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Evidence for Y_1 and Y_2 subtypes of Neuropeptide Y receptors linked to opposing postjunctional effects observed in rat cardiac myocytes

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Abstract

The aim of this study was to confirm the existence of and identify the receptor subtypes for neuropeptide Y that are present post-junctionally in myocardium. The effects of the selective agonists, [Leu^{31}, Pro^{34}] neuropeptide Y (neuropeptide Y Y_1 receptors), neuropeptide Y-(13–36) and peptide YY-(3–36) (neuropeptide Y Y_2 receptors), and neuropeptide Y and the related peptide YY, which have differential action at neuropeptide Y Y_3 receptors, on amplitudes of contraction of adult rat ventricular cardiomyocytes were studied. Also, the effect of the neuropeptide Y Y_1-selective antagonist, bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} on neuropeptide Y-mediated changes in myocyte contraction was investigated. Neuropeptide Y, peptide YY, neuropeptide Y-(13–36) and peptide YY-(3–36) attenuated the isoprenaline (10^{-7} M)-stimulated contractile response, and the EC_{50} values were 9.0×10^{-9}, 4.3×10^{-10}, 3.1×10^{-11} and 8.5×10^{-11} M, respectively. [Leu^{31}, Pro^{34}] neuropeptide Y increased the contractile response of cardiomyocytes, and the EC_{50} values were 8.1×10^{-9} and 1.5×10^{-9} M, in the absence and presence of isoprenaline, respectively. Since [Leu^{31}, Pro^{34}] neuropeptide Y caused a positive effect on ventricular myocyte contraction and neuropeptide Y-(13–36) and peptide YY (3–36) produced the most potent negative effects, it is proposed that both neuropeptide Y Y_1 and neuropeptide Y Y_2 receptors, linked respectively to the positive and negative responses, are expressed in cardiomyocytes. The finding of receptors with neuropeptide Y Y_2 characteristics on cardiomyocytes represents a further example of a postjunctional location for this subtype. As there was no significant discrepancy between the potencies of peptide YY and neuropeptide Y to attenuate the contractile response, it appears that neuropeptide Y Y_3-like receptors are not linked principally to contractile function in rat cardiomyocytes. Bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} antagonised the neuropeptide Y-mediated stimulation of contractile activity through neuropeptide Y Y_1 receptors, but the compound also inhibited the attenuation of isoprenaline-stimulated contraction, apparently by acting as a partial agonist at the neuropeptide Y Y_2 receptors. © 1997 Elsevier Science B.V.

Keywords: Neuropeptide Y; Receptor subtype; Contraction; Cardiomyocyte

1. Introduction

Neuropeptide Y and the related peptide YY have been found to activate at least five different receptor populations in a wide array of tissues and cells in the central and peripheral nervous systems (for review, see Playford and Cox, 1996). Identification of subtypes of receptors for neuropeptide Y in the cardiovascular system has been based largely on the rank order of affinities or potencies of neuropeptide Y analogues and fragments in radioligand binding studies or bioassay systems (for review, see McDermott et al., 1993). The existence of at least three of the subtypes of neuropeptide Y receptors (Y_1, Y_2 and Y_3) has been established using [Leu^{31}, Pro^{34}] neuropeptide Y or [Pro^{34}] neuropeptide Y, that are selective agonists at neuropeptide Y Y_1 receptors (Fuhlendorff et al., 1990; Potter and McCloskey, 1992), neuropeptide Y-(13–36), an agonist with some selectivity for neuropeptide Y Y_2 receptors (McCloskey and Potter, 1991), peptide YY-(3–36) that has a high affinity for neuropeptide Y Y_2 receptors (Grandt et al., 1992; Dumont et al., 1994) and peptide YY which, in a number of cultured cell and tissue preparations, is several

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orders of magnitude less effective than neuropeptide Y, leading to a definition of the neuropeptide Y Y_3 subtype (Balasubramaniam et al., 1990; Michel, 1991; Wahlestedt et al., 1992).

In the cardiovascular system, there appears to be selective distribution of neuropeptide Y receptor subtypes in some cell types. Mainly neuropeptide Y Y_1 receptors have been found in most blood vessels, mediating an increase in arterial pressure and coronary resistance (Grundemar et al., 1992; Potter and McCloskey, 1992), and a homogeneous neuropeptide Y Y_1 population has been identified in aortic tissue (Shigeri et al., 1991) and smooth muscle cells in culture (Mihara et al., 1990; Shen et al., 1991). Neuropeptide Y Y_2 receptors are associated with neuropeptide Y's effect on cardiac vagal action (McCloskey and Potter, 1991; Potter and McCloskey, 1992), with vasoconstriction in some vascular beds (Lundberg et al., 1988; Michel et al., 1990; McAuley and Westfall, 1992; Grundemar et al., 1992) and have been located in platelets (Myers et al., 1990). In the heart, binding sites of both high and low affinities were identified in rat cardiac ventricular membranes (Balasubramaniam et al., 1990) and since neuropeptide Y-(13–36) bound with a lower affinity than did neuropeptide Y, a neuropeptide Y Y_2 receptor population was distinguished. Furthermore, since peptide YY had a lower binding affinity than did neuropeptide Y, the presence of a neuropeptide Y Y_3 subclass was suggested. It would be important, however, to characterize the receptors for neuropeptide Y on isolated and purified cardiomyocytes, which should make clear the nature of the postjunctional receptors in heart muscle.

