Naunyn-Schmiedeberg's Arch Pharmacol (2002) 365 : 106–111
DOI 10.1007/s00210-001-0516-8

ORIGINAL ARTICLE

Rickard E. Malmström · Jon O. N. Lundberg
Eddie Weitzberg

Effects of the neuropeptide Y Y2 receptor antagonist BIIE0246 on sympathetic transmitter release in the pig in vivo

Received: 5 September 2001 / Accepted: 10 November 2001 / Published online: 18 December 2001

Abstract This study investigated the effects of BIIE0246, a novel neuropeptide Y (NPY) Y_2 receptor antagonist, on renal sympathetic nerve-evoked release of noradrenaline and NPY-like immunoreactivity (-LI) in the pig in vivo.

Stimulation (5 Hz, 60 s) of renal sympathetic nerves evoked vasoconstriction and overflow of noradrenaline and NPY-LI. Infusion of peptide YY (PYY; 1 μg/kg per min) strongly inhibited the stimulation-evoked overflow of noradrenaline and NPY-LI. BIIE0246 (100 nmol/kg) abolished the inhibitory effect of PYY. BIIE0246 did not however affect the stimulation-evoked overflow of noradrenaline and NPY-LI, or basal cardiovascular parameters, per se. The α_2-adrenoceptor antagonist yohimbine (0.2 mg/kg) significantly elevated both basal plasma levels and the nerve stimulation-evoked overflow of noradrenaline and NPY-LI. Subsequent administration of BIIE0246 did not further alter these effects. However, BIIE0246 caused splenic vasodilatation per se when given after yohimbine.

It is concluded that the renal sympathetic nerves in the pig possess NPY Y_2 receptors, which upon activation inhibit transmitter (noradrenaline and NPY) release. Furthermore, when circulating levels of NPY were enhanced after blockade of α_2-adrenoceptors, an involvement of endogenous NPY, acting on the NPY Y_2 receptor, in regulation of basal splenic vascular tone was unveiled.

Keywords BIIE0246 · Neuropeptide Y Y_2 receptor · Noradrenaline · Prejunctional · Sympathetic transmission

R.E. Malmström (✉) · J.O.N. Lundberg
Department of Physiology and Pharmacology,
Karolinska Institute, 17177 Stockholm, Sweden
e-mail: rickard.malmstrom@fyfa.ki.se,
Tel.: +46-8-7287961, Fax: +46-8-332278

E. Weitzberg
Department of Anaesthesiology and Intensive Care,
Karolinska Hospital, 17176 Stockholm, Sweden

Introduction

Neuropeptide Y (NPY; Tatemoto et al. 1982) is co-localized with noradrenaline in most sympathetic fibres within the cardiovascular system (see Lundberg 1996). NPY is co-released with noradrenaline upon sympathetic nerve activation to act on both postjunctional and prejunctional receptors. At the postjunctional level, NPY may evoke vasoconstriction (Lundberg and Tatemoto 1982) and enhance the effects of other vasoactive substances (Ekblad et al. 1984). Vascular (postjunctional) responses to NPY are predominantly mediated via the NPY Y_1 receptor subtype (see Malmström 1997). However, in some tissues, e.g. in pig spleen, there is evidence for a contribution of both NPY Y_1 and Y_2 receptors to NPY-evoked vasoconstriction (Modin et al. 1991; Malmström 2001a). At the prejunctional level, NPY may inhibit transmitter release, e.g. from sympathetic (Lundberg et al. 1982; Lundberg and Stjärne 1984; Pernow et al. 1986; Stjärne et al. 1986; Pernow and Lundberg 1989) and parasympathetic (Potter 1985, 1987) nerves. The inhibition of transmitter release has been attributed to activation of a prejunctional NPY Y_2 receptor (Wahlestedt et al. 1986, 1990). However, there is now evidence also for the existence of inhibitory prejunctional NPY Y_1 receptors (Doods et al. 1995). The role of the NPY Y_1 receptor at the pre- and postjunctional level has been established by the use of selective antagonists. NPY Y_2 receptor-mediated effects have until now however been classified on the basis of the actions of a number of peptide analogues acting like agonists (Wahlestedt et al. 1986) or the lack of effects of receptor antagonists selective for subtypes other than Y_2. Recently, Doods et al. (1999) reported on the development of BIIE0246, (*S*)-*N*2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6h)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-*N*-[2-[1,2-dihydro-3,5 (4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl]-argininamide, as the first non-peptide antagonist selective for the NPY Y_2 receptor subtype. 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 (or 60 other receptor types and enzymes) in var-

