Regulation of intracellular calcium in human esophageal smooth muscles

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Sims, Stephen M., Yang Jiao, and Harold G. Preiksaitis. Regulation of intracellular calcium in human esophageal smooth muscles. Am. J. Physiol. 273 (Cell Physiol. 42): C1679–C1689, 1997.—We have investigated sources of Ca2+ contributing to excitation of human esophageal smooth muscle, using fura 2 to study cytosolic free Ca2+ concentration ([Ca2+]i) in dispersed cells and contraction of intact muscles. Acetylcholine (ACh) caused an initial peak rise of [Ca2+]i, followed by a plateau accompanied by reversible contraction. Removal of extracellular Ca2+ or addition of dihydropyridine Ca2+-channel blockers reduced the plateau phase but did not prevent contraction. Caffeine also caused elevation of [Ca2+]i, and blocked responses to ACh. Undershoots of [Ca2+]i were apparent after ACh or caffeine. Blockade of the sarcoplasmic reticular Ca2+-ATPase by cyclopiazonic acid (CPA) reduced the ACh-activated increase of [Ca2+]i and abolished the undershoot, indicating involvement of Ca2+ stores. When contraction was studied in intact muscles, removal of Ca2+ or addition of nifedipine reduced, but did not abolish, carbachol (CCh)-induced contraction. Elevation of extracellular K+ caused contraction that was inhibited by nifedipine, although CCh still elicited contraction and suppressed the CCh-induced contraction, whereas ryanodine reduced CCh-induced contraction. Our studies provide evidence that muscarinic excitation of human esophagus involves both release of Ca2+ from intracellular stores and influx of Ca2+.

Acetylcholine; muscarinic receptors; caffeine; fura 2

ESOPHAGEAL PERISTALSIS is dependent on the integration of inhibitory and excitatory neuronal mechanisms. In humans and cats, atropine potently blocks peristalsis in the distal esophagus, indicating a prominent role for muscarinic cholinergic excitation and contraction of esophageal smooth muscle (6, 12). The activation of muscarinic receptors controls contraction of smooth muscle in part by regulating the concentration of cytosolic free Ca2+ ([Ca2+]i) (22, 25). Increase of [Ca2+]i can occur due to entry through ligand- or voltage-activated channels or by release from intracellular stores such as the sarcoplasmic reticulum (SR). The second messenger inositol 1,4,5-trisphosphate (IP3) is generated after activation of some receptors and mediates the release of Ca2+ from stores in many cell types, including gastrointestinal smooth muscles (3, 22). A number of Ca2+-influx pathways have been described in smooth muscles. For example, agonist-mediated depolarization activates dihydropyridine-sensitive Ca2+ channels (17), and Ca2+ window currents or nonselective cation channels have also been described (9, 18).

The source of Ca2+ mediating excitation in the esophagus has been the subject of investigation in animal models. Studies of cat esophageal body point to cholinergic excitation being mediated by the muscarinic M2-receptor subtype, with an absolute requirement for Ca2+ influx. In contrast, cholinergic excitation in the lower esophageal sphincter utilizes intracellular stores of Ca2+ via a mechanism involving the M1-receptor subtype and generation of IP3 (4, 14, 21). However, recent studies in our laboratory reveal a dominant role for M3-receptor subtype in excitation of cat esophageal body (16a), in support of the general view that contraction of most gastrointestinal smooth muscles is mediated by the M3 subtype (7).

The sources of Ca2+ involved in cholinergic excitation of human esophageal tissues have not, to our knowledge, been studied. We have recently characterized muscarinic receptors in human esophageal smooth muscle using a combination of in vitro muscle contraction studies, receptor autoradiography, and reverse transcriptase-polymerase chain reaction approaches (16b). Although a mixed receptor population exists in the human esophagus, contraction is mediated by the M3-receptor subtype. Cholinergic signaling via M3 receptors suggests a pathway involving IP3-mediated Ca2+ release from stores (7). To investigate the regulation of [Ca2+]i in human esophageal smooth muscles, we used the Ca2+-sensitive fluorescent dye fura 2 to monitor [Ca2+]i in freshly dispersed cells, together with contractile studies of intact tissues. We present evidence that cholinergic excitation involves both Ca2+-influx and release from intracellular stores. Some aspects of this work have been reported previously in an abstract (26).

