Temporal and spatial dynamics underlying capacitative calcium entry in human colonic smooth muscle

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Short Title: CCE and calcium hotspots in human colonic smooth muscle

Abbreviations Used: Caff, caffeine; CCE, capacitative calcium entry; CCh, carbachol; CPA, cyclopiazonic acid; CM, circular muscle; EB, esophageal body; IbTx, iberiotoxin; LM, Longitudinal muscle; mN, milli-Newtons; NANC, Non-adrenergic Non-cholinergic; NKA, Neurokinin A; NSC, non-selective cation; SMC, smooth muscle cell; SOC, store operated channels; SERCA; sarco-endoplasmic reticulum Ca\(^{2+}\) -ATPase; TG, thapsigargin

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ABSTRACT

Following smooth muscle excitation and contraction, depletion of intracellular Ca\textsuperscript{2+} stores activates capacitative Ca\textsuperscript{2+} entry (CCE) to replenish stores and sustain cytoplasmic Ca\textsuperscript{2+} elevations. The objectives of the present study were to characterize CCE and the cytosolic Ca\textsuperscript{2+} (Ca\textsuperscript{2+}\textsubscript{i}) dynamics underlying human colonic smooth muscle contraction using tension recordings, fluorescent Ca\textsuperscript{2+} indicator dyes and patch-clamp electrophysiology. The neurotransmitter acetylcholine (ACh) contracted tissue strips and, in freshly isolated colonic smooth muscle cells (SMCs), caused elevation of Ca\textsuperscript{2+}\textsubscript{i} as well as activation of non-selective cation currents. To deplete Ca\textsuperscript{2+}\textsubscript{i} stores, the sarcoplasmic reticulum Ca\textsuperscript{2+} -ATPase (SERCA) inhibitors thapsigargin and cyclopiazonic acid were added to a Ca\textsuperscript{2+}-free bathing solution. Under these conditions, addition of extracellular Ca\textsuperscript{2+} (3 mM) elicited increased tension that was inhibited by the cation channel blockers SKF-96365 (10 µM) and Lanthanum (100 µM) – suggestive of CCE. In a separate series of experiments on isolated SMCs, SERCA inhibition generated a gradual and sustained inward current. When combined with high-speed Ca\textsuperscript{2+} imaging techniques, the CCE-evoked rise of Ca\textsuperscript{2+}\textsubscript{i} was associated with inward currents carrying Ca\textsuperscript{2+} that were inhibited by SKF-96365. Regional specializations in Ca\textsuperscript{2+} influx and handling during CCE were observed. Distinct ‘hotspot’ regions of Ca\textsuperscript{2+} rise and plateau were evident in 70% of cells, a feature not previously recognized in smooth muscle. We propose that store-operated Ca\textsuperscript{2+} entry occurs in ‘hotspots’ contributing to localized Ca\textsuperscript{2+} elevations in human colonic smooth muscle.
INTRODUCTION

Regulation of smooth muscle function is critically dependent on Ca\(^{2+}\) (42). Excitatory neurotransmitters initiate contraction by interacting with cell surface receptors to generate inositol 1,4,5-trisphosphate (InsP\(_3\)), which binds to receptors on the sarcoplasmic reticulum to trigger Ca\(^{2+}\) release (29, 42). In turn, store depletion activates plasma membrane localized, Ca\(^{2+}\) permeable, store-operated channels (SOCs) in a process termed capacitative Ca\(^{2+}\) entry (CCE) that both replenishes stores and sustains cytoplasmic Ca\(^{2+}\) elevations to allow contraction (42).

In colonic smooth muscles, rhythmic variations of membrane potential, slow waves, underlie peristaltic contractions (22). Interstitial cells of Cajal initiate and propagate depolarizations and, through interaction with smooth muscle cells (SMCs), regulate contraction (22). Input from neurotransmitters to SMCs then modulates the amplitude and duration of slow wave activity (22). For example, in canine colonic SMCs, acetylcholine (ACh) and the tachykinin neurokinin A (NKA) activate an inward, non-selective cation (NSC) current (I\(_{NSC}\)) that depolarizes SMCs to cause Ca\(^{2+}\) influx and contraction (17). Receptor-independent activation of CCE with sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) blockers like thapsigargin (TG) or cyclopiazonic acid (CPA) results in a sustained, Ca\(^{2+}\) entry through I\(_{NSC}\) (10, 20, 39, 40). Multiple SOCs have been described, with unitary channel conductance ranging from 1.5-7 pS (5). Moreover, contractions can be evoked independent of L-type Ca\(^{2+}\) channel activation (25) suggesting that additional pathways contribute to the regulation of peristalsis.

Initial identification of CCE in smooth muscles was made in mouse anococcygeus (40). Evidence has since emerged for CCE and SOCs in a variety of cell types, including vascular (8), airway (2, 10), lower esophageal sphincter and esophageal body (39) smooth muscles. While whole-cell Ca\(^{2+}\) elevations have been well described in colonic SMCs both in response to
excitation (4, 29) and at rest in the form of Ca\(^{2+}\) puffs (3), the contribution of CCE and the mechanisms underlying Ca\(^{2+}\) signalling are still incompletely understood.

A developing view in smooth muscle physiology is that local events can contribute, or sum to, produce global Ca\(^{2+}\) changes (42). Indeed, the spatial and temporal integration of Ca\(^{2+}\) transients is a key issue since ultimately it is the pattern of global Ca\(^{2+}\) elevations that shapes contraction (28). Pioneering studies of neurons during the early 1990’s noted that L-type Ca\(^{2+}\) channels clustered in sub-cellular domains, giving rise to Ca\(^{2+}\) ‘hotspots’ (30). SERCA can be localized functionally to cellular regions where Ca\(^{2+}\) entry occurs (1) suggesting a possible relationship between CCE, agonist-evoked contraction and Ca\(^{2+}\) hotspots.

In the present study our objectives were to characterize CCE and Ca\(^{2+}\) dynamics in human colonic SMCs by recording contraction of intact tissues and responses of freshly isolated muscle cells. Using high-speed Ca\(^{2+}\) imaging techniques combined with patch-clamp electrophysiology, we correlate CCE with inward currents carrying Ca\(^{2+}\). In addition, we illustrate the temporal and spatial Ca\(^{2+}\) dynamics underlying CCE and cholinergic-evoked contractions. Store operated Ca\(^{2+}\) entry occurs in ‘hotspots’ and contributes to localized Ca\(^{2+}\) elevations; features not previously recognized in smooth muscle. Our studies provide insight into the mechanisms whereby CCE contributes to regulation of human colonic smooth muscle.
MATERIALS AND 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. Transverse, descending and sigmoid colon were obtained from patients undergoing colonic resection due to cancer, with specimens selected from disease-free margins. Samples of the entire thickness of bowel were removed, placed in oxygenated Krebs solution (composition given below in Solutions), kept at 4°C and transported to the laboratory. Circular muscle was carefully dissected based on morphology and orientation, cleaned of nerves, blood vessels, fat and fascia.