ious receptor binding assays (Doods et al. 1999; Dumont et al. 2000), and this compound also displays selectivity for the NPY Y_2 receptor in several in vitro bioassays (Doods et al. 1999; Dumont et al. 2000). Moreover, BIIE0246 potently antagonizes NPY Y_2 receptor-mediated vascular responses in vivo (Malmström 2001b). Recent studies have shown that BIIE0246 antagonizes the NPY-induced inhibition of transmitter release in hippocampal and hypothalamic slices in vitro (King et al. 2000; Weiser et al. 2000), as well as the NPY Y_2 receptor-mediated inhibition of cholinergic and purinergic transmission in the rat heart and guinea-pig vas deferens in vitro, respectively (Smith-White et al. 2001).

The present study was undertaken to determine if the NPY Y_2 receptor is indeed involved in prejunctional inhibition of sympathetic transmission. To this end, we investigated the effect of BIIE0246 on renal sympathetic transmitter release in the pig in vivo.

Materials and methods

This study was approved by the local ethics committee for animal research.

Surgical preparation. Domestic pigs (15–19 kg) of either sex were premedicated with ketamine (20 mg/kg i.m.) and atropine (0.02 mg/kg i.m.), and thereafter anaesthetized with sodium pentobarbitone (20 mg/kg i.v.), tracheotomized and artificially ventilated by a respirator (Servo ventilator 900; Siemens-Elema, Sweden). Skeletal muscle relaxation was induced with pancuronium (0.5 mg/kg i.v.) after sufficient anaesthesial depth was checked by pinching the interdigital skin. A catheter was inserted into the left femoral vein for infusion of drugs. For measurement of mean arterial pressure (MAP), a catheter, connected to a Statham P23 AC pressure transducer, was inserted into the left femoral artery. A tachograph unit triggered by the blood pressure recorded heart rate. A brachial artery was cannulated for arterial blood sampling. A catheter was also placed in the left renal vein for collection of venous effluent. The blood flows of the splenic and left renal arteries were measured by ultrasonic flow probes (2RB) connected to a Transonic flowmeter (T206; Transonic Instruments, Ithaca, N.Y., USA). All parameters were continuously recorded on Grass polygraphs. For electrical stimulation, a bipolar platinum electrode was placed around the distal branches of the cut sympathetic nerves accompanying the left renal artery. The abdomen was closed and the pigs were allowed to recover for 1 h before further experimental procedures were undertaken. Throughout the experiments, drugs were given to maintain anaesthesia (fentanyl, 10 μg/kg per h, and midazolam, 0.2 mg/kg per h), skeletal muscle relaxation (pancuronium, 0.5 mg/kg per h), fluid balance (sodium chloride 154 mM and glucose 28 mM, 2 ml/min), and for prevention of intravascular coagulation (heparin, 250 IU/kg per h).

