British Journal of Pharmacology (2008) 153, 1185–1194
© 2008 Nature Publishing Group All rights reserved
0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

ZP120 causes relaxation by pre-junctional inhibition of noradrenergic neurotransmission in rat mesenteric resistance arteries

U Simonsen[1], BE Laursen[1] and JS Petersen[2]

[1]*Department of Pharmacology, Faculty of Health Sciences, University of Aarhus, Aarhus, Denmark and* [2]*Zealand Pharma A/S, Glostrup, Denmark*

Background and purpose: ZP120 (Ac-RYYRWKKKKKKK-NH_2), is a new partial nociceptin/orphanin FQ (NOP) receptor agonist with sodium-potassium sparing aquaretic effects. The mechanisms of vasodilatation of ZP120 were examined in rat mesenteric resistance arteries.
Experimental approach: Arterial segments (internal diameters $206 \pm 4\,\mu m$, $n = 224$) were mounted in microvascular myographs for isometric tension recordings and electrical field stimulation (EFS).
Key results: ZP120 and the endogenous NOP receptor ligand, N/OFQ, did not relax arteries contracted with noradrenaline or adenosine-triphosphate. EFS-evoked contractions were inhibited by a purinoceptor antagonist, suramin, and the α_1-adrenoceptor antagonist prazosin. N/OFQ inhibited, concentration-dependently, EFS-evoked contractions with a maximal effect of $52 \pm 3\%$ ($n = 8$) at $1\,\mu M$. The maximal effect of $1\,\mu M$ ZP120 was lower ($27 \pm 5\%$, $P < 0.05$, $n = 9$) than for N/OFQ. Endothelial removal or pretreatment with capsaicin did not influence the vasodilator effects of ZP120 and N/OFQ. ZP120 and N/OFQ responses were preserved in the presence of suramin. The α_2-adrenoceptor antagonist, rauwolscine, antagonized the effect of clonidine and brimonidine, but ZP120 and N/OFQ inhibition of EFS-evoked contraction was unaltered. The competitive NOP receptor antagonist, UFP-101 ($10\,\mu M$), prevented the inhibitory effect of N/OFQ, but not ZP120 suggesting that N/OFQ and ZP120 have distinct modes of interaction with the NOP receptor.
Conclusions and implications: Our findings suggest that the vasodilator effect of ZP120 and N/OFQ in rat mesenteric resistance arteries is mediated by prejunctional inhibition of adrenergic neurotransmission. These properties, that promote diuresis and attenuate the cardiovascular consequences of increased sympathetic nerve activity, make ZP120 a promising drug candidate.
British Journal of Pharmacology (2008) **153,** 1185–1194; doi:10.1038/sj.bjp.0707688; published online 14 January 2008

Keywords: endothelium; nociceptin/orphan FQ peptide; noradrenaline; sympathetic neurotransmission; ZP120

Abbreviations: EFS, electrical field stimulation; N/OFQ, nociceptin/orphan FQ peptide; NOP, nociceptin/orphanin FQ receptor; ZP120, Ac-RYYRWKKKKKKK-NH2

Introduction

Nociceptin/orphan FQ peptide (N/OFQ), the endogenous peptide ligand that binds to the nociceptin/orphanin FQ (NOP) receptor (previously known as opioid receptor-like 1), exerts prominent effects on the neural and humoral mechanisms that control arterial blood pressure. Intravenous administration of N/OFQ to anaesthetized (Champion and Kadowitz, 1997; Bigoni *et al*., 1999) or conscious (Kapusta *et al*., 1996; Giuliani *et al*., 1997b; Madeddu *et al*., 1999; Kapusta, 2000) rats or mice lowers heart rate and systemic blood pressure, and recording of peripheral sympathetic nerve activity after intracerebral administration suggests that central N/OFQ may affect blood pressure by modulating autonomic neurotransmission at multiple levels of the central nervous system (see Kapusta, 2000).

Studies on isolated arteries or isolated hind limb preparations suggest that N/OFQ has direct vasodilator actions (Czapla *et al*., 1997a, b; Gumusel *et al*., 1997; Champion *et al*., 1998, 2002; Xu *et al*., 2004), but data on the vascular mode of action are conflicting. Early studies demonstrated that N/OFQ decreased tension in feline-isolated arteries with endothelium, suggesting that N/OFQ exerts a post-junctional effect on the vascular smooth muscle (Gumusel *et al*., 1997). However, in the rat-isolated tail artery N/OFQ inhibited electrical field stimulation (EFS)-evoked noradrenaline release (Bucher, 1998), and a pre-junctional inhibitory

Correspondence: Professor U Simonsen, Department of Pharmacology, Faculty of Health Sciences, University of Aarhus, 8000 Aarhus C, Aarhus 8000, Denmark.
E-mail: us@farm.au.dk
Received 1 November 2007; revised 6 December 2007; accepted 14 December 2007; published online 14 January 2008

effect of N/OFQ was also found in the rat anococcygeus muscle (Ho *et al*., 2000). In pithed and vagotomized rats, N/OFQ inhibited by about 60% the vasopressor response produced by electrical stimulation of the preganglionic sympathetic nerve fibres at T2-L6 vertebral level, but had no effect on vasopressor responses evoked by noradrenaline (Malinowska *et al*., 2000). In anaesthetized guinea pigs, the N/OFQ-induced hypotension and bradycardia are positively correlated with the decrease in the plasma noradrenaline level (Hashiba *et al*., 2003). Together, these studies suggest that the inhibitory pre-junctional effect of N/OFQ could be particularly, therapeutically relevant during clinical conditions in which an attenuation of noradrenergic stimulation is warranted.

ZP120 (Ac-RYYRWKKKKKKK-NH_2) is a new, highly selective, chemically stable, functional partial NOP receptor agonist (Hadrup *et al*., 2004; Kapusta *et al*., 2005). Animal studies have demonstrated that this novel NOP agonist is a sodium–potassium sparing aquaretic. ZP120 is currently being evaluated in clinical phase II trials for safety, tolerance and efficacy in patients with acute decompensated heart failure, and the powerful aquaretic effect of ZP120 is expected to reduce fluid overload in the lungs, facilitate pulmonary gas exchange and alleviate dyspnoea. Moreover, ZP120 also produces dose-dependent reductions in blood pressure in rats (Kapusta *et al*., 2005). Thus, ZP120-induced dilatation of resistance vessels could facilitate an increase in cardiac output and contribute to emptying of the overfilled lungs in acute heart failure patients. Considering the potential use of ZP120 in acute decompensated heart failure, it is of particular interest that this new compound lowers blood pressure in conscious rats, without causing reflex tachycardia (Kapusta *et al*., 2005).

The present study hypothesized that ZP120 regulates vascular tone in resistance vessels by inhibition of noradrenaline release during conditions with increased sympathetic nerve activity. The vascular effect of ZP120 was compared with that of N/OFQ and the α_2-adrenoceptor agonists, clonidine and brimonidine in arteries contracted with EFS or noradrenaline. To examine possible vascular interactions with purine receptors, ZP120- and N/OFQ-induced modulation of EFS-induced contractions was also studied in the presence of the P2 purinoceptor antagonist suramin. Release of sympathetic neurotransmitters in vascular preparations can be modulated by endothelium-derived relaxing factors (Tesfamariam and Cohen, 1988; Kun *et al*., 2003) and peptides released from sensory nerve fibres (Kakuyama *et al*., 1998; Zhang and McDougall, 2006), and therefore ZP120-induced vasorelaxation was also evaluated in preparations without endothelium and in preparations treated with capsaicin. Finally, the pharmacological effect of ZP120 was characterized using the selective NOP receptor antagonist, UFP-101 ([Nphe^{1}Arg^{14}Lys^{15}]N/OFQ-NH_2) (Calo *et al*., 2002).

