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α-Latrotoxin-induced transmitter release in feline oesophageal smooth muscle: focus on nitric oxide and vasoactive intestinal peptide

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1 The effects of α-latrotoxin (αLTX) on muscle tone, resting membrane potential, cyclic nucleotide content, and ultrastructure were examined in feline oesophageal smooth muscle, including the lower oesophageal sphincter (LOS).

2 In circular smooth muscle strips from LOS developing active tone, αLTX (1 nM) induced a 94±3% ($n=16$) relaxation. Intermittent treatment with αLTX for 4 h abolished the response. Pretreatment with N^G-nitro-L-arginine (L-NOARG; 0.1 mM) attenuated the relaxation.

3 In carbachol-contracted circular smooth muscle strips from the LOS and oesophageal body (OB), αLTX induced a 95±5% ($n=6$) and 73±9% ($n=8$) relaxation, respectively. The relaxations were attenuated by L-NOARG, and in LOS strips, the relaxation was abolished by the combination of L-NOARG and vasoactive intestinal peptide (VIP)-antiserum (1:25). At resting tension in circular smooth muscle strips from the OB, αLTX induced a scopolamine sensitive contraction in the presence of L-NOARG.

4 In circular LOS and OB preparations, αLTX changed the resting membrane potential from −49±2 mV to −59±3 mV ($n=4$), and −62±2 mV to −71±3 mV ($n=4$), respectively.

5 The αLTX-induced relaxation of LOS and OB muscle was associated with a 138% and 72% increase in cyclic GMP levels, respectively. No changes in cyclic AMP levels were observed.

6 Ultrastructural analysis of LOS and OB revealed a rich supply of nerve profiles containing small synaptic and large dense core vesicles. αLTX treatment resulted in a loss of both types of vesicle.

7 These results suggest that αLTX induces relaxation of oesophageal circular smooth muscle associated with NO-generation and transmitter release from synaptic vesicles. Beside NO, VIP seems to be involved in the relaxant effects of αLTX on the LOS. In addition, αLTX may have contractile effects by release of acetylcholine.

Keywords: Acetylcholine; α-latrotoxin; cyclic nucleotides; nitric oxide; oesophagogastric junction; oesophagus; vasoactive intestinal peptide

Introduction

α-Latrotoxin (αLTX) is a neurotoxic protein isolated from the venom of the black widow spider *Latrodectus mactans tredecimguttatus* (Frontali *et al*., 1976). The action of αLTX is considered to be initiated by an interaction with synaptotagmin and neurexin, proteins involved in the docking process of neurotransmitter containing synaptic vesicles and the presynaptic plasma membrane (Petrenko *et al*., 1991). After docking of the vesicles, a formation of cation channels and an interaction with intracellular processes may take place, leading to calcium influx into the cell. Subsequently, this leads to massive neurotransmitter release from the nerve terminal (Petrenko, 1993). Many different transmitters have been demonstrated to be released by αLTX, i.e., acetylcholine, noradrenaline, dopamine, glutamate and enkephalin (Rosenthal & Meldolesi, 1989). Most of the studies with αLTX have involved release from small synaptic vesicles (SSVs; known to contain acetylcholine, Gabella, 1994), as identified by ultrastructural analysis. Release from large dense core vesicles (LDCVs) in motoneurones at the neuromuscular junction, containing the neuropeptide calcitonin gene-related peptide (CGRP), has been suggested not to be affected by αLTX (Matteoli *et al*., 1988). However, recently Waterman and Maggi (1995) demonstrated αLTX-induced release of tachykinins and CGRP from sensory and enteric neurones in the guinea-pig, indicating that SSVs and LDCVs may share properties not previously appreciated.

The feline oesophagus and the lower oesophageal sphincter (LOS) are richly innervated (Ny *et al*., 1994), and the innervation is considered to consist mainly of cholinergic excitatory and non-adrenergic non-cholinergic (NANC) inhibitory components (e.g. Daniel, 1992). In the feline LOS, the NANC control is exerted by nitric oxide (NO), and by as yet unidentified transmitter(s) (Ny *et al*., 1995a). However, data has been obtained suggesting that vasoactive intestinal peptide (VIP) may be involved (Ny *et al*., 1995a). In some parts of the intestine, NO and VIP have been demonstrated to enhance and facilitate the release of each other (Makhlouf & Grider, 1993), but there are also systems where the two mediators seem to be released in parallel and not to interact (Keef *et al*., 1994).

In the present study, we used αLTX as a tool to elucidate further the role of vesicle stored transmitters in neurotransmission in feline lower oesophageal smooth muscle, including the LOS. Furthermore, the interaction between such transmitters, released by αLTX, and the NO-system was investigated.

Methods

Tissue Preparation

Adult male cats (with a weight of approximately 4.0 kg), were anaesthetized by i.v. α-chloralose (40 mg kg^{-1}), and killed by an i.v. injection of air or a saturated KCl solution. This procedure was approved by the Animal Ethics Committee, Lund

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University. The distal two thirds of the oesophagus, with adjacent parts of the stomach, were removed and opened along the longitudinal axis of the oesophagus. Tissue specimens were immediately placed in ice cold Na^+-Krebs solution (for composition, see below). The specimens were pinned flat with the mucosal side up and stretched to their *in vivo* length. The mucosa was removed by sharp dissection and the muscularis externa was dissected free. Preparations were taken from two levels, the LOS and the oesophageal body (OB), 2 cm above LOS. The LOS was identified as a thickening of the circular smooth muscle layer at the oesophagogastric junction as described in detail previously (Ny *et al*., 1995a).

