Evidence for Y1-Receptor-Mediated Facilitatory, Modulatory Cotransmission by NPY in the Rat Anococcygeus Muscle

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ABSTRACT

The potential role of neuropeptide Y (NPY) as a neuromodulatory cotransmitter was investigated in rat anococcygeus muscle. The effects of NPY on contraction to norepinephrine or adrenergic nerve stimulation and on relaxation to nonadrenergic, noncholinergic nerve stimulation were analyzed. Norepinephrine-induced contraction was enhanced by NPY (0.1 μM). The Y1 receptor antagonist BIBP 3226 (1 μM) completely reversed this effect. NPY (0.01 or 0.1 μM) increased contractions induced by electrical field stimulation of sympathetic nerves. This increase was reduced by BIBP 3226 (1 μM), indicating Y1 receptor involvement. NPY (13–36), a Y2 receptor agonist, at 0.1 μM but not 0.01 μM, caused an increase of the nerve-induced contraction, which was reversed by BIBP 3226 (1 μM), indicating no Y2 receptor involvement. BIBP 3226 (1–10 μM) produced a concentration-dependent attenuation of nerve-mediated but not norepinephrine-mediated contraction. The reduction in nonadrenergic, noncholinergic nerve-induced relaxation to nerve stimulation by NPY (0.1 μM) was not affected by BIBP 3226 (1 μM). It is concluded that 1) exogenous NPY increases excitatory nerve-induced contraction mainly via a Y1 receptor-mediated effect on smooth muscle with a small non-Y1 receptor component due to blocking inhibitory nitrergic nerves and 2) endogenous NPY is a modulatory cotransmitter, which facilitates the primarily noradrenergic contractile responses to sympathetic nerve stimulation via smooth muscle Y1 receptors.

Neuropeptide Y (NPY) is a 36-amino-acid peptide (Tatemoto et al., 1982) that is widely distributed in the central and peripheral nervous systems (Dumont et al., 1991; Morris and Gibbins, 1992). It is released from postganglionic sympathetic nerve terminals in combination with norepinephrine (Lundberg et al., 1990). Because NPY produces little or no contractile responses in smooth muscle preparations but can enhance contractile responses elicited by agonists or nerve stimulation (Edvinsson et al., 1984; Lopez et al., 1989) and can inhibit catecholamine release (Pernow et al., 1986; Grundemar et al., 1992), it is regarded as a modulator of sympathetic transmission and could play this role when coreleased with norepinephrine (Burnstock and Ralevic, 1996). However, there is a lack of conclusive evidence of cotransmission (Lundberg, 1996).

Rat anococcygeus muscle is a smooth muscle preparation with a dense intramural plexus of sympathetic nerve terminals that produces α-adrenergic-mediated contraction (Gillespie, 1980) and a nonadrenergic, noncholinergic (NANC), putatively nitrergic, innervation (Gillespie et al., 1989; Liu et al., 1991), whose activation produces relaxation if the sympathetic nerves are blocked. Vila et al. (1992) reported that in rat anococcygeus muscle NPY could increase nerve-induced contraction but not norepinephrine-induced contraction, without itself producing contraction or altering transmitter release. This paradoxical preferential potentiation of nerve-mediated rather than norepinephrine-mediated contraction by NPY was explained, at least in part, by inhibition of the NANC nerve-induced relaxation. This provided no support for a cotransmitter role for NPY in this preparation.

Since then, two developments encouraged us to revisit the role of NPY in the rat anococcygeus muscle: 1) the observation by Iravani and Zar (1997) that NPY can increase contraction to norepinephrine in rat anococcygeus muscle when, in contrast to the study of Vila et al. (1992), blockers of the neuronal and extraneuronal uptake of catecholamines were absent and 2) the characterization of NPY receptor subtypes and the availability of pharmacological tools for their study.

