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The presence and the effects of neuropeptide Y in rat anococcygeus muscle

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Abstract

Isolated anococcygeus muscle from male rats was examined for the presence of neuropeptide Y-immunoreactive nerves and for the effects of neuropeptide Y on its tone and its contractile/relaxant responses to electrical field stimulation, acetylcholine, guanethidine and noradrenaline. Using peroxidase anti-peroxidase immunohistochemistry in stretch preparation of the anococcygeus, neuropeptide Y-immunoreactive nerve fibres were observed, in abundance, running along both vascular as well as non-vascular smooth muscle cells. Neuropeptide Y (> 250 nM) evoked phentolamine and tetrodotoxin-resistant contractile response. Neuropeptide Y, even in subspasmogenic concentrations, potentiated contractions evoked by acetylcholine, guanethidine and noradrenaline. Electrical field stimulation (trains of 3–4 pulses, 0.1 ms, 10 Hz) of the isolated anococcygeus preparation produced robust, phentolamine and tetrodotoxin sensitive contractions. Neuropeptide Y (< 10 nM) exerted a biphasic effect on the electrical field stimulation-evoked contractions; an early potentiation was followed by a delayed and progressive inhibition. Neuropeptide Y (> 10 nM) caused a concentration-dependent potentiation of electrical field stimulation-evoked contraction alone, matching its potentiation of noradrenaline-evoked contraction. Electrical field stimulation (5 pulses, 0.1 ms, 10 Hz) of guanethidine (50 μM)-contracted anococcygeus induced a relaxant response and neuropeptide Y (1–100 nM) exerted a concentration-related slight and variable effect on the electrical field stimulation-evoked relaxant response (1 nM, augmentation; 10 nM, no effect; 100 nM, reduction). It is concluded that rat anococcygeus muscle has a rich neuropeptide Y-containing innervation and neuropeptide Y is mostly stored within adrenergic nerves. The main functional roles of neuropeptide Y in the anococcygeus muscle are likely to be post-junctionally mediated facilitation and prejunctionally mediated inhibition of adrenergic motor transmission. © 1997 Elsevier Science B.V.

Keywords: Drenergic transmission; Anococcygeus muscle; Neuropeptide Y; NANC (non-adrenergic non-cholinergic) transmission

1. Introduction

Neuropeptide Y, a 36-amino-acid peptide first isolated from porcine brain by Tatemoto et al. (1982), is now known to have an extensive presence both in the CNS and in the peripheral autonomic nervous system. As yet the physiological role of neuropeptide Y and the pharmacotherapeutic implications arising out of its presence in many autonomic nerves remain uncertain.

The anococcygeus is a paired thin band of smooth muscle, lying between the terminal part of the colon and coccygeal vertebrae and is present in several mammalian species including rat. It has an adrenergic motor innervation originating from the thoracolumbar vertebrae T_{11}–L_3 and a relaxant innervation arising from the lumbrosacral segments L_5–S_2 (Gillespie, 1972; Gillespie and McGrath, 1973). The identity of the inhibitory transmitter is believed to be nitric oxide (NO) or some other compound that can liberate NO or has effects similar to NO (Gillespie et al., 1989; Gillespie and Sheng, 1990; Gibson et al., 1990; La et al., 1996).

In the peripheral autonomic nerves, neuropeptide Y is frequently present co-localized with noradrenaline in adrenergic nerves and therefore presence of neuropeptide Y in the adrenergic innervation of the anococcygeus might be expected. The physical location of the anococcygeus, as a distinct band of smooth muscle fibres lying between the colon and coccyx, allows it, unlike other adrenergically-innervated smooth muscle preparations to be dissected out intact with virtually no trauma to individual muscle fibres. Assuming that neuropeptide Y-containing innervation is

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present in anococcygeus, the thinness of the anococcygeus muscle preparation, facilitating ready diffusion of added drugs and the minimum trauma to the tissue during its isolation render it an ideal isolated smooth muscle preparation for ascertaining the role of neuropeptide Y in peripheral adrenergic and non-adrenergic, non-cholinergic (NANC) neuromuscular transmission. To date there have not been any published reports of a detailed investigation for the presence of neuropeptide Y in the anococcygeus and there is only one publication on the effects of neuropeptide Y on adrenergic and non-adrenergic, non-cholinergic responses in this tissue (Vila et al., 1992). The present investigation was therefore undertaken to examine this tissue for the presence of neuropeptide Y-containing nerves. The study was extended to look into the effects of neuropeptide Y upon the contractility of the anococcygeus and upon its contractile and relaxant transmission.

2. Materials and methods

2.1. Animals

Adult male albino Wistar rats, weighing from 200–250 g, were stunned by a sharp blow on the head and decapitated. The lower abdomen was opened and the pair of anococcygeus muscles were isolated and dissected out according to the method of Gillespie (1972).

