

Mini Review

Gut Pacemaker Cells: the Interstitial Cells of Cajal (ICC)

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Abstract

This review will focus on the pacemaker mechanisms underlying gastrointestinal autonomic rhythmicity in an attempt to elucidate the differences and similarities between the pacemaker mechanisms in the heart and gut. Interstitial cells of Cajal (ICC) form networks that are widely distributed within the submucosal (ICC-SM), intra-muscular (ICC-IM, ICC-DMP) and inter-muscular layers (ICC-MY) of the gastrointestinal tract from the esophagus to the internal anal sphincter. The ICC generate spontaneously active pacemaker currents that may be recorded as plateau and slow potentials. These pacemaker currents drive the spontaneous electrical and mechanical activities of smooth muscle cells. The enteric nervous system, composed of both the myenteric (inter-muscular) plexus and the submucosal plexus, is also distributed in the gastrointestinal tract from the esophagus to the internal anal sphincter. The role of the ICC and the enteric nervous system in the integrative control of gastrointestinal function and especially of spontaneous rhythmic activity, is still unknown. Nevertheless, at least from the results presented in this review of studies of the jejunum, ileum and proximal colon of the mouse, it is convincing that the ICC drive spontaneous rhythmic motility, although a role for the enteric nervous system in the regulation of spontaneous rhythmic motility cannot be overlooked. Furthermore, intracellular Ca^{2+} handling has a critical role in the generation of pacemaker activity in the gut and heart, although respective players such as the Ca^{2+} -ATPase of the sarcoplasmic reticulum (endoplasmic reticulum), IP_3 receptors, ryanodine receptors and plasma membrane ion channels may have divergent roles in the Ca^{2+} -release refilling cycles. In conclusion, intracellular Ca^{2+} handling plays a key role in the gut pacemaker responsible for spontaneous rhythmicity, as well as in the cardiac pacemaker responsible for spontaneous beating. Pharmacotherapeutic targeting of intracellular Ca^{2+} handling mechanisms may be a promising approach to the treatment and cure of gut motility dysfunction.

Key words: pacemaker, slow wave, enteric nervous system, interstitial cells of Cajal, embryonic stem cell

Introduction

The mechanisms involved in the generation of spontaneous motility in the gut have been discussed for many years. Although Hukuhara *et al.* (1961; 1962) have discussed “myogenic”

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and “neurogenic” hypotheses, convincing evidence for either mechanism has not yet been obtained. They were however able to show that aganglionic intestinal loops still exhibited rhythmic spontaneous contractions, although intestinal intrinsic reflexes disappeared (Hukuhara *et al.*, 1962). Within the past 10 years, there has been increasing evidence that the interstitial cells of Cajal (ICC) are the pacemaker cells of spontaneous motility in the gut (Kobayashi *et al.*, 1996; 1995; Huizinga *et al.*, 1995; 1997; Sanders *et al.*, 1999). In rats and guinea pigs, Hukuhara and Neya (1968) have reported that a specific boundary region exists between the proximal and distal colon. This region has been believed to be a pacemaker region. In the rat proximal colon, indeed, rhythmic spontaneous contractions were observed (Yoneda *et al.*, 2001). Two different frequencies of spontaneous motilities were also reported in the mouse proximal colon (Yoneda *et al.*, 2002b; Takaki and Yoneda, 2003; Takaki *et al.*, 2003).

Plateau potentials were recorded from ICC along the submucosal surface of the circular smooth muscle (ICC-SM) that correlate with smaller amplitude (but more frequent) circular muscle contractions in the mouse (Yoneda *et al.*, 2002b). The plateau potentials were abolished by nifedipine (1 μ M) and inhibited by modulators of internal Ca^{2+} stores such as ryanodine (10 μ M), an inhibitor of Ca^{2+} -ATPase at the internal Ca^{2+} stores, cyclopiazonic acid (CPA; 1 μ M) (Uyama *et al.*, 1992), caffeine (3 mM), an intracellular Ca^{2+} chelator, bis-(aminophenoxy) ethane-N,N,N',N'-tetraacetic acid acetoxyethyl ester (BAPTA-AM; 20 μ M) (Michelangeli *et al.*, 1987) and an inhibitor of IP_3 receptor-mediated release of Ca^{2+} from internal stores, 2-aminoethoxy-diphenyl borate (2-APB; 10 μ M) (Cui and Kanno, 1997; Maruyama *et al.*, 1997) (Yoneda *et al.*, 2002a).

Bursts of action potentials in circular smooth muscle cells and bursts of membrane oscillations in longitudinal smooth muscle cells correlate with larger amplitude but less frequent longitudinal and circular smooth muscle contractions in the mouse proximal colon (Yoneda *et al.*, 2002b; Takaki and Yoneda, 2003; Takaki *et al.*, 2003).

In the mouse proximal colon, peristalses would occur both proximally from the pacemaker region and distally towards the anus. On the basis of the frequency of these spontaneous contractions, the more frequent circular smooth muscle contractions appeared to be related to peristalsis moving proximally from the pacemaker region (“antiperistalsis”). The less frequent longitudinal and circular smooth muscle contractions appeared to be related to peristalsis distally towards the anus. Furthermore, two similar types of motilities with different frequencies have also been found in the rat colon (Plujà *et al.*, 2000).

It is possible that under physiological conditions, more than two pacemakers could be active in the gut, whereas only a single pacemaker is likely to be active in the heart. Furthermore, in the *W/W^v* mutant mouse ileum where the original pacemaker ICC (ICC-MY) are deficient, it was shown that they could be switched to the other type of pacemaker ICC (ICC-DMP) in which the frequency of pacemaker activity is similar to ICC-MY (Nakagawa *et al.*, 2003). In the heart, after complete atrio-ventricular (AV) blockade, the AV node cells could act as a secondary pacemaker in which the frequency of pacemaker activity is much less than that in the sino-atrial (SA) node cells. Accordingly, there are many differences and similarities between pacemaker cells in the gut and heart.

To gain insight into pacemaker cells in the gut and heart, this review will concentrate on

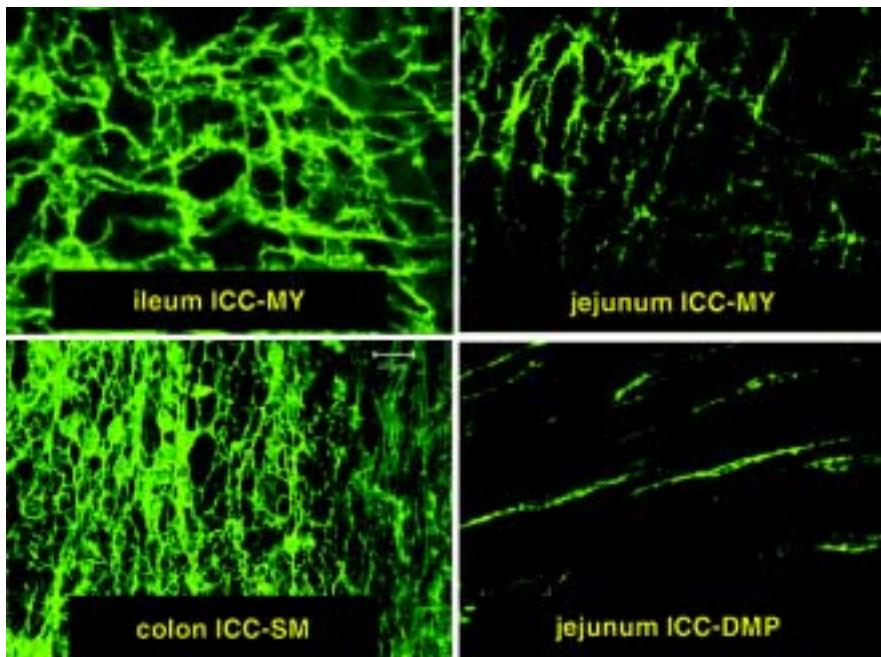


Fig. 1. Confocal images demonstrating c-Kit-like immunoreactivity stained with ACK2 in a whole mount preparation of the mouse jejunum and ileum. ICC-MY: myenteric (intermuscular) interstitial cells of Cajal (ICC). ICC-SM: submucosal ICC. ICC-DMP: deep muscular plexus ICC.

pacemaker cells in the gut wall of the mouse small intestine and proximal colon, as well as in the ES-gut which has differentiated from embryonic stem (ES) cells. It is composed of ICC cells and smooth muscle cells and could thus express spontaneously motility (Yamada *et al.*, 2000).

Classification of ICC

Pacemaker ICC networks vary both in different regions of the gut, such as stomach, small intestine and large intestine and in different species such as mice, rats, guinea pigs and dogs. ICC networks are widely distributed within submucosal (ICC-SM), intramuscular (ICC-IM, ICC-DMP) and inter-muscular layers (ICC-MY) (Sanders *et al.*, 1999). ICC-SM are distributed on the submucosal surface of the circular muscle of the colon. ICC-MY are distributed on the myenteric region of the stomach, small intestine and colon. In the myenteric region, the myenteric neural plexus also exists. ICC-DMP are found in the deep muscular plexus region of the small intestine. ICC-IM are found intramuscularly. The classification of ICC is still confusing. I propose the following nomenclature for ICC networks for easier understanding: ICC-SM, submucosal ICC; ICC-MY, myenteric (intermuscular) ICC; ICC-IM and ICC-DMP, intramuscular ICC. Figure 1 shows confocal images of whole mount preparations of the mouse jejunum and ileum exhibiting c-Kit-like immunoreactivity stained with ACK2.

