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Pharmacological characterization of human $NPFF_1$ and $NPFF_2$ receptors expressed in CHO cells by using NPY Y_1 receptor antagonists

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Abstract

Neuropeptide FF (NPFF) belongs to an opioid-modulatory system including two precursors (pro-$NPFF_A$ and pro-$NPFF_B$) and two G-protein coupled receptors ($NPFF_1$ and $NPFF_2$). The pharmacological and functional profiles of human $NPFF_1$ and $NPFF_2$ receptors expressed in Chinese hamster ovary (CHO) cells were compared by determining the affinity of several peptides derived from both NPFF precursors and by measuring their abilities to inhibit forskolin-induced cAMP accumulation. Each NPFF receptor recognizes peptides from both precursors with nanomolar affinities, however, with a slight preference of pro-$NPFF_A$ peptides for $NPFF_2$ receptors and of pro-$NPFF_B$ peptides for $NPFF_1$ receptors. BIBP3226 ((*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)-methyl]-argininamide) and BIBO3304 ((*R*)-N^2-(diphenylacetyl)-*N*-[4-(aminocarbonylaminomethyl)-benzyl]-argininamide trifluoroacetate), two selective neuropeptide Y (NPY) Y_1 receptor antagonists, display relative high affinities for NPFF receptors and exhibit antagonist properties towards $hNPFF_1$ receptors. The structural determinants responsible for binding of these molecules to NPFF receptors were investigated and led to the synthesis of $hNPFF_1$ receptor antagonists with affinities from 40 to 80 nM. Our results demonstrate differences in pharmacological characteristics between $NPFF_1$ and $NPFF_2$ receptors and the feasibility of subtype-selective antagonists.

Keywords: Neuropeptide FF; Neuropeptide Y; BIBP3226; BIBO3304; G-protein coupled receptor; cAMP assay; Neuropeptide Y Y_1 receptor antagonist

1. Introduction

Neuropeptide FF (NPFF) (FLFQPQRFa) and NPAF (AGEGLSSPFWSLAAPQRFa) are mammalian amidated neuropeptides, originally isolated from bovine brain (Yang et al., 1985) and characterized as pain-modulating peptides (Roumy and Zajac, 1998; Yang et al., 1985). Many arguments suggest that they act mainly through the regulation of the opioid system (for review, see Roumy and Zajac, 1998). In vivo, supraspinal injection of NPFF or analogues decreases morphine-induced analgesia in rat (Dupouy and Zajac, 1997; Yang et al., 1985), whereas intrathecal injection potentiates morphine-induced antinociception or produces long-lasting analgesia (Altier et al., 2000; Gouarderes et al., 1996; Xu et al., 2001), probably by increasing the release of spinal cord enkephalin (Mauborgne et al., 2001).

Modulation of the opioid system by NPFF is also observed at the cellular level. NPFF, and analogues, attenuate the opioid-induced inhibition of calcium conductance in isolated neurones of the rat dorsal root ganglion and dorsal raphe (Rebeyrolles et al., 1996; Roumy and Zajac, 1999) and decrease the excitatory effect of morphine on pyramidal neurones of the rat hippocampus (Miller and Lupica, 1997).

Beside its action on pain modulation, NPFF is also implicated in hormonal modulation, cardiovascular and thermal regulation and in food intake (for review, see Panula et al., 1996).

The distribution of NPFF receptors, studied by autoradiography in the rodent nervous system (Zajac and Gouarderes, 2000), reveals the presence of specific binding

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sites for NPFF in areas involved in pain perception (the dorsal horn of the spinal cord, the dorsal raphe nucleus, the parafascicular nucleus of the thalamus) and in several nuclei of the hypothalamus and brainstem known to play a role in endocrine and vegetative functions.

In the last 4 years, a growing set of new molecular informations concerning the NPFF system came from cloning techniques (Zajac, 2001).

Genes encoding two precursors, pro-$NPFF_A$ (Perry et al., 1997; Vilim et al., 1999) and pro-$NPFF_B$ (Hinuma et al., 2000; Liu et al., 2001), were identified in several mammalian species. Processing of the pro-$NPFF_A$ precursor at basic proteolytic sites should generate (i) an NPFF-containing peptide with three additional N-terminal amino acids different from one species to another, along with (ii) an NPSF (SLAAPQRFa)-containing peptide, the length of which depends on the species. Both type of peptides have been biochemically isolated in rodents (Bonnard et al., 2001) and are endowed with anti-opioid properties (Gelot et al., 1998; Roumy et al., 2000). Pro-$NPFF_B$, recently identified as a precursor for RFamide peptides (Hinuma et al., 2000), contains (i) an NPFF-related peptide containing the PQRFa sequence, such as NPVF (VPNLPQRFa) in human (Liu et al., 2001), and (ii) an LPLRFa-containing peptide, also found in avian precursors (Satake et al., 2001). Mammalian LPLRFa-related peptides were shown to stimulate prolactin secretion (Hinuma et al., 2000) and to decrease morphine-induced analgesia (Liu et al., 2001) in rat.

In parallel to the identification of two precursors for NPFF-related peptides, two orphan G-protein coupled receptors, $NPFF_1$ (Bonini et al., 2000; Hinuma et al., 2000; Liu et al., 2001) and $NPFF_2$ (Bonini et al., 2000; Elshourbagy et al., 2000; Kotani et al., 2001; Liu et al., 2001), were characterized as specific NPFF receptors. $NPFF_1$ and $NPFF_2$ receptors are about 50% identical and are most similar to neuropeptide Y and orexin receptors (30–35% homology). Variants of the $NPFF_2$ receptor in N- and/or C-terminal ends have also been reported (Cikos et al., 1999; Parker et al., 2000). A preliminary work suggested that peptides from the pro-$NPFF_A$ precursor display a high affinity for the $NPFF_2$ receptor and, conversely, peptides from the pro-$NPFF_B$ precursor slightly prefer the $NPFF_1$ receptor (Liu et al., 2001). The $NPFF_1$ receptor has been shown to inhibit adenylate cyclase, without mediating Ca^{2+} liberation or arachidonic acid release in transfected Chinese hamster ovary (CHO) cells (Hinuma et al., 2000). Similarly, a preferential coupling to $G_{i/o}$ proteins has been described for the $NPFF_2$ receptor cotransfected with promiscuous G-proteins (Bonini et al., 2000; Elshourbagy et al., 2000; Kotani et al., 2001; Liu et al., 2001). At this time, the endogenous intracellular signalling pathways activated by NPFF receptors in neurones are still unknown, while high NPFF concentrations could stimulate adenylate cyclase activity in membranes of mouse olfactory bulb (Gherardi and Zajac, 1997). Therefore, data from transfected cells constitute the first indication of a coupling of NPFF receptors to cyclase-inhibitory G-proteins.

