Title: Modulation of contractile function through neuropeptide Y receptors during development of cardiomyocyte hypertrophy

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Abstract

Severity of left ventricular hypertrophy (LVH) correlates with elevated plasma levels of neuropeptide Y (NPY) in hypertension. NPY elicits positive and negative contractile effects in cardiomyocytes through Y_1 and Y_2 receptors, respectively. **Hypothesis:** NPY receptor-mediated contraction is altered during progression of LVH. **Methods:** Ventricular cardiomyocytes were isolated from spontaneously hypertensive rats (SHRs) pre-LVH (12 weeks), during development (16 weeks) and at established LVH (20 weeks) and age-matched normotensive WKY rats. Electrically-stimulated (60V, 0.5Hz) cell shortening was measured using edge detection and receptor expression determined at mRNA and protein level. **Results:** NPY and Y_1 receptor-selective agonist, Leu^{31}Pro^{34}NPY, stimulated increases in contractile amplitude, which were abolished by Y_1 receptor-selective antagonist, BIBP3226, confirming Y_1 receptor-involvement; potencies of both agonists were enhanced in SHR cardiomyocytes at 20 weeks (2300- and 380-fold versus controls); maximum responses were not attenuated. BIBP3226 unmasked a negative contraction effect of NPY, elicited over the concentration range (10^{-12} -3\times10^{-9}\text{mol/L}) in which NPY and PYY_{3-36} attenuated the positive contraction effects of isoproterenol, the potencies of which were increased in cardiomyocytes from SHRs at 20 weeks (175- and 145-fold versus controls); maximum responses were not altered. Expression of NPY-Y_1 and NPY-Y_2 receptor mRNAs was decreased (55% and 69%) in left ventricular cardiomyocytes from 20 week old SHRs versus age-matched WKY rats; parallel decreases (32% and 80%) were observed at protein level. **Conclusion:** enhancement of NPY potency, producing (opposing) contractile effects on cardiomyocytes together with unchanged maximum response despite reduced receptor number, enables NPY to contribute to regulating cardiac performance during compensatory LVH.
Introduction

Mammalian myocardium contains large quantities of neuropeptide Y (NPY) (Onuoha et al., 1999), mainly co-localised with noradrenaline in perivascular sympathetic neurons innervating cardiac tissue (Franco-Cereceda et al., 1985; Allen et al., 1986). NPY has been implicated in left ventricular hypertrophy (LVH), an initial compensatory response of the heart to pressure overload precipitated by hypertension (Agabiti-Rosei and Muiesan, 2001) since increased plasma levels of the peptide are found in hypertension, myocardial infarction and heart failure (Maisel et al., 1989), and correlate with severity of LVH (Hulting et al., 1990).

NPY can both decrease and increase the contractile response of electrically-stimulated rat ventricular cardiomyocytes (Piper et al., 1989; Millar et al., 1991). The negative effect, observed in isoproterenol-treated cells, is due primarily to stimulation of the transient outward current (I\textsubscript{to}) and mediated through an inhibitory G protein / adenylate cyclase pathway (Kassis et al., 1987; Piper et al., 1989; Millar et al., 1991). Use of the selective Y\textsubscript{2} receptor agonists, PYY\textsubscript{3-36} and NPY\textsubscript{13-36} inferred Y\textsubscript{2} receptor involvement (McDermott et al., 1997), but the finding that long C-terminal fragments of both PYY and NPY also exhibit high affinity for the Y\textsubscript{3} receptor subtype (Hu et al., 1996) emphasizes the need for clarification of receptor subtypes involved in NPY-stimulated cardiomyocyte contraction. NPY alone does not influence the basal level of contraction of cardiomyocytes, but in the presence of 4-aminopyridine, which inhibits selectively I\textsubscript{to} in these cells, a positive response to NPY is unmasked (Millar et al., 1991). This has been observed also in chicken cardiomyocytes in the absence of rectifier current blockade (Jacques et al., 2000) and both effects are attributed to influx of Ca\textsuperscript{2+} via L-type channels and mediated through Y\textsubscript{1} receptor stimulation. Furthermore, Y\textsubscript{1} receptors are also coupled to mobilisation of intracellular calcium stores in cardiomyocytes by a phospholipase C-dependent mechanism (Heredia et al., 2005).
The number of postsynaptic α₁-adrenoceptors and the amount of 1, 4, 5 IP₃ accumulated in the myocardium in response to α₁-adrenoceptor stimulation are markedly increased in hypertension (Hanna and Khairallah, 1986). The release of NPY from the cardiac sympathetic innervation would also be expected to be enhanced during the pathogenesis of hypertension-induced myocardial hypertrophy, indeed the plasma concentration (Zukowskagrojec et al., 1993; Bohm et al., 1995) and platelet content (Ogawa et al., 1992; Zukowskagrojec et al., 1993; Chen and Han, 1995) of NPY are increased, and alterations in NPY-mediated myocardial contractile responses may be anticipated, since receptors for the peptide couple to signal transduction processes known to be altered in hypertrophied cardiomyocytes (Kawaguchi et al, 1992; 1993; Bohm et al, 1994; Cerbai et al., 1994; Xiao and McArdle, 1994; Yokoshiki et al, 1997).

