Role of gap junctions in structural arrangements of interstitial cells of Cajal and canine ileal smooth muscle

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Daniel, Edwin E., Yu-Fang Wang, and Francisco S. Cayabyab. Role of gap junctions in structural arrangements of interstitial cells of Cajal and canine ileal smooth muscle. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G1125–G1141, 1998.—We examined the structural and functional basis for pacemaking by interstitial cells of Cajal (ICC) in circular smooth muscle of the canine ileum. Gap junctions were found between ICC of myenteric plexus (MyP), occasionally between MyP ICC and outer circular smooth muscle cells, between individual outer circular smooth muscle cells, between them and ICC of the deep muscular plexus (DMP), and between DMP ICC. No visible gap junctions connected MyP ICC to longitudinal muscle cells or inner circular muscle cells. Occasionally contacts occurred between the two muscle layers. No special structures were found to connect MyP and DMP ICC networks. Octanol concentration dependently reduced the amplitude and frequency of, but did not abolish, slow waves in circular muscle in isolated ileum recorded near the MyP or the DMP. Slow waves triggered from MyP ICC by a current pulse also persisted. Contractile activity was abolished, cells were depolarized, and fast inhibitory junction potentials were reduced by octanol. We conclude that ICC pacemakers of the MyP and DMP utilize gap junctional conductances for pacemaking function but may not require them. Coupling between the two ICC networks may utilize the circular muscle syncytium.

Pacemaking and neural activity in a slab of canine ileal muscularis externa, either intact or without longitudinal muscle and myenteric plexus (MyP), has recently been studied in our laboratory (5–7, 20, 21). Both the MyP region and the deep muscular plexus (DMP) of circular muscle were found to be capable of initiating pacemaker activity (slow waves and concomitant contractions) in circular muscle. The wave forms initiated at the two sites differed; those from MyP usually consisted of a fast upstroke from a stable baseline followed by a plateau before repolarization, whereas those from the DMP consisted of a triangular or sinusoidal wave form. In the intact slabs, the wave form from the MyP dominated, spreading to cells close to the DMP, which, in isolated circular muscle, would have shown slow waves of the DMP configurations (5, 20). Slow waves from the MyP also dominated in the sense that they were able to drive DMP pacemaking activity, and premature slow waves triggered by a single 50- to 100-ms square wave depolarization or after an inhibitory junction potential (IJ P) resembled those initiated near the MyP and appeared to be initiated at the MyP and to spread to the DMP (5, 6). In isolated circular muscle, neither IJ Ps nor long-duration pulses triggered slow waves.

After inhibition of IJ Ps by TTX or \( \text{N}^6\text{-nitro-L-arginine (L-NNA)} \), the same pulse trains that initiated IJ Ps caused immediate triggering of slow waves. \( \alpha \)-Conotoxins abolished IJ Ps but not the delay before a triggered slow wave; subsequent L-NNA did abolish the lag before a triggered slow wave following a stimulus train after \( \alpha \)-conotoxin had abolished IJ Ps (6, 7). The conotoxin-insensitive source of nitric oxide (NO) was not identified. Not only endogenous but also exogenous NO appeared to modulate pacemaking activity, hyperpolarizing cell membranes and decreasing the amplitude and increasing the frequency of slow waves (7). In isolated circular muscle, in contrast, block of endogenous NO synthase by L-NNA enhanced slow wave amplitude.

Slow waves were shown to be dependent on extracellular Ca\(^{2+}\), but neither \( \text{L}- \) nor \( \text{N}-\text{Ca}^{2+}\) channel blockers affected slow waves significantly. However, \( \text{Ni}^{2+} \), a nonselective \( \text{Ca}^{2+}\) channel blocker, reduced slow wave frequency and amplitude and prolonged their duration (5). Slow waves were also affected by inhibition of the sarcoplasmic reticulum \( \text{Ca}^{2+}\) pump with cyclopiazonic acid, suggesting that intracellular \( \text{Ca}^{2+}\) levels in pacemaking cells influence slow waves.

These data are consistent with a model in which networks of interstitial cells of Cajal (ICC), known from studies in other regions of the intestine to be located in the MyP (4, 11, 33, 42, 43) or in the DMP (13, 14, 18, 32, 34, 42–45), can provide pacemaking activity either independently or in a coupled fashion, with the network in the MyP dominant when both plexuses are present. Moreover, these networks of pacemakers are readily modulated by NO from adrenergic or cholinergic nerves and possibly from the ICC themselves (7, 30, 35).

The structural basis for such a model has not been reported in canine ileum. There is, however, clear evidence of extensive gap junction contact between ICC of DMP and the outer circular muscle in the upper intestine of dogs and several other species (14, 18, 32, 33, 42–45). In electron microscopic studies of mouse intestine (3, 42, 43), ICC of the MyP were found to be closely innervated and to have gap junctions between them; however, gap junctions were not seen between ICC and longitudinal muscle or between ICC and circular muscle. ICC of DMP are coupled by gap junctions to the outer circular muscle, as in the canine upper intestine (14, 18, 45). In canine colon (3, 4), ICC of the MyP and the submucosal border are closely innervated, coupled to one another extensively only at the latter locus, and in MyP ICC are coupled to both longitudinal and circular muscle by rare gap junctions.
However, in this tissue, pacemaking activity of the ICC of MyP and those at the inner border of circular muscle differ (36–38, 43), and the circular muscle slow waves are dominated by the activity generated at the submucosal border. The ICC network in the MyP and pacemaking in the longitudinal muscle appear to function partially independently of the activity generated at the submucosal ICC network. Nevertheless, evidence suggests that the two muscle layers are coupled based on both spread of electrical activity (15, 37, 38) and spread of dye injected into one cell of a given layer (15).