In situ hybridization data revealed differences in the localization of $NPFF_1$ and $NPFF_2$ receptors mRNA in human and rat (Bonini et al., 2000). In the rat central nervous system, a quantitative autoradiographic study showed that $NPFF_2$ receptors are predominantly expressed (Gouarderes et al., in press), suggesting that nearly all the known pharmacological activities of NPFF analogues, mainly studied in rodents, are probably due to the activation of $NPFF_2$ receptors.

The physiological role of both receptors and precursors should be now investigated. This requires a precise in vitro pharmacological characterization of each receptor and, above all, the availability of agonists and antagonists selective for each type of receptor, which are still lacking. In the present study, we have compared the binding and functional properties of several natural and synthetic ligands on $NPFF_1$ and $NPFF_2$ receptors transfected in CHO cells. In an attempt to obtain a selective antagonist, we have synthesized and screened for their agonist or antagonist activity, various analogues of BIBP3226, a neuropeptide Y Y_1 receptor antagonist (Rudolf et al., 1994), previously shown to exhibit a low affinity antagonist activity towards $NPFF_2$ receptors (Mollereau et al., 2001).

2. Materials and methods

2.1. Materials

NPFF-related peptides and frog pancreatic polypeptide were synthesized in the laboratory by using an automated peptide synthesizer (Applied Biosystems model 433A). FMRF-NH_2 and bovine serum albumin were from Sigma (France). GR231118 (also known as 1229U91 or GW1229), homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$ was a gift from GlaxoWellcome (Research Triangle Park, USA). BIBP3226, (*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)-methyl]-argininamide and BIBO3304, (*R*)-N^2-(diphenylacetyl)-*N*-[4-(aminocarbonylaminomethyl)-benzyl]-argininamide trifluoroacetate were generously provided by H. Doods (Boehringer-Ingelheim, Biberach, Germany).

The BIBP3226 derived compounds were synthesized according to general liquid phase procedures. Amino acids were purchased from Bachem (France) and Fluka (France), tetramethylfluoroformamidiniumhexafluorophosphate (TFFH) was from Applied Biosystem (France). The carboxylic function of Fmoc-amino acids and of diphenylacetic acid was activated by TFFH to generate acid fluorides. The reaction was carried in dimethylformamide under conditions compatible with normal protocols used for solid or liquid phase synthesis, and in the presence of diisopropylethylamine according to White and Chan (2000). The purity of the final products was assessed by analytical high pressure liquid chromatography and their integrity was checked by electrospray mass spectrometry on a TSQ 700 (Finnigan-Mat, San José, CA). P**1**—(*R*)-N^2-(diphenylacetyl)-*N*-(benzyl)-argininamide, P**2**—(*R*)-N^2-(diphenylacetyl)-*N*-(phe-

nylalaninamide)-argininamide, P**4**—(*R*)-N^2-(diphenylacetyl)-*N*-(tyrosinamide)-argininamide, P**5**—(*R*)-N^2-(diphenylacetyl)-*N*-(tryptophanamide)-argininamide, P**11**—(*R*)-N^2-(diphenylacetyl)-*N*-[(phenyl)-ethyl]-argininamide, P**13**—(*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)-ethyl]-argininamide, P**14**—(*R*)-N^2-(diphenylacetyl)-*N*-[(phenyl)-glycinamide]-argininamide, P**16**—(*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)-glycinamide]-argininamide.

[^{125}I]EYF and [^{125}I]YVP were obtained by iodination of EYWSLAAPQRFa (EYW-NPSF) and YVPNLPQRFa, respectively, by electrophilic substitution as previously described (Gouarderes et al., 2002, in press). The specific activity was assumed to be identical to that of $Na^{125}I$ (80.5 TBq/mmol, 2175 Ci/mmol, Amersham). Radioiodinated peptides were stored at 4 °C in the presence of 0.1% bovine serum albumin.

2.2. Cell lines

The cDNA encoding the human $NPFF_1$ receptor was obtained by amplification of human spinal cord cDNA in a polymerase chain reaction (PCR) experiment using forward (5′-CCGGAATTCACCATGGAGGGGGAGCCCTCC-CAG-3′) and reverse (5′-CTAGTCTAGATCAGATATCC-CAGGCTGGAATG-3′) primers under the following conditions: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, 4 cycles; 94 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min, 32 cycles. PCR products were cloned in pCR 4blunt-TOPO (Invitrogen) and sequenced on both strands.

The cDNA encoding the human $NPFF_2$ receptor was obtained as described previously (Kotani et al., 2001).

CHO-K1 cells were transfected with the bicistronic vector pEFIN3 containing $hNPFF_1$ or $hNPFF_2$ receptor cDNA using, respectively, polybrene (Sigma) and Fugene 6 (Roche Molecular Biochemicals). Cells were grown in nutrient mixture Ham's F12 medium supplemented with 7% foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate (Gibco-BRL, France). About 400 μg/ml G418 (Gibco-BRL, France) was added to the medium for selection of the recombinant cells. For subsequent studies, the clone $hNPFF_1$C3 and the clone hNPPFF2S#2 (obtained by limit dilution) were chosen.

2.3. Binding experiments

For membrane preparation, CHO cells expressing hNPFF receptors were harvested in phosphate buffer saline (PBS), frozen at least for 1 h at −70 °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. Membranes were aliquoted and stored at −80 °C in Tris 50 mM, pH 7.4 and the protein concentration was determined by the Lowry method.

Binding of [^{125}I]YVP ([^{125}I]YVPNLPQRFa) and [^{125}I]EYF ([^{125}I]EYWSLAAPQRFa) was measured by rapid filtration. Briefly, 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 (Sigma), 0.1% bovine serum albumin and the radioligand at the desired concentration. The non-specific binding was determined in the presence of 1 μM YVPNLPQRFa (for $hNPFF_1$ receptor) and EYWSL-AAPQRFa (for $hNPFF_2$ receptor). After 1 h incubation at 25 °C, samples were rapidly filtered on Whatman GF/B filters preincubated in 0.3% polyethylenimine for the binding of [^{125}I]YVP and in 50 mM Tris–HCl, pH 7.4, 0.1% bovine serum albumin for the binding of [^{125}I]EYF. The filters were rinsed three times with 4 ml of ice cold buffer containing 0.1% bovine serum albumin, and the bound radioactivity was counted in a γ counter (Packard).

