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Synthesis and characterization of a selective peptide antagonist of neuropeptide Y vascular postsynaptic receptors

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1 A cyclic dimeric nonapeptide neuropeptide Y (NPY) receptor antagonist, 1229U91, was synthesized by Fmoc chemistry and dimerised in solution. Its effects were assayed in mesenteric arteries from rats and mice, and in rat vas deferens.

2 Mesenteric arteries were cannulated and pressurised to 55 mmHg and the external diameters continuously measured. NPY, PYY, $Leu^{31}Pro^{34}$NPY and NPY(13–36) each caused concentration-related contractions with the order of potency PYY $\geq$ $Leu^{31}Pro^{34}$NPY = NPY > NPY (13–36), consistent with the Y_1 receptor subtype.

3 1229U91 had no agonist activity in the arteries but caused a concentration-related rightward shift of NPY (mouse arteries) or $Leu^{31}Pro^{34}$NPY (rat) concentration-response curves. The antagonism was competitive with pK_Bs of 7.69 ± 0.15 and 7.47 ± 0.13 in the mouse and rat arteries, respectively.

4 Sympathetic nerves in the vas deferens were stimulated with a single electrical field pulse every 20 s and the twitch responses recorded. NPY, PYY, $Leu^{31}Pro^{34}$NPY and NPY(13–36) inhibited the twitches with the order of potency PYY > NPY > NPY(13–36) > > $Leu^{31}Pro^{34}$NPY, consistent with the Y_2 receptor subtype.

5 1229U91 inhibited the vas deferens twitch with a shallow concentration-response curve and a time-course of inhibition distinct from that of NPY. 1229U91 (30 μM) did not cause a rightward shift of the NPY concentration-response curve. 1229U91 is at least 5 orders of magnitude less potent in the vas deferens than in rat brain Y_2 binding assays reported by others, suggesting that the brain and vas deferens Y_2 receptors are different.

6 It is concluded that 1229U91 is a competitive antagonist of NPY Y_1 vascular receptors and has additional properties that inhibit the electrically evoked twitch of the rat vas deferens.

Keywords: Neuropeptide Y antagonist; mesenteric artery; vas deferens

Introduction

Neuropeptide Y (NPY) is a 36-amino acid peptide with a widespread distribution in the brain and in the periphery, where it is present in sympathetic nerves. There appear to be at least three types of receptor for NPY (Y_1, Y_2 and Y_3) (Wahlestedt & Reis, 1993), but because the classification is presently only on the basis of binding and functional potencies of agonists this must be regarded as an interim classification (Kenakin *et al.*, 1992). Y_1 receptors mediate most of the vascular effects of NPY, although the involvement of other receptor types has been proposed (Tessel *et al.*, 1993). Y_2 receptors occur on sympathetic nerves where they mediate inhibition of neurotransmitter release. Y_3 receptors occur in the adrenal medulla, brain stem and heart, but their effects are not fully characterized.

NPY is a vasoconstrictor agonist in many arteries *in vitro* and *in vivo*, and several mechanisms of action have been proposed (Fredholm *et al.*, 1985; Lobaugh & Blackshear, 1990; Xiong *et al.*, 1993; Xiong & Cheung, 1994). The activity of NPY in many arteries is thought of as indirect vasoconstriction via potentiation of other vasoconstrictor stimuli (Edvinsson *et al.*, 1984; Lundberg *et al.*, 1985; Oshita *et al.*, 1989; Adriantsitohaina & Stoclet, 1990; Gustaffson & Nilsson, 1990; Saville *et al.*, 1990; Xiong *et al.*, 1993). It should be noted that many vasoconstrictor agonists cause apparent potentiation of other vasoconstrictors where there is a significant level of stimulation needed to reach the threshold for measurement of tissue responses (Ariëns *et al.*, 1960; Stupecky *et al.*, 1986), so the difference between describing NPY as a vasoconstrictor or a potentiator of other vasoconstrictors is possibly only semantic.

