

Rapid Report

Pacemaking in interstitial cells of Cajal depends upon calcium handling by endoplasmic reticulum and mitochondria

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1. Pacemaker cells, known as interstitial cells of Cajal (ICC), generate electrical rhythmicity in the gastrointestinal tract. Pacemaker currents in ICC result from the activation of a voltage-independent, non-selective cation conductance, but the timing mechanism responsible for periodic activation of the pacemaker current is unknown.
2. Previous studies suggest that pacemaking in ICC is dependent upon metabolic activity and Ca^{2+} release from intracellular stores. We tested the hypothesis that mitochondrial Ca^{2+} handling may underlie the dependence of gastrointestinal pacemaking on oxidative metabolism.
3. Pacemaker currents occurred spontaneously in cultured ICC and were associated with mitochondrial Ca^{2+} transients.
4. Inhibition of the electrochemical gradient across the inner mitochondrial membrane blocked Ca^{2+} uptake and pacemaker currents in cultured ICC and blocked slow wave activity in intact gastrointestinal muscles from mouse, dog and guinea-pig.
5. Pacemaker currents and rhythmic mitochondrial Ca^{2+} uptake in ICC were also blocked by inhibitors of IP_3 -dependent release of Ca^{2+} from the endoplasmic reticulum and by inhibitors of endoplasmic reticulum Ca^{2+} reuptake.
6. Our data suggest that integrated Ca^{2+} handling by endoplasmic reticulum and mitochondria is a prerequisite of electrical pacemaking in the gastrointestinal tract.

Phasic gastrointestinal (GI) muscles contain pacemaker mechanisms for autorhythmicity that generate electrical slow waves in the absence of external stimuli from nerves, hormones or paracrine substances (e.g. Szurszewski, 1987). It is now recognized that a special group of cells within the tunica muscularis, known as interstitial cells of Cajal (ICC), are responsible for the origination of slow waves (Langton *et al.* 1989; Ward *et al.* 1994; Huizinga *et al.* 1995). Recent studies, using cultured ICC that retain the phenotype of autorhythmicity, have demonstrated that pacemaker currents generated by ICC result primarily from the activation of a non-specific cation conductance (Koh *et al.* 1998; Thomsen *et al.* 1998). Preliminary studies suggest that the pacemaker conductance is voltage independent and may conduct Na^+ and Ca^{2+} in physiological ionic gradients (Koh *et al.* 1998). At present the intracellular signals that regulate the opening of pacemaker current channels are unknown.

The mechanism that initiates slow wave activity has been a subject of investigation for several decades. ICC contain an abundance of mitochondria (e.g. Faussonne-Pellegrini, 1985), but the significance of these organelles to pacemaker function has not been determined. Slow waves may be tied to metabolic activity of ICC, and some investigators have proposed that the ionic mechanism for pacemaker current is regulated metabolically (Connor *et al.* 1976; Prosser & Mangel, 1982; Nakayama *et al.* 1997). Others have suggested that pacemaker activity depends upon Ca^{2+} release from intracellular stores (Liu *et al.* 1995). The link between Ca^{2+} release, metabolic activity and current induction in the plasma membrane, however, remains a mystery. We studied the relationships between Ca^{2+} release from IP_3 receptor-operated stores, Ca^{2+} uptake by mitochondria and initiation of pacemaker currents in cultured ICC from the murine small bowel and in intact GI smooth muscles. These

experiments indicate that integrated Ca^{2+} handling by endoplasmic reticulum (ER) and mitochondria is a prerequisite of electrical pacemaking in the GI tract and provide a novel concept of how pacemaker currents are initiated in ICC.

METHODS

The use and treatment of animals was approved by the Institutional Animal Use and Care Committee at the University of Nevada. Cells were dispersed from murine jejunal muscles and cultured for 1–3 days as described previously (Koh *et al.* 1998). The mice were rendered unconscious with carbon dioxide and killed by cervical dislocation. ICC were identified by live immunostaining for Kit protein (Ward *et al.* 1994; Huizinga *et al.* 1995; Koh *et al.* 1998) using a monoclonal Kit antibody (ACK2) labelled with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). Patch clamp experiments were performed as previously described (Koh *et al.* 1998), except that the equilibrium potential for chloride ions was adjusted to -80 mV.

