Enhanced capacitative calcium entry and TRPC channel gene expression in human LES smooth muscle

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Wang, Jian, Lisanne G. Laurier, Stephen M. Sims, and Harold G. Preiksaitis. Enhanced capacitative calcium entry and TRPC channel gene expression in human LES smooth muscle. Am J Physiol Gastrointest Liver Physiol 284: G1074–G1083, 2003; 10.1152/ajpgi.00227.2002.—Transient receptor potential channel (TRPC) genes encode Ca2+-permeable channels mediating capacitative Ca2+ entry (CCE), which maintains intracellular Ca2+ stores. We compared TRPC gene expression and CCE in human esophageal smooth muscle (EB) and lower esophageal sphincter (LES), because these smooth muscles have distinct contractile functions that are likely associated with different Ca2+ regulatory mechanisms. Circular layer smooth muscle cells were grown in primary culture. Transcriptional expression of TRPC genes was compared by semiquantitative RT-PCR. CCE was measured by fura 2 Ca2+ fluorescence after blockade of sarcoplasmic reticulum Ca2+-ATPase with thapsigargin. mRNA for TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 was identified in EB and LES. TRPC3 and TRPC4 were more abundant in LES than EB. Basal concentration of free intracellular Ca2+ ([Ca2+]i) was similar in cells from LES (138 ± 8 nmol/l) and EB (110 ± 6 nmol/l) and increased with ACh (10 μmol/l; 650 ± 28 and 590 ± 21 nmol/l, respectively). With zero Ca2+ in bath, thapsigargin (2 μmol/l) increased [Ca2+]i more in LES (550 ± 22 nmol/l) than EB (250 ± 15 nmol/l, P < 0.001). Subsequent external application of 1 mmol/l Ca2+ increased [Ca2+]i more in LES (585 ± 35 nmol/l) than EB (285 ± 21 nmol/l, P < 0.001), indicating enhanced CCE in LES. This demonstrates CCE and TRPC transcriptional expression in human esophageal smooth muscle. In LES cells, enhanced CCE and expression of TRPC3 and TRPC4 may contribute to the physiological characteristics that distinguish LES from EB.

esophagus; sphincter; store-operated Ca2+ influx

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may differ between esophageal body (EB), which is phasically active, and LES, which displays tonic contraction, since these distinct contractile functions are likely to be associated with distinct Ca\textsuperscript{2+} regulatory mechanisms.

**MATERIALS AND METHODS**

*Tissue retrieval and SMC culture.* Muscle samples were obtained from disease-free portions of human esophagus being removed because of cancer, as previously described (18, 23, 30). Smooth muscle samples were obtained from the distal third of the EB and from the clasp portion of the LES, as previously described in detail (18). The correct identity of LES smooth muscle was confirmed on the basis of the ability of this muscle to develop spontaneous, myogenic tone and relax with activation of intrinsic nerves (18). Portions of the muscle were frozen on dry ice and stored at -70°C for RNA extraction. Primary cultures of cells derived from the EB and LES were established as previously described (30). Briefly, cells were isolated by digestion with collagenase (1.7 mg/ml), elastase (0.5 mg/ml), and 1 mg/ml BSA and plated at a density of ~400 cells/cm\textsuperscript{2} onto 13-mm coverslips (for fluorescence microscopy) or directly in 10-cm petri dishes (for RNA extraction) in DMEM (GIBCO-BRL). Cells were grown in a humidified atmosphere of 5% CO\textsubscript{2} in air at 37°C.

Muscle samples were obtained from disease-free portions of human esophagus being removed because of cancer, as previously described (18, 23, 30). Mouse monoclonal anti-α-smooth muscle actin (1:50; Boehringer) was used with Cy3-linked tosaase (0.5 mg/ml), and 1 mg/ml BSA and plated at a density of ~400 cells/cm\textsuperscript{2} onto 13-mm coverslips (for fluorescence microscopy) or directly in 10-cm petri dishes (for RNA extraction) in DMEM (GIBCO-BRL). Cells were grown in a humidified atmosphere of 5% CO\textsubscript{2} in air at 37°C with 10% fetal bovine serum.

