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Non-competitive binding of the nonpeptide antagonist BIBP3226 to rat forebrain neuropeptide Y_1 receptors

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Abstract

[^{3}H]Neuropeptide Y labelled neuropeptide Y receptors in rat forebrain membranes as a homogenous class of high-affinity sites. Between 80 and 85% of these receptors showed high affinity for Y_1-selective antagonists such as (R)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-arginine amide (BIBP3226). While competitive in functional studies, BIBP3226 produced parallel shifts of the Scatchard plots of [^{3}H]neuropeptide Y saturation binding in rat forebrain membranes. Mechanisms which are routinely invoked to explain non-competitive binding do not apply to BIBP3226. Wash-out experiments, involving successive treatment of the membranes with BIBP3226, buffer (wash-out step) and [^{3}H]neuropeptide Y, argue against irreversible or a pseudo-irreversible binding of the antagonist. Allosteric inhibition is also unlikely since BIBP3226 did not affect the rate of dissociation of [^{3}H]neuropeptide Y in isotope dilution experiments. The non-hydrolyzable guanine nucleotide, 5′-guanylylimidodiphosphate (Gpp(NH)p), abolished the binding of [^{3}H]neuropeptide Y and increased its rate of dissociation in isotope dilution experiments. This suggests that the initial [^{3}H]neuropeptide Y-receptor association is a low affinity process and that the observed binding of [^{3}H]neuropeptide Y is related to the formation of a ternary [^{3}H]neuropeptide Y-receptor-G protein complex. Two- or even multistate models (in which BIBP3226 could potentially behave as an inverse agonist) could therefore be needed to explain the non-competitive antagonism of BIBP3226 in broken cell preparations. © 1997 Elsevier Science B.V.

Keywords: Neuropeptide Y; Neuropeptide Y Y_1 receptor; Brain, rat; BIBP3226; Radioligand binding

1. Introduction

The neurotransmitter neuropeptide Y is released both by central and peripheral neurons (Lundberg et al., 1982; O'Donohue et al., 1985; Stanley and Leibowitz, 1985). It is part of a family of homologous regulatory peptides (each 36 amino acids in length) which also includes peptide YY and pancreatic polypeptide (Tatemoto and Mutt, 1980; Tatemoto et al., 1982; Tatemoto, 1982). Neuropeptide Y exerts its effects by stimulating different receptor subtypes. Initial radioligand binding and functional assays made it possible to distinguish between neuropeptide Y Y_1, neuropeptide Y Y_2 and neuropeptide Y Y_3 receptors on the basis of differences in their pharmacological profile: high affinity of peptide YY and the synthetic analogue [Leu^{31},Pro^{34}]neuropeptide Y for the neuropeptide Y Y_1 receptor; high affinity of peptide YY and C-terminal fragments such as neuropeptide Y-(18–36) for the neuropeptide Y Y_2 receptor; very low affinity of peptide YY for the neuropeptide Y Y_3 receptor (Wahlestedt et al., 1992). More recent molecular cloning experiments have provided evidence of the existence of neuropeptide Y Y_1 and neuropeptide Y Y_2 receptors and of their belonging to the G protein-coupled seven transmembrane domain receptor family. This approach has also unveiled the existence of additional receptor subtypes; i.e., pancreatic polypeptide$_1$/neuropeptide Y Y_4 and neuropeptide Y Y_5 receptors (Larhammar et al., 1992; Bard et al., 1995; Lundell et al., 1995; Gerald et al., 1995, 1996).

In the periphery, neuropeptide Y coexists with noradrenaline in perivascular sympathetic fibers (Lundberg et al., 1990; Grundemar and Håkanson, 1993, 1994). In vascular smooth muscle, neuropeptide Y-stimulated neuropeptide Y Y_1 receptors provoke contraction directly as well as indirectly by potentiating the pressor response to

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other vasoconstrictors (Walker et al., 1991; Wahlestedt and Reis, 1993; Grundemar and Håkanson, 1994; Evequoz et al., 1994). To counteract these effects, much effort has already been spent in developing neuropeptide Y Y_1-selective receptor antagonists and, in this vein, a number of low molecular weight but potent nonpeptidic neuropeptide Y Y_1 antagonists, BIBP3226 and (1-[2-[2-(2-naphtylsulphamoyl)-3-phenylpropionamido]-3-[4[*N*-[4-(dimethylaminomethyl)-*trans*-cvclohexylmethyl] amidino]phenyl]propionyl]-pyrrolidine, (R,R) stereoisomer (SR120107A) and (1-[2-[2-(-naphtylsulfamoyl)-*cis*-cyclohexylmethyl]amidino]phenyl]propionyl]pyrrolidine (SR120819A)), have recently been synthesised (Rudolf et al., 1994; Serradeil-Le Gal et al., 1994, 1995; Lundberg et al., 1996).

Neuropeptide Y is also highly abundant in the central nervous system, where it participates in the control of a wide variety of functions including psychomotor activities, cognitive functions, sexual behavior, food intake, blood pressure regulation, circadian rhythmicity and neuroendocrine regulation (O'Donohue et al., 1985; Stanley and Leibowitz, 1985). Neuropeptide Y receptors have already been studied in the central nervous system of rats, pigs, cows and humans by radioligand binding and autoradiographic approaches. From these studies, it appears that the abundance of neuropeptide Y receptors varies from one region to another and, for the same brain region, even from one species to another (Busch-Sorensen et al., 1989; Widdowson and Halaris, 1990; Dumont et al., 1990, 1993; Czerwiec et al., 1996). Neuropeptide Y Y_2 receptors clearly predominate in the hippocampus of all investigated species. Neuropeptide Y Y_1 receptors predominate in most other brain regions of rats, pigs and cows, but their concentration is appreciably lower than that of the neuropeptide Y Y_2 receptors in the human frontal cortex. Less is known about the neuropeptide Y Y_3 receptor subtype that has been identified in the brainstem of the rat (Grundemar et al., 1991a,b).

The initial aim of the present study was to use the novel nonpeptidic neuropeptide Y Y_1-selective antagonist BIBP3226 as a tool to distinguish these receptors from the other neuropeptide Y receptor subtypes on membrane preparations from rat forebrain. This antagonist has been shown to be a competitive antagonist of neuropeptide Y Y_1 receptors in a number of functional assays in cells lines and isolated organs (Abounader et al., 1995; Doods et al., 1995; Wieland et al., 1995). In the present study, BIBP3226 produced unexpected parallel shifts of the Scatchard plots of the [^{3}H]neuropeptide Y saturation binding data. The lack of related radioligand binding data in the literature prompted us to perform a number of kinetic experiments to investigate the potential reasons for this apparent non-competitivity of BIBP3226. These experiments shed light on the hitherto unexpected complexity of the binding of [^{3}H]neuropeptide Y to the neuropeptide Y Y_1 receptors in rat forebrain membranes.

