British Journal of Pharmacology (2004) 143, 976–986
www.nature.com/bjp

Heterogeneity of the neuropeptide Y (NPY) contractile and relaxing receptors in horse penile small arteries

*,[1]Dolores Prieto, [1]Luis Rivera de los Arcos, [2]Pilar Martínez, [1]Sara Benedito, [1]Albino García-Sacristán & [1]Medardo Hernández

[1]Departamento de Fisiología, Facultad de Farmacia, Universidad Complutense de Madrid, 28040-Madrid, Spain and [2]Departamento de Anatomía y Anatomía Patológica, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040-Madrid, Spain

1 The distribution of neuropeptide Y (NPY)-immunorective nerves and the receptors involved in the effects of NPY upon electrical field stimulation (EFS)- and noradrenaline (NA)-elicited contractions were investigated in horse penile small arteries.

2 NPY-immunoreactive nerves were widely distributed in the erectile tissues with a particularly high density around penile intracavernous small arteries.

3 In small arteries isolated from the proximal part of the corpora cavernosa, NPY (30 nM) produced a variable modest enhancement of the contractions elicited by both EFS and NA. At the same concentration, the NPY Y_1 receptor agonist, [Leu^{31}, Pro^{34}]NPY, markedly potentiated responses to EFS and NA, whereas the NPY Y_2 receptor agonist, NPY(13–36), enhanced exogenous NA-induced contractions.

4 In arteries precontracted with NA, NPY, peptide YY (PYY), [Leu^{31}, Pro^{34}]NPY and the NPY Y_2 receptor agonists, *N-acetyl*[Leu^{28,31}]NPY (24–36) and NPY(13–36), elicited concentration-dependent contractile responses. Human pancreatic polypeptide (hPP) evoked a biphasic response consisting of a relaxation followed by contraction. NPY(3–36), the compound 1229U91 (Ile-Glu-Pro-Dapa-Tyr-Arg-Leu-Arg-Tyr-NH2, cyclic(2,4′)diamide) and eventually NPY(13–36) relaxed penile small arteries.

5 The selective NPY Y_1 receptor antagonist BIBP3226 ((*R*)-*N*^{2}-(diphenacetyl)-*N*-[(4-hydroxyphenyl)methyl]D-arginineamide) (0.3 μM) shifted to the right the concentration–response curves to both NPY and [Leu^{31}, Pro^{34}]NPY and inhibited the contractions induced by the highest concentrations of hPP but not the relaxations observed at lower doses.

6 In the presence of the selective NPY Y_2 receptor antagonist BIIE0246 ((*S*)-*N*2-[[1-[2-[4-[(*R,S*)-5,11-dihydro-6(6h)-oxodibenz[b,e]azepin-11-y1]-1-piperazinyl]-2-oxoethyl]cyclo-pentyl-*N*-[2-[1,2-dihydro–3,5 (4*H*)-dioxo-1,2-diphenyl-3*H*-1,2, 4-triazol-4-yl]ethyl]-argininamide) (0.3 μM), the Y_2 receptor agonists NPY(13–36) and *N-acetyl*[Leu^{28,31}]NPY (24–36) evoked potent slow relaxations in NA-precontracted arteries, under conditions of nitric oxide (NO) synthase blockade.

7 Mechanical removal of the endothelium markedly enhanced contractions of NPY on NA-precontracted arteries, whereas blockade of the neuronal voltage-dependent Ca^{2+} channels did not alter NPY responses.

8 These results demonstrate that NPY can elicit dual contractile/relaxing responses in penile small arteries through a heterogeneous population of postjunctional NPY receptors. Potentiation of the contractions evoked by NA involve both NPY Y_1 and NPY Y_2 receptors. An NO-independent relaxation probably mediated by an atypical endothelial NPY receptor is also shown and unmasked in the presence of selective antagonists of the NPY contractile receptors.
British Journal of Pharmacology (2004) **143,** 976–986. doi:10.1038/sj.bjp.0706005

Keywords: Neuropeptide Y; nerves; NPY Y_1 and Y_2 receptors; noradrenaline; potentiation; relaxation; endothelium; penile small artery

Abbreviations: BIIE0246, (*S*)-*N*2-[[1-[2-[4-[(*R,S*)-5,11-dihydro-6(6h)-oxodibenz[b,e]azepin-11-y1]-1-piperazinyl]-2-oxoethyl]cyclo-pentyl-*N*-[2-[1,2-dihydro –3,5 (4*H*)-dioxo-1,2-diphenyl-3*H*-1,2, 4-triazol-4-yl]ethyl]-argininamide; BIBP3226, (*R*)-*N*^{2}-(diphenacetyl)-*N*-[(4-hydroxyphenyl)methyl]D-arginineamide; EFS, electrical field stimulation; hPP, human pancreatic polypeptide; 1229U91, (Ile-Glu-Pro-Dapa-Tyr-Arg-Leu-Arg-Tyr-NH2, cyclic(2,4′)diamide); L-NOARG, N^G-nitro-L-arginine; NA, noradrenaline; NPY, neuropeptide Y; PYY, peptide YY

Introduction

Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) are 36-amino acid closely related peptides that activate a class of G-protein-coupled receptors called the NPY receptors. To date, five classes of NPY receptors have been cloned and classified as Y_1, Y_2, Y_4, Y_5 and Y_6 on the basis of their molecular and pharmacological profiles (Gehlert, 1998; Michel *et al*., 1998). The NPY Y_1, Y_2 and Y_5 receptors

*Author for correspondence; E-mail: dprieto@farm.ucm.es
Advance online publication: 22 November 2004

preferentially interact with the endogenous ligands NPY and PYY, whereas the NPY Y_4 receptor is characterized by its high affinity for PP of the same species (Michel *et al*., 1998). Structure–activity relationships for the NPY receptors were initially established on the basis of the action of several analogues and fragments of NPY, PYY and PP. However, the development of highly selective nonpeptide NPY receptor antagonists, first for the Y_1 receptor (Doods *et al*., 1995), and more recently for the Y_5 (Daniels *et al*., 2002) and Y_2 (Doods *et al*., 1999) receptors, has allowed a more precise pharmacological characterization of the NPY receptor subtypes.

From the members of the NPY family, NPY is primarily localized in central and peripheral neurons and is involved in multiple physiological functions such as feeding and anxiety in the central nervous system and control of smooth muscle contractility in the periphery (for a review, see Gehlert, 1998; Michel *et al*., 1998). The cloned NPY Y_1 receptor is the predominant receptor in the cardiovascular system and mediates vasoconstrictor actions of the peptide both *in vivo* and *in vitro* (Abounader *et al*., 1995; Prieto *et al*., 1995; 1997b; 1998a; 2000; Malmström, 1997). However, presynaptic NPY Y_2 receptors have been shown to inhibit noradrenaline (NA) release from sympathetic perivascular nerves (Malmström *et al*., 2002) and postsynaptic NPY Y_2 receptors can be involved in angiogenesis (Zukowska-Grojec *et al*., 1998) and in the vasoconstriction induced by NPY in certain vascular preparations (Tessel *et al*., 1993; Malmström, 2001).