2.4. Assay for intracellular cAMP

Two hundred thousand recombinant cells were seeded in glass tubes and incubated overnight as usual. Culture medium was then removed and replaced by fresh one (200 μl) containing 0.1 μM adenine and 0.6 μCi [^{3}H]adenine (26 Ci/mmol, Amersham, France). After 1 h incubation at 37 °C in the incubator under 5% CO_2 atmosphere, cells were rinsed two times with 400 μl of HEPES-buffered Krebs-Ringer saline (KRH) (124 mM NaCl, 5 mM KCl, 1.25 mM $MgSO_4$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM HEPES, 8 mM glucose, 0.5 mg/ml bovine serum albumin; pH 7.4). Prewarmed KRH, 150 μl, was added to each tube and the reaction was initiated by the addition of 50 μl KRH containing 8 μM Forskolin (Sigma), 0.4 mM IBMX (3-isobutyl-1-methylxanthine) (Sigma), 0.4 mM Ro-20 1724 (4-(3-butoxy-4methoxyphenyl)methyl-2-imidazolidone) (Fisher, France) and the ligands to be tested. The final concentration of dimethylsulfoxide was 0.1%. After 10 min at 37 °C, the reaction was stopped by addition of 20 μl HCl 2.2N and rapid mixing. The [^{3}H]cAMP content of each tube was isolated by chromatographic procedure on acid alumina columns (Sigma) and counted in a liquid scintillation analyzer (Packard).

2.5. Analysis of the data

Nonlinear regression analysis of the data were performed using Prism 2.0 (GraphPad Software, USA).

3. Results

3.1. Binding characteristics of $hNPFF_1$ and $hNPFF_2$ receptors transfected in CHO cells

The binding properties of the human $hNPFF_1$ receptor was studied by using the radioiodinated ligand [^{125}I]YVP

([^{125}I]YVPNLPQRFa) which displays a very high affinity for $NPFF_1$ receptors (Gouarderes et al., in press; Hinuma et al., 2000). As shown in Fig. 1 (left panel), in membrane preparation from CHO cells expressing the human $NPFF_1$ receptor (CHO-h$NPFF_1$), [^{125}I]YVP labelled one class of binding sites ($B_{max} = 3.8 \pm 0.8$ pmol/mg protein, $n = 3$) with a high affinity ($K_d = 0.14 \pm 0.01$ nM, $n = 3$), the value of which is close to that observed ($K_d = 0.19$ nM) by Hinuma et al. (2000) on CHO cells expressing the rat $NPFF_1$ receptor. No specific binding was detectable using this radioligand in the same range of concentrations on CHO cells expressing the $NPFF_2$ receptor (data not shown), indicating that [^{125}I]YVP is selective for $NPFF_1$ receptors.

The characterization of the human $NPFF_2$ receptor was performed by using [^{125}I]EYF, a high affinity radioligand selective towards $NPFF_2$ receptors (Gouarderes et al., in press), the properties of which have been recently described (Gouarderes et al., 2001). In membrane preparation from CHO cells expressing the human $NPFF_2$ receptor, [^{125}I]EYF labelled one class of binding sites ($B_{max} = 0.626 \pm 0.069$ pmol/mg protein, $n = 5$) with a very high affinity ($K_d = 0.072 \pm 0.014$ nM, $n = 5$) (Fig. 1, right panel).

3.2. Pharmacological profiles of human $NPFF_1$ and $NPFF_2$ receptors transfected in CHO cells

In order to evaluate the specificity of human $NPFF_1$ and $NPFF_2$ receptors towards peptides derived from pro-$NPFF_A$ and pro-$NPFF_B$ precursors, the ability of several synthetic peptides to displace the specific binding of [^{125}I]YVP or [^{125}I]EYF on human $NPFF_1$ and $NPFF_2$ receptors, respectively, have been compared. Data are presented in Table 1 and some binding profiles are shown in Fig. 2 (left panel).

The peptides displaying the highest affinities (K_i from 0.6 to 1.3 nM) for $NPFF_1$ receptors derived from pro-$NPFF_B$: VPNLPQRFa (NPVF), YVPNLPQRFa and MPHSFANLPLRFa (h$RFRP_1$). However, peptides generated from the pro-$NPFF_A$ precursor and containing the FLFQPQRFa sequence such as NPFF, SQA-NPFF, NPA-NPPF and the stable analogues 1DMe and 3D possessed also a good affinity for $NPFF_1$ receptors (K_i between 1 and 4 nM), indicating that $NPFF_1$ receptors did not discriminate strictly between peptides from pro-$NPFF_B$ precursor and those from pro-$NPFF_A$ precursor containing the NPFF sequence. Nevertheless, NPSF (SLAAPQRFa)-related peptides issued from pro-$NPFF_A$ such as hNPAF, NPSF, EFW-NPSF and EYW-NPSF were three- to eightfold less potent ($K_i = 10-30$ nM) than NPFF-containing peptides to displace the binding of [^{125}I]YVP on $NPFF_1$ receptors and could be considered as the less active ligands for $NPFF_1$ receptors among the endogenous peptides tested.

In contrast, all peptides issued from the pro-$NPFF_A$ precursor, except NPSF, displaced the specific binding of [^{125}I]EYF on $NPFF_2$ receptors with high affinities. The K_i values (from 0.05 to 0.2 nM) were 10- to 100-fold lower than those observed on $NPFF_1$ receptors (Table 1). On the other hand, peptides originating from the pro-$NPFF_B$ precursor (h$RFRP_1$, NPVF, YVPNLPQRFa) displayed lower affinities for $NPFF_2$ receptors (K_i between 4 and 20 nM), as compared to peptides contained in the pro-$NPFF_A$ precursor, indicating that $NPFF_2$ receptors are pro-$NPFF_A$-related peptides preferring receptors (Fig. 2, left panel).

Considering the ratio between the apparent affinities for $NPFF_1$ receptors over $NPFF_2$ receptors ($S_{1/2}$, Table 1), NPA-NPFF, NPAF and EFW-NPSF ($S_{1/2}$ around 100) exhibited the higher selectivity towards $NPFF_2$ receptors, whereas NPFF and the stable analogues, 1DMe and 3D, were poorly selective (4–13 times). Conversely, the most selective ligand for $NPFF_1$ receptors was NPVF, although it was only 30 times better for $NPFF_1$ than for $NPFF_2$ receptors. The apparent selectivity ($S_{1/2} = 75$) of EYW-NPSF for $NPFF_2$ receptors confirmed the use of [^{125}I]EYF as a judicious tool for specific labelling of $NPFF_2$ receptors.

As for $NPFF_2$ receptors (Mazarguil et al., 2001), the C-terminal RFamide part of peptides appeared to be important for interacting with h$NPFF_1$ receptors since the removal of the amide group (NPFF-OH) caused a dramatic loss of binding, and the replacement of the carboxamide group by an alcohol function (1DMe-ol) or the change of the phenylalanine by a tyrosine residue (NPFY) produced a profound decrease in affinity for each receptor (Table 1).

