Modulation of Contractile Function through Neuropeptide Y Receptors during Development of Cardiomyocyte Hypertrophy

Adrian R. Allen, Elizabeth J. Kelso, David Bell, YouYou Zhao, Paula Dickson, and Barbara J. McDermott

Cardiovascular Research Group, School of Medicine and Dentistry, Queen’s University Belfast, Belfast, United Kingdom

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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 Y1 and Y2 receptors, respectively. This study tested the hypothesis that NPY receptor-mediated contraction is altered during progression of LVH. 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 Wistar Kyoto (WKY) rats. Electrically stimulated (60 V, 0.5 Hz) cell shortening was measured using edge detection and receptor expression determined at mRNA and protein level. The NPY and Y1 receptor-selective agonist, Leu5Pro34NPY, stimulated increases in contractile amplitude, which were abolished by the Y1 receptor-selective antagonist, BIBP3226 [(R-N2-(diphenyl-acetyl)-(4-hydroxyphenyl)methyl-argininamide)], confirming Y1 receptor involvement. Potencies of both agonists were enhanced in SHR cardiomyocytes at 20 weeks (2300- and 380-fold versus controls). Maximal responses were not attenuated. BIBP3226 unmasked a negative contraction effect of NPY, elicited over the concentration range (10^-12 to 3 x 10^-9 M) in which NPY and PYY3-36 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); maximal responses were not altered. Expression of NPY-Y1 and NPY-Y2 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. Enhancement of NPY potency, producing (opposing) contractile effects on cardiomyocytes together with unchanged maximal response despite reduced receptor number, enables NPY to contribute to regulating cardiac performance during compensatory LVH.

Mammalian myocardium contains large quantities of neuropeptide Y (NPY) (Onuoha et al., 1999), mainly colocalized 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) because increased plasma levels of the peptide are found in hypertension, myocardial infarction, and heart failure (Maisel et al., 1989) and correlated 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 (Ito) 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 Y2 receptor agonists, PYY3–36 and NPY13–36, inferred Y2 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 Y2 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 selectively inhibits Ito 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 mobilization of intracellular calcium stores in cardiomyocytes by a phospholipase C-dependent mechanism (Heredia et al., 2005).

The number of postsynaptic α\textsubscript{1}-adrenoceptors and the amount of inositol 1,4,5-triphosphate accumulated in the myocardium in response to α\textsubscript{1}-adrenoceptor stimulation are markedly increased in hypertension (Hanna and Khairallah, 1986). The release of NPY from the cardiac sympathetic innervation would also be anticipated 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., 1993; Bohm et al., 1995) of NPY are increased, and alterations in NPY-mediated myocardial contractile responses may be anticipated because receptors for the peptide couple to signal transduction processes known to be altered in hypertrophied cardiomyocytes (Kawaguchi et al., 1992, 1993; Chen and Han, 1995).

The spontaneously hypertensive rat (SHR) is a useful model of pressure overload (Okamoto and Aoki, 1963; Pfeffer et al., 1994; Xiao and McArdle, 1994; Yokoshiki et al., 1997). Processes known to be altered in hypertrophied cardiomyocytes (Bell et al., 2002). Expression of NPY Y\textsubscript{1} and Y\textsubscript{2} 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 to 20 weeks, which encompasses baseline and developmental characteristics at cardiomyocyte level, has been identified (Bell et al., 2004). Furthermore, the Y\textsubscript{2} 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 hypothesized 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 Ca\textsuperscript{2+}, the positive inotrope, isoproterenol, and NPY. In combination with Ca\textsuperscript{2+} or isoproterenol, use of selective NPY receptor agonists (Y\textsubscript{1} receptor, Leu\textsuperscript{31}Pro\textsuperscript{34}NPY; Y\textsubscript{2} receptor, PYY\textsubscript{3-36}, Y\textsubscript{2} receptor, d-Trp\textsuperscript{38}NPY) and antagonists (Y\textsubscript{1} receptor, BIBP3226; Y\textsubscript{2} receptor, BIIE246) allowed differentiation of receptor-subtype mediated effects. Expression of NPY Y\textsubscript{1} and NPY Y\textsubscript{2} receptors was examined at mRNA and protein level.

