A Novel Pacemaker Mechanism Drives Gastrointestinal Rhythmicity

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Electric pacemaker activity drives peristaltic and segmental contractions in the gastrointestinal tract. Interstitial cells of Cajal (ICC) are responsible for spontaneous pacemaker activity. ICC remain rhythmic in culture and generate voltage-independent inward currents via a nonselective cation conductance. Ca²⁺ release from endoplasmic reticulum and uptake by mitochondria initiates pacemaker currents. This novel mechanism provides the basis for electric rhythmicity in gastrointestinal muscles.

The rhythmoneuromuscular apparatus of the gastrointestinal (GI) tract is more complicated than a syncytium of smooth muscle cells innervated by motor neurons. For many years, morphological studies of the tunica muscularis noted the presence of additional specialized cells that are commonly referred to as interstitial cells of Cajal (ICC). ICC were frequently found in close association with nerves, and in many cases they were described as “intercalated” between nerve terminals and smooth muscle cells (2). ICC were also observed to form gap junction connections with each other and with neighboring smooth muscle cells. Thus the electric syncytium of the tunica muscularis of the GI tract is composed of at least two cell types. Morphology also suggested that the innervation of the smooth muscle might be indirect and mainly occur via synapse-like structures between nerves and ICC. These studies were interesting and provocative, but it was only possible to speculate about the function of ICC from morphological analyses.

Intensive work on animal models (primarily mouse, guinea pig, and dog) during the past decade has provided physiological evidence that ICC provide the pacemaker activity typical of phasic GI muscles of the stomach, small bowel, and colon (i.e., electric slow waves; cf. Ref. 9). Pacemaker ICC are generally found in the region of the myenteric plexus in the space between the circular and longitudinal muscle layers. We refer to the cells in the myenteric region as IC-MY. ICC along the submucosal surface of the circular muscle layer in the colon (IC-SM) are also able to generate pacemaker activity, particularly in larger animals such as the dog. IC-MY and IC-SM form extensive networks within pacemaker regions. These cells also extend into the bulk of the muscle layers in the septa that divide bundles of smooth muscle cells. Thus pacemaker activity is not necessarily confined to the myenteric and submucosal pacemaker regions, but these pacemakers are dominant in intact muscles. Fine processes of pacemaker ICC interconnect via gap junctions, and electrical connections are also made with neighboring smooth muscle cells. Thus electric events occurring in ICC are capable of conducting to smooth muscle cells. Simultaneous recordings of electric activity from IC-MY and nearby smooth muscle cells have demonstrated that electric activity occurs first in IC-MY and then initiates electric responses in the smooth muscle cells (3). Connections between ICC are necessary for regenerative propagation of slow waves, and extension of ICC networks into the septa between muscle bundles may provide propagation pathways for transmission of slow waves through the tunica muscularis (perhaps analogous to the Purkinje fibers in the heart). Networks of IC-MY and some of the ultrastructural features of IC-MY are shown in Fig. 1.

Other types of ICC can also be found in GI muscles. ICC are intermingled with the fibers of the circular and longitudinal muscle layers in the esophagus, stomach, colon, and sphincters. We have called these intramuscular ICC (IC-IM). IC-IM are extensively and closely associated with nerve fibers of the enteric nervous system (1) and form very close (<20 nm) synapse-like connections with varicose nerve terminals of excitatory and inhibitory motor neurons. Cells with similar characteristics, called IC-DMP, are likely to be a specialized type of IC-IM and are found in the region of the deep muscular plexus in the small intestine. IC-IM and associations with enteric neurons are illustrated in Fig. 1, E–I.

IC-IM and IC-DMP are functionally innervated and appear to mediate a significant part of the motor input from the enteric nervous system. In mutant mice lacking IC-IM, field stimulation of intrinsic neurons in the stomach resulted in greatly reduced postsynaptic responses to cholinergic and nitrergic nerve stimulation (1, 14). Although the role of ICC in neurotransmission is extremely important in GI motility, this short review will focus on new findings about how ICC generate electric rhythmicity. IC-IM and IC-DMP may or may not have the capability of generating pacemaker-like currents, but these cells play an extremely important role in regulating the smooth muscle response to pacemaker activity.

