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Neuropeptide Y potentiates noradrenaline-induced contraction through the neuropeptide Y Y_1 receptor

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Abstract

To elucidate which neuropeptide Y receptor subtype is responsible for the neuropeptide Y-induced potentiation of the noradrenaline-evoked contraction in human omental arteries we used antisense oligodeoxynucleotide (Antisense), the new selective neuropeptide Y Y_1 receptor antagonist, BIBP3226 {(*R*)-*N*2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl) methyl]-D-arginine-amide} and the reverse transcriptase-polymerase chain reaction (RT-PCR). Neuropeptide Y significantly potentiated the noradrenaline-induced contraction in non-incubated vessels (pEC_{50} 6.4 ± 0.2 vs. 5.9 ± 0.2) and in vessels incubated with 1 μM Sense oligodeoxynucleotide (Sense) (pEC_{50} 6.0 ± 0.1 vs. 5.6 ± 0.2). In vessels incubated with 1 μM Antisense the potentiating effect of neuropeptide Y was completely abolished. BIBP3226 (1 μM) inhibited the neuropeptide Y-induced potentiation in human omental arteries (pEC_{50} 5.8 ± 0.3 vs. 6.4 ± 0.2). Finally, messenger RNA for the neuropeptide Y Y_1 receptor was detected using RT-PCR. On the basis of our results we conclude that the neuropeptide Y-induced potentiation of the noradrenaline-induced contraction is mediated by the neuropeptide Y Y_1 receptor.

Keywords: Artery, human; Noradrenaline; Neuropeptide Y; Antisense oligodeoxynucleotide; BIBP3226; Reverse transcriptase-polymerase chain reaction

1. Introduction

Neuropeptide Y is co-stored with noradrenaline in sympathetic nerves supplying the cardiovascular system (Edvinsson et al., 1987) and is a potent constrictor of human blood vessels both in vitro (Edvinsson et al., 1985) and in vivo (Pernow et al., 1987) . In addition neuropeptide Y, at subthreshold concentrations, has been shown to potentiate the noradrenaline-induced contraction (Edvinsson et al., 1984, 1985; Ekblad et al., 1984). So far the human neuropeptide Y Y_1, Y_2 and Y_4 receptors have been cloned and mRNA for the neuropeptide Y Y_1 receptor has been shown to be present in smooth muscle cells from human arteries (Bard et al., 1995; Herzog et al., 1993; Larhammar et al., 1992; Rose et al., 1995). The physiological effects of the postjunctional neuropeptide Y Y_1 and prejunctional Y_2 receptors have been characterized by means of truncated neuropeptide Y receptor agonists (Wahlestedt et al., 1990). However, the lack of neuropeptide Y receptor antagonists has made pharmacological characterization difficult and the physiological significance of neuropeptide Y has not yet been established (Edvinsson et al., 1994). In 1993, Erlinge and co-workers demonstrated one way to circumvent this problem (Erlinge et al., 1993) using an antisense oligodeoxynucleotide corresponding to a coding region near the N-terminus of the human neuropeptide Y Y_1 receptor. After incubation with antisense oligodeoxynucleotide the direct contractile effect of neuropeptide Y in human blood vessels was reduced (Erlinge et al., 1993). Recently, a potent and selective neuropeptide Y Y_1 receptor antagonist, {(*R*)-*N*2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-D-arginine-amide} (BIBP3226), was described which presumably will make future neuropeptide Y receptor characterization easier (Jacques et al., 1995; Rudolf et al., 1994). We adressed the following questions: (i) does the neuropeptide Y Y_1 receptor mediate the potentiation of noradrenaline-induced contraction and can this be characterized by these antagonists?; (ii) is there a difference in inhibitory mechanisms between the neuropeptide Y Y_1 receptor antagonist, BIBP3226, and

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Antisense oligodeoxynucleotide?; (iii) can the presence of mRNA corresponding to the human neuropeptide Y Y_1 receptor be detected by use of the reverse transcriptase-polymerase chain reaction (RT-PCR)?

