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Discrimination between neuropeptide Y and peptide YY in the rat tail artery by the neuropeptide Y_1-selective antagonist, BIBP 3226

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1 The ability of the novel, nonpeptide, neuropeptide Y (NPY) Y_1-selective antagonist, BIBP 3226 {(**R**)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-arginine amide}, to antagonize the increase in perfusion pressure induced by NPY and peptide Y (PYY) was tested in the perfused rat tail artery, a postjunctional Y_1-receptor bioassay, precontracted by 1 μM phenylephrine.

2 NPY and PYY produced a concentration-dependent enhancement of the vasoconstrictor response evoked by 1 μM phenylephrine. Although NPY and PYY are roughly equipotent, the maximal contractile response elicited by PYY was about twice that elicited by NPY.

3 Increasing concentrations of BIBP 3226 caused a parallel and rightward shift in the NPY concentration-response curve without depressing the maximal response. The contractile effect of NPY was potently inhibited in a competitive manner. The pA_2 value for BIBP 3226 was 7.01 ± 0.08, a value equivalent to that observed in the rabbit saphenous vein. Although increasing concentrations of BIBP 3226 shifted the concentration-response curve of PYY to the right without any significant decrease in the maximal vasoconstrictor response, the antagonism appeared non-competitive as the slope of the Schild plot was significantly different from unity (0.58 ± 0.04).

4 In conclusion, these data confirm that BIBP 3226 is a potent and selective nonpeptide Y_1 receptor antagonist. Moreover, they show that complex interactions occur between BIBP 3226 and postjunctional receptors activated by PYY. We postulate that BIBP 3226 might discriminate between the effects of NPY and PYY at the postjunctional level in the rat tail artery. It may be that distinct receptors for NPY and PYY exist; these may or may not allosterically interact with each other. Another working hypothesis would be that there is a single receptor complex with allosterically interacting binding sites for the two peptides.

Keywords: Neuropeptide Y; peptide YY; neuropeptide Y Y_1 antagonist; vasoconstriction; rat tail artery

Introduction

Neuropeptide Y (NPY) and peptide YY (PYY) are 36 amino acid peptides belonging to the pancreatic polypeptide family (Larhammar *et al.*, 1993). Three G protein-coupled NYP receptor subtypes (Y_1, Y_2, Y_3) have been pharmacologically identified based mainly on the rank order of potency exhibited by the native peptides, C-terminal fragments and various analogues of the native peptides (for review see Wahlestedt & Reis, 1993; Grundemar & Håkanson, 1994; Gehlert, 1994). Both the Y_1 (Eva *et al.*, 1992; Herzog *et al.*, 1992; Larhammar *et al.*, 1992) and Y_2 (Gerald *et al.*, 1995; Rose *et al.*, 1995) receptor subtypes have been cloned. Nevertheless, recently, a growing body of evidence based upon pharmacological characterization and functional expression of cloned receptors suggests that there is an additional subtype of NPY receptor Y_4 (Bard *et al.*, 1995) or PP_1 (Lundell *et al.*, 1995).

In the cardiovascular system, a variety of functional roles have been attributed to NPY (for review see Edvinsson *et al.*, 1987; Wahlestedt & Reis, 1993). In peripheral blood vessels, these effects are mediated via two pharmacologically distinct receptors termed Y_1 and Y_2 (Wahlestedt *et al.*, 1986; Sheikh *et al.*, 1989). Originally it was postulated that the Y_1-type receptor, which is involved in the direct and indirect effects on contractile activity, was located postjunctionally and required the whole NPY/PYY amino acid sequence for activation. The Y_2-type receptor seemed to be prejunctional and recognized not only NPY/PYY but also C-terminal fragments of both NPY and PYY (Wahlestedt *et al.*, 1986). However, this restricted functional localization has not been observed in recent studies. In the rat mesenteric aterial bed (McAuley & Westfall, 1992) and caval vein (Grundemar *et al.*, 1992), the presence of postjunctional Y_1 and Y_2 receptors was suggested and there is evidence for the existence of prejunctional Y_1 receptors (McAuley & Westfall, 1992).