To monitor mitochondrial $[\text{Ca}^{2+}]_m$, cells were loaded with reduced rhod-2 AM ($4.4 \mu\text{M}$; 50 min at 37°C) prepared according to the manufacturer's recommendations (Molecular Probes) and cultured

for an additional 12–18 h to enhance specific loading into mitochondria (Hajnóczky *et al.* 1995). Some cultures were co-loaded with MitoTracker Green FM (Molecular Probes; 100 nM), a dye which binds covalently to the inner mitochondrial membrane and fluoresces independently of mitochondrial $[\text{Ca}^{2+}]$ and membrane potential thus providing an independent test of mitochondrial localization and specificity of rhod-2 fluorescence.

Time series experiments were performed using a video fluorescence imaging system (Ionoptix Corp., Milton, MA, USA) coupled to a Nikon Diaphot inverted microscope equipped with a $\times 40$, 1.3 NA oil-immersion objective and filter sets appropriate for rhod-2 and Alexa Fluor 488 fluorescence (XF34 and XF23, respectively; Omega Optical, Inc., Brattleboro, VT, USA). Dye-loaded cells were excited with a xenon lamp and an intensified CCD camera collected fluorescence. Video signals were digitized at 5–20 Hz and analysed by IonWizard (version 4.4; Ionoptix) and smoothed using Microcal Origin 4.1 (Microcal Software, Inc., Northampton, MA, USA). Patch clamp and imaging experiments were performed at 30°C .

Confocal imaging was performed with a Bio-Rad MRC 600 (Hercules, CA, USA) coupled to a Nikon Diaphot inverted microscope. Images were acquired with a Nikon PlanApo $\times 60$, 1.4 NA or a Nikon Fluor $\times 100$, 1.3 NA oil-immersion objective using excitation wavelengths of 488 nm (for MitoTracker Green and Alexa Fluor 488) and 568 nm (for rhod-2). Confocal micrographs,

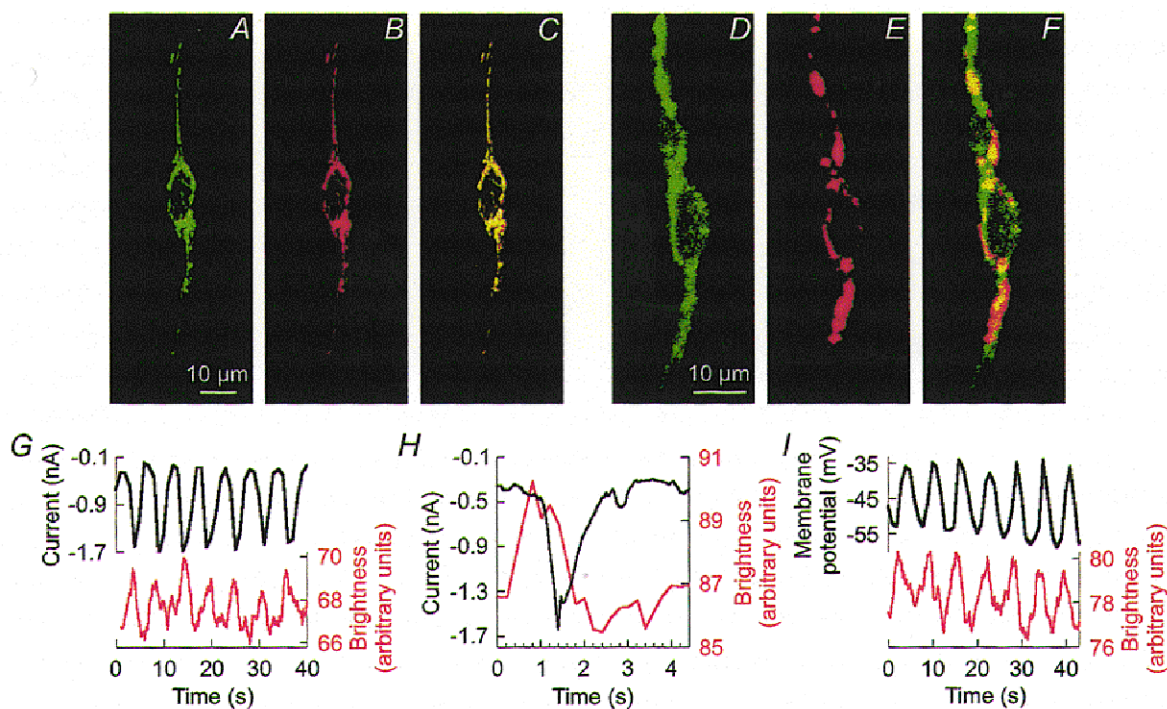


Figure 1. Oscillations in $[\text{Ca}^{2+}]_m$ in mitochondria and pacemaker activity in ICC

