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Neuropeptide Y Y_1 receptor regulates protein turnover and constitutive gene expression in hypertrophying cardiomyocytes

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Abstract

Increased levels of neuropeptide Y correlate with severity of left ventricular hypertrophy in vivo. At cardiomyocyte level, hypertrophy is characterised by increased mass and altered phenotype. The aims were to determine the contributions of increased synthesis and reduced degradation of protein to neuropeptide Y-mediated increase in mass, assess effects on gene expression, and characterise neuropeptide Y Y receptor subtype involvement. Neuropeptide Y (10 nM) increased protein mass of adult rat ventricular cardiomyocytes maintained in culture (24 h) (16% > basal) and de novo protein synthesis (incorporation of [^{14}C]phenylalanine) (18%>basal). Neuropeptide Y (100 nM) prevented degradation of existing protein at 8 h. Actinomycin D (5 μM) attenuated increases in protein mass to neuropeptide Y (≤ 1 nM) but not to neuropeptide Y (10 nM). [Leu^{31}, Pro^{34}]neuropeptide Y (10 nM), an agonist at neuropeptide Y Y_1 receptors, increased protein mass (25%>basal) but did not stimulate protein synthesis. Neuropeptide Y-(3–36) (10 nM), an agonist at neuropeptide Y Y_2 receptors, increased protein mass (29%>basal) and increased protein synthesis (13%>basal), respectively. Actinomycin D (5 μM) abolished the increase in protein mass elicited by neuropeptide Y-(3–36) but not that by [Leu^{31}, Pro^{34}]neuropeptide Y. BIBP3226 [(*R*)-*N*2-(diphenylacetyl)-*N*-(4-hydroxyphenylmethyl)-D-arginine amide] (1 μM), a neuropeptide Y Y_1 receptor subtype-selective antagonist, and T_4 [neuropeptide Y-(33–36)]$_4$, a neuropeptide Y Y_2 receptor subtype-selective antagonist, attenuated the increase in protein mass to 100 nM neuropeptide Y by 68% and 59%, respectively. Neuropeptide Y increased expression of the constitutive gene, myosin light chain-2 (MLC-2), maximally at 12 h (4.7-fold>basal) but did not induce ($t \leq 36$ h) expression of foetal genes (atrial natriuretic peptide (ANP), skeletal-α-actin and myosin heavy chain-β). This increase was attenuated by 86% and 51%, respectively, by BIBP3226 (1 μM) and T_4 [neuropeptide Y-(33–36)]$_4$ (100 nM). [Leu^{31}, Pro^{34}]neuropeptide Y (100 nM) (2.4-fold>basal) and peptide YY-(3–36) (100 nM) (2.3 fold>basal) increased expression of MLC-2 mRNA at 12 h. In conclusion, initiation of cardiomyocyte hypertrophy by neuropeptide Y requires activation of both neuropeptide Y Y_1 and neuropeptide Y Y_2 receptors and is associated with enhanced synthesis and attenuated degradation of protein together with increased expression of constitutive genes but not reinduction of foetal genes. © 2002 Published by Elsevier Science B.V.

Keywords: Neuropeptide Y; (Rat); Cardiomyocyte; Hypertrophy; Protein turnover; Receptor subtype; MLC-2 (myosin light chain-2); ANP (atrial natriuretic peptide); Skeletal-α-actin; MHC-β (myosin heavy chain-β)

1. Introduction

Increased plasma levels of neuropeptide Y, an autonomic neurotransmitter co-stored with noradrenaline in the sympathetic innervation of the heart and vessels, have been detected in patients with a variety of cardiovascular diseases including hypertension and heart failure (Maisel et al., 1989; Ullman et al., 1990; Hulting et al., 1990). Although a causal role for circulating neuropeptide Y in these diseases has not been established, a consistent finding is the correlation between plasma concentration of the peptide and the severity of left ventricular hypertrophy (Hulting et al., 1990).

Neuropeptide Y and the related peptide YY and pancreatic polypeptide can activate at least six different receptor populations in a wide array of tissues and cells in the central and peripheral nervous systems (Balasubramaniam, 1997; Michel et al., 1997). Identification of subtypes of receptors for neuropeptide Y has been based largely on the rank order of affinities or activities of neuropeptide Y analogues and fragments in radioligand binding studies or bioassay systems (McDermott et al., 1993). [Leu^{31}, Pro^{34}]neuropeptide Y is an agonist with some selectivity for neuropeptide Y Y_1 and neuropeptide Y Y_4 receptors relative to other neuropeptide Y

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Y receptor subtypes (Fuhlendorff et al., 1990; Dumont et al., 1994, 2000; Gehlert et al., 1997; Michel et al., 1997), while neuropeptide Y-(3–36) and peptide YY-(3–36) are selective for neuropeptide Y Y_2 and neuropeptide Y Y_5 receptors relative to other neuropeptide Y Y receptor subtypes (Michel et al., 1997; Dumont et al., 2000). Pancreatic polypeptide has high affinity at neuropeptide Y Y_4 receptors but much lower affinity relative to neuropeptide Y and peptide YY at other receptor subtypes including neuropeptide Y Y_1 receptors (Lundell et al., 1996). The peptide analogue, T_4 [neuropeptide Y-(33–36)]$_4$, is a selective antagonist at neuropeptide Y Y_2 receptors (Grouzmann et al., 1997; Pheng et al., 1999). The recent development of nonpeptide neuropeptide Y Y receptor antagonists has assisted greatly in receptor classification (reviewed in Cabrele and Beck-Sickinger, 2000). BIBP3226 [(*R*)-*N*2-(diphenylacetyl)-*N*-(4-hydroxyphenylmethyl]-D-arginine amide] is a highly selective nonpeptide antagonist at neuropeptide Y Y_1 receptors (Doods et al., 1999).

At the cellular level, myocardial hypertrophy is based on the increase in total mass, not number, of myocardial cells since adult cardiomyocytes do not undergo cell division. Increases in mass of myocardial cells are achieved by increased synthesis of de novo protein, due primarily to increases in ribosomal RNA content, and to reduced degradation of existing protein (reviewed in Schluter et al., 1995a). Hypertrophying cardiomyocytes also exhibit an altered pattern of gene expression characterised by the enhanced expression of constitutive genes including myosin light-chain-2 (MLC-2) and the reinduction of genes such as atrial natriuretic peptide (ANP), creatine kinase-B, myosin heavy chain-β (MHC-β) and skeletal-α-actin that are normally only expressed in the foetal state (reviewed in Schluter et al., 1995a).

Neuropeptide Y increases cellular protein mass by attenuating protein degradation in healthy cardiomyocytes maintained in short-term serum-free culture, a model of relevance to the initiation of hypertrophy in vivo, but does not influence protein synthesis or reinduce the foetal isoform of creatine kinase, creatine kinase-BB (Millar et al., 1994; Goldberg et al., 1998). In contrast, after 1 week in serum-supplemented media, cardiomyocytes lose their rod-shaped appearance and obtain a spread morphology (redifferentiated form) (Schluter and Piper, 1992; Pinson et al., 1993), which is thought to parody the maintenance of established hypertrophy in vivo; these cells have been shown to acquire responsiveness to the peptide for protein synthesis and reexpression of creatine kinase-BB (Millar et al., 1994; Goldberg et al., 1998). A role for the cytokine, transforming growth factor beta (TGF-β), in the acquisition of this responsiveness of the cells to the peptide has been proposed (Schluter et al., 1995b; Goldberg et al., 1998).

The aims of the present study were firstly to investigate the role of the neuropeptide Y Y_1 receptor subtype specifically in the initiation of cardiomyocyte hypertrophy through the accumulation of cellular protein in the absence of enhanced protein synthesis, and secondly determine whether neuropeptide Y, acting at neuropeptide Y Y_1 receptors exclusively, could *initiate* alterations in expression of the constitutive gene, myosin light chain-2, and of the foetal genes ANP, skeletal α-actin and myosin heavy chain-β.

2. Materials and methods

2.1. Solutions

Serum-free 'creatinine–carnitine–taurine' (CCT) medium for the culture of cardiomyocytes consisted of modified glutamine-free Medium M199 supplemented with Earle's salts, HEPES (15 mM), creatinine (5 mM), *l*-carnitine (2 mM), taurine (5 mM), ascorbic acid (100 μM), penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}). Medium was also supplemented with cytosine β-D-arabinofuranoside (10 μM) to prevent growth of nonmyocytes. The composition of the Ca^{2+}-free Krebs–Ringer solution used in the isolation of cardiomyocytes was as follows: NaCl (110 mM); KCl (2.6 mM); $NaHCO_3$ (25 mM); $MgSO_4$ (1.2 mM); KH_2PO_4 (1.2 mM); glucose (11 mM). This solution was gassed with 95% O_2/5% CO_2 and maintained at a pH of 7.4 at 37 °C. The composition of the phosphate-buffered saline (PBS) was as follows: NaCl (137 mM); KH_2PO_4 (1.5 mM); Na_2PO_4 (1.0 mM), pH 7.4. The composition of DNA assay solution was NaCl (1.985 M), Na_2HPO_4 (25 mM), at pH 7.4. Bisbenzamide was dissolved in water (0.2 mg ml^{-1}). This stock solution was diluted 1:200 with DNA assay solution to give a working concentration of 1 μg ml^{-1}. Solutions of bisbenzamide are very sensitive to light and were therefore kept in the dark prior to use. The stock solution was stable for 6 months in the dark at 4 °C. Dilute solutions were prepared daily.