2.2. Immunohistochemistry

After removal, anococcygeus muscles were rapidly placed into a Petri dish containing 0.1 M phosphate-buffered saline (PBS) at 4°C. Excess connective tissue was trimmed off and muscles were fixed as stretch preparations according to the method of Costa et al. (1980) in Zamboni's fixative (Stefanini et al., 1967) for 16 h at 4°C. After fixation and washing in 80% ethanol the stretch preparations were processed for peroxidase–antiperoxidase (PAP) immunohistochemistry following the protocol of Costa et al. (1980). The primary antiserum employed was raised in rabbit and obtained commercially from Peninsula Europe. The tissue was incubated with the primary antiserum at a dilution of 1:1000 in PBS containing 0.1% bovine serum albumin and 0.01% sodium azide at room temperature for 24 h. The secondary antibody was goat anti-rabbit serum, conjugated with peroxidase–anti-peroxidase complex. This was applied at a dilution of 1:300 with 0.1% bovine serum albumin containing PBS (no azide present), for 1 h at room temperature. 3,3-Diaminobenzidine (DAB) (Sigma) served as the colour reagent 3,3-diaminobezidine solution was prepared by dissolving 50 mg in 100 ml PBS containing 50 μl of 30% hydrogen peroxide (Sigma).

In order to check for non-specific staining, some stretch preparations were incubated with neuropeptide Y antiserum that had been pre-absorbed with excess amount of antigen (100 μg synthetic porcine neuropeptide Y per 1 ml of diluted antiserum).

2.3. Organ bath studies

2.3.1. General

Each anococcygeus muscle was suspended between a pair of parallel platinum electrodes at a resting tension of 0.5 g in a 1 ml organ bath containing Krebs–Henseleit solution as the bathing medium (Composition in mM: NaCl, 113; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25 and glucose, 11.5). The resting tension was kept constant throughout the experiment by appropriate adjustments whenever needed. The bathing medium was maintained at 37°C and was gassed with a 95% O_2 + 5% CO_2 mixture. For recording the tension of the muscle, the preparation was connected to an isometric transducer and a potentiometric chart recorder. An equilibration period of 30 min was allowed before the start of the experimentation; during this period the preparation was repeatedly washed with fresh bathing medium.

The muscular contractions were evoked either directly by injecting smooth muscle spasmogen into the organ bath medium, bathing the isolated preparation of anococcygeus or indirectly by stimulation of the embedded nerves by electrical field stimulation. In order to conduct experiments on the inhibitory transmission, the motor adrenergic transmission was blocked and the tone raised by addition of 50 μM guanethidine.

2.3.2. Electrical field stimulation

Whenever required, electrical field stimulation was delivered through a square-wave electronic stimulation (Bell and Stein, 1971). The indirect (nerve-induced) muscle contractions were evoked by delivering trains of 3–40 pulses at 10 Hz, at a supramaximal voltage of 30 V and at a pulse duration of 0.1 ms. In order to evoke quantifiable relaxant responses on activation of inhibitory transmission by electrical field stimulation, guanethidine 50 μM was used to paralyse prejunctionally the motor (adrenergic) transmission and to raise the smooth muscle tone (Gillespie, 1972). The parameters of electrical field stimulation for evoking relaxant transmission were identical to those for motor transmission, save the number of pulses per train, which ranged between 3 to 5. In preliminary experiments, it was established that the contractile and relaxant responses to electrical field stimulation so obtained, were fully susceptible to blockade by tetrodotoxin, 0.5 μM, and were therefore solely neurogenic.

2.4. Chronic treatment with 6-hydroxydopamine

6-hydroxydopamine bitartrate was dissolved in saline containing 0.1% ascorbic acid and was administered i.p. in 4 doses of 50 $mg \cdot kg^{-1}$ on days 1, 2, 4 and 6. The animals were sacrificed one week after the last injection.

2.5. Drugs

The drugs used and their sources were: acetylcholine chloride, noradrenaline bitartrate, 6-hydroxydopamine bitartrate and propranolol hydrochloride (all from Sigma), guanethidine sulphate and phentolamine mesylate (Ciba-Geigy), tetrodotoxin (Sankyo), neuropeptide Y (Cambridge Research Biochemicals), rabbit anti-porcine neuropeptide serum and goat anti-rabbit immunoglobulin-G (Peninsula Laboratories). Solutions of drugs were made in deionised water, fresh on the day of their use with two exceptions, namely tetrodotoxin, whose stock solution was stored at −20°C and neuropeptide Y which was dissolved in bovine serum albumin, aliquoted and freeze-dried according to the method of Stretton and Barnes (1988). Each aliquot containing 25 μg neuropeptide Y was reconstituted in distilled water shortly before use. All glassware including the organ baths were pre-treated with silicon coating (Repelcoat, BDH). This precaution minimises the problem of drugs particularly peptides adhering to glassware.

2.6. Statistics

Results are expressed as the arithmetical means ± standard error of means (S.E.M.). Statistical significance was assessed with the Student's *t*-test for paired or unpaired comparisons as appropriate.