Immunocytochemistry of cultured cells was carried out as previously described (30). Mouse monoclonal anti-α-smooth muscle actin (1:50; Boehringer) was used with Cy3-linked anti-mouse secondary antibody (1:100; Jackson Labs, West Grove, PA). TO-PRO-1 dimeric cyanine dye (Molecular Probes, Eugene, OR) was used to stain nuclei. Primary anti-actin was omitted in controls. Coverslips were mounted on slides with FluoroGuard Antifade (Bio-Rad, Hercules, CA).

*Coverslips were then transferred to a 0.75-ml chamber containing 0.2 nmol/l fura 2-AM (30 min, 25°C) then transferred to fresh DMEM for 30 min at 37°C for dye cleavage.*

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*Sequencing of PCR products for verification was done in the Robarts Research Institute Core Molecular Biology Facility (London, ON, Canada).*

**Table 1. PCR Primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession no.</th>
<th>Primer Pair Sequence, sense/antisense</th>
<th>Product Size, bp</th>
<th>Location in Sequence, nt</th>
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</thead>
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<tr>
<td>TRPC1</td>
<td>U31110</td>
<td>5'-CAAGATTTTGGAAAAATTCCTTG-3'</td>
<td>372</td>
<td>2238–2709</td>
</tr>
<tr>
<td>TRPC3</td>
<td>Y13758</td>
<td>5'-TTTGCTTTCTATGTTTCTAT-3'</td>
<td>317</td>
<td>2316–2633</td>
</tr>
<tr>
<td>TRPC4</td>
<td>X90697</td>
<td>5'-TCTGAAGCCCTCTCTTCTGC-3'</td>
<td>415</td>
<td>1–415</td>
</tr>
<tr>
<td>TRPC5</td>
<td>AF054568</td>
<td>5'-ACCTCTCATCAGACCATGCTCA-3'</td>
<td>444</td>
<td>3351–3794</td>
</tr>
<tr>
<td>TRPC6</td>
<td>U49069</td>
<td>5'-AAAGATATCCTAACCATCTTCATG-3'</td>
<td>327</td>
<td>2181–2507</td>
</tr>
<tr>
<td>TRPC7</td>
<td>AF139923</td>
<td>5'-TACTGACCCATGCACCTGACGCTCA-3'</td>
<td>767</td>
<td>1621–2387</td>
</tr>
<tr>
<td>β-actin</td>
<td>M10277</td>
<td>5'-GAGCGGTCACCCACACTGTGGCCCATCTA-3'</td>
<td>661</td>
<td>2134–3000</td>
</tr>
</tbody>
</table>

All primers were designed based on human gene sequences except for TRPC7, which was based on the mouse gene sequence (17).
mounted on a Nikon inverted microscope and superfused at 2–5 ml/min at room temperature. Individual cells were illuminated with alternating 345- and 380-nm light using a Deltascan system (Photon Technology International, London, ON, Canada), with emission detected by a photomultiplier at 510 nm. Following correction for background fluorescence, $[\text{Ca}^{2+}]_i$ was calculated as described previously (23, 30). The superfusion buffer had the following composition (in mmol/l): 130 NaCl, 5 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 20 HEPES, and 10 D-glucose, adjusted to pH 7.4. Ca$^{2+}$-free buffer contained 0.5 mmol/l EGTA to chelate residual Ca$^{2+}$.

**Drugs and materials.** Fura 2-AM (Molecular Probes) was prepared in dimethylsulfoxide. All drugs used were obtained from Sigma (St. Louis, MO). Other reagents were obtained from VWR (Mississauga, ON, Canada) or as indicated above. Drugs were prepared as concentrated stock solutions and diluted into the appropriate bathing solution before the addition to cells.

