23
	B_{max} (pmol/mg)	62.6 ± 3.0	56.4 ± 1.6	60.3 ± 7.5	49.9 ± 6.4
$NPFF_2$	K_H (nM)		0.22 ± 0.08		
	B_H (pmol/mg)		13.1 ± 5.3		
	K_L (nM)	1.41 ± 0.10	2.02 ± 1.22	7.18 ± 1.23	0.83 ± 0.04
	B_L (pmol/mg)	66.0 ± 4.5	51.9 ± 4.9	57.3 ± 8.0	58.0 ± 2.3

Data are mean ± S.E.M. of four independent experiments. [^{35}S]GTPγS binding (0.066 nM) to CHO cell membranes (1.5 ($NPFF_1$) or 1.4 ($NPFF_2$) μg protein) was inhibited with GTPγS in the absence (Basal) or presence of 10 μM 1DMe. Resulting isotherms were fitted by non-linear regression analysis by using homologous competitive binding curve with one or two classes of binding sites.

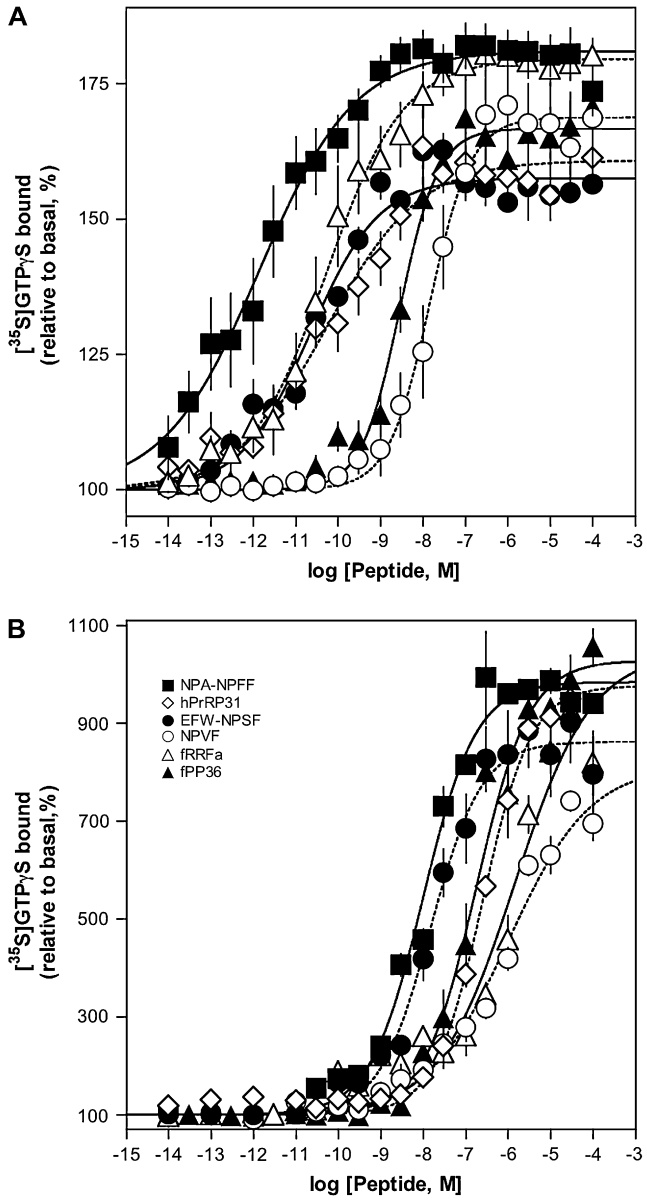

Fig. 3. Effects of some RFamide-related peptides on [^{35}S]GTPγS binding in CHO-hNPFF$_2$ cell membranes. [^{35}S]GTPγS binding was measured in the presence of 20 mM NaCl (A) or 150 mM NaCl (B) and various concentrations of ligands under optimized assay conditions. Results are expressed as mean ± S.E.M. of percentage values of [^{35}S]GTPγS bound relative to basal level (absence of peptides) obtained from three to eight independent experiments, each performed in duplicate.

they were to compete for [^{125}I]YVP binding. In contrast, in CHO-hNPFF$_2$ cell membranes, there was no correlation in low sodium between EC_{50} and K_I ($r = 0.399, P = 0.140$). Agonists stimulated [^{35}S]GTPγS binding to NPFF$_2$ receptors with EC_{50} values much smaller than their K_I values, resulting in EC_{50}/K_I ratios ranging from 0.0004 (hRFRP-1) to 0.626 (NPVF).

