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Neuropeptide Y neuromodulation of sympathetic co-transmission in the guinea-pig vas deferens

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1 We examined the neuromodulatory effects of neuropeptide Y (NPY) on purinergic and adrenergic co-transmission in the guinea-pig vas deferens.

2 In superfused vas deferens preparations, NPY (0.3 μM) inhibited the stimulus-evoked overflow of both ATP and [^{3}H]-noradrenaline ([^{3}H]-NA) at 2 Hz, but only the stimulus-evoked release of [^{3}H]-NA at 20 Hz.

3 Postjunctionally, NPY greatly enhanced responses to α,β-methylene ATP and to a lesser extent to exogenous NA.

4 Preparations stimulated in organ baths showed frequency-dependent contractions to field stimulation. NPY abolished responses to field stimulation at low frequency and a small number of pulses. At high frequency (20 Hz), NPY abolished responses elicited by 10 pulses, inhibited responses by 50% at 20 pulses and had little effect on preparations stimulated for 240 pulses.

5 Our study suggests that NPY neuromodulates co-transmission in the vas deferens by inhibiting the release of ATP and NA and that these effects predominate over the postjunctional enhancement by NPY. These results also show that the physiological effect of NPY will be determined both by the frequency at which the nerves are discharging and the duration of their firing.

Introduction

Neuropeptide Y (NPY), a 36 amino acid peptide originally isolated from porcine brain (Tatemoto *et al.*, 1982), has subsequently been shown to co-exist with noradrenaline (NA) and adenosine 5'-triphosphate (ATP) in sympathetic nerves in several tissues including the vas deferens (Lagercrantz, 1976; Fried *et al.*, 1978; 1985; Stjärne *et al.*, 1986). Recently, NPY has been shown to be concomitantly released along with the co-transmitters ATP and NA in the guinea-pig vas deferens (Kasakov *et al.*, 1988).

ATP and NA have been shown to be co-transmitters in the vas deferens. Upon release they produce a biphasic contraction, with ATP mediating the fast twitch phase and NA the sustained second contraction (Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984; Stjärne & Åstrand, 1985). NPY upon release does not produce a contraction, but rather it acts prejunctionally to inhibit NA release, whereas postjunctionally, NPY modulates the action of NA and ATP by enhancing their action on their receptors (Lundberg & Stjärne, 1984; Lundberg *et al.*, 1984; Stjärne & Lundberg, 1986; Stjärne *et al.*, 1986). In this respect, NPY acts as a neuromodulator rather than a transmitter in the vas deferens. This appears to be the case too in many blood vessels, although NPY has been claimed to be a transmitter in some (Lundberg & Tatemoto, 1982; Fried *et al.*, 1986; Edvinsson *et al.*, 1987).

The aim of this study was to examine pre- and postjunctional effects of NPY on both purinergic and adrenergic transmission. As the pre- and postjunctional effects of NPY are antagonistic, it was also hoped to examine which action of NPY predominated at various stimulation parameters in order to ascertain possible physiological roles for NPY.

Methods

Superfusion studies

Male guinea-pigs (250–400 g) were stunned and exsanguinated. The prostatic portions of the vasa deferentia were excised and stripped of connective tissue. Following incubation (60 min) with [^{3}H]-NA, the isolated preparations (10–20 mg wet weight) were placed between pairs of platinum ring electrodes (15 mm apart) and were allowed to equilibrate, with one end tied to the holder and the other attached to a Grass force-displacement transducer (FT03 C). Contractions were measured as changes in isometric tension by a Washington data recording system. The preparations, at an initial tension of 0.5–1 g, were superfused by a Watson-Marlow peristaltic pump at a flow rate of 2.5 ml min^{-1} with a modified Krebs solution (MKS) (mM: NaCl 133, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 2.5, NaH_2PO_4 1.4, $NaHCO_3$ 16.3, glucose 7.7 and ascorbic acid 0.1 and gassed continuously with 5% CO_2 in O_2 at 36°C). After a 90 min equilibration period the superfusate was collected before, during and after stimulation in polypropylene vials for subsequent determination of [^{3}H]-NA and ATP levels. The preparations were stimulated electrically twice, at 9 and 45 min with trains of 240 rectangular pulses of 0.1 ms duration, 50 V, and either at 2 or 20 Hz, delivered by Grass SD9 stimulators. Perfusate was collected for periods corresponding to the length of the stimulus train which was 120 s at 2 Hz and 12 s at 20 Hz. Results are expressed as the ratio of responses to the second stimulation (S_2) versus responses to the first stimulation (S_1), that is, the S_2/S_1 ratio. Control values for S_2/S_1 ratios were obtained when the second stimulation was carried out in the absence of any drug. Experimental data were obtained by superfusion of the preparation with MKS containing NPY, 6 min before the second stimulation. This superfusion continued until the end of the experiment. Tissues from different animals, but from the same batch, were used to assess the effects of NPY.

