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Characterization of nitrergic neurotransmission during short- and long-term electrical stimulation of the rabbit anococcygeus muscle

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1 Isolated preparations of rabbit anococcygeus muscle were exposed to electrical field stimulation (EFS; 50V, 0.3 ms duration, 0.08–40 Hz) for periods of 1–60 s (short-term EFS) or 10 min–2 h (long-term EFS).

2 Both short- and long-term EFS caused a contractile response which was enhanced by the nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine (L-NOARG), showing that it is modulated by endogenous NO.

3 In preparations treated with scopolamine and guanethidine and in which a constrictor tone was induced by histamine, both short- and long-term EFS resulted in relaxation of the tissue.

4 Such relaxations were reversed by tetrodotoxin (TTX), ω-conotoxin, inhibitors of NO synthase and the NO scavenger, oxyhaemoglobin, indicating that they are neuronal in origin and nitrergic in nature.

5 The relaxations to long-term EFS persisted for the duration of the stimulation and were associated with sustained release of oxidation products of NO (NO_x). The EFS-induced release of NO_x was decreased by N-iminoethyl-L-ornithine (L-NIO), an inhibitor of NO synthase, and by TTX.

6 Inhibitors of NO synthase, in addition, increased the basal tone of the tissue and reduced the basal output of NO_x. The basal output of NO_x was also reduced by TTX.

7 Long-term EFS which induces ~50% of the maximum relaxation could be enhanced by addition of L-, but not D-, arginine to the perfusion medium.

8 These data show that there is a continuous basal release of NO from nitrergic nerve terminals which maintains a relaxant tone in the rabbit anococcygeus muscle.

9 In addition, NO is released during short- and long-term EFS which further relaxes the preparation and modulates sympathetic transmission. Activation of the L-arginine: NO pathway for periods up to 2 h does not exhaust nitrergic transmission in any appreciable way.

Keywords: Nitrergic neurotransmission; nitric oxide; rabbit anococcygeus muscle; long-term stimulation; nitric oxide synthase inhibitors

Introduction

It is widely accepted that nitric oxide (NO) synthesized from L-arginine acts as a neurotransmitter in the central as well as in the peripheral nervous system (Moncada *et al.*, 1991; Rand, 1992; Sanders & Ward, 1992); accordingly the term 'nitrergic neurotransmission' has been introduced (Rand & Li, 1990). Pharmacological, physiological and immunohistochemical studies have provided evidence that nitrergic neurotransmission occurs in a large variety of autonomically innervated tissues from many species including man (Brookes, 1993; Leone *et al.*, 1994; Timmermans *et al.*, 1994). Some of these studies include the rat and mouse anococcygeus muscles, which are now well characterized (Gillespie *et al.*, 1989; Gibson *et al.*, 1990) and the rabbit anococcygeus muscle which has been identified more recently (Graham & Sneddon, 1993). Purification and characterization of NO synthase from the rat colorectum shows that the NO synthase in peripheral neurones is probably the same as that occurring in central neurones (Seo *et al.*, 1994).

Previous studies suggest that, at least in some preparations, NO is continuously released from the nerve terminals to maintain a nitrergic dilator tone (Ward *et al.*, 1992; Wiklund *et al.*, 1993). So far, all investigations on nitrergic preparations have been carried out using periods of stimulation of not more than 5 min (Li & Rand, 1990) and thus the nature of this continuous release of NO has not been characterized. We therefore decided to investigate the effect of long-term (10 min–2 h) electrical field stimulation (EFS) on the rabbit anococcygeus muscle preparation. Furthermore, we have characterized the effects of NO synthase inhibitors, the substrate of NO synthase L-arginine (L-Arg), the NO scavenger oxyhaemoglobin, the NO donor sodium nitroprusside (SNP), neurotoxins (tetrodotoxin and ω-conotoxin), adenosine 5'-triphosphate (ATP) and vasoactive intestinal peptide (VIP) in this preparation. In addition we have quantified the release of NO from these tissues by measuring the concentrations of nitrite and nitrate (NO_x) in the effluent.

