Rhythmic Spontaneous Contractions in the Rat Proximal Colon

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Abstract: C-kit immunoreactive cells are known to be interstitial cells of Cajal (ICCs), and they generate pacemaker activity of the gastrointestinal tract. Recently a large number of special smooth muscle cells corresponding to c-kit immunoreactive cells were found in the proximal colon of the guinea pig. We learned that the rat proximal colon showed tetrodotoxin-insensitive regular rhythmic spontaneous contractions (RSCs) and hypothesized that RSCs are generated and/or regulated by ICCs. To prove our hypothesis, we investigated whether RSCs are absent in homozygous Ws/Ws mutant rats, since c-kit positive ICCs along the submucosal surface of the circular muscle (ICCSM) and myenteric plexus (ICCMY) are lacking. In contrast to our hypothesis, we found that RSCs were still present in the proximal colon of the Ws/Ws mutant rats. A recent study has reported that c-kit negative ICCSM remains in Ws/Ws mutant rats. Taken together, RSCs may be generated by c-kit negative ICCSM in the rat proximal colon. The blockade of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase by cyclopiazonic acid (CPA) (10\textsuperscript{-6} M) or by thapsigargin (10\textsuperscript{-6} M) increased the frequency of RSCs. The increasing effects of CPA on the frequency of RSCs were more prominent in Ws/Ws mutant rats than in +/+ rats. We concluded that the functional coordination between c-kit negative ICCSM and other mutationally impaired c-kit positive ICCMY and ICCSM may be required for moderate regulation in the frequency of spontaneous activity. [Japanese Journal of Physiology, 51, 717–723, 2001]

Key words: cyclopiazonic acid, interstitial cells of Cajal, proximal colon, sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase, Ws/Ws mutant rats.

Pulsating contraction waves started from “the turning portion of the colon” in the rat and the guinea pig and propagated both orally as antiperistalsis and anally as peristalsis [1]. Hukuhara and Neya [1] named this portion the “pacemaker area.” C-kit immunoreactive cells are known to be interstitial cells of Cajal (ICCs) and to generate pacemaker activity of the gastrointestinal tract [2, 3]. Recently in the colonie “pacemaker area” [1] and in the proximal colon in guinea pig, a larger number of special smooth muscle cells corresponding to c-kit immunoreactive cells were found than in the distal colon [4]. We learned that tetrodotoxin (TTX)-insensitive rhythmic spontaneous contractions (RSCs) are present in the rat proximal colon and hypothesized that RSCs are generated and/or regulated by ICCs. To prove our hypothesis, we investigated whether these RSCs are absent in homozygous Ws/Ws mutant rats. A Ws/+ mutant rat with white spots and coat color dilution was first found in the inbred colony of the BN/fMai strain [5]. Because homozygous Ws/Ws rats were not obtained in the genetic background of BN/fMai strain, spotted BN/fMai-Ws/+ rats were crossed with +/+ rats of the Donryu strain. Rats of

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+/-, Ws/+ rats. The genotypes were obtained by crossing male and female Ws/+ rats from a closed colony. The genotypes were identified by their coat color. The Ws mutant locus shows a deletion of 12 bases at the tyrosine kinase of c-kit, and the c-kit kinase activity was severely impaired in Ws/Ws mutant rats [6]. Consequently, the development of c-kit positive ICCs was impaired in Ws/Ws mutant rats.

In the present study, we found RSCs in the proximal colon of Wistar, Ws/Ws mutant, and +/- rats. Although c-kit positive ICCs along the submucosal surface of the circular muscle bundles (ICC_Sm) and myenteric plexus (ICC_MY) in the colon are surely deficient in the Ws/Ws rat colon, c-kit negative ICC_Sm, which has many gap junctions, are present [7]. It seems likely that RSCs are generated by c-kit negative ICC_Sm. Cyclopiazonic acid (CPA) is a well-known potent and specific inhibitor of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase in some smooth muscles [8–12]. Thapsigargin (TG) is also a selective inhibitor of SR Ca^{2+}-ATPase [13]. The blockade of SR Ca^{2+}-ATPase by CPA or TG increased the frequency of RSCs in Wistar, +/-, and Ws/Ws mutant rats. The increasing effects of CPA on the frequency of RSCs were more prominent in Ws/Ws mutant rats than in +/- rats. We concluded that the functional coordination between c-kit negative ICC_Sm and other mutationally impaired c-kit positive ICC_MY and ICC_Sm may be required for moderate regulation in the frequency of spontaneous activity.

