Discrimination by benextramine between the NPY-Y₁ receptor subtypes present in rabbit isolated vas deferens and saphenous vein

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1 In order to characterize the neuropeptide Y (NPY) Y₁ receptors known to be present in rabbit isolated vas deferens and saphenous vein, the pharmacological activity of the selective NPY Y₁ receptor agonists, [Leu³¹,Pro³⁴] NPY and various other peptide agonists, together with the putative NPY antagonist, benextramine, were compared in the two tissues.

2 In rabbit isolated saphenous vein, cumulative dose-response curves to various NPY agonists were obtained. All the peptides tested caused contractions which developed quite slowly. The rank order of potency obtained was: PYY > NPY > [Leu³¹,Pro³⁴] NPY = NPY₂₋₃₆ > hPP >> NPY₁₃₋₃₆ = NPY₁₈₋₃₆. Incubation with benextramine (BXT) at 100 µM for 30 min irreversibly abolished the contractile response to [Leu³¹,Pro³⁴] NPY but was ineffective against NPY₁₈₋₃₆-induced contractions.

3 Cumulative dose-response curves to [Leu³¹,Pro³⁴] NPY were performed in the same preparation before and after incubation with 100 µM BXT for 20 min in order to inactivate NPY Y₁ receptors. The pK_A (–logK_A) estimation for [Leu³¹,Pro³⁴] NPY was 7.60 ± 0.30 using the operational model and 7.20 ± 0.33 using the null method; the difference between the two methods was not statistically significant (*P* = 0.36).

4 Prostatic segments of rabbit vas deferens were electrically stimulated with single pulses. Immediately after stabilization of the contractile response, a cumulative dose-response curve to various NPY agonists was obtained in each tissue. The rank order of potency for twitch inhibition was: PYY > [Leu³¹,Pro³⁴] NPY ≥ NPY₂₋₃₆ > hPP > NPY₁₃₋₃₆ >> NPY₁₈₋₃₆ which indicates the presence of a prejunctional NPY Y₁ receptor. BXT at 100 µM incubated for 10 or 60 min did not antagonize the response to [Leu³¹,Pro³⁴] NPY.

5 We conclude that rabbit isolated saphenous vein contains a population of post-junctional NPY Y₁ receptors irreversibly blocked by BXT, as well as a population of post-junctional NPY Y₂ receptors, which are insensitive to BXT. In contrast, the rabbit isolated vas deferens express a pre-junctional NPY Y₁ receptor subtype which is not blocked by BXT. Tetramine disulphides such as BXT could be useful tools in classifying NPY receptors.

Keywords: Rabbit isolated vas deferens and saphenous vein; [Leu³¹,Pro³⁴] NPY; NPY₁₈₋₃₆; benextramine; receptor inactivation methods; K_A

Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide belonging to the pancreatic polypeptide family, which also includes peptide YY (PYY) and the pancreatic polypeptide (PP). NPY is co-stored with noradrenaline (NA) and adenosine triphosphate (ATP) in noradrenergic sympathetic terminals of the mouse vas deferens (Stjärne *et al.*, 1986). In sympathetic nerves innervating most blood vessels NPY is co-released with NA and participates in the maintenance of smooth muscle tone (Ekblad *et al.*, 1984). It is generally accepted that NPY exerts its pharmacological effects via at least two distinct receptor subtypes, namely Y₁ and Y₂. Originally, it was proposed that Y₁ receptors are located exclusively post-junctionally and mediate vasoconstriction and potentiation of the NA response in many blood vessels, whereas Y₂ receptors are located exclusively pre-junctionally and are involved in the suppression of the electrically stimulated twitch contractile response of the isolated vas deferens (Wahlestedt *et al.*, 1986). However, this view has not been supported by some recent findings. For example, in the rat mesenteric arterial bed (McAuley & Westfall, 1992) and rat isolated caval vein (Grundemar *et al.*, 1992) the presence of post-junctional Y₂ and pre-junctional Y₁ receptors was suggested. Furthermore, from the rank order of potency

exhibited by NPY and some truncated analogues, the presence of pre-junctional Y₁ receptors in rabbit isolated vas deferens was proposed (Doods & Krause, 1991).

