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Neuropeptide Y is a prejunctional inhibitor of vagal but not sympathetic inotropic responses in guinea-pig isolated left atria

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1 The effects of NPY and related peptides were examined on basal contractile force and nerve-mediated inotropic responses to electrical field stimulation of the guinea-pig isolated left atrium.

2 Electrical field stimulus (EFS)-inotropic response curves were constructed by applying 1-64 trains of four field pulses (200 Hz, 0.1 ms duration, 100 V) across isolated left atria (paced at 4 Hz, 2 ms, 1–4 V) within the atrial refractory period. Curves were constructed in presence of vehicle, propranolol (1 μM) or atropine (1 μM) to determine appropriate stimulus conditions.

3 The effects of PYY (1–10,000 nM), NPY (0.01–10 μM), *N*-Ac-[Leu^{28,31}]NPY(24–36) (*N*-A[L]NPY(24–36); 0.01–10 μM) and clonidine (0.1–1000 nM) were examined on the positive and negative inotropic responses to EFS (eight trains, four pulses per refractory period).

4 NPY-related peptides had no effect on basal force of contraction nor on the inotropic concentration-response curves to bethanechol or isoprenaline. All three peptides inhibited vagally-mediated negative inotropic responses; rank order of potency PYY > NPY $\geqslant$ *N*-A[L]NPY(24–36) was consistent with an action at prejunctional Y_2-receptors. Clonidine concentration-dependently inhibited sympathetic inotropic responses. However, PYY, NPY and *N*-A[L]NPY(24–36) failed to mediate any significant inhibition of the positive inotropic response to EFS.

5 These data demonstrate that NPY is an effective inhibitor of vagal but not sympathetically-mediated inotropic responses in the guinea-pig isolated left atria. This may suggest that endogenously co-released NPY is important in mediating cross talk between efferent components of the autonomic nervous system modulating cardiac contractility, acting overall to sustain positive inotropic responses.

Keywords: Neuropeptide Y; cardiac contractility; neurotransmission; inotropic; sympathetic; vagal

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

Introduction

Extensive networks of neuropeptide Y (NPY)-immunoreactive nerve fibres exist in the mammalian heart, innervating both the coronary vasculature and myocardium (Allen *et al*., 1986). This has led to suggestions that NPY may have a functional role in the regulation of coronary blood flow and cardiac contractility. In light of this hypothesis, there has been extensive investigation into the effects of NPY on modulation of cardiac function. At least three subtypes of NPY receptors have been demonstrated to occur postjunctionally on the myocardial cell membrane, namely Y_1 (McDermott *et al*., 1997), Y_2 (McDermott *et al*., 1997) and the putative Y_3 receptor that is insensitive to PYY (Balasubramaniam *et al*., 1990). The localization of these receptors on the postjunctional cell membrane of myocardial tissues may suggest that NPY is capable of modulating cardiac function by direct effects on myocardial contractility. In support of a direct negative inotropic effect of NPY, the peptide causes inhibition of isoprenaline stimulated accumulation of cyclic AMP in rat isolated cardiomyocytes (Millar *et al*., 1988; Piper *et al*., 1989; Millar *et al*., 1991) and contractile responses to electrical stimulation (Piper *et al*., 1989). Both responses are sensitive to preincubation of the cells with pertussis toxin, suggesting coupling of NPY receptors to adenylate cyclase *via* an inhibitory G-protein (Millar *et al*., 1988; Piper *et al*., 1989). However, exogenous NPY has been shown to exert variable effects on myocardial contractility in isolated cardiac muscle preparations of several species. NPY elicits concentration-dependent inhibition of basal contractile force in rat (Balasubramaniam *et al*., 1988) and canine (Rigel *et al*., 1989) isolated cardiac muscle. However, others have reported no direct effect (Allen *et al*., 1986; Franco-Cereceda *et al*., 1987; Wahlestedt *et al*., 1987; Michel *et al*., 1989; Scott *et al*., 1990; Amerini *et al*., 1991; Woo *et al*., 1994; Woo & Ganguly, 1995), or even a positive inotropic effect (Lundberg *et al*., 1984; Franco-Cereceda *et al*., 1985) of NPY on myocardial force of contraction.

In the guinea-pig myocardium, highest levels of detectable NPY-like immunoreactivity are found in the atrial tissue, in particular the left atrium (Allen *et al*., 1986). Furthermore, exogenous NPY has been shown to exert direct positive inotropic and chronotropic effects in guinea-pig isolated right atrial preparations (Lundberg *et al*., 1984; Franco-Cereceda *et al*., 1985). In the same tissue, NPY has also been shown to mediate inhibition of both chronotropic and inotropic nerve-mediated responses to field stimulation (Lundberg *et al*., 1984; Franco-Cereceda *et al*., 1985; Amerini *et al*., 1991). However, these studies examining the effects of NPY on nerve-mediated inotropic responses utilized only a single concentration of NPY and therefore characterization of the receptor mediating this effect has not been performed. Recently, we have demonstrated that NPY and the Y_2-receptor selective agonist N-Ac-[Leu^{28,31}]NPY(24–36) caused stable inhibition of vagal and sympathetic chronotropic responses to field

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stimulation (Serone *et al*., 1998). However, the relatively selective Y_1-receptor agonist [Leu^{31},Pro^{34}]NPY was also able to cause transient inhibition of sympathetic nerve-mediated increases in atrial rate that was competitively antagonized by the Y_1-receptor selective antagonist GW1229 (Serone *et al*., 1998). Therefore, in light of the heterogeneity of prejunctional NPY receptors mediating inhibition of chronotropic responses to field stimulation, the present study was designed to further explore the effects of NPY and related peptides on cardiac autonomic control by examining their effects on basal contractility and nerve-mediated inotropic responses of the guinea-pig isolated left atrium. In order to characterize the receptor or receptors mediating these effects, full agonist concentration-response curves were constructed.