At present, six NPY receptors (Y1–Y6) have been described. Y1 and Y2 receptors have been best characterized (Michel, 1991; Gehlert, 1994; Grundemar and Hakanson, 1994; Michel et al., 1998). Both Y1 and Y2 receptors have been implicated in postjunctionally procontractile effects and inhibition of transmitter release (Wahlestedt et al., 1986; Grundemar et al., 1992; McAuley and Westfall, 1992). The

**ABBREVIATIONS:** NPY, neuropeptide Y; NANC, nonadrenergic, noncholinergic; TTX, tetrodotoxin.

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physiological effects of NPY on Y1 and Y2 receptors can be distinguished by using truncated neuropeptide agonists (Wahlestedt et al., 1990; Michel, 1991). The Y1 receptor possesses a high affinity for full-length NPY (Fuhlenhoff et al., 1990), and the Y2 receptor exhibits a high affinity for short [e.g., NPY (13–36)] fragments (Wieland et al., 1995a). Furthermore, a nonpeptide ligand BIBP 3226 [R]-N\(^2\)-diphenylacetyl)-N\(^-\)[(4-hydroxyphenyl)methyl]arginanimide] has been developed (Rudolf et al., 1994) and described as a selective and potent antagonist of Y1 receptors (Doods et al., 1996).

The aim of this study was to clarify whether endogenous NPY can increase adrenergic nerve responses, to characterize the subtype of NPY receptor involved, and to establish whether endogenous NPY released with norepinephrine plays a role in excitatory transmission.

**Experimental Procedures**

Male Sprague-Dawley rats (300–350 g) were sacrificed by decapitation. The anococcygeus muscle was dissected as described by Gillespie (1972) and set up in 20-ml organ baths containing physiological salt solution composed of 112.0 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\(_2\), 1.1 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 25.0 mM NaHCO\(_3\), and 11.1 mM glucose maintained at 37°C and continuously gassed with 95% O\(_2\), 5% CO\(_2\). A resting tension of 4.90 mN was placed on the tissue, and changes in tension were recorded with a PIONE (UF-1; Pionen Controls, Kent, UK) isometric transducer attached to an Omniscrieber pen recorder (Allen Datagraph, Salem, NH). The preparations were left to equilibrate for 45 min with frequent washes. Tension was readjusted if necessary. After equilibration, the preparation was contracted three or four times with KCl (60 mM) every 5 min until the contractile responses were similar. The tissue was then washed, and when the initial tension was achieved, it was left to equilibrate for an additional 30-min period before starting the experiments.

**Responses to Norepinephrine.** The effects of NPY alone and in the presence of BIBP 3226 were assessed on norepinephrine-induced contraction in the absence of neuronal and extraneuronal uptake blockers. In preliminary experiments, we found that the maximum contraction in the second concentration-response curve to norepinephrine was slightly smaller than in the first. Therefore, second curves were compared throughout. Two cumulative concentration-response curves to norepinephrine (0.001–30 μM) were constructed in each of three sets of preparations. In the first set, two control concentration-response curves were constructed. In the other two sets, before the second concentration-response curve, in either the absence or the presence of 1 μM BIBP 3226 (30 min), NPY (0.01 or 0.1 mM) was applied for 7 min. The effects of NPY in the presence or absence of BIBP 3226 were then assessed against the control experiments.

**Effects of Agonists and Antagonists on Contractile Responses to Electrical Field Stimulation.** Three sets of experiments were performed. In a first set, three control frequency-response curves in the presence of 0.1% BSA (the vehicle used to dissolve NPY) were carried out as time controls. In the second and third sets, after a control frequency-response curve, paired anococcygeus muscles were incubated with 0.01 and 0.1 μM NPY or NPY (13–36) for 7 min before a further frequency-response curve with or without a 30-min preincubation with BIBP 3226 (1 μM).

**Effects of NPY and BIBP 3226 on NANC Relaxations to Electrical Field Stimulation.** We assessed whether BIBP 3226 could influence the previously observed inhibition of NANC responses by NPY (Vila et al., 1992). NANC-induced relaxation to field stimulation was obtained after raising muscle tone and eliminating the sympathetic nerve-induced contraction. The sympathetic drugs phenolamine (1 μM) and guanethidine (30 μM) were added for 40 and 30 min, respectively; then, tone was raised to a submaximal plateau by carbachol (50 μM). Two frequency-response curves separated by a 30-min period were then constructed. In control experiments, inhibitory responses were greater in the second frequency-response curve compared with the first curve. Thus, comparisons were made between second curves. NPY (0.1 μM, 7 min) was added before the second frequency-response curve in either the absence or the presence of BIBP 3226 (1 μM, 30 min). Results obtained in the second frequency-response curve in the presence of the vehicle, NPY, or NPY in the presence of BIBP 3226 were compared. Field stimulation was applied at supramaximal voltage, 1-ms duration at 0.25 to 5 Hz applied for 10 s every 3 min, according to the protocol established by Gillespie (1972). Under these conditions, the inhibitory responses were abolished by TTX (1 μM) as previously reported (Gillespie, 1972).