ATP overflow

ATP overflow from the sympathetic nerves supplying the vas deferens was assayed by the luciferin-luciferase technique described by Stanley & Williams (1969). Briefly, 50 mg of luciferin-luciferase was dissolved in 12.5 ml of sterile water and left in the dark for 2 h to equilibrate. Under these conditions the assay was capable of measuring ATP levels as low as 30 fmol. Before the experiment, a calibration curve for ATP was prepared to be used as a standard. Automatic injection of 200 μl of luciferin-luciferase solution to 100 μl of superfusate or

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standards produced bioluminescence which was measured by a Packard Picolite Luminometer. The MKS was assayed for ATP content. This was used as a blank and subtracted from each sample.

[^3H]-NA overflow

After preparation (described above), the tissues were incubated for 60 min in continuously gassed MKS containing 0.1 nM tritium-labelled noradrenaline ([^{3}H]-NA) (two preparations in 5 ml medium) at 36°C. After being rinsed five times with MKS, the preparations were placed between the stimulating electrodes and superfused to wash out the excess [^{3}H]-NA during the 90 min equilibration period. Samples (2.5 ml for 2 Hz and 0.25 ml for 20 Hz) were added in a ratio of 1:3 to Optiphase mp (LKB) scintillation cocktail and ^{3}H-radioactivity in the samples was counted in a Beckman 7500 liquid scintillation system. The superfusion was stopped immediately after the end of the experiment and the preparations weighed and dissolved in 0.5 ml Optisolv (LKB) tissue solubilizer. The total tissue [^{3}H]-NA radioactivity was counted as described above. The results were calculated as the percentage fractional rate (FR %; mean ± s.e.mean) of the basal release and stimulation-evoked release of [^{3}H]-NA, which were calculated according to Alberts *et al.* (1981). Previous investigations in rodent vas deferens have shown that [^{3}H]-NA is the major constituent of the total radioactivity released upon stimulation of the tissue (Beattie *et al.*, 1986; Kapocsi *et al.*, 1987). Hence, the evoked efflux of [^{3}H]-NA reflected a quasi-physiological release of NA (Reichenbacher *et al.*, 1982).

Pharmacological study in the organ bath

Prostatic portions of the vas deferens were tied to tissue holders and placed in 5 ml organ baths containing MKS and equilibrated for 90 min.

Responses to α,β-methylene ATP and NA After the equilibration period either NA or α,β-methylene ATP (α,β-mATP) at bath concentrations of 30 μM and 1 μM respectively were added for short periods every 15 min. The tissues were washed as soon as a maximum response was reached to prevent desensitization. α,β-mATP was used as it is a stable analogue of ATP. After the control responses to agonists had been established, NPY was added 6 min before a further addition of NA or α,β-mATP. Results were calculated as the percentage response to each agonist relative to the previous response when no drug was present.

Responses to field stimulation After the equilibration period the preparations were electrically stimulated at 50 V, 0.1 ms at varying frequencies and duration of stimulation. The effects of NPY were tested in four different sets of experiments: (1) the frequency of stimulation was varied from 4 to 20 Hz and the number of pulses kept constant at 20. Concentrations of NPY used varied from 0.3 nM to 0.3 μM; (2) the frequency was kept constant at 20 Hz and the number of pulses altered from 10 to 240. Concentration of NPY used was 0.3 μM; (3) the frequency was kept constant at 4 Hz and the number of pulses altered from 10 to 240. Concentration of NPY used was 0.3 μM; (4) the tissues were stimulated every 30 s, 20 pulses at either 4 or 20 Hz. The NPY (0.3 μM) was added between stimulation trains immediately before the next stimulation train.