The spontaneously hypertensive rat (SHR) is a useful model of pressure overload, (Okamoto and Aoki, 1963; Pfeffer et al., 1976) in which hypertension, evident at ≥7 weeks of age, is followed by development of ventricular hypertrophy; a ‘hypertrophic window’ at 12-20 weeks, which encompasses baseline and developmental characteristics at cardiomyocyte level, has been identified (Bell et al., 2004). Furthermore, the Y₅ receptor becomes coupled transiently to the stimulation of protein synthesis by NPY during the active phase of development of ventricular cell hypertrophy in the SHR although receptor number is unaltered (Bell et al., 2002).

It was hypothesised that NPY receptor-mediated contraction responses become altered during progression of LVH. Focusing on the hypertrophic window above, effects on electrically-stimulated contractile function were investigated in cardiomyocytes under basal conditions and when the cells were stimulated using Ca²⁺, the positive inotrope, isoproterenol, and NPY. In combination with Ca²⁺ or isoproterenol, use of selective NPY receptor agonists (Y₁ receptors – Leu³¹Pro³⁴NPY; Y₂ receptors – PYY₃₋₆₆; Y₅ receptors – D-Trp³⁴NPY) and antagonists (Y₁ receptors – BIBP3226; Y₂ receptors - BIIE246) allowed differentiation of receptor-subtype
mediated effects. Expression of NPY Y1 and NPY Y2 receptors was examined at mRNA and protein level.

Methods

Experimental Model: Male spontaneously hypertensive rats (SHRs) and age- and sex-matched Wistar Kyoto normotensive rats (WKY) were obtained from Harlan (Blackthorn, Oxon, UK) at 4 weeks of age and maintained at the Laboratory Service Unit, QUB, prior to sampling at 12, 16 and 20 weeks of age. The study was performed in accordance with Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty’s Stationary Office, London.

Isolation and culture of cardiomyocytes: After deep anaesthesia of the rat using isoflurane (Abbott Laboratories, Kent, UK), the heart was excised quickly and placed in ice-cold 0.9% (w/v) NaCl. The heart was cannulated through the ascending aorta and ventricular cardiomyocytes were isolated by the Langendorff retrograde perfusion method described previously (Bell et al., 2002). After purification, cells were suspended at a concentration of 1.5 x 10^5 viable cardiomyocytes.ml^-1 in CCT medium.

Analysis of contraction function: An aliquot of myocyte suspension (=150 µl) was placed in a transparent recording chamber mounted on an inverted phase-contrast microscope (Axiovert IM35, Carl Zeiss, Germany), and allowed to settle for 10 min before being bathed with oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit buffer. Cells were field-stimulated at 0.5 Hz with biphasic pulses of 0.5 ms duration at 60 V via Ag/AgCl_2 wires embedded in the wall of the chamber. After initial stimulation, contractile amplitude was determined after incubation for 4 minutes (under basal conditions or with isoproterenol) or after 15 minutes (with NPY receptor...
agonists or antagonists). Responses to NPY receptor agonists were demonstrated in the presence of elevated Ca^{2+} (3 \times 10^{-3} \text{ mol/L}) or a sub-maximal concentration of isoproterenol (10^{-8} \text{ mol/L}), as indicated. Concentration - effect relationships were obtained in a cumulative manner. The video edge detection equipment and the procedure used in the measurement of contraction amplitude were those described by Kelso et al. (2000).

**Real-Time PCR:** Total cellular RNA was isolated using acid guanidinium thiocyanate-phenol-chloroform extraction and alterations in mRNA expression determined by RT-PCR and expressed relative to GAPDH mRNA. Reported sequences for each gene (Table 1) were used to design on Primer Express software (PE Applied Biosystems), rat specific primers adapted to RT-PCR conditions, which were synthesized by Invitrogen. RT-PCR and subsequent analysis were performed as described previously (Zhao et al., 2006).

**Immunodetection and quantification of membrane protein:** membranes were prepared from viable left ventricular cardiomyocytes as described previously (Zhao et al., 2006). Membrane protein concentration was determined by the method of Lowry. Protein samples were separated by 12\% SDS-PAGE (80\mu g protein per lane) as described previously (Zhao et al., 2006) and transferred to PVDF membrane (0.45\mu m, Millipore, UK). The PVDF membrane was washed with phosphate buffered saline (PBS) containing 0.1\% v/v Tween 20 (Sigma, UK) and blocked overnight in PBS/0.1\% v/v Tween 20 solution containing 5\% w/v Marvel. Immunoblotting was performed using primary antibodies directed specifically against rodent NPY Y_1 and Y_2 receptors (Santa Cruz Biotechnology, sc-21992 and sc-14736 respectively, raised in goat) used at a dilution of 1:500. Immunocomplexes were detected using secondary antibodies conjugated to horseradish peroxidase (donkey anti-goat sc-2020 used at a dilution of 1:20000, Santa-Cruz Biotechnology) and ECL plus (Amersham Biosciences, UK) as substrate, and quantified by densitometry.
(Analytical Imaging System) normalized for protein loading using β-actin (Santa Cruz Biotechnology, sc-1616 raised in goat).

