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Evidence against nitrergic neuromodulation in the rat vas deferens

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Abstract

Electrical field stimulation (60 V, 1 ms, single pulses or 20 s trains of 1–10 Hz) of the nerve terminals within the rat vas deferens produced biphasic contractions in preparations oriented to measure either longitudinal or circular muscle contractions. In confirmation of earlier reports, these contractions were blocked by tetrodotoxin (1 μM). The initial fast purinergic contraction was dominant in prostatic halves of the vas deferens while the second slower noradrenergic contraction was greater in epididymal halves. Although previous studies have shown nitric oxide synthase immuno-positive nerves in the vas deferens, electrical field stimulation-induced contractions were unaffected by L-arginine, sodium nitroprusside, *N*-nitro-L-arginine methyl ester (L-NAME) or superoxide dismutase in concentrations up to 1 mM. In concentrations above 1 mM, L-NAME reduced the size of the field stimulation-induced contractions but this effect could not be reversed by either L-arginine or sodium nitroprusside. Furthermore, L-arginine, sodium nitroprusside and L-NAME did not affect the contractions induced by exogenous application of noradrenaline (10 μM), ATP (1 mM) or $BaCl_2$ (1–10 mM). We conclude that nitric oxide does not act as a neuromodulator in isolated preparations of rat vas deferens. © 1997 Elsevier Science B.V.

Keywords: Vas deferens, rat; Nitric oxide (NO); L-Arginine; Sodium nitroprusside; *N*-nitro-L-arginine methyl ester (L-NAME); (Neurotransmission)

1. Introduction

There is ample evidence that nitric oxide is involved in neurotransmission in a variety of peripheral tissues where it has generally been shown to have inhibitory effects (Rand, 1992; Lincoln et al., 1997).

There is immunohistochemical evidence that nitric oxide synthase exists in the nerves innervating the rat vas deferens (Ceccatelli et al., 1994; Ventura and Burnstock, 1996) and in the nerve fibres innervating the guinea-pig vas deferens stained with nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase (Song et al., 1994). In our previous studies we showed that there is a regional variation in the distribution of nitric oxide synthase-immunoreactive nerve fibres with their density being greater in the prostatic end than in the epididymal end of the tissue (Ventura and Burnstock, 1996). Similar observations have been made in the rat vas deferens with other classes of nerves such as acetylcholinesterase-containing nerves (Dixon and Gosling, 1972) and peptide-containing nerves (Lamano Carvalho et al., 1986).

The rodent vas deferens is a sympathetically innervated tissue with noradrenaline and ATP being the principal excitatory neurotransmitters (Burnstock and Sneddon, 1985). Recently nitric oxide has also been implicated in the neurotransmitter processes of the rat vas deferens, but although nitric oxide has been shown to play a role in the sexual function of male rats (Hull et al., 1994), the effects of drugs affecting nitrergic mechanisms in the rodent vas deferens are still uncertain. Nitric oxide has been proposed to participate in excitatory neurotransmission in the rat vas deferens (Vladimirova et al., 1994). Their proposal was based on pharmacological experiments that claimed that the nitric oxide synthase inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) reduced the size of electrically-evoked contractions by a mechanism which can be reversed by administration of the nitric oxide precursor, L-arginine. In the guinea-pig vas deferens exogenous nitric oxide has been shown to affect neurotransmission in a more complex fashion without altering noradrenaline release (Cederqvist and Gustaffsson, 1994; Cederqvist et al., 1994). Excitatory effects of the nitric oxide donor sodium nitroprusside have been reported in isolated guinea-pig vas deferens preparations, where sodium nitroprusside has been reported to induce spontaneous contractions and potentiate the contractions induced by noradrenaline, carbachol and K^+ (Sunano, 1983). On the other hand, K^+-induced contrac-

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tions have also been reported to be inhibited by sodium nitroprusside in the rat vas deferens (Schultz et al., 1977). Meanwhile, others report that sodium nitroprusside has no effect on contractions induced by noradrenaline, phenylephrine or K^+ (Kreye et al., 1975; Diamond and Janis, 1978). Furthermore, the inhibitory effects of the nitric oxide synthase inhibitor, 7-nitroindazole on the rat vas deferens, have been reported to be unrelated to the inhibition of nitric oxide synthase (Allawi et al., 1994) and biochemical studies have also reported that the rat vas deferens does not show any nitric oxide synthase activity whatsoever (Mitchell et al., 1991).