Because it is co-stored and released with noradrenaline and ATP in sympathetic nerve varicosities, NPY has the potential to play a role in vascular responses to sympathetic nerve activity. Without an antagonist of NPY receptors, it has been difficult to produce definitive evidence for such a role of NPY. Recently Laher *et al.* (1994) have shown that the responses of rabbit basilar arteries to sympathetic nerve stimulation were selectively inhibited (80%) by antibodies to NPY, suggesting that neurally released NPY is responsible for most of the sympathetic nerve responses in that tissue.

There are several molecules that have been claimed to act as antagonists of NPY, some that appear likely to be competitive, and others that are probably non-specific. Wahlestedt *et al.* (1992) found that α-trinositol selectively inhibits vasoconstriction evoked by NPY and ATP, but the mechanism was non-competitive and so α-trinositol could be used in the characterization of NPY receptors. Tatemoto proposed that PYX-2 (Ac-[3-(2,6-dichlorobenzyl)-$Tyr^{27,36}$, D-Thr^{32}]NPY 27–36) is an NPY antagonist (Tatemoto, 1990), but it has not been found to act consistently. Benextramine has been shown to block the binding of NPY to brain binding sites, and at least partially inhibits vasoconstrictor effects of NPY both *in vivo* and *in vitro* (Doughty *et al.*, 1990; Tessel *et al.*, 1993; Melchiorre *et al.*, 1994). However, the affinity of benextramine for many other receptors (Lew & Angus, 1984) and particularly for L-type calcium channels (Plotek & Atlas, 1983) makes interpretation of its actions in vascular tissues difficult.

Recently, studies of two new competitive antagonists of NPY Y_1 receptors have been published; one a non-peptide,

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BIBP3226 (Rudolf *et al.*, 1994), and the other a peptide ([2',4],[2,4'] homodimer of Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$ where Dpr is diaminopropionic acid) designated peptide 1A (Daniels *et al.*, 1994) or 1229U91 (Daniels *et al.*, 1995). In this paper we report a different synthesis of 1229U91 from that reported by Daniels *et al.* and its characterization on putative Y_1 and Y_2 receptors in isolated tissue assays.

Methods

Chemistry

All agonist peptides used in this study (NPY, peptide YY (PYY), NPY(13–36) and $Leu^{31}Pro^{34}$NPY) were synthesized in our laboratory by continuous-flow Fmoc methodology, using 2-(1H-benzotriazole-1yl)1,1,3,3-tetramethyluronium hexafluorophosphate/di-isopropylethylamine (HBTU/DIEA) activation of suitably-protected Fmoc-amino acids. The peptides were purified to a single peak detected using both high performance liquid chromatography (h.p.l.c.) and capillary zone electrophoresis. The open-chain monomer of 1229U91 was synthesized on Polyhipe PR500 resin (Calbiochem-Novabiochem), derivatised with modified Rink linker with a substitution level of 0.36 mmol g^{-1}. The final Fmoc group (on Ile1) was left attached to the peptide. After cleavage from the resin and removal of sidechain protecting groups, the Fmoc-monomer was purified by preparative h.p.l.c. The Fmoc-monomer was dissolved in dimethylformamide at a concentration of 0.1 M, and cyclisation was accomplished in 2 h at room temperature by the addition of 2 equivalents of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluoro phosphate (PyBOP) and 12 equivalents of DIEA. The reaction products from this cyclisation reaction consisted of 75% cyclic dimer and 25% cyclic monomer, which were readily separated by preparative h.p.l.c. After removal of the Fmoc group by dissolution in 20% piperidine in methanol at room temperature for 20 min, the peptide was purified by preparative h.p.l.c. Homogeneity of the product was confirmed by analytical h.p.l.c. and capillary electrophoresis, and its identity confirmed by matrix-assisted laser-desorption mass spectrometry.