2.2. Isolation and culture of cardiomyocytes

All work 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. Ventricular cardiomyocytes were isolated as described previously (Bell et al., 1995). Briefly, adult (12 weeks) male Sprague–Dawley rats were subjected to deep isoflurane anaesthesia and their hearts excised. The excised hearts from two rats were immediately immersed in ice-cold saline to remove residual blood and reduce the risk of clotting, slow the metabolic rate and delay the onset of hypoxia, and then subjected to simultaneous perfusion using a Langendorff apparatus with Ca^{2+}-free Krebs Ringer solution containing collagenase (0.4 mg ml^{-1}) until they became flaccid. The two hearts were chopped finely and the mince was pooled and agitated gently in the same medium to dissociate individual cells. The resulting cell suspension was filtered to remove undigested material and the cells were sedimented at 750 rpm for 4 min. Ca^{2+} tolerance of

the cells was restored gently by resuspending the sediment in Krebs–Ringer solution containing a progressively higher concentration of Ca^{2+} to a final concentration of 1 mM. The cell suspension (3–4 ml) was then layered gently onto a 4% (w/v) albumin solution (12.5 ml), contained in a tube of length 20 cm and internal diameter 1 cm, in order to sediment viable cardiomyocytes and effectively remove nonmuscle cells and cell debris. The resultant sediment was resuspended in serum-free CCT medium. Cells derived from the two hearts were pooled, mixed thoroughly and resuspended at a concentration of 1.5×10^5 viable cardiomyocytes ml^{-1}. Petri dishes were preincubated for 2 h with foetal calf serum (4% v/v) in M199. Aliquots of cell suspension (1 ml) were pipetted gently onto Petri dishes of 35 mm diameter. After 1 h, viable cardiomyocytes had attached to the surface of the dish. The dishes were washed with fresh CCT medium to remove nonattached cells and cell debris and the attached cells were incubated at 37 °C for 24 h in CCT medium (1 ml) containing the appropriate concentrations of the various hypertrophic stimuli and/or antagonists and inhibitors as specified in the experimental protocols. Under all experimental conditions, cardiomyocytes remained mechanically quiescent.

2.3. Incorporation of l-U-[^{14}C]phenylalanine and total mass of cellular protein and total content of cellular DNA

The extent of de novo synthesis of protein in the cell cultures was estimated by measuring uptake of radiolabelled amino acid into cellular protein. The cells were exposed for 24 h to *l*-U-[^{14}C]phenylalanine (0.1 μCi ml^{-1} culture medium). Incorporation of radioactivity into the acid-insoluble cell fraction was determined. At the end of the chosen period of incubation, experiments were terminated by removal of the supernatant medium from the dishes. The attached cells were washed with an aliquot (1 ml) of ice-cold PBS, prior to the addition of an aliquot (1 ml) of ice-cold trichloroacetic acid (10% w/v). After storage overnight at 4 °C, the acid containing the intracellular precursor pool was removed from the dishes and the attached cells were washed with an aliquot (1 ml) of PBS. The precipitate remaining on the culture dishes was dissolved in an aliquot (1 ml) of NaOH (0.1 M)/sodium dodecyl sulphate (0.01% w/v) by overnight incubation at 37 °C. In these samples, concentration of protein was determined by the colorimetric method of Lowry, the concentration of DNA in the neutralized sample was determined by a spectrophotometric method in which bisbenzamide dye was incorporated into DNA (Mullan et al., 1997), and the radioactivity was counted. The DNA content of the cultures was not altered by any of the treatments described. The ratio of protein to DNA per culture served, therefore, as a parameter of cell mass and the ratio of *l*-U-[^{14}C]phenylalanine incorporated to DNA per culture served as a measure of de novo synthesis of protein. The concentration of DNA is a reliable measure of cell number as adult cardiomyocytes do not proliferate in culture (Jacobson and Piper, 1986); also, nonmyocytic cells are virtually absent from these fresh cultures, and the very few present cannot proliferate, since they would be killed by the presence of cytosine β-D-arabinofuranoside (Piper and Volz, 1990).

2.4. Inhibition of protein degradation

For pulse chase experiments, indicating the extent of protein degradation (Bell and McDermott, 2000), cardiomyocytes were preincubated for 24 h in the presence of *l*-U-[^{14}C]phenylalanine (0.1 μCi ml^{-1} culture medium) as described above. After removal of the radioactive amino acid by a medium change, cultures were incubated for another period of time ($0 < t \leq 48$ h) in the absence or presence of peptide. The radioactivity that remained incorporated in the acid-insoluble cell mass after that time was determined as described above.

2.5. Semiquantitative reverse transcriptase polyacrylamide chain reaction (RT-PCR)

Total cellular RNA was isolated by a modification of the acid guanidinium thiocyanate–phenol–chloroform method of Chomczynaki and Sacchi (1987). First Strand cDNA was synthesised from 2 μg total RNA in a 20 μl reaction volume using random decamers and M-MLV reverse transcriptase (Reverse-iT kit, Abgene, Surrey, UK). The resultant cDNA was amplified by PCR following a standard PCR protocol. Each PCR reaction contained 2 μl cDNA, 1 × reaction buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1.25 U DNA polymerase (*Thermus 'Icelandicus'*, Red Hot DNA polymerase, Abgene) and between 0.5 and 1 μM of each gene specific primer. The gene specific primers (Table 1) were based upon those previously reported in the literature (Henderson et al., 1988; Kobayashi et al., 1998; Makino et al.,

Table 1
Sequences of upstream and downstream oligonucleotide primers

MRNA	Forward	Reverse	Number of cycles	T_m (°C)	Size (bp)
MLC-2	GAATTCTGCAGATGTCA CCAAAGAAAGCCAAG	GAATTCTGCAGTCAGT CCTTCTCTTCTCCGTG	24	55	457
β-MHC	GCCAACACCAACCTGTCCAAGTTC	TGCAAAGGCTCCAGGTCTGAGGGC	28	56	171
ANP	CTGCTAGACCACCTGGAGGAG	CCAGGAGGGTATTCACCACCTC	30	55	438
Skeletal α-actin	CTCTCTCTCCTCAGGACAA	TGGAGCAAAACAGAATGGCTGG	30	56	218
GAPDH	GCCATCAACGACCCCTTCATTG	TGCCAGTGAGCTTCCCGTTC	29	64	29

1999; Yu et al., 1996). After an initial denaturation at 94 °C for 4 min, the following cycling profile was used: denaturation at 94 °C for 30 s, annealing at each suitable temperature (Table 1) for 40 s, and extension at 72 °C for 60 s. Amplification was performed over several cycles (Table 1) and was ended with a final extension at 72 °C for 5 min and cooling to 15 °C for 5 min. All PCR reactions were performed in duplicate. In preliminary experiments, the amplification cycles for each gene were determined in a range such that the amount of PCR product was in proportion to the amplification cycle. The PCR products were electrophoresed on a 2.0% agarose gel and stained with ethidium bromide. The gels were visualised under ultraviolet illumination, photographed and analysed using a GeneGenius Gel documentation system with Gene Tools analysis software (Syngene, Cambridge, UK). Band intensity was expressed as the target mRNA to GAPDH mRNA ratio.

2.6. Data analysis

In each experiment, the total population of cells contained in culture plates was obtained from a pooled suspension prepared from two hearts. Under each condition (in the absence/presence of peptide at various concentrations, with or without inhibitor), the average value measured in three culture plates was calculated for each parameter ([^{14}C]phenylalanine incorporation or protein/DNA content). Replicate data were obtained for n preparations ($4 \leq n \leq 10$) and the mean value ± S.E.M. was calculated. Data were analysed statistically using a one or two factor repeated measures analysis of variance (SPSS-PC, version 8.0). If $P < 0.05$ for the overall effect of concentration under a particular condition, differences between the mean values at a particular concentration (x_1) and at baseline (x_0) were tested by calculation of the t-statistic as $(x_1 - x_0)/\sqrt{}$ residual mean square $(2/n)$.

2.7. Materials

Neuropeptide Y (human, rat), [Leu^{31}, Pro^{34}]neuropeptide Y (human, rat), peptide YY-(3–36) (human, rat) and neuropeptide Y-(3–36) (human, rat) were supplied by Bachem (UK) (St. Helen's, Merseyside, UK). The neuropeptide Y Y_1 receptor antagonist, BIBP3226, was obtained from Research Biochemicals International (MA, USA). The neuropeptide Y Y_2 receptor antagonist, T_4 [neuropeptide Y-(33–36)]$_4$, was a gift from Dr. Eric Grouzmann (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). Bovine serum albumin (Cat. No. A7030), *l*-carnitine, creatine, taurine, cytosine-β-arabinofuranoside, DNA (sodium salt, from calf thymus), actinomycin D, Tri reagent and assay kits for the determination of microprotein were obtained from Sigma (Poole, Dorset, UK). Liquid scintillation fluid was obtained from BDH Chemicals (UK). Collagenase B was purchased from Boehringer Mannheim (Mannheim, Germany). Medium M199 (glutamine-free with Earle's salts), foetal bovine serum and penicillin (5000 IU)/streptomycin (5 mg ml^{-1}) were supplied by GIBCO (UK). Bisbenzamide (H 33258) was purchased from Riedel-de-Haen (Germany). Plastic Petri dishes were obtained from Falcon (Becton-Dickinson, UK). *l*-U-[^{14}C]phenylalanine was supplied by Amersham International (Buckinghamshire, UK). *Thermus Icelandicus* (Red Hot Taq Polymerase) and Reverse-iT kits were obtained from Abgene, and dNTPs were obtained from Bioline (UK). Primers were custom synthesised by the Oligonucleotide Synthesis Unit, QUB. All other chemicals were of analytical grade and purchased from BDH Chemicals.

