ICC pacing mechanisms in intact mouse intestine differ from those in cultured or dissected intestine

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Boddy, Geoffrey, Alicia Bong, WooJung Cho, and E. E. Daniel. ICC pacing mechanisms in intact mouse intestine differ from those in cultured or dissected intestine. Am J Physiol Gastrointest Liver Physiol 286: G653–G662, 2004. First published December 4, 2003; 10.1152/ajpgi.00382.2003.—Pacing of mouse intestine is driven by spontaneous activity of a network of interstitial cells of Cajal in the myenteric plexus (ICC-MP). So far, highly dissected circular muscle (CM) strips from control and mutant mice lacking ICC-MP and isolated, cultured ICC from newborn control mice were used to analyze its properties. Using intact circular and longitudinal segments of intestine, we recently reported that there were both significant similarities and differences between pacing studied in segments and from isolated, dissected tissues. Here, we report additional similarities and differences in our model from those in highly reduced systems. Similar to cultured or dissected intestine, blockade of sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\) pumps with thapsigargin or cyclopiazonic acid reduced pacing frequency, but thapsigargin was less effective than in isolated, cultured ICC. Moreover, inhibition of inositol 1,4,5-trisphosphate (IP\(_3\)) receptors with xestospongin C, a putative inhibitor of IP\(_3\) receptors, failed to affect pacing but successfully blocked increased pacing frequency by phorbol ester. 2-Aminothoxy-diphenylborate, a putative blocker of IP\(_3\)-mediated calcium release, caused a significant decrease in the amplitude and frequency of contractions. The mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone blocked pacing and KCl-induced contractions at a concentration of 1 \(\mu\)M. The cyclic nucleotide agonists sodium nitroprusside (SNP), forskolin, and 8-bromo-cGMP inhibited pacing in CM. In longitudinal muscle (LM), SNP and forskolin had little effect on pacing. Furthermore, dibutyryl-cAMP did not affect pacing in CM or LM. These results suggest that pacing in intact intestine is under partly similar regulatory control as in more reduced systems. However, pacing in intact intestine is not affected by xestospongin C, which abolishes pacing in isolated, cultured ICC and exhibits attenuated responses to thapsigargin. Also, major differences between LM and CM suggest a separate pacemaker may drive LM.

Interstitial cells of Cajal; intestinal pacing; sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\) pump block; xestospongin C; 2-aminoethoxy-diphenylborate

MOTILITY OF THE GASTROINTESTINAL tract is achieved by the coordinated contractions of both circular (CM) and longitudinal muscle (LM). These muscles are paced by a network of cells located in the myenteric plexus called interstitial cells of Cajal (ICC-MP), which are derived from the same mesenchymal precursors as LM (21, 29, 41, 42). ICC-MP produce characteristic, spontaneous cyclic depolarizations termed slow waves (8, 18, 20, 28). Slow waves are produced in ICC located throughout the gastrointestinal tract, except in esophagus, gastric fundus, and some sphincters. ICC-MP, located in the myenteric plexus, are thought to be the main ICC required for smooth muscle pacing in the stomach and small intestine (12, 13, 38). ICC are also found within the deep muscular plexus (ICC-DMP) and in some regions intramuscularly (ICC-IM). ICC-IM help to provide a secondary regenerative component of the slow wave in gastric antrum, and there is some suggestion that ICC-DMP may function as a secondary pacemaker to the ICC-MP in small intestine (7, 8, 10). ICC-MP are coupled through gap junctions and form networks that run along both the LM and CM (5, 6).

It is assumed that slow waves initiated by ICC are spread electrotonically through gap junctions into adjacent smooth muscle cells (8), but no structural gap junctions have been found between ICC and smooth muscle in mouse intestine (5, 6, 34). Depolarization of the muscle opens voltage-gated calcium channels that lead to an influx of calcium and muscle contraction. Pacing in cultured ICC has been reported to be driven by calcium cycling between the endoplasmic reticulum (ER), mitochondria, and extracellular space (11, 22, 32, 37, 39). Generation of a slow wave appears to involve the coupling of inositol 1,4,5-trisphosphate (IP\(_3\))–dependent calcium release from the ER with extracellular calcium influx through transient receptor potential (TRP)-like channels and mitochondrial calcium uptake (22, 32, 35, 37, 39). The frequency of slow waves may be regulated also by the enteric nervous system through the release of excitatory ACh and inhibitory nitric oxide (NO).