3. Results

3.1. Immunohistochemistry

Immunohistochemical examination of the stretch preparation of the anococcygeus revealed a dense network of neuropeptide Y-like immunoreactive nerve fibres distributed throughout the tissue (Fig. 1A and B). Varicose immunoreactive processes were observed as bundles in nerves and also individually ramifying in the tissue. Numerous neuropeptide Y-immunoreactive neuronal varicosity's were located on the surface of the non-vascular smooth muscle (Fig. 1B). Many blood vessels could also be seen, surrounded by a network of neuropeptide Y-immunoreactive axons (Fig. 1A and B). In preparations, obtained from 6-hydroxydopamine pre-treated rats, neuropeptide Y-immunoreactive nerve fibres could be detected but was greatly reduced (Fig. 1C).

3.2. Organ bath studies

3.2.1. Effect of neuropeptide Y on resting tone

Neuropeptide Y up to a concentration of 25 nM brought about no change in the resting tone of the anococcygeus muscle. Higher concentrations of neuropeptide Y produced a rise in the resting tension. The contractions produced by neuropeptide Y were slow in onset, reaching their peak 5–10 min after the introduction of neuropeptide Y into the bath and were concentration-related (Fig. 2). Prior treatment of the preparation with phentolamine 5 μM (Fig. 2F) or with tetrodotoxin 0.5 μM (Fig. 2G) failed to modify the contractile effect of neuropeptide Y.

3.2.2. Effect of neuropeptide Y on noradrenaline-evoked contractions

Noradrenaline-evoked contractions of the anococcygeus were augmented by the presence of neuropeptide Y. Neuropeptide Y even in concentrations low enough to have no effect on the tone of the preparation potentiated contractions evoked by noradrenaline. The effect of neuropeptide Y 25 nM, a subspasmogenic concentration was determined on the contractile response to a range of noradrenaline concentration (0.1–300 μM). Neuropeptide Y produced a leftward shift of noradrenaline concentration response curve (ED50 mean ± S.E.M. = before neuropeptide Y, 4.5 ± 0.5 μM; after neuropeptide Y, 1.6 ± 0.3 μM; $n = 7$, $P < 0.05$, paired *t*-test) and also enhanced the maximum contractile response (Fig. 3).

3.2.3. Effect of neuropeptide Y on acetylcholine-evoked contractions

In preparations pre-treated for 30 min with 5 μM phentolamine and 5 μM propranolol, in order to exclude the possibility of any adrenergic involvement, acetylcholine 1 μM produced a sub-maximal contraction of 221 ± 28 mg ($n = 4$). Neuropeptide Y at 25 nM potentiated these responses by 130 ± 14% to 509 ± 81 mg (mean ± S.E.M.; $P < 0.001$, $n = 4$, paired *t*-test).

3.2.4. Effect of neuropeptide Y on electrically evoked contractions

Electrical field stimulation (trains of 3 pulses, once every minute) evoked reproducible brief contractions (mean tension ± S.E.M., 560 ± 60 mg, $n = 37$). Such electrically-evoked contractions were readily and fully blocked by 0.5 μM tetrodotoxin, indicating their neurogenic origin, and were also completely abolished by 5 μM phentolamine or by 5 μM guanethidine, confirming their adrenergic credentials.

Neuropeptide Y in sub-spasmogenic concentrations (0.075–25 nM) exerted a biphasic effect on the electrically-evoked contraction; an early potentiation was followed by a slowly-developing, gradual and progressive inhibition of the motor transmission (see Section 3.2.5). The onset of potentiation was rapid, beginning 30–60 s after introduction of neuropeptide Y and the degree of potentiation of the electrically-evoked contraction was related to the concentration of neuropeptide Y used (Fig. 4). The time taken for neuropeptide Y to attain its peak potentiation was also directly related to the concentration of neuropeptide Y used, increasing with rising neuropeptide Y concentrations (Fig. 5). In a further four experiments, a higher concentration of 6.25 μM neuropeptide Y