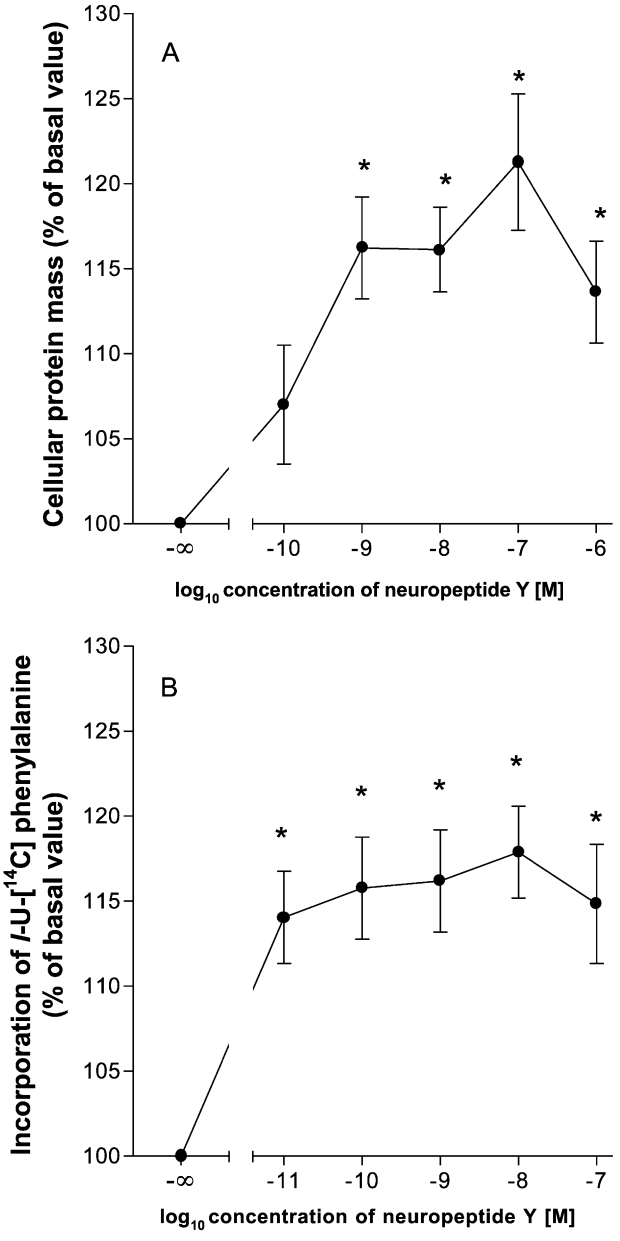

Fig. 1. Effect of neuropeptide Y on (A) total mass of cellular protein and (B) incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein of ventricular cardiomyocytes, isolated from the hearts of adult rats and maintained in short-term (24 h) serum-free culture. Data are expressed as percentage differences from basal values and are the means ± S.E.M. of 12 experiments. Significant difference from basal value (* $P < 0.05$).

3. Results

3.1. Neuropeptide Y-mediated protein turnover: effect of actinomycin D

Neuropeptide Y increased the total mass of cardiomyocyte protein over the concentration range 100 pM–1 μM; the maximum increase was observed at 100 nM and was 21.0% greater ($P<0.05$) than in the absence of peptide (55.3 ± 4.1 μg/μg DNA, $n=12$) (Fig. 1A). Unexpectedly, neuropeptide Y also increased the incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein over the concentration range 10 pM–100 nM, indicating an effect on de novo protein synthesis; the maximum increase was observed at 10 nM and was 18.0% greater ($P<0.05$) than in the absence of peptide (446.3 ± 21.8 dpm/μg DNA) (Fig. 1B). In pulse-chase experiments to assess protein degradation, cardiomyocytes were preincubated for 24 h in the presence of radiolabelled phenylalanine. After removal of the radioactive amino acid by a

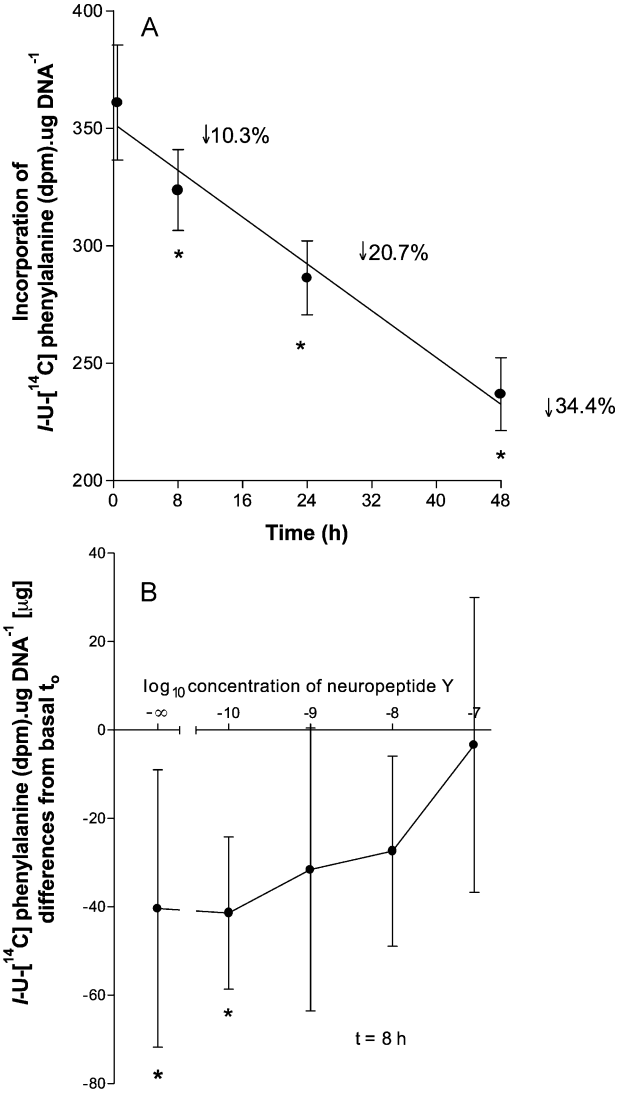

Fig. 2. Temporal dependence of protein degradation in the absence of peptide (A). Attenuation by neuropeptide Y of protein degradation after 8 h compared to time-matched control value (B). In pulse-chase experiments, cardiomyocytes were first labelled for 24 h by incorporation of *l*-U-[^{14}C]phenylalanine. After removal of the radioactive amino acid by a medium change (t_0), cells were incubated for a further period of time ($0<t<48$ h) with nonradioactive medium. Radioactivity remaining in the acid-insoluble protein mass was determined and corrected for DNA content. Data are expressed as absolute differences from basal value at t_0 and are the means ± S.E.M. of three experiments. Significant difference from basal value at t_0 (* $P<0.05$).