A–C, confocal images demonstrating mitochondrial localization of rhod-2. Dense clusters of mitochondria were labelled with MitoTracker Green FM (A) in the perinuclear region and within processes. Rhod-2 fluorescence (B) overlapped with MitoTracker Green FM as indicated by the yellow colour of the overlaid images (C). D–F, representative confocal images of an ICC labelled with an antibody for Kit protein (ACK2) conjugated with a fluorescent marker, Alexa Fluor 488 (D), and loaded with rhod-2 (E). F shows a composite image. Mitochondrial labelling in cells other than the Kit-positive cells was digitally removed in A–F. G–I, representative recordings illustrating the temporal relationship between $[\text{Ca}^{2+}]_m$ oscillations and rhythmic inward currents (G and H) or membrane potential (I). Recordings in G and I were obtained from the same cell. Black traces, pacemaker currents recorded under voltage clamp (-60 mV holding potential) or membrane potential under current clamp (holding current = 0). Red traces, $[\text{Ca}^{2+}]_m$ measured by rhod-2 fluorescence. Note that the increase in $[\text{Ca}^{2+}]_m$ preceded the onset of the inward current (H).

constructed with CoMOS software (version 7.0a; Bio-Rad), are digital composites of Z-series scans of 8–12 optical sections through a depth of 2.8–3.6 μm using a confocal aperture 20% of maximum. Confocal time series experiments were performed using the line scan option of the Bio-Rad MRC 600 (acquisition rate, 4 Hz) and traces were smoothed as described above. All imaging experiments were performed in the presence of 3 μM nifedipine. In most pharmacological experiments the excitation light source was turned off for 10 min after application of drugs to avoid photobleaching and to reduce phototoxicity.

Intracellular electrophysiological experiments were performed on intact muscles from the murine jejunum, guinea-pig antrum and canine proximal colon. Guinea-pigs were killed by the same method as mice, and dogs were killed by a lethal overdose of pentobarbital sodium (100 mg kg⁻¹ i.v.). Strips of jejunum and colon were prepared as previously described (Smith *et al.* 1987; Koh *et al.* 1998). Sheets of antral muscle (1 cm \times 1 cm) from the guinea-pig stomach were pinned in a recording chamber after removal of the mucosa. Impalements of circular muscle cells were made near the submucosal surface of all preparations.

For statistical analyses, various parameters of rhythmic currents, slow waves or mitochondrial Ca²⁺ transients were expressed as a percentage of control. Comparisons were made using Mann-Whitney rank sum test or Kruskal-Wallis one-way ANOVA on ranks followed by multiple comparisons (Dunn's test for comparisons *vs.* control and Dunnett's test for all-pairwise comparisons). $P < 0.05$ was used as the cut-off for statistical significance.

Heparin was obtained from Sigma (St Louis, MO, USA). Antimycin, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), oligomycin and nifedipine (all from Sigma) were dissolved in absolute ethanol (1–50 mM) and further diluted in the superfusion buffer (see Koh *et al.* 1998) to the concentrations indicated in the text. Stock solutions (1–10 mM) of RU-360, rotenone and xestospongine C (from Calbiochem-Novabiochem Corp., La Jolla, CA, USA) and thapsigargin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; from Sigma) were prepared in DMSO.