Methods

General

Guinea-pigs of either sex (445 ± 17 g, range 300 – 715 g, $n = 52$) were used in this study. This study was approved by the University of Melbourne Animal Ethics and Experimentation Committee in accordance with the guidelines of the National Health and Medical Research Council of Australia. Guinea-pigs were killed by cervical dislocation. The heart was rapidly removed, placed in oxygenated Krebs' solution (see below) and maintained at 37°C. The left 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.

Atria rested against two punctate platinum electrodes (3 mm apart) and were stimulated with square wave pulses (4 Hz, 2 ms, twice threshold voltage) delivered *via* one channel of a Grass S88C dual stimulator. The signal from the punctate stimulation was 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* the second channel of the 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 force that were linear with respect to the number of applied field pulses. The signal from both channels of the stimulator were monitored on a dual beam 10 MHz storage oscilloscope. The signal from the force transducer was amplified and atrial force of contraction continuously recorded on a chart recorder (Neotrace 600ZF).

Protocol

Determination of field stimulation parameters Atria were washed every 5 min for 30 min with Krebs' solution before examining the inotropic responses to electrical field stimulation (EFS). Control stimulus-response curves to EFS were constructed (stimulus conditions above) by applying between 1-64 trains where each train consisted of four field pulses per refractory period following the punctate or driving pulse. Atria were then incubated with either vehicle (water 15 μl), propranolol (1 μM) or atropine (1 μM) for 30 min and a second stimulus-response curve constructed. The stimulus that elicited approximately 50% of the maximum positive and negative inotropic response to EFS was chosen to study the effect of NPY and related peptides on vagal and sympathetic neurotransmission in subsequent experiments.

Sympathetic inotropic responses to EFS Atria were washed every 5 min for 30 min in Krebs' containing 1 μM atropine. At the end of this equilibration period the response to electrical field stimulation (EFS) was then assessed (as above) by applying four field pulses per refractory period (0.1 ms duration, 200 Hz, 100 V on S88 dial) for eight consecutive trains (see Results section for choice of stimulus). The subsequent increase in atrial force (g) was measured. A second control stimulus (C2) was performed 15 min after the initial response to EFS to test the reproducibility of the inotropic response. The effect of agonists on the inotropic response to EFS was then examined by constructing a single cumulative concentration-response curve to either clonidine (0.1 – 1000 nM), PYY (0.01 – 10 μM), NPY (single experiment only; 0.1 – 10 μM) or N-Ac-[Leu^{28,31}]NPY(24 – 36) (*N*-A[L]NPY(24 – 36); single experiment only; 0.01 – 10 μM). Atria were incubated with each concentration of peptide or clonidine for 10 min (Serone *et al*., 1998) and the inotropic response to EFS reassessed between concentrations. In time control experiments the response to EFS was assessed after the appropriate drug-free incubation time. After assessing the effects of PYY or time on the positive inotropic response to EFS, a single cumulative concentration-inotropic response curve to the β-adrenoceptor agonist isoprenaline (1 – 1000 nM) was constructed at the end of the experiment.

In separate experiments, the effects of PYY and NPY on the positive inotropic response to one and two trains of field pulses were examined. Atria were set up as above. The inotropic response to one and two trains EFS (four and eight pulses, respectively) was then assessed, with 10 min rest between stimuli. This regimen was then repeated after 10 min to test reproducibility of responses before incubating the atria with increasing concentrations of NPY (0.1 – 10 μM) or PYY (0.1 – 10 μM). The positive inotropic response to four and eight pulses was assessed after incubation of each peptide concentration.

Vagal inotropic responses to EFS Atria were washed every 5 min for 30 min in Krebs' containing 1 μM propranolol and 1 μM clonidine. Clonidine was included in the Krebs' solution because a small but significant residual positive inotropic response to eight trains EFS was observed with propranolol alone (Figure 1). The concentration of propranolol was not increased further because in an anecdotal experiment 3 μM propranolol appeared to decrease the vagal response to EFS. Clonidine had no effect on the negative inotropic response to EFS but appeared to further decrease the residual positive inotropic response (Figure 1). At the end of the equilibration period, the response to electrical field stimulation (EFS) was then assessed (as above) by applying a train of four field pulses per punctate stimulation (0.1 ms duration, 200 Hz, 100 V on S88 dial) for eight consecutive trains. The subsequent decrease in atrial force (g) was measured. A second control stimulus was performed 15 min after the initial response to EFS to test the reproducibility of the inotropic response (C2). A single cumulative concentration-response curve to either PYY (0.001 – 1 μM), NPY (0.01 – 10 μM) or N-Ac-[Leu^{28,31}]NPY (24 – 36) (*N*-A[L]NPY(24 – 36); 0.01 – 10 μM) was then con-