Penile erection results from a complex interaction between nervous and local factors that regulate the tone of trabecular smooth muscle and penile vasculature (Andersson & Wagner, 1995; Simonsen *et al*., 2002). Sympathetic nerves cause detumescence of the erect penis and also maintain the penis in a flacid state (Giuliano *et al*., 1993; Andersson & Wagner, 1995). NA released from nerves contracts trabecular smooth muscle through α_1-adrenoceptors, and penile cavernous and helicine arteries through a heterogenous population of α_1- and α_2-adrenoceptors (Andersson & Wagner, 1995; Simonsen *et al*., 1997; 2002). NPY is usually colocalized with NA in sympathetic perivascular nerves and contributes to the vasoconstriction elicited by activation of sympathetic nerves (Edvinsson *et al*., 1984). Immunohistochemical studies have demonstrated the presence of numerous NPY-immunoreactive nerves in the erectile tissues of the penis, with a high density around helicine arteries (Wespes *et al*., 1988; Schmalbruch & Wagner, 1989; Kirkeby *et al*., 1991). Although NPY was initially suggested to play a role in detumescence (Giuliano *et al*., 1993), *in vitro* studies showed either no effect of NPY in strips of human corpum cavernosum and cavernous artery (Hedlund & Andersson, 1985) or a limited contraction in two out of eight corpum cavernosum strips and in five out of eight penile circumflex vein segments (Kirkeby *et al*., 1991). Moreover, intracavernous injection of NPY increased intracavernous pressure and led to penile tumescence in rabbits (Kirkeby *et al*., 1992).

Despite the evidence for a rich NPY-peptidergic innervation in penile resistance or helicine arteries, no information is available concerning the role of NPY in the regulation of the tone in these arteries. Penile resistance arteries play a main role in the physiology of erection since they act as sphincters by regulating the blood flow between the systemic circulation and the cavernous sinusoids (Andersson & Wagner, 1995; Simonsen *et al*., 2002). We have earlier shown that nitric oxide (NO) from both neural (Simonsen *et al*., 1995) and endothelial (Prieto *et al*., 1998b) origin is one of the main vasodilators in these arteries, whereas NA released from sympathetic nerves is a powerful vasocontrictor (Simonsen *et al*., 1997). The aim of the present study was two-fold. First, to localize NPY-peptidergic nerves and evaluate the effects of the peptide on the contractions induced by both exogenous and EFS-released NA. Second, to characterize the receptors involved in the contractile action of NPY with the aid of selective NPY receptor agonists and antagonists in penile small arteries of the horse.

Methods

Dissection and mounting

Penises from normal horses were obtained once a week at the local slaughterhouse immediately after death and placed in cold physiological saline solution (PSS) of the following composition (mmol l^{-1}): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 1.5, ethylenediaminetetraacetic acid (EDTA) 0.027 and glucose 11. The solution was gassed with 5% CO_2–95% O_2 to maintain pH at 7.4. The proximal part of the corpus cavernosum was opened and penile small arteries, second- or third-order branches of the deep penile artery having a normalized lumen diameter of 200–500 μm, were dissected carefully removing the adhering trabecular tissue, as previously described (Prieto *et al*., 1998b). Segments (2 mm) of the small vessels were mounted as ring preparations in a myograph for isometric force recording (Danish Myo Technology, Denmark). The arteries were allowed to equilibrate in PSS, 37°C, pH 7.4 for 30 min. The relationship between resting wall tension and internal circumference was determined, and from this the internal circumference, L_{100}, corresponding to a transmural pressure of 100 mmHg for a relaxed vessel *in situ*, was calculated. The vessels were set to the normalized internal circumference $L_1 = 0.9 \times L_{100}$. Preliminary experiments showed that force development is close to maximal at this internal circumference (Simonsen *et al*., 1997). The effective internal lumen diameter was determined as $l_1 = L_1\ \pi^{-1}$.

Immunohistochemistry

Specimens of the proximal part of the corpus cavernosum from normal horse penises were immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 at 4°C for 24–48 h and then placed in a cryoprotective PB solution containing 30% sucrose at 4°C for 24 h.

Free-floating sections 40–50 μm thick were obtained with a freezing microtome and processed for immunohistochemistry following the avidin–biotin–peroxidase complex (ABC) method (Hsu *et al*., 1981). The samples were treated for 30 min with a mixture of H_2O_2 and 90% methanol in distilled water for endogenous peroxidase inhibition. The sections were preincubated in PB with 10% normal goat serum and 0.3% Triton X-100 for 3 h, and then incubated for 42 h at 4°C in the presence of rabbit antisera against NPY 1 : 250; Chemicon International Inc.). After rinsing in PB (three rinses of 10 min), the sections were incubated for 2 h at room temperature with biotinylated goat anti-rabbit immunoglobulins (1 : 400, Chemicon International Inc.). The specimens were incubated with

avidin–biotin complex (ABC, Vector) 1 : 100 for 90 min and the immunocomplex was visualized with 0.05% 3,3 diaminobenzidine (DAB) and 0.001% H_2O_2 in PB. In control experiments in which incubation of tissue sections with the anti-NPY antiserum was avoided, no immunoreactivity could be detected.

Experimental procedure for the functional studies

After normalization, the viability of the arteries was tested by stimulating twice with a high-potassium solution, KPSS (equivalent to PSS but NaCl exchanged with KCl on equimolar basis giving a final concentration of 123.7 mM K^+). The effects of NPY and NPY analogues were tested on the contractions elicited by both exogenous NA and electrical field stimulation (EFS).

EFS was performed as decribed previously (Simonsen *et al*., 1997). Briefly, arteries were mounted in a double myograph between a pair of platinum electrodes connected to an electrical stimulator (Cibertec CS20, Barcelona, Spain) with constant current output adjusted to 35 mA. Frequency–response curves to EFS were performed at resting tension, over a range of 0.5–32 Hz (0.3 ms pulse width, 20-s trains) at 5 min intervals. After a first control curve, the arteries were washed for 60 min, at 15 min intervals, and stimulated in between with 10 μM NA for 5 min and relaxed in PSS to replenish amine stores. Single concentrations of the peptides (10 or 30 nM) were added 5–8 min before the construction of a second frequency–response curve. These experiments were performed in the presence of propranolol (3 μM) and cocaine (1 μM) to block β-adrenoceptors and NA neuronal uptake, respectively. Since EFS releases NO from perivascular nerves of penile small arteries (Simonsen *et al*., 1995), all the experiments were carried out in the presence of the NOS inhibitor, L-NOARG (N^G-nitro-L-arginine) (50 μM).

Two consecutive NA concentration–response curves (CRC) were constructed at a 60 min interval in the presence of propranolol (3 μM), cocaine (1 μM) and L-NOARG (50 μM). After the first control CRC, the arteries were washed every 15 min for 1 h and single concentrations of the peptides (10 or 30 nM) were added 5–8 min before a second CRC was constructed.

The receptors involved in the NPY-induced potentiation of NA responses were characterized in NA-precontracted arteries by using different concentrations of agonists and antagonists, in order to establish a rank order of potency for the agonists and apparent pK_B values for the antagonists when possible (Gicquiaux *et al*., 1996; Prieto *et al*., 2000). Briefly, the arteries were stimulated with a single concentration of NA (0.1–0.5 μM) giving a response about 25–30 % of the KPSS response in the presence of propanolol and L-NOARG. Thereafter, CRCs for NPY and the NPY analogues were constructed by adding cumulative concentrations of the peptides to the NA-precontracted arteries, in the absence and presence of the selective NPY Y_1 or Y_2 receptor antagonists, BIBP 3226 (0.3 μM) or BIIE0246 (0.3 μM), respectively. Owing to the development of tachyphylaxis, only one CRC to NPY or the NPY analogues was constructed in each artery. BIBP 3226 and BIIE0246 were added 30 min before the addition of the agonists and kept in the bath during the construction of the CRCs. For each treated artery, a control CRCs was run in parallel in a consecutive arterial segment.