Pharmacological experiments have shown a regional variation in the responses of the vas deferens to electrical field stimulation (Duncan and McGrath, 1976; Anton et al., 1977; Brown et al., 1979) and exogenously added drugs (Kasuya and Suzuki, 1979; French and Scott, 1983). These differences in response along the length of the vas deferens may well be a consequence of regional variation in the distribution of various nerve types along the length of this tissue. Using the two ends of the rat vas deferens separately, the pharmacological studies presented here attempted to determine whether nitric oxide was involved in neurotransmission in the rat vas deferens and whether the responses to drugs affecting nitrergic mechanisms varied in different regions of the rat vas deferens. Regional variation in the responses may explain the conflicting results obtained in earlier studies using whole segments of the rat vas deferens.

2. Materials and methods

2.1. Isolated organ bath experiments

Male Sprague–Dawley rats, weighing approx. 250 g, were killed by CO_2 overdose. The abdomen was opened and the vasa deferentia from each side of the animal were removed. The vasa deferentia were divided into prostatic and epididymal halves of 1–2 cm in length. In some experiments the vasa deferentia were cut longitudinally into strips and the epithelium peeled away from one vas deferens while the other longitudinally split vas deferens was left intact to serve as a control. The longitudinal strips of vas deferens were then divided into prostatic and epididymal portions.

In order to study the effects of drugs and nerve stimulation the sections were mounted in separate 10 ml organ baths. Organ baths contained a modified Krebs–Bülbring solution of the following composition (mM): NaCl 133.0, KCl 4.7, $NaHCO_3$ 16.4, $MgSO_4$ 0.6, NaH_2PO_4 1.4, glucose 7.7 and $CaCl_2$ 2.5. This solution was maintained at 37°C and bubbled with 5% CO_2 in 95% O_2. Preparations were attached by a thread through two platinum ring electrodes to a tissue holder at one end. The other end of each preparation was attached by another thread to an FT03C transducer and isometric contractions were displayed on a Grass polygraph (Model 79D). Tissues were positioned so that the longitudinal muscle fibres were oriented vertically under approximately 1.0 g initial resting force. In some experiments, two entomological pins were threaded through the lumen so that the preparations could be suspended as rings with the circular muscle oriented vertically between two platinum electrodes. Preparations were allowed to equilibrate for 60 min, before the effects of drugs affecting nitrergic mechanisms or of electrical field stimulation were investigated.

2.2. Responses to electrical field stimulation

Intramural nerve terminals were field stimulated by pulses applied from a Grass SD9 stimulator via two platinum electrodes (10 mm apart) incorporated in the tissue holder. The stimulation parameters were 60 V and 1.0 ms duration. Stimulation was either by single pulses or by trains at a frequency of 1 or 10 Hz for 20 s every 20 min. Control responses to electrical field stimulation were first obtained, then the drugs to be tested were added to the organ baths sequentially 1 min before each train of stimuli. Tissues were washed with four times the bath volume following each train of stimulation. The height of the electrically evoked contractions in the presence of the various concentrations of drugs were compared with the control responses. Time control experiments were run concurrently with some of the drug tests. At the conclusion of some experiments the voltage-dependant Na^+ channel blocker tetrodotoxin (1 μM) was added to the organ bath to determine whether the electrically evoked contractions were of a neuronal origin.

Pilot experiments in which drugs were added at differing times before stimulation, indicated that the effects of nitrergic drugs achieved their maximum effect within 20 s of drug administration, and that the response remained stable for up to 5 min.

2.3. Postjunctional effects

In order to test whether the nitrergic agents were acting postjunctionally, in some groups of experiments the trains of field stimulation were omitted and standard concentrations of either noradrenaline (10 μM, approximate EC_{75}) or ATP (1 mM, approximate EC_{50}) were added. In experiments where the nitrergic agents were tested against contractions to $BaCl_2$, $BaCl_2$ (1–10 mM) was added to the organ bath and nitrergic agents were administered during the $BaCl_2$-induced tonic contraction.

2.4. Superoxide dismutase

Experiments using superoxide dismutase which increases available nitric oxide (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986) were carried out with su-

peroxide dismutase (30 U/ml) in the bathing solution for the duration of the experiment.

