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Characterization of neuropeptide Y (NPY) receptors in human cerebral arteries with selective agonists and the new Y_1 antagonist BIBP 3226

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1 We have characterized pharmacologically the receptor subtype(s) responsible for the neuropeptide Y (NPY)-induced vasoconstriction in human cerebral arteries. NPY, PYY and several of their derivatives with well defined affinities at the known Y_1 and Y_2 receptor subtypes were used. Moreover, we tested the ability of the new Y_1 receptor antagonist, BIBP 3226, to antagonize the NPY-induced cerebral vasoconstriction.

2 NPY, PYY and their agonists with high affinities at the Y_1 receptor subtype ([Leu^{31}-Pro^{34}]-NPY and [Leu^{31}-Pro^{34}]-PYY) elicited strong, long lasting and concentration-dependent contractions of human cerebral arteries. Compounds with Y_2 affinity such as PYY_{3-36} or NPY_{13-36} either elicited a submaximal contraction at high concentrations or failed to induce any significant vasomotor response. Also, the application of NPY or the specific Y_1 agonist, [Leu^{31}-Pro^{34}]-NPY, to human cerebral vessels pretreated with the Y_1 agonist, NPY_{13-36}, resulted in contractile responses identical to those obtained when these compounds were tested without prior application of NPY_{13-36}.

3 The order of agonist potency at the human cerebrovascular receptor was: [Leu^{31}-Pro^{34}]-NPY = [Leu^{31}-Pro^{34}]-PYY $\geqslant$ NPY > PYY > PYY_{3-36} > > > NPY_{13-36}, which corresponded to that reported previously at the neuronal and vascular Y_1 receptors.

4 Increasing concentrations (10^{-9}-10^{-6} M) of the Y_1 receptor antagonist, BIBP 3226, to human cerebral vessels caused a parallel and rightward shift in the NPY dose-response curves without any significant change in the maximal contractile response. The calculated pA_2 was 8.52 ± 0.13, a value compatible with the reported affinity at the rodent and human Y_1 receptor.

5 We conclude that Y_1 receptors exclusively, mediate the NPY-induced contraction in human cerebral arteries and we show that BIBP 3226 is a potent and competitive antagonist of this Y_1-mediated vasoconstriction.

Keywords: Cerebral blood flow; human cerebral arteries; vasoconstriction; neuropeptide Y (NPY); NPY-receptor agonists; NPY-receptor antagonists; Y_1-receptors; Y_2-receptors

Introduction

Neuropeptide Y (NPY) belongs to a peptide family which also includes peptide YY (PYY) and pancreatic polypeptide (PP) (Tatemoto, 1982). NPY has a wide distribution in the nervous system (Adrian *et al.*, 1983; De Quidt & Emson, 1986) and NPY-containing fibres are associated with blood vessels in both peripheral (Lundberg *et al.*, 1982) and cerebral vascular beds of several species (Edvinsson *et al.*, 1983; 1987; Mikkelsen *et al.*, 1993). NPY is involved in multiple functions including the regulation of blood flow through its potent contractile action on the vascular smooth muscle. In peripheral blood vessels, NPY elicits vasoconstriction by itself (Lundberg & Tatemoto, 1982) and by potentiating the effect of noradrenaline with which it is often colocalized (Ekblad *et al.*, 1984). In the cerebrovascular bed, NPY causes a strong and long lasting vasoconstriction in different species, including man (Edvinsson *et al.*, 1983; 1987; 1991; Meija *et al.*, 1988; Dacey *et al.*, 1988).

At least two different NPY receptors, referred to as Y_1 and Y_2 subtypes have been identified (Wahlestedt *et al.*, 1986; 1990). The Y_1 receptor which has recently been cloned from rat and man (Herzog *et al.*, 1992; Larhammar *et al.*, 1992) appears to be located mainly postjunctionally and belongs to the family of G protein-coupled receptors. This receptor is activated by the complete NPY molecule, PYY and the Y_1 agonists [Pro^{34}]-NPY, [Leu^{31}, Pro^{34}]-NPY and [Leu^{31}, Pro^{34}]-PYY (Wahlestedt *et al.*, 1986; Krstenansky *et al.*, 1990; Fuhlendorff *et al.*, 1990; Dumont *et al.*, 1994). In contrast, the Y_2 receptor seems to be located mainly prejunctionally and is activated by NPY, PYY and truncated C-terminal fragments of NPY and PYY (Wahlestedt *et al.*, 1986; Grandt *et al.*, 1994; Dumont *et al.*, 1994). The existence of a third receptor subtype (Y_3), which does not recognize PYY has also been well documented (Grundemar *et al.*, 1991; Wahlestedt *et al.*, 1992; Dumont *et al.*, 1994).