Methods

Preparation of tissues

Male New Zealand rabbits (2.8–3.5 kg, Recal Rabbits, U.K.) were killed by an overdose of pentobarbitone (Euthesate, Willows Francis Veterinary, U.K.) injected into the ear marginal vein after local anaesthesia (Xilocaine Gel 2%, Astra Pharmaceuticals, U.K.) and the bilateral anococcygeus muscles were excised. The preparations isolated from the region between the sacrum and rectum (3 mm width, 15 mm length; 29.5 ± 1.5 mg wet tissue weight, $n = 110$) were placed horizontally between two ring electrodes (4 mm diameter) located at a distance of 10 mm and 26 mm from the outlet of 1 ml plastic double-jacketed tissue chambers (37°C). The

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tissues were perfused at a constant flow of 0.6 ml min^{-1}, or 1.2 ml min^{-1} in the experiments with intermittent drug application, by means of peristaltic pumps (Miniplus 2, Gilson) with a medium of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgSO_4$ 0.6, $NaHCO_3$ 11.9, KH_2PO_4 0.5, glucose 11.5 containing (mg l^{-1}) albumin 25, ascorbic acid 100, EDTA 10 and gassed with 5% CO_2 in O_2 (pH 7.4–7.6). Bacitracin (30 μg ml^{-1}), bestatin (10 μg ml^{-1}), captopril (100 μg ml^{-1}) and D,L-thiorphan (10 μg ml^{-1}) were added in the experiments using VIP, in order to inhibit peptidases. One end of the preparation was tied to a Grass FT 03C force-displacement transducer connected to a Linearcorder WR 3101 (Graphtec) for registration of isometric changes in tension. The preparations were stretched (2 to 5 mN) until they reached approximately the *in situ* length (~15 mm) and allowed to equilibrate for 90 min. The preparations were stimulated electrically for 1 to 60 s in the short-term stimulation experiments or for 10 to 120 min in the long-term stimulation experiments with trains of rectangular pulses of 50V, 0.3 ms pulse duration and frequencies ranging from 0.08 to 40 Hz, delivered by Grass S88 stimulators every 130 s in the short-term stimulation experiments, or twice at 45–60 min intervals in the long-term stimulation experiments. Drugs were added to the medium reservoir (continuous treatment) or infused via a cannula close to the inlet of the tissue chamber into the perfusate at a flow rate of 0.3 ml min^{-1} by means of a peristaltic pump (intermittent treatment).

Determination of NO_x levels in the perfusate

Samples of the perfusate (0.12–0.15 ml) were collected at 15 s intervals directly from the outlet of the tissue chamber in ice-cold plastic tubes and frozen in liquid nitrogen. Nitrite (NO_2^-) and nitrate (NO_3^-) concentrations (expressed as NO_x) were measured by chemiluminescence after reduction in boiling acidic vanadium-III, as described previously (Bush *et al.*, 1992). Briefly, samples (100 μl) were injected into 100 ml of 0.1 M vanadium-III chloride in 2 N HCl at 98°C in a stream of nitrogen. NO_2^- and NO_3^- were reduced to NO in boiling acidic vanadium-III which was then quantified by a chemiluminescence detector (Mark II, The Wellcome Technology Group, Beckenham, U.K.) after reaction with ozone. The signals from the chemiluminescence detector were analysed with an integrator (Shimadzu, model C-R6A, Shimadzu Corp., Japan) as the area under the curve. Linear regression of the standard curves for NO_2^- and NO_3^- gave correlation coefficients of 1.0 and 0.99 respectively. The concentrations of NO_x are expressed as pmol per gram of tissue (wet weight) per minute or per second as appropriate. N^G-nitro-L-arginine (L-NOARG) or N^G-nitro-L-arginine methyl ester (L-NAME) could not be used as inhibitors of nitrergic neurotransmission for NO_x measurements because these inhibitors break down into equimolar concentrations of NO_2^- and NO_3^- during chemiluminescence detection (our unpublished observations). N-iminoethyl-L-ornithine (L-NIO) and tetrodotoxin (TTX) were used to assess the changes in the NO_x concentrations in the presence of inhibitors. NO_x concentrations due to possible contamination found in solutions treated with L-NIO and TTX were subtracted from the concentrations detected.