For isolation of smooth muscle cells (SMCs), segments of colon were cut into strips (~2 mm wide, 10 mm long) and placed in 2.5 ml of dissociation solution (see Solutions). Tissues were stored in dissociation solution overnight at 4°C. On the next day, tissues were warmed to room temperature for 20-60 minutes and placed in a gently shaking water bath at 31°C for 45-60 minutes, followed by dispersion of cells by trituration with fire-polished Pasteur pipettes. All freshly isolated SMCs were studied within 8 hours of dispersion. In total, muscle was obtained from 22 specimens (13 female, 9 male; mean age of 65 years)

Measurement of \([Ca^{2+}]_i\). Dissociated cells were loaded with fura-2-acetoxymethyl ester (fura-2 AM, 0.2 µM) or fluo-4-acetoxymethyl ester (fluo-4 AM, 5 µM) with 0.05% pluronic and incubated at room temperature for 40 minutes as previously described (15, 16). During incubation and throughout all experiments, care was taken to minimize light exposure to preserve fluorescence intensity. 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 (Nikon Eclipse TE2000-U, Tokyo, Japan) equipped with a water immersion lens (X60, NA 1.2). A Na+-HEPES bathing solution (see Solutions) was perfused (1-3 ml/min, room temperature) except where otherwise noted.

To estimate changes of Ca\(^{2+}\) concentration we used the ratiometric dye fura-2. The ratio of fluorescence emission at 510 nm with alternate excitation wavelengths of 345 nm and 380 nm was measured using a Photon Technology International (PTI) Deltascan system (PTI Inc., Birmingham, NJ), as previously described (15, 16). \([\text{Ca}^{2+}]_i\) was calibrated according to the methods of Grynkiewicz and coworkers (9), with \([\text{Ca}^{2+}]_i = (K_d(R-R_{\text{min}})/(R_{\text{max}}-R)) \frac{Sf_2}{Sb_2}\), where \(R_{\text{min}}\) and \(R_{\text{max}}\) are the ratio of fluorescence intensity at 345/380, with \(\text{Ca}^{2+}\)-free and saturated conditions, respectively. \(\frac{Sf_2}{Sb_2}\) is the ratio of fluorescence values for \(\text{Ca}^{2+}\)-free/\(\text{Ca}^{2+}\)-bound indicator measured at 380 nm. We used a dissociation constant of 225 nM for binding of \(\text{Ca}^{2+}\) to fura-2 (9) and a viscosity factor of 0.8. Data were corrected for background fluorescence. The calculation of \([\text{Ca}^{2+}]_i\) involves a number of assumptions and factors such as homogeneity of \(\text{Ca}^{2+}\) within cells that may introduce uncertainty in values.

To complement the fura-2 studies we used the single wavelength dye fluo-4, which is more appropriate for high speed imaging. Cells loaded with fluo-4 were illuminated with 488 nm of light from a multi-line argon ion laser and emissions were detected at 510 nm. Images were acquired at 40-65 Hz using a wide-field digital fluorescence imaging system (Photon Technology International Inc., Birmingham, NJ) with a Cascade Photometrics 650 cooled charge-coupled device (CCD) camera (653 x 492 pixels; Roper Scientific Inc., Tucson, Arizona) and ImageMaster 5 Software (PTI Inc.). With the X60 lens, each pixel represented an area of 196 x 196 nm. The spatial resolution, assessed as the 10-90% edge response, was 0.5 \(\mu\)m. Cells selected for study were solitary, initially relaxed and phase bright. Exposure to the excitation
laser was controlled by an electronic shutter that was closed during recovery from agonists. Results are reported as a change in fluorescence (ΔF/ΔF₀ (%) ) which is a relative measure of the free intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ). Image processing was performed off-line with all Ca²⁺ images shown presented as baseline subtracted images. This was achieved, pixel by pixel, using the equation ΔF/ΔF₀ (%) = [F - F₀]/F₀ x 100 where ‘F’ represents the fluorescence at each point in an experimental time course and ‘F₀’ the baseline level (as determined using the average of 20 consecutive images preceding each experimental treatment). Representative images are shown in pseudo-color, although saturation of the images limits the dynamic range compared to accompanying data traces.

**Electrophysiological recordings.** Dispersed cells were allowed to settle and adhere to the bottom of a perfusion chamber and perfused with a Na⁺-HEPES bathing solution (see Solutions) at 1-3 ml/min. 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 9 software.

To resolve colonic SMC currents, initial experiments were performed in a potassium chloride (KCl) electrode solution, and where indicated, a cesium chloride (CsCl) electrode solution was used to block K⁺ currents and characterize inward currents. Glutamate was substituted for Cl⁻ to give the Cs⁺-glutamate electrode solution (see Solutions for composition). Pipette resistance before seal formation ranged from 1-9 MΩ. Whole cell recordings were initiated when access resistance stabilized at <40 MΩ to allow series resistance compensation of
up to 80% to be used. Capacitive currents were compensated on-line using amplifier circuitry and linear leakage corrected off-line as assessed at negative potentials.

**Tissue bath studies.** Colonic circular muscle strips (~2 mm wide, 10 mm long) were dissected and mounted individually in tissue baths containing 10 ml Krebs solution continuously bubbled with 5% CO₂-95% O₂ at 37°C. Using 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). Output from the transducer was digitized (Metrabyte, Taunton, MA) and sampled at 2.5 Hz (Labtech Notebook, Laboratory Technologies, Wilmington, MA). After a 1 hour equilibration period, the length of each strip was adjusted to produce maximal tension upon application of 10 μM ACh. For quantification, tension responses were expressed as a percentage of the largest ACh-evoked response obtained prior to the commencement of the experiment.