Functional studies

For recording of mechanical activity in tissue baths, strips ($1 \times 1 \times 5$ mm) were dissected in the direction of the muscle fibres from the circular and the longitudinal smooth muscle layer. The strips were transferred to thermostatically controlled (37°C) 5 ml tissue baths containing Na^+-Krebs solution constantly bubbled with 5% CO_2 and 95% O_2 (pH 7.4), and mounted between two hooks. One hook was attached to a force transducer (Grass FT03) for recording of mechanical activity, and the other was connected to a sledge, which allowed adjustment of the passive tension of the strip. The recordings were made on a Grass polygraph, 7D or E. Strips from the circular smooth muscle layer of the LOS were stretched from resting length (L_R) until a length of about 160% of L_R was reached (Ny *et al*., 1995a). Preparations from the circular smooth muscle layer of the OB and from the longitudinal smooth muscle layer of both LOS and OB, were stretched from L_R until a length of about 130% of L_R was reached (Ny *et al*., 1995a). After being mounted, the strips were allowed to equilibrate for 1 h. During this period, the LOS circular smooth muscle preparations developed a spontaneous tone. Only LOS preparations that developed spontaneous tone were used. The passive tension of the LOS circular muscle strips was determined by exposure to a 'Ca^{2+}-free' Krebs solution containing 0.1 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA; for composition, see below). The base-line tension level obtained was considered as 100% relaxation; all calculations of relaxant responses were related to this level. Preparations from the OB and the longitudinal smooth muscle layer of the LOS never developed active tension. To test viability, strips from regions not developing a spontaneous tone were exposed to a 124 mM K^+-solution (for composition, see below).

Electrical field stimulation (EFS) was applied through platinum wire electrodes placed in parallel with the strips. The stimulation frequency was varied between 1–20 Hz for circular smooth muscle strips, and 1–40 Hz for longitudinal smooth muscle strips. Five-second trains of square wave pulses (0.8 ms duration, supramaximal voltage) were applied every 2 min. In some experiments, continuous stimulation for 2 min at 10 Hz was performed.

Electrophysiology

Electrophysiological experiments were performed on preparations from the circular smooth muscle layer of the LOS and OB. Segments measuring 2×5 mm were prepared and pinned on a sylgard bottom in a 2 ml tissue bath. The tissue bath was continuously superfused (1 ml min^{-1}) with Na^+-Krebs solution bubbled with a mixture of 95% O_2 and 5% CO_2. The preparations were allowed to equilibrate for at least 1 h before the experiments were started. The headstage, where the electrode was attached, was mounted on a Burleigh Inchworm motor (Burleigh Instruments, Fishers, NY, U.S.A.) and used to penetrate the tissue. The electrodes were made in a Flaming/Brown micropipette puller, model P-87 (Sutter Instruments Co, Novato, CA, U.S.A.), and filled with 3 M KCl yielding an electrode resistance of 30–50 MΩ. The amplifier was an Axoprobe-1A (Axon Instruments, Foster City, CA, U.S.A.). The membrane potential was simultaneously displaced on an oscilloscope and registered on a pen recorder. The electrode was advanced through the tissue with 0.5 μm steps and very fast acceleration. An acceptable impalement was characterized by a sharp decrease in potential and a stable recording of the membrane potential. The electrophysiological procedure has been described in detail previously (Waldeck *et al*., 1995).

Measurement of cyclic nucleotide concentrations

Guanosine 3′:5′-cyclic monophosphate (cyclic GMP) and adenosine 3′:5′-cyclic monophosphate (cyclic AMP) concentrations were analysed in LOS (at the spontaneously developed tension level) and OB (at the basal tension level) strips (control), and after exposure to αLTX (1 nM). When tissue strips were exposed to αLTX in the tissue baths, mechanical activity was recorded simultaneously. Approximately 2–3 min after exposure to αLTX, the LOS preparations had relaxed to a maximum and stable tension level, and the strips were rapidly removed from the tissue bath and put into liquid nitrogen (less than 2 s from tissue bath to liquid nitrogen). Muscle strips from OB were exposed to αLTX for 3 min at basal tension level, and then removed from the tissue bath and further treated as the LOS strips. Time-matched tissue strips without treatment served as controls. No phoshodiesterase inhibitor was used in the present experiments. The strips were then stored at −20°C until analysed. The tissue was homogenized in 10% trichloracetic acid (TCA) in water with a glass-glass homogenizer, and centrifuged at 1500 *g* (4°C) for 10 min. The protein content in the pellets was determined by the method described by Bradford (1976). The supernatants were extracted 5 times with 5 ml of water-saturated diethyl ether. The aqueous phase was evaporated and the residue stored at −20°C. Residues were dissolved in 0.05 M sodium acetate, and the amounts of cyclic GMP and cyclic AMP were quantified by using [^{125}I]-cyclic GMP and [^{125}]-cyclic AMP RIA kits (RIA-NEN, Du Pont Company, Boston, MA, U.S.A.). [^{3}H]-cyclic AMP was added to the TCA tissue homogenate in order to determine the recovery of cyclic GMP and cyclic AMP during the ether extraction. The mean recovery was 83% for LOS and 77% for OB preparations.

Electron microscopy

The ultrastructure of nerve profiles in LOS and OB strips from the circular muscle layer was examined by transmission electron microscopy. Strips not exposed to αLTX served as controls; LOS strips exposed to αLTX (1 nM) were treated for approximately 15 min or 4 h, whereas OB strips were exposed to αLTX (1 nM) for only 4 h. The strips were immersion-fixed in 2.5% glutaraldehyde for 1 h, rinsed in phosphate buffered saline (pH 7.2), post-fixed in 1.5% osmium tetroxide, and dehydrated in ethanol (50, 75, 96, and 100%) solutions. The specimens were embedded in Agar 100 (Link Corp, Essex, U.K.), and ultrathin sections were made in an ultramicrotome (LKB, Stockholm, Sweden) and mounted on copper grids. Ultrathin sections were contrasted with uranyl acetate and lead citrate, and examined in a JEOL JM100 electron microscope (Tokyo, Japan) or a Philips CM 10 electron microscope (Eindhoven, Netherlands). SSVs and LDCVs were identified in LOS tissue as described by Uddman *et al*. (1978), and Tsumori *et al*. (1994). From each of four different animals, strips were taken and exposed to αLTX (1 nM), in LOS preparations for 15 min and 4 h, and from OB preparations for 4 h. Controls for each tissue and exposure time were investigated in parallel. In each strip 100 nerve profiles, associated with the smooth muscle, were studied for vesicle content in 1–2 grids, i.e. a total number of 2400 nerve profiles were analysed.

Solutions

The Krebs solutions used had the following composition (in mM). Na^+-Krebs solution: NaCl 119, KCl 4.6, $NaHCO_3$15,

$CaCl_2$ 1.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2 and glucose 11; Ca^{2+}-free-Krebs solution: NaCl 119, KCl 4.6, $NaHCO_3$ 15, $MgCl_2$ 1.2, NaH_2PO_4 1.2, glucose 11 and EGTA 0.1; 124 mM K^+-solution: KCl 124, $NaHCO_3$ 15, $CaCl_2$ 1.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2 and glucose 11.