METHODS

Tissue retrieval and isolation of cells. Tissue collection was carried out in accordance with guidelines of the University Review Board for Research Involving Human Subjects. Tissues were obtained from 34 patients undergoing esophageal resection because of cancer, including 27 male and 7 female patients, ages 37–77 yr (mean 65 ± 11 yr) with the following tumor types: 13 squamous cell carcinomas, 16 adenocarcinomas, 2 undifferentiated carcinomas, and 3 with severe dysplasia. After resection, specimens were immediately cooled on ice. A sample of the entire thickness of the muscularis propria (~1 cm2) was removed from a disease-free region in the distal third of the esophagus and placed in ice-cold, oxygenated Krebs bicarbonate solution (see below) for transport to the laboratory. Portions of muscle were dissected for preparation of dispersed cells and others for tissue bath studies. Muscle cells were dispersed as previously described (19). Briefly, segments of esophagus (~1 mm wide, 1 cm long) were
placed in 2.5 ml of dissociation solution consisting of 135 mM K\textsuperscript{+} solution plus the following: 0.2 mg/ml collagenase (Sigma blend type F), 2 mg/ml bovine albumin (ICN Biomedicals), 2.5 mg/ml papain, 0.4 mg/ml 1,4-dithio-L-threitol, 10 mM tau-rine, and 0.5 mM EDTA (adjusted to pH 7.0). Tissues were generally stored in dissociation solution at 4°C overnight. On the following day, tissues were warmed to room temperature for 30–60 min, then placed in a gently shaking water bath at 31°C for 60 min, and dispersed by trituration with fire-polished Pasteur pipettes. Cells were studied within 8 h.

Solutions. The Krebs bicarbonate solution for retrieval of tissues and contraction studies consisted of (in mM) 116 NaCl, 5 KCl, 2.5 CaCl\textsubscript{2}, 1.2 MgSO\textsubscript{4}, 2.2 NaH\textsubscript{2}PO\textsubscript{4}, 25 NaHCO\textsubscript{3}, and 10 D-glucose, equilibrated with 5% CO\textsubscript{2}-95% O\textsubscript{2} (pH 7.4). The Na\textsuperscript{+}-N,2-hydroxyethylpiperazine-N',N'-ethanesulfonic acid (HEPES) solution used for single cell fluorescence studies contained (in mM) 130 NaCl, 5 KCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 20 HEPES, and 10 D-glucose (adjusted to pH 7.4 with NaOH). To minimize changes in membrane potential of cells in many experiments, we used bathing solution in which all NaCl was replaced with KCl, giving 135 mM K\textsuperscript{+} solution, which resulted in the membrane potential being clamped close to 0 mV. In all cases in which the concentration of K\textsuperscript{+} was raised, we adjusted the concentration of Na\textsuperscript{+} to maintain the osmolality of the solution. Ca\textsuperscript{2+}-free solutions had the same composition as above, except for the omission of CaCl\textsubscript{2} and the addition of 0.5 mM ethylene glycol-bis(\beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA).

Measurement of [Ca\textsuperscript{2+}]. Ca\textsuperscript{2+} transients were measured from single cells using fura 2. Cells were loaded by incubation with 0.2 µM fura 2-acetoxymethyl ester (AM) for 20–40 min at 29°C and then allowed to settle onto a glass coverslip that comprised the bottom of a perfusion chamber (0.75 ml vol). The chamber was mounted on a Nikon inverted microscope and continuously perfused with bathing solution at 2–5 ml/min at room temperature. Cells chosen for study were solitary, initially relaxed, and contracted reversibly after stimulation with acetylcholine (ACh). Cells were illuminated with alternating 345- and 380-nm light using a Deltascan system (Photon Technology International), with the 510-nm emission detected using a photometer. Exposure of cells to excitation light was minimized by closing the shutter during recovery from agonists. [Ca\textsuperscript{2+}] was calibrated according to the methods of Grynkiewicz et al. (13), using a dissociation constant of 225 nM for binding of Ca\textsuperscript{2+} to fura 2 and a viscosity factor of 15%. Data were corrected for background fluorescence.