**Statistics.** Data are expressed as means ± SE. The number of cells tested is designated by an $n$. All experiments were repeated with at least three different esophageal specimens. Statistical analyses were performed using Student’s $t$-test. Differences were considered to be significant when $P < 0.05$.

**RESULTS**

**EB and LES SMCs in culture.** Dispersed SMCs from EB and LES showed signs of growth after 2–3 days in culture and displayed similar growth characteristics, morphology, and $\alpha$-actin immunostaining (Figs. 1 and 2). These primary cultures are composed of >96% SMCs without contamination by other cell types, based on the absence of significant immunostaining for markers for endothelial cells, nerve cells, and interstitial cells of Cajal, as previously described (30). Individual SMCs were phase bright with a larger central area containing the nucleus and were spindle shaped (Fig. 1). At confluence, cells assumed a typical hill-and-valley appearance (Fig. 1), as described for other smooth muscles (5). Immunostaining for $\alpha$-smooth muscle actin revealed positive staining in virtually all cells seen as intracellular strands (Fig. 2).

**RT-PCR identifies TRPC channel mRNA in LES and EB.** PCR primers were designed to amplify sequences specific for six of seven mammalian TRPC channels.

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Fig. 1. Phase contrast microscopy of human lower esophageal sphincter (LES) and esophageal body (EB) smooth muscle cells (SMCs) cultured for 5 days, 8 days, and at confluence (14 days). LES and EB cells showed similar morphology and growth characteristics. Calibration bar at bottom right refers to all micrographs.
that have been cloned to date (7, 17, 19, 32). TRPC2 is a pseudogene in the human and was not examined in this study (28). mRNA for five TRPC channels was detected in cultured cells from both the EB and LES, including TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 (Fig. 3). The PCR products were of the expected size, and in each case the identity of the PCR product was confirmed by direct sequencing. The presence of a single band for β-actin in each sample rules out genomic DNA contamination. mRNA for TRPC7, which was recently cloned from mouse brain (17), was readily detected in rat brain and human brain and heart (Fig. 3) but could not be identified in any of four esophageal specimens tested. Primer sets for all of the TRPC genes except TRPC7 were based on the known human gene sequences. In the case of TRPC7, the primers were based on the mouse sequence (17), but the gene product was homologous to the human sequence that has been identified subsequently (GenBank accession no. AJ272034).

Our initial assessment of TRPC subtypes in EB and LES revealed specific differences between LES and EB. mRNA for TRPC3 and TRPC4 appeared to be more abundant in LES compared with EB cells, whereas TRPC1, TRPC5, and TRPC6 showed similar levels of transcription in both tissues (Fig. 3). To investigate this in more detail, semiquantitative RT-PCR was carried out using primers for 18S RNA (competimers) in each PCR reaction as a reference standard. Samples of the PCR reaction mixture were removed at 2- to 3-cycle intervals, subjected to electrophoresis on agarose gels, and stained with ethidium bromide (Fig. 4A). Samples from these reactions were subjected to capillary electrophoresis, and amplification curves were constructed to verify linearity of the PCR reactions with respect to cycle number (Fig. 4B) using relative fluorescence intensity as a guide to the amount of PCR product. Serial dilution of the template cDNA yielded similar results (Fig. 4, C and D).

Based on these results, the amount of input cDNA (5 μl) and cycles of amplification (28 cycles) was selected for semiquantitative PCR. Figure 5 shows these results for TRPC1, TRPC3, and TRPC4 in EB and LES SMCs obtained from four separate esophageal specimens. Both ethidium bromide-stained agarose gels (Fig. 5, left) and quantification by capillary electrophoresis (Fig. 5, right) showed greater levels of TRPC3 and TRPC4 mRNA in SMCs from the LES compared with
EB, whereas TRPC1 mRNA expression was not different. To address the possibility that these differences in TRPC expression might be unique to SMCs in culture, we next carried out similar experiments using intact tissues from EB and LES, where the pattern of TRPC mRNA expression was confirmed (Fig. 6). These data validate the primary culture model and confirm the physiological relevance of this observation.