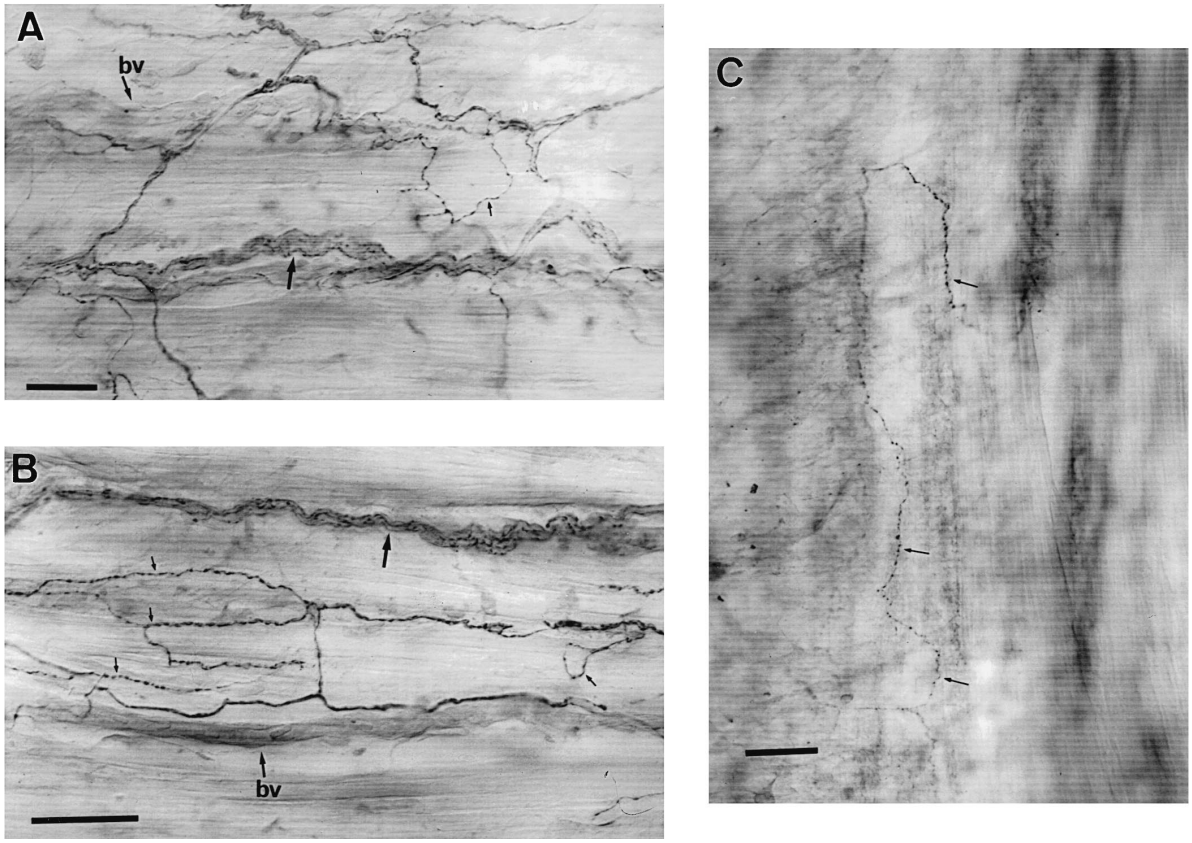

Fig. 1. Immunohistochemical demonstration of neuropeptide Y-immunoreactive nerves in the stretch preparation of rat anococcygeus muscle in control (A and B) and 6-hydroxydopamine treated animals (C). Blood vessel capillaries are indicated as bv. Small arrows indicate fine varicose neuropeptide Y-immunoreactive nerve bundles. Scale bars represent 50 μm.

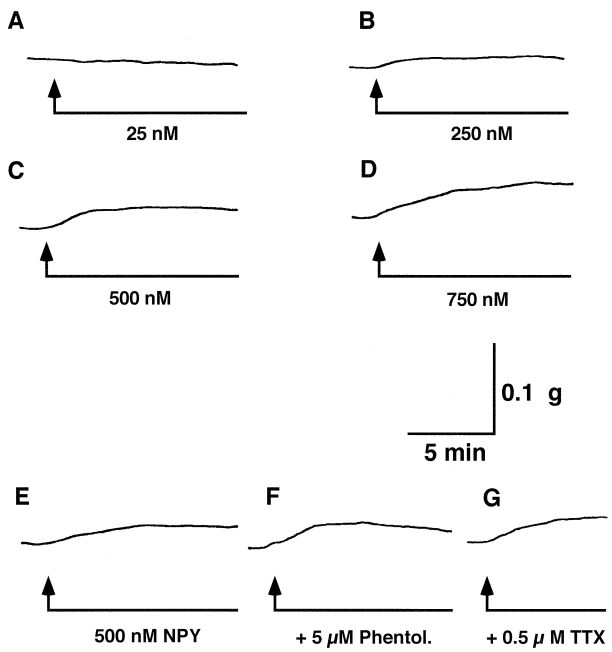

Fig. 2. Contractile effects of neuropeptide Y on isolated preparation of rat anococcygeus muscle. Neuropeptide Y at concentrations greater than 25 nM and up to 750 nM led to concentration dependent contraction of the smooth muscle (A–D). Contractile effect of 500 nM neuropeptide Y was unaffected by prior 30 min preincubation of the anococcygeus muscle preparation with 5 μM phentolamine (E) or 0.5 μM tetrodotoxin (F).

was used. This concentration of neuropeptide Y contracted the anococcygeus muscle and evoked a massive potentiation of the electrically-evoked contractions (response as % of control: 2370 ± 620, $n = 4$).

