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Noradrenergic-nitrergic interactions in the rat anococcygeus muscle: evidence for postjunctional modulation by nitric oxide

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1 The distribution of NADPH-diaphorase positive and catecholamine-containing nerve structures, and functional noradrenergic-nitrergic interactions, were studied in the rat anococcygeus muscle.

2 The morphological findings demonstrated NADPH-diaphorase positive neurones mostly as aggregates in intramural ganglia, nerve tracts and few single nerve fibres forming plexus-like structures.

3 The nitric oxide synthase inhibitor N^G-nitro-L-arginine (L-NOARG) inhibited concentration-dependently the nitrergic relaxation, an effect reversed by L-arginine. The drug had dual effects on noradrenergic contractile responses: at lower concentrations (0.1–10 μM) it decreased the amplitude of contractions and this was not affected by L-arginine; higher concentrations (50–500 μM) potentiated the contractions, an effect that was prevented by L-arginine.

4 The electron acceptor, nitro blue tetrazolium (NBT) produced a rapid inhibition of the noradrenergic contractile responses (EC_{50} 0.178 ± 0.041 μM). The drug decreased the tone of the preparations. However, it potentiated concentration-dependently the nitrergic relaxations.

5 NBT (1 μM) had no significant effect on the relaxations induced by exogenously applied nitric oxide (NO)-donor sodium nitroprusside (SNP, 0.01–50 μM). However, the effect of NBT (0.1–10 μM) on the electrically induced relaxation was significantly decreased by L-NOARG (10 and 50 μM). The inhibition was of a non-competitive type.

6 Neither L-NOARG (100 μM) nor NBT (1 μM) had any effect on the spontaneous or electrically-induced release of ^{3}H-radioactivity from the tissues preincubated in [^{3}H]-noradrenaline.

7 It is concluded that L-arginine-NO pathway can modulate noradrenergic transmission in the rat anococcygeus muscle at postjunctional, but not prejunctional site(s).

Keywords: Anococcygeus muscle (rat); NADPH-diaphorase; N^G-nitro-L-arginine; nitro blue tetrazolium; [^{3}H]-noradrenaline release.

Introduction

Following the identification of the endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) as the free radical of nitric oxide ($NO^{\cdot}$; Palmer *et al.*, 1987; Ignarro *et al.*, 1987) the so-called L-arginine-NO pathway has been suggested to represent a widespread mechanism for the regulation of cell function and communication (Moncada *et al.*, 1989; 1991; Moncada, 1992). The action of NO is mediated by the stimulation of a soluble guanylyl cyclase (Arnold *et al.*, 1977) leading to the elevation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels within the target cells (Snyder & Bredt, 1991). It is well documented that elevation of cyclic GMP can increase the release of acetylcholine (ACh) from PC12 cells and noradrenaline (NA) from the adrenal chromaffin cells, or decrease release of ACh in the hippocampus, cerebral cortex, and skeletal neuromuscular junction, and that of NA from adrenergic nerves and PC12 cells (for review see: Garthwaite, 1991). Recently, NO has been implicated as an inhibitor transmitter-like substance in the non-adrenergic, non-cholinergic (NANC) neurotransmission in various preparations (Bult *et al.*, 1990; Rand, 1992; Sanders & Ward, 1992). However, the interaction of NO with other types of neurotransmission needs further study. Controversial results have been reported that NO inhibits the release of NA from the adrenergic nerves in canine blood vessels (Cohen & Weisbrod, 1988; Greenberg *et al.*, 1989; 1990), that NO increases the release of NA in rat mesenteric vasculature (Yamamoto *et al.*, 1992), or that NO has no effect on either NA release from the noradrenergic nerve terminals in the vasculature (Cederquist *et al.*, 1991; Toda & Okamura, 1992), or ACh release from the cholinergic nerves in guinea-pig ileum (Gustafsson *et al.*, 1990b).

The rat anococcygeus muscle is a suitable non-vascular autonomically-innervated tissue to study the noradrenergic-nitrergic interactions. The electrical stimulation of intramural nerves evokes a fast high-amplitude contractile response which is largely mediated by NA (Gillespie, 1980); after guanethidine the application of identical stimulation evokes a rapid relaxation of the precontracted anococcygeus muscle (for review see Gillespie, 1980) which is mediated mainly by NO-related mechanisms (Hobbs & Gibson, 1990; Martin & Gillespie, 1990). The nerve terminals supplying the anococcygeus muscle comprise a dense network in which 60–70% of the fibres are noradrenergic and up to 40% are considered NANC terminals, with only about 5% of all nerve terminals having cholinergic characteristics (Burnstock *et al.*, 1978). More data are required about the presence and pattern of distribution of NO-containing nerve fibres and neurones in the rat anococcygeus muscle.

