0022-3565/95/2751-0136$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 275, No. 1

Printed in U.S.A.
JPET 275:136–142, 1995

Pharmacological Characterization of the Selective Nonpeptide Neuropeptide Y Y1 Receptor Antagonist BIBP 3226

HENRI N. DOODS, WOLFGANG WIENEN, MICHAEL ENTZEROTH, KLAUS RUDOLF, WOLFGANG EBERLEIN, WOLFHARD ENGEL and HEIKE A. WIELAND

Preclinical Research, Dr. Karl Thomae GmbH, D-88397 Biberach, Germany

Accepted for publication June 6, 1995

ABSTRACT

The present study was undertaken to investigate the *in vitro* and *in vivo* pharmacological profile of the novel, nonpeptide neuropeptide Y (NPY) Y1-selective antagonist, BIBP 3226 {(*R*)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-arginineamide}, and a recently described peptidic structure [Ile-Glu-Pro-Orn-Tyr-Arg-Leu-Arg-Tyr-NH_2, cyclic (2,4'), (2',4)-diamide]. BIBP 3226 antagonized the NPY Y1 receptor-mediated decrease in the twitch response in the rabbit vas deferens preparation with a pK_b value of 6.98 ± 0.06 (n = 16). It showed no affinity (EC_{50} > 1 μM) for NPY Y2 receptors in the rat vas deferens. NPY-induced increases in perfusion pressure in the isolated perfused rat kidney and rabbit ear preparations were antagonized with IC_{50} values of 26.8 ± 4.5 (n = 4) and 214 ± 30 nM (n = 4), respectively. The NPY-mediated potentiation of the noradrenaline elicited increase in perfusion pressure in the rat mesenteric bed was antagonized with an IC_{50} value of 976 (542–1760) nM. The NPY-induced increase in blood pressure in the pithed rat was inhibited by BIBP 3226 dose-dependently (ED_{50} = 0.11 ± 0.03 mg/kg i.v.), whereas no effect of BIBP 3226 (1 mg/kg i.v.) was observed for the noradrenaline-, angiotensin-, endothelin- or vasopressin-induced pressor response. The data presented demonstrate that BIBP 3226 is a competitive and NPY Y1-selective antagonist. The peptidic compound proved to possess high potency for NPY Y1 receptors, but showed both agonistic as well as antagonistic properties. BIBP 3226 in doses up to 3 mg/kg i.v. did not lower blood pressure in conscious spontaneously hypertensive rats. This might indicate that NPY or the NPY Y1 receptor do not play a relevant role in the maintenance of blood pressure in the spontaneously hypertensive rat.

NPY, a 36 residue peptide discovered by Tatemoto *et al.* (1982), is found both in peripheral nerves and in the circulation. The cardiovascular effects of NPY are profound and have received widespread attention (Walker *et al.*, 1991; Grundemar and Håkanson, 1993; McDermott *et al.*, 1993; Zukowska-Grojec and Wahlestedt, 1993). NPY is able to produce vasoconstriction and also is able to potentiate the vasoconstriction of other vasoactive agents such as noradrenaline (Edvinsson *et al.*, 1984; Pernow *et al.*, 1987; Grundemar *et al.*, 1992). Accordingly, when administered i.v. NPY increases vascular resistance in a variety of vascular beds and produces an increase in blood pressure by stimulating postjunctionally located NPY receptors (MacLean and Hiley, 1990; Modin *et al.*, 1993). In addition, NPY can stimulate prejunctionally located receptors and thereby suppress the release of noradrenaline (Pernow *et al.*, 1986, 1987). The effects of NPY are mediated by at least two different receptor subtypes classified as Y1 and Y2 (Michel, 1991; Grundemar and Håkanson, 1994; Gehlert, 1994). Until recently these NPY receptor subtypes could only be distinguished on the basis of the selectivity pattern of peptide analogs or C-terminal fragments of NPY in terms of affinity and their agonistic properties for these subtypes. As has been described for most receptor systems known so far, receptor subclassification is most reliable when nonpeptide antagonists are used. Therefore, the nonpeptide NPY antagonist BIBP 3226 {(*R*)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-arginineamide}, which displays high affinity and selectivity for the Y1 receptor (Rudolf *et al.*, 1994a,b; Wieland *et al.*, 1995) may be an important tool to study the heterogeneity of the NPY receptor.

In addition, because studies using selective Y1 or Y2 agonists revealed that most cardiovascular actions of NPY, such as vasoconstriction, appear to involve Y1 receptors, BIBP 3226 will be an interesting pharmacological tool to elucidate the (patho)physiological role of Y1 receptors in the cardiovascular system.

Accordingly, it was the aim of the present study to characterize the antagonistic properties of BIBP 3226 in several functional *in vitro* pharmacological models and to investigate the effects of the compound on blood pressure in rats. Moreover, because recently an interesting series of peptide NPY antagonists have been published in a patent of the Wellcome Foundation, Ltd. (Daniels *et al.*, 1994), we compared the pharmacological profile of one of those antagonists [in this paper referred to as EXBP 68 [Ile-Glu-Pro-Orn-Tyr-Arg-Leu-

Received for publication January 17, 1995.

ABBREVIATIONS: NPY, neuropeptide Y; SH, spontaneously hypertensive.

Arg-Tyr-NH_2, cyclic (2,4'), (2',4)-diamide)] with that of BIBP 3226.

Methods

Rat and rabbit vas deferens. Prostatic segments of rat (Chbb: Thom, 300 g) and rabbit (Chbb: NZW, 3–3.5 kg, live-stock breeding of Dr. Karl Thomas GmbH, Biberach, Germany) vasa deferentia were dissected and suspended in 25-ml organ baths at 37°C containing a modified Krebs' buffer solution of the following composition (millimolar); NaCl, 118; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 2.5; NaH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 11.1; gassed continuously with 5% CO_2-95% O_2. Yohimbine (1 μM) was added to the buffer solution to block prejunctional *alpha* receptors. Before the start of the experiments the preparations were allowed to equilibrate for about 60 min with an initial tension of 10 mN. Electrical field stimulation (square-wave pulses at 0.15 Hz, 1 msec duration and submaximal voltage) was applied to stimulate smooth muscle contraction. After 15 min of electrical field stimulation, cumulative concentrations of NPY (0.1 nM-3 μM) were added to the organ bath. Twitch responses were recorded isometrically and inhibition was expressed in percentages. EC_{50} values were determined by nonlinear regression analysis. Antagonist affinity was determined by obtaining concentration-response curves to NPY in the presence of BIBP 3226 {(*R*)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-arginine-amide} (0.3–10 μM) allowing a 15-min equilibration of the antagonist. In order to determine the pA_2 value for BIBP 3226, linear regression plots according to Arunlakshana and Schild (1959) were constructed. The slopes of the A-S plots were not significantly different from unity, so pK_b values were obtained from plots constrained to a slope of 1 (Tallarida and Jacob, 1979).