Chemicals: Medium M199 (glutamine-free with Earle’s salts) and penicillin (5000 IU)/streptomycin (5mg.ml⁻¹) were supplied by Gibco BRL (Paisley, Renfrewshire, UK). Carnitine, creatine, taurine, 4-aminopyridine (4-AP), isoproterenol hydrochloride and BIBP3226 (R-N²-(diphenyl-acetyl)-N-(4-hydroxyphenyl)methyl-argininamide) were obtained from the Sigma Chemical Company Ltd. (Poole, Dorset, UK). NPY (human, rat), Leu³¹Pro³⁴NPY (human, rat), and PYY₃₋₃₆ were purchased from Bachem (St Helens, Merseyside, UK). BIIE246 ((S)-N²-[(1-[2-(4-[(r,s)-5,11-dihydro-6(6H)-oxodibenz(b,e)azepin-11-yl]-1-piperazinyl)-2-oxoethyl]cyclopentyl]acetyl)-N-(2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl)-argininamide was kindly supplied by Dr. Henri Doods (Boehringer Ingelheim Pharmaceuticals, Biberach, Germany). D-Trp³⁴NPY was synthesised in the laboratory of Professor Ambikaipakan Balasubramaniam (University of Cincinnati Medical Center, Cincinnati, USA). All other chemicals were of analytical grade and purchased from BDH Chemicals (UK).

Solutions: Serum-free ‘creatinine-carnitine-taurine’ (CCT) medium consisted of modified glutamine-free Medium M199 supplemented with Earle’s salts and containing (in mmol/L): HEPES (15); creatine (5); l-carnitine (2); taurine (5); and penicillin (100µg. ml⁻¹) / streptomycin (100 IU.ml⁻¹). The composition of the Krebs-Henseleit solution was (in mmol/L): NaCl (120); KCl (4.7); NaHCO₃ (25); MgSO₄ (0.97); KH₂PO₄ (1.2); glucose (11); Ca²⁺ (1 or 3). Prior to use, this solution was gassed with 95% O₂ / 5% CO₂ for 20 minutes and maintained at pH 7.4.

Data analysis: All data are presented as mean ± SEM. Contraction amplitude was measured in µm and expressed as a percentage change from the diastolic length, as an absolute value (%δL) or
as a change from the baseline ($\Delta\%\delta L$) and $n$ denotes the number of cell preparations used. Concentration-response data were analysed by nonlinear regression using GraphPad Prism® (v.4). Statistical analyses were performed by analysis of variance to detect significant differences for between group (age, strain) or within group (treatment) effects and post-hoc comparisons by Bonferroni or an unpaired Student’s $t$ test as appropriate, using an SPSS package (v.11.5). Values of $p<0.05$ were considered significant.
Results

Control experiments for the effect of NPY and associated compounds on contractile function

The following agonists had no effect per se with Ca\(^{2+}\) (10\(^{-3}\) mol/L) on contractile amplitude (δL%) of cardiomyocytes (data are given as mean±SEM and were obtained in cardiomyocytes from 12 week old animals following 15 minutes incubation with agonist vs. time-matched baseline values): NPY (10\(^{-7}\) mol/L : 3.86±0.86 vs. 4.04±0.35, n=6 WKY; 3.38±0.96 vs. 3.98±1.07, n=6 SHR; 3x10\(^{-9}\) mol/L: 3.84±0.50 vs. 3.79±0.75, n=6 WKY; 2.00±0.42 vs. 1.56±0.40, n=6 SHR), Leu\(^{31}\)Pro\(^{34}\)NPY (10\(^{-7}\)mol/L : 3.43±0.66 vs. 3.40±0.57 n=6 WKY; 2.84±0.48 vs. 3.19±0.42, n=6 SHR), PYY\(_{3-36}\) (3x10\(^{-9}\) mol/L: 5.57±1.69 vs. 5.53±1.68, n=5 WKY; 2.83±0.60 vs. 2.79±0.46, n=6 SHR), D-Trp\(^{34}\)NPY (10\(^{-7}\) mol/L: 1.00±0.29 vs. 1.25±0.30, n=5 WKY; 2.04±0.26 vs. 2.22±0.21, n=6 SHR). Similarly, there were no effects of the antagonists, BIBP3226 (10\(^{-7}\) mol/L: 4.19±1.35 vs. 4.29±1.22, n=4 WKY; 6.57±1.01 vs. 6.67±1.18, n=4 SHR) or BIIE246 (10\(^{-7}\) mol/L: 5.92±1.38 vs. 6.03±1.06, n=4 WKY; 3.26±1.29 vs. 3.37±1.30, n=4 SHR) per se with Ca\(^{2+}\) (10\(^{-3}\) mol/L); also BIIE246 (10\(^{-7}\) mol/L) did not antagonise the response to isoproterenol (10\(^{-8}\) mol/L) (4.79±0.78 in presence of BIIE246 vs. 3.70±0.63 alone, n=4 WKY; 4.24±0.56 in presence of BIIE vs. 3.63±0.35 alone, n=4 SHR). BIBP3226 and BIIE246 can be used appropriately, therefore, to test the Y\(_1\) and Y\(_2\) receptor subtype specificity, respectively, of NPY responses. Leu\(^{31}\)Pro\(^{34}\)NPY (10\(^{-7}\) mol/L) with Ca\(^{2+}\) (10\(^{-3}\) mol/L) did not alter the positive effect of isoproterenol (10\(^{-8}\) mol/L) on amplitude of contraction (5.53±0.41 in presence of Leu\(^{31}\)Pro\(^{34}\)NPY vs. 5.46±0.49 alone, n=6 WKY; 4.40±0.77 in presence of Leu\(^{31}\)Pro\(^{34}\)NPY vs. 4.01±0.58 alone, n=4 SHR). It is apparent, therefore, that the Y\(_1\) receptor-selective compound does not have activity at Y\(_2\) receptors in this experimental system. For experiments with BIIE246, concentrations of NPY and PYY\(_{3-36}\) that produced maximal attenuation of isoproterenol (10\(^{-8}\) mol/L)-stimulated contraction amplitude were initially determined; because potency increased
with increasing age of SHRs, the concentration used in 16 and 20 week old rats was $3 \times 10^{-10}$ mol/L, but was $3 \times 10^{-9}$ mol/L in 12 week old SHRs and all WKY rats.