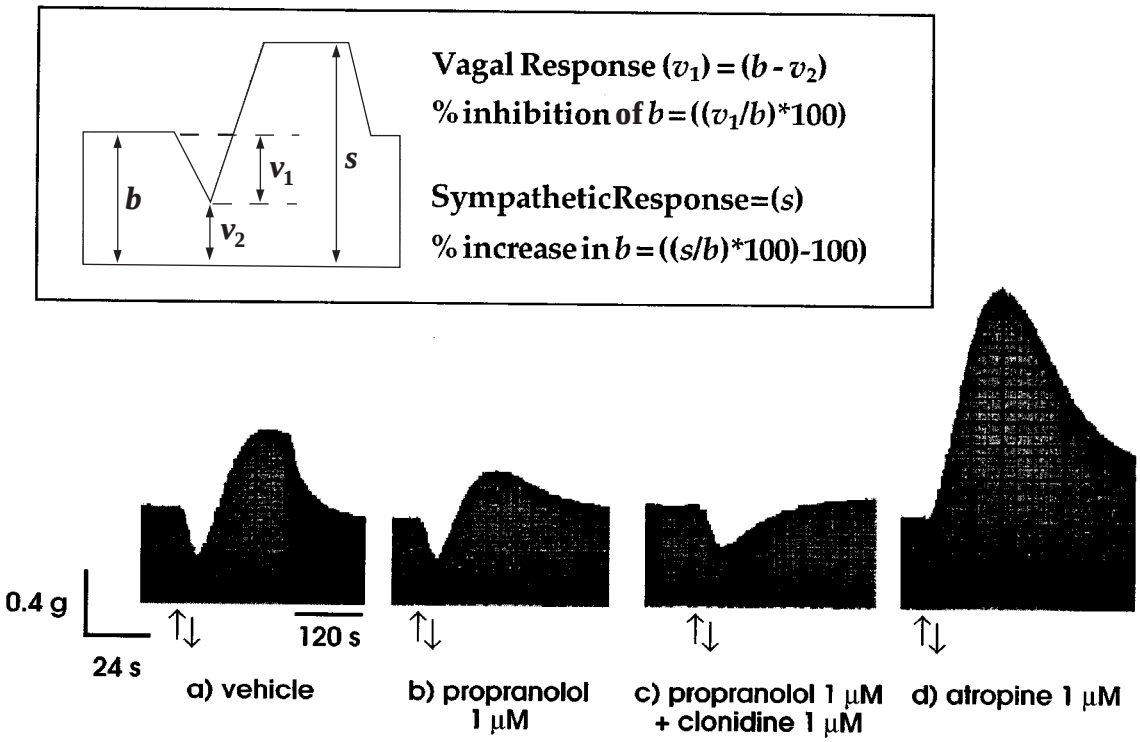

Figure 1 Representative trace recordings of the inotropic response to eight trains electrical field stimulation (EFS; each train was four pulses per refractory period, see Methods) in four separate guinea-pig isolated left atria. EFS was passed across the atria as indicated by the upward and downward arrows. (a) Control (vehicle) inotropic response to EFS. Second calibration bar on (a) indicates slowing of the chart speed from 25 to 5 mm min^{-1}; (b) inotropic response to EFS in the presence of 1 μM propranolol; (c) inotropic response to EFS in the presence of 1 μM propranolol + 1 μM clonidine; (d) inotropic response to EFS in the presence of 1 μM atropine. Insert shows a schematic representation of the analysis of the inotropic responses to EFS (see Methods). b is the baseline force of contraction immediately prior to EFS. V_1 is the vagally-mediated decrease in punctate force of contraction, calculated as the difference between b and the peak force of contraction at the end of EFS (V_2). The sympathetically-mediated positive inotropic response to EFS is measured as the difference between the maximum increase in the force of contraction following EFS (s) and b.

structed. Each concentration of NPY or related peptides was incubated for 10 min (Serone *et al*., 1998) before reassessing the negative inotropic response to EFS. Time control experiments had no drug administered but the responses to EFS were assessed after the appropriate incubation time. At the end of each experiment a single cumulative concentration-inotropic response curve to the muscarinic agonist bethanechol (0.1–100 μM) was constructed.

Drugs

Drugs used were freshly prepared in ultra-filtered water (Milli Q UV) and included atropine sulphate (Sigma, St Louis, MO, U.S.A.), carbamyl-β-methylcholine chloride (bethanechol chloride, Sigma), clonidine hydrochloride (Boehringer, Ingelheim), (−)isoprenaline hydrochloride (Sigma) and propranolol hydrochloride (Sigma). Peptides, neuropeptide Y, *N*-Acetyl-[Leu^{28,31}]NPY(24–36) and peptide YY were synthesized by Dr Roger Murphy, Department of Pharmacology, University of Melbourne, Victoria, Australia. Homogeneity of the peptides were confirmed by analytical H.P.L.C. and capillary electrophoresis (Lew *et al*., 1996). Stock solutions of peptides were made prior to use and frozen as aliquots. Each aliquot was used only once, immediately after thawing.

