Regional differences in L-type Ca\textsuperscript{2+} channel expression in feline lower esophageal sphincter

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Muinuddin, Ahmad, Youhou Kang, Herbert Y. Gaisano, and Nicholas E. Diamant. Regional differences in L-type Ca\textsuperscript{2+} channel expression in feline lower esophageal sphincter. Am J Physiol Gastrointest Liver Physiol 287: G772–G781, 2004. First published June 3, 2004; 10.1152/ajpgi.00102.2004.—In humans and cats, muscle from the lower esophageal sphincter (LES) circular region exhibits greater spontaneous tone than LES sling muscle, whereas the sling muscle is much more responsive to cholinergic stimulation. Despite physiological and pharmacological evidence for the presence of L-type Ca\textsuperscript{2+} channel current ($I_{\text{Ca,L}}$) activity in LES circular muscle, the identity of this channel has not been demonstrated biochemically or electrophysiologically fingerprinted. Furthermore, there is no information on the channel’s presence and role in the sling region of the LES. We hypothesized that regional differences in the expression of $I_{\text{Ca,L}}$ between LES circular and sling muscles, if present, could contribute to the functional asymmetry observed within the LES. $I_{\text{Ca,L}}$ expression was compared between circular and sling regions of the LES by Western blot analysis. The patch-clamp technique was used to study $I_{\text{Ca,L}}$. Muscle strip studies assessed $I_{\text{Ca,L}}$ contribution to contractile activity. We found both protein expression of $I_{\text{Ca,L}}$ and $I_{\text{Ca,L}}$ density to be greater in LES circular muscle than sling muscle. $I_{\text{Ca,L}}$ voltage- and time-dependent activation and inactivation curves were similar in cells from both regions. $I_{\text{Ca,L}}$ blockade with nifedipine inhibited spontaneous tone and ACh-induced contractions only in circular muscle but was able to abolish depolarization (KCl)-induced contractions in both sling and circular muscles. In contrast, La\textsuperscript{3+} inhibited tone and ACh-induced contractions in muscles from both regions. Therefore, regional myogenic differences in $I_{\text{Ca,L}}$ expression within the LES circular and sling muscle exist and provide one explanation for the differential contribution of sling and circular muscle to LES contractility.

Voltage-dependent Ca\textsuperscript{2+} channels have been identified in virtually all gastrointestinal smooth muscles including the stomach (33), intestine (10), and colon (19). They play a key role in action potential generation (6), modulation of slow-wave activity (31), and initiation of muscle contractions (36). In the esophagus, L-type Ca\textsuperscript{2+} channel current ($I_{\text{Ca,L}}$) has been studied using patch-clamp techniques in rabbit esophageal muscularis mucosa (1) and esophageal circular smooth muscle from the esophageal body of the opossum (2), cat (22, 35), and human (17a). In the esophageal body, $I_{\text{Ca,L}}$ plays an important role in excitation-contraction coupling of the smooth muscle. Nifedipine, a specific blocker of $I_{\text{Ca,L}}$, reduces cholinergically induced contractions in human and feline esophageal smooth muscle, suggesting that muscarinic excitation of the circular smooth muscle esophagus involves influx of Ca\textsuperscript{2+} through the $I_{\text{Ca,L}}$ (22, 34). In the LES circular muscle of all species studied, spontaneous muscle tone is supported by ongoing influx of extracellular Ca\textsuperscript{2+} through plasmalemmal Ca\textsuperscript{2+} channels as demonstrated by reduction in LES tone in the presence of $I_{\text{Ca,L}}$ channel blockers in opossum (11), cat (4, 24), dog (3, 30), and human LES (17a, 38). However, despite this physiological and pharmacological evidence for the presence of $I_{\text{Ca,L}}$ channel activity in the LES, this LES channel has not been definitively identified at the protein level or fully electrophysiologically fingerprinted. Furthermore, there is no information on the channel’s presence and role in the sling region of the LES.

In light of the differences in tonic and ACh-induced contractility between the sling and circular muscles, which could, in part, be explained by possible differences in $I_{\text{Ca,L}}$ expression or regulation, we employed complementary assays including, biochemical, electrophysiological, and contractile studies on the sling and circular muscle groups of the feline LES. With these assays, our aims were to: 1) characterize $I_{\text{Ca,L}}$ in LES smooth muscles, 2) assess regional differences in the expression of $I_{\text{Ca,L}}$, and 3) determine whether regional differences in the expression of $I_{\text{Ca,L}}$, if present, contribute to the contractile responses of LES muscles. A portion of this study has been reported elsewhere in abstract form (23).

METHODS

Tissue preparation and cell dissociation. Smooth muscle cells were isolated from feline LES circular muscle and from the oblique sling muscle as described previously (29). Briefly, adult cats of either sex were killed by intravenous injection of pentobarbital sodium overdose following a protocol approved by the University Health Network.