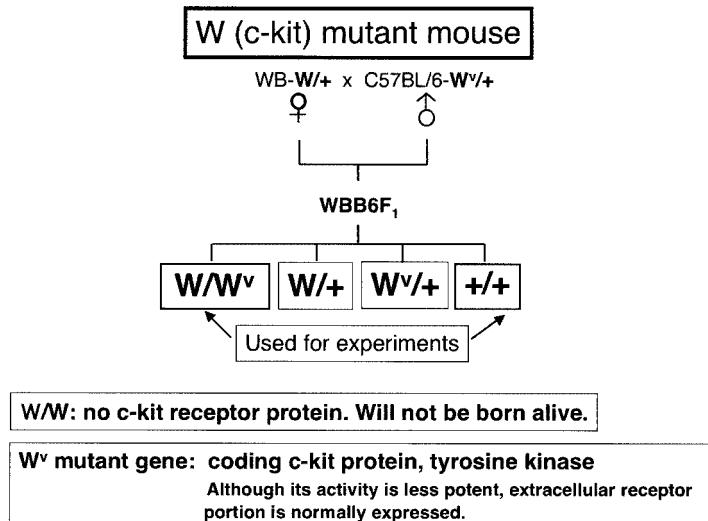


Fig. 2. Protocol for breeding *W/W^v* mutant mice.

ICC in *W (c-kit)* Mutant Mouse

As noted in Fig. 2, *W/W* mice do not express the c-Kit receptor protein at all and thus will not be born alive. However, those mice carrying the *W^v* mutant gene which codes the c-Kit protein, normally express the extracellular receptor portion of tyrosine kinase, although with lower activity. *W/W^v* mice are born alive and can be used as an ICC-deficient animal model (Huizinga *et al.*, 1995; Ward *et al.*, 1994), compared to the *+/+* mouse that normally expresses the c-Kit receptor protein and thus develops ICC. In my laboratory, we were able to demonstrate ICC-MY and ICC-DMP in the jejunum and ileum of *+/+* mice by c-Kit-LI stained with ACK2. On the other hand, in *W/W^v* mice, ICC-MY immunoreactivity was invisible in the jejunum and ileum, while ICC-DMP was still present at normal levels (Nakagawa *et al.*, 2003).

Using the apparatus as shown in Fig. 3, simultaneous recordings were made of spontaneous electrical and mechanical activities in murine (*+/+* and *W/W^v*) small intestine. Extracellular recording of electrical activity was performed from the serosa at the middle of a 1–2 cm long segment. Longitudinal smooth muscle contractions are recorded via an isometric force transducer and circular smooth muscle contractions are recorded via a pressure transducer with a hydrostatic pressure of 0–3 cmH₂O.

In the jejunum and ileum of *+/+* mice, regular electrical slow waves were recorded continuously, sometimes with spike potentials superimposed on the slow waves. Longitudinal and circular smooth muscle contractions were synchronous, although the amplitude of each contraction was rather variable (Fig. 4). Tetrodotoxin (TTX, 0.1 μM) (Fig. 5) or L-NAME (an NO synthase inhibitor; 10 μM) increased the amplitude of each contraction and of the slow waves, and increased the occurrence of spike potentials superimposed on the slow waves, but did not change the frequency of contractions and slow waves in either the jejunum or ileum of *+/+* mice. These results indicate that while the amplitude of the spontaneous motility was

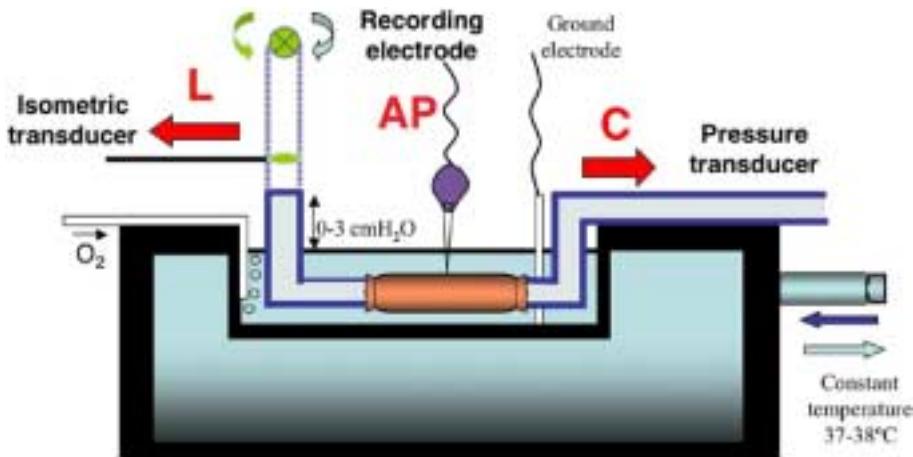


Fig. 3. The apparatus used for the simultaneous recording of spontaneous electrical and mechanical activity in the murine small intestine. AP: action potential. L: longitudinal muscle contractions. C: circular muscle contractions.

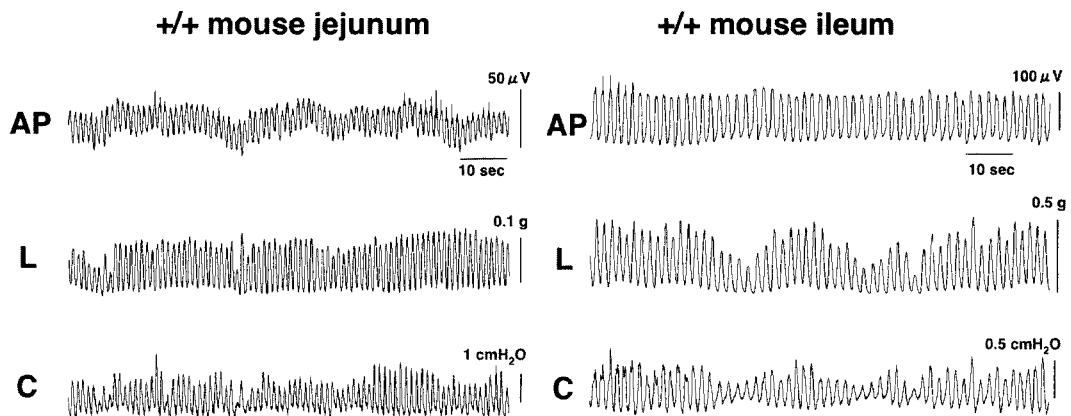


Fig. 4. Simultaneous recordings of spontaneous electrical activity and both longitudinal muscle contractions and circular muscle contractions in +/- mouse jejunum. AP: action potential. L: longitudinal muscle contractions. C: circular muscle contractions.

inhibited by nitrergic enteric neurons, the frequency of spontaneous motility was not affected by enteric neurons.

As shown in the ileum of *W/W^v* mice in Fig. 6, electrical slow waves could not be found and no regular contractions were found in either of the longitudinal and circular smooth muscle layers. This result indicates that ICC-MY are essential for generating electrical slow waves and a regular rhythm of contraction. Interestingly, however, after treatment with TTX (0.1 μ M) or L-NAME (10 μ M) (Fig. 6), fairly regular contractions appeared associated with spike potentials, although electrical slow waves were still not evident (Nakagawa *et al.*, 2003; Takaki *et al.*, 2003). This indicates that spontaneous contractions could still occur after blocking the nitrergic

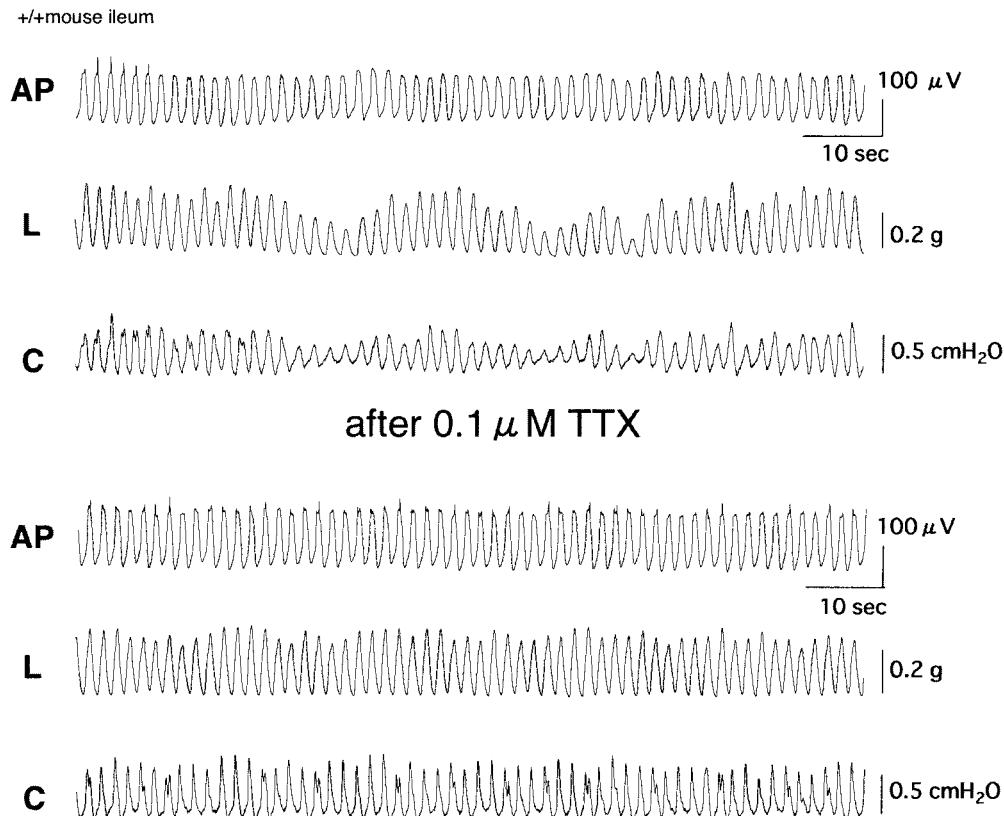


Fig. 5. Effects of tetrodotoxin on simultaneous spontaneous electrical activity and both longitudinal muscle contractions and circular muscle contractions in $+/+$ mouse ileum. AP: action potential. L: longitudinal muscle contractions. C: circular muscle contractions.

