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Nitrergic modulation of cholinergic responses in the opossum lower oesophageal sphincter

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1 Electrical field stimulation (EFS) of the superfused lower oesophageal sphincter from opossum (*Monodelphis domestica*) elicited biphasic responses. The first phase (relaxation) was strictly dependent on the duration of the EFS. The second phase (contraction) started following termination of the EFS ($\leqslant$15 Hz). EFS at frequencies above 15 Hz led only to contraction, which started immediately upon initiation of the stimulation.

2 In the presence of N^G-nitro-L-arginine (L-NOARG; 0.1–300 μM), the relaxation phase was abolished and the contractile response started with the initiation of EFS (at all frequencies) and was greater in magnitude. The contractile response to EFS was completely blocked with scopolamine (10 μM).

3 Exogenous acetylcholine (1–100 μM) elicited concentration-dependent contractions of the sphincter in the presence of botulinum toxin. These contractions were abolished when EFS was applied during administration of acetylcholine. This inhibitory effect of EFS was completely reversed when the tissue was treated with L-NOARG (100 μM).

4 These results suggest that the cholinergic response in the opossum lower oesophageal sphincter is under nitrergic control.

Keywords: Nitrergic; opossum; oesophageal sphincter; cholinergic

Introduction

Early indications of the existence of non-adrenergic non-cholinergic (NANC) neurotransmission were obtained towards the end of the last century when 'atropine-resistant' excitation of the bladder in response to pelvic nerve stimulation was demonstrated (Langley & Anderson, 1895) and relaxation of the stomach during vagal nerve stimulation was revealed following atropine treatment (Langley, 1898).

Since the discovery of the biological actions of nitric oxide (NO; for a review, see Moncada *et al*., 1991), it has been shown that the L-arginine:NO pathway is involved in inhibitory NANC neurotransmission in many organs from different species (for a review, see Rand & Li, 1995). The term 'nitrergic', which applies to nerves whose transmitter function depends on the release of NO or to transmission mechanisms that are brought about by NO, has now been accepted for NO-mediated neurotransmission (Moncada *et al*., 1997).

In the human oesophagus, electrical field stimulation (EFS) has been shown to cause relaxation following inhibition of cholinergic transmission by atropine and elevation of the tone (Tottrup *et al*., 1990). This relaxation was later shown in the opossum oesophageal sphincter to be NO-dependent (Murray *et al*., 1991; Tottrup *et al*., 1991). Following administration of an NO synthase inhibitor, EFS-induced relaxations are inhibited, revealing an atropine-sensitive contractile response (Tottrup *et al*., 1991; Preiksaitis *et al*., 1994). Relaxation of the lower oesophageal sphincter induced by vagal stimulation and swallowing is also reduced by inhibitors of NO synthase (Paterson *et al*., 1992; Yamato *et al*., 1992). Moreover, the presence of nitrergic nerves and of the NO synthase has been demonstrated in this tissue (Fang & Christensen, 1994; Murray & Clark, 1994; Christensen *et al*., 1995). Thus, pharmacological, biochemical and morphological studies indicate that nitrergic transmission operates in the lower oesophageal sphincter as an inhibitory system.

Interaction between nitrergic and cholinergic systems has not been studied widely. A modulatory role for nitrergic transmission in intestinal motility has been suggested (Gustafsson *et al*., 1990; Knudsen & Tottrup, 1992). Inhibitors of NO synthase have been shown to enhance EFS-induced cholinergic contractions *in vitro* in the rat gastric fundus (Lefebvre *et al*., 1992), rabbit gastric muscle (Baccari *et al*., 1993) and guinea pig trachea (Belvisi *et al*., 1991; Brave *et al*., 1991) and to enhance vagal nerve stimulation-induced contractions of the rabbit stomach and proximal colon *in vivo* (Iversen *et al*., 1997).

In a previous study we described nitrergic control of noradrenergic responses in the rabbit anococcygeus muscle and rabbit and human corpus cavernosum (Cellek & Moncada, 1997). In these tissues, NO released from nitrergic nerves during EFS controls the action of concomitantly released noradrenaline, so that noradrenergic contraction starts only after termination of the EFS. In the present study we investigated whether the nitrergic control mechanism operates in the lower oesophageal sphincter, this time against the cholinergic system.