Since peptides issued from both precursors contain a C-terminal PQRFa or PLRFa sequence, the binding profile of

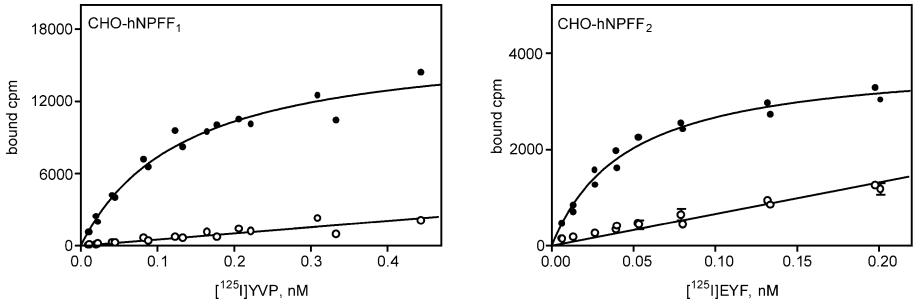


Fig. 1. Saturation binding assays on membranes of CHO cells expressing the human $NPFF_1$ (CHO-h$NPFF_1$) and $NPFF_2$ (CHO-h$NPFF_2$) receptors, using respectively [^{125}I]YVP and [^{125}I]EYF as radioligands. (●) specific binding, ○, nonspecific binding, determined in the presence of 1 μM YVPNLPQRFa for CHO-h$NPFF_1$ or EYW-NPSF for CHO-h$NPFF_2$. Each point represents the mean ± S.E.M. of triplicate determination from two representative experiments.

Table 1
Apparent affinities (K_i) and potency (EC_{50}) of diverse peptides of the NPFF and neuropeptide Y (NPY) families on human $NPFF_1$ and $NPFF_2$ receptors expressed in CHO cells

	CHO $hNPFF_1$			CHO $hNPFF_2$			$S_{1/2}$
	K_i (nM)	EC_{50} (nM)	R	K_i (nM)	EC_{50} (nM)	R	
Pro-$NPFF_A$-derived peptides							
NPFF (FLFQPQRFa)	2.82 ± 0.06	236 ± 43	84	0.21 ± 0.03	2.3 ± 0.5	15	13
1DMe (dYL(NMe)FQPQRFa)	1.09 ± 0.03	71 ± 14	65	0.18 ± 0.04	2.7 ± 0.5	15	6
3D (dYdL(NMe)FQPQRFa)	4.2 ± 0.7	231 ± 58	55	1.02 ± 0.12	10 ± 2	10	4
SQA-NPFF (*h*)	4.16 ± 0.31	153 ± 27	37	0.16 ± 0.02	0.56 ± 0.05	3.5	26
SPA-NPFF (*b*,*m*)				0.047 ± 0.003	0.84 ± 0.25	18	
NPA-NPFF (*r*)	3.4 ± 0.2	166 ± 40	49	0.033 ± 0.003	0.64 ± 0.05	19	103
NPAF (*h*)	13 ± 2	324 ± 30	25	0.14 ± 0.01	0.53 ± 0.03	3.8	93
NPAF (*b*)				0.16 ± 0.02	1.5 ± 0.5	9.4	
NPSF (SLAAPQRFa)	32 ± 6	876 ± 10	27	20 ± 2	222 ± 26	11	1.6
QFW-NPSF (*m*)				0.19 ± 0.01	1.5 ± 0.4	7.9	
EFW-NPSF (*r*)	20.8 ± 0.8	nd		0.21 ± 0.01	2.2 ± 0.3	10.5	94
EYW-NPSF	18 ± 3	nd		0.24 ± 0.03	nd		75
NPFF-OH	>10 000			>1000	>1000		
1DMe-ol	80 ± 11	>1000	>12	17.6 ± 0.5	397 ± 88	22	4
NPFY (FLFQPQRYa)	102 ± 26	>10 000	>100	39 ± 7	361 ± 75	9	3
Pro-$NPFF_B$-derived peptides							
hRFRP1 (MPHSFANLPLRFa)	1.27 ± 0.08	9.6 ± 0.7	7.5	3.9 ± 0.6	21 ± 4	5.4	0.3
NPVF (VPNLPQRFa)	0.6 ± 0.1	12 ± 2	20	17.4 ± 1.7	133 ± 11	7.6	0.03
YVPNLPQRFa	0.69 ± 0.09	8.2 ± 1.1	12	8.9 ± 1.5	nd		0.08
Others							
FMRFa	1.8 ± 0.2	391 ± 113	217	6.6 ± 1.1	517 ± 91	78	0.3
PQRFa	8.7 ± 0.2	2372 ± 100	272	6.8 ± 1.2	309 ± 27	45	1.3
PLRFa	0.83 ± 0.02	116 ± 16	140	0.51 ± 0.05	6.5 ± 0.9	12.7	1.6
LPLRFa	1.7 ± 0.1	84 ± 18	49	10.6 ± 0.5	129 ± 23	12	0.16
NPY-related peptides							
NPY (*p*)	>1000	nd		>1000	nd		
fPP	>1000	nd		7 ± 2	115 ± 5	16	>150
BIBP3226	12 ± 1	>10 000	antag	84 ± 12	>10 000	antag	0.14
BIBO3304	57 ± 7	>10 000	antag	288 ± 69	>10 000		0.2
GR231118	96 ± 26	>10 000		47 ± 5	3024 ± 370	64	2

Data represent mean ± S.E.M. of two to six experiments.
$K_i = IC_{50}/[1 + L/K_d]$ in which IC_{50} is the concentration of competitor required to displace 50% of specific binding of the radioligand, L the concentration of the radioligand and K_d is the affinity constant of the radioligand for the receptor. $NPFF_1$ receptors were labelled with 0.05 nM [^{125}I]YVP and $NPFF_2$ receptors were labelled with 0.05 nM [^{125}I]EYF.
EC_{50} is the concentration of agonist that inhibits 50% of the intracellular cAMP production induced by 2 μM forskolin in recombinant CHO cells. A full inhibition of the forskolin-induced cAMP production was observed for all agonists.
$R = EC_{50}/K_i$ for the activity index of the ligand, $S_{1/2} = K_i$ $NPFF_1/K_i$ $NPFF_2$ for the selectivity index of the ligand.
nd: not determined; (*b*): bovine, (*h*): human, (*m*): mouse, (*p*): porcine.

these tetrapeptides was compared to those of the molluscan FMRFa and of the chicken LPLRFa peptides (Table 1). PLRFa interacted similarly with both receptors with a high affinity ($K_i = 0.83$ and 0.51 nM for $hNPFF_1$ and $hNPFF_2$ receptors, respectively). Extending the N-terminal side with leucine, like in the chicken peptide or in $hRFRP_1$, conferred a slight selectivity towards the $NPFF_1$ receptor: K_i of 1.7 nM for $NPFF_1$ and 10 nM for $NPFF_2$ receptors. PQRFa and FMRFa were not selective (K_i about 2–8 nM for both receptors). Interestingly, additional sequence upstream PQRFa directed the selectivity of peptides either towards $NPFF_1$ (VPNLPQRFa, YVPNLPQRFa), either towards $NPFF_2$ (FLFQPQRFa and related peptides, SLAAPQRFa-related peptides) receptors, with the exception of NPSF (SLAAPQRFa) which exhibited a low affinity ($K_i = 20–30$ nM) for both receptors. As already mentioned (Gouarderes et al., 2001; Roumy et al., 2000), NPSF, although it has been biochemically isolated (Bonnard et al., 2001), is probably not biologically active on NPFF receptors since it cannot be matured from the precursor at consensus sites of cleavage but could result rather from a degradation process.

