Nitric oxide activation of a potassium channel (BK\text{Ca}) in feline lower esophageal sphincter

Marie-Claude L’Heureux, Ahmad Muinuddin, Herbert Y Gaisano, Nicholas E Diamant

Abstract

AIM: To assess the effect of nitric oxide (NO) on the large conductance potassium channel (BK\text{Ca}) in isolated circular (CM) and sling (SM) muscle cells and muscle strips from the cat LES to determine its regulation of resting tone and relaxation.

METHODS: Freshly enzymatically-digested and isolated circular smooth muscle cells were prepared from each LES region. To study outward K\textsuperscript{+} currents, the perforated patch clamp technique was employed. To assess LES resting tone and relaxation, muscle strips were mounted in perfused organ baths.

RESULTS: (1) Electrophysiological recordings from isolated cells: (a) CM was more depolarized than SM (-39.7 ± 0.8 mV vs -48.1 ± 1.6 mV, P < 0.001), and maximal outward current was similar (27.1 ± 1.5 pA/pF vs 25.7 ± 2.0 pA/pF, P > 0.05); (b) The NO donor sodium nitroprusside (SNP) increased outward currents only in CM (25.9 ± 1.9 to 46.7 ± 4.2 pA/pF, P < 0.001) but not SM (23.2 ± 3.1 to 27.0 ± 3.4 pA/pF, P > 0.05); (c) SNP added in the presence of the BK\text{Ca} antagonist iberiotoxin (IbTX) produced no increase in the outward current in CM (17.0 ± 2.8 vs 13.7 ± 2.2, P > 0.05); and (d) L-NNA caused a small insignificant inhibition of outward K\textsuperscript{+} currents in both muscles; and (2) Muscle strip studies: (a) Blockade of the nerves with tetrodotoxin (TTX), or BK\text{Ca} with IbTX had no significant effect on resting tone of either muscle; and (b) SNP reduced tone in both muscles, and was unaffected by the presence of TTX or IbTX.

CONCLUSION: Exogenous NO activates BK\text{Ca} only in CM of the cat. However, as opposed to other species, exogenous NO-induced relaxation is predominantly by a non-BK\text{Ca} mechanism, and endogenous NO has minimal effect on resting tone.

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Key words: Circular smooth muscle; Feline; K\textsuperscript{+} channel; Lower esophageal sphincter; Nitric oxide; Sling; Tone

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INTRODUCTION

Nitric oxide (NO) is the main neurotransmitter for active inhibition and relaxation of the lower esophageal sphincter (LES) [6-10]. In the esophageal body and in the LES circular muscle, NO activates the large conductance potassium channel (BKCa) and causes membrane hyperpolarization as one potential mechanism leading to LES relaxation. NO has also been shown to be one putative neurotransmitter responsible for the membrane hyperpolarization of the inhibitory junction potential (IJP) in the LES circular muscle of the opossum, guinea pig, dog and mouse [8,10-15]. Activation of the BKCa by NO is a factor in production of the IJP membrane hyperpolarization [16].

In the cat, the BKCa channel plays a role in setting the resting membrane potential (RMP) in LES muscles [17]. This modulation of the RMP by the BKCa is also present in the opossum LES circular muscle and blockade of the BKCa results in an increase in tone attributed to a balance between the calcium-activated BKCa and chloride (Cl-) channels that raise the RMP to the point where ongoing spiking activity is produced [18]. In the dog LES circular muscle, the activation of the BKCa in setting the RMP involves at least an endogenous source of NO [19,10]. It is not known to what extent endogenous NO is similarly involved in BKCa regulation of LES tone in other species.

The LES is composed of at least two separate muscle components, the circular and the oblique sling muscles [20]. In humans, the circular muscle forms only a partial ring (or semicircular clasp), whereas in other species including the cat, the circular muscle fully encircles the distal esophagus [21,22]. The circular and the sling muscles are functionally different with unique motor and electrophysiological properties. The circular muscle has significant spontaneous myogenic resting tone but is poorly responsive to cholinergic stimulation, whereas the sling muscle has little intrinsic myogenic tone but contracts vigorously to cholinergic stimulation, whereby it maintains its resting tone in vivo [23-25]. There are regional differences within the feline LES in terms of distribution, nature and function of ion channels [23,27-29]. In particular, the BKCa density is greater in circular muscle than in the sling muscle [30].