Drugs

The chemicals were obtained from the following sources: αLTX, ω-conotoxin GVIA (Almone Labs Ltd., Jerusalem, Israel), α-chymotrypsin, α,β-methylene ATP, carbachol, haloperidol, methylene blue, N^G-nitro-L-arginine (L-NOARG), (±)-propranolol, scopolamine, scorpion venom (*Leirus Quinquestratus Hebr*, V1755), tetrodotoxin (TTX), VIP (Sigma Chemical Company, St Louis, MO, U.S.A.). Rabbit VIP antiserum and normal rabbit serum (J. Fahrenkrug, Department of Clinical Biochemistry, Bispebjerg Hospital, Copenhagen, Denmark). The VIP-antiserum has been described in detail (Fahrenkrug *et al*., 1995). All drugs were dissolved in and diluted with saline. The preincubation time before αLTX administration for all drugs was 30 min. Only one substance was tested versus αLTX on every LOS/OB strip. A saturated solution of NO was prepared as described previously (Ny *et al*., 1995a).

Calculations and statistics

Statistical results are expressed as mean ± s.e.mean. When a statistical difference between two means was determined, a paired or unpaired two-tailed Student's *t* test was performed; for multiple comparisons a one-way analysis of variance, ANOVA, followed by Bonferroni/Dunn post-hoc test, was used. $P<0.05$ was regarded as significant. Outliers were checked for by Dixon's gap test. *n* is the number of strips tested, and, when not specifically indicated, refers to tissues from different animals. When statistical analyses were performed, all values refer to different animals.

Results

Functional studies

One hundred and forty six circular smooth muscle strips of the LOS were tested; 114 generated an active tension amounting to 13.5 ± 2.1 mN and were accepted for study. αLTX 1–10 pM did not affect muscle tone ($n=6$). At a concentration of 0.1 nM, a 25% and 33% unstable relaxation was seen in two out of six preparations; muscle tone in the other four strips was not affected. αLTX (1 nM) induced a 94 ± 3% ($n=16$) relaxation with an onset time of 45–60 s (Figures 1 and 2). Pretreatment with methylene blue (30 μM) or L-NOARG (0.1 mM), before addition of αLTX (1 nM), resulted in a relaxation amounting to 52 ± 5% ($n=6$; $P<0.001$) and 64 ± 4% ($n=6$; $P<0.001$) respectively, and caused a two-fold increase in onset time (Figures 1 and 2). Methylene blue itself increased the tone by 33 ± 8% ($n=6$), whereas L-NOARG did not affect muscle tone. TTX (1 μM; $n=6$) increased the onset time, but did not affect the amplitude of relaxation (Figures 1 and 2). α,β-methylene ATP (30 μM; $n=3$), α-chymotrypsin (2 units ml^{-1}; $n=3$), haloperidol (1 μM; $n=3$), phentolamine (1 μM; $n=3$), propranolol (1 μM; $n=3$), ω-conotoxin GVIA (0.1 μM;

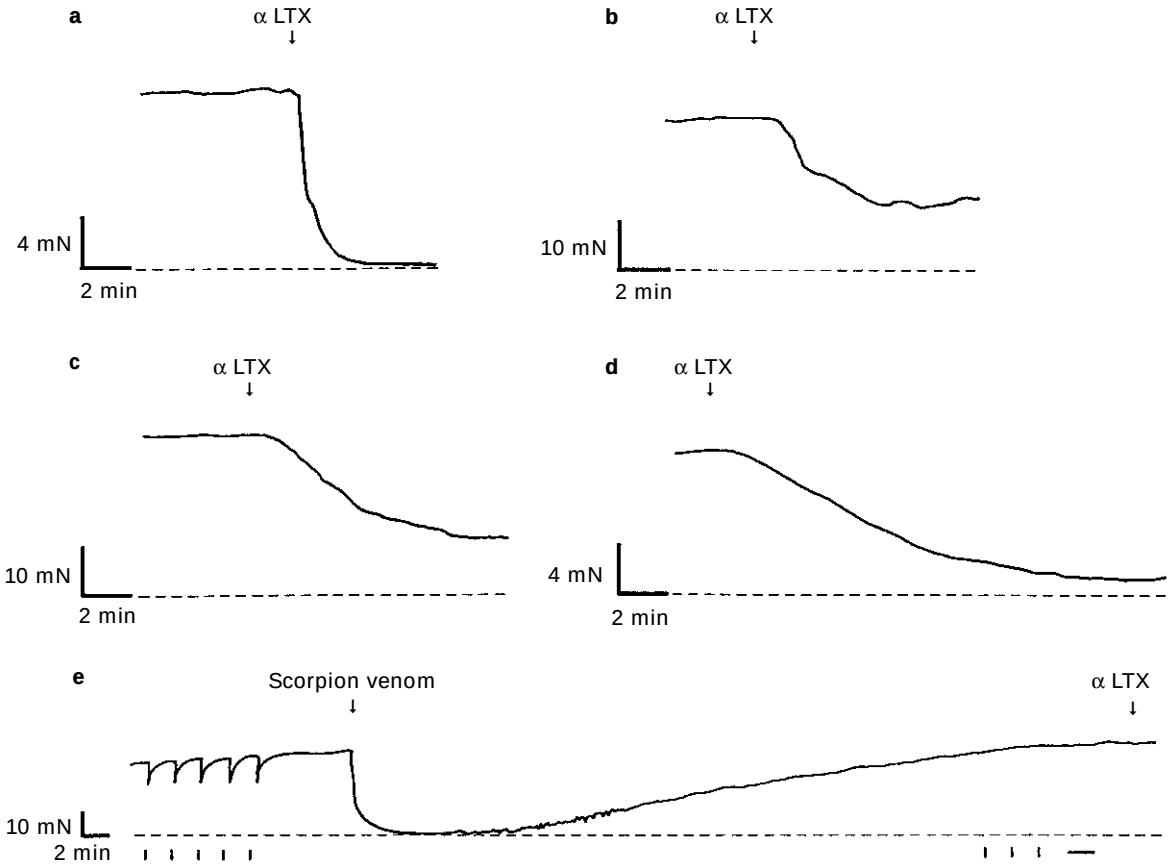

Figure 1 Original tracings of the effects of α-latrotoxin (αLTX) on tone of circular smooth muscle strips from the lower oesophageal sphincter developing active tone. Strips were exposed to αLTX (1 nM), (a) without any pretreatment, (b) in the presence of methylene blue (30 μM), (c) in the presence of L-NOARG (0.1 mM), and (d) in the presence of tetrodotoxin (1 μM). In (e), a strip was stimulated electrically (indicated by bars; 10 Hz, supramaximal voltage, 0.8 ms pulses, 5 s trains), and then exposed to scorpion venom (20 μg ml^{-1}). After 45 min the muscle tone was restored, and again the strip was electrically stimulated (indicated by bars; 10 Hz, supramaximal voltage, 0.8 ms pulses, 5 s trains or continuous stimulation for 2 min) and exposed to αLTX (1 nM).