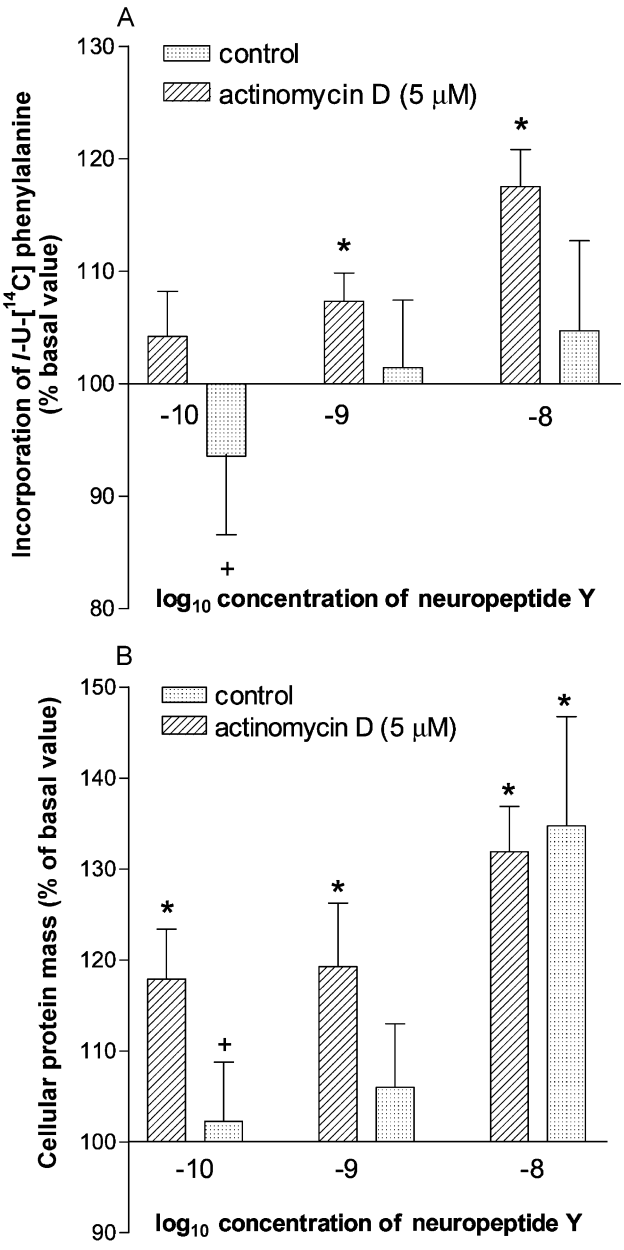

Fig. 3. Effect of actinomycin D (5 μM) on (A) incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein and (B) total mass of cellular protein elicited in response to neuropeptide Y in ventricular cardiomyocytes maintained in short-term (24 h) serum-free culture. Data are expressed as percentage differences from basal values (± actinomycin D) and are the means ± S.E.M. of seven experiments. Significant variation between paired data (+$P<0.05$); from basal response (* $P<0.05$).

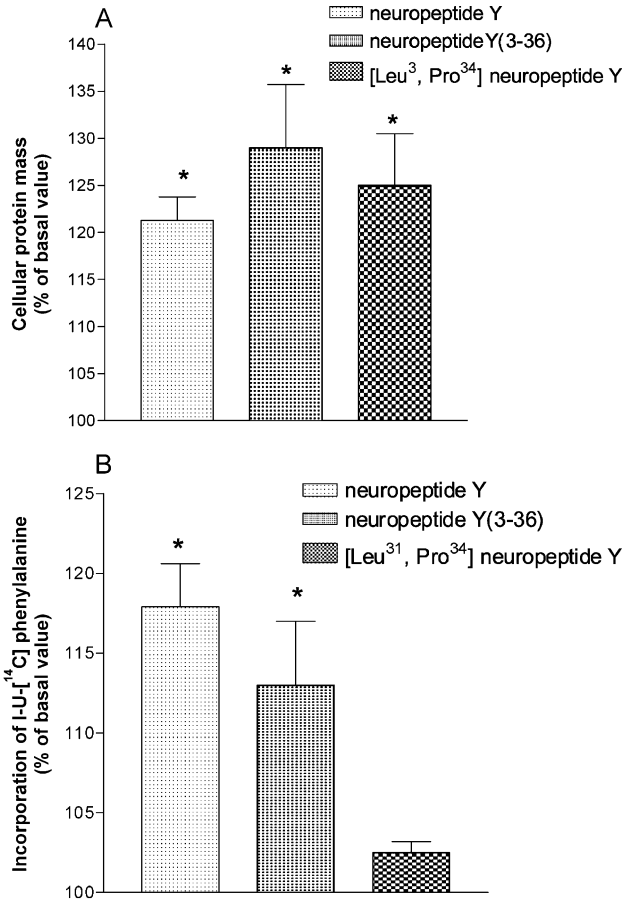

Fig. 4. Effect of neuropeptide Y, (10 nM), neuropeptide Y-(3–36) (10 nM) and [Leu^{31}, Pro^{34}] neuropeptide Y (10 nM) on (A) total mass of cellular protein and (B) incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein of ventricular cardiomyocytes isolated from the hearts of adult rats and maintained in short-term (24 h) serum-free culture. Data are expressed as percentage differences from basal values and are the means ± S.E.M. of 6 and 12 experiments, respectively. Significant difference from basal value (* $P < 0.05$).

medium change, cultures were subsequently incubated for a further period of time ($0 < t < 48$ h). Forty-eight hours after this medium change, the DNA content of the cultures did not differ from that obtained at the time of the medium change (4.1 ± 0.5 vs. 4.2 ± 0.5 μg culture^{-1}). Under control conditions, degradation of cellular protein synthesised during the previous 24 h period, as evidenced by the decline in the amount of the radiolabel that remained associated with the acid-insoluble cell mass, decreased in a linear fashion with respect to time (Fig. 2A): 34.4% of the radiolabelled protein had been metabolised after 48 h. Neuropeptide Y attenuated the degradation of this radiolabelled protein in a concentration-dependent manner: degradation was completely inhibited by neuropeptide Y (100 nM) at 8 h (Fig. 2B). In the presence of actinomycin D (5 μM), an inhibitor of protein synthesis, the increased incorporation of *l*-U-[^{14}C]phenylalanine in response to neuropeptide Y (100 pM) was abolished, while that to neuropeptide Y (1 and 10 nM) was attenuated markedly (Fig. 3A). The increase in total mass of cardiomyocyte protein in response to neuropeptide Y (100 pM) was attenuated by >85% ($P < 0.05$), while that to neuropeptide Y (1 nM) was attenuated by 60% but that to neuropeptide Y (10 nM) was unaltered (Fig. 3B).

3.2. Evidence for neuropeptide Y Y_1 and neuropeptide Y Y_2 receptor subtype involvement in protein turnover

[Leu^{31}, Pro^{34}]neuropeptide Y (10 nM), which displays some selectivity for neuropeptide Y Y_1 receptors relative to other neuropeptide Y Y receptor subtypes, increased the total mass of cardiomyocyte protein significantly to 25.0% greater than in the absence of peptide (64.3 ± 10.4 μg/μg DNA, $n = 6$) (Fig. 4A) but did not increase the incorporation of *l*-U-[^{14}C]phenylalanine above basal values (798.2 ± 46.5 μg/μg DNA, $n = 12$) (Fig. 4B). The neuropeptide Y Y_1 receptor subtype-selective antagonist, BIBP3226 (1 μM), attenuated the increase in total mass of cellular protein in response to neuropeptide Y ≥ 10 nM; the response to 100 nM neuropeptide Y was attenuated significantly by 1 μM BIBP3226. The response to 1 nM neuropeptide Y was not reduced by the antagonist (Fig. 5). This concentration of BIBP (1 μM) was sufficient to completely abolish the effect of [Leu^{31}, Pro^{34}]-neuropeptide Y (10 nM) on cellular protein mass (data not shown).

Neuropeptide Y-(3–36) (10 nM), which displays some selectivity for neuropeptide Y Y_2 and neuropeptide Y Y_5

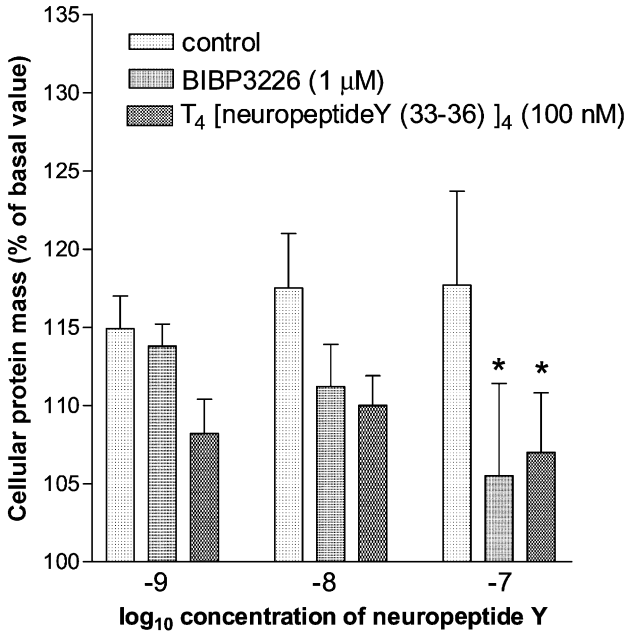

Fig. 5. Inhibition by the neuropeptide Y Y_1 subtype-selective antagonist, BIBP3226 (1 μM), and the neuropeptide Y Y_2 subtype-selective antagonist, T_4 [neuropeptide Y-(33–36)]$_4$ (100 nM), of the effect of neuropeptide Y on the total mass of cellular protein of ventricular cardiomyocytes isolated from the hearts of adult rats and maintained in short-term (24 h) serum-free culture. Data are expressed as percentage differences from basal values (± antagonist) and are the means ± S.E.M. of four experiments. Significant difference between paired data (* $P < 0.05$).

