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Heterogeneity of prejunctional NPY receptor-mediated inhibition of cardiac neurotransmission

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1 Neuropeptide Y (NPY) has been proposed as the candidate inhibitory peptide mediating interactions between sympathetic and vagal neurotransmission in several species, including man. Here, we have defined the NPY receptors involved in modulation of cardiac autonomic neurotransmission using receptor-selective agonists and antagonists in the rabbit and guinea-pig isolated right atria.

2 In isolated atrial preparations, sympathetically-mediated tachycardia (ST; with atropine 1 μM) or vagally-mediated bradycardia (VB; with propranolol 0.1–1 μM) in response to electrical field stimulation (EFS, 1–4 pulses) were tested 0–30 min after incubation with single concentrations of vehicle, NPY (0.01–10 μM), the Y_2 receptor agonist N-Acetyl-[Leu^{28,31}]NPY(24–36) (termed *N*-A[L]NPY(24–36)) or the Y_1 receptor agonist [Leu^{31},Pro^{34}]NPY (LP). The effect of NPY on the concentration-chronotropic response curves to isoprenaline and bethanechol were also assessed.

3 Guinea-pig atria: NPY and *N*-A[L]NPY(24–36) caused concentration-dependent inhibition of VB and ST to EFS. Both peptides caused maximal inhibition of VB and ST within 10 min incubation and this remained constant. LP caused a concentration-dependent, transient inhibition of ST which was antagonized by the Y_1-receptor antagonist GR231118 (0.3 μM), with apparent competitive kinetics. Rabbit atria: NPY (1 or 10 μM) had no effect on VB at any time point, but both NPY and LP caused a transient ($\sim$10 min) inhibition of sympathetic tachycardia. This inhibition could be prevented by 0.3 μM GR231118. *N*-A[L]NPY(24–36) had no effect on ST. NPY had no effect on the response to β-adrenoceptor stimulation by isoprenaline nor muscarinic-receptor stimulation by bethanechol in either species.

4 Thus, in the guinea-pig, NPY causes a stable inhibition of both VB and ST to EFS *via* Y_2 receptors and transient inhibition of ST *via* Y_1 receptors. In contrast in the rabbit, NPY has no effect on the cardiac vagus and prejunctional inhibition of ST is transient and mediated by a Y_1-like receptor (rather than Y_2). Therefore it would be surprising if NPY plays a functional role in modulation of cardiac neurotransmission in the rabbit.

Keywords: Neuropeptide Y; cardiac neurotransmission; sympathetic; vagus; desensitization; isolated right atrium

Abbreviations: EFS, electrical field stimulation; LP-NPY, [Leu^{31},Pro^{34}]NPY; *N*-A[L]NPY(24–36), *N*-Ac-[Leu^{28,31}]NPY(24–36); NPY, neuropeptide Y; PYY, peptide YY

Introduction

The peptide co-transmitter neuropeptide Y (NPY) may be an important modulator of cardiac autonomic neurotransmission in mammalian species. Dense populations of NPY-immunoreactive fibres are found throughout the myocardium (Gu *et al*., 1984; Sternini & Brecha, 1985; Allen *et al*., 1986) with particularly high levels of innervation in the region of the sinoatrial and atrioventricular nodes (Gu *et al*., 1983; 1984; Sternini & Brecha, 1985; Warner & Levy, 1990). High frequency electrical stimulation of cardiac sympathetic nerves (Potter, 1985; Warner & Levy, 1989b) or physiological stimuli that increase sympathetic activity (Potter, 1985) cause a subsequent inhibitory action on vagally-mediated falls in heart rate. This vagal modulation following sympathetic stimulation can be mimicked by exogenous NPY (Potter, 1985; Warner & Levy, 1989a) supporting the underlying hypothesis that endogenous NPY released from cardiac sympathetic nerves mediates the inhibitory effect on the vagus. In further support of this hypothesis, in the guinea-pig isolated right atrium, NPY is an inhibitor of vagally-mediated bradycardia where micromolar concentrations decrease the bradycardia elicited by electrical field stimulation to less than 50% of control (Potter, 1987). Similarly, in the anaesthetized rat, 0.2 pmole NPY is sufficient to inhibit the vagally-induced increase in pulse interval by more than 90% (Potter *et al*., 1989). However, in contrast to most other species studied, NPY fails to modulate the vagal component of the baroreceptor-HR reflex in the conscious rabbit (Minson *et al*., 1990; Serone *et al*., 1998).

In addition to the action of NPY on the cardiac vagus *in vivo* and *in vitro*, this peptide has been reported to inhibit the release of tritiated noradrenaline from sympathetic nerve terminals *via* a prejunctional mechanism (Lundberg & Stjarne, 1984). Previously, we have reported that exogenous NPY caused a decrease in the range of the sympathetic component of the baroreceptor-HR reflex in the absence of vagally-mediated bradycardia, in conscious rabbits (Serone *et al*., 1998). This effect on the baroreflex was also elicited by infusion of the Y_1-selective agonist [Leu^{31},Pro^{34}]NPY, initially suggesting that NPY may have been mediating a prejunctional inhibition *via* Y_1-receptors. However, administration of the α_1-adrenoceptor agonist methoxamine could effectively mimic this effect of both peptides on the baroreflex, indicating that the decreased range of sympathetically-mediated tachycardia may have been a non-specific consequence of the increase in blood pressure.

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The lack of any obvious direct effect of NPY on neurotransmission in our earlier experiments in conscious rabbits (Serone *et al*., 1998) may have been due to desensitization of the prejunctional NPY receptors. Maynard & Burnstock (1994) have shown that prejunctional inhibition by NPY in the rabbit isolated ear artery rapidly shows tachyphylaxis following continuous exposure up to 10 min. In our previous study, the rabbits were infused with NPY for 30 min before assessment of the baroreceptor-HR reflex. Thus desensitization may have occurred.

In the current work, we have explored the potential role of NPY receptor subtypes modulating neurotransmission and the potential for time dependent desensitization of prejunctional receptors at the cardiac sympathetic and vagal neuroeffector junction. Our findings indicate that Y_2-receptors inhibit both vagal and sympathetic responses in guinea-pig but not in rabbit isolated right atria. A transient and weak Y_1- or Y_1-like receptor mediated inhibition of sympathetic tachycardia occurs in both species.

Methods

General

Guinea-pigs of either sex (323 ± 5 g, range 200–463 g, $n = 114$) were anaesthetized in a mixture of 80% oxygen and 20% carbon dioxide and killed by decapitation. Rabbits of either sex (1.2 ± 0.03 kg, range 0.89–1.5 kg, $n = 33$) were killed by an overdose of pentobarbitone sodium (150 mg kg^{-1} i.v.; Virbac, NSW, Australia). The heart was then rapidly removed, placed in oxygenated Krebs' solution (see below) at 37°C. The right atrium was dissected free of surrounding vessels and connective tissue and suspended vertically on stainless steel S-shaped hooks attached to a Grass FT03C force transducer. Atria were maintained at a resting force of approximately 1 g. The Krebs' solution had the following composition (in mM): NaCl 119, KCl 4.7, CaCl 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, EDTA 0.026 and glucose 11. All experiments were performed in 15 ml jacketed organ baths that were silanized (Sigmacote, Sigma, St Louis, MO, U.S.A.) prior to experimentation.