Mesenteric artery preparations

Male Sprague-Dawley rats (273±25 g), or Swiss outbred white mice (34±2 g) were killed by carbon dioxide anaesthesia (80% CO_2 20% O_2) followed by decapitation, and a portion of intestine with attached mesentery was removed and placed in cold physiological saline solution (PSS) of the following composition (mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, sodium edetate 0.026, and glucose 5.5, saturated with carbogen (5% CO_2 in O_2). A second or third branch mesenteric artery was dissected free of adherent adipose tissue under a stereo microscope. The artery was pulled on to a small glass cannula (tip diameter approximately 200 μm, made using a custom-built microforge similar to the Stoelting 51526 R) and tied in place using a strand of thread teased from a piece of dental floss (Johnson & Johnson). The artery was flushed with just enough PSS to remove the red blood cells before the distal end of the artery was tied off. The tissue was then transferred to a standard glass photometry cuvette immersed in a heated glass-walled water-bath (37°C). The cannula was connected to elevated manometer filled with PSS to give a distending pressure of 55 mmHg. The cuvette was filled with PSS (2.5 ml) agitated with carbogen bubbles.

The arteries were observed using a horizontally mounted microscope (Olympus HSC) fitted with a video camera (JVC TK10). The arterial diameter (external) was constantly measured using an IBM compatible computer with a video capture card (FG 303, Dindima, Vermont, Victoria, Australia) and software written by Dr T.O. Neild (Neild, 1989) and recorded on a chart recorder.

Vas deferens preparation

Vasa deferentia were set up in 5 ml organ baths at 37°C with the prostatic end between platinum electrodes and the epididymal end attached to a Grass FT03 force transducer. The tissues were stimulated with single electrical field pulses (0.2 ms duration, 100 V) every 20 s and the twitches recorded on a chart recorder. These responses can be abolished by tetrodotoxin (0.1 μM) or guanethidine (10 μM) and so appear to be mediated by sympathetic nerves. $MgSO_4$ was not included in the physiological salt solution for the vas deferens experiments.

Experimental protocols

A single cumulative concentration-response curve for NPY or one of its analogues was constructed in each artery, as the vascular NPY receptors exhibit a marked tachyphylaxis. Rat arteries were precontracted by noradrenaline (concentration titrated to give a contracture of about 10%) before the concentration-response curves, because this was found to produce more robust and reliable reponses to NPY. The mouse arteries developed spontaneous contracture and so no additional precontraction was needed. For the determination of antagonist actions of 1229U91 the drug was left in contact with the tissues for 30 min before the start of the agonist concentration-response curve.

The responses of the vas deferens to NPY were easily reversed by washout, and were very reproducible upon reapplication of NPY, but a single concentration-response curve was conducted in most tissues. For the determination of antagonist actions of 1229U91 the drug was left in contact with the tissues for 30 min before the start of the agonist concentration-response curve. The inhibitory action of 1229U91 on the twitch of the vas deferens was observed with cumulative drug additions with 5 min between concentration increments.

Analysis

NPY concentration-response curves were fitted with a symmetrical logistic curve:

$$pEC_{50} = c - \log([B] + 10^{-pK_B})$$

where a is the resting level of response, b is the response range, c is the pEC_{50}, d is a slope and curvature parameter, and e is the base of the natural logarithm.

The effect of 1229U91 on the NPY concentration-response curves was analysed using the method described in Lew & Angus (1995). Briefly, the effect of 1229U91 on the slope and maximum of the NPY curve was assessed using ANOVA (no differences were found, so no *post-hoc* tests were performed). Next the pK_B of 1229U91 was estimated from the pEC_{50} with a non-linear regression of the pEC_{50} values against the antagonist concentration. This regression provides the pK_B estimate from the formula:

$$pEC_{50} = c - \log([B] + 10^{-pK_B})$$

where c is a constant and [B] is the antagonist concentration.

