Intracellular Ca\textsuperscript{2+} release from endoplasmic reticulum regulates slow wave currents and pacemaker activity of interstitial cells of Cajal

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Zhu MH, Sung TS, O’Driscoll K, Koh SD, Sanders KM. Intracellular Ca\textsuperscript{2+} release from endoplasmic reticulum regulates slow wave currents and pacemaker activity of interstitial cells of Cajal. Am J Physiol Cell Physiol 308: C608–C620, 2015. First published January 28, 2015; doi:10.1152/ajpcell.00360.2014.—Interstitial cells of Cajal (ICC) provide pacemaker activity in gastrointestinal muscles that underlies segmental and peristaltic contractions. ICC generate electrical slow waves that are due to large-amplitude inward currents resulting from anoctamin 1 (ANO1) channels, which are Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels. We investigated the hypothesis that the Ca\textsuperscript{2+} concentration, suggesting that pacemaker activity depends on Ca\textsuperscript{2+} dynamics in restricted microdomains. Our data from studies of isolated ICC differ somewhat from studies on intact muscles and suggest that release of Ca\textsuperscript{2+} from both IP\textsubscript{3} and ryanodine receptors is important in generating pacemaker activity in ICC.

ANO1 channel; SERCA pump; ryanodine receptor; IP\textsubscript{3} receptor

INTERSTITIAL CELLS OF CAJAL (ICC) are pacemaker cells that generate electrical slow waves in the gastrointestinal (GI) tract (7, 13, 24, 36, 41, 47, 49). Slow waves drive phasic contractions that are the basic contractions of segmentation and gastric peristalsis. The underlying pacemaker event in ICC is spontaneous transient inward currents (STICs). STICs generate depolarization, activate Ca\textsuperscript{2+} entry, and synchronize the openings of channels responsible for STICs, generating slow wave currents. STICs and slow wave currents are due to the activation of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels (CaCC) encoded by Ano1 (11, 14, 53, 54). While the mechanism and ionic conductance responsible for STICs and slow wave currents have been described, the mechanisms responsible for delivering Ca\textsuperscript{2+} to ANO1 channels are not fully understood and have not been studied directly at the level of single ICC.

Previous studies have investigated the potential role of Ca\textsuperscript{2+} release in driving pacemaker activity in GI muscles using whole muscle preparations or bundles of muscle. Thapsigargin and cyclopiazonic acid (CPA), endoplasmic reticulum (ER) Ca\textsuperscript{2+}-ATPase inhibitors, slowed the generation of slow waves (30). Xestospongin C, a membrane-permeable blocker of inositol 1,4,5-trisphosphate (IP\textsubscript{3}) receptor-operated Ca\textsuperscript{2+} channels, also disrupted slow wave activity. Another study showed loss of slow waves in gastric muscles when IP\textsubscript{3} receptors were genetically deactivated (45). In whole muscles, ryanodine did not have significant effects on slow wave frequency (30). Spontaneous transient depolarizations (STDs) in small bundles of gastric muscle are the result of Ca\textsuperscript{2+}-dependent conductances and are blocked by intracellular Ca\textsuperscript{2+} chelation (48). These authors also found inhibition of STDs after exposure to CPA and by heparin loading using reversible permeabilization techniques. STDs were initiated by depolarization, and this led to the idea that Ca\textsuperscript{2+} release from IP\textsubscript{3} receptors was controlled by voltage in ICC. These studies provided evidence of the importance of IP\textsubscript{3} receptors in regulation of a Ca\textsuperscript{2+}-dependent conductance involved in the pacemaker system in GI muscles. However, studies on whole muscles are complicated by the multiplicity of effects of reagents effective in many different cell types. In the case of GI muscles, at least three cell types are electrically coupled, forming a syncytium of smooth muscle cells, ICC, and cells labeled with antibodies to platelet-derived growth factor receptor α (PDGFR\textalpha) cells that we have referred to as the SIP syncytium (41). Each type of cell in the SIP syncytium generates membrane currents due to Ca\textsuperscript{2+} release from stores, and activation or suppression of conductances in any of the SIP cells is likely to affect the excitability of the other coupled cells. Thus, the question of regulation of CaCC in ICC needs to be studied in isolated cells.

For the experiments in this study, we used cells from Kit\textsuperscript{+}/copGFP mice, which have constitutive expression of copGFP in ICC, to investigate the effects of Ca\textsuperscript{2+} store-active drugs on STICs and slow wave currents (53, 54). We studied the effects of blocking Ca\textsuperscript{2+} uptake into stores and of blocking release of Ca\textsuperscript{2+} from IP\textsubscript{3}-dependent and ryanodine-sensitive channels to test directly the hypothesis that Ca\textsuperscript{2+} release from the endoplasmic reticulum (ER) is fundamental to pacemaker activity in ICC.

METHODS

Animals. Kit\textsuperscript{+}/copGFP mice (P8 to P12) were used for the experiments as described previously (53). Mice were anesthetized with isoflurane (Vetdepot, Encinitas, CA), killed by decapitation, and the small intestines were removed. The Institutional Animal Care and Use...
Committee at the University of Nevada approved all procedures regarding animal care and usage.