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Animal Care Committee. The esophagus was quickly excised and placed in Krebs solution. The specimen was freed of surrounding fascia and stretched to its in situ length. The excised esophagus was then opened along the greater curvature, and the mucosa was removed by sharp dissection. The circular and sling fibers of the LES were readily visible (26). Muscle from each region was dissected out and cut into squares of ~2 mm². These squares were placed in a test tube with 1-ml dissociation solution composed of (in mM): 110.0 NaCl, 5.0 KCl, 0.16 CaCl₂, 2.0 MgCl₂, 10.0 HEPES, 10.0 NaHCO₃, 0.5 KH₂PO₄, 0.5 NaH₂PO₄, 0.49 EDTA, and 10.0 glucose (pH 7.0). Papain (2 mg/ml) as well as collagenase blend F (1.3 mg/ml), 1.4-dithiothreitol (154 μg/ml), and bovine serum albumin (1 mg/ml) were added to the test tube that was then incubated in a water bath at 35°C for 60 min. Tissues were then rinsed with enzyme-free dissociation solution and gently agitated with a glass pipette. Spindle-shaped single smooth muscle cells were then used for patch-clamp study within the following 5 h after dissociation.

Whole cell patch clamp. Isolated muscle cells suspended in dissociation solution were placed in a 1-ml glass-bottom dish mounted on the stage of an inverted microscope and allowed to adhere to the bottom for 30 min. The chamber was perfused with the external solution composed of (in mM): 20 BaCl₂, 90 NaCl, 5 CsCl, 1 MgCl₂, 20 tetraethylammonium (TEA)-Cl, and 10 HEPES (pH 7.4). Barium was used rather than Ca²⁺ as the charge carrier to amplify the Ca²⁺ channel current and minimize Ca²⁺-dependent rundown (13, 40). Pipette resistance was between 2–4 MΩ after being filled with the pipette solution containing the following components (in mM): 105 Cs-aspartate, 1 MgCl₂, 5 EGTA, 4 Mg-ATP, and 20 HEPES (pH 7.2). Recordings were performed by using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Whole cell voltage-clamp protocols were generated by pClamp8 software (Axon Instruments). Experiments were only carried out on cells that had achieved a stable access resistance of <20 MΩ. All signals were filtered at 1 kHz by an onboard eight-pole Bessel filter before digitization with a Digidata 1320 analog-to-digital converter (Axon Instruments) and sampled at 10 kHz. Cell capacitance was determined by integration of the capacitive transient. All experiments were performed at room temperature of 22–25°C.

Immunoblotting. Membrane protein samples were prepared from the circular and sling muscle regions of the LES. Muscle tissues, isolated as described above and stored at ~80°C, were minced into small pieces (~1 mm³) and homogenized on ice using an electrotomogenizer (Ultra-Turrax T25 Basic, IKA Labor Technical, Staufen, Germany) at 16,000 rpm for 1 min × 5 in 600 μl homogenization buffer containing 250 mM sucrose, 50 mM MOPS, 0.1 mM phenylmethanesulfonyl fluoride, 5 mg/ml leupeptin, 5 g/ml antipain, and 5 mg/ml aprotinin A (pH 7.4 adjusted with NaOH). This was followed by sonication using a Vibra Cell sonicator (Sonic Materials, Danbury, CT) at output control 40, 30 s/ml × 3 on ice. The large tissue debris and nuclei were then removed by a low-centrifuge spin (1,000 g for 10 min) at 4°C, followed by a ultracentrifugation at 100,000 g at 4°C for 30 min to obtain the membrane pellet. The protein concentration of the membrane pellet was determined by the Lowry method using bovine serum albumin as a standard. Equivalent amounts of total protein (15 μg) from LES circular and sling muscle tissue samples obtained from four cats were loaded and electrophoretically size separated on a 15% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Subsequently, the membranes were incubated for 2 h at room temperature with a rabbit anti-α₁Csubunit (1:200), which is a polyclonal antibody raised against residues 818–835 of the intracellular loop between domains II and III of α₁C-subunit of the L-type Ca²⁺ channel (Alomone Laboratories, Jerusalem, Israel). Detection was made possible by enhanced chemiluminescence (Amersham, Arlington Heights, IL), and the specific bands were quantified by densitometric scanning of the blots.