Multiple subtypes of the cardiac neuropeptide Y receptor in ventricular cells may be inferred from studies in rat cardiomyocytes (Millar et al., 1991), in which we showed that the negative effect of neuropeptide Y on the contractile response, mediated by stimulation of the transient outward current and possibly by attenuation of cyclic AMP accumulation, was inhibited by pertussis toxin and by the partial peptide, neuropeptide Y-(18–36). In the absence of isoprenaline, but in the presence of a blocking agent of the transient outward current, 4-aminopyridine, neuropeptide Y exerted a positive contractile response which could not be abolished by pretreatment with pertussis toxin or attenuated by neuropeptide Y-(18–36). The positive effect was, however, abolished by treatment with the calcium antagonist, verapamil, indicating the involvement of an L-type Ca^{2+} current, and also the peptide fragment, neuropeptide Y-(17–36) (Millar et al., 1992). We proposed, therefore, that two postjunctional subtypes of the neuropeptide Y receptor exist on rat ventricular cardiomyocytes. It has been difficult to utilize information regarding signal transduction mechanisms in support for receptor subclassification, since specific links between individual neuropeptide Y receptor subtypes, GTP-binding proteins and second messenger substances have not been established (for review, see Wan and Lau, 1995). Although it was demonstrated that the negative action of neuropeptide Y through the transient outward current is sensitive to pertussis toxin whereas the positive action through an L-type Ca^{2+} current on cardiomyocyte contraction is not (Millar et al., 1991), this does not distinguish receptor subtypes, since it has been proposed that neuropeptide Y Y_2 receptors can activate both pertussis toxin-sensitive and -insensitive mechanisms in the same cell. In a human neuroblastoma cell line, CHP-234, possessing a homogeneous population of neuropeptide Y Y_2 receptors, pertussis toxin did not affect the neuropeptide Y-stimulated intracellular Ca^{2+} increase (Lynch et al., 1994; Lemos and Takeda, 1995), although it abolished the neuropeptide Y-dependent inhibition of cyclic AMP production (Lynch et al., 1994). The evidence from experiments with cloned and expressed receptor subtypes has demonstrated clearly that neuropeptide Y receptor subtypes do not differ substantially in their coupling to second messenger substances. In cells that expressed transfected cloned human neuropeptide Y Y_2 receptors (Rose et al., 1995), human neuropeptide Y Y_1 receptors (Herzog et al., 1992; Larhammar et al., 1992) and mouse neuropeptide Y Y_1 receptors (Nakamura et al., 1995), neuropeptide Y induced both increases in cytosolic Ca^{2+} and inhibition of forskolin-stimulated cyclic AMP accumulation. The findings that neuropeptide Y has negative effects on isoprenaline-stimulated accumulation of cyclic AMP and on contractile response, and that the positive effect is mediated by an L-type Ca^{2+} current (Millar et al., 1991), therefore, do not lend support to the hypothesis that there are distinct receptor subtypes on cardiac myocytes. However, the neuropeptide Y C-terminal fragment, neuropeptide Y-(18–36), which inhibits the negative but not the positive contractile action (Millar et al., 1991) does distinguish receptor subpopulations, but does not identify which receptor subtype is linked to each response, since the receptor specificity of neuropeptide Y-(18–36) is not clear. The fragment was found to act as an antagonist through partial agonism at neuropeptide Y Y_1 receptors in some systems, while in others it was a full agonist at neuropeptide Y Y_2 receptors (Michel et al., 1990). Yet it has been demonstrated that neuropeptide Y-(18–36) is a competitive antagonist at neuropeptide Y receptors in cardiac tissue (Balasubramaniam and Sheriff, 1990), which have been identified as being of the neuropeptide Y Y_2, and possibly, neuropeptide Y Y_3 subtype (Balasubramaniam et al., 1990). Although there have been no antagonists identified as acting specifically at neuropeptide Y Y_2 or neuropeptide Y Y_3 receptors, a number of neuropeptide Y Y_1 receptor-selective compounds, including the nonpeptide, BIBP3226 (Rudolf et al., 1994; Entzeroth et al., 1995; Jacques et al., 1995), the cyclic peptide 1229U91 (Hegde et al., 1995) and, most recently, the C-terminal hexapeptide analogue, bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} (Balasubramaniam et al., 1996), have been described. Application of such an antagonist to a study of neuropeptide Y-mediated effects in

cardiac cells should add to the information obtained previously using neuropeptide Y-(18–36) (Balasubramaniam and Sheriff, 1990; Millar et al., 1991).

So it appears that there are multiple neuropeptide Y receptors on cardiomyocytes, but it remains to be established which subtypes are present and if their characteristics are the same as the neuropeptide Y receptor subtypes that have been distinguished by the differential effects of neuropeptide Y and related compounds. The aim of this study was, therefore, to determine the rank order of potency of neuropeptide Y, peptide YY and the selective agonists, neuropeptide Y-(13–36), peptide YY-(3–36) and [Leu^{31}Pro^{34}] neuropeptide Y, on the contractile response of isolated ventricular cardiomyocytes, and to investigate the effects of bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} on neuropeptide Y-mediated responses, in order to identify the subtypes of the neuropeptide Y receptor which exist post-junctionally in cardiac tissue.