An unequivocal pharmacological characterization of NPY receptor subtypes has been hampered by the lack of selective NPY receptor antagonists and by the fact that all agonists are chemically related to NPY. The synthesis of subtype-specific NPY receptor antagonists has indeed proven difficult and the proposed molecules (Doughty *et al.*, 1990; Edvinsson *et al.*, 1990; Michel & Motulsky, 1990; Tatemoto *et al.*, 1992) did not fulfil the criteria of high affinity, specifity and competitiveness (Michel, 1991; Edvinsson *et al.*, 1994). Recently, however, a non-peptide compound (BIBP 3226) has been synthesized and shown to behave as a competitive, specific and selective Y_1 receptor subtype antagonist (Rudolf *et al.*, 1994; Jacques *et al.*, 1995).

Within these pharmacological limitations, Y_1 receptors have been identified by binding studies as the predominant subtype in vascular smooth muscle (Sheikh *et al.*, 1991; Grundemar *et al.*, 1992). This subtype has been shown to mediate the NPY-induced vasoconstriction in several peripheral vascular beds (Cadieux *et al.*, 1992; Grundemar & Häkanson, 1993;

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McDermott *et al.*, 1993), and expression of mRNA for the Y_1 receptor has been shown in smooth muscle cells from human peripheral arteries (Larhammar *et al.*, 1992). However, in some vascular beds, more detailed pharmacological studies indicate that Y_2 receptors are also involved in the NPY-induced contractile response (Grundemar *et al.*, 1992; Tessel *et al.*, 1993). Based on the use of [Pro^{34}]-NPY and the non selective antagonist PP56, it has been suggested that Y_1 receptors participate in the NPY-induced vasoconstriction in human and rodent cerebral arteries (Edvinsson *et al.*, 1991).

In the present study, we have taken advantage of several NPY and PYY derivatives with selective affinites at Y_1 or Y_2 receptor subtypes and of the new non-peptide Y_1 antagonist, BIBP 3226, to perform a detailed characterization of the receptor subtype(s) responsible for the NPY-induced vasoconstriction in human cerebral arteries. Altogether, the agonist and antagonist results suggest that Y_1 receptors are the exclusive mediators of the contractile response to NPY in human cerebral arteries. Furthermore, BIBP 3226 was found to be a potent and competitive antagonist of this Y_1-mediated vasoconstriction. Some of these data have appeared in abstract form (Abounader *et al.*, 1994).

Methods

Functional assay

Pial arteries (temporal ramifications of the middle cerebral artery) were obtained from 13 human subjects (10 women and 3 men), either post-mortem (delay 6–12 h) from patients who died from diseases not affecting the central nervous system (mainly cardiopulmonary failure) or post-operatively from epileptic patients undergoing temporal lobe surgery. Circular segments (internal diameter of $\simeq$ 0.8 mm and a length of 3–5 mm) were cut under a dissecting microscope, mounted on two L-shaped metal prongs in a temperature controlled (37°C) tissue bath (volume of 5 ml) for recording of the isometric tension developed by the smooth muscle as described previously (Högestätt *et al.*, 1983). The baths containing a Krebs-Ringer solution (in mM: NaCl 118, KCl 4.5, $MgSO_4.7H_2O$ 1.0, KH_2PO_4 1.0, $NaHCO_3$ 25, $CaCl_2.2H_2O$ 2.5, and glucose 6) which was replaced regularly every 15 min and bubbled with a gas mixture of 95% O_2 and 5% CO_2 to maintain a pH of 7.4. The vessels were given a mechanical tension of 0.4 g and allowed to stabilize at this level for 60 min. Changes in muscle tension were measured with a force displacement transducer and recorded on a Grass Polygraph coupled to a computer for automatic data acquisition and analysis (for details see Hamel & Bouchard, 1991; Hamel *et al.*,1993).

