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SPECIAL REPORT

Agonist and antagonist activities on human $NPFF_2$ receptors of the NPY ligands GR231118 and BIBP3226

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Neuropeptide FF (NPFF) is a part of a neurotransmitter system acting as a modulator of endogenous opioid functions. At this time, no non-peptide or peptide NPFF-antagonists have been discovered. Here, we demonstrate that Neuropeptide Y (NPY) ligands, in fact possess significant ability to interact with the human $NPFF_2$ receptors. NPY Y_1 antagonist BIBP3226 and mixed Y_1 antagonist/Y_4 agonist GR231118 are able to displace with low affinity, 50–100 nM, the specific binding on NPFF receptors expressed in CHO cells as well as in rat dorsal spinal cord, an affinity however superior to those determined against Y_2, Y_4 or Y_5 receptors. Furthermore, BIBP3226 which is unable to inhibit the forskolin-stimulated cyclic AMP production mediated by $NPFF_2$ receptors, antagonizes the effect of NPFF, revealing the first antagonist of NPFF receptors. These properties of NPY ligands on Neuropeptide FF receptors must be considered when evaluating pharmacological activities of these drugs.
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Abbreviations: BIBP3226, R-N^2-(Diphenylacetyl)-N-(4-hydrophenyl)-methyl argininamide; GR231118, homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$

Introduction Neuropeptide FF (NPFF) is an amidated neuropeptide acting as a modulator of endogenous opioid functions (for review Roumy & Zajac, 1998). Pharmacological studies suggested the possible existence of NPFF receptor subtypes. Indeed, two different complementary DNA encoding G-protein coupled receptors ($NPFF_1$ and $NPFF_2$ receptors) have recently been cloned (Bonini *et al*., 2000; Elshourbagy *et al*., 2000; Hinuma *et al*., 2000). Interestingly, both receptors share about 30–35% identity with the orexin and Neuropeptide Y (NPY) receptors. Moreover, Bonini *et al*. (2000) reported that BIBP3226 (Y_1 antagonist; (Rudolf *et al*., 1994)) and frog Pancreatic Polypeptide (fPP), two ligands of the NPY receptor family, were able to compete for specific binding of [^{125}I]-1DMe, an analogue of NPFF, on recombinant NPFF receptors, and were selective for $NPFF_1$ and $NPFF_2$ receptors, respectively.

The possible interaction between NPFF- and NPY-preferential ligands with NPFF and NPY receptors led us to investigate the capacity of several ligands belonging to the NPY family (i) to displace the specific binding of the [^{125}I]-EYF, an analogue of NPFF on human $NPFF_2$ receptors expressed in CHO cells, as well as on NPFF receptors of the rat spinal cord and (ii) to inhibit adenylate cyclase activity in $hNPFF_2$ receptor transfected cells. Our results indicate that the selective NPY Y_1 receptor antagonist BIBP3226 (Rudolf *et al*., 1994; Schober *et al*., 1998), the mixed NPY Y_1 receptor antagonist/ Y_4 receptor agonist GR231118 (Dumont *et al*., 2000b; Parker *et al*., 1998; Schober *et al*., 1998) and the fPP not only display a relatively high affinity for NPFF receptors, but exhibit also antagonistic (BIBP3226) or agonistic (GR231118, fPP) activity at the $hNPFF_2$ receptor expressed in CHO cells. These features suggest that the use of these molecules requires careful interpretation, as a potential contribution from NPFF receptors may be difficult to exclude.

Methods Peptides of the NPFF family and frog PP were synthesized using an automated peptide synthesizer (Applied Biosystems model 433A). PYY, [Leu^{31},Pro^{34}]-PYY, PYY_{3-36} and hPP were synthesized as previously described (Forest *et al*., 1990). BIBP3226, BIBP3445 and BIIE0246 were generously provided by Boehringer Ingelheim (Germany). GR231118 was a gift from GlaxoWellcome (Research Triangle Park, NC, U.S.A.) while CGP71683A and JCF109 were generously obtained from Servier (Paris, France). The rat Y_1, Y_2, Y_4 and Y_5 receptor cDNA were generously provided by Dr Herbert Herzog (Sydney, Australia).

Transfected HEK 293 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum and amphotericin B, (Gibco-BRL, Canada). All NPY binding assays were performed as previously described (Dumont *et al*., 2000a).