2. Materials and methods

2.1. Materials

Porcine neuropeptide Y, neuropeptide Y-(13–36), peptide YY, peptide YY-(3–36) and [Leu^{31}, Pro^{34}]-neuropeptide Y were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland), isoprenaline, 4-aminopyridine and bovine serum albumin from Sigma (Poole, UK), laminin from Gibco-BRL (Paisley, UK) and collagenase from Serva (Heidelberg, Germany). Bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)]} was synthesised according to Balasubramaniam et al. (1996). Petri dishes (35 mm diameter) were supplied by Nunc (Roskilde, Denmark). All other chemicals were of analytical grade and supplied by BDH (Poole, UK).

Tyrode's solution (pH 7.4) consisted of: NaCl (125 mM), KH_2PO_4 (1.2 mM), KCl (2.6 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (1.0 mM), glucose (11 mM) and HEPES (10 mM). Modified Tyrode's solution consisted of Tyrode's solution supplemented with KCl (1.5 mM) and $CaCl_2$ (1 mM).

2.2. Cell isolation and purification

Ventricular cardiomyocytes were isolated from male Sprague–Dawley rats (12 weeks old) by perfusion of the hearts with a collagenase solution (0.4 mg/ml) according to the method of Piper et al. (1982). The total ventricular cells were placed in Tyrode's solution at a concentration of 1×10^5 viable cardiomyocytes/ml. Aliquots (1 ml) of cell suspension were pipetted gently onto Petri dishes precoated with laminin (1 $\mu g/cm^2$) and incubated for 2 h, by which time viable cardiomyocytes had attached selectively to the surface of the dish.

2.3. Specific experimental protocols

The attached cardiomyocytes were washed with Tyrode's solution and were then incubated for 15 min at 37°C in modified Tyrode's solution (2.5 ml) containing various concentrations of neuropeptide Y, neuropeptide Y-(13–36), [Leu^{31}, Pro^{34}] neuropeptide Y, peptide YY or peptide YY-(3–36) with or without a subsequent addition of isoprenaline (10^{-9} M) for 5 min prior to electrical stimulation. This concentration of isoprenaline was chosen as under similar experimental conditions, it was shown previously that 10^{-7} M produced a submaximal (approximately 80%) response in these cells (Piper et al., 1989). 4-Aminopyridine was used at a concentration of 5×10^{-4} M, which selectively blocks the transient outward current (Giles and Van Ginneken, 1985) and was added to cardiomyocytes for 15 min, with or without various concentrations of peptide YY-(3–36). In experiments to investigate the effect of bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} on the positive effect of neuropeptide Y, the peptide was used at a submaximal concentration (10^{-7} M) along with 4-aminopyridine (5×10^{-4} M) and various concentrations of the antagonist, and incubation was carried out for 15 min. In experiments to investigate the effect of bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} on the negative effect of neuropeptide Y, cardiomyocytes were incubated with various concentrations of the antagonist, without or with neuropeptide Y (10^{-7} M) for 15 min prior to the addition of isoprenaline (10^{-7} M) for 5 min.

2.4. Measurement of cell contraction

At the end of the appropriate incubation period, the Petri dish was placed on the thermostatically controlled (37°C) stage of the microscope. Ensuring that the visual field was maintained in their middle, two silver chloride electrodes were immersed to a distance of 5 mm into the incubation buffer. Biphasic electrical stimuli (S88 Grass Stimulator, Qunicy, MA, USA), composed of two equal but opposite rectangular 50 V stimuli of 0.5 ms duration, were applied at a frequency of 0.5 Hz for 3 min. After a 30 s period, the microscopic picture in phase contrast was recorded on tape (Sony KSP-60) at a rate of 25 frames/s using a CCD-video camera (Panasonic WV-BL600) and a video cassette recorder (Sony U-matic VO-9600P).

2.5. Analysis of cell contractions

Images each including 4–8 individual cells were visualised on a video monitor screen at 500-fold magnification. Only those cardiomyocytes found to contract at both ends of their longitudinal axis were selected for measurement. Cells found to contract at only one end of their longitudinal axis, or along their horizontal axis, or cells which were

bent or of other distorted appearance, were excluded from the study. The cells to be investigated were selected using a mouse, by marking points just beyond each end of the longitudinal axis of each cell. A straight line was interpolated between these two points and cell length was determined by edge detection using the software, HEARTBEAT, developed by the Parallel Processing Unit, Department of Computer Science, The Queen's University of Belfast (Belfast, UK). A transputer-based Parsys Supernode and Meiko Computing Surface multiprocessor computers were used to perform the computations. The sequence of 25 frames during which the contractile event had occurred was analysed one frame at a time. The data file created was inspected visually and the maximum diastolic length (A), which was the most commonly occurring length within each group of 25 frames, and the fully contracted, systolic, length of each cell (B) contracting in synchrony with the electrical stimuli, were obtained from these data. The contractile response (dL) was expressed as the percentage $(A-B)\times 100/A$. Cells displaying spontaneous contractile activity, detected in a frame or frames distinct from those during which contraction of the majority of the cells in the field was observed, were identified and the data rejected.