Preparation of dispersed ICC. Strips of intestinal muscle were prepared and equilibrated in Ca$$^{2+}$$-free Hanks' solution for 10 to 20 min. Cells were dispersed from the muscle strips using an enzyme solution containing (per ml) collagenase (1.3 mg; Worthington Type II), bovine serum albumin (2 mg; Sigma, St. Louis, MO), trypsin inhibitor (2 mg; Sigma), and ATP (0.27 mg; Sigma). Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 mg/mL, Falcon/BD) in 35-mm culture dishes. The cells were allowed to stabilize 2–5 h in culture media at 37°C in a 95% O$_2$–5% CO$_2$ incubator in smooth muscle medium (Clonetics, San Diego, CA) supplemented with 2% antibiotic-antimycotic ( Gibco, Grand Island, NY) and stem cell factor (5 ng/mL, Sigma) before electrophysiological experiments were begun.

Expression of Ano1 in HEK-293 cells. An expressed sequence tag (IMAGE Consortium cDNA clone no. 30547439) homologous to Ano1 (A variant) from mouse was subcloned into pcDNA3.1 (Invitrogen). Ano1 was ligated into the pkmKate2-N vector (Evrogen, Moscow, Russia) containing a far-red fluorescent protein mKate2. The Ano1 stop codon was removed and EcoRI and ApaI restriction sites were placed at the termini by PCR, so the Ano1 transcript was inserted in frame and resulted in a plasmid encoding a COOH-terminal mKate2 tagged ANO1 fusion protein. The Ano1 splice variant AC, which is highly expressed in gastrointestinal muscles (14), was produced by inserting the 12-nucleotide “C” segment into the coding region using a QuickChange XL site-directed mutagenesis kit (Agilent Technologies). The final plasmids were sequenced by the Nevada Genomics Center to confirm sequence and insertion of the alternative exon.

The AC splice variant of Ano1 was expressed in human embryonic kidney (HEK)-293 cells (American Type Culture Collection, Manassas, VA). HEK-293 cells were seeded in 12-well plates and maintained in DMEM (Gibco) medium with FBS (10%, vol/vol, Gibco), penicillin-streptomycin (1%, vol/vol, Gibco), and glutamax (1%, vol/vol; Gibco). The plasmid DNA containing Ano1 tagged with mKate2 (0.5 µg/well) was transfected into cells using 1.5 µL/well of FuGENE 6 transfection reagent (Promega, Madison, WI). Expression of ANO1 was monitored by mKate2 fluorescence in cells.

Patch-clamp experiments. Intestinal cells of Cajal (ICC) and transfected HEK-293 cells were identified by the presence of green fluorescent protein, using an inverted microscope (TE 200-S; Nikon, Japan) attached with Lambda DG4 fluorescence (Sutter Instrument, Novato, CA). The conventional dialyzed whole cell patch-clamp configuration was used to record membrane currents (voltage clamp) and potentials (current clamp, I = 0) from cells. Membrane currents or transmembrane potentials were amplified with an Axopatch 200B patch-clamp amplifier (Molecular Devices) and digitized with a 16-bit analog-to-digital converter (Digidata 1322A, Molecular Devices) and stored directly on-line using pCLAMP software (version 9.2, Molecular Devices). Data were sampled at 4 kHz and filtered at 2 kHz for whole cell experiments. Mini-Digi with Axoscope (version 9.2, Molecular Devices) was used to monitor changes in holding currents (basal currents) throughout experiments. All data were analyzed with clampfit (version 9.2, Molecular Devices) and GraphPad Prism (version 3.0, GraphPad Software, San Diego, CA) software. The pipette tip resistance ranged between 3 and 6 MΩ for whole cell recordings, and experiments on ICC were conducted at 30°C with the use of a Thermoclip-1 (Automate Scientific, Berkeley, CA). External solutions were perfused and changed within 1 min with a fast bath perfusion system (AutoMate Scientific, Berkeley, CA). Whole cell configuration experiments were also used to study ANO1 currents expressed in HEK-293 cells, and these experiments were conducted at room temperature.

Solutions for patch-clamp experiments. The external solution for whole cell recordings was a Ca$$^{2+}$$-containing physiological salt solution (CaPSS) containing (in mM) 135 NaCl, 5 KCl, 2 CaCl$_2$, 1.2 MgCl$_2$, 10 glucose, and 10 HEPES adjusted to pH 7.4 with Tris. For the HEK-293 cell experiments, the NMDG external solution contained (in mM) 147.7 NMDGCl, 10 EGTA, 10 HEPES, and 4.14 CaCl$_2$, adjusted to pH 7.4 with Tris. The pipette solutions used for these experiments are described in Table 1. Free Ca$$^{2+}$$ concentrations were calculated by Maxchelator software (http://maxchelator.stanford.edu).