The majority of work done on the gastrointestinal tract has been done on cultured or dissected CM of mouse or canine intestine and guinea pig antrum (12, 30). However, examination of the applicability of these data to intact intestines remains an important question. We have demonstrated that pacing in undisseminated segments of mouse intestines after nerve block is similar in several respects to the pacing observed in cultured or dissected mouse intestines (4). Both exhibit TTX and L-type Ca\(^{2+}\) channel independence, high temperature dependence, jejunum faster than ileum, and in W/W\(^{v}\) intestine, lacking ICC-MP, CM is poorly active. However, there are also some differences. In our intact muscle preparations, LM pacing is faster than CM from adjacent sites, nicardipine decreases the frequency of contractions, and LM segments of W/W\(^{v}\) mice exhibit robust, regular pacing activity.

Our aim was to determine whether there are further similarities and differences between our model and other more reduced intestinal models. In particular, we evaluated whether the model of pacing dependent on calcium handling between the ER and mitochondria in ICC corresponds to the situation in vivo.

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MATERIALS AND METHODS

Preparation of Tissue

Male, 6- to 12-wk-old BALB/c or W/V mice (from Jackson Laboratories) were killed by cervical dislocation following the guidelines of our institutional Animal Ethics Committee. After the opening of the abdominal wall, the digestive tract beginning from the level of the stomach to the rectum was removed from the mouse. The tissue was immediately placed into a beaker of Krebs-Ringer solution that had been bubbled at room temperature (21–22°C) with carbogen (95% O₂ and 5% CO₂) for ~5 min before the dissection. In a petri dish filled with Krebs solution, continuously bubbled with carbogen, ileal (considered to be the distal third of the intestine) or jejunum (considered to be half of the intestine and located proximal to the ileum) tissue was isolated and cut into ~0.5 (for CM) or 1–1.5 cm (for LM) segments in preparation to mount onto glass holders with electrodes.

To study LM contractions, a tissue segment was placed between two platinum concentric circular electrodes and tied to a hook at the bottom of the electrode holder with silk suture thread. The top of the tissue was also tied with thread and attached to a strain gauge. To study CM contractions, one side of a thin metal triangle was slid through the lumen of the tissue segment. The triangle was then hooked together. A stainless steel rod attached to the bottom of the electrode holder was inserted into the lumen of the tissue under the metal triangle. Suture thread, attached at the apex of the triangle opposite to tissue, was tied to the strain gauge. Two thin platinum rods, situated parallel to and on either side of the tissue, were used to stimulate the tissue electrically.

Two longitudinal and two circular preparations were mounted and placed into muscle baths, filled with 10 ml Krebs solution, and bubbled continuously throughout the experiment with 95% O₂-5% CO₂ and maintained at a temperature of 37°C. The thread connected to each tissue segment was then tied to a strain gauge (GRASS FT-03). The tissues were subjected to slight tension just sufficient to remove slack in the thread and allowed to stabilize for 10 min. Tissue contractile activities were recorded on a Beckman Dynograph 611.

Experimental Protocol

The tissues were electrically field stimulated (EFS) to test nerve activity (parameters: 50 V/cm, 0.5 ms, and 5 pulses/s). LM always responded with contraction, whereas CM usually responded with relaxation. TTX (10⁻⁶ M) and N-Nitro-1-arginine (L-NNA, also known as L-NOARG; 10⁻⁴ M) were added to the bath to eliminate enteric nerve function and NO production. Within 1 min, CM contraction amplitudes increased. After 5 min, nerve activity was tested again by EFS. If there was any response in the tissue, TTX was added again until all enteric nerve function was blocked. The frequencies of slow wave-driven contractions were measured before and after TTX and L-NNA. After nerve activity was blocked by TTX and L-NNA, the experiment was carried out using the pharmacological agents under investigation. In some experiments, EFS was carried out at 50 V/cm, 50- or 100-ms pulses, and 60 Hz to pace segments electrically. At the end of each experiment, all tissues were washed twice with 10 ml Ca²⁺ free Krebs to relax tissues to basal passive tension and abolish spontaneous contractions.