The objective of the present study was to determine in the cat, whether the BKCa in LES circular and sling muscles is influenced by exogenous and/or endogenous NO to regulate muscle resting tone and/or relaxation. These studies utilized isolated smooth muscle cells and strips taken from the feline LES circular and sling regions. The effect of endogenous or exogenous sources of NO on the outward K+ currents was also assessed and compared. A portion of this work has appeared in abstract form [31].

MATERIALS AND METHODS

Animal model

The cat was chosen as our animal model because of several important similarities between the cat and human esophagus. These similarities include (1) a significant portion of the distal esophageal body is composed of smooth muscle [32]; (2) the cholinergic sensitivity of the smooth muscle esophagus and LES are similar [22,25]; and (3) the placement of the gastroesophageal junction is similarly placed relative to the diaphragm [33].

Animal preparation

Experiments were approved by the University Health Network Animal Care Committee. Fasted, adult cats of either sex, weighing 2.5 to 5.0 kg, were anesthetized with ketamine hydrochloride (0.15 mL/kg iv, Bimeda-MTC, Cambridge, ON, Canada) and euthanized with pentobarbital sodium (0.5 mL/kg iv; Bimeda-MTC). At laparotomy, an esophago-gastric segment from 5 cm above the LES and including a 4 cm cuff of the stomach was carefully excised and placed into Krebs solution equilibrated with 95% O2/5% CO2 and maintained at pH 7.40 ± 0.05. The tissue was freed from surrounding fascia, stretched to its in situ length, and then cut along the greater curvature of the stomach. The mucosa was then gently removed to expose the LES circular and sling regions [23]. To consider regional differences within the LES circular vs sling muscles, muscle strips and isolated smooth muscle cells were prepared from each region.

Muscle strip studies

Muscle strips, 2 mm wide and 8 mm long, were obtained from the long axis of the circular and oblique sling muscles. Muscle strips were individually mounted in a 25 mL water-jacketed tissue bath. For isometric tension measurement (transducer model FT-03; Grass Instruments, Quincy, MA, USA) Force transducer data were acquired (Digidata 1200B analog-to-digital converter, Axon Instruments, Union City, CA, USA) and analyzed using pCLAMP software (version 8; Axon Instruments, Union City, CA, USA). Transmural electrical field stimulation (EFS) was delivered (Grass stimulator SP-9) through platinum wire electrodes with 0.5 ms square-wave pulses in a 5 s train at a frequency of 10 Hz and a strength of 70 V.

Initially muscle strips were hung with 0.5 g tension for a 1 h equilibration period, and the length was then measured and defined as L0 (initial length). Strips were then slowly stretched twice at increments of 25% of L0 with 15 min between each stretch [24]. At study length of 150% L0, EFS of the sling muscle resulted in an initial contraction in all strips and EFS of circular muscle resulted in relaxation in all strips studied [25].

The relative contribution of the BKCa channel to tension as affected by an exogenous or endogenous myogenic source of NO was assessed with the nerves intact or blocked with tetrodotoxin (TTX) and using the following protocols: (1) TTX alone (10-6 mol/L) or with blockade of the BKCa channel with iberiotoxin alone (IbTX, 10-7 mol/L), or the two in combination TTX (10-6 mol/L) + IbTX (10-7 mol/L); and (2) TTX (10-6 mol/L) + SNP sodium nitroprusside (10-7 mol/L) ± IbTX (10-6 mol/L) or TTX (10-7 mol/L) + IbTX (10-7 mol/L) ± SNP (10-7 mol/L). The chemicals were successively added and allowed 15-30 min to act, the strips were not washed in between experimental steps.

L’Heureux MC et al. NO activation of K+ channel in LES
The data were normalized and expressed as tension: tension (mmol/L per mm²) = [tone (g) × 9.81 m/s²]/[cross-sectional area (mm²)]; where the cross-sectional area (mm²) = [tissue weight (mg)]/[1.05 mg/mm² × study length (mm)], and where 1.05 mg/mm² is the density of smooth muscle. The unstimulated tension was referred to as baseline resting tension. After an experiment, each muscle strip was blotted onto a filter paper and weighed.