EB and LES SMCs show CCE. TRPC channels have been implicated in a number of cellular functions including CCE, the putative mechanism for refilling of Ca\(^{2+}\) stores following store depletion (7, 19, 32). To explore the functional correlate of the difference in TRPC channel expression in LES and EB cells, we next compared CCE in these cell types. CCE was quantified using an approach previously applied in a number of other cell types (2, 10). The application of ACh produced a transient increase in \([\text{Ca}^{2+}]_{i}\) (Fig. 7), which has been previously shown to be due largely to \([\text{Ca}^{2+}]_{i}\) release from intracellular stores (30). The application

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**Fig. 4.** Effect of cycle length and cDNA concentration on TRPC amplicon accumulation. RT-PCR was carried out using primers for 18S RNA and TRPC1, TRPC3, and TRPC4 using cDNA samples from cultured LES and EB SMCs. PCR amplicon accumulated in a nearly linear manner with the number of PCR cycles (A and B) and the amount of input cDNA (C and D). On the basis of these data, we used 5 μl cDNA and 28 cycles of amplification for subsequent semiquantitative RT-PCR. Straight lines are best-fits to the data, with solid lines for filled symbols and dashed lines for open symbols.

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**Fig. 5.** LES and EB show quantitative differences in TRPC subtype transcriptional expression. A: cDNA from cultured SMCs derived from 4 independent esophageal LES and paired EB specimens (S1–S4) was amplified with primers for TRPC1, TRPC3, TRPC4, and 18S RNA using reaction conditions described in Fig. 4 and in MATERIALS AND METHODS. PCR products were electrophoresed on agarose gels and stained with ethidium bromide (left). Aliquots of each reaction were subjected to capillary electrophoresis, peak heights were obtained, and ratios of TRPC1/18S RNA mRNA expression were calculated. B: relative differences in the amounts of TRPC1, TRPC3, and TRPC4 in LES and EB (means ± SE; *P < 0.001).
The L-type voltage-gated Ca\(^{2+}\) channel blocker nifedipine (1 \(\mu\)mol/l; Fig. 7A). A similar transient rise in [Ca\(^{2+}\)]\(i\), on application of external Ca\(^{2+}\) following TG was also observed in freshly isolated cells (data not shown). In addition, we previously showed that blockade of the SR-Ca\(^{2+}\)-ATPase in intact muscle strips in the presence of extracellular Ca\(^{2+}\) results in prolonged contraction, consistent with Ca\(^{2+}\) influx (23). Together, these findings are characteristic of the presence of SOC\(s\) that mediate CCE (2).

Comparison of CCE in LES and EB SMCs. Despite the fact that a defining characteristic of LES smooth muscle is the development of sustained tonic contraction, resting [Ca\(^{2+}\)]\(i\) was not significantly different in SMCs from the LES (138 ± 8 nmol/l, n = 21) compared with EB (110 ± 6 nmol/l, n = 18) (Fig. 8). Furthermore, the application of ACh elicited a transient increase in [Ca\(^{2+}\)]\(i\) of similar amplitude in both cell types (650 ± 28 nmol/l and 590 ± 21 nmol/l, respectively) (Fig. 8). However, the TG-associated increases in [Ca\(^{2+}\)]\(i\) were about twofold greater in LES SMCs (550 ± 22 nmol/l, n = 21) compared with EB SMCs (250 ± 15 nmol/l, n = 18, P < 0.001) (Fig. 8D). CCE, as measured by the peak increase in [Ca\(^{2+}\)]\(i\) on application of external Ca\(^{2+}\), was also significantly greater in LES (585 ± 35 nmol/l, n = 21) than in EB SMCs (295 ± 21 nmol/l, n = 19, P < 0.001) when assessed by application of 1 mmol/l Ca\(^{2+}\) and measuring the peak increase in [Ca\(^{2+}\)]\(i\) (Fig. 8, C and D). This difference is maintained when the results are normalized to the peak transient [Ca\(^{2+}\)]\(i\) elicited by ACh, minimizing possible effects of differences in cell size (Fig. 8E).