Contractility studies. Muscle strips measuring 2 mm in width and 8 mm in length were obtained from the circular and sling muscle regions. A silk thread was tied to each end, and the strips were transferred to 25-ml organ baths containing Krebs solution bubbled with 5% CO₂/95% O₂ at 37°C and maintained at pH 7.4 ± 0.05. One end of the strip was fixed to an electrode holder and the other end to an isometric force transducer (model FT-03; Grass Instruments, Quincy, MA) coupled to a chart recorder (model 79E; Grass Instruments). The force transducer was supported on a rack-and-pinion clamp (Harvard Apparatus, Holliston, MA), which facilitated accurate length adjustment of the muscle strips. Transmural electrical field stimulation (EFS) was delivered by a Grass S89 stimulator through platinum wire electrodes placed on either side of the tissue strips. EFS consisted of 0.5-ms square-wave pulses in a 5-s train at 10 Hz and a strength of 50 V. Tissue strips were hung loosely, with no tension being applied to them in the organ baths, for a 1-h equilibration period before commencing studies. Each strip was gently stretched to initial length (Lₒ), which was first determined with a micrometer as the length at which a rapid stretch caused a small transient deflection of the recorder pen (~50 mg of tension), and allowed to equilibrate for 30 min. At this level of stretch, any slack in the strip or silk ties was eliminated, and further stretch began to produce active tension. Muscle strips were then slowly stretched and tested at increments of 25% of their Lₒ until optimal response to cholinergic stimulation with ACh at 10⁻³ M was achieved, after which point the experiments commenced. ACh was readily washed out, and separate experiments examining the effect of repeated exposure to ACh did not demonstrate tachyphylaxis over time. Subsequent experiments were all carried out in the presence of TTX (1 μM) to ensure the myogenic response was being assessed. At the completion of all experiments, muscle strips were bathed in Ca²⁺-free Krebs for 30 min to abolish all active contraction and record baseline tension for determination of LES tone. Afterwards, the strips were blotted on filter paper and the weight was determined. Tension was then expressed in milliNewtons per milligram.

Toxins, antibodies, and chemicals. Antibodies to the subunits of the L-type Ca²⁺ channels were obtained from Alomone Labs. Bay K 8644, nifedipine, ACh, TTX, and all enzymes and other chemical reagents were obtained from Sigma (St. Louis, MO). Both Bay K 8644 and nifedipine were prepared in DMSO with a final DMSO concentration in solution of 0.1%. Separate experiments confirmed that this concentration of vehicle did not significantly contribute to observed responses investigated. TTX and ACh were prepared as a 10 mM stock in double-distilled water.

Statistical analysis. Data are presented as means ± SE. A Student’s t-test was used to compare data between two groups, whereas ANOVA was used among three or more groups followed by the Tukey-Kramer posttest. P values <0.05 were considered to have significant difference. In the electrophysiological studies, n is the number of cells studied. In muscle strip studies, n is the number of muscle strips studied.

RESULTS

Freshly isolated esophageal LES smooth muscle cells were spindle shaped, of variable length, and appeared phase bright under phase-contrast microscopy. Reversible contractions were observed when these cells were exposed to acetylcholine (10–100 μM) in Ca²⁺-containing external solution (2.5 mM), indicating that the cells are viable and have retained contractile function after enzymatic digestion.

Identification of I_Ca,L in LES circular and sling muscle cells. We electrophysiologically identified and characterized the inward I_Ca,L in the LES circular and sling muscle cells. To examine this depolarization-activated inward current in isolation, K⁺ currents were blocked with TEA in the external bath
solution and Cs\(^+\) in the external and pipette solutions. Ba\(^{2+}\) was added to the external bath solution to enhance the \(I_{\text{Ca,L}}\) conductance and minimize Ca\(^{2+}\)-dependent rundown (13, 40).

As shown in Fig. 1A, when LES circular muscle cells were held at \(-50\text{mV}\) and stepped to more positive test potentials, inward current first became apparent at \(-30\text{mV}\) and reached maximal value at \(+10\text{mV}\). The reversal potential was at approximately \(+50\text{mV}\) (Fig. 1B). The inward current showed a transient component, first peaking then declining (Fig. 1A), as well as a sustained component (see Fig. 4B). With respect to Ca\(^{2+}\) (2.5 mM) as the charge carrier, Ba\(^{2+}\) (20 mM) increased the magnitude of the transient and sustained components of the inward currents. Figure 1A shows a family of inward currents in response to depolarization from a holding potential of \(-50\text{mV}\) in an LES circular muscle cell. Stimulation of the cells with the dihydropyridine Ca\(^{2+}\)-channel agonist Bay K 8644 (1 \(\mu\text{M}\)) caused an increase in inward current, with a scaling up of both the transient and sustained components of the current. The peak inward current was augmented over the entire voltage range with peak inward current occurring at a 10-mV less depolarized holding potential than control, as demonstrated by performing a standard \(I-V\) curve before and after addition of Bay K 8644 to the bath (Fig. 1B). The dihydropyridine Ca\(^{2+}\)-channel blocker nifedipine (1 \(\mu\text{M}\)) significantly inhibited both transient and sustained components of the inward current (Fig. 1). Enhancement with Bay K 8644 and suppression of the Ca\(^{2+}\) current with nifedipine, together with the high-voltage activation, is consistent with the inward current being carried primarily by L-type Ca\(^{2+}\) channels (13, 20, 33). Although another type of Ca\(^{2+}\)-channel current, the T type (\(I_{\text{Ca,T}}\)), was previously identified in circular smooth muscle from the esophageal body of the cat (35), we did not observe this current when stepping at low voltages from \(-80\text{ to } -30\text{mV}\) from a holding potential of \(-80\text{ mV}\) in our LES preparations. In sling muscle cells, we also identified an inward current that was qualitatively identical to the characteristics of the \(I_{\text{Ca,L}}\) of the circular muscle cells.