2. Materials and methods

2.1. Materials

N-[propionyl-^{3}H] neuropeptide Y ([^{3}H]neuropeptide Y) (80 Ci/mmol) was obtained from Amersham (Little Chalfont, UK). Neuropeptide Y (neuropeptide Y, porcine), polypeptide Y (peptide YY, porcine) and the analogues [Leu^{31},Pro^{34}]neuropeptide Y (porcine) and neuropeptide Y-(18–36) (porcine) were from Serva (Heidelberg, Germany). Bovine serum albumin (BSA, Fraction V) was from Sigma (St. Louis, MO, USA). BIBP3226 ((R)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-D-arginine amide) and SR120107A ((1-[2-[2-(2-naphtylsulphamoyl)-3-phenylpropionamido]-3-[4[*N*-[4-(dimethylaminomethyl)-*trans*-cvclohexylmethyl] amidino]phenyl]propionyl]-pyrrolidine, (*R,R*) stereoisomer) were from Albany Molecular Research (Albany, NY, USA). All other chemicals were of the highest grade commercially available.

2.2. Membrane preparations

Frozen rat forebrains were obtained from Iffa Credo (Belgium). The subsequent steps were carried out at 0–4°C. The brain samples were homogenized with an Ultraturrax and Potter–Elvejhem homogenizer in Krebs–Ringer buffer (137 mM NaCl/2.68 mM KCl/2.05 mM $MgCl_2$/1.80 mM $CaCl_2$/20 mM HEPES (pH 7.4)). The homogenate was centrifuged at $30\,000 \times g$ for 20 min and pellets were resuspended in the same buffer. This procedure was repeated twice and the final pellet was resuspended in Krebs–Ringer buffer containing 10% glycerol (v/v). The obtained suspensions were stored in Eppendorf tubes in 1 ml batches and kept at −80°C until used. Batches were thawed and homogenized in Krebs–Ringer buffer and washed by two centrifugations ($30\,000 \times g$, 20 min) prior to use.

2.3. Protein concentration determination

Protein concentrations were determined by a modification of the Sopachem ultra sensitive total protein assay, based on the pyragallol red–molybdate complex method (Watanabe et al., 1986) with bovine serum albumine (BSA) as standard.

2.4. Radioligand binding

Assays were performed in 200 μl Krebs–Ringer buffer containing 0.1% w/v BSA in plastic 96-well plates. Unless otherwise stated, rat forebrain membrane suspensions (100–200 μg protein/assay) were incubated for 90 min at 30°C with [^{3}H]neuropeptide Y (0.1 to 2.5 nM in saturation experiments and 0.5 nM in competition experiments). The incubation was stopped by rapid filtration through glass fiber filters (Whatman GF/C, incubated in an aqueous

solution of 0.3% (v/v) polyethyleneimine 15 min prior to filtration and prewashed with ice-cold Krebs–Ringer buffer) by a Skatron Cell Harvester. Filters were washed 4 times with ice-cold buffer, the first time for 2 s and then for 1 s each time. Filters were dried for 10 s in the Harvester and for 1 h at 40°C in an oven. Filters were sealed together with MeltiLex in a sample bag and radioactivity was counted in a Betaplate™.

2.5. Data analysis

Non-specific binding of [^{3}H]neuropeptide Y to the membranes was assessed in the presence of 0.1 μM neuropeptide Y. At a final concentration of 0.5 nM total binding of [^{3}H]neuropeptide Y was approximately 700 cpm per assay, whereas non-specific binding was 140 cpm per assay. The latter value was subtracted from the total binding to yield specific binding. The amount of binding was converted in fmol/mg protein, according to a counting efficiency of 50% of the betaplate counter. All binding experiments were performed in triplicate. Kinetic parameters, IC_{50}- and K_d values, were calculated by non-linear regression analysis, using GraphPad Prism. Values are given as means ± standard error of the mean (S.E.M.).

3. Results

Saturation binding experiments with [propionyl-^{3}H]neuropeptide Y ([^{3}H]neuropeptide Y) revealed that rat forebrain membranes contain an apparently homogeneous class of high-affinity sites for this radioligand ($B_{max} = 202 \pm 15$ fmol/mg protein, $K_d = 1.07 \pm 0.15$ nM, $n_H = 1.02 \pm 0.02$, $n = 3$).

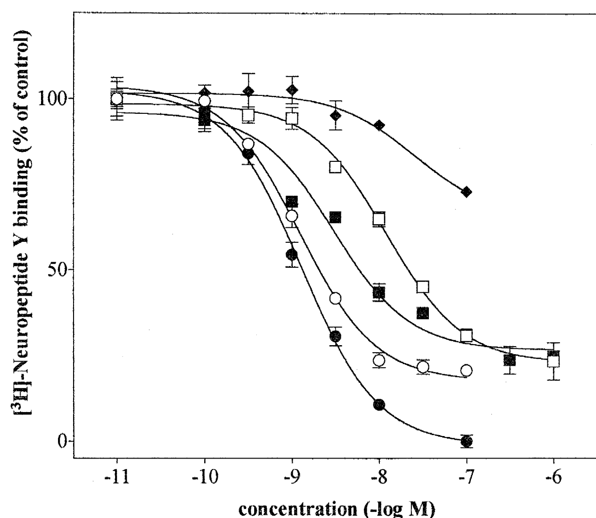

Fig. 1. [^{3}H]Neuropeptide Y competition binding to rat forebrain membranes. Membranes were incubated with 0.5 nM [^{3}H]neuropeptide Y for 90 min in the presence of increasing concentrations of porcine neuropeptide Y (●), porcine [Leu^{31},Pro^{34}]neuropeptide Y (○), BIBP3226 (■), SR120107A (□) and neuropeptide Y-(18–36) (▼). pIC_{50}- and n_H values are listed in Section 3.

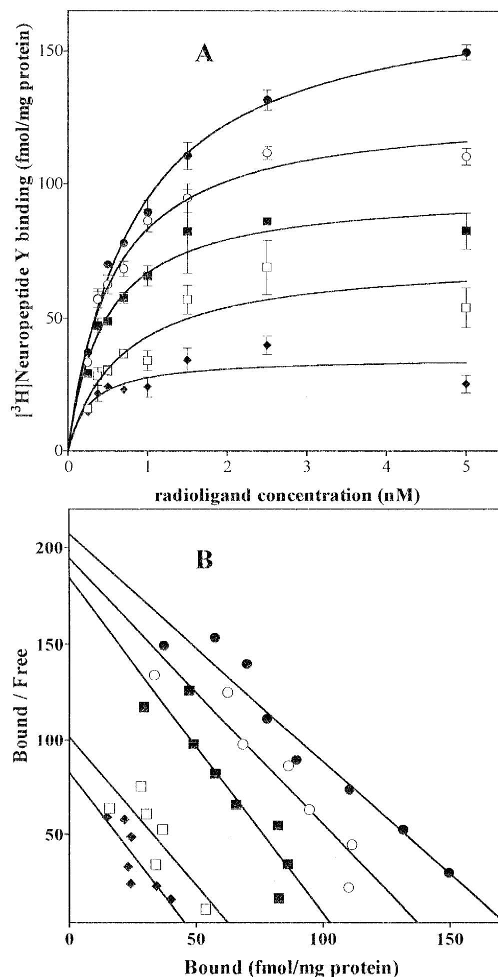

Fig. 2. Saturation binding of [^{3}H]neuropeptide Y to rat forebrain membranes in the absence (●) or presence of 0.1 nM (○), 1 nM (■), 10 nM (□) or 100 nM (♦) BIBP3226. (A) Specific binding of [^{3}H]neuropeptide Y (as fmol/mg protein) was calculated by subtracting non-specific binding in the presence of 0.1 mM unlabelled neuropeptide Y from the total binding. (B) Scatchard plot of the [^{3}H]neuropeptide Y saturation binding data in (A). Bound, specific binding; free, concentration free [^{3}H]neuropeptide Y. Linear regression analysis yielded the following B_{max} values: 175 (●), 140 (○), 105 (■), 65 (□) and 48 (♦) fmol/mg protein, respectively.