3.3. Functional profiles of human $NPFF_1$ and $NPFF_2$ receptors in transfected CHO cells

All peptides from both precursors induced a maximal inhibition of the forskolin-induced accumulation of cAMP,

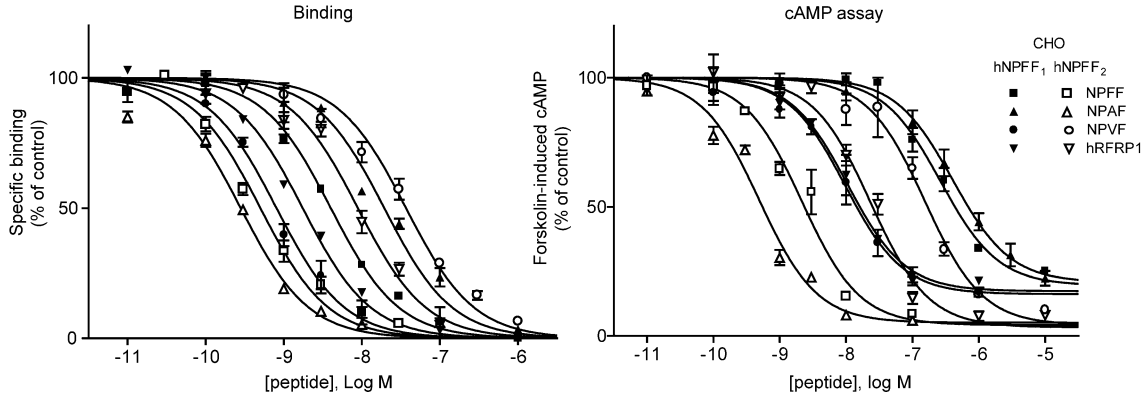


Fig. 2. Binding and functional profiles of representative NPFF ligands on human $NPFF_1$ (CHO-h$NPFF_1$) and $NPFF_2$ (CHO-h$NPFF_2$) receptors expressed in CHO cells. Curves are the mean of at least three experiments performed in triplicate. For binding experiments, $NPFF_1$ receptors were labelled with 0.05 nM [^{125}I]YVP and $NPFF_2$ receptors were labelled with 0.05 nM [^{125}I]EYF. For functional assay, the intracellular cAMP production was stimulated 10 min at 37 °C by 2 μM forskolin (100%).

corresponding to 75–80% in CHOh$NPFF_1$ cells and 90–100% in CHOh$NPFF_2$ cells (Fig. 2, right panel and Table 1). In each clone, all peptides tested exhibited the same maximal efficacy and appeared as full agonists.

Concerning h$NPFF_2$ receptors, the apparent binding affinities (K_i) were related to functional potencies (EC_{50}) for nearly all peptides tested, indicating that agonist activity reflected binding affinity. The mean of the ratio (R) between EC_{50} and K_i was around 11, a value often described for other receptors, which could result from differences in experimental conditions between binding studies performed on membranes in Tris buffer, and functional assays performed on intact cells in KRH buffer. The smallest difference between functional and binding parameters on human $NPFF_2$ receptors were observed for peptides (SQA-NPFF and (*h*)NPAF) processed from the human precursor at consensus sites of cleavage.

For h$NPFF_1$ receptors, the pro-$NPFF_B$-derived peptides exhibited EC_{50} (about 10 nM) in accordance with binding affinities. On the other hand, the pro-$NPFF_A$-derived peptides, even those exhibiting nanomolar affinity, were full agonists but with only weak potency. The ratios EC_{50} over K_i for pro-$NPFF_A$-derived peptides for $NPFF_1$ receptors were higher (from 25 to 80) than those for pro-$NPFF_B$-derived peptides (from 7 to 20), indicating that the selectivity of the $NPFF_1$ receptor is conferred on the basis of agonist activity rather than binding affinity, the pro-$NPFF_B$-derived peptides being the most potent agonists on $NPFF_1$ receptors. This is illustrated in Fig. 2 (right panel) where differences between curves for pro-$NPFF_A$- and pro-$NPFF_B$-derived peptides on h$NPFF_1$ receptors are larger for cAMP than for binding assay. A disproportion between affinity and functional parameters has been already reported in several systems and discussed as a «ligand paradox» (Kenakin and Onaran, 2002; Rosenkilde and Schwartz, 2000).

Interestingly, FMRFa and PQRFa which displayed a relative high affinity for $NPFF_1$ and $NPFF_2$ receptors (K_i = 2–8 nM) were weak agonists on both receptors (EC_{50} = 300–2300 nM). Similarly, PLRFa (K_i = 0.83 nM) was also a poor agonist towards h$NPFF_1$ receptors (EC_{50} = 116 nM). This indicates that the C-terminal end of peptides confers a high affinity without selectivity for both $NPFF_1$ and $NPFF_2$ receptors, but is not sufficient to produce a potent agonistic effect. Furthermore, NPFY, which contains a tyrosine instead of a phenylalanine residue, retained a binding capacity on both receptors but displayed agonist activity only on $NPFF_2$ receptors, suggesting that the presence of a hydroxyl group on the C-terminal phenyl ring prevents agonist activity on $NPFF_1$ but not on $NPFF_2$ receptors.

3.4. Binding and functional properties of ligands from the neuropeptide Y (NPY) family on human $NPFF_1$ and $NPFF_2$ receptors

Since it has been previously reported that some ligands of the NPY family cross-react with $NPFF_2$ receptors (Bonini et al., 2000; Mollereau et al., 2001), the ability of NPY ligands to interact with human $NPFF_1$ and $NPFF_2$ receptors was investigated.