2.6. Statistical analysis of data

Twenty cells were analysed under each experimental condition in each heart cell preparation and the mean value was obtained. Data of contractile response are given as mean values ± S.E.M. of a number of heart cell preparations. Data obtained for the relationship between concentration and response were fitted using the non-linear regression programme of Prism, Version 2.0 (Graphpad™, San Diego, CA, USA) and EC_{50} values with 95% confidence intervals (CI) generated. Analysis of the differences between responses due to the peptides and controls (under unstimulated or isoprenaline-stimulated conditions, as appropriate) were performed using a one-way analysis of variance (SPSS-PC) and a Dunnett's multiple range test (Winer, 1971). Differences with $P<0.05$ were regarded as significant.

3. Results

The contractile response of cells under basal conditions, in the absence of peptide, in the different sets of experiments were 10.46 ± 0.58% (neuropeptide Y), 10.28 ± 0.33% (neuropeptide Y-(13–36)), 9.34 ± 0.33% (peptide YY), 13.89 ± 0.27% (peptide YY-(3–36) and 8.25 ± 0.75% ([Leu^{31}, Pro^{34}] neuropeptide Y).

Neuropeptide Y, neuropeptide Y-(13–36) and peptide YY, over the concentration range, 10^{-10}–10^{-6} M, did not have any effect on basal levels of contraction (data not shown), whereas peptide YY-(3–36) produced at maximum a 34% inhibition of the basal response (at 10^{-6} M). Pre-incubation of the cells with increasing concentrations of neuropeptide Y, neuropeptide Y-(13–36), peptide YY or peptide YY-(3–36) (10^{-10}–10^{-6}M) attenuated the isoprenaline-stimulated contractile response in a manner which was dependent on concentration (Fig. 1). Comparison of the one- and two-component fits to the data obtained for neuropeptide Y, peptide YY and peptide YY-(3–36) indicated in each case that no difference existed, and so the simpler one-component model was chosen (Table 1). On fitting the data for neuropeptide Y-(13–36), however, the models produced significantly different fits and the goodness of fit statistic indicated that the two-component was the more appropriate model (Table 1). Therefore neuropeptide Y-(13–36), acting at high affinity receptors, had at least equal potency to peptide YY-(3–36), and both of these C-terminal fragments were more potent than neuropeptide Y in attenuating the isoprenaline-stimulated contraction. Also, peptide YY was more potent in inhibiting this response than neuropeptide Y, but was not as potent as neuropeptide Y-(13–36) or peptide YY-(3–36). Both neuropeptide Y and peptide YY, at the maximum concentration used (10^{-6} M), abolished completely the increase in contractile response induced by isoprenaline (10^{-7} M), which was inhibited by 106.3 ± 7.2% and 103.2 ± 8.4%, respectively. In contrast, pre-incubation of the cells with peptide YY-(10^{-6} M) did not abolish completely the isoprenaline-stimulated contractile response of the cells, in that only 72.7 ± 5.3% maximum inhibition was observed. On the other hand, peptide YY-(3–36) not only abolished

Table 1
Analysis of concentration–response curves for the attenuation of isoprenaline-stimulated contraction by neuropeptide Y, neuropeptide Y-(13–36), peptide YY and peptide YY-(3–36)

	One- vs. two-component fit (F value, p value)	Goodness of fit (R^2)		EC_{50} value (M)	95% CI (M)
		one-component	two-component		
Neuropeptide Y	2.42, 0.41			9.0×10^{-9}	1.0×10^{-9}–8.0×10^{-8}
Neuropeptide Y-(13–36)	2750, 0.01	0.86	1.00	3.1×10^{-11}	1.2×10^{-11}–8.1×10^{-11}
				7.2×10^{-8}	4.3×10^{-8}–1.2×10^{-7}
Peptide YY	0.35, 0.76			4.3×10^{-10}	8.4×10^{-11}–2.2×10^{-9}
Peptide YY-(3–36)	1.52, 0.50			8.5×10^{-11}	2.3×10^{-11}–3.1×10^{-10}

Data obtained for the relationship between concentration and response were fitted using non-linear regression to generate the statistics of the fits to a one- or two-component model and the appropriate EC_{50} values with 95% confidence intervals (CI).

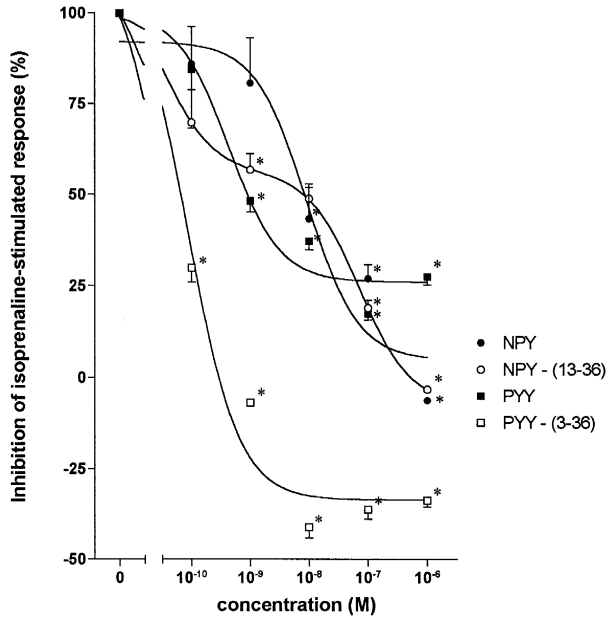

Fig. 1. Effect of neuropeptide Y (NPY), neuropeptide Y-(13–36), peptide YY (PYY) and peptide YY-(3–36) (10^{-10}–10^{-6} M) on contractile activity in isoprenaline (10^{-7} M)-stimulated rat ventricular cardiomyocytes ($n = 4$–5 experiments in each case). Contractile response was calculated as the maximum shortening relative to the pre-stimulated cell length and values are expressed as a percentage in relation to the isoprenaline stimulated response (set at 100%). * $P < 0.05$ by comparison with the isoprenaline-stimulated value in the absence of peptide.