**Effects of BIBP 3226 on Norepinephrine and Adrenergic and NANC Relaxations to Electrical Field Stimulation.** After a control concentration-response curve, increasing concentrations of BIBP 3226 (1–10 μM) were applied for 30 min before a second concentration-response or frequency-response curve, in either the absence or the presence of BIBP 3226 (1 μM, 30 min). Results obtained in the second frequency-response curve in the presence of the vehicle, NPY, or NPY in the presence of BIBP 3226 were compared. Field stimulation was applied at supramaximal voltage, 1-ms duration at 0.25 to 5 Hz applied for 10 s every 3 min, according to the protocol established by Gillespie (1972). Under these conditions, the inhibitory responses were abolished by TTX (1 μM) as previously reported (Gillespie, 1972).
Each individual data set for the concentration-response relationship to norepinephrine was fitted to a logistic function of the form:

\[ E = \frac{\alpha \cdot [A]^n}{[EC_{50}]^n + [A]^n} \]

where \( E \) and \([A]\) are the pharmacological effect and the concentration of agonist, respectively, and \( \alpha, EC_{50}, \) and \( n \) are the asymptote, location, and slope parameters, respectively. Location parameters were estimated as \( pEC_{50} \) (the negative logarithm of the concentration required to cause 50% of the maximum response).

The statistical significance for the estimated parameters (\( pEC_{50}, \alpha \)) was assessed by the two-tailed Student’s \( t \) test for paired or unpaired observations as appropriate. The dependence of contractile response on treatment and concentration/frequency was studied by a two-way ANOVA within the framework of the general linear model approach (Littell et al., 1991). Planned contrasts were used to test for differences among the levels of treatment factor at selected frequencies or concentrations (vertical pairwise comparisons). In all cases, significance was set at \( P < .05 \). Statistical analyses were carried out with the SAS/STAT package (SAS Institute Inc., Cary, NC).

**Materials.** Porcine NPY and BIBP 3226 were a gift from Dr. Karl Thomae GmbH (Biberach, Germany). NPY (13–36) was purchased from Calbiochem (England). (−)-Norepinephrine bitartrate, phenylephrine, desipramine HCl, normetanephrine HCl, and BSA were purchased from Sigma Chemical Co. (St. Louis, MO). Phenoxybenzamine HCl was obtained from Research Biochemicals International (Natick, MA). Stock solutions of 0.1 mM NPY and NPY (13–36) were dissolved in BSA (0.1%) and 0.4 mM BIBP 3226 in distilled water, aliquoted, and stored at −70°C in silicon-coated tubes. This procedure prevents the adsorption of the peptide to the plastic and glass tubes. Stock phenoxybenzamine solution (1 mM) was dissolved in 2 mM tartaric acid and kept at −20°C. Further dilutions of phenoxybenzamine were prepared in physiological salt solution. Norepinephrine was prepared daily in 23 mM Na₂EDTA. All chemicals used were of analytical grade.