2.5. Data and statistical analysis

From each series of experiments, responses to electrical field stimulation, noradrenaline or ATP were expressed as mean contraction ± S.E.M. Fast twitch and tonic phase responses to electrical field stimulation were both evaluated in this manner. Analysis of variance was used to evaluate the significance of differences between mean values. In all cases the criterion of statistical significance was taken as $P < 0.05$. Mean log concentration–response curves were constructed by pooling data from individual log concentration–response curves.

2.6. Drugs

The following drugs were used: adenosine 5′-triphosphate (disodium salt) (ATP), L-arginine hydrochloride, L-arterenol bitartrate (noradrenaline), N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), sodium nitroprusside, superoxide dismutase and tetrodotoxin. All these agents were obtained from Sigma (Poole, UK).

Noradrenaline was dissolved and diluted to the required concentrations in ascorbic acid (0.1 mM). All other drugs were dissolved and diluted to the required concentrations in distilled water.

3. Results

3.1. Longitudinal muscle preparations

3.1.1. Effects of electrical field stimulation

Electrical field stimulation of isolated intact vas deferens segments, using the protocol outlined in the methods, caused biphasic contractions of the isolated segments. Following a single pulse stimulation, this contraction was made up of two distinct peaks. The initial faster peak was

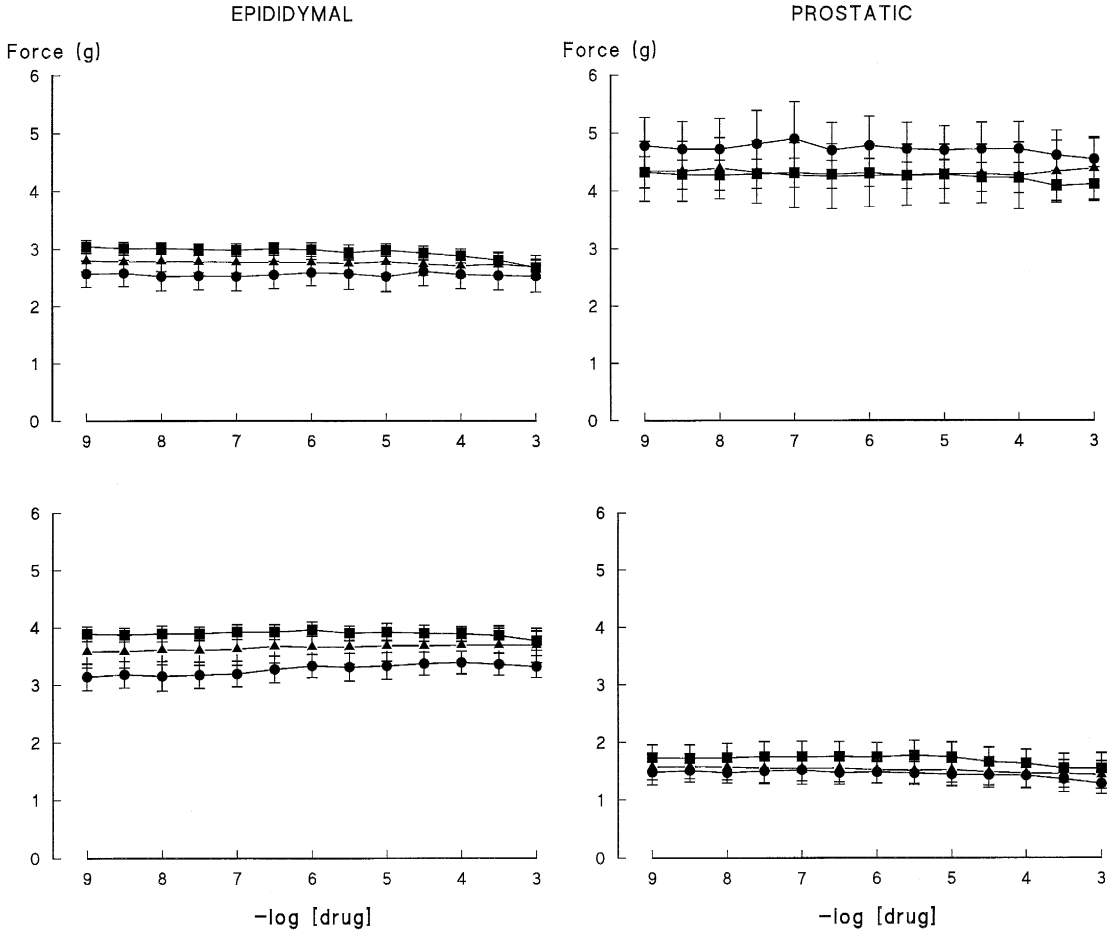

Fig. 1. Mean-log concentration–response curves for: L-NAME (●), L-arginine (▲) or sodium nitroprusside (■) applied sequentially to epididymal segments (left panels) or prostatic segments (right panels) of isolated rat vas deferens preparations, 1 min before trains of electrical field stimulation (10 Hz, 1 ms, 60 V, 20 s trains delivered every 20 min). Points represent the means of estimates for the height of the fast initial twitch phase (upper panels) and for the slower tonic second phase (lower panels) from six experiments. Bars represent S.E.M.

dominant in preparations of the prostatic half of the vas deferens while in the epididymal half of the vas deferens the second slower peak was more prominent. During trains of either 1 or 10 Hz stimulation, the biphasic contraction consisted of a fast twitch contraction followed by a slower tonic contraction. Longitudinally opened strips of vas deferens yielded similar