Isolated perfused rat kidney. Male rats (Chbb Thom, 300–350 g, live-stock breeding of Dr. Karl Thomas GmbH, Biberach, Germany) were anesthetized with pentobarbital sodium (60 mg/kg i.p.), and the right kidneys were taken for isolated perfusion. After the abdomen was incised at the midline, the abdomen was opened and the left renal artery, renal vein and ureter were prepared free and cannulated. Perfusion was started immediately upon interruption of aortic blood flow. The kidney was then removed and placed on a thermostated plastic holder. Perfusion pressure was kept constant at 100 mm Hg by adjustment of the pump rate (approximately 6 ml/min). The kidneys were perfused at 37°C with a modified Krebs' buffer (for composition see above). After an equilibrium period of 60 min, NPY was administered by a bolus injection (0.1 ml) at a concentration of 0.1 nM at 15-min intervals. Two control applications were performed before the antagonist was investigated. Antagonists were infused during 5 min (0.025 ml/min) in the kidney followed by NPY administration. In each kidney, three to five different concentrations of the antagonist were examined. IC_{50} values for the antagonists were calculated by plotting the percentage of inhibition of the NPY-induced increase in perfusion pressure against the concentration of the antagonist.

Isolated perfused rat mesentery. Male rats (Chbb: Thom 300–350 g, live-stock breeding of Dr. Karl Thomas GmbH, Biberach, Germany) were anesthetized with pentobarbital sodium (60 mg/kg i.p.). The abdomen was opened and the mesenteric artery was cannulated at its origin with a PE-50 tubing. The mesenteric artery bed was excised, placed on a thermostated plastic holder and perfused at a rate of 5.0 ml/min. The modified Krebs' perfusion solution (for composition see above) was maintained at 37°C and aerated constantly with 95% O_2-5% CO_2. The tissue was allowed to equilibrate for 45 to 60 min. After this equilibrium period, noradrenaline was given twice (5-min interval) at a concentration of 10 nmol/0.1 ml/bolus. The mean increase in perfusion pressure was taken as control value. After the second noradrenaline administration, vehicle or BIBP 3226 were infused for 15 min into the mesenteric artery followed by a bolus injection of NPY (1.0 nmol/0.1 ml). Five minutes after the NPY administration, noradrenaline was injected again. The increase in perfusion pressure produced by the second noradrenaline application was measured and calculated as percentage of increase compared to the first (before NPY injection) application. Because the NPY-mediated potentiation of noradrenaline-evoked increase in perfusion pressure was long-lasting, only one concentration of antagonists could be investigated in each preparation. Three concentrations (0.1, 1.0 and 10 μM), using four preparations for each concentration of BIBP 3226, were investigated.

Isolated perfused rabbit ear. Rabbits (Chbb: NZW, 3 kg, live-stock breeding of Dr. Karl Thomas GmbH, Biberach, Germany) were sacrificed by a blow on the neck and the ears were removed rapidly. The auricular artery was cannulated with a PE-50 tube and the ear was flushed with a heparin solution (100 IU/ml, approximately 7 ml). The ear was placed on a thermostated plastic holder and perfused (for composition of perfusion solution, see above) at a rate of 3.5 ml/min. After 15 min the perfusion pressure was adjusted to 75 mm Hg. After an equilibrium period of 45 min, NPY at a concentration of 1.0 nmol/0.1 ml was administered as bolus injections (0.1 ml) at 30-min intervals. After two control (vehicle) injections the antagonist was infused for 5 min in the ear artery before the next bolus injection of NPY. In each preparation, three to four different concentrations of BIBP 3226 were examined.

***In vivo* pithed rat preparation.** Male rats (Chbb:Thom, 300–325 g, live-stock breeding of Dr. Karl Thomas GmbH, Biberach, Germany) were anesthetized with hexobarbitone sodium (150 mg/kg i.p.). The trachea was cannulated and the animals were pithed by introducing a blunt needle into the spinal canal *via* the orbit. The animals were respirated with oxygenated room air by means of a positive pressure pump (80 strokes/min, 10 ml/kg). The left jugular vein was cannulated for injections of drugs. A cannula was inserted into the left common carotid artery and blood pressure was measured *via* a Statham pressure transducer. In the first series of experiments, the animal received NPY (0.1–1000 μg/kg) in a cumulative manner. Either vehicle or different doses of BIBP 3226 (0.01–1.0 mg/kg i.v.) were given 1 min before the administration of NPY. In a second series of experiments, the animals received i.v. bolus injections of NPY (10 μg/kg), angiotensin II (0.1 μg/kg), noradrenaline (2.0 μg/kg), vasopressin (0.03 IU/kg) or endothelin (1.0 μg/kg). With the exception of endothelin, the agonists were given at 15-min intervals. After two to three injections of the agonist, the observed hypertensive response attained a constant value and increasing doses of BIBP 3226 were given 1 min before the next application of the agonist. In the case of endothelin, separate animals for vehicle or BIBP 3226 (1 mg/kg i.v.) treatment were used. In the third series of experiments, the effects of i.v. administration of the peptidic compound EXBP 68 [Ile-Glu-Pro-Orn-Tyr-Arg-Leu-Arg-Tyr-NH_2, cyclic (2,4'), (2',4)-diamide] on blood pressure as well as its ability to antagonize the effects of NPY (10 μg/kg i.v.) were investigated.

Effects on blood pressure in conscious SH rats. Male SH rats (SHR/NCrlBL, 380 g, Charles River, Sulzfeld, Germany) were anesthetized with hexobarbitone sodium (150 mg/kg i.p.). After anesthesia, polyethylene catheters (PE-50) were placed in the right jugular vein and into the left carotid artery. The catheters were passed s.c. and exteriorized at the back of the neck. Each catheter was filled with a polyvinylpyrrolidone (40%)/heparin (500 IU/ml) solution. The animals were allowed to recover for 2 to 4 days. On the day of the experiments, the arterial catheter was connected to a Statham pressure transducer to measure blood pressure. Drugs were administered *via* the jugular vein.

Materials. BIBP 3226 {(R)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-arginine-amide} and EXBP 68 [Ile-Glu-Pro-Orn-Tyr-Arg-Leu-Arg-Tyr-NH_2, cyclic (2,4'), (2',4)-diamide] were provided by the Department of Chemistry of Dr. Karl Thomae GmbH (Biberach, Germany). NPY was obtained from Saxon Biochemicals (Frankfurt, Germany). Yohimbine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). Hexobarbitone sodium was obtained from Bayer (Leverkusen, Germany) and pentobarbital sodium was from Sanofi (Bordeaux, France).

Data analysis. All data are given as mean values ± S.E.M. and were analyzed using Students's *t* test; a P value of < .05 was taken as the significance level.