Recently, the tetramine disulphide, benextramine, originally developed as an irreversible α -adrenoceptor blocking agent (Melchiorre, 1981; Plotek & Atlas, 1983), was claimed to be a selective Y₁ ligand in a rat brain binding assay (Doughty *et al.*, 1992), to bind to a Y₂ receptor in bovine hippocampus (Li *et al.*, 1991) and to be a non-competitive antagonist of post-junctional Y₁ and Y₂ receptors in the rat isolated femoral artery (Tessel *et al.*, 1993). The aim of this study was, therefore, to characterize the putative Y₁ receptor present in the rabbit isolated vas deferens and to compare it with the post-junctional Y₁ receptor present in rabbit isolated saphenous vein, a tissue recently found to express mainly or exclusively this receptor subtype (Cadieux *et al.*, 1993). The experiments were performed by using NPY, peptide YY (PYY), human pancreatic polypeptide (hPP) and some truncated analogues of NPY together with the selective Y₁ agonist, [Leu³¹,Pro³⁴]NPY (Fuhlendorf *et al.*, 1990). Furthermore, the antagonist activity of benextramine and its potential irreversible blocking properties on NPY receptors expressed in these tissues have been evaluated. A preliminary account of some of these results was presented to the British Pharmacological Society Meeting, Victoria University of Manchester, 13th–15th April, 1994 (Palea *et al.*, 1994).

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Methods

Rabbit isolated vas deferens

Male New Zealand white rabbits weighing 2.5–2.8 kg were anaesthetized by intravenous administration of sodium pentobarbitone (60 mg kg⁻¹) and killed by exsanguination. Bilateral prostatic segments of vasa deferentia (cut as near as possible to the prostate) were removed and placed in a Petri dish containing cold, oxygenated Krebs solution. After dissection of connective tissue and blood vessels, the entire prostatic segment (2 cm long) was mounted vertically between two platinum electrodes (separated by 3 cm) and placed in a 2 ml syralized organ bath filled with modified oxygenated (95% O₂ and 5% CO₂) Krebs solution of the following composition (mM): NaCl 133, NaHCO₃ 16.3, KCl 4.7, MgCl₂ 1.0, KH₂PO₄ 1.4, CaCl₂ 2.0 and glucose 7.8. Experiments were performed at 33°C. Only two preparations were obtained from each animal and only one peptide was tested in each preparation.

The mechanical activity was recorded isometrically with a Grass FT 03 force displacement transducer connected to a Linseis model 7025 polygraph. Isolated vasa deferentia were allowed to equilibrate for 90 min with a tension of 1 g applied four times during the equilibration period and washed with fresh Krebs solution every 15 min. Electrical field stimulation (EFS) with single pulses (25 V, 1 ms, 0.15 Hz) was applied through a pair of platinum electrodes connected to a Grass S88 stimulator. As soon as a series of pulses gave identical responses (basal contractile response) a single cumulative concentration-response curve (CRC) to each peptide was obtained for each tissue. The resulting inhibition of the twitch response was expressed as the percentage of the basal contractile response.

Evaluation of benextramine antagonism

Benextramine tetrahydrochloride (BXT) was incubated at 100 µM for 10 or 60 min after the contractile response to EFS was stabilized. After 10 min EFS was stopped, tissues were washed out and allowed to recover for 15 min, then EFS was reapplied and tissues were challenged, as soon as the contractile response was stabilized, with [Leu³¹,Pro³⁴]NPY.

