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Neuropeptide Y accounts for sympathetic vasoconstriction in guinea-pig vena cava: evidence using BIBP 3226 and 3435

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Abstract

The ability of the novel, non-peptide, neuropeptide Y Y_1 receptor antagonist, BIBP 3226 ((*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide), to antagonize neuropeptide Y- and sympathetic-mediated vasoconstriction was examined in isolated segments of the thoracic vena cava of guinea-pigs. Increasing concentrations (10^{-9}–10^{-6} M) of BIBP 3226 caused a parallel and rightward shift in the neuropeptide Y dose-response curve but did not significantly change the effect of noradrenaline. The calculated pA_2 value for BIBP 3226 was 8.0 ± 0.08, a value fully compatible with the reported affinity at rodent and human neuronal Y_1 receptors. BIBP 3226 (10^{-6} M) also readily reversed the established vasocontraction induced by neuropeptide Y. BIBP 3226 (10^{-6} M) markedly inhibited the slow long-lasting contraction evoked by high frequency electrical field stimulation, leaving a rapid component which was abolished by phentolamine. Its enantiomer, BIBP 3435 ((*S*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide), which exerts a much weaker action on neuropeptide Y Y_1 receptors, had no such inhibitory effect. In propranolol-pretreated vessels, the vasoconstriction evoked by nerve stimulation was enhanced; then BIBP 3226 inhibited the peak response by 44%, and the integrated contractile effect by 90%. We conclude that BIBP 3226 is a potent and competitive antagonist of neuropeptide Y Y_1 receptor-mediated vasoconstriction in guinea-pig vena cava and that endogenous neuropeptide Y acting on the neuropeptide Y Y_1 receptor is likely to account for the long-lasting component of the sympathetic vasoconstriction in response to high-frequency stimulation in this vessel.

Keywords: Neuropeptide Y; Neuropeptide Y Y_1 receptor antagonist; BIBP 3226; Vasoconstriction, sympathetic; Vena cava, guinea-pig

1. Introduction

Neuropeptide Y belongs to a peptide family which also includes peptide YY and pancreatic polypeptide (Tatemoto, 1982). Neuropeptide Y is contained in most sympathetic vasoconstrictor neurons, and can be released together with noradrenaline in response to electrical stimulation of sympathetic pathways, especially at high-frequency stimulation. In vivo, neuropeptide Y elicits vasoconstriction (Lundberg and Tatemoto, 1982), while in vitro only some blood vessels are contracted by neuropeptide Y. In addition, especially in larger vessels, neuropeptide Y potentiates the effect of noradrenaline (Lundberg et al., 1990). In many blood vessels, as well as in other autonomically innervated organs, neuropeptide Y can act presynaptically to inhibit transmitter release (Lundberg and Stjärne, 1984). At least two different neuropeptide Y receptors, referred to as Y_1 and Y_2, have been pharmacologically characterized (Wahlestedt et al., 1986, 1990). The neuropeptide Y Y_1 receptor appears to be located mainly postjunctionally and cloning experiments have shown that it belongs to the family of G-protein-coupled receptors (Larhammar et al., 1992; Herzog et al., 1993). Neuropeptide Y Y_2 receptors have also been reported to evoke vasoconstriction in some blood vessels but seem to be located mainly prejunctionally, and to mediate inhibition of autonomic neurotransmitter release.

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The existence of a third receptor subtype (Y_3) has also been proposed (Grundemar et al., 1991; Dumont et al., 1993).