Analysis of results

The effects of NPY were assessed by the comparison of S_2/S_1 ratios for the experimental data versus S_2/S_1 ratios for the control data. The results for ATP overflow, [^{3}H]-NA overflow and mechanical responses were evaluated by Student's *t* test for unpaired observations. Results from the pharmacological study were evaluated by Student's *t* test for paired observations. In both cases, a probability less than 0.05 was considered significant.

Drugs

Neuropeptide Y (NPY), (−)-noradrenaline HCl (NA), α,β-methylene ATP Li (α,β-mATP) and luciferin-luciferase were obtained from Sigma. Tritiated (−)-noradrenaline [^{3}H]-NA (specific activity 1624.3 Gbq mmol^{-1}) was obtained from New England Nuclear. NPY and α,β-mATP were first prepared as stock solutions in distilled water. NA was prepared in a 0.4% ascorbic acid solution to prevent oxidation. Luciferin-luciferase was prepared in sterile water.

Results

Field stimulation on superfused preparations

Control responses The contractile response of the vas deferens to electrical field stimulation (EFS) at 2 Hz (stimulation period lasting 2 min) exhibited two distinct phases: an initial twitch response, which reached its maximum in 2–3 s and then faded rapidly, and a second slow phase which reached its maximum in 10–15 s and whose amplitude slowly decreased, but which lasted for the duration of the stimulation period. The second stimulation period, 30 min later, exhibited similar contractions but both phases were slightly reduced in amplitude. However, this reduction was not significant (S_2/S_1 ratio for the first phase was 0.81 ± 0.09, and for the second phase was 0.84 ± 0.06, $n = 6$) (Figure 1). During both stimulation periods there was a marked rise in [^{3}H]-NA and ATP overflow over basal levels. [^{3}H]-NA overflow differed little between the two stimulation periods (S_2/S_1 ratio was 1.04 ± 0.04, $n = 8$) (Figure 2). However, ATP overflow during the second phase was reduced (S_2/S_1 ratio was 0.51 ± 0.04, $n = 11$) (Figure 3), indicating that the amount of ATP released during the first period of stimulation is far greater than that actually required to elicit a contraction.

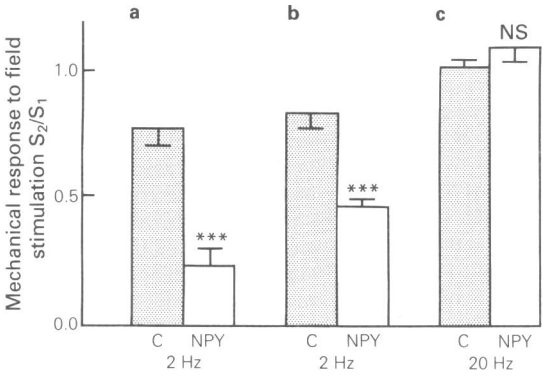

Figure 1 Effects of neuropeptide Y (NPY) on the mechanical response of the guinea-pig superfused vas deferens to electrical field stimulation. Alterations in the mechanical responses are shown by comparing the control S_2/S_1 ratio when no drug was present throughout the experiment with the S_2/S_1 ratio obtained when the drug was added to the superfusate 6 min before the second stimulation. (a) The purinergic response at 2 Hz in the presence of NPY (0.3 μM) compared with control (C, stippled columns). (b) The adrenergic response at 2 Hz in the presence of NPY (0.3 μM) compared with control. (c) The total response at 20 Hz in the presence of NPY (0.3 μM) compared with control. Each column is the mean of at least 6 observations and vertical lines indicate s.e.mean. Significance of drug effects was determined by Student's *t* test for unpaired observations (NS = not significant, *** $P < 0.001$).