Atria rested against two punctate platinum electrodes (3 mm apart) that detected the surface electrogram (monitored on a dual beam 10 MHz storage oscilloscope). This signal was amplified and passed to a field pulse controller that differentiated the signal and triggered a square wave pulse. This pulse, when allowed, triggered the start of a train of electrical field pulses, delivered *via* a Grass S88C dual stimulator to a pair of platinum wire field electrodes that were positioned parallel to the atrium. This equipment could deliver field pulses across the tissue in the atrial refractory period (40–60 ms long) to avoid conduction disturbances but allow depolarization of the autonomic varicosities and the release of neurotransmitters (Angus & Harvey, 1981). This method elicited graded changes in atrial period (interval between atrial contractions) that were linear with respect to the number of applied field pulses. The signal from the force transducer was also amplified and used to trigger a period meter. Atrial period and force of contraction were continuously recorded on a chart recorder (Neotrace 600ZF).

Protocol

Vagal responses to EFS: guinea-pig isolated right atria Atria were repeatedly washed for 30 min and then incubated for a further 30 min with propranolol (1 μM; a higher concentration of propranolol was used in guinea-pig atria due to the presence of a residual tachycardia following EFS, observed when only 0.1 μM propranolol was present in the incubation medium). The response to electrical field stimulation (EFS) was then assessed (as above) by applying 1–4 field pulses per atrial refractory period (2 ms duration, 100 Hz, 100 V on S88 dial). The subsequent increase in atrial period (ms) was measured. The tissues were then incubated with a single concentration of either vehicle (water, 15 μl, NPY (0.01–1 μM), the NPY Y_2 receptor selective agonist, *N*-Acetyl-[$Leu^{28,31}$]NPY(24–36) (Potter *et al*., 1994) (*N*-A[L]NPY(24–36); 0.01–1 μM) or the relatively selective NPY Y_1 receptor agonist [Leu^{31},Pro^{34}]NPY (Fuhlendorff *et al*., 1990; Potter & McCloskey, 1992) (1–10 μM). The response to four pulses per refractory period was then tested at 1–20 min after addition of peptide/vehicle. After 30 min incubation the full (1–4 field pulses) stimulus-response relationship was reassessed. In a separate group of experiments the effect of the NPY Y_1 receptor selective antagonist GR231118 (Lew *et al*., 1996) was tested on the response to [Leu^{31},Pro^{34}]NPY. GR231118 (0.3 μM) was added 30 min prior to addition of Y_1 receptor selective peptide or vehicle and then the sympathetic or vagal responses assessed as above.

The effect of NPY (1 μM) or vehicle (water; 15 μl) on postjunctional cardiac muscarinic receptor stimulation was examined in a separate group of atria by constructing cumulative chronotropic concentration-response curves to the muscarinic receptor agonist bethanechol 30 min after incubation with either peptide or vehicle.

Sympathetic response to EFS: guinea-pig isolated right atria Atria were frequently washed for 30 min before incubation for a further 30 min with atropine (1 μM). The response to electrical field stimulation (EFS) was then assessed (as above) by applying 1–4 field pulses per atrial refractory period (2 ms duration, 100 Hz, 100 V on S88 dial). The subsequent decrease (sympathetic response) in atrial period (ms) was measured. The tissues were then incubated with a single concentration of either vehicle (water, 15 μl), NPY (0.1–10 μM), the NPY Y_2 receptor selective agonist, *N*-Acetyl-[$Leu^{28,31}$]NPY(24–36) (Potter *et al*., 1994) (*N*-A[L]NPY(24–36); 0.1–10 μM) or the relatively selective NPY Y_1 receptor agonist [Leu^{31},Pro^{34}]NPY ((Fuhlendorff *et al*., 1990; Potter & McCloskey, 1992); 1–10 μM). The response to four pulses per refractory period was then tested at 1–20 min after addition of peptide/vehicle. After 30 min incubation the full (1–4 field pulses) stimulus-response relationship was reassessed. In a separate group of experiments the effect of the NPY Y_1 receptor selective antagonist GR231118 (Lew *et al*., 1996) was tested on the response to [Leu^{31},Pro^{34}]NPY. GR231118 (0.3 μM) was added 30 min prior to addition of Y_1 receptor selective peptide or vehicle and then the sympathetic responses assessed as above.

The effect of NPY (10 μM) or vehicle (water; 15 μl) on postjunctional cardiac β-adrenoceptor stimulation was examined in a separate group of atria by constructing cumulative chronotropic concentration-response curves to the β-adrenoceptor agonist isoprenaline 30 min after incubation with either peptide or vehicle.

Vagal responses to EFS: rabbit isolated right atria Atria were washed every 5 min for 30 min before incubating the tissue with propranolol (0.1 μM) for a further 30 min to prevent tachycardia responses to field stimulation. The response to electrical field stimulation (EFS) was then assessed (as above)

by applying 1–4 field pulses per atrial refractory period (2 ms duration, 100 Hz, 100 V on S88 dial). The subsequent increase in atrial period (ms) was measured. After a 30 min rest period, a second series of control responses was performed before construction of a control concentration-response curve to the muscarinic agonist bethanechol (0.1–31.6 μM). Concentrations were added cumulatively after the bradycardia to the previous dose had come to a plateau. The atria were then repeatedly washed for 30 min and re-incubated with propranolol (1 μM). At this time NPY (1 μM) was also added and incubated for 30 min. An incubation time of 30 min was chosen to reflect the duration of intravenous infusion of NPY that has been previously shown to have no effect on the vagal component of the cardiac baroreceptor-heart rate reflex in conscious rabbits (Serone *et al*., 1998). After incubation, the bradycardia to field pulse stimulation and bethanechol were reassessed as initially described. In the case of time control experiments, vehicle (15 μl; Milli Q water) was added in place of NPY. To ensure desensitization had not occurred in the rabbit atria, in two separate experiments, the effect of NPY (1 or 10 μM) on the response to four pulses per period was examined at 1–20 min after addition of the peptide.

Sympathetic responses to EFS: rabbit isolated right atria Atria were repeatedly washed for 30 min before construction of a concentration-response curve to the β-adrenoceptor agonist isoprenaline (0.1–1000 nM; vehicle and NPY-treated atria only). Concentrations were added cumulatively after the response to the previous concentration had come to a plateau. Atria were then washed for 30 min before incubating the tissue with atropine (1 μM) for a further 30 min to prevent bradycardic responses to EFS. After assessing the sympathetic response to EFS (1–4 pulses, 1 Hz, 2 ms, 100 V), atria were incubated with either vehicle (water, 15 μl), NPY (10 μM) or [Leu^{31},Pro^{34}]NPY (10 μM) and the response to four pulses tested at 1–20 min. The full stimulus-response relationship and the concentration-response curve to isoprenaline were then reassessed after 30 min incubation. In a separate experimental group, atria were pretreated with GR231118 (0.3 μM) for 30 min before control periods of stimulation were performed and the response to NPY (10 μM) tested as above. In a single experiment, the effect of *N*-A[L]NPY(24–36) on the sympathetic response to EFS was assessed as above.