Chemicals and drugs. Thapsigargin, tetracaine, 2-aminoethoxyphenyl borate (2-APB), xestospongin C, heparin sodium salt, and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) were purchased from Sigma. Cyclopiazonic acid (CPA) and ryanodine were purchased from Tocris (Ellisville, MO). Stock solutions were made by dissolving tetracaine (tetracaine HCl) in water, ryanodine in ethanol, 2-APB in methanol, and CPA, thapsigargin, NPPB, and xestospongin C in dimethyl sulfoxide (DMSO), as per the manufacturer’s recommendations. Adding stock solutions to the extracellular solutions used in specific experiments made final dilutions. Final concentrations of ethanol and DMSO were <$0.1%. Heparin was dissolved in the Cs$$^{+}$$-rich pipette solution (solution II, Table 1).

For the purposes of this study, CPA and thapsigargin were used to inhibit the ER Ca$$^{2+}$$-ATPase. IC$_{50}$ concentrations for these drugs on this transporter have been reported to be 0.2–3 µM and 0.03 µM, respectively (1, 18). Ryanodine and tetracaine were used to block ryanodine receptor ER Ca$$^{2+}$$ channels. IC$_{50}$ concentrations for these drugs on this ion channel have been reported to be 3.5 µM and 200–2000 µM, respectively (9, 25). 2-APB, xestospongin C, and heparin were used to inhibit Ca$$^{2+}$$ release from inositol IP$_3$ receptors. IC$_{50}$ concentrations for these drugs on these receptors have been reported to be 42 µM, 350 nM, and 20 µg/mL, respectively (10, 31, 32).

Statistical analysis. Data are expressed as means ± SE. The n values given represent the number of cells in patch-clamp experiments. Differences between data sets were evaluated by Student’s paired t-test and considered statistically significant when P < 0.05.

RESULTS

Effects of CPA and thapsigargin on STICs and slow wave currents in ICC. ICC were studied under voltage clamp to investigate the spontaneous transient inward currents (STICs) and slow wave currents occurring in these cells (53, 54). We used a Cs$$^{+}$$-rich pipette (solution II, Table 1) to prevent contamination from K$$^+$$ conductances and CaPSS, as the external solution, in these experiments. Slow wave currents were elicited by stepping from a holding potential of −80 mV to −35 mV (53). CPA (30 µM), an inhibitor of the ER Ca$$^{2+}$$-ATPase (SERCA pump) (16), decreased the amplitude and frequency of STICs (Fig. 1A) and slow wave currents (Fig. 1B). At the holding potentials of −80 mV, STICs were decreased from −47 ± 7.2 to −15 ± 2.2 pA (P < 0.01) in

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All pipette solutions were adjusted to pH 7.2 with Tris.
amplitude and from 34.1 ± 2.4 to 12.9 ± 1.8 cycles per minute (cpm) in frequency by CPA (30 μM; n = 6, P < 0.001; Fig. 1, A, E, and F). CPA (30 μM) decreased peak slow wave current from −1,052 ± 116.2 to −270 ± 116.0 pA (P < 0.001; Fig. 1B). Similar results were obtained with thapsigargin, another SERCA pump inhibitor (46). Thapsigargin (1 μM) decreased STIC amplitude from 34.1 to 270 cpm (P < 0.01; Fig. 1A and C) and frequency from 35.0 ± 2.5 to 6 ± 1.4 cpm (n = 7; P < 0.001; Fig. 2, A and D). Peak amplitude of slow wave currents was reduced from −1,127 ± 113.2 to −250 ± 20.6 pA (P < 0.001; Fig. 2B). Ryanodine (50 μM) had dual effects on STICs. Initially, within 3–6 min after application of ryanodine, STICs increased from −50 ± 8.4 to −169 ± 62.9 pA (P < 0.001; Fig. 3, A and E) in amplitude and from 32.7 ± 2.3 to 70.5 ± 8.2 cpm (n = 6; P < 0.01; Fig. 3, A and F) in frequency. Slow wave currents were unaffected during this initial period of ryanodine exposure (Fig. 3, B and D). After the initial stimulation, STICs decreased to −24 ± 6.9 pA (P < 0.01; Fig. 3, A and E) in amplitude and 6.8 ± 1.4 cpm (n = 6; P < 0.01; Fig. 3, A and F) in frequency. Peak slow wave current was also reduced by ryanodine, from −1,004 ± 82.7 to −230 ± 34.0 pA (P < 0.001; Fig. 3, C and D). These results suggest that ryanodine receptors contribute to the Ca²⁺ delivered to ANO1 channels during pacemaker activity in ICC.

Effects of store-active drugs on ANO1 currents. Results above show that store-active drugs block STICs and slow wave currents in ICC, and previous studies have shown that these currents are mediated by ANO1 channels in these cells (14, 53, 54). Therefore, we performed control studies to test whether...
Ca\(^{2+}\) store-active drugs have direct effects on ANO1 currents. Currents were elicited under voltage clamp by step depolarizations from −80 mV to +70 mV in 10-mV increments from a holding potential of −80 mV in HEK-293 cells expressing the AC splice variant of murine ANO1. The NMDG external solution was used for these experiments. The cells were dia-
lyzed with pipette solution V (Table 1) containing 100 nM free Ca\(^{2+}\), which is sufficient to activate the AC variant of ANO1 (Fig. 4, A, C, and E). Ryanodine (50 μM; \(n = 6\)), CPA (30 μM; \(n = 5\)), and thapsigargin (1 μM; \(n = 6\)) had no effect on the magnitude or kinetics of activation and deactivation of ANO1 (Fig. 4, B, D, and F).