**MATERIALS AND METHODS**

Twenty-seven male Wistar rats obtained from Charles River Japan Inc. (Yokohama, Japan), weighing 250–350 g, were killed by cervical dislocation following anesthesia with sodium pentobarbital (50 mg/kg I.P.). The abdomen was cut open along the midline, and approximately 2 cm of proximal colon 1–3 cm distal to the ileocecal sphincter was excised and flushed clean with Tyrode solution. The preparation was immediately suspended parallel to the longitudinal direction at a constant preload 0.3 g in the bath filled with 20-ml Tyrode solution, which was kept at a temperature ranging from 35 to 37°C and bubbled with 100% oxygen gas. The composition of the Tyrode solution was (mmol/l): NaCl, 136; KCl, 5.4; MgCl_2, 1.0; NaH_2PO_4, 0.33; CaCl_2, 1.8; D-glucose, 15.0; and HEPES, 5.0 (pH=7.4 throughout the experiment). The proximal colon preparation was left to settle in the organ bath for 1 h. The maintenance of animals and the experimental procedures both followed the guidelines of the local animal ethics committee.

**Recording of responses to CPA and TG in isometric longitudinal muscle contractions of isolated proximal colon in Wistar rats.** One side of the preparation was attached to the bottom of the bath and the other to an isometric force transducer (SS-1930, Nihon Kohden, Tokyo, Japan) to record force development in the longitudinal muscle direction. The response to CPA (10^{-6} M) or TG (10^{-6} M) was evaluated after the control regular RSCs were recorded for 30 min. The frequency and amplitude of RSCs reached a steady state within 15 min after the administration of CPA or TG. We evaluated the mean frequency and amplitude of RSCs for 5 min (before the drug application) and 15 min to 20 min (after application). Either an L-type Ca^{2+} channel blocker, nicardipine (10^{-11} M), or a capacitative Ca^{2+} entry inhibitor, SK&F 96365 (10^{-8} M), was added 30 min before the application of CPA after a 30-min recording of RSCs in control. The effect of either nicardipine or SK&F 96365 was evaluated 5 min before CPA application. We must perform these experiments in different preparations because each effect of CPA and TG is not completely reversible.

**Recording of responses to CPA in isometric longitudinal muscle contractions of proximal colon in Ws/Ws mutant and +/- rats.** Homozygous Ws/Ws mutant rats and sibling control +/- rats obtained from Nippon SLC (Shizuoka, Japan) were used. The mean body weight was 240±18 and 258±29 g, respectively. RSCs before and after CPA (10^{-6} M) were recorded and evaluated by use of the same protocol as that in Wistar rats.

**Intracellular Ca^{2+} level measurement.** The intracellular Ca^{2+} level was measured according to the method reported by Ozaki et al. with the fluorescent Ca^{2+} indicator fura-PE3 [14]. Wistar rat proximal colonic muscle strip preparations (2 mm in width, 7 mm in length) were treated with the acetoxyethyl ester of fura-PE3 (fura-PE3/AM, 20 M) for 4 h at 23°C. Pluoronic F-127 (0.06%) was added to increase the solubility of fura-PE3/AM. After being loaded, the preparations were washed with modified Tyrode’s solution at 37°C for 15 min to remove free fura-PE3/AM. Each preparation was held horizontally in a temperature-controlled organ bath (5 ml) connected by one end to a force transducer. The preparation was illuminated by dual wavelength (340 and 380 nm) excitation light applied alternatively (128 Hz). The intensity of fluorescence at 500 nm (F_{340} and F_{380}) was measured with fluorometer (CAF-110, JASCO, Tokyo, Japan), and the ratio of F_{340} to F_{380} (F_{340}/F_{380}) was treated as an indicator of intracellular Ca^{2+}. The ratios obtained baseline level before the application of
chemicals, and in the presence of 142.4 mmol/l K⁺ they were taken as 0 and 100%, respectively.