Rabbit isolated saphenous vein

Male New Zealand white rabbits weighing 2.0–2.4 kg were anaesthetized by intravenous administration of sodium pentobarbitone (60 mg kg⁻¹) and killed by exsanguination. A segment of 2 cm of the vena saphena lateralis was immediately dissected and placed in cold, oxygenated (95% O₂ and 5% CO₂) Krebs solution maintained at 37°C. The vein was cleaned of fat and connective tissue, cut into ring segments (3 mm of length) and mounted in 2 ml syralized organ baths containing a modified Krebs solution of the following composition (mM): NaCl 118, NaHCO₃ 25.0, KCl 4.6, MgCl₂ 1.2, NaH₂PO₄ 1.2, CaCl₂ 2.5 and glucose 11.1. The mechanical activity was recorded isometrically by a Grass FT 03 force displacement transducer connected to a Linseis model 7025 polygraph. Ring segments were allowed to equilibrate for 60 min with a tension of 1 g applied four times during the first 20 min of the equilibration period and washed with fresh Krebs solution every 10 min. Then, vein rings were challenged with KCl 80 mM twice every 20 min in order to establish tissue viability. The contractile response of NPY agonists was then referred to as % of the response to the second application of KCl. Thirty min after the second dose of KCl 80 mM, a single cumulative CRC to one agonist was performed on each tissue. For the experiments evaluating the antagonistic properties of BXT, two cumulative CRC to [Leu³¹,Pro³⁴]NPY or NPY₁₈₋₃₆ were performed on each tissue with an interval of 60 min between them, during which tissues were washed-out with fresh Krebs solution.

Benextramine (100 µM) was added before the second CRC when the tissues had reached the basal tone and was left in contact for 15, 20 or 30 min. Then, tissues were washed out three times every 10 min and the CRC to [Leu³¹,Pro³⁴]NPY was repeated. In some experiments benextramine was washed out for 2 h (washouts every 10 min) to ascertain the irreversibility of the antagonism.

Data analysis

Agonist potency was estimated by fitting the CRC data by the logistic equation:

$$Effect = \frac{E_{max} \times [A]^n}{[A]^n + [A_{50}]^n}$$

where, E_{max} is the maximal effect, $[A]$ is the agonist concentration, and n is the slope parameter. For the experiments with the rabbit vas deferens A_{50} represents the concentration of the agonist that reduced by 50% the basal contractile response induced by electrical stimulation (IC_{50}), whereas in the rabbit saphenous vein A_{50} is the concentration of the agonist that induces 50% of the maximal effect (EC_{50}). Data are expressed as mean ± s.e.mean.

K_A estimation for [Leu³¹,Pro³⁴]NPY was performed as described in the operational method by Black *et al.* (1985) and in the null method by Furchgott (1966) and McKay (1966).

Operational method

Experimental data from control and BXT-treated CRC were simultaneously fitted to the equation:

$$Effect = \frac{Em \times \tau^n \times [A]^n}{(K_A + [A])^n + \tau^n \times [A]^n}$$

providing a common estimation of E_m (maximum possible effect), n (steepness of the occupancy-effect relation), and K_A (agonist dissociation constant) and a value of τ (efficacy of the agonist) for each curve in the pair. Mean values of the parameters estimated are quoted with s.e.mean.

Figure 1 Effect of various agonists for neuropeptide Y (NPY) receptors on the twitch response of the rabbit isolated vas deferens. Data are expressed as % of the stabilized contractile response just before addition of agonists to the organ bath. Each curve is the mean of 6 experiments carried out in different tissues. The lines drawn through the data are derived from the fitting procedure using the logistic equation (see Methods). PYY (◆), [Leu³¹-Pro³⁴]NPY (●), NPY (■), hPP (▽), NPY₂₋₃₆ (▲), NPY₁₃₋₃₆ (▼) and NPY₁₈₋₃₆ (×).