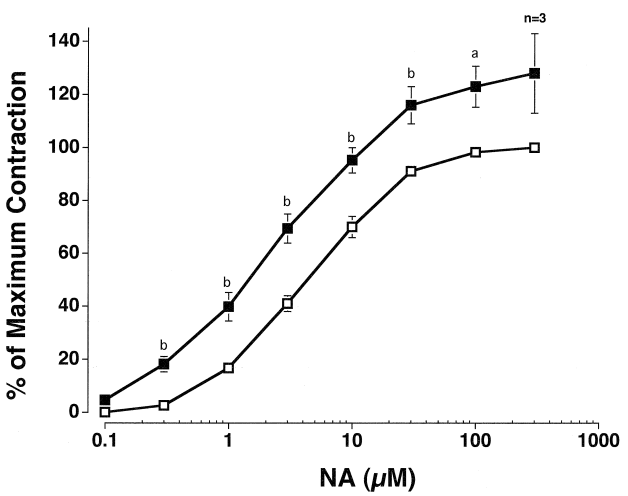

Fig. 3. Non-cumulative concentration response curves of noradrenaline-induced contraction of rat anococcygeus muscle in the absence (□) or in the presence of 25 nM neuropeptide Y (■). The magnitude of the contractions by the maximum concentration of noradrenaline in control preparations was taken as 100%. Each point represents the mean ± S.E.M. (vertical bars); $n = 7$ (or 3); $^{a}P < 0.05$, paired t-test.

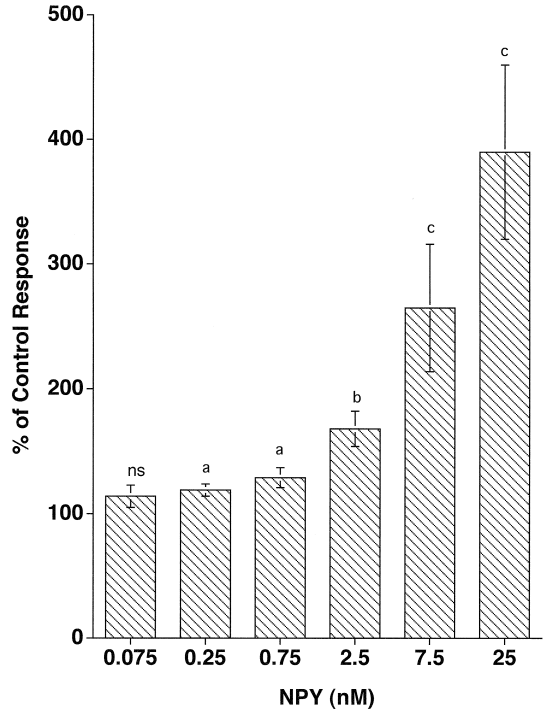

Fig. 4. Concentration dependent potentiation of the electrically evoked contraction of the rat anococcygeus muscle by neuropeptide Y. Each histograms represent mean ± S.E.M. (vertical bars) of muscle contractions evoked by 3 to 4 electrical pulses expressed as % of control electrically-evoked contraction in the absence of neuropeptide Y. $n = 7$; $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.005$, paired *t*-test.

The degree of potentiation of the motor transmission by neuropeptide Y appears to be inversely related to the train length of the electrical stimulation, and the potentiation was most marked at shorter train-lengths (response in presence of neuropeptide Y, 0.75 nM as % control: mean ± S.E.M. = 131 ± 7 and 112 ± 2 with 3 and 40 pulses/train, respectively; $n = 4$). In order to determine whether the potentiating action of neuropeptide Y on transmission was pre- or postjunctional, noradrenaline-evoked contractions representing postjunctional activity and electrically-evoked contractions, representing both pre- and postjunctional activity were obtained first in the absence of neuropeptide Y and then in its presence. The electrically-evoked and noradrenaline-evoked contractions were both potentiated; the degree of potentiation was almost identical in both cases indicating that the potentiation of motor transmission was fully accounted for by a postjunctional potentiation of noradrenaline-evoked contraction.

3.2.5. Inhibition of electrically-evoked contractions by neuropeptide Y

At low concentrations, neuropeptide Y as well as producing a potentiation of the motor transmission also produced a late appearing, gradual inhibition of the motor transmission. The inhibitory effect was found to be progressive with the length of exposure to neuropeptide Y and therefore to validate comparison of results from different experiments, this effect was studied by limiting the duration of exposure to neuropeptide Y to 60 min. At the end of the 60 min period of exposure to neuropeptide Y, there was a very marked inhibition of the transmission by 0.75 μM neuropeptide Y but not by 7.5 μM neuropeptide Y. The relationship between neuropeptide Y concentration and inhibitory potency was studied at neuropeptide Y concentration of 0.75 to 25 nM. The inhibitory activity of neuropeptide Y was greatest at the lowest concentration and declined with increasing concentrations (Fig. 6).

