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Heterogeneities in ICC Ca²⁺ activity within canine large intestine

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Abstract

Background & Aims—In human and canine colon, both slow (slow waves, 2–8/min) and fast (myenteric potential oscillations; MPOs, 16–20/min) electrical rhythms in the smooth muscle originate at the submucosal and myenteric borders, respectively. We used Ca²⁺ imaging to investigate whether ICC at these borders generated distinct rhythms.

Methods—Segments of canine colon were pinned submucosal or myenteric surface uppermost, or cut in cross-section. Tissues were loaded with a Ca²⁺ indicator (fluo-4) and activity was monitored at 36.5±0.5°C using a CCD camera.

Results—Rhythmic, biphasic Ca²⁺ transients (5–8/min), similar in waveform to electrical slow waves, propagated without decrement as a wave front through the ICC-SM network (2–5mm/s), decaying exponentially through the thickness of the CM. In contrast, rhythmic intracellular Ca²⁺ waves (~16/min) and spontaneous reductions in Ca²⁺ were observed in ICC-MY. Normally, intracellular Ca²⁺ waves were unsynchronized between adjacent ICC-MY, although excitatory nerve activity synchronized activity. In addition, spontaneous reductions in Ca²⁺ were observed that inhibited Ca²⁺ waves. L-NA (100µM; NO antagonist) blocked the reductions in Ca²⁺ and increased the frequency (~19/min) of intracellular Ca²⁺ waves within ICC-MY.

Conclusions—ICC-SM form a tightly coupled network that is able to generate and propagate slow waves. In contrast, Ca²⁺ transients in ICC-MY, which are normally not synchronized, have a similar duration and frequency as MPOs. Like MPOs, their activity is inhibited by nitrergic nerves and synchronized by excitatory nerves.

INTRODUCTION

Deficiencies in interstitial cells of Cajal (ICC) have been implicated in severe slow transit colonic motor disorders, including those observed in diabetes mellitus.^{1–3} Therefore, it is important to clearly understand the role of ICC in the large bowel.

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There are important differences in the organization of electrical pacemaker activity between the small and large intestine. In the small bowel, electrical slow waves are generated by pacemaker interstitial cells of Cajal at the myenteric border (ICC-MY) between the circular (CM) and longitudinal muscle (LM) layers.^{3–6} In addition, rhythmic electrical spiking activity originates in ICC in the inner CM, adjacent to the deep muscular plexus.⁴ Slow waves consist of an upstroke phase followed by a variable plateau phase. The active upstroke phase, which appears to result from opening of voltage dependent T-type Ca^{2+} channels, conducts rapidly through the ICC-MY network, where it synchronizes ICC-MY and initiates the plateau phase. The plateau phase involves Ca^{2+} induced Ca^{2+} release (CICR) from intracellular stores. As slow waves spread through the ICC-MY network they also conduct into the LM and CM where they depolarize smooth muscle cells, leading Ca^{2+} entry and phasic contractions.^{3–6}

Slow waves in the colon, in contrast to the small intestine, originate in ICC-SM lying on the submucosal surface of the CM (frequency: human, 2–4/min⁷; pig, 0.5–3.5/min⁸; dog, 5–7/min^{9–12}; cat, 4.7–5.8/min¹³). Slow waves actively propagate along the submucosal surface, conducting into the CM toward the myenteric border.^{9,10}

At the myenteric border of the LM and CM, higher frequency electrical depolarizations (frequency: human, 17.8/min⁷; pig, 43/min⁸; dog, 15–20/min^{9–12}; cat, 28–43/min¹³) occur, that likely originate in ICC-MY lying between the CM and LM.¹⁰ This electrical activity waxes and wanes in amplitude in a sinusoidal manner, and doesn't, like slow waves, exhibit a defined upstroke phase. It is often referred to as myenteric potential oscillations (MPOs), in order to distinguish this pacemaker activity from regular slow waves.^{9,10} Both MPOs and slow waves conduct into the CM, where they can summate to time and define the contractile behavior of colonic muscles.⁷

The activity of ICC networks in the colon hasn't been studied previously, so we used dynamic video imaging of a Ca^{2+} indicator to visualize the characteristics of intracellular Ca^{2+} transients in ICC-SM and ICC-MY networks in the canine proximal colon. Our results demonstrate that rhythmic Ca^{2+} transients occurred at the frequency of slow waves in the ICC-SM network, and propagated coherently across a well coupled network of ICC. In contrast, spontaneous Ca^{2+} transients in ICC-MY occurred at a similar frequency to MPOs, but their activity was highly unsynchronized within the network of ICC-MY. These observations demonstrate fundamental differences in the functional phenotype of the two populations of pacemaker ICC at either surface of the CM in the colon.