Null methods (or double reciprocal method)

The agonist K_A value was calculated from the equation:

$$\frac{1}{[A]} = \frac{1}{[A']} \times \frac{1}{q} + \frac{1}{K_A} \times \frac{(1-q)}{q}$$

where $[A]$ and $[A']$ are the equiactive concentrations of the agonist before and after treatment with BXT respectively, q is the fraction of functional receptor and K_A is the agonist dissociation constant. A straight line of slope $1/q$ and intercept $(1-q)/(q K_A)$, should be obtained on a plot $1/[A]$ against $1/[A']$. The value of q can be calculated from $1/\text{slope}$ and K_A from $(\text{slope}-1)/\text{intercept}$.

Drugs

All peptides were obtained from Peninsula Laboratories Europe Ltd (St. Helens, Merseyside, U.K.). Benextramine tetrahydrochloride (approximately 90% pure) and tetradotoxin were from Sigma. All peptides were dissolved, at a concentration of 100 μM , in twice distilled water containing bovine serum albumine (BSA) 0.5 mg ml^{-1} and stored in aliquotes frozen at -20°C . On the day of the experiment each peptide was diluted in a Krebs solution containing BSA 0.5 mg ml^{-1} .

Figure 2 Experimental recording of a dose-response curve to $[\text{Leu}^{31},\text{Pro}^{34}]\text{NPY}$ in the rabbit isolated vas deferens electrically stimulated with single pulses (25 V, 1 ms, 0.15 Hz).

Figure 3 Lack of effect of benextramine incubation (100 μM for 30 min) on the dose-response curve to $[\text{Leu}^{31},\text{Pro}^{34}]\text{NPY}$ in the rabbit isolated vas deferens electrically stimulated with single pulses (25 V, 1 ms, 0.15 Hz). Control curve (●), benextramine-treated curve (■).

Results

Agonist activity in rabbit vas deferens

Basal contractile responses to EFS were 1.22 ± 0.06 g ($n = 45$). These responses were completely abolished by 1 μM TTX, confirming their neurogenic origin (data not shown). The rank order of potency for twitch inhibition in rabbit vas deferens was: $\text{PYY} > [\text{Leu}^{31},\text{Pro}^{34}]\text{NPY} \geq \text{NPY} \geq \text{hPP} > \text{NPY}_{2-36} \gg \text{NPY}_{13-36} \gg \text{NPY}_{18-36}$ (Figure 1).

PYY, the most potent agonist tested, was about 3 times more potent than NPY and $[\text{Leu}^{31},\text{Pro}^{34}]\text{NPY}$. hPP was equipotent to NPY and NPY_{2-36} was only 13.6 times less potent than PYY. All these peptides had a rather slow time course of inhibition of the twitches, with no sign of spontaneous recovery during prolonged contact of the agonists with the tissue (Figure 2). In Table 1, IC_{50} values ($\pm$ s.e.mean) and equipotent molar ratios are given for all the peptides tested except NPY_{18-36} , for which the response obtained at 10 μM (the highest concentration that could be tested) was only 45% of the basal response.

Effect of benextramine in rabbit vas deferens

Preincubation with BXT at 100 μM for 10 min had a consistent inhibitory effect which partially recovered after 15 min of resting, during which tissues were washed twice. The basal contractile response after BXT treatment was reduced by $25.2 \pm 1.3\%$ compared with the response before BXT treatment ($n = 3$).

BXT treatment did not antagonize the $[\text{Leu}^{31},\text{Pro}^{34}]\text{NPY}$ effect (Figure 3) but on the contrary, lowered the IC_{50} value (2.15 ± 0.07 nM versus 5.04 ± 0.63 nM for controls; $P = 0.008$ by the F -test). Increasing the concentration of BXT to 300 μM and the incubation time to 120 min resulted in an IC_{50} value for $[\text{Leu}^{31},\text{Pro}^{34}]\text{NPY}$ (3.72 ± 0.52 nM) that was not significantly different from control value ($P = 0.086$ by the F -test).

BXT at 100 μM for 10 min was ineffective versus hPP ($n = 5$; data not shown).