## Materials and Methods

### Experimental Model

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

### Isolation and Culture of Cardiomyocytes

After deep anesthesia 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  \times 10\textsuperscript{6} viable cardiomyocytes/mL in creatinine-carnitine-taurine 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 GmbH, Jena, Germany) and allowed to settle for 10 min before being bathed with oxygenated (95% O\textsubscript{2}, 5% CO\textsubscript{2}) Krebs-Henseleit buffer. Cells were field-stimulated at 0.5 Hz with biphasic pulses of 0.5-ms duration at 60 V in Ag/AgCl wires embedded in the wall of the chamber. After initial stimulation, contractile amplitude was determined after incubation for 4 min (under basal conditions or with isoproterenol) or after 15 min (with NPY receptor agonists or antagonists). Responses to NPY receptor agonists were demonstrated in the presence of elevated Ca\textsuperscript{2+} (3  \times 10\textsuperscript{-3} M) or a submaximal concentration of isoproterenol (10\textsuperscript{-8} M), 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 were determined by RT-PCR and expressed relative to glyceraldehyde-3-phosphate dehydrogenase mRNA. Reported sequences for each gene (Table 1) were used to design on Primer Express software (Applied Biosystems, Foster City, CA), rat-specific primers adapted to RT-PCR conditions, which were synthesized by Invitrogen (Carlsbad, CA). 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-polyacrylamide gel electrophoresis (80 μg of protein per lane) as described previously (Zhao et al., 2006) and transferred to polyvinylidene difluoride membrane (0.45 μm; Millipore Corporation, Billerica, MA). The polyvinylidene difluoride membrane was washed with phosphate-buffered saline containing 0.1% (v/v) Tween 20 (Sigma Chemical Co. Ltd., Poole, Dorset, UK) and blocked overnight in phosphate-buffered saline/0.1% (v/v) Tween 20 solution containing 5% (w/v) milk. Immunoblotting was performed using primary antibodies directed specifically against rodent NPY Y\textsubscript{1} and Y\textsubscript{2} receptors (Santa Cruz Biotechnology; sc-21992 and sc-14736, respectively, raised in goat) used at a dilution of 1:500. Immunoblot complexes were detected using secondary antibodies conjugated to horseradish peroxidase (donkey anti-goat sc-2020 used at a dilution of 1:20,000; Santa-Cruz Biotechnology) and ECL plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as substrate and quantified by densitometry (Analytical

### Table 1

<table>
<thead>
<tr>
<th>Primer sequences</th>
<th>Accession numbers from the European Molecular Biology Laboratory (EMBL) data base, which is part of the International Nucleotide Sequence Database Collaboration.</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5'-GAAACCACATCACCACCTTCCA-3' (21 bp; 248–268) 5'-ACCCCACCTTTGATTTGCGG-3' (20 bp; 280–299)</td>
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<tr>
<td>AB017801</td>
<td></td>
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<tr>
<td>NPY-Y, ZI1504</td>
<td>5'-TGCAAACTCTGAGTTAGAACA-3' (20 bp; 232–251) 5'-ACTCGGACGAGAAAGCTGA-3' (20 bp; 317–336)</td>
</tr>
<tr>
<td>NPY-Y, AY004257</td>
<td>5'-ACGGTTAAGGCTCGAGGAA-3' (21 bp; 746–766) 5'-AGCAAGGTTGAGGACCATGGA-3' (20 bp; 851–870)</td>
</tr>
</tbody>
</table>
Imaging System) normalized for protein loading using β-actin (Santa Cruz Biotechnology; sc-1616 in goat).