Experimental procedures. Electrical stimulation of the renal sympathetic nerves was performed at 5 Hz for 60 s (5 ms, 25 V) by a Grass stimulator (model S11). To determine overflow as an estimate of release of noradrenaline and NPY-like immunoreactivity (-LI) upon sympathetic nerve activation, arterial and renal venous blood samples were collected before, during (at 30 s and 60 s) and at 1 min and 2 min after the electrical nerve stimulation. The overflow of noradrenaline and NPY-LI was studied upon nerve stimulation under control conditions and subsequently repeated 4 min after the onset of a 5-min infusion of peptide YY (PYY; 1 μg/kg per min). Two hours later the NPY Y_2 receptor antagonist BIIE0246 (100 nmol/kg equal to 101 μg/kg) was administered as an i.v. injection, 10 min after which the nerve stimulations were repeated in the absence and presence of PYY (as above). A further 2 h later, the α_2-adrenoceptor antagonist yohimbine (0.2 mg/kg) was given. The nerve stimulation was then repeated 20 min after administration of yohimbine, and again 10 min after a second dose of BIIE0246 (100 nmol/kg). The 2-h intervals between the three pairs of nerve stimulations were chosen according to the duration of action of BIIE0246 in vivo (Malmström 2001b), in order to study the effect of yohimbine in the absence and presence of BIIE0246. The dose of BIIE0246 was chosen according to a previous study (Malmström 2001b), in which a corresponding dose of BIIE0246 completely antagonized the postjunctional vascular responses evoked in pig spleen by moderate doses of an NPY Y_2 receptor agonist. An approximate 50% inhibition of the vasoconstrictor response evoked by PYY in spleen (a vascular response mediated by both NPY Y_1 and Y_2 receptors; Malmström 2001a) was used as indication of an established NPY Y_2 receptor blockade in the present study. The overflow of noradrenaline and NPY-LI is fully reproducible and not susceptible to any spontaneous decline as has been demonstrated in earlier studies (Pernow and Lundberg 1989; Modin et al. 1994). The doses of PYY and yohimbine were chosen according to a study by Pernow and Lundberg (1989).

Determination of noradrenaline and NPY-LI in plasma. The blood samples were collected in prechilled tubes containing EDTA (final concentration 10 mM), centrifuged 10 min (+4°C) and the plasma was pipetted off and stored at –20°C or –80°C. NPY-LI was determined with radioimmunoassay (using antibody N1) after ethanol extraction. The determination limit was 7.8 pM. The N1 antiserum shows no (<0.1%) cross-reactivity to structurally related peptides such as PYY. For further details see Theodorsson-Norheim et al. (1985). Noradrenaline was measured using a noradrenaline-ELISA kit (ICN Pharmaceuticals, Calif., USA).

Calculations. To give a measure of transmitter release from the kidney, the total overflows of noradrenaline and NPY-LI were calculated as the integrated area of the renal veno-arterial plasma concentration differences, multiplied by the local arterial plasma flow at each sample point. The hematocrit was determined after centrifugation of the blood samples. The vascular responses are expressed as changes in vascular conductance, calculated as blood flow divided by MAP (Stark 1968). Data in the text are given as means ± SEM, and statistical significance was calculated with the multiple analysis of variance (ANOVA) followed by the post hoc test of Tukey, or with the Student's *t*-test (paired samples) where applicable.

Drugs. Ketamine (Parke-Davis, Calif., USA), sodium pentobarbitone (NordVacc, Sweden), atropine and sodium heparin (KabiVitrum, Sweden), pancuronium bromide (Organon, The Netherlands), fentanyl (Pharmalink, Sweden), midazolam (Roche, Sweden), PYY (Auspep, Australia), yohimbine hydrochloride (Sigma, Mo., USA), and BIIE0246, (*S*)-*N*2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6h)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-*N*-[2-[1,2-dihydro-3,5 (4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl]-argininamide (Boehringer Ingelheim Pharma, Biberach, Germany). All drugs were dissolved in either saline or glucose (5%, w/v) solution.

Results

Basal splenic and renal blood flow, MAP and heart rate were 165±20 ml/min, 125±6 ml/min, 114±5 mmHg, and 125±15 beats/min, respectively. Basal arterial and renal venous plasma levels of noradrenaline were 0.9±0.2 nM and 1.0±0.2 nM, respectively, and those of NPY-LI were 25±2 pM and 22±2 pM, respectively (Fig. 1). Electrical sympathetic nerve stimulation (5 Hz for 60 s) evoked vasoconstriction in the kidney, reducing renal vascular conductance by 89±2%. This response was accompanied by