Drugs used

The drugs used were: adenosine 5'-triphosphate (ATP), albumin (bovine, fraction V), L-ascorbic acid, D-arginine, L-arginine, bacitracin, bestatin, captopril, ω-conotoxin GVIA (CTX), ethylenediaminetetraacetic acid disodium salt (EDTA), guanethidine, histamine hydrochloride, N^G-nitro-L-arginine (L-NOARG), N^G-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), scopolamine, tetrodotoxin (TTX), D,L-thiorphan, vasoactive intestinal peptide (VIP), all from Sigma, UK; vanadium-III chloride from Aldrich, U.K.; and oxyhaemoglobin, N-iminoethyl-L-ornithine (L-NIO) from the Wellcome Research Laboratories, U.K.. All substances were freshly prepared in twice distilled water.

Statistical analysis

Quantitative data are expressed as means ± s.e.mean and the differences between two means were evaluated by a 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. Analysis of variance for repeated measurements was performed for the appropriate results where stated. A statistical programme package was used for the statistical analysis of all the data (GraphPAD InStat, 1990, version 1.11a, GraphPAD Software, U.S.A.).

Results

Characteristics of mechanical responses

Short-term EFS-induced contractions Short-term EFS caused reproducible contractions of the rabbit anococcygeus muscle. The contractile response, which was dependent on the frequency and train duration, started following the period of stimulation and lasted for about 45 s (Figure 1a). In the presence of L-NOARG (300 μM) the lag period between stimulation and contraction was not observed and the contractile response was considerably larger (Figures 1b and 2b). L-NOARG 1, 10, 100 and 500 μM caused 7.7 ± 6.4%, 94.6 ± 31.8%, 449.6 ± 144.5% and 585.2 ± 132.7% respectively greater contractile responses than the control responses ($n = 6$). In the presence of 1 mM L-arginine (L-Arg), the increment in the contraction to nerve stimulation produced by addition of 500 μM L-NOARG was reduced to 84.4 ± 12.0% ($P < 0.01$; $n = 6$).

Long-term EFS-induced contractions Long-term EFS elicited a contraction which lasted for as long as the electrical stimulation was applied, was smaller than that due to short-term EFS and was converted into a sharp, short-lasting contraction at the end of the stimulation period (Figure 2a). In the presence of L-NOARG (300 μM) the contraction elicited by long-term EFS was considerably greater and not sustained at its maximum. Following treatment with L-NOARG the single contractions elicited by short-term EFS

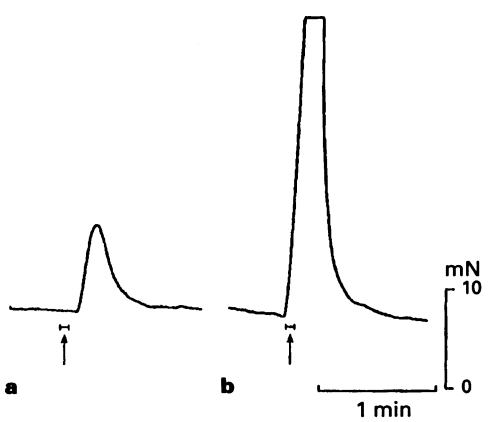

Figure 1 (a) Control contractile response to short-term EFS (indicated by arrow) (5 Hz, 50 V, 0.3 ms duration, 5 s train duration). (b) Contractile response of same tissue to identical short-term EFS in the presence of N^G-nitro-L-arginine (L-NOARG, 300 μM). The mechanogram is an original recording of the responses of one preparation and is representative of all experiments in this series ($n = 10$).

were usually reduced after termination of a long-term EFS (Figure 2c).

Effects of scopolamine, guanethidine and histamine Treatment of the preparation with scopolamine (10 μM) induced an increase in the contractile response to short-term EFS (Figure 3a,b). Addition of guanethidine (10 μM) abolished the contractile responses (Figure 3c). Once this was achieved and the tone of the tissue was increased by histamine (0.5 μM, ~EC_{70}, Figure 3d), short-term EFS induced reproducible relaxations consisting of a rapid loss of tone, which started immediately (< 1 s) after initiating the stimulus, and a slower recovery phase (Figure 3d,e).