**Chemicals.** Medium M199 (glutamine-free with Earle’s salts) and penicillin (5000 IU)/streptomycin (5 mg/ml) were supplied by Gibco BRL (Paisley, Renfrewshire, UK). Carnitine, creatine, taurine, 4-aminopyridine, isoproterenol hydrochloride, and BIBP3226 were obtained from the Sigma Chemical Co. Ltd. NPY (human, rat), Leu31Pro34NPY (human, rat), and PYY3–36 were purchased from Bachem (St. Helens, Merseyside, UK). BIIE246 was kindly supplied by Dr. Henri Doeds (Boehringer Ingelheim Pharmaceuticals, Biberach, Germany). d-Trp34NPY was synthesized in the laboratory of Professor Ambikaipakan Balasubramaniam (University of Cincinnati Medical Center, Cincinnati, OH). All other chemicals were of analytical grade and purchased from BDH Chemicals (Poole, Dorset, UK).

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

**Data Analysis.** All data are presented as mean ± S.E.M. Contractile amplitude was measured in micrometers and expressed as a percentage change from the diastolic length, as an absolute value (%ΔL), or as a change from the baseline (Δ%ΔL) and n denotes the number of cell preparations used. Concentration-response data were analyzed by nonlinear regression using GraphPad Prism (version 4; GraphPad Software Inc., San Diego, CA). Statistical analyses were preformed 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 (version 11.5; SPSS Inc., Chicago, IL). 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 Ca2+ (10⁻³ M) on contractile amplitude (%ΔL) of cardiomyocytes (data are given as mean ± S.E.M. and were obtained in cardiomyocytes from 12-week-old animals following 15-min incubation with agonist versus time-matched baseline values): NPY (10⁻⁷ M: 3.0 ± 0.68 versus 4.9 ± 0.55, n = 6 WKY; 3.38 ± 0.96 versus 3.98 ± 1.07, n = 6 SHR; 3 × 10⁻⁸ M: 3.84 ± 0.50 versus 3.79 ± 0.75, n = 6 WKY; 2.00 ± 0.42 versus 1.56 ± 0.40, n = 6 SHR), Leu3¹Pro3⁴NPY (10⁻⁷ M: 3.43 ± 0.66 versus 3.40 ± 0.57, n = 6 WKY; 2.84 ± 0.48 versus 3.19 ± 0.42, n = 6 SHR), PYrå³–rå⁶ (3 × 10⁻⁹ M: 5.57 ± 1.69 versus 5.53 ± 1.68, n = 5 WKY; 2.83 ± 0.60 versus 2.79 ± 0.46, n = 6 SHR), and d-Trp³⁴NPY (10⁻⁷ M: 1.00 ± 0.29 versus 1.25 ± 0.30, n = 5 WKY; 2.04 ± 0.26 versus 2.22 ± 0.21, n = 6 SHR). Likewise, there were no effects of the antagonists, BIBP3226 (10⁻⁷ M: 4.19 ± 1.35 versus 4.29 ± 1.22, n = 4 WKY; 6.57 ± 1.01 versus 6.67 ± 1.18, n = 4 SHR) or BIIE246 (10⁻⁷ M: 5.92 ± 1.38 versus 6.03 ± 1.06, n = 4 WKY; 3.26 ± 1.29 versus 3.37 ± 1.30, n = 4 SHR) per se with Ca²⁺ (10⁻³ M); also BIIE246 (10⁻⁷ M) did not antagonize the response to isoproterenol (10⁻⁸ M) (4.79 ± 0.78 in the presence of BIIE246 versus 3.70 ± 0.63 alone, n = 4 WKY; 4.42 ± 0.45 in the presence of BIIE versus 3.63 ± 0.35 alone, n = 4 SHR).