The inhibitory effect of neuropeptide Y was inversely related to the number of pulses in the stimulus train (Table 1). In contrast to the inhibitory effect of neuropeptide Y on electrically-evoked contractions, noradrenaline-evoked contractions were not inhibited by 60 min exposure to neuropeptide Y (Table 1).

Re-application of neuropeptide Y to preparations already exposed to neuropeptide Y for 60 min still elicited a potentiating response of a magnitude and duration indistinguishable from the original exposure. On washing-out neuropeptide Y after 60 min exposure, the responses to electrical stimulation recovered but despite repeated washings, the recovery remained incomplete.

3.2.6. Effect of neuropeptide Y on the relaxant transmission

For examining this effect the relaxant transmission was unmasked by guanethidine, 50 μM which blocked the

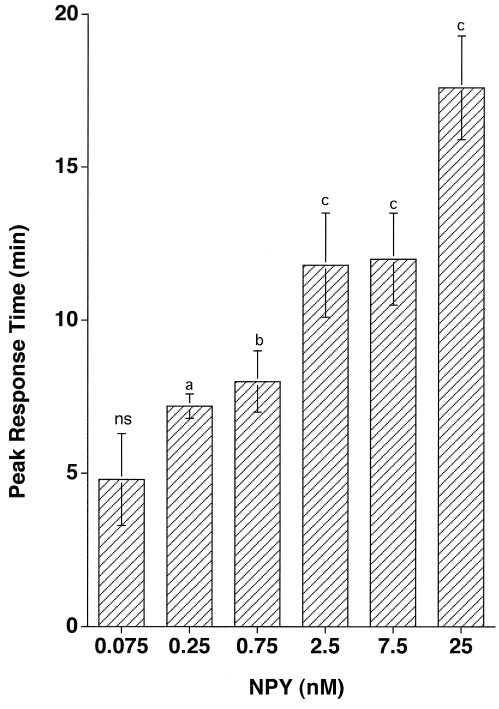

Fig. 5. Concentration dependent potentiation of the peak response time for neuropeptide Y to achieve maximum potentiation of the electrically-evoked contractions. Each histograms represent mean ± S.E.M. (vertical bars) of time in minutes. $n = 7$; $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.005$, paired *t*-test.

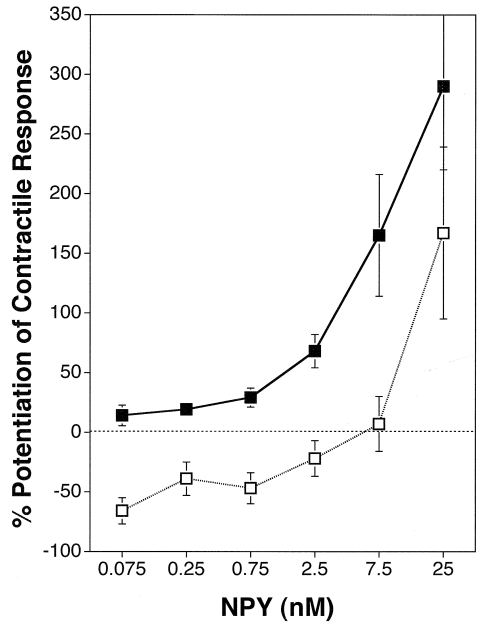

Fig. 6. Comparison of the effects of neuropeptide Y (0.075 to 25 nM) on the initial transient potentiation of the electrically-evoked anococcygeus contractions with the inhibition of the electrically-evoked contractions following 60 min neuropeptide Y exposure. Each data point is the mean ± S.E.M. (vertical bars) of 4 to 5 experiment. Responses are represented as % potentiation of the control (0) response. As the initial potentiation of the contractions were potentiated by increasing concentrations of neuropeptide Y, the secondary inhibition of contractions at 60 min neuropeptide Y exposure was diminished. At concentrations greater than 7.5 nM, neuropeptide Y at 60 min exposure had no inhibitory effect on electrically-evoked contractions.

adrenergic motor transmission and raised the muscle tone (Gillespie, 1972). Electrical field stimulation (trains of 5 pulses), now evoked consistent smooth and reproducible muscle relaxation of the pre-contracted muscle. Neuropeptide Y (10–100 nM) caused further enhancement of the tone of the muscle already raised by guanethidine. 10 min exposure to neuropeptide Y led to either augmentation (1 nM) or reduction (100 nM) of the relaxant response. Neuropeptide Y, 10 nM, had no effect.