Rabbit isolated saphenous vein

Mean magnitude of the KCl 80 mM-induced contraction for the rabbit isolated saphenous vein was 2.04 ± 0.08 g ($n = 57$).

$[\text{Leu}^{31},\text{Pro}^{34}]\text{NPY}$ and all the NPY analogues tested induced strong contractions (responses at the highest concentration tested were normally greater than the KCl-induced contraction) which developed much more slowly at the lowest concentrations (Figure 4). TTX at 1 μM was without effect on the contractile effect of $[\text{Leu}^{31},\text{Pro}^{34}]\text{NPY}$, indicating that contraction was due to an interaction with an NPY receptor located on smooth muscle cells (data not shown).

The rank order of potency for the agonists tested was the

Table 1 IC_{50} and EC_{50} (nM, $n = 6$) and equipotent molar ratio (e.p.m.r.) (relative to peptide YY, PYY) exhibited by neuropeptide Y (NPY) and some analogues in rabbit isolated vas deferens and saphenous vein

	Vas deferens		Saphenous vein	
	IC_{50}	e.p.m.r.	EC_{50}	e.p.m.r.
PYY	1.76 ± 0.11	1	1.88 ± 0.05	1
$[\text{Leu}^{31},\text{Pro}^{34}]\text{NPY}$	5.04 ± 0.63	2.9	9.04 ± 0.27	4.8
NPY	5.72 ± 0.21	3.2	3.77 ± 0.30	2.0
hPP	6.41 ± 1.05	3.6	26.3 ± 3.8	14.0
NPY_{2-36}	24.0 ± 2.1	13.6	8.70 ± 0.75	4.6
NPY_{13-36}	1368 ± 20.4	777	188 ± 4.2	100
NPY_{18-36}	$>10 \mu\text{M}$	—	204 ± 13.1	108

Values are mean $\pm$ s.e.mean for IC_{50} and EC_{50} .

following: PYY > NPY > [Leu³¹,Pro³⁴]NPY = NPY₂₋₃₆ > hPP >> NPY₁₃₋₃₆ = NPY₁₈₋₃₆ (Figure 5). EC₅₀ values and equipotent molar ratios (e.p.m.r.) are presented in Table 1. Two consecutive CRC to [Leu³¹,Pro³⁴]NPY on the same tissue were reproducible after 60 min of wash-out (Figure 6a). The mean logistic parameters ($\pm$ s.e.; $n = 6$) of these curves were as follows: first curve: $E_{max} = 116.9 \pm 6.3$; $pA_{50} = 7.88 \pm 0.14$; $n = 2.88 \pm 0.63$. Second curve: $E_{max} = 117.7 \pm 3.3$; $pA_{50} = 7.69 \pm 0.11$; $n = 2.91 \pm 0.62$.

BXT (100 μ M) incubated either at 10, 20 or 30 min, depressed the maximal response to the selective NPY-Y₁ receptor agonist, [Leu³¹,Pro³⁴]NPY, in a time-dependent manner, 30 min of contact being sufficient to abolish completely the agonist's response (Figure 6b-c-d). Washout for 2 h did not modify the magnitude of BXT inhibition of the Y₁ receptor (Figure 6e).

BXT at 100 μ M and 20 min of contact did not modify significantly the response to NPY₁₈₋₃₆ ($n = 3$; Figure 7a-b).

Figure 4 Experimental recording of a dose-response curve to [Leu³¹,Pro³⁴]NPY (1–100nM) in a ring segment of rabbit isolated saphenous vein.

Figure 5 Effect of various neuropeptide Y (NPY) receptor agonists on ring segments of rabbit isolated saphenous vein. Data are expressed as % of the response induced by KCl 80 mM. Each curve is the mean of 6 experiments carried out in different tissues. The lines drawn through the data are derived from the fitting procedure using the logistic equation (see Methods). PYY (▲), NPY (▼), [Leu³¹,Pro³⁴]NPY (●), NPY₂₋₃₆ (■), hPP (◆), NPY₁₃₋₃₆ (○), NPY₁₈₋₃₆ (X).