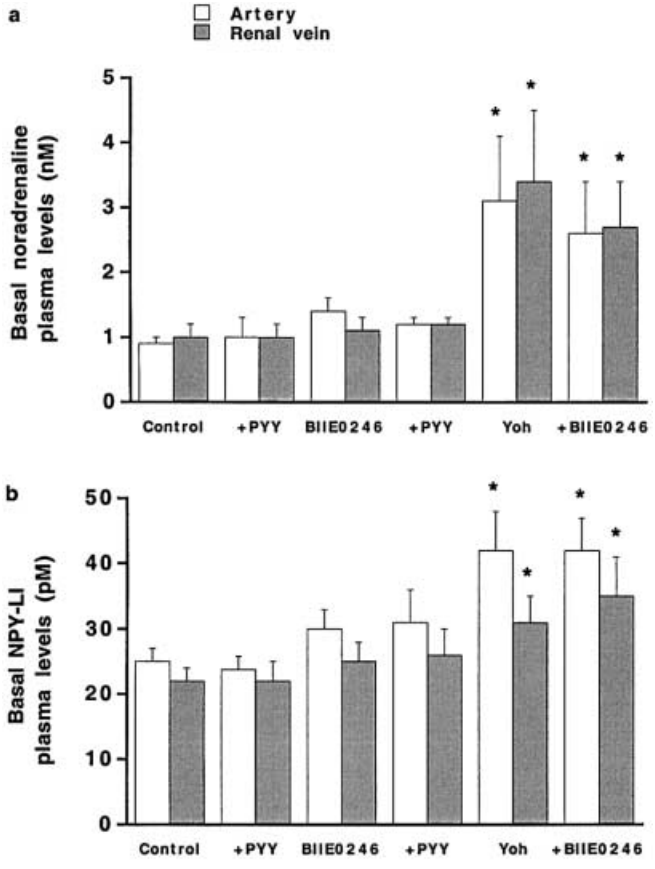

Fig. 1 Arterial and renal venous plasma levels of **a** noradrenaline and **b** NPY-LI are shown under control conditions and in the presence of PYY (1 μg/kg per min), after treatment with the NPY Y_2 receptor antagonist BIIE0246 (100 nmol/kg) and in the presence of PYY, and after administration of yohimbine (0.2 mg/kg) with addition of BIIE0246. Plasma levels were observed 4 min after the onset of a 5-min infusion of PYY, and 10 min and 20 min after BIIE0246 and yohimbine were given, respectively (i.e. immediately before nerve stimulations). Data are given as means ± SEM, n=6. *P<0.05 significant differences compared to control