**Solutions.** The Krebs solution used for retrieval of tissues and contraction studies consisted of (in mM): 116 NaCl, 5 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 2.2 NaH₂PO₄, 25 NaHCO₃ and 10 D-glucose, equilibrated with 5% CO₂-95% O₂ (pH 7.4). The 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 D-glucose (adjusted to pH 7.4 with NaOH). The dissociation solution contained a 135 mM K⁺ solution (in which the NaCl of the HEPES bathing solution was replaced with KCl) plus the following: 0.4 mg/ml collagenase (Sigma blend type F), 1.8 mg/ml bovine serum albumin, 1 mg/ml papain, and 0.125 mg/ml 1, 4-dithio-L-threitol. Ca²⁺-free solutions had the same composition as above with the omission of CaCl₂ and the addition of 0.5 mM EGTA.
For patch clamp recording, the KCl recording electrode solution contained (in mM) 140 KCl, 20 HEPES, 1 MgCl\(_2\), and 0.1 EGTA (adjusted to pH 7.2 with KOH). CsCl recording electrode solution contained (in mM): 130 CsCl, 20 HEPES, 1 MgCl\(_2\), 10 TEACl, 0.4 CaCl\(_2\), and 1 EGTA (adjusted to pH 7.2 with CsOH). Cs-glutamate electrode solution contained (in mM): 40 CsCl, 100 glutamate, 20 HEPES, 1 MgCl\(_2\), 10 TEACl, 0.4 CaCl\(_2\), and 0.01 EGTA (adjusted to pH 7.2 with CsOH).

**Chemicals.** Fura 2-AM, fluo-4 AM, pluronic and thapsigargin were obtained from Molecular Probes (Eugene, OR, USA). Caffeine and cyclopiazonic acid were from RBI Research Biochemicals International (Natick, MA, USA). SKF-96365 was from Tocris Cookson (Bristol, UK) and iberiotoxin was obtained from Bachem (King of Prussia, Pennsylvania, USA). All other agents were obtained from Sigma (St. Louis, MO, USA). Test substances were prepared from stock solutions in distilled water or dimethyl sulfoxide (DMSO), diluted into the appropriate bathing solution and applied either by bath perfusion or pressure ejection from glass micropipettes (Picospritzer II, General Valve Corp., Fairfield, NJ). Pipettes were positioned 25-75 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 the means ± SEM with sample sizes (n) indicating the number of cells or muscle strips studied. All traces shown are representative of at least 3 experiments on muscle or cells from 2 or more colon specimens. Comparisons were made using the Student’s paired \(t\)-tests with \(P < 0.05\) considered significant.
RESULTS

Ca\(^{2+}\)_i stores and CCE in human colonic smooth muscle.

We first established the importance of Ca\(^{2+}\)_i stores in human colon by analyzing contraction of smooth muscle strips. ACh evoked a reproducible increase in tension (Fig. 1A, C) verifying the response predicted from an earlier study (29). Following removal of Ca\(^{2+}\) from the bathing solution, a similar transient increase in tension could be elicited. However, with successive applications of ACh in Ca\(^{2+}\)-free solution, contractions gradually diminished until absent -- consistent with the depletion of Ca\(^{2+}\)_i stores (Fig. 1A). Recovery occurred following re-addition of extracellular Ca\(^{2+}\) (Fig. 1A, at right), confirming responses were not due to run-down, desensitization or tissue death.

Distinct Ca\(^{2+}\)_i stores activated by ryanodine and/or ryanodine and InsP\(_3\) are reported in some muscles (7). Accordingly, we applied ACh (10 \(\mu\)M, InsP\(_3\) receptors) together with caffeine (5 mM, ryanodine receptors) to fully deplete Ca\(^{2+}\)_i stores. In a series of paired experiments, the contractions evoked by ACh plus caffeine (n=12, 0.1 ± 0.01 N) were found to be similar to those observed with ACh alone (n=12, 0.09 ± 0.009 N; Fig. 1B, 1C). After Ca\(^{2+}\) was eliminated from the bathing solution, minimal responses were observed to control applications of extracellular Ca\(^{2+}\) (Ca\(^{2+}\)_o, 3 mM) when Ca\(^{2+}\)_i stores were not yet depleted (based on Fig. 1A). Throughout these studies, 3 mM Ca\(^{2+}\)_o was used in the presence of 0.5 mM EGTA to yield an effective free Ca\(^{2+}\) concentration of 2.5 mM -- a value similar to that seen under physiological conditions. Subsequent store depletion with the SERCA inhibitor CPA (10 \(\mu\)M) resulted in emptying of stores, as evidenced by the lack of response to subsequent stimulation with ACh/Caffeine (Fig. 1B). A role for CCE was noted when, in the presence of CPA, addition of Ca\(^{2+}\)_o evoked contraction (Fig. 1B). The contractions produced by Ca\(^{2+}\)_o in the presence of CPA were
reproducible and significantly greater than control applications of Ca\(^{2+}\) alone (p<0.05, Fig. 1D). These findings were suggestive of CCE in human colonic smooth muscles and prompted us to investigate the underlying signalling mechanisms.

**Cholinergic excitation of colonic SMCs and rise of Ca\(^{2+}\).**

To confirm the viability of our freshly isolated SMCs and to validate the results seen in our tissue strip experiments on a cellular level, we studied Ca\(^{2+}\) responses to cholinergic agonist. Initially, cells ranged in length from 75 \(\mu\)m to 250 \(\mu\)m and appeared spindle shaped with a bright periphery (Fig. 2A). Rapid and reproducible contraction of SMCs occurred following ACh stimulation. SMCs returned to ~90% of their resting length following a 10 minute washout.

Cells were loaded with the Ca\(^{2+}\) -sensitive dye fluo-4 to monitor regional change of Ca\(^{2+}\). To capture a representative trace of the Ca\(^{2+}\) responses, an area of interest was selected (Fig. 2A). (This area was chosen since it maintained position during contraction.) From a low basal Ca\(^{2+}\) level there was a rapid increase upon stimulation with ACh followed by a gradual return to baseline levels following washout (Fig 2A, 2B, n=33) in agreement with previous studies in colonic smooth muscle (4, 29, 35) and our studies of human esophageal muscle (16).

Regional changes in Ca\(^{2+}\) were examined (Fig. 2C) in chosen areas of interest (9 x 9 pixels). Initial onset of Ca\(^{2+}\) rise occurred in regions a and c (Fig. 2C) prior to the global Ca\(^{2+}\) elevations described above (Fig. 2A, 2B). The spatial dynamics suggested certain regions of the cell exhibited a more rapid onset of the rise of Ca\(^{2+}\) (e.g. a and c compared to b). In these studies the bathing solution contained Ca\(^{2+}\), so the ACh-evoked hotspots could have originated from store release or influx. The CCE experiments discussed below were carried out in Ca\(^{2+}\)-free conditions and with stores depleted to allow us to explore the different sources of Ca\(^{2+}\).
Electrophysiological characteristics of colonic SMCs.

We examined agonist-activated ion channels in human colonic SMC contraction using the nystatin perforated patch configuration. When currents were recorded with KCl in the electrode solution, outwardly rectifying K⁺ currents were evident, and were blocked with tetraethylammonium (5 mM, n=4) and iberiotoxin (100 nM, n=3, data not shown), supporting the existence of KCa channels, as we have previously characterized in human esophageal muscle (11). In human colonic SMCs, ACh enhanced K⁺ mediated outward currents, providing a further functional role for Ca²⁺ in colonic SMCs (data not shown).