The maximal contractile capacity of each vessel segment was determined in the presence of 124 mM K^+, by replacing NaCl with an equimolar concentration of KCl in the Krebs-Ringer solution. After washing, cumulative concentrations of 5-hydroxytryptamine (5-HT) (10^{-9} to 10^{-4} M) were added to each vessel and the maximal contractile response to 5-HT (5-HT E_{Amax}) was determined. These receptor-mediated responses to 5-HT were chosen as reference contractile effect based on their proven reliability and reproducibility in both post-mortem and post-operative tissues (Hamel & Bouchard, 1991; Hamel *et al.*, 1993) and because they appear as the best indicator of the vessels ability to respond to G protein-coupled receptor activation. Indeed, vessels that did not constrict in response to 5-HT were discarded from further analysis in spite of a positive response to K^+ depolarization. Using these criteria, all vessel segments tested reacted to NPY or other NPY agonists. In the present study, the 5-HT E_{Amax} corresponded to 65.7 ± 2.8% (n = 74) of the contraction elicited by 124 mM K^+, in agreement with our previous findings (Hamel & Bouchard, 1991; Hamel *et al.*, 1993). The responses were also similar whether the vessels were obtained post-mortem (68.6 ± 4.6%; n = 26) or post-operatively (64.6 ± 3.5%, n = 48).

Responses to agonists

Following the dose-response curve to 5-HT, the vessels were washed three times with fresh buffer and allowed to recover for at least 30 min. Then, log-concentration response curves were generated for each agonist by cumulative addition (10^{-12}–10^{-6}/10^{-5} M) of either NPY, PPY, [Leu^{31}-Pro^{34}]-NPY, [Leu^{31}-Pro^{34}]-PYY, NPY_{13-36} or PYY_{3-36} to individual vessels. Since preliminary studies showed that NPY and related peptides dissociated very slowly from the cerebrovascular receptor, only one agonist was tested on each vessel segment.

The affinities of the different agonists were determined and expressed as pD_2 values (or $-\log EC_{50}$) and calculated according to Van den Brink (1977) as described in detail elsewhere (Hamel & Bouchard, 1991; Hamel *et al.*, 1993). For comparison of agonist potencies, the maximal response to each agonist (E_{Amax}) was expressed as a percentage of 5-HT E_{Amax} in the same vascular segments.

In a different series of experiments, vessels were first exposed to graded concentrations (10^{-12}–10^{-6} M) of the selective Y_2 receptor agonist, NPY_{13-36} and then dose-response curves (10^{-12}–10^{-6} M) to either NPY or the Y_1 receptor agonist, [Leu^{31}-Pro^{34}]-NPY were generated. The pD_2 values and maximal responses (expressed as % of 5-HT E_{Amax}) were then calculated for NPY and [Leu^{31}-Pro^{34}]-NPY and compared to those obtained when these peptides were tested alone.

Effect of the Y_1 receptor antagonist BIBP 3226

To test the ability of the Y_1 receptor antagonist, BIBP 3226 to antagonize the NPY-induced vasoconstriction in human cerebral arteries, different concentrations of BIBP 3226 (10^{-9}–10^{-6} M) were applied to different sets of vessels for 30 min. Then, in the presence of the antagonist, a dose-response curve to NPY was obtained as described in 'Responses to agonists'. In each case, one or two vessels served as controls as they were not exposed to the antagonist. The antagonist potency was expressed as pA_2 (or the negative logarithm of the molar antagonist concentration, in the presence of which twice the original agonist concentration is needed to cause an effect that is relatively equal to the original effect) and calculated according to Van den Brink (Van den Brink, 1977), as described in length previously (Hamel & Bouchard, 1991; Hamel *et al.*, 1993).

The competitive nature of the antagonism was assessed by determination of the slope of the Schild plot (Arunlakashana & Schild, 1959) and pA_2 obtained from the Schild analysis was compared to the pA_2 calculated as described above.

Drugs

NPY, PYY, [Leu^{31}, Pro^{34}]-NPY, NPY_{13-36} (Serva Biochemica GmbH, Germany) and the non-peptide Y_1-receptor antagonist, BIBP3226 [(**R**)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-argininamide] (developed by Dr Karl Thomae GmbH, Germany) were kindly provided by Dr Henri Doods (Dr Karl Thomae GmbH, Germany). [Leu^{31}-Pro^{34}]-PYY and PYY_{3-36} were synthesized and generously offered to us by Drs A. Fournier and S. St.-Pierre (INRS-Santé, Pointe-Claire, QC, Canada).