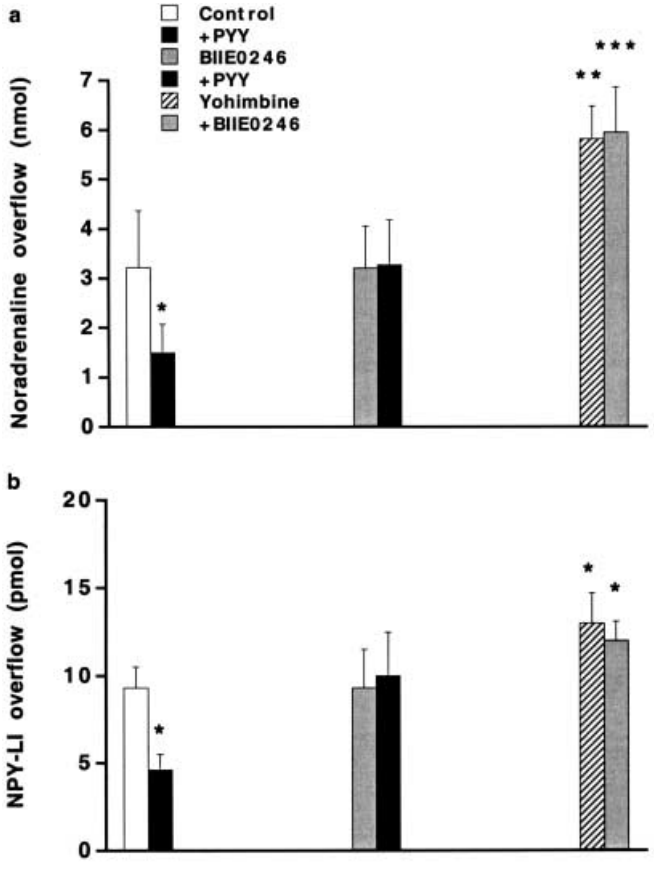

Fig. 2 Overflow of **a** noradrenaline and **b** NPY-LI from the pig kidney upon renal sympathetic nerve stimulation (5 Hz, 60 s) is shown under control conditions and in the presence of PYY (1 μg/kg per min), after treatment with the NPY Y_2 receptor antagonist BIIE0246 (100 nmol/kg) and in the presence of PYY, and after administration of yohimbine (0.2 mg/kg) with addition of BIIE0246. Data are given as means ± SEM, n=6. *P<0.05, **P<0.01, ***P<0.001 significant differences compared to control

overflow of noradrenaline and NPY-LI of 3.2±1.1 nmol and 9.3±1.3 pmol, respectively (Fig. 2).

Infusion of PYY (1 μg/kg per min) evoked splenic and renal vasoconstriction, elevation of MAP, and a decrease in heart rate (see Table 1). Basal plasma levels of noradrenaline and NPY-LI were not affected (Fig. 1). The nerve stimulation-evoked overflow of noradrenaline and NPY-LI was significantly reduced (Fig. 2), whereas the vasoconstrictor response (vascular conductance reduced by 90±2%) was unaltered during the PYY infusion.

Administration of the NPY Y_2 receptor antagonist BIIE0246 (100 nmol/kg) did not alter basal cardiovascular parameters (Table 1) or basal plasma levels of noradrenaline and NPY-LI (Fig. 1). Furthermore, the overflow of noradrenaline and NPY-LI (Fig. 2), as well as the vasoconstrictor response (vascular conductance reduced by 81±5%), upon nerve stimulation were not different from the control stimulation.

Infusion of PYY (1 μg/kg per min), subsequently to the injection of BIIE0246, evoked renal vasoconstriction, elevation of MAP, and reduction in heart rate not different from those seen during the infusion of PYY in the absence of BIIE0246 (Table 1). However, the splenic vasoconstrictor response to PYY was inhibited by 45±5% in the presence of BIIE0246 (Table 1). In addition, the overflow of noradrenaline and NPY-LI (Fig. 2) upon nerve stimulation during the infusion of PYY was not different from those seen upon the control nerve stimulation or after BIIE0246 treatment. Renal vascular conductance was reduced by 82±3% upon this nerve activation, and this was not different from control.

Administration of yohimbine (0.2 mg/kg) induced a marked tachycardia but did not significantly alter other basal parameters (Table 1). Basal arterial and renal venous plasma levels of noradrenaline and NPY-LI increased significantly (Fig. 1). Furthermore, the nerve stimulation-evoked overflow of noradrenaline and NPY-LI was significantly enhanced after yohimbine (Fig. 2). Renal vascular conductance was reduced by 80±4% upon the nerve activation, and this was not different from control.

Administration of BIIE0246 (100 nmol/kg), subsequently to the injection of yohimbine, evoked clear-cut

Table 1 Cardiovascular effects induced by i.v. administration of PYY (as seen 4 min after the onset of a 5-min infusion), BIIE0246 and yohimbine (10 min and 20 min post-injection, respectively). Results are given as means ± SEM, n=6. The effects of PYY (1 μg/kg per min) are shown in the absence and presence of the NPY Y_2 receptor antagonist BIIE0246. The effects of BIIE0246 (100 nmol/kg) are shown in the absence and presence of the α_2-adrenoceptor antagonist yohimbine (0.2 mg/kg)

	Spleen	Kidney	MAP (ΔmmHg)	Heart rate (Δbeats/min)
	(% change in vascular conductance)			
PYY	–60±4***	–34±2***	+25±3***	–18± 6*
BIIE0246	+1±1	–2±2	0±1	+5± 2
+ PYY	–33±4***,†	–35±6***	+18±2***	–31± 8**
Yohimbine	–6±5	–6±3	+3±5	+40±13**
+ BIIE0246	+21±5**,††	+2±1	–1±1	+5± 2