In preliminary experiments we found that the inhibition or potentiation of the relaxant responses produced by N^G-nitro-L-arginine (L-NOARG) or nitro blue tetrazolium (NBT) respectively, which are chemical agents that may alter the activity of nitric oxide synthesis (EC 1.14.23; NOS) (Palmer *et al.*, 1988; Gibson *et al.*, 1990; Hobbs & Gibson, 1990; Hope *et al.*, 1990), corresponded to marked reciprocal effects on the contractile responses (Kasakov *et al.*, 1991). The aim of the present investigation was to characterize further the effects of L-NOARG and NBT on the neurogenic responses of the rat anococcygeus muscle and to investigate the effect of L-NOARG amd NBT on the basal, as well as stimulus-

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evoked, release of tritium (^3H) from the noradrenergic terminals in the muscle preincubated with [^3H]-NA. In addition we have examined the distribution and localization of NADPH-diaphorase containing neuronal structures in the rat anococcygeus muscle.

Methods

General

Male Sprague-Dawley rats (200–250 g) were killed by an overdose of CO_2 and exsanguination and the anococcygeus muscles were excised. The isolated preparations were placed horizontally between pairs of platinum ring (4 mm diameter) electrodes (10 mm apart) in 1 ml plastic (high density polythene) double-jacketed organ baths (37°C). Tissues were perfused at a constant flow of 1.2 ml min^{-1} (0.6 ml min^{-1} in ^3H release experiments) by means of a peristaltic pump (Watson-Marlow) with a medium of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.6, $NaHCO_3$ 11.9, KH_2PO_4 0.5, glucose 11.5, containing (mg l^{-1}) albumin 25, ascorbic acid 100, atropine 0.7, bacitracin 30, EDTA 10, and gassed with 5% CO_2 in O_2 (pH 7.4–7.6). The preparations were stretched to a 5 mN initial tension and allowed to equilibrate for 90 min. One end of the preparation was tied to a Grass FT 03C force-displacement transducer connected to a Gould 2200S recorder for registration of the isometric changes of the tension. The preparations were stimulated electrically for 10 s (60 s in ^3H release experiments) with trains of rectangular pulses of 0.6 ms duration, 20 Hz and 60 V delivered by Grass SD9 stimulators triggered by a D100 Digitimer (Digitimer Ltd, Herts) every 130 s (twice at 45 min intervals in ^3H release experiments). Drugs were added to the medium reservoir (continuous treatment) or infused for short periods of time into the perfusate at a flow rate of 0.3 ml min^{-1} close to the preparation via a side-way canula by means of a peristaltic pump (intermittent treatment).

Fluorescence histochemistry

The anococcygeus muscles were dissected and stretched on a Slygard silicone rubber plate, and fat and connective tissues were cleared carefully. The stretched tissues were immersed in a 2% glyoxylic acid solution in 0.1 M phosphate buffer, pH 7.4, for 1.5 h at room temperature. After incubation the tissue samples were transferred onto clean glass slides, dried until translucent and placed in an oven at 80°C for 4 min. The tissues were then mounted with liquid paraffin and catecholamine containing nerve fibres were viewed under a Zeiss photomicroscope fitted for epifluorescence with ultraviolet filters.

NADPH-diaphorase staining

Stretched and cleaned tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2 h at 4°C. The tissues were then washed 10 times (each for 10 min) with 80% alcohol, dehydrated, rehydrated and washed 3 times (each for 5 min) with PBS containing 0.1% Triton X-100. For NADPH-diaphorase staining the tissues were incubated in 0.1 M Tris. HCl (pH 7.4) containing 1.2 mM β-NADPH, 2.4 mM NBT and 15.2 mM L-malic acid at 37°C for 1.5–2 h. After incubation the tissues were transferred onto clear glass slides, mounted with citifluor (glycerol:PBS) and the neurones and nerve fibres stained for NADPH-diaphorase were viewed with a light microscope.

Radiolabelled release experiments

The changes in NA-levels were monitored by the levels of ^3H-radioactivity in the perfusate and in the tissue. After isolation (as described above) the tissues were incubated for 60 min in continuously gassed medium containing 0.1 μM tritium labelled (−)-noradrenaline ([^3H]-NA, Amersham; Sp. Act. 1850 GBq $mmol^{-1}$; two preparations in 2 ml medium) at 37°C. After rinsing 5 times with 3 ml medium each preparation was placed in an organ bath between stimulating electrodes as described above. The bath chambers were perfused (0.6 ml min^{-1}) with a cocaine-containing (10 μM) medium.