RESULTS

Small networks and single ICC cultured from the murine small intestine were identified with fluorescent Kit antibody (ACK2-Alexa Fluor 488; see Koh *et al.* 1998). Under voltage clamp, ICC networks displayed spontaneous inward currents averaging -595 ± 37 pA (holding potential, -60 mV) in amplitude and 13.7 ± 0.5 cycles min⁻¹ ($n = 20$). The inward currents were associated with voltage oscillations under current clamp, averaging 22 ± 1.4 mV in amplitude. Spontaneous voltage oscillations in cultured ICC were similar to the electrical slow waves recorded from intact intestinal muscles (Koh *et al.* 1998). Individual ICC also generated spontaneous inward currents, at 13.7 ± 1.0 cycles min⁻¹ and averaging -112.7 ± 32.8 pA in amplitude. As previously reported, pacemaker currents were insensitive to nifedipine (3 μM) and blocked by gadolinium (10 μM) (Koh *et al.* 1998; data not shown).

The relationship between pacemaker activity and mitochondrial Ca²⁺ uptake was investigated in Kit-positive ICC loaded with dihydro-rhod-2. Mitochondrial localization of rhod-2 was verified by co-labelling with MitoTracker

Green FM (Fig. 1A–F). Ca²⁺-dependent fluorescence of rhod-2 was monitored by video or confocal microscopy and revealed rhythmic oscillations in mitochondrial [Ca²⁺]_m ([Ca²⁺]_m) at a frequency of 13.2 ± 0.5 cycles min⁻¹ ($n = 12$; Fig. 1G–J). [Ca²⁺]_m oscillations were insensitive to 3 μM nifedipine. These properties are similar to the spontaneous membrane currents recorded by patch clamp.

The temporal relationship between [Ca²⁺]_m oscillations and pacemaker activity was studied by monitoring rhod-2 fluorescence and membrane currents in eight voltage-clamped and four current-clamped ICC. There was a unitary relationship between [Ca²⁺]_m oscillations and pacemaker currents, and the onset of the [Ca²⁺]_m transients preceded inward currents and voltage transients (Fig. 1G–I).

We tested the relationship between mitochondrial respiration, [Ca²⁺]_m oscillations and pacemaker currents. The mitochondrial uncouplers FCCP (1 μM ; $n = 5$) and

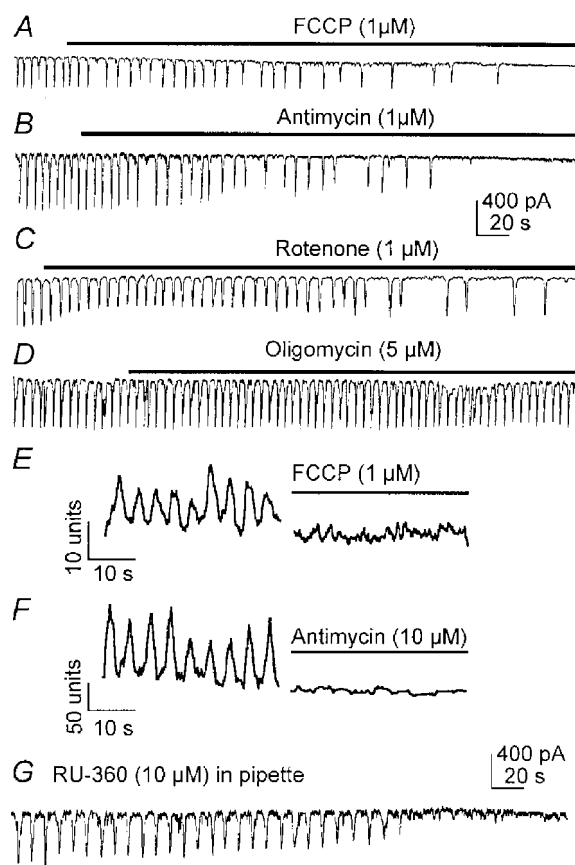


Figure 2. Pacemaker currents in ICC are associated with mitochondrial Ca²⁺ uptake

A–E show spontaneous currents recorded from ICC under voltage clamp conditions (holding potential, -60 mV). FCCP (1 μM , A), antimycin (1 μM , B) and rotenone (1 μM , C) slowed frequency and blocked pacemaker currents. Oligomycin (5 μM , D) had no effect on pacemaker currents. FCCP (E) and antimycin (F) also blocked oscillations in [Ca²⁺]_m. Records of [Ca²⁺]_m show oscillations before addition of drug and 10 min after application of the drug. RU-360 also inhibited spontaneous pacemaker currents in ICC (G).