Results

Mesenteric arteries

NPY was a very weak vasoconstrictor in the rat mesenteric arteries in the absence of some precontraction by another vasoconstrictor. To overcome this the rat arteries were preactivated to about 10% with noradrenaline before concentration-response curves to NPY or the analogues were constructed. In contrast the mouse arteries consistently developed spontaneous contracture of about 20%, and so no

additional preactivation was needed for the expression of NPY vasoconstrictor activity. In both arteries NPY, peptide YY, $Leu^{31}Pro^{34}NPY$ and NPY(13–36) all had similar maximum responses ($P>0.05$, ANOVA) which were about half the maximum effect of noradrenaline plus 100 mM K^+. The order of potency of these agonists was similar in both tissues, and was consistent with the currently used definition of a Y_1 receptor (Table 1).

1229U91 did not elicit any vasoconstriction or vasodilatation in the rat or mouse mesenteric arteries. It caused a concentration-dependent rightward shift of NPY concentration-response curves in the mouse artery and of $Leu^{31}Pro^{34}NPY$ in the rat artery (Figure 1). There was no significant change in the maximum (Table 2) or slope of the agonist concentration-response curves ($P>0.05$, ANOVA), and the spacing of the agonist curves was consistent with a competitive interaction with a pK_B for 1229U91 of 7.69 ± 0.15 in the mouse arteries and 7.47 ± 0.13 in the rat arteries.

1229U91 did not change the concentration of noradrenaline needed for precontraction of the rat arteries (−logM 6.59 ± 0.19, control and 6.86 ± 0.36, 1229U91 1 μM) and so seems devoid of α_1-adrenoceptor antagonist activity. 1229U91 at a concentration of 100 μM also failed to antagonize responses to vasopressin, bradykinin, calcitonin gene-related peptide (CGRP) and acetylcholine in the rat artery (data not shown).

Rat vas deferens

In the rat vas deferens the order of potency for the agonists was consistent with the currently used definition of Y_2 receptors (Table 1). Both PYY and NPY were able to inhibit completely the responses to nerve stimulation but the inhibition was incomplete at the highest concentrations of NPY(13–36) and $Leu^{31}Pro^{34}NPY$ used.

1229U91 inhibited the twitch responses of the vas deferens (threshold 0.3 μM) by about 50% at the highest concentration applied (30 μM). Compared to NPY, the inhibition was more rapid in onset, with a peak inhibition after only about 1 min, and faded noticeably over 5–10 min (Figure 2). 1229U91 did not cause any inhibition of NPY responses at 2 and 30 μM (Figure 3).

Discussion

Arteries

1229U91 competitively inhibited the effects of NPY and $Leu^{31}Pro^{34}NPY$ in the mesenteric arteries of the mouse and rat respectively. It was reasonably potent with a pK_B of about 7.6, and did not exhibit any agonist activity. It did not inhibit α_1-adrenoceptors, or the receptors for vasopressin, bradykinin, CGRP and acetylcholine in the rat artery at a concentration sufficient for a reasonable degree of NPY Y_1 receptor antagonism. Thus 1229U91 can be classified as a selective antagonist of NPY receptors.

Vas deferens

In contrast to its vascular effects, 1229U91 had some apparent agonist activity but no detectable antagonist activity in the rat deferens assay. The concentration-inhibition curve of 1229U91 had a shallow slope compared to NPY, and while the maximum effect was not reached at the highest concentration applied, it seems likely that the maximum inhibition of the twitch by 1229U91 is less than 100%. This might be consistent with 1229U91 being a partial agonist at Y_2 receptors, but other aspects of the behaviour of 1229U91 differ from what might be expected from a partial agonist. First, 1229U91 caused no rightward shift of the NPY concentration-response curve in the vas deferens even at the highest concentration used, 30 μM, which is about 100 times the threshold concentration for the inhibitory effect of 1229U91. If the inhibitory action of 1299U91 was partial agonist activity at the NPY receptor, then this concentration should be enough to occupy most of the