*P<0.05, **P<0.01, ***P<0.001 significant differences from basal values
†P<0.001 significant difference between PYY-evoked response seen in the absence and presence of BIIE0246
††P<0.05 significant difference between effect of BIIE0246 in the absence and presence of yohimbine

splenic vasodilatation without significantly altering other basal parameters (Table 1). This splenic vasodilatation was significantly different from the marginal splenic effect of BIIE0246 seen in the absence of yohimbine (Table 1). BIIE0246 given after yohimbine did not further alter basal plasma levels, or nerve stimulation-evoked overflow, of noradrenaline or NPY-LI, as compared to what was seen after yohimbine only (Figs. 1, 2). Renal vascular conductance was reduced by 82±3% upon the nerve activation, and this was not different from control.

Discussion

In the present study, evidence is presented for a prejunctional NPY Y_2 receptor that modulates the sympathetic release of noradrenaline and NPY. In addition, a possible involvement of postjunctional NPY Y_2 receptors in the basal regulation of splenic vascular tone is revealed when circulating NPY levels are enhanced after blockade of prejunctional α_2-adrenoceptors by yohimbine.

In 1982, it was demonstrated that NPY inhibited sympathetic nerve-evoked responses in rat vas deferens (Lundberg et al. 1982). The inhibition of contractions was paralleled by inhibition of noradrenaline release (Lundberg and Stjärne 1984), and NPY also inhibited the rapid stimulus-evoked excitatory junction potentials (EJPs) in smooth muscle cells that are caused by ATP (Stjärne et al. 1986). Therefore, NPY was suggested to prejunctionally inhibit the release of the sympathetic transmitters noradrenaline and ATP. This was supported by studies on noradrenaline release from perivascular nerves (Pernow et al. 1986; Lundberg et al. 1989). Later studies showed that a prejunctional NPY receptor may reduce the release of noradrenaline, and also of NPY, in the pig kidney in vivo (Pernow and Lundberg 1989). In vitro experiments using NPY-analogue agonists, predominantly in rat vas deferens, revealed that the prejunctional inhibitory effect of NPY on the release of sympathetic transmitters was usually NPY Y_2 receptor-mediated (Wahlestedt et al. 1986; Grundemar and Håkanson 1990). However, evidence was later presented for a prejunctional inhibitory NPY Y_1 receptor in rabbit vas deferens (Doods et al. 1995). Recently, functional in vitro experiments showed that the inhibition by NPY of electrically evoked contractions and EJPs in rat and guinea-pig vas deferens, respectively, was antagonized by the novel NPY Y_2 receptor antagonist BIIE0246 (Smith-White et al. 2001). These findings are consistent with the notion that NPY Y_2 receptors may reduce the release of respectively noradrenaline and ATP in these tissues, although the actual release of transmitter was not studied. In the present study the NPY receptor inhibiting sympathetic transmitter release in the pig kidney was further characterized using BIIE0246. In accord with the previous study by Pernow and Lundberg (1989), PYY reduced, and the α_2-adrenoceptor antagonist yohimbine enhanced, the overflow of both noradrenaline and NPY-LI. The PYY-evoked inhibition of transmitter release was fully antagonized by BIIE0246, indicating an NPY Y_2 receptor-mediated effect. Thus, the prejunctional NPY receptor at the sympathetic nerves of the pig kidney, inhibiting the release of noradrenaline and NPY, is of the Y_2 subtype.