the isoprenaline-stimulated increase in contractile amplitude, but this fragment, at concentrations in excess of approximately 5×10^{-10} M, also depressed myocyte contraction to below the basal level. The data in Fig. 2 demonstrate that under conditions when the transient outward current is blocked in these cells using 4-aminopyridine, peptide YY-(3–36) over the concentration range, 10^{-10}–10^{-6} M, does not attenuate the basal contraction and, moreover, does not stimulate contractile activity.

[Leu^{31}, Pro^{34}] neuropeptide Y increased the amplitude

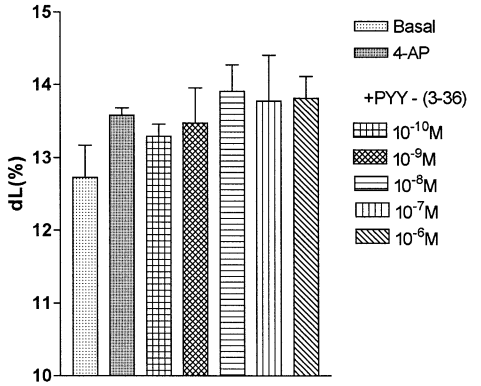

Fig. 2. Effect of peptide YY(PYY)-(3–36) (10^{-10}–10^{-6} M) on contractile activity in rat ventricular cardiomyocytes in the presence of 4-aminopyridine (4-AP, 5.0×10^{-4} M) ($n = 5$ experiments in each case). Contractile response (dL) is expressed as a percentage, i.e., the maximum shortening relative to the pre-stimulated cell length.

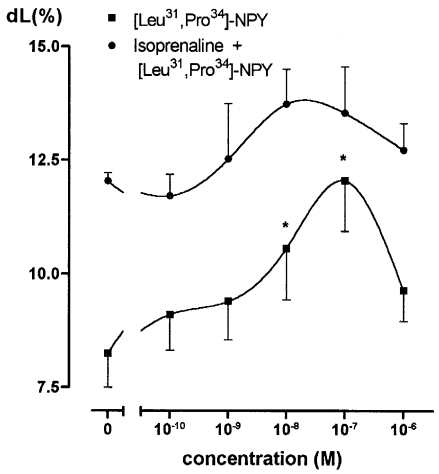

Fig. 3. Effect of [Leu^{31}, Pro^{34}] neuropeptide Y (NPY) (10^{-10}–10^{-6} M) on contractile activity in rat ventricular cardiomyocytes under basal conditions or stimulated with isoprenaline (10^{-7} M) ($n = 4$ experiments in each case). Contractile response is expressed as given for Fig. 2. * $P < 0.05$ by comparison with the contractile response in the absence of [Leu^{31}, Pro^{34}] neuropeptide Y, without or with isoprenaline as appropriate.

of the contractile response under basal conditions (Fig. 3). A maximum increase was observed at [Leu^{31}, Pro^{34}] neuropeptide Y (10^{-7} M) and at a concentration of 10^{-6} M, contractile amplitude decreased to 37% of the maximum response. In the presence of isoprenaline (10^{-7} M), the addition of [Leu^{31}, Pro^{34}] neuropeptide Y tended to increase further the contractile activity of the cells (Fig. 3). Even at the maximum response obtained at a 10^{-8} M concentration of the neuropeptide Y analogue, the effect was not significantly different from that obtained under control conditions with isoprenaline, since only relatively small increases in contractile activity can be obtained when the cells are already being stimulated to a large extent. Similar to the finding under basal conditions, at a concentration of 10^{-6} M, contractile amplitude decreased, in this case to 40% of the maximum response. The results obtained both under basal and isoprenaline-stimulated conditions were insufficient for fitting to a 'bell-shaped' mathematical model as described by Rovati and Nicosia (1994) and so the simple four-parameter logistic curve was applied to the data that defined the stimulatory portions. EC_{50} values are given in Table 2.

The stimulation of contractile activity by neuropeptide

Table 2
Analysis of concentration–response curves for potentiation of basal and isoprenaline-stimulated contraction by [Leu^{31}, Pro^{34}]-neuropeptide Y

	EC_{50} value (M)	95% CI (M)
Basal	8.1×10^{-9}	3.1×10^{-10}–2.2×10^{-7}
+Isoprenaline	1.5×10^{-9}	4.1×10^{-11}–5.7×10^{-8}

Data obtained for the relationship between concentration and response were fitted using non-linear regression (four-parameter logistic equation) to generate EC_{50} values with 95% confidence intervals (CI).