Competition curves with unlabeled neuropeptide Y (Fig. 1) were steep ($n_H = 0.93 \pm 0.05$) and, therefore, also compatible with the presence of a uniform population of sites with high affinity for neuropeptide Y and peptide YY ($pIC_{50} = 9.0 \pm 0.1$ and 8.3 ± 0.2, respectively). In contrast, the neuropeptide Y Y_1 selective compounds [Leu^{31},Pro^{34}]neuropeptide Y, BIBP3226 and SR120107 A displayed high affinity for only 80 to 85% of neuropeptide Y-displaceable sites ($pIC_{50} = 8.9 \pm 0.1$, 8.2 ± 0.1 and 7.9 ± 0.1 and $n_H = 0.84 \pm 0.06$, 0.83 ± 0.06, 0.80 ± 0.04, respectively) (Fig. 1), neuropeptide Y-(18–36), the neuropeptide Y Y_2 selective C-terminal fragment of neuropeptide Y, had only a limited ability to displace the binding (Fig. 1). Although indicative of the heterogeneity of the neuropeptide Y receptors, these data indicate that the neuropeptide Y Y_1 receptors constitute the majority of the

Table 1
Saturation binding parameters of [^{3}H]neuropeptide Y to rat forebrain membranes: effect of pre- and co-incubation with 10 nM BIBP3226

Condition	K_d (nM)	B_{max} (% of control value [a])	n_H
Control	1.5 ± 0.3	100	1.02 ± 0.02
Coincubation BIBP3226	1.6 ± 0.3	41 ± 10	1.01 ± 0.03
Preincubation + wash 5 min	2.6 ± 0.1	91 ± 10	1.12 ± 0.03
Preincubation + wash 60 min	2.1 ± 0.1	93 ± 13	1.03 ± 0.02

Membranes were incubated for 90 min with increasing concentrations of radioligand either in the absence (control) or presence of 10 nM BIBP3226 (co-incubation). Alternatively, membranes were preincubated with 10 nM BIBP3226 for 20 min at 30°C, centrifuged for 5 min at 9000× g, resuspended in buffer and incubated at 30°C for 5 min (wash-out of 5 min) or 60 min (washout of 60 min), centrifuged for 5 min at 9000× g and finally resuspended in buffer and incubated at 30°C for 90 min with increasing concentrations of [^{3}H]neuropeptide Y. The K_d and B_{max} values were calculated by non-linear regression analysis and are the average ± S.E.M. of 3–6 independent experiments with triplicate determinations.

[a] The control B_{max} value was 175 fmol/mg protein.

[^{3}H]neuropeptide Y binding sites in rat forebrain membranes.

To establish the nature of the antagonism exerted by BIBP3226, its effect on the saturation binding of [^{3}H]neuropeptide Y was determined. As shown in Fig. 2, the addition of BIBP3226 produced concentration-dependent, almost parallel shifts of the Scatchard plots of [^{3}H]neuropeptide Y saturation binding. The effects of the different concentrations of BIBP3226 on the B_{max} and K_d values of [^{3}H]neuropeptide Y (calculated from different experiments by non-linear regression) are summarised in the legend of Fig. 2. Competition binding experiments, performed with concentrations of [^{3}H]neuropeptide Y ranging between 0.25 and 2.5 nM, also indicated that radioligand concentration has little impact on the IC_{50} of BIBP3226 (Table 1).

Association experiments were performed to assess whether the decrease in B_{max} observed in saturation experiments could be attributed to BIBP3226 affecting the rate of [^{3}H]neuropeptide Y receptor binding. As shown in Fig. 3, [^{3}H]neuropeptide Y receptor association was rather slow at 30°C, with a pseudo-first-order rate constant (k_{obs}) of 0.033 ± 0.009 min^{-1} (for 0.5 nM [^{3}H]neuropeptide Y). At concentrations, ranging between 0.1 and 100 nM, BIBP3226 did not markedly affect the k_{obs} for [^{3}H]neuropeptide Y binding (Table 2). Instead, the antagonist caused a profound, concentration-dependent decrease in radioligand binding for each incubation period.

To investigate possible allosteric interactions, the dissociation of [^{3}H]neuropeptide Y from rat forebrain membranes was evaluated by the addition of an excess of unlabeled neuropeptide Y with or without BIBP3226. The rationale for this experiment is based on the fact that (a) a supramaximal concentration of neuropeptide Y prevents the (re)association of [^{3}H]neuropeptide Y to the receptor

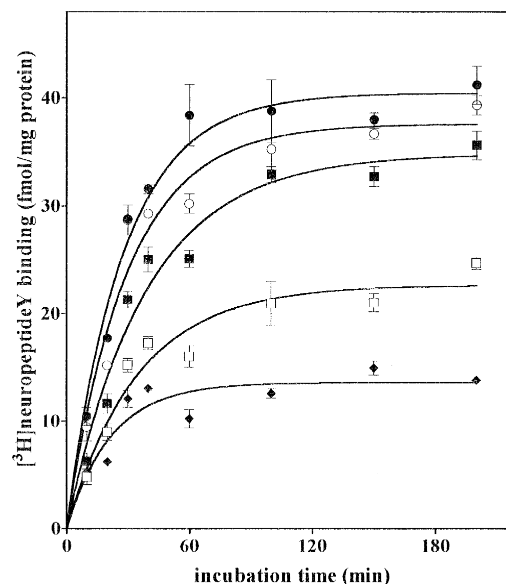

Fig. 3. Association binding of [^{3}H]neuropeptide Y to rat forebrain membranes. Specific binding of [^{3}H]neuropeptide Y (as fmol/mg protein) was measured after different periods of time (abscissa) in the absence (●) or presence of 0.1 nM (○), 1 nM (■), 10 nM (□) or 100 nM (♦) BIBP3226. The corresponding pseudo-first-order rate constants for association (k_{obs}) are given in Table 2.

sites and that (b) if the addition of BIBP3226 alters the dissociation rate of the radioligand, it must do so by interacting at a different allosteric site. As shown in Fig. 4 and Table 3, dissociation of the radioligand was very slow in the presence of excess unlabeled neuropeptide Y (first-order dissociation rate constant, $k_2 = 0.050 \pm 0.012$ min^{-1}) and was not further accelerated by the presence of 10 to 1000 nM BIBP3226. However, when BIBP3226 was replaced by 1 mM 5′-guanylylimidodiphosphate (GppNHp) in the same experiments, this non-hydrolyzable analogue of guanosine 5′ triphosphate (GTP) produced a net increase in the dissociation rate of [^{3}H]neuropeptide Y ($k_2 = 0.53 \pm 0.11$ min^{-1}, Fig. 4). In agreement with these kinetic experiments, the specific binding of [^{3}H]neuropeptide Y was almost completely abolished (decline of 83 ± 8%) when 5′-guanylylimidodiphosphate (GppNHp) was present in the medium form the very start of the incubation.