Analysis and statistical methods

Parameter measurement and agonist concentration-responses curves Data are presented as mean ± 1 standard error of the mean (s.e.mean). The decrease in force of contraction of the atria (V_1, vagal response, Figure 1) following EFS was calculated by subtracting the force of contraction (in g) at the peak of the negative inotropic response to EFS (V_2) from the force of contraction at baseline immediately prior to EFS (b). The decrease in force was then expressed as percentage inhibition of baseline contraction. The sympathetically-mediated inotropic response to EFS (S) was measured as the peak increase in contractile force of the atrial following EFS (in g). The positive inotropic response to EFS was then expressed as percentage increase in atrial force (see Figure 1). Individual agonist concentration-response curves were fitted to a sigmoid logistic equation:

$$Response = \frac{a+b}{1+e^{-d(c+x)}}$$

where a is the resting level of response, b is the response range, c is the $-\log_{10}$ of the molar concentration that elicits 50% of the maximum response (pEC_{50}), d is the slope and curvature parameter and e is the base of the natural logarithm (Lew & Angus, 1995). Changes in basal contractile force with time were compared between treatment groups by repeated measures ANOVA. The effect of time (control) and PYY treatment on the positive inotropic response to eight trains EFS was compared between groups by repeated measures ANOVA. The Greenhouse-Geisser estimate of epsilon was used as a correction for correlation (Ludbrook, 1994). In control atria, the negative or positive inotropic response to eight trains EFS with time was

compared by 2-way ANOVA. C2 responses were compared between treatment groups by 1-way ANOVA. The potencies (pIC_{50}) of PYY, NPY and *N*-A[L]NPY(24–36) at inhibiting the negative inotropic response to EFS were compared between treatment groups by 1-way ANOVA with the Bonferroni *post-hoc* test for multiple comparisons. The maximum inhibition of vagally-mediated inotropic responses was compared between NPY and *N*-A[L]NPY(24–36) by Students' *t*-test for unpaired data. In all cases, statistical significance was accepted when $P<0.05$.

Results

Basal contractility of left atria and effect of time and drug treatment

Initial force of contraction was not different among experimental groups therefore the data was pooled. Average contractile force immediately prior to the first electrical field stimulus was 0.42 ± 0.02 g, range 0.15–0.80 g ($n=48$).

In the experiments examining sympathetic nerve responses, there was a small but significant fade of basal contractile force over time in all three experimental groups pretreated with atropine (1 μM) ($P=0.0043$, $n=15$). However this time dependent fade in basal contractile strength was not affected by incubation of the atria with increasing concentrations of PYY or clonidine when compared to the vehicle time controls ($P=0.1957$).

In experiments examining vagal stimulation, atria pretreated with propranolol (1 μM) and clonidine (1 μM) showed a significant decline in the basal force of contraction with time ($P=0.0001$, $n=20$). However, there was no significant difference in the rate of fade of basal contractility between agonist treatment groups and the corresponding time controls ($P=0.1263$).

Inotropic responses to electrical field stimulation of left atria

Electrical field stimulation of the left atrium elicited stimulus-dependent biphasic responses in contractile force. There was an initial negative inotropic response to field stimulation that reached a maximum inhibition of baseline force to the punctate stimulus immediately following EFS, followed by a more sustained positive inotropic response that reached a maximum increase in basal force of contraction between 5–15 s after cessation of EFS (Figure 1). In the presence of atropine, the negative inotropic response to 1-64 trains EFS was completely abolished whilst the positive inotropic response was significantly enhanced (Figures 1 and 2). The maximum increase in force of contraction to 64 trains EFS was 216% in vehicle treated atria and 338% in the presence of atropine (Figure 2). Incubation of the atria with propranolol depressed the positive inotropic response to EFS at all train lengths whilst only increased the negative inotropic response to EFS at 32 and 64 trains EFS (Figure 2). The maximum negative inotropic response to 64 trains EFS was a 59 and 70% inhibition of baseline force in the absence and presence of propranolol respectively, however this difference was not significant ($P=0.3916$). Propranolol (1 μM) did not completely inhibit positive intropic responses to field stimulation (Figure 2). From these preliminary experiments we chose EFS of eight trains of four pulses per train that elicited approximately 50% of the maximum positive and negative

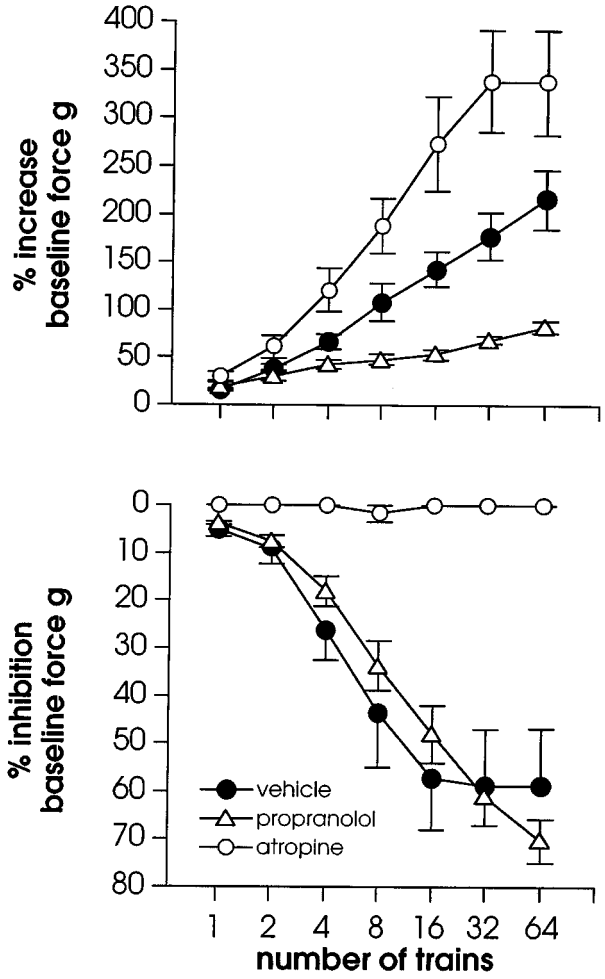

Figure 2 Electrical field stimulus-inotropic response curves in guinea-pig isolated left atria pretreated for 30 min with either vehicle ($n=4$), propranolol ($n=4$) or atropine ($n=4$). Top panel is the positive inotropic response to 1-64 trains EFS (see Methods). Bottom panel is the negative inotropic response to EFS. Error bars are $\pm$s.e.mean.

