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NPY receptor subtype in the rabbit isolated ileum

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1 The purpose of this work was to verify the hypothesis that the rabbit ileum is a selective preparation for the NPY Y5 receptor by using new selective antagonists recently synthesized. Spontaneous contractions of the rabbit isolated ileum were recorded and binding experiments were performed in cells expressing the human NPY Y1, Y2, Y4 or Y5 receptor subtype.

2 NPY analogues produced a concentration-dependent transient inhibition of the spontaneous contractions of the rabbit ileum with the following order of potency hPP > rPP > PYY ⩾ [Leu^{31},-Pro^{34}]-NPY > NPY > > NPY_{13-36}. Pre-exposure to rPP, PYY, [Leu^{31},Pro^{34}]-NPY or NPY (but not NPY_{13-36}) inhibited the effect of subsequent administration of hPP suggesting cross-desensitization of the preparation. The apparent affinity of the various agonists studied was correlated to the affinity reported for the human Y4 receptor subtype (and to a lesser extent for the rat Y4 subtype) but not to the affinity for the Y5 receptor subtype.

3 BIBO 3304, a selective NPY Y1 receptor antagonist, and CGP 71683A, a selective NPY Y5 receptor antagonist, did not affect the response to hPP. JCF 109, another NPY Y5 receptor antagonist, produced an inhibition of the response to hPP but only at the highest dose tested (10 μM) which also, by itself, produced intrinsic inhibitory effects.

4 1229U91, a non-selective ligand for Y1, Y2, Y4 and Y5 receptors with high affinity toward the Y1 and Y4 receptor subtypes, produced a concentration-dependent transient inhibition of the spontaneous contractions of the rabbit ileum and a dose-dependent inhibition of the response to hPP (apparent pK_B: 7.2).

5 These results suggest that in the rabbit ileum, the NPY receptor involved in the inhibition of the spontaneous contractile activity is a NPY Y4 receptor subtype.

Keywords: Neuropeptide Y; binding; isolated organ; CGP 71683A; BIBO 3304

Abbreviations: BIBO 3304, R-N-[4-(Aminocarbonylaminomethyl)phenylmethyl-N^2-(diphenylacetyl)arginamide; CGP 71683A, N1-[(4-{[(4-amino-2-quinazolinyl)amino]methyl}cyclohexyl)methyl]-1-naphtalenesulphonamide; hPP, human pancreatic peptide; JCF 109, N-(4-trans-{[(Naphtalen-2-ylmethyl)-amino]-methyl}-2-nitro-benzenesulphonamide; NPY, neuropeptide Y; PYY, peptide YY; rPP, rat pancreatic peptide; TTX, tetrodotoxin; 1229U91, Ile-Glu-Pro-Dapa-Tyr-Arg-Leu-Arg-Tyr-NH2, cyclic(2,4′) (2,4′) diamide

Introduction

The Y-receptor family is a growing family of poorly related proteins (Larhammar, 1997) activated by structurally related peptides: neuropeptide Y, peptide YY and pancreatic polypeptide (NPY, PYY and PP, respectively). Six receptors (Y1–Y6) have been described, five of which have been cloned and characterized (Blomqvist & Herzog, 1997). The NPY Y5 receptor subtype which has been strongly suggested to be involved in feeding behaviour is mainly expressed in the brain (Gerald *et al*., 1996; Dumont *et al*., 1998). However, the expression of this subtype has also been demonstrated in the periphery (Félétou *et al*., 1998; Goumain *et al*., 1998). Therefore, the identification of a selective preparation for the NPY Y5 receptor subtype has aroused considerable interest in order to study the functional sites which may be involved in the control of food intake. Pheng *et al*. (1997) have recently suggested that the rabbit ileum was such a preparation. However, this suggestion was based on the rank order of potency of various NPY analogues and the inhibition produced by a first generation Y5 antagonist which had a low affinity and a poor selectivity for the NPY Y5 receptor subtype (Félétou *et al*., 1998). As second generation non-peptidic compounds, with high affinity and good selectivity towards some of the NPY receptor subtypes, have been recently synthesized (such as CGP 71683A, Criscione *et al*., 1998), the purpose of this work was to verify the hypothesis that the rabbit ileum is indeed a selective preparation for the NPY Y5 receptor.