**Isolated smooth muscle cell studies**

Freshly enzymatically-digested and isolated circular smooth muscle cells were prepared from each LES region. Two to three pieces (about 2 mm²) of LES tissues were cut and placed into a test tube with 1 mL of dissociation solution stored at 4°C for up to 36 h. For cell dissociation of the cells, each of the following chemicals was added to the test tube: papain (2 mg/mL), collagenase Sigma blend type F (1.3 mg/mL), 1,4-dithio-L-threitol (154 μg/mL) and bovine serum albumin (1 mg/mL) were added and the tube was incubated at 35°C for 30-60 min. Following rinsing in enzyme-free dissociation solution and gentle mechanical agitation, isolated spindle-shaped single smooth muscle cells were allowed to settle and adhere to a 1 mL glass bottom recording chamber, mounted on the stage of an inverted microscope. After 30 min, cells were washed with external solution and used within 4 h of isolation. Recordings were performed at room temperature (22-25°C).

To study outward K⁺ currents, the perforated patch clamp technique was employed, with the pipette tip resistance between 2-4 MΩ. The pipettes were front-filled with the pipette solution and back-filled with the nystatin solution. With a giga ohm seal the cell was held at -50 mV and a whole-cell configuration was achieved within 5 min. Once a stable access resistance (i.e. below 20 MΩ) had been achieved and maintained, the RMP was measured. To study the effect of NO, two approaches were employed: (1) puffing sodium nitroprusside (SNP, NO donor, 10⁻⁴ mol/L) onto the muscle cell with a picospritzer positioned 100 μm from the cell (to mimic NO release from nerves); and (2) adding N⁶-nitro-L-arginine (L-NNa; NO synthase inhibitor, 10⁻⁷ mol/L) to the recording dish (to assess the potential contribution of endogenous NO). BKCa channel currents were blocked by the addition of iberiotoxin (IbTX, 200 μmol/L).

To record outward K⁺ currents, voltage-ramp protocols (starting and ending with the holding potential of -50 mV), from -140 to +60 mV or from -70 to +70 mV over 500 ms were generated by a pClamp 9.0 software (Axon Instruments, Union City, CA, USA) and recorded by an Axopatch 200B amplifier (Axon Instruments). Before digitization, all signals were sampled at 10 kHz, filtered at 1 kHz by an on board eight-pole Bessel filter, and digitized (Digidata 1320 converter, Axon Instruments). Cell capacitance was determined by integration of the capacitance transient, and the maximal current density was normalized for cell size. Peak outward currents were compared after each pharmacological manipulation. Recordings were analyzed using Clampfit 9.0 software (Axon Instruments).

**Chemicals and solutions**

Atropine (atropine sulfate), TTX (Alomone Labs, Jerusalem, Israel), IbTX (Alomone Labs) and the dissociation enzymes were reconstituted into deionized distilled water. SNP was diluted into the external solution. Seventy-five mg of L-NNa was added into 5 mL of 0.1 mol/L HCl, heated gently until dissolved, and then brought up to 10 mL with water. For nystatin, 0.006 g of the powder was dissolved into 200 μL DMSO and 13 μL of that stock was further diluted into 2 mL of pipette solution. If not otherwise stated, all chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

For the muscle strip studies, the Krebs solution (115 mmol/L NaCl, 4.6 mmol/L KCl, 1.2 mmol/L MgSO₄·7H₂O, 1.2 mmol/L NaH₂PO₄·H₂O, 22 mmol/L NaHCO₃, 2.0 mmol/L CaCl₂·2H₂O and 11 mmol/L dextrose) was maintained at 37°C with 95% O₂/5% CO₂ in the organ bath. For the isolated smooth muscle studies, the dissociation solution (135 mmol/L KCl, 10 mmol/L HEPES, 10 mmol/L glucose, 10 mmol/L taurine, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 0.25 mmol/L EDTA; pH 7.0 adjusted with 1 mol/L KOH), the external solution (130 mmol/L NaCl, 5 mmol/L KCl, 20 mmol/L HEPES, 10 mmol/L glucose, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂; pH 7.4 adjusted with 1 mol/L NaOH) and the pipette solution (140 mmol/L KCl, 20 mmol/L HEPES, 10 mmol/L glucose, 0.1 mmol/L EGTA, 1 mmol/L MgCl₂; pH 7.2 adjusted with 1 N KOH) all had an osmolality between 285 and 310 mOsm.