contractions to that seen with intact tissues, although the size of the contractions were reduced by approximately 30–40% at all test parameters and in both halves of the vas deferens. Epithelium removal produced no further change in response. The magnitude of the electrically evoked contractions remained unchanged throughout the duration of the experiments (4–5 h), and were totally abolished by tetrodotoxin administration (1 μM).

3.1.2. Effects of L-NAME

L-NAME (1 nM–1 mM) was without effect on the magnitude of contractions evoked by electrical field stimulation of isolated intact preparations of both the epididymal and prostatic halves of the vas deferens (Fig. 1, $P > 0.05$, $n = 6$ for each test). At concentrations above 1 mM, the second peak of the contraction generated by single pulse stimulation and both phases of the contraction yielded by trains of stimulation were inhibited (Fig. 2, $P < 0.05$, $n = 6$). This inhibition caused by very high concentrations of L-NAME (3–10 mM) could not be reversed by treatment of the tissues with either the nitric oxide precursor L-arginine (1–10 mM) or the nitric oxide donor sodium nitroprusside (0.1 mM) (Fig. 2, $P > 0.05$, $n = 6$). Addition of concentrations of L-arginine greater than 10 mM potentiated the inhibition induced by high concentrations of L-NAME rather than reversing this effect. Similar effects of L-NAME were obtained using longitudinal strips of the vas deferens halves and also in longitudinal strip preparations with the epithelium removed.

3.1.3. Effects of L-arginine or sodium nitroprusside

Addition of L-arginine or sodium nitroprusside (both 1 nM–1 mM) to the isolated vas deferens preparations did not affect the contractile responses to field stimulation in either half of intact vas deferens preparations (Fig. 1, $P > 0.05$, $n = 6$ for each test). Electrically-evoked contractions of longitudinal strip preparations with or without the epithelium were also unaltered by the administration of either L-arginine or sodium nitroprusside ($P > 0.05$, $n = 6$ for each test). Concentrations of L-arginine greater than 1 mM (3–10 mM) also had no effect on the electrically-evoked contractions of either prostatic or epididymal halves of the vas deferens ($P > 0.05$, $n = 6$), whereas concentrations of sodium nitroprusside above 1 mM (3–10 mM) had a slight inhibitory effect which was insensitive to incubation of the tissues with L-NAME (0.1 mM) ($P < 0.05$, $n = 6$).