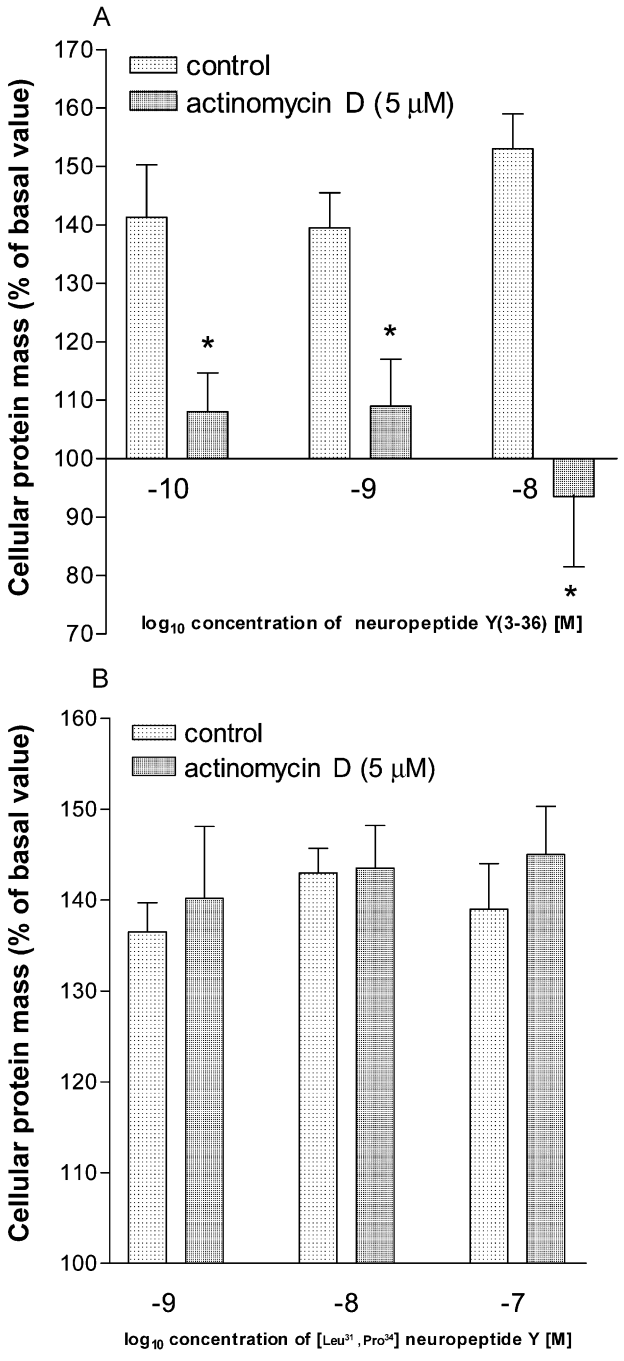

Fig. 6. Inhibition by actinomycin D (5 μM) of the increase in total mass of cellular protein elicited in response to neuropeptide Y-(3–36) (A) but not of that to [Leu^{31}, Pro^{34}] neuropeptide Y (B) in ventricular cardiomyocytes maintained in short-term (24 h) serum-free culture. Data are expressed as percentage differences from basal values (± actinomycin D) and are the means ± S.E.M. of four experiments. Significant variation between paired data (* $P<0.05$).

receptors relative to other neuropeptide Y Y receptor subtypes, increased the total mass of cardiomyocyte protein to 29.0% greater than basal value (Fig. 4A); this increase was accompanied by increased incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein to 13.5% greater than basal value (624.9 ± 70.9 dpm/μg DNA, $n=12$) (Fig. 4B). The neuropeptide Y Y_2 receptor subtype-selective antagonist, T_4 [neuropeptide Y-(33–36)]$_4$, (100 nM), attenuated the increase in total mass of cellular protein in response to neuropeptide Y (1 nM–100 nM); the response to 100 nM neuropeptide Y was attenuated significantly by 100 nM T_4 [neuropeptide Y-(33–36)]$_4$ (Fig. 5). The incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein in response to neuropeptide Y (10 nM)

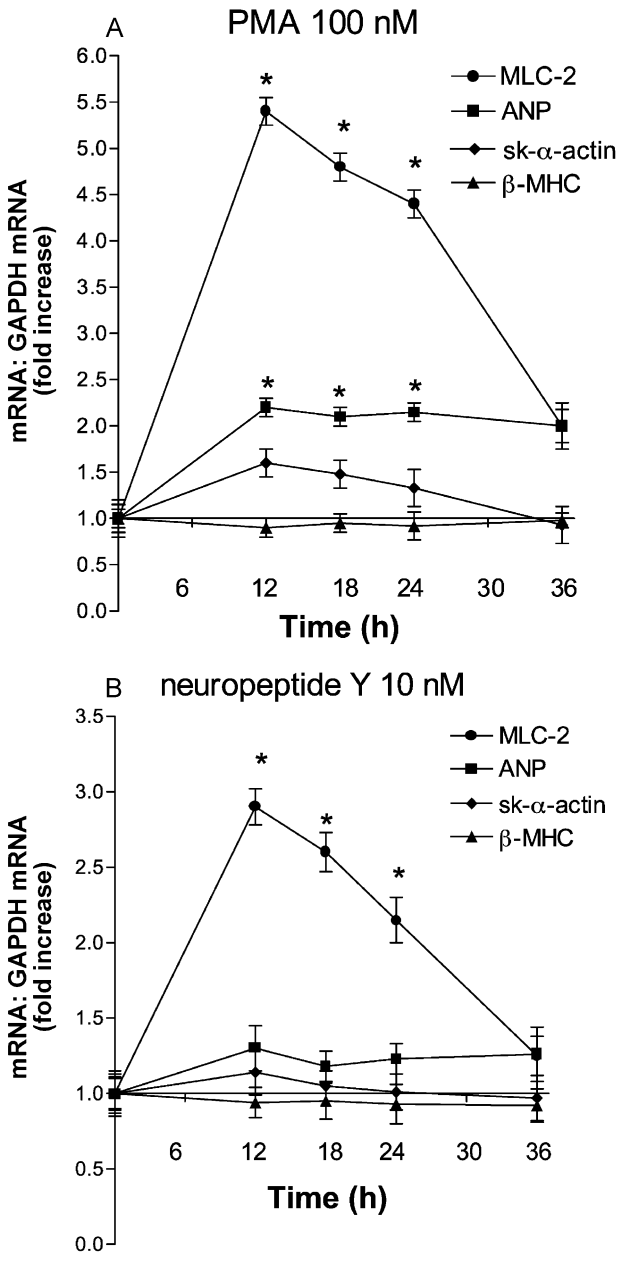

Fig. 7. Temporal dependence of the expression of the constitutive gene, myosin light chain-2 (MLC-2) and of the foetal genes, ANP, myosin heavy chain-β and skeletal-α-actin in response to PMA (100 nM) (A) and neuropeptide Y (10 nM) (B) in ventricular cardiomyocytes maintained in short-term (≤ 36 h) serum-free culture. mRNA expression was assessed by semiquantitative RT-PCR and normalised to GAPDH mRNA expression. Data are expressed as fold increase of basal response and are the means ± S.E.M. of three experiments. Significant difference from basal value (* $P<0.05$).

and to neuropeptide Y-(3–36) (10 nM) was abolished by T_4 [neuropeptide Y-(33–36)]$_4$ while the effect of [Leu^{31}, Pro^{34}] neuropeptide Y (10 nM) on cellular protein mass was unaltered (data not shown).

Actinomycin D (5 μM) abolished the increase in mass of cardiomyocyte protein initiated by neuropeptide Y-(3–36) (Fig. 6A). In contrast, the increase in total mass of cellular protein in response to [Leu^{31}, Pro^{34}]neuropeptide Y was not attenuated by actinomycin D (Fig. 6B).

3.3. Temporal dependence of gene expression

The phorbol ester, phorbol 12 myristate 13 acetate (PMA) (100 nM), employed as a positive control, increased the expression of mRNA encoding the constitutive gene, MLC-2, and the foetal gene, ANP, maximally after 12 h to values 5.4-fold and 2.2-fold greater ($P<0.05$) than respective basal levels. PMA also tended to increase the expression of skeletal-α-actin mRNA, maximally at 12 h, to a value 1.6-fold greater than basal although this increase was not significant. The expression of myosin heavy chain-β mRNA was not increased by PMA (Fig. 7A). Neuropeptide Y (10 nM) increased the expression of MLC-2 mRNA maximally at 12 h to a value 2.9-fold greater ($P<0.05$) than basal level but did not increase the expression of the foetal genes, ANP, skeletal-α-actin and myosin heavy chain-β (Fig. 7B).