**Statistical analysis**

All data are expressed as mean ± SE, where “n” represents the number of muscle strips (n = 6-11) or cells (n = 6-13) studied per group of 2-5 cats per experiment. Each muscle strip or cell served as its own control. SAS software (SAS Institute, Cary, NC, USA) was used to determine statistical differences between groups by a repeated measure ANOVA, followed by a post hoc Bonferroni adjusted paired-wise comparison test. A Student’s t-test was also used to determine statistical differences between means. An alpha value of 0.05 was considered significant.

**RESULTS**

**Effects of NO on the BKCa channel in LES isolated smooth muscle cells**

The RMP of the LES circular smooth muscle cells (no added drugs) was significantly more depolarized than the sling smooth muscle cells (-39.7 ± 0.8 mV vs -48.1 ± 1.6 mV, P < 0.001, data not shown). Ramp protocols elicited outward potassium currents at potentials greater than -35 mV. The maximal total outward current was similar in isolated smooth muscle cells from the LES circular and sling regions (27.1 ± 1.5 pA/pF vs 25.7 ± 2.0 pA/pF, P > 0.05).

To mimic the effect of NO release from nerves, the NO donor, SNP, was puffed onto the smooth muscle cells, and a ramp protocol from -140 to +60 mV was used. SNP increased outward potassium currents of LES circular...
smooth muscle cells from 25.9 ± 1.9 to 46.7 ± 4.2 pA/pF ($P < 0.001$, Figure 1); an 80% increase, and hyperpolarized the cells (from -42.2 ± 1.3 to -63.4 ± 5.0 mV, $P < 0.05$, Table 1). In contrast, SNP did not cause a significant increase in the outward K$^+$ currents (from 23.2 ± 3.1 to 27.0 ± 3.4 pA/pF, $P > 0.05$, Figure 1) of sling cells, and no significant change in the RMP (Table 1) was observed. SNP also activated outward potassium currents in circular smooth muscle from both proximal (21.9 ± 3.0 to 38.5 ± 9.6 pA/pF, $n = 5$) and distal (23.5 ± 2.3 to 39.1 ± 6.0 pA/pF, $n = 8$) esophageal body sites.

In the LES, since the effect of the exogenous NO donor (SNP) was seen only in the circular muscle cells, we then pharmacologically isolated the portion of the recorded outward potassium currents attributed to the BK$_{Ca}$ channel currents only in the circular muscle cells.$^{[30]}$ The BK$_{Ca}$ channel was blocked with 200 nmol/L IbTX, and a voltage-ramp protocol, from -140 to +60 mV, was employed where the different steps of the protocol were performed on the same LES circular smooth muscle cells. SNP induced a significant increase in elicited outward potassium currents in control current density (37.9 ± 4.9 pA/pF vs 26.7 ± 3.5 pA/pF, $P < 0.05$, $n = 8$, Figure 2). IbTX alone blocked a significant portion of the control outward potassium currents (13.7 ± 2.2 pA/pF vs 26.7 ± 3.5 pA/pF, $P < 0.05$, $n = 8$, Figure 2). SNP added in the presence of IbTX, produced no significant increase in the outward currents (17.0 ± 2.8 vs 13.7 ± 2.2, $P > 0.05$ for IbTX+SNP vs IbTX alone, $n = 8$, Figure 2). In separate experiments, blockade of K$_{Ca}$ with DTX (200 nmol/L) did not inhibit SNP induced outward potassium currents. Thus, for the circular smooth muscle cells, SNP, an exogenous source of NO, produced a significant activation of the BK$_{Ca}$ channel.