Table 1
Effect of 60 min exposure to 0.75 nM neuropeptide Y on contractile responses of rat anococcygeus to electrical field stimulation (2–40 pulses/train) and to 1 μM noradrenaline

No. of pulses/train	Contractile response (g)	
	controls	neuropeptide Y-treated
2	0.68 ± 0.12	0.18 ± 0.06[a]
3	1.34 ± 0.11	0.18 ± 0.13[a]
4	1.71 ± 0.13	1.21 ± 0.17[a]
5	1.94 ± 0.17	1.50 ± 0.19[a]
10	2.45 ± 0.21	2.15 ± 0.19
20	2.95 ± 0.27	2.73 ± 0.25
40	3.31 ± 0.27	3.24 ± 0.24
Noradrenaline	1.24 ± 0.16	1.46 ± 0.21

Results are shown as the mean ± S.E.M. ($n = 5$). In each experiment, contractile responses to electrical field stimulation and to noradrenaline were obtained before and following 60 min exposure to neuropeptide Y.
[a] $P < 0.5$, paired t-test.

4. Discussion

The investigation has demonstrated a plentiful presence of neuropeptide Y-containing nerves in the anococcygeus muscle of the rat. Since the rat anococcygeus has a very rich adrenergic innervation (Gillespie and Maxwell, 1971) and since neuropeptide Y has been shown to be present in many peripheral adrenergic nerves (Lundberg et al., 1982), its presence in the innervation of the anococcygeus should not come as a surprise. Drastic reduction in neuropeptide Y-immunoreactivity following chronic treatment with 6-hydroxydopamine indicates that the vast majority of neuropeptide Y-storing nerves in the anococcygeus are capable of taking up to 6-hydroxydopamine, a property regarded as characteristic of adrenergic nerves (Kostrzewa and Jacobowitz, 1973). This finding suggests that in the rat anococcygeus, neuropeptide Y is present mainly colocalised with noradrenaline in the adrenergic nerves. However persistence of the presence of some neuropeptide Y-immunofluorescent nerve fibres despite treatment with 6-hydroxydopamine may imply either that some neuropeptide Y-containing nerve fibres are non-adrenergic or that some adrenergic fibres escaped the destructive effects of 6-hydroxydopamine treatment. It is interesting to note that neuropeptide Y-containing nerves were distributed not only as perivascular nerve fibres, but also were located on the surface of the anococcygeus smooth muscle cells. That these nerves are actually innervating the anococcygeal smooth muscle cells and are not simply on their way to the vascular smooth muscle, is evidenced by the presence of neuropeptide Y-containing varicosities on the surface of the non-vascular anococcygeal smooth muscle. Presence of neuropeptide Y-containing neuronal varicosities amongst the non-vascular smooth muscle cells, raises the question of the functional role of this neuropeptide in these cells. The richness of its presence is perhaps indicative of the importance of its role in the physiology of anococcygeus muscle. The results of the present investigation have shown that neuropeptide Y was capable of exerting three types of effects on the non-vascular, anococcygeal smooth muscle cells. These were:

(i) Contraction of the smooth muscle.

(ii) Potentiation of the contractile action of smooth muscle spasmogens.

(iii) Inhibition of adrenergic motor transmission.

It seems reasonable to assume that these effects should provide some clues towards identifying the functional role(s) of neuropeptide Y in this tissue. We shall, therefore, discuss each of these effects at length in order to evaluate their possible physiological relevance.

4.1. Contractile effect

Neuropeptide Y in relatively high concentrations (in excess of 250 nM) caused the smooth muscle to contract. The contraction was not prevented by antagonism of α-adrenoceptors and muscarinic cholinoceptors or by the cyclooxygenase-inhibitor, indomethacin (unpublished observations, but see Iravani and Zar, 1994) indicating that the contraction evoked by neuropeptide Y was not mediated through noradrenaline, acetylcholine or eicosanoids. A direct contractile effect of neuropeptide Y has long been known in many vascular smooth muscle preparations (Hellstrom et al., 1985; Mabe et al., 1985; Kahan et al., 1988) and presumably the contractile effect of neuropeptide Y in the anococcygeus is another example of its direct action on the smooth muscle. The ability of neuropeptide Y to contract the anococcygeus, coupled with its rich presence in the nerves supplying this muscle might suggest that it may act as a major motor neurotransmitter in addition to noradrenaline. However, it is unlikely that a direct contractile effect of endogenous neuropeptide Y plays a significant physiological role under the present conditions of electrical stimulation. Complete blockade of electrical field stimulation-evoked contractions of anococcygeus by adrenoceptor antagonists has been repeatedly demonstrated (Gillespie, 1972; Ambache et al., 1975), whereas neuropeptide Y-evoked contractions remained unaffected by α-adrenoceptor antagonist phentolamine in the present investigation. If endogenous neuropeptide Y release from intrinsic nerves contributed to electrical field stimulation-evoked contraction independently of noradrenaline, the nerve-evoked contraction should have been in part phentolamine-resistant. Low sensitivity of the anococcygeus to the contractile action of neuropeptide Y would seem to be the likeliest explanation for the failure of endogenously release neuropeptide Y to evoke a contractile response. In our experiments the threshold concentration of exogenous neuropeptide Y for this effect was 250 nM and it is possible that endogenous neuropeptide Y concentration in response to electrical field stimulation did not reach this level in the synaptic cleft. The alternative explanation for the higher potency of neuropeptide Y to enhance spasmogen action, than to cause a direct contractile effect may be due to a threshold synergy phenomenon.