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Methods

Organ bath experiments

Male Sprague-Dawley rats and male New-Zealand rabbits (200–220 g and 6–7 weeks old respectively; Charles River: France), were killed with an overdose of pentobarbital (250 mg kg^{-1}, i.p and 40 mg kg^{-1}, i.v.). The ileum and the saphenous vein of the rabbit as well as the vas deferens (epididymal portion) of the rat were excised, immersed in cold (4°C) modified Krebs-Ringer salt solution of the following composition (mM) : NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, calcium-disodium EDTA 0.026 and glucose 11.1 and cleaned of adherent connecting tissue. The preparation of the rabbit saphenous vein and the rat vas deferens has been described elsewhere (Félétou *et al*., 1998). The rabbit ileum was cut into segments (2 cm in length) for isometric tension recording, bathed in modified Krebs-Ringer solution (37°C, bubbled with a 95% O_2, 5% CO_2 gas mixture;

pH: 7.4) and submitted to 0.5 g of passive tension. Ileal segments were repeatedly rinsed and after stabilization of the spontaneous contractile activity, were tested with a single concentration of hPP (10 nM). The effects of hPP were transient, suggestive of receptor desensitization. However, the inhibitory effects obtained during the test (i.e. application of a single concentration of hPP (10 nM) or the inhibitory effects produced by the same concentration of hPP (10 nM) during the course of cumulative concentration-response curves) were not significatively different (38 ± 5 and $43 \pm 5\%$, $n = 24$, respectively). Therefore, in the present study unless otherwise mentioned, cumulative concentration-response curves were performed. Antagonists were allowed to equilibrate for 30 min before the cumulative addition of agonist. Experiments were performed in parallel.

Isolation of human Y4 receptor subtype

RNA from human total brain (Clonetech) was reverse transcribed with oligo $(dT)_{12-18}$ and Reverse Transcriptase Superscript II (Gibco BRL). First strand cDNA (corresponding to 1 μg of total RNA) was amplified with oligonucleotide primers based on GenBank entries (accession number U35232) for human Y4 receptor (forward 22–42, reverse 1146–1167). Amplification was carried out for 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 3 min with a preincubation and postincubation of 94°C for 1 min and 72°C for 7 min, respectively. The expected 1155 base pair fragment was isolated and ligated into the pcDNA3 expression vector (Invitrogen). The recombinant plasmid pY4 was sequenced on both strands by automated sequencing (ABI377).

Establishment of a CHO-Ki cell line stably expressing the human Y4 receptor subtype

CHO-K1 cells grown in complete Ham-F12 medium were seeded at 5×10^6 cells in a T75 cm^2 culture flask and transfected 24 h later with 10 μg of the pY4 plasmid using lipofectamine as described by the manufacturer (Life Technologies). Twenty-four hours after the beginning of the transfection, cells were placed in complete Ham-F12 containing 800 $\mu g/ml^{-1}$ of active geneticin during 2 weeks. At the end of this selection period, isolated clones were picked, amplified and further characterized by binding experiments. One positive clone, the CHO-K1-hY4 clone 4, was subcloned in limited dilution before being used (subclone CHO-K1-hY4- 4.2) for all the binding experiments.

Binding experiments

Y1, Y2 and Y5 binding assays have been performed as previously described (Félétou *et al*., 1998) on SK-N-MC, KAN-TS, and transiently transfected COS7 cells, respectively.

Y4 binding assay was performed with the same radioligand, [^{125}I]-PYY and the same buffer (mM): HEPES/NaOH 20 (pH = 7.4), NaCl 10, KH_2PO_4 0.22, $CaCl_2$ 1.26, $MgSO_4$ 0.81 and 0.1% BSA. CHO-K1-hY4-4.2 cell membranes were incubated 90 min at 37°C with 50 pM [^{125}I]-PYY and drugs at various concentrations in 250 μl (final volume) of binding buffer. Incubation was terminated by rapid filtration on GF/B Unifilters (Packard). Non specific binding was measured in the presence of 1 μM unlabelled PYY.

Drugs

hNPY analogues (PP, PYY, NPY, NPY_{13-36}, [Leu^{31}, Pro^{34}]-NPY), rPP and Ile-Glu-Pro-Dapa-Tyr-Arg-Leu-Arg-Tyr-NH2, cyclic(2,4′) (2,4′) diamide (1229U91): Neosystem, Strasbourg, France; atropine, papaverine and tetrodotoxin: Sigma, La Verpillère, France; R-N-[4-(Aminocarbonylaminomethyl)phenylmethyl-N^2-(diphenylacetyl)arginamide (BIBO 3304, Doods *et al*., 1997), IdRS, Suresnes, France; N-(4-trans-{[(Naphtalen-2-ylmethyl)-amino]-methyl}-2-nitro-benzenesulphonamide (compound 34 described in patent # 97/319425 by Synaptic Pharmaceut. Corp., Dhanoa *et al*., 1997, has been synthesized by the Peptide Chemistry Department, IdRS, France and termed JCF 109), N1-[(4-{[(4-amino-2-quinazolinyl) amino] methyl} cyclohexyl) methyl] - 1-naphtalenesulphonamide (CGP 71683A, Criscione *et al*., 1998; IRIS, Courbevoie, France).