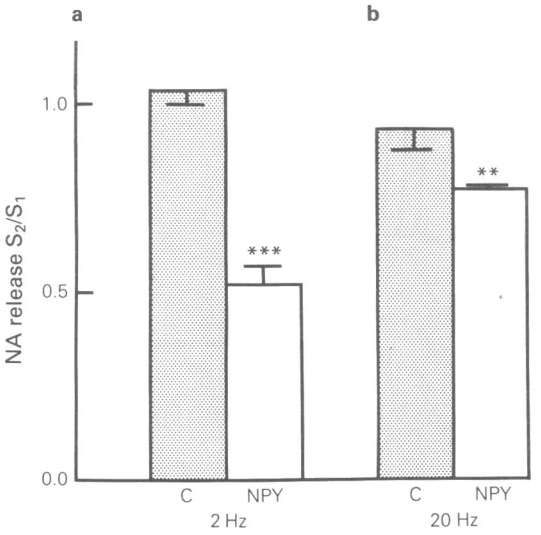

Figure 2 Effects of neuropeptide Y (NPY) on the overflow of [^{3}H]-noradrenaline ([^{3}H]-NA) from the superfused guinea-pig vas deferens induced by electrical field stimulation. Alterations in the overflow of [^{3}H]-NA are shown by comparing the control S_2/S_1 ratio when no drug was present throughout the experiment with the S_2/S_1 ratio obtained when the drug was added to the superfusate 6 min before the second stimulation. (a) Overflow at 2 Hz in the presence of NPY (0.3 μM) compared with control (C, stippled columns). (b) Overflow at 20 Hz in the presence of NPY (0.3 μM) compared with control. Each column is the mean of at least 6 observations and vertical lines indicate s.e.mean. Significance of drug effects was determined by Student's t test for unpaired observations (** $P < 0.01$, *** $P < 0.001$).

At 20 Hz (stimulation period lasting 12 s) the distinction between the two phases was not clear, therefore mechanical responses are given as the overall magnitude of the response and not divided into two distinct phases. The magnitude of the response elicited by the second stimulation was unchanged (S_2/S_1 ratio was 1.02 ± 0.03, $n = 6$) (Figure 1). Again, during both stimulation periods, there was a marked rise in [^{3}H]-NA and ATP overflow over basal levels. [^{3}H]-NA overflow elicited by stimulation was unchanged during the second stimulation (S_2/S_1 ratio was 0.93 ± 0.05, $n = 9$) (Figure 2). ATP overflow was only slightly diminished during the second stimulation (S_2/S_1 ratio was 0.78 ± 0.09, $n = 11$) (Figure 3).

Neuropeptide Y The addition of NPY (0.3 μM) to the superfusate 6 min before the second stimulation had no effect on the baseline tension of the preparations. At 2 Hz NPY inhibited both the purinergic and adrenergic phases of the response. The S_2/S_1 ratio for the purinergic phase of the response was 0.24 ± 0.07 ($n = 6$) (Figure 1) and for the adrenergic phase it was 0.51 ± 0.05 ($n = 10$) (Figure 1). At this frequency NPY inhibited the stimulus-evoked overflow of both [^{3}H]-NA (S_2/S_1 ratio was 0.40 ± 0.09, $n = 10$) (Figure 2), and ATP (S_2/S_1 ratio was 0.28 ± 0.07, $n = 6$) (Figure 3). At 20 Hz NPY had no effect on the overall magnitude of the response (S_2/S_1 was 1.10 ± 0.06, $n = 8$) (Figure 1). At this frequency NPY significantly inhibited the overflow of [^{3}H]-NA (S_2/S_1 ratio was 0.77 ± 0.01, $n = 8$) (Figure 2). However, it was without significant effect on ATP overflow (S_2/S_1 ratio was 0.72 ± 0.08, $n = 6$) (Figure 3).

Responses in the organ bath

Responses to exogenously added NA (30 μM) and α,β-mATP (1 μM) did not show tachyphylaxis, provided that once the tissues had reached their maximum response they were washed immediately. NPY (0.3 μM) added 6 min before the addition of NA produced a significant increase in the response to a value $109 \pm 1\%$ ($n = 6$) (Figure 4) of the control level. NPY produced a larger increase in the response to α,β-mATP to $153 \pm 11\%$ of the control value ($n = 6$) (Figure 4).