Results

NPY produced a concentration-dependent inhibition of the twitch response of rabbit and rat vas deferens with EC_{50} values of 2.45 ± 0.13 (n = 5) and 4.1 ± 0.95 nM (n = 7), respectively. The concentration-response curve for NPY in the rabbit vas deferens was shifted to the right in a parallel fashion by BIBP 3226 {(*R*)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-arginine-amide} (0.3–30 μM), and no decrease of the maximum response was observed (fig. 1). Moreover, the slope of the Schild plot (0.87) was not significantly different from unity, which indicates competitive antagonism. A mean pK_b value of 6.98 ± 0.06 (n = 16) was calculated. BIBP 3226 (10 μM) did not produce a rightward shift of the concentration-response curve for NPY in the rat vas deferens. In contrast, EXBP 68 [Ile-Glu-Pro-Orn-Tyr-Arg-Leu-Arg-Tyr-NH_2, cyclic (2,4', (2',4)-diamide] showed agonist-like properties in both vas deferens preparations. However, a 175-fold selectivity for Y1 receptors was observed in the rabbit vas deferens. The corresponding EC_{50} values for the rabbit and rat vas deferens were 5.26 ± 1.86 (n = 4) and 918 ± 329 nM (n = 4), respectively. The effect of 0.1 nM of EXBP 68 could be antagonized by 1 μM of BIBP 3226 by approximately 89% (fig. 2). Antagonistic properties were not observed for EXBP 68.

NPY (0.1 nmol/0.1 ml) caused an increase in perfusion pressure of 146.3 ± 8.6 mm Hg (n = 4) in the isolated perfused rat kidney. BIBP 3226 could antagonize the NPY-mediated increase in perfusion pressure with an IC_{50} = 26.8 ± 4.5 nM (n = 4) (fig. 3). In this preparation, EXBP 68 showed no agonistic properties in the concentration range investigated (0.1–100 μM). On the contrary, it proved to be an antagonist of the NPY-induced increase in perfusion pressure. A mean IC_{50} value of 6.23 ± 3.12 nM (n = 4) was calculated (table 1).

In the isolated rabbit ear preparation, NPY administration (1 nmol/0.1 ml) resulted in an increase in perfusion pressure of 57.9 ± 6.7 mm Hg (n = 4). A concentration-dependent inhibition was observed with BIBP 3226, resulting in an IC_{50} value of 214 ± 30 nM (n = 4) (fig. 3). In the isolated perfused rat mesenteric bed, noradrenaline (10 nmol/0.1 ml) increased the perfusion pressure by 42.1 ± 3.2 mm Hg (n = 24). NPY itself showed no significant effect on the perfusion pressure. However, NPY (1 nmol/0.1 ml) administration 5 min before noradrenaline produced a potentiation of the noradrenaline response by 147.3 ± 14% (n = 6). This potentiating effect of NPY could be antagonized by BIBP 3226 in a concentration-dependent manner (fig. 3C). A 50% inhibition was calculated at 976 nM (confidence interval, 542-1760 nM) of the drug (table 1). BIBP 3226 had no significant effect on the increase in perfusion pressure elicited by noradrenaline.

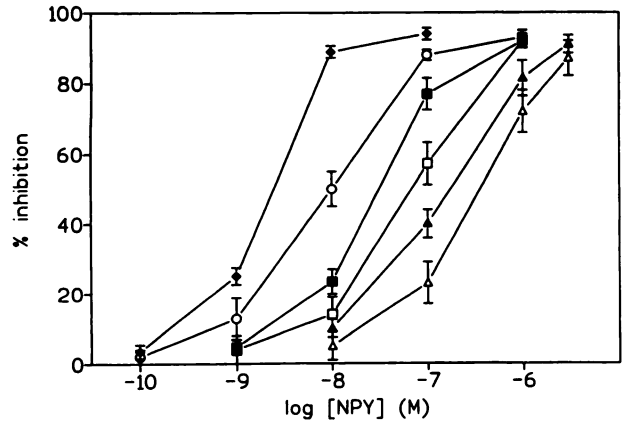

Fig. 1. Concentration-response curves of NPY. Inhibition of the twitch response elicited by electrical field stimulation in rabbit vas deferens in the absence or presence of BIBP 3226. Data are presented as mean values ± S.E.M. (n = 3–4). (◆, control; ○, 0.3 μM; ■, 1 μM; □, 3 μM; ▲, 10 μM; △, 30 μM)

Cumulative i.v. administration of NPY (0.10–1000 μg/kg) in the pithed rat produced a dose-dependent increase in diastolic blood pressure. The maximal effects amounted to 66.9 ± 1.2 mm Hg (fig. 4). Pretreatment with BIBP 3226 (0.01–1.0 mg/kg i.v.) resulted in a dose-dependent rightward shift of the NPY curve without influencing the maximum effect as depicted for the 0.1- and 1.0-mg/kg doses. In separate experiments, a fixed i.v. dose of 10 μg/kg of NPY was used to increase diastolic blood pressure by 57 ± 4 mm Hg (n = 8). BIBP 3226, given 1 min before NPY, caused a dose-dependent inhibition of the NPY-induced pressor response. The ID_{50} value was 0.11 ± 0.03 mg/kg i.v. (n = 8). An even approximately 10-fold higher dose of 1 mg/kg i.v. did not attenuate the pressor response to angiotensin II (0.1 μg/kg), noradrenaline (2 μg/kg), endothelin (1 μg/kg) or vasopressin (0.03 IU/kg) (fig. 5). EXBP 68 antagonized the pressor response elicited by 10 μM NPY in this model very effectively with a mean ID_{50} value of 0.0095 ± 0.0015 mg/kg i.v (n = 4). However, beside its antagonistic properties, the compound also was able to increase blood pressure dose-dependently (1.0–100 μg/kg i.v.). The increase in diastolic blood pressure amounted to 50.3 ± 5.5 mm Hg at a dose of 100 μg/kg given i.v. The increase in blood pressure evoked by EXBP 68 (10 μg/kg i.v.) was short-lasting and could be attenuated by prior administration of BIBP 3226 (1 mg/kg i.v.) (fig. 2).

Conscious SH rats had a basal mean arterial blood pressure of 144 ± 6 mm Hg (n = 13). BIBP 3226, in doses up to 3 mg/kg i.v. (n = 5), had no effect on blood pressure. EXBP 68 produced a dose-dependent (1.0–100 μg/kg i.v.) increase in diastolic blood pressure with a maximal effect of 32.1 ± 1.5 mm Hg (n = 8). At the dose of 100 μg/kg, a biphasic response was observed. An initial increase in blood pressure was followed by a decrease of 44 ± 6.0 mm Hg (n = 8) in blood pressure.