2. Materials and methods

2.1. Production of oligodeoxynucleotides

The oligodeoxynucleotides, Sense, (hY_1-S = 5′-CAACATTATTTTCCCAGG-3′) and Antisense, (hY_1-AS = 5′-CCTGGGAAAATAATGTTG-3′), corresponding to a region near the N-terminus of the human neuropeptide Y Y_1 receptor mRNA, were synthesized on a Biosearch Cyclone DNA Synthesizer (Scandinavian Gene Synthesis, Sweden). After deprotection with 30% ammonium hydroxide, the oligodeoxynucleotides were lyophilized and redissolved in water. These oligodeoxynucleotides were run on 15% acrylamide gels to verify their sizes. The DNA sequence of the oligodeoxynucleotides was checked for similarities against all human DNA sequences present in the GENEMBL database.

2.2. Vasomotor studies

Omental arteries from patients who underwent abdominal surgery for non-vascular diseases were removed and cut into cylindrical segments (2–3 mm long). The vessels were used either directly or after incubation in Dulbecco's modified essential medium (DMEM, Sigma, St. Louis, MO, USA), supplemented with streptomycin (100 mg/ml) and penicillin (100 U/ml), in the presence of the oligodeoxynucleotides, Antisense or Sense (1 μM) for 48 h at 37°C in humified 5% CO_2 and 95% air. The cylindrical segments were mounted on two metal prongs, one of which was connected to a force-displacement transducer (FT03C) attached to a Macintosh Plus computer and the other to a displacement device. The data from the experiments were continuously recorded and registrated by the Macintosh software program Chart. The mounted specimens were immersed in temperature-controlled tissue baths containing a buffer solution of the following composition (mmol): NaCl 119; $NaHCO_3$ 15; KCl 4.6; $MgCl_2$ 1.2; NaH_2PO_4 1,2; $CaCl_2$ 1,5; glucose 11. The solution was continuously gassed with 5% CO_2 in O_2, giving a pH of 7.4. A tension of 4–6 mN was applied to the vessel segments and they were allowed to stabilize at this tension for 1 h. The contractile capacity of each vessel segment was examined by exposure to a potassium-rich (60 mM) buffer solution which had the same composition as the standard solution except that some of the NaCl was exchanged for an equimolar concentration of KCl. Concentration-response curves for noradrenaline were obtained by adding the drug in a cumulative fashion. Neuropeptide Y was added 2 min before noradrenaline. The controls were an equal number of matched vascular segments that received noradrenaline without prior exposure to neuropeptide Y. The antagonist was added 20 min before neuropeptide Y. The maximal contraction in percentage of the 60 mM KCl buffer solution is expressed as E_{max}. The potency of the agonist used is expressed as pEC_{50} values (negative logarithm of the molar concentration of the drug inducing a half-maximum response).

2.3. Oligonucleotide design

RT-PCR assay for neuropeptide Y Y_1 receptor mRNA was performed using the following primers (Scandinavian Gene Synthesis, Sweden): neuropeptide Y Y_1 forward; 5′-CTCTTGCTTATGGA/GGCTGTGA-3′ and neuropeptide Y Y_1 reverse; 5′-CTGGAAGTTTTTGTTCAG-GAAC/TCCA-3′.

The primers are based on published nucleotide sequences of the human neuropeptide Y Y_1 receptor (Herzog et al., 1993; Larhammar et al., 1992).