Experimental Procedures

Several types of procedures were used, and they are described in depth in RESULTS. In general, for each experiment, one LM and one CM segment was used as an experimental tissue and another of each as a time control. Frequency and amplitude measurements were made every 5 min. Frequencies were measured over a period of at least 20 s. Contractions were regular and stable in time controls over time (see RESULTS). Amplitudes of contractions were determined as the values above the passive tension determined at the end of the experiment. They were measured by evaluating individual contractions over at least nine contractions and calculating a mean and a standard error. In many cases, only the data before and after final measurements for each experimental intervention are presented. In the case of contraction amplitudes, the results were normalized to the mean values in the control periods, set as 100%. The n values represent the number of mice whose intestine provided segments for study.

Analysis of data. Measurements were entered into Graph Pad Instat and analyzed by paired comparison, using Student’s paired t-test or ANOVA as appropriate. Figures were plotted in Prism 3.

Immunohistochemistry

Immunofluorescent labeling for cryosection. For cryosection, the jejunum was fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) overnight at 4°C. The fixed tissue was washed in PBS for 30 min × 4, cryoprotected in graded sucrose solution (10%, 20% sucrose in PBS) for 2 h each, placed in 30% sucrose in PBS for 24 h at 4°C, and was then stored at −80°C until used. For whole mount preparation, the jejunum was fixed as mentioned above. The fixed tissue was washed in PBS for 30 min × 4, dehydrated and cleared in DMSO for 10 min × 3, and was rehydrated in PBS for 15 min × 4 at room temperature.

Frozen jejunum was sectioned by a cryostat (Leitz 1720 digital cryostat) to make sections of 10-μm thickness. The sections were attached on slide glasses coated with 2% 3-aminopropyltriethoxysilane (Sigma A3648) in acetone and were dried for at least 1 h or overnight at room temperature. The sections were washed in PBS containing 0.4% Triton X-100 (TBS) for 15 min × 3. To reduce nonspecific binding proteins, the sections were blocked with 10% normal sera that were raised in the host of secondary antibody for 1 h at room temperature. For immunohistochemistry, primary antibodies, Na⁺/Ca²⁺ exchanger 1 (NCX1; obtained from Dr. J. Lytton, Univ. of Calgary), and c-kit, mouse anti-CD117 (Zymed) were incubated for 18-19 h at 4°C. The sections were washed in TBS for 15 min × 3. Secondary antibodies, immunoglobulins conjugated with Cy3 and F1TC were incubated for 1 h at room temperature. The sections were washed in TBS for 15 min × 2 and then were washed in PBS for 15 min × 1. The sections mounted with aquamount medium were observed with confocal laser scanning microscope (CLSM; LSM 1500, Zeiss).

Immunofluorescent labeling for whole mount preparation. Muscle layers, CM and LM, of the jejunum were separated from the mucosa and the submucosa layer under the dissection microscope. The muscle layers were vigorously washed in TBS for 15 min × 4 on orbital shaker and were then blocked with 10% normal sera that were raised in the host of secondary antibody for 1 h at room temperature. For immunohistochemistry, primary antibodies examined in the immunofluorescent labeling for cryosection were incubated for 48 h at 4°C. The muscle layers were washed in TBS for 15 min × 4. For immunofluorescent labeling, secondary antibodies, immunoglobulins conjugated with Cy3 and FITC, were incubated for 2 h at room temperature. The muscle layers were washed in TBS for 15 min × 3 and then were washed in PBS for 15 min × 1. The muscle layers mounted with aquamount medium were observed with CLSM.

Drugs and Solutions

Pharmacological agents. TTX and tanspargin were from Alomone Laboratories (Jerusalem, Israel), L-NNA, sodium nitroprusside (SNP), cyclopiazonic acid (CPA), and nicardipine were from Sigma-Aldrich (Oakville, ON). Forskolin, xestospongin C, PMA, and 2-aminoethoxy-diphenylborate (2-APB) were from Calbiochem-Neo- vibiochem (San Diego, CA). SEA0400 (22) was a gift from TAIHO Pharmaceutical (Toshino-ku, Tokyo, Japan). Because some drugs were dissolved in DMSO, the solvent was added to time-control tissues in equivalent amounts. The final concentration of DMSO did not exceed 0.1% and had no significant effects on control tissues. DMSO was from Sigma-Aldrich.