K_A estimation for [Leu³¹,Pro³⁴]NPY in rabbit saphenous vein

Assuming BXT to be an irreversible antagonist of the post-junctional Y₁ receptor, the K_A for [Leu³¹,Pro³⁴]NPY using both the operational method and the null method has been estimated (see Methods). Only pairs of CRC carried out in the same tissue in the absence and presence of BXT 100 μ M (incubated for 20 min) were used in this analysis. BXT depressed the maximal response to the agonist to $20 \pm 5.6\%$ of the KCl 80 mM-induced contraction ($n = 8$). The estimated parameters by using the two methods are shown in Table 2. The estimated pK_A ($-\log K_A$) for the selective Y₁ agonist was 7.60 ± 0.30 using the operational method and 7.20 ± 0.33 for the null method, the difference not being statistically significant with the Student's t test ($P = 0.36$).

Discussion

Rabbit vas deferens

The current classification of NPY receptors is based upon the rank order of potency of the agonists, because no selective antagonists are available. A number of studies support the proposal that there are at least three receptor subtypes recognized by NPY (Y₁, Y₂ and Y₃) and a different receptor which binds selectively the pancreatic polypeptide (PP) (see Wahlestedt & Reis, 1993, for a review). In rabbit vas deferens several lines of evidence suggest the presence of a pre-junctional Y₁ receptor which seems to be somewhat different from the Y₁ post-junctional receptor characterized in vascular preparations. The selective Y₁ receptor agonist, [Leu³¹,Pro³⁴]NPY, was found to be equipotent to NPY and to abolish completely the twitch response at a concentration of 30 nM. In contrast, in the rat isolated vas deferens, a tissue known to functionally express the Y₂ receptor (Wahlestedt *et al.*, 1986) [Leu³¹,Pro³⁴]NPY at this concentration was completely ineffective (unpublished results).

The Y₃ receptor subtype has been recently postulated to be present both in the central nervous system (Grundemar *et al.*, 1991a,b; Foucart *et al.*, 1993) and in the periphery (Balasubramaniam *et al.*, 1990; Wahlestedt *et al.*, 1992). The rank order of agonist potency for this receptor in rat superior cervical ganglia neurones is rPP = NPY₁₃₋₃₆ > NPY = [Leu³¹,Pro³⁴]NPY whereas PYY is almost inactive, confirming previous reports that this peptide is ineffective on the Y₃ receptor type (Grundemar *et al.*, 1991a,b). So, our finding that PYY was the most potent agonist is clearly inconsistent with the involvement of a Y₃ receptor in the inhibition of electrically-induced contraction of the rabbit vas deferens.

The presence of the postulated PP receptor in rabbit isolated vas deferens is a matter of further discussion. In our paradigm we found an unusually high agonist activity of hPP, which was nearly equipotent to NPY, whereas in binding studies rPP was reported to be a very poor ligand of the Y₁ receptor expressed in PC12 cells (Schwartz *et al.*, 1987; 1990). To explain our results we advance the hypothesis that the contractile response to single pulse stimulation in rabbit vas deferens is controlled not only by the Y₁ receptor but also by a PP receptor, which is probably present also in the rat isolated vas deferens (Jorgensen *et al.*, 1990).