While recording ionic currents we observed ACh-evoked I_{NSC} (Fig. 3). With cells held at -60 mV, ACh (10 µM) activated a transient inward current (Fig. 3A) similar to that seen in human esophageal SMCs (15). Responses were reproducible and recovered following washout. Voltage ramp commands (-100 to 50 mV) were periodically applied to evaluate the current-voltage relationship and reversal potential of ACh-evoked current. With Cs⁺ in the electrode solution to block K⁺ currents, reversal potentials were -6 ± 5 mV (n=5). When chloride (Cl⁻) was replaced with glutamate, shifting the Cl⁻ equilibrium potential from 0 to -30 mV, the reversal potential was unaltered (-1 ± 2 mV, n=7) indicating a negligible contribution for Cl⁻.

Tachykinins, present in neurons of the gastrointestinal tract, induce contraction of human colonic smooth muscle strips (24) as well as contraction and I_{NSC} in human esophageal smooth muscle (15). We examined the effects of NKA on colonic SMCs. An I_{NSC} similar to that evoked by ACh was observed and found to reverse at -2.9 ± 9.5 mV (n=3) with Cs⁺ in the electrode solution. In addition, we looked for evidence of purinergic regulation in human colon. The purinergic receptor agonist (BzATP, 300 µM) evoked a I_{NSC} with reversal potentials of -8.6 ± 6.0
mV (CsCl electrode solution, n=4) and -5.5 ± 5.3 mV (CsGlutamate electrode solution, n=5) (data not shown). These findings are consistent with the activation of an $I_{NSC}$ in response to muscarinic, tachykinin and purinergic agonists in human colonic SMCs. However, we did not study interactions between the agonists to explore potential synergy.

**Depletion of Ca$^{2+}$i stores activates CCE.**

We developed a protocol to reproducibly elicit CCE in isolated SMCs while measuring global changes in Ca$^{2+}$i concentration with fura-2. ACh (10 µM) evoked a transient rise in $[Ca^{2+}]_i$, presumably through InsP$_3$-sensitive Ca$^{2+}$ stores (Fig. 4A). Perfusion of a Ca$^{2+}$-free bathing solution resulted in diminished responses consistent with a gradual, but not complete, depletion of intracellular stores. Re-addition of Ca$^{2+}$ into the bathing solution restored ACh-evoked transients (Fig. 4A). These data support a role for both the release of Ca$^{2+}$ from intracellular stores as well as influx - results similar to those seen in esophageal SMCs (15, 16).

Next, using a protocol similar to that in the tissue strip experiments (Fig. 1), we concurrently applied ACh (10 µM) and caffeine (5 mM) to deplete both InsP$_3$- and ryanodine-sensitive stores (7). A transient $[Ca^{2+}]_i$ rise similar to that seen with ACh alone (Fig. 4B) was observed. Repetitive applications of ACh and caffeine in a Ca$^{2+}$-free bathing solution resulted in abolition of Ca$^{2+}$i elevations, suggesting complete depletion of intracellular stores (Fig. 4B). Once stores were emptied, extracellular Ca$^{2+}$ (Ca$_{2o}$, 3 mM) was applied focally from a puffer pipette. A small but consistent increase in $[Ca^{2+}]_i$ was observed, reflecting CCE, in response to prior applications of ACh (n=4) or ACh plus caffeine (n=7, Fig. 4C). No marked decline in the magnitude of responses was observed in control experiments involving repetitive applications of ACh or ACh plus caffeine in a 2.5 mM Ca$^{2+}$-bathing solution (3 cells), as we have shown earlier.
for human esophageal SMCs (32). Depletion of intracellular stores occurred at a faster rate in isolated SMCs (Fig. 4B) when compared to whole tissues (Fig. 1B), perhaps reflecting the minimal diffusion barriers in isolated cells.

Store depletion can also be induced through inhibition of SERCA pumps (2, 8, 10). To refine our CCE-induction protocol, we used SERCA inhibitors. Application of one such inhibitor, thapsigargin (TG, 2 µM) in a Ca$^{2+}$-free bathing solution induced a slow rise of [Ca$^{2+}$]$_i$, indicating rapid store depletion (n=5, Fig. 4D). Subsequent cholinergic stimulation failed to evoke contraction (data not shown), confirming store depletion occurred under these conditions.

**SERCA blockade induces CCE.**

We next used SERCA inhibition to deplete Ca$^{2+}$$_i$ stores and study changes of Ca$^{2+}$$_i$ in single SMCs. Cells were loaded with fluo-4 and bathed in an extracellular medium containing 0 Ca$^{2+}$, 0.5 mM EGTA and the reversible SERCA inhibitor CPA (10 µM). Fluorescence intensity was recorded from a stationary region of interest (Fig. 5A), chosen to minimize changes arising from SMC contraction. Basal Ca$^{2+}$$_i$ levels were initially low due to the Ca$^{2+}$-free solution and SERCA inhibition (Fig. 5A, 4D). Following application of 3 mM Ca$^{2+}$$_o$, a prompt increase in Ca$^{2+}$$_i$ was observed (Fig. 5). After a 5 minute washout, Ca$^{2+}$$_i$ levels returned to baseline whereupon a second application of Ca$^{2+}$$_o$ resulted in a reproducible Ca$^{2+}$$_i$ elevation (Fig. 5A, B). Application of Ca$^{2+}$$_o$ for longer durations yielded further rise of Ca$^{2+}$ (Fig. 5C) suggesting that CCE did not desensitize. A separate observation was that after the second application of Ca$^{2+}$$_o$, two distinct regions of Ca$^{2+}$$_i$ rise were observed (Fig. 5A, panel iv) – a result discussed below.

The nature of the Ca$^{2+}$ influx was explored by treating cells with the cation channel blocker SKF-96365, which inhibits CCE in other cell types (19) including smooth muscle (2).
3 minute perfusion of SKF-96365 had minimal effects on basal Ca\(^{2+}\) levels but significantly reduced CCE evoked by a 15 s application of Ca\(^{2+}\)\(_o\) (Fig. 5).

Consistent with the activation of CCE in other cell types (10), electrophysiological recordings revealed that SERCA-inhibition induced a gradual and sustained inward current (Fig. 6A, n=8). In separate recordings, voltage ramp protocols (-100 to 50 mV) run during TG application revealed a current with reversal potential at -10 ± 1 mV (n=4, Fig. 6B). The reversal potential of this current was suggestive of an NSC current, contrasting with the L-type Ca\(^{2+}\) current previously described in colonic (43) and human esophageal SMCs (16).