The ability of NPY to inhibit responses to electrical field

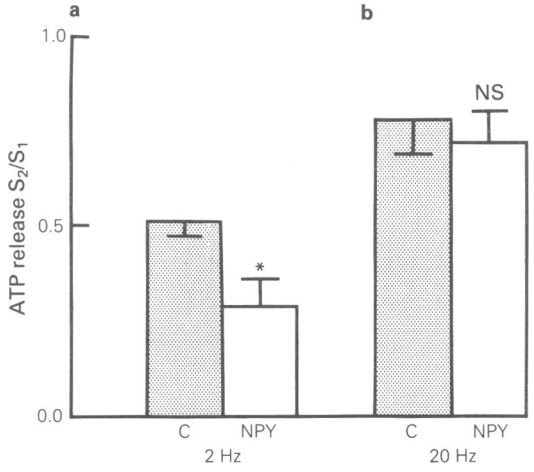

Figure 3 Effects of neuropeptide Y (NPY) on the overflow of ATP from the guinea-pig superfused vas deferens induced by electrical field stimulation. Alterations in the overflow of ATP are shown by comparing the control S_2/S_1 ratio when no drug was present throughout the experiment with the S_2/S_1 ratio obtained when the drug was added to the superfusate 6 min prior to the second stimulation. (a) Overflow at 2 Hz in the presence of NPY (0.3 μM) compared with control (C, stippled columns). (b) Overflow at 20 Hz in the presence of NPY (0.3 μM) compared with control. Each column is the mean of at least 6 observations and vertical lines indicate s.e.mean. Significance of drug effects was determined by Student's t test for unpaired observations (NS = not significant, * $P < 0.05$).

stimulation (EFS), 4, 10 and 20 Hz for 20 pulses, was tested over a range of concentrations (0.3 nM–0.3 μM). In six different tissues NPY, from a threshold concentration of 3 nM, inhibited responses to EFS in a concentration-dependent manner (Table 1). Thus, NPY (0.3 μM) added 6 min before the

Table 1 % inhibition produced by neuropeptide Y (NPY) of responses to electrical field stimulation at various frequencies (0.1 ms, 50 V, 20 pulses)

	NPY concentration			
Frequency	0.3 nM	3 nM	30 nM	0.3 μM
4	17 ± 5	22 ± 2	78 ± 11	100 ± 0
10	5 ± 3	12 ± 4	38 ± 7	51 ± 11
20	5 ± 3	8 ± 3	31 ± 5	35 ± 5

Values are mean $\pm$ s.e.mean, $n = 4$.

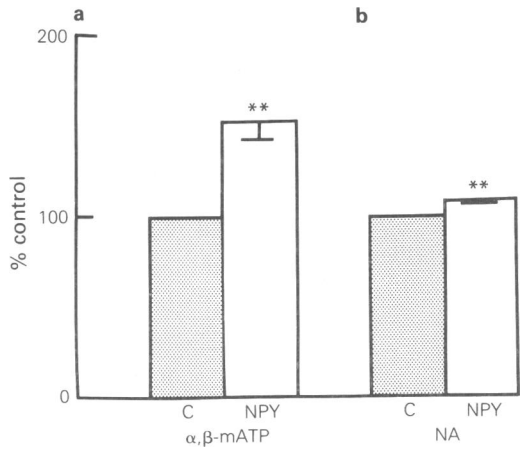

Figure 4 Effects of neuropeptide Y (NPY) on the response of guinea-pig isolated vas deferens to noradrenaline (NA) and exogenous α,β-methylene ATP (α,β-mATP). Responses to drugs are compared with the control value (C, stippled columns). (a) Responses to α,β-mATP in the presence of NPY (0.3 μM). (b) Responses to NA in the presence of NPY (0.3 μM). Each column is the mean of 6 observations and vertical lines indicate s.e.mean. Significance of drug effects was determined by Student's t test for paired observations (** $P < 0.01$).

