Functional and molecular analysis of L-type calcium channels in human esophagus and lower esophageal sphincter smooth muscle

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Kovac, Jason R., Harold G. Preiksaitis, and Stephen M. Sims. Functional and molecular analysis of L-type calcium channels in human esophagus and lower esophageal sphincter smooth muscle. Am J Physiol Gastrointest Liver Physiol 289: G998–G1006, 2005. First published July 14, 2005; doi:10.1152/ajpgi.00529.2004.—Excitation of human esophageal smooth muscle involves the release of Ca2+ from intracellular stores and influx. The lower esophageal sphincter (LES) shows the distinctive property of tonic contraction; however, the mechanisms by which this is maintained are incompletely understood. We examined Ca2+ channels in human esophageal muscle and investigated their contribution to LES tone. Functional effects were examined with tension recordings, currents were recorded with patch-clamp electrophysiology, channel expression was explored by RT-PCR, and intracellular Ca2+ recorded with fura-2 fluorescence. LES muscle strips developed tone that was abolished by the removal of extracellular Ca2+ and reduced by the application of the L-type Ca2+ channel blocker nifedipine (to 13 ± 6% of control) but was unaffected by the inhibition of sarcoplasmic reticulum Ca2+-ATPase by cyclopiazonic acid (CPA). Carbodil increased tension above basal tone, and this effect was attenuated by treatment with CPA and nifedipine. Voltage-dependent inward currents were studied using patch-clamp techniques and dissociated cells. Similar inward currents were observed in esophageal body (EB) and LES smooth muscle cells. The inward currents in both tissues were blocked by nifedipine, enhanced by Bay K8644, and transiently suppressed by acetylcholine. The molecular form of the Ca2+ channel was explored using RT-PCR, and similar splice variant combinations of the pore-forming α1C-subunit were identified in EB and LES. This is the first characterization of Ca2+ channels in human esophageal smooth muscle, and we establish that L-type Ca2+ channels play a critical role in maintaining LES tone.

patch clamp; Ca2+ current; muscle tone; polymerase chain reaction; Ca,1,2

A PATHOPHYSIOLOGICAL ABNORMALITY contributing to gastroesophageal reflux disease (GERD) is failure of the lower esophageal sphincter (LES) smooth muscle to maintain tone. By remaining tonically contracted, the LES separates the esophagus from the gastric environment, protecting it from damage. The mechanisms underlying generation of this spontaneous tone in humans are incompletely understood, and discovery of how tone is maintained could lead to new drug targets in the treatment of GERD and spastic esophageal smooth muscle disorders such as achalasia (5).

The LES is a complex structure, and both myogenic and neurogenic mechanisms contribute to the tonic contraction (24). In contrast to LES smooth muscle, esophageal body (EB) smooth muscle develops little spontaneous tone and is normally phasically active during peristalsis. Several structural and biochemical specializations may account for the differences between these anatomically adjacent, yet physiologically distinct, smooth muscle types, including the regulation of intracellular Ca2+ concentration ([Ca2+]i) and different contractile protein isoforms (8, 31, 32). We (28) previously examined Ca2+ regulation in human EB smooth muscle and found that cholinergic excitation involved both the release of Ca2+ from intracellular stores and influx via L-type Ca2+ channels. Cholinergic regulation of human LES smooth muscle has not previously been studied.

Systemic administration of the dihydropyridine (DHP) L-type Ca2+ channel blocker nifedipine decreases LES pressure in healthy volunteers (16) and in achalasia patients (5, 35). These findings suggest a role for Ca2+ channels in the genesis and maintenance of LES tone in humans. However, pharmacological intervention is ineffective in some patients experiencing achalasia, suggesting that still other mechanisms may contribute to the development of tone (1). In vitro experiments on canine (27), feline (22), and opossum (41) models have indicated that LES tone involves nifedipine-sensitive Ca2+ influx. In contrast, Biancarni and co-workers (2) reported that in feline LES muscle, intracellular Ca2+ stores were critical to the maintenance of LES tone. However, no studies to date have identified or characterized the Ca2+ channels involved in regulating human esophageal smooth muscle tone and contraction.