Table 2
BIBP3226/[^{3}H]neuropeptide Y competition binding parameters at different radioligand concentrations

[^{3}H]NPY concentration (nM)	pIC_{50}	n_H
0.25	8.4 ± 0.05	0.73 ± 0.10
0.50	8.2 ± 0.09	0.83 ± 0.06
1.00	8.2 ± 0.04	1.04 ± 0.12
2.5	8.2 ± 0.08	1.00 ± 0.12

BIBP3226 competition binding experiments were performed by using the listed concentrations of [^{3}H]neuropeptide Y. pIC50- and n_H values were calculated by non-linear regression analysis and are the average ± S.E.M. of 3–4 independent experiments with triplicate determinations.

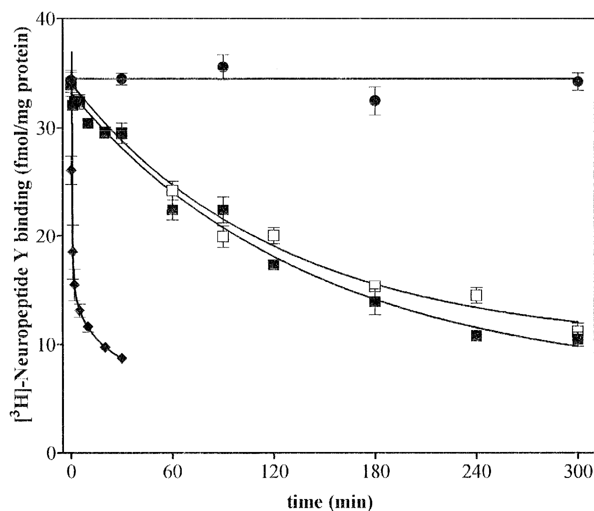

Fig. 4. Dissociation of $[^3H]$neuropeptide Y from rat forebrain membranes. Membranes were incubated for 60 min with 0.5 nM $[^3H]$neuropeptide Y, after which dissociation was initiated by addition of 0.1 μM unlabelled neuropeptide Y in the absence (■) or presence of 0.1 mM BIBP3226 (□) or 1 mM Gpp(NH)p (♦). As a control for the stability of the binding sites, binding of $[^3H]$neuropeptide Y was continued without addition of unlabelled neuropeptide Y (●). Specific binding of $[^3H]$neuropeptide Y (as fmol/mg protein) was measured after different times (abscissa). $[^3H]$neuropeptide Y dissociation constants are given in Table 3.

To determine whether BIBP3226 binds to the receptor in an irreversible or slowly reversible manner, rat forebrain membranes were equilibrated with or without 10 nM BIBP3226, centrifuged to remove free antagonist, incubated with buffer for various periods of time (wash-out step), centrifuged again and finally incubated for 90 min with increasing concentrations of $[^3H]$neuropeptide Y. The results, depicted in Table 4, indicate that, even after a very short wash-out step, the $[^3H]$neuropeptide Y saturation binding curve of the BIBP3226-treated membranes almost

Table 3
Association rate constants (k_{obs}) of $[^3H]$-neuropeptide Y binding to rat forebrain membranes in the absence or presence of different concentrations of BIBP3226

BIBP3226 concentration (nM)	k_{obs} (min^{-1})	Maximal binding (% of control [a])
0	0.039 ± 0.008	100
0.1	0.036 ± 0.007	97 ± 4
1	0.034 ± 0.007	83 ± 5
3	0.033 ± 0.005	67 ± 3
10	0.033 ± 0.003	50 ± 4
30	0.046 ± 0.010	33 ± 3
100	0.056 ± 0.017	25 ± 4

The rate constants and the maximal specific binding (as % of binding in the absence of BIBP3226) were calculated by non-linear regression analysis and are given as the average ± S.E.M. of 3–4 independent experiments with triplicate determinations.
[a] Control specific binding of $[^3H]$neuropeptide Y after an incubation time of 60 min was 34 fmol/mg protein.

Table 4
Dissociation rate constants (k_2) of $[^3H]$neuropeptide Y binding to rat forebrain membranes in the absence or presence of different concentrations of BIBP3226

BIBP3226 concentration (nM)	k_2 (min^{-1})
0	0.0050 ± 0.0012
10	0.0046 ± 0.0015
100	0.0077 ± 0.0012
1000	0.0046 ± 0.0010

The experiments were performed as described in the legend of Fig. 4. The rate constants were calculated by non-linear regression analysis and are given as the average ± S.E.M. of 3 independent experiments with triplicate determinations.

matched the curve for the control membranes (i.e., which were not pretreated with BIBP3226). When BIBP3226 was also present during the incubation step, there was a decline in the binding of about 60% for each $[^3H]$neuropeptide Y concentration.

4. Discussion

Previous radioligand binding and autoradiographic experiments have revealed that neuropeptide Y receptors of the neuropeptide Y Y_1 subtype prevail over their neuropeptide Y Y_2 counterparts in most of the forebrain areas of the rat, with the hippocampus being the most noteworthy exception (Busch-Sorensen et al., 1989; Dumont et al., 1990, 1993; Widdowson and Halaris, 1990; Czerwiec et al., 1996). These studies were carried out with selective analogues of neuropeptide Y such as the neuropeptide Y Y_1 (versus neuropeptide Y Y_2)-selective [Leu^{31}, Pro^{34}]neuropeptide Y and neuropeptide Y Y_2 (versus neuropeptide Y Y_1) selective C-terminal fragments neuropeptide Y-(13–36) and neuropeptide Y-(18–36) and, based on this approach, the present study confirms the prevalence of neuropeptide Y Y_1 receptors in rat forebrain membranes (Fig. 1). However, additional neuropeptide Y receptor subtypes have emerged recently and their pharmacological characterisation in transfected cell lines has shed light on the limited neuropeptide Y Y_1 or neuropeptide Y Y_2 selectivity of the above compounds (Gerald et al., 1996). The resulting doubts about the nature and distribution of neuropeptide Y receptor subtypes in complex tissues such as the CNS can partly be overcome by using BIBP3226, a newly developed nonpeptidic antagonist which displays pronounced neuropeptide Y Y_1 selectivity when compared to the other neuropeptide Y receptor subtypes known to date (Jacques et al., 1995; Gerald et al., 1996). In rat forebrain membranes, this antagonist displaced 80 to 85% of the binding of $[^3H]$neuropeptide Y with high affinity (Fig. 1). The IC_{50} of BIBP3226 was comparable to values previously reported for neuropeptide Y Y_1 receptors in rat and human (Rudolf et al., 1994; Gerald et al., 1996) and this confirms the opinion that neuropeptide Y Y_1 receptors constitute the

most abundant neuropeptide Y receptor subtype in rat forebrain membranes.