MATERIALS AND METHODS

Animals and tissue preparation

Mongrel dogs of either sex (0.5–2 years of age) were killed with an overdose of pentobarbital sodium (100mg/kg) administered into the femoral vein. The abdomen was opened and a segment of proximal colon (6–14cm from the ileocecal sphincter) was removed. The colon was opened along the mesenteric border and luminal contents were removed by washing with Krebs' solution (see below). The use and treatment of animals were reviewed and approved by the Institutional Animal Use and Care Committee at the

University of Nevada. Cross-sectional preparations (0.5–1.0mm thick, 10mm long): thin strips were cut parallel to the LM with a double-bladed scalpel.^{7,9,10} Flat-sheet preparations (15×15mm square; ~1mm thick): for visualization of the submucosal region, the mucosa was removed and the preparation pinned LM down. For visualization of the myenteric region, tissues were pinned submucosal side down and strips of LM were peeled away. All preparations were continuously perfused with oxygenated Krebs' solution at 36.5±0.5°C.

Visualization of Ca²⁺ transients

The methods for dye loading ICC have been published in detail elsewhere.^{5,6} In brief, after equilibration, tissues were incubated with fluo-4 (~10μM; Molecular Probes, Eugene, OR) dissolved in a Krebs' solution with 0.8% dimethyl sulfoxide and 0.2% Cremophor EL for 20 min at 25°C. Following incubation, tissues were re-perfused with Krebs' solution and then illuminated by a 100-W high-pressure mercury burner and viewed with a BX50WI upright microscope fitted with epi-fluorescence using water immersion objectives [×4: numerical aperture (NA) 0.1, diffraction depth of field (DOF) 40.4μm; ×10: NA 0.3, DOF 7.5μm; ×20: NA 0.5, DOF 2.6μm; ×40: NA 0.8, DOF 0.9μm] (Olympus UMPlanF; Olympus, Melville, NY). Appropriate filters produced excitation of fluo-4 between 460–490nm, and passed emissions >515nm. Image sequences (500–1800 frames) were captured (15.6 frames/s) using a Cascade 512B camera (Roper Scientific Inc., Trenton, NJ) and MetaMorph 6.26 software (Molecular Devices Corp, Downingtown, PA). For pharmacological experiments, control Ca²⁺ activity was recorded for 1 min, a particular drug solution was perfused for 10 min during a period of non-illumination, and then Ca²⁺ activity was recorded again for 1 min.

Simultaneous recording of electrical activity and Ca²⁺ fluorescence in ICC-SM

Microelectrode recordings were made from the CM at the submucosal surface of a flat-sheet preparation while simultaneously recording the Ca²⁺-fluorescence in ICC-SM. These methods have been published in detail elsewhere.^{5,6}

Image analysis

Image sequences were analyzed using custom written software (Volumetry G6a, G.W.H.).^{5,6} Ca²⁺ transients in individual cells were extracted and the amplitude, duration (10% of peak amplitude), and frequency were measured. All frames showing spread of Ca²⁺ activity were subtracted from the average background intensity. Ca²⁺ activity within the whole field of view (FOV) or in the region of interest (ROI) was visualized using spatiotemporal maps (ST-maps). ST-maps were generated by stacking the intensity lines of summed pixels orthogonal to the direction of propagation in the ROI for each frame.

Immunohistochemical identification of ICC

Colonic tissues were fixed in paraformaldehyde and labeled with kit as previously described.¹⁴ Briefly, cryostat sections (10–100μm thick, cut transverse or parallel to the CM layer) were incubated in anti-human SCF R/c-kit antibody (R&D Systems, Minneapolis, MN; 5μg/ml) for 24 hours at 4°C. Sections were washed in PBS and incubated in Alexa Fluor 488 coupled donkey anti-goat (1:1000, for 1 hour at room temperature). Sections were

mounted in Aquamount (VWR, West Chester, PA) and examined with a LSM 510 Meta confocal microscope (Zeiss, Thornwood, NY) using excitation wavelengths appropriate for Alexa Fluor 488. Z-series scans were taken using $\times 20$ – $\times 63$ objectives @ 1 μm z-steps. Images were constructed using Zeiss LSM-5 Image Examiner software and converted to tiff files for final processing.