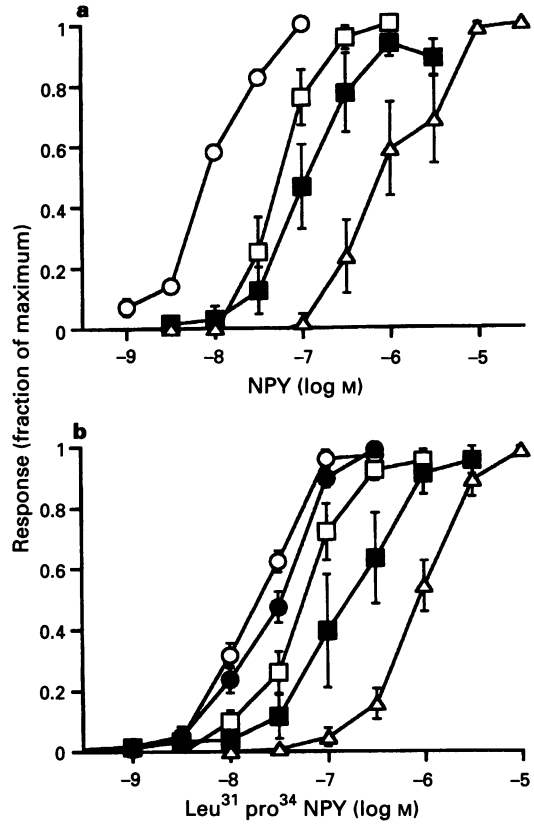

Figure 1 Effect of 1229U91 (0 μM, ○; 0.03 μM, ●; 0.1 μM, □; 0.3 μM, ■; 1 μM, △) on (a) neuropeptide Y (NPY) concentration-response curves in mouse mesenteric arteries ($n=5-10$) and (b) $Leu^{31}Pro^{34}NPY$ concentration-response curves in rat mesenteric arteries ($n=4-8$). Points show mean±s.e.mean.

Table 1 Agonist concentration-response curve locations and maxima in mouse and rat small mesenteric arteries, and the rat vas deferens

	Mouse arteries		*Rat arteries*		*Rat vas deferens*	
Agonist	*pEC_{50}*	*Max (%)†*	*pEC_{50}*	*Max (%)†*	*pEC_{50}*	*Max (%)††*
NPY	8.02±0.02	42±4	7.67±0.07	52±2	6.82±0.05	100
$Leu^{31}Pro^{34}NPY$	8.13±0.15	45±3	7.68±0.05	54±4	<5	§
NPY (13–36)	6.67±0.19	35±3	5.75±0.11	34±4*	6.12±0.09	§
PYY	8.12±0.09	41±2	7.99±0.19	42±2	7.35±0.06	100

Data shown are means±s.e.mean (n = 3–10, mouse arteries; n = 6–8, rat arteries; n = 4–5, rat vas deferens).
*Maximum significantly different from NPY. §Maximum effect not achieved at highest concentration applied. †Responses are expressed as a percentage of the maximal tissue response to noradrenaline plus 100 mM KCl. ††Responses expressed as % inhibition of the twitches.

Table 2 Maximal responses to $Leu^{31}Pro^{34}NPY$ (rat arteries) and NPY (mouse arteries) in the presence of 1229U91 at concentration indicated

		Leu³¹Pro³⁴NPY or NPY			
	Control	*0.03 μM*	*0.1 μM*	*0.3 μM*	*1 μM*
Rat	44.8±3.7	38.4±3.9	40.3±2.3	43.4±3.4	42.7±2.2
Mouse	37.5±3.4	–	36.6±6.7	28.1±3.1	32.8±4.8

Responses are expressed as a percentage of the maximal tissue response to noradrenaline plus 100 mM KCl and are means±s.e.mean.