**Regional difference in \(I_{\text{Ca,L}}\) density.** Figure 2A demonstrates two families of inward current traces from cells from the circular and sling regions of the LES. These two cells had similar cell capacitance to avoid cell size variation. The \(I-V\) relationship of peak inward currents for the cells from these two regions is plotted in Fig. 2B. The threshold potential (approximately \(-30\text{mV}\)) and the reversal potential (approximately \(50\text{mV}\)) were virtually identical. However, the averaged inward current density from the circular muscle region (\(n=8\)) was significantly greater than that from the sling region (\(n=8\)) at voltages from \(-10\text{ to } +20\text{mV}\). The inward current density was \(-0.88 \pm 0.14\text{ pA/pF}\) in LES circular muscle at...
+10 mV, whereas only $-0.31 \pm 0.09\ pA/pF$ in cells from the sling region ($P < 0.05$). Thus the inward current density in the circular tissue was almost three times that of the sling muscle. In the presence of Bay K 8644, the inward current density in the circular tissue was almost three times that of the sling muscle.

Voltage-dependent activation of the $Ca^{2+}$ current was investigated with standard activation protocols. Voltage-dependent activation curves were determined by plotting normalized peak tail current amplitudes against test pulse voltages (Fig. 3). Activation could be described by a Boltzmann equation of the form $I_{max} = \left\{1 + \exp\left\{\left(V_m - V_{1/2}\right) k^{-1}\right\}\right\}^{-1}$, where $I_{max}$ is the relative current, $V_m$ is membrane potential during the prepulses, $V_{1/2}$ is the half-activation potential, and $k$ is the slope factor. From the best-fit curve, the half-maximal activation potential was determined to be $-3.8 \pm 2.8\ mV$ in sling cells and $-6.4 \pm 3.9\ mV$ in circular muscle cells ($n = 4$ for each region; $P > 0.05$). Slope factor ($k$) was similar in both regions (sling $k = 23.9 \pm 2.5$, circular muscle $k = 20.4 \pm 2.5$; $P > 0.05$). Voltage-dependent inactivation was investigated with 10-s prepulses from $-100$ to $10\ mV$ followed by a test pulse to $+10\ mV$. The size of the peak inward current declined with more positive prepulses (Fig. 3). Inactivation could be described by a Boltzmann equation with half-maximal inactivation potentials of $-23.4 \pm 0.7\ mV$ in sling cells and $-26.5 \pm 1.2\ mV$ in circular muscle ($n = 4$ for each region; $P > 0.05$). Slope factor ($k$) was similar in both regions (sling $k = -9.0 \pm 0.6$, circular muscle $k = -10.0 \pm 1.2$; $P > 0.05$). In cells from both regions, the activation and inactivation curves overlapped, suggesting the existence of some noninactivating window current (12), which peaked at $-16\ mV$ reaching 29% of the maximum $I_{Ca,L}$ (Fig. 3).