Methods

Male adult opossums (*Monodelphis domestica*, 100–150 g, supplied by Biological Services Unit, University College London, U.K.) were sacrificed by cervical dislocation. The distal oesophagus, together with the proximal part of the stomach, was excised and placed in modified Krebs' solution at room temperature. The composition of the modified Krebs' solution was (in 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, gassed with 5% CO_2 in O_2 (pH 7.4–7.6). A circular muscle strip (2 × 6 mm) from the gastro-oesophageal junction was isolated after removal of the adjacent connective tissue. Each preparation was placed horizontally between two platinum ring electrodes (4 mm diameter) in horizontal superfusion chambers which were constantly heated by circulating water at 37°C. The chambers were perfused with the modified Krebs' solution at a constant flow of 1.0 ml min^{-1} by means of peristaltic pumps (Minipuls 2, Gilson). 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

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in tension. The mechanical responses were also recorded on a computer using specialized software (Axotape, U.S.A.). A tension of 0.5 g was applied to the tissue, which was then allowed to equilibrate for 90 min. The preparations were stimulated electrically for 5 s to 10 min with trains of rectangular pulses of 50 V, 0.3 ms pulse duration and frequencies ranging from 1 to 25 Hz, delivered by Grass S88 stimulators. The concentration-response studies with N^G-nitro-L-arginine (L-NOARG) were performed by cumulative addition of the drug into the medium reservoir; the tissues were allowed to incubate with each concentration for at least 20 min. Acetylcholine was administered directly into the chamber at a rate of 50 μl in 30 s with a Hamilton syringe (Hamilton Co., U.S.A.).

Presentation of the results and statistics

Results are expressed as mean $\pm$ s.e.mean. Statistical analysis was performed using Student's t test. A probability value (P) < 0.05 was taken as significant.

Results are presented as a point or column plot where each point or column represents the mean of at least three separate experiments in that series. Vertical bars represent s.e.mean.

The contraction and relaxation responses were measured as area under the curve. The effect of L-NOARG on these responses is expressed as percentage of the control responses.

Chemicals

All chemicals used in the experiments were from Sigma, U.K.

Results

Characterisation of EFS-induced responses

The tissues developed spontaneous tension ($\sim$0.5 g) during the incubation period. This tension was not affected by either scopolamine (10 μM) or botulinum toxin A (0.1 μg ml^{-1}). In 11 of the 14 oesophageal sphincters studied, EFS (50 V, 1–15 Hz, 0.3 ms pulse duration for 5 s to 10 min) elicited a relaxation which was maintained for as long as the EFS was applied, even during periods of up to 10 min. The relaxation was followed by a contraction which started after the EFS was terminated (Figure 1a, b). In the remaining three tissues there was no response during EFS but a contraction was observed after termination of the stimulation. The optimum frequency for relaxation was 5 Hz. At frequencies above 15 Hz, however, EFS elicited a contractile response which started with the initiation of the stimulation. In all tissues the contractile response (at all frequencies) was blocked with scopolamine (10 μM) while tetrodotoxin (TTX, 2 μM) blocked both the relaxation and the contraction.

In the presence of L-NOARG (100 μM), the relaxation observed in 11 tissues was abolished and in all tissues a contraction started with the initiation of EFS (50 V, 0.3 ms pulse duration, 1–15 Hz, 5 s to 10 min) and was greater in magnitude (Figure 1a). The effect of L-NOARG was concentration-dependent ($EC_{50} = 46.1 \pm 8.5$ μM, $n = 4$ for the contractions; $IC_{50} = 17.7 \pm 2.4$ μM, $n = 4$ for the relaxations; $P < 0.05$; Figure 2). In the presence of 300 μM L-NOARG, the magnitude of contraction was 188.8 ± 13.0% of control ($n = 4$). The effect of 100 μM L-NOARG could be completely reversed by 250 μM L-arginine (data not shown).

The contractions elicited by EFS with frequencies above 15 Hz were also enhanced by L-NOARG. In the presence of 300 μM L-NOARG, the contraction was 201.2 ± 20.3% of control ($n = 3$).