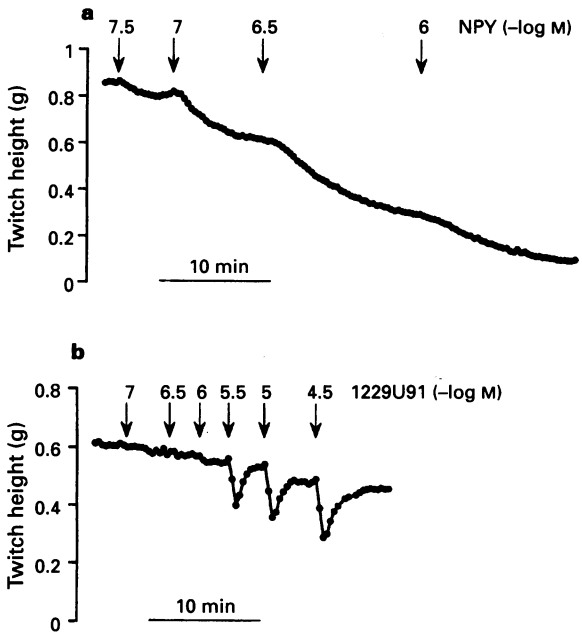

Figure 2 Time-courses of the effects of neuropeptide Y (NPY) (a) and 1229U91 (b) on the rat vas deferens twitches.

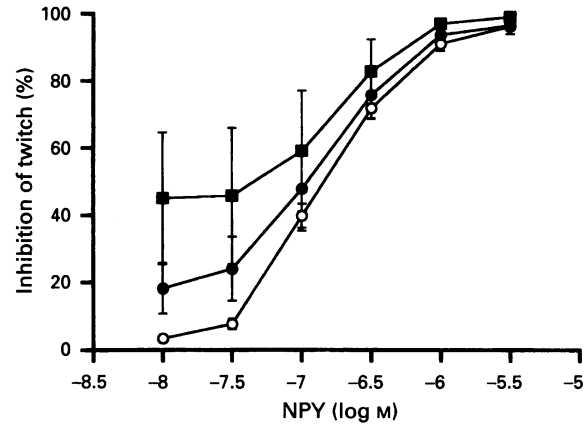

Figure 3 1229U91 failed to inhibit the effect of neuropeptide Y (NPY) on the rat vas deferens at 2 (●) and 30 (■) μM. (○) Control responses. Points show mean±s.e.mean ($n=4-7$).

receptors, and thus shift the NPY concentration-response curve significantly. The second observation that may be inconsistent with partial agonist activity of 1229U91 is the different time-course of the inhibitory activity of 1299U91 and NPY in the vas deferens. The effect of 1229U91 is relatively rapid in onset (about 1 min to peak) and wanes slightly over 5 min. The effect of NPY has a slower onset (at least 5 min) and does not exhibit any significant fade. This difference in time-course might be taken as evidence for different sites of action (e.g. different prejunctional receptors). On the basis of these observations, we propose different sites of action for NPY and 1229U91 in the vas deferens.

Daniels *et al.* found that 1229U91 displaced [^{125}I]-NPY binding to rat brain membranes (nominally Y_2 receptors) with an IC_{50} of only 0.02 nM compared to its IC_{50} on human erythroleukemia cells (nominally Y_1 receptors) of 0.2 nM (Daniels *et al.*, 1995). This does not correlate well with our data, where 1229U91 did not seem to have significant affinity for the Y_2 receptors in the rat vas deferens. Even if the inhibition of vas deferens twitches by 1229U91 was mediated by Y_2 receptors, the least difference in potency for 1229U91 between the nominally Y_2 assay is 5 orders of magnitude. While the different conditions of binding and functional experiments may contribute to this discrepancy, it is suggestive of differences in receptor types between the nominally Y_2 assays of rat brain membrane binding and the rat vas deferens. If the brain and vas deferens Y_2 receptors are indeed different, then 1229U91 would appear to be a very selective agent for the brain type Y_2 receptors.

Conclusions

1229U91 is a competitive antagonist of the smooth muscle NPY Y_1 receptors in the rat and mouse mesenteric arteries. It has some inhibitory activity in the electrically-stimulated vas deferens of the rat, but this is probably not the result of agonist activity at presynaptic NPY Y_2 receptors. The difference between the potency of 1229U91 in our rat deferens assay and its reported potency in a brain Y_2 binding assay suggests that the nominally Y_2 receptors may be an heterogeneous class. This compound, like the recently described non-peptide antagonist of Rudolf *et al.* (1994) should be useful in the classification of NPY receptor subtypes and in the elucidation of the physiological and pathophysiological roles of NPY.

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