Recombinant CHO cells expressing the human $NPFF_2$ receptor were grown in Ham's F12 medium supplemented with 7% foetal calf serum and G418 400 μg ml^{-1}, (Gibco-BRL, France). For membrane preparation, cells were harvested in phosphate buffer saline, frozen at −70°C, and

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homogenized in 50 mM Tris-HCl, pH 7.4 in a Potter Elvehjem tissue grinder. The nuclear pellet was discarded by centrifugation at $1000 \times g$ for 15 min at 4°C, and the membrane fraction was collected upon centrifugation of the supernatant at $100{,}000 \times g$ for 30 min at 4°C. Binding of [^{125}I]-EYF ([^{125}I]-EYWSLAAPQRF-NH_2), a new specific radioligand for NPFF receptors (2000 Ci mmole; Gouardères *et al*., 2001) on membranes (2 μg) of hNPFF$_2$ receptor expressing CHO cells was measured by rapid filtration as described (Gouardères *et al*., 2001). Binding on NPFF receptors in rat (male Sprague-Dawley, 350 g; Depré, France) spinal cord sections was exactly as described in Gouardères *et al*. (2001).

Assay for intracellular cyclic AMP was performed essentially as described in (Mollereau *et al*., 1999). Briefly, 200,000 recombinant cells were incubated for 1 h at 37°C under 5% CO_2 with 0.6 μCi [^{3}H]-adenine (26 Ci/mmole Amersham) in Ham's F12 medium. Cyclic AMP production was stimulated by 2 μM Forskolin (Sigma, France) for 10 min at 37°C in 200 μl HEPES buffered Krebs-Ringer saline in the presence of 0.1 mM of the phosphodiesterase inhibitors, IBMX (Sigma, France) and Ro-20 1724 (Fisher, France). Ligands to be tested were added at the same time at the desired concentration. The reaction was stopped by addition of 20 μl HCl 2.2 N and intracellular [^{3}H]-cyclic AMP was isolated by chromatograhic procedure on acid alumina columns (Sigma, France).

Results Competition studies on HEK 293 cells transfected with either the rat NPY Y_1, Y_2, Y_4 or Y_5 receptor cDNA revealed that NPFF related peptides such as NPFF (FLFQPQRF-NH_2), NPA-NPFF, 1DMe (D.YL(N-Me)FQPQRF-NH_2), NPSF (SLAAPQRF-NH_2), EFW-NPSF, hNPAF (Perry *et al*., 1997; Vilim *et al*., 1999) were inactive up to 10 μM to compete against specific [^{125}I]-GR231118, [^{125}I]-PYY3-36, [^{125}I]-hPP or [^{125}I][Leu^{31},Pro^{34}]-PYY sites, respectively (Table 1 and data not shown). Conversely, ligands of the NPY family (Dumont *et al*., 2000c) including BIBP3445 (enantiomer of BIBP3226), BIIE0246 (Y_2 antagonist), CGP71683A (Y_5 antagonist) and JCF109 (Y_5 antagonist) were not recognized ($IC_{50} > 1$ μM) by the human NPFF$_2$ receptor expressed in CHO cells (not shown) except the frog pancreatic polypeptide (fPP), the Y_1 antagonist/Y_4 agonist GR231118 and the Y_1 antagonist BIBP3226. As shown in the Figure 1A, these molecules were able to completely inhibit the specific binding of [^{125}I]-EYF on the hNPFF$_2$ receptor with apparent affinities (K_i) in the nM range (Table 1). Similar values were obtained against endogenously expressed NPFF receptors in the rat spinal cord (Table 1), confirming the ability of some NPY ligands to interact with native NPFF receptors. Moreover, the binding of BIBP3226 to NPFF receptors exhibited the same stereoselectivity as on NPY receptors since the enantiomer BIBP3435 was inactive. It is interesting to add that the last C-terminal nine residues of fPP were sufficient to confer affinity of fPP$_{28\text{-}36}$ for NPFF receptors (Table 1).

Functional activity of fPP, GR231118 and BIBP3226 was investigated next by measuring the decrease of forskolin-induced cyclic AMP content as we found the hNPFF$_2$ receptor to be negatively coupled to adenylate cyclase activity in CHO cells (Figure 1B and data not shown). In recombinant hNPFF$_2$ transfected cells, NPFF inhibited the cyclic AMP production with EC_{50} of 3 nM (Table 1). fPP and GR231118 were found to be full agonists (Figure 1B) but with a rather low potency ($EC_{50} = 115$ nM and 3000 nM respectively, Table 1). On the other hand, BIBP3226 was inactive by itself at concentration up to 10 μM (Figure 1B). However, increasing concentrations of this compound reversed the inhibitory effect of 10 nM NPFF on adenylate cyclase activity demonstrating that BIBP3226 behaved as an antagonist at hNPFF$_2$ receptors (Figure 2).