Drugs

Drugs used were freshly prepared in ultra-filtered water (Milli Q UV) and included acetylcholine bromide (Sigma, St Louis, MO, U.S.A.), atropine sulphate (Sigma), carbamyl-β-methylcholine chloride (bethanechol chloride, Sigma), (−)isoprenaline hydrochloride (Sigma), neuropeptide Y, *N*-Acetyl-[Leu^{28,31}]NPY(24–36), [Leu^{31},Pro^{34}]NPY and GR231118 (Lew *et al*., 1996) synthesized by Dr Roger Murphy, Department of Pharmacology, University of Melbourne, Victoria, Australia) and propranolol hydrochloride (Sigma).

Analysis and statistical methods

Parameter measurement and agonist concentration-response curves Data are presented as mean ± s.e.mean. The chronotropic responses to EFS were measured as changes in atrial period (in ms) from resting and expressed as either a tachycardia or bradycardia. The changes in atrial period to four pulses EFS over time were expressed as a percentage of the initial control response to four pulses prior to addition of peptide/vehicle (100%). The effect of vehicle or NPY and related peptides on the sympathetic and vagal stimulus-response curves and the response to four pulses versus time were com-

Table 1 Values for resting atrial period (ms) in guinea-pig isolated right atria

	NPY treated atria			
	Sympathetic (n = 6)		*Vagus* (n = 6)	
Treatment	*control*	*treated*	*control*	*treated*
Vehicle	309 ± 13	313 ± 13	300 ± 14	305 ± 13
NPY (0.01 μM)	–	–	341 ± 17	343 ± 16
NPY (0.1 μM)	315 ± 8	326 ± 13	312 ± 8	319 ± 13
NPY (1 μM)	293 ± 8	293 ± 10	293 ± 8	293 ± 10
NPY (10 μM)	328 ± 11	334 ± 12	–	–
	N-*A[L]NPY(24–36) treated atria*			
	Sympathetic (*n* = 5)		*Vagus* (*n* = 5)	
	control	*treated*	*control*	*treated*
Vehicle	326 ± 24	324 ± 21	370 ± 31	360 ± 30
N-A[L]NPY(24–36) (0.01 μM)	–	–	326 ± 23	336 ± 18
N-A[L]NPY(24–36) (0.1 μM)	304 ± 15	301 ± 14	366 ± 22	375 ± 19
N-A[L]NPY(24–36) (11 μM)	297 ± 16	300 ± 21	332 ± 10	342 ± 15
N-A[L]NPY(24–36) (10 μM)	277 ± 9	278 ± 11	–	–
	LP-NPY treated atria			
	Sympathetic (*n* = 5)		*Vagus* (*n* = 5)	
	control	*treated*	*control*	*treated*
Vehicle	336 ± 13	332 ± 10	335 ± 13	332 ± 10
GR (0.3 μM)	329 ± 13	338 ± 8	319 ± 15	331 ± 10
LP-NPY (1 μM)	312 ± 7	314 ± 8	320 ± 8	327 ± 8
LP-NPY (10 μM)	350 ± 17	351 ± 21	328 ± 16	335 ± 12
LP-NPY (1 μM) + GR231118 (0.3 μM)	347 ± 9	341 ± 9	321 ± 20	318 ± 20
LP-NPY (10 μM) + GR231118 (0.3 μM)	334 ± 6	329 ± 7	334 ± 6	329 ± 7

Treatment groups shown are: NPY, neuropeptide Y; *N*-A[L]NPY(24–36), Y_2 receptor selective agonist N-Acetyl-]Leu^{28,31}]NPY(24–36); LP-NPY, Y_1 receptor selective agonist [Leu^{31},Pro^{34}]NPY; GR231118, selective Y_1 receptor antagonist. Values shown are resting arial period (interval between atrial beats, ms) before (control) and 30 min after (treated) incubation with a single concentration of peptide or vehicle (water). *n*, number of atria per group. Errors are ± s.e.mean.

pared between treatment groups by repeated measures ANOVA. The Greenhouse-Geisser estimate of epsilon was used as a correction for correlation (Ludbrook, 1994). In the case of vehicle-treated atria, the response to four pulses versus time was compared within group by two-way ANOVA. Values of resting atrial period at 0 or 30 min were compared between atrial groups for each peptide-treatment group by one-way ANOVA.

Sigmoid logistic concentration-atrial rate response curves for isoprenaline were constructed using absolute values (Nakashima *et al*., 1982) and values at effective concentration 50% (EC_{50}) were obtained from each fitted curve. Values for EC_{50} between atria were compared by Student's *t*-test for unpaired data. The full agonist concentration-response curves for both bethanechol and isoprenaline were also compared between treatment groups by repeated measures ANOVA as above. In all cases, statistical significance was accepted when $P<0.05$.

Results

Effect of NPY-related peptides on responses to EFS

Guinea-pig isolated right atria After 30 min incubation of the atria with NPY and related peptides or GR231118, there was no significant difference in the resting atrial period among all treatment groups when compared with their corresponding vehicle controls (Table 1). Application of field pulse stimulation to guinea-pig isolated right atria caused changes in atrial period that were proportional to the number of applied field pulses. In the presence of propranolol, incubation of the atria

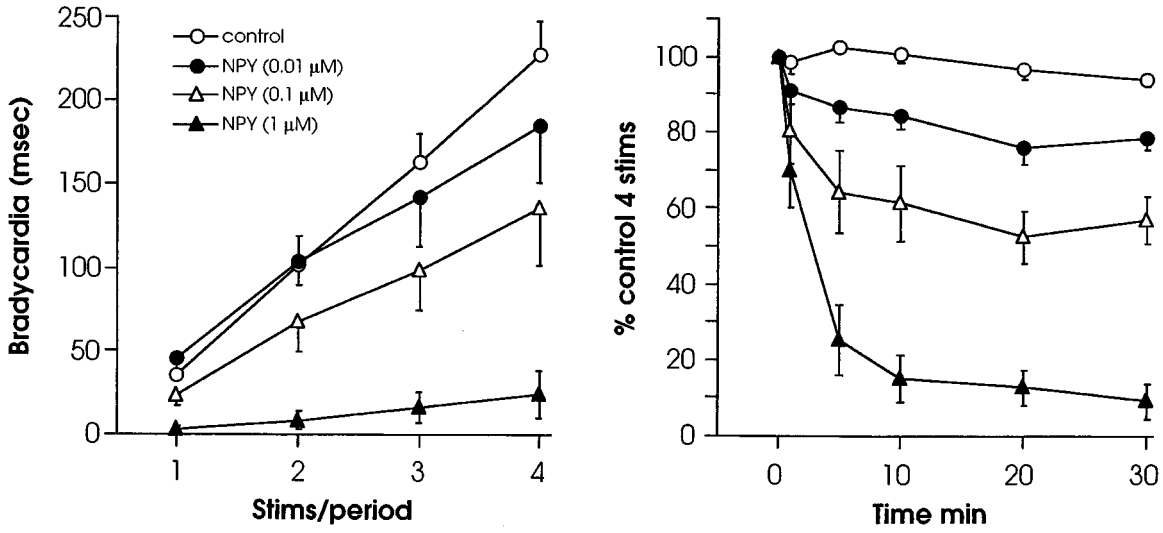

Figure 1 Left panel shows the effects of 30 min incubation with vehicle (water, $n=6$) or increasing concentrations of NPY (0.01 μM, $n=6$; 0.1 μM, $n=6$; 1 μM, $n=6$) on the bradycardic responses to electrical field stimulation in guinea-pig isolated right atria pretreated with propranolol (1 μM). Right panel shows the responses to four pulses of electrical field stimulation (expressed as a percentage of control) versus time for vehicle or each concentration of NPY. Error bars are s.e.mean.