**Drugs and chemicals.** The following drugs were used in the present study: CPA, TG, pluronic F-127, and nicardipine, which were purchased from Sigma (St. Louis, MO, USA). SK&F 96365 was purchased from Biol Res. Lab. (Plymouth Meeting, PA, USA). Fura-PE3/AM was purchased from Dojindo (Kumamoto, Japan). CPA and nicardipine were dissolved in DMSO to prepare stock solutions of 1 mmol/l and TG of 0.1 mmol/l. Stock solutions of SK&F 96365 were dissolved in distilled water to prepare stock solutions of 1 mmol/l.

**Statistical analysis.** Data were expressed as means±SE. (n, the number of animals). A statistical analysis was performed by a Student’s t-test or an analysis of variance (ANOVA) and a Fisher’s test. The p value of <0.05 was regarded as significant.

**RESULTS**

**Effects of CPA and TG on the amplitude and frequency of RSCs of isolated proximal colon in Wistar rats**

After equilibration for 30–60 min, the proximal colon showed regular RSCs with 2 to 5 cycles/min (cpm) in frequency and 1 to 2.5 g in amplitude. Tetrodotoxin (10⁻⁶ mol/l) did not affect RSCs, thus indicating that they were neither neurogenic nor conducted by nerve pathway. At a concentration of 10⁻⁷ mol/l, CPA did not increase the frequency and amplitude of RSCs in all preparations, but at concentrations of 10⁻⁶–10⁻⁴ mol/l, it markedly increased both in all preparations. We adopted a lowest effective concentration of 10⁻⁶ mol/l. CPA (10⁻⁶ mol/l) transiently increased muscle tone within 1 min, though this increase was restored within a few minutes. However, CPA persistently increased the frequency and amplitude of RSCs for 15 min (Fig. 1A). Figure 2 indicated the summarized data of the effects of CPA on the frequency (A) and amplitude (B) of RSCs. CPA significantly increased mean frequency to 5.5±1.9 cpm (50.0% of control), from 3.7±1.6, and mean amplitude to 2.0±0.7 g (42.9% of control), from 1.4±0.5, in all preparations (p<0.05, n=9).

We investigated the effect of TG on the frequency and amplitude of RSCs. TG (10⁻⁶ mol/l) also increased the frequency of RSCs without increasing their amplitude and muscle tone (Fig. 1B). This effect reached a steady state within 15 min. Figure 2 showed summarized data of the effects of TG on the frequency (A) and amplitude (B) of RSCs. TG significantly increased mean frequency to 5.7±1.1 cpm (43.7% of control, p<0.05), from 4.0±0.8, but it did not affect mean amplitude (1.2±0.2 vs. 1.1±0.3 g) in all preparations (n=6).

There was no significant difference between CPA and TG in the effect of increasing the frequency of RSCs. These results indicated that the blockade of SR Ca²⁺-ATPase increased, at least, the frequency of RSCs in rat proximal colon, but the CPA-induced increase in the amplitude of RSCs seems to be caused by any other mechanism.

**Effects of CPA on the frequency and amplitude of RSCs of isolated proximal colon in Ws/Ws mutant and +/+ rats**

The proximal colon in Ws/Ws mutant rats also showed RSCs similar to that in +/+ rats. The regularity of RSCs appeared to be slightly impaired in
Ws/Ws mutant rats, but their frequency was high (Fig. 3B) in comparison with those in Wistar (see Fig. 1) or 1/1 rats (Fig. 3A). CPA (10⁻⁶ mol/l) transiently increased the muscle tone within 1 min, although this increase was restored within a few minutes. However, CPA persistently increased the frequency and amplitude of RSCs for 15–20 min in Ws/Ws mutant and 1/1 rats (Fig. 3, C and D).