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Fig. 2. The effects of tetracaine on STICs and pacemaker currents. A: tetracaine (500 μM) inhibited the amplitude and frequency of STICs at a holding potential of −80 mV with external Ca\(^{2+}\)-containing physiological salt solution (CaPSS) and pipette solution containing CsCl. B: portions of the trace in A (designated as a and b) were expanded to show that slow wave currents decreased after application of tetracaine. C and D: summarized data showing averaged effects of tetracaine (500 μM) on STIC amplitude (C) and frequency (D). *** \(P < 0.001\).

Fig. 3. The effect of ryanodine on STICs and slow wave currents. A: ryanodine (50 μM) transiently increased the amplitude and frequency of STICs, then inhibited the amplitude and frequency of STICs at a holding potential of −80 mV with external CaPSS solution and pipette solution containing CsCl. B and C: ryanodine had no significant effect on slow wave currents within the first few minutes (B), but it inhibited slow wave currents in interstitial cells of Cajal (ICC) after several minutes (C). Traces in B and C are shown with expanded time scales from A (a, b, c, d). D–F: summarized data showing average effects of ryanodine (50 μM) on peak current (D), STIC amplitude (E), and STIC frequency (F). Rya1 describes STICs during the initial 3–6 min after application of ryanodine, and Rya2 indicates STICs after 6 min. ** \(P < 0.01\); *** \(P < 0.001\); ns, not significant.
Effects of IP$_3$ receptor antagonists on STICs and slow wave currents in ICC. Previous studies have shown loss of slow waves in gastric muscles in animals with genetic deactivation of IP$_3$ receptors (45). We tested the effects of 2-APB on STICs and slow wave currents in ICC. Cs$^+$-rich pipette solution (solution II, Table 1) and CaPSS, as the external solution, were used in these experiments. 2-APB (50 μM) decreased the amplitude of STICs from $-45 \pm 3$ to $-17 \pm 2$ pA ($P < 0.001$) and frequency from $29 \pm 2.0$ to $11.2 \pm 0.7$ cpm ($n = 6$, $P < 0.001$; Fig. 5, A, C, and D) at a holding potential of $-80$ mV. Peak slow wave current was reduced from $-1,034 \pm 112.9$ to $-368 \pm 67.5$ pA ($P < 0.001$; Fig. 5B).

In some experiments, heparin (100 and 300 μg/ml) was added to Cs$^+$-rich pipette solution (solution II, Table 1) and CaPSS was used as the external solution. Slow wave currents were elicited by stepping from $-80$ mV to $-35$ mV from a holding potential of $-80$ mV. A, C, and E: currents measured in control conditions. B, D, and F: store-active drugs such as ryanodine (B), CPA (D), and thapsigargin (F) had no significant direct effect on ANO1 currents.

Fig. 4. Effects of store-active drugs on anoctamin 1 (ANO1) currents. ANO1 was expressed in HEK-293 cells. Currents were recorded when cells were dialyzed with 100 nM free [Ca$^{2+}$], and stepped from $-80$ mV to $+70$ mV in 10-mV increments. Holding potential was $-80$ mV. A, C, and E: currents measured in control conditions. B, D, and F: store-active drugs such as ryanodine (B), CPA (D), and thapsigargin (F) had no significant direct effect on ANO1 currents.
holding potential of \(-80\) mV. Despite dialyzing cells with concentrations far exceeding the \(IC_{50}\) for blocking IP3 receptors (32), heparin had no effect on STICs or slow wave currents after 10 min of dialysis (\(n = 4\); data not shown).

Xestospongin C, a selective, reversible, and membrane-permeable inhibitor of IP3 receptors (34), was also tested. Cs\(^+\)-rich pipette solution (\textit{solution II}, Table 1) and CaPSS, as the external solution, were used for these experiments. Xestospongin C (350 nM) decreased the amplitude of STICs from \(-42 \pm 5.5\) to \(-12.6 \pm 2.0\) pA (\(n = 5\); \(P < 0.01\); Fig. 6, A and C) and frequency from \(28.2 \pm 3.5\) to \(5.8 \pm 1.5\) cpm (\(n = 5\); \(P < 0.01\); Fig. 6, A and D). Stepping from \(-80\) mV to \(-35\) mV from a holding potential of \(-80\) mV before and during xestospongin C exposure evoked slow wave currents. The peak amplitude of the slow wave currents was reduced by xestospongin C from \(-1,070 \pm 110.9\) to \(-509 \pm 95.4\) pA (\(P < 0.001\); Fig. 6B). These results are consistent with the involvement of IP3 receptors in the pacemaker activity of ICC.