Among the subtypes of NPY receptors proposed, previous studies described in the rat small intestine and adipocytes a receptor exhibiting a preference for PYY over NPY (Laburthe *et al.*, 1986; Castan *et al.*, 1992). In the rat jejunum, this 'PYY preferring' receptor (Gehlert, 1994) appears to be a 44 kDa glycoprotein (Voisin *et al.*, 1991) and a role in the epithelial cell growth has been attributed to this receptor (Voisin *et al.*, 1993). Moreover, a differential mechanism for PYY and NPY in inhibiting motility of the guinea-pig colon has been recently demonstrated (Sawa *et al.*, 1995) and it has also been suggested that more than one receptor phenotype is expressed by a human colonic epithelial cell line with high affinity for PYY (Cox & Tough, 1995).

In our *in vitro* studies with the rat tail artery, PYY elicited a vasoconstrictor response that was about twice that of NPY (Tschöpl *et al.*, 1993). Moreover, in this vessel, we suggeted that the Y_1-receptor may posses an allosteric binding site. Until recently, we have not been able to distinguish between the effects of these peptides and the absence of useful selective and potent competitive antagonists has limited the understanding of the physiological role of PYY and NPY in this artery.

Recently a non-peptide compound described as a selective and potent Y_1 subtype receptor antagonist, BIBP 3226 {(**R**)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-arginine amide}, has been synthesized (Rudolf *et al.*, 1994). In the present study, we have taken advantage of this new antagonist

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to perform a detailed pharmacological characterization of the receptor subtype responsible for the enhancement by NPY and PYY of the α-adrenoceptor-mediated vasoconstriction in the perfused isolated tail artery of the rat.

Methods

Rat tail artery

Male Wistar rats (12 weeks old) were killed by cervical dislocation and exsanguinated. A segment of about 2.0–2.5 cm of the proximal part of the ventral rat tail artery was dissected out as described previously (Tschöpl *et al.*, 1993) and kept in oxygenated (95% O_2 5% CO_2) medium of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11. The arteries were cannulated at both ends and suspended vertically in an organ bath containing 4 ml of medium and perfused via their proximal ends. Changes in the inflow perfusion pressure reflected changes in the resistance to flow, i.e. the degree of vasoconstriction. The arteries were allowed to equilibrate for 60 min before the contractile capacity was tested by exposure of the arterial segment to a concentration of 3 μM phenylephrine before being contracted every 20 min by addition of 1 μM phenylephrine before being contracted every 20 min by addition of 1 μM phenylephrine for about 10 min. The EC_{50} for phenylephrine in this vessel is about 2.5 μM. Functional endothelium was confirmed by the ability of the preparation precontracted with phenylephrine to relax in response to 10 μM acetylcholine at the beginning and at the conclusion of each experiment. Once the artery had equilibrated and responded with 3 comparable phenylephrine-elicited contractions, concentration-response curves to NPY and PYY were constructed non-cumulatively by the addition of a single concentration of the peptide to the arterial segment precontracted with 1 μM phenylephrine. When the contraction to NPY or PYY had reached equilibrium, both phenylephrine and the peptide were washed out and the preparation left for 30 min before the addition of the same concentration of phenylephrine and a different concentration of the peptide. Arterial segments were washed three times every 10 min with fresh medium between each incremental concentration. Two concentration-response curves for either NPY or PYY were determined in each artery, the first before, the second 60 min after a washing period in the presence of the antagonist or its solvent in the case of time controls. Antagonist was present for 10 min before the preparation was challenged again with the agonist. Because the NPY- or PYY-induced increase of phenylephrine-elicited vasoconstriction was long lasting, only one concentration of antagonist was investigated in each arterial preparation. All compounds were administered extraluminally to the bath fluid in a volume of 10 or 30 μl. Note that at the beginning of each experiment, the preparation precontracted with 1 μM phenylephrine was challenged with 100 nM NPY to ascertain tissue responsiveness to the peptide; this contraction served as an internal reference and was set at 100%. In control experiments, contractions to PYY were expressed as a percentage of the 100 nM NPY-induced response. At the end of all experiments, the same control contraction to 100 nM NPY was elicited: there was no significant difference between the first and last contractile responses.