Although NPY did not recognize h$NPFF_1$ and h$NPFF_2$ receptors, frog Pancreatic Polypeptide (frogPP), Y_1 antagonists such as BIBP3226 (Rudolf et al., 1994) and BIBO3304 (Wieland et al., 1998) and the mixed Y_1 antagonist/Y_4 agonist GR231118 (Dumont and Quirion, 2000; Parker et al., 1998; Schober et al., 1998) could displace the specific binding on human NPFF receptors with a relative high affinity (Table 1). FrogPP exhibited a high affinity on h$NPFF_2$ receptors (K_i = 7 nM) and was at least 150-fold less potent on h$NPFF_1$ receptors (K_i >1000 nM), appearing thus as the most selective ligand for $NPFF_2$ receptor at this time. BIBP3226 was more effective than BIBO3304 to displace the specific binding on both receptors. Moreover, the affinities of these compounds were higher for the h$NPFF_1$ receptor (K_i = 12 and 57 nM for BIBP3226 and BIBO3304, respectively) than for the h$NPFF_2$ receptor (K_i = 84 and 288 nM for BIBP3226 and BIBO3304,

respectively). In contrast, the mixed Y_1 antagonist/Y_4 agonist GR231118 was equipotent on both receptors (K_i = 96 and 47 nM on hNPFF1 and hNPFF2 receptors, respectively).

As shown in Fig. 3, BIBP3226, and in a lesser extent BIBO3304, could dose-dependently antagonize the NPVF and NPFF inhibition of forskolin-induced cAMP accumulation in CHO cells expressing the human $NPFF_1$ and $NPFF_2$ receptor, respectively. The potency of BIBP3226 and BIBO3304 was higher on $hNPFF_1$ than on $hNPFF_2$ receptors (Fig. 3 and Table 2). GR231118, which was a poor agonist on the $hNPFF_2$ receptor (Table 1 and Mollereau et al., 2001), was not active up to 10 μM on the hNPFF1 receptor. These results demonstrate that the NPY Y_1 receptor antagonists BIBP3226 and BIBO3304 are also antagonists for NPFF receptors, especially towards the $NPFF_1$ subtype.

3.5. Structure–activity studies of BIBP3226-derived compounds on human $NPFF_1$ and $NPFF_2$ receptors

In order to improve selectivity and affinity of antagonists for NPFF receptors, a series of analogues of BIBP3226 ((*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)-methyl]-argininamide) were synthesized and tested for their agonist/antagonist activity (Table 2). Since compounds with a monophenylacetyl moiety instead of a diphenylacetyl, or with an L-argininamide residue instead of a D-argininamide, exhibited weak affinities (data not shown), the N-terminal part ((*R*)-N^2-diphenylacetyl-argininamide) of BIBP3226 was not modified. Only the C-terminal end was derivatized by extension of the chain bearing the phenyl group, with or without a *para*-hydroxyl substitution, and by the addition of a carboxamide function.

All compounds tested on the $hNPFF_1$ receptor exhibited apparent affinities between 40 and 260 nM and were devoid of agonistic activity (up to 10 μM). For pseudopeptides containing only one carbon between NH and the phenyl group (BIBP3226, BIBO3304, P**1**, P**16**, P**14**), the presence of the *para*-hydroxyl group on the phenyl ring conferred the highest affinity for molecules without a C-terminal carboxamide (K_i = 12 nM for BIBP3226 versus K_i = 82 nM for P**1**), in contrast to those exhibiting a carboxamide group (K_i = 69 nM for P**16** versus K_i = 42 nM for P**14**). This suggests that OH and $CONH_2$ groups present on the C-terminal moiety could compete to form a hydrogen bond with the binding site of the receptor. Pseudopeptides possessing an ethyl-phenyl (P**11**) or an ethyl-hydroxyphenyl moiety (P**13**) exhibited also relatively high affinities (K_i = 45 and 78 nM, respectively). However, the addition of a carboxamide group on these compounds reduced about five times their apparent affinity (P**2** versus P**11**, P**4** versus P**13**). When tested for their antagonistic properties in CHO cells expressing the $hNPFF_1$ receptor, compounds with affinities inferior to 100 nM were able to reverse the inhibition of cAMP production induced by NPVF (100 nM) (Fig. 4 and Table 2), P**11** being the most potent, in addition to BIBP3226.

In contrast to the $hNPFF_1$ receptor, the $hNPFF_2$ receptor was less tolerant to modifications on BIBP3226 since nearly all analogues, except P**2**, P**5** and P**11**, displayed affinities higher than 300 nM (Table 2). An important constraint for binding to $NPFF_2$ receptors appeared to be the length of the carbon chain bearing the phenyl ring since the derivatives exhibiting the best affinities were those containing an ethyl–phenyl chain, like P**11** (K_i = 90 nM) and especially P**2** (K_i = 10 nM), or an indole group, like P**5** (K_i = 67 nM). A shorter chain (P**1** compared to P**11**; P**14** compared to P**2**) led to less active (5–50-fold) compounds, indicating that the position of the aromatic ring is important for a correct docking of the molecule within the binding pocket, by making probably hydrophobic interactions. Moreover, the presence of a car-

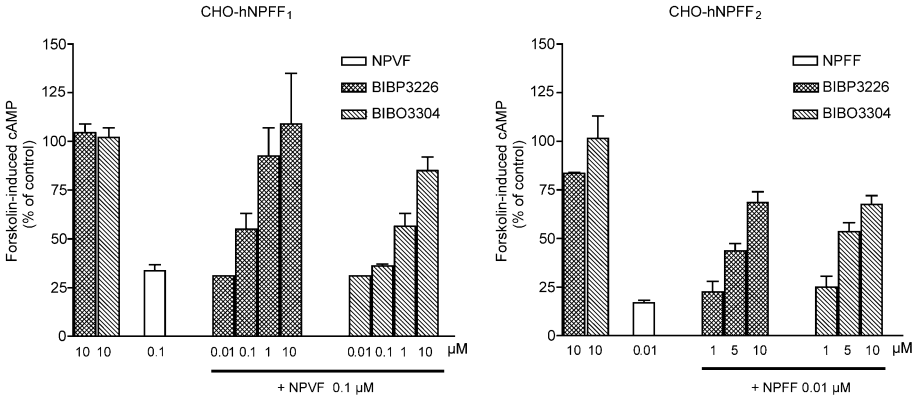
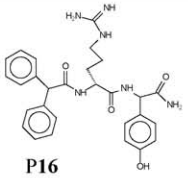
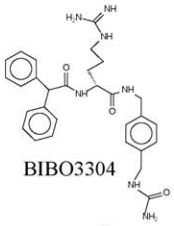
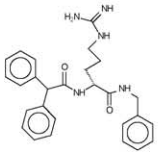
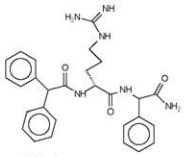
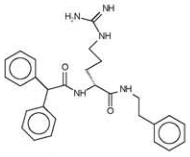
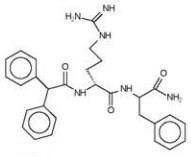
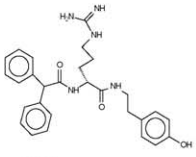
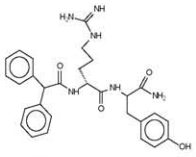
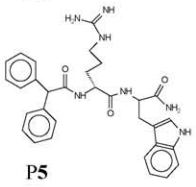