In rat forebrain membranes, the addition of BIBP3226 in [^{3}H]neuropeptide Y saturation binding experiments produced concentration-dependent, almost parallel shifts of the Scatchard plots (Fig. 2). The apparent non-competitive nature of BIBP3226 in the present radioligand binding experiments was unexpected as it contrasts with its reported competitivity in functional experiments with human SK-N-MC cells, human cerebral arteries and rabbit saphenous vein and vas deferens (Rudolf et al., 1994; Abounader et al., 1995; Doods et al., 1995; Jacques et al., 1995; Wieland et al., 1995). The reasons for this discrepancy may be multiple and could evidently be related to one of the many differences between the experimental systems such as buffer composition, incubation time, cellular integrity, species, etc. Since the prime purpose of our study was to investigate neuropeptide Y receptors in brain we investigated in depth the potential explanations for the binding properties of BIBP3226 in the present experimental system. [^{3}H]neuropeptide Y was used as the radioligand as it is structurally much closer to the endogeneous ligand for neuropeptide Y receptors than its radioiodinated analogues which possess a bulky iodine atom. Yet, useful complementary information can be gained by direct binding studies with [^{3}H]BIBP3226 (Entzeroth et al., 1995), but this approach is hampered by the high extent of non-specific binding of this radioligand to rat forebrain membranes (> 90% of total binding, data not shown). Explorative experiments were also done with membranes from various regions of human brain but, in agreement with previous studies (Widdowson and Halaris, 1990), they were shown to contain very few neuropeptide Y receptors of the Y_1 subtype so that no information could be gained about their interaction with BIBP3226.

A number of different mechanisms, including irreversible, slowly reversible (pseudo-irreversible) and allosteric inhibition are routinely invoked to explain non-competitive binding profiles (Robertson et al., 1992; Olins et al., 1994; Renzetti et al., 1995). Non-competitive inhibition can evidently occur when BIBP3226 forms an irreversible covalent bond with the receptor, such that the receptor number is effectively reduced. A less radical explanation is that the dissociation of BIBP3226 is very slow, such that [^{3}H]neuropeptide Y cannot reach equilibrium with the antagonist–receptor complex under the time constraints of the experiment (Olins et al., 1994). Under these conditions, the non-competitivity of the inhibition is only apparent. However, wash-out experiments, in which the membranes were successively treated with BIBP3226 (preincubation step), buffer only (wash-out step) and [^{3}H]neuropeptide Y (incubation step), revealed that the antagonist did not bind to the neuropeptide Y Y_1 receptor in an irreversible or a pseudo-irreversible fashion. Indeed, whereas 10 nM BIBP3226 produced a profound decrease in the B_{max} of [^{3}H]neuropeptide Y when present in the incubation step, the binding of [^{3}H]neuropeptide Y was completely restored in the wash-out experiments, even when the wash-out time was kept to a minimum (Table 4). This indicates that nearly all the BIBP3226-neuropeptide Y Y_1 receptor complexes had the possibility to dissociate during the incubation step with [^{3}H]neuropeptide Y and, hence, that the half-life of the complex is shorter than the standard incubation time of 90 min.

To further explore the possibility that the non-competitivity of BIBP3226 is only apparent and merely related to the kinetics of [^{3}H]neuropeptide Y and BIBP3226 binding, computer-assisted simulations were performed using the equation of Motulsky and Mahan (Motulsky and Mahan, 1983). This equation describes the binding of the radioligand as a function of time and provided that the radioligand and eventual inhibitor interact with a single class of sites (a condition which is apparently met for [^{3}H]neuropeptide Y and BIBP3226 binding to the neuropeptide Y Y_1-receptor) and in a competitive fashion, it allows the simulation of association, saturation and competition binding data even before equilibrium is reached. Based on the known association and dissociation rate constants of [^{3}H]neuropeptide Y ($k_1 = 5.6 \times 10^7$ M^{-1} min^{-1} and $k_2 = 5 \times 10^{-3}$ min^{-1}, respectively), iterative calculations with this equation revealed that a continuous range of paired association (k_3) and dissociation rate constants (k_4) of BIBP3226 may account for its experimental IC_{50} of 6.3 nM under the standard incubation conditions (Fig. 5). However, based on the wash-out experiments, it is reasonable to assume that the half-life of the BIBP3226-receptor complex is less than 45 min, so that the k_4-values for this

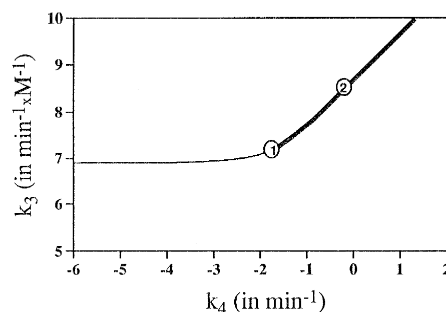

Fig. 5. Possible association (k_3) and dissociation rate constants (k_4) of BIBP3226 yielding an experimental IC_{50} of 6.3 nM (Fig. 1). Computer-assisted simulations of BIBP3226/[^{3}H]neuropeptide Y competition binding curves were performed using the equation of Motulsky and Mahan (Motulsky and Mahan, 1983) for k_3 values ranging between 1×10^5 and 1×10^{10} M^{-1} min^{-1} and for k_4 values ranging between 1×10^{-6} and 1×10^2 min^{-1}. The dissociation rate constant (k_2) of BIBP3226 was 5×10^{-3} min^{-1} (Table 3) and its association rate constant (k_1, calculated from its k_{obs} (Table 2) and k_2) was 5.6×10^7 M^{-1} min^{-1}. The [^{3}H]neuropeptide Y concentration was set at 0.5 nM and the incubation time at 90 min. The line represents all association/dissociation rate constant combinations; the thick line represents combinations with a half-life of the BIBP3226-receptor complex of 45 min or less. Encircled numbers correspond to a half-life of the BIBP3226-receptor complex of 45 min (1) and 0.5 min (2).

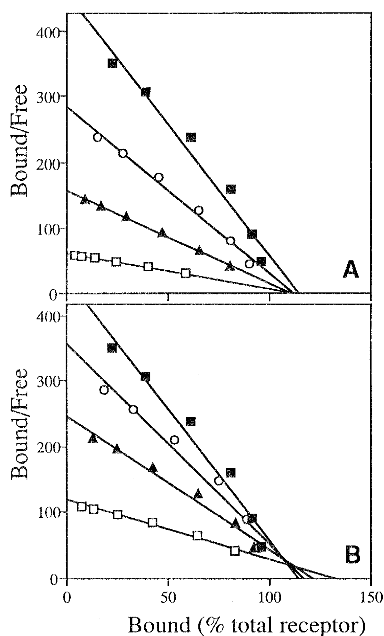

Fig. 6. Scatchard plots of theoretical [^{3}H]neuropeptide Y saturation binding curves: effect of BIBP3226. Computer-assisted simulations of [^{3}H]neuropeptide Y saturation binding curves done by using the equation of Motulsky and Mahan (Motulsky and Mahan, 1983). The incubation time was set at 90 min and the k_1 and k_2 values for [^{3}H] neuropeptide Y were 5.6×10^7 $M^{-1}min^{-1}$ and 5×10^{-3} min^{-1}, respectively (Fig. 5). The half-life of the BIBP3226-receptor complex was set to (A) 45 min (encircled 1 in Fig. 5, $k_3 = 1.8\times10^7$ $M^{-1}min^{-1}$ and $k_4 = 2.2\times10^{-2}$ min^{-1}) and to (B) 0.5 min (encircled 2 in Fig. 5, $k_3 = 3.5\times10^8$ M^{-1} min^{-1} and $k_4 = 1.4$ min^{-1}). BIBP3226 concentrations were set to 0 (control, ■), 1 nM (○), 3 nM (▲) and 10 nM (□). Lines correspond to linear regression analysis of the Scatchard plots; the calculated B_{max} and K_d-values are listed in Table 5.

antagonist are likely to exceed 0.015 min^{-1} (Fig. 5, thick line).