**Effects of ryanodine receptor antagonists (tetracaine and ryanodine) on STDs in ICC.** We also tested the effects of ryanodine receptor antagonists on membrane potential and STDs under current clamp (\(I = 0\)). K\(^+\)-rich pipette solution (\textit{solution I}, Table 1) and CaPSS, as the external solution, were used in these experiments. Resting membrane potential (RMP; most negative potentials between STDs) averaged \(-63 \pm 2.1\) mV (Fig. 7, A and C, \(n = 5\)), and STDs with average

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**Fig. 5. Effects of 2-aminoethoxydiphenyl borate (2-APB) on STICs and slow wave currents of ICC.**

A: 2-APB (50 \(\mu\)M) reduced the amplitude and frequency of STICs at a holding potential of \(-80\) mV with external CaPSS solution and pipette solution containing CsCl. B: portion of the trace in A (designated as \(a\) and \(b\)) were expanded to show that slow wave currents were decreased by 2-APB. C and D: summarized effects of 2-APB (50 \(\mu\)M) on STIC amplitude (C) and frequency (D). ***(P < 0.001.**

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**Fig. 6. Effects of xestospongin C on STICs and slow wave currents of ICC.**

A: xestospongin C (350 nM) transiently increased the amplitude and frequency of STICs, then inhibited STICs at a holding potential of \(-80\) mV with external CaPSS solution and pipette solution containing CsCl. B: slow wave currents evoked by stepping from \(-80\) mV to \(-35\) mV (holding potential was \(-80\) mV) were reduced by xestospongin C. B: traces from A (\(a\) and \(b\)) are shown at an expanded time scale. C and D: summarized data showing effects of xestospongin C (350 nM) on average STIC amplitude (C) and frequency (D). ***(P < 0.01.**
amplitudes of 55 ± 3.1 mV (Fig. 7, A and D, n = 5) were recorded during control conditions. Tetracaine (500 μM) depolarized ICC to −34 ± 2.5 mV (n = 10, P < 0.001; Fig. 7, A and C), and the amplitude of STDs decreased to 10 ± 2.3 mV in amplitude and from 24.8 ± 2.4 to 4.6 ± 1.3 cpm in frequency (n = 5, P < 0.001 for both parameters; Fig. 7, A, D, and E). With the magnitude of depolarization caused by tetracaine, the reduction in STDs could have been due to a collapse in the driving force for Cl−. Therefore, during tetracaine application, current was injected to repolarize membrane potential close to the control RMP (approximately −65 mV). Upon repolarization, STDs recovered slightly (i.e., from 10 ± 2.3 to 15 ± 1.8 mV in amplitude and from 4.6 ± 1.3 to 11.8 ± 8.4 in frequency; Fig. 7, B, D, and E).

Ryanodine (50 μM) was also tested, and this compound depolarized cells from −60 ± 1.8 mV to −24 ± 2.2 mV (n = 6, P < 0.001; Fig. 8, A and C). Ryanodine decreased the amplitude of STDs from 60 ± 1.4 to 13 ± 2.0 mV (n = 5, P < 0.001; Fig. 8, B and E) and the frequency from 21.2 ± 2.6 to 10 ± 0.8 cpm (n = 5, P < 0.05; Fig. 8, B and F). When current was applied to repolarize cells close to the control RMP, STDs were restored to some extent (Fig. 8B, n = 5). The amplitude of STDs recovered to 56 ± 1.8 mV; however, the frequency remained significantly reduced at 12.4 ± 1.1 cpm (n = 5, P < 0.01; Fig. 8, E and F). STDs occurring in cells exposed to ryanodine and repolarized by current injection were inhibited by a CaCC blocker, NPPB (100 μM; Fig. 8, B, E, and F). It should also be noted that NPPB also induced hyperpolarization of cells from −64 ± 2.0 mV to −70 ± 1.7 mV (Fig. 8, B and D), suggesting tonic activation of CaCC in these cells. STDs recovered when NPPB was removed (Fig. 8B).

**Effects of CPA and thapsigargin on STDs in ICC.** Results from studies designed to evaluate the importance of Ca2+ stores in pacemaker activity performed on whole GI muscles can be difficult to interpret, because drugs used to affect release of Ca2+ from stores might have indirect effects on slow waves through depolarization or hyperpolarization and it is unclear which cells of the SIP syncytium are affected (30). Therefore, to better relate our cellular studies to conditions in intact muscles, we tested the effects of SERCA pump inhibitors, CPA and thapsigargin, on STDs under current clamp (I = 0). K+–rich pipette solution (solution I, Table 1) and CaPSS, as the external solution, were used in these experiments. RMP of ICC averaged −59 ± 2.5 mV in these experiments. CPA (30 μM) depolarized cells to −36 ± 2.0 mV and reduced the amplitude of STDs from 60 ± 3.5 mV to 21 ± 1.4 mV (n = 6, P < 0.001; Fig. 9, A, C, and D). Thapsigargin (1 μM) depolarized cells from −63 ± 2.6 mV to −18 ± 1.6 mV and inhibited amplitude of STDs from 57 ± 3.9 mV to 7 ± 1.3 mV (n = 7, P < 0.001; Fig. 9, B–D). The frequency of STDs was also reduced by CPA (Fig. 9E) and thapsigargin (Fig. 9F).