$n=3$) or scopolamine (1 μM; $n=3$) did not affect the relaxation amplitude or the onset time for relaxation. Treatment with scorpion venom (20 μg ml^{-1}), known to cause massive transmitter release from nerves by inactivation of Na^+-channels (Koppenhöfer & Schmidt, 1968; Daniel & Posey-Daniel, 1984), induced an almost instant 100% relaxation ($n=6$). Fortyfive to sixty min after administration of the scorpion venom, the tone of the strips had been restored. αLTX administration during this state did not affect tone, nor did EFS (1–20 Hz, delivered in 5 s trains, or as continuous stimulation for 2 min at 10 Hz; Figure 1). Repeated exposure to αLTX during a 4 h period, with 10 min intermittent washing periods every 30 min, resulted in a progressive inhibition and a final abolition of the response to the toxin. The relaxations induced

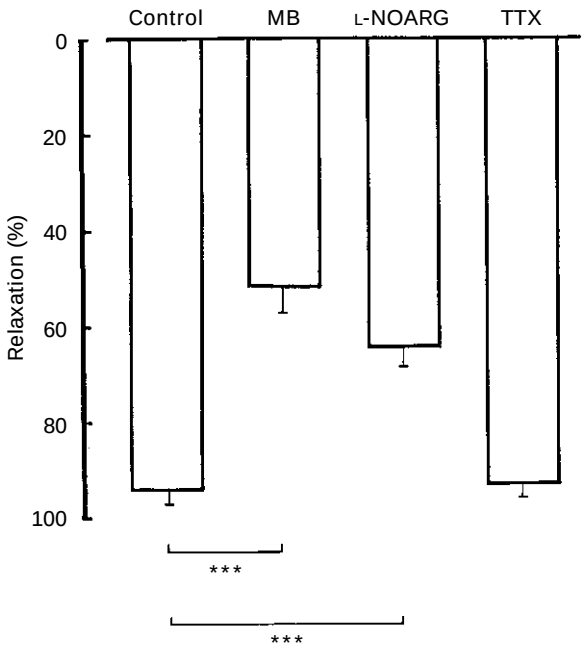

Figure 2 Effects of methylene blue (MB; 30 μM), L-NOARG (0.1 mM), and tetrodotoxin (TTX, 1 μM), respectively, on the α-latrotoxin (αLTX, 1 nM) induced relaxation in the lower oesophageal sphincter developing active tone. Values are expressed as mean ± s.e.mean. ***$P<0.001$.

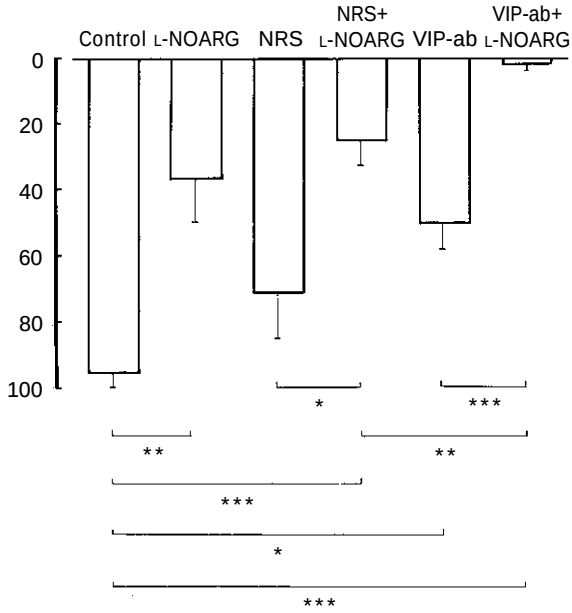

Figure 3 Effects of L-NOARG (0.1 mM), normal rabbit serum (NRS; dilution 1:25), vasoactive intestinal peptide (VIP)-antiserum (VIP-ab; dilution 1:25), and the combinations of NRS and L-NOARG, and VIP-ab and L-NOARG, respectively, on α-latrotoxin (αLTX, 1 nM)-induced relaxation in carbachol (1 μM) contracted circular strips from the lower oesophageal sphincter. Values are expressed as mean ± s.e.mean. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