To assess the potential contribution of an endogenous source of NO in the two LES regions, the NO synthase inhibitor, L-NNA, was added and a voltage-ramp protocol from -70 to +70 mV was used. Addition of L-NNA led to an insignificant 14% decrease in outward potassium currents in the circular smooth muscle cells (from 29.1 ± 2.6 to 25.0 ± 2.4 pA/pF, $P > 0.05$, Figure 3), however, L-NNA significantly depolarized the cell (from -40.5 ± 1.4 to -33.8 ± 1.5 mV, $P < 0.05$, Table 1), a 17% change in the

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**Table 1** Resting membrane potential (mV) of isolated cells from the lower esophageal sphincter circular and sling regions in the absence or presence of sodium nitroprusside or L-NNA.

| Condition          | Circular | Sling |
|--------------------|----------|-------|
| Control            | -42.2 ± 1.3 | -53.0 ± 1.2 |
| Sodium nitroprusside | -63.4 ± 5.0$^a$ | -50.3 ± 3.1 |
| Control            | -40.5 ± 1.4 | -44.0 ± 1.1 |
| L-NNA              | -33.8 ± 1.5$^a$ | -38.8 ± 2.3 |

$^aP < 0.05$ vs control.
In the sling smooth muscle cells, there was a smaller reduction in current density (from 28.2 ± 2.5 to 26.3 ± 2.7 pA/pF, P > 0.05, Figure 3), a 7% decrease, and only a 3% increase in the RMP (from -44.0 ± 1.1 to -38.8 ± 2.3 mV, P > 0.05, Table 1). Thus, an endogenous source of NO may modulate outward potassium currents and RMP, but to a small extent, and the effect is more pronounced in the circular muscle.

**Effects of NO on the BKCa channel in LES smooth muscle strips**

In view of the small effect of endogenous NO on the RMP of both circular and sling muscle cells, we investigated the myogenic contribution of the BKCa channel to basal resting smooth muscle tone. Nerves were blocked with TTX and the BKCa channel was inhibited with IbTX. For the circular muscle, TTX (tension 14.5 ± 3.4 mmol/L per mm² vs 18.2 ± 4.7 mmol/L per mm²) or IbTX (tension 14.5 ± 2.3 mmol/L per mm² vs 15.0 ± 2.3 mmol/L per mm²) did not significantly change the resting tension (P > 0.05, Figure 4A). The combined blockade of the nerves and the BKCa channel also resulted in no significant change of the overall muscle tone (P > 0.05). The combined blockade of the nerves and the BKCa channel also resulted in no significant change of the overall muscle tone (P > 0.05).

**Figure 3** Representative traces and quantification of the effect of L-NNA (10⁻⁵ mol/L) on elicited outward K⁺ currents using a ramp protocol from -70 to +70 mV of lower esophageal sphincter circular (n = 8) and sling (n = 8) smooth muscle cells. Addition of L-NNA led to an insignificant 14% decrease in outward potassium currents in the circular smooth muscle cells. In the sling smooth muscle cells, the addition of L-NNA led to an insignificant 7% reduction in current density.

**Figure 4** Effect of the blockade of the BKCa channel with iberiotoxin (10⁻⁵ mol/L) of lower esophageal sphincter (A) circular (n = 6-8) and (B) sling (n = 7-11) smooth muscle tone with intact nerves or blocked with tetrodotoxin (10⁻⁵ mol/L). A: For the circular muscle, tetrodotoxin (TTX) or iberiotoxin (IbTX) did not significantly change the resting tension (P > 0.05). The combined blockade of the nerves and the BKCa channel also resulted in no significant change of the overall muscle tone (P > 0.05); B: Similarly, for the sling muscle, TTX, IbTX or TTX and IbTX together did not significantly modulate the basal resting tone (P > 0.05). #: Separates different experiments.

To study the effect of exogenous NO on the BKCa channel in muscle strips, SNP was added in the presence or absence of IbTX, and *vice versa*, with the nerves blocked with TTX. In LES circular muscle, SNP decreased the resting tension by 17.4 ± 5.7 mmol/L per mm², the tension was unchanged with further addition of IbTX (P > 0.05, Figure 5). When IbTX was added first, there was no significant change in tension, while the subsequent addition of SNP (IbTX+SNP in the presence of TTX) significantly decreased the change in tension (1.1 ± 0.5 mmol/L per mm² vs -6.3 ± 1.2 mmol/L per mm², P < 0.001, Figure 5). For the sling muscle, similar changes were observed. SNP or SNP+IbTX decreased the tension to the same extent, IbTX alone produced no significant change in resting tension, and IbTX+SNP decreased the tension by 4.1 ± 1.0 mmol/L per mm² (P < 0.001, Figure 5). Therefore, in the cat, whether the BKCa channel is blocked or not, exogenous NO causes relaxation in both sling and circular smooth muscles.