Therefore, BIBP3226 and BIIE246 can be used appropriately to test the Y₁ and Y₂ receptor subtype specificity, respectively, of NPY responses. Leu³¹Pro³⁴NPY (10⁻⁷ M) with Ca²⁺ (10⁻³ M) did not alter the positive effect of isoproterenol (10⁻⁸ M) on amplitude of contraction (5.53 ± 0.41 in the presence of Leu³¹Pro³⁴NPY versus 5.46 ± 0.49 alone, n = 6 WKY; 4.40 ± 0.77 in the presence of Leu³¹Pro³⁴NPY versus 4.01 ± 0.58 alone, n = 4 SHR). Therefore, it is apparent that the Y₁ receptor-selective compound does not have activity at Y₂ receptors in this experimental system. For experiments with BIIE246, concentrations of NPY and PYrå³–rå⁶ that produced maximal attenuation of isoproterenol (10⁻⁸ M)-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 × 10⁻¹⁰ M but was 3 × 10⁻⁹ M 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 to 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 Fig. 1a. In the presence of Ca²⁺ (3 × 10⁻⁶ M), NPY (Fig. 2a) and Leu³¹Pro³⁴NPY (Fig. 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 to 10⁻⁷ M, and EC₅₀ values (in the 10⁻⁹ M range) were similar. The potency

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**Fig. 1.** Example recordings of positive contractile effect of NPY (10⁻⁷ M) (a) and negative contractile effect of NPY (10⁻⁹ M) on elevated response to isoproterenol (ISO; 10⁻⁸ M) (b) in electrically stimulated cardiomyocytes.

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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 Leu31Pro34NPY with increased contraction amplitude showed that the potencies were similar under all conditions. As such, the potency of Leu31Pro34NPY to increase contraction amplitude was unchanged in WKY rat cardiomyocytes but enhanced in cardiomyocytes obtained from SHRs at 20 weeks of age (380-fold versus age-matched controls). Maximal responses to NPY and to Leu31Pro34NPY did not differ significantly between SHR and WKY rats at any age. The Y1 receptor-selective antagonist, BIBP3226, abolished the positive contraction effects of NPY (Fig. 3a) and Leu31Pro34NPY (Fig. 3b) across the range of concentration used (10^{-13} to 10^{-7} M) in both SHR and WKY rat cardiomyocytes at all ages.

**Fig. 2.** Effects of NPY (a) and Leu31Pro34NPY (b) in the presence of Ca^{2+} (3 \times 10^{-6} M) to stimulate contraction amplitude of cardiomyocytes from WKY rats and SHRs at 12 (i), 16 (ii), and 20 (iii) weeks of age. Data are given as differences from basal value, expressed as a percentage of resting cell length (\%L) and are mean values \pm S.E.M. of n = 5 to 6 experiments. EC_{50} values (in moles per liter) are: a, (5.1 \pm 3.2) \times 10^{-10}, (7.2 \pm 6.5) \times 10^{-10}, and (1.1 \pm 0.5) \times 10^{-9}, in WKY rats and (1.2 \pm 0.22) \times 10^{-9}, (1.3 \pm 0.5) \times 10^{-9}, and (4.8 \pm 1.1) \times 10^{-10} \pm in SHRs; and b, (9.2 \pm 4.8) \times 10^{-10}, (3.1 \pm 6.5) \times 10^{-10}, (2.3 \pm 0.9) \times 10^{-10} in WKY rats; and (4.4 \pm 2.0) \times 10^{-10}, (1.2 \pm 0.3) \times 10^{-9}, (6.0 \pm 4.8) \times 10^{-10} \pm in SHRs, respectively. *, p < 0.05 versus strain-matched 12-week-old rats; †, p < 0.05 versus strain-matched 16-week-old rats; ‡, p < 0.05 versus age-matched WKY rats.

Temporal and Concentration Dependence of the Negative Contraction Response to NPY Unmasked in the Presence of BIBP3226. NPY in the presence of BIBP3226 elicited a negative, concentration-dependent effect in all cases (Fig. 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 \times 10^{-6} M) (Fig. 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} M 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 with that observed in cardiomyocytes from WKY rats at 20 but not at 12 and 16 weeks of age. In the presence of BIBP3226, Leu31Pro34NPY applied over the same concentration range, which, when used alone, had increased contraction...
tion amplitude in SHR and WKY rat cardiomyocytes, had no effect on amplitude of contraction (Fig. 3b).