inhibitory neural influences, even if the ICC-MY were absent. These spontaneous contractions may be generated by ICC-IM (according to my nomenclature, *i.e.* previously ICC-DMP).

The result obtained from W/W^{y} mice suggests the possibility that either ICC-MY and/or ICC-IM (ICC-DMP) are pacemakers in the mouse small intestine. If ICC-MY is deficient, ICC-IM (ICC-DMP) could be a primary pacemaker, although there is not much difference between the frequencies of the two ICC networks (compare the frequency in the traces in Fig. 5 with the lower traces after L-NAME in Fig. 6). This is markedly different from the pacemakers in the heart, where the frequency of the pacemaker potentials in SA node cells is higher than that in AV node cells.

Properties of Plateau Potentials in ICC-SM in the Mouse Proximal Colon

Electrophysiological properties of spontaneously active cells in the mouse proximal colon

Intracellular recordings were made from different types of smooth muscle preparations of the mouse proximal colon.

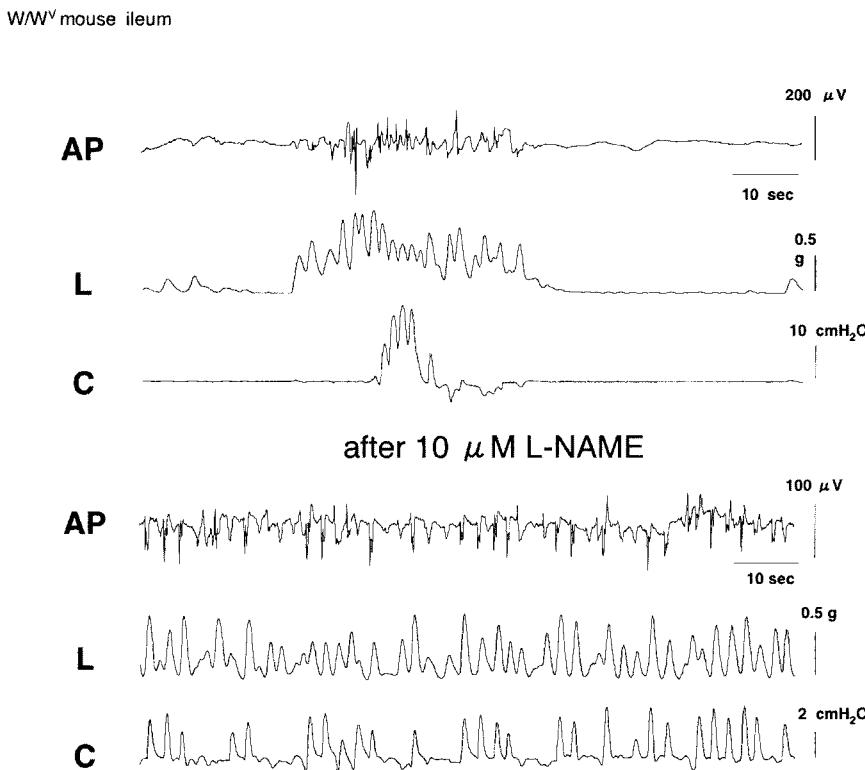


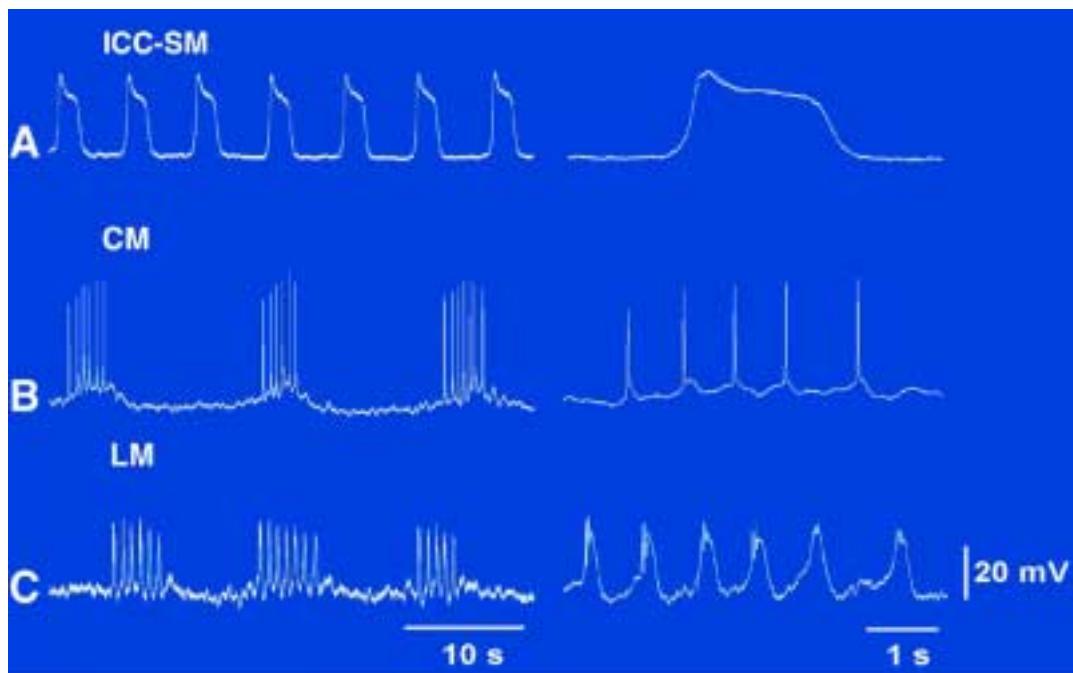
Fig. 6. Effects of an NO synthase inhibitor, nitro-L-arginine methyl ester (L-NAME) on simultaneous spontaneous electrical activity and both longitudinal muscle contractions and circular muscle contractions in the *W/W^v* mouse ileum. Upper traces: before L-NAME. Lower traces: after L-NAME (10 μ M).

Plateau potentials in ICC-SM cells

In preparations in which the submucosal layer was uppermost, but with the circular and longitudinal smooth muscle layers still attached, square-shaped potential changes consisting of initial fast rising and subsequent plateau components (plateau potentials) were recorded from cells in the submucosa (Fig. 7, A). These plateau potentials were generated at mean frequencies of $14.8 \pm 3.4 \text{ min}^{-1}$, with mean peak amplitudes of $19.6 \pm 6.9 \text{ mV}$. The mean rates of rise of the initial potentials were $148 \pm 61 \text{ mV/s}$ and the mean duration of the plateau potentials at the foot were $2.6 \pm 0.3 \text{ s}$. The mean resting membrane potentials of cells generating plateau potentials were $-48.2 \pm 5.9 \text{ mV}$. Neither TTX (1 μ M) nor atropine (1 μ M) altered the frequency, amplitude or resting membrane potentials of the plateau potentials (Yoneda *et al.*, 2002a).

Bursts of spike potentials in circular smooth muscle cells

In preparations with the circular smooth muscle layer exposed after removal of the submucosal layer, recordings were made from smooth muscle cells which exhibited rhythmic generation of bursts of spike potentials (Fig. 7, B) (Yoneda *et al.*, 2000a). The mean resting membrane potential of these circular smooth muscle cells was $-44.9 \pm 5.5 \text{ mV}$. Bursts of spike



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Fig. 7. Spontaneous electrical activity recorded from the mouse proximal colon. A: Plateau potentials from an ICC-SM (submucosal ICC). B: Bursts of action potentials in a circular smooth muscle cell. C: Bursts of oscillatory potentials in a longitudinal smooth muscle cell. From Yoneda *et al.*, (2002a).

potentials were generated with a mean frequency of $4.6 \pm 1.1 \text{ min}^{-1}$. Both TTX (1 μM) and L-NAME (10 μM) increased the frequency of the bursts of spike potentials, but atropine (1 μM) did not (Yoneda *et al.*, 2002b; Takaki and Yoneda, 2003; Takaki *et al.*, 2003).