Fig. 3. Antagonist properties of BIBP3226 and BIBO3304 on human $NPFF_1$ (CHO-$hNPFF_1$) and $NPFF_2$ (CHO-$hNPFF_2$) receptors expressed in CHO cells. Bars represent the mean ± S.E.M. of data from two to four experiments performed in triplicate. Intracellular cAMP content was stimulated by 2 μM forskolin, 10 min at 37 °C (control). 10 μM BIBP3226 and BIBO3304 alone were inactive, while 0.1 μM NPVF or 0.01 μM NPFF (white bars) inhibited about 75% and 80% of the stimulated cAMP in CHO-$hNPFF_1$ and CHO-$NPFF_2$ cells, respectively. Increasing the concentration of BIBP3226 or BIBO3304 in the presence of the agonist reversed the inhibitory effect in both cell line.

Table 2
Apparent affinities (K_i) and antagonist activities (IC_{50}) of diverse compounds derived from BIBP3226 on human $NPFF_1$ ($CHOhNPFF_1$) and $NPFF_2$ ($CHOhNPFF_2$) receptors expressed in CHO cells

-Carboxamide	Binding K_i, nM $CHOhNPFF_1$ $CHOhNPFF_2$	Antagonist activity IC_{50}, nM $CHOhNPFF_1$ $CHOhNPFF_2$	+Carboxamide	Binding K_i, nM $CHOhNPFF_1$ $CHOhNPFF_2$	Antagonist activity IC_{50}, nM $CHOhNPFF_1$ $CHOhNPFF_2$
 BIBP3226	12±1 84±12	332 ± 134 6600 ± 900	 **P16**	69 ± 4 1764 ± 260	8252 ± 3000 nd
 BIBO3304	57 ± 7 288 ± 69	1806 ± 865 10400 ± 3700			
 **P1**	82 ± 5 466 ± 40	2780 ± 557 nd	 **P14**	42 ± 3 574 ± 44	1922 ± 112 nd
 **P11**	45 ± 5 90 ± 10	690 ± 175 weak agonist	 **P2**	232 ± 30 10 ± 2	nd weak agonist
 **P13**	78 ± 12 843 ± 112	2044 ± 891 nd	 **P4**	265 ± 19 2000 ± 354	nd nd
			 **P5**	141 ± 19 67 ± 9	nd weak agonist

Data represent mean ± S.E.M. of two to five experiments.
$K_i = IC_{50}/[1 + L/K_d]$ in which IC_{50} is the concentration of competitor required to displace 50% of specific binding of the radioligand, L is the concentration of the radioligand and K_d is the affinity constant of the radioligand for the receptor. $NPFF_1$ receptors were labelled with 0.05 nM [^{125}I]YVP and $NPFF_2$ receptors were labelled with 0.05 nM [^{125}I]EYF.
IC_{50} is the concentration that produces a 50% reversion of the NPVF (0.1 μM) or NPFF (0.01 μM)-induced inhibition of forskolin-stimulated cAMP accumulation in CHO $hNPFF_1$ and CHO $hNPFF_2$ cells, respectively.
Low affinity compounds were inactive by themselves up to 10 μM on the forskolin-induced cAMP production and were not tested for their antagonist activity (nd).
For carboxamide pseudopeptides, the configuration of the asymmetric carbon carrying the $CONH_2$ group was *S*.

boxamide group at the C-terminal portion of these molecules appeared to be also essential for high affinity binding, since it induced 10-fold increase in affinity (P**2** versus P**11**). In contrast, except BIBP3226, the introduction of a *para*-hydroxyl group on the phenyl ring caused a dramatic loss of binding (P**13** compared to P**11**; P**4** compared to P**2**) and, as observed for $NPFF_1$ receptors, counteracted the effect of the carboxamide group (P**16** compared to BIBP3226; P**4** compared to P**2**). It is interesting to note that the derivatives with the highest affinities for $hNPFF_2$ receptors (P**2**, P**5**, P**11**)

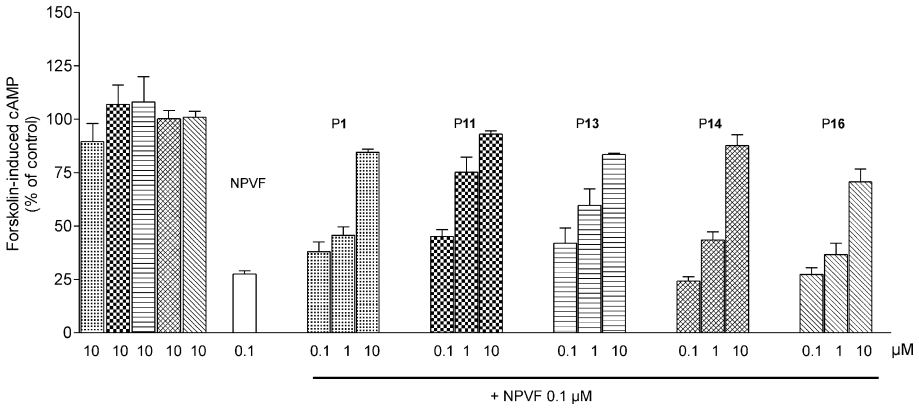


Fig. 4. Antagonist properties of diverse compounds derived from BIBP3226-derived on human $NPFF_1$ receptors expressed in CHO cells. Bars represent the mean ± S.E.M. of data from three experiments performed in triplicate. Intracellular cAMP content was stimulated by 2 μM forskolin, 10 min at 37 °C (control). The compounds, tested alone at 10 μM, were inactive. In the presence of NPVF (0.1 μM), which caused a 75% inhibition of the forskolin-induced cAMP production (white bar), increasing the concentrations of the BIBP3226 analogues from 0.1 to 10 μM reversed the NPVF inhibition.

exhibited a weak agonistic activity (Table 2), which prevented their use as antagonists.