In a different group of experiments, in which electrical stimulation was applied to tissues contracted with histamine (0.5 μM) but not treated with scopolamine or guanethidine, an initial relaxation, which occurred during the period of stimulation, followed by a longer lasting contraction was observed (not shown).

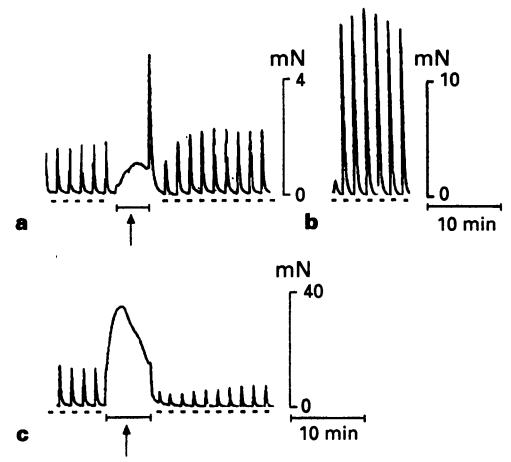

Figure 2 (a) Control contractile responses to short-term EFS (indicated by short lines) (5 Hz, 50 V, 0.3 ms duration, 5 s train duration, every 120 s) and long-term EFS (indicated by long line and arrow) (5 Hz, 50 V, 0.3 ms duration, 10 min). (b) Enhancement of short-term EFS-induced contractions by N^G-nitro-L-arginine (L-NOARG, 300 μM). (c) Contractile responses to short- and long-term EFS in the presence of L-NOARG (300 μM). The mechanogram is an original recording of the responses of one preparation and is representative of all experiments in this series ($n=4$).

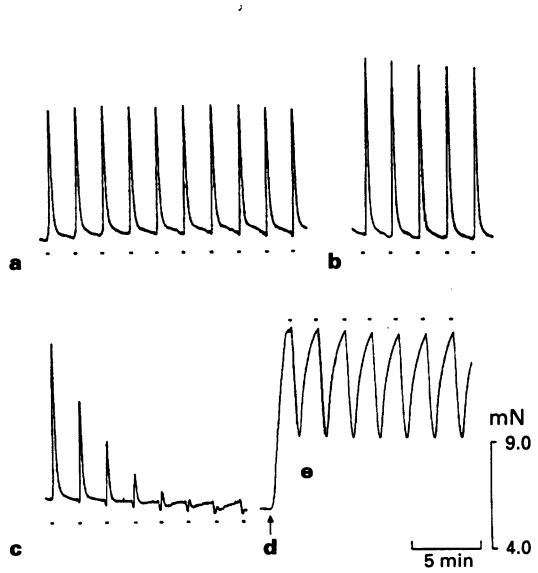

Figure 3 (a) Contractions elicited by short-term EFS (5 Hz, 50 V, 0.3 ms duration, 5 s train duration, every 120 s) in rabbit anococcygeus muscle. (b) Effect of scopolamine (10 μM) on contractions. (c) Inhibition of contractions by guanethidine (10 μM). (d) Histamine (0.5 μM)-induced increase in the tissue tone. (e) The relaxations produced by identical short-term EFS in the presence of scopolamine, guanethidine and histamine. Short lines indicate the time of EFS. The mechanogram is an original recording of the responses of one preparation and is representative of all experiments in this series ($n=60$).

Characterization of the short-term EFS-induced relaxation response in tissues treated with scopolamine, guanethidine and histamine The amplitude of the relaxation was frequency-dependent with a maximum relaxation observed at a frequency of 5 Hz.

Treatment of the tissues with L-NOARG, L-NAME and L-NIO (10 μM–500 μM) for 30 min prior to and during short-term EFS dose-dependently inhibited the relaxant responses to short-term EFS (maximum inhibition at 300 μM, 74.8 ± 9.6%, 58.3 ± 10.0% and 42.5 ± 5.4%, respectively). This inhibition could be prevented by pretreatment with L-, but not D-, Arg (500 μM) 30 min before the addition of inhibitors. The IC_{50} values for L-NOARG, L-NAME and L-NIO were 116 ± 19 μM, 163 ± 11 μM and > 500 μM respectively ($n=6$–8).