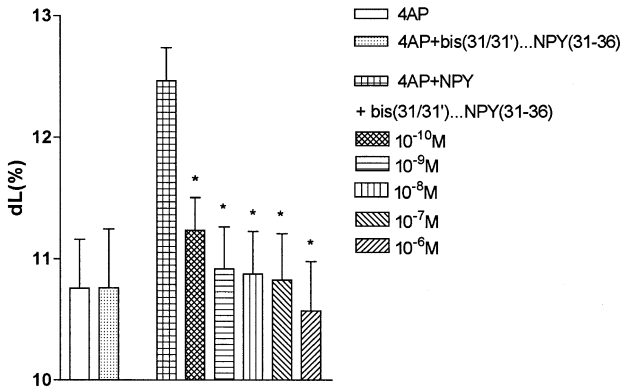

Fig. 4. Inhibition of the positive contractile response to neuropeptide Y (NPY, 4×10^{-7} M), elicited in the presence of 4-aminopyridine (4-AP, 5.0×10^{-7} M) by bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} (bis(31/31′)…NPY-(31–36), 10^{-10}–10^{-6} M) in rat ventricular cardiomyocytes ($n=5$ experiments in each case). Contractile response is expressed as given for Fig. 2. * $P<0.05$ by comparison with the contractile response to neuropeptide Y and 4-aminopyridine in the absence of bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)}.

Y in the presence of 4-aminopyridine, and its inhibition by the Y_1-selective antagonist, bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)}, is shown in Fig. 4. The addition of bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)}(10^{-6} M) did not have any effect on the contractile amplitude of cells that had been treated with 4-aminopyridine. When neuropeptide Y (10^{-7} M) was used in the presence of 4-aminopyridine, contractile amplitude increased to 116% of that obtained with the inhibitor

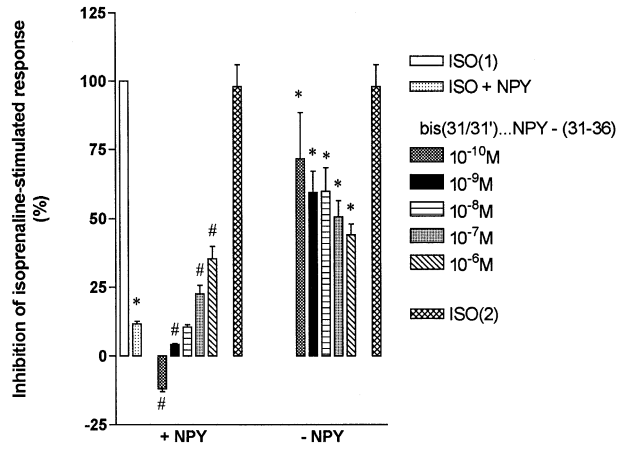

Fig. 5. Effect of bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} (bis(31/31′)…NPY-(31–36), 10^{-10}–10^{-6} M) on the negative effect of neuropeptide Y (NPY, 10^{-7} M) elicited in the presence of isoprenaline (10^{-7} M) and of the antagonist alone on the isoprenaline-stimulated response. Contractile response was calculated as for Fig. 1 and values were expressed relative to the response to isoprenaline at the beginning of the experiments (ISO(1)), whereas ISO(2) was the isoprenaline-stimulated response at the end of the experiments. As appropriate, * $P<0.05$ by comparison with the contractile response to isoprenaline at the beginning of the experiments or $^{\#}$ $P<0.05$ with respect to the response to isoprenaline and neuropeptide Y, in the absence of bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)}.

alone. The addition of bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} resulted in attenuation of the neuropeptide Y-stimulated response at all concentrations of the antagonist used (10^{-10}–10^{-6} M) and virtually complete inhibition was obtained at concentrations $\geq 10^{-9}$ M. The effect of bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} on the attenuation of isoprenaline-stimulated contractile activity by neuropeptide Y is shown in Fig. 5. Neuropeptide Y (10^{-7} M) almost completely abolished the effect of isoprenaline, such that the response was restored to just above the basal value. The addition of low concentrations of bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} (10^{-10} and 10^{-9} M) resulted in a significant decrease in contractile activity, but with increasing concentrations of the antagonist, contractile amplitude increased, and significant inhibition of the neuropeptide Y-mediated response was obtained at concentrations $\geq 10^{-7}$ M. In the absence of neuropeptide Y, bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} (10^{-6} M) alone attenuated the isoprenaline-stimulated response in a concentration-dependent manner over the range, 10^{-10}–10^{-6} M, and the EC_{50} value was 5.8×10^{-11} M (95% CI 4.7×10^{-12}–7.1×10^{-10} M). That these effects were not due to rundown of contractile activity was confirmed by the demonstration of similar responses to isoprenaline at the beginning and end of the experiments.

4. Discussion

The current classification of neuropeptide Y receptors has been based largely on the rank order of potency of the agonists, neuropeptide Y and its analogues and fragments, because there has been a lack of selective and high affinity antagonists available. The actions of peptide YY, neuropeptide Y, neuropeptide Y analogues and C-terminal fragments have been studied in the heart in vivo and in tissue preparations in vitro (McDermott et al., 1993), in which both pre- and postjunctional receptor interactions would be expected to contribute to the overall response. In this study, the effects of such peptides and also a recently described Y_1-selective antagonist (Balasubramaniam et al., 1996) on the purely post-junctional receptors that exist in rat ventricular cardiomyocytes have been studied in order to clarify the receptor subtypes present in heart muscle cells.