CCCP ($0.5\text{--}50\ \mu\text{M}$; $n = 10$) initially reduced the frequency of pacemaker currents and then blocked these events (e.g. Fig. 2A). The respiratory chain (complex III) inhibitor antimycin ($1\text{--}3\ \mu\text{M}$; $n = 5$; Fig. 2B) and complex I inhibitor rotenone ($1\text{--}10\ \mu\text{M}$; $n = 6$; Fig. 2C) also inhibited pacemaker currents. It is unlikely that these effects were due to depletion of ATP because the cells were dialysed with $5\ \text{mM}$ ATP, and oligomycin ($5\text{--}10\ \mu\text{M}$), an inhibitor of the F_1/F_0 ATP synthase, failed to block pacemaker currents ($n = 5$; Fig. 2D). FCCP ($1\text{--}10\ \mu\text{M}$; $n = 6$; Fig. 2E) and antimycin ($10\ \mu\text{M}$; $n = 6$; Fig. 2F) also inhibited $[\text{Ca}^{2+}]_m$ oscillations in cells loaded with rhod-2. Oligomycin ($10\ \mu\text{M}$; $n = 2$) did not affect $[\text{Ca}^{2+}]_m$ oscillations (not shown), suggesting that the effects of FCCP and antimycin on $[\text{Ca}^{2+}]_m$ oscillations were not due to depletion of ATP.

Consistent with blocking of pacemaker currents, mitochondrial uncouplers (FCCP and CCCP) and respiratory chain inhibitors (antimycin) also inhibited voltage oscillations in current-clamped ICC (not shown) and in intact muscles from murine small intestine, guinea-pig stomach and canine proximal colon. Intracellular microelectrode recording from circular muscle cells showed that intact muscles generated slow waves at average frequencies of $38.3 \pm 3\ \text{cycles min}^{-1}$ (jejunum; $n = 15$ muscles), $4.6 \pm 0.8\ \text{cycles min}^{-1}$ (stomach; $n = 3$) and $4.9 \pm 1.6\ \text{cycles min}^{-1}$ (colon; $n = 15$). Addition of FCCP ($1\ \mu\text{M}$), CCCP ($1\ \mu\text{M}$) and antimycin ($10\ \mu\text{M}$; with or without oligomycin) inhibited slow wave activity (Fig. 3). In most cases the loss in slow wave activity was

accompanied by a small depolarization of resting membrane potential. For example, canine colon depolarized from a control level of -77 ± 1 to $-67 \pm 1.6\ \text{mV}$ ($n = 15$) and murine jejunal muscles depolarized from -70 ± 2 to $-62 \pm 2\ \text{mV}$ ($n = 15$) in response to FCCP. This level of depolarization has been shown not to block slow wave activity in these muscles (Ward *et al.* 1994). Oligomycin had no effect on slow waves, suggesting the effects of FCCP and antimycin were not simply due to depletion of ATP.

The mitochondrial uncouplers FCCP and CCCP are protonophores that reduce mitochondrial transmembrane potential (Ψ_m ; Farkas *et al.* 1989). Similar effects on Ψ_m have been reported for respiratory chain inhibitors (Schinder *et al.* 1999; Tinel *et al.* 1999). These drugs would therefore reduce the driving force for Ca^{2+} uptake into mitochondria. A link between mitochondrial Ca^{2+} uptake and pacemaker currents was demonstrated by blocking the mitochondrial uniporter with RU-360 (Matlib *et al.* 1998). Dialysis of cells with RU-360 ($1\text{--}10\ \mu\text{M}$) blocked spontaneous inward currents and slow waves (under current clamp) within 2–5 min of cell membrane rupture (Fig. 2G; $n = 4$). Taken together, these data suggest that mitochondrial Ca^{2+} uptake, but not increased ATP production that might result from Ca^{2+} uptake (McCormack *et al.* 1990; Robb-Gaspers *et al.* 1998), is necessary for activation of pacemaker currents.