Results

Responses to agonists

With the exception of NPY_{13-36}, all NPY receptor agonists were found to induce potent and long lasting concentration-dependent contraction of human cerebral arteries (Figure 1; Table 1). NPY, PYY, [Leu^{31}-Pro^{34}]-NPY and [Leu^{31}-Pro^{34}]-PYY induced a full contraction which corresponded to about 100% of that elicited by 5-HT. The PYY derivative, PYY_{3-36} induced a vasocontractile response of smaller magnitude

(~70% of the 5-HT E_{Amax}). In some cases, a decrease in the contractile response was observed at the highest agonist concentration as shown by the decline in the dose-response curves (Figure 1). The Y_2 agonist, NPY_{13-36} failed to induce any significant vasocontractile response in human cerebral arteries with a contraction corresponding to only 2% of the 5-HT E_{Amax} measured at 10^{-6} M (Figure 1; Table 1). The selective Y_1 agonists, [Leu^{31}-Pro^{34}]-NPY and [Leu^{31}-Pro^{34}]-PYY, exhibited the highest pD_2 values at the cerebrovascular reporter, closely followed by NPY. PYY and PYY_{3-36} had lower pD_2 values. The overall rank order of agonist potency at the human cerebrovascular receptor corresponded to [Leu^{31}-Pro^{34}]-NPY = [Leu^{31}-Pro^{34}]-PYY $\geqslant$ NPY > PYY > PYY_{3-36} > > > NPY_{13-36} (Table 1).

In vessels incubated with up to 10^{-6} M NPY_{13-36}, the subsequent addition of NPY or the Y_1 agonist, [Leu^{31}-Pro^{34}]-NPY (Figure 2) resulted in strong concentration-dependent contractions with respective pD_2 values of 8.5±0.08 and 8.74±0.10 and maximal responses of 105±9.1 and 84±21% of the 5-HT E_{Amax} which were identical to those obtained when these compounds were tested without previous application of the Y_2 agonist (Table 1).

Effect of the Y_1 receptor antagonist, BIBP 3226

BIBP 3226 potently inhibited the NPY-induced vasoconstriction in human cerebral arteries. The application of increasing antagonist concentration led to a rightward shift of the dose-response curve without any significant decrease in the maximal contractile effect (Figure 3a). The mean pA_2 for BIBP 3226 (calculated at antagonist concentrations of 10^{-8} and 10^{-7} M) was 8.52±0.13 (n=15). The inhibition appeared competitive as the slope (1.10±0.02) of the Schild plot analysis (Figure 3b) was not significantly different from unity, with a correlation coefficient (r) of 0.99. The pA_2 value evaluated at the intercept (8.39) corresponded well to that calculated according to Van den Brink.

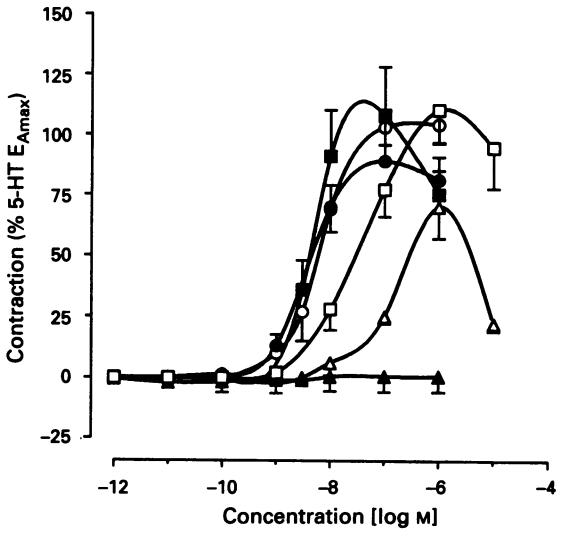

Figure 1 Concentration-response curves for neuropeptide Y (NPY, ○), [Leu^{31}, Pro^{34}]-NPY (●) PYY (□), [Leu^{31}, Pro^{34}]-PYY (■), PYY_{3-36} (△) and NPY_{13-36} (▲) on human cerebral arteries. The individual potencies, maximal responses and number of vascular segments are given in Table 1. Values are means±s.e.mean.