2.4. RNA preparation

Total cellular RNA was isolated from human omental arteries (obtained from patients who underwent abdominal surgery) by acid guanidininum thiocyanate/phenol/chloroform extraction (Chomczynski and Sacchi, 1987). Frozen tissues were homogenised with 4 M guanidinium isothiocyanate containing 2 β-mercaptoethanol and 0.5% sarcosyl, and further extracted with phenol and chloroform-isoamyl alcohol. RNA was precipitated at −20°C with isopropanol and dissolved in 20 μl RNAase-free water. The amount and the potency of RNA was evaluated by absorption at 260 nm, using a DU-64 spectrophotometer (Beckman Instruments, Sweden). The ratio of absorption (260 nm/280 nm) of all preparations was between 1.6 and 1.8.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The reverse transcription of total RNA to cDNA and subsequent PCR was carried out using the GeneAmp RNA PCR kit (Perkin Elmer, Sweden) in a Perkin Elmer DNA Thermal Cycler. First strand cDNA was synthesised from 1 μg total RNA in a 20 μl reaction volume following the standard reverse transcription protocol using random hexamers as primers. The reaction mixture was incubated at 42°C for 15 min, heated to 99°C for 5 min, and chilled to 5°C for 5 min. The resultant cDNA was amplified by PCR in a final volume of 100 μl following the standard PCR protocol. AmpliTaq DNA polymerase (Perkin-Elmer) was used as the thermostable enzyme. The PCR was carried out by using four linked files as follows: file 1, 2 min at 95°C for 1 cycle; file 2, 1 min at 95°C and 1 min at 60°C for 35 cycles; file 3, 7 min at 72°C for 1 cycle; file 4, incubation at 4°C for 5 min. To verify the absence of contamination, negative control samples with RNAase-free water were used.

2.6. Electrophoretic analysis of PCR products

After RT-PCR, a 10 μl aliquot from each PCR product was electrophoresed on a 1.5% Seakem LE agarose gel (FMC Bioproducts, Rockland, ME, USA), containing 1.0 μg/ml ethidium bromide, in TBE buffer (89 mmol Tris-borate, 2 mmol EDTA, pH 8.0) at 5 V/cm for 1.5 h. The 100-bp DNA Ladder (Promega, Scandinavian Diagnostic Service, Sweden) was used as the molecular weight marker. This analysis was performed in a GNA 200 electrophoresis apparatus (Pharmacia Biotech).

2.7. Statistics

Values represent means ± S.E.M. *n* refers to the number of patients from whom the vessels were collected. Stastistical analyses were done with the Wilcoxon signed-rank test using the StatView IIT program on a Macintosh IIcx computer. $P < 0.05$ was considered significant.

2.8. Drugs

Neuropeptide Y (Auspep, Australia), noradrenaline (Sigma, USA), BIBP3226 {(*R*)-*N*2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl) methyl]-D-arginine-amide} (a generous gift from Drs. H. Doods and K. Rudolf, Dr. Karl Thomae, Germany).

The study was approved by the Ethics Committee at the University of Lund.

3. Results

3.1. Non-incubated vessels

Noradrenaline induced a concentration-dependent contraction of human omental arteries. Neuropeptide Y (30 nM) did not per se cause any contraction while the same concentration significantly potentiated the noradrenaline-induced contraction, causing a leftward shift of the concentration-response curve without change of the maximum contractile effect (Table 1). BIBP3226 (1 μM) significantly inhibited the neuropeptide Y-induced potentiation (Fig. 1).

3.2. Incubated vessels

The effects of the Antisense and Sense oligodeoxynucleotides were examined in subsequent experiments. Despite the 48 h incubation in DMEM at 37°C, with or without oligodeoxynucleotides, arteries responded to 60 mmol KCl (K^+) with powerful contractions. However, the K^+-induced contractions of the incubated arteries were weaker than those of the non-incubated vessels (Table 1). In general, the K^+-induced contraction was reduced from 11.3 mN to 4.0 mN (64%, mean value, Table 1). At the same time the noradrenaline-induced contraction was reduced from 11.9 mN to 5.1 mN (57%, mean value, not shown). Noradrenaline induced a concentration-dependent contraction in all arteries incubated with DMEM. In arteries incubated with Sense oligodeoxynucleotides, neuropeptide Y (30 nM) potentiated the noradrenaline-induced con-

Table 1
The potentiating effect of 30 nM neuropeptide Y (NPY) on the noradrenaline (NA)-induced contraction of human omental arteries and the effect of BIBP3226 (1 μM), Antisense (1 μM) and Sense (1 μM) on the potentiation