3.5. Identification of G proteins associated with NPFF$_1$ and NPFF$_2$ receptors in CHO cells

Peptides corresponding to the carboxyl-terminus of the Gα subunits of G proteins have been reported to specifically uncouple several receptors from G proteins. The effects of increasing concentrations (1 to 100 μM) of $G\alpha_{i1,2}$ $G\alpha_{i3}$, $G\alpha_o$ and $G\alpha_s$ inhibitory peptides were tested on [^{35}S]GTPγS binding. We observed that [^{35}S]GTPγS basal binding on CHO-hNPFF$_2$ membranes increased with Gα peptide concentrations whereas it did not change or decreased in CHO-hNPFF$_1$. Therefore, the [^{35}S]GTPγS binding stimulated by NPFF$_2$ and NPFF$_1$ agonists was expressed as relative to their respective control, i.e. agonist-stimulated in the absence of Gα peptide.

The $G\alpha_{i3}$ and $G\alpha_s$ synthetic peptides inhibited (maximum 30% and 45%) the [^{35}S]GTPγS binding stimulated by 10 μM 1DMe in CHO-hNPFF$_1$ membranes (Fig. 4). In CHO-hNPFF$_2$, the binding stimulated by 0.1 μM 1DMe was inhibited by all $G\alpha_{i1,2}$, $G\alpha_{i3}$, $G\alpha_o$ and $G\alpha_s$ inhibitory peptides. A smaller inhibition (about 40%) was observed for $G\alpha_o$ peptide while this effect reached near 75% with the other Gα peptides (Fig. 4), indicating that NPFF$_2$ receptors interact with all these Gα subunits.

Fig. 5 shows the ability of all inhibitory peptides (100 μM) to modify the [^{35}S]GTPγS binding stimulated by several RFamide agonists used at concentrations inducing the maximal stimulating effect. In CHO-hNPFF$_1$, $G\alpha_{i1,2}$ and $G\alpha_0$ peptides had no effect whatever the agonist used. In contrast, $G\alpha_{i3}$ and $G\alpha_s$ peptides decreased significantly the stimulated [^{35}S]GTPγS binding induced by RFamide agonists by 20–30% and 40–50%, respectively. Some differences appeared significant (two-way ANOVA followed by Bonferroni's test) between $G\alpha_{i3}$ and $G\alpha_s$ for hPrRP31, fRRFa, NPSF and hRFRP-1, these ligands interacting significantly more with $G\alpha_s$ as compared to $G\alpha_{i3}$.

In CHO-hNPFF$_2$, all Gα peptides decreased significantly ($P < 0.001$) the RFa-stimulated control binding from 40% ($G\alpha_0$) to more than 90% ($G\alpha_{i3}$) and this decreased effect is similar whatever the RFamide peptide. However, differences appeared significant ($P < 0.001$) among the four Gα peptides $G\alpha_{i3} > G\alpha_{i1,2} = G\alpha_s > G\alpha_0$.

4. Discussion

A series of RFamide ligands with different affinities and selectivities toward NPFF$_1$ and NPFF$_2$ receptors was functionally analyzed using [^{35}S]GTPγS binding assay on membrane preparations from stably transfected CHO cell lines. The binding of [^{35}S]GTPγS stimulated by a receptor agonist measures a membrane-proximal event and is the most sensitive method to quantify agonist efficacy permitting to identify full, partial or inverse agonists. Furthermore, the agonist-induced [^{35}S]GTPγS response is a direct event without downstream signal amplification. This work is the first study comparing potencies of a large number of structurally diverse RFa-peptides at the two human Neuropeptide FF receptors. Using this test, only one previous report described limited data for some NPFF peptides and PrRP only on NPFF$_2$ receptor (Engstrom et al., 2003). In our study, six groups of RFamide peptides have been tested presenting different affinities and selectivities toward NPFF$_1$ and NPFF$_2$ receptors: peptides exhibiting SFRFamide, PLRFamide, VGRFamide, FMRFamide, RPRFamide and PQRFamide C-terminal sequences.