Effect of L-NOARG on the tone induced by histamine In tissues contracted with lower concentrations of histamine (0.25 μM, ~EC_{40}), L-NOARG (500 μM) elicited an increase in the tone of 182.5 ± 17.5% ($n=4$).

Characterization of the long-term EFS-induced relaxation response in tissues treated with scopolamine, guanethidine and histamine Long-term EFS induced a steady state relaxation followed by a rebound contraction on termination of the stimulation (Figure 4a). After the tissue had regained its original tone, the responses to short-term EFS were unchanged. The steady state relaxation remained unchanged for as long as the stimulus was applied (up to 2 h, not shown, $n=4$).

Infusion of L-NOARG (300 μM) during long-term EFS caused a rapid and complete reversal of relaxation which was followed by a rebound contraction on termination of stimulation. This inhibition of relaxation did not occur if L-Arg (1 mM) was present in the perfusion fluid (Figure 4d). The IC_{50} for L-NOARG was 56.2 ± 6.2 μM ($n=6$).

Effect of L-arginine

In the presence of L-Arg (500 μM), the baseline tone and the amplitude of relaxations to both short and long-term EFS were similar to control preparations (Figure 4a,b). However, when the tissue was stimulated continuously with an EFS of lower frequency (0.08 Hz, 0.3 ms duration, 50 V) to maintain the tone of the tissue at ~50% of that of its maximal potential relaxation, L-Arg (500 μM), but not D-Arg, caused a further fall in the tone (86.0 ± 7.4%) without significantly affecting the magnitude of these relaxations (13.3 ± 8.1% reduction, $P=0.13$) (Figure 5) ($n=4$).

Effect of an NO scavenger

Infusion of oxyhaemoglobin during long-term EFS inhibited the relaxation responses dose-dependently. Concentrations of 1, 2.5, 7.5 and 15 μM oxyhaemoglobin inhibited the relaxations by 0.5 ± 0.5%, 22.6 ± 4.1%, 37.1 ± 9.1% and 46.4 ± 2.9% ($n=6$) respectively.

Effect of sodium nitroprusside

Sodium nitroprusside (SNP) relaxed the preparation dose-dependently in the presence of guanethidine, scopolamine and histamine. The EC_{50} for SNP was 73.0 ± 10.5 nM. At a con-

centration of 1 μM, SNP caused a relaxation of the same magnitude as that elicited by short-term EFS (2.5 Hz, 0.3 ms duration, 50 V, for 5 s) ($n=4$).

Effects of neurotoxins

Treatment of the tissue with TTX (2 μM) for 15 min abolished the relaxation responses to short-term EFS. During long-term EFS, addition of TTX (3 μM) increased the tone, abolished the rebound contraction and completely prevented the relaxation to short-term EFS (not shown).

Infusion of ω-conotoxin GVIA (CTX, 0.5 μM) during long-term EFS caused a transient contractile response followed by loss of tone and a rebound contraction on termination of long-term EFS. When the original tone was regained, short-term EFS caused 75.5±2.8% smaller relaxation than during control conditions (not shown).