Bursts of oscillatory potentials in longitudinal smooth muscle cells

In preparations with the longitudinal smooth muscle layer exposed after removal of the submucosal layer, recordings were made from smooth muscle cells which exhibited periodic bursts of oscillatory potentials (Yoneda *et al.*, 2002a). The form of these oscillatory potentials differed from both the plateau potentials and spike potentials, as shown in Fig. 7, C. The mean resting membrane potential was $-48.1 \pm 7.2 \text{ mV}$. Bursts of oscillatory potentials were generated with a mean frequency of $4.3 \pm 1.3 \text{ min}^{-1}$. Both TTX (1 μM) and L-NAME (10 μM) increased the frequency of the bursts of oscillatory potentials, but atropine (1 μM) did not (Yoneda *et al.*, 2002b; Takaki and Yoneda, 2003; Takaki *et al.*, 2003).

The periodic generation of bursts of either spike potentials or oscillatory potentials in smooth muscle cells was not causally related to the ICC-SM, because the removal of the submucosal layer did not affect their periodic generation and their frequency was quite different from that of the plateau potentials of the ICC-SM. As none of these electrical activities were blocked by either TTX or atropine, it seems unlikely that excitation of cholinergic nerves triggers these electrical activities. It is convincing, however, that enteric nitrergic nerves

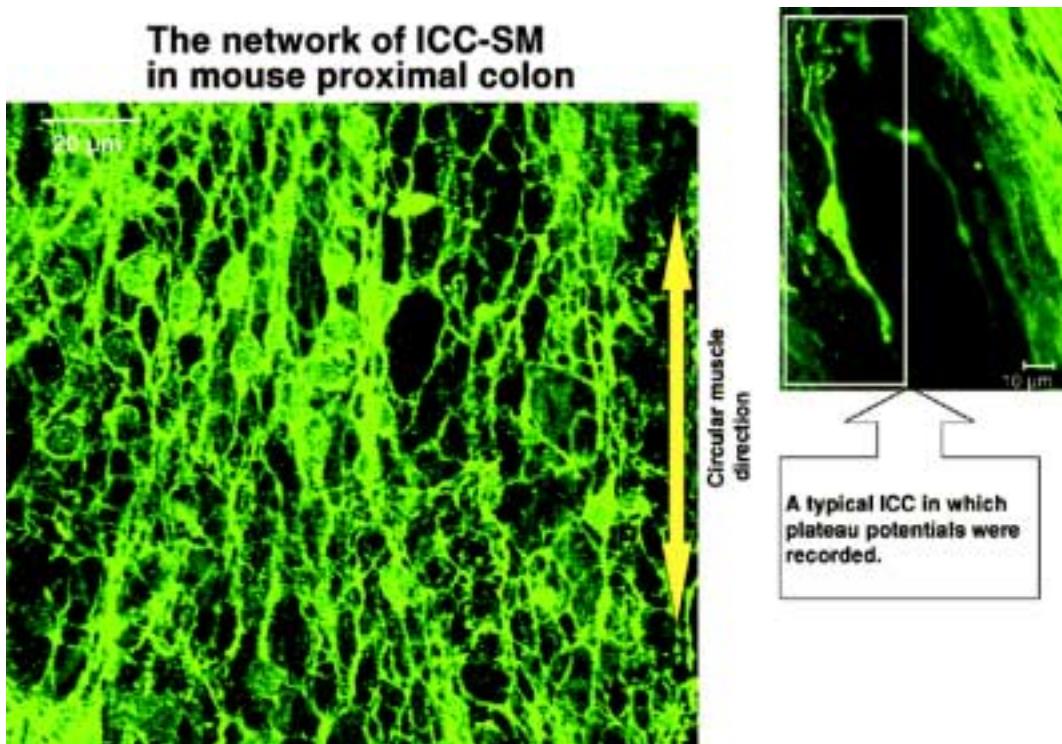


Fig. 8. The morphology of the ICC-SM network in the mouse proximal colon. A typical ICC in which plateau potentials were recorded is also shown.

tonically suppress these electrical activities in smooth muscle cells. This is in contrast to the plateau potentials of ICC-SM cells, in which no enteric nerves modulate the generation of plateau potentials.

Morphology of cells spontaneously generating plateau potentials in the proximal colon

Neurobiotin was injected into submucosal cells that had demonstrated the generation of plateau potentials. This was done in the presence of 18β -glycyrrhetic acid (18β -GA, 20 μ M), a known inhibitor of gap junctions in smooth muscle tissues (Yamamoto *et al.*, 1998). The stained cells had oval shaped cell bodies with a maximum diameter of 20–30 μ m and a thickness of 5–8 μ m. Each cell had bipolar processes of 50–150 μ m in length with the tip of the process often being bifurcated. These cells ran parallel to the circular smooth muscle bundles, suggesting the possibility that an electrical tonic excitation is conducted in the direction of the circular smooth muscle.

Immunohistochemical examination identified a dense network of c-Kit-positive interstitial cells in the submucosal layer at the boundary of the circular smooth muscle layer (Fig. 8), as had been reported by Vanderwinden *et al.* (2000). The c-Kit-positive cells were distributed in the same layer with the neurobiotin-filled cells and appeared to be similar in shape and size as detailed above. Thus, it would appear that the plateau potentials were recorded from the c-Kit

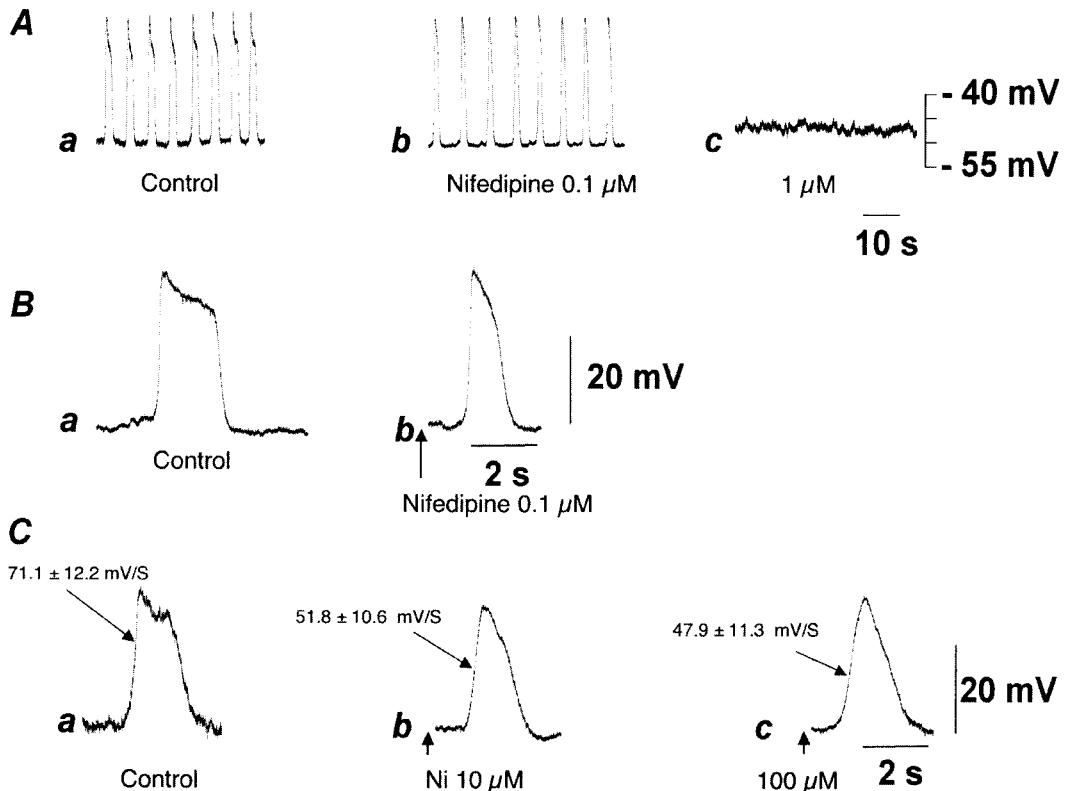


Fig. 9. Effects of nifedipine and nickel (Ni) on plateau potentials in an ICC. A: Dose-dependent effects of nifedipine. B: Nifedipine (0.1 μ M) decreased the duration of plateau potentials. C: Ni (10–100 μ M) decreased the rate of rise of initial phase of plateau potentials.

protein-expressing interstitial cells distributed in the submucosal layer (ICC-SM) of the proximal colon. However, the nifedipine sensitivity of the plateau potentials of the ICC-SM of the mouse proximal colon is quite different from that of the other ICC as described below.

Effects of nifedipine and Ni^{2+} on plateau potentials

The effects of nifedipine, an inhibitor of voltage-gated Ca^{2+} -channels (Nelson *et al.*, 1990), were investigated on the spontaneously generated plateau potentials in the ICC-SM (Yoneda *et al.*, 2002a). This was because pacemaker potentials (or currents) in ICC are considered to be nifedipine-resistant (Torihashi *et al.*, 2002). Plateau potentials were still generated with the same frequency in the presence of 0.1 μ M nifedipine, although the duration of the plateau component was markedly shortened (Fig. 9, Ab). Nifedipine (0.1 μ M) did not affect the rate of rise of the initial component of plateau potentials (155 ± 65 mV/s in control vs. 148 ± 69 mV/s in nifedipine) (Fig. 9, Bb), but nifedipine (1 μ M) did abolish plateau potentials (Fig. 9, Ac). These observations indicate that the plateau potentials are dependent upon activation of voltage-gated L-type Ca^{2+} -channels. The reason for the nifedipine-sensitivity of plateau potentials in the ICC-SM in the mouse proximal colon remains unknown.