**Receptor subtype-mediated effects on electrically-stimulated contraction of rat cardiomyocytes**

Data obtained in our laboratory establishing the temporal development of hypertension and cardiomyocyte hypertrophy in SHRs during the period 12-20 weeks employed in the contraction experiments below have been published previously (Bell et al., 2004).

**Temporal and concentration-dependence of the positive contraction response:** A representative original tracing of the stimulation of contractile activity by NPY is shown in Figure 1a. In the presence of Ca$^{2+}$ ($3 \times 10^{-6}$ mol/L), NPY (Figure 2a) and Leu$^{31}$Pro$^{34}$NPY (Figure 2b) stimulated concentration-dependent increases in contraction amplitude in cardiomyocytes from SHRs and WKY rats of all ages. At 12 weeks, increases were observed in the range, $10^{-10}$ - $10^{-7}$ mol/L, and EC$_{50}$ values (in the $10^{-9}$ mol/L range) were similar. The potency of NPY was unaltered with the progression of age in WKY rat cardiomyocytes, but significantly increased in cardiomyocytes from 20 week old SHRs (2500- and 2800- fold greater than observed at 12 and 16 weeks of age, respectively). Age-matched between strain comparisons showed that the potency of NPY was significantly elevated at 20 (2300-fold), but not at 12 and 16 weeks of age in cardiomyocytes from SHRs. Comparison of the effects of NPY and Leu$^{31}$Pro$^{34}$NPY to increase contraction amplitude showed that the potencies were similar under all conditions. As such, the potency of Leu$^{31}$Pro$^{34}$NPY to increase contraction amplitude was unchanged in WKY rat cardiomyocytes but enhanced in cardiomyocytes obtained from SHRs at 20 weeks of age (380-fold vs. age-matched controls). Maximum responses to NPY and to Leu$^{31}$Pro$^{34}$NPY did not differ significantly between SHR and WKY rats at any age. The Y$_1$ receptor selective antagonist, BIBP3226 abolished the
positive contraction effects of NPY (Figure 3a) and Leu$^{31}$Pro$^{34}$NPY (Figure 3b), across the range of concentration used (10$^{-13}$ to 10$^{-7}$ mol/L) in both SHR and WKY rat cardiomyocytes at all ages.

Temporal and concentration-dependence of the negative contraction response to NPY in the unmasked in the presence of BIBP3226: NPY in the presence of BIBP3226 elicited a negative, concentration-dependent effect in all cases (Figure 3a), observed over the same range of concentration in which application of NPY alone had increased contraction amplitude in the presence of Ca$^{2+}$ (3 x 10$^{-6}$ mol/L) (Figure 2a). In cardiomyocytes from SHRs and WKY rats at 12 weeks of age, decreases in contraction amplitude stimulated by NPY in the presence of BIBP3226 had similar EC$_{50}$ values (in the 10$^{-10}$ mol/L range). In WKY rat cardiomyocytes, the EC$_{50}$ value was unaltered with increasing age, but in SHR cardiomyocytes, increased significantly at 20 weeks of age (100- to 800-fold greater than observed at 12 and 16 weeks of age). In age-matched comparisons between strains, the potency value in SHR cardiomyocytes was significantly altered compared to that observed in cardiomyocytes from WKY rats at 20, but not at 12 and 16 weeks of age. In the presence of BIBP3226, Leu$^{31}$Pro$^{34}$NPY applied over the same concentration range which, when used alone, had increased contraction amplitude in SHR and WKY rat cardiomyocytes, had no effect on amplitude of contraction (Figure 3b).

Temporal and concentration-dependence of the negative contraction response to PYY$_{3-36}$ alone: PYY$_{3-36}$ (10$^{-12}$ to 3x10$^{-9}$ mol/L) elicited a concentration-dependent, negative contraction effect in cardiomyocytes from SHRs and WKY rats of all ages (Figure 4). Maximum responses to PYY$_{3-36}$ did not differ significantly between SHR and WKY rats at any age. The potency of PYY$_{3-36}$ was similar in cardiomyocytes from 12 week old rats of each strain, in the region of 3x10$^{-10}$ mol/L, and in WKY rat cardiomyocytes was unchanged with progression of age, whereas potency increased in SHRs, becoming significantly elevated (x400) in cardiomyocytes from 20 compared to 12 week old rats. The potency of PYY$_{3-36}$ was similar in cardiomyocytes from age-matched 12 week
old SHRs and WKY rats but was noticeably increased (x10) at 16 weeks, becoming significantly elevated (x400) in cardiomyocytes from 20 week old SHRs compared to that found in WKY rats. The potencies of PYY$_{3-36}$ and NPY in the presence of BIBP3226 (Figures 4 and 3, respectively) under any condition were not different.