Expression of results and statistical analysis

The contractile responses were expressed as percentages of the maximum contractile response to either NPY or PYY obtained in the first control concentration-response curve without antagonist. As a slight, non significant increase of the maximal contractile response to NPY or PYY without any significant change in the EC_{50} values was observed in the second time control curve, all second concentration-response curves were corrected for the increase that occurred without antagonist (1.10 and 1.19 fold for NPY and PYY respectively). The concentration-response curves, each containing at least four concentrations, were analysed by fitting sigmoidal functions to the experimental data, using iterative non-linear regression analysis with the Prism programme (GraphPAD Software, San Diego, California, U.S.A.). The calculation yielded the maximal effect and the EC_{50} for each curve, EC_{50} being the concentration producing 50% of the maximum effect of that curve. A Schild plot (Arunlakshana & Schild, 1959) for the antagonism of NPY or PYY by BIBP 3226 was constructed. The plot of log (CR − 1) where CR is the concentration-ratio (ratio of the EC_{50} value in the presence and absence of the antagonist) versus log [antagonist] was analysed by linear regression. Antagonism was considered to be simply competitive in nature if the slope of the regression line was not significantly different from unity. A mean pA_2 value (i.e. $-\log K_b$, K_b being the dissociation constant of the antagonist determined according to the equation 4 of Furchgott, 1972) was also calculated from individual vascular preparations.

All values in the text and figures are expressed as the mean ± s.e.mean of n experiments, i.e. each experiment refers to one artery taken from one rat. Statistical analysis was performed by the Mann-Whitney U test if Kruskall-Wallis analysis indicated a significant difference between multiple groups. Differences in mean EC_{50} and E_{max} values within a single tissue were tested for significance with Student's paired t test. Bonferroni correction was used for multiple comparisons to a single control as described by Wallenstein *et al.* (1980). A probability level of 0.05 or less was considered statistically significant.

Drugs

The following drugs were used: (−)-phenylephrine hydrochloride (Sigma, France); hNPY, hPYY (Neosystem, France); {(**R**)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-arginine amide} (BIBP 3226) was a kind gift from Dr Karl Thomae GmbH (Biberach, Germany).

Results

After the equilibration period, the mean increase in vasoconstriction upon exposure of the rat tail artery to 1 μM phenylephrine was 27.5 ± 0.9 mmHg (n = 112), with no significant differences between different series of experiments. It should be noted, however, that the maximal vasoconstrictor response (about 180–200 mmHg) produced by phenylephrine 10 μM was never attained by any combination of the peptides with 1 μM phenylephrine.

After the 60 min washout period following the first concentration-response curve, we observed an increase in the phenylephrine-elicited vasoconstriction which amounted at the end of the experiment to 39.6 ± 1.4 mmHg (n = 112; P < 0.01). In order to verify whether this increase had any influence on the enhancing effect of NPY or PYY, two successive concentration-response curves were constructed. Although we observed an increase of about 45% of the phenylephrine-elicited precontraction level, we noted only a slight but non significant increase in the maximal values of the second concentration-response curves for both NPY (20.7 ± 5.0 mmHg to 23.1 ± 4.4 mmHg; n = 6; P > 0.05) and PYY (48.6 ± 11.3 mmHg to 55.0 ± 6.0 mmHg; n = 6; P > 0.05) without any significant change in the EC_{50} values (39.7 nM ± 5.9 vs 43.7 ± 5.8 nM; n = 6; P > 0.05 for NPY and 30.5 ± 3.6 nM vs 27.5 ± 3.5 nM; n = 6; P > 0.05 for PYY). Thus, in this particular preparation and under this experimental protocol, the vasoconstriction induced by NPY and PYY is not dependent on the phenylephrine precontraction level.

NPY (0.010–0.3 μM) both elicited a concentration-dependent enhancement of the vasoconstriction evoked by previous exposure of the rat tail artery to 1 μM phenylephrine (Figure 1). When the contractions were expressed as a percentage of the initial 100 nM NPY-induced response, set as 100%, the

maximal effect elicited by PYY was about twice that elicited by NPY (185.6±6.9%; $n=55$ and 101.5±3.0; $n=57$; respectively; $P<0.01$), with EC_{50} values of 32.9±1.7 nM and 36.1±1.4 nM respectively. In subsequent experiments we observed that application of 100 nM PYY further increased the enhancement of vasoconstriction elicited by a submaximal concentration of 100 nM NPY, whereas 100 nM NPY had no effect when added after the response evoked by 100 nM PYY had reached its maximal effect (Figure 2).