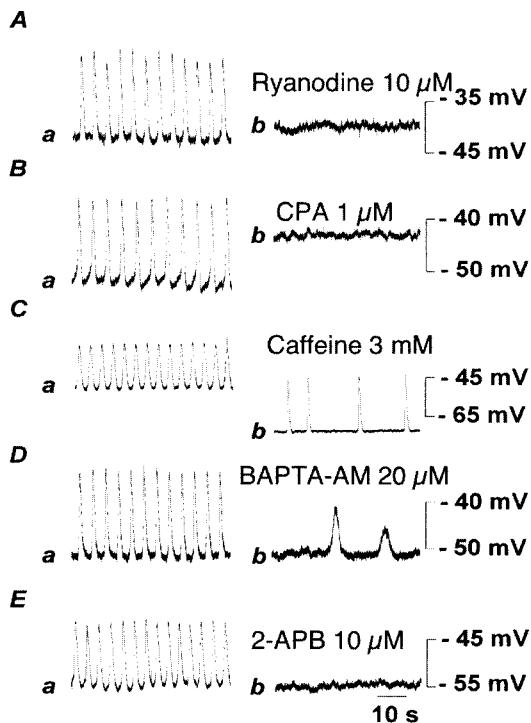


Fig. 10. Modulation of internal Ca^{2+} store and plateau potentials in ICC. A: Ryanodine (10 μM) abolished plateau potentials. B: cyclopiazonic acid (CPA; 1 μM) abolished plateau potentials with an associated membrane depolarization. C: Caffeine (3 mM) decreased the frequency but increased the amplitude of plateau potentials with an associated membrane hyperpolarization. D: BAPTA-AM (20 μM) markedly suppressed plateau potentials. E: 2-aminoethoxy-diphenyl borate (2-APB; 10 μM) abolished plateau potentials.

Furthermore, Ni^{2+} (10–100 μM), an inhibitor of voltage-gated T-type Ca^{2+} -channels, decreased the rate of rise of plateau potentials but did not abolish the plateau potentials (Fig. 9, C) (Yoneda *et al.*, 2003). These results indicate that two types of voltage-gated channel currents form the plateau potentials: the initial component is Ni^{2+} -sensitive and their plateau component is nifedipine-sensitive.

Effects of internal Ca^{2+} modulators on plateau potentials

Ryanodine (10 μM) completely abolished plateau potentials (Fig. 10, Ab). CPA (1 μM) also completely abolished plateau potentials but with an associated membrane depolarization (Fig. 10, Bb). The fact that high $[\text{K}^+]_o$ solution depolarized the membrane by about 25 mV, but did not abolish plateau potentials, showed that the inhibition of plateau potentials by CPA is not due to depolarization of the membrane. Caffeine (3 mM) largely decreased the frequency of plateau potentials also with an associated membrane hyperpolarization, although the amplitude increased (Fig. 10, Cb). BAPTA-AM (20 μM) also largely decreased the frequency of plateau potentials without any changes in the membrane potential (Fig. 10, Db). 2-APB (10 μM)

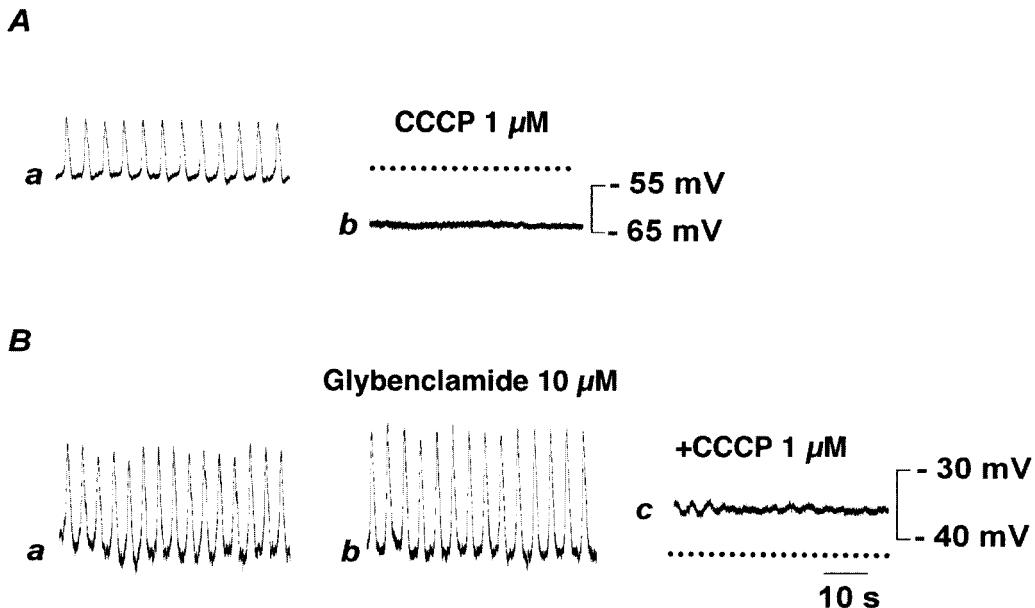


Fig. 11. Effects of CCCP, a mitochondria protonophore, on plateau potentials in ICC. A: CCCP (1 μ M) abolished plateau potentials with an associated membrane hyperpolarization. B: After pretreatment with glybenclamide, a K_{ATP} channel blocker, CCCP abolished plateau potentials without causing membrane hyperpolarization. J. Physiol. 542.3, 887–897, 2002 (with permission)

completely abolished plateau potentials Fig. 10, Eb). These results suggest that the Ca^{2+} release-refilling cycle determines the frequency of plateau potentials as suggested by Liu *et al.* (1995). IP_3 receptors may also be involved in the process of plateau potential generation as suggested by many researchers (Suzuki, 2000; Suzuki *et al.*, 2000; Hirst and Edwards, 2001; Fukuta *et al.*, 2002). Furthermore, the plateau potentials in the ICC-SM in the proximal colon are modulated by ryanodine receptors. This Ca^{2+} modulation by ryanodine receptors in the proximal colon is different from that of the IP_3 receptors in the mouse and guinea pig stomach (Suzuki, 2000; Suzuki *et al.*, 2000; Hirst and Edwards, 2001; Fukuta *et al.*, 2002).

Role of mitochondrial functions in the plateau potential

Recently, it was postulated that intracellular Ca^{2+} handling between an internal Ca^{2+} store and mitochondria produces the rhythmic activity in cultured myenteric ICC of both the mouse jejunum (Ward *et al.*, 2000) and the guinea-pig stomach (Fukuta *et al.*, 2002). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (1 μ M), a mitochondrial protonophore (Duchen, 2000), reduced the frequency of plateau potentials, and finally abolished them with an associated membrane hyperpolarization (Fig. 11, Ab). Glybenclamide (10 μ M), an inhibitor of ATP sensitive K^+ -channels (Cook, 1988), antagonized CCCP-induced hyperpolarization. In the presence of glybenclamide, CCCP abolished plateau potentials with an associated membrane depolarization (Fig. 11, Bc) (Yoneda *et al.*, 2002a). These results suggest that the inhibition of plateau potentials by CCCP is not causally related to the membrane hyperpolarization.