Development of ICC and pacemaking in the GI tract

A valuable approach used to determine the physiological significance of ICC in GI motility has been to manipulate the development of these cells (see reviews in Refs. 9 and 10). Knowledge of the factors that regulate the development of ICC may yield important therapeutic approaches if populations of ICC can be regulated (see Clinical consequences of defective ICC, below). Developmental studies have been performed on...
mice, which begin to express the receptor tyrosine kinase (Kit) in undifferentiated mesenchymal cells of the small intestine by embryonic day 12 (E12). By E15, Kit-positive cells aggregate into dense clusters and begin to form networks of ICC. At some time between E15 and E18, a lineage decision is made; some of the Kit-positive mesenchymal cells lose Kit expression, develop smooth muscle-like characteristics, and become the longitudinal muscle layer. Other Kit-positive cells retain Kit expression and become IC-MY. By E18–E19, mature appearing ICC networks are observed with Kit immunohistochemistry and slow wave activity can be recorded. An interesting proximal-to-distal developmental gradient exists, in which electric activity first develops in the duodenum and jejunum and then later (i.e., after birth) in the ileum. It should
be noted that if jejunal sections are removed from fetuses at E15 and placed into organ culture, ICC networks and slow waves develop within 5 days (i.e., at about the normal pace), but if a neutralizing antibody to Kit is included in the culture medium, ICC and slow wave activity are absent after 5 days of culture. These data suggest that Kit signaling is required for the lineage decision that determines whether Kit-positive cells will develop into ICC. The onset of slow wave activity closely follows the development of ICC networks.

After birth, the ultrastructure of ICC continues to develop as features recognized in mature cells become apparent. During this period, the amplitude and frequency of spontaneous electric activity increases, such that slow waves with adult characteristics are present within 10 days. IC-DMP continue to develop after birth in the mouse, and these cells emerge from precursors at the inner aspect of the circular muscle layer.

Kit signaling requires the presence of a ligand referred to as stem cell factor (SCF). This factor is expressed as either a membrane-bound or soluble isoform. The membrane-bound isoform is needed for proper development of ICC, and this protein is typically presented to cells expressing Kit by neighboring cells. Enteric neurons express SCF, but morphologically normal and functional ICC develop in animals lacking enteric neurons (see Ref. 10). Smooth muscle cells within the GI tract also express SCF, and these cells may be the main source of SCF required for ICC development. An interesting question remains about the spatial organization of ICC in designated locations within GI muscles. It is possible that localized expression of specific isoforms of SCF could be responsible for this organization.

Functionally mature cells can lose the ICC phenotype if Kit signaling is blocked shortly after birth (13). Neutralizing Kit antibodies have been used in neonatal animals and in organ cultures of GI muscles. These antibodies bind to the receptor moiety of Kit and inhibit binding of SCF. Losing signaling by the Kit pathway causes Kit-positive cells to disappear, and electron microscopy has verified that this is due to loss of ICC and not merely loss of Kit expression. When ICC disappear, electric rhythmicity ceases (9). Similar results occur when phosphatidylinositol-3-kinase, a downstream signaling molecule in the Kit pathway, is blocked (see Ref. 10).

An extremely interesting observation regarding the loss of Kit-positive cells is that the decrease in ICC does not result from cell death (13). When Kit receptors are blocked, ICC undergo transdifferentiation and take on smooth muscule-like characteristics. This plasticity between the ICC and smooth muscle phenotypes may be an extremely important phenomenon that might be exploited for therapeutic purposes when ICC numbers are reduced in human GI motility disorders (see Ref. 10).