To further investigate the properties of the CCE pathway in LES and EB SMCs, we used extracellular of thapsigargin (TG), an SR-Ca\(^{2+}\)-ATPase inhibitor, in the absence of extracellular Ca\(^{2+}\), resulted in an elevation of [Ca\(^{2+}\)]\(i\), reflecting the emptying of intracellular stores. Under these conditions, a Ca\(^{2+}\) entry pathway is activated, as illustrated by the transient rise in [Ca\(^{2+}\)]\(i\) seen on subsequent application of external Ca\(^{2+}\) (Fig. 7, A and B). This Ca\(^{2+}\) influx was not observed in control experiments when TG application was omitted, indicating that depletion of intracellular Ca\(^{2+}\) stores is essential for activating this Ca\(^{2+}\) influx pathway (Fig. 7B). Furthermore, Ca\(^{2+}\) influx was reversibly blocked by 10 \(\mu\)mol/l La\(^{3+}\) (Fig. 7C) but not by the L-type voltage-gated Ca\(^{2+}\) channel blocker nifedipine.
Mn\textsuperscript{2+} as a surrogate ion for Ca\textsuperscript{2+}. Others have shown that Mn\textsuperscript{2+} can enter the cell via SOCs and that the rate of entry can be determined by monitoring quenching of fura 2 fluorescence at an excitation wavelength of 360 nm, the isosbestic point (11). As seen in Fig. 9, the rate of quenching by Mn\textsuperscript{2+} was significantly more rapid in LES cells than in EB cells. In addition, the peak rise in [Ca\textsuperscript{2+}] was 2-fold greater in LES cells than in EB cells. This difference was also apparent when results of individual cells were normalized as the ratio of the CCE and ACh [Ca\textsuperscript{2+}], transients, suggesting that the differences were not due to cell size.

**DISCUSSION**

We have identified the expression of mRNA species encoding five of the seven known mammalian TRPC channel genes in human EB and LES smooth muscles. Using semiquantitative RT-PCR, we demonstrated enrichment of mRNA for TRPC3 and TRPC4 in muscle from the LES compared with muscle from the EB. We have demonstrated that CCE can be activated in these muscles by store depletion and that this pathway is more prominent in LES SMCs. These differences in the Ca\textsuperscript{2+} homeostatic mechanism may contribute to the characteristic property of LES smooth muscle to develop and maintain spontaneous tonic contraction.

Our previous studies have demonstrated that both Ca\textsuperscript{2+} influx and release from intracellular Ca\textsuperscript{2+} stores contribute to cholinergic excitation in human esophageal smooth muscle (23). We showed that cyclopiazonic acid, which acts similarly to TG and blocks the SR-Ca\textsuperscript{2+}-ATPase, produces a sustained contraction of esophageal smooth muscle, consistent with participation of intracellular Ca\textsuperscript{2+} stores and activation of a Ca\textsuperscript{2+} influx pathway. The present data confirms the presence of CCE as a functional Ca\textsuperscript{2+} entry pathway with characteristic features similar to those described in several other cell types. CCE was activated by inhibition of the SR-Ca\textsuperscript{2+}-ATPase, reversibly blocked by La\textsuperscript{3+} but insensitive to the L-type Ca\textsuperscript{2+} channel inhibitor nifedipine (2). Application of extracellular Ca\textsuperscript{2+} without prior depletion of intracellular stores did not produce a transient rise in [Ca\textsuperscript{2+}], indicating that Ca\textsuperscript{2+} store depletion is required for CCE activation.
To date, seven members of the TRPC family of genes have been identified, with recent evidence for the existence of splice variants of TRPC4 and TRPC7 (7, 17, 29, 32). These channels can be categorized into four groups based on structural and functional similarities as follows: TRPC1; TRPC2; TRPC3, -6, -7; and TRPC4, -5 (7). There remains some controversy over the regulation of each TRPC subtype and whether they function as ROCs, SOCs, or both. TRPC2 is a pseudogene in the human and has not been shown to form a functional channel (28). Of the remaining channels, TRPC1, TRPC4, and TRPC5 can be activated by store depletion and thus are suggested to mediate CCE (7).