Alternative splicing of Ca2+ channel gene products can give rise to a functional diversity of channels with distinct ionic properties and activation/inactivation characteristics, regulated in large part through the pore-forming, DHP-binding α1C-subunit (30). For example, the effectiveness of DHPs in cardiovascular diseases such as hypertension is due to a specific Ca2+ channel subunit splice variant in vascular smooth muscle (11). We considered whether expression of a specific combination of exons may give rise to the unique ability of the LES to generate tone. We therefore examined the expression of several splice variant combinations of the Ca2+ channel using RT-PCR and compared the channels in LES and EB. In conjunction with these molecular studies, we used functional, electrophysiological, and Ca2+ fluorescence studies to evaluate the roles for Ca2+ channels in human EB and LES smooth muscle.

METHODS

Tissue retrieval and isolation of cells. Tissue collection was carried out in accordance with the guidelines of the University of Western Ontario Review Board for Health Sciences Research Involving Human Subjects and conformed to the Helsinki Declaration. Tissues...
were obtained from patients undergoing esophageal resection due to cancer, as described previously (24, 28). In total, muscle was obtained from 52 specimens and studied using the different approaches.

For isolation of smooth muscle cells (SMCs), segments of the esophagus were cut into strips (~2 mm wide, 10 mm long) and placed in 2.5 ml of dissociation solution consisting of 135 mM K+ solution (composition given below in Solutions) as described previously (28). Cells were studied within 8 h of dispersion.

**Tissue bath studies.** Muscle strips were dissected from the circular muscle layer of the EB or the clasp portion of the LES, a region chosen because it develops greater spontaneous tension than sling muscle (24). Strips were mounted individually in water-jacketed muscle chambers. Cells were studied within 8 h of dispersion.

**Electrophysiological recordings.** Dispersed cells were allowed to settle and adhere to the bottom of a perfusion chamber mounted on the stage of a Nikon inverted microscope and perfused with bathing solution at 1–3 ml/min. Cells selected for study appeared phase bright and contracted in response to ACh. Whole cell recordings were made in the perforated-patch configuration with electrode solution containing nystatin (250 μg/ml). All currents were recorded at room temperature (21–24°C) with an Axopatch 200A amplifier (Axon Instruments; Foster City, CA) filtered at 1 kHz and sampled at 5 kHz using pCLAMP 6 software (Axon Instruments). To characterize inward currents, we blocked K+ currents using Cs+ electrode solution (see Solutions). Pipette resistance before seal formation ranged from 1 to 9 MΩ. Whole cell recording was initiated when access resistance had stabilized (<40 MΩ) to allow series resistance compensation of up to 80% to be used. Capacitive currents were compensated off-line using a capacitance circuit and linear leakage corrected off-line as assessed at negative potentials. In some traces, uncancelled capacitative currents were compensated off-line or blanked.

**Measurement of [Ca2+].** Cells were loaded by incubation with fluo-2 AM (0.2 μM) at room temperature for 40 min. Cells were allowed to settle onto a glass coverslip that comprised the bottom of a perfusion chamber (~0.75 ml volume). The chamber was mounted on a Nikon inverted microscope, and bathing solution was perfused (1–3 ml/min, room temperature) during the experiment. The ratio of fluorescence emission at 510 nm with alternate excitation wavelengths of 345 and 380 nm was measured using a Deltascan system (Photon Technology; London, Ontario, Canada), as previously described (28). Responses illustrated are from single cells and are representative of responses elicited in multiple samples from two or more patients.

**Solutions.** The Kres solution used for the retrieval of tissues and contraction studies consisted of (in mM) 116 NaCl, 5 KCl, 2.5 CaCl2, 1.2 MgSO4, 2.2 Na2HPO4, 25 NaHCO3, and 10 glucose equilibrated with 5% CO2-95% O2 (pH 7.4). The Na+-HEPES bathing solution used for electrophysiological recordings and fluorescence studies contained (in mM) 130 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 20 HEPES, and 10 glucose (adjusted to pH 7.4 with NaOH). Ca2+-free solutions had the same composition as above except with the addition of varying amounts of EGTA and omission of CaCl2. In electrophysiological recordings, the CsCl recording electrode solution contained (in mM) 130 CsCl, 20 HEPES, 1 MgCl2, 10 tetraethylammonium chloride, 0.4 CaCl2, and 1 EGTA (adjusted to pH 7.2 with CsOH).