Contraction was quantified from video recordings of cells using an interactive, computer-based video analysis system. Cell lengths were determined as the length of a line drawn through the central axis of each cell. Video images of contraction were recorded simultaneously with the fluorescence...
signals using a light-emitting diode to illuminate cells at >600 nm, together with a dichroic mirror in the emission path to direct light >550 nm to a charge-coupled device camera. Shorter wavelengths were filtered (510-nm band pass) and directed to the photomultiplier. Test agents were applied by bath perfusion or by glass micropipettes positioned ~50 µm from cells. Control applications of vehicle had no effect on \([\text{Ca}^{2+}]_i\).

**Tissue bath studies.** Muscle strips (~2 × 10 mm) were prepared from the longitudinal and circular muscle layers and mounted individually in water-jacketed tissue baths containing 10 ml of Krebs bicarbonate solution continuously bubbled with 5% CO2-95% O2 at 37°C. By use of silk ties, one end of the strip was attached to a Grass FT03 isometric force transducer coupled to a Grass 79E chart recorder (Grass Instruments, Quincy, MA). After a 1-h equilibration period, the length of each strip was adjusted to produce the maximal tension increase to 10^{-6} M carbachol (CCh). For quantification, tension responses are expressed as a percentage of that obtained in each individual strip with 10^{-5} M CCh, the concentration producing the maximal tension response.

**Drugs and materials.** All drugs and chemicals were obtained from Sigma Chemical (St. Louis, MO) or BDH (Toronto, ON, Canada) unless otherwise indicated. Fura 2-AM (Molecular Probes, Eugene, OR) was prepared in dimethyl sulfoxide. Nifedipine, nicardipine, and BAY K 8644 were from Research Biochemicals International (Natick, MA). All drugs were prepared as concentrated stock solutions and diluted into the appropriate bathing solution before addition to the tissue baths or application to isolated cells.

**Statistics.** Values are presented as means ± SE. Statistical comparisons were made using Student’s t-test where P < 0.05 was considered to indicate statistical significance. The number of individual esophagectomy specimens used is indicated by N, and the number of isolated cells studied is indicated by n. All traces are representative of at least three experiments on muscle from two or more patients.

**RESULTS**

Enzymatic dissociation of longitudinal and circular layers of esophageal body yielded individual spindle-shaped cells that contracted reversibly in response to ACh. Contraction of a circular muscle cell is illustrated for the relaxed cell (Fig. 1A, top) and then 20 s after stimulation with ACh (Fig. 1A, middle). Such contractions elicited by ACh were reversible, with relaxation occurring over the course of several minutes (Fig. 1A, bottom). The \([\text{Ca}^{2+}]_i\)-sensitive fluorescent dye fura 2 was
used to investigate the regulation of \([Ca^{2+}]_i\), in isolated cells. ACh caused elevation of \([Ca^{2+}]_i\), accompanied by contraction (Fig. 1B). The ACh-evoked rise of \([Ca^{2+}]_i\) was concentration dependent, with half-maximal elevation at \(\sim 2 \mu M\) ACh (Fig. 1, C and D). In the series of experiments illustrated in Fig. 1, cells were bathed in 135 mM K\(^+\) solution to clamp the membrane potential at 0 mV, but similar contractions, \([Ca^{2+}]_i\) transients, and basal \([Ca^{2+}]_i\) were observed for cells bathed in Na\(^+\)-HEPES solution. Basal \([Ca^{2+}]_i\) of longitudinal muscle cells was 149 ± 9 nM (\(n = 33\)) when bathed in Na\(^+\)-HEPES, similar to the value of 143 ± 6 nM (\(n = 11\)) for cells bathed in 135 mM K\(^+\) solution. Peak \([Ca^{2+}]_i\) in response to 100 µM ACh reached 410 ± 29 nM (\(n = 40\)) for longitudinal muscle cells. Levels of \([Ca^{2+}]_i\) in circular muscle cells were consistently lower than in longitudinal muscle, with basal levels of 94 ± 5 nM (cells in Na\(^+\)-HEPES solution) and peak \([Ca^{2+}]_i\) of 247 ± 12 nM (\(n = 8\)) in response to 100 µM ACh. Although cells of the circular and longitudinal layers differed in this quantitative sense, no qualitative differences in the regulation of \([Ca^{2+}]_i\) were apparent.