Statistical analysis

Results are expressed as mean $\pm$ standard error. Student's *t*-test and one way analysis of variance with Newman-Keuls tests were used.^{5,6} $P < 0.05$ was accepted as statistically significant. "n" refers to the number of tissues (1 or 2 per animal).

Solutions and drugs

The Krebs' solution used in this study contained (in mM): 120.4 NaCl, 5.9 KCl, 15.5 NaHCO_3 , 11.5 glucose, 1.2 MgCl_2 , 1.2 NaH_2PO_4 , and 2.5 CaCl_2 . This solution had a pH of 7.3–7.4 when bubbled with 97% O_2 –3% CO_2 . 2-aminoethoxydiphenyl borate (2-APB), atropine, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), cyclopiazonic acid (CPA), cremophor EL, DMSO, mibefradil, nicardipine, N ω -nitro-L-arginine (L-NA), ryanodine, and tetrodotoxin (TTX) were purchased from Sigma-Aldrich Co (St. Louis, MO).

RESULTS

Morphology of ICC

Immunohistochemical staining with Kit antibodies in preparations that were cut in cross-section or as whole mounts, labeled ICC in various locations throughout colonic tunica muscularis (Figure 1): on the serosal surface of the LM (ICC-SER); running parallel to and within LM bundles (ICC-IM_{LM}); a loose network of ICC-MY distributed throughout the myenteric region between and around ganglia; running parallel to and within CM fibers (ICC-IM_{CM}), and a dense layer of ICC-SM along the submucosal border. In cross-sectional preparations, the LM was $510.6 \pm 100.6 \mu\text{m}$ ($n=7$) thick and the CM was $1402.5 \pm 24.3 \mu\text{m}$ ($n=13$) thick. The width of a CM bundle was $852.9 \pm 54.7 \mu\text{m}$ ($n=13$).

Ca^{2+} transients in ICC-SM

In flat-sheet preparations pinned with the submucosal surface uppermost, rhythmic, biphasic Ca^{2+} transients could be resolved in individual ICC-SM (frequency $6.4 \pm 0.1/\text{min}$, range 5 to 8/min; duration 6.1 ± 0.3 s, $n=47$) that consisted of an upstroke phase followed by a more variable plateau phase (Figure 2A and B). The Ca^{2+} transients had a similar waveform to electrical slow waves recorded from CM at the submucosal border (Figure 2D and E).^{9,10} The upstroke phase propagated through the network of ICC-SM in its slow conducting axis as a distinct wave front orthogonal to the CM fibers ($3.4 \pm 0.9 \text{ mm/s}$; range 2.2–5.5 mm/s, $n=4$), as shown in the ST-map (Figure 2B; movie 1). The upstroke phase, which synchronized ICC-SM, was followed by a plateau phase, which was more variable between ICC-SM. Higher power objectives ($\times 20$ and $\times 40$) revealed that the plateau phase consisted of an intracellular Ca^{2+} wave that propagated throughout an individual ICC-SM. Even with low power imaging ($\times 4$ objective), the wave front of activity in the ICC-SM network extended across the entire FOV ($2 \times 2 \text{ mm}$) in the direction of the CM fibers, demonstrating

this is the fast conduction axis of the network. The spread of the wave parallel to the CM fibers was too fast to capture. Averaging Ca^{2+} over a number of frames and past ultrastructural studies^{22,23} demonstrated that the processes of ICC-SM had an overall orientation parallel to the CM, i.e., the direction of the fast conduction axis (Figure 2C). Previous electrophysiological studies have also observed anisotropic propagation of slow waves, 17mm/s in the long axis of the CM fibers and 6mm/s in the transverse axis of the CM fibers in the canine colon.^{11,15}

Collisions of wave fronts originating from different areas of the ICC-SM network within the FOV were also observed (Figure 3# and *). A collision annihilated wave fronts, suggesting that the upstroke phase was an active event exhibiting refractory properties.

Neither the amplitude nor frequency of spontaneous Ca^{2+} transients in ICC-SM were affected significantly by blocking neural inputs with TTX (1 μM) or atropine (1 μM), suggesting these events were non-neurogenic (Table 1).