inotropic response. All responses were nerve-mediated as indicated by complete inhibition of changes in contractile force following incubation of the atria with 0.1 μM tetrodotoxin (data not shown).

Effect of peptides and clonidine on the positive inotropic response to EFS

There was no significant difference in the control response (C2) to eight trains EFS between experimental groups (Figure 3). Incubation of the atria with increasing concentrations of PYY (up to 10 μM) had no significant effect on the positive inotropic response to electrical field stimulation. In contrast, clonidine caused a concentration dependent inhibition of inotropic responses (pEC_{50} of 7.7 ± 0.09 M). Clonidine (1 μM) was sufficient to cause near complete inhibition of the stimulus induced increase in force of contraction (Figure 3). In single experiments, the effects of NPY and the selective Y_2 receptor agonist *N*-A[L]NPY(24–36) were examined on eight trains EFS. Like PYY, NPY and *N*-A[L]NPY(24–36) also had no effect on sympathetic response to EFS up to 10 μM. Inotropic response to eight trains EFS was 133 and 244%, before, and 144 and 333% after 10 μM NPY and *N*-A[L]NPY(24–

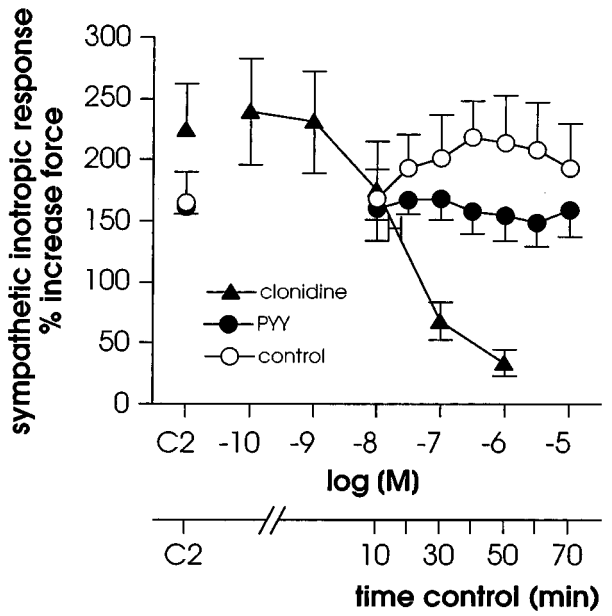

Figure 3 Effect of PYY and clonidine on the sympathetic response to field stimulation (EFS) of guinea-pig isolated left atria in the presence of atropine (1 μM). Curves are the positive inotropic response to eight trains EFS (each train was four pulses per refractory period; see Methods) expressed as percentage increase in baseline force (see Methods) following cumulative additions of either clonidine (0.1–1000 nM; $n=5$) or PYY (0.01–10 μM; $n=5$). The positive inotropic response to EFS with time in control atria ($n=5$) is shown for comparison. Vertical error bars are $\pm$s.e.mean. Horizontal error bars are ± 1 s.e.mean on the average fitted IC_{50} for clonidine. C2 is the second control inotropic response to EFS immediately prior to addition of the first concentration of peptide, clonidine or vehicle.

36) respectively. Furthermore, when the stimulus train duration was decreased to one and two trains EFS, neither PYY nor NPY had any effect on the positive inotropic responses. Responses to one and two trains EFS were 30 and 86%, before, and 35 and 93% after incubation with 10 μM PYY, respectively ($n=1$). In the NPY-treated atria, control responses to one and two trains EFS were 32 and 86%, respectively. After incubation with 10 μM NPY, responses were 21 and 67% ($n=1$). In time-control atria, the positive inotropic response to EFS increased significantly with time (Figure 3, $n=5$, $P=0.0001$). However, there was no significant difference in the positive inotropic response to EFS between control and PYY treated atria ($P=0.0992$). The presence of 10 μM PYY had no significant effect on the potency and maximum positive inotropic response to the β-adrenoceptor agonist isoprenaline compared with time control responses (Table 1).