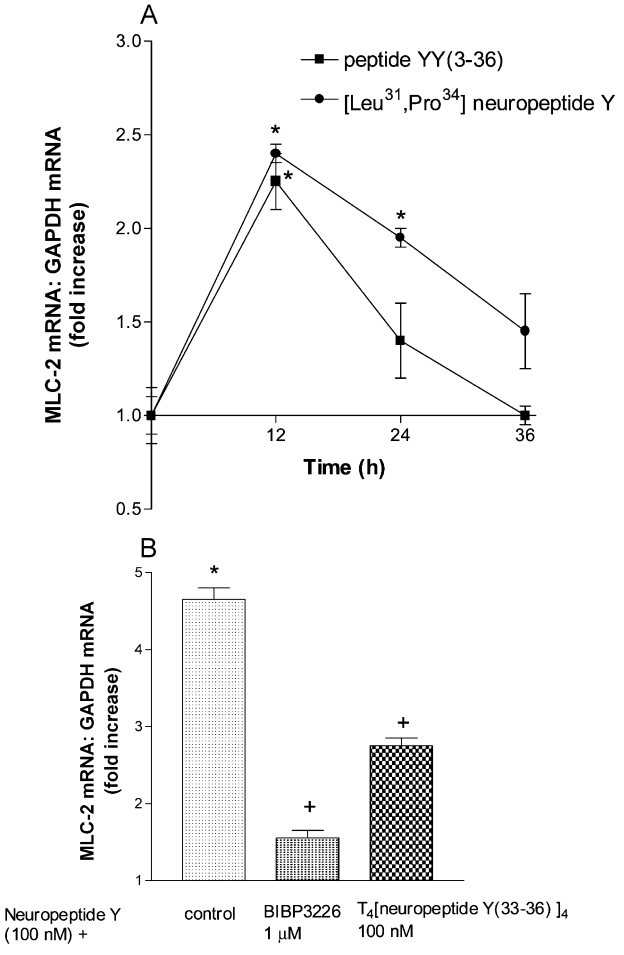

Fig. 8. Expression of MLC-2 mRNA in response to [Leu^{31}, Pro^{34}] neuropeptide Y (100 nM) and peptide YY-(3–36) (100 nM) (A); neuropeptide Y (100 nM) in the presence of BIBP3226 (1 μM) and T_4 [neuropeptide Y-(33–36)]$_4$ (100 nM) (B) in ventricular cardiomyocytes maintained in short-term (≤36 h) serum-free culture. MLC-2 mRNA expression was assessed by semiquantitative RT-PCR and normalised to GAPDH mRNA expression. Data are expressed as fold increase of basal response and are the means ± S.E.M. of three experiments. Significant difference from basal value (*$P<0.05$); significant variation between paired data ($^{+}P<0.05$).

3.4. Evidence for neuropeptide Y Y_1 and neuropeptide Y Y_2 receptor subtype involvement in constitutive gene expression

[Leu^{31}, Pro^{34}]neuropeptide Y (100 nM) and peptide YY-(3–36) (100 nM) each increased the expression of MLC-2 mRNA maximally at 12 h to values 2.4-fold and 2.3-fold greater, respectively ($P<0.05$) than basal level (Fig. 8A). BIBP3226 (1 μM) and T_4 [neuropeptide Y-(33–36)]$_4$ (100 nM) attenuated the increase in MLC-2 mRNA elicited in response to neuropeptide Y (100 nM), which was 4.7-fold greater than basal level, by 86% and 51%, respectively ($P<0.05$) (Fig. 8B).

4. Discussion

Neuropeptide Y initiated a hypertrophic effect, as evidenced by increased protein mass, in adult rat ventricular cardiomyocytes maintained in short-term culture. This finding is in agreement with published data (Millar et al., 1994). However, there are a number of important differences between the two studies. Firstly, a significant hypertrophic response was observed at picomolar concentrations, considerably lower than the micromolar concentrations applied by Millar et al., and consequently of greater physiological relevance (Grandt et al., 1992a,b; Ogawa et al., 1992; Zukowskagrojec et al., 1993; Bohm et al., 1995; Chen and Han, 1995). Secondly, in contrast to the previous study, initiation by neuropeptide Y of increased protein mass was accompanied by enhanced synthesis of de novo protein, as evidenced by the incorporation of radiolabelled amino acid. This finding indicates that de novo synthesis of protein also contributes to the initial increase in protein mass, in addition to confirmation of the attenuated degradation of preexisting protein, demonstrated in pulse-chase experiments (Millar et al., 1994). Attenuation by actinomycin D, an inhibitor of transcription, of the effects on protein mass of neuropeptide Y (≤1 nM), but not of neuropeptide Y (10 nM), also supports the contribution of two mechanisms, one dependent on the synthesis of new protein and the other on the attenuated degradation of existing protein. Previously, Millar et al. (1994) reported that neuropeptide Y did not initiate protein synthesis in freshly isolated cells, but acquired the ability to stimulate protein synthesis in redifferentiated cardiomyocytes, a model relevant to established hypertro-

phy. Induction of this mechanism has been attributed to autocrine signalling by TGF-β (Schluter et al., 1995b; Goldberg et al., 1998; Taimor et al., 1999). It is possible that the nonphysiological concentrations of the peptide utilised by Millar et al. masked or blunted the effect of neuropeptide Y on de novo protein synthesis in freshly isolated cells, or alternatively that in the cells utilised in the present study, the necessary signalling pathway between neuropeptide Y receptors and protein synthesis was already established.

Hypertrophying cardiomyocytes also exhibit alterations in gene expression (Schwartz et al., 1986; Izumo et al., 1987, 1988; Schluter et al., 1995a). Millar et al. (1994) reported that expression of the foetal gene, creatine kinase-B, was unchanged by neuropeptide Y in freshly isolated cells, but was induced by the peptide in redifferentiated cardiomyocytes. The results of the present study are in agreement with this finding. Neuropeptide Y did not induce the expression of the foetal genes, myosin heavy chain-β and skeletal-α-actin in freshly isolated cells and caused a marginal increase in the expression of the paracrine mediator, ANP, which is normally only expressed abundantly by ventricular cardiomyocytes during foetal development (Arai et al., 1988; Lee and Millar, 1998; Franch et al., 1988; Yu et al., 1996). In contrast, PMA, a direct activator of protein kinase C, induced the expression of ANP, and to a lesser extent, of skeletal α-actin. PMA was chosen as a positive control, as the receptors for many of the mediators identified as 'initiators' of cardiomyocyte hypertrophy, for example α-adrenoceptors and endothelin receptors are known to be coupled to protein kinase C-dependent mechanisms (Bell et al., 1995; Yamazaki et al., 1999; Cullen et al., 2001). The inability of neuropeptide Y to induce these genes initially, in contrast to the effect of PMA, indicates that the cells had not yet acquired the necessary signalling machinery to couple neuropeptide Y Y receptors efficiently to the mechanism responsible for the reinduction of foetal genes. Neither neuropeptide Y nor PMA, however, induced the expression of myosin heavy chain-β mRNA. This finding indicates that either the regulation of this gene is under the control of a different, non-protein kinase C-dependent mechanism, or that induction of the gene is not associated with the initiation of the hypertrophic process but is instead linked to the much later changes in cardiomyocyte phenotype normally associated with established hypertrophy. In contrast to the relative inability of the peptide to initially reinduce a foetal programme of gene expression, neuropeptide Y significantly enhanced the expression of the constitutive gene, MLC-2, to a similar extent as PMA. Induction of foetal genes and upregulation of constitutive genes, although each dependent in part on the activation of protein kinase C (Shubeita et al., 1992), may be associated with different downstream signalling mechanisms. Upregulated expression of MLC-2 mRNA is clearly an early marker of neuropeptide Y-mediated cardiomyocyte hypertrophy.

Neuropeptide Y Y receptor-subtype involvement in neuropeptide Y-mediated cardiomyocyte hypertrophy had not been investigated previously. The data obtained in the present study support the involvement of two neuropeptide Y Y receptor subtypes in the initiation of the hypertrophic response to the peptide. [Leu^{31}, Pro^{34}]neuropeptide Y, an agonist at neuropeptide Y Y_1 receptors (Fuhlendorff et al., 1990; Potter and McCloskey, 1992), increased cellular protein mass but did not stimulate protein synthesis. Furthermore, the increase in protein mass elicited by [Leu^{31}, Pro^{34}]neuropeptide Y was not inhibited by actinomycin D. In addition, the increased protein mass observed in response to neuropeptide Y was attenuated significantly, though not abolished, by BIBP3226, a selective antagonist at neuropeptide Y Y_1 receptors (Rudolf et al., 1994; Cabrele and Beck-Sickinger, 2000; Prieto et al., 2000; Zhang et al., 2000). Taken together, these findings support a contribution of neuropeptide Y Y_1 receptors to the NPY-mediated increase in cellular protein and indicate that these receptors are coupled to attenuated degradation rather than enhanced synthesis of protein. The selectivity of [Leu^{31}, Pro^{34}]neuropeptide Y as an agonist at neuropeptide Y Y_1 receptors has been questioned; this analogue also interacts at neuropeptide Y Y_4 receptors (Gehlert et al., 1997). The neuropeptide Y Y_4 receptor-preferring agonist, pancreatic polypeptide (Lundell et al., 1995), does increase cell protein mass, although this increase is accompanied by stimulation of de novo synthesis of protein in freshly isolated cardiomyocytes (Nicholl, unpublished observation). However, the relatively weak interaction of endogenous neuropeptide Y with neuropeptide Y Y_4 receptors would probably preclude a role for this subtype in neuropeptide Y-mediated cardiomyocyte hypertrophy in vivo.

Neuropeptide Y-(3–36), which displays selectivity for neuropeptide Y Y_2 (and neuropeptide Y Y_5) receptors relative to other neuropeptide Y Y receptor subtypes (Michel et al., 1997; Dumont et al., 2000), stimulated de novo protein synthesis and increased cellular protein mass. The effect of neuropeptide Y-(3–36) was inhibited by actinomycin D but not by the neuropeptide Y Y_1 receptor antagonist, BIBP3226 (1 μM). T_4 [neuropeptide Y-(3–36)]$_4$, a neuropeptide Y Y_2 receptor antagonist (Grouzmann et al., 1997; Pheng et al., 1999), attenuated significantly but did not abolish the increased protein mass observed in response to neuropeptide Y. These data indicate that stimulation of de novo protein synthesis by neuropeptide Y, possibly acting through neuropeptide Y Y_2 receptors, also contributes to the observed increase in cellular protein mass initiated in cardiomyocytes response to the peptide. Conclusive evidence for the involvement of the neuropeptide Y Y_2 receptor in this regard and exclusion of a possible effect of neuropeptide Y-(3–36) mediated via neuropeptide Y Y_5 receptors will however require the use of a selective antagonist at neuropeptide Y Y_5 receptors; a suitable antagonist is not commercially available at present.