**Temporal and concentration-dependence of the negative contraction response to NPY in isoproterenol-stimulated cells:** A representative original tracing of the effect of NPY to attenuate isoproterenol-stimulated contractile activity in cardiomyocytes is shown in Figure 1b. NPY at $10^{-12}$ to $3 \times 10^{-9}$ mol/L reduced the positive contraction effect of isoproterenol in cardiomyocytes from both rat strains at all ages (Figure 5 a, b). In cardiomyocytes from 12 week old rats, the potency of NPY was in the region of 100 pmol/L and was unaltered with increasing age in WKY rats, but increased (60-fold) in SHRs, being significant in 20 compared to 12 week old rats. When cardiomyocytes from age-matched WKY rats and SHRs were compared, the potency of NPY was significantly increased, 175-fold, only at 20 weeks.

**Temporal and concentration-dependence of the negative contraction responses to PYY$_{3-36}$ in isoproterenol-stimulated cells:** Comparison of the effects of PYY$_{3-36}$ and NPY to attenuate isoproterenol-stimulated contraction amplitude showed that, in all cases, the potencies of both peptides were similar. As such, the potency of PYY$_{3-36}$ to decrease contraction amplitude was unchanged in WKY rat cardiomyocytes but enhanced in cardiomyocytes obtained from SHRs at 20 weeks of age (145-fold vs. age-matched controls). PYY$_{3-36}$ was equally potent in attenuating isoproterenol-stimulated contraction amplitude (Figure 5) and in eliciting decreased contraction amplitude under elevated extracellular Ca$^{2+}$ conditions (Figure 4). BIIE246, applied at a concentration ($10^{-7}$ mol/L) at which it has high affinity for the Y$_2$ receptor subtype but virtually no affinity for the Y$_1$, Y$_4$ and Y$_5$ receptor subtypes (Doods et al., 1999) abolished the maximal attenuation of isoproterenol-stimulated contraction amplitude elicited by NPY (and PYY$_{3-36}$) at all
ages in both strains of rat cardiomyocytes (Figure 6). Application of the Y5 receptor selective agonist, D-Trp34NPY, at a concentration (10^-7 mol/L) determined to yield a maximal hypertrophic effect on adult cardiomyocytes from both SHR and WKY rats (Bell et al., 2002) did not attenuate the effects of isoproterenol on amplitude (ΔL%) of cardiomyocyte contraction (5.12±0.87 in presence of D-Trp34NPY vs. 4.89±0.70 alone, n=6 WKY; 4.99±0.66 in presence of D-Trp34NPY vs. 4.31±0.63 alone, n=6 SHR); data are given as mean±SEM and were obtained in cardiomyocytes from 20 week old animals.

**Gene expression of NPY and receptors**

NPY-Y1 receptors were expressed more abundantly than NPY-Y2 receptors at mRNA and protein levels in left ventricular cardiomyocytes from WKY rats at 20 weeks of age (Figure 7). Expression of NPY-Y1 and NPY-Y2 receptor mRNAs was decreased by 55% (P<0.05) and 69% (P<0.05), respectively, in left ventricular cardiomyocytes from 20 week old SHRs relative to that of age-matched WKY rats; parallel decreases of 32% (P = ns) and 80% (P<0.05), respectively, were observed at protein level.
Discussion

**NPY receptor subtype-mediated effects on electrically-stimulated contraction of rat cardiomyocytes**

That the positive contraction effect of NPY in SHR and WKY rat cardiomyocytes is mediated solely *via* the Y\textsubscript{1} receptor subtype, as was found previously in Sprague Dawley rat cardiomyocytes (McDermott et al., 1997), was confirmed by the following observations: similar potencies of NPY and the Y\textsubscript{1} receptor-selective agonist, Leu\textsuperscript{31}Pro\textsuperscript{34}NPY in all strains/ages; abolition of these effects by BIBP3226, applied at a concentration determined to have no cross reactive effects on rat Y\textsubscript{2} receptors (Doods et al, 1996); no stimulation on application of the Y\textsubscript{2} receptor selective agonist, PYY\textsubscript{3-36}. The fact that Y\textsubscript{1} receptor blockade by BIBP3226 (10\textsuperscript{-7} mol/L) is complete, established by the total reversal of Leu\textsuperscript{31}Pro\textsuperscript{34}NPY’s positive contraction effects, indicates that the negative effect must arise from a population of receptors distinct from the Y\textsubscript{1} sub-type, possibly the Y\textsubscript{2} subtype. This was substantiated by the following findings: potencies and magnitudes of effect of PYY\textsubscript{3-36} were similar to those elicited by NPY in the presence of BIBP3226; NPY (and PYY\textsubscript{3-36}) attenuated the positive contraction effects of isoproterenol, exhibiting similar potencies within strains. Although PYY\textsubscript{3-36} was thought initially to be selective for the Y\textsubscript{2} receptor (Grandt et al., 1996), it has now been shown that long C-terminal fragments of either NPY or PYY also have high potency at the Y\textsubscript{5} receptor (Hu et al., 1996). However, the latter does not appear to be involved in mediating the negative contraction effects of NPY (or PYY\textsubscript{3-36}) since the Y\textsubscript{5} receptor selective agonist, D-Trp\textsuperscript{34}NPY, did not attenuate the effects of isoproterenol on amplitude of cardiomyocyte contraction. Abolition by BIIE0246 of the attenuation by NPY (and PYY\textsubscript{3-36}) of isoproterenol-stimulated contraction indicates that in the absence of Y\textsubscript{1} receptor activity, NPY and associated peptides act on Y\textsubscript{2} receptors to stimulate a negative contraction effect, confirming earlier studies in which the negative contraction effect of NPY has been attributed to Y\textsubscript{2} receptors (Millar et al., 1988; Hu et al., 1996).
Altered potency at NPY receptors during the development of hypertrophy