Table 3
Activity of the RFamide-related peptides at human $NPFF_1$ receptors expressed in CHO cells assessed by the [^{35}S]GTPγS binding assay

Peptides	20 mM NaCl			150 mM NaCl		
	EC_{50} (nM)	E_{max} (%)	IE	EC_{50} (nM)	E_{max} (%)	IE
NPFF	8.0 ± 1.1	179 ± 11	0.73	290 ± 65	219 ± 8	0.45
SQA-NPFF	132 ± 30	177 ± 20	0.55	2599 ± 717	265 ± 10	0.54
SPA-NPFF	39 ± 9	201 ± 33	0.65			
NPA-NPFF	122 ± 7	168 ± 5	0.52	897 ± 219	216 ± 8	0.44
dNPA-NPFF	60 ± 3	143 ± 2	0.45	645 ± 145	214 ± 24	0.43
1DMe	2.5 ± 0.3	182 ± 10	0.79	157 ± 24	255 ± 4	0.52
NPSF	56 ± 7	180 ± 10	0.85	6561 ± 2155	255 ± 20	0.52
hNPAF	689 ± 162	219 ± 21	0.67	5809 ± 1751	289 ± 44	0.59
bNPAF	179 ± 41	226 ± 24	0.72			
QFW-NPSF	105 ± 18	213 ± 17	0.69			
EFW-NPSF	172 ± 43	186 ± 23	0.63	5132 ± 1338	230 ± 22	0.47
EYF (EYW-NPSF)	300 ± 30	204 ± 12	0.65			
NPVF	3.0 ± 0.3	166 ± 5	0.60	158 ± 29	247 ± 5	0.50
YVP (YNPVF)	4.5 ± 0.4	161 ± 7	0.56			
FMRFa	19 ± 5	166 ± 4	0.55	262 ± 56	273 ± 5	0.56
fPP36	8261 ± 803	198 ± 28	–	>100000	n.e.	–
hPrRP31	236 ± 91	188 ± 37	0.67	7191 ± 1797	198 ± 30	0.40
h26Rfa	338 ± 47	160 ± 3	0.54	7802 ± 835	203 ± 9	0.41
LPLRFa	5.9 ± 0.6	198 ± 9	0.77	524 ± 120	276 ± 15	0.56
hRFRP-1	29 ± 4	193 ± 11	0.59	154 ± 24	253 ± 10	0.52
FRRFa	25 ± 2	167 ± 6	0.55	1249 ± 335	279 ± 26	0.57

Data represent the mean ± S.E.M. values of 3–7 independent experiments performed in duplicate. IE = (E_{max} Peptide/E_{max} NPVF) × [(K_I/EC_{50}) + 1] × 0.5. n.e., not estimated because of incomplete dose–response curve.

Saponin markedly increased the agonist-stimulated binding, resulting in a very high agonist-mediated signal. Na^+ ions were not essential to observe agonist-mediated stimulation of GTPγS binding but they improved the signal to noise ratio by favoring uncoupling of the receptor–G-protein complex. The inhibitory effect of Na^+ on G-protein activity should correspond to a decrease of the affinity of unliganded NPFF receptors for G protein and to a reduction of the activity of constitutively active

Table 4
Activity of the RFa-related peptides at the human $NPFF_2$ receptors expressed in CHO cells assessed by the [^{35}S]GTPγS binding assay