**Effects of the IP3 receptor antagonist (2-APB) on STDs in ICC.** The IP3 receptor antagonist, 2-APB, was also tested for effects on membrane potential and STDs under current clamp

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**Fig. 7. Effects of tetracaine on the resting membrane potential (RMP) and spontaneous transient depolarizations (STDs) of ICC in current-clamp mode. A and B:** tetracaine (500 μM) induced depolarization and decreased the amplitude of STDs in ICC. After repolarization by injection of current, STDs did not cause significant recovery of STDs (B). C–E: summarized effects of tetracaine on RMP (C), amplitude of STDs (D), and frequency of STDs (E). Tetracaine 1 describes the initial application of tetracaine (i.e., during I = 0 conditions) and tetracaine 2 indicates the tetracaine effect on STDs during I = C conditions. **P < 0.01; ***P < 0.001.
Fig. 8. Effects of ryanodine on RMP and STDs in ICC under current-clamp. A and B: ryanodine (50 μM) induced depolarization and decreased amplitude of STDs of ICC with external solution (CaPSS) and pipette solution (KCl). After repolarization of membrane potential by injection of current, STDs recovered and were blocked by 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB: B). C and D: summarized data of ryanodine effects on RMP. E and F: summarized data of ryanodine (50 μM) and NPPB (100 μM) effects on STD amplitude (E) and STD frequency (F). Rya1 describes STD during the initial application of ryanodine, during I = 0 conditions, and Rya2 indicates the ryanodine effect on STD, during I-C conditions. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant.

**DISCUSSION**

In this study we showed the importance of Ca^{2+} stores for activation of ANO1 currents that are responsible for STICs, the basic pacemaker activity of ICC, and slow wave currents, the currents resulting from synchronous activation of ANO1 channels, that are responsible for electrical slow waves (14, 43, 53, 54). When drugs known to inhibit SERCA pumps were tested, STICs and slow wave currents were blocked or inhibited. Similar results occurred with 2-APB and xestospongin C, inhibitors of IP3 receptor-operated channels. The effects of 2-APB and xestospongin C were not surprising as a previous study showed absence of slow wave in muscles of Itpr1−/− mice (45). It was interesting to note that dialysis of cells with a solution containing heparin did not inhibit STICs and slow wave currents. We believe that the ineffectiveness of heparin, but effective inhibition of pacemaker currents by membrane-permeable IP3 receptor inhibitors, may be due to localization of IP3 receptors in microdomains not easily accessible to heparin (see discussion of this topic below). An important observation was finding a role for ryanodine receptors in pacemaker activity, because previous studies on intact muscle strips showed no indication that ryanodine receptors have a role in the genera-
tion of slow waves (30, 48). We found that ryanodine and tetracaine significantly reduced STICs and slow wave currents in ICC. Another interesting observation was that store-active drugs caused significant depolarization when applied under conditions of current clamp. At present, the mechanism(s) for the change in membrane potential are not understood. No net inward current was observed under voltage clamp in response to the store-active compounds tested. Thus, the conductance responsible may be an, as yet unrecognized, Ca\textsuperscript{2+}/H\textsubscript{1001} and voltage-dependent inward current expressed by ICC.

Changes in membrane potential make it more difficult to interpret the effects of the store-active drugs on pacemaker activity, because membrane potential itself can affect the currents responsible for STICs and slow wave currents [e.g., by altering the availability of T-type Ca\textsuperscript{2+} channels and the driving force on Cl\textsuperscript{−} ions (52–54)]. Our results also suggest that the compartment into which Ca\textsuperscript{2+} is released to activate ANO1 channels is highly excluded and that cellular localization of ANO1 channels may be restricted, because a general increase in cytoplasmic Ca\textsuperscript{2+} via dialyzing patch-clamp conditions did not activate ANO1 channels tonically.

The question of the underlying “clock” mechanism in electrical rhythmicity in GI muscles has been debated for many years, and it was generally assumed that conditions that affected slow wave frequency would be linked to the mechanism driving pacemaker activity. Experiments performed on whole muscle strips with the sucrose gap method of voltage clamping and prior to the recognition that ICC are the source of pacemaker activity concluded that the underlying mechanism was voltage independent (6, 35). These experiments motivated several years of studies in which drugs, physical conditions, or ionic conditions were tested to determine their effects on slow wave frequency and to probe the clock mechanism responsible for electrical rhythmicity. Now it is understood that the slow wave mechanism includes voltage-dependent components [e.g., conductances for Ca\textsuperscript{2+} entry and/or a mechanism that might enhance IP\textsubscript{3}-dependent release of Ca\textsuperscript{2+} from stores that can affect frequency and propagation of slow waves (48, 52)], making it more difficult to interpret the effects of stimuli that alter membrane potentials in intact muscles. In addition, GI muscles are electrical syncytia in which at least two types of interstitial cells are electrically coupled to smooth muscle cells, forming the functional structure known as the SIP syncytium (40). Conductance changes in any of the SIP cells can lead to changes in membrane potential and the excitability of the other SIP cells.