The effect of various concentrations of BIBP3226 on [^{3}H]neuropeptide Y saturation binding was then simulated for two particular situations; one in which the antagonist dissociates slowly ($t_{1/2} = 45$ min, Fig. 6A) and the other in which the antagonist dissociates rapidly ($t_{1/2} = 0.5$ min, Fig. 6B). The Scatchard plots of the simulated saturation binding data were slightly bell-shaped (Fig. 6) and this may be explained by the fact that, at low concentrations, the incubation time is insufficient for binding to approach equilibrium. An important consequence of this shape is that the B_{max}-values, which are calculated either by linear regression analysis of the plots or by non-linear regression analysis of the binding data themselves (Table 5), will be overestimated, i.e., the actual receptor concentration is lower. Yet, these different types of calculations revealed that the addition of both fast- and slow-dissociating BIBP3226 would produce a dose-dependent increase in the K_d of the radioligand, without greatly affecting its B_{max} (Fig. 6, Table 5). The simulated data, which were based on the ability of BIBP3226 and [^{3}H]neuropeptide Y to bind reversibly to the same site according to the law of mass action, were thus unable to explain to the apparent non-competitive nature of BIBP3226.

Allosteric modulation of ligand binding has been shown to occur for several G protein-coupled receptors, including muscarinic, adrenergic, dopaminergic and adenosine receptors (Kostenis and Mohr, 1996). It is thus conceivable that, at least for the rat, BIBP3226 binds to a site on the neuropeptide Y Y_1 receptor or on a neuropeptide Y Y_1 receptor-containing macromolecular complex that is different from the neuropeptide Y binding site, and thereby induces a conformational change in the receptor that decreases its affinity for neuropeptide Y. Experimentally, such allosteric inhibition should involve a reduction in the association rate and/or an increase in the dissociation rate of [^{3}H]neuropeptide Y binding. Since an altered dissociation rate is unequivocally indicative of an allosteric interaction (Kostenis and Mohr, 1996), radioligand dissociation experiments are frequently performed to find out if an inhibitor acts allosterically. In this context, it is clearly shown in Fig. 4 and Table 3 that BIBP3226 did not affect

Table 5
Theoretical [^{3}H]neuropeptide Y saturation binding curves: effect of BIBP3226

BIBP3226 concentration (nM)	Non-linear regression		Scatchard plot analysis	
	K_d (nM)	B_{max} (% of control)	K_d (nM)	B_{max} (% of control)
0	0.22	109	0.22	110
$t_{1/2}$ for BIBP3226 dissociation = 45 min				
1	0.36	109	0.36	111
3	0.68	109	0.63	108
10	1.85	113	1.60	107
$t_{1/2}$ for BIBP3226 dissociation = 0.5 min				
1	0.28	112	0.33	118
3	0.42	115	0.50	122
10	1.00	127	1.10	133

Computer-assisted simulations of [^{3}H]neuropeptide Y saturation binding curves were done by using the equation of Motulsky and Mahan (Motulsky and Mahan, 1983). The parameters are the same as listed in the legend of Fig. 6. The half-life of the BIBP3226-receptor complex was set to 45 or 0.5 min and BIBP3226 concentrations were set to 0 (control), 1, 3 and 10 nM. B_{max} and K_d values for [^{3}H]neuropeptide Y were calculated by non-linear regression of the saturation binding curves and by linear regression of the Scatchard plots shown in Fig. 6.

the rate of dissociation of [^{3}H]neuropeptide Y from the neuropeptide Y Y_1 receptors in isotope dilution experiments. Accordingly, the dissociation experiments do not plead in favour of allosteric interactions between these ligands and the receptor. Yet, it cannot be excluded that BIBP3226 exerts allosteric control of [^{3}H]neuropeptide Y binding at the level of its association rate, but it is difficult to reveal such an effect in an unambiguous fashion (Kostenis and Mohr, 1996).

Since neuropeptide Y receptors belong to the G protein-coupled seven transmembrane domain receptor family, guanine nucleotides are required for the transduction of the neuropeptide Y receptor-mediated signal across the cell plasma membrane (Birnbaumer and Birnbaumer, 1995). Whereas intracellular guanosine 5′-triphosphate (GTP) is obviously present in functional assays, it is removed during the preparation of rat forebrain membranes. This difference led us to examine the possibility that the non-competitive behavior of BIBP3226 in forebrain membranes could be linked to the absence of guanine nucleotides in this system. We observed that the binding of [^{3}H]neuropeptide Y to its receptors in rat forebrain membranes was almost completely abolished in the presence of the non-hydrolyzable guanine nucleotide 5′-guanylylimidodiphosphate (GppNHp) and also that the nucleotide provoked a net increase in the rate of dissociation of [^{3}H]neuropeptide Y in isotope dilution experiments (Fig. 4). These findings indicate that 5′-guanylylimidodiphosphate (GppNHp) exerts allosteric control over [^{3}H]neuropeptide Y binding, a phenomenon which can easily be explained by assuming that the observed binding involves the formation of a ternary [^{3}H]neuropeptide Y-receptor-G protein complex. Such ternary complexes are likely to be formed since neuropeptide Y is an agonist and it is well known that guanosine 5′-triphosphate (GTP) and related guanine nucleotides are able to dissociate such complexes by interchanging with guanosine 5′-diphosphate (GDP) at the α-subunit of the G-protein (Birnbaumer and Birnbaumer, 1995). For monoamine receptors, such as adrenergic and dopamine receptors, it has been demonstrated that the receptor adopts a slow agonist-dissociating conformation when such ternary complexes are formed, a phenomenon which is usually referred to as ‘tight agonist binding’. Several models have been proposed in the past to describe this process (De Lean et al., 1980; Convents et al., 1987; Severne et al., 1986; Samama et al., 1993), the simplest being a two-step model: $L + R + G \Leftrightarrow LR + G \Leftrightarrow L.R.G$ (wherein L is the agonist ligand, R the receptor and G the G protein; $L \cdot R \cdot G$ complexes constitute the observable binding of radiolabelled L).