The role of the vascular endothelium in NPY responses was evaluated in arterial segments where the endothelial cells were mechanically removed by guiding a human hair through the vessel lumen and gently moving it forth and back several times. The lack of relaxation to acetylcholine was taken as a functional evidence of endothelium removal. In a last set of experiments, a possible involvement of neuronal NPY receptors was investigated in arteries incubated for 30 min with 0.5 μM of the inhibitor of neuronal voltage-dependent Ca^{2+} channels, ω-conotoxin GVIA.

Drugs

Porcine NPY, PYY, [Leu^{31}, Pro^{34}]NPY, NPY(13–36), NPY (3–36) and human pancreatic polypeptide (hPP), cocaine HCl, ω-conotoxin GVIA, (±)-noradrenaline HCl, (L-NOARG) and propranolol HCl were obtained from Sigma (Madrid, Spain). *N-acetyl*[Leu^{28,31}]NPY (24–36) and 1229U91 (Ile-Glu-Pro-Dapa-Tyr-Arg-Leu-Arg-Tyr-NH2, cyclic(2,4′)diamide) were provided by Neosystem (France). BIBP 3226 ((*R*)-N^2-(diphenacetyl)-*N*-[(4-hydroxyphenyl)methyl]D-arginineamide) and BIIE02246 ((*S*)-*N*2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6h)-oxodibenz[b,e]azepin-11-y1]-1-piperazinyl]-2-oxoethyl]cyclo-pentyl-*N*-[2-[1,2-dihydro–3,5 (4*H*)-dioxo-1,2-diphenyl-3*H*-1,2, 4-triazol-4-yl]ethyl]-argininamide) were kindly provided by Boerhinger Ingelheim (Germany).

Analysis of data and statistics

Mechanical responses of the arteries were measured as force and expressed as active wall tension, ΔT, which is the increase in force, ΔF, divided by twice the segment length (Newton per meter of vessel wall, Nm^{-1}). Responses to NA and EFS in the absence and presence of the peptides are given as a percentage of the maximal response obtained in the first control concentration– or frequency–response curve. Comparative responses to NPY and NPY analogues in NA-precontracted arteries are expressed relative to the response to either KPSS or NA in each artery. Results are expressed as mean ± s.e.m. and *n* represents the number of arteries. Sensitivity to the agonists is expressed in terms of pEC_{50} values, where $pEC_{50} = -\log EC_{50}$ [M]. Apparent pK_B values for BIBP3226 were calculated from a single concentration of this antagonist by use of the Gaddum equation, $pK_B = \log$ (CR-1)-log[antagonist]. Statistical differences between groups were tested by the use of Student's *t*-test for paired or unpaired observations where appropriate. When multiple comparisons were made, values were analysed by one-way analysis of variance (ANOVA), using the Bonferroni method as a *post hoc* test. Probability levels less than 5% were considered significant.

Results

Immunohistochemistry

NPY-peptidergic nerves were visualized by using specific antibodies against NPY in sections from horse penile corpus cavernosum. NPY-immunoreactive nerve fibres were distributed along the smooth muscle septa surrounding the cavernous sinusoids and forming dense perivascular plexuses around intracavernous arteries, with a particularly high density in the

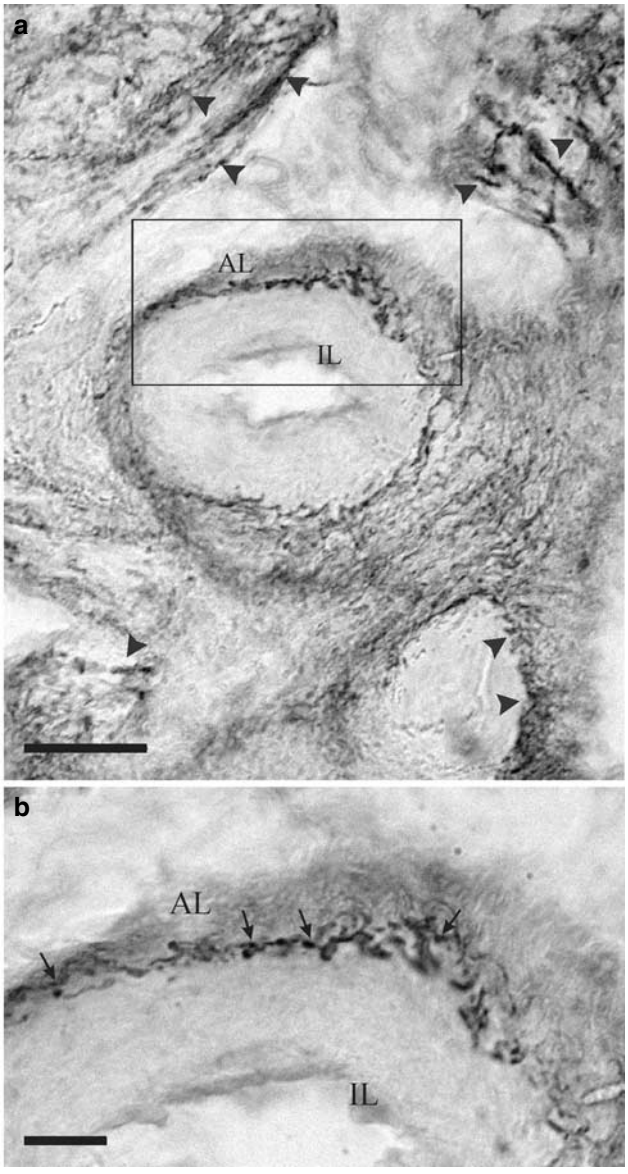

Figure 1 Photomicrographs showing neuropeptide Y (NPY) immunoreactivity in tissue sections of horse penile corpus cavernosum. (a) NPY-immunoreactive nerve fibres with dense varicosities between smooth muscle cells (arrow heads) of the trabecular septa and around the inner part of the adventitia layer (AL) of the thick-walled penile small arteries. IL: intima layer; calibration bar = 50 μm. (b) High magnification of (a) showing NPY-immunoreactive nerve fibres of dense varicosities (arrows) at the adventitia (AL)-media junction. IL: intima layer; calibration bar = 20 μm.

penile resistance or helicine arteries (Figure 1a). Numerous NPY- immunoreactive fibres, rich in varicosities, were mostly concentrated in the inner part of the adventitia close to the media of the small arterial vessels (Figure 1b).

Effects of NPY and NPY-receptor agonists on EFS- and NA-induced contractions

In the absence of blockers of NA neuronal uptake, β-adrenoceptors and NOS, NA contracted penile small arteries in a concentration-related manner, pEC_{50} and maximum response being 5.35 ± 0.03 and $17.0 \pm 2.7\,Nm^{-1}$ ($n = 5$), respectively. NPY (0.1 μM) applied at resting tension contracted penile small arteries by $0.6 \pm 0.1\,Nm^{-1}$ ($7.0 \pm 1.3\%$ of the KPSS-induced contraction, $n = 5$). In the presence of L-NOARG (50 μM), propranolol (3 μM) and cocaine (1 μM), the NA CRCs were shifted to the left ($pEC_{50} = 5.87 \pm 0.10$, $P < 0.05$, $n = 5$, paired t-test, and maximum response of $19.3 \pm 2.5\,Nm^{-1}$, ns), whereas the contraction induced by 0.1 μM NPY was unaffected ($0.5 \pm 0.1\,Nm^{-1}$ representing $6.7 \pm 0.9\%$ of the KPSS-induced response, $n = 8$). Under these conditions, EFS performed at resting tension caused frequency-dependent contractions, with EF_{50} values of 24.3 ± 3.1 Hz and maximum response at 32 Hz of $1.7 \pm 0.1\,Nm^{-1}$ ($26 \pm 3\%$ of the KPSS response, $n = 24$). Contractions elicited by EFS under these conditions are inhibited by guanethidine and α_1-adrenoceptor blockade, thus suggesting their noradrenergic nature (Simonsen *et al*., 1997).