3.1.4. Postjunctional effects

L-NAME, L-arginine or sodium nitroprusside (all 1 nM–1 mM) did not affect the excitatory responses of either half of the vas deferens in intact or longitudinal strip preparations with or without the epithelium attached, when contracted by exogenous application of either noradrena-

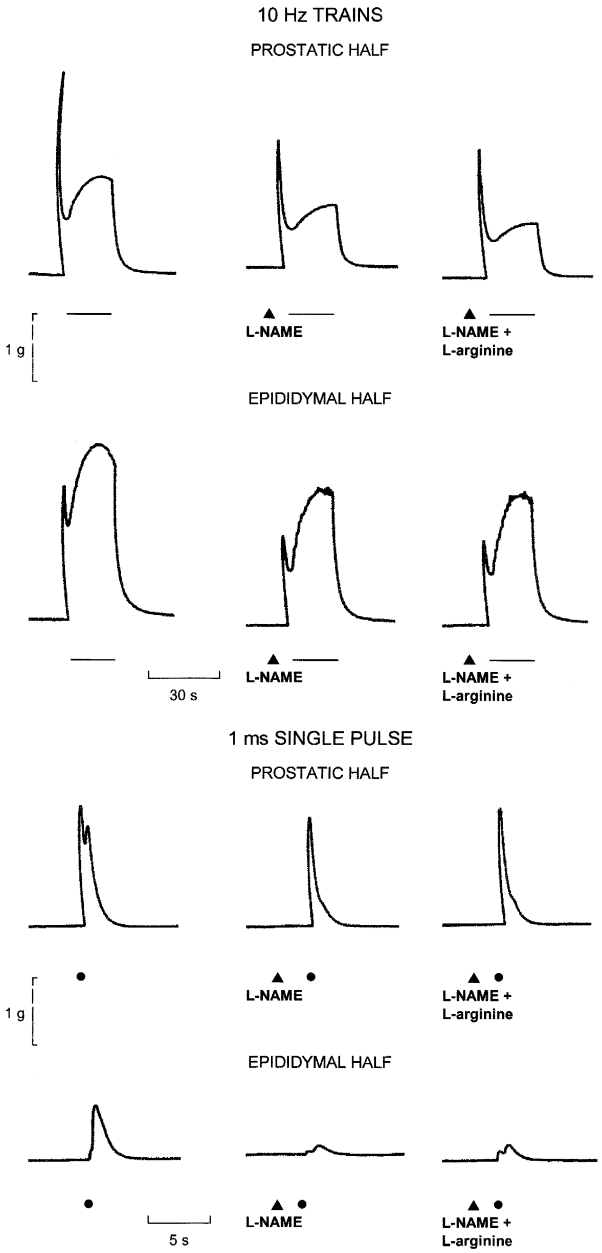

Fig. 2. Typical traces showing the effects of a high concentration of L-NAME (3 mM) (▲) or L-NAME (3 mM) and L-arginine (0.1 mM) in combination (▲) on responses to trains of electrical field stimulation (————; 10 Hz, 1 ms, 60 V, for 20 s) or single pulse field stimulation (●; 1 ms, 60 V) of isolated preparations of prostatic and epididymal halves of the rat vas deferens. Note that, while 3 mM L-NAME reduced the field stimulation-induced responses, this was not reversed by L-arginine.

line (10 μM) (Fig. 3) or ATP (1 mM) (Fig. 4, $P > 0.05$, $n = 6$ for each test).

3.1.5. Effects of superoxide dismutase

Incubation of preparations with superoxide dismutase (30 U/ml) did not affect the control responses to any of the test stimuli of any of the test tissues ($P > 0.05$, $n = 6$). Addition of superoxide dismutase also had no effect on the action of exogenous application of either L-arginine or sodium nitroprusside which were still inactive on the responses of the tissues ($P > 0.05$, $n = 6$).

3.2. Circular muscle preparations

As with the longitudinal muscle preparations, circular muscle preparations showed no change in response to either electrical field stimulation (1 ms, 60 V, 10 Hz) or exogenously applied ATP (1 mM), noradrenaline (10 μM) or $BaCl_2$ (10 mM) following the administration of either L-arginine, sodium nitroprusside or L-NAME in concentrations up to 1 mM ($P > 0.05$, $n = 6$ in each case).