In the present study, CCE was activated by store depletion following blockade of the SR-Ca\(^{2+}\)-ATPase and thus did not involve agonist-induced inositol 1,4,5-trisphosphate [Ins(1,4,5)P\(_3\)] or diacylglycerol (DAG) generation. Since TRPC4 can be activated by store depletion without stimulation by Ins(1,4,5)P\(_3\) or DAG, our finding that mRNA for TRPC4 is more abundant in LES compared with EB can be correlated with enhanced CCE in SMCs derived from LES.

Recent evidence suggests that TRPC4 may also function as an ROC that can be activated independently of store depletion in some conditions (22). The signaling pathway is not clearly defined, but others have shown that TRPC4 can bind and be activated by Ca\(^{2+}\)-calmodulin (25, 27). Ioune et al. (13) recently provided evidence that TRPC6 forms a ROC that mediates α-adrenoceptor activation of a nonselective cation current in vascular SMCs. A similar receptor-activated nonselective cation current that can cause smooth muscle depolarization has been demonstrated in opossum (1) and human (Sims SM and Freiksaitis HG, unpublished observations) esophageal smooth muscles and represents an additional candidate function for TRPC4, TRPC6, or other TRPC subtypes.

LES smooth muscle also showed greater transcriptional expression of TRPC3. Whether this may contribute to enhanced CCE as well is not known, since we did not study CCE during agonist stimulation, conditions that would result in accumulation of Ins(1,4,5)P\(_3\) or DAG, which activate TRPC3 (7, 32). Additional possible roles for TRPC3 mediating other Ca\(^{2+}\) entry pathways, such as ROCs mentioned above, will need to be considered. Moreover, emerging evidence suggests that the functional TRPC channel is a tetramer formed by four subunits and that these channels may exist as heteromultimers (15, 24, 31). For example, coassembly of TRPC1 and TRPC3 or TRPC1 and TRPC5 to form novel cation channels has been demonstrated in HEK-293 cells (15, 24), and TRPC1 and TRPC5 have similar distribution in the hippocampus (24). This raises the possibility that the physiologically active forms of these channels in intact tissues, including LES or EB smooth muscles, may result from a combination of TRPC subtypes.

The pattern of TRPC channel subtype mRNA expression in human esophageal smooth muscle found in the present study differs from that of other smooth muscle types. Walker et al. (29), recently showed that TRPC4, TRPC6, and TRPC7 were the predominant subtypes expressed in canine and murine smooth muscles. In contrast, we could not detect mRNA expression for TRPC7 in either cultured cells or tissue of the human esophagus, although our methods readily identified the gene product in rat brain and human brain and heart. We did not examine other human gastrointestinal smooth muscles for the expression of TRPC7. In dog and mouse, TRPC4 and TRPC7 showed a substantial quantitative variation in gene expression between different gastrointestinal smooth muscles (29), but the functional correlate of these differences has not been studied. On the other hand, TRPC3 mRNA was detected in EB and LES, whereas none was identified in canine or murine gastric, jejunal, or colonic smooth muscles (29) or in the rat or canine pulmonary artery (16, 29). The functional implications of these striking tissue-dependent differences in TRPC subtype expression remain to be elucidated. It should be recognized that these comparisons are based on detection of mRNA in these tissues and not functional channel proteins. Further studies using subtype-specific antibodies or blocking drugs are limited by the lack of general availability of such reagents. Moreover, EB and LES cells in culture assume a proliferative noncontractile phenotype similar to other SMC cultures (5, 30), raising the possibility that changes in TRPC channel expression may be related to cell phenotype. The fact that we identified a similar pattern of TRPC mRNA expression in whole tissues from the EB and LES indicates that this is unlikely.