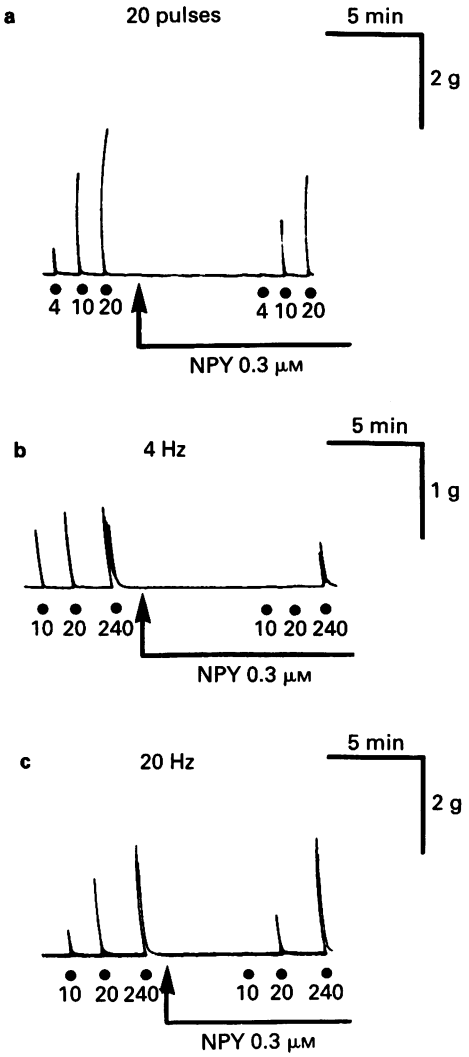

Figure 5 Effects of neuropeptide Y (NPY, 0.3 μM) on responses to electrical field stimulation (EFS) on isolated vas deferens in the organ bath. (a) Effect of NPY on responses with constant pulse number and with the frequency varying from 4 to 20 Hz. (b) Effect of NPY on responses at constant frequency (4 Hz) and the number of pulses varying from 10 to 240. (c) Effect of NPY on responses at constant frequency (20 Hz) and the number of pulses varying from 10 to 240.

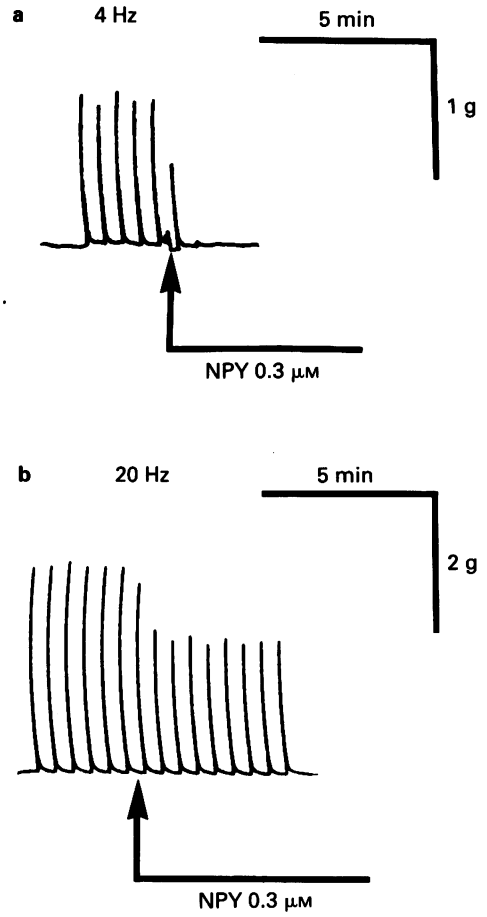

Figure 6 Effects of neuropeptide Y (NPY, 0.3 μM) on responses to continuously stimulated isolated vas deferens in the organ bath. (a) Effects of NPY added between stimulation trains at 4 Hz, 20 pulses. (b) Effects of NPY added between stimulation trains at 20 Hz, 20 pulses.

frequency-response curve, obtained by use of 20 pulses, abolished responses to 4 Hz, but inhibited responses at 10 and 20 Hz (Figure 5). NPY (0.3 μM) abolished responses to 4 Hz, using 10 and 20 pulses, and inhibited responses to 50% of control at this frequency for 240 pulses (Figure 5, Table 2). Against stimulation at 20 Hz, NPY abolished responses to 10 pulses, inhibited responses to 20 pulses to about 50% of the control level and slightly enhanced responses to stimulation for 240 pulses (Figure 5, Table 2).

Table 2 % inhibition produced by neuropeptide Y (NPY, 0.3 μM) of responses to electrical field stimulation (4, 20 Hz, 0.1 ms, 50 V, 10–240 pulses)

	Number of pulses		
Frequency	10	20	240
4	100 ± 0	100 ± 0	48 ± 6
20	100 ± 0	51 ± 5	−5 ± 2

Values are mean ± s.e.mean, $n = 4$.

The effects of NPY (0.3 μM) on six tissues stimulated every 30 s, 20 pulses at either 4 or 20 Hz, was tested. The NPY was added immediately before the next stimulation train. By employment of these parameters, NPY was shown to inhibit responses to EFS immediately. After 60 s, responses to 4 Hz were abolished, whereas at 20 Hz responses were inhibited to about 65% of the control level (Figure 6).