Peptides	20 mM NaCl			150 mM NaCl		
	EC_{50} (nM)	E_{max} (%)	IE	EC_{50} (nM)	E_{max} (%)	IE
NPFF	0.13 ± 0.02	163 ± 4	1.2	29.7 ± 5.0	609 ± 28	0.32
NPA-NPFF	0.0014 ± 0.0003	181 ± 5	16.0	12.3 ± 1.4	997 ± 12	0.52
dNPA-NPFF	0.0022 ± 0.0004	183 ± 4	6.6	12.8 ± 2.1	968 ± 18	0.50
1DMe	0.058 ± 0.011	180 ± 3	2.0	28.0 ± 5.6	747 ± 38	0.39
NPSF	0.38 ± 0.08	158 ± 5	23.1	1636 ± 396	835 ± 76	0.44
hNPAF	0.050 ± 0.014	178 ± 11	1.8	9.7 ± 1.4	510 ± 9	0.27
EFW-NPSF	0.022 ± 0.005	158 ± 1	4.6	14.8 ± 1.6	863 ± 27	0.45
NPVF	14.4 ± 2.7	169 ± 9	0.7	1023 ± 191	814 ± 53	0.43
FMRFa	2.1 ± 0.5	166 ± 6	2.1	1445 ± 242	1244 ± 74	0.65
fPP36	2.8 ± 0.4	167 ± 9	1.3	177 ± 15	1029 ± 31	0.55
hPrRP31	0.058 ± 0.016	161 ± 11	26.2	253 ± 9	977 ± 16	0.51
h26RFa	5.3 ± 1.1	181 ± 8	1.5	1363 ± 178	456 ± 13	0.24
LPLRFa	0.068 ± 0.018	162 ± 5	69.4	1524 ± 275	1283 ± 77	0.67
hRFRP-1	0.0011 ± 0.0003	173 ± 4	1204	257 ± 34	1149 ± 52	0.60
fRRFa	0.052 ± 0.010	180 ± 5	228	1423 ± 303	1044 ± 87	0.55

Data represent the mean ± S.E.M. values of three to eight independent experiments performed in duplicate. IE = (E_{max} Peptide/E_{max} NPVF) × [(K_I/EC_{50}) + 1] × 0.5.

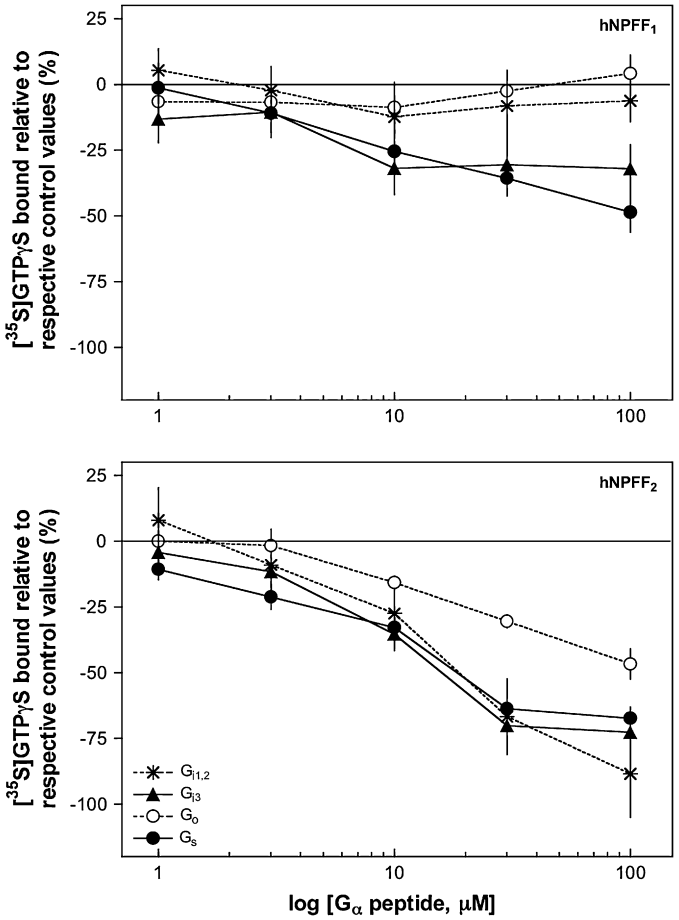

Fig. 4. Effects of Gα peptides on maximal 1DMe-stimulated [^{35}S]GTPγS binding in CHO-hNPFF$_1$ and CHO-hNPFF$_2$ cell membranes. Maximal 1DMe-stimulated [^{35}S]GTPγS binding was measured in the absence (control) or presence of increasing concentrations of $G\alpha_{i1,2}$, $G\alpha_{i3}$, $G\alpha_o$ and $G\alpha_s$ peptides. Data are expressed as a percentage of maximal control stimulation of [^{35}S]GTPγS binding induced by 1DMe at 0.1 (NPFF$_2$) or 10 μM (NPFF$_1$) in the absence of Gα peptides (0%). Results are means ± S.E.M. of triplicate determinations of one representative experiment among three giving similar results.

receptors as suggested for other receptors (Szekeres and Traynor, 1997; Harrison and Traynor, 2003). In the case of NPFF receptors, the uncoupling of the receptor–G-protein complex in the presence of sodium ions was revealed by the high proportion of low-affinity state of GTP-binding protein (Table 2). In 20 mM sodium, a precoupling of NPFF receptors could be observed more easily and in this case, the real efficacy of agonists to induce G-protein activity rather than a combination of affinity and ability of coupling could be detected. In contrast, the high concentration of sodium permits to measure [^{35}S]GTPγS binding in conditions where affinity of agonists is a conclusive factor in coupling with G protein.