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**Solutions.** Krebs solution was composed of (in mM) 115.5 NaCl, 21.9 NaHCO₃, 11.1 glucose, 4.6 KCl, 1.16 MgSO₄·H₂O, 1.16 NaH₂PO₄·H₂O, and 2.5 CaCl₂·H₂O. Ca²⁺-free Krebs solution was composed of (in mM) 115.5 NaCl, 21.9 NaHCO₃, 11.1 glucose, 4.6 KCl, 1.16 MgSO₄·H₂O, 1.16 NaH₂PO₄·H₂O, and 1.0 EGTA.

**RESULTS**

**Effects of Thapsigargin and Nicardipine**

The current theory of ICC pacing suggests that IP₃-dependent release from calcium stores is required for proper pacing (22, 37, 39). Therefore, blockade of the sarcoplasmic-endoplasmic reticulum Ca²⁺ (SERCA) pumps should affect normal pacing. We have previously demonstrated in intact mouse intestine that CPA slows both LM and CM pacing to one-half. However, reports have suggested that CPA also acts to depolarize smooth muscle cells in addition to its action on SERCA pumps (22). Thapsigargin, another inhibitor of SERCA pumps, was used to confirm that the effects of CPA were due to its inhibition of SR Ca²⁺ ATPases. Because many experiments on smooth muscle are done in the presence of L-type Ca²⁺ channel blockers, 1 μM nicardipine was added to one of the two segments of LM or CM before application of 1 μM thapsigargin. We previously reported (4) that nicardipine (1 μM) slightly but significantly reduced the frequency of contractions and decreased the amplitude of contractions to about one-half control values after 10 min in CM and LM segments. The application of 1 μM thapsigargin caused a decrease in the frequency of CM from 45.0 ± 1.4 to 32.0 ± 3.1 contractions/min after 20 min (n = 6, P ≤ 0.001). Frequency decreased in LM from 48.0 ± 1.1 to 39.5 ± 1.2 contractions/min (n = 6, P ≤ 0.05). Thus LM contraction frequencies were only reduced slightly. These decreases were less than those caused by CPA (4) and comparable with that observed after CPA only in CM.

Thapsigargin, similar to CPA (4), also caused an increase in the amplitude of LM contractions to 206.5 ± 85.7% of control (n = 6); however, this was not significant. Nicardipine before thapsigargin caused a greater decrease in CM frequencies and amplitudes than thapsigargin alone and prevented any increase in contraction amplitudes in LM. Figures 1B and 2 show the effects of nicardipine and thapsigargin. Figure 1A demonstrates the stability of pacing in CM and LM over time.

**Effects of CPA and SEA0400**

In addition to the SR Ca²⁺ ATPases, the NCX is another important calcium-handling protein. SEA0400, a novel inhibitor of NCX (22), was added to one of two segments of CM or LM before application of CPA. SEA0400 (3 μM) did not have any significant effect on frequencies or amplitudes of contraction when added. However, prior treatment with 3 μM SEA0400 blocked the increase in tone and amplitude in LM after 10 μM CPA (Fig. 3). SEA0400 (3 μM) did not affect the response of CM, which does not usually increase tone in response to 10 μM CPA. The lack of effect of block of Na⁺/Ca²⁺ exchange on ICC-driven contraction frequency might result from its absence from ICC-MP or smooth muscle.

**Immunohistochemistry for NCX1**

The expression of the NCX was confirmed using immunohistochemistry. NCX1 was found in both LM and CM as well as in the ICC of the myenteric plexus. ICC cells were also stained with antibodies for c-kit and exhibited double staining for NCX1 and c-kit (Fig. 4).