In the guinea-pig vena cava, neuronally released noradrenaline produces relaxation, acting via β-adrenoceptors and contraction, via α-adrenoceptors. Presynaptic α_2-adrenoceptors also mediate inhibition of autonomic neurotransmitter release (Morris, 1991). In this vessel, neuropeptide Y produces a long-lasting vasoconstriction which mimics the contractile response that is seen predominantly at higher frequencies of sympathetic stimulation. Furthermore, tachyphylaxis to the neuropeptide Y Y_1 receptor reduces the response to sympathetic nerve stimulation in this tissue (Morris, 1991). Attempts to establish the status of neuropeptide Y as a transmitter and to carry out pharmacological characterization of neuropeptide Y receptor subtypes have been hampered by the lack of selective neuropeptide Y receptor antagonists and by the fact that all available agonists are chemically related to neuropeptide Y. Within these pharmacological limitations, neuropeptide Y Y_1 receptors have been identified in binding studies as the predominant subtype in vascular smooth muscle (Sheikh et al., 1991; Grundemar et al., 1992), and studies using a range of neuropeptide Y analogues have shown the neuropeptide Y Y_1 receptor subtype to be the main subtype mediating contraction in guinea-pig vena cava (Morris and Sabesan, 1994). Recently a non-peptide compound, BIBP 3226 ((*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide), has been synthesized and shown to behave as a competitive, specific and selective neuropeptide Y Y_1 receptor antagonist (Rudolf et al., 1994; Doods et al., 1995; Wieland et al., 1995). In the present study we have taken advantage of this novel neuropeptide Y Y_1 receptor antagonist to characterize the receptor subtype responsible for the neuropeptide Y-induced vasoconstriction in guinea-pig vena cava and to obtain further evidence that endogenous neuropeptide Y is a sympathetic vasoconstrictor transmitter. The *S*-enantiomer of BIBP 3226, BIBP 3435 ((*S*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide) has no effects on neuropeptide Y Y_1 receptors (Rudolf et al., 1994) and was therefore used as control.

2. Materials and methods

2.1. Experimental procedure

Young adult guinea-pigs of either sex (250–450 g body weight) were killed with an intracardiac injection of sodium pentobarbital. The entire thoracic portion of the inferior vena cava was then removed. For measurement of isometric tension, circular vessel segments, with a length of 2 mm, were cut under a dissecting microscope and mounted on two L-shaped metal prongs. The segments were placed in a temperature-controlled (37°C) tissue bath (volume 2.5 ml) containing a Hepes-buffered Krebs-Ringer solution of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.0, KH_2PO_4 1.0, $NaHCO_3$ 25, $CaCl_2 \cdot 2H_2O$ 2.5, glucose 6 and Hepes 20. The solution was bubbled with a gas mixture of 93.5% O_2 and 6.5% CO_2 to maintain a pH of 7.4.

The vessels were given a mechanical tension of 1 g and allowed to stabilize at this level for 60 min. The bathing solution was replaced regularly every 15 min with fresh buffer. Tension was measured with Grass FT03 transducers, and was displayed on a Grass 7C polygraph.

Some vessels were stored overnight in Hepes-buffered solution at 4°C before use, without any detectable changes in response to exogenous agonists or transmural nerve stimulation the following day.

After a 60-min equilibration period, vessels were contracted with noradrenaline (10^{-5} M). Noradrenaline was applied and washed out at 30-min intervals, until consistent contractions were obtained. All contractions provoked by exogenous agonists were expressed as percentage of these noradrenaline contractions.

Perivascular nerves were stimulated transmurally via pairs of electrodes. Pulses of 1 ms duration were delivered for 10 s at frequencies of 10 or 40 Hz by a nerve stimulator.

Consecutive transmural electrical field stimulations were performed in the same vessel segment at least 40 min apart, the first of which served as a control. All drugs were added 30 min before electrical field stimulation. Nerve-mediated responses evoked by electrical field stimulation were calculated as areas under the curve and expressed as percentages of the control. The peak responses to electrical field stimulation were expressed as percentages of the maximal response in individual control stimulations.

Concentration-response curves for contractions produced by noradrenaline and neuropeptide Y were obtained by cumulative addition of noradrenaline (10^{-9}–10^{-6} M) and neuropeptide Y (10^{-10}–10^{-5} M), respectively. In order to test the ability of the neuropeptide Y Y_1 receptor antagonist, BIBP 3226, to antagonize the neuropeptide Y-induced vasoconstriction in guinea-pig vena cava, different concentrations of BIBP 3226 (10^{-9}–10^{-6} M) were applied to different sets of vessels for 30 min. Then, in the presence of the antagonist, concentration-response curves to neuropeptide Y were obtained as described above. In each series, one or two vessels served as controls; these were not exposed to the antagonist. The same procedure was undertaken to test the ability of BIBP 3226 to antagonize vasoconstriction induced by electrical field stimulation and by

noradrenaline. In a final series of experiments a combination of histamine H_1- (mepyramine) and H_2- (cimetidine) receptor antagonists was included to study whether neuronally released neuropeptide Y may degrade mast-cells as does the exogenous peptide (Grundemar and Håkanson, 1991), which could alter the contractile response.