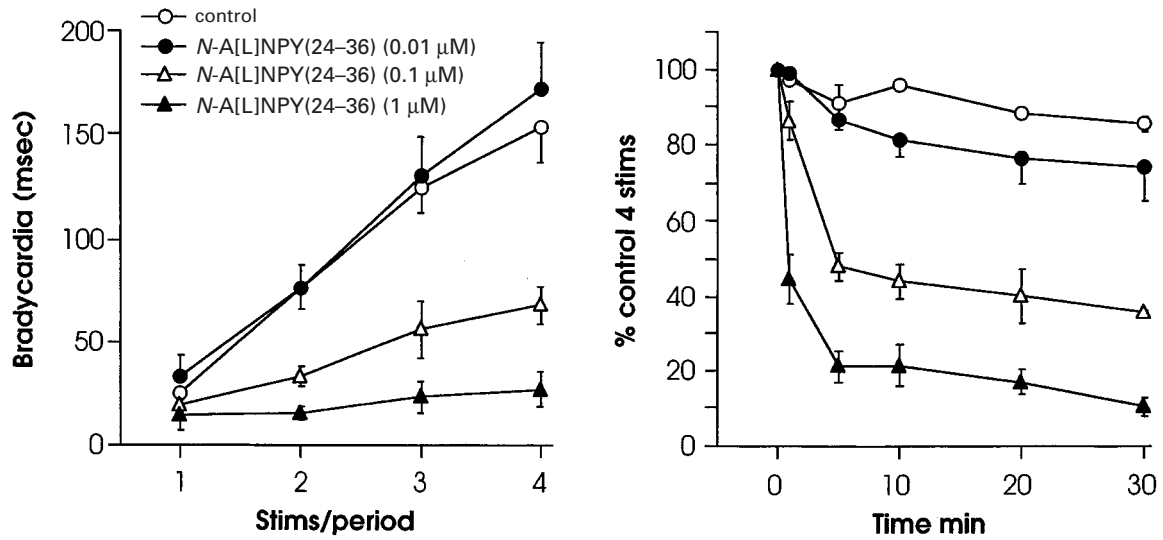

Figure 2 Left panel shows the effects of 30 min incubation with vehicle (water, $n=5$) or increasing concentrations of the Y_2 receptor agonist *N*-Acetyl-[$Leu^{28,31}$]NPY(24–36) (termed *N*-A[L]NPY(24–36); 0.01 μM, $n=5$; 0.1 μM, $n=5$; 1 μM, $n=5$) on the bradycardic responses to electrical field stimulation in guinea-pig isolated right atria pretreated with propranolol (1 μM). Right panel shows the responses to four pulses of electrical field stimulation (expressed as a percentage of control) versus time for vehicle or each concentration of *N*-A[L]NPY(24–36). Error bars are s.e.mean.

with increasing concentrations of NPY caused a concentration-dependent decrease in the degree of vagal slowing in response to field stimulation ($P=0.003$; Figure 1, left panel). The maximum concentration of NPY (1 μM) caused almost complete inhibition of the stimulation-induced increase in atrial period ($15\pm6\%$ of control, $n=6$, $P=0.001$; Figure 1, right panel). The inhibition of bradycardia by NPY reached a plateau after approximately 10 min incubation at each concentration and remained stable for the duration of the observation period (Figure 1, right panel). In the presence of vehicle, vagal responses showed a small time-dependent decrease of approximately 14% (*N*-A[L]NPY(24–36)-treatment group, $P=0.0015$, $n=5$) and 11% (LP-NPY-treatment group, $P=0.0131$, $n=5$) after 30 min.

Like NPY, the Y_2 receptor agonist *N*-A[L]NPY(24–36) was able to mimic the concentration-dependent inhibition of the vagally-mediated bradycardia (Figure 2, left panel, $P=0.016$), causing a similar degree of inhibition with the maximum concentration ($21\pm6\%$ with 1 μM; $n=5$) and following a similar time course with all concentrations (Figure 2, right panel). The Y_1 receptor agonist [Leu^{31},Pro^{34}]NPY also inhibited vagal responses to EFS (Figure 3). However, the maximum concentration applied (10 μM) decreased responses to only $71\pm7\%$ of control after 10 min ($P=0.0264$, $n=5$). This inhibition was relatively stable and not significantly different in the presence of the Y_1 receptor selective antagonist GR231118 (Figure 3, right panel; $81\pm6\%$ with 10 μM LP-NPY; $n=5$), suggesting that at the concentration used in these experiments, [Leu^{31},Pro^{34}]NPY was no longer acting selectively at Y_1 receptors and the inhibition observed was most likely due to agonist activity at Y_2 receptors.

In the presence of atropine, NPY caused a concentration-dependent inhibition of the sympathetically-mediated tachycardia ($P=0.0001$, Figure 4, left panel), however, the maximum concentration of NPY (10 μM) decreased the sympathetic response to EFS to only $37\pm5\%$ of control

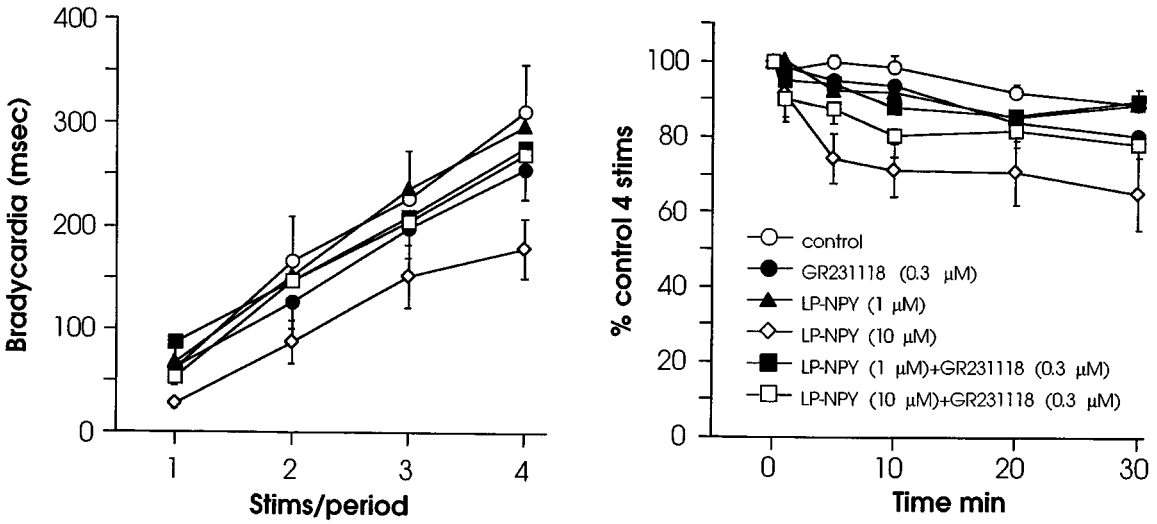

Figure 3 Left panel shows the effects of 30 min incubation with vehicle (water, $n=6$), the Y_1 receptor agonist [Leu^{31},Pro^{34}]NPY (LP-NPY), the Y_1 receptor antagonist GR231118 (0.3 μM) or [Leu^{31}, Pro^{34}]NPY in the presence of the Y_1 receptor antagonist GR231118 (0.3 μM) on the bradycardic responses to electrical field stimulation in guinea-pig isolated right atria pretreated with propranolol (1 μM). Right panel shows the responses to four pulses of electrical field stimulation (expressed as a percentage of control) versus time for vehicle or each treatment group. Error bars are s.e.mean. $n=5$ per group.