According to such a model, the present data suggest that neuropeptide Y receptors adopt a ‘tight neuropeptide Y binding’ conformation when the ternary complex is formed and, hence, that the observed binding of [^{3}H]neuropeptide Y reflects the process of receptor activation rather than merely representing the initial bimolecular association between [^{3}H]neuropeptide Y and the receptor. In this vein, several two-state models of the receptor (Gero, 1983; Kaumann and Frenken, 1985; De Chaffoy de Courcelles et al., 1986; Robertson et al., 1994) have been evoked to explain the ability of certain antagonists to decrease the maximal response of an agonist in functional studies, a phenomenon often referred to as ‘insurmountable antagonism’. Recently, much interest has been devoted to Gero’s model, wherein (a) the receptor is able to adopt an active and an inactive conformation, (b) the antagonist modifies the ratio active to inactive conformation and (c) their interconversion is too slow for the original equilibrium to be regained upon subsequent agonist exposure (Gero, 1983). This model has proven to be particularly useful for explaining the insurmountable nature of certain angiotensin-II receptor antagonists in functional studies (Robertson et al., 1994), but its extension to radioligand binding studies, as proposed by Renzetti et al. (1995), is questionable because the exposure of the receptor to the agonist occurs for a much longer period of time in these experiments.

The molecular basis for the apparent non-competitive nature of BIBP3226 binding in the present radioligand binding studies with [^{3}H]neuropeptide Y still remains to be established as it cannot be uncovered by the conventional tests for allosteric, irreversible or slowly reversible antagonist–receptor interactions. In this vein, our results with 5′-guanylylimidodiphosphate (GppNHp) suggest that the initial association between [^{3}H]neuropeptide Y and the uncoupled receptor is a low-affinity process and that the observed binding of [^{3}H]neuropeptide Y is related to receptor activation; i.e., the formation of a ternary [^{3}H]neuropeptide Y receptor-G protein complex. Two- or even multi-state models (in which BIBP3226 could potentially behave as an inverse agonist) are therefore needed to explain the non-competitive/insurmountable antagonism of BIBP3226 in rat brain membranes.

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References

Abounader, R., Villemure, J.-G., Hamel, E., 1995. Characterization of neuropeptide Y (NPY) receptors in human cerebral arteries with selective agonists and the new Y_1 antagonist BIBP 3226. Br. J. Pharmacol. 116, 2245–2250.

Bard, J.A., Walker, M.W., Branchek, T.A., Weinshank, R.L., 1995. Cloning and functional expression of a human Y_4 subtype receptor for pancreatic polypeptide, neuropeptide Y, and peptide YY. J. Biol. Chem. 270, 26762–26765.

Birnbaumer, L., Birnbaumer, M., 1995. Signal transduction by G proteins: 1994 edition. J. Recept. Signal Transduction Res. 15, 213–252.

Busch-Sorensen, M., Peikh, S.H., O'Hare, M., Tortoa, O., Schwartz, T.W., Gammeltoft, S., 1989. Regional distribution of neuropeptide Y and its receptor in the porcine central nervous system. J. Neurochem. 52, 1545.

Convents, A., De Backer, J.-P., Vauquelin, G., 1987. Tight agonist binding may prevent the correct interpretation of agonist competition binding curves of α_2-adrenergic receptors. Mol. Pharmacol. 32, 65–1152.

Czerwiec, E., De Backer, J.-P., Vauquelin, G., Vanderheyden, P.M.L., 1996. Neuropeptide Y receptors from calf brain: Effect of crude conus venom preparations on [^{3}H]neuropeptide Y binding. Neurochem. Int. 29, 669–676.

De Chaffoy de Courcelles, D., Leysen, J.E., Roevens, P., Van Belle, H., 1986. The serotonin-S2 receptor: A receptor, transducer coupling model to explain insurmountable antagonist effects. Drug Dev. Res. 8, 173–178.

De Lean, A., Stadel, J.M., Lefkowitz, R.J., 1980. A ternary complex model explains the agonist-specific properties of the adenylate cyclase-coupled β adrenergic receptor. J. Biol. Chem. 255, 7108–7117.

Dumont, Y., Fournier, A., St-Pierre, S., Schwattz, T.W., Quirion, R., 1990. Differential distribution of neuropeptide Y_1 and Y_2 receptors in the rat brain. J. Pharmacol. 191, 501–503.

Dumont, Y., Fournier, A., St-Pierre, S., Quirion, R., 1993. Comparative characterization and autoradiographic distribution of neuropeptide Y receptor subtypes in the rat brain. J. Neurosci. 13, 73–86.

Doods, H.N., Wienen, W., Entzeroth, M., Rudolf, K., Eberlein, W., Engel, W., Wieland, H.A., 1995. Pharmacological characterization of the selective nonpeptide neuropeptide Y Y1 receptor antagonist BIBP3226. J. Pharmacol. Exp. Ther. 275, 136–142.

Entzeroth, M., Braunger, H., Eberlein, W., Engel, W., Rudolf, K., Wienen, W., Wieland, H.A., Willim, K.-D., Doods, H.N., 1995. Labeling of neuropeptide Y receptors in SK-N-MC cells using the novel nonpeptide Y_1 receptor-selective antagonist [^{3}H]BIBP3226. Eur. J. Pharmacol. 278, 239–242.

Evequoz, D., Grouzmann, E., Beck Sickinger, A.G., Brunner, H.R., Waeber, B., 1994. Differential vascular effects of neuropeptide Y (NPY) selective receptor agonists. Experientia 50, 936–938.

Gerald, C., Walker, M.W., Vaysse, P.J., He, C., Branchek, T.A., Weinshank, R.L., 1995. Expression cloning and pharmacological characterization of a human hippocampal neuropeptide Y/peptide YY Y_2 receptor subtype. J. Biol. Chem. 270, 26758–26761.

Gerald, C., Walker, M.W., Criscione, L., Gustafson, E.L., Batzl-Hartmann, C., Smith, K.E., Vaysse, P., Durkin, M.M., Laz, T.M., Linemeyer, D.L., Schaffhauser, A.O., Whitebreadt, S., Hofbauer, K.G., Taber, R.I., Branchek, T.A., Weinshank, R.L., 1996. A receptor subtype involved in neuropeptide-Y-induced food intake. Nature 382, 168–171.

Gero, A., 1983. Desensitization, two-state receptors and pharmacological parameters. J. Theor. Biol. 103, 137–161.

Grundemar, L., Wahlestedt, C., Reis, D.J., 1991a. Long-lasting inhibition of the cardiovascular responses to glutamate and the baroreceptor reflex elicited by neuropeptide Y injected into the nucleus tractus solitarius. Neurosci. Lett. 122, 135–139.

Grundemar, L., Wahlestedt, C., Reis, D.J., 1991b. Neuropeptide Y acts at an atypical receptor to evoke cardiovascular depression and to inhibit glutamate responsiveness in brainstem. J. Pharmacol. Exp. Ther. 258, 633–638.

Grundemar, L., Håkanson, R., 1993. Multiple neuropeptide Y receptors are involved in cardiovascular regulation. Peripheral and central mechanisms. Gen. Pharmacol. 24, 785–796.

Grundemar, L., Håkanson, R., 1994. Neuropeptide Y effector systems: Perspectives for drug development. Trends Pharmacol. Sci. 15, 153–159.

Jacques, D., Cadieux, A., Dumont, Y., Quirion, R., 1995. Apparent affinity and potency of BIBP3226, a non-peptide neuropeptide Y receptor antagonist, on purported neuropeptide Y Y_1, Y_2 and Y_3 receptors. Eur. J. Pharmacol. 278, R3–R5.