IP_3 receptor-operated channels in ER are in close apposition to mitochondria, and Ca^{2+} release from IP_3 receptors can

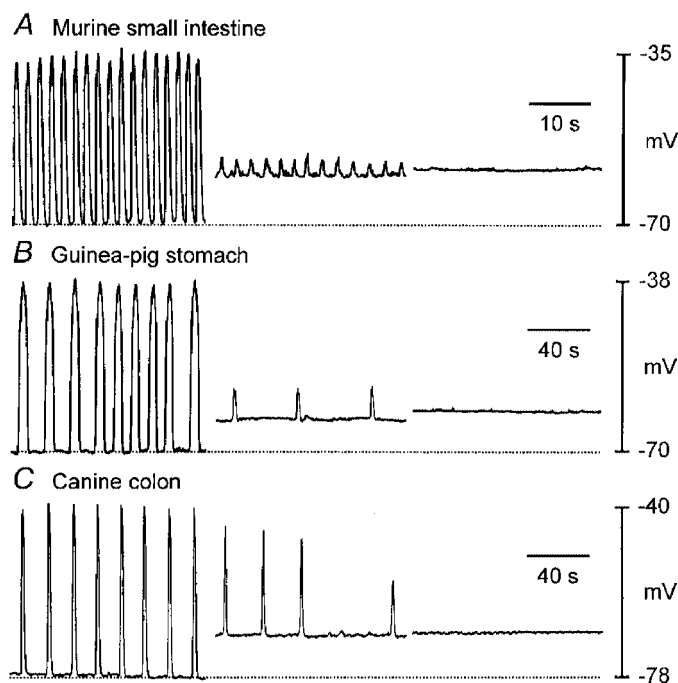


Figure 3. Intracellular recordings showing effects of FCCP ($1\ \mu\text{M}$) on slow wave activity from murine small intestine (A), guinea-pig stomach (B) and canine proximal colon (C)

FCCP inhibited slow waves in all three models of GI electrical rhythmicity. Loss of rhythmicity was associated with small depolarizations in resting potential (dotted lines denote most polarized level between slow waves). Traces are excerpts of continuous recordings from the same cells, with the second and third portions beginning 6 and 12 min in A, 7 and 9 min in B, and 6 and 8 min in C after addition of FCCP to the perfusion buffer.

trigger mitochondrial Ca^{2+} uptake (Sparagna *et al.* 1995; Litsky & Pfeiffer, 1997; Rizzuto *et al.* 1998; Csordás *et al.* 1999). Ca^{2+} uptake into mitochondria occurs via facilitated diffusion through the mitochondrial Ca^{2+} uniporter down a large transmembrane potential gradient (Ψ_m), and some investigators have presented evidence that the uniporter is gated by cytoplasmic Ca^{2+} (Sparagna *et al.* 1995; Litsky & Pfeiffer, 1997). We tested the relationship between Ca^{2+} release from stores and oscillations in $[\text{Ca}^{2+}]_m$. Xestospongine C (1–3 μM), a membrane-permeable blocker of IP_3 receptors (Gafni *et al.* 1997), reversibly inhibited $[\text{Ca}^{2+}]_m$ oscillations ($n = 10$; Fig. 4A). Xestospongine C also inhibited spontaneous pacemaker currents ($n = 3$; Fig. 4B). The involvement of IP_3 receptor-operated stores was also tested with intracellular heparin. When cells were dialysed with pipette solutions containing heparin (250 u ml^{-1}), pacemaker currents and voltage oscillations resulting from pacemaker activity, which were apparent immediately after cell membrane rupture, decreased in frequency and ceased within 2–5 min of breaking into the cells ($n = 4$; Fig. 4C). Before cessation of $[\text{Ca}^{2+}]_m$ oscillations and pacemaker currents, there was a slowing of frequency with IP_3 receptor blockers. In contrast to the dependence of $[\text{Ca}^{2+}]_m$ oscillations and pacemaker currents on IP_3 -dependent Ca^{2+} release, Ca^{2+} release via ryanodine receptors did not appear to participate in the regulation of pacemaker currents. Pacemaker activity was unaffected by ryanodine (50 μM ,

20 min, $n = 6$ cells; 10 μM , $n = 6$ intact muscles; not shown). Treatment with thapsigargin (0.5–1 μM), an inhibitor of Ca^{2+} uptake by the ER, also blocked pacemaker currents ($n = 4$; Fig. 4D). These data demonstrate an important role for Ca^{2+} release from IP_3 receptor-operated stores in initiating Ca^{2+} uptake by mitochondria and activation of pacemaker currents.