Pharmacology of ICC-SM

We tested the hypothesis that the biphasic Ca^{2+} transient in ICC-SM involve Ca^{2+} entry and Ca^{2+} release from intracellular stores (Table 1).^{3,5,6,16} The Ca^{2+} channel antagonists, nickel (100 μM) and mibefradil (1 μM) reduced both phases of the Ca^{2+} transients and reduced their frequency. Nicardipine (1 μM) significantly reduced the Ca^{2+} transient but did not affect the frequency of Ca^{2+} transients in ICC-SM. Ca^{2+} transients in ICC-SM were abolished by FCCP (mitochondrial ionophore; 1 μM), and by CPA (SERCA pump blocker; 10 μM). Ryanodine (10 μM) had neither a significant effect on the frequency or amplitude of spontaneous Ca^{2+} transients nor their propagation velocity (control $2.5 \pm 0.6 \text{ mm/s}$; ryanodine $2.3 \pm 0.7 \text{ mm/s}$, $p > 0.05$; $n = 4$). 2-APB (50 μM) reduced both the amplitude and frequency of Ca^{2+} transients in ICC-SM (Table 1). However, although 2-APB has been commonly used to inhibit IP_3 -induced Ca^{2+} release,^{3,5,6,16} it has been shown recently to have additional effects including the inhibition of store-operated Ca^{2+} entry.¹⁷

Decay of Ca^{2+} transients within CM

In preparations cut in cross-section parallel to the LM fibers, Ca^{2+} transients were observed to propagate slowly (velocity $3.2 \pm 0.3 \text{ mm/s}$, $n = 5$) along the entire length of the ICC-SM network at the submucosal border, which is the slow conduction axis (Figure 4A–C). However, the site of origin of the Ca^{2+} transients was could change from cycle to cycle (Figure s1).

As a Ca^{2+} transient propagated along the submucosal border, it also conducted rapidly into CM bundles without a detectable delay between the onsets of the transients (Figure 4A–C). In fact, the Ca^{2+} transients decayed exponentially from the submucosal border toward the myenteric border (Figure 4E and F), as do electrical slow waves and the resting membrane potential.⁹ Nicardipine (1 μM) significantly reduced the intensity and increased the rate of decay of Ca^{2+} transients in the CM, suggesting L-type Ca^{2+} channels were opened by the electrical slow wave as it conducted through the muscle (Figure 4E and F).

Ca²⁺ transients within LM

In cross-sectional preparations (7 out of 23), intercellular Ca²⁺ waves (duration 4.2 ± 0.3 s; frequency 5.4 ± 0.5 /min; $n=7$) were observed within the LM. Intercellular Ca²⁺ waves originated at the serosal surface and propagated (velocity of 0.9 ± 0.2 mm/s; $n=7$) towards the myenteric border in 5 preparations, whereas in the 2 other preparations, they also propagated from the myenteric border towards the serosal surface; each wave producing contraction of the LM (Figure 5A–D; movie 2). Intercellular Ca²⁺ waves were blocked by nicardipine (1 μ M), suggesting they resulted from Ca²⁺ action potentials propagating through the LM (Figure 5E).¹⁸

Ca²⁺ transients in ICC-MY

When imaging through the LM in flat-sheet preparations pinned with the serosal surface uppermost, it was difficult to distinguish between ICC-MY, ICC-IM_{LM}, and ICC-SER as confocal microscopy was not used. ICC in these regions exhibited similar patterns of Ca²⁺ transients (Figure 6A and B; movie 3). After peeling away the LM, multipolar shaped ICC were observed in the myenteric region between and upon ganglia (ICC-MY). Spindle shaped cells were observed in adjacent bundles of LM (ICC-IM_{LM}). The activity consisted of intracellular Ca²⁺ waves,¹⁶ that propagated (velocity 62.9 ± 3.9 μ m/s; 55 cells, $n=5$) from the cell body and along the processes (ICC-MY) or along the length of the cell (ICC-IM_{LM}). When Ca²⁺ waves propagated along processes of ICC-MY, longer duration transients were observed. The duration (3.4 ± 0.3 s; 103 cells, $n=5$) of these waves was directly proportional to the interval between them (Figure 7D). The frequency of Ca²⁺ transients in ICC were normally distributed (Figure 7E), and the mean frequency was 15.6 ± 0.8 /min (103 cells, $n=5$). This was the same frequency as the contractions of the muscle (i.e., 15.2 ± 1.5 /min; $n=5$, $p>0.5$). Ca²⁺ transients in ICC-MY/ICC-IM_{LM} were typically unsynchronized across the FOV (see random pattern in the ST-map; Figure 6B); in fact, even intracellular Ca²⁺ waves in adjacent ICC were unsynchronized (Figure 6C and D). An identical pattern of activity was observed in ICC-MY lying upon myenteric ganglia (Figure 7A and B).