We showed previously that neuropeptide Y can attenuate a stimulated contractile response in cardiomyocytes and when this negative effect is blocked, a positive contractile action is disclosed that can be observed under basal conditions (Millar et al., 1991). In the present study, the order of potency of the peptides to produce a decrease in the isoprenaline-stimulated contractile amplitude of cardiomyocytes, being neuropeptide Y-(13–36) ≥ peptide YY-(3–36) > peptide YY > neuropeptide Y > [Leu^{31},

Pro[34]] neuropeptide Y, indicates that this negative response is mediated by neuropeptide Y Y_2-like receptors. This conclusion has been reached on consideration that neuropeptide Y-(13–36) and peptide YY-(3–36) are neuropeptide Y Y_2 receptor-selective agonists that, at least in the latter case, are virtually inactive at Y_1 receptors, whereas the substituted analogue, [Leu[31], Pro[34]]-neuropeptide Y, is a full neuropeptide Y Y_1 receptor agonist, while being substantially inactive at neuropeptide Y Y_2 receptors. Indeed, these data show in addition that [Leu[31], Pro[34]] neuropeptide Y, but apparently none of the other peptides, has a positive effect on contractile amplitude, which would indicate that there is also a population of neuropeptide Y Y_1 receptors on rat cardiomyocytes. It would appear, therefore, that the positive effect of neuropeptide Y on contractile amplitude, observed previously in the presence of blockade of the transient outward current, is mediated by neuropeptide Y Y_1 receptors. Interestingly, the potency of [Leu[31], Pro[34]] neuropeptide Y to potentiate the basal contractile response (EC_{50} 8×10^{-9} M) was comparable to that obtained previously (Millar et al., 1991) for stimulation of a positive contractile response by neuropeptide Y in the presence of 4-aminopyridine, an inhibitor of the transient outward current (EC_{50} 2×10^{-9} M). This observation would argue against the identification on rat cardiomyocytes of the neuropeptide Y Y_4 receptor, a pancreatic polypeptide-preferring receptor, termed the PP1 receptor, which has been detected as mRNA in human coronary artery (Bard et al., 1995) and mouse heart (Gregor et al., 1996). Although [Leu[31], Pro[34]] neuropeptide Y binds with high affinity to rat neuropeptide Y Y_4/PP1 receptors (Gehlert et al., 1997), indications are that the native peptide has a lower affinity for this receptor population. Using recombinant murine neuropeptide Y receptors having 92% identity to rat neuropeptide Y Y_4/PP1 receptors, expressed in COS-7 cells, the IC_{50} value for neuropeptide Y was one order of magnitude greater than that of the analogue (Gregor et al., 1996). Also, using cloned and transiently expressed human neuropeptide Y Y_4/PP1 receptors, higher affinity binding was obtained with the analogue than with neuropeptide Y (Bard et al., 1995). Furthermore, although their pharmacological analysis (Hu et al., 1996) is similar to that of neuropeptide Y Y_1 receptors, the existence on cardiomyocytes of neuropeptide Y Y_5 receptors is unlikely on consideration that their mRNA distribution has been located primarily in the central nervous system (Gerald et al., 1996).

It had been suggested previously (Balasubramaniam et al., 1990), on the basis of a discrepancy between the binding affinities of neuropeptide Y and peptide YY in cardiac ventricular membranes, that the heart contains a peptide YY-insensitive group of neuropeptide Y receptors, designated neuropeptide Y Y_3, and it was proposed that such receptors could be expressed by the cardiomyocytes. Our finding that peptide YY was the more potent agonist is clearly inconsistent with the involvement of a neuropeptide Y Y_3 receptor in cardiomyocytes linked to the negative inotropic response to neuropeptide Y. Also, Xiang and Brown (1993) found no difference in the inhibitory effect of neuropeptide Y and peptide YY on inositol 1,4,5-trisphosphate formation in rat cardiomyocytes. It remains, however, that there may be a neuropeptide Y Y_3-like receptor for neuropeptide Y in cardiac tissue, that is not linked primarily to phosphatidylinositol turnover and contractile activity, or that is located on another cell type.