Discussion

Until recently, NPY research was hampered by the lack of suitable antagonists. Attempts to investigate the (patho)-physiological role of NPY were performed using antibodies or antisense oligonucleotides against NPY (Daly *et al.*, 1988; Akabayashi *et al.*, 1994; Dube *et al.*, 1994) or antisense oligonucleotides against the Y1 receptor (Wahlestedt *et al.*, 1992). Although antagonistic properties have been claimed for several compounds (Doughty *et al.*, 1990; Michel and Motulsky, 1990; Sun *et al.*, 1991; Adamsson *et al.*, 1992; Tatemoto *et al.*, 1992; Michel *et al.*, 1992; Balasubramaniam *et al.*, 1994), the low affinity, partial agonistic activity or unspecificity of these compounds limited their use as pharmacological tools (Grundemar and Håkanson, 1994). Re-

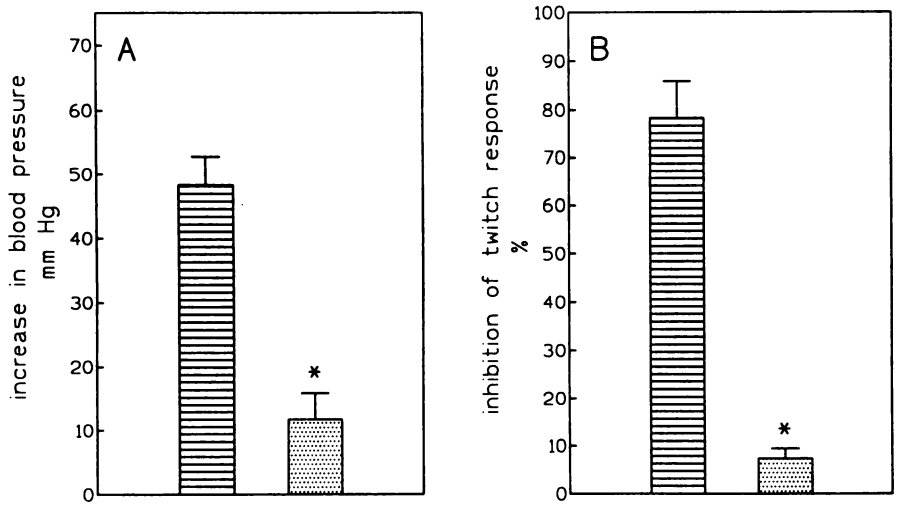

Fig. 2. Inhibition by BIBP 3226 of the agonistic effect of EXBP 68. Data are presented as mean values ± S.E.M. (n = 3). A, increase in diastolic blood pressure elicted by 10 μg/kg i.v. EXBP 68 and after pretreatment (spickled bar) with BIBP 3226 (1 mg/kg i.v.). B, inhibition of the twitch response by 0.1 nM EXBP 68 and the antagonism thereof by 1 μM BIBP 3226 (spickled bar).

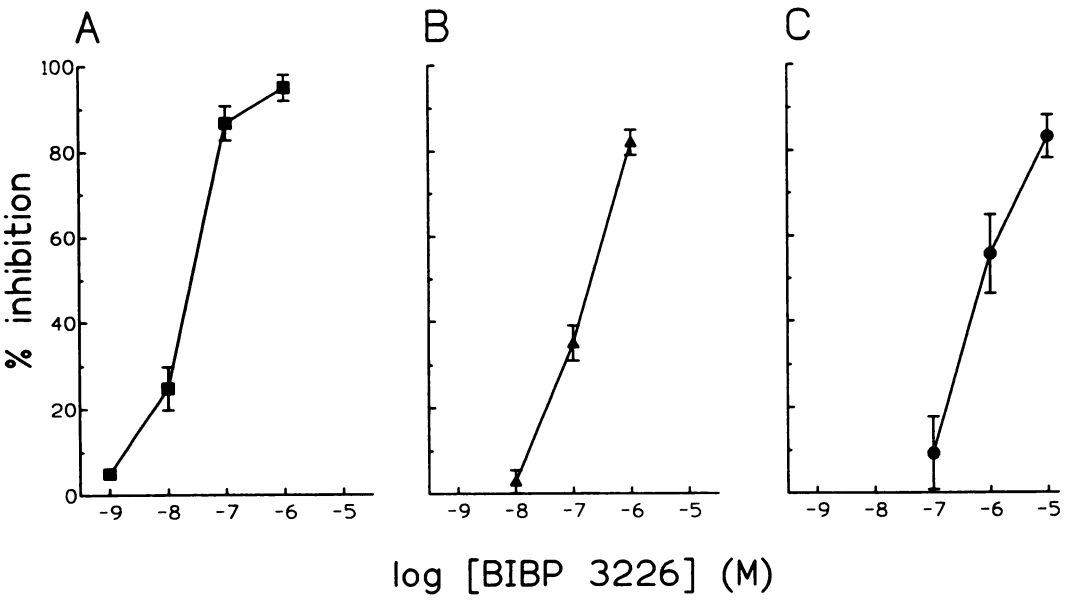

Fig. 3. Inhibitory effect of BIBP 3226 on the increase in perfusion pressure in the isolated rat kidney (A), isolated rabbit ear (B) and isolated rat mesentery (C). Data are presented as mean values ± S.E.M. (n = 4).

cently, peptide structures as well as nonpeptide NPY receptor antagonists have been disclosed that possess NPY antagonistic properties exemplified by EXBP 68 [Ile-Glu-Pro-Orn-Tyr-Arg-Leu-Arg-Tyr-NH_2, cyclic (2,4′), (2′,4)-diamide] (Daniels *et al.*, 1994) and BIBP 3226{(*R*)-N^2- (diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-arginine-amide} (Rudolf *et al.*, 1994a,b), respectively. These compounds display high affinity and selectivity for the Y1 receptor subtype in receptor binding studies (Wieland *et al.*, 1995). Moreover, functional experiments in the SK-N-MC cell line reveal that both compounds behave as full antagonists lacking any agonistic activity.

In order to investigate the selectivity of both compounds in functional experiments, the effects of the nonpeptide BIBP 3226 and the peptide EXBP 68 were tested in the isolated rat and rabbit vas deferens. Presynaptically located NPY receptors inhibit neurotransmitter release after electrical stimulation and consequently inhibit the twitch response in the isolated vas deferens. We have demonstrated previously that NPY receptors in the rabbit vas deferens belong to the Y1 subtype, whereas those present in the rat vas deferens are of the Y2 subtype (Doods and Krause, 1991). This characterization was based on the different rank order of potency of selective Y1 and Y2 agonists for both tissues. In accordance with our assumption that different NPY receptor subtypes modulate neurotransmitter release in these two species, we found that BIBP 3226 is a competitive antagonist for the prejunctionally located Y1 receptor in the rabbit vas deferens. The pK_b value of 6.98 is in agreement with the pK_b value of BIBP 3326 to antagonize the NPY-mediated increase in intracellular Ca^{++} in SK-N-MC cells (Wieland *et al.*, 1995). BIBP 3226 could not attenuate the inhibition of the twitch response mediated by NPY in the rat vas deferens. These findings not only underline the selectivity of BIBP 3226 for Y1 receptors, but also support the hypothesis that the prejunctionally located receptors in rabbit and rat vas deferens are different and belong to the Y1 and Y2 subtype, respectively. To our surprise, we observed that EXBP 68 displayed agonistic properties which were not seen with BIBP 3226. In accordance with the receptor binding data (Wieland *et al.*, 1995), a selectivity of EXBP 68 was observed for the Y1 receptor. The agonistic profile of EXBP 68, with