Time-dependent activation and inactivation of $I_{Ca,L}$ was examined in LES sling and circular muscle cells. Time constants during activation were obtained by fitting depolarization-evoked inward current traces with a monoexponential function. Figure 4A shows that the inward current was activated to a similar degree in cells from both circular and sling regions of the LES. By calculation, the activation time constant ($\tau$) was $2.7 \pm 0.2\ ms$ in circular muscle ($n = 4$) vs. $2.6 \pm 0.1\ ms$ in sling muscle ($n = 4$). There were no significant differences in time-dependent activation of $I_{Ca,L}$ as demonstrated by time constant values ($P > 0.05$). Time-dependent inactivation of the $I_{Ca,L}$ was studied using a long (9 s) depolarizing pulse to $10\ mV$ (Fig. 3B). Time constants ($\tau_1$ and $\tau_2$) of the inward current decay were calculated by fitting traces with a biexponential equation. In circular muscle, $\tau_1 = 4.046 \pm 620\ ms$ and $\tau_2 = 247 \pm 84\ (n = 4)$. In sling, $\tau_1 = 4.450 \pm 395\ ms$ and $\tau_2 = 287 \pm 65\ ms$ ($n = 4$). There were no significant differences in time-dependent inactivation of $I_{Ca,L}$ as demonstrated by time constant values ($P > 0.05$). $Ca^{2+}$ channel α1C-subunit expression. We next investigated the possibility that the different $I_{Ca,L}$ density within the circular and sling regions of the smooth muscle LES could be partly
explained by a possible difference in expression of the \( I_{Ca,L} \) channel protein in these tissues. Therefore, we prepared purified plasma membrane fractions from each of these muscle tissues and performed immunoblot analysis of the membrane \( I_{Ca,L} \) protein using an antibody generated against rabbit anti-\( \alpha \)IC888–835 (1:200), which is a polyclonal antibody raised against residues 888–835 of the intracellular loop between domains II and III of the \( \alpha \)1C-subunit of the L-type \( Ca^{2+} \) channel (Alomone Labs). Rat brain lysates were used as a positive control. Indeed, Fig. 5 demonstrates the characteristic immunoreactive band corresponding to the predicted size (~200 kDa) of the \( \alpha \)1C-subunit protein of the \( I_{Ca,L} \) (14), identical in size to that seen in the rat brain lysate (17). The blots were then reprobed with \( \alpha \)-actin to serve as an internal control for protein loading. Densitometric analysis of Western blots normalized to \( \alpha \)-actin expression were carried out against the \( \alpha \)1C-subunit. There was 2.4 times more expression of the \( \alpha \)1C-subunit in LES circular muscle than in the LES sling region (Fig. 5). The blot shown is representative of four experiments, with each experiment involving samples from three cats. We also performed separate experiments to examine for the presence of the \( \alpha \)1D-subunit; however, we were unable to detect its presence, indicating that \( \alpha \)1D is in low abundance in both the circular and sling LES muscles.

Effect of calcium channel blockade on tone-, KCl-, and ACh-induced contractions. Muscle strip studies in organ baths were carried out to examine the possibility that the regional differences observed in LES contractility might partly be due to different use of \( Ca^{2+} \) influx pathways. To abolish all neural influences, the muscle strips were all treated with TTX (1 \( \mu \)M), and electrical field stimulation confirmed that indeed the neural responses were abolished. Circular and sling muscle strips both developed spontaneous tone, but the spontaneous tone of the circular muscle was greater than the sling muscle (17.0 \( \pm \) 1.3 vs. 7.8 \( \pm \) 1.3 nM/mg; \( P < 0.02 \)). Because \( Ca^{2+} \) influx through the \( I_{Ca,L} \) contributes to the spontaneous tone, we examined the effect of nifedipine on the spontaneous tone of these tissues (Fig. 6A). The addition of nifedipine (1 \( \mu \)M) to the recording chamber resulted in great inhibition of tone in circular muscle (17.0 \( \pm \) 1.3 vs. 7.4 \( \pm \) 1.4 nM/mg, \( n = 8; P < 0.05 \)) in a time- and concentration-dependent manner, with no significant effect in sling (7.8 \( \pm \) 1.3 vs. 6.7 \( \pm \) 1.5 nM/mg, \( n = 8; P > 0.05 \)). The more general \( Ca^{2+} \) influx blocker La3+ (1 mM) inhibited tone in circular (15.5 \( \pm \) 2.0 vs. 7.3 \( \pm \) 1.8 mM/mg, \( n = 5; P < 0.05 \)) and sling (6.6 \( \pm \) 1.0 vs. 4.4 \( \pm \) 0.4 mM/mg, \( n = 5; P < 0.05 \)) muscles. ACh (10 \( \mu \)M) caused contractions in both circular and sling muscles. Whereas ACh-induced contractions were of greater amplitude in the sling muscle than in circular muscle (11.9 \( \pm \) 2.1 vs. 4.6 \( \pm \) 0.5 mM/mg, \( P < 0.05 \)), nifedipine (1 \( \mu \)M), effectively inhibited the ACh-induced contraction (Fig. 6B) of the circular muscle (4.6 \( \pm \) 0.5 vs. 1.9 \( \pm \) 0.6 mM/mg, \( n = 8; P < 0.05 \)) but had no significant effect on the sling muscle (11.9 \( \pm \) 2.1 vs. 10.7 \( \pm \) 1.6 mM/mg, \( n = 8; P > 0.05 \)). Even at higher concentrations of nifedipine (10 \( \mu \)M), neither ACh-induced contractions nor tone were significantly inhibited in sling. However, La3+ (1 mM) caused significant inhibition of ACh-induced contractions in both circular (3.7 \( \pm \) 0.8 vs. 1.5 \( \pm \) 0.6 mM/mg, \( n = 5; P < 0.05 \)) and slang (9.7 \( \pm \) 2.1 vs. 5.1 \( \pm \) 1.4 mM/mg, \( n = 5; P < 0.05 \)) muscles.