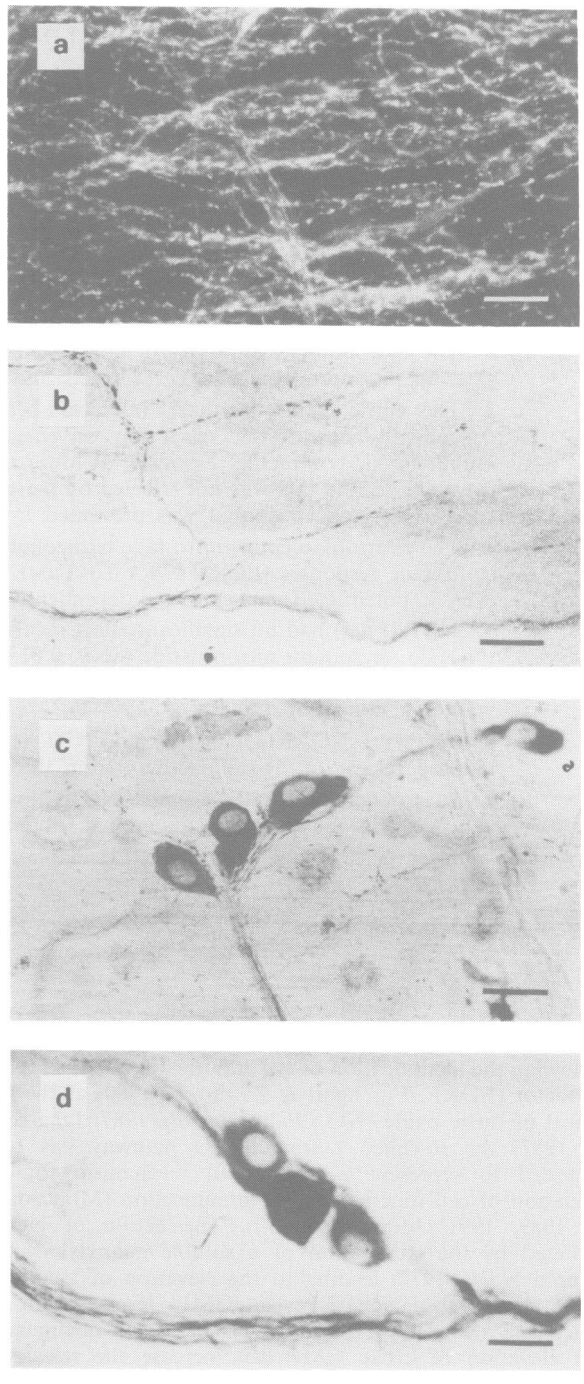

Figure 1 Microphotographs showing: (a) The distribution of catecholamine-containing nerve fibres on whole-mount preparation of the rat anococcygeus muscle. (b) NADPH-diaphorase containing single fibres forming plexus-like structures. (c) NADPH-diaphorase stained neurones in groups forming ganglion structures. Sometimes single neurones were seen lying between the ganglia structures. (d) Prominent nerve tracts which could be followed across the preparations and connecting ganglion structures. Calibration bars = 30 μm.

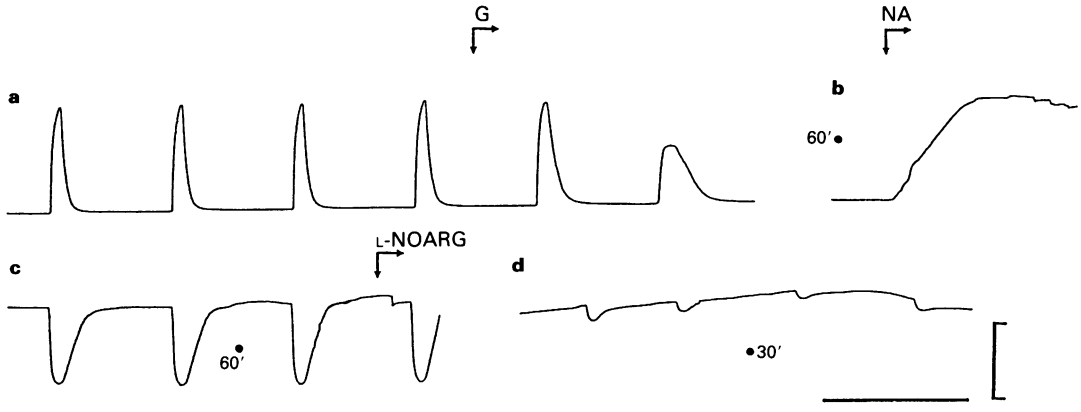

Figure 2 The neurogenic responses of the rat anococcygeus muscle induced by electrical field stimulation (60 V, 20 Hz, 0.6 ms, 10 s duration at 130 s interval). The mechanograms are original recordings of the consecutive responses of one preparation and are representative of all experiments of this series. (a) The electrically induced contractions in the presence of atropine (1 μM) an inhibition of the contractile response by guanethidine (G; 50 μM). (b) Complete block of the contractile response to electrical stimulation applied at 60th min of the guanethidine treatment and the effect of exogenously applied noradrenaline (NA; 1 μM). (c) The electrically induced relaxations (guanethidine 50 μM and atropine 1 μM present) of the precontracted (NA 1 μM) muscle and the application of N^G-nitro-L-arginine (L-NOARG, 100 μM). (d) The inhibition of relaxation after 30 min L-NOARG treatment. The time elapsed after each treatment is indicated by the dot. Arrows show the beginning and onward flow of the medium containing the respective drug(s). Note that atropine (1 μM) is present from (a) to (d). The bars represent 10 mN (vertical) and 2 min (horizontal).