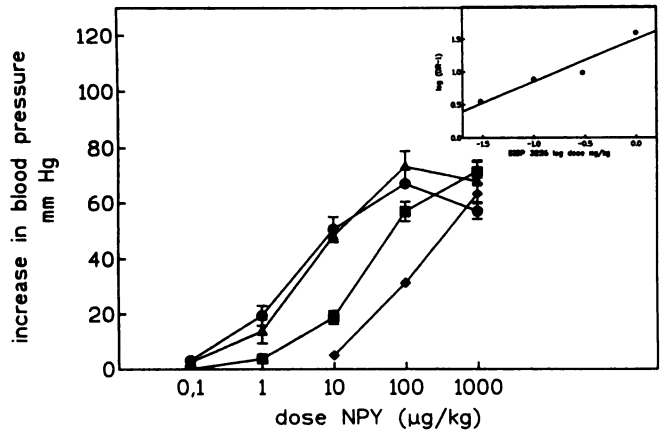

Fig. 4. Log dose-vasopressor-response curves of NPY after pretreatment with vehicle (●), 0.01 (▲), 0.1 (■) and 1.0 (◆) mg/kg (i.v.) of BIBP 3226. Insert: *in vivo* Schild plot of the BIBP 3226 antagonism (0.03–1.0 mg/kg i.v.). Data are presented as mean values ± S.E.M. (n = 8).

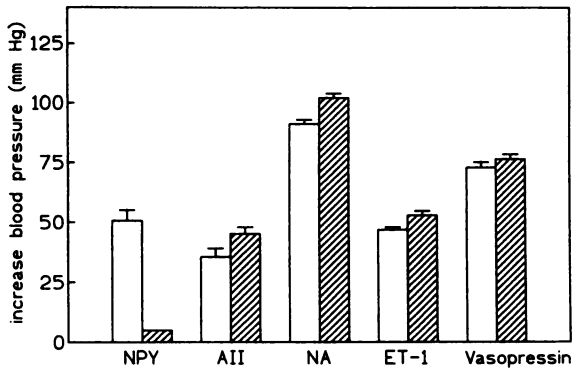

Fig. 5. *In vivo* selectivity of BIBP 3226 (1 mg/kg i.v.) in the pithed rat (open bars, control; filled bars, BIBP 3226). Data are presented as mean values ± S.E.M. (n = 4).

respect to activity (EC_{50} = 5 nM) and Y1 selectivity (175-fold), is comparable to that of the Y1-selective agonist [Leu^{31},Pro^{34}]NPY (Doods and Krause, 1991). An unspecific effect of EXBP 68 can be ruled out because the agonism observed with EXBP 68 in the rabbit vas deferens could be blocked by BIBP 3226. It is obvious from the paper of Wieland *et al.* (1995), as well as from the patent in which the peptide EXBP 68 is disclosed (Daniels *et al.*, 1994), that by using cell lines to characterize the compound only antagonistic properties of the compound are evident. The discrepancy between the results obtained with cell lines expressing the human Y1 receptor and the rabbit vas deferens preparation is difficult to explain at present. It can be speculated that the human Y1 receptor and the Y1 receptor present in the rabbit vas deferens are not identical. However, unfortunately, only the human and rat Y1 receptors have been cloned (Herzog *et al.*, 1992; Krause *et al.*, 1992; Larhammar *et al.*, 1992), and therefore the answer to the above speculation cannot yet be given.

In contrast to the experiments in the vas deferens preparations, those in the isolated perfused rat kidney revealed that the peptide EXBP 68 exhibited no agonistic properties but clearly antagonized the NPY-mediated increase in perfusion pressure, as did BIBP 3226 (table 1). EXBP 68 proved to be approximately 4-fold more potent than BIBP 3226 in this model, which is in agreement with the higher affinity of EXBP 68 for Y1 receptors (Wieland *et al.*, 1995). BIBP 3226 also antagonized the increase in perfusion pressure elicited by NPY in the isolated perfused rabbit ear preparation. The present study supports earlier findings using selective agonists that NPY increases perfusion pressure in the isolated rat kidney (Entzeroth *et al.*, 1994) and the perfused rabbit ear (Doods, 1992) by stimulating vascular Y1 receptors. NPY is not able to constrict vascular smooth muscle in all vascular beds. However, in these tissues, NPY can potentiate the effects of other vasoactive agents such as noradrenaline. The amplifying effect of NPY can be demonstrated convincingly in the rat perfused mesentery (McAuley and Westfall, 1992). In the rat mesenteric bed, the majority of vascular NPY receptors belong to the Y1 subtype, although also prejunctional Y1 and Y2 receptors modulating noradrenaline release appear to be present in this preparation (McAuley and Westfall, 1992). The potentiating effect of NPY could be antagonized completely by BIBP 3226. Accordingly, BIBP 3226 was able to antagonize *in vitro* both the direct vasoconstriction as well as the indirect vasoconstricting potentiating effects of NPY.

When administered i.v. to rats, NPY produces an increase in blood pressure (Maclean and Hiley, 1990; Michel *et al.*, 1992). The *in vivo* dose-response curve of NPY to increase diastolic blood pressure was shifted to the right in a parallel and dose-dependent manner. Although *in vivo* Schild analysis should be interpreted with caution, the Schild plot (fig. 4) indicates that the NPY-induced pressure response is mediated solely by Y1 receptors. This is supported further by the fact that the pressure response evoked by i.v. administration of 10 µg of NPY could be inhibited by 95% when 1.0 mg of BIBP 3226 was injected before NPY. In addition, we investigated whether BIBP 3226 shows specificity for NPY receptors in this *in vivo* model. A dose of 1.0 mg/kg i.v. could not attenuate the pressure response elicited by noradrenaline, angiotensin II, endothelin or vasopressin. This finding, together with the lack of BIBP 3226 to interact with some 60 other receptor types and 15 enzyme systems (unpublished

TABLE 1

Antagonistic and agonistic properties of BIBP 3226 and EXBP 68 in the different *in vitro* and *in vivo* models

n.d., not determined.

	In vitro ID_{50}			*In vivo* ID_{50}		
	nM			*mg/kg*		
	Rat vas deferens	Rabbit vas deferens	Rat kidney	Rat mesentery	Rabbit ear	Rat blood pressure
BIBP 3226	>1,000	105[a]	26.8	976	214	0.11
EXBP 68 antagonist			6.23	n.d.	n.d.	0.0095
EXBP 68 agonist	918	5.26		n.d.	n.d.	0.002[b]

[a] K_b.