**Results**

In the absence of uptake blockers, norepinephrine contracted the anococcygeus muscle in a concentration-related manner (Fig. 1). NPY (0.01 or 0.1 \( \mu M \)) caused no contraction. However, NPY (0.01 \( \mu M \)) significantly (\( P < .05 \)) increased the responses to norepinephrine in the range of 0.1 to 3 \( \mu M \) (Fig. 1a). This effect was fully reversed by 1 \( \mu M \) BIBP 3226. A higher concentration of NPY (0.1 \( \mu M \); Fig. 1b) clearly enhanced (\( P < .05–P < .001 \)) responses to all submaximal concentrations of norepinephrine. BIBP 3226 prevented this increase of norepinephrine-induced contractions. In the presence of neuronal and extraneuronal uptake blockers and after exposure to phenoxybenzamine, the norepinephrine concentration-response curve (\( pEC_{50} = 5.25 \pm 0.01, n = 6 \)) was shifted to the left (\( pEC_{50} = 5.64 \pm 0.08, n = 6; P < .001 \)) by NPY (0.1 \( \mu M \)) (Fig. 2a). This effect was prevented by BIBP 3226 (1 \( \mu M \); Fig. 2b).

Electrical field stimulation induced reproducible, frequency-dependent contraction of rat anococcygeus muscle (Fig. 3a). NPY (0.01 or 0.1 \( \mu M \)) significantly increased, in a concentration-dependent manner, the responses induced at all frequencies of electrical field stimulation (Fig. 3b). BIBP 3226 (1 \( \mu M \)) partly reversed the increase by NPY of electrical field stimulation-induced responses at all frequencies used (Fig. 3c). To evaluate whether Y2 receptors could contribute to the enhancement by NPY of responses induced by electrical field stimulation, the effect of the Y2 receptor agonist NPY (13–36) (0.01 \( \mu M \)) did not modify the frequency-response curve (Fig. 4a). However, a higher concentration (0.1 \( \mu M \)) produced small but significant increases (Fig. 4a) at all frequencies except 0.25 Hz. BIBP 3226 (1 \( \mu M \)) completely blocked this increase (Fig. 4b).

Electrical field stimulation in the presence of sympathetic nerve blockade elicited the known frequency-related NANC relaxation (Fig. 5). NPY (0.1 \( \mu M \)) significantly reduced the NANC responses at 0.25 and 0.5 Hz. BIBP 3226 (1 \( \mu M \)) had no effect on the inhibition by NPY of NANC-induced relaxation.

The effects of increasing concentrations of BIBP 3226 on electrical field stimulation-induced (Fig. 6a) and norepinephrine-induced (Fig. 6b) contractions were studied to evaluate the participation of endogenous NPY in the nerve-induced responses. BIBP 3226 (1 \( \mu M \)) decreased responses at 1 and 5 Hz.
Hz. Higher concentrations diminished responses at all frequencies of stimulation except at the lowest frequency, which was reduced only by the highest concentration of BIBP 3226 (Fig. 6a). BIBP 3226 had no effect on norepinephrine-induced contraction at 1 or 3 μM (results not shown) or at 10 μM (Fig. 6b). BIBP 3226 at 1 to 10 μM had no effect on NANC-induced relaxation (results not shown).

Discussion

This study shows that exogenous NPY increases contractile responses to stimulation of sympathetic, adrenergic nerves via postjunctional Y1 receptors. The participation of endogenous NPY via this mechanism, as a facilitatory, excitatory cotransmitter, is demonstrated.

NPY produced a concentration-dependent increase of the contractile responses to norepinephrine. This was clear cut at 0.1 μM NPY, and the threshold occurred at around 0.01 μM NPY, where there was a significant increase in individual norepinephrine concentrations. On its own, this suggests a postjunctional synergistic action that facilitates the contractile effect of norepinephrine. We tested and excluded an indirect origin for this effect through blockade of cellular uptake of norepinephrine by showing that NPY could still
cause an increase in the presence of neuronal and extraneuronal norepinephrine uptake blockers. This confirms an action of NPY to enhance the postjunctional effect of norepinephrine. This facilitatory action of NPY was completely blocked by 1 μM BIBP 3226. This was a selective action against NPY because the contraction to norepinephrine per se was unaffected by BIBP 3226. The affinity of BIBP 3226 for Y2 receptors (K_i = 10^{-6.8} \text{nM}) is 1000 times less than that for Y1 receptors (K_i \approx 10^{-7.9} \text{nM}) in rat (Wieland et al., 1995b). Thus, blockade by 1 μM BIBP 3226 of facilitation by NPY of the action of norepinephrine indicates an interaction at Y1 receptors.