Discussion

In the present study, we have used a series of NPY and PYY derivatives with agonist properties at either the Y_1 or Y_2 receptor subtype and the new non-peptide Y_1 receptor antagonist, BIBP 3226 (Rudolf *et al.*, 1994; Jacques *et al.*, 1995) to determine the receptor subtype(s) responsible for the NPY-induced vasoconstriction in human cerebral arteries. Altogether, the results point to the exclusive involvement of the Y_1 receptor subtype in this vasomotor response.

Our results show that NPY, PYY and their derivatives with Y_1 selectivity elicited potent vasoconstriction in human cerebral arteries. PYY_{3-36}, a recently developed PYY C-terminal fragment which posesses a higher potency at Y_2 than at Y_1 receptors (Dumont *et al.*, 1994), induced a submaximal contraction which was apparent only at relatively high concentrations (10^{-7}/10^{-6} M). This low potency corresponded very closely to PYY_{3-36} affinity at Y_1 receptors determined in rat frontoparietal cortex and rabbit saphenous vein (Dumont *et al.*, 1994), an observation which strongly suggests that Y_1 receptors are responsible for the constriction elicited by PYY_{3-36} in human cerebral arteries. This contention is supported by the failure of the selective Y_2 agonist, NPY_{13-36} (Wahlestedt *et al.*, 1986; 1990) to show any significant vasomotor effect on the human cerebral arteries tested. The involvement of Y_1 receptor in this effect is further substantiated by the fact that [Leu^{31}, Pro^{34}]-NPY or NPY tested after the application of the Y_2 receptor agonist, NPY_{13-36}, elicited vasocontractile responses with pD_2 and E_{Amax} values indistinguishable from those obtained when these compounds were tested alone. This finding indicates that both NPY and [Leu^{31}, Pro^{34}]-NPY induce contraction in human cerebral arteries via NPY_{13-36}-insensitive receptors, namely the Y_1 receptor subtype. Interestingly, in tissues where both Y_1 and Y_2 receptors have been found to participate in the NPY-induced contraction (Tessel *et al.*, 1993), the responsiveness to NPY was significantly diminished in vessels previously exposed to NPY_{13-36} or [Leu^{31}, Pro^{34}]-NPY whereas NPY_{13-36} and [Leu^{31}, Pro^{31}]-NPY, acting respectively on Y_1 and Y_2 receptors, did not interact with each other responsiveness. In the present study, the lack of selective desensitization by NPY_{13-36} on the vasomotor response to NPY and [Leu^{31}, Pro^{34}]-NPY provides no evidence for other than the postjunctional Y_1 receptors mediating vasoconstriction in human cerebral arteries.

Table 1 Potencies of various neuropeptide (NPY) and PYY derivatives for inducing contraction of human cerebral arteries

Agonist	n	*K^+ 124 mM* (g)	*Agonist E_{Amax}* (g)	*Agonist E_{Amax}* *(% of 5-HT-E_{Amax})*	*pD_2* *(-log EC_{50})*
NPY	17	1.13±0.11	0.79±0.11	106±6.6	8.35±0.16
PYY	8	0.59±0.10	0.46±0.10	111±14.0	7.55±0.15
[Leu^{31}, Pro^{34}]-NPY	5	1.63±0.46	0.83±0.19	90±12.3	8.72±0.14
[Leu^{31}, Pro^{34}]-PYY	4	1.31±0.18	1.01±0.32	108±20.4	8.72±0.14
PYY_{3-36}	4	2.1±0.39	0.77±0.29	70±13.0	6.67±0.13
NPY_{13-36}	10	0.75±0.18	0.07±0.03	1.4±1.7	ID

All values represent mean±s.e.mean of the number n of individual vessel segments. For each agonist, E_{Amax} values are given in tension developed by the smooth muscle (g) and as % of the maximal contractile response to 5-HT (5-HT E_{Amax}) measured in the same vessel segments. For information, the magnitude of the contractile response elicited by a depolarizing concentration of K^+ is also provided for each group of vessels. ID: impossible to determine.