Effect of EFS on acetylcholine-induced contractions

Following treatment of the oesophageal sphincter with botulinum toxin A (0.1 μg ml^{-1}), the contractile response to EFS was blocked but the relaxant phase was not affected. In the presence of botulinum toxin A, acetylcholine (1–100 μM) elicited concentration-dependent contractions ($n = 4$; Figures 3 and 4). When the same concentrations of acetylcholine were given during EFS, no contractile response was observed but there was a relaxation which lasted for as long as EFS was applied, followed by a sharp contraction upon termination ($n = 4$; Figure 3). In the presence of L-NOARG (300 μM), this effect of EFS was completely reversed and the sharp contraction was abolished ($n = 4$; Figures 3 and 4).

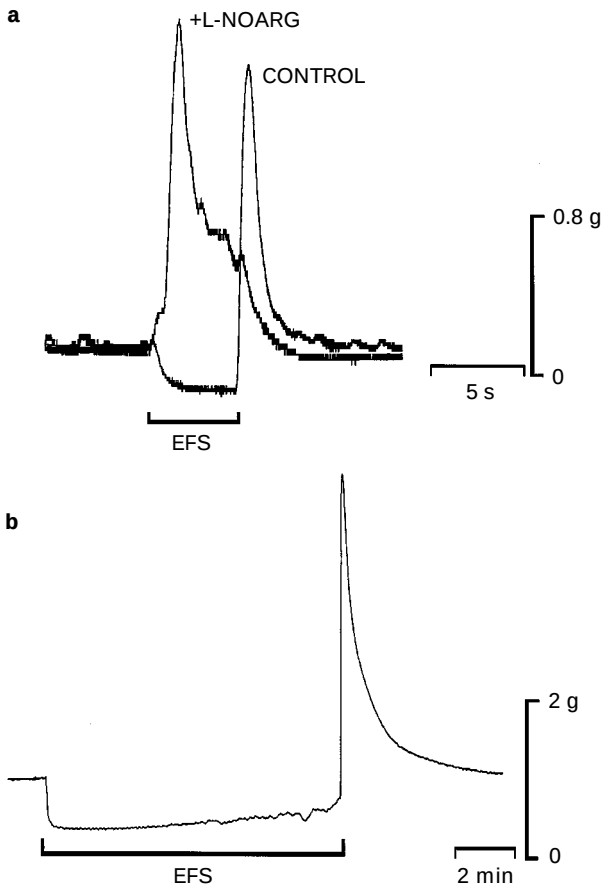

Figure 1 (a) Response of the opossum lower oesophageal sphincter elicited by EFS (50 V, 0.3 ms pulse duration, 5 Hz, for 5 s) in the absence and presence of 100 μM L-NOARG as indicated. (b) Response of the same tissue to EFS (50 V, 0.3 ms pulse duration, 5 Hz) for 10 min in control conditions. EFS is shown by horizontal bar. The mechanograms are original recordings of the responses of one preparation and are representative of all experiments in this series ($n = 14$).

Characterisation of nitrergic relaxations

After blockade of the cholinergic component with scopolamine (10 μM) and elevation of the tone with histamine (1 μM), EFS (50 V, 0.3 ms pulse duration, 1–25 Hz, for 5–10 s) elicited reproducible relaxation responses without a rebound contraction (data not shown). These relaxations were completely inhibited by L-NOARG (300 μM; $n = 3$) or TTX (2 μM; $n = 3$; data not shown).

Discussion

We have shown that EFS at low frequencies (< 15 Hz) led to a relaxation of the opossum lower oesophageal sphincter which lasted for as long as the stimulus was applied (up to 10 min) and was followed by a cholinergic contraction. In the presence of L-NOARG the contraction started immediately with EFS