After 90 min pre-collection period the perfusate was collected for 63 min divided into 21 consecutive intervals of 3 min each (collections 7, 8, 10, 11, 12, 19, 20 were discarded). Drugs were applied with the perfusion medium at the end of collection 9 (27th min) and were present throughout the next 12 collections. Samples (1 ml) from each collection were added to 2 ml Ready-gel (Beckman) scintillation cocktail and ^{3}H-radioactivity was counted in a Beckman 5300 liquid scintillation system. The perfusion was stopped immediately after the last collection, the preparations were weighed and dissolved in 0.2 ml Soluene 100 (Packard) tissue solubilizer, and the total tissue ^{3}H-radioactivity was counted as described above. The basal outflow, as well as stimulus-evoked overflow, of tritium was calculated as a percentage fractional rate (FR%) as described previously (Kasakov *et al.*, 1988). The FR values of the discarded collections were estimated by a linear approximation from d.p.m. (disintegrations per minute) values in the collections prior to and after the discarded period. The changes in the stimulus-evoked release or spontaneous efflux of ^{3}H were quantified as S_2/S_1 ratio (the integral increase of ^{3}H release over the basal level in collections 16, 17 and 18 versus the identical increase of ^{3}H release in collections 4, 5 and 6) or as B_{15}/B_3 ratio (the level of ^{3}H in collection 15 versus the level of ^{3}H in collection 3). The basal levels of ^{3}H release during S_1 and S_2 were estimated by a linear approximation from the values in collections 3 and 9 or collections 15 and 21.

Drugs used

The drugs used were: albumin (bovine, fraction V), L-ascorbic acid, atropine sulphate, bacitracin, cocaine hydrochloride, ethylenediamine tetraacetic acid disodium salt (EDTA), N^G-nitro-L-arginine (L-NOARG), nitro blue tetrazolium (NBT), noradrenaline hydrochloride, β-NADPH, sodium nitroprusside (all from Sigma) and guanethidine monosulphate (Ismelin, CIBA). All drugs were first prepared in distilled water as stock solutions.

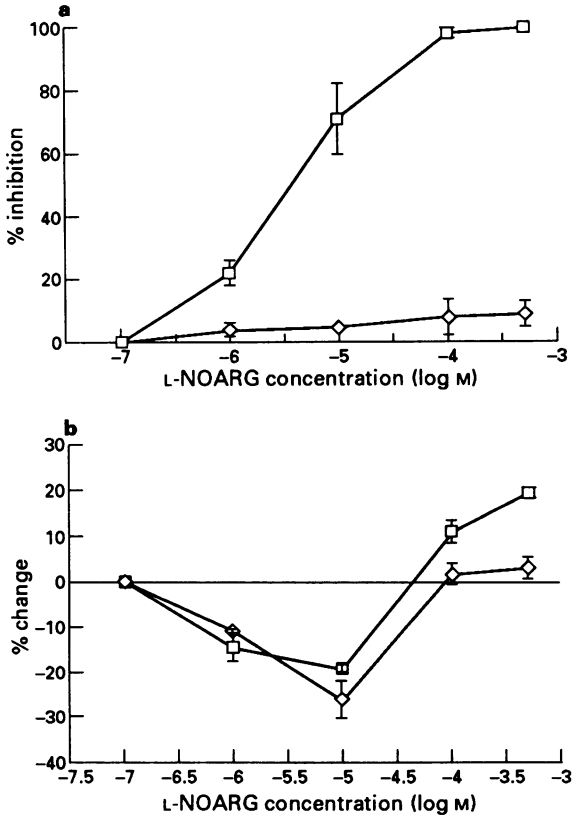

Figure 3 The effect of N^G-nitro-L-arginine (L-NOARG) on the neurogenic relaxation (a) and contraction (b) of the rat anococcygeus muscle induced by electrical field stimulation (see legend for Figure 2). The effect of L-NOARG in the absence (□) and presence (◇) of L-arginine is shown. Each point represents the mean ± s.e.mean of 4 to 7 experiments.

Statistical analysis

Quantitative data are expressed as mean ± s.e.mean and the differences between two means were evaluated by Student's two-tailed *t* test for paired or unpaired observations as appropriate. A probability of less than 0.05 was considered statistically significant. *n* denotes the number of preparations.