Discussion In the present study, we found that among several ligands of the NPY family, frog PP, GR231118 and BIBP3226, were able to compete for the specific NPFF binding expressed in CHO cells as well as in rat dorsal spinal cord. Interestingly, the apparent affinity of the selective NPY Y_1/Y_4 ligand GR231118 for NPFF$_2$ receptors ($K_i = 50$–70 nM) is comparable to its well established affinities for the NPY Y_2 ($K_i = 63$ nM) and Y_5 ($K_i = 100$ nM) receptors (Parker *et al*., 1998 and Table 1). Moreover, the apparent affinity (K_i about 100 nM) of the selective NPY Y_1 receptor antagonist BIBP3226 for NPFF$_2$ receptors is much greater than those reported on the NPY Y_2, Y_4 and Y_5 receptors, (Schober *et al*., 1998; Dumont *et al*., 2000c and Table 1). Furthermore, we observed relatively high affinities ($K_i = 1.5$–7 nM) for fPP and its truncated analogue fPP$_{28\text{-}36}$ for NPFF receptors. This is likely explained by the presence of Arg-Phe-

Table 1 Binding constants (K_i) and functional parameters (EC_{50}) of various ligands on NPFF receptors in rat spinal cord (rNPFF column) and CHO transfected cells (hNPFF$_2$ column), and on rat NPY receptors in HEK 293 transfected cells (rNPY Y_1, Y_2, Y_4, Y_5 columns)

	rNPFF	*hNPFF$_2$*		*rNPY Y_1*	*rNPY Y_2*	*rNPY Y_4*	*rNPY Y_5*
	K_i, (nM)	K_i, (nM)	EC_{50} (nM)	K_i, (nM)	K_i, (nM)	K_i, (nM)	K_i, (nM)
NPFF	0.28±0.02	0.20±0.03	3.2±0.9	>10,000	>10,000	>10,000	>10,000
GR231118	73±9	47±5	3024±370	0.36±0.05	145±45	0.27±0.04	236±109
BIBP3226	108±7	79±14	>10,000	1.1±0.4	>1000	>1000	>1000
FrogPP$_{1-36}$	3.2±0.2	7.2±2	115±5	–	–	–	–
FrogPP$_{28-36}$	6.6±0.8	1.5±0.3	874±48	–	–	–	–

Data represent the means±s.e.mean of 3–6 experiments performed in triplicate. NPFF receptors were labelled with 0.05 nM [^{125}I]-EYF. NPY receptors were labelled with 0.035 nM [^{125}I]-GR231118 (Y_1), [^{125}I]-PYY$_{3\text{-}36}$ (Y_2), [^{125}I]-hPP (Y_4), [^{125}I][Leu^{31},Pro^{34}]-PYY (Y_5). Experimental curves were analysed by non linear regression method using prism 2.0 (GraphPad software Inc., San Diego, CA, U.S.A.). $K_i = IC_{50}/[1 + L/K_D]$ in which IC_{50} is the concentration of competitor required to displace 50% of the 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.

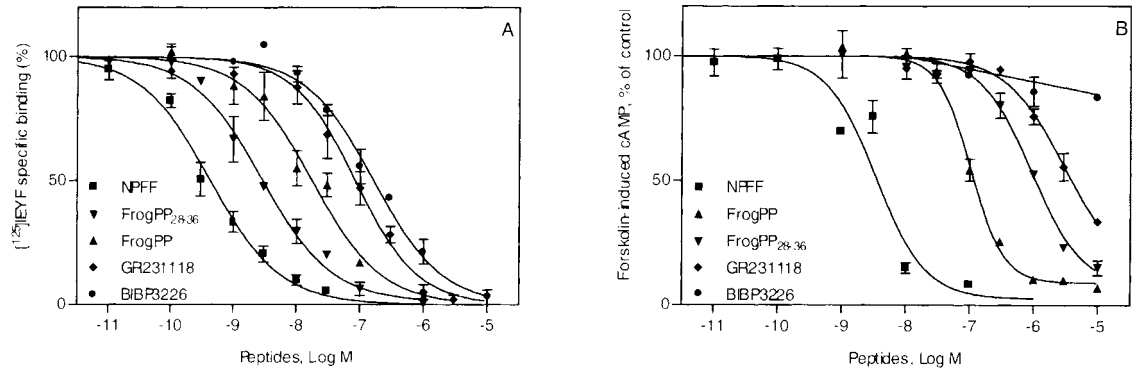

Figure 1 Displacement of [^{125}I]-EYF (0.05 nM) specific binding on membranes of recombinant CHO cells (A) and accumulation of intracellular cyclic AMP (B) in CHO cells expressing the human $NPFF_2$ receptor. Each curve represents the means ± s.e.mean of at least three experiments performed in triplicate.