**Effects of Xestospongin C and PMA**

As previously mentioned, IP₃-dependent calcium stores are assumed to be required for ICC pacing. Their blockade with xestospongin C (0.5–1 μM), a presumed membrane-permeable, noncompetitive IP₃-receptor antagonist, has been found to block pacing in cultured ICC and dissected smooth muscle. In the current study, the application of xestospongin C (1–3 μM) to intact preparations for 30 min did not affect LM or CM contraction frequencies or amplitudes (n = 8, 1 μM; n = 2, 3 μM). To determine whether xestospongin C was penetrating the tissue, PMA was added to stimulate IP₃ production. Application of 100 μM PMA caused a significant increase in contraction frequency in LM [from 57.5 ± 1.2 to 70.5 ± 2.9 contractions/min (n = 6, P ≤ 0.05)] and CM [from 51.5 ± 2.1 to 66.0 ± 3.4 contractions/min (n = 6, P ≤ 0.01)], which was successfully blocked with prior application of 1 μM xestospongin C for 30 min (n = 6; Fig. 5).

**Effect of 2-APB**

Despite having numerous actions, including block of store-operated Ca²⁺ entry and SERCA pumps as well as others (2, 3, 19, 26, 36), 2-APB has been widely used to study the role of IP₃-mediated calcium release. It is described sometimes as an inhibitor of IP₃-receptor functions and sometimes as an inhibitor of Ca²⁺ release from SR. In dissected tissues, it has been found to abolish slow waves in canine colon (37), guinea pig distal colon (23), and murine cecum and proximal colon (9). In addition to our undissected segments of mouse intestine, 2-APB caused a concentration-dependent reduction in contraction frequency and amplitude in both LM and CM (Fig. 6). After 10 min exposure to 100 μM 2-APB, contractions in two
of seven CM and zero of seven LM were completely blocked. Amplitudes were reduced to 34.6 ± 3.2 (n = 6, P = 0.0001) and to 40.4 ± 8.5% (n = 7, P = 0.01) of control values in CM and LM, respectively. Frequencies dropped from 51.0 ± 2.4 to 21.4 ± 6.3 contractions/min in CM (n = 6, P = 0.01) and from 58.0 ± 2.6 to 34.5 ± 3.2 contractions/min in LM (n = 7, P = 0.001). 2-APB similarly reduced frequencies of LM segments of W/W mice (not shown).

Effect of Carbonyl Cyanide p-Fluoromethoxyphenylhydrazone

Mitochondrial uncouplers carbonyl cyanide p-fluoromethoxyphenylhydrazone (FCCP) and carbonyl cyanide m-chlorophenylhydrazone have been found to inhibit slow waves in cultured ICC and dissected CM (39). FCCP inhibited contraction frequency and amplitude in LM and CM, primarily at 1 μM FCCP, which reduced contraction frequencies to 27.3 ± 14.7 and 17.1 ± 11.5% of control in CM (n = 8, P = 0.001) and LM (n = 10, P = 0.0001), respectively. Amplitudes were reduced to 0.2 ± 0.2% of control in CM (n = 5, P = 0.01) and 1.9 ± 1.4% of control in LM (n = 6, P = 0.001). To determine whether these effects were due to an action on mitochondrial calcium uptake in ICC, KCl-induced contractions, which are independent of ICC, were measured before and after FCCP. Application of 1 μM FCCP reduced KCl-induced contractions to 22.1 ± 3.3 and 10.9 ± 3.9% of control in CM (n = 6, P = 0.01) and LM (n = 6, P = 0.0001), respectively (Fig. 7).

Effect of Cyclic Nucleotides

ICC slow waves and intestinal smooth muscle contraction frequency are reported to be, in part, modulated by NO and cyclic nucleotides (16). We evaluated whether our experimen-
The model is under the same regulatory influences as the several reduced models. Application of the NO donor SNP (100 μM) caused a significant reduction in the frequency and amplitude of CM contractions over 10 min (Fig. 8, A and B). After 10 min, amplitude dropped to 55.9 ± 8.7% of control (n = 10, P ≤ 0.001), and frequency was reduced from 55.7 ± 1.6 to 38.3 ± 1.5 contractions/min (n = 10, P ≤ 0.01). In LM, 100 μM SNP slightly reduced frequency over 5 min, significantly reduced amplitude within the first minute, and significantly increased amplitude after 5 min (Fig. 8, A and B). The effects of SNP on LM were not significant after 10 min (n = 10).