Experiments were first carried out to examine whether \([Ca^{2+}]_i\) release from stores contributed to the cholinergic responses, as suggested by the contraction of cells bathed in 135 mM K\(^+\) solution (above). Because of variability in the basal \([Ca^{2+}]_i\), and peak transients seen among cells, we compared responses of individual cells before and after various treatments. Removal of extracellular \([Ca^{2+}]_i\) (perfusion with \([Ca^{2+}]_i\)-free solution with 0.5 mM EGTA) caused a reduction of basal \([Ca^{2+}]_i\) as well as reduced amplitude of the ACh-evoked \([Ca^{2+}]_i\) transient (Fig. 2A). However, ACh continued to elicit a substantial rise of \([Ca^{2+}]_i\), even with bathing in \([Ca^{2+}]_i\)-free solution for 30 min, indicating that intracellular stores of \([Ca^{2+}]_i\) are sufficient to maintain the ACh-mediated \([Ca^{2+}]_i\) transient in esophageal muscle (Fig. 2A). Similarly, caffeine, which acts on the ryanodine receptor to release \([Ca^{2+}]_i\) from intracellular stores (8), caused elevation of \([Ca^{2+}]_i\) in esophageal muscle (rise of \([Ca^{2+}]_i\) to peak level of 682 ± 95 nM, \(n = 8\) in response to 5 mM caffeine). Caffeine continued to elicit rise of \([Ca^{2+}]_i\) for many minutes in cells bathed in \([Ca^{2+}]_i\)-free solution (Fig. 2B). Contraction of cells also persisted in
Ca\textsuperscript{2+}-free solutions (not shown). We note that, although basal levels of [Ca\textsuperscript{2+}]i were restored after readdition of Ca\textsuperscript{2+}, there was incomplete recovery of the peak transients, which may have been due to rundown of the cells with repeated stimulation.

Because responses to ACh and caffeine persisted in Ca\textsuperscript{2+}-free solution, we investigated the contribution of Ca\textsuperscript{2+} stores. By use of cyclopiazonic acid (CPA, 10 µM) to block the SR Ca\textsuperscript{2+}-ATPase, removal of extracellular Ca\textsuperscript{2+} and stimulation with ACh led to rapid reduction of the ACh-evoked Ca\textsuperscript{2+} transient (Fig. 3A), indicative of store depletion (summarized in Fig. 3B). The effects of CPA were reversible, as shown by recovery of the response to ACh (Fig. 3A, right). Because the refilling of Ca\textsuperscript{2+} stores in some smooth muscles appears to involve voltage-dependent Ca\textsuperscript{2+} channels (15), we investigated this possibility in esophageal muscles. However, as assayed by stimulation with ACh, neither the Ca\textsuperscript{2+} channel blocker nifedipine nor the agonist BAY K 8644 significantly altered the rate or extent of refilling of stores (Fig. 3B). As illustrated in Fig. 3C, prominent undershoots of [Ca\textsuperscript{2+}]i after the ACh-induced transient were observed in many esophageal muscle cells. Such undershoots of [Ca\textsuperscript{2+}]i have been taken as evidence that stores participate actively in restoration of basal [Ca\textsuperscript{2+}]i in smooth muscle (1, 2, 11, 19). We examined the role of the SR Ca\textsuperscript{2+}-ATPase in the generation of undershoots using selective short-term exposure of cells to CPA while avoiding the depletion of stores resulting from longer term exposure. After a control response to ACh (Fig. 3C, left), cells were treated for 30 s with CPA. Although CPA had little effect on resting [Ca\textsuperscript{2+}]i, it reversibly abolished the undershoot after ACh (Fig. 3C, representative of responses observed in 5 cells). Thus Ca\textsuperscript{2+} stores participate in the elevation of [Ca\textsuperscript{2+}]i after agonist stimulation as well as the restoration of basal [Ca\textsuperscript{2+}]i in esophageal muscle.