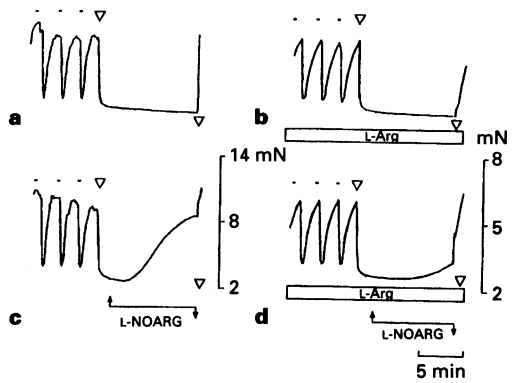

Figure 4 (a and b) Relaxation responses to long-term EFS in the absence (a) and presence (b) of L-arginine (L-Arg, 1 mM). (c) The effect of N^G-nitro-L-arginine (L-NOARG, 300 μM) on relaxation induced by long-term EFS (2.5 Hz, 50 V, 0.3 ms duration, 15 min train duration) in tissue (a). (d) The effect of L-NOARG (300 μM) on relaxation induced by long-term EFS in the presence of L-Arg (1 mM) in tissue (b) (△) Indicate the initiation and termination of long-term EFS. Short lines indicate the time of short-term EFS. Arrow bars denote the infusion of L-NOARG. The mechanograms are original recordings of the responses of two separate preparations and are representative of all experiments in this series ($n=10$).

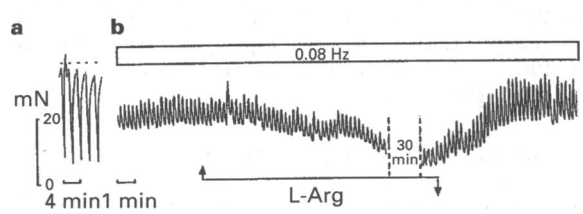

Figure 5 (a) Control relaxation responses elicited by short-term EFS (2.5 Hz, 50 V, 0.3 ms duration, 5 s train duration, every 120 s). (b) The same tissue was stimulated continuously with an EFS of lower frequency (0.08 Hz, 0.3 ms duration, 50 V) to maintain the tone of the tissue at a magnitude half that of control tissues. L-Arginine (L-Arg, 500 μM) induced further relaxation of the tissue without significant change in the amplitude of the responses to short-term EFS. Short lines in (a) indicate the time of short-term EFS. The mechanogram is an original recording of the responses of the preparation and is representative of all experiments in this series ($n=4$).

Effects of ATP and VIP

Addition of ATP (100 μM) or VIP (50 nM) caused a relaxation that was reversible following termination of the infusion. L-NOARG (300 μM) did not affect the responses to these agents (not shown).

NO_x concentrations

During short-term EFS, the concentrations of NO_x in the tissue effluent, measured as pmol g^{-1} s^{-1}, were significantly elevated 15 s after the initiation of the 5 s stimulation period. They decreased thereafter towards basal concentration, which was usually achieved at ~120 s after initiation of the EFS. This release was significantly inhibited by treatment with L-NIO (1 mM) and TTX (2 μM) (Figure 6).

The concentrations of NO_x following short-term and long-term EFS were significantly elevated compared with basal concentrations (Figures 6 and 7) ($P<0.01$, for paired observations). The difference between NO_x concentrations elicited by short- and long-term EFS was not significant ($P>0.05$, for paired observations). NO_x concentrations measured in the first 5 min of long-term EFS were not significantly

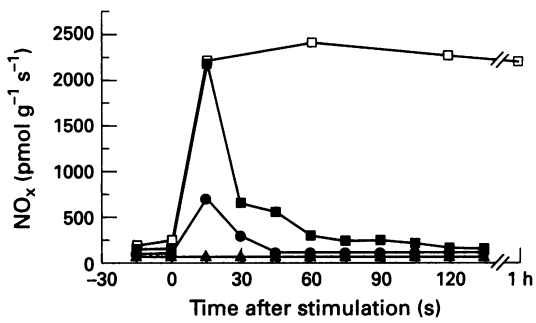

Figure 6 Time course of NO_x concentrations in the perfusate after short-term EFS (2.5 Hz, 50 V, 0.3 ms duration, 5 s train duration) and long-term EFS (2.5 Hz, 50 V, 0.3 ms duration, 60 min train duration) in the absence (control) and presence of N-iminoethyl-L-ornithine (L-NIO, 1 mM) or tetrodotoxin (TTX, 2 μM). (■) Control, short-term EFS; (●) short term EFS + L-NIO; (▲) short-term EFS + TTX; (□) control, long-term EFS. The figure represents the values of one preparation and is representative of all experiments in this series ($n=8$–12).