Statistics

Data are shown as means ± s.e.mean. *n* represents the number of animals from which tissues were taken. The changes in the contractile activity of the rabbit ileum could be expressed

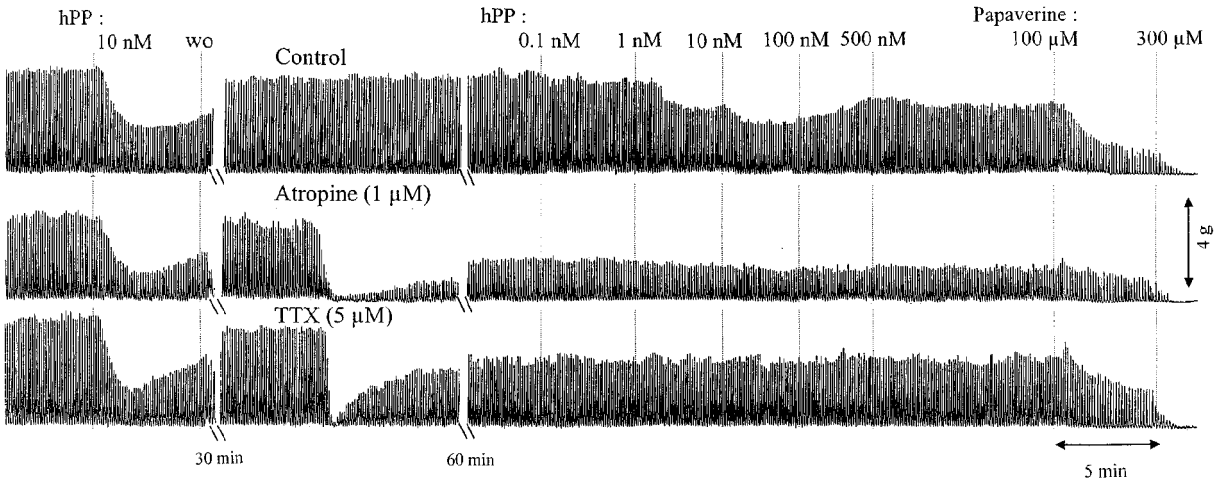

Figure 1 Original tracings showing the effects of atropine (1 μM) and tetrodotoxin (TTX, 5 μM) on the inhibition produced by hPP on the spontaneous contractions of the rabbit isolated ileum. Top tracing: control conditions, Medium tracing: presence of atropine, Bottom tracing: presence of tetrodotoxin. Note that the three ileal segments show similar responses to hPP (10 nM) prior treatment with drugs and that papaverine produces complete relaxation in all the tissues suggesting that the smooth muscle is still able to relax. wo: wash out.

either by the changes in the maximal amplitude of the spontaneous contractions or by the integrated contractile activity (Acknowledge III, Biopac System, Inc, Goleta, CA., U.S.A.). Both approaches to express data provided similar results.

Statistical evaluation was performed with a two-way analysis of variance with repeated measures on agonist concentration or on agonist concentration and antagonist concentration depending on protocols and pairing. When a significant interaction was observed ($P<0.05$) a complementary analysis was undertaken (Dunnett's parametric test) to identify differences among groups. EC_{50} calculation was performed with a linear regression within the two half log concentrations surrounding the 50% value. Apparent antagonist dissociation constants were determined according to the equation : K_B= [Ant]/(concentration ratio -1) where [Ant] is the concentration of the antagonist and concentration ratio is the EC_{50} in the presence of the antagonist divided by the EC_{50} of the agonist in the absence of the antagonist. These results were then expressed as the negative logarithm of the K_B i.e.: $-\log(K_B)=pK_B$.

Results

Isolated organs

NPY analogues inhibited the spontaneous contractions of the rabbit ileum with the following order of potency ($-\log EC_{50}$): hPP (8.85 ± 0.06, $n=24$) > rPP (8.01 ± 0.15, $n=7$) > PYY (7.55 ± 0.18, $n=7$) $\geqslant$ [Leu^{31},Pro^{34}]-NPY (7.39 ± 0.12, $n=7$) > NPY (7.18 ± 0.16, $n=10$) > > NPY_{13-36} (<6, $n=5$). The inhibition produced by the peptides was transient and reproducible (Figure 1). The maximal inhibitory responses (inhibition of the spontaneous contractile activity in per cent) were similar for hPP (43 ± 5, $n=24$), rPP (39 ± 7, $n=7$), PYY (44 ± 11, $n=7$) and [Leu^{31}, Pro^{34}]-NPY (43 ± 9, $n=7$). The maximal inhibition produced by NPY was significantly smaller than the maximal inhibition produced by hPP (31 ± 6, $n=10$; e.g. 70% of hPP maximal response, $P<0.05$) and NPY_{13-36} did not produce any significant inhibition (2 ± 3, $n=5$).