Under normal conditions when hypertensive disease is absent, the positive contraction effect of NPY was maintained at a constant level. This may arise because in the absence of pressure overload, there is no need to potentiate the contraction performance of cardiomyocytes. Plasma levels of circulating NPY in WKY rats are approximately 4x10^{-11} mol/L (Ogawa et al., 1989) which lies just outside the range (10^{-10} to 10^{-7} mol/L) in which the peptide stimulates increased contraction function in WKY rat myocytes. It appears, therefore, that modulation of contraction function by NPY is a redundant mechanism in normotensive animals. The SHR myocardium could undergo a stress-induced change in which the importance of peptidic regulation of function is of greater importance. The NPY level in SHR plasma (Ogawa et al., 1989) would certainly stimulate the maximal positive contraction effect of the peptide in 20 week old SHR cardiomyocytes. Therefore the hypertrophied myocardium may adapt, so that NPY may play a part in the modulation of contractile function via Y_1 receptors. Similarly, the concentration range (10^{-10} to 3x10^{-9} mol/L) in which NPY (and PYY_{3-36}) stimulated their negative contraction effects in WKY rat myocytes is less than the in vivo plasma concentration of NPY (Ogawa et al., 1989). Therefore, the Y_2 receptor-mediated negative contraction effect may also be a redundant mechanism in normotensive animals. However, the potency of both peptides to elicit this negative effect was up-regulated in cardiomyocytes from 20 week old SHRs to a level at which the observed circulatory concentration of NPY would be expected to elicit a maximal negative effect. Again this is consistent with the hypothesis that in hypertensive disease, peptidic regulation of the myocardium is enhanced. Augmented activity of the sympathetic neurotransmitter norepinephrine (possibly in combination with NPY acting via Y_1 receptors) would promote myocardial contractility during systole and help initially to maintain cardiac output despite increased after-load, working alongside the progressive unloading of muscle fibres achieved by the more gradual development of compensatory LVH in an attempt to normalise wall stress.
stress. Prolonged activation of sympathetic drive however would be expected to increase myocardial oxygen demand and energy expenditure of the hypertrophied myocardium, thereby contributing to cardiomyocyte ischemia and eventually cell death due to apoptosis or necrosis, resulting in impaired contractility of the residual myocardium and reduced ability to sustain the increased load since adult cardiomyocytes have only limited potential for self-renewal (Anversa et al., 2006). Enhancement of the negative contraction effect of NPY during early compensated hypertensive LVH could therefore represent a protective measure to help to counter the excessive shortening and energy expenditure of cardiomyocytes in response to norepinephrine. However, impaired intrinsic myocardial contractility has also been observed in patients with prolonged hypertension despite fully compensated ventricular hypertrophy, normal wall stress and apparently normal pump function (Aoyagi et al., 1993). The possibility should be considered that chronic enhancement of the negative contraction effects of NPY could instead represent a pathophysiological phenomenon, such that sustained depression of contractility mediated via Y2 receptor stimulation may exacerbate progressive mechanical dysfunction and impair ventricular emptying during systole and ultimately accelerate transition to overt failure.

**Reduced NPY Receptor Numbers in established cardiomyocyte hypertrophy**

Decreased Y2 and, to a lesser extent, Y1 receptor numbers in SHR cardiomyocytes at 20 weeks might reflect receptor down regulation which could arise by negative feedback as a consequence of increased receptor activity in SHR due to the observed increased potency of agonist-receptor complex interactions. However, maximum contractile responses to NPY and receptor-subtype selective antagonists were not reduced significantly, indicating either that residual receptor numbers are probably still surplus to functional requirements or that up-regulation of downstream signalling components coupled to Y receptors might offset any consequences of reduced receptor number. In regard to the latter: (1) phospholipase C-β mediated mobilisation of intracellular
calcium stores is enhanced in SHR myocardium (Kawaguchi et al., 1992; 1993). Since Y₁ receptors are known to couple to mobilisation of intracellular calcium stores in cardiomyocytes by a phospholipase C-dependent mechanism (Heredia et al., 2005), enhanced activity downstream of the receptor-involvement could compensate for, or alternatively may contribute to recruitment of processes responsible for the modest reduction in Y₁ receptor number. (2) Expression of G_i-α subunit protein is also enhanced and G_i-mediated inhibition of adenylate cyclase augmented (Bohm et al., 1994) implying enhanced activity downstream of the Y₂ receptor. Again, this could compensate for, or alternatively may contribute to the regulation of processes responsible for the modest reduction in Y₂ receptor number. However, I_to current is blunted (Cerbai et al., 1994; Yokoshiki et al., 1997) while conversely L-type calcium channel current is enhanced (Xiao and McArdle, 1994) in hypertrophied SHR myocardium; these effects would oppose the negative contraction effect of NPY, mediated via the Y₂ receptor subtype. This observation, together with the greater reduction in Y₂ than Y₁ receptor protein in hypertrophied cardiomyocytes, indicates that the balance between the opposing actions of the peptide may shift towards the Y₁ mediated positive contractile response, to assist the myocardium in potentiation of normal contractile performance, and attempt to offset a potentially detrimental influence of Y₂ receptor stimulation.