[b] Dose (milligrams per kilogram i.v.) to increase diastolic blood pressure by 25 mm Hg.

results), points to the high degree of selectivity of this compound. BIBP 3226 did not display any agonistic properties in the pithed rat. On the contrary, EXBP 68 proved to be a partial agonist in the rat. EXBP 68 inhibited dose-dependently the pressor response evoked by 10 μg/kg i.v. of NPY. An ID_{50} value of 0.0095 mg/kg i.v. was calculated. Accordingly, EXBP 68 proved to be 12-fold more potent than BIBP 3226 (ID_{50} = 0.11 mg/kg i.v.). However, EXBP 68 also increased blood pressure dose-dependently, which could be attenuated by pretreatment of the animals with BIBP 3226. The partial agonism might explain the diversity of effect, agonism in one model and antagonism in another one, observed with this compound in the *in vitro* assays. The receptor density in a given tissue might be decisive whether EXBP 68 acts as an agonist or antagonist. Similar equivocal pharmacological effects of peptidic structures also have been described, *e.g.*, for the neurotensin (Cusack *et al.*, 1993) and vasopressin receptor system (Ruffolo *et al.*, 1991). However, it should be mentioned that only one peptide of a series has been investigated in the present study. It might be that other compounds of this published series of peptides (Daniels *et al.*, 1994) show different pharmacological profiles.

Both compounds also were investigated after i.v. administration to conscious SH rats. As expected from the data of the pithed rat preparation, EXBP 68 increased mean arterial blood pressure. At the highest dose of 0.1 mg/kg, the initial increase in blood pressure was followed by a sharp drop in blood pressure. In separate experiments using anesthetized SH rats, the hypotensive effect could be blocked by pretreatment of the animals with the histamine H1 antagonist mepyramine (data not shown). Accordingly, the fall in blood pressure observed with EXBP 68 at higher doses is probably not due to NPY antagonism, but the result of histamine liberation. It has been reported that NPY and C-terminal fragments of NPY also are able to liberate histamine and consequently lower blood pressure (Grundemar and Håkanson, 1991; Shen *et al.*, 1991). Unexpected, however, was that BIBP 3226 in doses up to 3 mg/kg i.v. had no effect on mean arterial blood pressure in conscious SH rats. One explanation for the lack of effect on blood pressure could be that NPY is not involved in the maintenance of the high blood pressure in SH rats. We (H. A. Wieland, W. Wiemen and H. N. Doods, unpublished results) and others (Miller and Tessel, 1991; Ogawa *et al.*, 1992; Zukowska-Grojec *et al.*, 1993) found that plasma NPY levels are not increased in the SH rats compared to normotensive rats. It has been shown that the neuronal release of NPY occurs mainly during high intensity/frequency sympathetic nerve stimulation (Lundberg *et al.*, 1986; Zukowska-Grojec and Wahlestedt, 1993). Therefore, the physiological importance of NPY may be during stress situations and as such in stress-related cardiovascular pathology. Acute stress situations in humans, *e.g.*, exercise and cold stress (Lundberg *et al.*, 1985; Morris *et al.*, 1986), and in rats, *e.g.*, handling stress and cold stress (Castagné *et al.*, 1987; Zukowska-Grojec *et al.*, 1992), are accompanied by an increase in plasma NPY immunoreactivity which may explain in part the blood pressure responses observed under these conditions. BIBP 3226 is presently under investigation in experimental models for stress-induced hypertension. An alternative explanation for the failing antihypertensive effect of BIBP 3226 in SH rats might be that a second (non-Y1) NPY receptor subtype mediates the vascular effects of endogenously released NPY. Although most studies using Y1-selective agonists (Grundemar *et al.*, 1992; Grundemar and Högestätt, 1992; Xia *et al.*, 1992; Modin *et al.*, 1993) or Y1 antisense oligonucleotides (Erlinge *et al.*, 1993) support the hypothesis that vascular NPY receptors belong to the Y1 subtype, there are some hints for the presence of a second NPY receptor subtype on vascular smooth muscle (Modin *et al.*, 1991; Tessel *et al.*, 1993a,b). It could be speculated that the "classical Y1 receptor" is located extrasynaptically and mediates the effects of NPY released from platelets or the adrenal medulla, and that the second subtype is located intrasynaptically and is stimulated upon release from NPY by sympathetic nerves. Recently, a benextramine analog has been described that does not interact with vascular Y1 receptors stimulated by the selective Y1 agonist [Leu^{31},Pro^{34}]NPY, but showed high affinity for vascular NPY receptors activated by NPY(13–36) (Chaurasia *et al.*, 1994). Unfortunately, only data from *in vitro* experiments using the rat femoral artery were presented and effects on blood pressure or a detailed receptor binding characterization were not reported. The authors suggested that this second vascular NPY receptor is a Y2-like receptor not identical with the Y2 receptor found in the rat brain. However, this assumption needs further experimental confirmation.

It can be concluded that the nonpeptide compound BIBP 3226, in contrast to the peptide structure EXBP 68, is a competitive and highly selective antagonist for Y1 receptors, irrespective of the pharmacological model used. Even doses 30-fold higher than necessary to antagonize the pressor response induced by exogenously applied NPY by 50% did not alter the blood pressure in conscious SH rats, suggesting that NPY does not play an important role in the maintenance of the high blood pressure in this strain. Preliminary studies show that BIBP 3226 is able to attenuate stress (*e.g.*, cold stress)-induced hypertension. Accordingly, we hypothesize that the Y1 receptor does not modulate basal blood pressure, but is involved in blood pressure regulation under stress conditions. Alternatively, it could be that a novel NPY receptor subtype mediates the vascular effects of endogenously released NPY. Further experiments are certainly necessary to elucidate the role of NPY and its receptor subtypes in the cardiovascular system. The availability of nonpeptide compounds like BIBP 3226 could play a central role in answering these questions. After discovery of NPY and the cloning and characterization of the Y1 receptor, BIBP 3226 can be considered as the third major achievement in the field of NPY research.

Acknowledgments

The authors would like to acknowledge the skillful technical assistance of E. Schilling, E. Liebhardt, S. Hecht and R. Wiest.

References

Adamsson, M., Fallgren, B. and Edvinsson, L.: Inhibition of neuropeptide Y-induced potentiation of noradrenaline-induced vasoconstriction by PP56 (D-myo-inositol-1,2,6-tris-phosphate). Br. J. Pharmacol. **105:** 93–96, 1992.

Akabayashi, A., Wahlestedt, C., Alexander, J. T. and Leibowitz, S. F.: Specific inhibition of endogenous neuropeptide Y synthesis in arcuate nucleus by antisense oligonucleotides suppresses feeding behavior and insulin secretion. Mol. Brain Res. **21:** 55–61, 1994.