In a separate set of experiments, KCl was added to the bath to determine the effect of membrane depolarization on LES contractility. In sling muscle, addition of KCl (5–140 mM) resulted in a concentration-dependent sustained contraction above spontaneous tone (Fig. 7A). KCl (60 mM)-induced contractions were completely inhibited by nifedipine (1 \( \mu \)M; \( n = 5 \)). However, as before, on washout of KCl, nifedipine did not inhibit ACh-induced contractions in sling muscle (Fig. 7B). In LES circular muscle, the tissue response to KCl was dependent on dosage. Low and middle concentrations of KCl (5, 20, 60 mM) resulted in transient inhibition of spontaneous tone followed by subsequent gradual partial recovery (Fig. 7C). The addition of high KCl (140 mM) to the bath resulted in a large transient contraction followed by gradual inhibition of contraction and tone. Nifedipine abolished KCl (140 mM)-induced contraction (\( n = 5 \)) and, as before, caused severe inhibition of subsequent ACh-induced contractions (25.4% of control; Fig. 7D).

DISCUSSION

To our knowledge, these studies are the first to investigate and compare \( I_{Ca,L} \) in LES circular and sling muscle electrophysiologically and to identify the channel protein expression in these tissues. Remarkably, significant regional differences in \( I_{Ca,L} \) activity and protein expression were found, which we believe can account, at least in part, for the differences in contractile properties of these muscle tissues. In this study, we showed that 1) \( I_{Ca,L} \) is present, and its activation and inactivation profiles are similar in both muscular equivalents of the LES; 2) \( I_{Ca,L} \) density is greater in circular muscle than sling.
muscle, and the $I_{\text{Ca,L}}$ protein is expressed in more abundant levels in the circular muscle region; and 3) selective inhibition of $I_{\text{Ca,L}}$ reduces spontaneous tone and also ACh- and KCl-induced contractions in the LES circular muscle but only reduces KCl-induced contractions in LES sling muscle. This identification of $I_{\text{Ca,L}}$ in LES smooth muscle is in keeping with the previous pharmacological evidence for its presence in contractility studies (24, 30). In this study, we report that $I_{\text{Ca,L}}$ density is greater in LES circular muscle than in LES sling muscle (at 10 mV, $-0.88 \pm 0.14$ vs. $-0.31 \pm 0.09$ pA/pF; $P < 0.05$). Previously, it was reported (22) that there is a greater expression of $I_{\text{Ca,L}}$ in the proximal circular smooth muscle portion of the feline esophageal body as opposed to more distal regions (at 10 mV, $-2.3 \pm 0.2$ vs. $-1.2 \pm 0.2$ pA/pF; $P < 0.05$). When comparing current densities in those studies with the present investigation, our results are consistent with a decreasing gradient in $I_{\text{Ca,L}}$ expression from the proximal region of the circular smooth muscle esophageal body into the LES circular muscle and then sling muscle.

The electrophysiological assessments of voltage-dependent activation and inactivation properties of $I_{\text{Ca,L}}$ were similar in LES sling and circular muscles and are consistent with those of other gastrointestinal smooth muscles including the esophageal body (22, 35), stomach (33), and intestine (10). Peak amplitude of window current was obtained at $-16$ mV and is similar to what has been reported in other smooth muscles (10). LES

![Fig. 4](http://ajpgi.physiology.org/)

**A**

-50 mV

15 ms

40 pA

**B**

-50 mV

1 s

50 pA

**Fig. 4.** Time-dependent kinetics of $I_{\text{Ca,L}}$ in LES circular and sling muscle cells. **A:** representative inward current traces from LES circular and sling cells. Inward currents were evoked in response to a test pulse of 10 mV for 300 ms from a holding potential of $-50$ mV and normalized to the same amplitude to compare their activation time course more efficiently. Only initial segments of current traces are displayed. **B:** representative inward current traces were elicited by a 9-s test pulse to 10 mV from a holding potential of $-50$ mV. Current traces were superimposed based on the same amplitude to compare their decay more conveniently. Dotted lines are the zero current level. Open circle, circular muscle; filled circle, sling muscle.