A submaximal concentration of NPY (30 nM) caused a variable enhancement of both the duration and the magnitude of the contractions elicited by EFS, rendering a moderate average potentiation of the EFS responses (Figures 2a and 3a; Table 1). NPY (30 nM) modestly increased the sensitivity of the NA CRCs, without altering maximal responses (Figures 2b and 3b; Table 1). The NPY Y_1 receptor agonist, [Leu^{31}, Pro^{34}]NPY (30 nM), markedly potentiated both EFS- and NA-induced contractions (Figures 2c, d and 3c, d, Table 1). The NPY Y_2 receptor agonist, NPY(13–36) (30 nM), induced a small enhancement of the contractions to the lowest frequencies of EFS (0.5–4 Hz) and a marked potentiation of the responses to exogenous NA (Figure 3e, f, Table 1).

Effect of NPY and NPY receptor agonists on NA-precontracted arteries

The NPY receptor subtypes involved in the vasoactive actions of NPY on NA responses were characterized by constructing CRCs for NPY and NPY analogues in arteries precontracted with NA. In the presence of L-NOARG (50 μM) and propranolol (3 μM), a single concentration of NA (0.1–0.5 μM) contracted helicine arteries by $1.58 \pm 0.19\,Nm^{-1}$ averaging $25 \pm 3\%$ of the KPSS-induced response ($n = 27$). NPY, PYY and [Leu^{31}, Pro^{34}]NPY elicited moderate concentration-dependent contractions on NA-precontracted arteries with no significant differences in either potency or magnitude (about 15% of the K-PSS-induced contraction) (Figure 4a; Table 2). In some arteries, an initial relaxation was observed at the lowest concentrations of the peptide agonists and also at the highest doses. The selective NPY Y_2 receptor agonist, *N-acetyl*[Leu^{28,31}]NPY (24–36), elicited potent contractions on NA-precontracted arteries, significantly smaller than those elicited by NPY (Figure 4a; Table 2). The C-terminal fragment NPY(13–36) evoked contractions in eight out of 14 arteries (Figure 4a; Table 2) and a potent relaxation averaging $42 \pm 6\%$ of the NA-induced contraction in six out of 14 arteries (Figure 4b; Table 2). The long C-terminal fragment NPY (3–36) also relaxed by $33 \pm 6\%$ ($n = 6$) penile small arteries (Figure 4; Table 2). hPP evoked a biphasic response consisting of a modest relaxation at the lowest doses followed by a contraction at higher doses (29 ± 9 and $116 \pm 14\%$ of the NA-induced contraction, at 0.1 and 0.3 μM, respectively, $n = 7$) (Figures 4b, Table 2). The compound 1229U91, potent agonist at NPY Y_4 receptors, elicited relaxations only at the highest concentrations used (Figure 4b).

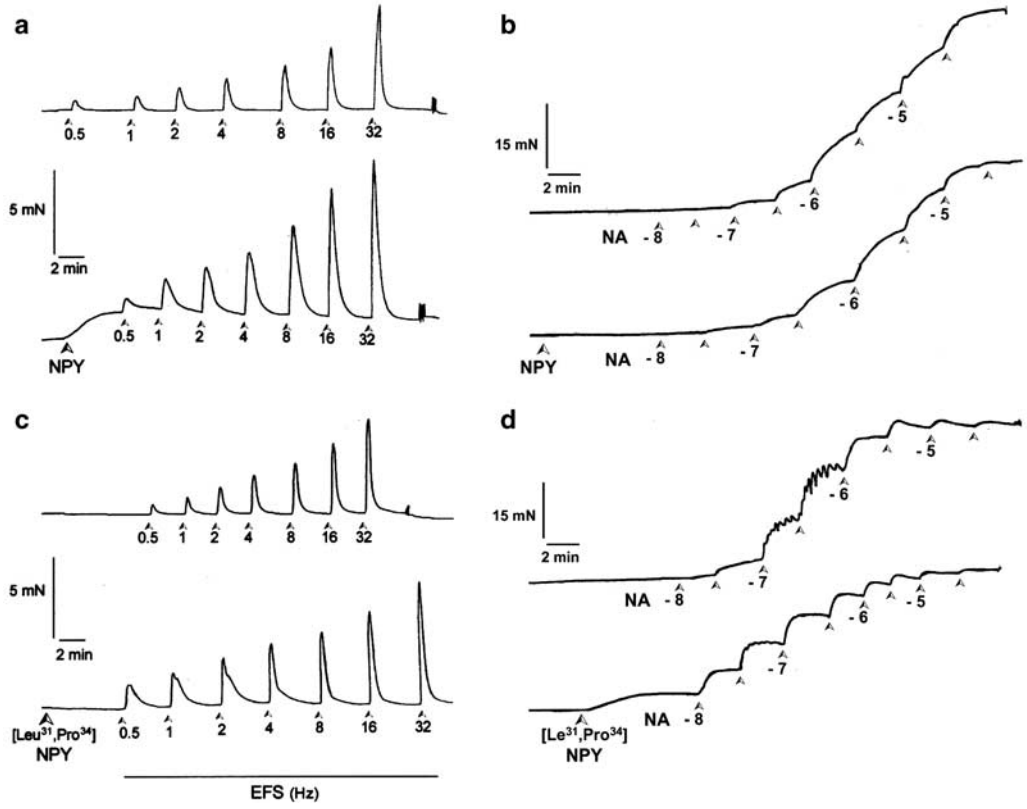

Figure 2 Isometric force recordings showing the effects of a submaximal concentration (30 nM) of (a, b) NPY and (c, d) [Leu^{31}, Pro^{34}]NPY on the contractile responses elicited by (a, c) EFS (0.5–32 Hz, 0.3 ms pulses, 20 s trains, 35 mA output current) performed at resting tension and (b, d) noradrenaline (NA) in horse penile small arteries. Arteries were preincubated with 50 μM L-NOARG, 3 μM propranolol and 1 μM cocaine to inhibit NO synthesis, β-adrenoceptors and NA neuronal uptake, respectively. After a first frequency– (a, c) or concentration– (b, d) response curve, the arteries were washed in PSS every 15 min for 1 h, and a single concentration (30 nM) of either NPY (a, b) or [Leu^{31}, Pro^{34}]NPY (c, d) was added 5–8 min before the construction of the second frequency– or concentration–response curve. Internal lumen diameter of the artery (l_1) was (a) 373 μm, (b) 412 μm, (c) 482 μm and (d) 567 μm. Vertical bars represent force (mN) and horizontal bar represent time (min).

Effect of BIBP 3226 on the responses to NPY and NPY receptor agonists

The Y_1 receptor-selective antagonist BIBP3226 (0.3 μM) induced parallel rightward shifts of the CCRs to both NPY and [Leu^{31}, Pro^{34}]NPY on NA-precontracted arteries (Figure 5a, b). The apparent calculated pK_B values for the antagonism of BIBP3226 against the NPY- and [Leu^{31}, Pro^{34}]NPY-induced responses were 7.38 ± 0.09 ($n=6$) and 7.64 ± 0.12 ($n=6$), respectively. In the presence of BIBP3226, the initial relaxant response observed at the lowest concentrations of [Leu^{31}, Pro^{34}]NPY was significantly enhanced (Figure 5b). Thus, maximal relaxation induced by this substituted NPY analogue was $8 \pm 2\%$ at 0.3 nM ($n=6$) and $17 \pm 3\%$ at 1 nM ($n=6$, $P < 0.05$ unpaired t-test) of the NA-induced contractions, in the absence and presence of 0.3 μM BIBP, respectively.