It has been reported that the NPY Y₁ receptor found in vascular tissues is stringent in its requirement for the N-terminal end of NPY since deletion of Tyr¹ results in a marked loss of potency (Wahlestedt & Reis, 1993). For example, it was shown that NPY₂₋₃₆ was about 30 times less potent than NPY in increasing arterial blood pressure in anaesthetized rats (Grundemar & Hakanson, 1993) and was about 16 times less potent than NPY in eliciting increases in myocardial perfusion pressure in isolated heart of the guinea-pig (Rioux *et al.*, 1986). In rabbit vas deferens we found that NPY₂₋₃₆ was only about 4 times less potent than NPY, itself

suggesting the possibility of a heterogeneity between Y_1 receptors expressed in vascular tissues (located postjunctionally) and Y_1 receptors found in our preparation, which are probably expressed at a pre-junctional level. To verify

this hypothesis we decided to test the antagonistic activity of BXT, which was reported to block non-competitively the post-junctional Y_1 receptor mediating contraction of the rat isolated femoral artery (Tessel *et al.*, 1993). The tetramine

Figure 6 (a) Cumulative dose-response curves to $[Leu^{31},Pro^{34}]$ NPY generated in the same tissue with an interval of 60 min. First curve (●) and second curve (▲). The response to the agonist appears to be quite reproducible. (b) Effect of incubation with a single dose of benextramine (100 μM) for 15 min; (c) for 20 min; (d) for 30 min; (e) for 20 min followed by 2 h of washout. Control curve (●), benextramine-treated curve (■). The lines through the data are the result of the operational model fitting using the receptor inactivation method.

Figure 7 (a) Cumulative dose-response curves to NPY₁₈₋₃₆ generated in the same tissue with an interval of 60 min. First curve (●) and second curve (■). (b) Effect of incubation with a single dose of benextramine (100 μM) for 20 min. Control curve (●), benextramine-treated curve (■).

disulphide BXT was originally described as an irreversible α -adrenoceptor antagonist. We found that an incubation time of 10 min at 100 μM partially inhibited the twitch response to electrical stimulation of the isolated vas deferens. However, after two washouts, to remove the excess of this compound from the organ bath, we found a partial recovery of the contractile response. So, the single pulse stimulation we used probably elicited the release of a purinergic neurotransmitter instead of an adrenergic one. In fact, we found that the P₂ antagonist, suramin, at 300 μM, completely abolished the contractile response to electrical stimulation (unpublished results). This seems to indicate that the inhibitory effect of BXT is not due to its α -adrenergic blocking properties but to other properties of this drug e.g. blockade of K⁺ activated Ca²⁺ channels (Plotek & Atlas, 1983) which could be related to purinergic neurotransmission (Illes & Norenberg, 1993).

BXT (up to 300 μM) was completely ineffective in antagonizing [Leu³¹,Pro³⁴]NPY-induced suppression of the twitch response. This result is in contrast with both functional studies in rat isolated femoral artery (Tessel *et al.*, 1993) and ligand binding studies with rat brain membranes (Doughty *et al.*, 1992), both of which demonstrated an antagonism of BXT at 10 μM versus the putative NPY Y₁ receptor. The most plausible explanation is that rabbit vas deferens expresses a NPY Y₁ receptor subtype different from that mediating contraction of the rat femoral artery. Furthermore, it could be speculated that mammalian tissues can express two NPY Y₁ receptor subtypes, one pre-junctional and insensitive to BXT, the other post-junctional and sensitive to BXT. To confirm this hypothesis we decided to test

Table 2 pK_A estimation and related parameters obtained for [Leu³¹,Pro³⁴]NPY in the presence of benextramine 100 μM incubated for 20 min using both the operational method and the null method

	Operational method	Null method
pK _A	7.60 ± 0.30	7.20 ± 0.33
τ ₁	7.4 ± 4.2	—
τ ₂	0.81 ± 0.19	—
E _{max}	114 ± 13.9	—
n	4.78 ± 2.24	—
q	—	0.14 ± 0.04

Values are reported as mean ± s.e.mean (n = 5).

BXT antagonism in another rabbit tissue, the isolated saphenous vein, where NPY was reported to exert a direct post-junctional effect (Cadieux *et al.*, 1993).