4. Discussion

The aim of this study was to characterize and compare the pharmacological profiles of human $NPFF_1$ and $NPFF_2$ receptors. Our results clearly indicate that $NPFF_2$ receptors exhibit more selectivity than $NPFF_1$ receptors. Indeed, $NPFF_2$ receptors preferred, 10 to 100 times, peptides derived from the pro-$NPFF_A$ precursor, in contrast to $NPFF_1$ receptors which did not discriminate strictly peptides from the two precursors. However, in functional assay, $hNPFF_1$ was preferentially activated by pro-$NPFF_B$ peptides (Table 1), as previously observed by Fukusumi et al. (2001) but not by Liu et al. (2001). Conversely, peptides from the pro-$NPFF_A$ precursor displayed clearly higher (up to 100 times) affinity for $NPFF_2$ than for $NPFF_1$ receptors, whereas the pro-$NPFF_B$-derived peptides exhibited only slightly (3- to 20-fold) higher affinity towards $hNPFF_1$ receptors, as previously suggested by Liu et al. (2001). Some discrepancies exist between data from previous reports (Bonini et al., 2000; Elshourbagy et al., 2000; Hinuma et al., 2000; Liu et al., 2001), including the present study. They are probably due to the use of different radioligands in binding assays and of transfected cells (CHO or human embryonic kidney HEK293) expressing modified G-protein and/or reporter system for rapid screening investigations. Notwithstanding, it is important to mention that we have obtained similar binding parameters in HEK293 transfected cells (Gouarderes et al., in press) and, above all, that the affinities determined in recombinant cells are close to those observed for NPFF receptors in rat spinal cord slices, labelled either with [^{125}I]EYF or [^{125}I]1DMe, which are assumed to be $NPFF_2$ receptors (Gouarderes et al., 2001; Mazarguil et al., 2001; Roumy et al., 2000).

In a previous study, we have shown that some ligands belonging to the neuropeptide Y family are able to bind to the $hNPFF_2$ receptor and that BIBP3226, a NPY Y_1 receptor selective antagonist, behaves also as a weak $NPFF_2$ receptor antagonist (Mollereau et al., 2001). Our present data show, similarly, that BIBP3226, as well as BIBO3304, a more potent Y_1 receptor antagonist (Dumont et al., 2000a; Wieland et al., 1998), display antagonist activities on $hNPFF_1$ receptors, with apparent affinities (K_i = 12 and 57 nM, respectively) higher than those for $hNPFF_2$ receptors and largely greater than those measured on the other NPY receptor types (all superior to 1000 or 10 000 nM) (Dumont et al., 2000b; Mollereau et al., 2001; Schober et al., 1998; Wieland et al., 1998). Moreover, BIBP3226 exhibits an affinity towards $hNPFF_1$ receptors close to those previously reported (from 0.5 to 14 nM) for human and rat NPY_1 receptors (Dumont and Quirion, 2000; Mollereau et al., 2001; Wieland et al., 1995). This result indicates that BIBP3226 could bind to and antagonize NPFF receptors, specially the $NPFF_1$ subtype, as well as NPY receptors, in vivo, and suggests therefore to be careful when evaluating the pharmacological properties of this compound.

While NPFF-related peptides did not recognize NPY receptors (Mollereau et al., 2001) and NPY did not bind to NPFF receptors (this study and Bonini et al., 2000), NPFF and NPY systems share common structural features that could explain the interaction of some NPY ligands with NPFF receptors: (i) the related C-terminal end (RYamide or RFamide) of the endogenous peptides is crucial for the binding to both receptors and (ii) receptor sequences are 30–35% identical. Interestingly, nearly all the residues shown, in mutagenesis studies, to contribute specifically to the binding of BIBP3226 to NPY Y_1 receptors (Y211) or to be involved in the binding of both BIBP3226 and NPY (W163, D287) (Sautel et al., 1995, 1996; Walker et al., 1994), are also present in NPFF receptors and may be responsible for the interaction with BIBP3226. Other amino

Table 3
Affinities (K_i) and antagonist activities (IC_{50}) of BIBP3226 derivatives on NPY Y_1 receptors

	NPY Y_1 receptors	
	K_i (nM)	IC_{50} (nM)
P1	70[a]	110[b]
P11	16[c]	
P13	290[a]	
P14	3900[d]	7500[d]
P16	4200[d]	8100[d]

[a] (Rudolf et al., 1997).
[b] (Aiglstorfer et al., 1998).
[c] NPY Y_1 receptors of rat brain membranes were labelled by [^{125}I][Leu^{31}, Pro^{34}]PYY, as described earlier (Dumont et al., 2000a).
[d] (Aiglstorfer et al., 2000).

acids at positions F173, Q219 and N283 important for binding to NPY receptors are, however, lacking, preventing therefore a high affinity for BIBP3226, and probably BIBO3304, on NPFF receptors. On the other hand, amino acids specifically involved in the binding of NPY and not of BIBP3226, such as D104, W288 and Y100, H298, supposed to interact respectively with the carboxamide group and the OH of the Tyr^{36} in NPY (Sautel et al., 1996), are not present in NPFF receptors. This probably explains the poor affinity of NPY for NPFF receptors. According to the model of Sautel et al. (1996), we hypothesize that BIBP3226 binding on NPFF receptors is based on (i) an ionic interaction between the guanidino group of BIBP3226 (corresponding to Arg^7 of NPFF) and D295 in $hNPFF_1$ or D298 in $hNPFF_2$ receptors, and on (ii) hydrophobic interactions between the diphenylacetyl moiety and Y216 or Y219 (in $hNPFF_1$ and $hNPFF_2$ receptors, respectively), between the benzyl group and W166 ($hNPFF_1$) or W168 ($hNPFF_2$). Mutagenesis of NPFF receptors has to be performed now to confirm this model and to determine the structural features in $hNPFF_1$ and $hNPFF_2$ receptors responsible for the selectivity of BIBP3226 binding.

The interesting properties of BIBP3226 on NPFF receptors led us to consider this molecule as a lead compound from which modifications were expected to increase affinity or selectivity towards NPFF receptors. In a first round of synthesis, we found several compounds with relative high affinity for NPFF receptors. As observed in competition studies with endogenous peptides, the $hNPFF_1$ receptor was less restrictive for binding than the $hNPFF_2$ receptor since nearly all the tested molecules exhibited affinities under 100 nM. Selectivity towards $NPFF_2$ receptors was observed only for molecules (**P2**, **P5**) possessing a C-terminal end close to the phenylalaninamide of endogenous peptides, confirming that this part, either in natural or synthetic molecules, plays a crucial role for high affinity binding to the $NPFF_2$ receptors. As it also confers a weak agonist activity (Table 2), possibility to dissociate high affinity and agonist activity will be restrained for $NPFF_2$ selective ligands. Among the $hNPFF_1$ receptor antagonists, either a weak agonist activity was detected on the $hNPFF_2$ receptor (**P11**), either a relative high affinity or an antagonist property towards NPY Y1 receptors were described (Table 3), giving additional support to a close structural homology between NPFF and NPY Y_1 receptors.

Efforts to synthesize new molecules devoid of cross-reactivity and selective for one NPFF receptor subtype have to be carried on since specific high affinity antagonists for NPFF receptors are still lacking.

Acknowledgements

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