BIBP3226 did not inhibit the contractile responses elicited by NPY(13–36) on NA-contracted arteries. pEC50 values and maximal contractions were 8.03 ± 0.18 and 0.9 ± 0.2 and 8.44 ± 0.33 and 1.1 ± 0.3 Nm^{-1} ($n=7$), in the absence and presence, respectively of 0.3 μM BIBP2336 (Figure 5c). The selective Y_1 receptor antagonist did not affect either the relaxant responses elicited by NPY(13–36) on NA-contracted arteries ($n=3$, results not shown). BIBP3226 slightly enhanced the relaxations elicited by hPP at the lowest concentrations and significantly inhibited the contraction at the highest concentration of the peptide from 128 ± 15 to $84 \pm 11\%$ of the NA-induced contraction ($P < 0.05$, $n=5$, unpaired t-test) (Figure 5d).

Effect of BIIE0246 on the responses to NPY Y_2 receptor agonists

In the presence of the selective NPY Y_2 receptor antagonist BIIE0246 (0.3 μM), the Y_2 agonists NPY(13–36) and *N-acetyl*[Leu^{28,31}]NPY (24–36) evoked potent slow relaxations (Figure 6). pEC_{50} values and maximal responses for the relaxant effects of NPY(13–36) (Figure 6b) and *N-acetyl* [Leu^{28,31}]NPY (24–36) (Figure 6c) averaged 9.26 ± 0.23 and $30 \pm 6\%$ ($n=6$), and 8.22 ± 0.48 and $39 \pm 4\%$ ($n=5$), respectively, of the NA-induced contractions.

Effect of endothelial cell removal and ω-conotoxin GVIA on the NPY contractile responses

Mechanical removal of the vascular endothelium did not significantly affect KPSS-induced contractions (6.7 ± 0.9 Nm^{-1}, $n=5$, and 6.0 ± 0.3 Nm^{-1}, $n=5$, in endothelium-intact and denuded arteries, respectively), but abolished the relaxations elicited by 3 μM acetylcholine ($98 \pm 0.3\%$ of the

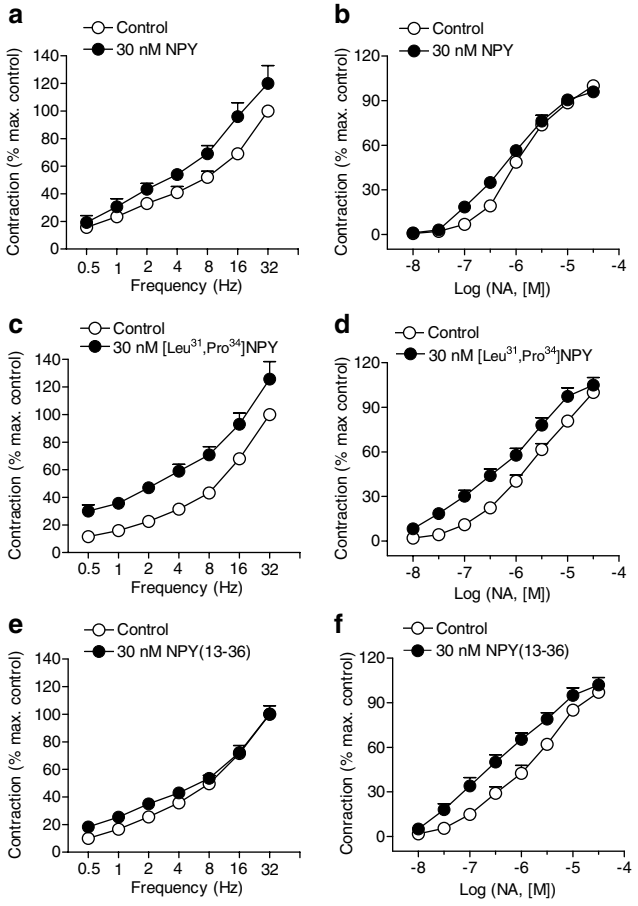

Figure 3 Average effects of (a, b) NPY, (c, d) [Leu^{31}, Pro^{34}]NPY and (e, f) NPY(13–36) on the EFS- and NA-induced contractions in horse penile small arteries. (a, c, e) The responses to EFS (0.5–32 Hz) in the absence and presence of (a) 30 nM NPY, (c) 30 nM [Leu^{31}, Pro^{34}]NPY and (e) 30 nM NPY(13–36). (b, d, f) The responses to increasing concentrations of NA in the absence and presence of (b) 30 nM NPY, (d) 30 nM [Leu^{31}, Pro^{34}]NPY and (f) 30 nM NPY(13–36). Experiments were performed in the presence of L-NOARG (50 μM), propranolol (3 μM) and cocaine (1 μM). Results are expressed as percentage of the maximal response in the control frequency– or NA concentration–response curve. Each symbol represents the mean and vertical bars s.e.m. of 5–10 arteries.

contraction evoked by NA, $n=5$) and greatly enhanced the NPY-elicited contractile responses on NA-precontracted arteries, without affecting sensitivity to the peptide (Figure 7a). Thus, pEC_{50} values and maximal responses were 8.30 ± 0.09 and $13\pm3\%$ ($n=6$), and 8.18 ± 0.09 and 33 ± 3 of the KPSS-induced contraction ($P<0.001$, unpaired t-test $n=6$), in endothelium-intact and -denuded arteries, respectively.

The blocker of neuronal voltage-gated Ca^{2+} channels ω-conotoxin GVIA (0.5 μM) did not significantly alter contractions evoked by NPY (Figure 7b), pEC_{50} values and maximal responses being 8.31 ± 0.09 ($n=6$) and 8.18 ± 0.15 ($n=5$), and $13\pm3\%$ and 11 ± 1, respectively, of the KPSS-induced contraction.

Discussion

The present study shows for the first time a dual modulatory action of NPY on the noradrenergic vasoconstriction of horse penile small arteries. This is supported by a rich distribution of perivascular NPY-peptidergic nerves and by the fact that NPY can elicit both contractile and relaxing responses through a heterogenous population of NPY receptors. Postjunctional potentiation of NA-induced contractions by NPY involves activation of both NPY Y_1 and Y_2 receptors, whereas the relaxant effects of the peptide, which are NO-independent, are mediated by a receptor with an atypical pharmacological profile probably located at the endothelium.

The density of NPY-immunoreactive nerve fibres was particularly high at the adventitia-media junction of the horse penile small arteries. This finding confirms previous immunohistochemical reports in humans (Wespes *et al*., 1988), monkey (Schmalbruch & Wagner, 1989) and rat (Carrillo *et al*., 1991) and suggests a relevant vasoactive role for the peptide in these arteries. However, when applied at baseline tension NPY induced little contractions and submaximal concentrations of the peptide caused an average modest enhancement of the contractile responses to both stimulation of noradrenergic nerves and exogenous NA. The low magnitude of this potentiating effect is in contrast to that reported for NPY in small arteries from other vascular beds (Andriantsitohaina & Stoclet, 1988; Prieto *et al*.,1991; 1997b), but would be in accordance with the inconsistent constrictor effects found for NPY in penile erectile tissues (Hedlund & Andersson, 1985; Kirkeby *et al*., 1991).