DISCUSSION

Our experiments suggest that Ca^{2+} release from Ca^{2+} stores in ICC drives the initiation of pacemaker currents. Using drugs to block specific Ca^{2+} release channels, we have provided data that Ca^{2+} release from IP_3 receptor-operated stores (and not ryanodine receptor-operated stores) is linked to initiation of pacemaker current. It is well known that Ca^{2+} release from ER is capable of oscillatory and even regenerative activity (e.g. Berridge, 1993), and factors such as Ca^{2+} pump (SERCA pump) activity and luminal ER Ca^{2+} regulate the frequency of Ca^{2+} release events (Petersen *et al.* 1993; Koizumi *et al.* 1999). We also found that blockers of Ca^{2+} uptake into the ER (i.e. SERCA pump inhibitors) inhibited spontaneous pacemaker currents suggesting that cycling of Ca^{2+} into and out of the ER may be the primary oscillator regulating pacemaker activity.

A second major finding of this study was the requisite integration between ER Ca^{2+} release and mitochondrial

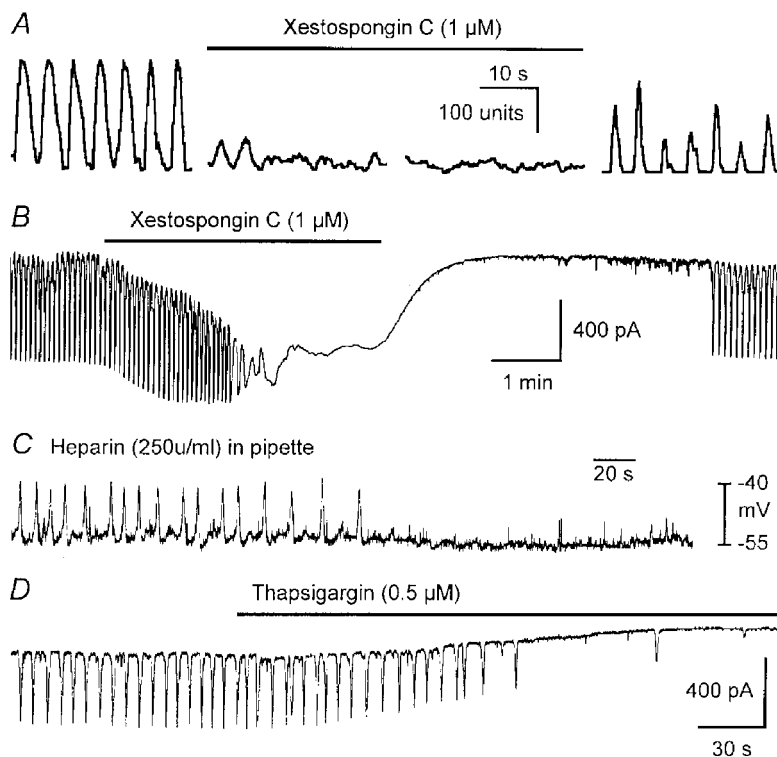


Figure 4. Relationship between Ca^{2+} stores and oscillations in $[\text{Ca}^{2+}]_m$ and pacemaker currents

A shows oscillations in $[\text{Ca}^{2+}]_m$ before (left trace), during (middle two traces) and after (right trace) exposure to xestospongine C (1 μM). B shows that pacemaker currents were also reversibly inhibited by xestospongine C (1 μM). C shows pacemaker activity (recorded under current clamp) inhibited by dialysis with heparin (250 u ml^{-1}). Thapsigargin (0.5 μM) also inhibited pacemaker currents (D).