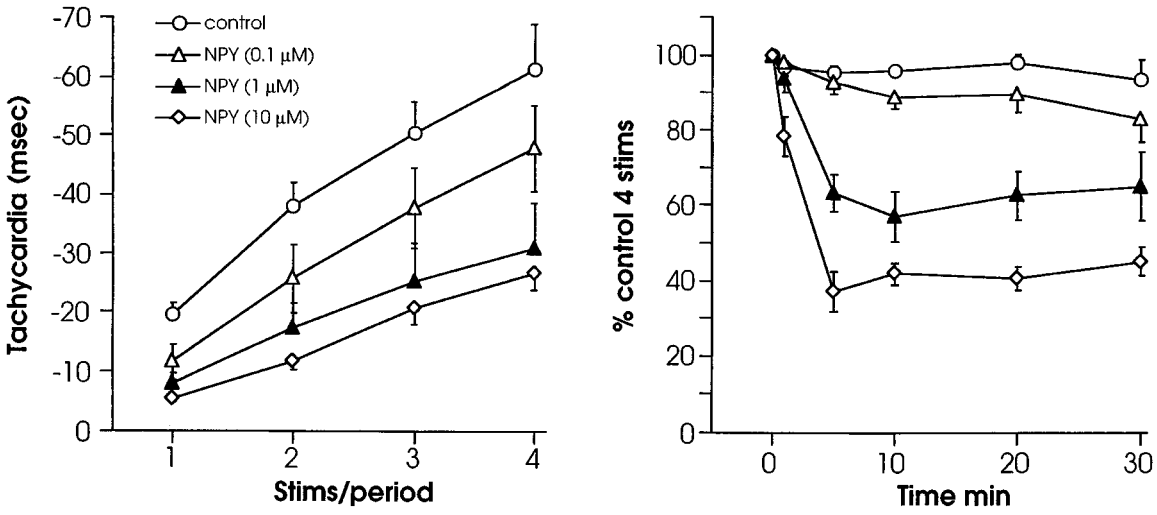

Figure 4 Left panel shows the effects of 30 min incubation with vehicle (water, $n=6$) or increasing concentrations of NPY (0.1 μM, $n=6$; 1 μM, $n=6$, 10 μM, $n=6$) on the tachycardic responses to electrical field stimulation in guinea-pig isolated right atria pretreated with atropine (1 μM). Right panel shows the responses to four pulses of electrical field stimulation (expressed as a percentage of control) versus time for vehicle or each concentration of NPY. Error bars are s.e.mean.

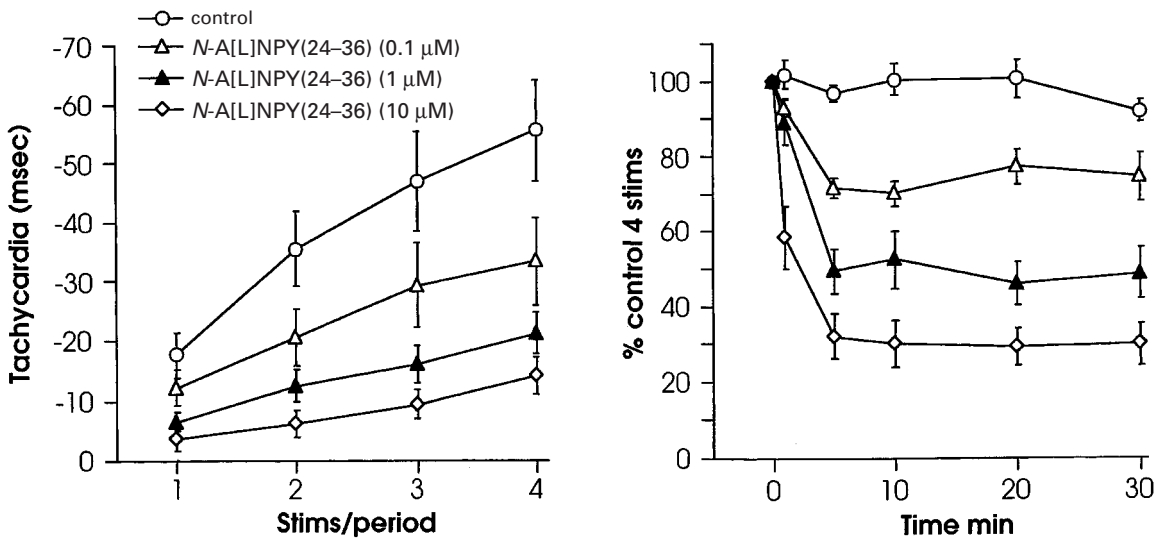

Figure 5 Left panel shows the effects of 30 min incubation with vehicle (water, $n=5$) or increasing concentrations of the Y_2 receptor agonist *N*-Acetyl-[Leu^{28,31}]NPY(24–36) (termed *N*-A[L]NPY(24–36); 0.1 μM, $n=5$; 1 μM, $n=5$; 10 μM, $n=5$) on the tachycardic responses to electrical field stimulation in guinea-pig isolated right atria pretreated with atropine (1 μM). Right panel shows the responses to four pulses of electrical field stimulation (expressed as a percentage of control) versus time for vehicle or each concentration of *N*-A[L]NPY(24–36). Error bars are s.e.mean.