A number of Ca\textsuperscript{2+} entry pathways exist in smooth muscles, including ligand and voltage-gated channels. To evaluate the contribution of L-type Ca\textsuperscript{2+} channels in
ACh-evoked elevation of [Ca^{2+}], we examined the effects of Ca^{2+} channel blockers and activators. We examined multiple responses in individual cells, as described above, and in this series of experiments bathed cells in Na^{+}-HEPES solution to allow for normal membrane potential and voltage-dependent responses. Figure 4A shows typical reproducible elevations of [Ca^{2+}] in response to repeated applications of ACh in one cell (prominent undershoots of [Ca^{2+}] are evident after ACh). In another cell, 10 µM nifedipine caused a prompt reduction of basal [Ca^{2+}], but resulted in little change of the subsequent response to ACh (Fig. 4B). Similarly, treatment of cells with the L-type Ca^{2+} channel activator BAY K 8644 did not alter ACh-evoked Ca^{2+} transients (summarized in Fig. 4C). Because the effect of nifedipine may be diminished due to degradation by ultraviolet excitation light, we also examined effects of nicardipine, a Ca^{2+} channel blocker without such light sensitivity. In all six cells tested, nicardipine (10 µM) caused similar reduction in basal [Ca^{2+}], but did not diminish the amplitude of ACh-evoked transients or contractions, confirming the findings with nifedipine.

To this point, our observations indicate that Ca^{2+} release from stores contributes to the acute elevation of [Ca^{2+}] and contraction in esophageal muscle cells. Longer exposure to ACh revealed a plateau elevation of [Ca^{2+}] after the transient (Fig. 5A), which was in part due to Ca^{2+} influx. The plateau phase was graded and was dependent on the continued presence of agonist, as apparent from three overlapping responses elicited by ACh from a representative cell (Fig. 5B). Short-duration stimulations (10 s, as used for most responses described above) elicited only a transient component (as well as an undershoot of [Ca^{2+}]), whereas the plateau was evident with longer exposures to ACh. Removal of extracellular Ca^{2+} reduced the plateau phase and slightly lowered basal [Ca^{2+}] (Fig. 5C). This reduction of the plateau phase remained even after the responses were aligned by correcting for differences in basal [Ca^{2+}] (Fig. 5C, inset). In contrast, caffeine caused a transient elevation of [Ca^{2+}], but no plateau phase (Fig. 5D), and responses were not altered by removal of Ca^{2+}. Nicardipine reduced the plateau in a similar manner (10 µM, 4 cells, not shown), indicating that Ca^{2+} influx was mediated in part by L-type Ca^{2+} channels.

To examine whether ACh and caffeine acted on a common Ca^{2+} store, we examined responses to both applied sequentially to cells. Pretreatment with ACh abolished the response to caffeine (Fig. 6A), and when applied in the reverse order, caffeine reduced the response to ACh (Fig. 6B). This interaction could indicate that IP_{3} and ryanodine receptors act to release

![Fig. 5. Ca^{2+} influx during plateau elevation of [Ca^{2+}].](http://ajpcell.physiology.org/)

A: control response to ACh (100 µM) illustrates peak elevation of [Ca^{2+}] followed by plateau during prolonged stimulation with ACh. B: plateau is dependent on duration of agonist application, with undershoots of [Ca^{2+}], apparent with shorter applications of ACh. C: perfusion of cell with Ca^{2+}-free solution (with 0.5 mM EGTA) reduced amplitude of ACh-induced plateau, which was still apparent when basal [Ca^{2+}] was aligned (inset). D: caffeine (5 mM) caused transient elevation of [Ca^{2+}] without a plateau phase. All responses from cells of longitudinal muscle layer. Peaks of [Ca^{2+}] transients in B–D were blanked to emphasize plateau.
Ca²⁺ from a common store. However, the reduced response to ACh by caffeine (Fig. 6B) may also be due to block of IP₃ production (23) or block of the IP₃ receptor by caffeine (8). To test this, we applied caffeine during the plateau, where it caused [Ca²⁺]ᵢ to drop to basal levels (Fig. 6C), indicating that it can inhibit muscarinic signaling, independent of its effect on the Ca²⁺ store. Similarly, prolonged stimulation with caffeine prevented the subsequent action of ACh (Fig. 6D). The mechanism by which caffeine blocks muscarinic signaling in esophageal muscle is not known, but its effects do provide evidence for involvement of stores in muscarinic regulation of [Ca²⁺].