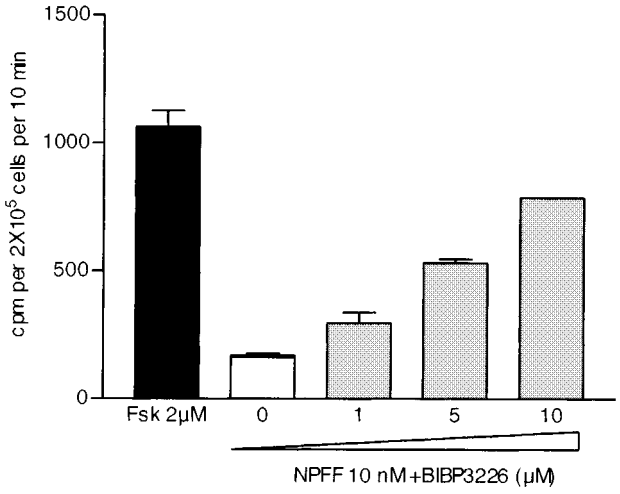

Figure 2 Effect of increasing concentrations of BIBP3226 on the inhibition by NPFF of forskolin-induced cyclic AMP accumulation in CHO cells expressing the human $NPFF_2$ receptor. Intracellular cyclic AMP content induced by 2 μM forskolin (black bar) was inhibited (80–90%) by 10 nM NPFF (white bar), and increasing concentrations of BIBP3226 (grey bar) reversed this effect. The bars represent the means ± s.e.mean of triplicate determinations of one representative experiment among three.

amide residues at the C-terminus of the peptides instead of the usual Arg-Tyr-amide residues present in all mammalian pancreatic polypeptides as well as in NPY and PYY. To our knowledge, no data on the affinity of fPP for NPY receptor subtypes is available in the literature. Interestingly, we observed that the affinities of BIBP3226 and fPP for the $hNPFF_2$ receptors expressed in CHO cells and for the rat spinal cord receptors (suspected to be of the $NPFF_2$ receptor subtype; Bonini *et al*., 2000), were 10 fold better than those reported on human and rat $NPFF_2$ receptors expressed in HEK 293 cells (Bonini *et al*., 2000). This apparent discrepancy remains to be explained.

The functional properties of fPP, GR231118 and BIBP3226 were investigated next on the basis of cyclic AMP accumulation assays in $hNPFF_2$ receptors transfected cells. Interestingly, fPP and GR231118 exhibit agonistic activity. Although the potency of GR231118 is 500–1000 fold lower than that observed for NPY Y_4 receptors (Parker *et al*., 1998; Schober *et al*., 1998), it is in the same range order than those described for Y_2 and Y_5 NPY receptors (Parker *et al*., 1998). On the other hand, the Y_1 antagonist BIBP3226 which is inactive by itself at up to 10 μM, is able to antagonize in a concentration-dependent manner the inhibition of forskolin-stimulated cyclic AMP production induced by NPFF (10 nM). Hence, BIBP3226 is the first antagonist to be reported for $NPFF_2$ receptors and could therefore be considered as a lead compound in an effort to develop more potent antagonists for the $NPFF_2$ receptor subtype.

Taken together our data suggest that NPFF receptors are related to NPY (most particularly Y_1 and Y_4) receptors not only on sequence homology but also on binding affinity and functional properties. Both families may have conserved an ancestral binding pocket that has evolved towards the Arg-Phe-amide or Arg-Tyr-amide interactions. This hypothesis should be explored in detailed mutagenesis and structure-activity studies.

NPY agonists are known to stimulate appetite (Dumont *et al*., 2000c). In contrast, the only report on the effect of NPFF on ingestive behaviour described reduction of food intake in rats (Murase *et al*., 1996). Similarly, GR231118 although acting as a NPY Y_4 agonist, has been found to decrease food intake in rats (Schober *et al*., 1998). Whether this effect is due to a possible interaction with a NPFF receptor subtype should be investigated in future studies.

In conclusion, our results describe the first NPFF receptors antagonist (BIBP3226) and suggest cross-reaction between BIBP3226 and GR231118 with NPFF receptors when using these compounds to investigate the NPY receptors.

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