In the rabbit intestine (8, 40), the two sets of pacemakers appear to be tightly coupled, but the two muscle layers are not, i.e., electrotonic currents injected into one layer spread only within that layer, as do the IJP initiated only within circular muscle. In this tissue, it was proposed that ICC of the MyP drive both layers, but an independent pacemaking role for ICC of the DMP was not evaluated (8).

Thus it is unclear what structures provide for coupling or dominance between networks of ICC and the muscle layers and between the pacemaking networks themselves. In the canine colon, there appears to be no special pathway between the MyP and the submuscular ICC plexuses (4). In this tissue and in intestine (3, 18), some ICC are located within the body of circular muscle, but there is currently no evidence that a string of such ICC couple the two networks.

The objectives of this study were to evaluate, using ultrastructural analysis, 1) whether in the canine ileum ICC of the MyP are joined to one another and to the two muscle layers by visible gap junctions, or if the two muscle layers are directly connected; 2) the possible existence of a special structural basis for coupling between ICC networks or MyP and DMP; and 3) the relationships between ICC of DMP and inner and outer circular muscle. We also examined the effects of a gap junction coupler on intestinal slow waves and IJPs recorded from near MyP or DMP to evaluate the possible relationship between the observed frequencies of gap junctions and the dependencies of electrical events on them.

MATERIALS AND METHODS

Preparation of Tissues for Electron Microscopy

Seven unselected mongrel dogs (20–50 kg) were anesthetized with pentobarbital sodium (30 mg/kg iv). The animals were cared for in accordance with the principles of the Canadian Council on Animal Care (Guideto the Care and Use of Experimental Animals, vols. 1 and 2). From each dog, a terminal ileal artery was locally perfused, first with Krebs-Ringer solution and then with 2% glutaraldehyde in 0.075 M sodium cacodylate buffer, pH 7.4, containing 4.5% sucrose and 1 mM CaCl2. Just before perfusion fixation, each dog was killed by injection of pentobarbital sodium (100 mg/kg iv). The perfused segment was isolated with ligatures, and the draining vein was severed. After 5 min of initial perfusion fixation, the ileal segment was removed, opened, pinned flat in a petri dish to a silicon-rubber mat, mucosa side up, and immersed into the same fixative. The mucosa and submucosa were carefully dissected, and well-fixed longitudinal strips (1.5 × 10 mm) were prepared and immersed in the fresh fixative for an additional 4 h at room temperature. After fixation, all tissues were washed overnight in 0.1 M sodium cacodylate buffer containing 6% sucrose and 1.24 mM CaCl2 (pH 7.4) at 4°C, postfixed with 2% OsO4 in 0.05 M sodium cacodylate buffer (pH 7.4) at room temperature for 90 min, stained with saturated uranyl acetate for 60 min at room temperature, dehydrated in graded ethanol and propylene oxide, and embedded in Epon 812 or Spurr. To locate suitable areas, sections 0.5 μm thick were cut and stained with 2% toluidine blue. After examination of the toluidine blue-stained sections, ultrathin sections were cut, mounted on either 200-mesh grids or 400-mesh Ultra Light transmission grids (Marivac, Halifax, NS), and double stained with uranyl acetate and lead citrate. The grids were examined in a J EOL-1200 EX Biosystem electron microscope at 80 kV or in a Phillips 301 electron microscope at 60 kV.

Preparation of Ileal Strips for Electrophysiological Study

Tissue Dissection. Healthy adult mongrel dogs of either sex, ranging from 10 to 25 kg, were euthanized using intravenous pentobarbital sodium (100 mg/kg). This procedure was approved by the McMaster University Animal Care Committee. The abdomen was immediately opened along the midline, and a segment of ileum (10 cm) was removed from a position about 10 cm oral to the ileocecal junction. The dissection was made at room temperature in normal oxygenated Krebs solution.

The segment of ileum was cleaned of external fat and connective tissue and opened along the mesenteric border. The mucosa and submucosa were removed, taking care not to damage the circular muscle. The longitudinal muscle was also removed in the isolated circular strips using the same technique already described (5, 7, 9, 20, 21). Electron micrographs of this preparation confirmed that the longitudinal muscle and the MyP were completely removed and the DMP undamaged.