4.2. Potentiation of spasmogens

Neuropeptide Y potentiated contractions of anococcygeus evoked by acetylcholine, noradrenaline, or nerve stimulation and probably therefore acted through a mechanism which did not distinguish between agonists acting through different receptors. The non-discriminatory nature of the potentiating effect might give rise to the suspicion that it is the result of the contractile effect of neuropeptide Y summating with the contractile effects of other spasmogens. This explanation seems improbable since the potentiating effect was obtained even at concentrations of neuropeptide Y many folds lower than the threshold concentration of neuropeptide Y required for evoking a contractile response. Thus, in the present investigation, threshold concentration of neuropeptide Y for contraction was 500 nM whereas it, in a twenty-fold lower concentration of 25 nM, caused a significant augmentation of electrically-evoked neurogenic contractions.

Our findings, firstly, that neuropeptide Y potentiated contractions evoked by acetylcholine and noradrenaline which are known to act directly on anococcygeal smooth muscle (Gillespie, 1980) and secondly that nerve-evoked contractions were potentiated by neuropeptide Y to the same degree as noradrenaline-evoked contractions, are consistent with a postjunctional smooth muscle site for its potentiating action. The exceptionally high sensitivity of the anococcygeus to the potentiating effect of neuropeptide Y renders it highly probably that the intrinsically released neuropeptide Y would augment significantly the adrenergic nerve-mediated contraction of this muscle.

4.3. Inhibition of motor transmission

Exogenous neuropeptide Y, in low concentrations (< 10 nM), following an initial, transient potentiation, induced a slow and gradually progressive inhibition of the motor transmission. The inhibition of the motor transmission was not associated with a concomitant inhibition of noradrenaline-evoked contraction, suggesting that the inhibition was caused by a decline in noradrenaline release and was not due to a diminished response of the smooth muscle to noradrenaline. Train-length dependence of the inhibitory effect, with greater inhibition of contractions evoked by shorter trains, is also probably best explained on the basis of a prejunctional action of neuropeptide Y. An inhibitory effect of neuropeptide Y on release of neurotransmitters, including noradrenaline, has been widely reported (for review see Zukowska-Grojec and Wahlestedt, 1993). However, Vila et al. (1992) have reported that neuropeptide Y did not inhibit electrically-evoked noradrenaline release in rat anococcygeus. The apparent discrepancy between the findings of Vila et al. (1992) and our results might be explained on the assumption that in the present investigation, it was not neuropeptide Y itself but a degradation product of neuropeptide Y which was responsible for the inhibition of the motor transmission. The delayed onset and the slowly progressive nature of the neuropeptide Y-induced inhibition of transmission, lend further support to such a postulate. Therefore, since Vila et al. (1992) studied the effects of a range of concentrations of neuropeptide Y in a cumulative manner, it is conceivable that anococcygeus preparations in their study were not exposed to the low concentrations of neuropeptide Y for a sufficient length of time for the inhibitory effects of neuropeptide Y to be unmasked.

Irrespective of whether the prejunctional inhibitory ef-

fect on adrenergic transmission in rat anococcygeus is produced by neuropeptide Y itself or by a breakdown product of neuropeptide Y, the physiological relevance of this effect is beyond doubt since it is produced by exceptionally low concentrations of neuropeptide Y, concentrations which would almost certainly be reached in synaptic cleft following its release from neuropeptide Y-containing neuronal varicosities (Lundberg et al., 1989).

The effect of neuropeptide Y on electrical field stimulation-evoked relaxations also merits discussion. Vila et al. (1992) had reported some reduction of these relaxations by neuropeptide Y. In our experiments, neuropeptide Y did not exert any consistent effect on relaxant transmission. Thus, while neuropeptide Y (1 nM) slightly potentiated the relaxant transmission, neuropeptide Y (100 nM), on the other hand, slightly reduced the relaxant response. Since the guanethidine-induced tone of the anococcygeus preparation was further augmented by neuropeptide Y (100 nM) but not by neuropeptide Y (1 nM), it remains unclear whether the reduction in relaxant transmission by neuropeptide Y (100 nM) was the result of a genuine inhibitory effect on relaxant transmission or was merely secondary to the rise in tone of the muscle.

In conclusion, neuropeptide Y-containing nerves are present in abundance amongst the smooth muscle of the rat anococcygeus. Neuropeptide Y raises the tone of anococcygeus muscle in concentration > 0.25 μM and in the subspasmogenic concentrations, it potentiates contractions evoked by acetylcholine, guanethidine, noradrenaline and electrical field stimulation. Prolonged exposure to low concentrations of neuropeptide Y (< 1 nM) causes a prejunctionally-mediated inhibition of electrically-evoked contractions; further investigations are needed to confirm or exclude the possibility that this effect is due to a breakdown product of neuropeptide Y.

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