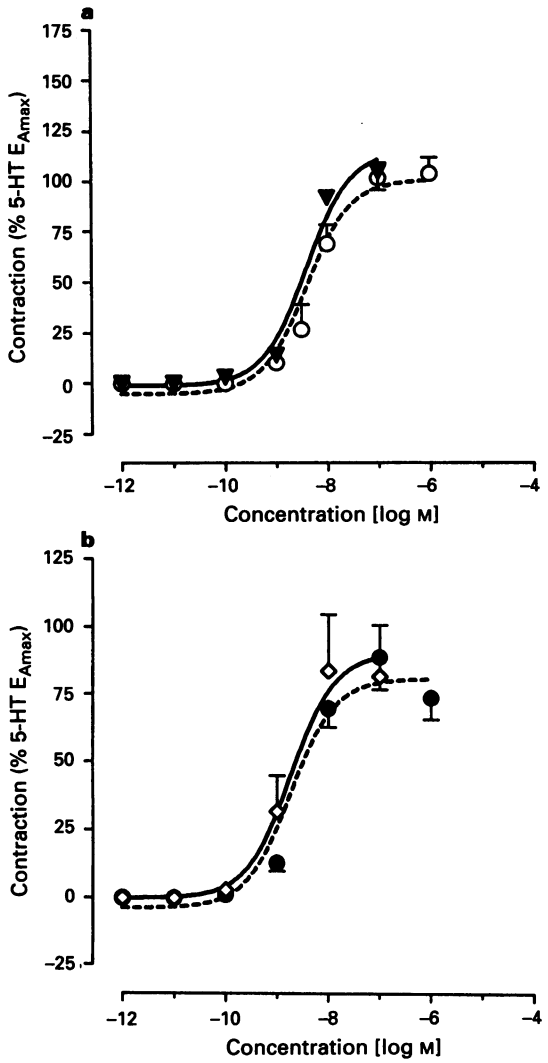

Figure 2 Concentration-response curves on human cerebral vessels for (a) neuropeptide Y (NPY) in the absence (○) and presence (▼) of 10^{-6} M NPY_{13-36} and (b) [Leu^{31}, Pro^{34}]-NPY in the absence (●) and presence (◇) of 10^{-6} M NPY_{13-36}. Values are means ± s.e.mean.

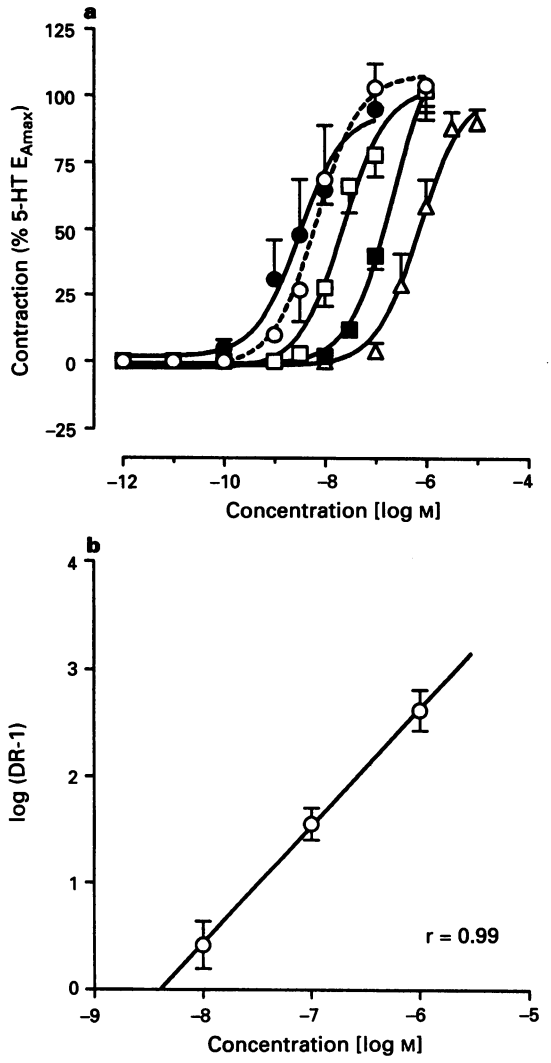

Figure 3 (a) Concentration-response curves for neuropeptide Y (NPY) on human cerebral arteries in the absence (○, control) and presence of 10^{-9} M (●), 10^{-8} M (□), 10^{-7} M (■) or 10^{-6} M (△) BIBP 3226, $n = 17$, 7, 8, 7 and 4 respectively. (b) Schild plot analysis of BIBP 3226 antagonism (○) on human cerebral arteries. Values are means ± s.e.mean.