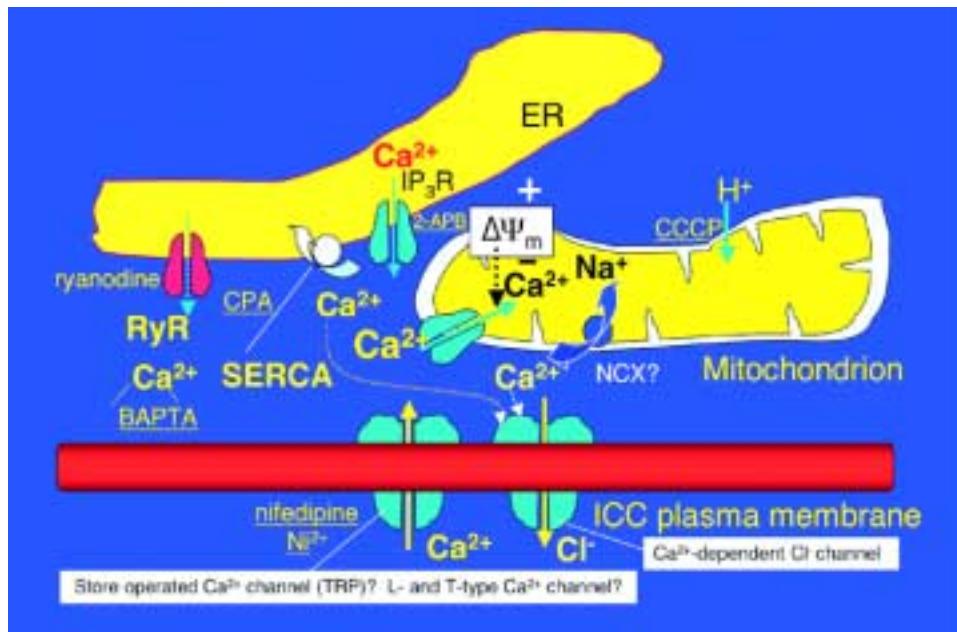


Fig. 12. Model proposed as an explanation for how plasma membrane-endoplasmic reticulum-mitochondrial Ca^{2+} handling drives pacemaker currents in ICC. Ca^{2+} is released from ER via IP_3R and RyR . Some of the Ca^{2+} is taken-up by mitochondria driven by the negative membrane potential (Ψ_m). This Ca^{2+} uptake by mitochondria activates nonselective cation channels (TRP) in the plasma membrane, resulting in inward pacemaker currents. Voltage-gated Ca^{2+} channels may also be activated. Ca^{2+} entry via these channels is partially taken up by ER via SERCA to reset the cycle. NCX removes Ca^{2+} from mitochondria. Ca^{2+} -dependent Cl^- channels are also activated by intracellular Ca^{2+} to generate pacemaker currents. Which of these components has the primary role is not yet determined, but this is a possible generation mechanism for pacemaker currents. ER: endoplasmic reticulum. IP_3R : inositol 1,4,5-triphosphate receptor. NCX: $\text{Na}^+/\text{Ca}^{2+}$ exchange. RyR: ryanodine receptor. SERCA: ER- Ca^{2+} ATPase. $\Delta\Psi_m$: negative membrane potential difference.

The proposal that intracellular Ca^{2+} handling between an internal Ca^{2+} store and mitochondria produces the rhythmic activity is also supported in the ICC-SM in the mouse proximal colon. The plateau potentials are generated through activation of voltage-gated Ca^{2+} channels, which are coupled to the release of Ca^{2+} from the internal stores and the handling of Ca^{2+} in mitochondria. In addition to voltage-gated Ca^{2+} channels, several types of plasmalemmal ionic channels that are periodically activated to generate a pacemaker current have been proposed, such as non-selective cation channels including transient receptor potential subfamily ion channels (TRPs), Ca^{2+} -dependent Cl^- channels, and/or Ca^{2+} activated K^+ channels (Huizinga *et al.*, 1995; Kölbel *et al.*, 1998; Horowitz *et al.*, 1999; Fujita *et al.*, 2001; Huizinga *et al.*, 2002; Torihashi *et al.*, 2002) (Fig. 12).

The possible role of plateau potentials in ICC-SM

The frequencies of spontaneous electrical activity differs between ICC-SM cells and circular

and longitudinal smooth muscle cells in the mouse proximal colon (Yoneda *et al.*, 2002a). Therefore, the role of plateau potentials in the ICC-SM remains unknown. A variety of driving mechanisms have been reported for the ICC networks in both different tissues and in different species as follows: 1) ICC-SM and circular smooth muscle cells form networks of gap junctions in the canine proximal colon and thus the ICC-SM drives circular muscle activity (Smith *et al.*, 1987a; Berezin *et al.*, 1988; Sanders and Smith, 1989; Kobayashi *et al.*, 1995). 2) The ICC-MY drives smooth muscle activities in the colon, stomach or small intestine (Sanders, 1996; Huizinga *et al.*, 1997; Sanders *et al.*, 1999). 3) In the rat colon, two independent and distinct pacemakers (ICC-SM and ICC-MY) drive the longitudinal and circular smooth muscle contractions: slow waves derived from the ICC-SM trigger circular smooth muscle contractions and the cyclic oscillatory potentials derived from the ICC-MY trigger both circular and longitudinal smooth muscle contractions (Plujà *et al.*, 2000). 4) Summed signals from ICC-SM and ICC-MY excite circular smooth muscle electrical and mechanical activities in the canine proximal colon (Smith *et al.*, 1987b; Keef *et al.*, 1997).

The mouse proximal colon may differ from other parts of the gastrointestinal tract but may be similar to the rat proximal colon (Plujà *et al.*, 2000). Although peristalsis distally towards the anus could also occur in this pacemaker region, “antiperistalsis” is the first activity to appear in this region in both the rat and guinea pig proximal colon (Hukuhara and Neya, 1968). Thus, it seems likely that plateau potentials in the ICC-SM contribute to the generation of this “antiperistalsis”, because the frequency of the plateau potentials is similar to that of the regular contractions at the mesenteric border of the pacemaker area in the guinea-pig proximal colon (Kobayashi *et al.*, 1996).

Two Different Pacemaker Activities Regulate Peristalsis and “Antiperistalsis” in the Mouse Proximal Colon

The next study was aimed to elucidate how the electrical activities of the ICC-SM and circular and longitudinal smooth muscle cells control spontaneous contractions such as peristalsis and “antiperistalsis” (circular rings of contraction rippling proximally from the pacemaker region) of the mouse proximal colon. The results indicate that the ICC-SM controls the spontaneous small faster circular muscle contractions (possibly corresponding to “antiperistalsis”) and another pacemaker (possibly ICC-MY and/or ICC-IM) independently controls the spontaneous big slower circular and longitudinal contractions (possibly corresponding to peristalsis). The pacemaker activity in the ICC-SM during “antiperistalsis” is not regulated by the enteric nervous system, but another possible pacemaker activity during peristalsis is regulated by enteric nitrergic inhibitory nerve.

Properties of mechanical activities in mouse proximal colon

Periodical rhythmic contractions (big contractions) were recorded in the circular muscle direction of preparations with the submucosa attached. These contractions had a mean frequency of 4 ± 2 times/min and a mean amplitude of 0.12 ± 0.07 g. Between such big contractions, rhythmic smaller contractions (small contractions) were observed. The mean

frequency of these small contractions was 16 ± 3 times/min and was significantly higher than that of the big contractions, but the amplitude of these contractions was one-tenth of the big contractions (Yoneda *et al.*, 2002b; Takaki and Yoneda, 2003; Takaki *et al.*, 2003).

Periodical rhythmic contractions (big contractions) were also recorded in the direction of the longitudinal smooth muscle. The frequency and amplitude of these contractions corresponded to those in the circular muscle direction. Small contractions were not recorded in the longitudinal smooth muscle direction. Small contractions in the circular muscle were not observed after removal of the submucosal layer where the ICC-SM is distributed, but the frequency and amplitude of the big contractions in the circular and longitudinal directions were not affected (Yoneda *et al.*, 2002b; Takaki and Yoneda, 2003; Takaki *et al.*, 2003).

Simultaneous recordings of mechanical and electrical activities in the proximal colon

The faster small circular contractions were generated synchronously with the plateau potentials in the ICC-SM. The big contractions in the direction of the circular or longitudinal smooth muscle directions were generated asynchronously with the plateau potentials, but synchronously with bursts of action potentials in the circular smooth muscle cells or with bursts of oscillatory potentials in longitudinal smooth muscle cells. The frequency of the big contractions was completely different from that of the plateau potentials in the ICC-SM (Yoneda *et al.*, 2002b; Takaki and Yoneda, 2003; Takaki *et al.*, 2003). The frequency of the big contractions and the frequency of both bursts of action potentials and oscillatory potentials were not changed after removal of the submucosal layer, indicating that the big contractions are not causally related to the ICC-SM. These results suggest that the electrical and mechanical activities of the smooth muscle layers are derived from two pacemaker activities (ICC-SM and another possible pacemaker such as ICC-MY and/or ICC-IM) and that these two or three pacemakers regulate the mechanical activity of the smooth muscle coats.

Role of cholinergic and nitrergic nerves on the electrical and mechanical activities in circular and longitudinal smooth muscle cells

The electrical activities recorded from the ICC-SM were not affected by TTX or atropine, suggesting that enteric nerves do not control these activities as mentioned above (Yoneda *et al.*, 2002a). To reveal possible neural control mechanisms for the slower big contractions possibly generated by another pacemaker activity, the effects of TTX, atropine and a nitric oxide (NO) synthase inhibitor, L-NAME were tested on the big contractions in circular and longitudinal muscle directions and on corresponding bursts of action potentials in circular muscle cells and on bursts of oscillatory potentials in longitudinal muscle cells.

While TTX ($0.1 \mu\text{M}$) increased the frequency of the big contractions from 3 to ~4–5 times/min and also increased the frequency of both bursts of action potentials and oscillatory potentials without changes in the resting membrane potential, atropine ($1 \mu\text{M}$) did not. L-NAME ($10 \mu\text{M}$) increased the frequency of the big contractions from 3–4 to 5–6 times/min and also increased the frequency of both bursts of action potentials and oscillatory potentials without changes in the resting membrane potential. These inhibitory effects of nitrergic nerves on another possible pacemaker activity are consistent with those in the canine proximal colon

(Keef *et al.*, 1997) and in the ileum of the *W/W^v* mutant mouse (Nakagawa *et al.*, 2003; Takaki *et al.*, 2003), where the spontaneous electrical and mechanical activities might be tonically suppressed by basal release of NO.