Tissue strips (1 × 10–15 mm) were cut parallel with the circular muscle fibers and placed in a 5-ml organ chamber for electrophysiological recordings. A small portion of each strip was meticulously pinned to the floor of the chamber to immobilize regions to be used for recording of intracellular electrical activity. About 1 cm of unpinned region was connected to a force transducer for recording of mechanical activity. This unpinned region was stretched by 2 g once, and the whole preparation was allowed to equilibrate for 2–3 h before impalements were attempted. The strips were superfused with normal Krebs at a rate of 3 ml/min (37°C). The Krebs solution (in mM: 115.5 NaCl, 1.6 NaH2PO4, 21.9 NaHCO3, 4.2 KCl, 2.5 CaCl2, 1.2 MgSO4, and 11.1 glucose) was continuously aerated with 95% O2-5% CO2 to maintain pH of ~7.4. Glass electrodes filled with 3 M KCl with resistances ranging from 30 to 80 MΩ were used to impale the cells. Membrane potential changes were measured using a standard electrometer (World Precision Instruments KS-700). The signal was monitored on a dual-beam oscilloscope (Tektronix D13; SA22N differential amplifier; 5B12N dual time base) and recorded on 1/4-in. magnetic tape with a Hewlett-Packard Instrumentation Recorder and on chart paper (Gould 2200). A microscope (M3C, Wild Leitz) with a calibrated eyepiece graticule was used to select accurately the position of the recording electrode. The electrical activity was studied in the following areas of the circular muscle: 1) near the MyP (0–10% of the total width close to the longitudinal muscle) and 2) near the DMP (60–90% of the width from the MyP). The number of strips from at least three different animals...
used for each type of experiment is indicated by n, and a total of 12 animals were used in this study. When octanol was applied to the bath, it was left in contact with the tissue for 15–20 min or more, but the reported data were obtained after 15–20 min.

**Electrical Field Stimulation**

Electrical field stimulation (EFS) was applied using a pore-type silver electrode in contact with the tissue on one side of the strip, and a silver ground electrode on the other side. Stimuli were provided by a Grass S88 stimulator through a stimulus isolation unit (Grass SIU5). A range of parameters was used to obtain the maximal IJP in each strip. The pulse rate was 25–30 pulses/s, the train duration was 300 ms, and supramaximal voltage was 120–150 V, with 0.3- to 0.4-ms pulses. Obtaining a typical IJP recording ensured the integrity of the neural networks.

**Recordings and Statistical Analysis**

The resting membrane potential; frequency, duration, and amplitude of slow waves; and the durations and amplitudes of IJPs were analyzed for each record. Triggered slow waves were differentiated from spontaneous slow waves by their occurrence, advanced occasionally or delayed in time relative to the expected occurrence of the next spontaneous slow wave. Also, when recorded in regions with slow waves characteristic of the DMP region, the triggered slow wave had a different configuration, typical of slow waves from the MyP. These parameters were analyzed during the control period (20 min) and every 5 min after the infusion of octanol (30 min). Frequencies of slow waves were determined by averaging the number of slow waves occurring over a period of 3 min. Data are means ± SE. Ordinary ANOVA (with Bonferroni correction) or Student’s t-tests, as appropriate, were performed to check for statistical significance. Mean values were considered significantly different when P < 0.05.

**RESULTS**

**Structural Relationships in MyP Region and Between ICC and Muscle Layers**

In the MyP, the ICC had typical structures, as shown in Fig. 1. ICC in this plexus had some typical features (see Fig. 1), such as cell bodies with several long processes, nuclei that filled a large fraction of the cell body, and with dispersed heterochromatin except at the periphery, Golgi apparatus, and rough and smooth endoplasmic reticulum. Caveolae were more common in processes; basal laminae were sparse or absent. Also present were actin and intermediate filaments, microtubules, condensed mitochondria that were elongated in appropriate sections, and occasional secondary liposomes. ICC were close to nerve profiles. Dense bodies were very rare and myosin filaments were not observed.

In nonganglionated regions of the MyP, ICC cell bodies with nuclei were present at intervals of 100–300 µm (Fig. 2). The cell bodies of ICC in this region were sometimes nearly devoid of caveolae but had them on processes (Fig. 2B). They also had sparse and incomplete basal lamina (Fig. 2, B and inset) and a very thin layer of cytoplasm around the nucleus. These features and their close relationships to smooth muscle and sometimes to nerve profiles and the lesser arrays of rough endoplasmic reticulum distinguished the ICC from fibroblasts. These cell bodies were found more frequently in contact with the circular muscle layer (Fig. 2) but were less commonly juxtaposed to the longitudinal muscle layer or located between the muscle layers, away from close contact with muscle layers (Figs. 3A and 6B). Several putative ICC near or in close contact with nerve axons compared with ICC in deep muscular plexus (DMP) (see Fig. 8). Scale bar, 200 nm.
contact with other ICC (labeled “ICC?” in Fig. 3A) or with longitudinal smooth muscle cells (labeled fibroblast-like in Fig. 4B and ICC in Fig. 6B) could not be definitively identified as ICC, since they lacked or had questionable caveolae. This was one qualitative distinction between fibroblasts and ICCs. This may imply that more than one type of ICC exists in MyP, fibroblast-like ICC and typical ICC.

The ICC near the two muscle layers were presumed to be connected by the numerous bundles of ICC processes (Fig. 5B and Fig. 7) found intermediate between the two muscle layers. A complete connection across the plexus was not observed in a single thin section. However, short processes from ICC cell bodies in several instances connected them to circular muscle by small gap junctions (Fig. 2A) or gap junction-like connections (Fig. 2B). Those ICC near circular muscle were frequently near nerve bundles (Fig. 2A). Longitudinal and circular muscle were occasionally close to one another (Figs. 3B and 6A) but were usually separated by nerve and ICC profiles and were never connected by visible gap junctions.