Previous investigations of store-active drugs on intact GI muscles had no way of determining which cells were affected by the drugs applied, and this was the major motivation for testing these drugs on single, isolated, and identified ICC. There are opposing Ca\textsuperscript{2+}-activated conductances in ICC and PDGFR\textalpha\textsuperscript{+} cells; ANO1 channels generate high current density...
inward currents in ICC, and SK3 channels generate high current density outward currents in PDGFrα+ cells (23, 53). Both conductances are activated by release of Ca2+ from intracellular stores [(2); and current study], and intracellular recordings from these cells in intact muscles demonstrate opposing voltage-responses that can summate in the cells of the SIP syncytium (19). Smooth muscle cells also express a variety of ionic conductances that are regulated by Ca2+ (21) and would also be likely to contribute to the integrated effects of manipulating Ca2+ stores in the SIP syncytium. Thus, altering Ca2+ uptake into stores, deactivation of the Ca2+ stores’ ability to regulate cytoplasmic Ca2+ concentration, or blocking Ca2+

Fig. 10. Effects of 2-APB on RMP and STDs of ICC in current-clamp mode. A, 2-APB (50 μM) reduced the amplitude and induced depolarization of STDs of ICC with external solution (CaPSS) and pipette solution (KCl). B–D: summarized effects of 2-APB (50 μM) on RMP (B), STD amplitude (C), and STD frequency (D). **P < 0.01; and ***P < 0.001.

Fig. 11. Effects of different concentrations of [Ca2+]i on ANO1 currents. ICC dialyzed with solutions containing different concentrations of Ca2+ were held at −80 mV and stepped to potentials from −80 mV to −35 mV. Large-amplitude slow wave currents (ANO1 current) were activated by depolarization, and no evidence of tonic activation of ANO1 (as observed in HEK-293 cells) was observed even when high cells were dialyzed with 1 μM Ca2+. A–C: traces are representative of 3 cells each dialyzed with [Ca2+]: <10 nM (low; A), 500 nM (B), or 1 μM (C). Changing [Ca2+] over this range did not affect the need for voltage-dependent activation of slow wave currents. D: summarized data of peak slow wave currents elicited at concentrations of [Ca2+].
release from stores could have complicated and nonlinear effects on membrane potential and pacemaker activity. For example, CPA and thapsigargin both reduced the frequency of slow waves in the mouse small intestine; however, the effects of CPA were accompanied by depolarization while thapsigargin did not cause significant effects on membrane potential (30). CPA also caused substantial depolarization and abolition of STDs in strips of guinea pig pyloric circular muscle (48).

Most investigators have recognized the importance of IP3 receptors in slow wave generation since it was shown that slow waves were absent in Ipprl−/− mice (45) and xestospongion C was shown to block spontaneous electrical rhythmicity (30, 50). These findings were supported by the use of 2-APB, another inhibitor of IP3 receptors (31). 2-APB reduced frequency and eventually blocked slow waves in guinea pig antral muscles; however, these effects were accompanied by about 9 mV depolarization of cells (12). Depolarization, noted in the responses of whole muscles to this drug, also occurred in single ICC in response to 2-APB. Our data support a role for IP3 receptors in generating STICs and slow wave currents in ICC because these currents were blocked by 2-APB and xestospongion C. However, it should also be noted that nonspecific effects have been reported for 2-APB, including inhibition of store-operated Ca2+ (SOC) entry, for example, in pancreatic acinar cells at a concentration of 30 μM (5). The role of SOC entry in pacemaker activity of ICC is yet to be investigated, but since SOC entry may be a factor in refilling Ca2+ stores, blockade of this mechanism could affect the ability of ICC to sustain STICs and slow wave currents.

The role of ryanodine receptors in generation of slow waves has been controversial. For example, caffeine has complicated effects on intact muscles of the murine small intestine, causing transient hyperpolarization and then eventual marked depolarization and loss of slow waves (30). Ryanodine, at 50 μM, caused depolarization and reduced the frequency of slow waves, but the effects of ryanodine in intact muscles could not be distinguished from the effects of depolarization, and it was concluded that ryanodine receptors have little role in regulating slow waves (30). Results from experiments on gastric muscles also suggested that STDs were insensitive to ryanodine (48).

Our voltage-clamp experiments suggest a role for ryanodine receptors in the pacemaker activity of ICC, because STICs and slow wave currents were inhibited by ryanodine and tetracaine. However, it should be noted that an initial increase in STICs was observed upon addition of ryanodine, and then STICs were blocked after the initial stimulation. Both agents also reduced slow wave currents. The initial enhancement in STICs may have been due to the concentration-response effects of ryanodine on Ca2+ release channels; studies have shown activation of ryanodine receptors at low concentrations (<10 μM) and block at higher concentrations (33). Thus, as ryanodine entered cells after its addition to the bath, there might have been an initial phase of increased Ca2+ release before channels were blocked. No such phenomenon was observed with tetracaine, however, another inhibitor of Ca2+ release from ryanodine receptors (51). Under current clamp the responses to ryanodine and tetracaine were more complex in that both compounds caused significant depolarization. We repolarized cells to the approximate level of the most negative potentials before addition of the drugs to determine whether the reduction in STDs was due to a reduction in the driving force on Cl− ions. The block of STDs was retained upon repolarization; however, STDs were partially restored by repolarization in the presence of ryanodine. This observation may have something to do with the block imposed on ryanodine receptors by these antagonists, but these experiments underscore the problems with analyzing the effects of store-active drugs under conditions in which membrane potential is at liberty to change.