Superimposed on the pattern of intracellular Ca²⁺ waves were periodic reductions in average basal Ca²⁺ (6 out of 13 preparations), which depressed their activity (Figures 6B and 7B). Averaging the traces together showed the drop in baseline Ca²⁺ more clearly (Figures 6B and 7B; black traces). During reductions in basal Ca²⁺, we noted occasional clustering of intracellular Ca²⁺ waves (Figure 6B*). In two preparations, we found that intracellular Ca²⁺ waves in ICC-MY became strongly synchronized when the LM and CM layers elicited intercellular Ca²⁺ waves at the same time (Figure 7C). Intercellular Ca²⁺ waves in muscle cells are elicited by Ca²⁺ action potentials.^{6,18} TTX (1 μ M) appeared to desynchronize intracellular Ca²⁺ waves in ICC-MY and blocked the synchronous firing of both muscles (Figure 7C). Previous studies have shown that synchronous bursts of activity in LM and CM occurs when excitatory nerves discharge.¹⁸

Effects of blocking nitric oxide synthesis on activity in ICC-MY

Myenteric potential oscillations that occur within the LM and CM adjacent to the myenteric border are interrupted by periodic hyperpolarizing events that suppress their activity.^{10,12} The hyperpolarizations are neurally mediated since they are blocked by TTX and L-NA (NO

synthesis inhibitor), and are due to NO release from spontaneously active inhibitory motor neurons.¹² Similarly, we found that the periodic reductions in average Ca^{2+} in ICC-MY/ICC-IM_{LM}, including ICC-MY lying over ganglia, were abolished by inhibiting NO synthesis with L-NA (100 μM , $n=3$; Figures 6C–E and 7E), which didn't increase coupling between adjacent ICC (Figure 7E). L-NA also significantly decreased the duration (from 3.4 ± 0.3 s to 2.5 ± 0.1 s; 108 cells, $p<0.05$, $n=5$) and increased the overall mean frequency of intracellular Ca^{2+} waves from $15.6\pm0.8/\text{min}$ to $18.7\pm0.6/\text{min}$ (108 cells, $p<0.05$, $n=5$), but did not change their velocity (from $62.9\pm3.9\mu\text{m/s}$ to $64.4\pm4.0\mu\text{m/s}$; 55 cells, $p>0.5$, $n=5$), suggesting that activity in ICC-MY/ICC-IM_{LM} was inhibited by NO release from nitrergic neurons (Figures 6C–E and 7B, D, E).

DISCUSSION

Previous studies have demonstrated distinctly different pacemaker activity originating at the submucosal and myenteric borders of the CM layer in many mammals, including the human colon.^{7–13} In the present study, we have found that this is due to fundamental differences in the behaviors of ICC at the two borders. ICC-SM are well coupled and produce pacemaker events like we have observed in the small intestine in our previous imaging studies.^{5,6} In contrast, Ca^{2+} transients in ICC-MY are poorly coordinated and thus not capable of producing propagated pacemaker activity. Ca^{2+} transients in ICC-SM ($\sim 6/\text{min}$) and ICC-MY/ICC-IM_{LM} ($\sim 16/\text{min}$) have different frequencies and durations, suggesting that the pacemaker mechanism differs between these cells.

The ICC-SM network in the colon has many properties in common with the ICC-MY network in the small intestine. Both networks consist of a dense layer of ICC that are well coupled to each other by numerous gap junctions that allows the propagation of slow waves.^{3,5,6,19,20} Ca^{2+} wave fronts in the ICC-SM network propagated, in the slow conduction axis, perpendicular to the CM fibers, whereas, in the small intestine, the direction of propagation through the ICC-MY network is more variable and propagation of wave fronts can occur at angles.^{5,6} These differences in propagation are likely related to the morphology and orientation of ICC. ICC-MY in the small intestine consist largely of stellate shaped cells with no obvious orientation,^{3,5,6} whereas ICC-SM in the colon run primarily parallel to the CM fibers.^{16,17} This likely explains why slow waves in the colon naturally produce propulsive rings of contraction, as observed by Cannon (1902).²²