**Simultaneous patch-clamp recording and Ca\(^{2+}\)\(_i\) imaging during CCE.**

To explore the relationship between Ca\(^{2+}\) influx and ionic current, we used combined patch-clamp electrophysiology and Ca\(^{2+}\) fluorescence. Cells were loaded with fluo-4 and immersed in the Ca\(^{2+}\)-free bathing solution designed to evoke CCE (0 Ca\(^{2+}\), 0.5 mM EGTA, 10 µM CPA). Cellular [Ca\(^{2+}\)]\(_i\) was initially very low and uniform across the cell. Following initiation of whole-cell recording, addition of Ca\(^{2+}\)\(_o\) resulted in a prompt rise of Ca\(^{2+}\)\(_i\), indicative of CCE (Fig. 7). The rise of Ca\(^{2+}\)\(_i\) was associated with the development of a net outward current (n=9) which likely represented reduction of the inward current carried by Na\(^+\), a feature previously described in Ca\(^{2+}\) permeable channels (26). Both Ca\(^{2+}\)\(_i\) and current levels returned to baseline following washout of the Ca\(^{2+}\)\(_o\) (Fig. 7B). In separate cells, the current-voltage relationships were characterized by voltage ramp protocols (-100 to 50 mV) (Fig. 7C). The current modulated by addition of Ca\(^{2+}\) reversed direction at -17 ± 2 mV (n=3) and was apparent as a reduction of current, hence the negative slope. Taken together, these observations suggest that CCE occurs in SMCs through NSC currents activated by store depletion.
CCE and cholinergic excitation results in localized $\text{Ca}^{2+}$ elevations at ‘hotspots’.

During our initial fluorescence recordings of CCE, spatial variations in $\text{Ca}^{2+}$ were apparent (Fig. 5A, panel $iv$). As a result, we hypothesized that regional specialization in $\text{Ca}^{2+}$ influx could occur with CCE. While early studies have hinted at this phenomenon (23, 41), little is reported in SMCs due to limited availability of rapid $\text{Ca}^{2+}$ imaging systems. Following SERCA inhibition (described above), CCE was activated with $\text{Ca}^{2+}$ resulting in $\text{Ca}^{2+}$ elevations (Fig. 8A). Prolonged $\text{Ca}^{2+}$ (60 s) resulted in contraction (Fig. 8A) similar to results observed in intact muscle strips (Fig. 1). SMCs returned to resting lengths following a 10 minute washout.

With high-speed fluorescence imaging, distinct regions of $\text{Ca}^{2+}$ rise were evident (Fig. 8). The rapid rise and onset of $\text{Ca}^{2+}$ influx in Fig. 8B (region $iv$) is suggestive of a $\text{Ca}^{2+}$ influx hotspot. The delayed and more subdued rise in regions $i$ and $ii$ might reflect diffusion of $\text{Ca}^{2+}$. Onset of the $\text{Ca}^{2+}$ rise occurred at different rates in the varying regions (Fig. 8B). Hotspots seen in response to induction of CCE occurred in 26 of 37 cells observed (70%). In the other instances, no distinct hotspots were observed and $\text{Ca}^{2+}$ rose uniformly (11 of 37 cells, 30%). No reliable pattern of hotspot distribution was noted between different cells. Within individual cells, specific regions of $\text{Ca}^{2+}$ entry were consistently visualized upon repeated applications of extracellular $\text{Ca}^{2+}$. Based on these observations, we propose that CCE-evoked $\text{Ca}^{2+}$ entry originates at distinct ‘$\text{Ca}^{2+}$ hotspots’ within human smooth muscle cells.

Regional specialization of $\text{Ca}^{2+}$ was also apparent in other conditions. Our earlier data revealed both global changes in response to ACh (Fig. 2) and changes in discrete areas of interest (Fig. 2C). Indeed, when distinct regions were examined, it was clear that $\text{Ca}^{2+}$ rise occurred initially in regions $a$ and $c$ with region $b$ exhibiting a delay in onset but higher overall $\Delta F/F_0$ (Fig.
2C) – demonstrating regional specialization in \( \text{Ca}^{2+} \) handling following both CCE and cholinergic excitation.

**CCE blockers reduce contraction in colonic smooth muscle strips.**

We returned to tissue strip experiments to extend and explore the findings from isolated SMCs. Addition of \( \text{Ca}^{2+} \) to tissues depleted of \( \text{Ca}^{2+} \) resulted in a rise of tension that was inhibited by addition of the NSC channel blockers SKF-96365 (Fig. 9A, n=30) and Lanthanum (La, 100 µM, Fig. 9B, n=12). The blockade was significant (Fig. 9C), and supported the notion that CCE occurred in human colonic smooth muscle.

**DISCUSSION**

Our objective was to characterize CCE and \( \text{Ca}^{2+} \) dynamics in human colonic SMCs using several complimentary approaches. Patch-clamp recordings of freshly isolated cells identified ACh-activated non-selective cation currents and SOCs activated by SERCA inhibition. Under conditions where SERCA \( \text{Ca}^{2+} \) pumps were inhibited, CCE contributed to contraction of colonic muscle. High-speed \( \text{Ca}^{2+} \) imaging techniques combined with patch-clamp electrophysiology allowed us to correlate CCE with inward currents carrying \( \text{Ca}^{2+} \). Analysis of the temporal and spatial \( \text{Ca}^{2+} \) dynamics underlying CCE revealed that store operated \( \text{Ca}^{2+} \) entry occurred in localized \( \text{Ca}^{2+} \) ‘hotspots’, a feature not previously recognized in smooth muscle. When taken together, our studies establish the existence of CCE in human colonic smooth muscle.
ACh, the primary excitatory neurotransmitter in the gastrointestinal tract, is critical to colonic smooth muscle contraction in a wide variety of species (24, 29, 35). The present study confirms the ability of ACh to contract human colonic smooth muscle and further demonstrates that removal of extracellular Ca\(^{2+}\) results in depletion of intracellular stores and diminished contraction. Furthermore, we provide evidence that CCE can mediate Ca\(^{2+}\) entry that can contribute to Ca\(^{2+}\) homeostasis. Indeed, CCE contributes to contraction of ileum (25) and gall bladder (20) smooth muscle strips. Given that renal, mesenteric and coronary artery smooth muscles are reported to be functionally unaffected by CCE (33), it was important to characterize the effect in human muscles. Indeed, NSC channel inhibitors also exhibit species specificity. For example, in organ bath studies on mouse anococcygeus, the cation channel blocker SKF-96365 inhibited CCE while another blocker, lanthanum, exhibited no effect (40). In contrast, CCE-evoked in gall bladder smooth muscle was not inhibited by SKF-96365 (20). We report here that both SKF-96365 and Lanthanum inhibit CCE in human colon smooth muscle.