Arunlakshama, A. D. and Schild H. O.: Quantitative uses of drug antagonists. Br. J. Pharmacol. **14:** 48–58, 1959.

Balasubramaniam, A., Sheriff, S., Johnson, M. E., Pradhakaran, M., Huang, Y., Fischer, J. E. and Chance, W. T.: [D-Trp^{32}]Neuropeptide Y: A competitive antagonist of NPY in rat hypothalamus. J. Med. Chem. **37:** 811–815, 1994.

Castagné, V., Corder, R., Gaillard, R. and Mormède, P.: Stress-induced changes of circulating neuropeptide Y in the rat: Comparison with catecholamines. Regul. Pept. **19:** 55–63, 1987.

Chaurasia, C., Misse, G., Tessel, R. and Doughty, M. B.: Nonpeptide peptidomimetic antagonists of the neuropeptide Y receptor: Benextramine analogs with selectivity for the peripheral Y2 receptor. J. Med. Chem. **37:** 2242–2248, 1994.

Cusack, B., Richelson, E., Pang, Y.-P., Zaidi, J. and Kozikowski, A. P.: Pharmacological studies on novel neurotensin mimetics: Discovery of a pharmacologically unique agent exhibiting concentration-dependent dual effects as antagonists and agonist. Mol. Pharmacol. **44:** 1036–1040, 1993.

Daly, R. N., Roberts, M. I., Ruffolo, R. R. and Hieble, J. P.: The role of neuropeptide Y in vascular sympathetic neurotransmission may be enhanced in hypertension. J. Hypertens. **6:** S535–S538, 1988.

Daniels, A., Heyer, D., Landavazo, A., Leban, J. J. and Spaltenstein, A.: Neuropeptide Y antagonists, International Publisher's No. WO 94/00486, 1994.

Doods, H. N.: Effects of NPY and selective analogs in the isolated perfused rabbit ear. Naunyn-Schmiedeberg's Arch. Pharmacol. **345:** Suppl. 1, R115, 1992.

Doods, H. N. and Krause, J.: Different neuropeptide Y receptor subtypes in rat and rabbit vas deferens. Eur. J. Pharmacol. **294:** 101–103, 1991.

Doughty, M. B., Chu, S. S., Miller, D. W., Li, K. and Tessel, R.: Benextramine: A long lasting NPY receptor antagonist. Eur. J. Pharmacol. **185:** 114–114, 1990.

Dube, M. G., Xu, B., Crodley, W. R., Kalra, P. S. and Kalra, S. P.: Evidence that neuropeptide Y is a physiological signal for normal food intake. Brain Res. **646:** 341–344, 1994.

Edvinsson, L., Emson, P., Häkanson, R. and Wahlestedt, C.: Neuropeptide Y potentiates the effects of various vasoconstrictor agents on rabbit blood vessels. Br. J. Pharmacol. **83:** 519–525, 1984.

Entzeroth, M., Wieland, H. A., Wienen, W. and Doods, H. N.: The increase in renal perfusion pressure by neuropeptide Y is mediated via receptors of the Y1 subtype. Can. J. Physiol. Pharmacol. **72:** Suppl. 1, 170, 1994.

Erlinge, D., Edvinsson, L., Brunkwall, J., Yee, F. and Wahlestedt, C.: Human neuropeptide Y Y1 receptor antisense oligodeoxynucleotide specifically inhibits neuropeptide Y-evoked vasoconstriction. Eur. J. Pharmacol. **240:** 77–80, 1993.

Gehlert, D.: Subtypes of receptors for neuropeptide Y: Implications for the targeting of therapeutics. Life Sci. **55:** 551–562, 1994.

Grundemar, L. and Häkanson,R.: Neuropeptide Y, peptide YY and C-terminal fragments release histamine from rat peritoneal mast cells. Br. J. Pharmacol. **104:** 776–778, 1991.

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

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

Grundemar, L. and Högestätt, E. D.: Unmasking the vasoconstrictor response to neuropeptide Y and its interaction with vasodilating agents *in vitro*. Eur. J. Pharmacol. **221:** 71–76, 1992.

Grundemar, L., Mörner, S. E. J. M., Högestätt, E. D., Wahlestedt, C. and Häkanson, R.: Characterization of vascular receptors for neuropeptide Y. Br. J. Pharmacol. **105:** 45–50, 1992.

Herzog, H., Hort, Y. J., Ball, H. J., Hayes, G., Shine, J. and Selbie, L. A.: Cloned human neuropeptide Y receptor couples to two different second messenger systems. Proc. Natl. Acad. Sci. U.S.A. **89:** 5794–5798, 1992.

Krause, J., Eva, C., Seeburg, P. and Sprengel, R.: Neuropeptide Y1 subtype pharmacology of recombinantly expressed neuropeptide receptor. Mol. Pharmacol. **41:** 817–821, 1992.

Larhammar, D., Blomquist, A. G., Yee, F., Jazin, E., Yoo, H. and Wahlestedt, C.: Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1-type. J. Biol. Chem. **267:** 10935–10938, 1992.

Lundberg, J. M., Martinsson, A., Hemsén, A., Theodorson, E., Svedenhag, J., Ekblom, B. and Hjemdahl, P.: Corelease of neuropeptide Y and catecholamines during physical exercise in man. Biochem. Biophys. Res. Commun. **133:** 30–36, 1985.

Lundberg, J. M., Rudehill, A., Sollevi, E., Theodorsson-Norheim, E. and Hamberger, B.: Frequency- and reserpine-dependent chemical coding of sympathetic transmission: Differential release of noradrenaline and neuropeptide from pig spleen. Neurosci. Lett. **63:** 96–100, 1986.

MacLean, M. R. and Hiley, C. R.: Effect of neuropeptide Y on cardiac output, its distribution, regional blood flow and organ resistances in the pithed rat. Br. J. Pharmacol. **99:** 340–342, 1990.

McAuley, M. and Westfall, T. C.: Possible location and function of neuropeptide Y receptor subtypes in the rat mesenteric arterial bed. J. Pharmacol. Exp. Ther. **261:** 863–868, 1992.

McDermott, B. J., Millar, B. C. and Piper, H. M.: Cardiovascular effects of neuropeptide Y receptor interactions and cellular mechanisms. Cardiovasc. Res. **27:** 893–905, 1993.

Michel, M. C.: Receptors for neuropeptide Y: Multiple subtypes and multiple second messengers. Trends Pharmacol. Sci. **611:** 392–394, 1991.

Michel, M. C., Gaida, W., Beck-Sickinger, A. G., Wieland, H. A., Doods, H. N., Duerr, H., Jung, G. and Schnorrenberg, G.: Further characterization of neuropeptide Y receptor subtypes using centrally truncated analogs of neuropeptide Y: Evidence for sub-type-differentiating effects on affinity and intrinsic efficacy. Mol. Pharmacol. **42:** 642–648, 1992.