Our data are consistent with the model of agonist action proposed by Breivogel et al. (1998) in which agonists alter G-protein affinity for guanine nucleotide; thus, the effect of agonists in our assay is to increase the ability of G protein to bind low concentrations of [^{35}S]GTPγS and we used sodium ions as modulator of constitutive activity.

Our data suggest that the efficacy of coupling could be very different from the affinity of ligands to receptors. Consequently, drug intrinsic efficacy in a homogeneous population of NPFF receptors should yield information about how drugs might evoke effects of different magnitude while occupying the same proportion of target receptors without interference of the real affinity to binding sites.

From the potency and relative efficacy obtained together with the affinity measured under similar conditions, the relative intrinsic efficacy could be calculated according to the equation of Ehlert (1985), which could be only relative since the binding assay conditions differ slightly from those of GTP binding, especially in terms of sodium and nucleotide concentrations. Thus, only a relative efficacy of the agonists used was evaluated by using the EC_{50} and maximal effect values for each compound; this relative index does not depend upon the total number of NPFF binding sites.

All of the NPFF agonists studied on NPFF$_1$ receptors had a low level of intrinsic efficacy, producing a 50% response at about 45% and 60% receptor occupancy in 150 and 20 mM NaCl, respectively. Our data show that the ternary complex model easily describes the behavior of NPFF$_1$ receptors; despite a large difference in agonist binding affinity from 0.59 nM to 45 nM, the efficacy of agonists to stimulate GTP binding was correlated to their affinities. When the G protein and receptor were uncoupled, in the presence of 150 mM NaCl, the intrinsic efficacy of the different agonists varied from 0.40 to 0.59. Selective NPFF$_1$ agonists such as NPVF displayed a similar intrinsic efficacy as 1DMe which behaves as a weak NPFF$_2$ selective agonist. In the presence of 20 mM NaCl, the precoupling of NPFF$_1$ receptors to G-protein increased the intrinsic efficacy values from 0.45 to 0.85 producing for NPVF a 50% response at about 98% receptor occupancy.

Only NPAF, FMRFamide, fRRFamide, SQA-NPFF and LPLRFamide exhibited, in 150 mM NaCl, a relative intrinsic efficacy higher than NPVF. In low sodium concentration, i.e. in a precoupling situation, several compounds had higher intrinsic efficacy than the referent agonist NPVF. In particular, NPFF, 1DMe, NPSF, NPAF, LPLRamide (possessing PQRFamide or PLRFamide as C-terminal) exhibited the highest intrinsic efficacy to stimulate GTP binding. Clearly, the intrinsic efficacy has no relationship with the affinity or selectivity since NPSF behaved as a full agonist with a high potency and efficacy, which was not observed for binding affinity. In fact, this compound has been recently described as a potent agonist in pharmacological studies (Bonnard et al., 2001; Jhamandas et al., 2006) despite its low affinity for NPFF$_1$ and NPFF$_2$ receptors.

The situation is somewhat different for NPPF$_2$ receptors. In high Na^+ concentrations, only RFRP-1, FMRFamide, LPRFamide, fPP36 and fRRFamide exhibited a higher intrinsic efficacy than dNPA-NPFF but in similar range. These agonists displayed, however, only low potency with high EC_{50} values as compared to high-affinity agonists and so high intrinsic efficacy corresponds to a high efficacy. In low sodium concentrations, these agonists exhibited a very high relative intrinsic efficacy since RFRP-1 was 1000-fold more efficacious than