2.2. Calculations

Antagonist potency was calculated as pA_2 according to the following formula (Furchgott, 1972): $pA_2 = \log([E']/[E] - 1) - \log[B]$, where $[E']$ and $[E]$ are the concentrations which caused half-maximal effects in the presence and absence, respectively, of the antagonist. $[B]$ is the concentration of the antagonist. The competitive nature of the antagonism was assessed by determination of the slope of the Schild plot (Arunlakshana and Schild, 1959), and pA_2 obtained from the Schild analysis was compared with the pA_2 calculated as described above.

2.3. Statistics

Means ± S.E.M. and the number of experiments (n) are given throughout. Student's t-test was used for comparison of the means.

2.4. Drugs

Neuropeptide Y (Cambridge Research Biochemicals, UK), phentolamine (Regitin®, Ciba-Geigy, Switzerland), propranolol hydrochloride, α,β-methyleneadenosine 5'-triphosphate, cimetidine (Sigma, St Louis, MO, USA), mepyramine maleate (Smith Kline and French Laboratories, Herts, UK), sodium pentobarbital (Apoteksbolaget, Sweden), BIBP 3226 ((*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide) and BIBP 3435 ((*S*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide) (Karl Thomae GmbH, Biberach, Germany). All substances were dissolved in Krebs-Ringer solution.

3. Results

Neuropeptide Y caused concentration-dependent contraction of the inferior vena cava with an EC_{50} value of 1.3×10^{-8} M (Figs. 1 and 2). The maximum contraction produced by neuropeptide Y was 230% of the effect of noradrenaline (10^{-5} M). BIBP 3226 (10^{-6} M), given at the neuropeptide Y-induced maximal vasocontraction (10^{-7} M), caused reversal of the established contraction (Fig. 1). The application of increas-

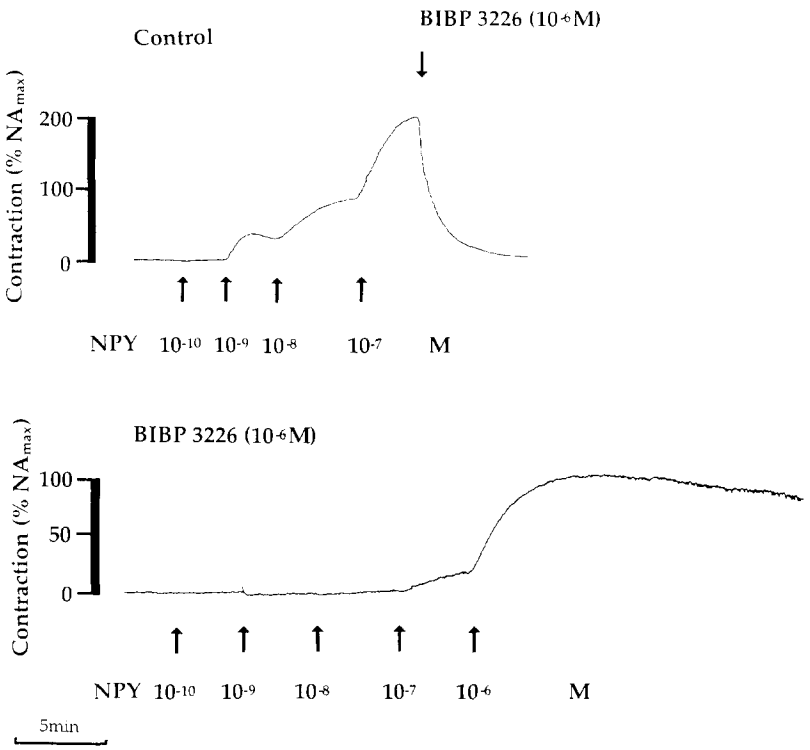

Fig. 1. Neuropeptide Y evokes concentration-dependent contraction of guinea-pig vena cava. The neuropeptide Y-induced contraction was reversed when BIBP 3226 (10^{-6} M) was added (top panel). Preincubation with BIBP 3226 (10^{-6} M) for 30 min led to inhibition of the contractile effect of neuropeptide Y (lower panel).