The NPY Y_1 receptor is the predominant receptor subtype in the vasculature (Malmström, 1997; Prieto *et al*., 1998a; 2000), although both presynaptic inhibitory and postsynaptic vasoconstrictor receptors belonging to the Y_2 subtype are present in some vascular beds (Tessel *et al*., 1993; Malmström, 2001; Malmström *et al*., 2002). In the present study, the clearcut potentiation elicited by the NPY Y_1 receptor agonist [Leu^{31}, Pro^{34}]NPY on the contractions evoked by both EFS and exogenous NA indicates the involvement of an NPY Y_1 receptor in the postjunctional facilitatory action of NPY on NA responses. Similarly to the Y_1 agonist, the Y_2 receptor agonist NPY(13–36) induced a marked enhancement of the contractile responses to exogenous NA but not to EFS, which suggests an additional involvement of NPY Y_2 receptors. The apparent discrepancy between the effects of NPY(13–36), on the responses to NA- and EFS-induced contractions, suggests that this agonist might have dual opposite actions at the neuroeffector junction, that is, prejunctional inhibitory and postjunctional potentiating effects on NA release and contractions, respectively.

The protocol applied to further characterize the NPY receptor subtypes in penile small arteries confirms the involvement of NPY Y_1 and Y_2 receptors in the potentiating action of NPY on NA-induced responses and unmasks an additional inhibitory/relaxant effect. The contractions elicited by NPY and the NPY analogues on NA-precontracted arteries were concentration-dependent and larger than those evoked at resting tension, thus showing the postjunctional synergistic action of these peptides on adrenergic contractions. Responses evoked by the Y_1 agonist [Leu^{31}, Pro^{34}]NPY and the Y_2 agonists NPY(13–36) and *N-acetyl*[Leu^{28,31}]NPY(24–36) were equipotent to those evoked by the endogenous agonists, NPY and PYY, which suggests the involvement of both NPY Y_1 and Y_2 receptors. This is further confirmed by the inhibition exerted by the selective NPY receptor antagonists. Thus, the pK_B values obtained for the antagonism of BIBP3226 against

Table 1 Effect of NPY and NPY-receptor agonists on the contractions elicited by EFS and exogenous NA in horse penile small arteries

	EFS			
	EF_{50} (Hz)	E_{max} (Nm^{-1})	n	l_l (μm)
Control	26.9±6.0	2.1±0.2	6	435±72
30 nM NPY	12.5±0.5[a]	2.4±0.1	6	
Control	27.9±5.4	1.6±0.4	6	461±78
30 nM [Leu[31]Pro[34]]NPY	6.4±0.8[a]	2.1±0.5	6	
Control	14.6±2.7	1.3±0.1	6	405±28
30 nM NPY(13–36)	8.3±0.6	1.3±0.1	6	
	NA			
	pEC_{50} ($-logEC_{50}$)	E_{max} (Nm^{-1})	n	l_l (μm)
Control	5.96±0.05	13.7±3.6	4	435±72
30 nM NPY	6.19±0.05[a]	12.9±3.3	4	
Control	5.53±0.18	13.7±3.6	4	384±89
30 nM [Leu[31]Pro[34]]NPY	6.32±0.16[a]	12.9±3.3	4	
Control	5.74±0.13	8.1±1.0	10	457±23
30 nM NPY(13–36)	6.68±0.15[b]	8.1±0.7	10	

Values represent mean±s.e.m. of the number *n* of individual arteries. EF_{50} is the frequency of electrical field stimulation (EFS) giving half-maximal contraction. pEC_{50} is $-logEC_{50}$, EC_{50} being the concentration of noradrenaline giving half maximal contraction (E_{max}). E_{max} is the maximum contraction at either 32 Hz or 30 μM NA expressed as Nm^{-1}. l_l is the effective lumen diameter of penile resistance arteries at which experiments were performed, determined as $l_l = L_l\pi^{-1}$. Significant differences from controls were analysed by a paired *t*-test.
[a]$P < 0.05$;
[b]$P < 0.01$.

the NPY- and [Leu[31], Pro[34]]NPY-induced enhancement of NA-mediated vasoconstriction, 7.38 and 7.64, respectively, are in agreement with the affinity values around 8 reported for BIBP3226 at vascular NPY Y_1 receptors (Abounader *et al*., 1995; Doods *et al*., 1995; Malmström, 1997; Prieto *et al*., 1998a; 2000). However, the lower potency of BIBP3226 against the NPY contractions compared to that obtained against the more selective Y_1 agonist [Leu[31], Pro[34]]NPY suggests that NPY may act on a heterogeneous population of contractile NPY receptors. Activation of an additional receptor subtype is confirmed by the results obtained with BIIE0246, since this NPY Y_2 receptor antagonist inhibited the small vasoconstrictor responses elicited by the Y_2 agonists and unmasked an obvious relaxant effect of these compounds on NA-precontracted arteries. BIIE0246 possesses high affinity for the NPY Y_2 receptor and is devoid of affinity for NPY Y_1, Y_4 and Y_5 receptors (Doods *et al*., 1999; Malmström, 2001). Therefore, the present results demonstrate the involvement of a heterogeneous population of NPY Y_1 and Y_2 receptors in the vasoconstrictor responses of NPY in penile small arteries.

NPY has traditionally been regarded as a sympathetic vasoconstrictor, which usually induces indirect contractions by either enhancing other vasoconstrictors responses or by inhibiting vasodilatation (Edvinsson *et al*., 1984; Prieto *et al*., 1991; 1997a; 2000; Malmström, 1997; Michel *et al*., 1998). However, evidence has emerged recently suggesting a vasodilator role for the peptide in certain vascular beds both *in vivo* and *in vitro* models. Thus, NPY relaxes precontracted renal arteries of diabetic and nondiabetic rats (Torffvit & Edvinsson, 1997), human subcutaneous arteries (Nilsson *et al*., 2000) and mesenteric small arteries (Gradin *et al*., 2003), and induces vasodilatation in cerebral arteries from different species (Kobari *et al*., 1993; You *et al*., 2001). In the present study, several results provide evidence for the existence of NPY vasodilator receptors. Thus, the enhancing effect of NPY on NA-induced responses was modest compared to that reported in other small arteries and vascular preparations (Prieto *et al*, 1995; 1998a; 2000; Gicquiaux *et al*., 1996;). Furthermore, a slight relaxant effect was eventually observed at the lowest and highest concentrations of the peptides, and agonists such as hPP, NPY(3–36) and occasionally NPY(13–36) elicited potent and more pronounced relaxations in NA-precontracted arteries. Finally, blockade of NPY Y_2 contractile receptors unmasked an obvious relaxant effect of the Y_2 agonists. Since the experiments were performed in the presence of L-NOARG to block NOS activity, these relaxations appear not to be mediated by NO. These findings in principle suggest the presence of vasodilator NPY receptors which might be counteracting the peptide contractile effects and therefore account for the moderate magnitude of the constrictor responses elicited by NPY in penile small arteries. Moreover, the present results showing *in vitro* relaxant effects of the NPY analogues would be consistent with *in vivo* studies showing that intracavernous injection of NPY increases intracavernous pressure and causes penile tumescence in rabbits (Kirkeby *et al*., 1992).

The pharmacological profile found for the relaxations elicited by the NPY analogues in penile small arteries suggests the involvement of an atypical NPY receptor subtype. Thus, the high potency of the relaxations elicited by hPP would initially suggest a role for NPY Y_4 receptors. However, the relaxant effect of the nonselective ligand 1229U91 was not in the nanomolar range shown for this compound at the Y_4 receptor (Gehlert, 1998; Michel *et al*., 1998; Schober *et al*., 1998). Moreover, relaxations were also observed with the long C-terminal fragment NPY(3–36) and occasionally with NPY(13–36), which have low affinity for NPY Y_4 receptors (Gehlert, 1998; Michel *et al*., 1998).