	K^+	*n*	E_{max} (%)	pEC_{50}
NA	10.7 ± 1.4	6	104.6 ± 3.6	5.9 ± 0.1 [a]
NA + NPY	12.3 ± 1.5	7	114.6 ± 11.7	6.4 ± 0.2 [a,b]
NA + NPY + BIBP3226	11.0 ± 1.9	6	116.4 ± 6.4	5.8 ± 0.3 [b]
Sense + NA	6.3 ± 1.7	6	134.2 ± 21.4	5.6 ± 0.2 [c]
Sense + NA + NPY	5.3 ± 1.9	6	143.6 ± 18.2	6.0 ± 0.1 [c]
Antisense + NA	2.5 ± 0.5	6	138.0 ± 13.3	5.9 ± 0.1 [d]
Antisense + NA + NPY	2.0 ± 0.8	5	138.8 ± 19.0	6.0 ± 0.1 [d]

Neuropeptide Y potentiated significantly the noradrenaline-induced contration of human omental arteries. In the presence of BIBP3226 the potentiation was abolished. Neuropeptide Y did not potentiate the noradrenaline-induced contraction in vessels incubated with Antisense but induced a significant potentiation in vessels incubated with Sense. Values represent means ± S.E.M.; values with the same superscript are significantly (a, b, c) or non-significantly (d) different ($P < 0.05$; Wilcoxon signed-rank test); *n* denotes number of patients from whom segments were used; K^+ denotes potassium-induced contraction in milli-Newton; E_{max} (%) denotes the agonist-induced contraction in percentage of K^+.

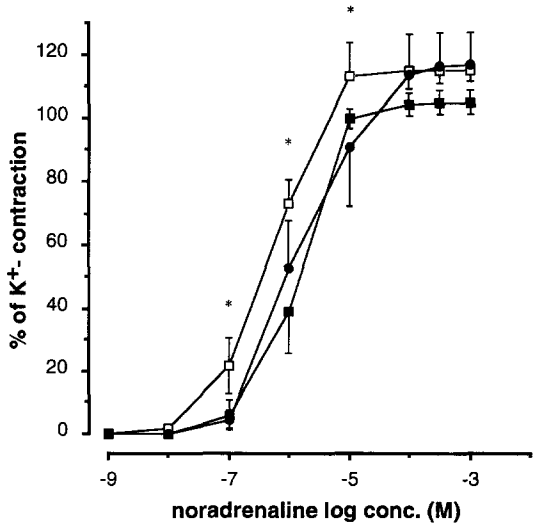

Fig. 1. Noradrenaline-induced contraction in human omental arteries in the absence of neuropeptide Y (■), in the presence of neuropeptide Y 30 nM (□) and in the presence of neuropeptide Y 30 nM and BIBP3226 1 μM (●). The results are expressed as percentages of the potassium-induced contraction. Each point represents the mean ± S.E.M. A leftward shift of the concentration-response curve was noted in the presence of neuropeptide Y 30 nM (□), compared to noradrenaline alone (■) and to noradrenaline and neuropeptide Y 30 nM in the presence of BIBP3226 1 μM (●), pEC_{50} values of the contractions are shown in Table 1.

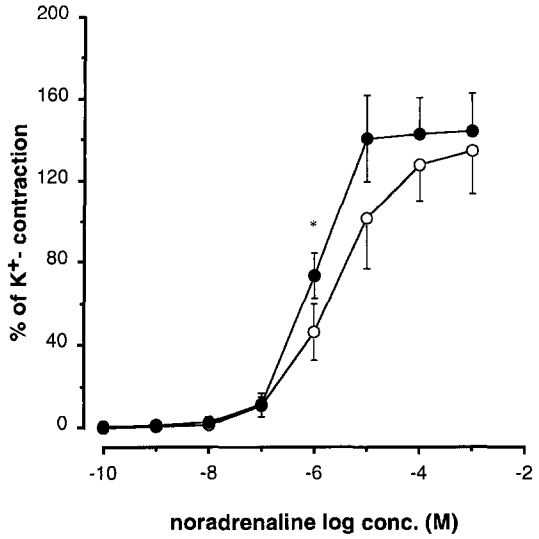

Fig. 2. Human omental arteries incubated in DMEM in the presence of Sense. The noradrenaline-induced contraction (○) was significantly potentiated by neuropeptide Y 30 nM (●). The results are expressed as percentages of the potassium-induced contraction. Each point represents the mean ± S.E.M; a leftward shift was found in the presence of neuropeptide Y 30 nM (●), pEC_{50} values of the noradrenaline-induced contractions are shown in Table 1.

traction without change in maximal contraction (Table 1, Fig. 2). The neuropeptide-Y-induced potentiation of the noradrenaline-evoked contraction did not differ between Sense-incubated vessels and vessels incubated in DMEM without oligodeoxynucleotides added (not shown). In omental arteries incubated with Antisense, the contractile response to noradrenaline in the presence of 30 nM neuropeptide Y was not potentiated (Table 1, Fig. 3).