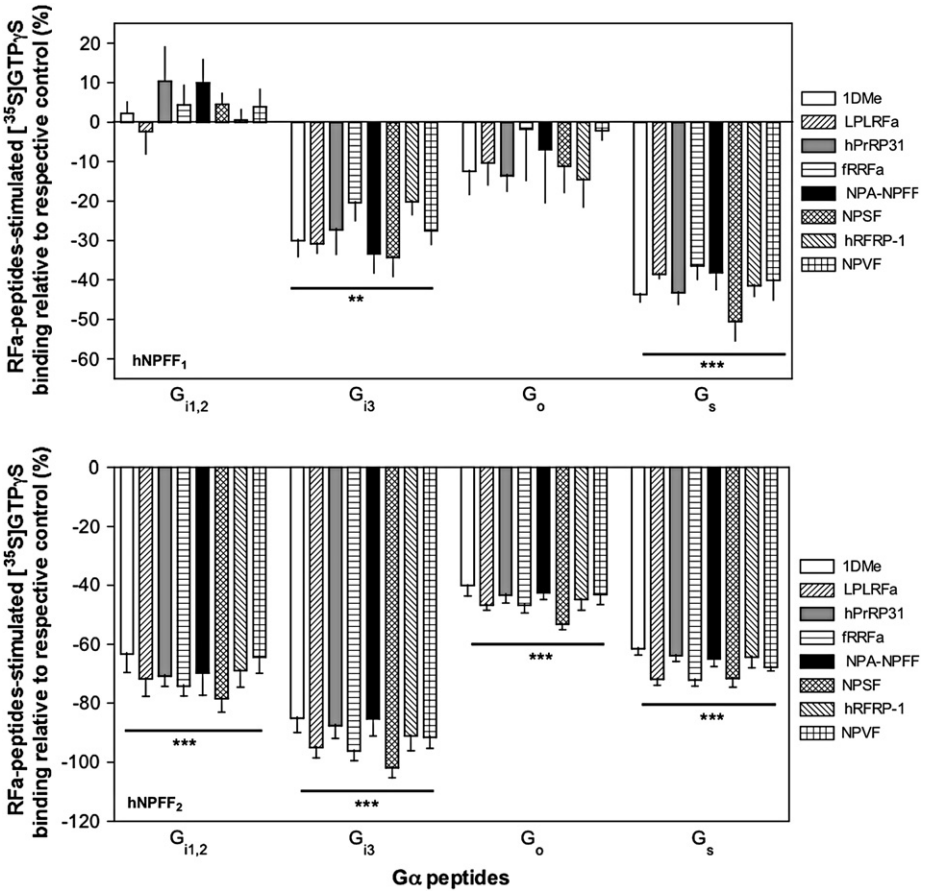

Fig. 5. Changes of the maximal RFa-peptides-stimulated [^{35}S]GTPγS binding induced by $G\alpha_{i1,2}$, $G\alpha_{i3}$, $G\alpha_{o}$ and $G\alpha_{s}$ peptides in CHO-hNPFF1 and CHO-hNPFF2 cell membranes. Membranes were incubated under optimized assay conditions in the absence (control) or presence of 100 μM Gα peptides with 0.1 μM ($NPFF_2$) or 10 μM ($NPFF_1$) RFa-peptides. Columns represent mean % ± S.E.M. of agonist-stimulated [^{35}S]GTPγS binding in the presence of indicated Gα peptide relative to respective control agonist-stimulated binding (absence of Gα peptide) from at least six independent experiments performed in duplicate. $^{**}P < 0.01$, $^{***}P < 0.001$, significant difference as compared to respective control (absence of Gα peptide = zero). ANOVA analysis followed by Bonferroni multiple comparison tests: for hNPFF1, $G\alpha_{i3} < G\alpha_{s}$ ($P < 0.05$) only for hPrRP31, fRRFa, NPSF and NPVF; for hNPFF2, $G\alpha_{i3} > G\alpha_{s} = G\alpha_{i1,2} > G\alpha_{o}$ ($P < 0.01$) for all RFa-peptides.

NPFF in stimulating GTPγS binding. This corresponds in fact to a very high activity; dNPA-NPFF, for example, displayed an EC_{50} value of 0.0022 nM, about 10 fold inferior to its binding K_I value.

It has been previously reported that hPrRP31 in 20 mM NaCl had higher efficacy compared with 1DMe at $NPFF_2$ receptors (Engstrom et al., 2003). We did not observe such a difference and in CHO-$NPFF_1$ membranes, hPrRP31 was 45–100 times less potent than 1DMe in high or low sodium concentrations.