Effect of peptides on the negative inotropic response to EFS

All three peptides examined caused concentration-dependent inhibition of the negative inotropic response to EFS (Figure 4) in the presence of propranolol and clonidine. The rank order of potency was PYY > NPY $\geqslant$ *N*-A[L]NPY(24–36) (7.26 ± 0.12, 6.63 ± 0.12 and 6.55 ± 0.15; pIC_{50}'s for PYY, NPY and *N*-A[L]NPY(24–36) respectively). The significant difference in potency ($P=0.0045$) was not due to the initial level of negative inotropism as there was no significant difference between C2 responses to eight trains EFS between experimental groups (Figure 4). PYY (1 μM) caused an almost complete blockade of the negative inotropic response

Table 1 Effect of NPY-related peptides on the cumulative concentration-inotropic response curves to exogenous agonists in guinea-pig isolated left atria

Treatment group	*pIC_{50}*	*Isoprenaline CRC's Maximum % increase baseline force g*
Vehicle ($n=5$)	7.97 ± 0.10	491 ± 145
PYY (10 μM; $n=5$)	7.87 ± 0.41	365 ± 105
		Bethanechol CRC's
Vehicle ($n=5$)	5.32 ± 0.12	99 ± 2
PYY (1 μM; $n=5$)	5.20 ± 0.08	93 ± 4
NPY (10 μM; $n=5$)	5.40 ± 0.05	94 ± 2
N-A[L]NPY(24–36) (10 μM; $n=5$)	5.37 ± 0.05	94 ± 3

Values are mean$\pm$s.e.mean. *n*, number of atria; PYY, peptide YY; NPY, neuropeptide Y; *N*-A[L]NPY(24–36), Y_2-receptor selective agonist; *N*-Acetyl[$Leu^{28,31}$]NPY(24–36); Vehicle, water; CRC's, concentration-response curves.

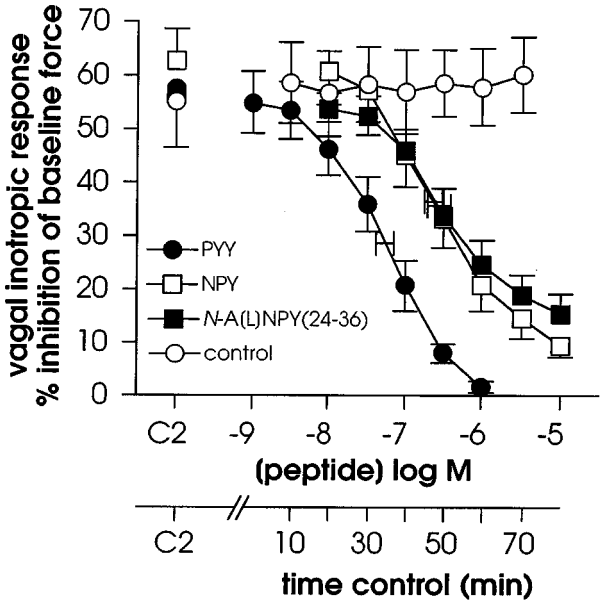

Figure 4 Effect of peptides on vagal response to field stimulation (EFS) of guinea-pig isolated left atria in the presence of propranolol (1 μM) and clonidine (1 μM). Curves are the effect of cumulative additions of either PYY (1–1000 nM; $n=5$), NPY (0.01–10 μM; $n=5$) or N-Ac-[$Leu^{28,31}$]NPY(24–36) (*N*-A[L]NPY(24–36); 0.01–10 μM; $n=5$) on the negative inotropic response to eight trains EFS expressed as percentage inhibition of baseline force (each train was four pulses per refractory period; see Methods). The negative inotropic response to EFS with time in control atria ($n=5$) is shown for comparison. C2 is the second control response to eight trains EFS expressed as a percentage inhibition of baseline force for each of the treatment groups. Vertical error bars are $\pm$s.e.mean for each concentration of peptide. Horizontal error bars indicate ± 1 s.e.mean of the average fitted IC_{50} for each curve.

to eight trains EFS (2.5 ± 1.6% of C2) whilst both NPY and *N*-A[L]NPY(24–36) (at 10 μM) were unable to achieve a maximum inhibition of the vagal response (14.7 ± 2.5 and 28.1 ± 6.5% of C2, respectively). However, there was no significant difference in the location (potency) nor maximum response achieved between NPY and *N*-A[L]NPY(24–36). There was no effect of the peptides on the location or maximum negative inotropic response to bethanechol (Table 1). The negative inotropic response to eight trains EFS was

stable over time, with no evidence of time-dependent changes in the response to electrical stimulation (Figure 4).

Discussion

We assessed the effects of NPY and related peptides on basal contractility and nerve-mediated inotropic responses to electrical field stimulation of the guinea-pig isolated left atrium. The NPY-receptor non-selective peptides PYY and NPY and the Y_2 receptor-selective agonist N-Ac-[Leu^{28,31}]NPY(24 – 36) (first described by Potter *et al*., 1994) caused selective inhibition of vagally-mediated inotropic responses. Agonist rank order of potency was consistent with activity at a Y_2 receptor (Balasubramaniam, 1996). Sympathetically-mediated positive inotropic responses were unaffected by PYY, NPY or N-Ac-[Leu^{28,31}]NPY(24 – 36). For comparison, the α_2-adrenoceptor agonist clonidine was able to cause near complete inhibition. There was no direct effect of the peptides studied on basal contractility of the atrial myocardium. Sensitivity of the inotropic responses elicited by postjunctional cardiac β-adrenoceptor or muscarinic receptor stimulation were also unaffected. Therefore, the inhibitory effects of the peptides examined on inotropic responses to electrical field stimulation reflect a prejunctional action on neurotransmitter release.