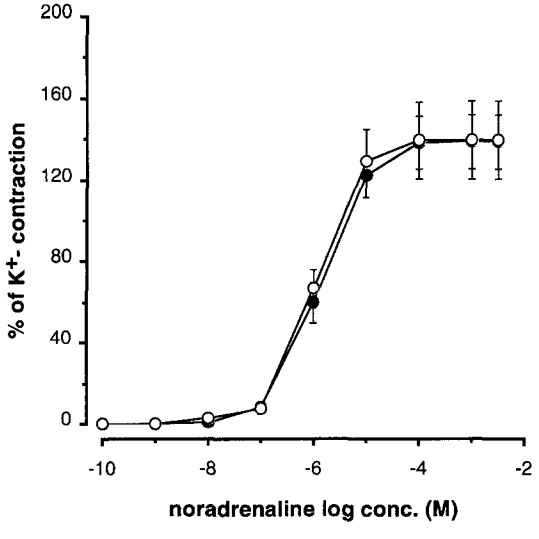

Fig. 3. Human omental arteries incubated in DMEM in the presence of Antisense. No difference was seen in the noradrenaline-induced contraction in the absence (○) or in the presence of 30 nM neuropeptide Y (●). The results are expressed as percentages of the potassium-induced contraction. Each point represents the mean ± S.E.M. pEC_{50} values of the noradrenaline-induced contractions are shown in Table 1.

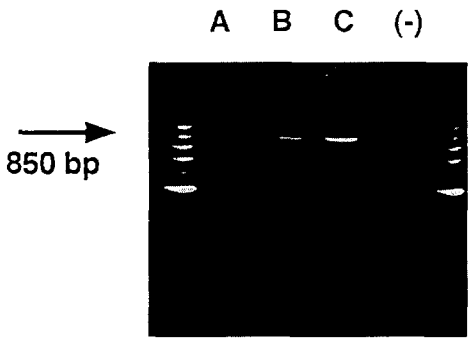

Fig. 4. Agarose gel showing the presence of amplified mRNA RT-PCR products in omental arteries from two patients (A and B) and the neuropeptide Y Y_1 expressing neuroblastoma cell line SK-N-MC (C). The 850 base-pair amplification product corresponds to the TM1 to TM7 of the neuropeptide Y Y_1 receptor. RNAase-free water was used as a negative control (−).

3.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The presence of RT-PCR products of the expected size (850 base pairs), corresponding to mRNA encoding the human neuropeptide Y Y_1 receptor, was detected in the positive control SK-N-MC (a neuropeptide Y Y_1 receptor-expressing neuroblastoma cell line) as well as in the two human omental arteries used (Fig. 4).

4. Discussion

The aim of the present study was to elucidate which neuropeptide Y receptor subtype mediates the potentiation of noradrenaline-induced contraction and how two different ways of inhibiting the neuropeptide Y Y_1 receptor would affect the potentiation profile of the noradrenaline-induced dose–response curve.

As demonstrated before in the guinea-pig mesenteric arteries, the neuropeptide Y Y_1 receptor mediated the potentiation of noradrenaline-induced contraction (Nilsson et al., 1996a). Due to the previous absence of neuropeptide Y antagonists (Edvinsson et al., 1994) we designed an antisense 18-base oligodeoxynucleotide corresponding to the N-terminus of the human neuropeptide Y Y_1 receptor in order to down-regulate the neuropeptide Y Y_1 receptor. Wahlestedt and colleagues demonstrated a 50% down-regulation of neuropeptide Y Y_1 receptor binding in rat cerebral cortex by use of a corresponding oligodeoxynucleotide (Wahlestedt et al., 1993). In human subcutaneous arteries the contractile effect of neuropeptide Y was attenuated after incubation with Antisense (Erlinge et al., 1993).