As in the small bowel, the upstroke phase of the underlying slow wave also propagates as a wave front through the ICC-SM network to initiate the variable plateau phase in ICC.^{5,6} The upstroke phase must be an active event, since it propagates without decrement and is annihilated when it collides with another wave front of ICC activity. The regenerative upstroke phase is produced by Ca^{2+} influx through a T-type Ca^{2+} channel (reduced by nickel and mibefradil). Ca^{2+} influx during the upstroke phase likely produces the plateau phase by causing CICR to form the intracellular Ca^{2+} wave underlying the plateau phase.^{3,5,6} In addition, the upstroke and plateau phases may also involve activation of L-type Ca^{2+} channels since the Ca^{2+} transients were reduced by nicardipine.³ Our pharmacological studies are also consistent with the idea that the pacemaker “clock” mechanism in ICC-SM involves an interaction between mitochondria (blocked by FCCP) and intracellular Ca^{2+}

stores (blocked by CPA and reduced by 2-APB) as elsewhere in the gastrointestinal tract.^{3,5,6,19}

We have previously shown that as the slow wave declines in amplitude from the submucosal border there is a proportional depolarization in resting membrane potential.^{19,10} Similarly, we observed that as Ca^{2+} transients conducted into the CM they also decayed exponentially in amplitude from the submucosal border. These Ca^{2+} transients are likely to be due to the opening of L-type Ca^{2+} channels (reduced by nifedipine) and probably T-type channels (reduced by mibefradil). Therefore, the decline in the resting membrane potential from the submucosal border likely ensures that slow waves within the bulk CM are near threshold for opening voltage sensitive Ca^{2+} channels.

Ultrastructurally, the ICC network at the myenteric border (ICC-MY) is quite different from the ICC-SM network of the colon since it forms a loose network of ICC.^{8,15,19,21,22} ICC-MY make occasional contact with each other and with the CM and LM via small gap junctions. On rare occasions dye injection into LM cells has been observed to spread through ICC-MY to the CM, implying that ICC are connected to and pace the muscle.¹⁹ In consideration of morphological studies, it is significant that intracellular Ca^{2+} waves in ICC-MY do not couple with adjacent ICC.

Regularly occurring intracellular Ca^{2+} waves have been observed in urethra ICC where they produce depolarizing events by opening Ca^{2+} activated Cl^- currents.¹⁶ It is possible that intracellular Ca^{2+} waves cause similar depolarizing events in ICC-MY/ICC-IM. There are several indications that intracellular Ca^{2+} waves in ICC-MY generate myenteric potential oscillations (MPOs) in LM and CM: 1) they have a similar frequency and duration to MPOs and contractions of the muscle; 2) like MPOs, they don't have a defined upstroke phase; 3) like MPOs, they exhibit periodic inhibition from the release of NO from myenteric neurons; 4) blocking NO synthesis also increases their overall frequency and amplitude, and 5) ICC-MY are ideally located to generate MPOs in both the LM and CM. The fact that ICC-MY do not generate a propagating upstroke phase may also be a reason why the ICC-MY network lacks coupling. Therefore, activity in ICC-MY likely influences only a few LM or CM cells, and this might explain the more random waveforms and waxing and waning of MPOs.^{7,10,12}

However, neural activity appears to coordinate the activity of ICC-MY and ICC-IM_{LM}. When inhibitory nerves are active, reductions in Ca^{2+} occur synchronously in all ICC-MY. Reduced Ca^{2+} transients in ICC-MY correlate with inhibition of intracellular Ca^{2+} waves and muscular activity. Excitatory nerve activity synchronizes intracellular Ca^{2+} waves across the ICC-MY network, leading to intercellular Ca^{2+} waves in CM and LM and contraction. An important finding is that all ICC-MY that lie on and between ganglia are affected by release of transmitter from both inhibitory and excitatory nerves. Coordination of activity in ICC-MY might also occur if a slow wave depolarization conducts across the CM to influence ICCMY.^{9,10,12} This might occur if a slow wave depolarization activates voltage dependent Ca^{2+} entry to stimulate CICR.

We also found that intercellular Ca^{2+} waves in LM were most often initiated first at the serosal surface and then propagated through the thickness of the LM towards the myenteric

plexus. This is consistent with the observation that MPOs elicit action potentials more readily near the serosal surface of the LM,¹⁰ suggesting they could be generated by ICC-SER. This is the first indication of a possible functional role of ICC-SER, although these cells have been identified before in morphological studies of the colon.^{23,24}

In slow transit constipation, there is an increase in myenteric nitroergic neurons, but a decrease in cholinergic neurons,²⁵ as well as increased NO production and a reduction in cholinergic responses.²⁶ Increased NO would likely lead to an increased inhibition of ICC-MY and a lack of tone in the LM. In addition, a reduced cholinergic activity would translate into a lack of excitatory nervous coupling between ICC-MY and the LM and CM, as well as a reduced summed MPO and slow wave activities within the bulk CM, leading to compromised colonic propulsive motor patterns.^{18,27}