Functional interaction between positive and negative contractile responses to electrical field stimulation

Incubation of the atria with the muscarinic receptor antagonist, atropine, completely inhibited the negative inotropic response to field stimulation. Therefore, negative inotropic responses following stimulation of cardiac intramural nerves of the guinea-pig atrium appear to be mediated solely through vagal release of acetylcholine. In the presence of atropine, positive inotropic responses to field stimulation were greatly enhanced at all levels of stimulation suggesting initial negative inotropic responses functionally antagonize the slower developing maximal increases in contractile force. Conversely, vagal responses remained unopposed until stimulation exceeded 16 trains in length (approximately 4 s). When the atria were stimulated with trains longer than 4 s in duration, in the absence of propranolol, negative inotropic responses began to plateau as the onset of positive inotropic responses became evident. The degree of this interaction was demonstrated by incubation of the tissue with propranolol. In the presence of this β-adrenoceptor antagonism, the negative inotropic response to field stimulation was slightly enhanced, with no further evidence of a plateau in the decrease in force of contraction. This suggests that for vagal responses in the left atrium, only supramaximal levels of sympathetic stimulation caused a temporal interaction on the negative inotropic responses to EFS. In contrast, temporal separation of sympathetic responses from the initial vagal response does not occur, as indicated by an enhancement of the positive inotropic response to even a single train of field pulses. This temporal separation of vagal and sympathetic responses has been demonstrated previously in both left (Hong & Chang, 1995) and right (Lew & Angus, 1983) atrial preparations of the guinea-pig. The duration of stimulus that was chosen to examine the negative inotropic responses in these experiments should not have been complicated by positive inotropic interactions.

In our experiments, the majority of the positive inotropic response to EFS is sympathetically-mediated as demonstrated by a depression of the inotropic stimulus-response curve in the presence of propranolol. However, there is a significant proportion of the positive inotropic response to EFS that is resistant to propranolol. Clonidine (1 μM) effectively inhibited the residual inotropy in the majority of atria. It may be that under these conditions of stimulation sufficient noradrenaline is being released to compete with propranolol and elicit positive inotropic responses. The subsequent addition of clonidine may have been sufficient to overcome the stimulation-induced release of NA. Indeed, in the absence of propranolol, clonidine effectively elicited concentration-dependent inhibition of sympathetic responses to EFS (see Results).

Effect of NPY and related peptides on cardiac contractility

We found that neither antagonist pretreatment, nor NPY and related peptides affected basal contractility of the paced left atria. Previous findings from investigations into direct inotropic effects of NPY on myocardial contractility are confounding and often contradictory depending on the species and preparation examined. NPY has been shown to have negative inotropic effects *in vivo* (Allen *et al*., 1986; Minson *et al*., 1987; 1989; 1990; Zukowska-Grojec *et al*., 1987; Awad *et al*., 1991) and *in vitro* (Allen *et al*., 1983; Franco-Cereceda *et al*., 1985; Rioux *et al*., 1986; Jolly *et al*., 1991) however, these decreases in contractility are suggested to be secondary to impaired myocardial perfusion and ischaemia. When the effects of NPY are examined in preparations devoid of the confounding effects of changes in myocardial perfusion, there is still disagreement in results. In isolated atrial or ventricular muscle, NPY has been shown to exert a negative inotropic effect on basal contractility (Balasubramaniam *et al*., 1988; Rigel *et al*., 1989) whilst others demonstrate no effect (Allen *et al*., 1986; Franco-Cereceda *et al*., 1987; Wahlestedt *et al*., 1987; Michel *et al*., 1989; Scott *et al*., 1990; Amerini *et al*., 1991; Woo *et al*., 1994; Woo & Ganguly, 1995).

To add to the controversy, the guinea-pig appears to be the only mammalian species that exhibits a positive inotropic response to NPY (Lundberg *et al*., 1984; Franco-Cereceda *et al*., 1985). It has been suggested that this positive inotropic effect of NPY is *via* activation of a slow inward calcium current (Millar *et al*., 1991). However, this positive inotropic response has not been observed by us (this manuscript, Serone *et al*., 1998) and others (Allen *et al*., 1986; Wahlestedt *et al*., 1987; Amerini *et al*., 1991).

Effect of NPY and related peptides on nerve stimulation-evoked inotropic responses

Neither the non-selective NPY receptor agonists PYY or NPY, nor the Y_2 selective agonist N-Ac-[Leu^{28,31}]NPY(24 – 36) mediated inhibition of positive inotropic responses to electrical field stimulation of guinea-pig isolated left atria. However, all three agonists caused inhibition of vagally-mediated decreases in force of contraction. The inability of NPY and related peptides to inhibit sympathetic inotropic responses was not due to an inability of sympathetic nerves to be modulated by agonists in this preparation, as indicated by an almost complete inhibition of responses to EFS by clonidine. The lack of effect of PYY may suggest that sympathetic nerve-mediated responses are modulated by a non-Y_2 receptor in the guinea-pig left atrium. The putative Y_3 receptor has no or very low affinity for PYY and has been demonstrated to occur postjunctionally on rat cardiac cell membranes (Balasubramaniam *et al*., 1990). However, the lack of effect of either NPY or N-Ac-[Leu^{28,31}]NPY(24 – 36) discounts the possibility of Y_3

receptors mediating inhibition of sympathetic responses. Prejunctional Y_1 receptors mediate transient inhibition of chronotropic responses to sympathetic stimulation in the guinea-pig and rabbit isolated right atrium (Serone *et al*., 1998). Both PYY and NPY are Y_1 receptor agonists. Therefore, in the absence of a Y_2 receptor-mediated stable inhibition of inotropic responses, Y_1 receptor-mediated inhibition should still have been evident at 10 min after incubation if it was to occur.