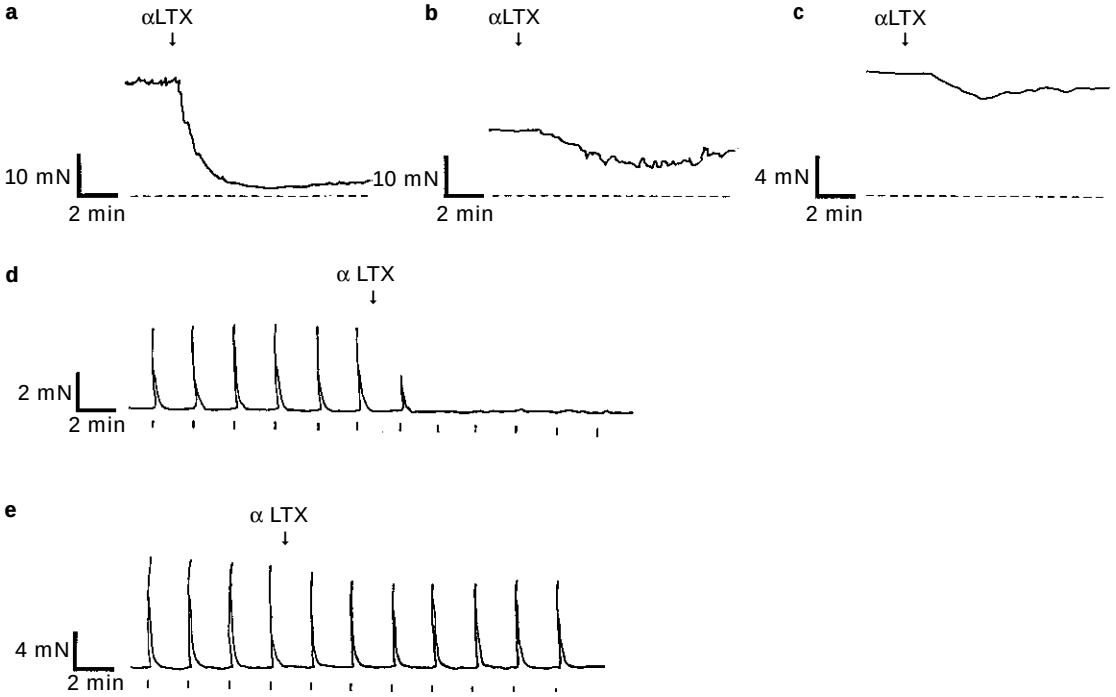

Figure 4 Original tracings of the effects of α-latrotoxin (αLTX) on tone and electrically induced contractions in circular smooth muscle strips from the oesophageal body. Carbachol (1 μM) contracted strips were exposed to αLTX (1 nM), (a) without any pretreatment, (b) in the presence of methylene blue (30 μM), and (c) in the presence of L-NOARG (0.1 mM). In (d) and (e) the effect of αLTX (1 nM) on contractions induced by electrical field stimulation (10 Hz, supramaximal voltage, 0.8 ms pulses) is shown.

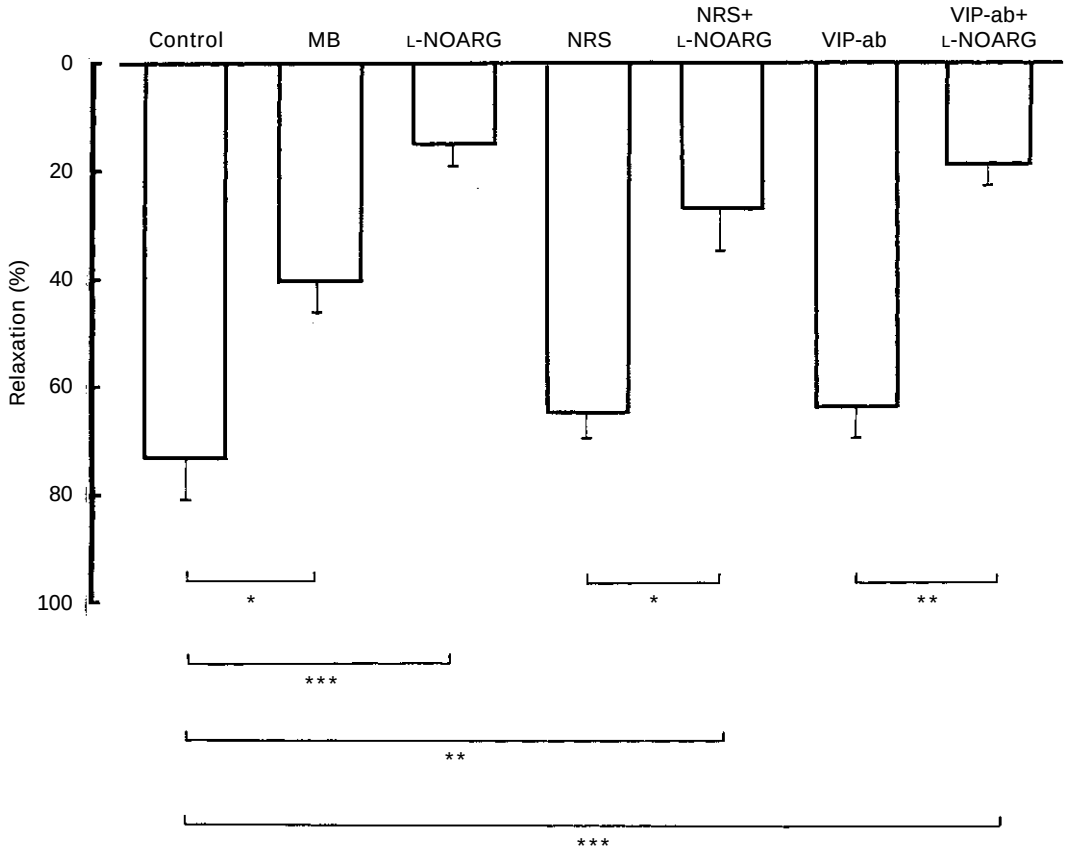

Figure 5 Effects of methylene blue (MB; 30 μM), L-NOARG (0.1 mM), normal rabbit serum (NRS; dilution 1:25), vasoactive intestinal peptide (VIP)-antiserum (VIP-ab; dilution 1:25), and the combinations of NRS and L-NOARG, and VIP-ab and L-NOARG, respectively, on the α-latrotoxin (αLTX, 1 nM) induced relaxation in carbachol (1 μM) contracted circular strips from the oesophageal body. Values are expressed as mean ± s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

by EFS (1–20 Hz, delivered in 5 s trains, or as continuous stimulation for 2 min at 10 Hz) was not affected after 15 min treatment with αLTX. However, after 4 h intermittent treatment, the relaxations were reduced, and in the presence of L-NOARG (0.1 mM), they were abolished.

Rabbit VIP antiserum (dilution 1:25) and normal rabbit serum (dilution 1:25) induced an unstable, often rhythmic variation in tension when administered after development of spontaneous, active tone. LOS strips were therefore contracted with carbachol (1 μM), which induced a stable muscle tension, before administration of αLTX. In these carbachol-contracted preparations, αLTX induced a relaxation amounting to $50\pm8\%$ ($n=6$; $P<0.05$) in the presence of VIP antiserum, and $71\pm14\%$ ($n=6$) in the presence of normal rabbit serum (Figure 3). The corresponding value without treatment was $95\pm5\%$ ($n=6$), and with previous L-NOARG (0.1 mM) treatment $37\pm13\%$ ($n=6$; $P<0.01$). The combination of L-NOARG and VIP-antiserum abolished the relaxation ($n=6$; $P<0.001$), whereas the effect of a combination of L-NOARG and normal rabbit serum did not differ from the effects of L-NOARG itself ($n=6$).