function in the initiation of pacemaker activity. Several drugs known to inhibit mitochondrial respiration or to collapse Ψ_m inhibited spontaneous pacemaker currents. Treatment with mitochondrial uncouplers and respiratory chain inhibitors has been shown to decrease Ψ_m and Ca^{2+} uptake (Farkas *et al.* 1989; Schinder *et al.* 1999; Tinel *et al.* 1999). We found that both classes of drugs blocked the spontaneous oscillations in $[\text{Ca}^{2+}]_m$ that were associated with pacemaker currents. Inhibition of ATP production was apparently not responsible for inhibition of pacemaker activity because: (i) cells dialysed with 5 mM ATP were not protected from inhibition of pacemaker activity by mitochondrial uncouplers and respiratory chain inhibitors; (ii) oligomycin did not affect spontaneous pacemaker currents; (iii) glycolytic pathways were left intact when cells were treated with mitochondrial uncouplers and respiratory chain inhibitors; and (iv) there was no hyperpolarization or outward current noted in ICC due to activation of ATP-dependent K^+ channels (present in ICC and activated by reduced cytoplasmic ATP; S. D. Koh & K. M. Sanders, unpublished observations).

Reduction of the electrochemical gradient across the mitochondrial membrane affects Ca^{2+} uptake. Imaging of mitochondrial rhod-2 fluorescence showed oscillations in $[\text{Ca}^{2+}]_m$ at the same frequency as pacemaker currents. In experiments in which $[\text{Ca}^{2+}]_m$ was monitored in voltage-clamped cells we found that the $[\text{Ca}^+]_m$ oscillations preceded the onset of pacemaker currents. We also found that dialysis of cells with RU-360, an inhibitor of the Ca^{2+} uniporter, inhibited pacemaker currents. These data suggest that Ca^{2+} uptake by mitochondria may be an important link in the initiation of pacemaker currents. How mitochondrial Ca^{2+} uptake is coupled to activation of non-selective cation channels in the plasma membrane is not understood at the present time, but dynamic changes in Ca^{2+} in microdomains near ER, mitochondria and the plasma membrane could be important.

The importance of mitochondrial function in electrical slow wave generation has been considered in previous studies of intact GI muscles. Prosser & Mangel (1982) proposed an elaborate model for slow waves in which mitochondrial ATP production was regulated by mitochondrial Ca^{2+} uptake. These authors believed that oscillations in Na^+ pump activity were the mechanism for slow waves (see Connor *et al.* 1974), and oscillations in ATP production were considered as a means of driving Na^+ pump oscillations. Tomita and coworkers showed that cyanide inhibited slow wave activity in guinea-pig stomach muscles (Huang *et al.* 1993), and iodoacetate, an inhibitor of glycolysis, also inhibited slow waves (Nakayama *et al.* 1997). In a more recent study they concluded that the inhibitory effect of iodoacetate may have been due to accumulation of metabolites rather than block of ATP production (Ogino *et al.* 1999). All of these studies suggested that ATP was the major link between mitochondrial function and slow waves. The present study

suggests that ATP (while obviously important for restoration of ionic gradients and resequestration of Ca^{2+} into the ER) is not the primary link between mitochondrial function and pacemaker activity. Our data are more compatible with the idea that localized changes in Ca^{2+} concentration may be important in regulating pacemaker current channels. Future studies will be required to more clearly characterize the channels responsible for pacemaker currents and to discover the intracellular messenger(s) that regulate these channels before it will be possible to determine the link between mitochondrial Ca^{2+} uptake and pacemaker currents.

In summary, ICC generate pacemaker currents in GI muscles. We propose the following model for activation of the non-selective cationic conductance responsible for pacemaker currents. Activation of pacemaker currents depends upon periodic release of Ca^{2+} from IP_3 receptors. Ca^{2+} release from IP_3 receptors triggers Ca^{2+} uptake by mitochondria, possibly by gating the rapid uptake mode of the uniporter (Sparagna *et al.* 1995; Litsky & Pfeiffer, 1997). Mitochondrial Ca^{2+} uptake is linked in an unknown way to activation of pacemaker currents. The pacemaker cycle may be completed and reset by uptake of Ca^{2+} into the ER (i.e. via a thapsigargin-sensitive Ca^{2+} pump). Uptake and periodic release of Ca^{2+} from IP_3 receptor-operated stores appears to be the main oscillatory process responsible for GI autorhythmicity. Thus the pacemaker mechanism is a highly integrated process requiring physical proximity and coordination between ion channels and transport proteins in the ER, mitochondria and plasma membrane. Uncouplers of mitochondrial proton transport and respiratory chain inhibitors blocked slow waves in several intact GI muscles suggesting this mechanism may be commonly employed to generate pacemaker activity in visceral smooth muscles.

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