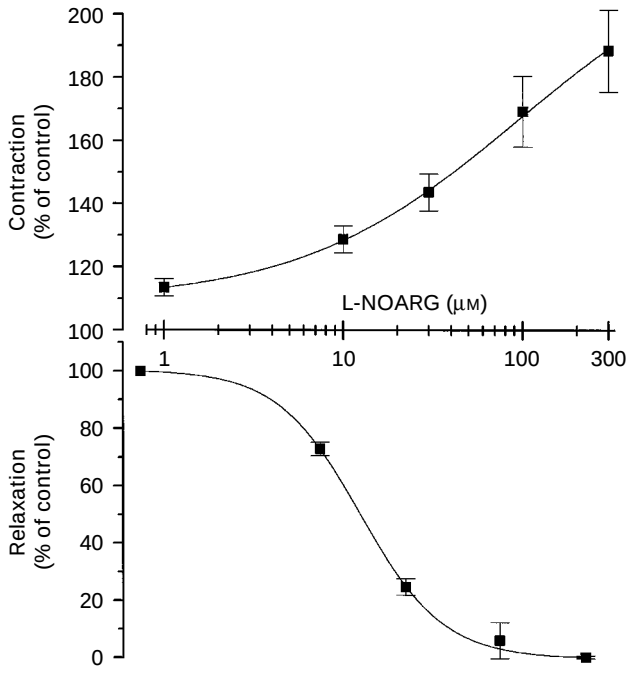

Figure 2 Effect of L-NOARG (1–300 μM) on the contraction (upper) and relaxation (lower) elicited by EFS (50 V, 0.3 ms pulse duration, 5 Hz, for 5 s) of the opossum lower oesophageal sphincter. Each point represents the mean of contraction or relaxation, measured as area under the curve. Vertical bars = s.e.mean ($n=4$).

and its magnitude was enhanced. The lack of contractile response during the stimulation period is due to release of NO, which also modulates the magnitude and the duration of the subsequent cholinergic contraction after EFS. The net biological response during the stimulation period is nitrergic rather than cholinergic in nature.

In order to investigate further this nitrergic control of cholinergic responses, we administered exogenous acetylcholine in pharmacological concentrations to tissues treated with botulinum toxin A. Acetylcholine elicited concentration-dependent contraction of the preparations. When EFS was applied during acetylcholine injections, the contraction to exogenous acetylcholine was abolished and a relaxation response was observed during the stimulation period; this effect was reversed completely in the presence of L-NOARG. This experiment shows that the nitrergic system is capable of suppressing the contractions elicited by even pharmacological concentrations of acetylcholine.

Spike-type contractions, observed after termination of EFS in the experiments with exogenous acetylcholine (see Figure 3b), were not cholinergic as they still occurred in the presence of botulinum toxin. Thus, the nature of this short lasting, spike-type contraction remains to be clarified.

In other studies, an 'off-contraction', which occurred only after cessation of the stimulation, has been reported in the opossum oesophagus (Lund & Christensen, 1969). This 'off-contraction' was TTX-sensitive but could not be inhibited by atropine, suggesting a NANC nature (Lund & Christensen, 1969). The magnitude of the 'off-contraction' and the delay between stimulation and its occurrence were reduced with NO synthase inhibitors, suggesting a role for nitrergic transmission in the regulation of this 'probably rebound contraction' (Knudsen *et al.*, 1991). In our study, however, we did not observe any NANC 'off-contraction' and the contractile responses that occurred after EFS in our experiments were inhibited by scopolamine or TTX.

Contractions elicited by EFS are enhanced by inhibitors of NO synthase *in vitro* in the rat gastric fundus (Lefebvre *et al.*, 1992), rabbit gastric muscle (Baccari *et al.*, 1993) and guinea