![Fig. 5](http://ajpgi.physiology.org/)

**Fig. 5.** Comparison of the expression of the $\alpha_{1C}$-subunit in membranes fractions from circular and sling regions of the LES. **A:** each lane was loaded with 15 μg protein. Rat brain lysates overexpressing $\alpha_{1C}$-subunit were used as the positive control. The density of the 200-kDa band corresponding to the $\alpha_{1C}$-subunit was less in the sling region compared with the circular muscle region. This figure is representative of 4 separate experiments. **B:** blot was reprobed with anti-$\alpha$-actin antibody to serve as an internal control for protein loading. Densitometric analysis of Western blots normalized to $\alpha$-actin expression were carried out against the $\alpha_{1C}$-subunit and demonstrated that the $\alpha_{1C}$-subunit was expressed more abundantly in the circular muscle LES tissues than in muscle from the sling region. *$P < 0.05$. 

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circular and sling smooth muscle cells also responded similarly to the dihydropyridine agonist Bay K 8644, a Ca\(^{2+}\)/H\(_{11001}\)-channel opener that acts directly on the \(\alpha 1C\)-subunit protein of \(I_{Ca,L}\) to increase its open-state probability. Bay K 8644 increased the peak amplitude of \(I_{Ca,L}\) and concurrently induced a characteristic hyperpolarizing shift of \(-10\) mV in the \(I-V\) curve of cells from both regions. Although the ratio of the increase in peak \(I_{Ca,L}\) in response to Bay K 8644 was similar in circular and sling cells, the maximal peak current was still significantly greater in the circular than in the sling cells. The greater \(I_{Ca,L}\) in the circular region of the LES occurs in association with more abundant expression of the \(I_{Ca,L}\) \(\alpha 1C\)-subunit protein in the same region. Thus, together, the electrophysiological findings and immunoassessment of \(I_{Ca,L}\) expression indicate that the difference in the level of Ca\(^{2+}\) channel current observed in the circular and sling regions of the LES results primarily from a difference in the number of L-type Ca\(^{2+}\) channel proteins rather than from any differences in the functional characteristics of the channel. This interpretation, however, does not rule out the possibility that these channels are differentially modulated by other regulatory mechanisms.

The regional differences in \(I_{Ca,L}\) expression are also accompanied by functional differences, as reflected in the muscle strip studies conducted in this investigation and in previous studies (20, 21, 25, 26). Spontaneous tone is much higher in LES circular muscle. Early studies of the cat LES circular muscle suggested that spontaneous tonic contraction is due to continuous release of Ca\(^{2+}\) from intracellular stores (4). However, the \(I_{Ca,L}\) blocker nifedipine and/or Ca\(^{2+}\)-free medium have also been shown to inhibit tone in this muscle in several species including opossum (11), dog (30), cat (24), and human (17a, 38). This observation, combined with the current study in the cat, indicates that continuous Ca\(^{2+}\) influx through \(I_{Ca,L}\) is essential for maintenance of tone in LES circular muscle, presumably through feeding the intracellular stores as one major mechanism (8, 24). The greater expression of \(I_{Ca,L}\) in the LES circular muscle is consistent with the greater tone in this muscle compared with the sling. Because LES circular muscle is more depolarized (approximately \(-41\) mV) than the sling region (approximately \(-54\) mV) (28), the resting membrane potential is closer to a range where continuous low-level \(I_{Ca,L}\) activation could also occur, facilitating tone development. The mechanisms whereby calcium entry could occur at a more negative resting membrane potential require further study (8).

Unlike LES circular muscle, we found that tone in the LES sling muscle was not significantly inhibited by nifedipine, even at high concentrations (10 \(\mu M\)), but was partially inhibited by the more general Ca\(^{2+}\) influx blocker La\(^{3+}\). However, contractions induced with KCl were abolished by nifedipine in the sling muscle, demonstrating that membrane depolarization can activate \(I_{Ca,L}\), resulting in contraction in sling tissue as well. Therefore, as opposed to the circular muscle, the smaller...
amount of tone in sling muscle relies only partially on influx of extracellular Ca^{2+}, and this occurs via nifedipine-insensitive Ca^{2+}-entry pathways. Though I_{Ca,T} has previously been reported in cat esophageal smooth muscle (35), we did not find it in these LES smooth muscles. Furthermore, it is unlikely that I_{Ca,T} plays a major role in tonic or sustained agonist-induced contractions in these muscles, because they are rapidly inactivated. The lower level of I_{Ca,L} expression and I_{Ca,L} density in sling muscle suggests that a relative absence of this current may contribute to the reduced tone. It is also possible that reduced tone in the LES sling muscle is a result of less Ca^{2+} entry through I_{Ca,L}. Recently, the expression of transient receptor potential (TRP) channels have been reported in human esophageal and LES circular smooth muscle (41). These channels are believed to be involved in the refilling of depleted intracellular Ca^{2+} stores. The role of TRP channels and other nonselective cation channels in LES contractility have yet to be determined.