**Pacemaking in GI muscles is unique**

Investigators of GI smooth muscles during the past century have often considered pacemaker activity in GI muscle to be analogous to the mechanisms in the heart. The spontaneous electric activity of the GI tract (most commonly referred to as slow waves) is dissimilar to cardiac rhythmicity in many respects. Slow waves 1) are generated at lower frequencies (at a maximum of ~40 cycles/min and more typically at 3–10 cycles/min), 2) have lower amplitudes (maximum amplitude of 10–50 mV, so slow waves do not overshoot 0 mV), and 3) have much longer durations than cardiac action potentials (up to many seconds per event). Even after ICC were recognized as specialized pacemaker cells (6), most investigators still considered the role of the pacemaker to be a source of currents that depolarize smooth muscle cells to a threshold at which the slow wave could be regenerated and propagated. This concept is inconsistent with some of the basic properties of GI smooth muscles and the slow wave mechanism. First, an ionic apparatus capable of generating slow waves has not been demonstrated in voltage clamp studies of isolated GI muscle cells. Studies of isolated and cultured ICC have demonstrated spontaneous electric rhythmicity and specialized conductances (5, 6, 11). Second, tissues lacking ICC cannot be electrically paced to generate slow waves (4). Thus it appears that smooth muscle cells do not express the mechanism needed to generate slow waves, and the absence of this mechanism in isolated cells is not a result of cell dispersion per se. Third, slow waves do not propagate through regions of muscle lacking ICC (4, 9). Slow waves, generated in intact tissues coupled to regions lacking ICC, rapidly decay as a function of distance. These findings suggest that the slow wave mechanism is a unique property of ICC and that smooth muscle cells lack the ability to actively generate or regenerate a slow wave response.

At present, it appears that the role of ICC is to depolarize the smooth muscle syncytium to increase the open probability of voltage-dependent ion channels expressed by smooth muscle cells. Depolarization of smooth muscle cells activates Ca$^{2+}$ entry mechanisms. The most important group of channels, in terms of linking slow waves to mechanical responses, are L-type Ca$^{2+}$ channels, which are abundantly expressed in GI muscle cells (4). In some cases, increasing the open probability of Ca$^{2+}$ channels results in fast Ca$^{2+}$ action potentials, but in other GI muscles, voltage-dependent K$^+$ channels prohibit the cells from reaching a threshold for action potentials, and a quasi-stable “plateau potential” occurs that results from a balance between inward and outward currents. Although the open probability of Ca$^{2+}$ channels is lower during the plateau potential than during the rising phase of an action potential, the long duration of enhanced open probability during the plateau potential (up to many seconds) facilitates enough Ca$^{2+}$ entry to activate the contractile apparatus.

Slow wave propagation occurs through networks of electrically coupled ICC. The ICC that generate and propagate slow waves are distributed throughout continuous pacemaker networks that extend the length and circumference of the phasic
regions of the gut. Pacemaker ICC may also extend into the muscle layers in septa between muscle bundles. To maintain the discrete electric activities of each organ there are discontinuities in the ICC networks between organs. When pacemaker networks are disrupted, slow wave propagation is blocked and events decay exponentially in the surrounding smooth muscle (4). The electric impedance of the smooth muscle syncytium tends to prohibit regenerative propagation of action potentials, and the cell-by-cell smooth muscle response to the conducting slow wave depolarization is therefore likely to be a relatively localized response. Local smooth muscle responses can be modulated by neural inputs to IC-IM and IC-DMP that are electrically coupled to the smooth muscle cells. Neurotransmitters alter input impedance and activate conductances that generate inward or outward currents in IC-IM and IC-DMP, but the electric coupling between these cells and smooth muscle cells means that the ICC-smooth muscle syncytium is electrically conditioned by neural inputs via ICC. Neural regulation affects the spatial decay of conducted slow waves or modulates the local action potential threshold. Either effect impacts the open probability of Ca\(^{2+}\) channels and regulates the excitability response of muscle cells to the slow wave depolarization. Thus the electric behavior of GI muscles is a composite of the distinct electric behaviors of at least four cell types: pacemaker ICC (IC-MY and IC-SM), smooth muscle cells, enteric motor neurons, and neurotransmission ICC (IC-IM and IC-DMP). We refer to these functionally integrated populations of cells as the rhythmoneuromuscular apparatus of GI motility. A conceptualization of the interplay between nerves, ICC, and smooth muscle cells is illustrated in Fig. 2.