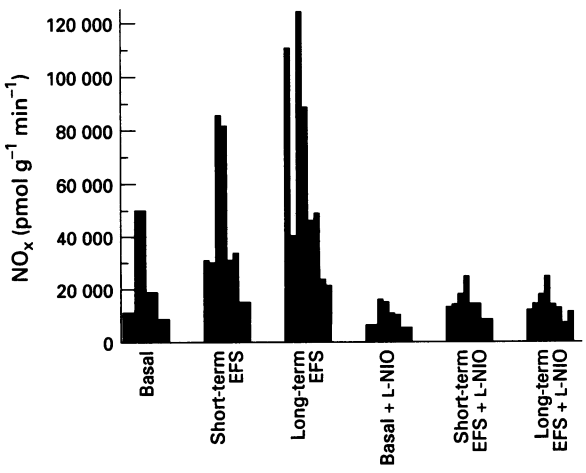

Figure 7 Basal NO_x concentrations and nerve-evoked release of NO_x by short- and long-term EFS in the absence and presence of N-iminoethyl-L-ornithine (L-NIO, 1 mM). The individual results from 8 separate preparations are shown consecutively in each group.

different from those measured at the 30th and 60th min of long-term EFS ($P > 0.05$, for paired observations). In the presence of L-NIO (1 mM) and TTX (2 μM), basal NO_x concentration and NO_x release following short-term and long-term EFS were decreased significantly ($P < 0.05$, for paired observations). The basal NO_x concentrations and those released by short- and long-term EFS in the absence and presence of L-NIO for each single experiment are presented in Figure 7. Analysis of variance for these results showed that the effect of EFS or treatment with inhibitors was significant in spite of the high variability within the groups ($F = 11.9$ and $P = 0.0009$).

Discussion

We have shown that short-term EFS (< 60 s) of untreated rabbit anococcygeus muscle induces a reproducible, monophasic, contractile response which starts at the end of the stimulation period and lasts for approximately 45 s. Long-term EFS (> 1 min), on the other hand, induces a smaller but sustained contractile response which lasts for the duration of stimulus and is followed by a sharp further contraction after cessation of electrical stimulation. These contractions are mediated by the release of noradrenaline (Gillespie, 1980) and down-regulated by concomitant release of NO, since contractions to both short-and long-term EFS were enhanced by treatment of the preparation with an inhibitor of NO synthase, which also abolished the lag period between stimulation and contraction. That the lag period is likely to be due to NO release is further supported by the fact that, in preparations in which tone is increased by treatment with histamine, the contraction due to EFS is preceded by a relaxation which lasts for the duration of the stimulus. Modulation of autonomic neurotransmission by concomitant release of NO has been reported to occur in several preparations (Li & Rand, 1989; Baccari *et al.*, 1994; Kilbinger & Wolf, 1994). NO might be acting prejunctionally by inhibiting the release of autonomic neurotransmitters, as has been suggested previously (Cohen & Weisbrod, 1988) or it could be acting postjunctionally by a direct effect on the smooth muscle (Kasakov *et al.*, 1994). We did not attempt to determine the site of the modulatory action of NO; however, what is evident is that the down-regulation by NO is extremely effective and able to override the sympathetic response to a large extent.

Contractile responses due to short-term EFS were enhanced by treatment with scopolamine. This phenomenon, which has been reported previously in rabbit jejunum (Manber & Gershon, 1979), is due to reversal of inhibition of catecholamine release induced by acetylcholine on presynaptic muscarinic receptors. Further treatment of the preparation with guanethidine abolished the contractile responses, thus confirming their sympathetic nature (Gillespie, 1980).

The remaining relaxant response to EFS following guanethidine and scopolamine treatment in histamine-contracted preparations is nitrergic in nature, since it could be blocked by inhibitors of NO synthase (L-NOARG, L-NAME, L-NIO) and by oxyhaemoglobin. Furthermore, this response is neuronal in origin, since treatment with TTX reversed the relaxation. Interestingly, the preparation remained relaxed as long as nerve stimulation was applied (up to 2 h) without any sign of exhaustion. Moreover, elevated NO_x concentrations in the effluent were present as long as EFS was maintained.