To determine whether the effects of SNP were via cGMP production, a membrane-soluble analog of cGMP was added to the muscle baths. 8-bromo-cGMP (100 μM) caused a significant reduction in contraction frequency from 43.8 ± 1.6 to 25.2 ± 6.6 contractions/min in CM (n = 5, P ≤ 0.05) and from 51.9 ± 3.5 to 43.9 ± 2.4 contractions/min in LM (n = 7, P ≤ 0.05) after 10 min.

The effect of cAMP was also examined using forskolin, a putative activator of adenylate cyclase and dibutyryl (db)-cAMP. Application of 1 μM forskolin caused a reduction in CM contraction frequency from 54.0 ± 2.3 to 21.7 ± 7.1 contractions/min after 10 min (n = 10, P ≤ 0.01; Fig. 8C). It had no significant effect on LM contraction frequency. After 1 μM forskolin, amplitudes were reduced to 30.2 ± 16.6 and 27.0 ± 4.3% of controls in CM (n = 10, P ≤ 0.01) and LM (n = 10, P ≤ 0.0001), respectively (Fig. 8D). The application of the membrane permeable cAMP analog, db-cAMP (100 μM), caused no significant reduction in contraction amplitude or frequency in LM (n = 6) or CM (n = 5) over 10 min and longer intervals.

**DISCUSSION**

We have developed a simple model of pacing in mouse intestine for LM as well as CM, and we have previously demonstrated that this model has similar properties expected of ICC-paced contractions: ileum slower than jejunum, inhibited but not abolished by nicardipine, reduced in frequency by CPA, abolished by Ca^{2+}-free media, and high temperature dependence (4). In this study, we showed that pacing in mouse intestine is influenced by agents that disrupt the calcium-handling abilities of ICC and smooth muscle, similar to other reports in mouse intestine. However, there were also some differences: pacing was rarely blocked completely, no response to xestospongin C, FCCP may have blocked due to actions independent of ICC such as on KCl-induced contractions, and there were major discrepancies between LM vs. CM pacing. The reason for these differences is unclear.

IP_{3}-mediated calcium release is reported to be required for ICC pacing based on studies of mice with their IP_{3} receptor knocked out (32) and on pharmacological studies with isolated ICC and CM strips (18, 22, 39). However, studies done on IP_{3}-receptor type 1 knockout mice have focussed on gastric...
antrum CM and not on intestinal CM or LM (32). It is also not clear whether all of the developmental effects of IP$_3$-receptor knockout have been elucidated. In regard to pharmacological studies with cultured ICC, there is growing evidence that cultured cells have differences in membrane ion-channel conductance and gene expression. Intracellular recordings recently made from ICC-MP in situ exhibit major differences from cultured ICC (15). Unlike cultured ICC, pacemaker potentials from in situ ICC-MP are sensitive to depolarization, although the methods used vary as well. Because many of the pharmacological agents used to demonstrate the role of IP$_3$ receptors in producing slow waves also affect membrane potential, a closer examination of the contribution of IP$_3$ receptors to intestinal smooth muscle pacing is required.

Our results suggest that intracellular calcium stores play a role in ICC pacing, but the role of IP$_3$-induced calcium release is unclear. We have previously demonstrated that CPA reduces pacing frequency in CM and LM by one-half (4), but it has also been found to increase pacemaker potential frequency in cultured ICC (18). This was assumed to be due to CPA’s effect on the SERCA pump. However, CPA may also act to depolarize CM (22) and hyperpolarize cultured ICC (18), suggesting that CPA may have an effect on ion-channel conductances. Compared with CPA, thapsigargin also impaired pacing; however, this was slower in onset and not as effective. Malysz et al. (22) showed that thapsigargin took 400 min to completely abolish slow waves in mouse intestine. We also observed a gradual increase in the effect of thapsigargin over time (20 min); however, after 400 min, the sarcoplasmic reticulum of the muscle may become depleted. In cultured ICC, thapsigargin acted within 2–3 min (39). In that same study (22), xestospongin-C was reported to abolish slow waves at 1 µM, but no

Fig. 5. Effect of xestospongin C and PMA. Xestospongin C (1 µM) or vehicle was applied 30 min before PMA. PMA (100 µM) was applied for 20 min. PMA consistently increased frequency, and the increase was blocked by xestospongin C in CM (A) and LM (B; n = 6). *P ≤ 0.05; **P ≤ 0.01.