The interpretation of the action of NPY on the contractile responses to nerve stimulation is now straightforward. The adrenergic component of the response to sympathetic nerve activation is enhanced by a Y1 receptor-mediated action, such as potentiation of the responses to norepinephrine. In addition, the inhibition of the NANC nerve-mediated relaxation response by a non-Y1 receptor-mediated mechanism contributes to NPY enhancement of the contractile response. This component of the NPY effect explains why BIBP 3226 can completely block NPY enhancement of noradrenergic contractile responses but incompletely blocks its enhancement of contraction to nerve stimulation.

A further question is whether the non-Y1 receptor-mediated effect of NPY on nerve stimulation might be mediated by the Y2 receptors. Evidence against this was that NPY (13–36) inhibited the contractile response to nerve stimulation.
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36) produced no BIBP 3226-resistant facilitatory action against the nerve responses. Given the greater affinity and efficacy of this peptide for Y2 than for Y1 receptors (Wieland et al., 1995b), this can be taken as clear evidence for the lack of a Y2 receptor capable of facilitatory action in this tissue. It follows from this interpretation that in contrast to some other tissues, there is no evidence for a prejunctional Y2 receptor (Wahlestedt et al., 1986) or Y1 receptor (McAuley and Westfall, 1992).

Taken together, these results provide positive evidence for a functional postjunctional Y1 receptor that facilitates nor- epinephrine responses or adrenergic transmission and for a non-Y1 action of NPY that is inhibitory to NANC transmis- sion. Its non-Y1 site of action (prejunctional or postjunc- tional) cannot be conclusively identified. However, because relaxation to sodium nitroprusside is attenuated by NPY (Vila et al., 1992), at least part of this action seems to be postjunctional. These data require us to modify the conclusions from our earlier study of NPY in rat anococcygeus muscle. We missed the facilitatory effect of NPY on norpi- nphrine contraction, which was subsequently shown by Ira- vani and Zar, (1997), leading us to argue against a postjunc- tional modulatory effect of NPY (Vila et al., 1992). The present results show that NPY does increase contraction to exogenous norepinephrine but that this effect is not seen if the norepinephrine neuronal and extraneuronal uptake mechanisms are blocked. This raised the question of whether the absence of the effect of NPY in the presence of uptake blockers is because it blocks norepinephrine uptake. This possibility was eliminated by demonstrating that after phe- noxybenzamine, NPY still caused enhancement of norepi- nphrine-induced contractions when uptake blockers were present. This allows us to conclude that NPY can enhance the response to exogenous norepinephrine by a direct postjunc- tional action on smooth muscle.

The final important question raised by our observations is whether NPY is a functional excitatory cotransmitter. NPY- immunoreactive nerve fibers are present in rat anococcygeus muscle (Iravani and Zar, 1997). Thus, if NPY is present in the same nerves as norepinephrine, as in other tissues, and is coreleased with norepinephrine, NPY would be expected to facilitate transmission by the Y1 receptor-mediated action that we have demonstrated.

BIBP 3226 produced a concentration-related inhibition of electrical field stimulation-induced contraction. Even the highest concentration of BIBP 3226 had no effect on exogenous norepinephrine. This suggests that BIBP 3226 was acting via blockade of the effects of endogenous NPY core- leased with norepinephrine. Excitatory transmission in rat anococcygeus muscle can be entirely blocked by α-adrenocep- tor antagonists (Gillespie and McGrath, 1974; Docherty and Starke, 1981) in contrast to adrenergic/purinergic cotrans- mission, where blockade of responses to either norepineph- rine or ATP leaves the response to the other transmitter intact (Blakeley et al., 1981; Sneddon and Westfall, 1984; Bulloch and McGrath, 1988). NPY may, therefore, be more accurately described as a modulatory cotransmitter.

In conclusion, in rat anococcygeus muscle, exogenous NPY can act through Y1 receptors on smooth muscle to increase the contractile effect of norepinephrine. Endogenous NPY coreleased with norepinephrine enhances α-adrenergic transmission through this Y1 receptor. This tissue provides a model system for the further study of the cotransmitter role of NPY, and the approach that was necessary to illustrate this may prove fruitful in other tissues.

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References


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