Incubation of human omental arteries with DMEM resulted in a general reduction of the potassium- and noradrenaline-induced contractions. This phenomenon has been seen before (Adner et al., 1994) and is thought to be

a consequence of the incubation procedure. No differences in this behaviour were seen between vessels incubated with or without an oligodeoxynucleotide.

The Antisense oligodeoxynucleotide completely inhibited the potentiation induced by neuropeptide Y. The inhibited neuropeptide Y response was probably due to down-regulation or to reduced numbers of neuropeptide Y Y_1 receptors (Wahlestedt et al., 1993). The selectivity of the Antisense seems very high since incubation with an oligodeoxynucleotide sequence, complementary Sense, did not affect the potentiation.

To further understand if the neuropeptide Y-induced potentiation of the noradrenaline-evoked contraction was mediated by the neuropeptide Y Y_1 receptor we used BIBP3226, a recently developed, selective and potent neuropeptide Y Y_1 receptor antagonist (Rudolf et al., 1994). So far, BIBP3226 has shown selective binding affinity for neuropeptide Y Y_1 receptors in human neuroblastoma cells and in rat cerebral cortex. In rat colon, a Y_3 bioassay, and in rat vas deferens, a Y_2 receptor bioassay, however, BIBP3226 failed to alter the contractile effect of neuropeptide Y (Doods et al., 1995a,b; Jacques et al., 1995; Wieland et al., 1995). Furthermore, BIBP3226 has displayed highly antagonistic properties in vitro against vasopressor responses evoked by neuropeptide Y and Y_1 receptor agonists in isolated rat kidney and in vivo in pithed rats, antagonizing the neuropeptide Y induced increase in blood pressure (Doods et al., 1995a). Further, a physiological effect, mediated by the neuropeptide Y Y_1 receptor, has been reported using BIBP3226 (Zukowska-Grojec et al., 1996). Doods et al. (1995a) have shown that BIBP3226 antagonizes the neuropeptide-Y-mediated potentiation of the noradrenaline-elicited perfusion pressure in the rat mesenteric bed. Their results in the rat are thus consistent with our results in human, since BIBP3226 signficantly inhibited the neuropeptide Y-induced potentiation of the noradrenaline-evoked contraction of human omental arteries.

In the present study the neuropeptide Y_1 receptor was characterized with two different inhibitors; however, the inhibitory mechanism of BIBP3226 and Antisense is of different nature. The non-peptide inhibitor, BIBP3226, works in a competitive way causing a rightward shift of the neuropeptide Y-induced potentiation while Antisense treatment seems to work in a down-regulatory way (Wahlestedt et al., 1993). The use of Antisense still has the disadvantages of administration. Interestingly, however, it has been shown that long-term intravenous infusion of a stabilized phosphorothioated Antisense oligodeoxynucleotide to the rat selectively reduces the pressor response to neuropeptide Y administration (Sun et al., 1996). However, non-specific oligodeoxynucleotides (Sense and Mismatch) had various effects on the mean arterial blood pressure that could not be explained. Thus, at the present time oligodeoxynucleotide treatment with Antisense seems to be an experimental tool; however, once distributed in the cell, it has the powerful effect of turning off its target molecule.

Finally we used specific human neuropeptide Y Y_1 primers, confirming the presence of the Y_1 receptor in SK-N-MC (a neuropeptide Y Y_1-expressing neuroblastoma cell line) and, in addition, PCR products of the same size were detected in human omental arteries from two patients. The human neuropeptide Y Y_1 receptor in vascular smooth muscle cells have been detected before in, e.g., human coronary artery, subcutaneous and cerebral arteries (Erlinge, 1994; Larhammar et al., 1992; Nilsson et al., 1996b).

In conclusion, our findings demonstrate that the neuropeptide Y-induced potentiation of the noradrenaline-evoked contraction in human omental arteries is mediated by the neuropeptide Y Y_1 receptor. Two different ways to inhibit specifically the neuropeptide Y Y_1 receptor are demonstrated: (i) the use of BIBP3226 and (ii) the use of Antisense oligodeoxynucleotide.

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