**RT-PCR.** Total RNA was extracted from EB and LES tissue by phenol-chloroform extraction using frozen muscle samples. The integrity of the RNA was confirmed using agarose gel electrophoresis and ethidium bromide staining. Four micrograms of total RNA were reverse transcribed with random hexamers using a first-strand cDNA synthesis kit (Pharmacia Biotech; Madison, WI). PCR was performed in 50 μl of PCR buffer containing 2 mM MgCl2, 200 μM dNTPs, 0.1 mM each primer, 2 units Taq DNA polymerase (Quigian; Valencia, CA), and 10–15 μl cDNA reaction mixture. PCR was carried out in a GeneAmp 2400 PCR thermal cycler (Perkin-Elmer; Norwalk, CT) for 35 cycles with cycling parameters of 1 min at 94°C, 1 min at 54°C, 2 min at 72°C, and a final 10-min extension at 72°C. The PCR primers used to amplify cDNA are shown in Table 1. PCR primers for β-actin were used to confirm the fidelity of the PCR and to detect genomic DNA contamination. The amplified products were analyzed by electrophoresis on 1% agarose-Tris-acetic acid-EDTA [10 mM Tris (pH 7.5), 5.7% glacial acetic acid, and 1 mM EDTA] gels and visualized by ethidium bromide staining. Sequencing of PCR products was done at the Robarts Research Institute Core Molecular Biology Facility (London, Ontario, Canada).

**Chemicals.** Chemicals were obtained from Sigma (St. Louis, MO), BDH (Toronto, Ontario, Canada), or Calbiochem (San Diego, CA) unless otherwise stated. Test substances were prepared from stock solutions in distilled water or DMSO, diluted into the appropriate bathing solution, and applied either by bath perfusion or pressure ejection from glass micropipettes (Picospritzer II, General Valve; Fairfield, NJ). Pipettes were positioned 25–100 μm from cells with the concentration reported being that in the application pipette. Control studies carried out with vehicle alone had no effect.

**Statistics.** Values are means ± SE with sample sizes (n) indicating the number of cells or muscle strips studied. All traces shown are representative of at least three experiments on muscle or cells from two or more esophageal specimens. For patch-clamp and Ca2+ fluorescence experiments, only one recording was obtained per cell. Comparisons were made using the Student’s paired and unpaired t-tests, ANOVA, and Tukey’s test, with P < 0.05 considered significant.

## RESULTS

**Roles for Ca2+ in maintaining LES tone and in cholinergic contraction.** We first investigated a role for extracellular Ca2+ and Ca2+ channels in the maintenance of tone in human LES using tissue bath studies. LES muscle strips developed spon-

### Table 1. Primer sequences for identification of Ca2+ channel sequences in human esophageal muscle

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession No.</th>
<th>Primer Pair Sequence</th>
<th>Location</th>
<th>Exons Present</th>
<th>Predicted Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca.1.2α[α1c(IS-6)]</td>
<td>M92269</td>
<td>Sense: 5′-CAAGCAGGCGATCACCACACT-3′&lt;br&gt;Antisense: 5′-TCCGCTAAGACACCGGAGA-3′</td>
<td>1330–1531</td>
<td>8 or 8a</td>
<td>202</td>
</tr>
<tr>
<td>Ca.1.2α[α1c(HIS-2)]</td>
<td>M92269</td>
<td>Sense: 5′-CAACAACCTGATCTTCCTTCTC-3′&lt;br&gt;Antisense: 5′-GACATGTGTCATCGCCAG-3′</td>
<td>3019–3353</td>
<td>21 or 22 plus 23</td>
<td>275</td>
</tr>
<tr>
<td>Ca.1.2α[α1c(ISV-3)]</td>
<td>M92269</td>
<td>Sense: 5′-CAACATTGCTTCTACTTGCCCTC-3′&lt;br&gt;Antisense: 5′-GGAACCAGGGAAGAAGTTGATG-3′</td>
<td>4135–4451</td>
<td>31 or 32 plus 33</td>
<td>234</td>
</tr>
<tr>
<td>β-Actin</td>
<td>X00351</td>
<td>Sense: 5′-CAGTCTCCACGGCTTCTCTC-3′&lt;br&gt;Antisense: 5′-CTCTGATATCTCTGCTGTCG-3′</td>
<td>820–1133</td>
<td>314*</td>
<td></td>
</tr>
</tbody>
</table>

See Fig. 6 for the locations of exons studied in detail. *The β-actin PCR primer pair was selected to span a 206-bp intron.