In circular muscle strips, nifedipine also inhibited cholinergic contractions by 60%, demonstrating the importance of Ca^{2+} entry via I_{Ca,L} in response to cholinergic stimulation in this muscle. Similar nifedipine sensitivity as well as reduction of contraction in Ca^{2+}-free medium was seen in muscle strips of the dog LES circular muscle (30). These authors postulated the presence of a special extracellular Ca^{2+} source located near the plasma membrane from which Ca^{2+} enters through I_{Ca,L} channels when a cholinergic contraction is induced in the absence of extracellular Ca^{2+}. Because ACh has been shown to affect several different Ca^{2+} influx and release pathways, more selective experiments with KCl were carried out in the current investigation to further explore the role of voltage-dependent L-type Ca^{2+} channels in LES contractility. Stimulation of LES muscle with KCl had distinct effects in these two diverse tissues. In LES sling muscle, KCl caused sustained contractions at low and high concentrations that were abolished in the presence of nifedipine. Because the resting membrane potential in sling muscle is relatively less depolarized than the circular muscle (28), this contractile activity is likely due to membrane depolarization leading to activation of I_{Ca,L}. However, in the more depolarized LES circular muscle, further depolarization with KCl (5–60 mM) caused rapid inhibition of tone that could be explained in at least two ways. Membrane depolarization due to continuous stimulation of the muscle with KCl may inactivate I_{Ca,L}, and hence inhibit the continuous influx of Ca^{2+} required for tone maintenance. Another explanation is that membrane depolarization results in release of nitric oxide (NO) by a muscle cell NO synthase associated with caveolin-1, resulting in subsequent inhibition of tone (7). With high KCl (140 mM), LES circular muscle responded with an initial nifedipine-sensitive contraction followed by subsequent inhibition of muscle tone. This response is consistent with a previous study in the LES circular muscle of the cat (5). The initial transient contraction may be the result of calcium-induced-calcium release from intracellular stores due to substantial depolarization with KCl, with subsequent reduction in tone due to I_{Ca,L} inactivation by the continued depolarizing effect of KCl. Further studies, however, are required to test this hypothesis.

The implications of these regional differences are of considerable interest. A greater I_{Ca,L} in the circular muscle would be consistent with using this channel to maintain virtually all of its elevated level of myogenic tone. A lower I_{Ca,L} in the sling region provides a basis for the relatively low resting tone in this muscle. It is also possible that smooth muscle from this region has a greater reliance on release of Ca^{2+} from intracellular Ca^{2+} stores for contraction, as is seen in many smooth muscles (34). In this case, the modulation of the level of contraction through the muscarinic receptor inositol triphosphate link for release of Ca^{2+} from the sarcoplasmic reticulum would provide a mechanism for greater neural excitatory cholinergic control of the LES sling muscle, including maintenance of its resting tone in vivo. Because the LES pressure profile in vivo shows a higher pressure in the left lateral-posterior aspect in both the human (27, 32, 37) and the cat (26), this aspect being
most sensitive to atropine, it is likely that the sling, in addition to the circular muscle, is an integral and important physical (18) and physiological contributor to the LES. Furthermore, the sling would lend itself to pharmacological manipulation of this cholinergic control and gives credence to the use of cholinergic agonists in raising LES pressure in patients with gastroesophageal reflux disease (9). Our findings indicate the effect would be largely due to stimulated contraction of the sling muscle. On the other hand, the reduction in LES pressure in vivo with the administration of nifedipine would, in large part, be due to relaxation of the circular muscle portion (39).

Recently, attention has been paid to the two muscle regions and their potential role in the pathogenesis of LES disorders including achalasia (32) and gastroesophageal reflux disease (37). Schneider et al. (32) have raised the importance of regional LES differences in patients with achalasia. In achalasia, Schneider et al. noted that the LES pressure was equal in all quadrants and equivalent to the higher pressure in the left lateral quadrant. That is, presumably absence of the inhibitory innervation to the LES and removal of some tonic inhibition of the circular muscle equalized the LES pressure profile, whereas the intact cholinergic innervation (15) maintained contraction of the sling muscle. This raises the possibility that the type of surgical myotomy for achalasia should be revisited. Perhaps cutting only the circular muscle will serve to relieve the functional obstruction but will leave some physiological cholinergic sling activity to prevent reflux. Therefore, there is good reason to further explore regional differences in Ca²⁺ handling in health and disease and how these differences dictate different responses to cholinergic and nitric (NO) innervation. Such studies hold the potential for more specific therapeutic targets.

In summary, this study provides the first demonstration of regional differences in ICa,L expression through biochemical, electrophysiological, and contractile assays of LES smooth muscle. These differences in ICa,L expression allow for differential muscular responses to innervation and varied muscular contribution to LES contractility.

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