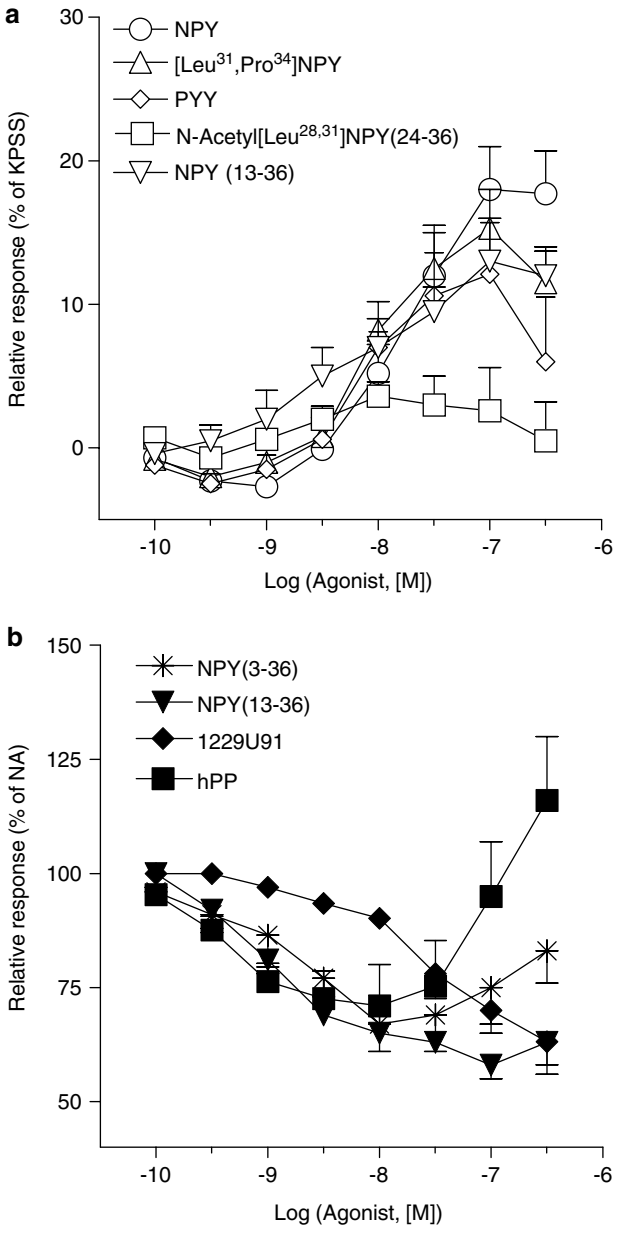

Figure 4 Average effects of NPY and NPY analogues on penile small arteries precontracted with NA (0.1–0.5 μM). (a) CRCs for the contractile effects of NPY, PYY, [Leu^{31}, Pro^{34}]NPY, *N-acetyl* [Leu^{28,31}]NPY (24–36) and NPY(13–36) on arteries precontracted with NA by about 25% of the KPSS-induced response. Responses are expressed as percentage of the contraction elicited by KPSS in each artery. (b) CRCs for the relaxing effects of NPY(3–36), NPY(13–36) and 1229U91, and dual relaxing/contractile effect of hPP. Responses are expressed as a percentage of the tone induced by NA. Each symbol represents mean and vertical bars s.e.m. of 5–14 arteries.

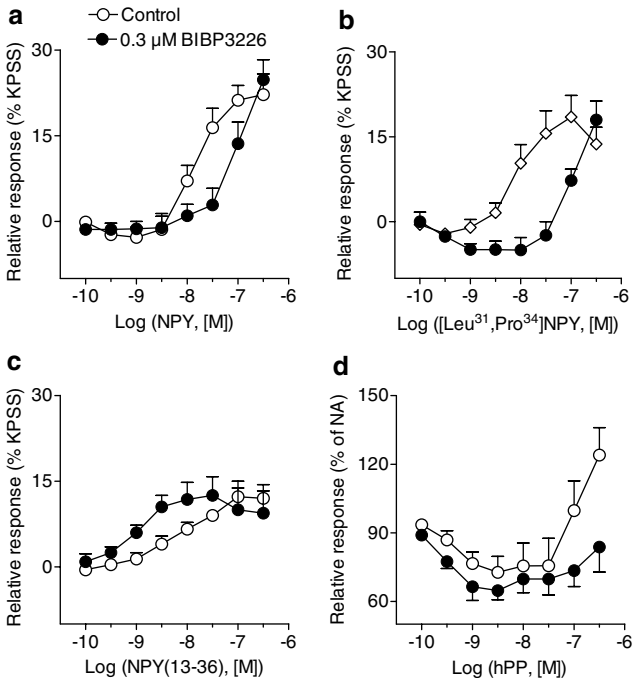

Figure 5 Effects of the NPY Y_1 receptor antagonist, BIBP 3226 (0.3 μM) on the responses to (a) NPY, (b) [Leu^{31}, Pro^{34}]NPY, (c) NPY(13–36) and (d) hPP in arteries precontracted with NA (0.1–0.5 μM). Experiments were performed in the presence of propranolol (3 μM) and L-NOARG (50 μM). Since CRCs for the peptide agonists could not be repeated in the same artery, two consecutive arterial segments from the same animal were used, one serving as a control and the other being incubated for 30 min with 0.3 μM BIBP 3226. Responses are expressed as (a, b, c) percentage of the contraction to KPSS in each artery and (d) percentage of the tone induced by NA. Points represent mean $\pm$ s.e.m. of 5–7 arteries.

Since NPY Y_1 receptors have been localized in endothelial cells (Bao *et al*., 1997; Jackerott & Larsson, 1997) and both NPY Y_1 and Y_2 receptors are expressed in the human endothelium (Zukowska-Grojec *et al*., 1998), these receptor subtypes could be expected to mediate the vasodilator effects of NPY. However, BIBP3226 did not inhibit the relaxant response elicited by hPP or NPY(13–36), but tended to enhance those to the lowest concentrations of hPP and [Leu^{31}, Pro^{34}]NPY. On the other hand, selective blockade of NPY Y_2 receptors with BIIE0246 unmasked a relaxant effect of NPY(13–36) and *N-acetyl*[Leu^{28,31}]NPY (24–36), which suggests that NPY Y_2 receptors are involved in vasoconstriction but not in vasodilatation. The lack of inhibitory effect of BIBP3226 on the relaxant responses elicited by NPY analogues is in agreement with that reported for rat middle cerebral arteries, where vasodilatations evoked by luminal application of either [Leu^{31}, Pro^{34}]NPY or NPY(13–36) were unaffected by the selective Y_1 receptor antagonist (You *et al*., 2001). The present results therefore rule out an involvement of either NPY Y_1 or Y_2 receptors in the relaxant effects of NPY in penile small arteries and confirm an atypical pharmacological profile for the receptor mediating NPY vasodilator actions (You *et al*., 2001).