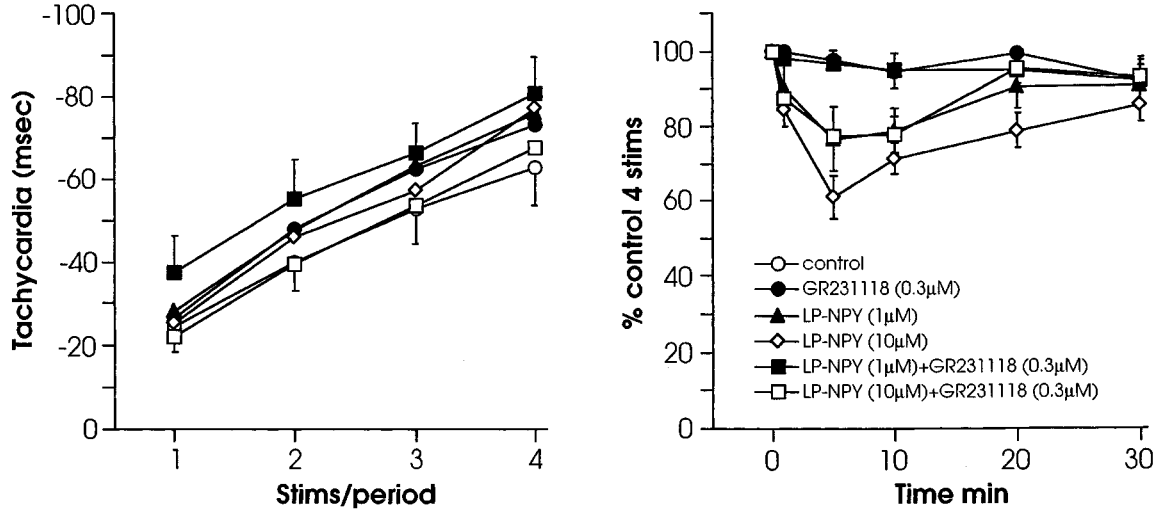

Figure 6 Left panel shows the effects of 30 min incubation with vehicle (water, $n=6$), the Y_1 receptor agonist [Leu^{31},Pro^{34}]NPY (LP-NPY), the Y_1 receptor antagonist GR231118 (0.3 μM) or [Leu^{31},Pro^{34}]NPY in the presence of the Y_1 receptor antagonist GR231118 (0.3 μM) on the tachycardic responses to electrical field stimulation in guinea-pig isolated right atria pretreated with atropine (1 μM). Right panel shows the responses to four pulses of electrical field stimulation (expressed as a percentage of control) versus time for vehicle or each treatment group. Error bars are s.e.mean. $n=5$ per group.

Table 2 Values for resting atrial period (ms) in rabbit isolated right atria

	Sympathetic ($n=5$)		*Vagus* ($n=6$)	
Treatment group	*control*	*treated*	*control*	*treated*
Vehicle	402±16	421±17	391±16	407±19
NPY (1–10 μM)	377±18	404±26	363±13	388±16
LP-NPY (10 μM)	363±11	397±15	–	–
NPY (10 μM)+GR231118 (0.3 μM)	368±18	379±22	–	–

Treatment groups shown are: NPY, neuropeptide Y; LP-NPY, Y_1 receptor selective agonist [Leu^{31},Pro^{34}]NPY; GR231118, selective antagonist. Values shown are resting arial period (interval between atrial beats, ms) before (control) and 30 min after (treated) incubation with a single concentration of peptide or vehicle (water). *n*, number of atria per group. Errors are ±s.e.mean.

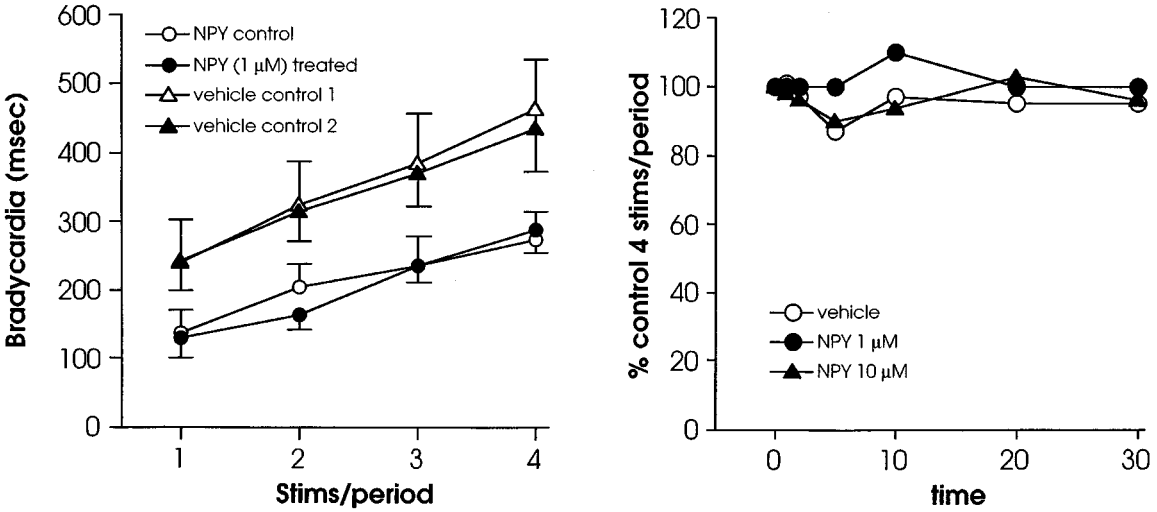

Figure 7 Left panel shows the bradycardic response to electrical field stimulation in rabbit isolated right atria, pretreated with propranolol (1 μM) before and 30 min after incubation with vehicle (water, $n=6$) or NPY (1 μM, $n=6$). Right panel shows the responses to four pulses of electrical field stimulation (expressed as a percentage of control) versus time for single atria pretreated with either vehicle or NPY (1 or 10 μM). Error bars are s.e.mean.

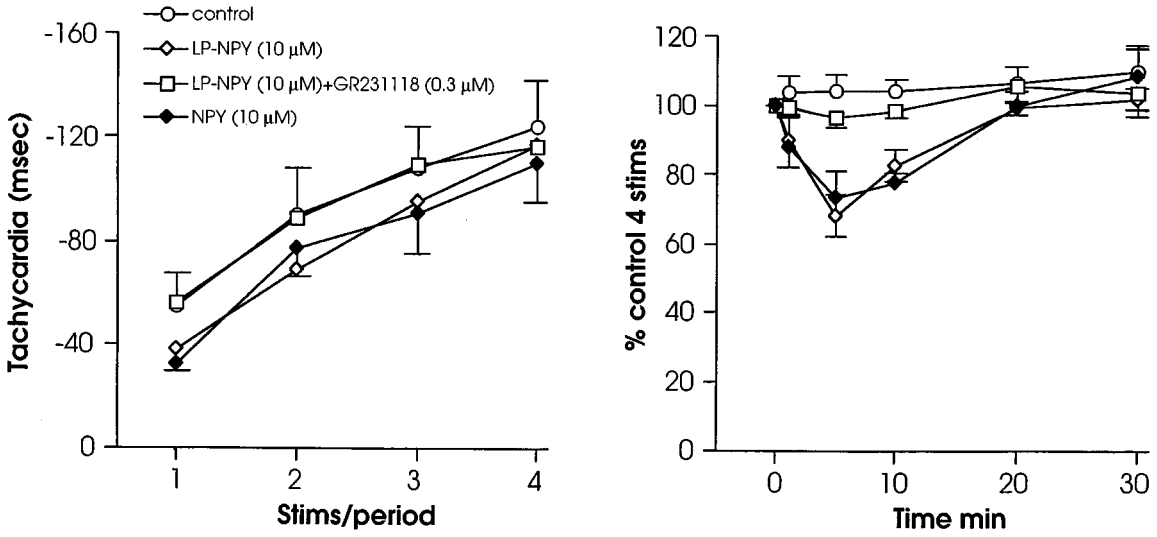

Figure 8 Left panel shows the effects of 30 min incubation with vehicle (water, $n=6$), NPY (10 μM), $n=5$), the Y_1 receptor agonist [Leu^{31},Pro^{34}]NPY (10 μM, $n=5$) or NPY (10 μM) in the presence of the Y_1 receptor antagonist GR231118 (0.3 μM, $n=5$) on the tachycardic responses to electrical field stimulation in rabbit isolated right atrial pretreated with atropine (1 μM). Right panel shows the responses to four pulses of electrical field stimulation (expressed as a percentage of control) versus time for vehicle or each treatment group. Error bars are s.e.mean

(Figure 4, right panel). The time course of inhibition was similar to NPY's action on the vagus, with the inhibition caused by each concentration reaching a plateau after approximately 10 min. Incubation of the tissue with *N*-A[L]NPY(24–36) caused a similar degree of sympathetic inhibition to NPY ($P=0.0165$, 30 ± 6% of control, 10 μM *N*-A[L]NPY(24–36)) and followed a similar time course (Figure 5, right panel).