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Fig. 1. Lower esophageal sphincter (LES) smooth muscle tone and contraction is dependent on extracellular Ca$^{2+}$. A: LES muscle strips characteristically developed spontaneous tension with stretch and relaxed to electrical field stimulation (EFS; 0.5 ms, 10 Hz, 40–80 mV) or the nitric oxide donor sodium nitroprusside (SNP; 100 µM). B: removal of extracellular Ca$^{2+}$ resulted in the gradual loss of tension and abolition of carbachol (CCh; 10 µM)-evoked contraction. Recovery of tone is shown at right after the readdition of Ca$^{2+}$, followed by relaxation induced by SNP. C: nifedipine (10 µM) significantly reduced tone, indicating a role for L-type Ca$^{2+}$ channels in the regulation of LES tone.

taneous tension and relaxed in response to activation of intrinsic nerves by electrical field stimulation or in response to the nitric oxide donor SNP (100 µM; Fig. 1A), all features characteristic of LES smooth muscle (13, 24). Removal of Ca$^{2+}$ from the bathing solution (plus the addition of 0.5 mM EGTA) abolished LES tone (reduced to 12 ± 1% of control, n = 7), with recovery upon the readdition of Ca$^{2+}$ (Fig. 1B). Because previous studies have suggested that low-Ca$^{2+}$ chelator concentrations failed to completely block Ca$^{2+}$ influx, thus influencing tension development in canine LES smooth muscles (27), we varied EGTA concentrations (0.05 mM, n = 10; 0.1 mM, n = 11; and 0.2 mM, n = 6; data not shown) and found that basal tone was inhibited comparably under all conditions.

To further examine a role for Ca$^{2+}$ entry, we applied the DHP Ca$^{2+}$ channel blocker nifedipine (10 µM), which significantly decreased LES tension (13 ± 6% of control, P < 0.01, n = 11; Figs. 1C and 2C). Application of vehicle alone had no effect in these or any further experiments. These data support a role for Ca$^{2+}$ entry through L-type Ca$^{2+}$ channels in the maintenance of LES tone.

Because intracellular stores of Ca$^{2+}$ are proposed to participate in maintenance of LES tone in feline LES (2, 22), we investigated the effects of sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase blockade with cyclopiazonic acid (CPA). CPA (10 µM) did not affect basal LES tone (n = 6; Fig. 2, B and C). As a positive control, we examined the effects of CPA on EB muscle, where CPA did cause an increase in tension (Fig. 2D). The lack of effect of CPA on LES tone, coupled with the marked sensitivity to Ca$^{2+}$ channel blockers, supports the conclusion that differences in Ca$^{2+}$ handling exist between LES and EB. In LES smooth muscle, CCh-evoked contractions were inhibited by perfusion with a Ca$^{2+}$-free solution (0.5 mM EGTA, 3 ± 3% of control, n = 4; Fig. 1B) or by the addition of nifedipine (5 ± 4% of control, n = 5; Fig. 2, A and C). In addition, CPA decreased CCh-evoked contractions (37 ± 12% of control, n = 4; Fig. 2, B and C). The actions of cholinergic agonists are mediated by muscarinic receptors, as indicated in previous studies using selective antagonists (17, 25). Although multiple muscarinic receptor subtypes are present, contraction of EB occurs predominantly through the M$\text{3}$ receptor subtype (25, 38), although the subtypes mediating contraction in LES have not previously been examined. Taken together, these results are consistent with cholinergic contraction of LES smooth muscle being dependent on Ca$^{2+}$ influx and release from stores, whereas tone was dependent on influx.