In the presence of 1 μM phenylephrine, BIBP 3226 up to 10 μM, had no enhancing effect on the vasoconstriction induced by the α-adrenoceptor agonist. The addition of 100 nM, 200 nM, 300 nM, 1 μM and 3 μM BIBP 3226 progressively shifted the concentration-response curve elicited by NPY in the absence of BIBP 3226 to the right in a parallel manner with no significant depression in maximum response (Figure 3a). The contractile effect of NPY was potently inhibited by BIBP 3226 in a competitive fashion yielding a Schild plot slope not significantly different from unity (0.97±0.09), with a correlation coeffecient (r) of 0.98. The pA_2 value for BIBP 3226 estimated at the intercept of the abscissa scale was 7.01±0.08 (Figure 3b) and is practically identical to that calculated according to the equation of Furchgott: 6.99±0.04 ($n=49$).

Similarly, BIBP 3226 shifted the concentration-response curves of PYY to the right without depressing significantly the maximal response (Figure 4a). However, the Schild plot analysis of these rightward shifts revealed a regression line with a slope which was significantly different from unity (0.58±0.04; $P<0.01$), so that the pA_2 value could not be assessed in this condition (Figure 4b); apparent pA_2 values calculated for 0.1, 0.3, 1.0 and 3.0 μM BIBP 3226 were 7.28±0.05 ($n=11$), 7.02±0.04 ($n=12$), 6.88±0.04 ($n=12$) and 6.62±0.06 ($n=14$), respectively.

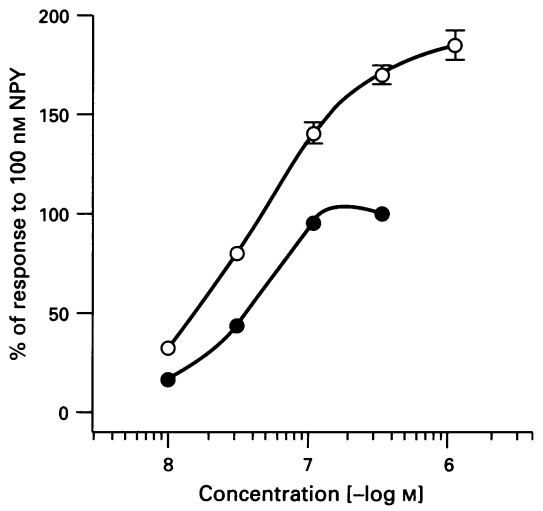

Figure 1 Concentration-dependent contractile responses produced by neuropeptide Y (NPY) and peptide YY (PYY) in rat tail arteries precontracted with 1 μM phenylephrine. Responses are expressed as a percentage of the contractile response elicited by 100 nM NPY. Symbols indicate mean±s.e.mean: (●) NPY (57); (○) PYY (55).

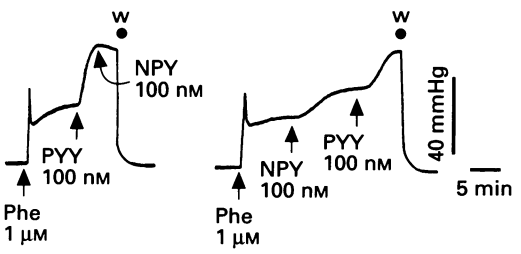

Figure 2 Representative traces showing the enhancement of the perfusion pressure produced by either NPY or PYY alone or after plateau has been reached by the further addition of one of the peptides in the perfused rat tail artery precontracted with 1 μM phenylephrine (Phe).