No inhibitory effects of intracellular Ca²⁺ modulators on electrical activities in circular and longitudinal smooth muscle cells

CPA (10 μM) depolarized the membrane potentials in circular and longitudinal smooth muscle cells and consequently increased the frequency or abolished both the bursts of action potentials in circular muscle cells and the oscillatory potentials in longitudinal muscle cells. BAPTA (20 μM) did not change the frequency of both bursts of action potentials and oscillatory potentials, and resting membrane potentials. 2-APB (10 μM) depolarized the membrane potentials in circular and longitudinal smooth muscle cells and increased the frequency of both bursts of action potentials and oscillatory potentials, and finally continuously generated action potentials and oscillatory potentials (Yoneda *et al.*, 2002b). The results of 2-APB, BAPTA and CPA indicated that the release of Ca²⁺ from internal stores through IP₃ receptors does not play a major role in the generation of electrical activity in circular and longitudinal smooth muscle cells.

Role of two different pacemaker activities in the mouse proximal colon

In conclusion, in the mouse proximal colon, the ICC-SM regulates only the spontaneous faster rhythmic small contractions in the circular muscle direction and another pacemaker (possibly ICC-MY and/or ICC-IM) independently regulates the spontaneous slower periodical rhythmic big contractions in the circular and longitudinal muscle directions. No enteric nerves regulate pacemaker activity in the ICC-SM, whereas nitric oxide (NO) inhibitory enteric nerves control another pacemaker activity. Intracellular Ca²⁺ release from the internal stores through IP₃ receptors and ryanodine receptors play a major role in the generation of pacemaker activity in the ICC-SM, but not for the generation of another pacemaker activity recorded in the circular and longitudinal smooth muscle cells. These results are in contrast to the dog proximal colon, where the activity of smooth muscle cells is the result of the summation of inputs from two discrete populations of pacemakers, the ICC-SM and the ICC-MY (Smith *et al.*, 1987b; Barajas-Lopez and Huizinga, 1989; Keef *et al.*, 1997).

Peristalsis and “antiperistalsis” in the mouse proximal colon

In rats and guinea pigs, “antiperistalsis” is initiated in a special region of the proximal colon, termed the “pacemaker area” (Hukuhara and Neya, 1968). This area has been reported to contain bipolar or multipolar cells in the submucosa which express c-Kit proteins, in addition to the ICC-MY (Nahar *et al.*, 1998). “Antiperistalses” are composed of circular muscle contractions which ripple proximally from the pacemaker area and thus contribute to the retention and mixing of the contents as well as preventing a too rapid transportation of the intraluminal contents by peculiar constriction rings (tonic and local circular muscle contractions) in the proximal colon. Consequently, the intraluminal contents would stay longer in the proximal colon and allow more effective water absorption and the formation of solid feces.

As described above, the two different mechanical activity patterns of the mouse proximal colon are similar to those in the rat colon (Plujà *et al.*, 2000). Rhythmic contractions with a frequency of 10–12 times/min are generated in the pacemaker region of the guinea-pig proximal colon, with a different frequency to other regions of the colon (Kobayashi *et al.*, 1996). The frequency of these rhythmic contractions is comparable to that of the plateau potentials in the ICC-SM and to the small faster contractions in the circular muscle direction in the mouse proximal colon. Small contractions in the circular muscle direction driven by the ICC-SM may correspond to the “antiperistalsis” observed *in situ* by Hukuhara and Neya (1968). Moreover, peculiar constriction rings moving distally are also observed in the proximal colon of rats and guinea pigs (Hukuhara and Neya, 1968). Big contractions driven by another pacemaker may be related to these peculiar distally moving constriction rings observed *in situ*. When these small faster and slower big contractions are well coordinated, solid feces could be made from semi-liquid intraluminal contents in the mouse proximal colon and propelled to the distal colon.

Gut-like Clusters Differentiated from Embryonic Stem Cells as a Good Model for Exploring Generation Mechanisms of Gut Spontaneous Motility

Hukuhara *et al.*, (1961, 1962) have shown that enteric ganglion cells are essential for the intestinal intrinsic reflex but not for spontaneous motility of the intestine. Enteric nerves, however, could modulate some types of spontaneous motility such as slower big contractions in the mouse proximal colon (Yoneda *et al.*, 2002b; Takaki and Yoneda, 2003). Moreover, in the *W/W^r* mutant mouse ileum where the ICC-MY is deficient, enteric nerves could modulate spontaneous motility (Nakagawa *et al.*, 2003; Takaki *et al.*, 2003). Therefore, the contribution of the enteric neural plexus and the ICC networks to spontaneous rhythmic activity in the gut is still not certain.

Recently, embryonic stem (ES) cells gave rise to a functional organ-like unit, the “gut” (ES-gut), which demonstrated peristalsis-like motility and was morphologically well-organized with enteric derivatives of all three embryonic germ layers: epithelial cells (endoderm), smooth muscle cells and interstitial cells of Cajal (ICC) (mesoderm), and enteric neurons (ectoderm) (Yamada *et al.*, 2002). On approximately day-21 of outgrowth culture, the ES-gut showed distinct and highly coordinated contraction patterns with regular rhythms. This mechanical activity was composed of periodic contraction and relaxation. It was very similar to gastrointestinal (GI) motility, i.e. peristalsis and local back-and-forth and segmenting movements. The ICC and/or enteric neurons could coordinate GI motility.

Many types of ES clusters can form, because ES cells have a pluripotent ability to differentiate into a wide range of cell types. Therefore, to obtain a good model for exploring generation mechanisms of gut spontaneous motility, specific differentiation needs to be induced by adding exogenous factors. Before doing this, physiological and morphological properties of spontaneously differentiated ES clusters need to be investigated.

In order to gain insight into the mechanisms involved in the generation of spontaneous motility as in the mammalian GI (Suzuki *et al.*, 2000; Ward *et al.*, 2000; Torihashi *et al.*, 2002), physiological and morphological properties of the ES-gut on approximately day-21 of embryoid

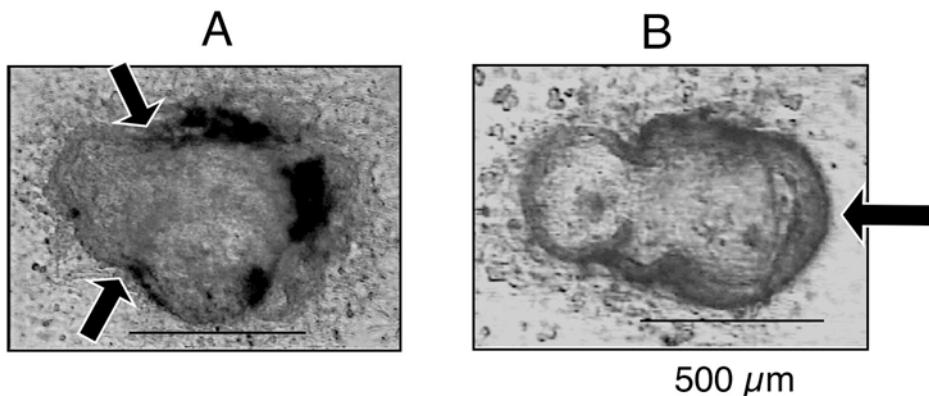


Fig. 13. Two gut-like organs differentiated from embryonic stem cells. Arrows showing the actively moving sites.

body (EB) outgrowth culture were investigated by analyzing spontaneous motility, electrical activity and intracellular Ca^{2+} movements as well as by immunohistochemistry and electron microscopy.

Spontaneous motilities in ES-gut

A variety of motility patterns are observed in ES clusters. A tubular (but hemispherical) cluster showed distinct patterns of highly coordinated peristalsis-like contractions (data not shown) and a dome-like cluster showed local back-and-forth contractions (Fig. 13B). Each mechanical activity was composed of a periodic contraction (Fig. 13A) and relaxation.

The mean frequency of spontaneous motility in ES clusters was ~14 cpm on day-21 of embryoid body (EB) outgrowth culture, but this was temperature-dependent. A voltage-gated L-type Ca^{2+} channel blocker, nifedipine (0.1–10 μM) dose-dependently decreased the frequency of the motility. An agent inhibiting neural activity via Na^+ channel blockade (TTX; 0.1–1 μM) did not affect the frequency of spontaneous periodic contractions. Ryanodine (0.1–10 μM) dose-dependently decreased the frequency, whereas 2-APB (1–10 μM) only slightly decreased the frequency (Ishikawa *et al.*, 2003).

Electrophysiological studies

Plateau potentials, which are possibly pacemaker potentials in ICC, were recorded from a twin hemispherical cluster as shown in Fig. 14 (Ishikawa *et al.*, 2003). The mean amplitude of these potentials was approximately 43 mV. The mean rate of rise of these potentials was approximately 30 mV/s. After treatment with an L-type Ca^{2+} -channel blocker, nifedipine (5 μM), the plateau potentials unchanged. Electrical slow waves at a frequency range of 1–18 cpm (mean: ~8 cpm) were frequently recorded at 35°C from what were possibly smooth muscle cells.