Fig. 2. Cholinergic excitation of LES smooth muscle involves influx and release of Ca$^{2+}$ from stores. A: nifedipine (10 µM) inhibited LES tone and CCh-evoked contraction. B: LES tone persisted, but CCh-evoked contractions were attenuated in the presence of the sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase blocker cyclopiazonic acid (CPA; 10 µM). C: summary of the experiments shown in A and B with means ± SE presented as a percentage of basal tension (left) and of control CCh responses (right). Spontaneous tension was inhibited by nifedipine (n = 11) but unaffected by CPA (n = 6), indicating a critical role for L-type Ca$^{2+}$ channels in the maintenance of tone. CCh-evoked contractions were reduced by nifedipine (n = 5) and CPA (n = 4), indicating the involvement of both Ca$^{2+}$ influx and intracellular stores in contraction (*P < 0.05; **P < 0.01). D: similar experiment as in B except carried out using circular layer of esophageal body (EB) smooth muscle. Notably, CPA caused a slow development of tension and abolished subsequent CCh-evoked contraction, distinct from that observed in the LES.
**L-type Ca\textsuperscript{2+} current in EB and LES SMCs.** In view of the demonstrated involvement of Ca\textsuperscript{2+} entry, we next used patch-clamp electrophysiology to characterize voltage-dependent Ca\textsuperscript{2+} currents in SMCs isolated from EB and LES. Enzymatic dissociation of SMCs yielded spindle-shaped cells that appeared phase bright and ranged in length from 50 to 150 \(\mu\)m. Cells isolated from EB and LES smooth muscle were of similar size with whole cell capacitance values of 61 ± 4 (n = 26) and 66 ± 7 pF (n = 22), respectively. We used the Cs\textsuperscript{+} electrode solution to block K\textsuperscript{+} currents (37) and allow resolution of inward currents. From a holding potential of -60 mV, depolarization elicited a rapid, transient inward current in both EB and LES SMCs (Fig. 3). The current-voltage relationship for EB SMCs revealed a peak inward current of 35 ± 7 pA at 0 mV (n = 13; Fig. 3B). An inward current with essentially the same kinetics and voltage dependence was observed in LES SMCs, with a peak inward current of 31 ± 7 pA at 0 mV (n = 10; Fig. 3C).

**Fig. 3.** Voltage-dependent Ca\textsuperscript{2+} current in human EB and LES smooth muscle cells (SMCs). A: EB SMCs were held at -60 mV and depolarized to the potentials indicated. Depolarization induced a transient inward current that increased with positive potentials. Dotted lines indicate zero current level in this and subsequent records. In all current recordings, Cs\textsuperscript{+} electrode solution was used to block K\textsuperscript{+} currents. B: average current-voltage relationship for EB SMCs. Peak inward current was recorded at 0 mV (n = 13; means ± SE). C: LES SMCs exhibited a similar average current-voltage distribution with equivalent peak inward current at 0 mV (n = 10). Whole cell capacitance measurements revealed that EB and LES SMCs were of similar size.

**Fig. 4.** L-type Ca\textsuperscript{2+} current in human EB and LES SMCs. A: representative traces show that depolarizing voltage steps (-60 to 0 mV) elicited inward current in EB SMCs that was increased with the L-type Ca\textsuperscript{2+} channel agonist Bay K8644 (10 \(\mu\)M) and inhibited with nifedipine (10 \(\mu\)M). B: summary of data in A. Peak control current at 0 mV (n = 9) was enhanced with Bay K8644 (n = 6) and inhibited with nifedipine (n = 5). C: representative traces from LES SMCs showing Bay K8644 and nifedipine effects similar to those observed in EB. D: summary of data obtained in C. Peak inward current at 0 mV was significantly enhanced with Bay K8644 (n = 6) and inhibited with nifedipine (n = 5) (*P < 0.05; **P < 0.01).