The involvement of both neuropeptide Y Y_1 and neuropeptide Y Y_2 receptors in neuropeptide Y-mediated cardiomyocyte hypertrophy is compatible with the finding that

both receptor subtypes are coupled to the activation of MAPK activity, which is associated with vascular smooth muscle growth (Nic and Selbie, 1998). We have also reported previously that neuropeptide Y Y_1 and neuropeptide Y Y_2 receptors are linked to opposing effects on the amplitude of cardiomyocyte contraction (McDermott et al., 1997). This conclusion was based on the comparative activity of [Leu^{31}, Pro^{34}]neuropeptide Y and peptide YY-(3–36) for stimulation of a positive and a negative contractile effect, respectively, and antagonism of the positive contractile effect of neuropeptide Y by bis(31/31′)[Cys^{31}, Trp^{32}, Nva^{34}]neuropeptide Y-(31–36), a putative neuropeptide Y Y_1 receptor subtype-selective antagonist (Balasubramaniam et al.,1996). Peptide YY-(3–36) and [Leu^{31}, Pro^{34}]neuropeptide Y enhanced the expression of MLC-2 mRNA to a similar extent in hypertrophying cardiomyocytes. Furthermore, the increase in expression of this gene elicited in response to neuropeptide Y was attenuated significantly by BIBP3226 and by T_4 [neuropeptide Y-(3–36)]$_4$. These data implicate both neuropeptide Y Y_1 and neuropeptide Y Y_2 receptors, though not necessarily acting through an identical intracellular signalling pathway, in the enhanced expression of the constitutive gene. It is not clear why two receptors should regulate the expression of the same gene. However, since neuropeptide Y Y_1 receptors have been implicated in attenuated degradation of existing protein rather than enhanced synthesis of new protein, it is also possible that the peptide acting through neuropeptide Y Y_1 receptors may stabilise existing MLC-2 mRNA rather than actively increasing the transcription of the gene. This conclusion is compatible with the observation that the increased level of MLC-2 mRNA declines more slowly in the presence of [Leu^{31}, Pro^{34}]neuropeptide Y than in the presence of peptide YY-(3–36). It should also be noted that the effects of neuropeptide Y both in regard to protein turnover and MLC-2 mRNA expression are less than would be predicted from the additive effects of neuropeptide Y_1 receptor stimulation by [Leu^{31} Pro^{34}]neuropeptide Y and neuropeptide Y Y_2 receptor stimulation by neuropeptide Y-(3–36). This observation may indicate a degree of cross-talk and reciprocal regulation between the hypertrophic mechanisms regulated by the two neuropeptide Y Y receptor subpopulations.

In conclusion, neuropeptide Y initiates cardiomyocyte hypertrophy in vitro, as evidenced by increased mass of cellular protein and enhanced expression of the constitutive gene, MLC-2. The findings of the present study provide evidence for the contribution of both neuropeptide Y Y_1 and possibly also neuropeptide Y Y_2 receptors to the hypertrophic response to the peptide. Neuropeptide Y Y_1 receptors and neuropetide Y Y_2 receptors are coupled to attenuated degradation of existing protein and enhanced synthesis of de novo protein, respectively. The concentration-dependencies and the magnitudes of these effects are compatible with a pathophysiological role for the peptide in the onset of left ventricular hypertrophy in vivo and afford the possibility that neuropeptide Y, in concert with other known hypertrophic growth factors, may contribute to cardiac growth and remodelling.

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References

Arai, H., Nakao, K., Sito, Y., Morin, N., Suguwara, A., Yamada, T., Itoh, H., Shiono, S., Mykoyama, M., Ohkubo, H., Nakanishi, S., Imura, H., 1988. Augmented expression of atrial natriuretic polypeptide gene in ventricles of spontaneously hypertensive rats (SHR) and stroke-prone SHR. Circ. Res. 62, 926–933.

Balasubramaniam, A., 1997. Neuropeptide Y family of hormones: receptor subtypes and antagonists. Peptides 18, 445–457.

Balasubramaniam, A., Zhai, W., Sheriff, S., Tao, Z., Chance, W.T., Fischer, J.E., Eden, P., Taylor, J., 1996. Bis(31/31′) [Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36): a specific neuropeptide Y Y1 receptor antagonist. J. Med. Chem. 39, 811.

Bell, D., McDermott, B.J., 2000. Contribution of de novo protein synthesis to the hypertrophic effect of IGF-1 but not of thyroid hormones in adult ventricular cardiomyocytes. Mol. Cell. Biochem. 206, 113–124.

Bell, D., Schluter, K.D., Zhou, X.J., McDermott, B.J., Piper, H.M., 1995. Hypertrophic effects of CGRP and amylin in adult mammalian ventricular cardiomyocytes. J. Mol. Cell. Cardiol. 27, 2433–2443.

Bohm, M., Grabel, C., Knorr, A., Erdmann, E., 1995. Treatment in hypertensive cardiac hypertrophy: 1. Neuropeptide Y and β-adrenoceptors. Hypertension 25, 954–961.

Cabrele, C., Beck-Sickinger, A.G., 2000. Molecular characterisation of the ligand–receptor interaction of the neuropeptide Y (Review). J. Pept. Sci. 6, 97–122.

Chen, S.H., Han, Q.D., 1995. Increase of release of neuropeptide Y in vitro from platelets of spontaneously hypertensive rat. Acta Pharmacol. Sin. 16, 149–151.

Chomczynaki, P., Sacchi, N., 1987. Single step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Cullen, J.P., Bell, D., Kelso, E.J., McDermott, B.J., 2001. Use of A-19261 to provide evidence for involvement of endothelin ETB-receptors in endothelin-1 mediated cardiomyocyte hypertrophy. Eur. J. Pharmacol. 417, 157–168.

Doods, H.N., Gaida, W., Weiland, H.A., Dollinger, H., Schnorrenberg, G., Esser, F., Engel, W., Eberlein, W., Rudolf, K., 1999. BIIE0246: a selective and high affinity neuropeptide Y Y_2 receptor antagonist. Eur. J. Pharmacol. 384, R3–R5.

Dumont, Y., Cadieux, A., Pheng, L.H., Fournier, A., 1994. Peptide YY derivatives as selective neuropeptide Y/peptide YY Y_1 and Y_2 agonists devoid of activity for the Y_3 receptor subtype. Mol. Brain Res. 26, 320–326.

Dumont, Y., Cadieux, A., Doods, H., Pheng, L.H., Abounader, R., Hamel, E., Jacques, D., Regoli, D., Quirion, R., 2000. BIIE0246, a potent and highly selective non-peptide neuropeptide Y Y_2 receptor antagonist. Br. J. Pharmacol. 129, 1075–1088.

Franch, H.A., Dixon, R.A.F., Blaine, E.H., Siegel, P.K.S., 1988. Ventricular atrial natriuretic factor in the cardiomyocyte hamster model of congestive heart failure. Circ. Res. 62, 31–36.

Fuhlendorff, J., Gether, U., Aakerlund, L., Langeland-Johansen, N., Thogersen, H., Melberg, S.G., Olsen, U.B., Thastrup, O., Schwartz, T.W.,

1990. Leu[31] Pro[34]-NPY: a specific Y1 receptor agonist. Proc. Natl. Acad. Sci. U.S.A. 187, 182–186.

Gehlert, D.R., Schober, D.A., Gackenheimer, S.L., Beavers, L.S., Gadski, E., Lundeu, I., Larhammar, D., 1997. ^{125}I Leu[31] Pro[34] PYY is a high affinity radioligand for rat PP1/Y_4 and Y_1 receptors: evidence for heterogeneity in pancreatic polypeptide. Peptides 18, 397–401.

Goldberg, Y., Taimor, G., Piper, H.M., Schluter, K.D., 1998. Intracellular signalling leads to the hypertrophic effect of neuropeptide Y. Am. J. Physiol. 275, C1207–C1215.

Grandt, D., Schimiczek, M., Feth, F., Rascher, W., Goebell, H., Reeve, J.R., Eysselein, V.E., Michel, M.C., 1992a. Peptide YY_{3-36} is an endogenous Y_2 selective agonist and a major molecular form of PYY in circulating blood in man. Gastroenterology 102, A743 (Abstract).

Grandt, D., Teyssen, S., Schimiczek, M., Reeve, J.R., Feth, F., Rascher, W., Hirche, H., Singer, M.V., Layer, P., Goebell, H., 1992b. Novel generation of hormone receptor specificity by amino terminal processing of Peptide YY. Biochem. Biophys. Res. Commun. 186, 1299–1306.