Discussion

NPY and PYY both enhance phenylephrine-mediated vasoconstriction of the rat isolated caudal artery in a concentration-dependent manner. This vascular preparation expresses almost exclusively NPY Y_1-like receptors since our previous *in vitro*

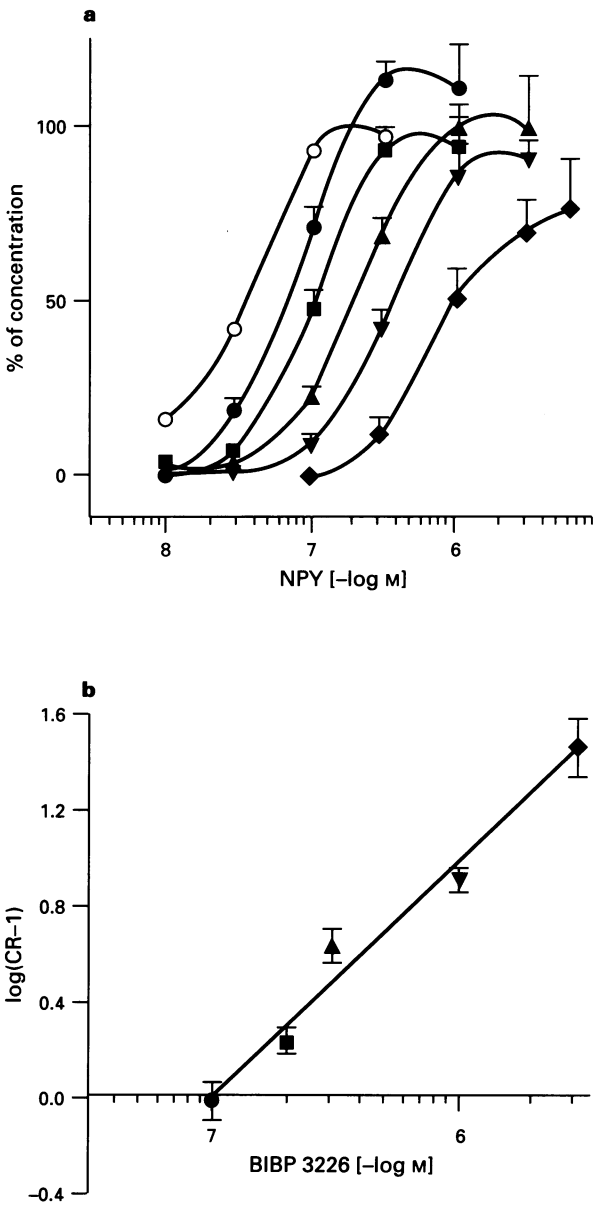

Figure 3 (a) Log concentration-response curves for neuropeptide (NPY)-elicited enhancement of the vasoconstrictions evoked by previous exposure of rat tail arteries of 1 μM phenylephrine in the absence (○; $n=51$) and presence of 100 nM (●; $n=13$), 200 nM (■; $n=13$), 300 nM (▲; $n=13$), 1 μM (▼; $n=6$) or 3 μM (◆; $n=6$) BIBP 3226. (b) Schild plot regression analysis for antagonism of NPY-induced vasoconstrictor responses in rat tail arteries by BIBP 3226. Slope was 0.97±0.09 and the intercept with the abscissa scale was 7.01. Data are means±s.e.mean. Error bars falling within the area covered by a symbol are not shown.