Materials and methods

Dissection and mounting

All experiments were conducted in agreement with Danish law on the use of laboratory animals. Male Wistar rats (12–16 weeks old), were killed by a blow to the head followed by exsanguination. The mesenteric bed was removed and placed in cold physiological salt solution (4 °C) of the following composition (mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 1.6, EDTA 0.027 and glucose 5.5. The solution was gassed with 5% CO2 in air to maintain pH at 7.4

Vascular segments (approximately 2 mm long) that were third order of the rat superior mesenteric artery were mounted in wire-myographs equipped with electrodes. After equilibration in oxygenated (5% CO_2 in air) physiological salt solution, pH 7.4, at 37 °C for 30 min, a standardized normalization procedure was performed (Lei *et al*., 1999; Simonsen *et al*., 2001). This defines the lumen diameter (d_{100}) that the artery would have had *in vivo*, when relaxed and under a transmural pressure of 100 mm Hg. The arteries were then set to the lumen diameter $d_1 = 0.9 \times d_{100}$, where active force development is maximal. To test tissue viability, all arteries were subjected to 10 μM noradrenaline for 2 min. If active force development exceeded 13 kPa, the artery was considered viable and used for further experiments.

To investigate the role of the endothelial cell layer for the inhibition evoked by ZP120 and N/OFQ on contractions induced by EFS, vascular segments with and without endothelium were examined. The endothelial cells were removed by introducing into the lumen a human scalp hair and rubbing it back and forth several times. The effectiveness of this procedure was assessed by the absence of relaxation to acetylcholine in noradrenaline-contracted arteries (Buus *et al*., 2000).

Electrical field stimulation was performed with platinum electrodes (Danish Myotechnology, Aarhus, Denmark), measuring 2 × 2 mm, secured in plastic mounting heads on either side of the artery, approximately 1 mm from the vessel wall, as described previously (Lei *et al*., 1999; Simonsen *et al*., 2001). The electrodes were connected to an electrical stimulator with constant current output (Cibertec CS20, Barcelona, Spain). To obtain reproducible contractions to EFS, neuronal noradrenaline uptake and β-adrenoceptors were blocked, respectively, with cocaine (1 μM) and propranolol (1 μM), and EFS (0.3-ms pulse, 16 Hz, 10-s trains, supramaximal constant current) was applied with 5-min intervals. In one separate series of experiments, it was demonstrated that EFS-induced contractions were abolished after treatment with guanethidine (10 μM). To characterize the EFS-induced contractions further, the arterial segments were incubated with vehicle, a blocker of voltage-dependent sodium channels, tetrodotoxin (1 μM), the P_2 purinoceptor antagonist, suramin (100 μM), the α_1-adrenoceptor antagonist, prazosin (0.1 μM), the NPY Y_1 receptor antagonist, BIBP3226 (0.3 μM), the α_2-adrenoceptor antagonist, rauwolscine (0.1 μM), or combinations of prazosin and suramin, prazosin and rauwolscine, suramin and BIBP3226, or prazosin, suramin, and BIBP3226 for at least10 min, and the effect on EFS-evoked contraction evaluated.

To investigate the effect of ZP120 on neurogenic contractions, increasing cumulative concentrations (10^{-13}–10^{-6} M) of ZP120, N/OFQ, prazosin and vehicle were added. In a first series of experiments, ZP120 responses were compared when the compound was applied with 5 or 10-min intervals. These

studies demonstrated that the response to ZP120 was maximal already after 5 min and therefore the 5-min protocol was used in all subsequent studies with ZP120. Prazosin was applied with 12-min intervals. In a separate set of experiments, all arteries were incubated with suramin to exclude contribution from purinoceptors to EFS-evoked contractions, and increasing concentrations of ZP120 or N/OFQ were added. Prazosin (1 μM) was added at the end of the experiments to confirm contraction induced by EFS was abolished.

To investigate whether the effect of ZP120 and N/OFQ was at the smooth muscle, preparations were contracted with noradrenaline (5 μM) or U46619 (0.1 μM), and when a stable contraction was obtained, increasing concentrations of vehicle, ZP120, N/OFQ or prazosin were added. To evaluate the effect on post-junctional P2 purinoceptors, preparations were stimulated with ATP (100 μM) with 10-min intervals, which gave reproducible responses up to six times. Increasing concentrations (10^{-9}–10^{-6} M) of ZP120, N/OFQ or vehicle were added 5 min before each stimulation, or in case of suramin (10^{-7}–10^{-4} M).

To investigate whether modulation of vasodilator neurotransmitters from sensory nerves are involved in the effect of N/OFQ or ZP120, preparations were desensitized by pretreatment with capsaicin (10 μM) for 20 min, followed by 30 washing (Kakuyama *et al*., 1998), and the effect of ZP120 and N/OFQ on EFS-evoked contractions was examined.

The inhibitory effect of ZP120 was compared with that of α_2-adrenoceptor agonists, clonidine and brimonidine (10^{-13}–10^{-6} M). To investigate whether the effect of ZP120 on EFS-evoked contractions was through α_2-adrenoceptors, arterial segments were incubated with rauwolscine (0.1 μM) and increasing concentrations (10^{-9}–10^{-6} M) of clonidine, brimonidine, ZP120, N/OFQ and vehicle were added. In the presence of rauwolscine (0.1 μM), EFS-evoked contractions were only reproducible up to six times, and in cases where the vehicle control was reduced by more than 10% during the protocol, the experiment was excluded.

To characterize the interactions of ZP120 with the NOP receptors, concentration–response curves for ZP120 and N/OFQ were plotted in the absence and presence of the selective, competitive peptide NOP receptor antagonist, UFP-101 (1 and 10 μM) (Calo *et al*., 2002; McDonald *et al*., 2003), and the non-selective opioid receptor antagonist, naloxone (1 μM).

Drugs

Acetylcholine HCl, BIBP3226 ((R)-*N*2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)-methyl]-argininеamide), brimonidine, capsaicin, clonidine, noradrenaline hydrochloride, prazosin hydrochloride, propranolol and U46619 (9,11-dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$) were from Sigma (St Louis, MO, USA). Naloxone was from Tocris Cookson (Bristol, UK). N/OFQ and ZP120 were synthesized by Zealand Pharma A/S (Glostrup, Denmark). UFP-101 ([Nphe1, Arg14, Lys15] N/OFQ-NH2) was kindly provided by Dr G Calo', University of Ferrara, Italy. Prazosin was dissolved in warm water (50 °C) at pH 4–5 with constant agitation. The other drugs were dissolved in distilled water.

Data calculations and analysis

Mechanical responses of the arteries were measured as force and expressed as active wall tension, ΔT ($N\,m^{-1}$), which is the increase in force, ΔF (mN), divided by twice the segment length. The magnitude of inhibition evoked by ZP120, N/OFQ, prazosin and vehicle of contraction evoked by EFS was expressed relative to an average of two control responses evoked by EFS. Relaxations evoked in noradrenaline-contracted preparations are expressed as percentage of the contraction just prior to addition of the agonist.

Differences in dose–response relationships between treatments were analysed using two-way analysis of variance followed by Bonferroni correction when more than one comparison was performed. Differences at the $P<0.05$ level were considered statistically significant.