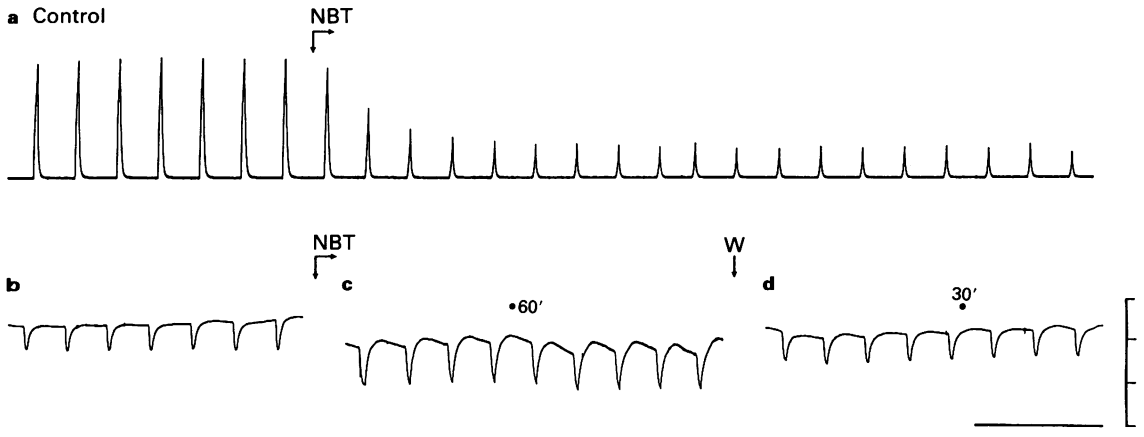

Figure 4 The effect of nitro blue tetrazolium (NBT) on the electrically induced responses of the rat anococcygeus muscle induced by electrical field stimulation (see legend to Figure 2). The mechanographs are original recordings from two experiments and are representative of all experiments of this series. (a) Neurogenic contractions and the inhibitory effect of NBT (1 μM) in the absence of guanethidine. (b) Neurogenic relaxations of the precontracted muscle (by noradrenaline, NA, 1 μM) in the presence of guanethidine (50 μM) and atropine (1 μM). (c) Neurogenic relaxations in the presence of NBT (1 μM). (d) Restoration of the initial tone and neurogenic relaxations after the wash out of NBT (W). Note that atropine (1 μM) is present from (a) to (b). The bars represent 30 mN (vertical) and 5 min (horizontal). The time elapsed after NBT and W is indicated by the dot.

Results

NADPH-diaphorase and catecholamine staining

The NADPH-diaphorase reaction used for the histochemical studies is based on the NADPH-dependent reduction of NBT (an electron accepting substrate for NADPH-diaphorase and one of the constituent chemicals of the staining solution) to produce a visible formazan product (Hope *et al.*, 1990). There were single nerve fibres, nerve tracts and neuronal cell bodies that stained for NADPH-diaphorase (Figure 1b,c,d). The neurones were found mostly in aggregates as intramural ganglia with some single neurones lying between the ganglion structures (Figure 1c,d). There were few single nerve fibres forming plexus-like structures (Figure 1b). However, several thin and thick nerve tracts could be followed across the preparations connecting ganglionic structures (Figure 1c,d).

The fluorescence histochemistry revealed a dense innervation of the rat anococcygeus muscle by catecholamine-containing nerve fibres (Figure 1a).

Effect of L-NOARG and NBT on the contractile and relaxant responses

In atropine-containing medium the preparations responded to electrical stimulation with fast high-amplitude contractions which increased in amplitude as stimulation continued and rapidly relaxed to the prestimulation level at its cessation (Figure 2a). After application of guanethidine (50 μM) the contractile responses rapidly attenuated and in 46–60 min were almost completely inhibited (Figure 2b). The application of NA (1 μM) in the presence of guanethidine (1 μM) and atropine (1 μM) induced a sustained increase of the tone that reached similar or higher amplitude than the neurogenic contractions and remained unchanged until NA was present (Figure 2b). Applied at that level, the electrical stimulation with identical parameters evoked fast high-amplitude relaxations which reached the maximum before the stimulation terminated followed by a slow restoration of the prestimulation tone (Figure 2c). The relaxations were inhibited by L-NOARG (100 μM). This effect was combined in many preparations with a slight increase of the tone (Figure 2d).

The effect of L-NOARG on the mechanical responses of the rat anococcygeus muscle was studied in a concentration range of 0.1 to 500 μM. It was found that L-NOARG inhibited concentration-dependently the relaxant responses

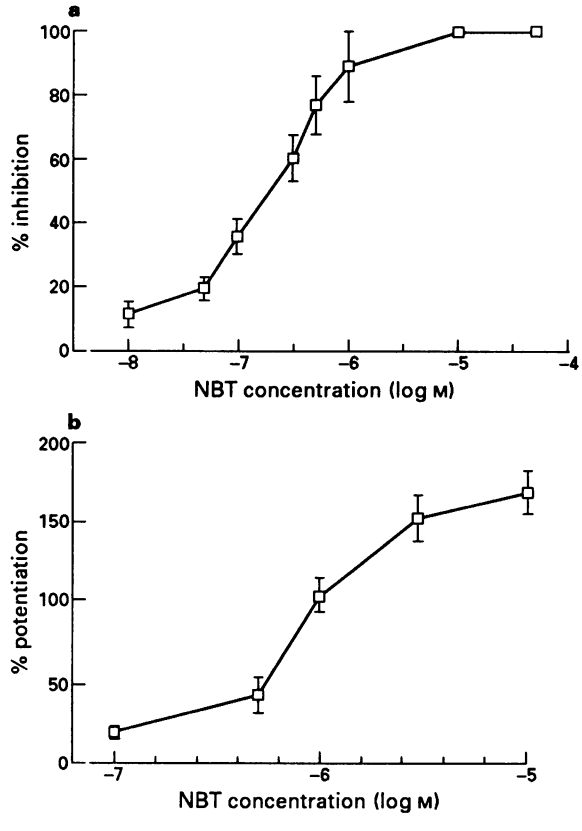

Figure 5 The effect of nitro blue tetrazolium (NBT) on neurogenic contraction (a) and relaxation (b) of the rat anococcygeus muscle induced by electrical field stimulation (see legend to Figure 2). Each point represents the mean ± s.e.mean of 7 to 9 experiments.