Calcium oscillation in ES clusters

Synchronous $[\text{Ca}^{2+}]_i$ oscillations propagate widely over the ES clusters. The

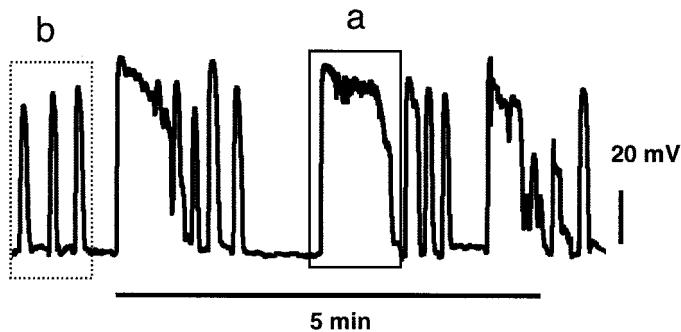


Fig. 14. Plateau potentials possibly within an ICC of a gut-like organ differentiated from embryonic stem cells. a: a single long-duration plateau potential. b: several short-duration plateau potentials.

pharmacological properties of these $[Ca^{2+}]_i$ oscillations were investigated. In the presence of nifedipine ($10 \mu M$), the $[Ca^{2+}]_i$ oscillations over the cluster appeared to decrease but were not abolished, whereas spontaneous contractions were abolished. When additional $40 \mu M$ Ni^{2+} was applied, the remaining $[Ca^{2+}]_i$ oscillations frequently almost disappeared (Ishikawa *et al.*, 2003). Application of $10 \mu M$ ryanodine almost always significantly decreased the frequency of $[Ca^{2+}]_i$ oscillation.

Immunohistochemistry

Immunoreactivity for c-Kit, a useful marker for ICC (Der-Silaphet *et al.*, 1998; Thomsen *et al.*, 1998; Torihashi *et al.*, 1997), was detected in clusters on day-21 of EB culture that had spontaneous contractions at a regular rhythm and generated electrical slow waves (Yamada *et al.*, 2002; Ishikawa *et al.*, 2003). The hemispherical dome-like cyst showed a large number of *c-Kit* immunopositive (*c-Kit*+) cells on the wall of the dome-like structure surrounding the lumen. The *c-Kit*+ cells were mostly multipolar, and formed a distinct and dense network. This network structure of *c-Kit*+ cells was similar to that of ICC at the level of the myenteric plexus (ICC-MY) in the pylorus, small intestine, and colon of a murine embryo or neonate, as described by Torihashi *et al.* (1997). The *c-Kit*+ cells did not form a single layer, but were scattered throughout the muscle layer.

Ultrastructure of ICC within the musculature of the contracting ES clusters

As described above, the wall of the cluster was constituted of three layers, *i.e.* epithelium, submucosa and musculature (Yamada *et al.*, 2002). The musculature consisted of smooth muscle cells. Although the musculature was not well organized, several smooth muscle cells ran in the same direction and the musculature was divided into two or more layers (Ishikawa *et al.*, 2003). Interstitial cells showing some ultrastructural features of ICC were confirmed within the musculature, as previously reported by Yamada *et al.* (2002). The ICC frequently formed close contacts with neighboring smooth muscle cells. Gap junctions were also observed between a variety of components (Yamada *et al.*, 2002; Ishikawa *et al.*, 2003; Takaki *et al.*, 2003).

ES gut as a good model for exploring generation mechanisms of spontaneous rhythm

Although the ES-gut lacked some components, such as lymphatic vessels and vascular formation, we have shown that the ES-gut has the essential transmural morphological organization found in a normal intestine (Yamada *et al.*, 2002). Thus, the ES cell-derived gut could be a good model to clarify the mechanism by which GI motility is regulated.

In this study, we have confirmed that an organized gut is generated *in vitro* from developing EBs in the absence of exogenously added growth factors. This suggests the possibility that in the presence of exogenously added growth factors we could differentiate a particular ES-gut with uniform physiological and morphological properties of its cellular components such as being ICC-deficient, or having well differentiated enteric neurons, or well differentiated endocrine glands, or with well differentiated IP₃ receptors and/or ryanodine receptors.

Intracellular Ca²⁺ circuit

It has been proposed that the periodic activation of plasmalemmal ion channels to generate pacemaker currents triggers the intracellular release of Ca²⁺ from endoplasmic reticulum (ER). This Ca²⁺ release is mediated through inositol triphosphate (IP₃) type 1 receptors in the ER with subsequent entry of Ca²⁺ into mitochondria (Suzuki *et al.*, 2000; Ward *et al.*, 2000). These processes are very similar to those observed in cultured cell clusters isolated from mouse intestine (Nakayama and Torihashi, 2002; Torihashi *et al.*, 2002). Ryanodine, however, largely decreased the frequency of both Ca²⁺ oscillations and spontaneous motility in the ES clusters on 21-day outgrowth, suggesting the possibility that ryanodine receptors are differentiated and contribute to intracellular Ca²⁺ movements. Differentiation of either ryanodine or IP₃ receptors or both types of receptors is expected in the ES-gut.

Plasmalemmal ionic channels

In contrast to the evidence for an intracellular Ca²⁺ circuit, plasmalemmal ionic channels that are periodically activated to generate pacemaker currents have not been demonstrated. Several candidates for the role of plasmalemmal ionic channels such as non-selective cation channels which include a transient receptor potential subfamily ion channel (TRPs), Ca²⁺-dependent Cl⁻ channels, and/or Ca²⁺ activated K⁺ channels have been reported (Kölbl *et al.*, 1998; Horowitz *et al.*, 1999; Fujita *et al.*, 2001; Huizinga *et al.*, 2002; Torihashi *et al.*, 2002). Furthermore, it has been found that L-type and T-type Ca²⁺ channels contribute to the generation of the pacemaker potentials in the ICC-SM of the mouse proximal colon (Yoneda *et al.*, 2002a; 2003). T-type Ca²⁺ channels may contribute to the generation of the pacemaker potential in ICCs of the ES cluster.

Conduction of pacemaker currents from ICC

Effective electrical coupling between ICC and smooth muscle cells in the ES cluster is likely to occur, because gap junctions were observed between a variety of cellular components (Yamada *et al.*, 2002; Ishikawa *et al.*, 2003; Takaki *et al.*, 2003). Indeed, the frequency of electrical slow waves and of the widely propagated Ca²⁺ oscillations corresponds to that of the spontaneous motility of the ES clusters, suggesting that there is an efficient conduction of pacemaker currents between the ICC and smooth muscle cells in the ES clusters. However, the

Table 1 Summary of autonomic rhythmicity in the gut

organ	Rhythm (mean cpm)	Neural regulation	Intracellular Ca ²⁺ modulation
	Electrical Mechanical		
+/- mouse			
Jejunum	47	47	No regulation
Ileum	40	40	No regulation
W/W ^v mouse			
Jejunum	Less rhythmicity	Slight inhibition	
Ileum	No rhythmicity	Nitregic inhibition	
			Not tested
BALB/C mouse			
Proximal colon			
ICC-SM	15	16 ^a	No regulation
Circular muscle cells	5	5	Nitregic inhibition
Longitudinal muscle cells	4	5	Nitregic inhibition
ES-gut	8	14	No regulation (?) ^b
			Important

a, Antiperistalsis, ripple circular muscle contractions transmitted from anal to oral side. b, Enteric neurons are not well-differentiated.

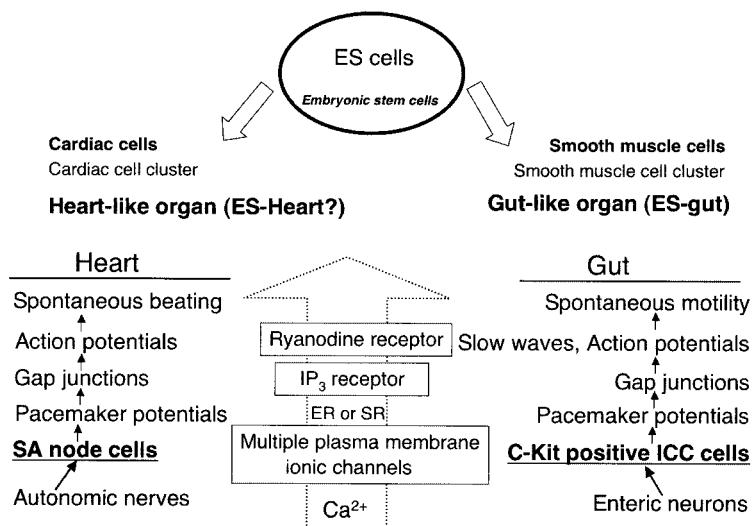


Fig. 15. Summary of the generation of spontaneous motility in the gut compared with that of spontaneous beating in the heart. The similarity between the gut and the heart including that of Ca²⁺ handling mechanisms is emphasized.

frequency of the electrical slow waves, the Ca²⁺ oscillations and of the spontaneous motility exhibited considerable variation. Well differentiated gap junctions between the ICC, smooth muscle cells and enteric neurons would be expected in the ES gut by adding exogenous growth factors or by gene transfer. Table 1 shows summary of autonomic rhythmicity, neural regulation and intracellular Ca²⁺ modulation on the rhythmicity of the gut in this review.

In conclusion, neural regulation of rhythmicity occurs in the gut as it does in the heart, and intracellular Ca²⁺ handling mechanisms plays a key role in the gut pacemaker responsible for spontaneous rhythmicity as it does for the cardiac pacemaker responsible for spontaneous

beating, as shown in Fig. 15. Although detailed Ca^{2+} handling mechanisms for the generation of rhythmicity are different, the role-players are very similar for both gut pacemaker and cardiac pacemaker cells.

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