The studies of isolated cells provided evidence for both Ca²⁺ release from stores and Ca²⁺ influx. To evaluate the functional contributions of these pathways to contraction, we measured tension development in strips of muscle. The muscarinic agonist CCh caused reversible contraction of both longitudinal and circular esophageal muscles (Fig. 7) that was concentration dependent and blocked by atropine (not shown). To evaluate the contribution of Ca²⁺ influx, we first removed Ca²⁺ from the bathing solution. CCh initially elicited contraction, but the amplitude gradually diminished with repeated applications of agonist, so that virtually no contraction was evoked after 10 min of exposure to Ca²⁺-free solution (Fig. 7A). Complete recovery of the response occurred within 1 min of readdition of Ca²⁺ to the bath (Fig. 7A, right). The longitudinal and circular muscles behaved qualitatively the same in all respects, although quantitative differences were apparent (Fig. 7C, average values of contraction given as a percentage of control contraction elicited by 10 μM CCh in same strip). Blockade of Ca²⁺ channels with nifedipine (1 μM) decreased the response to CCh to 33 ± 6% in longitudinal muscle (N = 4) and 19 ± 1% in circular muscle (N = 3, Fig. 7, B and C; significant decreases, P < 0.001). Nicardipine (1 μM) caused a similar reduction in CCh-evoked contraction (not shown, N = 2).

Further evidence for Ca²⁺ influx was obtained when muscles were depolarized by raising bath K⁺ (substitution for Na⁺). Elevation of K⁺ to 100 mM caused an initial phasic increase in tension followed by prolonged contraction (Fig. 8), both of which were completely blocked by removal of extracellular Ca²⁺ or addition of 1 μM nifedipine (not shown). Both the initial and prolonged phases occurred with 20 and 50 mM K⁺ and were maximal at 100 mM K⁺. After prolonged elevation of K⁺, CCh still elicited contraction, although the amplitude was reduced to 49 ± 6% of control in longitudinal muscle (P < 0.0001, N = 7) and to 30 ± 2% in circular muscle (P < 0.0001, N = 7) when determined relative to the increased baseline tension that persisted with 100 mM K⁺. When the baseline tension was disregarded, the peak tension elicited by CCh in 100 mM K⁺ was 66 ± 5% for longitudinal and 45 ± 3% of control, indicating a significant reduction in the
contraction during persistent depolarization. Nifedipine (1 µM) reduced the tonic contraction but did not affect the residual CCh-evoked contraction (Fig. 8B).

The finding that CCh continued to elicit contraction when Ca\(^{2+}\) influx was minimized was consistent with intracellular stores contributing to contraction. We further examined the contribution of Ca\(^{2+}\) stores using CPA (10 µM), which itself caused a marked and persistent contraction (Fig. 9A) comprising 43 ± 6% (N = 5) of the maximal contraction elicited by 10\(^{-5}\) M CCh for longitudinal muscle and 40 ± 4% (N = 6) for circular muscle. CPA (1 µM) had negligible effects on basal tone. In the presence of 10 µM CPA, CCh-evoked contractions (10\(^{-5}\) M CCh) were reduced to 69 ± 6% of the maximal CCh response for longitudinal muscle (P < 0.001, N = 5) and 58 ± 8% for circular muscle (P < 0.005, N = 6) when measured from the baseline before addition of CPA. When measured from the increased level of tension due to CPA, contraction was reduced to 25 ± 5% of the maximal CCh response for longitudinal muscle and 18 ± 7% for circular muscle. Suppression of the CCh response persisted after washout of CPA, even after recovery of the baseline (Fig. 9A, right).

Ryanodine (10 µM) caused a small reduction of the CCh-induced contraction in normal Krebs bicarbonate solution (Fig. 9B), decreasing the contraction to 89 ± 2% of control in longitudinal muscle and 89 ± 2% for circular muscle (P < 0.001, N = 7 for both layers). When Ca\(^{2+}\) influx was minimized by depolarizing the muscle with 100 mM K\(^{+}\), the effect of ryanodine was more prominent (Fig. 9B, with quantification in Fig. 9C).

DISCUSSION

We have investigated muscarinic signaling in human esophageal smooth muscle using Ca\(^{2+}\) fluorescence methods in dispersed cells and contraction studies of intact muscle strips. Studies of dispersed cells reveal that cholinergic elevation of [Ca\(^{2+}\)]\(_i\) arises from both Ca\(^{2+}\) influx and release from intracellular stores. Muscle contraction studies confirm the involvement of multiple sources of Ca\(^{2+}\) contributing to the cholinergic tension response. These data provide the first characterization of Ca\(^{2+}\) regulation in human esophageal muscle cells. Removal of extracellular Ca\(^{2+}\) or addition of nifedipine caused significant reduction of CCh-induced contraction. Recovery periods of 3–5 min were allowed between stimulation with CCh.