Near the longitudinal muscle, putative ICC cell bodies were less frequent than near the circular muscle, were irregularly present, and sometimes lacked detectable caveolae, similar to fibroblasts, as noted above. However, they had otherwise similar structures (Figs. 3A, 4B, 6B, and 7B). Processes of ICC and putative ICC were commonly found near longitudinal muscle (Figs. 4A and 5B; Fig. 6 and Fig. 7). These were often found as bundles of processes, sometimes connected by gap junctions (Fig. 5 and Fig. 6A). ICC and their processes were often near nerve processes and sometimes close to bare nerve profiles (Figs. 3A and 4A). Gap junctions...
junction connections between putative ICC and their processes and longitudinal muscle were not found, but close contacts were (Figs. 4B, 6B, and 7A). Figure 6, inset b, shows the closest approach to a gap junction contact between a putative ICC and a longitudinal muscle cell observed in this study.

ICC were rarely seen to penetrate into the circular muscle layer and never penetrated the longitudinal muscle layer. In those cases where ICC were observed near the MyP inside the circular muscle layer, they were within one or two cell layers of the plexus (not shown). Thus in canine ileum the ICC of the MyP were observed to form a network coupled by occasional gap junctions to the tightly coupled syncytium of the outer circular muscle.

DMP and ICC

The structure of the ICC in this plexus of the canine intestine has been described previously (14, 18), when ICC cells were called “hybrid cells” or “specialized smooth muscle cells” (45). As in those studies, in this study ICC had typical structures, all qualitatively similar to those described in the MyP. Caveolae were more frequent in the cell bodies of DMP ICC compared with MyP ICC, as were very close contacts with nerves.
and lobate nuclei (Figs. 8, 9, and 10). As in earlier studies, ICC were connected by gap junctions to one another (Fig. 9) and to outer but not to inner circular muscle (Fig. 10). They were very frequently within 40–60 nm of bare nerve profiles. ICC were close to cells of the inner circular muscle layer (Fig. 10A), composed of smaller, more densely packed smooth muscle cells (not shown); however, they were never observed to be in gap junction contact with these cells. In addition, although gap junctions were common between outer circular muscle cells, they were never observed between inner circular muscle cells, in agreement with previous reports (12, 14, 18, 43–45).

Connections Between ICC of MyP and DMP

No specialized connections between the two networks of ICC were found. ICC were not present in arrays at septa between circular muscle bundles or within these bundles. ICC were never found branching into the circular muscle from the MyP or DMP. However, many gap junctions were present between outer circular muscle cells (Fig. 2A).

Coupling and Pacemaking in the Canine Ileum

In slabs of canine ileum circular muscle, the effects of octanol on slow waves were recorded after 15–20 min at various sites of the circular muscle. Near the MyP, where slow waves usually arise from a stable baseline and always have a plateau, 0.5 mM octanol significantly reduced slow wave amplitudes by ~20% and also insignificantly inhibited their frequencies by ~11% (Tables 1 and 2). Effects of 0.5 mM octanol on slow wave amplitude seemed greater near the DMP, and slow waves recorded near the MyP remained larger than...
those recorded near the DMP (Table 2). Figures 11B and 12B depict the effects of 0.5 mM octanol on slow wave amplitude and frequency near the MyP and the DMP, respectively. Note also that octanol at this concentration abolished phasic contractions associated with slow waves and reduced or abolished tone. At 1.0 mM octanol, slow waves recorded near the MyP were reduced in frequency by 31% and further reduced in amplitude by 60%, becoming insignificantly different in amplitude from slow waves recorded near the DMP (Tables 1 and 2). Figures 13B and 14B depict the effects of octanol at 1 mM on slow waves recorded near the MyP and DMP, respectively. Note that at 1 mM octanol all contractile activity was lost (Figs. 13 and 14). Table 3 shows that the changes in slow wave frequency and amplitude produced after 0.5 or 1 mM octanol were accompanied by significant depolarization, a change that might have increased the size of hyperpolarization toward the $K^+$ equilibrium potential caused by NO release during the IJP (7, 9).

The fast IJPs and the slow waves triggered after them recorded in circular muscle near the MyP (20) were reduced or abolished by 0.5 mM octanol and abolished by 1 mM octanol (Figs. 11 and 13, A and B). However, after 0.5 mM octanol (Fig. 11B) there was a prolonged hyperpolarization on electrical field stimulation (EFS), and after 1 mM octanol there was a delayed hyperpolarization on EFS (Fig. 13B).
or delayed hyperpolarizing responses were abolished by 0.1 mM L-NNA (Fig. 11C). Sometimes, as in this case, abolition of the hyperpolarization by octanol and L-NNA unmasked an excitatory junction potential. Near the DMP in the control conditions, a fast IJP is usually followed by a slow hyperpolarization (20); both are susceptible to inhibition by L-NNA (7). After 0.5 mM octanol both fast and slow components were reduced in amplitude, despite marked membrane depolarization (Figs. 11B and 12B). After 0.1 mM L-NNA, the residual fast component was abolished in both the MyP and DMP regions (Figs. 11C and 12C), but there was a persistent, small, delayed hyperpolarization in the DMP region (Fig. 12C).