Circular smooth muscle strips from OB had a resting tension of 4.5 ± 1.0 mN ($n=44$). EFS (1–20 Hz) induced scopolamine- and TTX-sensitive contractions, with a maximal response at 8–10 Hz. In 4 out of 8 preparations, αLTX (1 nM) abolished the EFS-induced contractions without affecting basal tension (Figure 4d); in the other 4 preparations αLTX (1–10 nM) produced a 10–20% inhibition of the contraction (Figure 4e). No effect on tone was seen when αLTX was administered at the basal tension level. In the presence of L-NOARG (0.1 mM) αLTX (1 nM) induced a contraction at basal tension level. This response was abolished by scopolamine (1 μM) treatment.

In circular OB strips, carbachol (1 μM) evoked a stable contraction, amounting to 12.4 ± 0.9 mN ($n=36$). In these strips, αLTX (1 nM) induced a $73\pm9\%$ ($n=8$) relaxation (Figures 4 and 5). The corresponding results were $64\pm6\%$ ($n=4$) after pretreatment with VIP antiserum (dilution 1:25), $65\pm5\%$ ($n=4$) after normal rabbit serum (dilution 1:25), $41\pm6\%$ ($n=6$; $P<0.05$) after methylene blue (30 μM) and $15\pm4\%$ ($n=6$; $P<0.001$) after L-NOARG (0.1 mM). No differences were seen with the combination of L-NOARG and VIP-antiserum ($n=4$) or L-NOARG normal rabbit serum ($n=4$) compared to L-NOARG treatment itself. Methylene blue itself increased muscle tone by $11\pm6\%$ ($n=6$).

Longitudinal smooth muscle preparations of LOS had a tension of 4.1 ± 0.6 mN ($n=4$); the corresponding figure for longitudinal preparations from the OB was 3.7 ± 1.0 mN ($n=4$). αLTX (1 nM) did not affect resting muscle tension, or contractions induced by EFS (1–40 Hz). In carbachol (1 μM) contracted LOS strips, αLTX (1 nM) induced a $35\pm8\%$ ($n=4$) relaxation, whereas it did not affect the tension in carbachol-contracted OB preparations.

Electrophysiology

The influence of αLTX on resting membrane potential was studied in circular LOS and OB tissue. Technically, it was difficult to obtain satisfactory measurements from the LOS. In 4 out of 10 LOS preparations successful impalements were made and a resting membrane potential of -49 ± 2 mV ($n=4$) was recorded. αLTX (3 nM) produced a hyperpolarization from this level to -59 ± 3 mV ($n=4$; $P<0.05$; Figure 6a).

In OB preparations, a resting membrane potential of -61 ± 2 mV ($n=8$) was recorded. αLTX (3 nM) produced a hyperpolarization from -62 ± 2 mV to -71 ± 3 mV ($n=4$;

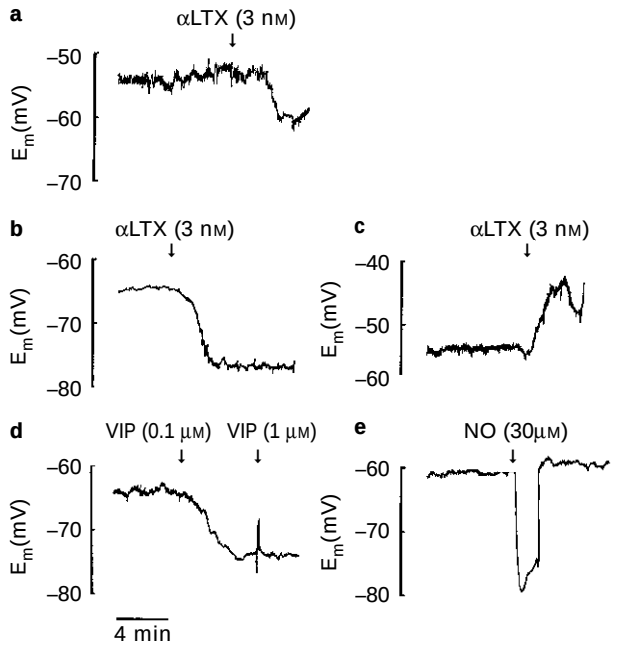

Figure 6 Original tracings of the effects of α-latrotoxin (αLTX) on resting membrane potential in circular smooth muscle strips from the lower oesophageal sphincter (LOS) and oesophageal body (OB). Effect of αLTX (3 nM), (a) without any pretreatment in LOS, and (b) the effect of αLTX (3 nM) without any pretreatment in OB, (c) in the presence of L-NOARG (0.1 mM) on OB. For a comparison, the effects of treatment with exogenously administered VIP 0.1–1 μM (d) and NO 30 μM (e) are demonstrated in OB tissue.

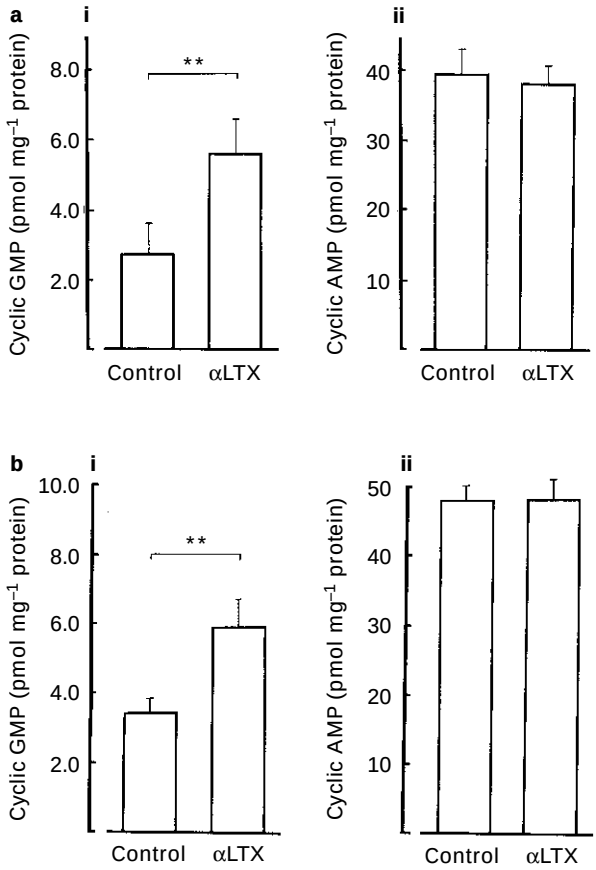

Figure 7 Effect of α-latrotoxin (αLTX) on cyclic nucleotide concentrations in (a) circular smooth muscle strips from the lower oesophageal sphincter developing active tone, and (b) oesophageal body on basal tension level. Tissue concentrations of (i) cyclic GMP and (ii) cyclic AMP were measured before (control), and after exposure to αLTX (1 nM). Values are expressed as mean ± s.e.mean. $^{**}P<0.01$.