Slow rhythmic variations in membrane potential underlie peristaltic contractions in colonic smooth muscle (22). In addition to Ca\(^{2+}\), regulation is dependent upon various ionic currents. In agreement with our findings, previous studies on canine colonic SMCs identified \(K_{Ca}\) channels (6) inhibited by extracellular TEA (36). In canine SMCs, cholinergic excitation resulted in inhibition of \(K_{Ca}\) activity with subsequent depolarization, voltage-dependent Ca\(^{2+}\) channel activation and contraction (6). This is in contrast to our studies on human colonic SMCs and previous work in tracheal SMCs (37) where \(K_{Ca}\) was acutely activated by ACh. Such activation of a voltage-dependent \(K_{Ca}\) current would limit depolarization and hasten repolarization of SMCs following contraction.
Excitatory cholinergic stimulation can depolarize SMCs via activation of $I_{\text{NSC}}$, as shown in the present studies as well as, among others, jejunum, gastric corpus (31), tracheal (13), and human esophageal (15) SMCs. The nonadrenergic, noncholinergic neurotransmitter NKA contracts human colonic smooth muscle strips (24) and, in agreement with our studies, activates an $I_{\text{NSC}}$ in canine SMCs that demonstrates a reversal potential of $\sim 0 \text{ mV}$ (18). Given these findings, further studies into the signaling underlying excitation by tachykinins are warranted.

The membrane currents underlying CCE-evoked SOCs in smooth muscle have not been widely recorded, due in part to their small size. Indeed, single channel conductance of 3 pS has been described in vascular SMCs with conductances of 1.5-7 pS emerging as a distinguishing feature of SOCs (5). SOCs most generally exhibit permeability for $\text{Ca}^{2+}$ as well as $\text{Na}^{+}$ (26). Accordingly, our finding that addition of extracellular $\text{Ca}^{2+}$ caused a reduction of inward current could reflect the blockade of $\text{Na}^{+}$ entry by $\text{Ca}^{2+}$, a phenomenon previously described in cation channels (26).

While SERCA inhibitors evoke CCE, the lack of effect on SOCs in excised plasma membrane patches (where intracellular stores are absent) suggests that they do so only indirectly (5). In airway SMCs, the SERCA inhibitor CPA activated a linear current with reversal potential of 0 mV resulting in $\text{Ca}^{2+}$ elevations (2, 10) similar to CCE-evoked SOCs identified in the present study. Given the $\text{Na}^{+}$ and $\text{Ca}^{2+}$ permeability of SOCs, indirect effects of a $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger cannot be discounted (5). However, in smooth muscles, the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger has been suggested to make only minor contributions to $\text{Ca}^{2+}$ homeostasis (14) and CCE-evoked $\text{Ca}^{2+}$ influx was insensitive to the selective $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger inhibitor KBR-7943 (27) suggesting a negligible role in CCE.
In agreement with our studies, whole-cell Ca\textsuperscript{2+} elevations in response to cholinergic excitation have been described in cultured human colonic SMCs (35) as well canine SMCs (4, 29). Localized Ca\textsuperscript{2+} puffs have been described in cells at rest (3) with widely dispersed, propagating Ca\textsuperscript{2+} waves identified in murine colonic SMCs (28). Spatially and functionally distinct Ca\textsuperscript{2+} stores have been identified in colonic SMCs (7): one with both ryanodine and InsP\textsubscript{3} receptors and one with only InsP\textsubscript{3} receptors (12). Depletion of the InsP\textsubscript{3}-sensitive store reduced, but did not abolish, responses to caffeine in colonic SMCs (7). In other studies, thapsigargin depleted Ca\textsuperscript{2+} stores that were functionally and structurally distinct from those depleted by caffeine (34). In our hands, depletion of stores with a combination of ACh and caffeine was more effective than ACh alone, although the magnitude of CCE was similar. Thus, while the existence of distinct Ca\textsuperscript{2+} stores in human colonic SMCs is suggested, this specialization does not appear to play a critical role in activation of CCE.

In pioneering studies, Williams et al. (41) provided an initial illustration of the spatial dynamics underlying Ca\textsuperscript{2+} signalling in gastric SMCs of *Bufo marinus*. Expanding on these observations, others described focal Ca\textsuperscript{2+} elevations that propagated throughout the cytoplasm as ‘Ca\textsuperscript{2+} waves’ (23) or existed in the resting SMCs as highly localized Ca\textsuperscript{2+} transients (‘sparks’) (4). Ca\textsuperscript{2+} sparks can modulate local cellular processes but do not appear to serve as the elementary events responsible for global Ca\textsuperscript{2+} elevation and contraction (28). However, localized Ca\textsuperscript{2+} events can contribute, or even sum, to produce contraction (42). Our results suggest that in both CCE and ACh-evoked colonic SMC responses, regional Ca\textsuperscript{2+} hotspots exist and may serve as a foundation for global Ca\textsuperscript{2+} elevations or store refilling.

Clustering of channels into the specific sub-cellular domains needed for localized responses has been described for Ca\textsuperscript{2+} (30) and transient receptor potential (TRP) channels (1,
21). TRP proteins are hypothesized to be components of the store-operated channels responsible for CCE (1) and are expressed in colonic smooth muscle (38). TRP channels have been localized within caveolae and signalling microdomains (1) suggesting a possible relationship between CCE, Ca\(^{2+}\) hotspots and TRP channels.

In summary, the present study reveals that CCE is present in human colonic SMCs and contributes to Ca\(^{2+}\) influx. We describe the temporal and spatial Ca\(^{2+}\)\(_i\) dynamics underlying CCE and cholinergic-evoked contractions and propose that store operated Ca\(^{2+}\) entry occurs in localized Ca\(^{2+}\) ‘hotspots’. Given that Ca\(^{2+}\) hotspots are target cell and contact type specific (1, 30) it is tempting to speculate that \textit{in vivo}, they could underlie the interactions between neighbouring SMCs or between SMCs and interstitial cells of Cajal to aid propagation of smooth muscle contraction.