More generally, under 20 mM NaCl, $NPFF_2$ receptors exhibited an extraordinary activity to transduce binding to stimulation of GTPγS binding since all compounds tested exhibited EC_{50} values of GTPγS binding lower than their K_I values. This suggests that under these experimental conditions, which reveal precoupling of receptor with G protein, an important receptor reserve could be detected, i.e. the maximal stimulation of [^{35}S]GTPγS binding is obtained only when a small fraction of the receptors is occupied by agonists. This was clearly observed with LPRFamide since only 0.63% of the receptor need be occupied to produce 50% of stimulation of GTP binding. In contrast, NPVF occupied 38% of the receptors to induce the same [^{35}S]GTPγS response, evidencing considerable difference in the ability of agonists to stimulate efficaciously receptor G-protein response. All agonists with a LPLRFamide C-terminal sequence possessed an extraordinary intrinsic efficacy: 1204, 228 and 69 for hRFRP-1, fRRFamide and LPLRFamide, respectively. This indicates that the LPRFamide sequence is more efficacious than the PQRFamide one in inducing coupling to the G-protein. This observation will be decisive in synthesizing new agonists exhibiting a high activity rather than a high affinity to their binding sites.

Our study provides additional evidence that the molecular interactions of each NPFF receptor with G proteins could be different. Peptides corresponding to the last ten carboxyl terminus residues of the α subunits of G proteins represent an important site of interaction with G-protein coupled receptors and have been reported to specifically uncouple receptors from G proteins in several systems (Gilchrist et al., 1998). Such competitor peptides represent useful probes to investigate the ability of receptors to interact with specific G-protein subunits (Mazzoni et al., 2000).

CHO cells express $G\alpha_{i3}$, $G\alpha_{i2}$ (Law et al., 1993; Gettys et al., 1994), and low levels of $G\alpha_{o}$ and $G\alpha_{s}$ are also present (Burford

and Nahorski, 1996; Brink et al., 2000) but $G\alpha_{i1}$ is undetectable (Law et al., 1993; Gettys et al., 1994; Newman-Tancredi et al., 2002). Our data show that the binding of G protein stimulated through $NPFF_2$ receptors, whatever the agonist used, was inhibited by all $G\alpha_{i1,2}$, $G\alpha_{i3}$, $G\alpha_o$ and $G\alpha_s$ inhibitory peptides, suggesting that this receptor contains domains that are specifically recognized by the carboxyl terminus of α_{i2}, α_{i3}, α_o and α_s subunits. In CHO-h$NPFF_1$, only $G\alpha_{i3}$ and $G\alpha_s$ peptides decreased significantly the stimulated [^{35}S]GTPγS binding, thus clearly indicating a qualitative difference between $NPFF_1$ and $NPFF_2$ coupling in CHO cells; $G\alpha_{i3}$ and $G\alpha_s$ are the main transducers of $NPFF_1$ receptors while $NPFF_2$ are probably coupled with $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$ and $G\alpha_s$ proteins.

A great variety of experimental data indicate in fact that receptors could couple to different G-protein types that transduce divergent signaling pathways. Thus, it is not surprising to observe that $NPFF_2$ can couple to both $G\alpha_{io}$ and $G\alpha_s$.

This is reminiscent of a recent report demonstrating that the specific binding to $NPFF_2$ receptors in SH-SY5Y, is preferentially coupled with $G\alpha_{i1,2}$ and $G\alpha_o$ proteins (Mollereau et al., 2005). Furthermore, our results are in accordance with previous experiments performed in HEK 293 and COS7 cells co-transfected with chimeric G proteins, showing an interaction between NPFF receptors with $G\alpha_{q/i2}$ and $G\alpha_{q/o}$ (Elshourbagy et al., 2000) as well as $G\alpha_{q/i3}$ and $G\alpha_{q/s}$ (Bonini et al., 2000).

In conclusion, we established an optimized methodology to assess NPFF-receptor mediated G-protein activity under conditions where signal-to-noise ratio is significantly improved. These studies indicate that under the assay conditions, $NPPF_1$ and $NPFF_2$ receptors are differently coupled to G proteins and that $NPFF_2$ agonists exert a complex functional activity.

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