The effects of IbTX on neural responses induced by Electrical Field Stimulation (EFS) were assessed. The addition of TTX was omitted in these studies. In circular muscle, EFS caused transient relaxation of resting tone...
Furthermore, exogenous NO, whether provided by SNP or tert NO was significant only in the LES circular muscle. In some species, the present study explored this role in the LES in the modulation of resting tone and relaxation of the sling and circular muscle components of the cat LES.

**DISCUSSION**

In view of the part played by the activation of the BK_{Ca} by NO in the modulation of resting tone and relaxation of the LES in some species, the present study explored this role in the sling and circular muscle components of the cat LES. The BK_{Ca} responsiveness to the inhibitory neurotransmitter NO was significant only in the LES circular muscle. Furthermore, exogenous NO, whether provided by SNP or released by EFS, produced its inhibitory effect to relax LES smooth muscle predominantly through other mechanisms than the activation of the BK_{Ca} channel. There may be a small endogenous source of NO that has a small inhibitory effect to slightly reduce resting tone in both muscles. These findings add to the other known physiological properties of the two muscles and their regional differences. Any NO-BK_{Ca} relaxant effect, if needed, would be directed primarily to facilitate relaxation of the high intrinsic myogenic tone of the LES circular muscle.

Since SNP produced a large and significant activation of the BK_{Ca} channel only in the circular smooth muscle cells, a difference in sensitivity of the BK_{Ca} to NO is one possible explanation for this disparity, assuming that the two muscles express a similar number of channels. This disparity could also reflect regional differences in K+ channel molecular constitution through expression of different regulatory subunits leading to channel isofoms with different channel activity and sensitivity to voltage and intracellular calcium. In the human esophagus there are at least four different splice variants of BK_{Ca} with differences in expression at various sites of the esophageal body and LES.

It is generally accepted that in vivo, NO is the main neurotransmitter for active inhibition of the LES. The circular muscle with its high intrinsic tone relaxes predominantly due to the neural release of the inhibitory neurotransmitter NO as well as some non-NO inhibitory influence. The sling muscle with its tone maintained by cholinergic excitation, presumably relaxes predominantly by switching off this excitatory influence, although NO can also relax the sling when contracted by cholinergic stimulation.

The primary mechanism for NO-mediated relaxation is considered to be via a cyclic guanosine monophosphate (cGMP) pathway. There are several potential intracellular pathways whereby NO can lead to smooth muscle relaxation. Activation of the BK_{Ca} by NO could potentially occur directly or indirectly through interaction with a number of these mechanisms. For example, the PKG-pathways are accepted intracellular messengers that can lead ultimately to LES circular muscle relaxation in dog, opossum and cat. In addition, an involvement of the contractile proteins has been proposed in the sequence of events targeted by NO for LES relaxation in opossum. The BK_{Ca} channel integrates changes in both intracellular Ca2+ and membrane potential, and the cGMP pathway can in counter-part influence these latter parameters. Moreover, cross-talk between NO and the ion channel may be facilitated by a close spatial relationship between ion channels and NOS sequestered in or in the vicinity of caveolae, seen in dog LES circular muscle. Although it is unclear why endogenous NO had minimal effect on resting LES tone in this study of the cat, variability in the effect of different NO donors and NO states on gut smooth muscle physiology as well as species differences have also been reported and are likely contributing factors. The apparent species and smooth muscle differences open the door to further experimentation. This aim in our paper was not

**Figure 5.** Effect of sodium nitroprusside (10^{-6} M) ± iberiotoxin (10^{-7} M) and iberiotoxin (10^{-7} M) ± sodium nitroprusside (10^{-6} M) on smooth muscle tone relative to basal tone in the presence of tetrodotoxin (10^{-6} M) in muscle strips of lower esophageal sphincter (A) circular (n = 6) and (B) sling (n = 6-9) muscles. 
A: In lower esophageal sphincter (LES) circular muscle, sodium nitroprusside (SNP) decreased the resting tension and this tension was then unchanged with further addition of iberiotoxin (IbTX) (P > 0.05). When IbTX was added first, there was no significant change in tension, while the subsequent addition of SNP [IbTX+SNP in the presence of tetrodotoxin (TTX)] significantly decreased the tension (P < 0.001); B: In LES sling muscle, SNP or SNP+IbTX decreased the tension to the same extent, and IbTX alone produced no significant change in resting tension. IbTX+SNP decreased the tension (P < 0.001). II: Separates different experiments. P < 0.001.