Michel, M. C. and Motulsky, H. Y.: HE90481: A competitive nonpeptidergic antagonist at NPY receptors. Ann. N.Y. Acad. Sci. **611:** 389–394, 1990.

Miller, D. W. and Tessel, R.: Age-dependent hyperresponsiveness of spontaneously hypertensive rats to the pressor effects of intravenous neuropeptide Y (NPY): Role of mode of peptide administration and plasma NPY-like immunoreactivity. J. Cardiovasc. Pharmacol. **18:** 647–656, 1991.

Modin, A., Pernow, J. and Lundberg, J. M.: Evidence for two neuropeptide Y receptors mediating vasoconstriction. Eur. J. Pharmacol. **203:** 165–171, 1991.

Modin, A., Pernow, J. and Lundberg, J. M.: Sympathetic regulation of skeletal muscle blood flow in the pig: A non-adrenergic component likely to be mediated by neuropeptide Y. Acta Physiol. Scand. **148:** 1–11, 1993.

Morris, M. J., Russell, A. E., Kapoor, V., Cain, M. D., Elliott, J. M., West, M. J., Wing, L. M. H. and Chalmers, J. P.: Increases in plasma neuropeptide Y concentrations during sympathetic activation in man. J. Auton. Nerv. Syst. **17:** 143–149, 1986.

Ogawa, T., Kitamura, K., Kangawa, K., Matsuo, H. and Eto, T.: Platelet neuropeptide Y in spontanenously hypertensive rats. J. Hypertens. **10:** 765–771, 1992.

Pernow, J., Saria, A. and Lundberg, J. M.: Mechanisms underlying pre- and postjunctional effects of neuropeptide Y in sympathetic vascular control. Acta Physiol. Scand. **126:** 239–249, 1986.

Pernow, J., Svenberg, T. and Lundberg, J.: Actions of calcium antagonists on pre- and postjunctional effects of neuropeptide Y on human peripheral blood vessels *in vitro*. Eur. J. Pharmacol. **136:** 207–218, 1987.

Rudolf, K., Eberlein, W., Engel, W., Mihm, G., Doods, H. N., Wieland, H. A., Willim, K., Krause, J., Dollinger, H., Esser, F., Schnorrenberg, G., Entzeroth, M. and Wienen, W.: Aminoacid Derivates, Medicaments Containing These Compounds and Process for Preparing the Same, Patent No. WO 94/17035, 1994a.

Rudolf, K., Eberlein, W., Engel, W., Wieland, H. A., Willim, K.-D., Entzeroth, M., Wienen, W., Beck-Sickinger, A. G. and Doods, H. N.: The first highly potent and selective nonpeptide Y1 receptor antagonist: BIBP 3226. Eur. J. Pharmacol. **271:** R11–R13, 1994b.

Ruffolo, R. R., Brooks, D., Huffman, W. F. and Poste, G.: From vasopressin antagonist to agonist: A saga of surprise! Drugs News Perspect. **4:** 217–222, 1991.

Shen, G. H., Grundemar, L., Zukowska-Grojec, Z., Häkanson, R. and Wahlestedt, C.: C-terminal neuropeptide Y fragments are mast cell-dependent vasodepressor agents. Eur. J. Pharmacol. **204:** 249–256, 1991.

Sun, X., Dahlöf, C., Edvinsson, L. and Hedner, T.: D-myo-inositol-1,2,6-triphosphate is a selective antagonist of neuropeptide Y-induced pressor responses in the pithed rat. Eur. J. Pharmacol. **204:** 281–286, 1991.

Tallarida, R. J. and Jacob, L. S.: The Dose-Response Relation in Pharmacology, Springer-Verlag, New York, 1979.

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

Tatemoto, K., Mann, M. J. and Shimizu, M.: Synthesis of receptor antagonists of neuropeptide Y. Proc. Natl. Acad. Sci. U.S.A. **89:** 1174–1178, 1992.

Tessel, R. E., Miller, D. W., Misse, G. A., Dong, X. and Doughty, M. B.: Characterization of vascular postsynaptic neuropeptide Y receptor function and regulation. 1. NPY-induced constriction in isolated rat femoral artery rings is mediated by both Y1 and Y2 receptors: Evidence from benextramine protection studies. J. Pharmacol. Exp. Ther. **265:** 172–177, 1993a.

Tessel, R. E., Miller, D. W., Misse, G. A., Dong, X. and Doughty, M. B.: Characterization of vascular postsynaptic NPY receptor function and regulation and differential sensitivity of Y1 and Y2 receptor function to changes in extracellular calcium availability and prior peptide exposure. Neuropeptides **25:** 289–298, 1993b.

Wahlestedt, C., Pick, E. M., Koob, G. F., Yee, F. and Heilig, M.: Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligonucleotides. Science (Wash. DC) **259:** 528–531, 1992.

Walker, P., Grouzman, E., Burnier, M. and Waeber, B.: The role of neuropeptide Y in cardiovascular regulation. Trends Pharmacol. Sci. **12:** 111–115, 1991.

Wieland, H. A., Willim, K. D., Entzeroth, M., Wienen, W., Rudolf, K., Eberlein, W., Engel, W. and Doods, H. N.: Subtype selectivity and antagonistic profile of the nonpeptide Y1 receptor antagonist BIBP 3226. J. Pharmacol. Exp. Ther. **275:** 143–149, 1995.

Xia, J., Neild, T. O. and Kotecha, N.: Effects of neuropeptide Y and agonists selective for neuropeptide Y receptor subtypes on arterioles of the guinea-pig small intestine and the rat brain. Br. J. Pharmacol. **107:** 771–776, 1992.

Zukowska-Grojec, Z., Golczynska, M., Shen, G. H., Torres-Duarte, A., Haass, M., Wahlestedt, C. and Myers, A. K.: Modulation of vascular function by neuropeptide Y during development of hypertension in spontaneously hypertensive rats. Pediatr. Nephrol. **7:** 845–852, 1993.

Zukowska-Grojec, Z., Shen, G. H., Deka-Starosta, A., Myers, A. K., Kvetnansky, R. and McCarty, R.: Neuronal, adrenomedullary and platelet-derived neuropeptide Y responses to stress in rats. *In* Stress, Neuroendocrine and Molecular Approaches, ed. by R. Kvetnansky, R. McCarty and J. Axelrod, pp. 197–209, Gordon and Beach Science Publishers S. A., New York, 1992.

Zukowska-Grojec, Z. and Wahlestedt, C.: Origin and actions of neuropeptide Y in the cardiovascular system. *In* The Biology of Neuropeptide Y and Related Peptides, ed. by W. F. Colmers and C. Wahlestedt, pp. 315–388, Humana Press Inc., Totowa, New Jersey, 1993.

Send reprint requests to: Dr. Henri Doods, P.O. Box 1755, D-88397 Biberach, Germany.