ω-Conotoxin GVIA (CTX) is a potent and specific inhibitor of the N-type voltage-sensitive Ca^{2+} channels in neurones but not in smooth muscle (Miller, 1987) and it has been shown to block EFS-induced non-adrenergic, non-cholinergic relaxation in the guinea-pig taenia caeci, rat gastric fundus and rat anococcygeus muscle (De Luca *et al.*, 1990) and reduce the release of nitrergic neurotransmitter in response to EFS from canine ileocolonic junction (Boeckxstaens *et al.*, 1993). In our study, CTX reduced both short- and long-term EFS-induced relaxations, indicating that EFS-induced relaxations in our preparations are evoked by presynaptic Ca^{2+}-dependent release of NO. This might suggest that CTX inhibits either Ca^{2+}-dependent exocytosis of NO from synaptic vesicles, as has been postulated by others (Li & Rand, 1989), or stimulation of presynaptic Ca^{2+}-dependent NO synthase. It is unlikely that the former is the case since, if NO is stored in the form of a precursor in vesicles, its release would be expected to be exhausted during long-term stimulation of *in vitro* preparations, as has been shown for other neurotransmitters (Kernell & Sedvall, 1964; Chang & Chang, 1965).

The potencies of NO synthase inhibitors on relaxations elicited by short-term EFS were found to be different, so that L-NOARG > L-NAME > L-NIO. This rank order of potencies is similar to that observed for inhibition of neuronal NO synthesis from rat brain homogenates (R.G. Knowles, personal communication); however, in our study higher concentrations of inhibitors were required to produce maximum inhibition. This difference might be due to low accessibility of inhibitors into intact tissue. L-NOARG and L-NAME (both at 500 μM) inhibited the relaxations induced by short-term EFS by 74.8 ± 9.6% and 60.0 ± 3.8% respectively, while these same concentrations completely inhibited the relaxations induced by long-term EFS. The IC_{50} values of L-NOARG for short- and long-term EFS were also found to be different. The reason why NO synthase inhibitors are more potent during long-term EFS requires further investigation.

Infusion of L-Arg during long-term EFS to preparations which were relaxed to ~50% of their potential relaxation caused a fall in the tone of the tissue. A similar effect of L-Arg has been observed by others in rat (Gibson *et al.*, 1990) and mouse anococcygeus muscle (Li & Rand, 1989). This finding demonstrates that, when stimulated, the L-Arg: NO pathway can utilize exogenously-administered L-Arg as a substrate.

ATP caused relaxation of the preparation which was not affected by L-NOARG. This suggests that the relaxations induced by this nucleotide are not NO-mediated. It has been shown that ATP relaxes rabbit anococcygeus muscle via P_2 purinoceptors and nitrergic nerve-mediated responses have been reported not to be affected by purinoceptor antagonists (Graham & Sneddon, 1993). These findings, considered together, indicate that stimulation of nitrergic neurones in the rabbit anococcygeus muscle does not cause release of ATP and that ATP does not induce NO-dependent relaxation. It has been shown that VIP released from neurones is capable of stimulating NO production from muscle cells (Grider *et al.*, 1992). Our finding that relaxation elicited by VIP was not affected by L-NOARG indicates that VIP does not induce release of NO in the rabbit anococcygeus muscle. We conclude therefore that ATP and VIP are not involved as final neurotransmitters in this muscle.

In summary, we have shown that the release of nitrergic transmitter by EFS is non-exhaustible and correlates with a continuous relaxation of the preparation. Furthermore, the basal tone of the tissue could be raised by treatment with L-NOARG and the detected basal release of NO was also inhibited by L-NIO and TTX. We therefore conclude that NO is released constantly from the nerve endings, thus maintaining a sustained relaxant tone in the smooth muscle, as suggested by previous studies (Ward *et al.*, 1992; Wiklund *et al.*, 1993). This is akin to the tone maintained in the vasculature by NO released from the vascular endothelium (Rees *et al.*, 1989; Cederqvist *et al.*, 1991) and probably from perivascular nitrergic nerves (Toda & Okamura, 1992). The physiological regulators of this basal tone remain to be studied in detail.

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