Tetrodotoxin (5 μM) and atropine (1 μM) induced a complete but transient inhibition of the spontaneous contractions of the rabbit isolated ileum followed by a slow partial recovery of the spontaneous contractile activity (Figure 1). In the presence of tetrodotoxin, the inhibitory effect of hPP was abolished (maximal inhibitory effect of hPP in presence of tetrodotoxin: $-5\pm2\%$, $n=6$, $P<0.05$). However, in the presence of atropine, the inhibitory effect of hPP was only partially but significantly suppressed ($10\pm5\%$, $n=6$, $P<0.05$; Figure 1).

rPP, PYY, [Leu^{31}, Pro^{34}]-NPY and NPY (3 μM) induced a transient inhibition of the spontaneous contractions of the rabbit ileum. Once the contractile activity was returned to control values, the subsequent addition of hPP (10 nM: a concentration which by itself produced the maximal inhibitory effect) evoked no or minor inhibition of the spontaneous contractions. In contrast, the effect of hPP (10 nM) was not significantly different in presence or absence of NPY_{13-36} (3 μM; Figure 2).

BIBO 3304 was devoid of any intrinsic properties (up to 10 μM) in the rat vas deferens as well as in the rabbit ileum and saphenous vein. It was a potent antagonist of the NPY-induced contraction of the rabbit saphenous vein but up to 10 μM did not show any antagonistic properties in the rat deferens (agonist: PYY). In the rabbit ileum, BIBO 3304 (1