Results

Mesenteric vessels with an internal diameter of 206 ± 3 μm ($n=224$) were mounted and noradrenaline (10 μM) induced contractions of 2.7 ± 0.2 Nm^{-1} ($n=224$) in these arteries. In the presence of propranolol (1 μM) and cocaine (1 μM), 16 Hz EFS induced reproducible contractions in arteries without endothelium. In the presence of the P2 purinoceptor antagonist, suramin (100 μM), and the α_1-adrenoceptor antagonist, prazosin (0.1 μM), EFS-evoked contractions were reduced by 56 ± 6 ($n=8$) and 77 ± 3% ($n=9$), respectively. The combination of prazosin and suramin reduced EFS-evoked contractions by 90 ± 3% ($n=5$), and in the presence of prazosin, suramin and the NPY Y_1 receptor antagonist, BIBP32216 (0.3 μM), EFS-evoked contraction was reduced by 94 ± 2% ($n=4$) (Figure 1a). In the presence of the α_2-adrenoceptor antagonist, rauwolscine (0.1 μM), EFS-evoked contractions were markedly enhanced and the neurogenic response, measured as sensitivity to tetrodotoxin (1 μM), persisting in the presence of prazosin was increased (Figure 1b).

Agonist study

In the presence of propranolol (1 μM) and cocaine (1 μM), 16 Hz EFS applied with 5-min intervals induced reproducible contractions in arteries without endothelium (Figure 2a). N/OFQ concentration dependently inhibited contractions induced by EFS (Figures 2a and 4a). Thus, in the presence of 1 μM N/OFQ, EFS-induced contractions were reduced by 52 ± 3% ($n=8$), which was comparable to the effect of the α_1-adrenoceptor antagonist, prazosin (59 ± 13%, $n=4$; Figure 3a). ZP120 also produced concentration-dependent inhibition of EFS-induced contractions. However, the ZP120-induced inhibition was significantly attenuated (27 ± 5% at 1 μM ZP120, $n=9$) relative to N/OFQ (Figures 2c and 3a). In the presence of the P2 purinoceptor antagonist, suramin (100 μM), EFS evoked reproducible contractions, which were inhibited concentration dependently by ZP120 and N/OFQ. The maximal ZP120-induced inhibition at 1 μM was similar in the absence (27 ± 5%; Figure 3a) and in the presence (19 ± 3%; Figure 3b) of suramin.

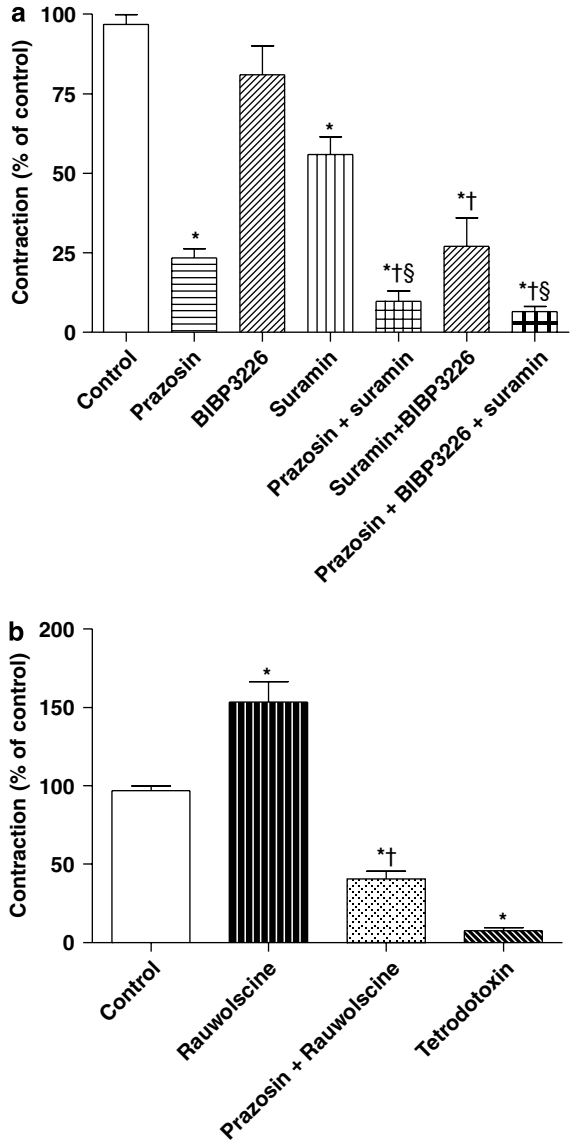

Figure 1 Average contraction evoked by induced by EFS (16 Hz, 0.3 ms, 10-s trains) in mesenteric small arteries. (**a**) EFS responses were obtained in arterial preparations incubated with vehicle (control); the NPY Y_1 receptor antagonist, BIBP3226 (0.3 μM); the P2 purinoceptor antagonist, suramin (100 μM); the α_1-adrenoceptor antagonist, prazosin (0.1 μM); the combination of prazosin and suramin, suramin and BIBP3226, or prazosin, BIBP, and suramin. (**b**) EFS-evoked contractions in the presence of vehicle (control), the α_2-adrenoceptor antagonist, rauwolscine (0.1 μM), the combination of prazosin and rauwolscine, and in the presence of tetrodotoxin (TTX, 1 μM). Values represent means ± s.e.mean of arteries from six animals. *$P<0.05$ versus vehicle control; $^{\dagger}P<0.05$ versus EFS-evoked contraction in the presence of (**a**) suramin and (**b**) rauwolscine, respectively; $^{\S}P<$ 0.05 versus prazosin alone. EFS, electrical field stimulation.

In arteries without endothelium and contracted with noradrenaline (5 μM), prazosin caused marked inhibition of the contraction (Figure 3c). In contrast, ZP120 and N/OFQ had no effect on contractility in vessels that were pre-contracted with noradrenaline (Figure 3c). ATP (100 μM) contracted rat mesenteric small arteries by 68 ± 6% ($n=22$) of the contraction observed in the presence of 5 μM noradrenaline. Suramin (100 μM) reduced ATP (100 μM)-evoked contraction by 79 ± 9% ($n=5$), whereas ZP120 (10^{-9}–10^{-6} M) and N/OFQ did not change ATP-evoked contraction ($n=5$ experiments for each agonist, results not shown).

The inhibitory effect of ZP120 was compared to the effect of α_2-adrenoceptor agonists in rat mesenteric arteries. The general α_2-adrenoceptor agonist, clonidine, and the selective α_{2A}-adrenoceptor agonist, brimonidine, concentration dependently inhibited EFS-induced contractions at concentrations up to 100 nM, the efficacy of ZP120 being similar to that of clonidine and brimonidine (Figure 4a). In mesenteric resistance vessels without endothelium and contracted with noradrenaline, ZP120 and brimonidine had no effect on vascular tone, whereas clonidine blocked noradrenaline-induced vasocontractions at 100 nM and 1 μM (Figure 4b).

Antagonist study

To determine whether α_2-adrenoceptors are involved in the inhibitory effect of ZP120 on EFS-evoked contractions, the preparations were incubated with rauwolscine, which significantly antagonized the effects of clonidine and brimonidine (Figures 5a and b). In contrast, incubation with rauwolscine did not alter the inhibitory effect of N/OFQ and ZP120 on EFS-evoked contractions in rat mesenteric arteries (Figures 5c and d).

The NOP receptor antagonist, UFP-101 (1 and 10 μM), did not change the inhibitory effects of ZP120 on EFS-induced contractions in mesenteric resistance arteries (Figure 6a). However, UFP-101 (10 μM) counteracted the inhibitory effect of N/OFQ on EFS contraction (Figure 6b). In contrast, the opioid receptor antagonist, naloxone (1 μM), did not affect the N/OFQ-induced inhibition of EFS-evoked contractions in rat mesenteric resistance arteries (Figure 6b).