Discussion

This study presents direct evidence that NPY is capable of modulating purinergic and adrenergic transmission both pre- and postjunctionally in the guinea-pig vas deferens. This is in agreement with an earlier study by Lundberg *et al.* (1984) in the guinea-pig, which also demonstrated that NPY inhibited adrenergic and purinergic transmission in the vas deferens by a prejunctional action.

There has been much discussion on the possible physiological roles of NPY in sympathetic transmission. One popular hypothesis, especially for blood vessels, is that NPY promotes economy of the motor transmitters ATP and NA. This is achieved in that the initial effect of NPY has been suggested to be an enhancement of the responses of the transmitters via a postjunctional effect and a secondary delayed prejunctional effect to inhibit the release of the co-transmitters, ATP and NA (Stjärne *et al.*, 1986).

However, the results in this study on the guinea-pig vas deferens do not fit in with this hypothesis. NPY added during a stimulation train showed no enhancement of response, but rather a successive inhibition of responses at both low and high frequency. Our results show that at nearly all the stimu-

lation parameters employed, NPY caused an inhibition of sympathetic transmission, indicative that the prejunctional inhibition predominated over postjunctional enhancement. Only at 20 Hz and 240 pulses was NPY shown not to inhibit, but to enhance slightly responses to EFS.

Another possible mechanism of action for NPY is that the postjunctional enhancement of transmitter action predominates at low concentrations of NPY and that prejunctional inhibition is not seen until higher concentrations of NPY are used (Wong-Dusting & Rand, 1988). However, this was shown not to be the case in this study, as over the range of concentrations tested (0.3 nM–0.3 μM), NPY was not shown to enhance responses. What was shown was that from a threshold concentration of about 3 nM, NPY concentration-dependently inhibited responses to EFS. Similar results were found in the mouse vas deferens (Stjärne *et al.*, 1986), where it was demonstrated that even at low concentrations NPY did not enhance responses to EFS, although in this tissue postjunctional enhancement was more marked than in the present study. This is in contrast to the situation in many blood vessels where NPY has been shown to enhance responses to EFS, but to inhibit the release of NA (Pernow *et al.*, 1986; Wong-Dusting & Rand, 1988).

The inhibition of transmission by NPY has been shown in this study to be dependent on both the frequency and duration of stimulation. Thus, NPY inhibition is maximal at low frequency and a low number of pulses. This is likely to be due to the decreased modulation of transmitter release seen at higher frequencies and duration of stimulation for both purinergic (Ellis & Burnstock, 1988) and adrenergic transmission (Vizi, 1979).

The question still remains to be answered on the physiological function of NPY neuromodulation. Lundberg *et al.* (1986) found that NPY release from pig spleen was favoured by high frequency and short bursts of stimulation, although Lundberg and his colleagues still demonstrated that NPY release was seen at low frequency (2 Hz) stimulation. NPY release has also been shown to occur from the guinea-pig vas deferens at 9 Hz, 540 pulses (Kasakov *et al.*, 1988). It therefore seems likely that NPY is co-released with ATP and NA over a range of stimulation frequencies and that the physiological effect of NPY in the guinea-pig vas deferens will predominantly be one of inhibition of sympathetic transmission.

The observed action of NPY is likely to be the resultant enhancement of transmission via its postjunctional action and reduction of transmission via its prejunctional action. The resultant response may depend on the geometry of the neuroeffector junction. Where the minimum neuromuscular separation of the junction is relatively large (60–600 nm), as is the case in blood vessels, and the co-transmitters need to diffuse a long way to reach receptors on the effector cells, one could argue that it makes physiological sense to have a mechanism that enhances the postjunctional actions of the transmitters with NPY, a more slowly degradable molecule. On the other hand, where the junction is narrow (10–20 nm), for example in the vas deferens, one could argue that inhibition of transmitter release is a more physiologically viable action for NPY release from the nerve. Thus the physiological role for NPY at sympathetic junctions could be considered as a device for conservation of co-transmitters, enhancing transmitter action in the blood vessels and inhibiting their release in densely innervated tissues such as the vas deferens.

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