Membrane potentials in both regions, near MyP and near DMP, were affected by both concentrations of octanol (Table 3). As reported earlier (20), there is a membrane potential gradient across the intestinal circular muscle, from about −70 mV near the MyP to −60 mV near the DMP. At 0.5 mM octanol, the membrane potential was reduced by 6.5 mV near the MyP and by 13.3 mV near the DMP, increasing the potential gradient between the two plexuses from 9.7 to 16.5 mV (Table 3). There was no further significant change at any site when 1 mM octanol was used, but the mean values of the decreases at the two plexuses became similar (−8 mV), and the potential gradient did not increase (−7 mV).

Slow waves were triggered or delayed after IJPs but were always triggered after a single long 100-ms square wave (which did not induce an IJP) when the square wave was applied before the expected
arrival of the next slow wave (20). After 0.5 or 1 mM octanol, slow waves were no longer triggered or delayed after IJP (shown for 0.5 mM octanol in Figs. 11B and 12B). However, slow waves triggered by single long-duration pulses still occurred.

DISCUSSION

A recent review summarized the growing evidence that ICC networks in the MyP of the intestine are essential for and associated with pacing of slow waves and that they may play a role in neurotransmission (35). However, there is little structural information about the organization of this network in canine ileum and its coupling to the muscle layers and to the other ICC network in the DMP. The important observations reported here include the following: 1) small, rare gap junctions appear to couple ICC of the canine ileal MyP as in the mouse intestine (42–44), and 2) these cells are occasionally coupled by similar gap junctions on their processes to circular muscle. Outer circular muscle cells are well coupled to one another and to ICC of DMP by numerous gap junctions in other intestinal regions (12, 14, 18, 44–45), and this was confirmed for the ileum. In contrast, longitudinal muscle did not have detectable gap junctions connecting its cells to other muscle cells or to ICC. Cells of this layer did have multiple close appositions to ICC and other muscle
cells, as well as many intermediate contacts with circular muscle cells and ICC. The most significant result was that octanol reduced but did not abolish slow waves near either the MyP or DMP regions. Thus coupling of ICC to circular muscle may utilize but not require gap junctions.

A previous study in canine ileum (23) of the distribution of mRNA gap junction protein, connexin 43 (Cx43), suggested that, despite their absence in ultrastructural studies such as this one, some gap junctions might be synthesized within longitudinal muscle. In contrast to the finding of low levels in longitudinal muscle, an abundance of Cx43 mRNA and Cx43 was found in circular muscle, consistent with the abundance of gap junctions in this layer (23). In our experience, a thin layer of outer circular muscle often cleaves with the longitudinal muscle and the MyP. Therefore, the longitudinal muscle may have been slightly contaminated with Cx43 mRNA from ICC or outer circular muscle gap junctions. Mikkelsen et al. (27) failed to find immunocytochemical evidence of Cx43 in the longitudinal muscle layer. In any case, this muscle layer in canine jejunum did have coupled slow waves, which were coordinated with those of circular muscle (17).

Moreover, the canine colon was recently reported to have slight dye coupling between longitudinal muscle cells, good dye coupling between ICC of MyP and circular smooth muscle, and some dye coupling, all inhibited by 1 mM octanol, between the two muscle layers (15). Ultrastructural studies showed a paucity of gap junctions between smooth muscles near the MyP in canine colon (4).

If the slow waves in both ileal muscle layers are driven by pacemaker activity within the ICC network of the MyP, as we suggest (20), there must be adequate coupling both between ICC and to muscle cells of each layer. So far, no experimental evidence suggests that close appositions or intermediate contacts can provide such coupling. However, an alternate explanation to coupling through low-resistance contacts is field coupling (see Refs. 1, 10, 11, 30, and 39 for review), where the existence of extensions of processes of longitudinal smooth muscle cells within adjacent cells (18), separated by intermediate contacts, may provide the physical basis (1).

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network would leak away into the circular muscle and could not be controlled independently if the network were tightly coupled to circular muscle syncytium through low-resistance pathways (22, 30). Coupling at intervals along the outer circular muscle through small gap junctions with significant impedance might reduce the current required from ICC to induce voltage change and drive slow waves in coupled circular muscle (see Ref. 43 for a similar suggestion).

The cardiac sinus node may provide an example (22, 28); it has few gap junctions and poor coupling of cells within the central portion of the node and appears to be protected from the hyperpolarizing current of the atrium by the limited coupling between the two regions. In the canine ileum there may be a hyperpolarizing input from the region of the MyP to the circular muscle. When octanol was applied (this study) or the MyP was dissected away from the isolated circular muscle (20), outer muscle cells had a lower membrane potential.