$P<0.05$; Figure 6b). Pretreatment with L-NOARG (0.1 mM) resulted in a slight depolarization to -54 ± 1 mV ($n=4$; $P<0.05$). Addition of αLTX (3 nM) at this state resulted in a further depolarization to -41 ± 1 mV ($n=4$; $P<0.001$; Figure 6c). VIP (0.1 μM) induced a hyperpolarization from -60 ± 3 mV to -69 ± 2 mV ($n=4$; $P<0.001$; Figure 6d). Addition of VIP (1 μM) did not produce further effects on the membrane potential. NO (30 μM) induced a shortlasting hyperpolarization from -60 ± 2 mV to -78 ± 1 mV ($n=4$; $P<0.01$; Figure 6e).

In experiments on LOS tissue, we had difficulty recording the resting membrane potential for periods as long as the extended experimental protocol required. Electrophysiological experiments with VIP, NO, and L-NOARG in combination with αLTX, were therefore performed only on OB tissue.

Effects of αLTX on cyclic nucleotide content

Exposure to circular smooth muscle preparations from the LOS to αLTX (1 nM) induced a 138% ($P<0.01$) increase in cyclic GMP concentration (2.36 ± 0.41 before, and 5.61 ± 0.70 pmol mg^{-1} protein after αLTX exposure; $n=8$). The cyclic AMP concentration remained unchanged (39.4 ± 3.5 before and 38.1 ± 2.3 pmol mg^{-1} protein after αLTX exposure; $n=8$; Figure 7).

Exposure of circular smooth muscle preparations from the OB to αLTX (1 nM) induced a 72% ($P<0.01$) increase in cyclic GMP concentration (3.41 ± 0.36 before, and 5.88 ± 0.83 pmol mg^{-1} protein after αLTX exposure; $n=6$). The cyclic AMP concentration remained unchanged (48.3 ± 1.5 before and 48.4 ± 2.0 pmol mg^{-1} protein after αLTX exposure; $n=6$; Figure 7).

Effects of αLTX on nerve profile ultrastructure

Ultrastructural analysis by transmission electron microscopy of the circular smooth muscle layer from LOS and OB revealed an abundance of nerve profiles supplying the smooth muscle. The nerve structures contained both SSVs and LDCVs (Figure 8a). The two types of vesicle occurred separately or together. In the control tissue from LOS, $72\pm3\%$ ($n=4$) of the nerve profiles contained LDCVs After 15 min αLTX treatment, $37\pm2\%$ ($n=4$) of the nerve profiles in LOS contained LDCVs. At this stage, no obvious loss of SSVs could be detected. After 4 h αLTX treatment in LOS and OB, there was a major, in some cases complete, loss of both types of vesicles (Figure 8b). No ultrastructurally visible effect of αLTX on smooth muscle was observed.

Discussion

This study demonstrates that the neurotoxic protein, αLTX, produces relaxation in circular smooth muscle from the feline OB and LOS. This relaxation seems to be accompanied by release of NO and of synaptic vesicle stored neurotransmitters. In previous studies, αLTX effects have been shown to be exclusively neuronally mediated by an exocytotic release of neurotransmitters through an interaction with docking proteins, located at the plasma membrane of the presynaptic nerve cell (e.g. Rosenthal & Meldolesi, 1989). Possibly, also an induction of cation channels, leading to an increase in intracellular calcium may take place. Also in the feline lower oesophagus, the αLTX effects appeared to be of neuronal origin, since αLTX did not affect muscle tone after treatment with a scorpion venom known to cause massive release of

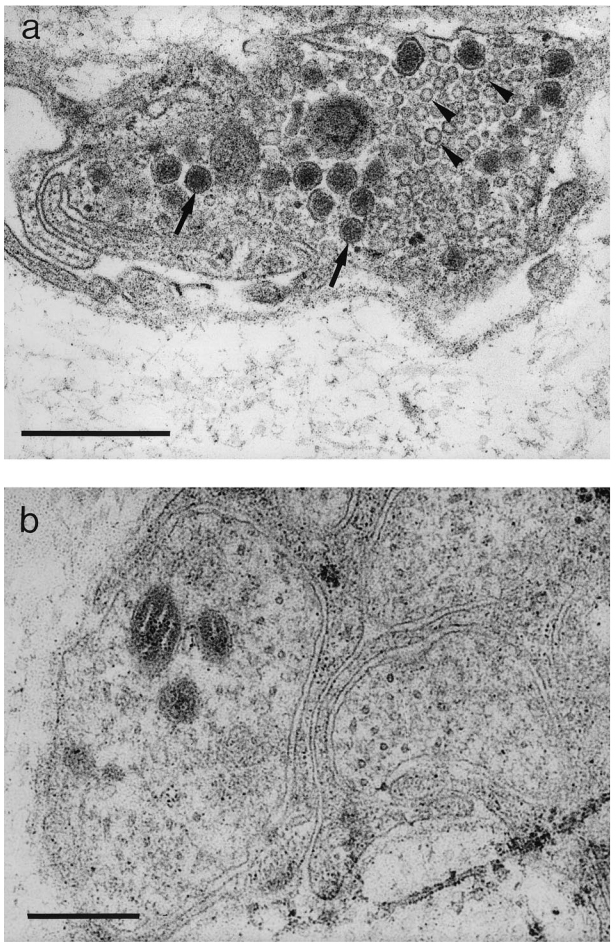

Figure 8 Ultrastructure of nerve profiles in the circular smooth muscle layer from the lower oesophageal sphincter. (a) A nerve profile with an abundance of both small synaptic vesicles (SSVs, arrowheads) and large dense core vesicles (LDCVs; arrows). (b) Nerve profiles after treatment with α-latrotoxin (αLTX, 1 nM) for 4 h. Practically no SSVs or LDCVs were observed. Bar 500 nm.

neurotransmitters from nerve terminals by inactivation of Na^+-channels (Koppenhöfer & Schmidt, 1968; Daniel & Posey-Daniel, 1984), and αLTX did not affect smooth muscle ultrastructure.