Role of endothelium and vasodilator neurotransmitters

Acetylcholine (10 μM) relaxed noradrenaline-contracted arteries with endothelium 93 ± 1% ($n=20$), but did not induce relaxations in arteries without endothelium ($n=20$). EFS (16 Hz) increased tension by 1.02 ± 0.07 ($n=20$) and 0.92 ± 0.08 N m^{-1} ($n=20$) in arteries with and without endothelium, respectively. The inhibitory effects of ZP120 and N/OFQ on EFS-evoked contractions were similar in arteries with and without endothelium (Figures 7a and b). Noradrenaline (5 μM) increased tension by 3.1 ± 0.2 ($n=20$) and 2.9 ± 0.2 N m^{-1} ($n=20$) in arteries with and without endothelium, respectively. In contrast to acetylcholine, ZP120 and N/OFQ up to 1 μM did not relax noradrenaline-contracted preparations with endothelium ($n=5$ for each agonist, results not shown).

U46619 (0.1 μM) contracted mesenteric small arteries by 2.2 ± 0.2 N m^{-1} ($n=20$ arteries from five animals). Increasing concentrations of ZP120 failed to change U46619-induced contractions compared with vehicle control, whereas N/OFQ evoked small relaxations of 18 ± 10% ($n=5$) at 1 μM (results not shown).

To determine whether vasodilator neuropeptides are involved in the effect of ZP120 and N/OFQ, preparations

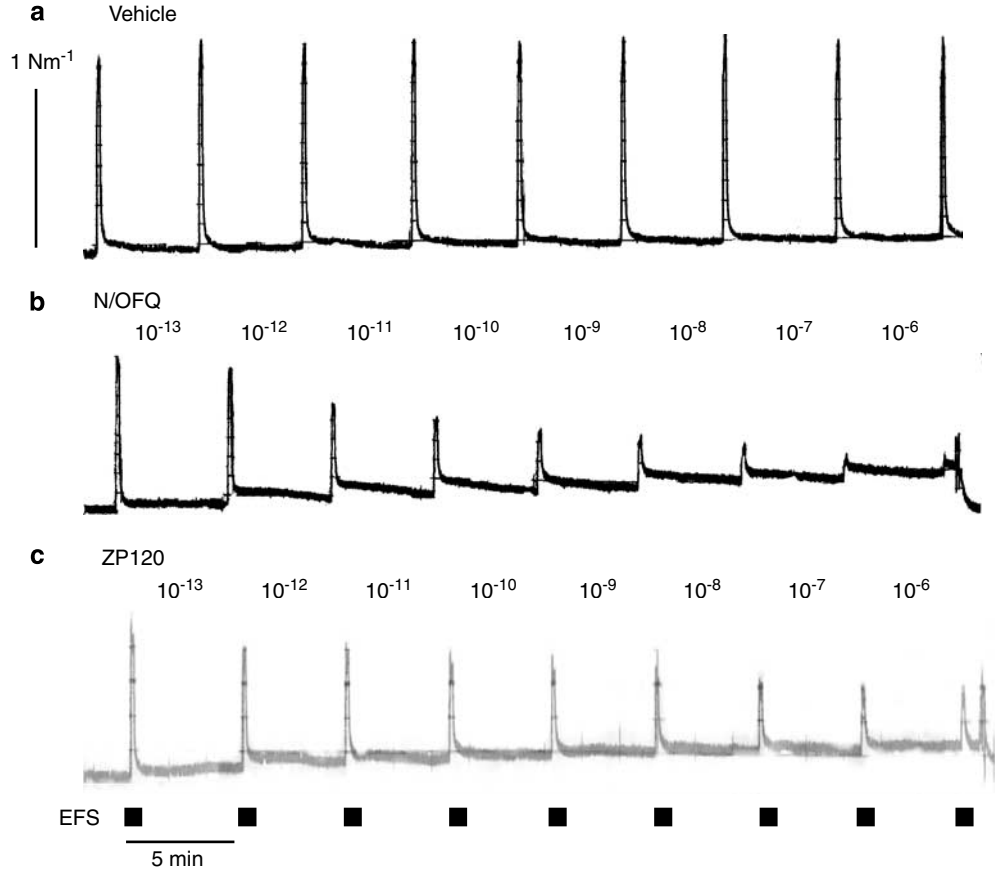

Figure 2 Representative original traces showing contractions in rat mesenteric resistance arteries evoked by EFS (16 Hz, 0.3 ms, 10-s trains with 5-min intervals) in the presence of vehicle (**a**), increasing concentrations of N/OFQ (**b**) and ZP120 (**c**). The experiments were performed in the presence of cocaine (1 μM) and propranolol (1 μM). Horizontal bar shows time and vertical bar tension. EFS, electrical field stimulation; N/OFQ, nociceptin/orphan FQ peptide; ZP120, Ac-RYYRWKKKKKKK-NH_2.

were pretreated with capsaicin. In capsaicin-treated preparations with endothelium, ZP120 and N/OFQ inhibited EFS-evoked contractions to the same extent as in control segments (Figures 7a and b).

Discussion

The main findings of the present study were that ZP120 and N/OFQ inhibited prazosin-sensitive, EFS-evoked contractions in mesenteric resistance arteries, while the vasorelaxing properties of both NOP receptor agonists were absent in noradrenaline-contracted preparations with or without endothelium. Removal of the endothelial cell layer did not affect the effect of ZP120 or N/OFQ. The vasodilator responses to ZP120 and N/OFQ during neurogenic contraction were preserved in the presence of the P2 purinoceptor antagonist suramin. These findings indicate that the vasorelaxing effects of ZP120 and N/OFQ are mediated by a pre-junctional neuromodulatory effect on adrenergic neurotransmission at the postganglionic nerve endings in rat mesenteric resistance vessels.

The mechanism behind the vasorelaxing effect of N/OFQ has only been examined in a few studies in isolated arteries. In feline renal, mesenteric, carotid and femoral rings with intact endothelium, N/OFQ produced vasorelaxation in phenylephrine-contracted vessels (Gumusel *et al*., 1997). In porcine-isolated coronary arteries, N/OFQ produced endothelium-dependent relaxation, which was sensitive to nitric oxide synthase inhibition with N^{ϖ}-nitro-L-arginine (Xu *et al*., 2004). In rat-isolated aortic rings, 1 μM N/OFQ had no effect on vascular tone in phenylephrine-contracted vessels (Hugghins *et al*., 2000). However, in rat mesenteric resistance arteries, which were pressurized and contracted with U46619, N/OFQ produced vasodilatation and the response was insensitive to muscarinic blockade with atropine, α-adrenoceptor blockade with phentolamine, nitric oxide synthase inhibition with N^{ϖ}-nitro-L-arginine and removal of the endothelium (Champion *et al*., 1998). In contrast, in the present study, N/OFQ failed to relax noradrenaline-contracted preparations and only induced small relaxations in U46619-contracted arteries. It is possible that these differences relate to the rat strain used or to the isometric recording technique employed in the present study and the isobaric recording technique used by Champion *et al*. (1998). In summary, studies on pigs have shown endothelium-dependent, N/OFQ-induced vasodilatation, whereas experiments on rat-isolated arteries suggest that