Previously we showed that the circular muscle of the canine ileum, isolated from the MyP, also produced regular slow waves, perhaps driven by the ICC network of the DMP, which differed in configuration from slow waves paced from the MyP (20). We also found evidence suggesting that in the intact muscularis externa both pacemakers influenced slow wave configuration in their vicinity, but that the pacemaker of the MyP was dominant (5, 7). We need to consider whether the structural arrangements reported here are consistent with these findings, as well as the observation that slow waves could be triggered by electrical excitation of the intact muscularis but not of the isolated circular muscle. These triggered slow waves, which can pace circular muscle contractile activity, everywhere resembled in configuration those recorded near the MyP in the intact muscularis (5, 7, 20). Does the presence of a few gap junctions between ICC of MyP and a few intermittently spaced gap junctions between MyP ICC and circular muscle explain why this network of ICC seems to
dominate that in the DMP? Our earlier ultrastructural studies in duodenum (14, 18) and our studies in ileum show that ICC of the DMP are coupled by abundant and large gap junctions to one another and to circular muscle cells. Gap junctions of ICC in the MyP are scarcer and smaller. The DMP ICC network may have such excellent coupling that it functions as a syncytial unit with circular muscle. Such a large syncytium would require a much larger depolarizing stimulus to be activated, because of the larger total capacitance of its membranes. This may cause various electrical

Fig. 10. A: gap junction (arrow) between a process of an OCM cell and an ICC process. Note bare axons very close (20–50 nm) to ICC. Note also intermediate contacts between ICC profile and ICM. Scale bar, 200 nm. B: two ICC bodies in gap junction contact (arrows) with OCM. Arrowhead shows a gap junction between two cells of OCM. In these two ICC bodies, note unusual lobate nuclei (n). No gap junctions were found between any ICC and ICM. Scale bar, 200 nm.

Table 1. Octanol effects on frequencies of slow waves

<table>
<thead>
<tr>
<th></th>
<th>Near MyP</th>
<th>n</th>
<th>Near DMP</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.5 ± 0.3</td>
<td>4</td>
<td>8.9 ± 0.7</td>
<td>4</td>
</tr>
<tr>
<td>0.5 mM Octanol</td>
<td>7.6 ± 0.6 (NS)</td>
<td>4</td>
<td>6.4 ± 0.9 (NS)</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>9.3 ± 0.45</td>
<td>7</td>
<td>8.8 ± 0.6</td>
<td>7</td>
</tr>
<tr>
<td>1.0 mM Octanol</td>
<td>6.4 ± 0.6†</td>
<td>7</td>
<td>7.2 ± 0.4*</td>
<td>7</td>
</tr>
</tbody>
</table>

Control 21.0 ± 1.4 4 12.5 ± 2.8* 4
0.5 mM Octanol 16.6 ± 2.1† 4 6.5 ± 2.0*† 4
Control 18.8 ± 1.7 7 14.3 ± 0.1* 3
1.0 mM Octanol 7.5 ± 1.1† 7 6.5 ± 2.25‡ 3

Data are means ± SE. MyP, myenteric plexus; DMP, deep muscular plexus. *Significantly different from control (P < 0.05). †Significantly different from control (P < 0.01). NS, not significantly different from control.

Data are means ± SE. *Significantly different from values recorded near MyP (P < 0.05). †Significantly different from control (P < 0.01). ‡Significantly different from control (P < 0.01).
stimuli, which appear to trigger slow waves from the MyP, to fail to trigger slow waves from the isolated circular muscle (5, 7, 20). Reduced gap junction density leading to reduced electrical coupling of circular muscle to ICC of the MyP compared with ICC of the DMP may isolate its pacemaker network, enabling it to function autonomously and to respond to electrical stimulation.

Additional factors may contribute to the observed heterogeneity of ileal slow waves in circular muscle. As we have shown elsewhere (5, 7, 20), the membrane potentials of the circular muscle cells near the MyP are more polarized than membrane potentials of cells near the DMP. Also, the upstroke velocity of the MyP slow wave is greater. Finally, there appears to be a persis-

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**Fig. 11.** Recordings made near MyP and effects of 0.5 mM octanol and 0.1 mM N\(\text{G}\)-nitro-L-arginine (L-NNA). A: control slow waves and triggered slow waves induced after an inhibitory junction potential (IJP; curved arrow; left) induced by electrical field stimulation (EFS) and after a single 100-ms square wave (curved arrow; middle). Spontaneous slow waves at slower recording speed are also shown (right); note association of a contraction with each slow wave. B: after 0.5 mM octanol was applied for 15 min the fast IJP was inhibited and replaced by an excitatory junction potential (EJP; straight arrow; left), followed by a prolonged hyperpolarization, which reached only \(-70\) mV, compared with \(-80\) mV in A. There was no triggered slow wave after this hyperpolarization. A slow wave was still triggered (curved arrow; middle) after a single long square wave. A longer recording of spontaneous slow waves is also shown (right); note reduced amplitude and frequency but similar configuration compared with A and absence of contractile activity. C: after 0.1 mM L-NNA, delayed hyperpolarization was abolished and EJP was enhanced in amplitude, but no slow wave was triggered (left). A single long pulse still triggered a slow wave (curved arrow; middle). L-NNA did not alter slow wave frequency, amplitude, or configuration compared with octanol alone and did not restore contractile activity (right).