In support of our findings, in dog isolated blood-perfused atrium, NPY (3 nmol) injected into the sinus node artery inhibited only the negative chronotropic and inotropic responses to concommitant stimulation of sympathetic and parasympathetic nerves (Ren *et al*., 1991). Furthermore, when sympathetic responses were examined in the presence of atropine to prevent vagally-mediated changes in rate and force, NPY still had no effect on positive inotropic or chronotropic responses (Ren *et al*., 1991). We have previously demonstrated that sympathetically-mediated chronotropic responses to EFS can be successfully modulated (up to 70% inhibition of positive chronotropic responses) by both NPY and N-Ac-[$Leu^{28,31}$]NPY(24–36) acting at a Y_2 receptor in the guinea-pig isolated right atria (Serone *et al*., 1998). The lack of effect of NPY at inhibiting the chronotropic responses to nerve stimulation demonstrated by Ren *et al*. (1991) may have been due to the relatively low concentrations utilized in their study. Early studies by Lundberg and co-workers (Franco-Cereceda *et al*., 1985) demonstrated NPY (0.5 μM) effectively inhibited the positive inotropic response to EFS by approximately 30% in guinea-pig isolated right atria when stimulated at 2 Hz for 2 s (i.e. total of four pulses). However, when the duration of stimulation was extended to 20 s (i.e. total of 40 pulses), NPY had no observable effect on either inotropic or chronotropic responses to EFS. The inability to demonstrate a functional decrease in the sympathetic response to stimulation was despite a 40% decrease in the measured stimulus-induced efflux of [^{3}H]-NA (Franco-Cereceda *et al*., 1985). These results appear to be analogous to the results we have found. The stimulus conditions of experiments performed previously by us in guinea-pig isolated right atria (Serone *et al*., 1998) were of comparable intensity, however, the duration of stimulation was approximately an eighth of the experiments presented above. Therefore, during prolonged intense stimulation, an excessive amount of NA may be released such that any small inhibitory effect of Y_2 receptor stimulation at decreasing the stimulus-evoked secretion of NA is not sufficient to elicit a measurable decrease in the functional response. The apparent need for both propranolol and clonidine to be present in the bath medium in micromolar concentrations gives further indication of the level of NA being released following field stimulation. However, when the duration of sympathetic stimulation was reduced to a level comparable to that in our previous right atria experiments, neither PYY, NPY nor *N*-A[L]NPY(24–36) were able to elicit an inhibition of the positive inotropic response to EFS. This would suggest that the observed lack of effect of these peptides was not due to a saturation of the synaptic cleft with noradrenaline, rather, it is most likely due to a lack of efficacy. This may be due to either poor coupling of existing prejunctional Y_2 receptors on sympathetic nerve terminals innervating the myocardium in the left atrium or a deficit in receptor number.

In contrast to the effects of PYY, NPY and *N*-A[L]NPY(24–36) on sympathetic responses, all three peptides examined elicited concentration-dependent inhibition of the vagally-mediated negative inotropic response to EFS. Agonist rank order of potency was PYY > NPY $\geqslant$ *N*-A[L]NPY(24–36), consistent with activity at a Y_2 receptor (Balasubramaniam, 1996). The approximate 5 fold greater potency between PYY (pIC_{50}, 7.26 ± 0.12) and NPY/*N*-A[L]NPY(24–36) (pIC_{50}, 6.63 ± 0.12 and 6.55 ± 0.15, respectively) at inhibiting the negative inotropic response to EFS was not due to discrepancies in the level of initial response as there was no difference in the degree of inhibition of basal contractility of the C2 stimulation between experimental groups. In the rat isolated vas deferens preparation (nominally Y_2 receptor assay), PYY and NPY inhibit the electrically-evoked twitch response with similar potency to their inhibition of the vagus described above (pIC_{50} 7.35 ± 0.06 and 6.82 ± 0.05, respectively) (Lew *et al*., 1996), which is consistent with classification of the presynaptic Y_2 receptor (Michel, 1991; Balasubramaniam, 1996). Our results also demonstrate that PYY was capable of eliciting full inhibition of vagal responses whilst both NPY and *N*-A[L]NPY(24–36) had a lower efficacy. This difference cannot be due to differences in receptor number as the agonists were compared in the same assay system.

Implications for modulation of cardiovascular function by NPY and related peptides

These data provide some insight into the possible functional significance of NPY in the regulation of cardiac function. In contrast to the reported negative inotropic effects of exogenous NPY, NPY released from cardiac sympathetic varicosities during sustained sympathetic stimulation, may act to maintain cardiac contractility by inhibiting both aspects (chronotropic and inotropic) of vagal transmission in the heart. However, NPY also elicits an inhibition of sympathetically-mediated increases in heart rate, although this effect of NPY is less potent that its effects on vagal activity. Therefore, another function of endogenous NPY may be to normalize heart-rate to the intrinsic rate of the sinoatrial node by preventing the chronotropic influences of extrinsic autonomic effectors. The greater potency of exogenous NPY at inhibiting vagal chronotropic responses may reflect the necessity for endogenous NPY to diffuse from its site of release (sympathetic varicosity) to the prejunctional effector site (vagal varicosity). By maintaining heart rate below tissue maximum, in conjunction with sustained cardiac contractility, NPY would serve to maintain cardiac output at an optimum level by preventing tachycardia from shortening ventricular filling time.

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