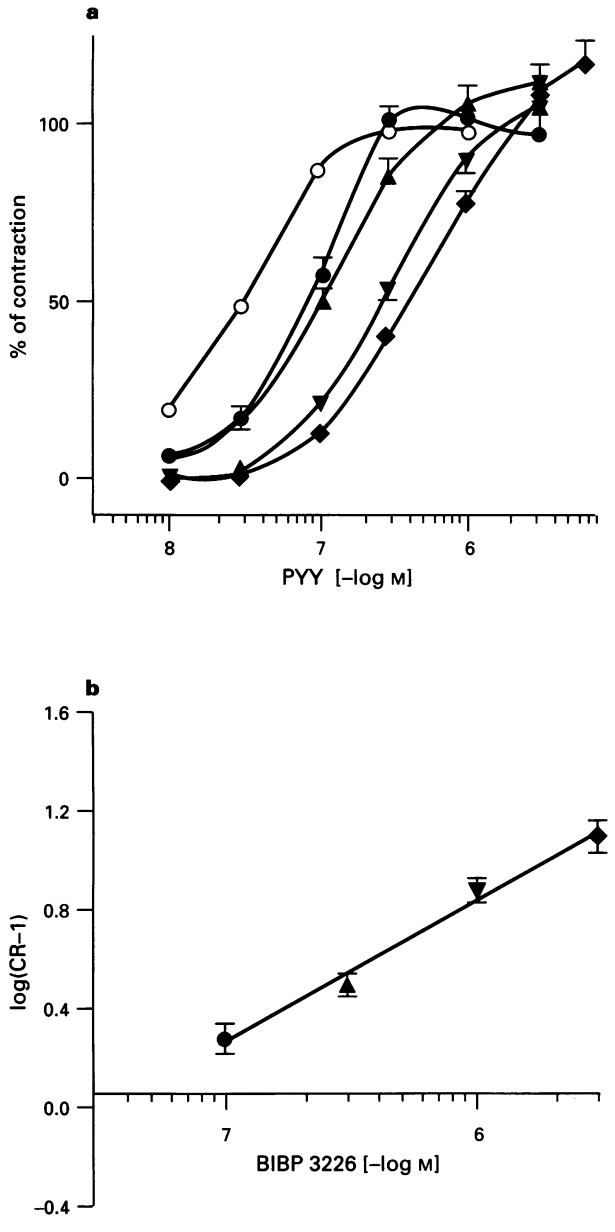

Figure 4 (a) Log concentration-response curves for peptide YY (PYY)-elicited enhancement of the vasoconstrictions evoked by previous exposure of rat tail arteries to 1 μM phenylephrine in the absence (○; $n=49$) and presence of 100 nM (●; $n=11$), 300 nM (▲; $n=12$), 1 μM (▼; $n=12$) or 3 μM (◆; $n=14$) BIBP 3226. (b) Schild plot regression analysis for antagonism of PYY-induced vasoconstrictor responses in rat tail arteries by BIBP 3226. Slope was 0.58 ± 0.04. Data are means ± s.e.mean. Error bars falling within the area covered by a symbol are not shown.

study had shown that NPY, PYY and [Leu^{31}, Pro^{34}]NPY were practically equipotent and NPY 13-36 was negligibly active (Tschöpl *et al.*, 1993). The contractile responses found here agree with our earlier observations and with the enhancement of contractile responses to a variety of vasoactive agents by NPY or PYY in arteries from different species being mediated predominantly by NPY Y_1 receptors (Wahlestedt & Reis, 1993). The fact that PYY produced a maximal response about twice that observed for NPY might suggest that NPY acts as a partial agonist and PYY behaves as a full agonist on a unique Y_1 receptor. However, this possibility is not supported by our previous data (Tschöpl *et al.*, 1993) showing that the effect of NPY on the concentration-response curve to PYY was not that expected for a partial agonist, i.e., producing a response and blocking the effect of the full agonist (Kenakin, 1993).

In the present study we examined the effect of BIBP 3226, the first described nonpeptide antagonist of neuropeptide Y (Rudolf *et al.*, 1994) in the rat tail artery precontracted with phenylephrine. This compound was recently reported to display potent and highly subtype Y_1-selective antagonistic properties both in *in vitro* and *in vivo* models (Rudolf *et al.*, 1994; Abounader *et al.*, 1995; Doods *et al.*, 1995; Entzeroth *et al.*, 1995; Lundberg & Modin, 1995; Malmström & Lundberg, 1995; Wieland *et al.*, 1995; Nilsson *et al.*, 1996). We have further confirmed and extended the evidence that BIBP 3226 is a selective competitive antagonist of NPY Y_1-mediated effects in the rat tail artery. BIBP 3226 antagonized the NPY-elicited enhancement of phenylephrine-mediated vasoconstriction with a potency essentially identical to that reported in the rabbit saphenous vein (Jacques *et al.*, 1995). The reported pA_2 values obtained in human cerebral arteries of 8.58 (Abounader *et al.*, 1995) and 8.38 (Nilsson *et al.*, 1996) are slightly higher. Recently, functional vascular studies in different animal species and in man were compared in order to determine whether BIBP 3226 shows species selectivity (Doods *et al.*, 1996). The measured pK_B values were only slightly different from each other, strongly suggesting that BIBP 3226 does not discriminate between vascular Y_1 receptors of different species.