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FIGURE LEGENDS

Figure 1: Role for intracellular Ca\textsuperscript{2+} stores and CCE in contraction of human colonic smooth muscle. A: Acetylcholine (ACh, 10 µM, applied at black arrowhead) evoked contraction of intact smooth muscle strips (left). On removal of Ca\textsuperscript{2+} from the bathing solution, ACh continued to evoke contractions however successive responses were greatly diminished – consistent with a role for Ca\textsuperscript{2+}i stores in excitation of the muscle. B: To initiate complete release of Ca\textsuperscript{2+} from stores, ACh and caffeine were applied simultaneously (10 µM and 5 mM respectively; open arrowhead, left) and evoked contractions similar to ACh alone (For summary see panel C). Addition of Ca\textsuperscript{2+}o to the bath (3 mM, duration indicated by arrows) prior to store depletion evoked no contraction. However, following treatment with CPA (10 µM), stores were readily emptied as indicated by the lack of response to ACh + Caffeine. Subsequent application of Ca\textsuperscript{2+}o (3 mM) in the presence of CPA evoked contraction, suggestive of CCE. ACh + Caffeine responses returned following washout (right) confirming tissue viability. C: Summary of responses illustrated above expressed as mean ± SEM. In a series of paired experiments, contractions evoked by ACh alone (n=12) were not different to those evoked by ACh plus caffeine (n=12). D: CCE was evaluated using this protocol and found to be reproducible, with responses shown as a percentage of control cholinergic-evoked rise of tension. Addition of Ca\textsuperscript{2+} alone caused only a minor rise in tension whereas addition of Ca\textsuperscript{2+} after pre-treatment with CPA caused significantly greater tension (* p<0.05, n=12).

Figure 2: ACh evokes global and regional elevations of Ca\textsuperscript{2+}i and contraction in colonic SMCs. A: A bright field image (left) illustrates a freshly isolated colonic SMC at rest. As visualized with the Ca\textsuperscript{2+} dye fluo-4, low basal Ca\textsuperscript{2+}i levels (i) increased on stimulation with ACh (ii, iii) (10
µM, 5 s). The transient rise of Ca\(^{2+}\)\(_i\) was accompanied by contraction (iii). Recovery to basal Ca\(^{2+}\)\(_i\) levels was apparent following 5 minute washout (iv). These images are representative of responses recorded in 12 cells. B: The time course for the Ca\(^{2+}\)\(_i\) rise depicted in A. Values were obtained from an ‘area of interest’ represented by the large black box in A. ACh, applied by pressure ejection from a pipette resulted in a rapid rise of Ca\(^{2+}\)\(_i\) that gradually returned to baseline levels following washout - consistent with a role for Ca\(^{2+}\)\(_i\) in the regulation of SMC contraction. Superimposed numerals (i-iv) represent the time points where images in A were obtained. To account for baseline regional variations, in these and future traces, fluorescence (F) values were expressed as ΔF/ΔF\(_o\)(%) = ((F-F_\_o)/ F_\_o)x100. Baseline fluorescence values (F_\_o) were obtained prior to agonist application as an average of 20 frames. Fluorescence images were collected at 30 to 50 frames per second. C: To evaluate regional changes in Ca\(^{2+}\), areas of interest (9 x 9 pixels) were selected (a, b, c) represented by the small colored boxes superimposed on the image in A. Initial onset of Ca\(^{2+}\)\(_i\) rise occurred in regions a and c and after 5 seconds a global Ca\(^{2+}\)\(_i\) elevation was observed (see above). Spatial dynamics of regional Ca\(^{2+}\) hotspots are illustrated with regions a and c exhibiting a more rapid Ca\(^{2+}\)\(_i\) onset compared to region b. Region b achieved a higher overall ΔF/ΔF\(_o\) in spite of the gradual onset. Cells were bathed in 2.5 mM bathing solutions and Ca\(^{2+}\)\(_i\) elevations were likely a combination of Ca\(^{2+}\) influx and release from stores.

**Figure 3:** Agonist activated NSC currents (I\(_{NSC}\)) in human colonic SMCs. A: Representative trace from a SMC held at -60 mV using the perforated patch configuration. With Cs\(^+\) in the electrode solution to block K\(^+\) currents, ACh (10 µM, applied for duration of bar) activated a transient inward current. Voltage ramp commands (-100 to 50 mV) were applied periodically to evaluate the current-voltage relationship of the ACh-evoked current. Chloride (Cl\(^-\)) was replaced
with glutamate (shifting the Cl⁻ equilibrium potential from 0 to -30 mV). Subtraction of the control current (Cont) from the agonist-evoked current revealed the activated current (ΔI, right), which reversed at -1 ± 2 mV (n=7), consistent with activation of INSC. B: A similar current, with reversal potential of -3 ± 10 mV (n=3) was evoked by the tachykinin agonist Neurokinin A (NKA, 1 µM).

**Figure 4:** Depletion of Ca²⁺ stores activates CCE in colonic SMCs. A: The Ca²⁺ dye fura-2 was used to measure global [Ca²⁺]ᵢ. ACh (10 µM, applied for 10 s at black arrowhead) evoked a transient rise in [Ca²⁺]ᵢ, presumably through InsP₃-sensitive Ca²⁺ stores. Perfusion of a Ca²⁺-free bathing solution resulted in diminished responses consistent with a gradual, but not complete, depletion of intracellular stores. Reperfusion with a Ca²⁺-containing solution resulted in partial recovery of the ACh-evoked transients (right). B: Concurrent administration of ACh (10 µM) and caffeine (Caff; 5 mM), to deplete both InsP₃- and ryanodine-sensitive stores, produced a transient rise in [Ca²⁺]ᵢ similar to that seen with ACh alone. Repetitive applications of ACh and Caff in a Ca²⁺-free bathing solution resulted in abolition of Ca²⁺ᵢ elevations, suggesting complete depletion of intracellular stores. Once stores were emptied, 3 mM extracellular Ca²⁺ was applied (Ca²⁺ₒ; indicated by the bar above the trace). A small but consistent increase in [Ca²⁺]ᵢ was observed – suggestive of CCE. C: Summary of experiments in A and B. Store depletion with ACh alone (n=4 cells, Ca²⁺ₒ applied following lowest [Ca²⁺]ᵢ peak evoked by agonists), or in combination with caffeine (n=7 cells), was effective in depleting the stores sufficiently to allow CCE to be visualized. Values represent average peak response to Ca²⁺ₒ obtained following intracellular store depletion through the respective protocols, shown as a percentage change from basal levels. No significant difference in the magnitude of evoked CCE was noted. D: In the
presence of a Ca$^{2+}$-free bathing solution, use of SERCA inhibiting agents (thapsigargin, TG; 2 
µM, 60 s, n=5 cells) resulted in a gradual rise of Ca$^{2+}$ levels as stores were depleted.