**Future studies and clinical significance**

The sympathetic nervous system co-transmitter, NPY, can elicit in SHR cardiomyocytes, an increase in the amplitude of contraction through the Y₁ receptor subtype, and also a negative effect on contraction parameters through the Y₂ receptor subtype, which both involve modulation of intracellular Ca^{2+} signalling. The potency of NPY to elicit positive and negative contraction effects is dramatically increased during the established phase of hypertrophic growth. As a compensatory strategy, the positive effect of NPY may contribute to maintenance of normal circulatory function, at least initially, in hypertension, whilst the negative contraction effect may contribute to the pathophysiological changes occurring within the myocardium which ultimately
contribute to the transition to overt failure. This study has focused on the early phase of compensated LVH in the SHR. Clearly, it would now be important to explore NPY-mediated contraction responses in aged SHR during the transition into overt failure to determine if the alterations identified in regard to each NPY receptor subpopulation persist and are enhanced. To further elucidate the respective roles of pressure-loading and LVH on the changes observed parallel studies in an appropriate model of volume-overload LVH should also be conducted. Intervention with suitable Y_2 selective antagonists in SHRs in vivo together with transgenic over-expression or knockout of the Y_2 receptor in aortic banded mice should help to clarify whether the influence of this receptor is cardio-protective or ultimately detrimental in the setting of pressure overload and, provided such findings can be extrapolated to humans, might indicate the rationale for therapeutic intervention with agents targeting this receptor subpopulation.
References


Footnotes: This work was funded by the British Heart Foundation.
Legends for Figures

**Figure 1.** Example recordings of: (a) positive contractile effect of NPY (10^{-7}M); (b) negative contractile effect of NPY (10^{-9}M) on elevated response to isoproterenol (ISO, 10^{-8}M) in electrically-stimulated cardiomyocytes.

**Figure 2.** Effects of (a) NPY and (b) Leu^{31}Pro^{34}NPY, in the presence of Ca^{2+} (3 x 10^{-6} mol/L), to stimulate contraction amplitude of cardiomyocytes from WKY rats and SHRs at (i) 12, (ii) 16 and (iii) 20 weeks of age. Data are given as differences from basal value, expressed as a percentage of resting cell length, δL% and are mean values ± SEM of n=5-6 experiments. EC_{50} values (mol/L) are: (a) (5.1 ± 3.2) x 10^{-10}, (7.2 ± 6.5) x 10^{-10}, (1.1 ± 0.5) x 10^{-9}, in WKY rats and (1.2 ± 0.22) x 10^{-9}, (1.3 ± 0.5) x 10^{-9}, (4.8 ± 1.1) x 10^{-13}*,†‡ in SHRs; (b) (9.2 ± 4.8) x 10^{-10}, (3.1 ± 6.5) x 10^{-10}, 2.3 ± 0.9) x 10^{-10} in WKY rats and (4.4 ± 2.0) x 10^{-10}, (1.2 ± 0.3) x 10^{-9}, (6.0 ± 4.8) x 10^{-13}*,†‡ in SHRs, respectively. *p<0.05 vs. strain-matched 12 week old rats, †p<0.05 vs. strain-matched 16 week old rats, ‡p<0.05 vs. age-matched WKY rats.

**Figure 3.** Effect of the Y_{1} receptor selective antagonist, BIBP3226 (10^{-7} mol/L) on amplitude of contraction stimulated by (a) NPY and (b) Leu^{31}Pro^{34}NPY, in the presence of Ca^{2+} (3 x 10^{-6} mol/L), in cardiomyocytes from WKY rats and SHRs at (i) 12, (ii) 16 and (iii) 20 weeks of age. Data are given as differences from basal value, expressed as a percentage of resting cell length, δL% and are mean values ± SEM of n=6 experiments. EC_{50} values (mol/L) are: (a) (6.0 ± 2.7) x 10^{-10}, (6.7 ± 2.6) x 10^{-10}, (7.9 ± 2.6) x 10^{-11}, in WKY rats and (1.9 ± 0.69) x 10^{-10}, (1.0 ± 0.5) x 10^{-9}, (1.4 ± 0.67) x 10^{-12}*,‡ in SHRs, respectively. *p<0.05 vs. strain-matched 12 week old rats, ‡p<0.05 vs. age-matched WKY rats.
**Figure 4.** Effect of PYY3-36, in the presence of Ca²⁺ (3 x 10⁻⁶ mol/L), to attenuate contraction amplitude of cardiomyocytes from WKY rats and SHRs at (a) 12, (b) 16 and (c) 20 weeks of age. Data are given as differences from basal value, expressed as a percentage of resting cell length, δL% and are mean values + SEM of n=6 experiments. EC₅₀ values (mol/L) are: (2.2 ± 0.71) x 10⁻¹⁰, (1.7 ± 0.98) x 10⁻¹⁰, (2.1 ± 0.31) x 10⁻¹⁰, in WKY rats and (3.2 ± 1.8) x 10⁻¹⁰, (2.5 ± 0.29) x 10⁻¹¹, (7.5 ± 4.5) x 10⁻¹³*†‡ in SHRs, respectively. *p<0.05 vs. strain-matched 12 week old rats, †p<0.05 vs. strain-matched 16 week old rats, ‡ p<0.05 vs. age-matched WKY rats.