In summary, ICC-SM form a tightly coupled network that generates and propagates colonic slow waves, but pacemaker activity of ICC-MY is poorly coordinated under basal conditions and synchronous activity is elicited by neural regulation. These functional differences in pacemaker phenotypes are integrated in the whole tissue to regulate colonic contractions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CM	circular muscle
FOV	field of view
ICC	interstitial cells of Cajal
LM	longitudinal muscle
MPO	myenteric potential oscillation
ROI	region of interest
SMB	submucosal border
ST-map	spatiotemporal map

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Figure 1. Distribution of colonic ICC with Kit labeling

A. Cross-sectional preparation stained with Kit antibody. ICC were observed in several locations throughout the colon: 1) on the serosal surface of the LM (ICC-SER); 2) in parallel to and within LM fibers (ICC-IM_{LM}); 3) throughout the myenteric region between and around ganglia (ICC-MY); 4) within CM fibers (ICC-IM_{CM}), and 5) as a dense layer along the submucosal border (ICC-SM). **B.** ICC-MY around a myenteric ganglion. **C.** Flat-sheet preparation shows a loosely coupled ICC-MY network (cell bodies *). **D.** Dense ICC-SM along the submucosal border (SMB).

Figure 2. Spontaneous Ca^{2+} transients in ICC-SM

A. Flat-sheet preparation with ICC-SM network uppermost. **B.** Biphasic Ca^{2+} transients in several ICC-SM (colored circles in A) are overlaid. Overlay (colored traces) and summed average (black trace) of traces show activity is tightly coupled. The corresponding ST-map created from the entire FOV in A shows that this activity propagated smoothly through the ICC-SM network. **C.** ICC-SM processes are largely orientated along the direction of the CM fibers (top to bottom). **D.** Simultaneous electrical recording from CM and Ca^{2+} imaging of ICC-SM network. **E.** ST-map shows that Ca^{2+} transients in ICC-SM propagated across the recording site (dotted line). The Ca^{2+} transients had a waveform that was similar to the electrical slow waves in the underlying CM.

Figure 3. Propagation of Ca^{2+} transients through the ICC-SM network

A. Flat-sheet preparation with ICC-SM network uppermost. ROIs (colored boxes) were taken from 3 areas over the ICC-SM network. **B.** ST-map of FOV in A, shows propagation of activity through the network of ICC-SM. Wave fronts of activity started on both sides of the preparation and propagated through the ICC-SM network (orthogonal to CM fibers) and collided with one another (see # and *). **C–E.** Series of frames showing collisions between opposing wave fronts through ICC-SM observed in B. In C and D, the wave front originated on the anal side and collided with a wave front initiated later on the oral side, and vice versa in E.

Figure 4. Conduction of pacemaker activity through the CM

A. Cross-sectional preparation shows several CM bundles. Ca²⁺ transients were sampled in ROIs (see colored boxes) throughout the thickness of two muscle bundles. **B.** Ca²⁺ transients in these ROIs declined in amplitude from the SMB into the CM bundle. In contrast, Ca²⁺ transients propagated along the SMB (compare upper and lower red traces in B). **C.** Compares ST-maps made of Ca²⁺ activity along the entire SMB (white long dotted rectangle in A-upper ST-map) with those through the thickness of the CM (white dotted rectangle over middle bundle-lower ST-map). Note that activity propagated along the SMB,

but occurred simultaneously through the thickness of the CM, where the Ca^{2+} transients declined in intensity from the SMB. **D.** ST-maps were constructed from the same regions as in C but after nicardipine. Note that the Ca^{2+} transients within the bulk muscle much diminished in intensity. **E and F.** Show the decay of the amplitude (E) and area under Ca^{2+} transients (F) through the thickness of the CM, from the SMB (0mm; n=6).