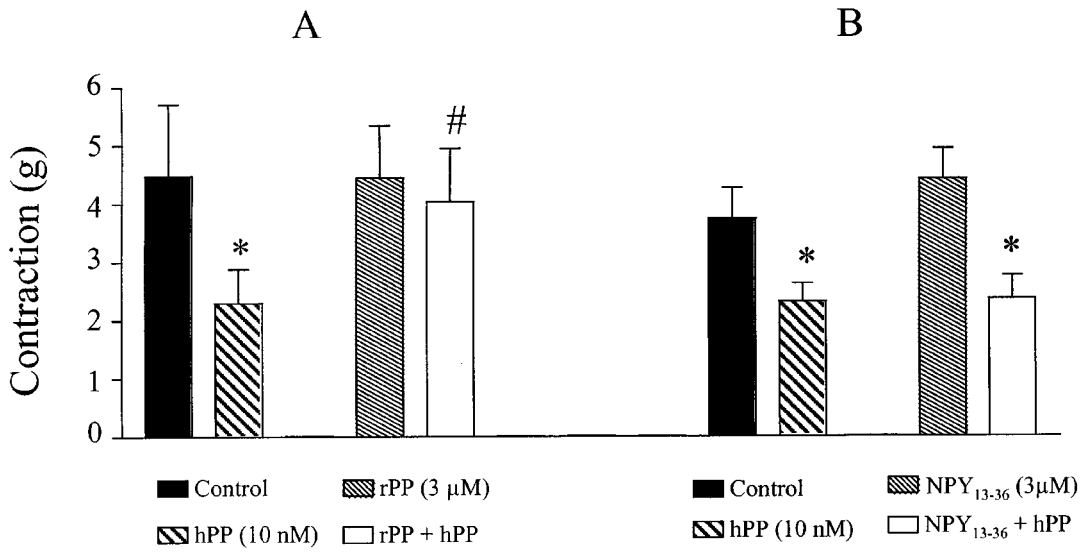

Figure 2 Cross-desensitization in the inhibitory response produced by NPY analogues in the rabbit isolated ileum. (A) Cross desensitization between rPP and hPP (left panel, $n=6$). The rabbit isolated ileum presents in control conditions a spontaneous contractile activity. In control conditions hPP (10 nM) produces a transient inhibition of the contractile response (maximal inhibition of the responses). hPP was washed out and thereafter the addition of rPP (3 μM) produces also an inhibition of the contractile response (not shown in this graph), which was transient and after a few minutes the amplitude of the contractions was no longer significantly different than the amplitude of the contractions observed in control conditions. In the presence of rPP the addition of hPP did not significantly influence the contractile activity. The presence of PYY, [Leu^{31}, Pro^{34}]-NPY and NPY also significantly affected the response to hPP (data not shown). (B) Absence of cross-desensitization between NPY_{13-36} and hPP (right panel, $n=5$). The rabbit isolated ileum presents in control conditions a spontaneous contractile activity. In control conditions hPP (10 nM) produces a transient inhibition of the contractile response (maximal inhibition of the responses). hPP was washed out and thereafter the addition of NPY_{13-36} (3 μM) did not produce any significant inhibition of the contractile response. In the presence of NPY_{13-36} the addition of hPP produces a significant inhibition of the contractile activity which was similar to the first inhibition produced by hPP in control conditions. Data are shown as means$\pm$s.e.mean. The asterisk indicates a significant effect produced by hPP. # indicates that the effect of hPP has been significantly affected by the presence of rPP.

and 10 μM) did not influence the inhibitory effect of hPP (Table 1) or NPY ($-\log EC_{50}$: 7.6 ± 0.4, 7.6 ± 0.4, 7.3 ± 0.3, $n=4$, in absence and presence of BIBO 3304 1 and 10 μM, respectively).

JCF 109 was devoid of any intrinsic properties (up to 10 μM) in the rat vas deferens and in the rabbit saphenous vein and did not show any antagonistic properties of the PYY-induced inhibition of the electrically stimulated rat vas deferens. JCF 109 (1 and 10 μM) potentiated the NPY-induced contraction of the rabbit saphenous vein. This compound at 1 μM did not influence the contraction of the rabbit ileum but at 10 μM produced a significant decrease in the spontaneous contractions and an inhibition (although not statistically significant) of the effect of hPP (Table 1). CGP 71683A at 10 μM (but not at lower concentrations) demonstrated an intrinsic activity in the three tissues studied. At this elevated concentration, CGP 71683A did not show any antagonistic activity in the three tissues studied but a statistically significant small potentiation of NPY-induced contraction of the rabbit saphenous vein (Figure 3, Table 1).

1229U91 showed potent intrinsic activity in the three tissues studied. In the rabbit ileum, non-cumulative addition of 1229U91 (1 nM – 2 μM) produced a concentration-dependent transient decrease in the spontaneous contractions ($-\log EC_{50}$: 7.3, $n=7$). The inhibition produced by 1229U91 was not observed during cumulative addition of the compound (Figure 4). 1229U91 was a potent antagonist in the three tissues studied (Table 1, Figure 4).

Binding

BIBO 3304 and JCF 109 are potent and selective ligands for the human Y1 and Y5 receptor subtypes, respectively. CGP 71683A was also a very potent ligand with nanomolar affinity for the Y5 receptor subtype. At supra micromolar concentrations this compound showed some affinity for the Y1 and Y4 receptor subtypes. However, although more potent than JCF

Table 1 Pharmacological profile of neuropeptide receptors: apparent affinities of antagonists: pK_B, or $-\log (IC_{50})$

	Y1		*Y2*		*Y4*	*Y5*	*Y?*
	RSV (pK_B)	*Binding* $-log$ (IC_{50})	*RVD* (pK_B)	*Binding* $-log$ (IC_{50})	*Binding* $-log$ (IC_{50})	*Binding* $-log$ (IC_{50})	*Rabbit ileum* (pK_B)
BIBO 3304	8.0	9.5	In.	<5	<5	<6	In.
JCF 109	Pot.	<5	In.	5.2	<5	8.0	≈ 5 (+)
CGP 71683A	Pot. (+)	5.5	In. (+)	5.9	5.4	8.8	In. (+)
1229U91	7.2 (++)	10.4	6.0 (++)	7.7	9.7	6.7	7.2 (+++)

Apparent affinities of antagonists are shown as pK_B for isolated organs and $-\log (IC_{50})$ for binding experiments. In. indicates that the compound has no antagonistic activity at 10 μM. (+), (++) and (+++) indicate an intrinsic activity of the compound at 10, 1 or 0.1 μM, respectively. Pot. indicates that the compound potentiated the effect of the antonist. RSV: rabbit saphenous vein, RVD: rat vas deferens.