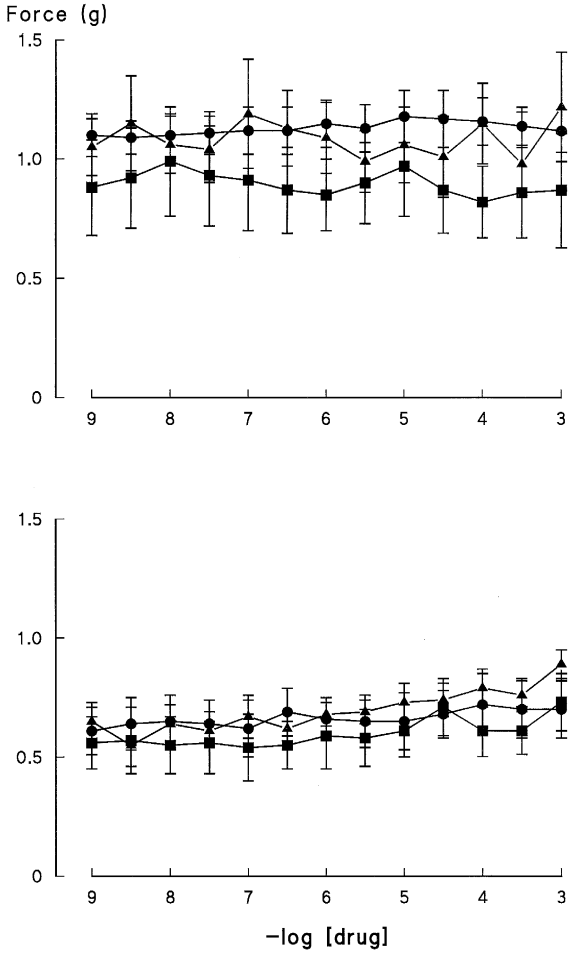

Fig. 3. Mean-log concentration–response curves for: L-NAME (●), L-arginine (▲) or sodium nitroprusside (■) applied sequentially to epididymal segments (upper panel) or prostatic segments (lower panel) of isolated rat vas deferens preparations, 1 min before exogenous application of noradrenaline (10 μM). Points represent the means of estimates for the height of contraction from six experiments. Bars represent S.E.M.

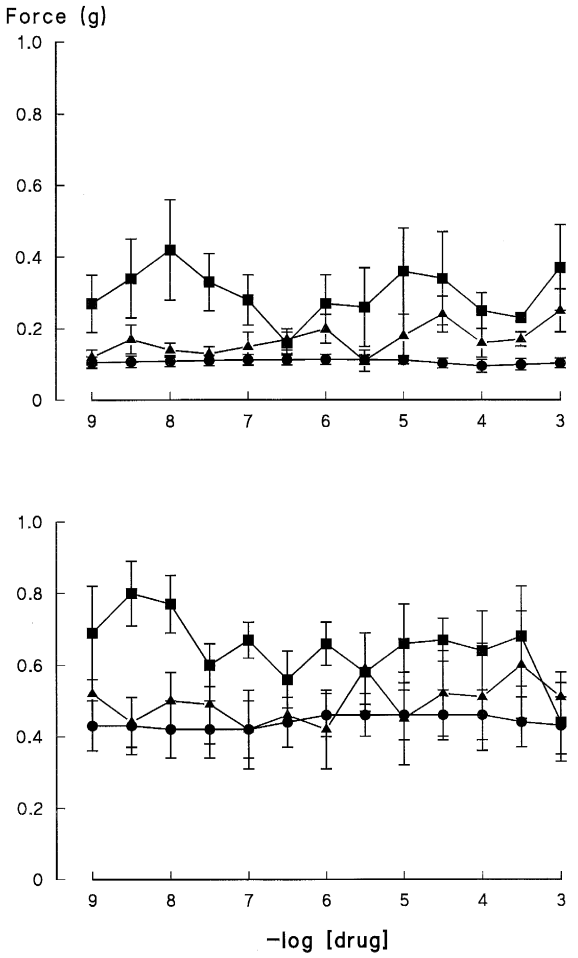

Fig. 4. Mean-log concentration–response curves for: L-NAME (●), L-arginine (▲) or sodium nitroprusside (■) applied sequentially to epididymal segments (upper panel) or prostatic segments (lower panel) of isolated rat vas deferens preparations, 1 min before exogenous application of ATP (1 mM). Points represent the means of estimates for the height of contraction from six experiments. Bars represent S.E.M.

4. Discussion

The results presented here show that drugs which affect nitrergic mechanisms do not appear to affect neurotransmission in the longitudinal or circular muscle of the isolated rat vas deferens. Contractions of vas deferens smooth muscle preparations oriented to show either contractions of the longitudinal or circular muscle layers evoked by electrical field stimulation or exogenous application of either noradrenaline or ATP were unaltered by either the nitric oxide synthase inhibitor L-NAME, the inhibitor of nitric oxide breakdown superoxide dismutase, the nitric oxide precursor L-arginine or the nitric oxide donor sodium nitroprusside. This suggests that despite the now well documented presence of nitric oxide synthase-im-

munoreactive nerves in this tissue (Ceccatelli et al., 1994; Ventura and Burnstock, 1996), their role does not appear to be a contractile one.