**The pacemaker current generated by ICC has unique properties**

The ionic permeability mechanisms responsible for electric slow waves have been investigated since the 1970s. Investigative groups working with Prosser and Tomita (see Ref. 12) used sucrose gap voltage clamp techniques to study intact strips of GI muscle. These authors observed rhythmic currents that occurred at a relatively constant frequency regardless of potential. The pacemaker mechanism was therefore said to lack voltage dependence. Recent studies have revealed such a current in cultured ICC from the small intestine of mice.

Isolated ICC retain a rhythmic phenotype. The first study to demonstrate that ICC generate electric slow waves was performed on cells from the canine colon (6). It is also possible to maintain ICC in culture for several days with preservation of spontaneous electric activity (5, 11). The patch clamp technique has been used to show that ICC from the canine colon and cultured ICC from the murine intestine generate regular slow wave events. Voltage clamp studies of murine ICC suggest that spontaneous inward currents cause slow waves. The pacemaker currents reversed near 0 mV and were decreased in amplitude or blocked when extracellular Na\(^{+}\) or Ca\(^{2+}\) was reduced (5). Pacemaker currents were blocked by Gd\(^{3+}\) but were not blocked by Cl\(^{-}\)channel blockers or L-type Ca\(^{2+}\) channel blocking drugs. An interesting aspect of the pacemaker currents generated in ICC was the lack of effect of depolarization on frequency (until current reversal; see Fig. 3 and Ref. 5). These experiments confirmed the notion that pacemaker currents were voltage independent and perhaps carried by a non-selective cation conductance that passed both Na\(^{+}\) and Ca\(^{2+}\). It was also found that ICC express a Ba\(^{2+}\)-sensitive inward rectifier conductance that might contribute to the relatively negative resting potentials of GI muscles (5). Additional ionic conductances may contribute to or amplify pacemaker currents in intact muscles because the pharmacology of slow waves in cultured ICC does not completely match the pharmacology of slow waves in intact muscles of the small intestine. Additional studies are needed to more fully characterize the ionic conductances expressed in ICC and to determine the single-channel conductance(s) responsible for the pacemaker current.
Pacemaker currents are activated by intracellular $\text{Ca}^{2+}$ handling mechanisms

Since open probability of the channels responsible for the pacemaker current does not appear to depend on membrane potential, intracellular signaling mechanisms must be responsible for gating the conductance(s) responsible for pacemaker current. The mechanism controlling the open probability of the pacemaker conductance must include a timing device to achieve the regular frequency of GI rhythmicity. The “clock” mechanism that lies at the heart of the rhythmoneuromuscular apparatus and initiates pacemaker current via a voltage-independent conductance has eluded investigators for many years.

ICC contain an abundance of mitochondria (see Fig. 1), and the extensive clusters of these organelles are one of the identifying features of ICC in ultrastructural studies. Active cells like ICC may require enhanced metabolic capability, but the specific importance of mitochondria in generating pacemaker activity has not been understood. Some investigators have suggested that cyclic changes in energy production by mitochondria might regulate ionic transporters used in slow waves.
wave generation or that the ionic conductances involved in pacemaker current are metabolically regulated (8). Others have suggested that pacemaker activity requires Ca\(^{2+}\) release from intracellular stores (7). Recent studies have suggested that pacemaker activity depends on a link between Ca\(^{2+}\) release from cellular stores, oxidative metabolism, and the pacemaker conductance in the plasma membrane (15).