(Figure 3a). This inhibitor effect was antagonized by L-arginine (500 μM) (Figure 3a). When L-NOARG was applied in concentrations higher than 10 μM it caused in 50% of all preparations an additional rise in tension which varied from 15 to 30% of the tone generated in the presence of NA. As is shown in Figure 3b, lower concentrations of L-NOARG (0.1–50 μM) attenuated concentration-dependently the con-

tractile responses. On the contrary higher concentrations (100–500 μM) L-NOARG induced a potentiation of the contractile responses. L-Arginine (500 μM) antagonized the L-NOARG-induced potentiation of the contractile responses but did not affect L-NOARG-induced decrease of the neurogenic contractions. NBT was applied in a concentration range of 0.01–50 μM. It was found that NBT produced a rapid inhibition of the contractile response (Figure 4a). The results of these experiments are summarized in Figure 5a. EC_{50} estimated for the inhibition of contractile response was 0.178 ± 0.041 μM. The response to exogenously applied NA (1 μM) was inhibited by NBT (0.5 μM) by 30–35% (not shown). In another series of experiments the effect of NBT on the neurogenic relaxation was investigated. It was found that NBT produced a rapid decline of the tone. Despite the lower tone of the preparation, relaxations evoked by electrical field stimulation were greatly potentiated in the presence of NBT (Figure 4c). The effect was concentration-dependent and eliminated soon after the wash out of the substance (Figure 4d). The results of these experiments are summarized in Figure 5b. The Effect of NBT on the relaxations induced by the exogenously applied NO-donor sodium nitroprusside (SNP) was investigated. It was found that NBT (1 μM) did not change significantly the relaxation induced by SNP. The results of these experiments are shown in Figure 6. However

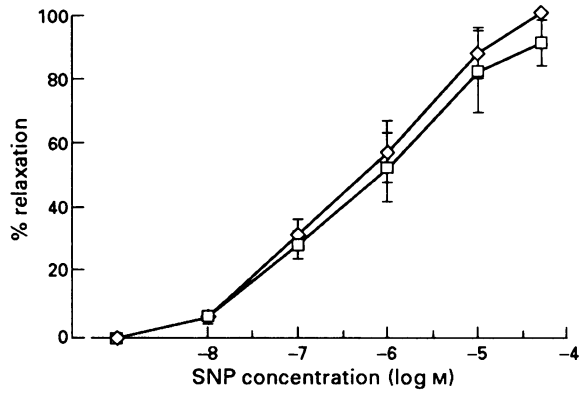

Figure 6 Sodium nitroprusside (SNP)-induced relaxation of rat anococcygeus muscle in the absence (□) and presence (◇) of nitro blue tetrazolium (NBT, 1 μM). SNP was applied intermittently for 4 min at 20 min intervals. The initial concentrations were appropriately adjusted to give the final concentrations required. With this experimental protocol no tachyphylaxis towards SNP was observed. Each point represents the mean ± s.e.mean of 7 experiments.

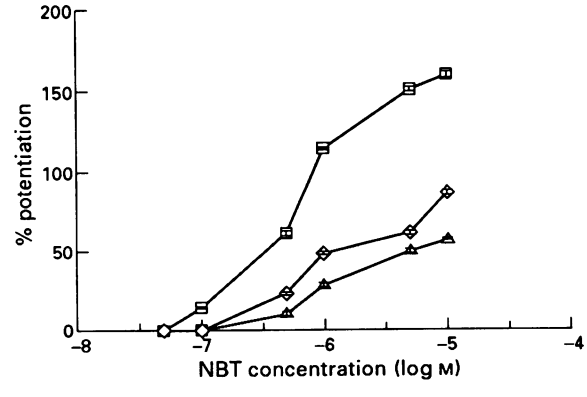

Figure 7 The effect of nitroblue tetrazolium (NBT) on the neurogenic relaxation of the rat anococcygeus muscle induced by electrical field stimulation (see legend to Figure 2) in the absence (□) and presence of N^G-nitro-L-arginine at concentrations of 10 μM (◇) and 50 μM (△). Each point represents the mean ± s.e.mean of 6 or 7 experiments.

the potentiating effect of NBT on the electrically evoked relaxation was significantly antagonized in a non-competitive manner by L-NOARG (Figure 7).

Effect of L-NOARG and NBT on ^3H release

The data presented above showed that L-NOARG and NBT may strongly inhibit and potentiate respectively the nitrergic relaxant responses in the rat anococcygeus muscle. At the same time L-NOARG and NBT were powerful modulators of the noradrenergic contractile responses. These effects might result from a pre-junctional or a postjunctional nitrergic modulation of the noradrenergic transmission in the anococcygeus muscle. Therefore the next step was to investigate the effect of L-NOARG and NBT on the spontaneous as well as the electrically induced release of ^3H in the anococcygeus muscle. The electrical stimulation of non-treated preparations induced a rapid substantial increase in the level of ^3H in the perfusate which corresponded to the twitch contractile response of the muscle. A second stimulation produced similar contractile response and ^3H release (Figure 8). L-NOARG applied at a concentration of 100 μM, which produced a nearly complete block of the relaxations, potentiated the contractile response but did not change the spontaneous or the electrically induced release of ^3H. NBT at a concentration of 1 μM, which strongly inhibited the contractile response, also did not change the release of ^3H. It was found that some 15% of the total ^3H activity which accumulated in the tissue during the incubation period was released in the superfusate under this experimental protocol. The total release was changed neither by L-NOARG nor NBT. The results of these experiments are summarized in Table 1.