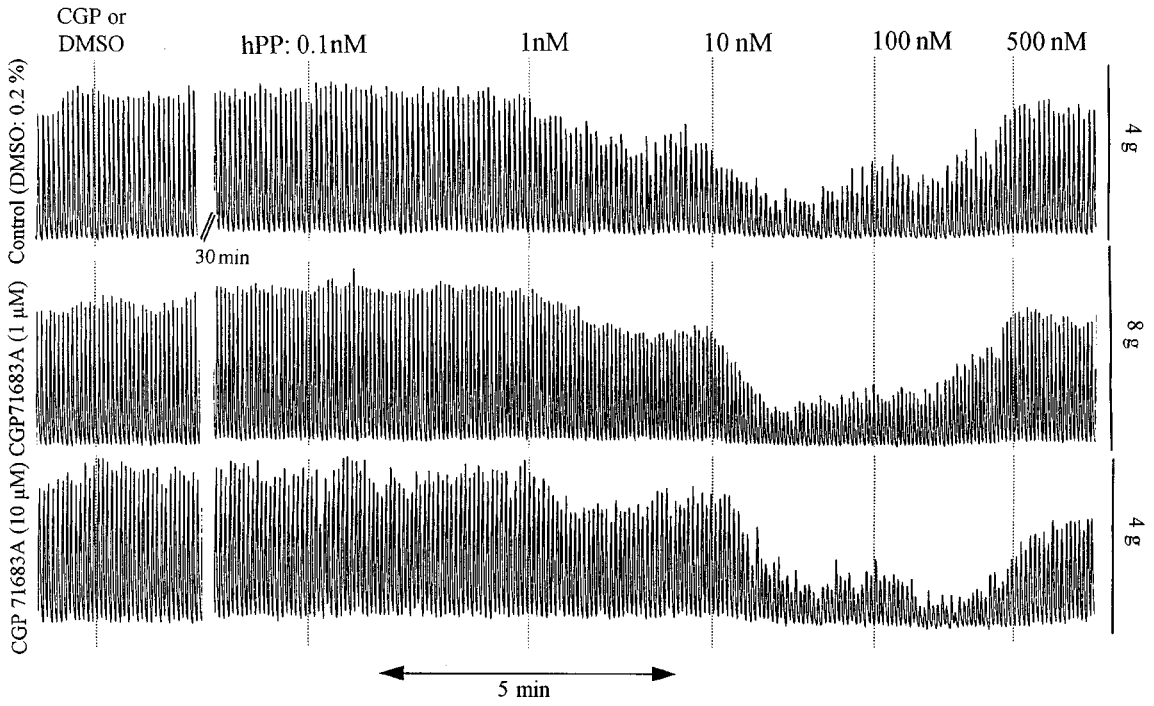

Figure 3 Original tracings showing the effects of CGP 71683A on the inhibition produced by hPP on the spontaneous contraction of the rabbit isolated ileum. Top tracing: control conditions, Medium tracing: presence of CGP 71683A (1 μM), Bottom tracing: presence of CGP 71683A (10 μM). DMSO: Dimethyl sulphoxide (CGP 71683A solvent).

109 toward the Y5 receptor subtype, the selectivity ratio of both coumpounds was similar. 1229U91 showed sub-nanomolar affinity for the Y1 and Y4 receptor subtypes, nanomolar affinity for Y2 and sub-micromolar affinity for the Y5 receptor subtype (Table 1).

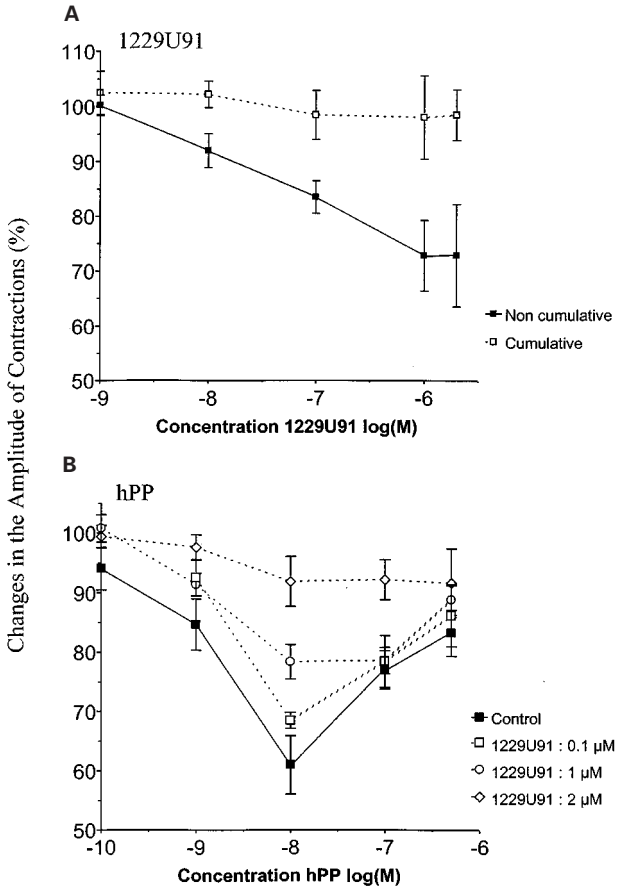

Figure 4 Effect of 1229U91 in the isolated ileum of the rabbit. (A) Concentration response curve to 1229U91 in a cumulative ($n=4$) or non-cumulative way ($n=2-9$). (B) Effect of the 1229U91 (0.1, 1 and 2 μM, $n=5-6$) on the concentration-response curves to hPP in the isolated ileum of the rabbit. Data are shown as means ± s.e.mean. n represents the number of animals from which tissues were taken.