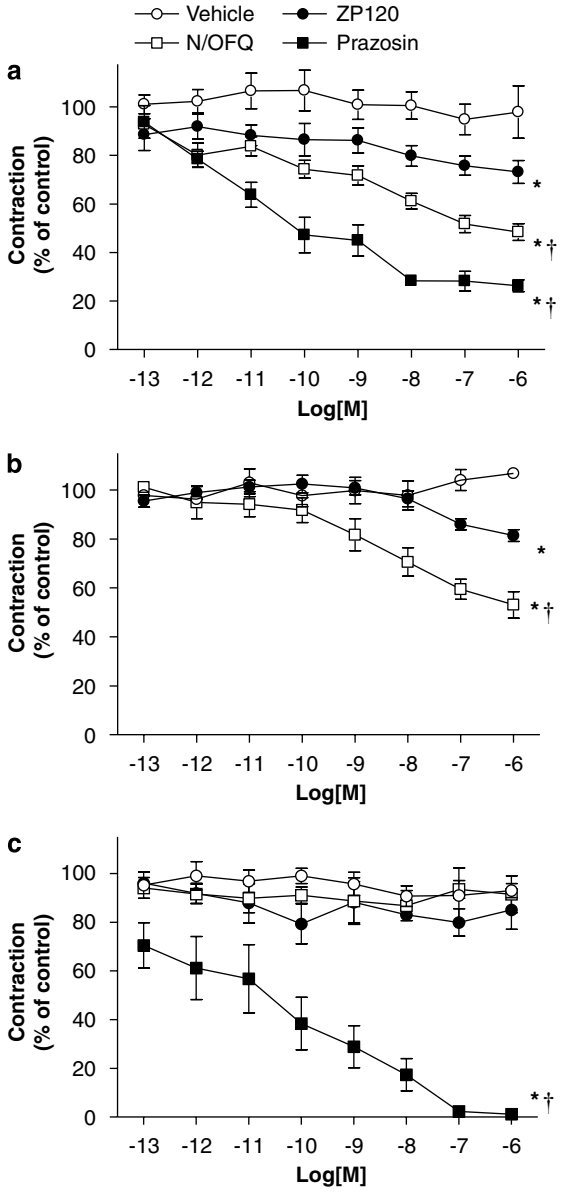

Figure 3 Average concentration–response curves for vehicle (control), N/OFQ (nociceptin), ZP120 and prazosin on contractions induced by, respectively, EFS (16 Hz, 0.3 ms, 5-min intervals) (**a**, **b**) and noradrenaline (5 μM) (**c**). EFS was performed in the absence (**a**) and presence (**b**) of the P2 purinoceptor antagonist, suramin (100 μM). Values represent means ± s.e.mean of arteries from 4–9 animals. The experiments were performed in the presence of cocaine (1 μM) and propranolol (1 μM). Differences were evaluated by two-way analysis of variance: $^{*}P<0.05$ versus curve for vehicle control; $^{\dagger}P<0.05$ versus curve for ZP120. EFS, electrical field stimulation; N/OFQ, nociceptin/orphan FQ peptide; ZP120, Ac-RYYRWKKKKKKK-NH_2.

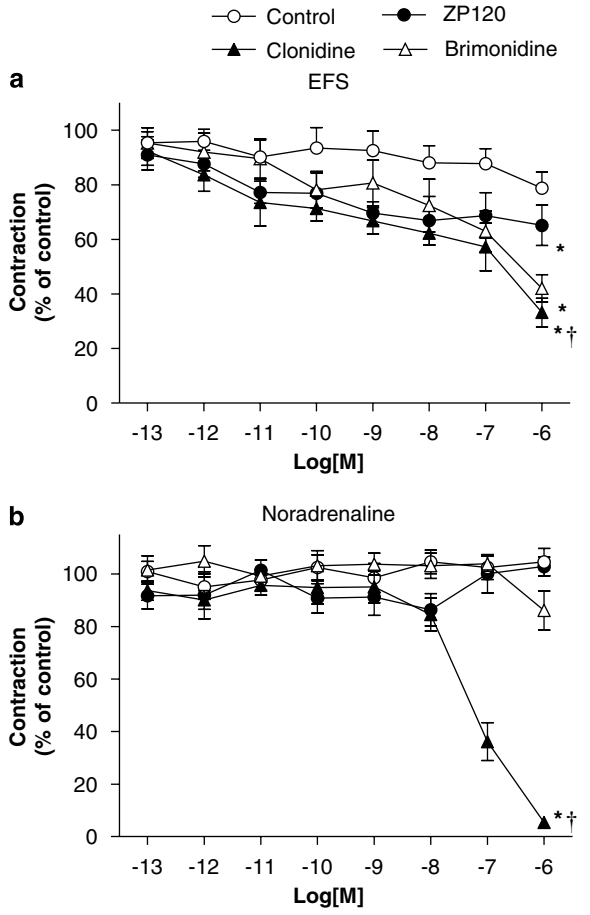

Figure 4 Comparison of the effects ZP120 with those of α_2-adrenoceptor agonists on (**a**) EFS and (**b**) noradrenaline in rat mesenteric arteries without endothelium. ZP120 inhibited EFS-evoked contraction to the same extent as a general α_2-adrenoceptor agonist, clonidine, and the selective α_{2A}-adrenoceptor agonist, brimonidine. The experiments were performed in the presence of cocaine (1 μM) and propranolol (1 μM). Values represent means ± s.e.mean of arteries from 5–9 animals. Differences were evaluated by two-way analysis of variance: $^{*}P<0.05$ versus curve for vehicle control; $^{\dagger}P<0.05$ versus curve for ZP120. EFS, electrical field stimulation; ZP120, Ac-RYYRWKKKKKKK-NH_2.

NOP-receptor-mediated pre-junctional inhibition of noradrenaline release is the most important mechanism for N/OFQ-mediated vasodilatation (Bucher, 1998; Champion *et al*., 1998).

Applying short trains of EFS at resting tension, as in the present study, mainly results in neurogenic contractions mediated by noradrenaline in mesenteric arteries (Lei *et al*., 1999). In the present study, the main component of the neurogenic contraction was also adrenergic and mediated through activation of α_1-adrenoceptors. However, purinergic neurotransmitters may also contribute to neurogenic contractions (Donoso *et al*., 1997), and contractions elicited with long trains of stimuli in preactivated arteries are mainly mediated by neuropeptide Y (Prieto *et al*., 2000; Gradin *et al*., 2003). Indeed the P2 purinoceptor antagonist, suramin, inhibited neurogenic contractions in the present study, whereas the NPY Y_1 receptor antagonist did not influence EFS-evoked contractions. Moreover, both ZP120 and N/OFQ had the same inhibitory effect on neurogenic contractions in the presence of suramin, and the NOP receptor agonists did not alter ATP-evoked contractions. Although these latter findings do not exclude the possibility that ZP120 and N/OFQ have an effect on purinergic neurotransmission, our results suggest that the vasodilator effect of N/OFQ and ZP120 in the rat mesenteric artery is mediated by a pre-junctional neuromodulatory effect on adrenergic neurotransmission at the postganglionic nerve endings.