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**Fig. 12.** Similar to Fig. 11 but recorded near DMP. Note that this is same preparation studied in Fig. 11 after 120 min of washing in 1 mM L-arginine-containing Krebs solution. A: in this region the response to EFS is a fast IJP followed by a slower hyperpolarization, which may be associated with a delayed or a triggered slow wave (curved arrow; left). A single 100-ms square wave clearly initiated a triggered slow wave (curved arrow; middle) of different configuration from spontaneous ones. Spontaneous slow waves and associated contractions are shown at right. B: after 15 min in 0.5 mM octanol, membrane was depolarized and slow wave amplitude and frequency were reduced, as were both components of the response to EFS (left). No slow wave was triggered after the response to EFS, but a single 100-ms stimulus was effective (curved arrow; middle). Reduced slow wave amplitudes and frequencies as well as the loss of phasic and tonic contraction are shown at right. C: after 0.1 mM L-NNA, fast component of the response to EFS was abolished and slower component was reduced (left). Again no slow wave was triggered after this stimulus. However, a slow wave was still triggered by a single 100-ms square wave (middle). At slower recording speed, compared with B, adding L-NNA had no effect on slow wave amplitude, frequency, or contractile function (right).
tent release of NO in the DMP (7), which can reduce slow wave amplitude. All of these differences can function in concert with those discussed above to increase the effectiveness of slow waves from the MyP to drive the whole muscularis externa, i.e., cells near the DMP may be nearer to the threshold of activation by depolarizing pulses and receive less signal (dV/dt lower) due to the abundant gap junctions. It is possible these factors combine to limit the ability of ICC of DMP to dominate pacing of slow waves and enhance the dominance of driving by impulses from the ICC of the MyP. However, the pacemaking activity of the DMP ICC appeared to determine the pattern of slow waves in circular muscle cells near it when both ICC networks were present and to determine the pattern for all cells when only the DMP ICC network was present (20, 21).

Although this study did not fully address the pathway whereby the ICC network of MyP sends a driving signal to the network in the DMP to couple the two networks, we found no evidence for arrays of ICC within the circular muscle connecting the ICC in the two plexuses. Definitive elimination of the existence of such arrays requires either detailed studies of serial sections along the ileum or light microscopic studies with a marker for ICC. Unfortunately, an antibody against c-Kit protein kinase, which recognizes ICC in some species (19, 35, 46), does not recognize canine ICC. An alternate route of coupling might be through circular muscle cells.

Hara and Szurszewski et al. (16, 17) showed in canine jejunum that outer circular muscle isolated from both MyP and DMP had no spontaneous slow waves, but slow waves of normal frequency could be initiated by acetylcholine (17). Apparently, ionic mechanisms of circular muscle cells are designed to respond to the appropriate slow wave frequencies. This suggests that these cells themselves can respond to and may be able to amplify or actively transmit pacemaking signals from ICC (see Ref. 35 for further discussion as applied to canine colon). However, as noted above, coupling from each ICC network influenced the shape of slow waves in adjacent circular muscle cells, which responded passively to pacemaker currents.

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*Fig. 13. Recordings from cells near the MyP. A: control recordings of spontaneous and triggered (curved arrow) slow waves and IJP (left). Spontaneous slow waves and associated contractions, at slower recording speed, are shown at right. B: after 1 mM octanol, fast IJP is lost and smaller delayed hyperpolarization occurs (arrow), which fails to trigger a slow wave (left). Slow wave frequency, amplitude, and cell membrane potential are all reduced by octanol (right). C: all effects of octanol were reversed 30 min after washing.*

*Fig. 14. Recording from cells near the DMP. A: control spontaneous slow waves and a triggered or delayed slow wave (curved arrow) after an IJP with associated mechanical activity (left). Spontaneous slow waves and associated contractions are shown at slower recording speed (right). B: recordings after 15–20 min of superfusion with 1 mM octanol, showing that spontaneous slow waves and there is membrane depolarization (left). Fast IJP is abolished and later phase of hyperpolarization (straight arrow) is reduced and slowed in onset and does not appear to trigger anything. Spontaneous slow waves occurring at reduced amplitude and frequency, with contractile activity abolished, are shown at right. C: after 30 min of washout, all effects of octanol were reversed.*
Table 3. Octanol effects on membrane potentials

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean</th>
<th>SE</th>
<th>n</th>
<th>Mean</th>
<th>SE</th>
<th>n</th>
<th>Gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>68.0</td>
<td>3.4</td>
<td>4</td>
<td>58.3</td>
<td>3.0</td>
<td>3</td>
<td>9.7</td>
</tr>
<tr>
<td>0.5 mM Octanol</td>
<td>61.5</td>
<td>3.1</td>
<td>4</td>
<td>45.0</td>
<td>2.3</td>
<td>3</td>
<td>15.15</td>
</tr>
<tr>
<td>Control</td>
<td>68.1</td>
<td>2.5</td>
<td>8</td>
<td>61.5</td>
<td>2.9</td>
<td>4</td>
<td>6.63</td>
</tr>
<tr>
<td>1.0 mM Octanol</td>
<td>59.9</td>
<td>2.5</td>
<td>8</td>
<td>53.25</td>
<td>4.2</td>
<td>4</td>
<td>6.63</td>
</tr>
</tbody>
</table>

Data are means ± SE. *Significantly different from values recorded near MyP (P < 0.05). †Significantly different from control (P < 0.01). ‡Significantly different from control (P < 0.001).