Incubation of the atria with [Leu^{31},Pro^{34}]NPY caused a concentration-dependent, transient inhibition of sympathetically mediated tachycardia ($P=0.0096$). Inhibition reached a maximum after approximately 5 mins (61 ± 6% with 10 μM, $n=5$) and then rapidly returned to baseline (Figure 6, right panel). This transient inhibition was antagonized by pretreatment of the tissue with GR231118 (0.3 μM) (Figure 6, right panel). A single estimate of the pA_2 for GR231118 was 7.5 which is consistent with activity at a Y_1-receptor (Lew *et al.*, 1996). After incubating the atria for 30 min with either [Leu^{31},Pro^{34}]NPY, vehicle or antagonist, sympathetic responses to 1–4 field pulses were no different between treatment groups (Figure 6, left panel).

Incubation of guinea-pig atria with NPY (1 or 10 μM) had no effect on postjunctional muscarinic receptor or β-adrenoceptor-mediated changes in atrial period as indicated by no change in sensitivity or range of the bethanechol or isoprenaline concentration-response curves, respectively (data not shown).

Rabbit isolated right atria In the rabbit right atrium, incubation of the tissue with 1 μM NPY had no effect on either resting atrial rate (Table 2), the EFS-induced vagal slowing (Figure 7, left panel, $n=6$) or the response to

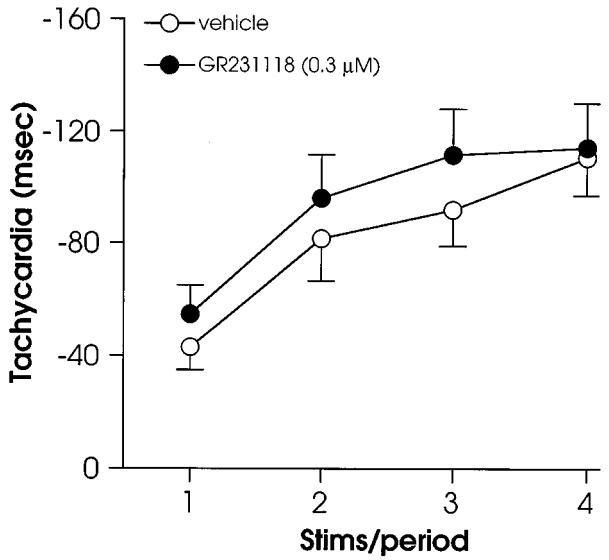

Figure 9 Tachycardic responses to electrical field stimulation in rabbit isolated right atria pretreated with atropine (1 μM). The data shown are the control stimulus response curves for the data set in Figure 7, prior to addition of peptide. Curves are 30 min after pretreatment with either vehicle (water, 15 μl, $n=5$) or GR231118 (0.3 μM, $n=5$). Error bars are s.e.mean.

muscarinic receptor stimulation caused by bethanechol (data not shown). NPY (1 or 10 μM, $n=1$, respectively) had no effect on the vagal response to four pulses at any time point (Figure 7, right panel) indicating that the lack of effect of NPY was not due to a desensitization of the prejunctional receptors. NPY (10 μM) did, however, transiently inhibit the sympathetic response to EFS, the effect reaching a maximum after approximately 5 min incubation and then rapidly fading to be no different from control at 30 min (Figure 8, right panel). This effect of NPY was most likely due to its activity at prejunctional receptors, NPY having no effect on the chronotropic response to β-adrenoceptor stimulation by isoprenaline (data not shown).

This effect of NPY was mimicked by [Leu^{31},Pro^{34}]NPY which caused a similar degree of inhibition ($73\pm8\%$ of control, $n=5$) and then rapidly desensitized (Figure 9, right panel). The inhibition by [Leu^{31},Pro^{34}]NPY was also completely abolished by the Y_1 receptor antagonist GR231118 (0.3 μM). *N*-A[L]NPY(24–36) (10 μM, $n=1$) had no effect on the sympathetic response to EFS (data not shown). Preincubation with GR231118 (0.3 μM) also had no effect on the sympathetic response to EFS compared to vehicle treated atria (Figure 9, $n=5$ control and GR231118-treated atria, respectively).

Discussion

We have found that NPY is not a universal prejunctional inhibitory agonist in cardiac autonomic transmission. In the guinea-pig isolated right atrium, exogenous NPY is a potent inhibitor of cardiac sympathetic and vagal neurotransmission. But in the rabbit isolated right atrium, exogenous NPY has only weak effects in inhibiting cardiac sympathetic responses and has no effect on vagal neurotransmission. Furthermore, NPY did not affect postjunctional receptor activation following concentration-atrial response curves to either isoprenaline or bethanechol in either the guinea-pig or rabbit isolated right atrium. Thus, the lack of effect of NPY on vagal neurotransmission, and its low potency at inhibiting sympathetically-mediated tachycardia in the rabbit atrium was not the result of any confounded effect from an enhanced postjunctional response to endogenous acetylcholine or noradrenaline. This would also suggest that the inhibitory effect of NPY on cardiac autonomic neurotransmission in the guinea-pig atrium is mediated *via* a prejunctional effect on neurotransmission. Furthermore, NPY (rabbit only) and LP-NPY transiently affected sympathetic transmission in the rabbit and guinea-pig atrium but only at high concentrations that are unlikely to be achieved in the intact animal. These data also provide evidence for the first time suggesting the possible existence of putative prejunctional Y_1 receptors mediating functional responses in the guinea-pig and rabbit isolated right atrium.