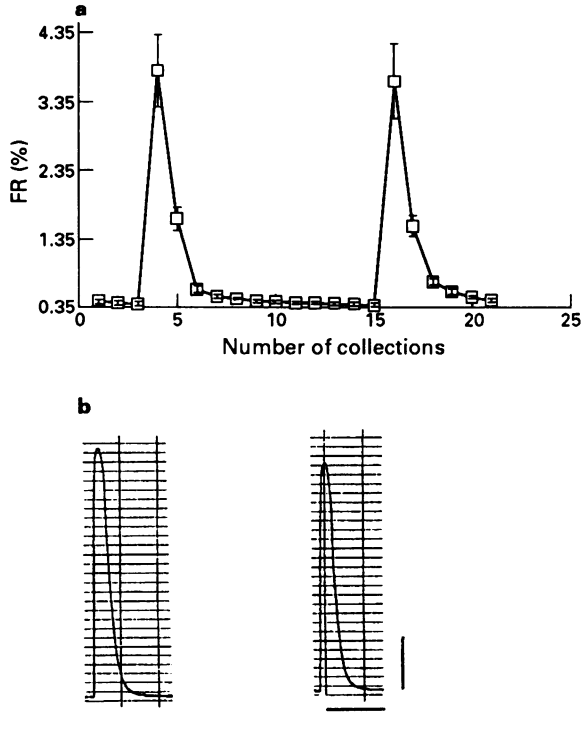

Figure 8 'No drugs' contractile response of the rat anococcygeus muscle induced by electrical field stimulation (b; 60 V, 20 Hz, 0.6 ms, 60 s duration twice at 45 min interval). The corresponding fractional rate (FR %) of ^3H release measured in the perfusate is shown in (a). The mechanogram is an original recording from one experiment and is representative of all experiments of this series. The bars represent 10 mN (vertical) and 5 min (horizontal). Note the different time scales; the mechanical activity is recorded at a paper advance of 3 mm min^{-1}. Each point in the ^3H fractional rate graph represents the values of ^3H-radioactivity in the superfusate of 21 consecutive 3 min collections of 4–8 experiments.

Table 1 The effect of N^G-nitro-L-arginine (L-NOARG) and nitro blue tetrazolium (NBT) on the contractile response and corresponding ^3H release in the rat anococcygeus muscle

	B_{15}/B_3	S_2/S_1	A_2/A_1	*Total release 0–63 min (%)*
Control	0.95 ± 0.05 ($n = 6$)	0.98 ± 0.03 ($n = 5$)	0.91 ± 0.02 ($n = 6$)	18.79 ± 1.22 ($n = 6$)
L-NOARG (100 μM)	0.09 ± 0.03 ($n = 8$)	0.93 ± 0.04 ($n = 8$)	1.22 ± 0.08** ($n = 8$)	15.46 ± 1.29 ($n = 8$)
NBT (1 μM)	0.91 ± 0.08 ($n = 4$)	1.01 ± 0.07 ($n = 4$)	0.67 ± 0.02** ($n = 4$)	15.2 ± 0.66 ($n = 4$)

The effect of L-NOARG and NBT on spontaneous (B_{15}/B_3, the ratio of ^3H-radioactivity in collections 15 and 3), electrically induced (S_2/S_1, the ratio of the integral increase of ^3H release over the basal level in collections 16, 17 and 18 and the identical increase of ^3H release in collections 4, 5 and 6) at 60 V, 20 Hz, 0.6 ms, 60 s duration twice at 45 min interval, percentage total release of ^3H and amplitude of the contractile responses (A_2/A_1, the ratio of the amplitude of contractile responses (mm) induced by S_2 and S_1), in the rat anococcygeus muscle. n denotes number of preparations. $^{**}P < 0.01$.

Discussion

The morphological findings in the present study have demonstrated the presence of neurones and nerve fibres that contain NADPH-diaphorase in the rat anococcygeus, similar to that reported by Dail *et al.* (1993). Hope *et al.* (1991) have revealed that neuronal NADPH-diaphorase is an NO-synthase (NOS) and the co-localization of NOS and NADPH-diaphorase has been shown by others, establishing that NADPH-diaphorase staining accounts for the presence of NOS in neuronal structures (Bredt *et al.*, 1991; Belai *et al.*, 1992; Bredt & Snyder, 1992). The brain tissue has been shown to contain a cystolic (soluble) isoform of NOS while vascular endothelium contains a particulate isoform (Bredt & Snyder, 1990; Schmidt *et al.*, 1991; Mitchell *et al.*, 1991a). Unlike brain and endothelium, the rat anococcygeus muscle is reported to contain both particulate and soluble NOS activity (Mitchell *et al.*, 1991b). The authors have demonstrated that NOS is localized specifically in the NANC neuronal components rather than smooth muscle cells, as the activity of NOS in the rat anococcygeus is dependent on the presence of calcium, unlike the inducible NOS which has calcium/calmodulin independent activity (Mitchell *et al.*, 1991b). These findings suggest that NO (or a related molecule) is one of the NANC neurotransmitters in the rat anococcygeus muscle. The presence of dense catecholamine-containing nerve fibres supports the previous findings that the majority of the fibres supplying the muscle are noradrenergic (Burnstock *et al.*, 1978).