Coupling between Ca\(^{2+}\) release from inositol 1,4,5-trisphosphate (IP\(_3\)) receptor-operated stores and Ca\(^{2+}\) uptake by mitochondria is linked to initiation of pacemaker currents in ICC (15). Pacemaker currents generated by ICC from the murine intestine were reduced in frequency and eventually inhibited by xeostospongin C, a membrane-permeable inhibitor of IP\(_3\) receptors. Simple release of Ca\(^{2+}\) from IP\(_3\) receptor-operated stores, however, did not initiate pacemaker currents. Mitochondrial uncouplers, such as carbonylcyanide m-chlorophenylhydrazone and carbonylcyanide p-trifluoro-methoxyphenylhydrazone, also slowed the frequency of pacemaker currents and then inhibited spontaneous activity. These effects were mimicked by respiratory chain inhibitors such as rotenone (complex I inhibitor) and antimycin (complex III inhibitor). It is unlikely that ATP depletion caused the inhibition of pacemaker activity in response to these agents because cells were dialyzed with millimolar concentrations of ATP, and oligomycin, an inhibitor of the F\(_1\)/F\(_0\) ATPase, did not affect pacemaker currents.

Oxidative metabolism generates a substantial electrochemical gradient (Ψ\(_m\)) across the inner mitochondrial membrane, and Ψ\(_m\) serves as the driving force for Ca\(^{2+}\) uptake into mitochondria. FCCP and CCCP are protonophores that reduce Ψ\(_m\) and similar effects have been observed on Ψ\(_m\) in response to respiratory chain inhibitors. Therefore, these drugs reduce the driving force for Ca\(^{2+}\) uptake into mitochondria. We tested the hypothesis that Ca\(^{2+}\) uptake, perhaps stimulated by release of Ca\(^{2+}\) from IP\(_3\) receptor-operated stores, may be a fundamental step in activating pacemaker currents. Ca\(^{2+}\) uptake into mitochondria occurs via the mitochondrial uniporter, and dialysis of cells with RU-360, an inhibitor of the uniporter, blocked pacemaker currents.

ICC loaded with rhod-2, a fluorescent indicator of mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_m\)), demonstrated oscillations in [Ca\(^{2+}\)]\(_m\) at the same frequency as the pacemaker currents. Rhod-2 localization in mitochondria was verified by double labeling with MitoTracker green FM. A temporal, one-to-one relationship was shown between the [Ca\(^{2+}\)]\(_m\) oscillations and pacemaker currents by monitoring [Ca\(^{2+}\)]\(_m\) oscillations in voltage-clamped ICC. The onset of the [Ca\(^{2+}\)]\(_m\) transients preceded the initiation of inward currents. These observations indicate that mitochondrial Ca\(^{2+}\) uptake, perhaps by regulating the local Ca\(^{2+}\) concentration near the cytoplasmic aspect of the pacemaker channels, initiates pacemaker currents. Mitochondrial Ca\(^{2+}\) uptake depends on release of Ca\(^{2+}\) from IP\(_3\) receptor-operated stores, and this was demonstrated by blocking the oscillations in [Ca\(^{2+}\)]\(_m\) (and pacemaker currents) with xeostospongin C. Thapsigargin, an inhibitor of Ca\(^{2+}\) uptake into stores, also inhibited pacemaker current, suggesting that cycle by cycle reloading of the IP\(_3\) receptor-operated store is important for sustaining rhythmicity.

These findings demonstrate that pacemaker currents are generated by integrated Ca\(^{2+}\) handling by the sarcoplasmic reticulum and mitochondria in ICC (Fig. 4; Ref. 15). The precise link between mitochondrial Ca\(^{2+}\) uptake and pacemaker current activation is not fully understood, but the results are consistent with the idea that local Ca\(^{2+}\) regulation is the key to controlling the open probability of the pacemaker channels. Future experiments will identify the single channel conductance(s) involved in pacemaker activity and determine the specific regulators that affect channel open probability.

How does propagation occur via a voltage-independent ionic conductance?