Fig. 6. Effect of 2-aminoethoxy-diphenylborate (2-APB) on pacing. Increasing concentrations of 2-APB were added progressively after incubations of 10 min each. A and C: the effect of 2-APB on frequency was concentration dependent. Time controls (left bars) for 10, 30, and 100 µM 2-APB effects after 10 min (right bars). B and D: time controls and effects of 100 µM 2-APB on amplitude in CM and LM (n = 6–7). ****P ≤ 0.0001.
duration of exposure or quantitative data were given, and the figure illustrating the effect showed that small slow waves persisted and that the tissue was depolarized by \( \pm 10 \text{ mV} \). Thus its efficacy on intestine in vitro is unclear.

Clearly, cultured cells are much more susceptible to any drug application, and this may account for some of the differences observed. The effects of CPA and thapsigargin to increase LM contraction amplitude are likely due to direct actions on the muscle, as the corresponding increase in tone suggests that intracellular \( \text{Ca}^{2+} \) concentration \( ([\text{Ca}^{2+}]_i) \) may be raised. This is unlikely to be the result of direct effects on the ICC, because there was no similar effect in CM, believed driven by the same ICC-MP. Blocking L-type \( \text{Ca}^{2+} \) channels or NCX prevented this increase in amplitude. One possibility is that the NCX, when SERCA pumps are inhibited, raising \( [\text{Ca}^{2+}]_i \) near the membrane, may be working in reverse. Although there was a high expression of the NCX in LM, CM, and ICC, its blockade did not affect pacing or contraction amplitudes. Therefore, the NCX may not be active during normal pacing conditions. However, SEA0400 is a new compound that has not been fully characterized (23, 33). It is possible that it is ineffective in blocking the NCX in this tissue. A role for the NCX in pacing cannot be ruled out without further study.

The evidence for a role of IP3 receptors in ICC is extensive (32, 37, 39). Therefore, our failure to replicate this evidence may be due to poor permeability and access of drugs to ICC within intact muscle preparations. Xestospongin C (1–3 \( \mu \text{M} \)) did not affect pacing in our intact segments of intestine over 30 min. However, xestospongin C was successful in blocking PMA-induced increases in contraction frequency, providing evidence suggesting that the compound was successfully blocking IP3-induced calcium release. In any case, this demonstrates that xestospongin C was accessing the tissue successfully. In cultured ICC of mouse intestine, \( 1 \mu \text{M} \) xestospongin C completely abolishes slow waves in a short period of time (<5 min) (39). It is unclear why cultured ICC appear to be much more sensitive to manipulation of IP3-sensitive calcium stores than both dissected and undissected muscle. This could be due to the ease with which drugs access cultured ICC or simply due to differences between cultured and in situ ICC. Cultured ICC do not form the same networks found with the intact tissue. Koh et al. (18) have reported that small clusters of ICC have more robust and regular pacemaker currents than isolated cells. In guinea pig ileum, xestospongin C blocks IP3-induced contractions only in permeabilized tissue previously treated with \( \alpha \)-toxin, and in nonpermeabilized tissue, it blocks voltage-dependent \( \text{Ca}^{2+} \) and \( K^+ \) currents (25). Therefore, the variable actions of xestospongin C are likely due to differences in tissue permeability, IP3-receptor inhibition, and/or voltage-gated ion-channel blockade.

Another pharmacological agent used to block IP3-induced calcium release is 2-APB. 2-APB blocks slow waves in canine, guinea pig, and mouse colon (9, 24, 37). In our experiments, 2-APB reduced contraction frequency and amplitude in LM and CM.