Temporal and Concentration Dependence of the Negative Contraction Response to PYY$_{3-36}$ Alone.

PYY$_{3-36}$ (10$^{-12}$ to 3 × 10$^{-9}$ M) elicited a concentration-dependent, negative contraction effect in cardiomyocytes from SHRs and WKY rats of all ages (Fig. 4). Maximal 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 3 × 10$^{-10}$ M, and in WKY rat cardiomyocytes was unchanged with progression of age, whereas potency increased in SHRs, becoming significantly elevated (∼400) in cardiomyocytes from 20-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 (∼33) at 16 weeks, becoming significantly elevated (∼400) in cardiomyocytes from 20-week-old SHRs compared with that found in WKY rats. The potency of PYY$_{3-36}$ and NPY in the presence of BIBP3226 (Figs. 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 Fig. 1b. NPY at 10$^{-12}$ to 3 × 10$^{-9}$ M reduced the positive contraction effect of isoproterenol in cardiomyocytes from both rat strains at all ages (Fig. 5, a and b). In cardiomyocytes from 12-week-old rats, the potency of NPY was in the region of 100 pM and was unaltered with increasing age in WKY rats but increased (60-fold) in SHRs, being significant in 20- compared with 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 versus age-matched controls). PYY3–36 was equally potent in attenuating isoproterenol-stimulated contraction amplitude (Fig. 5) and in eliciting decreased contraction amplitude under elevated extracellular Ca²⁺ conditions (Fig. 4). BIIIE246, applied at a concentration (10⁻⁷ M) at which it has high affinity for the Y₂ receptor subtype but virtually no affinity for the Y₁, Y₄, and Y₅ receptor subtypes (Doods et al., 1999), abolished the maximal attenuation of isoproterenol-stimulated contraction amplitude elicited by NPY (and PYY3–36) at all ages in both strains of rat cardiomyocytes (Fig. 6). Application of the Y₅ receptor-selective agonist, d-Trp³⁴NPY, at a concentration (10⁻⁷ M) 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 the presence of d-Trp³⁴NPY versus 4.89 ± 0.70 alone, n = 6 WKY; 4.99 ± 0.66 in the presence of d-Trp³⁴NPY versus 4.31 ± 0.63 alone, n = 6 SHR); data are given as mean ± S.E.M. 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 (Fig. 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 = N.S.) and 80% (p < 0.05), respectively, were observed at the 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 Y₁ 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 Y₁ receptor-selective agonist, Leu³¹Pro³⁴NPY in all strains/ages; abolition of these effects by BIBP3226, applied at a concentration determined to have no cross reactive effects on rat Y₂ receptors (Doods et al., 1996); and no stimulation on the total reversal of Leu³¹Pro³⁴NPY’s positive contraction effects, indicates that the negative effect must arise from a population of receptors distinct from the Y₁ subtype, possibly the Y₂ subtype. This was substantiated by the following findings. Potencies and magnitudes of effect of PYY3–36 were similar to those elicited by NPY in the presence of BIBP3226; NPY (and PYY3–36) attenuated the positive contraction effects of isoproterenol, exhibiting similar potencies within strains. Although PYY3–36 was thought initially to be selective for the Y₂ 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 Y₅ receptor (Hu et al., 1996). However, the latter does not seem to be involved in mediating the negative contraction effects of NPY (or PYY3–36) because the Y₅ receptor-selective agonist, d-Trp³⁴NPY, did not attenuate the effects of isoproterenol on amplitude of cardiomyocyte contraction. Abolition by BIIIE246 of the attenuation by NPY (and PYY3–36) of isoproterenol-stimulated contraction indicates that in the absence of Y₁ receptor activity, NPY and associated peptides act on Y₂ receptors to stimulate a negative contraction effect, confirming earlier studies in which the negative contraction effect of NPY has been attributed to Y₂ 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 approxi-
mately $4 \times 10^{-11}$ M (Ogawa et al., 1989), which lies just outside the range ($10^{-10}$ to $10^{-7}$ M) in which the peptide stimulates increased contraction function in WKY rat myocytes. Therefore, it seems 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