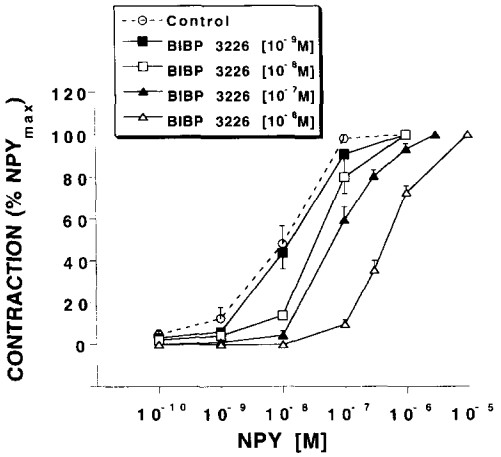

Fig. 2. Concentration-response curves for neuropeptide Y on guinea-pig vena cava in the absence (control) and presence of different concentrations of BIBP 3226. Increasing concentrations of BIBP 3226 led to a rightward shift of the neuropeptide Y concentration-response curve.

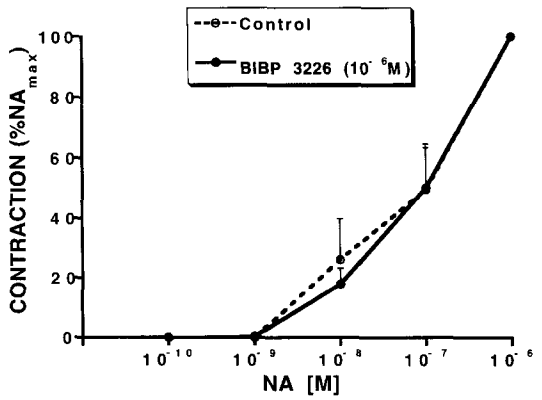

Fig. 4. Concentration-response curves for noradrenaline in the absence (control) and presence of BIBP 3226 (10^{-6} M). Pretreatment with BIBP 3226 (10^{-6} M) did not affect the contractile response to noradrenaline.

ing concentrations of BIBP 3226 led to a rightward shift of the concentration-response curve without any significant decrease in the maximal contractile effect (Fig. 2). The mean pA_2 for BIBP 3226 (calculated at antagonist concentrations of 10^{-8} and 10^{-7} M) was 8.0 ± 0.08 ($n = 14$). The inhibition appeared competitive as the slope (0.84 ± 0.07) of the Schild plot (Fig. 3) was not significantly different from unity, with a correlation coefficient (r) of 0.99. The pA_2 value evaluated at the intercept (8.05) corresponds well to that calculated according to Furchgott (1972).

Concentration-response curves for noradrenaline were made in the presence of propranolol. Pretreatment with BIBP 3226 (10^{-6} M) did not affect the contractile response to noradrenaline (Fig. 4). In a separate series of experiments, α,β-methylene ATP, up to a concentration of 10^{-5} M, did not evoke any contractile response ($n = 6$).

Transmural electrical field stimulation of the vena cava with 10 Hz for 10 s caused a biphasic contraction consisting of a transient rapid phase followed by a prolonged contraction (Fig. 5, top panel). A slight spontaneous reduction of the slow phase occurred upon repeated stimulation. Propranolol enhanced the contractions in response to electrical field stimulation (40 Hz for 10 s) in the control (+58% in our series) and the difference between the two phases then became less obvious (Fig. 5, lower panel). BIBP 3435 (10^{-6} M), the *S*-enantiomer of BIBP 3226, with significantly less effect on neuropeptide Y Y_1 receptor binding, did not affect the electrical field stimulation-evoked contrac-

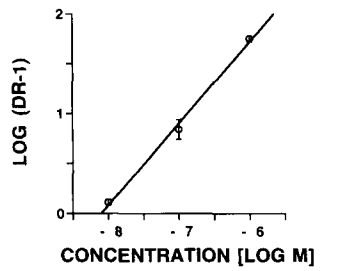

Fig. 3. Schild plot analysis of BIBP 3226 antagonism of neuropeptide Y-evoked contractions in guinea-pig vena cava.