Rabbit isolated saphenous vein

Isolated rings of the vena saphena lateralis were potently constricted by all the agonists tested. The agonist rank order of potency and the high efficacy exhibited by [Leu³¹,Pro³⁴]NPY clearly indicates the presence of the NPY Y₁ receptor. However, comparing EC₅₀ values with those obtained in the vas deferens, some differences can be noted. In the saphenous vein, NPY₁₃₋₃₆ and NPY₁₈₋₃₆ were nearly equipotent and only 100 times less potent than PYY itself, in contrast to the situation found in vas deferens where NPY₁₃₋₃₆ was over 700 times less potent than PYY. These data seem to indicate the presence, in the saphenous vein, of a heterogeneous population of receptors, probably Y₁ and Y₂, both mediating direct smooth muscle contraction.

BXT depressed the maximal response to [Leu³¹,Pro³⁴]NPY in a time- and concentration-dependent manner, so we hypothesize that this compound irreversibly binds to the post-junctional, Y₁ receptor subtype. Nevertheless, BXT was without effect on the dose-response curve to NPY₁₈₋₃₆ suggesting that this tissue expresses not only the 'classical' Y₁ receptor as described previously (Cadieux *et al.*, 1993) but also functional Y₂ receptors which are insensitive to BXT blockade.

The slow decline of the effect of BXT in this preparation allowed us to consider this antagonist irreversible and permitted a determination of the dissociation constant for [Leu³¹,Pro³⁴]NPY. The pK_A value is considered to be a drug-receptor-dependent parameter, therefore it may be used to classify receptors. Two different methods have been applied: the operational (Black & Leff, 1983) and the null method (Furchgott, 1966; MacKay, 1966), the difference occurring in the efficacy term that is introduced only in the former. The results obtained were comparable and not statistically different in terms of estimated pK_A (−log K_A) value. However, the derived dissociation constants for [Leu³¹,Pro³⁴]NPY (7.20–7.60) were at least 30 fold lower than that obtained by binding experiments in a human cell line (SK-N-MC) thought to express naturally the NPY Y₁ receptor (Michel *et al.*, 1990). A similar result was obtained in our laboratories where a pK_i of 8.52 for [Leu³¹,Pro³⁴]NPY was found in displacement experiments using [³H]-propionyl-NPY. Furthermore, BXT at 200 μM extensively decreased the binding of [³H]-propionyl-NPY in these cells, thus confirming the presence of the NPY Y₁ receptor (unpublished results). The differences in [Leu³¹,Pro³⁴]NPY affinity for the NPY Y₁ receptor found between rabbit saphenous vein and SK-N-MC cells could be due to a real heterogeneity of receptors or to problems arising from the level of expression of the receptor in this cell line. In fact, it is known that the level of receptors expressed at different cell passages may vary considerably (McKinney *et al.*, 1984) generating different receptor/G-protein ratios that, in turn, can influence the measurements of agonist affinity (Kenakin, 1993).

Moreover, the utilization of the operational method allowed us to show that [Leu³¹,Pro³⁴]NPY behaves as a full agonist. In fact the efficacy term (τ_1) was 7.4, quite a large value. However, this result was expected from the fact that the pK_A estimated was 3–7 times lower than the potency for the same agonist, therefore representing the classical behaviour of a full agonist.

In conclusion, this work seems to support the hypothesis that pre-junctional (rabbit vas deferens) and post-junctional (rabbit saphenous vein) NPY Y₁ receptors are different and that BXT may be considered a tool to discriminate between

them. Moreover, the rabbit saphenous vein probably also expresses a post-junctional NPY Y₂ receptor evoking smooth muscle contraction, which is insensitive to BXT. This fact and our observation that pre-junctional NPY Y₂ receptors located on rat vas deferens are also insensitive to BXT (Palea et al., 1994) seem to indicate that this compound is a useful tool to discriminate also between NPY Y₁ and Y₂ receptors.

We would like to thank Dr Francesca Graziani for providing the binding data for NPY receptor in SK-N-MC cells.

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(Received September 14, 1994

Revised December 20, 1994

Accepted December 22, 1994)