Additional similarities in the properties of the currents were noted when responses to DHP Ca\textsuperscript{2+} channel regulators were studied. The Ca\textsuperscript{2+} channel agonist Bay K8644 (10 \(\mu\)M) significantly increased voltage-dependent peak inward currents in both EB (-51 ± 11 pA, n = 6, vs. control -31 ± 7 pA, n = 9, P < 0.01; Fig. 4B) and LES (-48 ± 7 pA vs. control -24 ± 3 pA, n = 6, P < 0.01; Fig. 4D) SMCs. Furthermore, the L-type Ca\textsuperscript{2+} channel antagonist nifedipine essentially abolished peak inward current in both EB (-3 ± 5 pA, n = 5, vs. control -31 ± 7 pA, n = 9, P < 0.01; Fig. 4B) and LES (-1 ± 1 pA vs. control -25 ± 5 pA, n = 5, P < 0.01; Fig. 4D) SMCs.

**Fig. 4.** Additional traces show that depolarizing voltage steps (-60 to 0 mV) elicited inward current in EB SMCs that was increased with the L-type Ca\textsuperscript{2+} channel agonist Bay K8644 (10 \(\mu\)M) and inhibited with nifedipine (10 \(\mu\)M). B: summary of data in A. Peak control current at 0 mV (n = 9) was enhanced with Bay K8644 (n = 6) and inhibited with nifedipine (n = 5). C: representative traces from LES SMCs showing Bay K8644 and nifedipine effects similar to those observed in EB. D: summary of data obtained in C. Peak inward current at 0 mV was significantly enhanced with Bay K8644 (n = 6) and inhibited with nifedipine (n = 5) (*P < 0.05; **P < 0.01).
To study the physiological regulation of Ca\textsuperscript{2+} currents, we recorded responses to ACh, which has previously been shown to cause elevation of [Ca\textsuperscript{2+}], and contraction of human esophageal muscle cells (28). Cells were held under voltage clamp and periodically depolarized from \(-60\) to \(0\) mV to elicit Ca\textsuperscript{2+} current. Stimulation with ACh caused a marked inhibition of the Ca\textsuperscript{2+} current in both EB (Fig. 5, A and B) and LES (Fig. 5C) SMCs. As shown in Fig. 5D, peak inward current was reduced to \(-7 \pm 3\) pA from a control level of \(-35 \pm 7\) pA (n = 9) in EB and to \(-7 \pm 1\) pA from a control level of \(-39 \pm 8\) pA in LES (n = 8) SMCs. Inhibition of the Ca\textsuperscript{2+} current was rapidly reversible and consistent with the time course for Ca\textsuperscript{2+} inhibition of L-type Ca\textsuperscript{2+} channels previously established in other smooth muscles (37). Evidence to support the agonist-induced rise of [Ca\textsuperscript{2+}], in LES cells is shown below, which could account for the acute Ca\textsuperscript{2+} current inhibition. In addition to the inhibition of the Ca\textsuperscript{2+} current, ACh often elicited an inward nonselective cation current (Fig. 5, C). Moreover, ACh often elicited an inward nonselective cation current (Fig. 5, C). Peak inward current at 0 mV was significantly inhibited by ACh in both EB (n = 9) and LES (n = 8) SMCs (**P < 0.01).

Fig. 5. Acetylcholine (ACh) inhibits L-type Ca\textsuperscript{2+} current in human EB and LES SMCs. A: representative trace from an EB SMC held at \(-60\) mV and periodically stepped to 0 mV to elicit Ca\textsuperscript{2+} current. ACh (10 \(\mu\)M) elicited a small inward current and caused acute and transient inhibition of Ca\textsuperscript{2+} current, with recovery evident on washout (break in recording represents 5 min). Sections are amplified for display in the traces (i, control; ii, ACh; iii, washout) shown in B. C: representative traces from LES SMCs show similar inhibition of Ca\textsuperscript{2+} current in response to ACh. D: summary of data shown in B and C. Peak inward current at 0 mV was significantly inhibited by ACh in both EB (n = 9) and LES (n = 8) SMCs (**P < 0.01).

**DISCUSSION**

The contribution of Ca\textsuperscript{2+} to LES smooth muscle tone was examined using a combination of functional studies of contrac-
tion in intact muscle strips, freshly dispersed cells to characterize ionic currents and \([\text{Ca}^{2+}\]/i\) responses, and RT-PCR to study the transcriptional expression of L-type \([\text{Ca}^{2+}\]/i\) channels.