The vascular endothelium has been reported to play a variable role in the vasoconstrictor responses elicited by NPY. Thus, mechanical removal of endothelial cells does not alter NPY contractions in either retinal or coronary small arteries, but enhances and depresses the peptide contractions in coronary and retinal large arteries, respectively (Prieto *et al*., 1991; 1995). In addition, relaxations evoked by luminal application of NPY or the NPY analogues [Leu^{31}, Pro^{34}]NPY and NPY(13–36) were turned into contractions by removal of endothelium, respectively, in rat middle cerebral arteries (You *et al*., 2001). The results of the present study demonstrate a powerful role of the endothelium suppressing NPY

Table 2 Effects of NPY and NPY receptor agonists on NA-precontracted horse penile small arteries

	Contraction		*Relaxation*	
	pEC_{50} ($-logEC_{50}$)	E_{max} (Nm^{-1})	pEC_{50} ($-logEC_{50}$)	E_{max} (% of NA)
NPY	7.87±0.13	0.88±0.25 ($n=14$)	—	—
PYY	8.23±0.09	0.60±0.16 ($n=5$)	—	—
[Leu^{31}, Pro^{34}]NPY	8.02±0.08	1.04±0.17 ($n=8$)	—	—
N-acetyl[Leu^{28}, Pro^{28}] NPY(24–36)	8.46±0.23	0.26±0.08[a] ($n=5$)	—	—
NPY(13–36)	8.04±0.30	0.88±0.29 ($n=8$)	9.16±0.14	42±3 ($n=6$)
hPP	6.42±0.24[a,b,c,d]	1.02±0.12 ($n=7$)[e]	9.62±0.17	29±9 ($n=7$)[e]
NPY(3–36)	—	—	8.84±0.19	33±6 ($n=6$)
1229U91	—	—	7.62±0.11	49±6 ($n=6$)

Values are expressed as mean±s.e.mean. n is the number of arteries. pEC_{50} is $-logEC_{50}$, EC_{50} being the concentration of the peptide agonist giving half-maximal contraction or relaxation. E_{max} is the maximal contraction or relaxation. Contraction is expressed as Nm^{-1} of tension and calculated by substracting the initial precontraction of NA from that elicited by the peptide on the top of the NA response. Relaxation is expressed as percentage of the contraction induced by NA.
[a]$P<0.01$ *versus* NPY;
[b]$P<0.01$ *versus* PYY;
[c]$P<0.01$ *versus* [Leu^{31}, Pro^{34}]NPY;
[d]$P<0.01$ *versus* NPY(13–36); analysed by ANOVA followed by Bonferroni.
[e]For hPP, relaxation and contraction were measured in the same artery.

contractions, which along with the relaxant effect found for the NPY analogues suggests that dilator NPY receptors may be located at the vascular endothelium in penile small arteries. On the other hand, the lack of significant effect of ω-conotoxin GVIA initially rules out the possibility that relaxations elicited by the NPY analogues are due to activation of presynaptic

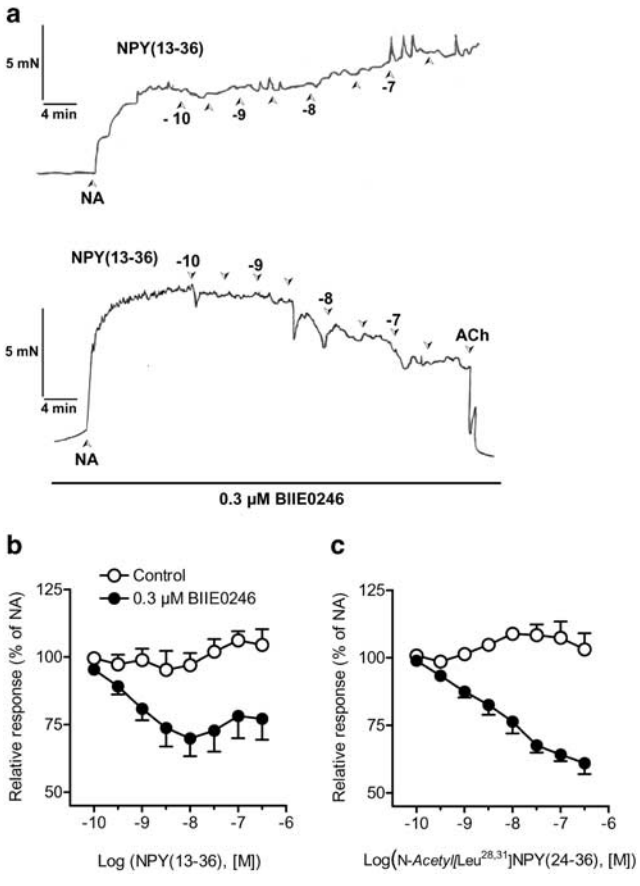

Figure 6 Effect of the NPY Y_2 receptor antagonist BIIE0246 (0.3 μM) on the responses elicited by the NPY Y_2 receptor agonists (a, b) NPY(13–36) and (c) *N-acetyl*[Leu^{28,31}]NPY (24–36), on NA-precontracted arteries. (a) Isometric force recordings showing examples of the effects of NPY(13–36) in the absence (upper trace) and the presence (lower trace) of 0.3 μM BIIE0246. Internal lumen diameter of the arteries (l_1) were 458 μm (upper trace) and 378 μm (lower trace). Acetylcholine (ACh, 10 μM) maximally relaxed the artery. Vertical bars represent force (mN) and horizontal bars represent time (min). (b, c) Average effects of 0.3 μM BIIE0246 on the responses to (b) NPY(13–36) and (c) *N-acetyl*[Leu^{28,31}]NPY (24–36). Experiments were performed in the presence of propranolol (3 μM) and L-NOARG (50 μM). Responses are expressed as a percentage of the tone induced by NA. Points represent mean± s.e.m. of 5–7 arteries.

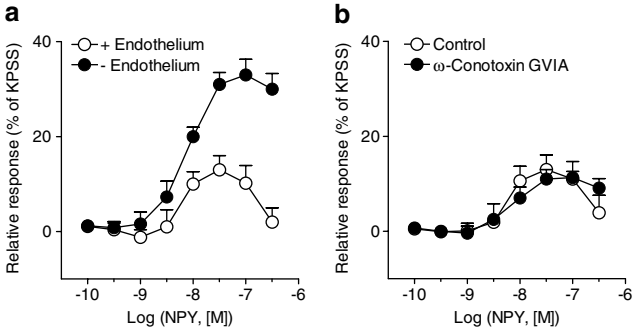

Figure 7 Effect of (a) mechanical endothelial cell removal and (b) blockade of neuronal voltage-gated Ca^{2+} channels with ω-conotoxin GVIA in the contractile responses induced by NPY on NA-precontracted arteries. Experiments were performed in paired consecutive arterial segments one of which served as a control and in the other (a) the endothelium was mechanically removed by guiding a human hair through the vessel lumen or (b) incubated with 0.5 μM of ω-conotoxin GVIA. Responses are expressed as percentage of the contraction elicited by KPSS in each artery. Points represent mean±s.e.m. of six arteries.

receptors mediating the release of inhibitory transmitters from nerve terminals (Lin *et al*., 2004).

In summary, the present study demonstrates a rich NPY-peptidergic innervation in penile small arteries and a dual facilitatory/inhibitory modulatory role of NPY in the arterial noradrenergic vasoconstriction. The ability of NPY to both enhance and decrease the NA-induced contractions is achieved through a heterogenous population of NPY receptors. Thus, both NPY Y_1 and Y_2 receptors are involved in the NPY-induced enhancement of NA contractions. An NO-independent relaxation not mediated by either the Y_1 or Y_2 subtypes and probably due to receptors located at the endothelium is also demonstrated. The latter could account for the *in vivo* proerectogenic effects reported for NPY when applied intracavernously.

This work was supported by Grant No. SAF2002/02923 from the Spanish Ministry of Science and Technology. We thank Dr Stephan Mueller and Dr Karl Rudolph from Boehringer Ingelheim Pharma KG, Biberach, Germany for generously supplying BIIE0246 and BIBP3226, respectively. We also thank Hugh Tyrrell-Gray for expert language editing, Manuel Perales and Francisco Puente for technical assistance and Villaviciosa de Odón and Segovia Slaughterhouses for kindly donating horse penises.

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(Received July 8, 2004
Accepted September 8, 2004)