from 14.5 ± 2.3 to 3.8 ± 1.9 mmol/L per mm², and IbTX had no effect on LES tone (15.0 ± 2.3 vs 14.5 ± 2.3, P > 0.05) or EFS induced LES relaxation (3.9 ± 1.9 mmol/L per mm² vs 3.8 ± 1.9 mmol/L per mm², P > 0.05). In the LES sling muscle, EFS caused a transient contractile response from resting tone of 7.3 ± 1.2 to 19.3 ± 4.1 mmol/L per mm². IbTX had no effect on resting tone (7.3 ± 1.3 mmol/L per mm² vs 7.3 ± 1.2 mmol/L per mm², P > 0.05) or EFS induced responses (19.3 ± 4.1 mmol/L per mm² vs 23.4 ± 5.8 mmol/L per mm², P > 0.05).
directed to a study of the potential intracellular pathways and mechanisms underlying these differences.

Although the BK\textsubscript{\textalpha} current density is greater in the circular muscle, the channel plays a role in setting the RMP in both LES muscles\cite{3,16}. IbTX causes significant membrane depolarization in both LES muscles, as confirmed in the present study. This modulation of the RMP by the BK\textsubscript{\textalpha} is also present in the opossum LES circular muscle\cite{17}. Our present experiments in isolated smooth muscle cells of the cat LES, show that this activation of the BK\textsubscript{\textalpha} in setting the RMP involves at least an endogenous source of NO in both LES regions, consistent with findings in dog LES circular muscle\cite{3,16}.

Although the BK\textsubscript{\textalpha} contributes to setting the RMP, and\cite{14,17}, in the current muscle strip studies, blockade of the BK\textsubscript{\textalpha} with IbTX did not result in a significant increase in muscle tension in either muscle. This is unlike the esophageal body where both IbTX and tetra ethyl ammonium increase tonic and phasic contractility\cite{28}. In the opossum LES circular muscle, blockade of the BK\textsubscript{\textalpha} results in an increase in tone when the RMP is raised to the point where ongoing spiking activity is produced. In those experiments, BK\textsubscript{\textalpha} blockade changed the RMP only slightly (from -43.4 to -37.8 mV). The further transient depolarizations associated with the spike-like action potentials then are associated with calcium (Ca\textsuperscript{2+}) entry through the L-type channel\cite{17}. The findings fit with the knowledge that Ca\textsuperscript{2+} is required for, and Ca\textsuperscript{2+} availability can alter LES circular smooth muscle motor response in dog and opossum\cite{7,14}.

The lack of major effect of IbTX on resting tone in both cat LES muscles in the present experiments would occur if membrane depolarization is unable to sufficiently activate channels for entry of extracellular calcium for tension development, either directly or through the stimulation of spiking activity. In the LES muscles, L-type calcium channel activation is only seen at membrane potentials more positive than about -20 mV\cite{27,57}, levels not reached in the sling (-24 mV) or in the circular muscle (-31 mV) with IbTX or other blockade of the BK\textsubscript{\textalpha}\cite{14,17}. Furthermore, we have shown that resting tone in both muscles utilizes both intracellular and extracellular calcium sources, the circular muscle more dependent on an extracellular, and the sling on an intracellular source\cite{28}. In addition, the two muscles utilize different calcium entry portals, the circular muscle using the L-type and the sling a non-L-type channel. Therefore, our findings in the cat indicate that if endogenous NO is acting on the BK\textsubscript{\textalpha} to modulate resting tone, this modulation is mainly through an indirect action, and augmented by its other mechanisms of action\cite{14,13,15,16,49}. Furthermore, there appears to be a more complex relationship in the LES between active changes in membrane potential such as spiking activity or slow wave activity and levels of LES tone\cite{3,16,49}. Our present findings, taken together with findings in other species, indicate that changes in resting tone are likely dependent on Ca\textsuperscript{2+} entry associated with the active changes in membrane potential rather than entry with any small changes in the RMP\cite{7}. Further studies are required to assess which mechanisms and pathways are involved.