Ryanodine receptors are an attractive element for models of the slow wave mechanism in ICC because they readily display the property of Ca2+-induced Ca2+ release (44). ICC also express T-type Ca2+ channels (52), which have been suggested as the Ca2+ entry mechanism that converts stochastic STICs into synchronized slow wave currents that can propagate actively through ICC networks (41). IP3 receptors also display properties of Ca2+-induced Ca2+ release in the presence of IP3 (8, 15). Thus, release of Ca2+ by one type of receptor could be amplified by Ca2+ released by the second type of receptor. The exact mechanisms of interaction between ryanodine receptors and IP3 receptors and the sequence of activation in ICC are not yet understood.

Microdomains are a fundamental feature of Ca2+ signaling in a variety of cells (3), and such structures (referred to as pacemaker units in ICC) appear to be a critical structural component of the pacemaker activity of ICC (41). There is ample ultrastructural evidence for close associations between cisternae of ER and the plasma membranes of ICC that could create the microdomain of pacemaker units (22, 38, 39). Ionic concentrations in microdomains may not reflect the concentrations of ions within the greater cell cytoplasm. For example, dialysis of HEK-293 cells transfected with the AC splice variant of Ano1 with 100 nM Ca2+ activated large-amplitude Ca2+-activated Cl− currents (CaCC) in our experiments (Fig. 4), but dialysis of ICC, which also express ANO1 channels, with high concentrations of Ca2+ did not tonically activate CaCC. Depolarizing steps were still required to activate slow wave currents in cells dialyzed with solutions containing 500 nM or 1 μM Ca2+. We also found that cells dialyzed with solutions containing heparin failed to show progressive inhibition of STICs and slow wave current while membrane-permeable inhibitors of IP3 receptors effectively blocked pacemaker activity. It is possible that heparin was unable to reach the IP3 receptors that deliver Ca2+ ANO1 channels. These observations are consistent with the following concepts: 1) pacemaker units in ICC represent excluded volumes that are functionally independent of cytoplasmic ion concentrations, likely due to vigilant ion pumps and exchange mechanisms guarding against diffusional equilibration; 2) expression of most ANO1 channels must be restricted to areas of the plasma membrane within microdomains; 3) IP3 receptors engaged specifically in regulating ANO1 channels may be closely associated with ANO1 channels in microdomains and have limited access to antagonists in the cytoplasm. Microdomain-specific localization of ANO1 channels and a very close association between IP3,R1 and ANO1 channels has been reported in nociceptive sensory neurons in which ANO1 channels are activated by receptor-operated Ca2+ release from stores but insensitive to a global increase in cytoplasmic Ca2+ due to activation of voltage-dependent Ca2+ channels (17). A similar structural arrangement between IP3,R1 and ANO1 might exist in the pacemaker unit microdomains of ICC. It should also be noted that no study to date has succeeded in
Ca2+ dynamics in the pacemaker unit microdomains.

In summary, results from our experiments on isolated and identified ICC support the model of pacemaker activity suggested by Michael Berridge (4) that is supported by experimental evidence from several labs (12, 20, 42, 48). Ca2+ release from endoplasmic reticulum results in transiently elevated concentrations of Ca2+ within pacemaker units under the plasma membrane. Pacemaker units represent microdomains of excluded volume in ICC. The rise in Ca2+, which occurs in a stochastic manner in individual pacemaker units, activates clusters of CaCC (encoded by Ano1), resulting in generation of STICs. STICs are inward currents and cause transient depolarizations (STDs). Transient depolarization synchronizes Ca2+ release in available pacemaker units either through Ca2+ entry and Ca2+-induced Ca2+ release or by effects on IP3 production or the sensitization of IP3 receptors. Synchronized Ca2+ release activates whole cell ANO1 currents (slow wave currents) that can propagate through ICC networks. Other channels and transporters regulating the Ca2+ dynamics in pacemaker microdomains, maintaining suitable ionic gradients, and restoring gradients cycle-to-cycle are yet to be determined.

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AUTHOR CONTRIBUTIONS

M.H.Z., T.S.S., K.E.O., S.D.K., and K.M.S. conceived and designed the experiments; M.H.Z., T.S.S., K.E.O., S.D.K., and K.M.S. performed the experiments; M.H.Z., K.E.O., S.D.K., and K.M.S. analyzed the data; M.H.Z., T.S.S., K.E.O., S.D.K., and K.M.S. drafted the manuscript; M.H.Z., T.S.S., K.E.O., S.D.K., and K.M.S. revised the manuscript; M.H.Z., T.S.S., K.E.O., S.D.K., and K.M.S. approved the manuscript.

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ER AND PACEMAKER ACTIVITY IN ICC


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