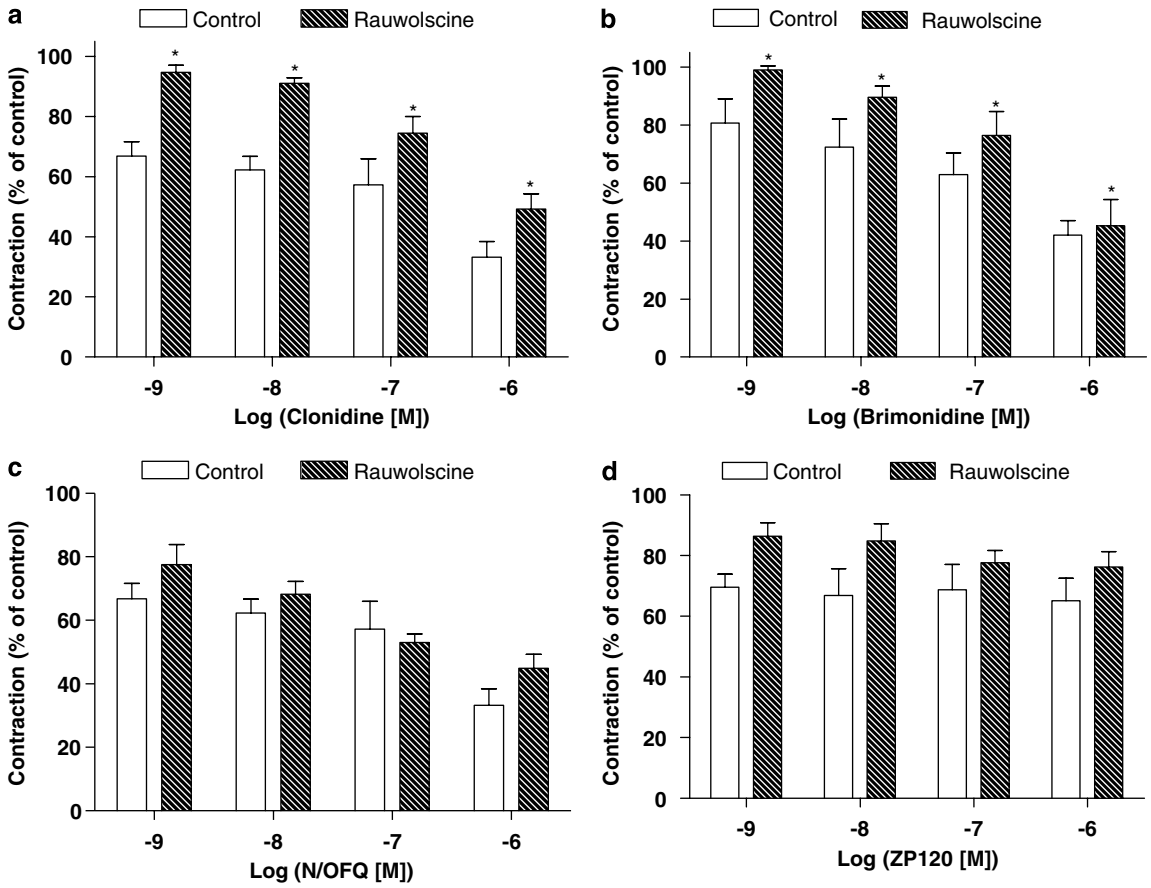

Figure 5 Effect of the α_2-adrenoceptor antagonist, rauwolscine, on EFS-evoked contractions obtained in the presence of increasing concentrations of (**a**) a general α_2-adrenoceptor agonist, clonidine, (**b**) a selective α_{2A}-adrenoceptor agonist, brimonidine, (**c**) N/OFQ (nociceptin) and (**d**) ZP120. Values represent means ± s.e.mean of arteries from 5–9 animals. Differences were evaluated by two-way analysis of variance: $^*P<0.05$ versus control responses for the drugs obtained in the absence of rauwolscine. EFS, electrical field stimulation; N/OFQ, nociceptin/orphan FQ peptide; ZP120, Ac-RYYRWKKKKKKK-NH_2.

Modulation of adrenergic neurotransmission by pre-junctional α_2-adrenoceptors is an important mechanism. This is also supported by the findings in the present study where the α_2-adrenoceptor antagonist, rauwolscine, increased neurogenic contractions to 150% of the control contractions and blocked the inhibitory effect of the α_2-adrenoceptor agonists, clonidine and brimonidine. Moreover, the effect of ZP120 and N/OFQ was as pronounced as for clonidine and brimonidine, suggesting that activation of pre-junctional NOP receptors is also an important mechanism for inhibition of sympathetic neurotransmission in rat resistance arteries.

In contrast to acetylcholine, ZP120 and N/OFQ did not relax noradrenaline-contracted arteries with endothelium and removal of the endothelium did not influence the vasodilator effects of ZP120 and N/OFQ in vessels with EFS-evoked contractions. These findings concur with those from studies in the rat-isolated perfused hind limb preparation, in which the vasodilator response to N/OFQ was not altered by nitric oxide synthase inhibition with N^G-nitro-L-arginine methyl ester, cyclooxygenase inhibition with meclofenamate or by K_{ATP} channel blockade with U-37883A (Champion *et al.*, 2002). Although the NOP receptor is present in the endothelium from rats and humans (Granata *et al.*, 2003), its functional role remains unclear. Together, these findings suggest that pre-junctional inhibition of noradrenaline release in resistance arteries is of key importance for the vasodilator effects elicited by NOP receptor agonists.

Neural control of vascular tone in mesenteric arteries is complex and involves both excitation by neurotransmitters released from sympathetic nerve terminals and inhibition by vasodilatator neurotransmitters released from sensory c-fibres (Kawasaki *et al.*, 1988; Kakuyama *et al.*, 1998). Moreover, N/OFQ has been demonstrated to increase sensory neuropeptide release and blood flow in the inflamed knee (Zhang and McDougall, 2006), and inhibit release of substance P and calcitonin gene-related peptide in rat trachea (Helyes *et al.*, 1997). However, in the present study capsaicin pretreatment failed to alter the inhibitory effect of ZP120 and N/OFQ on EFS-evoked contractions, suggesting that during normal physiological conditions modulation of sensory c-fibres is not involved in the pre-junctional effect of ZP120 and N/OFQ in rat mesenteric arteries.

It has previously been shown that ZP120 behaves as a functional partial agonist in the mouse vas deferens assay, inhibiting EFS-induced contractions with a pEC_{50} similar to that of N/OFQ, but with sub-maximal efficacy as compared

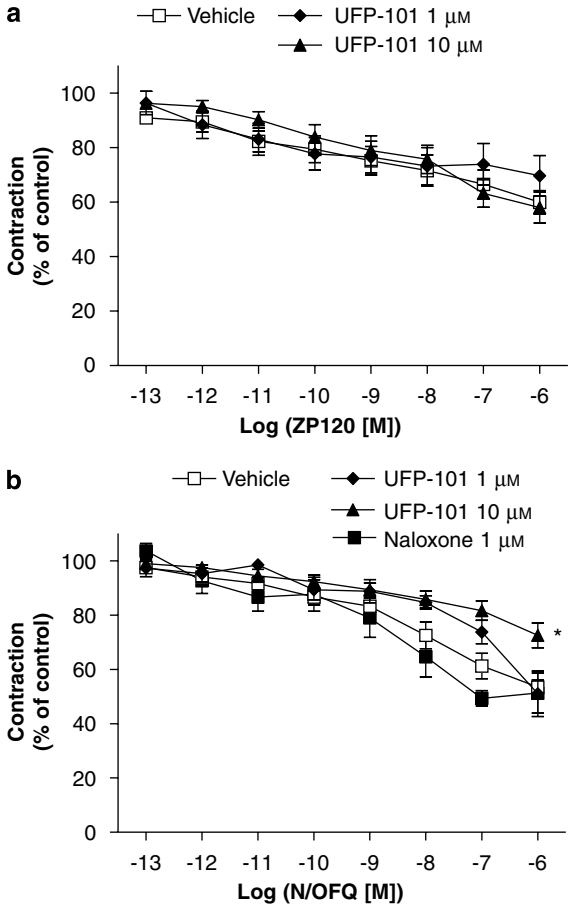

Figure 6 Average concentration–response curves for the inhibitory effect of (**a**) ZP120 and (**b**) N/OFQ on contractions induced by EFS in rat mesenteric resistance. The curves were constructed in the absence (vehicle control) and the presence of the NOP receptor antagonist, UFP-101 (1 and 10 μM), and in case of N/OFQ also in the presence of the opioid receptor antagonist, naloxone (1 μM). The experiments were performed in the presence of cocaine (1 μM) and propranolol (1 μM). Values represent means ± s.e.mean of arteries from 4–11 animals. Differences were evaluated by two-way analysis of variance: $*P<0.05$ versus ZP120. EFS, electrical field stimulation; N/OFQ, nociceptin/orphan FQ peptide; NOP, nociceptin/orphanin FQ; ZP120, Ac-RYYRWKKKKKKK-NH_2.