![Figure 5](image-url)

**Fig. 5.** Attenuation of isoproterenol ($10^{-8}$ M)-stimulated contraction amplitude by NPY (a) and PYY3–36 (b) under conditions of physiological Ca$^{2+}$ concentration ($10^{-6}$ M) in cardiomyocytes from WKY rats (i) and SHRs (ii) at 12, 16, and 20 weeks of age. Data are given as percentage inhibition of the isoproterenol response and are mean values ± S.E.M. of $n = 8$ experiments. EC50 values (in moles per liter) are: a, (5.8 ± 1.1) × $10^{-10}$, (2.8 ± 0.64) × $10^{-10}$, and (7.5 ± 2.9) × $10^{-10}$ in WKY rats and (2.4 ± 2.2) × $10^{-10}$, (6.1 ± 0.70) × $10^{-10}$, and (4.3 ± 3.7) × $10^{-12}$ in SHRs; b, (8.8 ± 6.7) × $10^{-12}$, (1.3 ± 1.7) × $10^{-12}$, and (6.1 ± 0.66) × $10^{-10}$ in WKY rats and (1.6 ± 0.66) × $10^{-10}$, (2.7 ± 0.41) × $10^{-11}$, and (4.2 ± 1.1) × $10^{-12}$ in SHRs; respectively. *, $p < 0.05$ versus strain-matched 12-week-old rats; ‡, $p < 0.05$ versus age-matched WKY rats.
modulation of contractile function via $Y_1$ receptors. Likewise, the concentration range ($10^{-10}$ to $3 \times 10^{-9}$ M) 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 fibers achieved by the more gradual development of compensatory LVH in an attempt to normalize wall stress. However, prolonged activation of sympathetic drive 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 because 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 $Y_2$ 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 $Y_2$ and, to a lesser extent, $Y_1$ receptor numbers in SHR cardiomyocytes at 20 weeks might reflect receptor down-regulation that 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, maximal 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 signaling components coupled to Y receptors might offset any consequences of reduced receptor number. In regard to the latter, phospholipase C-β-mediated mobili-

![Fig. 7. NPY-Y₁ and NPY-Y₂ receptor mRNA RT-PCR dissociation curves (a), cyclic numbers (b), and expression levels standardized to GADPH mRNA (c and d); representative immunoblots and receptor protein levels standardized to β-actin (e and f) in left ventricular cardiomyocytes isolated from SHR and WKY rats aged 20 weeks. Data are mean values ± S.E.M. of n = 7 to 9 experiments. *, p < 0.05 versus age-matched WKY rats.](image-url)
zation of intracellular calcium stores is enhanced in SHR myocardium (Kawaguchi et al., 1992, 1993). Because Y1 receptors are known to couple to mobilization 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. Expression of G\textsubscript{\alpha}\textsuperscript{\textgamma} subunit protein is also enhanced, and G\textsubscript{\alpha}\textsuperscript{\textgamma}-mediated inhibition of adenylyl cyclase is 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\textsubscript{Na} current is blunted (Cerbai et al., 1994; Yokoshiki et al., 1997); 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 toward the Y1-mediated positive contractile response to assist the myocardium in potentialization of normal contractile performance and attempt to offset a potentially detrimental influence of Y2 receptor stimulation.

**Future Studies and Clinical Significance.** The sympathethic nervous system cotransmitter, 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\textsuperscript{2+} signaling. 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, whereas 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 whether 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\textsubscript{\alpha} selective antagonists in SHR in vivo together with transgenic overexpression or knockout of the Y\textsubscript{\alpha} receptor in aortic banded mice should help to clarify whether the influence of this receptor is cardioprotective 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**


Address correspondence to: Dr. David Bell, Division of Medicine and Therapeutics, Whitla Medical Building, 97 Lisburn Road, Belfast BT9 7BL, UK. E-mail: d.bell@qub.ac.uk