Grouzmann, E., Buclin, T., Martire, M., Cannizzaro, C., Dorner, B., Razaname, A., Mutter, M., 1997. Characterisation of a selective antagonist of neuropeptide Y at the Y_2 receptor. J. Biol. Chem. 272, 7699–7706.

Henderson, S.A., Xu, Y.C., Chien, K.R., 1988. Nucleotide sequence of full length cDNAs encoding rat cardiac myosin light chain-2. Nucleic Acids Res. 16, 4722–4727.

Hulting, J., Saollevi, A., Ullman, B., Franco-Cereceda, A., Lundberg, J.M., 1990. Plasma neuropeptide Y on admission to a coronary care unit: raised levels in patients with left heart failure. Cardiovasc. Res. 24, 102–108.

Izumo, S., Lompre, A.M., Matsouka, R., Koren, G., Schwartz, K., 1987. Myosin heavy chain mRNA and protein isoform transition during cardiac hypertrophy. J. Clin. Invest. 79, 970–977.

Izumo, S., Nadal-Ginard, B., Mahdavi, V., 1988. Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. Proc. Natl. Acad. Sci. U.S.A. 85, 339–343.

Jacobson, S.L., Piper, H.M., 1986. Cell cultures of adult cardiomyocytes as models of the myocardium. J. Mol. Cell. Cardiol. 18, 439–448.

Kobayashi, T., Miyauchi, T., Sakai, S., Yamaguchi, I., Goto, K., Sugishita, Y., 1998. Endothelin-converting enzyme and angiotensin-converting enzyme in failing hearts of rats with myocardial infraction. J. Cardiovasc. Pharmacol. 31, S417–S420.

Lee, C.C., Millar, R.J., 1998. Is there really an neuropeptide Y Y_3 receptor? Regul. Pept. 75, 71–78.

Lundell, I., Blomqvist, A.G., Berglund, M.M., Schober, D.A., Johnson, D., Statnick, M.A., Gadski, R.A., Gehlert, D.R., Larhammar, D., 1995. Cloning of a human receptor of the neuropeptide Y receptor family with high affinity for pancreatic polypeptide and peptide YY. J. Biol. Chem. 270, 29123–29128.

Lundell, I., Statnick, M.A., Johnston, D., Schober, D.A., Gehlert, D.R., Larhammar, D., 1996. The cloned rat pancreatic polypeptide receptor exhibits profound differences to the orthologous human receptor. Proc. Natl. Acad. Sci. U.S.A. 93, 5111–5115.

Maisel, A.S., Scott, N.A., Motulsky, J.H., 1989. Elevation of plasma neuropeptide Y levels in congestive heart failure. Am. J. Med. 86, 43–48.

Makino, S., Fukuda, K., Miyoshi, S., Konishi, F., Kodama, H., Pan-Sano, M., Takahashi, T., Hori, S., Abe, H., Hata, J., Umezawa, A., Ogawa, S., 1999. Cardiomyocytes can be generated from marrow stromal cells in vitro. J. Clin. Invest. 103, 697–705.

McDermott, B.J., Millar, B.C., Piper, H.M., 1993. Cardiovascular effects of neuropeptide Y: receptor interactions and cellular mechanisms. Cardiovasc. Res. 27, 893–905.

McDermott, B.J., Millar, B.C., Dolan, F.M., Bell, D., Balasubramaniam, A., 1997. Evidence for Y_1 and Y_2 subtypes of neuropeptide Y receptors linked to opposing postjunctional effects observed in rat cardiac myocytes. Eur. J. Pharmacol. 336, 257–265.

Michel, M.C., Beck-Sickinger, A., Cox, H., Doods, H.N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., Westfall, T., 1997. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY and pancreatic polypeptide receptors. Pharmacol. Rev. 50, 143–150.

Millar, B.C., Schluter, K.D., Zhou, X.J., McDermott, B.J., Piper, H.M., 1994. Neuropeptide Y stimulates hypertrophy of adult ventricular cardiomyocytes. Am. J. Physiol. 266, C1271–C1277.

Mullan, D.M., Bell, D., Kelso, E.J., McDermott, B.J., 1997. Involvement of endothelin ETA and ETB receptors in the hypertrophic effects of ET-1 in rabbit ventricular cardiomyocytes. J. Cardiovasc. Pharmacol. 29, 350–359.

Nic, M., Selbie, L.A., 1998. Neuropeptide Y_1 and Y_2 receptor mediated stimulation of MAPK activity. Regul. Pept. 75, 207–213.

Ogawa, T., Kitamura, K., Kangawa, K., Matsuo, H., Eto, T., 1992. Platelet neuropeptide Y in spontaneously hypertensive rats. J. Hypertens. 10, 765–771.

Pheng, L.H., Perron, A., Quirion, R., Cadieux, A., Fauchere, J.L., Dumont, Y., Regoli, D., 1999. Neuropeptide Y-induced contraction is mediated by neuropeptide Y Y_2 and Y_4 receptors in the rat colon. Eur. J. Pharmacol. 374, 85–91.

Pinson, A., Schluter, K.D., Zhou, X.J., Schwartz, P., Kessler-Icekson, G., Piper, H.M., 1993. Alpha and beta-adrenergic stimulation in cultured adult ventricular cardiomyocytes. J. Mol. Cell. Cardiol. 25, 477–490.

Piper, H.M., Volz, A., 1990. Adult ventricular heart muscle cells. In: Piper, H.M. (Ed.), Cell Culture Techniques in Heart and Vessel Research. Springer-Verlag, Heidelberg, pp. 159–177.

Potter, E.K., McCloskey, M.J.D., 1992. [Leu[31],Pro[34]] neuropeptide Y, a selective functional postjunctional agonist at neuropeptide Y receptors in anaesthetised rats. Neurosci. Lett. 134, 183–186.

Prieto, D., Buus, C.L., Mulvany, M.J., Nilsson, H., 2000. Neuropeptide Y regulates intracellular calcium through different signalling pathways linked to a Y_1 receptor in rat mesenteric small arteries. Br. J. Pharmacol. 129, 1689–1699.

Rudolf, K., Eberlein, W., Engel, W., Weiland, H.A., Willim, K.D., Entzeroth, M., Wienen, W., Beck-Sickinger, A.G., Doods, H.N., 1994. The first highly potent and selective non-peptide neuropeptide Y Y_1 receptor antagonist. Eur. J. Pharmacol. 271, R11–R13.

Schluter, K.D., Piper, H.M., 1992. Trophic effects of catecholamines and parathyroid hormone on adult ventricular cardiomyocytes. Am. J. Physiol. 263, H1739–H1746.

Schluter, K.D., Millar, B.C., McDermott, B.J., Piper, H.M., 1995a. Regulation of protein turnover in adult ventricular cardiomyocytes. Am. J. Physiol. 269, C1347–C1355.

Schluter, K.D., Zhou, X.J., Piper, H.M., 1995b. Induction of hypertrophic responsiveness to isoproterenol by TGF-β in adult rat cardiomyocytes. Am. J. Physiol. 269, C1311–C1316.

Schwartz, K., de la Bastie, D., Bouveret, P., Oliviero, P., Alonso, S., Buckingham, M., 1986. α-Skeletal muscle actin mRNAs accumulate in hypertrophied adult rat hearts. Circ. Res. 59, 551–555.

Shubeita, H.E., Martinson, E.A., Vanbilsen, M., Chien, K.R., Brown, J.H., 1992. Transcriptional activation of the cardiac myosin light chain-2 and atrial natriuretic factor genes by protein kinase C in neonatal rat ventricular cardiomyocytes. Proc. Natl. Acad. Sci. U.S.A. 89, 1305–1309.

Taimor, G., Schluter, K.D., Frishkopf, K., Flesh, M., Rosenkranz, S., Piper, H.M., 1999. Autocrine regulation of TGF-β expression in adult cardiomyocytes. J. Mol. Cell. Cardiol. 31, 2127–2136.

Ullman, B., Franco-Cereceda, A., Hulting, J., Lundberg, J.M., Saollevi, A., 1990. Elevation of plasma neuropeptide Y-like immunoreactivity and noradrenaline during myocardial ischaemia in man. Int. Med. 228, 583–589.

Yamazaki, T., Komuro, I., Zou, Y., Yazaki, Y., 1999. Hypertrophic responses of cardiomyocytes induced by endothelin-1 through the protein kinase C-dependent but Src and Ras-independent pathways. Hypertens. Res. 22, 113–119.

Yu, J.Z., Bondy, G.P., Allard, M.F., Thompson, C.R., Levin, A., McManus, B.M., 1996. Serum from patients with chronic renal insufficiency alters

growth characteristics and ANP mRNA expression of adult cardiac myocytes. J. Mol. Cell. Cardiol. 28, 2429–2441.

Zhang, P., Zheng, J., Vorce, R.L., Hexum, T.D., 2000. Identification of an neuropeptide Y Y_1 receptor subtype in bovine chromaffin cells. Regul. Pept. 87, 9–13.

Zukowskagrojec, Z.G., Olczyska, M., Shen, G.H., Torresdurate, A., Haass, M., Wahlestedt, C., Myers, A.K., 1993. Modulation of vascular function by neuropeptide Y during the development of hypertension in spontaneously hypertensive rats. Pediatr. Nephrol. 7, 845–852.