In the light of the preceding discussion, it is proposed that probably only neuropeptide Y Y_1 and neuropeptide Y Y_2 receptors are expressed in cardiomyocytes. Since these receptor subtypes appear to be linked, respectively, to positive and negative contractile responses, there is a functional antagonism that can be seen to underlie the perceived actions of neuropeptide Y and related compounds. The observation of bell-shaped concentration–response curves for the effect of [Leu[31], Pro[34]] neuropeptide Y, under basal and isoprenaline-stimulated conditions, would indicate that, at high concentration, the analogue can interact with neuropeptide Y Y_2 receptors, resulting in attenuation of the positive response. Also, there are indications of interactions with both receptor subtypes in relation to the actions of peptide YY and neuropeptide Y in reducing the isoprenaline-stimulated response. In a purely neuropeptide Y Y_2 receptor system, peptide YY appears to be slightly more potent than neuropeptide Y, which exhibits one order of potency greater than neuropeptide Y-(13–36) (Grundemar and Håkanson, 1990; Wahlestedt et al., 1992). The fact that neuropeptide Y and peptide YY had apparently lesser potencies than neuropeptide Y (13–36) or peptide YY-(3–36) in reducing the isoprenaline-stimulated response of cardiomyocytes, however, can be explained by the activation by the full peptides of both the neuropeptide Y Y_2 receptor population that is coupled to the negative response and a neuropeptide Y Y_1 receptor population which mediates the opposing action, resulting in a reduced attenuating effect. This is in contrast to the apparent highly selective effect demonstrated here of peptide YY-(3–36) at neuropeptide Y Y_2 receptors which, having apparently no affinity for the opposing neuropeptide Y Y_1 receptors, can be observed to attenuate the basal myocyte contraction even at low concentrations. It is interesting to note, from the observation that neuropeptide Y-(13–36) produces a biphasic response, that the interaction of this C-terminal fragment with neuropeptide Y Y_2 receptors in cardiomyocytes may be coupled to two different mechanisms both of which mediate a negative effect on the isoprenaline-stimulated contractile response. In this respect, it has been argued that neuropeptide Y receptors, besides being coupled to adenylate cyclase, may interfere negatively with excitation-contraction by an additional mechanism that is also mediated by an inhibitory GTP-binding protein (Millar et al., 1991).

The identification of neuropeptide Y Y_1 and neuropeptide Y Y_2 receptor subclasses linked to positive and nega-

tive contractile effects of neuropeptide Y on rat ventricular cardiomyocytes does not accord favorably at first sight with our previous findings of the differential effect of the neuropeptide Y C-terminal fragment, neuropeptide Y-(18–36), which was found to inhibit the negative but not the positive response (Millar et al., 1991). It has been reported that neuropeptide Y-(18–36) acts as a full agonist at neuropeptide Y Y_2 receptors in some systems and, in other experimental settings, as an antagonist by virtue of its action as a partial agonist at neuropeptide Y Y_1 receptors (Michel et al., 1990). Certainly, there was some evidence for neuropeptide Y-(18–36) behaving as a partial agonist in the cardiomyocyte bioassay (Millar et al., 1991), in that at low concentrations of neuropeptide Y (10^{-10} to 10^{-8} M), neuropeptide Y-(18–36) increased, although not significantly, the positive contractile response, presumably by acting additively with neuropeptide Y at neuropeptide Y Y_1 receptors. Neuropeptide Y-(18–36), however, inhibited significantly the negative response to neuropeptide Y, over the concentration range, 10^{-10} to 10^{-6} M, and no evidence of agonist properties at these purported neuropeptide Y Y_2 receptors were observed. Furthermore, it has been found that neuropeptide Y-(18–36) can abolish neuropeptide Y-induced inhibition of adenylate cyclase activity in rat cardiac ventricular membranes (Balasubramaniam and Sheriff, 1990), which may be neuropeptide Y Y_2 receptor-mediated, since in rat cardiomyocytes the full peptide decreases isoprenaline-stimulated accumulation of cAMP (Millar et al., 1988) and this is associated with the negative contractile response to neuropeptide Y (Piper et al., 1989). Also, neuropeptide Y-(18–36) was found to block the decrease in inositol 1,4,5-trisphosphate levels in rat cardiomyocytes that may be implicated in the negative inotropic effect of neuropeptide Y (Xiang and Brown, 1993). It is possible that that the postjunctional neuropeptide Y Y_2 receptor identified here in rat heart muscle cells, at which neuropeptide Y-(18–36) had no activity (Millar et al., 1991), has some different characteristics to the pre-synaptic neuropeptide Y Y_2 receptors in serotinergic neurons and neuropeptide Y Y_2 receptors in cutaneous microvasculature, at which neuropeptide Y-(18–36) produced a potent response (Michel et al., 1990).

Use of the neuropeptide Y Y_1 selective antagonist, bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36) has demonstrated clearly that a neuropeptide Y Y_1 population of receptors mediate the positive response to neuropeptide Y in cardiomyocytes observed when the negative response through the transient outward current is inhibited using 4-aminopyridine. Under these conditions, this antagonist on its own does not influence myocyte contraction, even though the potential exists to attenuate at least the stimulatory effect of 4-aminopyridine; however, the compound did attenuate the isoprenaline-stimulated contraction and the pattern of response obtained when the antagonist was combined with neuropeptide Y is consistent with bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y-(31–36)} having properties of a partial agonist. In the original characterization of this compound, it was shown in radioligand binding studies that its affinity for neuropeptide Y Y_2 receptors on SK-N-BE2 cells was of the order of > 10 μM (Balasubramaniam et al., 1996), whereas its potency as a partial agonist for the purported neuropeptide Y Y_2 receptors in this study in cardiomyocytes was in the picomolar range, which indicates further that this population of neuropeptide Y Y_2 receptors may not be representative.

In conclusion, the findings of this study support the view that on cardiomyocytes, there are two populations of receptors for neuropeptide Y which have characteristics related to neuropeptide Y Y_1 and neuropeptide Y Y_2 receptors. The finding of neuropeptide Y Y_2 receptors on heart muscle cells represents a further example of a postjunctional location for this subtype. It may be that the population of neuropeptide Y Y_2 receptors on cardiomyocytes is atypical in comparison with previously identified pre-junctional neuropeptide Y receptors.

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