Similarly, the overall order of agonist potency at the human cerebrovascular receptor: [Leu^{31}-Pro^{34}]-NPY = [Leu^{31}-Pro^{34}]-PYY ≥ NPY > PYY > PYY_{3-36} > > > NPY_{13-36} compared very well with that reported for these compounds at the neuronal (Dumont *et al.*, 1993; 1994) and vascular (Sheikh *et al.*, 1991; Grundemar *et al.*, 1992; Shigeri *et al.*, 1991) Y_1 binding sites. The absolute pD_2 values for these agonists at human cerebrovascular receptors were very similar to their published affinities in the rabbit saphenous vein, a tissue enriched with Y_1 receptors (Cadieux *et al.*, 1993). The drop in the maximal contractile response observed for some agonists at high concentrations (10^{-6}/10^{-5} M) may be due to a toxic effect of such high levels of neuropeptides on the vessel wall.

The involvement of a putative Y_3 receptor in the NPY-induced cerebral vasoconstriction cannot be excluded but appears most unlikley in view of the relatively high potency of PYY and the lack of agonistic effect of NPY_{13-36} in human cerebral arteries (Grundemar *et al.*, 1991; Wahlestedt *et al.*, 1992; Dumont *et al.*, 1994). Altogether, the agonists data indicate that Y_1 receptors are most likely the only receptors responsible for the vasocontractile response induced by NPY in human cerebral arteries.

A non-peptide antagonist (BIBP 3226) has recently been described as a potent and selective antagonist at Y_1 receptors (Rudolf *et al.*, 1994; Jacques *et al.*, 1995). This compound represents a very interesting tool in the pharmacological characterization of NPY receptors since all previously synthesized NPY receptor antagonists (Doughty *et al.*, 1990; Edvinsson *et al.*, 1990; Michel & Motulsky, 1990; Edvinsson *et al.*, 1994). BIBP 3226, on the contrary, has proven its selectivity for Y_1 receptors in binding assays with human and rodent tissues and in its ability to block Y_1-mediated functional responses such as increases in intracellular calcium in SK-N-MC neuroblastoma human cell line or in perfusion pressure in the rat isolated perfused kidney (Rudolf *et al.*, 1994). More recently, the high potency and selectivity of BIBP 3226 for Y_1 receptors in the rabbit saphenous vein was demonstrated over Y_2 and Y_3 functional receptors in the rat vas deferens and rat colon selective bio-assays, respectively (Jacques *et al.*, 1995). In the present study, we further demonstrate that BIBP 3226 is a potent and competitive antagonist at the human Y_1 cerebrovascular receptor as shown by the rightward and parallel shift of the concentration curves with no change of the maximal contractile response. Its cerebrovascular affinity is high ($pA_2 = 8.52$) and in full agreement with that determined at the neuronal Y_1 receptors (Rudolf *et al.*, 1994), an observation which confirms the involvement of Y_1 receptors in the NPY-induced vasoconstriction of human cerebral arteries.

Although other NPY receptors may be present in the human cerebrovascular bed, our results agree with most binding

and functional studies (Sheikh *et al.*, 1991; Edvinsson *et al.*, 1991; Grundemar *et al.*, 1992; Dumont *et al.*, 1994) in showing that Y_1 receptors mediate vasoconstriction in human cerebral arteries. Recent studies relying on molecular biology techniques have used antisense oligonucleotides to block the expression of vascular Y_1 receptor in human peripheral blood vessels (Erlinge *et al.*, 1993). This treatment resulted in a loss of vasocontractile response to NPY, a finding which again underscores the importance of Y_1 receptors in the human vascular bed, albeit of peripheral origin.

We conclude that the vasoconstriction elicited by NPY in human cerebral blood vessels appears to be mediated exclusively by Y_1 receptors, as no evidence for the participation of other receptor subtype(s) could be provided. Furthermore, we show that BIBP 3226, a novel non-peptide Y_1 receptor antagonist, exhibits high affinity and competitiveness at the human Y_1 cerebrovascular receptor and thus appears as a new tool for the pharmacological investigation of functional NPY-mediated responses.

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