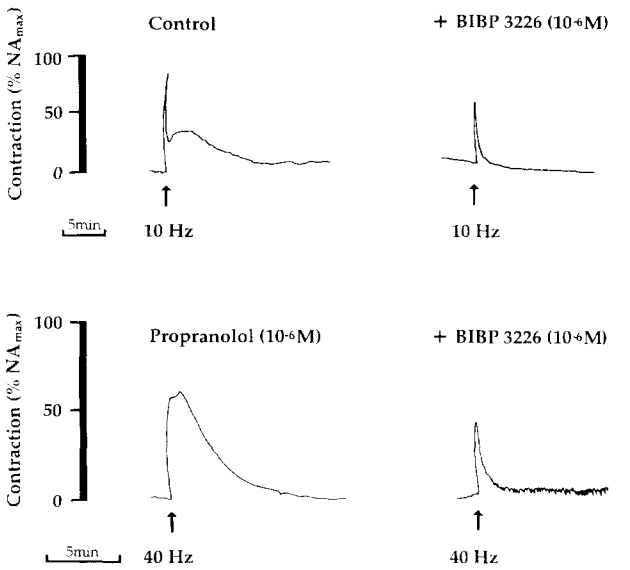

Fig. 5. Contractions of vena cava produced by electrical field stimulation (arrows) for 10 s at two different frequencies. Top panel: contractions evoked in a control vessel by electrical field stimulation (10 Hz) before and after pretreatment with BIBP 3226 (10^{-6} M). Note the reduction of the prolonged phase of the contraction. Lower panel: contraction evoked in propranolol (10^{-6} M)-pretreated vessel by electrical field stimulation (40 Hz), before and after addition of BIBP 3226 (10^{-6} M).

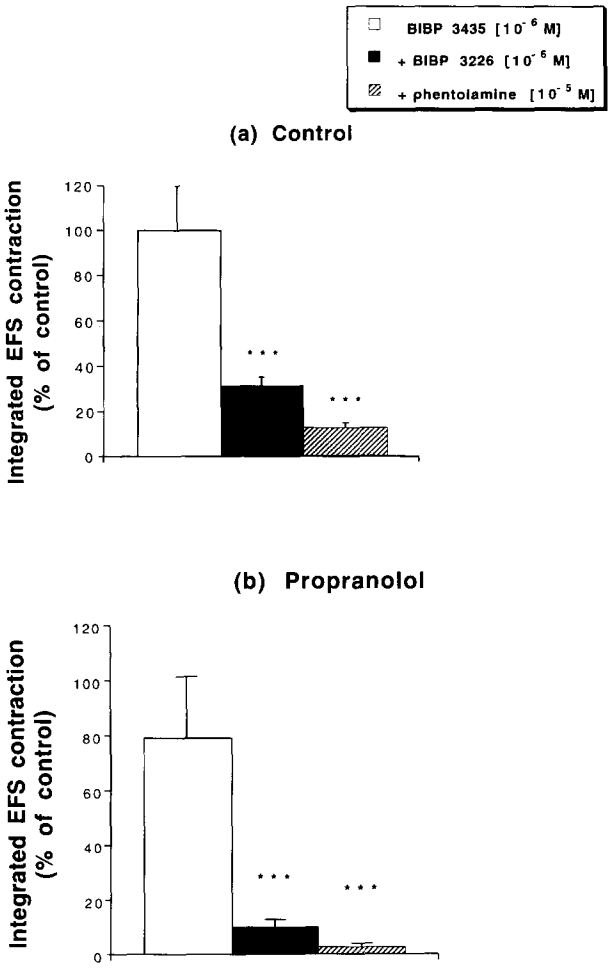

Fig. 6. Effects of BIBP 3435, BIBP 3226, and phentolamine, added cumulatively to the tissue bath, on the integrated constrictor responses to electrical field stimulation in guinea-pig vena cava in the (a) control (10 Hz for 10 s) and (b) propranolol (10^{-6} M) (40 Hz for 10 s)-pretreated series. Data are given as means ± S.E.M., (n = 5–8), and significant differences between the responses in the control and in the presence of respective drug are indicated, $^{***}P < 0.001$.

tions in either control or propranolol-treated vessels (n = 8 and 5, respectively) (Fig. 6a,b).