BIIE0246 alone did not, however, affect stimulated, or basal, release of noradrenaline or NPY-LI indicating that, under the conditions of the experiments, endogenous NPY does not play a role in modulating transmitter release in the pig kidney upon nerve activation (or systemic release during basal conditions). In line with this, BIIE0246 per se did not affect cholinergic or purinergic transmission in anaesthetized rats and guinea-pig vas deferens, respectively (Smith-White et al. 2001). As demonstrated earlier, in e.g. pig kidney (Pernow and Lundberg 1989), α_2-adrenoceptors play an important role in regulating sympathetic transmitter release. In fact, the α_2-adrenoceptor antagonist yohimbine enhanced both basal and stimulated release of noradrenaline and NPY-LI. Addition of BIIE0246 after yohimbine did not further enhance transmitter overflow. This may indicate that there were still too low levels of endogenous NPY released upon nerve activation to exert significant prejunctional effects. [In parallel, the role of endogenous NPY in sympathetic nerve-evoked postjunctional vascular effects is not clear-cut during control conditions (Lundberg and Modin 1995; Malmström and Lundberg 1996), or even after adrenoceptor blockade (unpublished observations).] Alternatively, any effect of NPY may have been still marginal in comparison with α_2-mediated autoinhibition, taking into account the possibility of an incomplete α_2-adrenoceptor blockade in combination with an enhanced release of noradrenaline. The presence of another prejunctional sympathetic NPY receptor subtype than the Y_2, activated exclusively by endogenous NPY, seems less likely considering that NPY Y_1 receptor antagonists do not affect sympa-

thetic transmitter release in the pig kidney (Lundberg and Modin 1995; Malmström and Lundberg 1996).

Confirming the results of Pernow and Lundberg (1989), neither PYY or yohimbine affected the functional responses (reduction in renal blood flow) upon nerve activation. A large release of transmitters under control conditions, saturating the postjunctional receptors, may explain the lack of effect of PYY on the functional response. A blockade of any possible postjunctional α_2-adrenoceptors would mask the functional effect of an enhanced noradrenaline release in the case of yohimbine. Furthermore, the functional response upon nerve activation under control conditions was nearly maximal, rendering difficulties in demonstrating an augmented response.

As demonstrated in an earlier study (Malmström 2001b), BIIE0246 did not significantly alter basal cardiovascular parameters per se. That a blockade of NPY Y_2 receptors had been established after injection of BIIE0246 was shown by the PYY-evoked vascular response in spleen, a vascular bed with a dual NPY receptor subtype population (Malmström 2001a). As anticipated, BIIE0246 antagonized approximately half of this splenic vasoconstrictor response (that is mediated by both NPY Y_1 and Y_2 receptors). Interestingly, when BIIE0246 was administered after yohimbine, the NPY Y_2 receptor antagonist evoked clear-cut splenic vasodilatation (without causing other significant effects). Antagonism of α_2-adrenoceptors with yohimbine increased circulating levels of NPY-LI and noradrenaline two- and threefold, respectively, without any significant splenic vascular effects. Addition of BIIE0246 did not further enhance these basal plasma levels, but markedly increased splenic blood flow. It is thus suggested that what is presumably the involvement of NPY Y_2 receptors in the regulation of basal splenic vascular tone was revealed upon the enhanced (as caused by yohimbine) circulating plasma levels of NPY. In line with this, another study (Ahlborg et al. 1992) described significant NPY-evoked renal and splanchnic vasoconstriction at an arterial plasma concentration of 55 pM in man (as detected 60 min after an NPY infusion), i.e. in the same range as in the present study. Since splenic venous plasma levels of NPY-LI were not measured in the present study, it cannot however be excluded that local splenic NPY levels were even higher than systemic levels, due to a possible local nerve discharge as the splenic sympathetic nerves were kept intact.

In summary, we have demonstrated, using the selective NPY Y_2 receptor antagonist BIIE0246 in the pig vivo, that renal sympathetic nerves possess prejunctional NPY Y_2 receptors that, upon activation, inhibit the release of both noradrenaline and NPY. Moreover, when basal release of NPY is enhanced upon blockade of prejunctional inhibitory α_2-adrenoceptors, endogenous NPY acting on NPY Y_2 receptors seems to be involved in the regulation of basal splenic vascular tone.

Acknowledgements The authors wish to thank Dr. Henri Doods at Boehringer Ingelheim Pharma (Biberach, Germany) for a generous supply of BIIE0246. Margareta Stensdotter and Carina Nihlén are acknowledged for expert technical assistance. This work was supported by grants from Karolinska Institutet, AstraZeneca R&D Mölndal, Magn. Bergvalls and Lars Hiertas Foundations, and the Swedish Medical Research Council (project nos. 12585 and 12586).

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