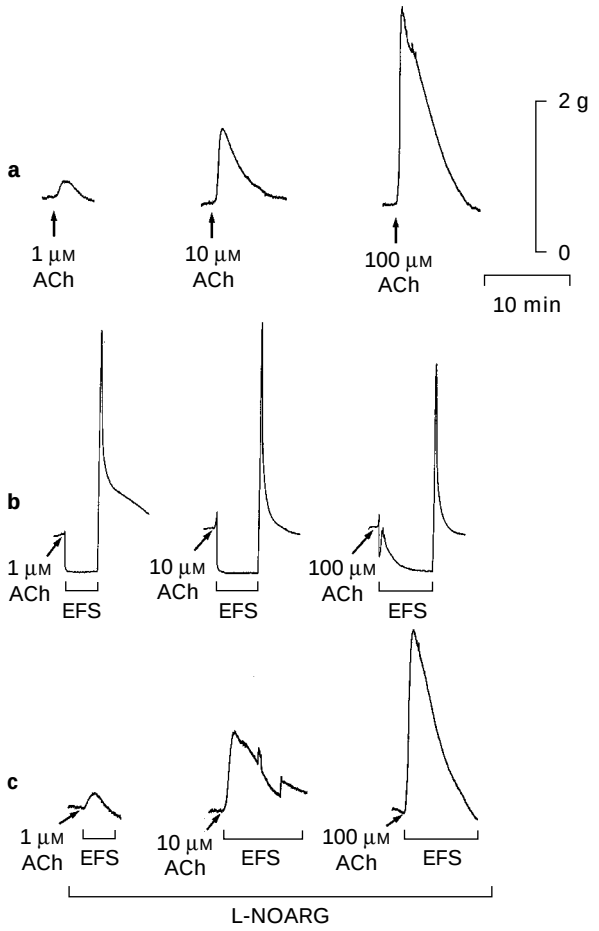

Figure 3 Effect of acetylcholine (1–100 μM; as indicated by arrows) on botulinum toxin A-treated tissue in control conditions (a), during application of EFS (50 V, 0.3 ms pulse duration, 5 Hz; as indicated by horizontal bars (b) and during application of EFS in the presence of L-NOARG (300 μM) (c). The mechanogram is an original recording of the responses of one preparation and is representative of all experiments in this series ($n=4$).

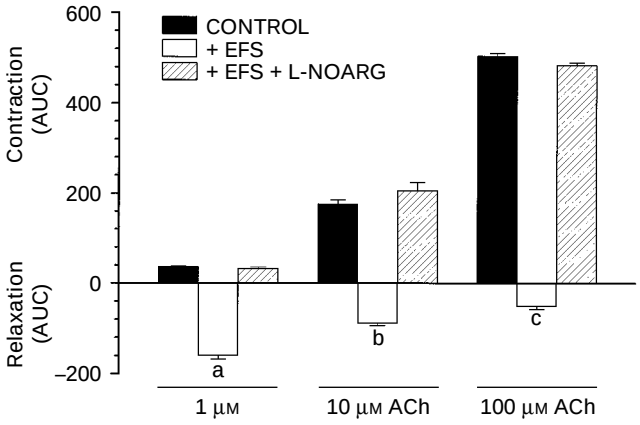

Figure 4 Concentration-dependent responses of botulinum toxin A-treated tissues to acetylcholine (1–100 μM) in control conditions, in the presence of EFS (50 V, 0.3 ms pulse duration, 5 Hz) and in the presence of EFS and L-NOARG (300 μM). Each column represents the mean of contraction or relaxation, measured as area under the curve (AUC, arbitrary units). Vertical bars represent s.e.mean ($n=4$). (a) Significantly different from control and +EFS+L-NOARG values at 1 μM ACh ($P<0.0001$). (b) Significantly different from control and +EFS+L-NOARG values at 10 μM ACh ($P<0.0001$), significantly different from +EFS value at 1 μM ACh ($P<0.001$). (c) Significantly different from control +EFS+L-NOARG values at 100 μM ACh ($P<0.0001$), significantly different from +EFS value at 10 μM ACh ($P<0.001$).

pig trachea (Belvisi *et al*., 1991). Vagal nerve stimulation-induced contractions of the rabbit stomach and proximal colon *in vivo* have also been shown to be enhanced by treatment with an NO synthase inhibitor (Iversen *et al*., 1997). Whether NO modulates the cholinergic contractile response prejunctionally or postjunctionally is still not known since acetylcholine release is not affected by NO synthase inhibitors in the guinea pig trachea (Brave *et al*., 1991) and human trachea (Ward *et al*., 1993) but is enhanced in the rat trachea (Sekizawa *et al*., 1993), guinea pig caecum (Knudsen & Tottrup, 1992) and dog duodenum (Toda *et al*., 1991). Our experiments with exogenous acetylcholine suggest that the interaction between the two opposing systems seems to be at a postjunctional site. The degree of control of parasympathetic responses by nitrergic transmission, however, seems to be dependent on the frequency of EFS because at higher frequencies, cholinergic contraction starts immediately with EFS. This contraction is still modulated by the nitrergic system as it is enhanced by NO synthase inhibitors. Thus, the way in which the interaction operates *in vivo* physiologically is worth investigating.

Previously we have demonstrated a similar modulation of sympathetic responses by nitrergic neurotransmission in the rabbit anococcygeus muscle and rabbit and human corpus cavernosum (Cellek & Moncada, 1997). This was different in the mouse and rat anococcygeus muscles where sympathetic responses were more predominant (Cellek & Moncada, 1997). These previous results, together with our present results showing that the modulation of parasympathetic responses is greater at low frequency stimulation, suggest that nitrergic neurotransmission may have a general modulatory role on excitatory responses in multi-innervated structures. The extent of this modulatory function seems to vary between species as well as between organs. The significance of this modulation in human physiology remains to be clarified.

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