Fig. 7. Ca\(^{2+}\) entry contributes to carbachol (CCh)-induced contraction of longitudinal and circular esophageal muscle. Contraction of intact strips was monitored as described in METHODS. CCh (10 µM final concentration, added at time indicated by arrows) caused increased tension of longitudinal and circular muscles (indicated here and in subsequent figures as LM and CM, respectively). A: control CCh-induced contraction is shown at left and then after removal of Ca\(^{2+}\) from bath solution, which caused gradual reduction of CCh-evoked contraction (middle). Recovery is shown at right after readdition of Ca\(^{2+}\). B: nifedipine (1 µM final concentration) reduced amplitude of evoked contraction, with partial recovery evident at right after washout. C: summary of experiments illustrated in A and B, with means ± SE presented as a percentage of control responses. Removal of extracellular Ca\(^{2+}\) or addition of nifedipine caused significant reduction of CCh-induced contraction. Recovery periods of 3–5 min were allowed between stimulation with CCh.
of [Ca$^{2+}$], and the accompanying contractions persisted for over 30 min in Ca$^{2+}$-free solution. This observation provides evidence for a store of Ca$^{2+}$ that is mobilized by ACh, probably involving IP$_3$ as a second messenger. When cholinergic contractions were examined in intact muscle strips, findings also supported a role for intracellular Ca$^{2+}$ stores. A portion of the CCh-induced contraction remained after voltage-dependent influx was blocked by high-K$^+$ depolarization or by addition of L-type Ca$^{2+}$ channel blockers. Additionally, disruption of intracellular Ca$^{2+}$ handling by blocking the SR Ca$^{2+}$-ATPase with cyclopiazonic acid, or inhibition of the ryanodine receptor, impaired the contraction response. Although the removal of extracellular Ca$^{2+}$ eventually suppressed CCh contractions, the change was time dependent, and we cannot rule out the possibility that intracellular Ca$^{2+}$ stores in muscle strips are more sensitive to alterations in the concentration of extracellular Ca$^{2+}$ than dispersed muscle cells.

Previous studies in cat have indicated a role for Ca$^{2+}$ stores in supporting the contraction of smooth muscle from the lower esophageal sphincter (4, 14, 21). However, both nerve-mediated contraction and the response to cholinergic stimulation in the esophageal body are reported to be dependent on Ca$^{2+}$ influx. These findings are supported by earlier studies on muscle strips from opossum esophagus and lower esophageal sphincter in which extracellular Ca$^{2+}$ was required for nerve-evoked responses (5, 10). However, because the removal of extracellular Ca$^{2+}$ would impair neurotransmitter release, interpretation of these studies at the cellular level is difficult.

Multiple muscarinic receptor subtypes have been identified by molecular cloning studies. Most gastrointestinal smooth muscles express both the M$_2$ and M$_3$ subtypes, although the M$_3$ subtype is generally found to mediate contraction (7). We have recently found that the M$_3$-receptor subtype mediates contraction in the human esophagus (16b), as well as messenger RNA for both M$_2$ and M$_3$ subtypes, indicating the coexistence of subtypes in the muscle, as described for other gastrointestinal muscles (7, 27). Others have identified regional differences in muscarinic receptor expression and signaling pathways in the gastrointestinal tract. For example, in the cat, M$_2$ receptors mediate contraction in the esophageal body, relying on Ca$^{2+}$ influx, whereas in the lower esophageal sphincter the M$_3$ receptor triggers Ca$^{2+}$ release from stores (21). In the small intestine, contraction of the longitudinal layer involves Ca$^{2+}$ influx, whereas in the circular layer, Ca$^{2+}$ release from stores contributes to contraction (16). Although [Ca$^{2+}$]$_{i}$ transients were larger in longitudinal muscles from the human esophagus, we found no evidence for differences in signaling between longitudinal and circular muscles.