**Figure 5.** Attenuation of isoproterenol (10⁻⁸ mol/L)–stimulated contraction amplitude by (a) NPY and (b) PYY3-36, under conditions of physiological Ca²⁺ concentration (10⁻⁳ mol/L), in cardiomyocytes from (i) WKY rats and (ii) SHRs at 12, 16 and 20 weeks of age. Data are given as percentage inhibition of the isoproterenol response and are mean values + SEM of n=8 experiments. EC₅₀ values (mol/L) are: (a) (5.8 ± 1.1) x 10⁻¹⁰, (2.8 ± 0.64) x 10⁻¹⁰, (7.5 ± 2.9) x 10⁻¹⁰, in WKY rats and (2.4 ± 2.2) x 10⁻¹⁰, (6.1 ± 0.70) x 10⁻¹¹, (4.3 ± 3.7) x 10⁻¹²*‡ in SHRs; (b) (8.8 ± 6.7) x 10⁻¹⁰, (1.3 ± 1.7) x 10⁻⁹, 6.1 ± 0.60) x 10⁻¹⁰ in WKY rats and (1.6 ± 0.66) x 10⁻¹⁰, (2.7 ± 0.41) x 10⁻¹¹, (4.2 ± 1.1) x 10⁻¹²*‡ in SHRs; respectively. *p<0.05 vs. strain-matched 12 week old rats, † p<0.05 vs. age-matched WKY rats.

**Figure 6.** Effect of the Y₂ receptor selective antagonist, BIIIE246 (10⁻⁷ mol/L) [n=6], on maximum (a) NPY- and (b) PYY- induced attenuation of isoproterenol (10⁻⁸ mol/L) stimulated amplitude of contraction in cardiomyocytes using the peptides at a maximally effective concentration of 3 x 10⁻⁹ mol/L in WKY rats at all ages and in 12 week old SHRs, and at 3 x 10⁻¹⁰ mol/L in 16 and 20 week old SHRs, to take account of increased potency of the peptides with advancing age in SHRs. Data are expressed as a percentage of the control isoproterenol response and are mean values + SEM of n=8 experiments.
**Figure 7.** NPY-Y<sub>1</sub> and NPY-Y<sub>2</sub> receptor mRNA RT-PCR dissociation curves (a), cyclic numbers (b) and expression levels standardised to GADPH mRNA (c, d); representative immunoblots and receptor protein levels standardised to β-actin (e, f) in left ventricular cardiomyocytes isolated from SHR and WKY rats aged 20 weeks. Data are mean values + SEM of n=7-9 experiments. *p<0.05 vs. age-matched WKY rats.
### Table 1: Primers Sequences

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<td>AGCAAGTTGGAAGGCATGGA 20 bp [851-870]</td>
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Accession Numbers are taken from the European Molecular Biology Laboratory (EMBL) database which is part of the *International Nucleotide Sequence Database Collaboration*. 
Figure 2

(i) Contraction amplitude (Δ%Δ L) for WKY and SHR.

(ii) Contraction amplitude (Δ%Δ L) for WKY and SHR.

(iii) Contraction amplitude (Δ%Δ L) vs. log_{10} [NPY] (M) for WKY and SHR.

(iv) Contraction amplitude (Δ%Δ L) vs. log_{10} [Leu^{31}Pro^{34}NPY] (M) for WKY and SHR.
Figure 4

(a) Contraction amplitude vs. log₁₀ [PYY <sub>3-36</sub>] (M)

(b) Contraction amplitude vs. log₁₀ [PYY <sub>3-36</sub>] (M)

(c) Contraction amplitude vs. log₁₀ [PYY <sub>3-36</sub>] (M)
Figure 5

(a) Attenuation of isoproterenol (10^-8 mol/L)-stimulated contraction amplitude (%) vs. 
\[ \log_{10}[\text{NPY}] \text{ (mol/L)} \]

(b) Attenuation of isoproterenol (10^-8 mol/L)-stimulated contraction amplitude (%) vs. 
\[ \log_{10}[\text{PYY}_{3-36}] \text{ (mol/L)} \]
Figure 7a

Delta Rn vs Cycle

Delta Rn

1.0e+01
1.0e+00
1.0e-01
1.0e-02
1.0e-03
1.0e-04

1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39

Cycle Number

GAPDH

NPY Y1

NPY Y2
Figure 7 c-f

(c) 

Y₁ mRNA

(d) 

Y₂ mRNA

(e) 

NPY Y₁ 42 KDa

Actin 42 KDa

Y₁ protein

(f) 

NPY Y₂ 60 KDa

Actin 42 KDa

Y₂ protein