In smooth muscle strips, removal of bath \([\text{Ca}^{2+}\]/i\) or application of nifedipine essentially abolished LES tone, indicating a critical role for \([\text{Ca}^{2+}\]/i\) entry in tonic LES contraction. Patch-clamp recordings characterized a DHP-sensitive, voltage-dependent, inward \([\text{Ca}^{2+}\]/i\) current in EB and LES smooth muscle. The L-type \([\text{Ca}^{2+}\]/i\) channel \(\alpha_{1C}\)-subunit was identified, and similar splice combinations were observed between EB and LES smooth muscles.

The present study provides the first direct in vitro evidence of the essential role for L-type \([\text{Ca}^{2+}\]/i\) channels in maintaining tone in human LES. Previous in vivo studies showed that \([\text{Ca}^{2+}\]/i\) channel blockade with nifedipine decreased LES tone in healthy volunteers (16). Indeed, \([\text{Ca}^{2+}\]/i\) channel blockers may predispose individuals to GERD and have been used, with moderate success, in the treatment of spastic esophageal disorders such as achalasia, a condition in which the LES fails to relax (1, 5).

In agreement with our findings, early pioneering in vitro studies by Tottrup and co-workers (34) on human esophageal muscle showed that removal of extracellular \([\text{Ca}^{2+}\]/i\) attenuated LES tone. More recently, a role for \([\text{Ca}^{2+}\]/i\) influx via L-type \([\text{Ca}^{2+}\]/i\) channels was demonstrated in several animal models, including canine (27), feline (22), and opossum (41) LES. Salapatek and co-workers (27) hypothesized that low EGTA concentrations may fail to completely block \([\text{Ca}^{2+}\]/i\) influx resulting in a residual contraction. We found similar inhibition by all concentrations of EGTA tested (0.05–0.5 mM), suggesting the presence of a small nifedipine-insensitive component to LES contraction. This small residual component may represent

![Schematic diagram and amino acid sequence of the L-type \([\text{Ca}^{2+}\]/i\) channel \(\alpha_{1C}\)-subunit. A: putative membrane topology of the \(\alpha_{1C}\)-subunit, with four domains (I–IV) each consisting of six transmembrane segments. The three shaded regions, labeled IS-6, IIIS-2, and IVS-3, indicate common sites of alternative splicing. B: relative positions of selected exons with respect to protein sequence. Solid arrows indicate alternatively spliced exons (exons 21 and 22 for IIIS-2 and 31 and 32 for IVS-3), which are mutually exclusive. Open arrows show the protein locations corresponding to the sense and antisense primers used for RT-PCR experiments (see Table 1).](http://ajpgi.physiology.org/).
a Ca\(^{2+}\)-independent contraction (7) or the release of Ca\(^{2+}\) from other sites, including intracellular stores. Given the pharmacological profiles of the Ca\(^{2+}\) currents observed, it does not appear that a DHP-insensitive Ca\(^{2+}\) channel contributes to human esophageal LES tone, such as that reported in some vascular smooth muscles (20).

[Ca\(^{2+}\)]\(_i\) stores are proposed to participate in the maintenance of tone in the feline LES (2, 22). We found that blockade of Ca\(^{2+}\)-entry exist in esophageal muscle, including nonselective cation channels of the transient receptor potential C (TRPC) family (39). It remains to be determined whether TRPC channels contribute to Ca\(^{2+}\) influx and the maintenance of tone in LES muscle.

To further assess the contribution of L-type Ca\(^{2+}\) channels in EB and LES smooth muscle, we examined the expression pattern of L-type Ca\(^{2+}\) channel \(\alpha_{1C}\)-subunit splice combinations. The \(\alpha_{1C}\)-subunit defines the Ca\(^{2+}\) conduction pore...
voltage sensor and confers DHP sensitivity (29). Indeed, the effectiveness of DHPs in cardiovascular disease is due to their influence on specific splice variants of the Ca\(^{2+}\) channel \(\alpha_{1C}\)-subunit in vascular smooth muscle (11). Expression is regulated through alternative splicing that generates Ca\(^{2+}\) channels with distinct gating, pharmacology, and activation/inactivation characteristics (19, 40). As such, we hypothesized that the functional diversity observed between tonic and phasic esophageal muscles could be the result of alternatively spliced Ca\(^{2+}\) channel transcripts. We examined the IS-6, IIIS-2, and IVS-3 variants because they have been identified in smooth muscle and confer sensitivity to DHPs (4, 26, 29, 42). Previous studies of the cat esophagus have demonstrated evidence for the \(\alpha_{1C}\)-subunit, based on an antibody to a conserved region common to L-type channels in many tissues, including cardiac and brain cells (21). We advanced this basic finding by examining several regions contributing to functional diversity and pharmacological sensitivity.