Table 2 Coefficient correlation (r^2) of the linear regression between the apparent affinities for the human and rat Y5 and Y4 receptor subtypes and the NPY receptor in the rabbit isolated ileum

r^2	*Ileum***	*Ileum*†	*hY4*‡	*rY4*‡	*hY5*#	*rY5*+
Rabbit ileum**		0.81*	0.91*	0.65	0.07	0.04
Rabbit ileum†	0.81*		0.65	0.38	0.20	0.20
Human Y4‡	0.91*	0.65		0.80*	0.01	0.01
Rat Y4‡	0.65	0.38	0.80*		0.11	0.07
Human Y5#	0.07	0.20	0.01	0.11		0.82*
Rat Y5+	0.04	0.20	0.01	0.07	0.82*	

Apparent affinities ($-\log EC_{50}$) of six NPY analogues (hPP, rPP, hPYY, [Leu^{31}, Pro^{34}]-NPY hNPY and NPY_{13-36}. ** and †Data from the present study and from Pheng *et al.* (1997), respectively: inhibition of the spontaneous contraction of the rabbit isolated ileum; ‡Data from Walker *et al.* (1997): inhibition of forskolin-induced stimulation of cyclic AMP in rat or human Y4-transfected L-M(TK-) cells; #Data from Félétou *et al.* (1998): binding in cell membranes in human Y5-transfected COS cells; +Data from Gerald *et al.* (1996): inhibition of forskolin-induced stimulation of cyclic AMP in human Y5-transfected L-M(TK-) cells. The asterisk indicates a slope significantly different from zero.

Discussion

This study confirms that NPY related peptides inhibit the spontaneous contractions of the rabbit isolated ileum (Pheng *et al.*, 1997). This effect is most likely linked to the activation of a single receptor as cross-desensitization occurs between the active peptides. The ineffectiveness of a specific NPY Y2 agonist (NPY_{13-36}) and of specific NPY Y1 and Y5 antagonists (BIBO 3304 on one hand and JCF 109 or CGP 71683A on the other hand), plus an order of potency of the NPY analogues consistent with the NPY Y4 subtype concur to suggest that a single NPY receptor of the Y4 subtype is involved.

As previously shown by Pheng *et al.* (1997), the spontaneous contractile activity of the rabbit ileum derives from continuous release of acetylcholine since it is eliminated by tetrodotoxin and atropine. However, alternative mechanisms, not identified in the present study, allow the recovery of the spontaneous activity either during the blockade of neuronal excitation by tetrodotoxin or during the inhibition of muscarinic receptors by atropine. The abolition by tetrodotoxin of the inhibitory effect induced by hPP indicates that the NPY receptor is located on the intrinsic inhibitory motoneurons of the rabbit ileum while the partial suppression by atropine shows that the activation of the pre-junctional NPY receptor mainly inhibits acetylcholine release.

The order of potency of the NPY analogues observed in the present study is consistent with the previous study of Pheng *et al.* (1997). Although the apparent affinity of the NPY analogues is slightly lower in the present study, the results of these two studies are highly correlated (Table 2). Furthermore, this study also confirms that NPY induced a smaller maximal response than the other active agonists tested.

BIBO 3304, a potent antagonist in the rabbit saphenous vein, a Y1 receptor enriched bioassay (Cadieux *et al.*, 1993), was inactive in the electrically stimulated rat vas deferens, a Y2 enriched bioassay (Martel *et al.*, 1990). In binding experiments, the specificity of BIBO 3304 was confirmed as this compound showed high affinity exclusively towards the human Y1 receptor (Doods *et al.*, 1997). In the rabbit ileum, BIBO 3304 did not influence the inhibitory effect of hPP or NPY indicating that the Y1 receptor subtype is not involved confirming earlier results (Pheng *et al.*, 1997) obtained with another Y1 receptor antagonist, BIBP 3226 (Rudolf *et al.*, 1994).