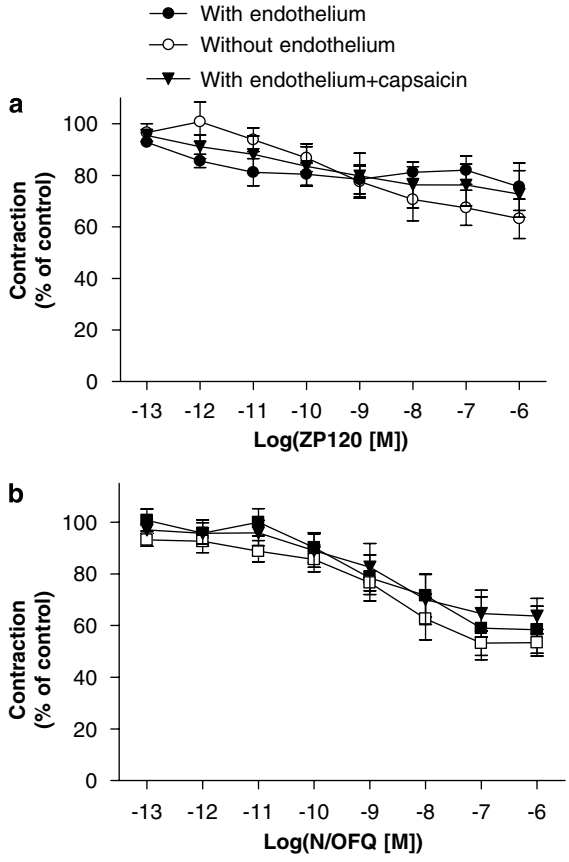

Figure 7 Average concentration–response curves for the inhibitory effect of (**a**) ZP120 and (**b**) N/OFQ on contractions induced by EFS in rat mesenteric resistance arteries with and without endothelium, and in preparations with endothelium pretreated with capsaicin (10 μM). The experiments were performed in the presence of cocaine (1 μM) and propranolol (1 μM). Values represent means ± s.e.mean of arteries from six animals. EFS, electrical field stimulation; N/OFQ, nociceptin/orphan FQ peptide; ZP120, Ac-RYYRWKKKKKKK-NH_2.

with N/OFQ (Rizzi *et al*., 2002; Kapusta *et al*., 2005). Moreover, ZP120 inhibited N/OFQ-induced smooth muscle relaxation in the mouse vas deferens and the bradycardic-hypotensive response to intravenous bolus administration of N/OFQ in conscious rats. In the present study, we found that the maximal inhibitory effect of ZP120 on EFS-induced contractions was less than the maximal effect of N/OFQ. These findings complement previous observations and indicate that ZP120 also behaves as a partial agonist in the rat mesenteric artery.

Whereas intravenous administration of N/OFQ produces hypotension associated with severe bradycardia, the haemodynamic responses to diuretic doses of ZP120 are less marked, and importantly not associated with changes in heart rate. Experiments on guinea pig-isolated left atrium suggest that N/OFQ is able to inhibit the inotropic responses produced by electrically evoked release of acetylcholine and noradrenaline from parasympathetic and sympathetic nerve endings, respectively (Giuliani and Maggi, 1997a). Thus, a possible pre-junctional effect of ZP120 on cardiac sympathetic nerve endings might be responsible for the lack of reflex tachycardia during ZP120-induced vasodilatation. For patients with acute decompensated heart failure who are not on β-blocker therapy at time of admission, attenuated cardiac sympathetic stimulation could potentially be of great clinical benefit.

UFP-101 is structurally related to N/OFQ and the competitive antagonist behaviour on the NOP receptor indicates that UFP-101 and N/OFQ share the same binding site on the NOP receptor (Calo *et al*., 2002; McDonald *et al*., 2003). ZP120 is structurally related to the family of hexapeptide partial agonists described by Dooley *et al*. (1997). Previous studies have indicated that N/OFQ and the hexapeptides have distinct modes of interaction with the NOP receptor (Bes and Meunier, 2003), and in agreement with these observations we found that whereas 10 μM UFP-101 inhibited the effect of N/OFQ on EFS-evoked contractions, UFP-101 had no effect on ZP120-induced vasodilatation.

In conclusion, ZP120 and N/OFQ inhibited prazosin-sensitive EFS-evoked contractions in mesenteric resistance arteries while the vasorelaxing properties of both compounds were absent in noradrenaline-contracted preparations with or without endothelium. Removal of the endothelium did not change the vasodilatation induced by ZP120 or N/OFQ. Our findings suggest that the vasorelaxing effect of ZP120 and N/OFQ in rat mesenteric resistance vessels is mediated by a pre-junctional inhibition of adrenergic neurotransmission. This mode of action may have important clinical implications for the treatment of cardiovascular disorders. Thus, increased sympathetic nerve activity elicits tachycardia and peripheral vasoconstriction with reduced diastolic coronary perfusion and increased cardiac metabolic demand. In heart failure patients, such changes may rapidly lead to further deterioration of cardiac pump function and shock. However, the combination of a powerful diuresis to alleviate pulmonary congestion and the ability to attenuate cardiovascular consequences of high sympathetic nerve activity makes ZP120 a promising new drug candidate for the treatment of cardiovascular diseases such as heart failure and hypertension.

Acknowledgements

We thank technician Henriette Gram Johansson for technical assistance. This work was supported by a grant from Zealand Pharma A/S.

Conflict of interest

Jørgen Søberg Petersen is employed in Zealand Pharmaceuticals, which also provided ZP120 and N/OFQ for the study.

References

Bes B, Meunier JC (2003). Identification of a hexapeptide binding region in the nociceptin (ORL1) receptor by photo-affinity labelling with Ac-Arg-Bpa-Tyr-Arg-Trp-Arg-NH2. *Biochem Biophys Res Commun* **310**: 992–1001.

Bigoni R, Giuliani S, Calo G, Rizzi A, Guerrini R, Salvadori S *et al*. (1999). Characterization of nociceptin receptors in the periphery: *in vitro* and *in vivo* studies. *Naunyn-Schmiedebergs Arch Pharmacol* **359**: 160–167.

Bucher B (1998). ORL1 receptor-mediated inhibition by nociceptin of noradrenaline release from perivascular sympathetic nerve endings of the rat tail artery. *Naunyn-Schmiedebergs Arch Pharmacol* **358**: 682–685.

Buus NH, Simonsen U, Pilegaard HK, Mulvany MJ (2000). Nitric oxide, prostanoid and non-NO, non-prostanoid involvement in acetylcholine relaxation of isolated human small arteries. *Br J Pharmacol* **129**: 184–192.

Calo G, Rizzi A, Rizzi D, Bigoni R, Guerrini R, Marzola G *et al*. (2002). [Nphe(1),Arg(14),Lys(15)]nociceptin-NH2, a novel potent and selective antagonist of the nociceptin/orphanin FQ receptor. *Br J Pharmacol* **136**: 303–311.

Champion HC, Bivalacqua TJ, Zadina JE, Kastin AJ, Hyman AL, Kadowitz PJ (2002). Role of nitric oxide in mediating vasodilator responses to opioid peptides in the rat. *Clin Exp Pharmacol Physiol* **29**: 229–232.

Champion HC, Kadowitz PJ (1997). Nociceptin, and endogenous ligand for the ORL(1) receptor, has novel hypotensive activity in the rat. *Life Sci* **60**: L241–L245.