BIBP 3226 (10^{-6} M) reduced the integrated electrical field stimulation-evoked contractions in the control group with 68.8 ± 3.8% (n = 7). In the propranolol-pretreated series, the integrated electrical field stimulation contractions were reduced by 90.1 ± 2.7% by BIBP 3226 (n = 8) (Figs. 5 and 6). Further addition of phentolamine (10^{-5} M) reduced the integrated contractions by 87.5 ± 2.2% in the control group (n = 6) and by 97.5 ± 1.4% in the propranolol-pretreated group (n = 8) (Fig. 6). The peak contractions elicited by electrical field stimulation were not affected by BIBP 3435 (n = 6) or BIBP 3226 (n = 8) in the control group; however, when phentolamine was added to the tissue bath containing BIBP 3226 (n = 7), the peak contraction was reduced to 12.2 ± 4.6% of the control (Fig. 7a). In the propranolol-pretreated series, BIBP 3435 (n = 4) slightly reduced the peak contraction to 80.0 ± 5.2%, BIBP 3226 (n = 7) caused a further reduction to 55.7 ± 14.0%, and a final addition of phentolamine (n = 7) almost abolished the response (Fig. 7b).

In a separate series of experiments we compared the influence of different concentrations of BIBP 3226 on vasocontraction evoked in propranolol-pretreated vessels by two types of stimulation: electrical field stimulation (40 Hz for 10 s) and neuropeptide Y (10^{-8} M). These conditions were chosen to give a roughly similar contractile amplitude. BIBP 3226 was 34 times more potent as an inhibitor of the contractions evoked by exogenous neuropeptide Y than of the electrical field stimulation-evoked response, judging from the IC_{50} values calculated (data not shown) (Fig. 8). In a final series of experiments, a combination of mepyramine and cimetidine (10^{-5} M each) did not influence the

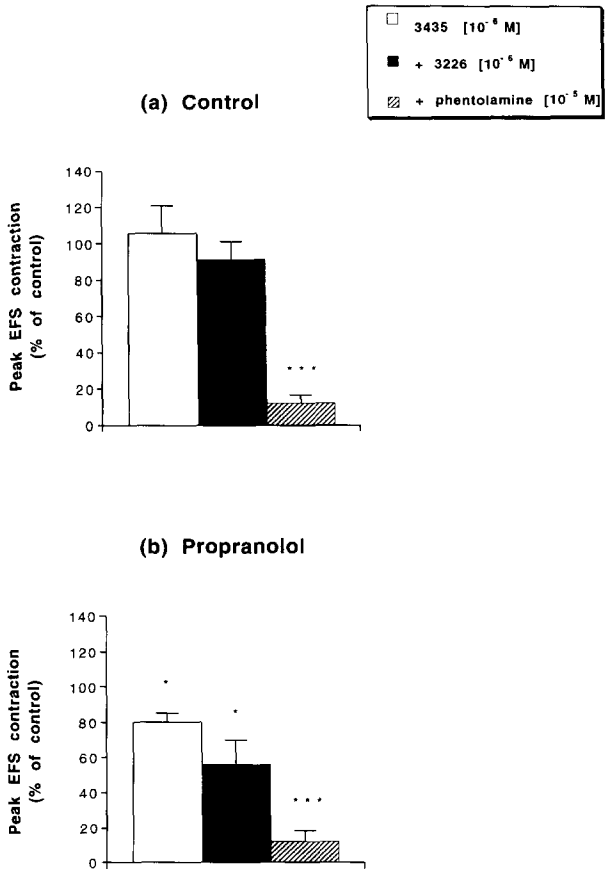

Fig. 7. Effects of BIBP 3435, BIBP 3226, and phentolamine, added cumulatively to the tissue bath, on the peak constrictor response to electrical field stimulation in guinea-pig vena cava in the (a) control (10 Hz for 10 s) and (b) propranolol (10^{-6} M) (40 Hz for 10 s)-pretreated series. Data are given as means ± S.E.M. (n = 4–8), and significant differences between the responses in the control and in the presence of respective drug are indicated, $^{*}P < 0.05$, $^{***}P < 0.001$.