The transient inhibitory effect of NPY on the cardiac sympathetic responses in the rabbit isolated right atrium was mimicked by the Y_1 receptor selective agonist [Leu^{31},Pro^{34}]NPY and inhibited by the Y_1 receptor selective antagonist GR231118. The lack of effect of the Y_2-receptor selective agonist *N*-A[L]NPY(24–36) would suggest that functional Y_2-receptors are absent prejunctionally in cardiac nerve varicosities of this species. Allen *et al.* (1993) have demonstrated expression of Y_1 receptors on rabbit cardiac postganglionic sympathetic neurons. This would concur with our findings in the rabbit isolated right atrium where the profile of relative agonist potencies is consistent with NPY's action at a Y_1-receptor. However, the antagonism by GR231118 was greater than would be expected for a pA_2 value of 7.5 and may suggest that the prejunctional receptor located on sympathetic varicosities in the rabbit heart may have different properties to that of vascular or postjunctional Y_1 receptors or even prejunctional Y_1 receptors of other species. Indeed, at the prejunctional receptor in the rabbit isolated right atrium, 0.3 mM GR231118 completely abolished the inhibitory response to 10 μM NPY and [Leu^{31},Pro^{34}]NPY. This is in contrast to the responses we observed in the guinea-pig isolated right atrium where GR231118 only shifted the inhibition elicited by [Leu^{31},Pro^{34}]NPY on sympathetically-mediated tachycardia. A single estimate of the pA_2 from this data was consistent with GR231118's interaction with the vascular Y_1 receptor in the rat (Lew *et al.*, 1996). Modulation of sympathetic neurotransmission by prejunctional Y_1-receptors has also been previously reported in the rabbit isolated vas deferens (Doods & Krause, 1991; Palea *et al.*, 1995). However, NPY appears to be a much more potent agonist in this tissue than in the rabbit isolated right atrium; both NPY and [Leu^{31},Pro^{34}]NPY inhibited the vas deferens twitch response with a $pEC_{50}>8$, suggesting that there is regional variation in the role for NPY in neuromodulation even between tissues of the same species. We have also demonstrated that NPY inhibits the twitch response to nerve stimulation in the rabbit isolated vas deferens *via* a receptor that is sensitive to GR231118 (unpublished observations), confirming the likely existence of a prejunctional Y_1-receptor (or non-Y_2 receptor) in this tissue. Prejunctional Y_1-receptors have also been shown to mediate an inhibition of noradrenaline overflow following sympathetic nerve stimulation of the portal vein in conscious rats (Coppes *et al.*, 1994) and in the rat isolated perfused mesenteric arterial bed preparation (Mangel *et al.*, 1991; McAuley & Westfall, 1992). Although the findings of these studies were based on agonist order of potency only (Coppes *et al.*, 1994) or in conjunction with the use of benextramine as a 'selective' Y_1-receptor antagonist (McAuley & Westfall, 1992), these previous reports, coupled with our

current findings in the guinea-pig atria suggest the possibility exists that NPY Y_2-receptors may not be the only receptors to mediate prejunctional effects of NPY in species other than the rabbit.

The low potency of NPY at the cardiac neuroeffector junction in the rabbit may be a reflection of low numbers of NPY receptors in the heart of this species. However, autoradiographic evidence suggests that there are high concentrations of binding sites for [^{125}I]-PPY in all chambers of the rabbit heart (Allen *et al*., 1993). These sites, which show an agonist potency profile in competition studies that is consistent with the Y_1-receptor, are only being associated with vascular smooth muscle, no detectable binding being observed on the myocardium itself (Allen *et al*., 1993). The absence of NPY receptors on the myocardial cell membrane is consistent with our observations that NPY had no direct effect on atrial rate nor potentiated agonist concentration-response curves. In this study, no attempt was made to quantitate the effect of NPY and related peptides on atrial force of contraction, however, qualitatively NPY had no observable effect on basal contractile force in either the rabbit or guinea-pig isolated right atrium.

The lack of enhanced sympathetic response to EFS in the presence of GR231118 may indicate that under these conditions of stimulation, autoinhibition by endogenous NPY plays no role in modulation of cardiac function in the rabbit isolated right atrium. Release of endogenous NPY from sympathetic nerves is reported to occur preferentially at higher frequencies of stimulation (Lundberg *et al*., 1986). We used low frequency stimulation (1 Hz) to elicit depolarization of sympathetic nerve varicosities in the experiments examining the effect of NPY on sympathetic responses to EFS in the rabbit isolated right atrium in order to minimize the possibility of NPY release and therefore minimize the potential for endogenous NPY to desensitize its own receptors. However, others have shown that endogenous NPY may be released even at physiological frequencies of stimulation (Warner & Levy, 1989b) and this may have influenced the tachyphylaxis we observed after incubation with a high concentration (10 μM) of exogenous NPY. Preincubation of the guinea-pig isolated atria with GR231118 also had no effect on the sympathetic or vagal response to field stimulation, even though higher frequencies of stimulation were used to elicit sympathetic responses. However, unlike in the rabbit, this does not necessarily rule out the possibility that endogenous NPY may modulate sympathetic and vagal neurotransmission, as GR231118 would not affect any modulation occurring at Y_2 receptors. The only conclusion that can be drawn from these observations is that Y_1 receptors, located prejunctionally on cardiac autonomic nerve terminals in the guinea-pig, do not appear to function as autoinhibitory receptors under these experimental conditions.

In the guinea-pig isolated right atrium, the Y_2-selective agonist *N*-A[L]NPY(24 – 36) had a time course and potency of inhibition of sympathetic and vagal responses to EFS very similar to NPY, consistent with the theory that prejunctional Y_2-receptors mediate NPY's inhibitory action on the cardiac sympathetic and vagal nerve varicosities. The stable inhibition of both sympathetic and vagal responses in the guinea-pig isolated atrium is consistent with the prolonged inhibition of the cardiac vagus that is observed after high-frequency stimulation of sympathetic nerves in the anaesthetized dog (Potter, 1985; 1987). Our experiments also show that NPY is a more potent agonist at prejunctional receptors on cardiac vagal than on cardiac sympathetic nerve terminals in the guinea-pig isolated right atrium. This raises questions as to the nature of the functional role for NPY acting at the cardiac neuroeffector junction. At the cardiac sympathetic synapse, autoinhibition of sympathetic transmission may only occur at high synaptic levels of NPY. This would be consistent with NPY release only occurring at high levels of sympathetic activity. During lower levels of sympathetic drive, neuronally released NPY may act primarily to inhibit the system that functionally opposes adrenergic effects on the heart in vagal neurotransmission. However, without a selective Y_2 receptor antagonist, the functional role for endogenous NPY, if any, in cardiac neurotransmission remains speculative.

The lack of postjunctional modulation or potentiation of the effects of exogenous agonists acting at the sinoatrial node has been reported by others in dog (Potter, 1987; Ren *et al*., 1991), rat (Pardini *et al*., 1992) and guinea-pig (Lundberg *et al*., 1984; Pardini *et al*., 1992). These experiments suggest that the site of action of NPY at the parasympathetic neuroeffector junction is most likely to be prejunctional. Pardini *et al*. (1992) have demonstrated that NPY's action may be at the level of preganglionic neurons whilst a study by Potter (1987) established that ganglion blockade had no effect on the inhibition elicited by NPY on vagal responses in the guinea-pig isolated right atrium, suggesting that prejunctional NPY receptors were located on postganglionic nerve terminals. In our study, we are unable to distinguish between the prejunctional sites of action of NPY. Clearly, further investigation is required to clarify these discrepancies.

These data confirm our previous findings that NPY has no effect on vagal neurotransmission in the conscious rabbit (Serone *et al*., 1998). The transient nature of the inhibition elicited by high concentrations of NPY and [Leu^{31},Pro^{34}]NPY supports the conclusion that the decrease in the range of the sympathetic component of the baroreflex after administration of peptides was most likely a consequence of the pressor response in the conscious rabbit (Serone *et al*., 1998). These data would also suggest there is substantial variation for the role of NPY in regulating cardiovascular function between species and also provide evidence for further variation in the localization of NPY-receptor subtypes. Due to the lack of effect of NPY on cardiac vagal neurotransmission and its low potency at inhibiting sympathetic responses, it would be surprising if NPY plays a functional role in modulating cardiac autonomic neurotransmission in the rabbit. The results of this study also indicate that NPY may be an important mediator of interactions between cardiac sympathetic and vagal neurotransmission in the guinea-pig.

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