Figure 5. Propagation of Ca^{2+} transients in LM

A. Cross-sectional preparation showing LM and portion of underlying CM. Ca^{2+} transients were measured in ROI at the serosal border (black rectangle) and near the myenteric border (white rectangle). **B.** A series of frames showing the propagation of an intercellular Ca^{2+} wave through the thickness of the LM. Note that the wave started at the serosal border. **C.** ST-map of the FOV in A. All the waves started at the serosal border and propagated towards the myenteric border. Ca^{2+} transients from the ROIs in A together with displacement of the tissue. **D.** In another preparation, an ST-map was constructed from the white dotted

rectangle in the FOV. **E.** The ST-map shows that the first 3 and the 5th intercellular Ca^{2+} waves were initiated from the serosal surface and propagated towards a septa within the middle of the LM, whereas the 4th wave started at the septa and propagated towards the serosal surface (*). These waves were blocked by nicardipine (lower ST-map).

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Figure 6. Spontaneous Ca^{2+} transients in ICC-MY and ICC-IM

A. Flat-sheet preparation with serosa uppermost and strips of LM removed. Averaged Ca^{2+} activity showed numerous ICC-MY lying over and between ganglia, as well as ICC-IM in LM. **B.** Superimposed Ca^{2+} transients from ICC at these different locations (colored circles in A), showing clusters of activity (*). After averaging all the individual traces together, the reductions in average Ca^{2+} were more clearly observed (black trace). The ST-map created from entire FOV in A demonstrates that activity was uncoordinated. **C.** Orange and blue rectangles each surround a multipolar ICC-MY cell. **D.** ST-maps and line traces of Ca^{2+} transients in these two adjacent ICC-MY (see C). The activities in these two adjacent ICC-MY lying between ganglia were uncoordinated, despite the fact that a process appeared to connect them (see C*; $\times 20$, DOF 2.6 μm). **E.** ST-maps and line traces from these same cells after L-NA (100 μM). L-NA increased the frequency of Ca^{2+} waves in these ICC, but their activity was still unsynchronized.

Figure 7. Spontaneous Ca^{2+} transients in ICC-MY

A. Flat-sheet preparation showing summed Ca^{2+} fluorescence, which showed active ICC-MY over myenteric ganglia. **B.** Ca^{2+} transients from individual ICC-MY (colored ellipses in A) lying along a myenteric ganglion were overlaid. When the traces were averaged together (black trace), spontaneous reductions in Ca^{2+} were clearly observed. L-NA increased the frequency of Ca^{2+} waves in these cells (colored traces) and blocked the reductions in Ca^{2+} (see black trace). Note that after L-NA, averaging all the traces together (black line) produces almost a straight line, implying the activity was uncoordinated. **C.** Clustering of

intracellular Ca^{2+} waves in ICC-MY during simultaneous Ca^{2+} transients in LM and CM. During activity in the muscle there was a corresponding displacement of the tissue. TTX reduced these clusters and increased the frequency of Ca^{2+} transients in ICC-MY but blocked those in both the LM and CM. **D.** Plot of duration versus interval of Ca^{2+} waves in ICC-MY/ICC-IM before (blue circles) and after L-NA (red circles; 32 cells in a single preparation). **E.** Plot of % ICC-MY/ICC-IM against frequency of Ca^{2+} transients before (blue bars; 103 cells, n=5) and after L-NA (red bars; 108 cells, n=5).

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Table 1

Pharmacology of ICC-SM in canine proximal colon

Drug	Concentration	N	Frequency (c/min)		Relative AUC (%)
			Before	After	
Vehicle	0.1 % DMSO	5	6.50 ± 0.56	6.40 ± 0.54	100
TTX	1 μM	5	6.40 ± 0.37	6.30 ± 0.42	98.5 ± 3.2
Atropine	1 μM	4	6.50 ± 0.53	6.38 ± 0.43	102.2 ± 6.2
Nickel	100 μM	4	6.38 ± 0.72	1.25 ± 0.55*	38.8 ± 16.1*
Mibefradil	1 μM	5	6.50 ± 0.50	0.60 ± 0.45*	29.8 ± 20.4*
Nicardipine	1 μM	6	6.42 ± 0.36	6.58 ± 0.59	55.9 ± 6.6*
Ryanodine	10 μM	4	6.38 ± 0.64	5.38 ± 0.43	83.4 ± 8.9
2-APB	50 μM	4	6.25 ± 0.37	0.75 ± 0.55*	34.9 ± 23.8*
CPA	10 μM	4	6.38 ± 0.43	0.00 ± 0.00*	0.0 ± 0.0*
FCCP	1 μM	4	6.13 ± 0.60	0.00 ± 0.00*	0.0 ± 0.0*

Frequency: paired Student's *t*-test.

* P<0.05 vs. each before value. AUC (area under curve); one way analysis of variance followed by Newman-Keuls test. * P<0.05 vs. vehicle value.