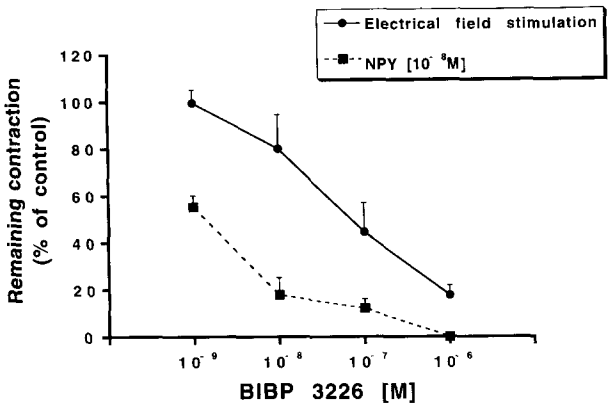

Fig. 8. Effects of BIBP 3226 at different concentrations, on constrictor responses to electrical field stimulation (40 Hz for 10 s) and exogenous neuropeptide Y (10^{-8} M) in guinea-pig vena cava, in the presence of propranolol (10^{-6} M).

contractions evoked by electrical field stimulation (n = 6). Furthermore, BIBP 3226 (10^{-6} M) inhibited the electrical field stimulation-evoked contractions to the same extent as in the absence of histamine receptor antagonists (data not shown).

4. Discussion

It has been proposed that neuropeptide Y receptors in guinea-pig vena cava are of the Y_1 subtype, based on the observation that the selective neuropeptide Y Y_1 receptor agonist, [Leu^{31},Pro^{34}]neuropeptide Y, has a potency similar to that of neuropeptide Y (Morris and Sabesan, 1994). Similar conclusions have been drawn from studies of several other blood vessels and vascular beds (Wahlestedt et al., 1986; Fuhlendorf et al., 1990; Modin et al., 1991; Sheikh et al., 1991; Grundemar et al., 1992). However, it had not been possible to test this hypothesis using specific antagonists. BIBP 3226, has recently been described as a potent, selective and competitive non-peptide neuropeptide Y Y_1 receptor antagonist (Rudolf et al., 1994). Since all previously described neuropeptide Y antagonists (Doughty et al., 1990; Edvinsson et al., 1990; Michel and Motulsky, 1990; Tatemoto et al., 1992) have failed to survive more detailed investigation, BIBP 3226 is a very interesting tool for the pharmacological characterization of neuropeptide Y receptors and of putative neuropeptide Y mechanisms. Binding assays with human, dog, pig and rat tissues have proven the selectivity of BIBP 3226 for neuropeptide Y Y_1 receptors as well as in its ability to block neuropeptide Y Y_1 receptor-mediated functional responses. Thus the increases in intracellular Ca^{2+} in SK-N-MC neuroblastoma human cell line, the increase in perfusion pressure in isolated rat kidney (Rudolf et al., 1994) and rabbit ear (Doods et al., 1995) and the vasoconstrictor effects in pig in vivo (Lundberg and Modin, 1995), all of which are mediated by the neuropeptide Y Y_1 receptor, are antagonized by BIBP 3226. BIBP 3226 also antagonizes the ability of neuropeptide Y to potentiate the noradrenaline-elicited increase in perfusion pressure in the rat mesenteric bed (Doods et al., 1995).