The present investigation has demonstrated that modulation of the NO-dependent relaxation of rat anococcygeus muscle corresponded to reciprocal modulatory effects on the neurogenic contractile responses of the muscle.

We utilized this finding to study the level at which the interaction between two neurotransmitter mechanisms may occur. The inhibition of the relaxation corresponded to an increase in the amplitude of the contractions evoked by electrical nerve stimulation or exogenously applied NA. However, the corresponding release of ^3H from the nerve terminals supplying the tissue remained unchanged. Furthermore, the potentiation of NO-related relaxation corresponded to a marked inhibition of the contractile response. Again there were no changes in the release of ^3H from the nerve terminals. These findings strongly suggest that NO-modulation of noradrenergic responses occurs at postjunctional but not prejunctional site(s). Similar findings have been reported recently for the rat tail artery (Bucher *et al.*, 1992), in which neurogenic vasoconstriction is modulated by NO and this modulation is not due to a prejunctional action of NO. In a recent investigation Brave *et al.* (1993) found that L-NOARG did not change the electrically-induced release of ^3H in the rat anococcygeus preloaded with [^3H]-NA. The authors concluded that endogenous nitrate NANC transmitter did not influence release of NA from the sympathetic nerves in the rat anococcygeus muscle. The negative postjunctional modulation of the contractile response of the rat anococcygeus muscle by NO is an example of the physiological antagonism between the excitatory (NA) and inhibitory (NO) transmitter mechanisms which may contribute to the balance of the excitatory and inhibitory inputs in the autonomically innervated tissues.

The data presented here show that inhibition of NOS by high concentrations of L-NOARG (0.1–0.5 μM) potentiates the neurogenic contraction. Similar potentiation of the contractile responses by NOS-inhibitors has also been described by other authors (Gustafsson *et al.*, 1990a; Belvisi *et al.*, 1991). This effect is most likely due to elimination of the relaxant effect of NO. This seems to be a specific effect since: (1) at the same concentrations, L-NOARG completely inhibited the neurogenic relaxations of the rat anococcygeus muscle that are mediated by NO-related mechanism(s) (Martin & Gillespie, 1990); (2) potentiation was reversed by L-arginine. This also implies that generation of NO may contribute to the continuous low, or negligible, tone of this smooth muscle. This is further supported by the observation that in a great number of preparations L-NOARG inhibited the relaxation and induced a further rise of the tone of the precontracted tissue. The existence of a physiologically significant continuous generation of NO has also been suggested in the gastrointestinal tract (Wiklund *et al.*, 1993). It may serve as an ubiquitous mechanism for a physiological adjustment of the tone of the smooth muscles. In the present study it has been additionally found that L-NOARG at lower concentrations (0.1–50 μM) attenuated the neurogenic contractions and that L-arginine did not reverse this effect. Similar results have been reported for dog mesenteric artery (Toda & Okamura, 1990). Recently it has been found that N^G-monomethyl-L-arginine but not L-NOARG (20 μM) was a partial agonist for NOS (Archer & Hampl, 1992). In the present investigation L-NOARG produced its maximal inhibitory effect on the contractile responses at concentrations of 1–50 μM. It is most likely to be a non-specific effect of which the mechanism has yet to be unravelled.

NBT is an electron acceptor in the NADPH-diaphorase reaction and a constituent of the recipes for the NADPH-diaphorase visualization (Hope *et al.*, 1990). We reported here that NBT dose-dependently potentiated the NO-mediated relaxation of the rat anococcygeus muscle. It is unlikely to be primarily due to an inhibition of the excitatory transmitter mechanisms since atropine and guanethidine were present throughout the experiment. NBT might potentiate the neurogenic relaxation in the rat anococcygeus muscle by a stimulation of NO synthesis/release. As NO is not stored in the nerve terminals, NOS is more likely to be the primary target for the action of NBT. This suggestion is supported by two findings: (1) NBT had no effect on the relaxation

induced by the NO-donor molecule SNP and (2) NBT-induced potentiation of the electrically induced relaxation of rat anococcygeus muscle was significantly decreased by the specific NOS inhibitor L-NOARG. This is however a non-competitive antagonist effect. Recently, Davisson *et al.* (1993) reported that NBT produced substantial hypotension and vasodilatation in rats. The authors suggested that these effects of NBT involve an augmentation of NO synthesis/release. It has been shown that NBT at a concentration of 50 μM completely inhibited NOS with a K_i, with respect to L-arginine, of 11 μM (Hope *et al.*, 1991). In the present investigation, NBT potentiated the relaxation at much lower concentrations (0.01–1 μM). The present study does not verify the exact mechanism of this activation but it seems plausible to suggest that at these lower concentrations NBT acts as a substrate activator of NOS, whereas at higher concentrations it is a competitive inhibitor of NOS. The finding that NBT is able, at lower concentrations, to potentiate the NO-dependent relaxation in the rat anococcygeus muscle could be a useful tool in future investigations.

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