Active propagation of slow waves is an extremely important property of GI motility. In the stomach, for example, a dominant pacemaker in the orad corpus drives gastric peristaltic waves that are important for reduction of particle size and regulation of gastric emptying. Slow waves propagate at rates of several to tens of millimeters per second, and the velocity in the long axis of the circular muscle layer exceeds the velocity in the long axis of the longitudinal muscle by severalfold. Anisotropic propagation allows slow waves to rapidly spread around the stomach and then to migrate as a band of activity toward the pyloric sphincter. One of the remaining mysteries about electric rhythmicity in GI muscles is how propagation occurs. If pacemaker current channels are voltage independent, then electrotonus, the typical means of initiating regenerative propagation, would be ineffective in active propagation. Taking into account the mechanism described above for activation of the pacemaker current, it is possible that intracellular Ca\(^{2+}\) waves, mitochondrial depolarization, or activation of voltage-dependent Ca\(^{2+}\) channels may contribute to slow wave propagation. We already know that slow waves propagate with fidelity in the presence of dihydropyridines, suggesting that L-type Ca\(^{2+}\) channels are not involved. The propagation velocity of Ca\(^{2+}\) waves is too slow to explain active propagation of slow waves. It is likely that novel conductances or novel applications of cellular processes will be required to understand the process of slow wave propagation.

Clinical consequences of defective ICC

Because of the critical roles of ICC in GI motility, loss of these cells (either pacemaker ICC or neurotransmission ICC) would be extremely detrimental. Such defects could result in pathologies such as achalasia, loss of gastric accommodation reflexes, weakened or absent postprandial phasic contractions (e.g., gastroparesis or gastric atony), electric arrhythmias, gastric emptying disorders, pseudoobstruction-like symptoms, small intestinal and colonic transit defects, and...
FIGURE 4. SR-mitochondrial Ca\textsuperscript{2+} handling drives pacemaker currents. Top: model proposed for slow wave mechanism. Ca\textsuperscript{2+} is released from SR via inositol 1,4,5-trisphosphate (IP\textsubscript{3}) receptors, which are physically close to mitochondria and specifically to Ca\textsuperscript{2+} uniporter channels in inner mitochondrial membrane. Negative membrane potential (\(\Phi_m\)) generated across mitochondrial membranes by components of the electron transport chain serves as the driving force for Ca\textsuperscript{2+} entry into mitochondria. Ca\textsuperscript{2+} uptake by mitochondria activates nonselective cation channels (\(x^+\)) in the plasma membrane, resulting in inward pacemaker currents. Ca\textsuperscript{2+} entry via this conductance is partially taken up by SR via SERCA pumps to reset the cycle. Ca\textsuperscript{2+} removal from mitochondria may be achieved by Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (NCE). Permeability transition pores (PTP) are also present and are thought to be important in apoptosis. PTP channels do not seem to contribute to the slow wave cycle. RyR, ryanodine receptor. Bottom: experimental evidence supporting the model. A: mitochondrial Ca\textsuperscript{2+} oscillations in an ICC. Note regular frequency of oscillation that matched slow wave frequency. Oscillations seem tightly linked to Ca\textsuperscript{2+} release from IP\textsubscript{3} receptor-operated stores because they were blocked by xestospongin C (an inhibitor of IP\textsubscript{3} receptors). This effect was reversible as shown. B: xestospongin C also blocked pacemaker currents recorded under voltage clamp. C: simultaneous recordings from a voltage-clamped cell with mitochondria loaded with rhod-2. Note one-to-one relationship between mitochondrial Ca\textsuperscript{2+} oscillations and pacemaker currents (left). As shown at right, mitochondrial Ca\textsuperscript{2+} oscillations precede pacemaker currents by several hundred milliseconds. D: pacemaker currents were inhibited by a mitochondrial uncoupler (FCCP) that reduces the driving force for Ca\textsuperscript{2+} uptake. E: FCCP also inhibited mitochondrial Ca\textsuperscript{2+} oscillations. Finally, the process is reset and reloaded by Ca\textsuperscript{2+} uptake into the SR, and thapsigargin, an inhibitor of Ca\textsuperscript{2+} uptake, blocked pacemaker currents (F). Parts of the figure are reproduced from Ref. 15.
constipation. Since ICC are reduced in number in many clinical disorders with these symptoms (see review in Ref. 10), research into the biology of ICC provides exciting new opportunities to understand the etiology of diseases that have long eluded understanding. Discovering ways to manipulate the development or to stimulate regeneration of ICC may provide dramatic new therapies for chronic GI diseases that result in life-long suffering.

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References