Similarly, the involvement of a Y2 receptor subtype is unlikely as NPY_{13-36}, a specific Y2 receptor agonist (Blomqvist & Herzog, 1997), did not elicit any significant inhibition of the contractions. Furthermore, in presence of NPY_{13-36} the response to hPP was not affected, showing that the truncated NPY analogue did not desensitize the preparation.

In the present study, PYY was not considerably less active than NPY, ruling out the involvement of the putative Y3 receptor subtype (Michel *et al.*, 1998).

In contrast, the involvement of a Y4 receptor subtype in the inhibition of the contraction of rabbit ileum appears most likely. The NPY Y4 receptor subtype is highly expressed in the gut. The principal characteristic of the Y4 receptor is its high affinity for the PP of the same species although PP homologues from other species may have lower affinities. [Leu^{31}, Pro^{34}]-NPY is also a potent agonist at the Y4 receptor (Blomqvist & Herzog, 1997; Michel *et al.*, 1998). In the present study, hPP is the most active compound and both rPP and [Leu^{31}, Pro^{34}]-NPY are also potent agonists. Furthermore, the EC_{50} values obtained in this study are highly correlated to the EC_{50} values observed for the human NPY Y4 receptor subtype (and to a

lesser extent toward the rat NPY Y4 subtype, Walker *et al*., 1997; Table 2). Functional and binding experiments in the present study confirmed that the 1229U91 compound is a non-selective ligand for Y1, Y2, Y4 and Y5 receptors with, however, high affinity toward hY1 and hY4, moderate affinity for the hY2 and only relatively minor affinity for the hY5 receptor subtypes. This compound has been described as a potent antagonist of the Y1 receptor and a potent agonist of the Y4 receptor (Matthews *et al*., 1997; Schober *et al*., 1998). Indeed, 1229U91 induced an inhibition of the contractile activity of the rabbit ileum, being as potent as [Leu^{31}, Pro^{34}]-NPY. The effect of 1229U91 was transient and could not be observed when the compound was applied in a cumulative manner. This suggests that either 1229U91 induced rapid desensitization of the receptor or that 1229U91 behaves as a partial agonist. Interestingly, 1229U91 produced a concentration-dependent inhibition of the effects of hPP, suggesting that hPP and 1229U91 share the same site of action. As the inhibitory effect of 1229U91 cannot be explained by the Y1 antagonistic properties of this compound, because BIBO 3304 is ineffective, and hPP is a poor Y1 ligand, it is tempting to attribute this effect to a partial agonistic property of 1229U91 toward the Y4 receptor.

In contrast to a previous suggestion (Pheng *et al*., 1997), the NPY Y5 receptor subtype is most unlikely to be involved in the inhibitory effects of NPY analogues in the rabbit ileum. The EC_{50} values of agonists found in the rabbit ileum either in the study performed by Pheng *et al*. (1997) or obtained in the present study do not show any correlation with the affinity reported for the human and rat NPY Y5 receptor subtypes (Gerald *et al*., 1996; Félétou *et al*., 1998, Table 2). Furthermore, the specific antagonist NPY Y5 receptor antagonist, CGP 71683A (Criscione *et al*., 1998) up to 10 μM, did not show any significant activity. JCF 109, another Y5 receptor antagonist (Dhanoa *et al*., 1997), at 1 μM, a concentration 100 times higher than the EC_{50} toward the human Y5 receptor, was ineffective. In two tissues, at 10 μM it produced a significant, long-lasting inhibition of the contractile activity and subsequently an inhibition of the response to hPP, suggesting a non-specific effect of this compound. JCF 105, a compound from an earlier generation was used in a previous study (Pheng *et al*., 1997) to propose the existence of Y5 receptors in the rabbit ileum. However, JCF 105 is a poorly specific antagonist, with low affinity for the Y5 receptor and possessing muscarinic antagonistic properties (Félétou *et al*., 1998). Altogether these data are not in favour of the involvement of NPY Y5 receptors.

The y6 gene encodes a functional receptor protein in mouse and rabbit in contrast to human and rat (Gregor *et al*., 1996; Matsumoto *et al*., 1996). In one murine clone the order of potency of agonist could have been similar to that which is observed in the rabbit ileum (Gregor *et al*., 1996). However, in a rabbit clone the order of potency indicates that NPY_{13-36} has a high ligand affinity while PP is a very poor ligand (Matsumoto *et al*., 1996), showing that the receptor in the rabbit ileum is not due to the activation of a NPY y6 receptor subtype. The results of the present study strongly suggest that the NPY receptor involved in the inhibition of the spontaneous contractile activity of the rabbit ileum, is a NPY Y4 receptor subtype.

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