Several kinds of transmitter, stored in SSVs, have been shown to be released by αLTX (Rosenthal & Meldolesi, 1989). Neuropeptides, stored in LDCVs, have been suggested not to be sensitive to the toxin (Matteoli *et al*., 1988). However, Waterman & Maggi (1995) demonstrated that CGRP and tachykinins were released by αLTX from guinea-pig sensory and enteric neurones. In the feline LOS, we observed a decrease of LDCV content in nerve terminals after αLTX treatment. The LDCVs observed in the nerve profiles in the human and canine LOS have been shown to contain VIP, sometimes stored within the same nerve terminals as neuropeptide Y (Berezin *et al*., 1987; Tsumori *et al*., 1994). Evidence for an involvement of VIP in the αLTX-induced inhibitory response of the LOS was found in the present experiments, showing that VIP antiserum in combination with the NO-synthesis inhibitor L-NOARG abolished the αLTX-induced relaxation. In contrast, the combination of VIP antiserum and L-NOARG on the αLTX-induced relaxation of the OB did not differ from L-NOARG treatment only. However, exogenously applied VIP hyperpolarized the membrane to the same level as αLTX in OB. If VIP is involved in the relaxant response, an increase in cyclic AMP levels would be expected, since VIP is know to activate adenylate cyclase. No such increase in cyclic AMP levels was seen. Previous investigations in the opossum LOS (Torphy *et al*., 1986) and the feline LOS (Ny *et al*, 1995a) have demonstrated that electrically evoked relaxations in these tissues are accompanied by an increase in cyclic GMP, but not cyclic AMP levels. In addition, in the feline LOS, an L-NOARG-insensitive relaxation in response to electrical stimulation of nerves has previously been identified. This was not associated with a significant increase in cyclic AMP levels, but was inhibited by VIP antiserum (Ny *et al*., 1995a). The peptidase, α-chymotrypsin, which has been used as a tool to demonstrate VIP-mediated responses, did not have any effect on the L-NOARG-insensitive electrically-induced relaxation of the feline LOS, but abolished the relaxant effect of exogenous VIP (Ny *et al*., 1995a). The possibility that α-chymotrypsin is unable to enter into the synaptic cleft should be considered, and may explain the lack of effect of α-chymotrypsin also on the αLTX-induced relaxation of the LOS.

The αLTX-induced relaxation in both LOS and OB was attenuated after pretreatment with the NO synthesis inhibitor L-NOARG. Also methylene blue, which inhibits soluble guanylate cyclase, reduced the amount of the αLTX-induced relaxation. This effect, however, may not be directly related to an inhibition of αLTX-induced transmitter release, since methylene blue also increased the muscle tone. Furthermore, the αLTX effects were associated with an increase of tissue cyclic GMP levels. These findings suggest that NO is produced in the tissue. Since NO is probably synthesized on demand and not stored in vesicles (Zhang & Snyder, 1995), the release of NO in these experiments may be as a result of an activation of the NO system by VIP, and possibly the pituitary adenylate cyclase activating peptide (PACAP), which is stored in LOS nerves together with VIP and may act on the same receptor (Ny *et al*., 1995b). Such a mechanism would be in agreement with the effects of VIP and PACAP observed in gastric smooth muscle, where these peptides have been shown to activated NO synthase in the smooth muscle cell. However, neurones may be an additional source of NO production (Makhlouf & Grider, 1993; Murthy & Makhlouf, 1994). Alternatively, an increase of intracellular calcium, independent of synaptic protein interaction produced by induction of cation channels, may cause a VIP-independent release of NO. This would mean separate independent transduction pathways for VIP and NO, as was suggested to be the case in the dog colon (Keef *et al*., 1994).

In LOS preparations, the neurotoxin TTX, which is considered to inactivate Na^+-channels, was observed to increase the onset time of the relaxation, but not to affect the maximum relaxation. Thus, normally functioning Na^+-channels seem to be of importance for the effects of αLTX. It may be speculated that αLTX partly depolarizes the nerves by initiating action potentials, and that this contributes to the release of relaxant transmitters.

In the ultrastructural studies, two different times of αLTX treatment were used, 15 min and 4 h. In the experiments in which 15 min αLTX treatment was used, we did not observe any clear differences between control and αLTX-treated tissues, especially regarding the release of SSVs. The limited effect of αLTX on ultrastructure after 15 min, despite marked effects on muscle tone already after 2–3 min, may be explained if only a small portion of synapses have to be activated to induce maximum relaxation. At the time this occurs, only a fraction of the synapses may have been exposed to αLTX, which is a large molecule likely to have problems with penetration into the tissue. Thus, after 2–3 min of treatment, a majority of the nerve terminals may have an intact supply of vesicles. After 4 h treatment with αLTX, not only loss of LDCVs was demonstrated by electron microscopy, but also depletion of SSVs. A depletion of SSVs by αLTX has previously been demonstrated at the frog neuromuscular junction (Hurlbut *et al*., 1990). The SSV type is considered to contain acetylcholine (Gabella, 1994), the major excitatory neurotransmitter in oesophageal tissue (Daniel, 1992). Contractile effects of αLTX, sensitive to scopolamine, could only be demonstrated in the experiments

with circular OB tissue, and in the presence of L-NOARG. Thus, it is likely that scopolamine-sensitive, acetylcholine-mediated EFS-induced contractions are counteracted by a release of inhibitory mediators. This release may vary between different preparations, and therefore possibly explain the variations in the effects of αLTX.

In the longitudinal smooth muscle of the OB, no effect of αLTX was seen. This may be explained by the sparse innervation of the longitudinal smooth muscle layer of the OB (Ny *et al*., 1994), and therefore, a lack of a suitable target for αLTX in this tissue.

In conclusion, these results show that αLTX induces relaxation of oesophageal circular smooth muscle. This response involves release of NO and of other relaxation-producing transmitters. In the LOS, one of these may be VIP. The αLTX-induced relaxation is associated with hyperpolarization, an increase in cyclic GMP content, and a depletion of synaptic vesicles. In addition, αLTX can produce contraction of oesophageal smooth muscle by release of acetylcholine.

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