We also provide evidence for Ca$^{2+}$ influx in human esophageal smooth muscle. In single cells, basal [Ca$^{2+}$]$_{i}$ was reduced on removal of extracellular Ca$^{2+}$ or addition of Ca$^{2+}$-channel blockers, consistent with the presence of a Ca$^{2+}$-window current (9). In addition, ACh-evoked increase in [Ca$^{2+}$]$_{i}$ displayed a plateau phase, a portion of which was due to Ca$^{2+}$ influx. In muscle strips, we confirmed the contribution of Ca$^{2+}$ influx to contraction in two ways. The contraction initiated by depolarization with high K$^+$ was totally dependent on Ca$^{2+}$ influx, since removal of extracellular Ca$^{2+}$ or addition of Ca$^{2+}$ channel blockers completely inhibited this contraction. Moreover, CCh-mediated contractions were attenuated by Ca$^{2+}$ channel blockade. Consistent with these functional studies, voltage-activated Ca$^{2+}$ currents have been described in cat (20) and human esophageal smooth muscle cells (26). Thus the present findings do support a role for Ca$^{2+}$ influx, consistent with earlier observations made in the cat (4, 14). However, as in most other smooth muscles (7, 17), multiple sources of Ca$^{2+}$ contribute to excitation in human esophagus.

Several additional features indicated a contribution of Ca$^{2+}$ stores to regulation of [Ca$^{2+}$]$_{i}$ in esophageal
Caffeine, which activates ryanodine receptors to release Ca\textsuperscript{2+} from the endoplasmic reticulum, caused transient elevation of [Ca\textsuperscript{2+}]\textsubscript{i} accompanied by contraction, and continued to elicit these responses in Ca\textsuperscript{2+}-free solution. The persistence of the ACh- and caffeine-evoked responses in Ca\textsuperscript{2+}-free solution suggested that intracellular cycling of Ca\textsuperscript{2+} by the SR was required to maintain responsiveness. Consistent with this, blockade of the SR Ca\textsuperscript{2+}-ATPase with CPA led to substantial reduction of the ACh-evoked rise of [Ca\textsuperscript{2+}]\textsubscript{i} and muscarinic contraction, consistent with depletion of the store. To determine whether IP\textsubscript{3} and caffeine mobilized a common pool of Ca\textsuperscript{2+}, we applied the two agents sequentially to cells. Each agent reduced the responses to the other, consistent with them acting on a common pool. However, in addition to activating ryanodine receptors, caffeine is also known to block production of IP\textsubscript{3} (23) or the IP\textsubscript{3} receptor (8), so that the failure of ACh to act after caffeine does not necessarily indicate store depletion. When applied during the plateau elevation of [Ca\textsuperscript{2+}]\textsubscript{i} induced by ACh, caffeine caused [Ca\textsuperscript{2+}] to drop promptly to basal levels. Thus caffeine does appear to inhibit muscarinic signaling independent of its effect on releasing Ca\textsuperscript{2+} from intracellular stores, although the precise mechanisms by which caffeine acts are not resolved in this study. Overall, we conclude that ACh and caffeine act on a common Ca\textsuperscript{2+} store, similar to that found in other muscles, such as trachea (19), but different from some vascular muscles, where IP\textsubscript{3}-sensitive stores have been reported to be functionally distinct from the caffeine-mobilized stores (e.g., Refs. 24, 25).

Many esophageal muscle cells exhibited an undershoot of [Ca\textsuperscript{2+}] after ACh or caffeine, which in other smooth muscles is thought to reflect sequestration of Ca\textsuperscript{2+} by the SR Ca\textsuperscript{2+}-ATPase (1, 2, 11). CPA reversibly blocked the undershoot, as described for tracheal muscle cells (19). Taken together, these data point to the involvement of SR not only as a source of Ca\textsuperscript{2+} during excitation of esophageal muscle but also as an important and active site for sequestration of Ca\textsuperscript{2+}.

In summary, the present studies demonstrate that muscarinic receptor activation causes contraction of human esophageal smooth muscle, in part by regulating [Ca\textsuperscript{2+}]\textsubscript{i}. In both muscle layers of the esophagus, elevation of [Ca\textsuperscript{2+}]\textsubscript{i} arises due to multiple sources of Ca\textsuperscript{2+}, including Ca\textsuperscript{2+} influx and release of Ca\textsuperscript{2+} from intracellular stores. Details of the signaling cascade initiated by muscarinic receptors in this tissue remain to be determined.

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