LES muscle is characterized by its ability to maintain spontaneous myogenic tension, whereas the adjacent muscle of the EB contracts in response to nerve activation during peristalsis. The unique features of LES smooth muscle that impart its characteristic behavior are incompletely understood. The regional differences in TRPC channel expression and CCE we identified in the present study add to the growing body of information characterizing the distinct physiological properties of LES and EB smooth muscles. For example, compared with EB, LES SMCs are larger, have a more irregular cell surface, a more depolarized resting membrane potential, greater SR density, more plentiful mitochondria, different contractile protein composition, and lower levels of cytochrome c oxidase (8, 12). The central role of Ca\(^{2+}\) in smooth muscle contraction provides a logical focus for exploring differences in muscle function. The findings of the present study provide additional evidence that fundamentally important differences exist between these muscle types in the regulation of cell Ca\(^{2+}\).

The relative importance of intracellular Ca\(^{2+}\) stores or influx from the extracellular space in the characteristic phasic contraction of EB smooth muscle during peristalsis vs. the sustained muscular contraction of LES remains controversial. Biancane et al. (3, 4) showed that LES tonic contraction in the cat was dependent on continuous low-level Ca\(^{2+}\) release from intracellular stores. Such a mechanism would require an efficient pathway for replenishing these stores. In
several other species, removing extracellular Ca\(^{2+}\) or blocking influx reduces LES tone (9, 21, 26), although it remains uncertain whether this represents a direct contribution of Ca\(^{2+}\) influx to [Ca\(^{2+}\)]\(_i\), or an indirect effect on intracellular Ca\(^{2+}\) stores. Influx of Ca\(^{2+}\) via L-type channels has been shown to contribute only partially to refilling of intracellular Ca\(^{2+}\) stores in the canine LES (21) and not at all in human esophageal smooth muscle (23). Previously, we demonstrated that removal of extracellular Na\(^+\) had no significant effect on basal Ca\(^{2+}\) levels or the response of [Ca\(^{2+}\)]\(_i\) to ACh stimulation in human esophageal cells, indicating that Na\(^+\)/Ca\(^{2+}\) exchange pathways did not contribute significantly to Ca\(^{2+}\) homeostasis in this tissue. Finally, enhanced CCE in LES cells was also reflected by enhanced Mn\(^{2+}\) entry. Mn\(^{2+}\) is a poor substrate for the SR-Ca\(^{2+}\)-ATPase and the Na\(^+\)/Ca\(^{2+}\) exchanger (11). Together, these observations suggest that the maintenance and refilling of intracellular Ca\(^{2+}\) stores requires additional influx pathways, most likely CCE.

In addition to enrichment in transcriptional expression of TRPC subtypes and enhanced CCE in smooth muscle from the LES, the acute increase in [Ca\(^{2+}\)]\(_i\) to ACh stimulation in human esophageal smooth muscle. This finding may be relevant to previous studies in the opossum, which have shown abundant SR in the LES compared with EB (6).

In summary, the present study demonstrates the expression of mRNA encoding multiple TRPC channel subtypes in smooth muscle of the human EB and LES. mRNA for TRPC3 and TRPC4 are enriched in smooth muscle from the LES, which also shows enhanced CCE. These differences may contribute to the unique physiological properties of these two smooth muscle types.

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