Schild analysis of the antagonism of BIBP 3226 against PYY yielded a slope different from unity, indicating a lack of equilibrium. This might be the consequence, at least in part, of the involvement of multiple receptor subtypes. Therefore, a speculative hypothesis of the present observations might be that NPY and PYY produce their biological effects selectively through two physically distinct receptors. Moreover, the fact that PYY is able to increase further the vasoconstriction induced by a submaximal concentration of NPY might indicate the involvement of another receptor not stimulated by NPY and argues against functional cross-desensitization between NPY and PYY at a common receptor. Thus our data are compatible with the idea that besides the Y_1 subtype receptor, a PYY-preferring receptor for which the affinity of BIBP 3226 is much lower than for the putative Y_1 subtype receptor, might contribute to the response of PYY in the rat tail artery. A clear cut answer to this hypothesis might be obtained by binding studies but unfortunately we were unable to define specific [^{125}I]-PYY or [^{125}I]-NPY binding sites on smooth muscle cell membranes from rat tail artery (Tschöpl *et al.*, 1993).

We are not aware of any reports proving the existence of such a receptor. However, it has been shown recently that PYY may act on receptors distinct from those for NPY that are located on the soma-dendritic region of postganglionic cholinergic neurones in the guinea-pig colon (Sawa *et al.*, 1995). Moreover, it has also been suggested recently that a 'new Y' subtype receptor with an unusual phenotype exists or that more than one receptor phenotype is expressed by a human colonic epithelial cell line (Cox & Tough, 1995). Based on cross-desensitization experiments, one subtype seems to be PYY-preferring, and another receptor population is Y_1-like (Cox & Tough, 1995). It should be noted that previous studies have described a PYY-preferring receptor in rat small intestine (Laburthe *et al.*, 1986), dog adipocytes (Castan *et al.*, 1992) and in a proximal tubule cell line (Voisin *et al.*, 1993). However, although this receptor has been solubilized from rat jejunal crypts (Voisin *et al.*, 1991), it has not yet been cloned.

Alternatively, another speculative interpretation of the present data might be that these two receptors are allosterically coupled, perhaps coexisting as an NPY/PYY-receptor complex, in rough analogy with the model of the opoid receptor complex postulated by Rothman & Westfall (1982a, b). It is therefore possible that the PYY-mediated enhancement of the phenylephrine-elicited vasoconstriction could be mediated either via the NPY-site associated with the receptor complex or by a PYY binding site not associated with the NPY-receptor complex or by both. Moreover, the results obtained in our previous study with truncated and stabilized NPY analogues

(Krstenansky *et al.*, 1989) also suggested that the Y_1-receptor subtype present in this vessel probably possesses an allosteric binding site (Tschöpl *et al.*, 1993).

The complexity of the interaction between PYY and BIBP 3226 might also be related to an intrinsic property of the antagonist. A definitive answer must await the development of further antagonists with high affinities for the different receptors involved in the observed responses. Note that the potent antagonist, 1299U91, recently described as a selective NPY antagonist, exhibits high affinities for both Y_1 and Y_2 receptors (Daniels *et al.*, 1995).

In accordance with recent studies, it is obvious that the putative subclassification of NPY/PYY receptors into four or more subtypes needs further corroboration in future; more subtypes may be pharmacologically characterized and their functional role defined. The concept of subclasses of NPY receptors will require additional study especially with the development of selective antagonists of the proposed receptor subtypes with different chemical structures.

In conclusion, our experimental results described for the first time straightforward evidence that the selective nonpeptide NPY Y_1 subtype antagonist, BIBP 3226, is able to discriminate between NPY- and PYY-mediated effects in the rat tail artery. PYY mediates the enhancement of the contractile response of the perfused tail artery through the NPY Y_1 subtype and possibly also through PYY-specific receptor or both. Moreover, the present results indicate that BIBP 3226 may be a useful tool to investigate further the pharmacological subclassification of the receptors of the peptides belonging to the pancreatic polypeptide family.

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