Other Ca\(^{2+}\) channel subunits (i.e., \(\alpha_{1}, \alpha_{\delta}, \beta, \) and \(\gamma\)) influence channel activity; however, they were not examined in the present study. Our findings of similar variants between the EB and LES did not account for the fundamental differences in the physiology of the muscles. This, combined with the similar profile of membrane currents, suggests other factors must account for the physiological specialization.

Another mechanism proposed to explain the maintenance of tone is that LES exhibits chronically increased \([Ca^{2+}]_{i}\), above the threshold to elicit contraction (10). Anatomically, LES SMCs exhibit larger cellular diameters, increased mitochondrial content, and a more developed sarcoplasmic reticulum compared with EB SMCs (8, 31, 32). In our in vitro studies, basal \([Ca^{2+}]_{i}\) levels were similar among EB and LES muscles, suggesting that these theories do not apply in the human esophagus under these conditions. It is possible that contraction and tone may occur under constant \([Ca^{2+}]_{i}\), levels through increased Ca\(^{2+}\) sensitivity, or sensitization, of the smooth muscle contractile apparatus. For example, downregulation of myosin light chain phosphatase (MLCP) activity either directly via Rho kinase phosphorylation of the MLCP targeting subunit or indirectly via Rho kinase and PKC phosphorylation of a MLCP inhibitor (6). Whether such a mechanism applies in human LES muscle remains to be determined.

Although the LES can autonomously generate tone, under physiological conditions it is also regulated by inhibitory and excitatory neurotransmitters (3). ACh is the primary excitatory neurotransmitter of the gastrointestinal tract and evokes contraction of human EB (28). A cholinergic contribution to LES contraction has been described (2, 22, 27); however, the sources of Ca\(^{2+}\) remain controversial. We provide evidence that cholinergic contractions are abolished on removal of extracellular Ca\(^{2+}\) or the addition of nifedipine, supporting a key role for influx in human LES tone.

We also report that cholinergic stimulation causes a transient inhibition of Ca\(^{2+}\) current in both EB and LES muscles, similar to that observed in guinea pig ileal, gastric, and tracheal muscles (36, 37). PKC-mediated suppression of Ca\(^{2+}\) current has been suggested (23); however, others have found that the initial suppression of Ca\(^{2+}\) influx was due to the release of Ca\(^{2+}\) from stores (36, 37). This negative feedback controls \([Ca^{2+}]_{i}\) levels and may be an intrinsic mechanism by which EB and LES smooth muscles regulate contraction. ACh evoked essentially identical elevations in \([Ca^{2+}]_{i}\), that were largely unaffected by nifedipine in both EB and LES SMCs. These results suggest that cholinergic regulation of contraction involves release from intracellular stores. However, we found that cholinergic contractions are chiefly dependent on Ca\(^{2+}\) influx with only a small contribution from intracellular stores. Several reasons may account for this apparent discrepancy: 1) dispersion of cells may alter \([Ca^{2+}]_{i}\), regulation (33); 2) ACh-evoked contraction may result in cross-talk between PKC-dependent and -independent pathways, resulting in other second messengers subsequently influencing tone and contraction (15); and 3) the existence of discrete \([Ca^{2+}]_{i}\); stores that are insoluble (1, 4, 5)trisphosphate insensitive and cannot be depleted by CPA (14).

In summary, these data provide the first identification and characterization of L-type Ca\(^{2+}\) channels in human esophageal smooth muscles. We establish a contribution for Ca\(^{2+}\) influx in the maintenance of LES tone and demonstrate the expression of smooth muscle-specific \(\alpha_{1C}\)-subunit splice combinations in EB and LES smooth muscles.

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