In terms of resting tone in the LES, our findings have potential functional significance. In the present in vitro study in the cat, TTX blockade of excitatory as well as inhibitory nerve activity failed to significantly affect basal tension in circular smooth muscle strips. This finding supports the concept that in vivo, intrinsic ongoing myogenic contraction underlies resting tone of the circular muscle. The intrinsic tone in the circular muscle is not significantly modulated by cholinergic excitation and/or nitrergic inhibition\cite{3,14}. On the other hand, cholinergic neural influence, normally vagally-driven, acts to augment and maintain the low intrinsic basal resting tone in the sling.

A better understanding of the asymmetries of both circular and sling LES muscles at the cellular level could give insights into the pathogenesis of and potential therapeutic usefulness for patients with motor disorders such as achalasia and gastroesophageal reflux disease. For instance, in human and cat, addition of atropine decreases the leftward resting LES pressure where the sling is located, with little effect in the other radial orientations\cite{3,53,56}. Although in the present cat experiments, exogenous NO causes smooth muscle relaxation of both the LES circular and sling smooth muscles, whether the BK\textsubscript{\textalpha} channel is blocked or not, activation of the BK\textsubscript{\textalpha} channel by NO provides another potential mechanism to augment the muscle relaxing effect of the cGMP pathway. Whether such a mechanism may become more important, for example in circumstances where NO release to produce relaxation of the circular muscle is decreased, as in patients with achalasia, requires further study\cite{84-86}. Hence, regional differences in muscle cell BK\textsubscript{\textalpha} responsiveness to NO hold the potential for selective modulation of LES function in health and disease.

**COMMENTS**

**Background**

The lower esophageal sphincter (LES) is normally closed to prevent reflux of gastric acid, but opens with a swallow to allow passage of the bolus into the stomach. The LES has two main components, the circular and sling muscles. To maintain closure, the circular muscle has significant resting tone but is poorly responsive to neural cholinergic excitation. On the other hand, the sling has little resting tone, and its closing contraction is maintained by vagal release of acetylcholine. Opening of the LES with a swallow requires relaxation of the two muscles. Relaxation of the circular muscle is therefore dependent on active relaxation induced by an inhibitory neurotransmitter such as nitric oxide (NO), while relaxation of the sling can readily occur with vagal excitation turned off.

**Research frontiers**

Although NO can relax smooth muscle by a number of cellular mechanisms, in some species activation of the BK\textsubscript{\textalpha} by NO is important for the modulation of resting tone and relaxation of the LES. The importance of this mechanism was assessed in isolated cells and in strips of muscle from the two muscle components of the cat LES. The cat esophagus is very similar to the human esophagus with a significant portion of the distal esophagus composed of smooth muscle.

**Innovations and breakthroughs**

The BK\textsubscript{\textalpha} responded to the inhibitory neurotransmitter NO, administered as sodium nitroprusside (SNP), only in the LES circular muscle, the muscle with high resting tone. Furthermore and as opposed to other species, exogenous NO, whether provided by SNP or released by electrical field stimulation, provided its inhibitory effect to relax LES smooth muscle predominantly through other mechanisms than the activation of the BK\textsubscript{\textalpha} channel.

**Applications**

Any NO-BK\textsubscript{\textalpha} relaxant effect, if needed, would be directed primarily to facilitate
relaxation of the high intrinsic myogenic tone of the LES circular muscle. Such a mechanism may become more important, for example in circumstances where NO release to produce relaxation of the circular muscle is decreased, as in patients with achalasia. Since NO provided its inhibitory effect to relax LES smooth muscle predominantly through other mechanisms than the activation of the BK<sub>Ca</sub> channel, further research should be directed to these other mechanisms. Such studies hold the potential for new therapeutic approaches applicable to the human esophagus.

**Terminology**

Achalasia: a condition where swallowing difficulty is due to obstruction of the esophagus at the LES resulting from failure of LES relaxation due to absence of the inhibitory neurotransmitter NO.

**Peer review**

This is a well-designed and interesting study that provides convincing evidence regarding the activation of large conductance potassium channels within the circular smooth muscle during administration of exogenous nitric oxide. The role of endogenous nitric oxide appears to be relatively less significant.

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