Fig. 7. Effects of carbonyl cyanide \( p \)-trifluoromethoxyphenylhydrazone (FCCP) on contraction frequencies and amplitudes in CM and LM. A–D: application of increasing concentrations of FCCP (0.01–1.0 \( \mu \text{M} \)) at 20 min vs. 95% ethanol control at 20 min in CM (A and B) and LM (C and D). FCCP significantly reduced contractions only at 1 \( \mu \text{M} \). E and F: the KCl sustained contraction amplitude of CM (E) and LM (F) treated with increasing concentrations of FCCP (0.1–1 \( \mu \text{M} \)) at 20 min. FCCP significantly reduced KCl-induced contractions at 1 \( \mu \text{M} \) (\( n = 5–10 \)). **\( P \leq 0.01 \); ***\( P \leq 0.001 \); ****\( P \leq 0.0001 \).
release in murine intestinal pacing, but the fact that 2-APB has numerous other actions must be considered. 2-APB has been found to inhibit IP3-induced calcium release, block store-operated calcium influx, decrease mitochondrial calcium uptake, activate calcium permeable cation channels, inhibit voltage-gated potassium currents, and inhibit the SERCA pump (2, 3, 18, 26, 36). So far, no evidence proves that it acts primarily on IP3-mediated Ca2+/H11001 release in ICC. For this reason, we are unable to confirm a role for IP3-induced calcium release in ICC-driven pacing of intestinal smooth muscle. Further study needs to be done to determine which actions of 2-APB account for its effects on ICC and smooth muscle.

Mitochondrial calcium uptake is proposed to be required for ICC pacing, because slow waves are sensitive to mitochondrial uncouplers and respiratory chain inhibitors (37, 39). We also found that FCCP, a mitochondrial uncoupler, inhibited pacing in a concentration-dependent manner in LM and CM. However, KCl-induced contractions, which are independent of ICC, were also impaired. It is therefore possible that the effect of FCCP may be due, at least in part, to an action on the muscle and not the ICC. Application of oligomycin, an inhibitor of ATP synthesis, fails to block ICC pacemaker currents or CM slow waves (39). Our results suggest that proper mitochondrial function is required for successful pacing in undissected intestine; however, there is also evidence that FCCP may impair the muscle’s ability to contract.

To determine whether our model is under the same regulatory control as the previously mentioned reduced models, we applied a number of cyclic nucleotide agonists or analogs. We found that SNP, 8-bromo-cGMP, and forskolin all inhibited CM pacing. The membrane-permeable analog of cAMP, db-cAMP, did not affect pacing in CM or LM. These results are consistent with previous experiments done in mouse intestine (17). However, this is the first time this work has been done in murine LM, and our results in LM differed from those in CM. Pacing in LM was insensitive to forskolin and only slightly reduced by SNP and 8-bromo-cGMP. These experiments suggest that pacing in LM and CM are under different regulatory
control. Previous experiments (17) have demonstrated a direct effect of SNP, 8-bromo-cGMP, and forskolin on ICC pacemaker currents. If in fact these currents are pacing the LM, then pacing in LM should have been similarly affected. A possible explanation for this discrepancy is that LM is paced by a separate pacemaker. Evidence for this comes from the many differences found between CM and LM pacing: response to SNP and forskolin, LM faster than CM, and robust LM pacing in W/W\(^v\) mice that is unresponsive to CPA and persists after L-type Ca\(^{2+}\)-channel blockade (4). It is possible that LM retains some pacemaker ability throughout development, because LM and ICC originate from the same mesenchymal progenitor cells (16, 29). Although LM and CM exhibit many differences in pacing, there are also many similarities. We suggest that LM and CM are paced by the same network of myenteric ICC, but LM may rely on its own pacemaker ability when ICC-driven pacing is compromised.

In summary, pacing in undissected intestine is not as dependent on calcium handling between the ER and mitochondria as in cultured and dissected intestine. This is likely due to reduced permeability and the presence of intact networks of muscle and ICC. We propose that pacing is not limited to one pacemaker system and that LM may be driven by a separate pacemaker to CM. Evidence for this is found throughout the literature: LM of guinea pig distal colon produces rhythmic depolarizations (slow waves?) even after separation from myenteric plexus and CM (31). Synchrony between myenteric ICC and LM is not always observed (40), and there are also reports of two pacemakers within the rat colon (27). However, similarities between CM and LM pacing suggest that if a secondary pacemaker current is indeed present, it can be driven in a similar manner by ICC-MP if they are present. LM and CM pacing in undissected mouse intestine is sensitive to SERCA inhibitors, 2-APB, FCCP, and the cyclic nucleotide agonists SNP, forskolin, and 8-bromo-cGMP but insensitive to the IP\(_3\)-receptor inhibitor xestospongin C.

REFERENCES