Champion HC, Pierce RL, Kadowitz PJ (1998). Nociceptin, a novel endogenous ligand for the ORL1 receptor, dilates isolated resistance arteries from the rat. *Regul Pept* **78**: 69–74.

Czapla MA, Champion HC, Kadowitz PJ (1997a). Decreases in systemic arterial and hindquarters perfusion pressure in response to nociceptin are not inhibited by naloxone in the rat. *Peptides* **18**: 1197–1200.

Czapla MA, Champion HC, Kadowitz PJ (1997b). Nociceptin, an endogenous ligand for the ORL1 receptor, has vasodilator activity in the hindquarters vascular bed of the rat. *Peptides* **18**: 793–795.

Donoso MV, Steiner M, HuidobroToro JP (1997). BIBP 3226, suramin and prazosin identify neuropeptide Y, adenosine 5′-triphosphate and noradrenaline as sympathetic cotransmitters in the rat arterial mesenteric bed. *J Pharmacol Exp Ther* **282**: 691–698.

Dooley CT, Spaeth CG, BerzeteiGurske IP, Craymer K, Adapa ID, Brandt SR *et al*. (1997). Binding and *in vitro* activities of peptides with high affinity for the nociceptin/orphanin FQ receptor, ORL1. *J Pharmacol Exp Ther* **283**: 735–741.

Giuliani S, Maggi CA (1997a). Prejunctional modulation by nociceptin of nerve-mediated inotropic responses in guinea-pig left atrium. *Eur J Pharmacol* **332**: 231–236.

Giuliani S, Tramontana M, Lecci A, Maggi CA (1997b). Effect of nociceptin on heart rate and blood pressure in anaesthetized rats. *Eur J Pharmacol* **333**: 177–179.

Gradin KA, Li JY, Andersson O, Simonsen U (2003). Enhanced neuropeptide Y immunoreactivity and vasoconstriction in mesenteric small arteries from spontaneously hypertensive rats. *J Vasc Res* **40**: 252–265.

Granata F, Potenza RL, Fiori A, Strom R, Caronti B, Molinari P *et al*. (2003). Expression of OP4 (ORL1, NOP1) receptors in vascular endothelium. *Eur J Pharmacol* **482**: 17–23.

Gumusel B, Hao QZ, Hyman A, Chang JK, Kapusta DR, Lippton H (1997). Nociceptin: an endogenous agonist for central opioid like(1) (ORL(1)) receptors possesses systemic vasorelaxant properties. *Life Sci* **60**: L141–L145.

Hadrup N, Petersen JS, Praetorius J, Meier E, Graebe M, Brond L *et al*. (2004). Opioid receptor-like 1 stimulation in the collecting duct induces aquaresis through vasopressin-independent aquaporin-2 downregulation. *Am J Physiol Renal Physiol* **287**: F160–F168.

Hashiba E, Hirota K, Kudo T, Calo' G, Guerrini R, Matsuki A (2003). Effects of nociceptin/orphanin FQ receptor ligands on blood pressure, heart rate, and plasma catecholamine concentrations in guinea pigs. *Naunyn-Schmiedebergs Arch Pharmacol* **367**: 342–347.

Helyes Z, Nemeth J, Pinter E, Szolcsanyi J (1997). Inhibition by nociceptin of neurogenic inflammation and the release of SP and CGRP from sensory nerve terminals. *Br J Pharmacol* **121**: 613–615.

Ho M, Corbett AD, McKnight AT (2000). Characterization of the ORL1 receptor on adrenergic nerves in the rat anococcygeus muscle. *Br J Pharmacol* **131**: 349–355.

Hugghins SY, Champion HC, Cheng G, Kadowitz PJ, Jeter JR (2000). Vasorelaxant responses to endomorphins, nociceptin, albuterol, and adrenomedullin in isolated rat aorta. *Life Sci* **67**: L471–L476.

Kakuyama M, Vallance P, Ahluwalia A (1998). Endothelium-dependent sensory NANC vasodilatation: involvement of ATP, CGRP and a possible NO store. *Br J Pharmacol* **123**: 310–316.

Kapusta DR (2000). Neurohumoral effects of orphanin FQ/nociceptin: relevance to cardiovascular and renal function. *Peptides* **21**: 1081–1099.

Kapusta DR, Sezen SF, Chang JK, Lippton H, Kenigs VA (1996). Diuretic and antinatriuretic responses produced by the endogenous opioid-like peptide, nociceptin (orphanin FQ). *Life Sci* **60**: L15–L21.

Kapusta DR, Thorkildsen C, Kenigs VA, Meier E, Vinge MM, Quist C *et al*. (2005). Pharmacodynamic characterization of ZP120 (Ac-RYYRWKKKKKKK-NH2), a novel, functionally selective nociceptin/orphanin FQ peptide receptor partial agonist with sodium-potassium-sparing aquaretic activity. *J Pharmacol Exp Ther* **314**: 652–660.

Kawasaki H, Takasaki K, Saito A, Goto K (1988). Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. *Nature* **335**: 164–167.

Kun A, Martinez AC, Tanko LB, Pataricza J, Papp JG, Simonsen U (2003). Ca^{2+}-activated K^+ channels in the endothelial cell layer involved in modulation of neurogenic contractions in rat penile arteries. *Eur J Pharmacol* **474**: 103–115.

Lei S, Orensanz LM, Mulvany MJ, Simonsen U (1999). Mechanisms involved in the vasorelaxant effect of (−)-stepholidine in rat mesenteric small arteries. *Eur J Pharmacol* **365**: 193–204.

Madeddu P, Salis MB, Milia AF, Emanueli C, Guerrini R, Regoli D *et al*. (1999). Cardiovascular effects of nociceptin in unanesthetized mice. *Hypertension* **33**: 914–919.

Malinowska B, Kozlowska H, Koneczny B, Schlicker E (2000). Nociceptin inhibits the neurogenic vasopressor response in the pithed rat via prejunctional ORL1 receptors. *Naunyn-Schmiedebergs Arch Pharmacol* **361**: 80–84.

McDonald J, Calo G, Guerrini R, Lambert DG (2003). UFP-101, a high affinity antagonist for the nociceptin/orphanin FQ receptor: radioligand and GTP gamma S-35 binding studies. *Naunyn-Schmiedebergs Arch Pharmacol* **367**: 183–187.

Prieto D, Buus CL, Mulvany MJ, Nilsson H (2000). Neuropeptide Y regulates intracellular calcium through different signalling pathways linked to a Y-1-receptor in rat mesenteric small arteries. *Br J Pharmacol* **129**: 1689–1699.

Rizzi A, Rizzi D, Marzola G, Regoli D, Larsen BD, Petersen JS *et al*. (2002). Pharmacological characterization of the novel nociceptin/orphanin FQ receptor ligand, ZP120: *in vitro* and *in vivo* studies in mice. *Br J Pharmacol* **137**: 369–374.

Simonsen U, Contreras J, Garcia-Sacristan A, Martinez AC (2001). Effect of sildenafil on non-adrenergic non-cholinergic neurotransmission in bovine penile small arteries. *Eur J Pharmacol* **412**: 155–169.

Tesfamariam B, Cohen RA (1988). Inhibition of adrenergic vasoconstriction by endothelial-cell shear–stress. *Circ Res* **63**: 720–725.

Xu PH, Chang M, Cheng LX, Cheng Q, Yan X, Wang R (2004). The relaxant effect of nociceptin on porcine coronary arterial ring segments. *Can J Physiol Pharmacol* **82**: 993–999.

Zhang C, McDougall JJ (2006). Stimulation of sensory neuropeptide release by nociceptin/orphanin FQ leads to hyperaemia in acutely inflamed rat knees. *Br J Pharmacol* **148**: 938–946.