**Figure 5:** SERCA blockade induces CCE in human colonic SMCs. A: A bright field image 
(left) illustrates a freshly isolated colonic SMC at rest. Cells were loaded with fluo-4 and bathed 
in an extracellular medium containing 0 Ca$^{2+}$, 0.5 mM EGTA and the SERCA blocker 
cyclopiazonic acid (CPA, 10 
µM) to deplete intracellular stores. Basal Ca$^{2+}$ levels (i) were 
initially low and increased promptly on application of 3 mM Ca$^{2+}$ (ii) reflecting CCE. After a 5 
minute washout, Ca$^{2+}$ levels returned to baseline (iii) whereupon a second application of Ca$^{2+}$ 
resulted in a reproducible Ca$^{2+}$ elevation (iv). 3 minute perfusion of the NSC channel blocker 
SKF-96365 (SKF) had no effect on basal Ca$^{2+}$ levels (v) but inhibited subsequent Ca$^{2+}$ increases 
evoked by addition of Ca$^{2+}$ (vi). B: Traces reveal fluorescence intensity obtained from a 9 x 9 
pixel region of interest (3.1 
µm$^2$), schematically illustrated by the black box in A. Superimposed 
numerals (i-vi) represent time points in which the panels in A were obtained. The change in 
fluorescence, ΔF/Fo (%) was a relative measure of free [Ca$^{2+}$]. C: Application of Ca$^{2+}$ for 
longer durations yielded reproducible, elevated levels of Ca$^{2+}$ fluorescence. D: Summary of the 
effects of NSC inhibition described in B. A 3 minute perfusion of SKF-96365 resulted in a 
significant blockade of CCE elicited by Ca$^{2+}$ applied for 15 s (*, p<0.05).

**Figure 6:** CCE-evoked inward currents in human colonic SMCs A: Application of the SERCA 
inhibitor thapsigargin (TG) induced a gradual and sustained inward current (2 
µM, applied for the 
time indicated by the bar, n=8 cells). B: In separate recordings, voltage ramp protocols (-100 to 
50 mV) were applied during SERCA inhibition to evaluate the current-voltage relationship of the
resulting current. Control (Cont) values were obtained prior to application and for each following minute. Traces shown are representative of currents obtained at 2 minutes with TG, revealing activation of a $I_{NSC}$ that reversed at -10 mV ($n=4$ cells).

**Figure 7:** Simultaneous patch-clamp and $Ca^{2+}_{i}$ imaging reveal $Ca^{2+}$ influx during CCE. **A:** Bright field image illustrates a human colonic SMC at rest following seal formation with a patch clamp pipette (left panel). Cells were immersed in a bathing solution optimized to evoke CCE (0 $Ca^{2+}$ solution containing 0.5 mM EGTA and 10 µM CPA). Addition of $Ca^{2+}_{o}$ (3 mM) resulted in a prompt rise of $Ca^{2+}_{i}$. **B:** Summary trace of an area of interest as depicted by the box shown above in panel i. Superimposed numerals correspond to time points in which panels i-iii were obtained. $Ca^{2+}_{o}$ was applied for the duration of the arrow. Concurrent patch-clamp recording reveals that the rise of $Ca^{2+}_{i}$ was closely associated with development of a net outward current that represents a reduction of the inward current carried by Na+. $Ca^{2+}_{i}$ and current levels returned to baseline following washout of the $Ca^{2+}_{o}$. **C:** In a separate cell, current-voltage relationships were characterized by voltage ramp protocols (-100 to 50 mV). The current modulated by addition of $Ca^{2+}$ reversed direction at -17 mV ($n=3$ cells) and was apparent as a reduction of current, hence the negative slope.

**Figure 8:** CCE yields localized $Ca^{2+}_{i}$ elevations at distinct ‘hotspots’ in human colonic SMCs. **A:** Bright field image (left) illustrating areas of interest (9 x 9 pixel, 3.1 µm²). Note that depicted boxes are enlarged for visibility. Intracellular stores were depleted and CCE was evoked using SERCA inhibition. Application of $Ca^{2+}_{o}$ (3 mM) resulted in focal $Ca^{2+}_{i}$ elevations (middle) and prolonged $Ca^{2+}_{o}$ (60 s) resulted in contraction (right) similar to results observed in
muscle strips. B: Time-course of the experiment shown in A with traces corresponding to individual areas of interest (i-iv). Regions exhibited distinct patterns of Ca\textsuperscript{2+} rise and plateaus. The rapid rise and onset of Ca\textsuperscript{2+} influx in region iv is suggestive of a Ca\textsuperscript{2+} influx hotspot, with delayed and more subdued rise in regions i and ii possibly reflecting diffusion of Ca\textsuperscript{2+}. Findings in B correspond to images in A. The boxed region (left) is shown on an expanded scale (right) to emphasize the distinct onset of regional changes. We propose that CCE-evoked Ca\textsuperscript{2+} entry occurs at ‘Ca\textsuperscript{2+} hotspot’ locations (ie. region iv) within cells. Hotspots seen in response to induction of CCE occurred in 26 of 37 cells observed (70%). In the other instances, no distinct hotspots were observed and Ca\textsuperscript{2+} rose globally (11 of 37 cells, 30%).

**Figure 9:** CCE-evoked contractions are inhibited by NSC channel blockade in human colonic muscle. A: Control applications of ACh (10 µM, black arrowhead) induced contraction of colonic muscle strips (left). Removal of Ca\textsuperscript{2+} from the bathing solution and addition of CPA caused store depletion. Under these conditions, CCE was evoked via addition of Ca\textsuperscript{2+} \textsubscript{o} (3 mM) that induced contraction. Treatment with the NSC channel blocker SKF-96365 (SKF, 10 µM) inhibited Ca\textsuperscript{2+} \textsubscript{o}-evoked contractions. ACh responses returned following washout of CPA and SKF along with the re-addition of Ca\textsuperscript{2+} to allow for store refilling (right). B: The NSC channel blocker Lanthanum (La, 100 µM) also reduced CCE-evoked contractions. C: Summary of experiments illustrated in A and B (mean ± SE) presented as a percentage of control responses. Perfusion of SKF (n=30 cells) and La (n=12 cells) resulted in significant inhibition (* p<0.05, ) of the CCE evoked tension increases.
Figure 1

A
2.5 mM Ca²⁺ → 0 Ca²⁺ + 0.5 mM EGTA → 2.5 mM Ca²⁺

Tension (0.1 N)

ACh

Time (min.)

B
2.5 mM Ca²⁺ → 0 Ca²⁺ + 0.5 mM EGTA → 2.5 mM Ca²⁺

Tension (0.1 N)

ACh + Caff

Time (min.)

C

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D

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Figure 3  Kovac

A

B

156x149mm (600 x 600 DPI)
Figure 8  Kovac

A  
Control  \[Ca^{2+}(3 \text{ s})\]  \[Ca^{2+}(60 \text{ s})\]  
\[\Delta F/\Delta F_0 (\%)\]

B  
\[\Delta F/\Delta F_0 (\%)\]

161x156mm (600 x 600 DPI)