These results are also contrary to earlier pharmacological reports that show nitric oxide to have effects in isolated preparations of the vas deferens from rats (Vladimirova et al., 1994) or guinea pigs (Cederqvist and Gustaffsson, 1994; Cederqvist et al., 1994). In the guinea-pig vas deferens, nitric oxide was reported to play a complex role in neurotransmission, inhibiting the faster initial twitch response while enhancing the second slower tonic response (Cederqvist and Gustaffsson, 1994; Cederqvist et al., 1994). In isolated preparations of the rat vas deferens, it was reported that nitric oxide has an excitatory effect on trains of electrical field stimulation at 10 Hz (Vladimirova et al., 1994). This report was based on the ability of the nitric oxide synthase inhibitor L-NAME to reduce the size of the electrically-evoked contractions at a high concentration above 1 mM, an effect which was reversible by addition of L-arginine (0.1 mM) to the preparation. However, this was not strictly the case, since they reported that L-arginine often caused inhibition on its own, and preparations where this was the case were discarded, and not included in their results. Whilst we observed that L-NAME at concentrations above 1 mM were able to inhibit field stimulation-induced contractions of the rat vas deferens, we found this inhibition to be insensitive to reversal by L-arginine or sodium nitroprusside. Our conclusion was based on experiments in which L-arginine sometimes yielded a slight reversal, sometimes a slight potentiation and sometimes no change in the effects of high concentrations of L-NAME. The mean net result being that L-arginine had no effect on the inhibition of nerve stimulation induced contractions caused by high concentrations of L-NAME. The inhibitory effects of L-NAME may be due to a non-specific effect unrelated to nitric oxide synthase inhibition such as its previously reported muscarinic receptor antagonist action (Buxton et al., 1993).

Another possibility for the discrepancy in observations is that drugs have poor access to nitrergic nerves of intact vas deferens preparations as the nerves are located only in the inner muscle layers of the rat vas deferens (Ceccatelli et al., 1994; Ventura and Burnstock, 1996). To test this theory, we also used longitudinal strips similar to those used by Vladimirova et al. (1994). The similar insensitivity to these preparations of the nitrergic drugs showed that drug access was not a cause for the observed inactivity of drugs acting on nitrergic mechanisms.

Although, all of the functional studies conducted on the effects of drugs affecting nitrergic mechanisms to date have been on longitudinal muscle preparations of the vas deferens, it is still under debate whether the nitric oxide synthase-containing nerve fibres identified so far are present in the circular or longitudinal muscle layers of the rat vas deferens. All previous reports show nitric oxide synthase-immunoreactive nerve fibres to be present in the inner layer of the vas deferens muscle coat, but previous reports claim this to be in the circular muscle layer of the guinea pig (Song et al., 1994) while we have previously described these nerve fibres in the rat, to be present in an inner longitudinal muscle layer present only at the prostatic end of the vas deferens (Ventura and Burnstock, 1996). The results presented in this study give no indication that nitric oxide is involved in the contractility of either muscle layer, in either region of the rat vas deferens examined.

Nitric oxide synthase is believed to be colocalized with vasoactive intestinal polypeptide (VIP) and acetylcholine in the postganglionic parasympathetic nerve fibres of the rat vas deferens (Ceccatelli et al., 1994). The roles that acetylcholine as well as VIP play in the contractile mechanisms of the rat vas deferens are still unclear, as it now seems is the role of nitric oxide in contractility in this tissue. In fact it appears that nitric oxide may be without a role in the contractility of the vas deferens in the rat despite the presence of nitric oxide synthase in the nerves innervating the innermost muscle layers of this tissue. Given the distribution of nitric oxide synthase-immunoreactive nerve fibres in the innermost muscle layers and lamina propria of the vas deferens, just beneath the epithelium, it may be that nitric oxide is more likely to be involved in secretory processes of the epithelial cells to assist the passage of sperm through this duct or it may play an as yet unknown trophic role.

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