Kaumann, A.J., Frenken, M., 1985. A paradox: The 5-HT_2 receptor antagonist ketanserin restores the 5-HT-induced contraction depressed by methysergide in large coronary arteries of calf. Naunyn-Schmiedebergs Arch. pharmacol. 328, 295–300.

Kostenis, E., Mohr, K., 1996. Two-point kinetic experiments to quantify allosteric effects on radioligand dissociation. Trends Pharmacol. Sci. 17, 280–283.

Larhammar, D., Blomqvist, A.G., Yee, F., Jazin, E., Yoo, H., Wahlestedt, C., 1992. Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y_1 type. J. Biol. Chem. 267, 10935–10938.

Lundberg, L.M., Terenius, L., Hokfelt, T., Martling, C.-R., Tatemoto, K., Polak, J., Bloom, S.R., Goldstein, M., 1982. Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and effects of NPY on sympathetic function. Acta Physiol. Scand. 116, 477–480.

Lundberg, L.M., Franco-Cereceda, A., Hemsen, A., Lacroix, J.S., 1990. Fundam. Clin. Pharmacol. 4, 373–391.

Lundberg, J., Modin, A., Malmström, R.E., 1996. Recent developments with neuropeptide Y receptor antagonists. Trends Pharmacol. Sci. 17, 301–304.

Lundell, I., Blomqvist, A.G., Berglund, M.M., Schober, D.A., Johnson, D., Statnick, M.A., Gadski, R.A., Gehlert, D.R., Larhammar, D., 1995. Cloning of a human receptor of the NPY receptor family with high affinity for pancreatic polypeptide and peptide YY. J. Biol. Chem. 270, 29123–29128.

Motulsky, H.J., Mahan, L.C., 1983. The kinetics of competitive radioligand binding predicted by the law of mass action. Mol. Pharmacol. 25, 1–9.

O'Donohue, T.L., Cornwall, B.M., Pruss, R.M., Mezey, E., Kiss, J.Z., Eiden, L.E., Massari, V.L., Tessel, R.E., Pickel, V.M., DiMaggio, D.A., 1985. Neuropeptide Y and peptide YY neuronal and endocrine systems. Peptides 6, 755–768.

Olins, G.M., Chen, S.T., McMahon, E., Palomo, M.A., Reitz, D.B., 1994. Elucidation of the insurmountable nature of an angiotensin receptor antagonist, SC-54629. Mol. Pharmacol. 47, 115–120.

Renzetti, A.R., Criscuoli, M., Salimbeni, A., Subisii, A., 1995. Molecular pharmacology of LR-B/081, a new non-peptide angiotensin A T_1 receptor antagonist. Eur. J. Pharmacol. 290, 151–156.

Robertson, M.J., Barnes, J.C., Drew, G.M., Clark, K.L., Marshall, F.H., Michel, A., Middlemiss, D., Ross, B.C., Scopes, D., Dowle, M.D., 1992. Pharmacological profile of GR117289 in vitro: A novel, potent and specific non-peptide angiotensin A T_1 receptor antagonist. Br. J. Pharmacol. 107, 1173–1180.

Robertson, M.J., Dougall, I.G., Harper, D., Mckechnie, K.C.W., Leff, P., 1994. Agonist–antagonist interactions at angiotensin receptors: Application of a two-state receptor model. Trends Pharmacol. Sci. 15, 364–369.

Rudolf, K., Eberlein, W., Engel, W., Wieland, H.A., Willim, K.D., Entzeroth, M., Wienen, W., Beck-Sickinger, A.G., Doods, H.N., 1994. The first highly potent and selective non-peptide Y Y_1 receptor antagonist: BIBP3226. Eur. J. Pharmacol. 271, R11–R13.

Samama, P., Cotecchia, S., Costal, T., Lefkowitz, R.J., 1993. A mutation-induced activated state of the β_2-adrenergic receptor, extending the ternary complex model. J. Biol. Chem. 268, 4625–4635.

Serradeil-Le Gal, C., Valette, G., Rouby, P.E., Pellet, A., Villanova, G., Foulon, L., Neliat, G., Chambon, J.P., Maffrand, J.-P., Le Fur, G., 1994. SR120107A and SR120819A: Two potent and selective, orally effective antagonists for NPY Y_1 receptors. Am. Soc. Neurosci. Abstr. 20, 907.

Serradeil-Le Gal, C., Valette, G., Rouby, P.E., Pellet, A., Oury-Dounat, F., Brossard, G., Lespy, L., Marthy, E., Neilat, G., de Cointet, P.,

Maffrand, J.-P., Le Fur, G., 1995. SR120819A, an orally active and selective neuropeptide Y Y_1 receptor antagonist. FEBS Lett. 362, 192–196.

Severne, Y., IJzerman, A., Nerme, V., Zimmerman, H., Vauquelin, G., 1986. Shallow agonist competition binding curves for β-adrenergic receptors: The role for tight agonist binding. Mol. Pharmacol. 31, 69–73.

Stanley, B.G., Leibowitz, S.F., 1985. Neuropeptide Y injected in the paraventricular hypothalamus: A powerful stimulant of feeding behaviour. Proc. Natl. Acad. Sci. USA 82, 3940–3943.

Tatemoto, K., 1982. Neuropeptide Y: The complete amino-acid sequence of the brain peptide. Proc. Natl. Acad. Sci. USA 79, 5485–5489.

Tatemoto, K., Mutt, V., 1980. Isolation of two novel candidate hormones using a chemical method for finding naturally occuring polypeptides. Nature 285, 417–418.

Tatemoto, K., Carlquist, M., Mutt, V., 1982. Neuropeptide Y: A novel brain peptide with structural similarities to polypeptide YY and pancreatic polypeptide. Nature 296, 659–660.

Wahlestedt, C., Reis, D.J., 1993. Neuropeptide Y-related peptides and their receptors: Are the receptors potential therapeutic drug targets?. Annu. Rev. Pharmacol. Toxicol. 32, 309–352.

Wahlestedt, C., Regunathan, S., Reis, D.J., 1992. Identification of cultured cells selectively expressing Y_1-, Y_2- or Y_3-type receptors for neuropeptide Y/peptide YY. Life Sci. 50, PL7–PL12.

Walker, P., Grouzmann, E., Burier, M., Waeber, B., 1991. The role of neuropeptide Y in cardiovascular regulation. Trends Pharmacol. Sci. 12, 111–115.

Watanabe, N., Kamei, S., Ohkubu, A., Yamanaka, M., Ohsawa, S., Makino, K., Tokuba, K., 1986. Urinary protein as measured with a pyrogallol red-molybdate complex, manually and in Hitachi 726 automated analyser. Clin. Chem. 32, 1551–1554.

Widdowson, P.A., Halaris, A.E., 1990. A comparison of the binding of $[^3H]$propionyl-neuropeptide Y to rat and human frontal cortical membranes. J. Neurochem. 55, 956–962.

Wieland, H.A., Willim, K.D., Entzeroth, M., Wienen, W., Rudolf, K., Eberlein, W., Engel, W., Doods, H.N., 1995. Subtype selectivity and antagonistic profile of the nonpeptide Y1 receptor antagonist BIBP3226. J. Pharmacol. Exp. Ther. 275, 143–149.