In the present study, we demonstrated that BIBP 3226 is a potent and competitive antagonist also at the neuropeptide Y Y_1 receptor in the guinea-pig vena cava as was shown by the rightward and parallel shift of the neuropeptide Y concentration curves without any significant change of the maximal contractile response. The affinity for BIBP 3226 in the vena cava is high ($pA_2 = 8.0 \pm 0.08$) and agrees with that determined at the neuronal neuropeptide Y Y_1 receptors (Rudolf et al., 1994). This observation confirms the involvement of neuropeptide Y Y_1 receptors in the neuropeptide Y-induced vasoconstriction in the guinea-pig vena cava. Our studies have, because BIBP 3226 and BIBP 3435 were used, given definitive proof that neuropeptide Y acting on neuropeptide Y Y_1 receptors accounts for the slow, long-lasting, non-adrenergic contractions evoked by electrical field stimulation in guinea-pig vena cava. Thus, the slow contractions produced by electrical field stimulation were nearly abolished in the presence of the neuropeptide Y Y_1 receptor antagonist, leaving only the initial rapid peak of contraction, which in turn was shown to be adrenergic since it was strongly inhibited by further addition of the α-adrenoceptor antagonist, phentolamine. In various other vascular beds, ATP mediates rapid, short-lasting contractions (see Von Kügelgen and Starke, 1991). In guinea-pig vena cava the lack of postjunctional response to α,β-methylene ATP clearly argues against any purinergic mechanisms in the neurogenic contraction. The rapid, phentolamine-sensitive (noradrenaline-mediated), component was still present after BIBP 3226 both in control and propranolol-pretreated vessels, which is consistent with the unchanged noradrenaline contractions in the present study. Phentolamine is an α_1- and α_2-adrenoceptor antagonist, acting both pre- and postsynaptically, resulting in prejunctional facilitation of neurotransmitter release and postjunctional inhibition of adrenergic contractions. Combined treatment with phentolamine and BIBP 3226 almost abolished electrical field stimulation-evoked contractions, further supporting the theory of noradrenaline and neuropeptide Y co-transmission in this vessel.

Previous studies (Morris and Murphy, 1988; Morris, 1991) have shown that exogenous neuropeptide Y causes inhibition of the slow non-adrenergic electrical field stimulation-evoked contractions due to receptor desensitization. However, these studies have not ruled out the possibility that blockade of neurogenic contrac-

tions by neuropeptide Y may be partly due to a presynaptic inhibitory action of the peptide; prejunctional neuropeptide Y Y_1 receptors inhibiting sympathetic transmitter release are known to be present in some species (Doods and Krause, 1991). Presynaptic effects of BIBP 3226 seem less likely in guinea-pig vena cava, since tachyphylaxis to exogenous neuropeptide Y in this preparation led to similar results. If there had been any substantial number of presynaptic neuropeptide Y Y_1 receptors through which endogenous neuropeptide Y could regulate noradrenaline release, an increase, especially of the initial rapid contraction, would have been anticipated. This was not the case, however.

It is not surprising that BIBP 3226 was a 34-fold less potent antagonist of the non-adrenergic response evoked by electrical field stimulation (40 Hz for 10 s) than of the response evoked by exogenous neuropeptide Y (10^{-8} M) considering the likelihood that the concentrations of endogenous neuropeptide Y at release sites activating 'innervated neuropeptide Y Y_1 receptors' are much higher than 10^{-8} M. Although it is difficult to estimate the concentration of released transmitter due to large variations in the neuromuscular gap distance, it has been calculated that noradrenaline is present at 10^{-3} M (Stjärne, 1989). Since the tissue content and overflow of neuropeptide Y upon stimulation is generally 100- to 1000-fold less than that of noradrenaline, concentrations of 10^{-6} M may occur close to release sites. It is also likely that neuropeptide Y can act at a certain distance due to limited degradation, with a long half-life, and diffusion within the tissue (Rudehill et al., 1987). A mainly similar finding has been reported, involving another non-peptide antagonist, the neurokinin 2 receptor antagonist, SR 48968. Thus, higher concentrations of a neurokinin 2 receptor antagonist are required for inhibition of the electrical field stimulation-evoked (tachykinin-mediated) non-adrenergic, non-cholinergic bronchoconstriction, than for inhibition of contractions evoked by exogenous neurokinin A (Lou et al., 1993).

We conclude that BIBP 3226, a selective non-peptide neuropeptide Y Y_1 receptor antagonist, exhibits high affinity and acts competitively regarding inhibition of neuropeptide Y-evoked contractions mediated by the neuropeptide Y Y_1 receptor in the guinea-pig vena cava. Furthermore, endogenous neuropeptide Y acting on the neuropeptide Y Y_1 receptor is likely to account for the slow, long-lasting non-adrenergic vasocontraction evoked by electrical field stimulation in guinea-pig vena cava.

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