The general model of coupling suggested by our structural studies is supported in part by our studies with the gap junction conductance inhibitor, octanol (15, 29, 36). However, octanol also inhibits L-Ca2⁺ and K⁺ channels (36, 29). Thus inhibition of contractile activity by octanol, as by nifedipine and Ni²⁺ (5), is likely a consequence of L-Ca²⁺ channel blockade. However, nifedipine did not affect slow waves or membrane potentials in this tissue, whereas Ni²⁺ inhibited slow waves and produced slight but insignificant membrane hyperpolarization (5). In addition, the slow waves in isolated circular muscle strips were minimally affected when the small-conductance K⁺ channel blocker, apamin (21), caused modest depolarization and inhibition of fast IJP (9, 21). The surprising finding that slow wave activity in 1 mM octanol persisted may be explained if ICC networks no longer pace them but they derive from the spontaneous slow action potentials of circular muscle cells which have been subjected to K⁺ channel blockade. In canine colon (24) lacking the ICC networks, initiation of action potentials resembling slow waves in shape and frequency after K⁺ channel blockade also required activation of L-Ca channels and, unlike slow waves, they were abolished by Ca²⁺ channel blockers. Thus the initiation of slow action potentials by ileal circular muscle after octanol seems unlikely if L-Ca²⁺ channels were blocked. Moreover, the observed effects of octanol on membrane potentials and slow waves likely involved depolarization by uncoupling from hyperpolarizing ICC networks and K⁺ channel blockade.

At 1 mM, octanol has been reported to block smooth muscle gap junction coupling (15, 36) rapidly and completely. It was surprising then that slow waves persisted, albeit at reduced amplitude and frequency (Tables 1 and 2), if pacemaking depended on cell-to-cell coupling through gap junctions. Conceivably, higher concentrations of octanol might have abolished slow waves. We have noted no instances in the literature in which concentrations higher than 1 mM were required. In addition, it is unclear whether effects at higher concentrations were due to uncoupling of gap junctions or the result of effects on ion channels. In any case, 1 mM octanol drastically reduced slow wave amplitudes recorded near the MyP by 70% and abolished the amplitude differential of MyP slow waves over DMP slow waves but reduced their frequency by only 35%. This implies either that gap junctional conductance plays an important but nonessential role in initiating or propagating ileal pacemaking currents to circular muscle or that these gap junctions are resistant to uncoupling by this agent. The persistence of some mode of coupling in the presence of 0.5–1 mM octanol is supported by the persistent ability of a single long-duration (100 ms) square wave to trigger a slow wave (Figs. 11 and 12). The failure of triggering of slow waves after IJPs (Figs. 13 and 14) may result from the abolition of fast IJPs by octanol, leading to reduced hyperpolarization and rebound, but in earlier studies (7) TTX or L-NNA completely abolished IJPs without inhibiting triggering of slow waves by the same stimuli.

Octanol (1 mM) also eliminated the fast IJP recorded near the MyP, leaving only a small delayed hyperpolarization, which was L-NNA sensitive like the fast IJP. The significant membrane depolarization induced by octanol, acting by itself, would have increased IJP amplitude because of the greater differential in response to the K⁺ equilibrium potential. Loss of coupling of smooth muscle cells to ICC might eliminate fast IJPs initiated by mediator release onto ICCs (see Refs. 7 and 34 for discussion). The disappearance of the fast IJP near the MyP could result if it normally is initiated elsewhere, such as in or near the DMP, and propagated through gap junctions to outer circular muscle. The fast IJP recorded near the DMP was also markedly reduced or abolished by octanol, consistent with its dependence on coupling for transmission. Failure of propagation of IJPs from their sites of origin also can explain the occurrence of delayed-onset, small hyperpolarizations after 1 mM octanol (Figs. 13 and 14), which were sensitive to L-NNA. A direct effect of octanol on apamin-sensitive K⁺ channels mediating the fast IJP (21) may also contribute. However, a recent report (26) showed that guinea pig vas deferens excitatory junction potentials were abolished rapidly and reversibly by 1 mM heptanol without any change in membrane resting potential or in nerve currents.

Recent studies in mouse intestine (19, 46) showed that a mutation in the gene for the Kit protein resulted in loss of ICC in the MyP and absence of pacemaking activity. Normally, these cells recognized an antibody against this protein. Both fibroblast-like and macrophage-like cells were increased in number and were found in areas normally occupied by ICC (25). In contrast, the ICC of the DMP were present with ultrastructural features and density unaltered (25). The function of ICC of the DMP in murine small intestine did not include pacemaking. In contrast, our studies of the canine ileum (5, 7, 20, 21) showed that physical removal of the ICC of the MyP still resulted in slow waves recorded in outer circular muscle, which were apparently generated from the ICC network of the DMP. However, no triggered slow wave activity could be evoked by the ending of an IJP or by a long-duration single pulse (20). In whole-thickness preparations, triggered slow wave amplitudes decayed away from the MyP (7), and only those recorded near the MyP region persisted in Ca²⁺-free media (5). Also the higher frequency slow waves from the MyP region entrained the...
DMP slow waves with a lower intrinsic frequency (20). We therefore argued that the ICC of the MyP dominat
ated the pacemaking activity of the ICC network of the DMP when the two pacemaking networks were intact, but that each pacemaking network could function independently to drive pacemaking. Thus in murine and canine small intestine, the ICC of the MyP are fully dominant in pacemaking, as suggested in a recent review (34), but in canine ileum the DMP can drive slow waves in the absence of MyP ICC and may be involved in facilitating inhibitory neurotransmission.

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