Figure 4 indicated the summarized data of control and CPA in Ws/Ws mutant and 1/1 rats. There were no significant differences in mean frequency or amplitude of RSCs between Ws/Ws and 1/1 rats. Both mean frequency and amplitude in Ws/Ws mutant and 1/1 rats corresponded to those in Wistar rats (control in Fig. 2). CPA significantly increased the mean frequency of RSCs by 72.9% of control and the mean amplitude of RSCs by 26.5% of control in Ws/Ws mutant rats (p<0.05, n=4). In contrast, CPA did not significantly increase the mean frequency of RSCs in 1/1 rats. Consequently, the mean frequency of RSCs increased by CPA in Ws/Ws mutant rats was significantly higher than in 1/1 rats (p<0.05).

**Effects of CPA and TG on intracellular Ca²⁺ of Wistar rats**

Both CPA (10⁻⁶ M, n=4; Fig. 5A) and TG (10⁻⁶ M, n=3; Fig. 5B) caused an increase in baseline intracellular free Ca²⁺ level in these preparations.

**Effect of nicardipine or SK&F 96365 on the CPA-induced actions in RSCs of Wistar rats**

To examine the contribution of extracellular Ca²⁺ entry mechanisms for CPA-induced actions in RSCs, we investigated the effect of nicardipine (10⁻¹ⁱ mol/l) or SK&F 96365 (10⁻⁸ mol/l) (Fig. 6). A high concentration (10⁻⁶ mol/l) of nicardipine abolished RSCs, but a low concentration (10⁻¹¹ mol/l) affected neither the frequency nor the amplitude of RSCs for 30 min (frequency, 3.8±1.1 vs. 3.5±0.5 cpm; amplitude, 1.2±0.2 vs. 1.2±0.4 g, n=6; Fig. 7). In the presence of nicardipine (10⁻¹¹ mol/l), CPA did not increase the amplitude of RSCs, though it increased the frequency.
SK&F 96365 affected neither the frequency nor the amplitude of RSCs for 30 min (frequency, 3.4 \pm 0.5 vs. 3.6 \pm 1.1 cpm; amplitude, 1.6 \pm 0.5 vs. 1.9 \pm 0.8 g; \( n = 6 \); Fig. 6A). In the presence of SK&F 96365 (10^{-8} \text{ mol/l}) CPA hardly increased the amplitude of RSCs, but it increased the frequency for 30 min (Fig. 6B).

Figure 7 showed that the low concentration of nicardipine (10^{-11} \text{ mol/l}) antagonized the CPA-induced increase in the amplitude of RSCs (panel B: 1.2 \pm 0.4 vs. 1.1 \pm 0.4 g; \( n = 6 \); Fig. 7). In the presence of SK&F 96365 (10^{-8} \text{ mol/l}), CPA hardly increased the amplitude of RSCs, but it increased the frequency for 30 min (Fig. 6B).

Figure 7 showed that the low concentration of nicardipine (10^{-11} \text{ mol/l}) antagonized the CPA-induced increase in the amplitude of RSCs (panel B: 1.2 \pm 0.4 vs. 1.1 \pm 0.4 g; \( n = 6 \); Fig. 7). In the presence of SK&F 96365 (10^{-8} \text{ mol/l}), CPA hardly increased the amplitude of RSCs, but it increased the frequency for 30 min (Fig. 6B).

DISCUSSION

In the present study, we found regular RSCs in the proximal colon of Wistar rats. In the Ws/Ws mutant rats, the c-kit kinase activity is severely impaired [6], and thus the development of ICCs is supposed to be impaired. We therefore hypothesized that RSCs are absent in the proximal colon of the Ws/Ws mutant rats. In contrast to our hypothesis, we also found that RSCs are actually generated in the proximal colon of the Ws/Ws mutant rats. Although c-kit positive ICC_{SM} and ICC_{MY} are surely deficient in the Ws/Ws rat colon, c-kit negative ICC_{SM}, which has many gap junctions, is present [7]. A previous study has demonstrated that the pacemaker component of the slow waves is generated within ICC_{SM} in the rat colon [15]. This result indicates that c-kit positive ICC_{MY} and ICC_{SM} are not involved in the generation of RSCs, but that RSCs may be generated by c-kit negative ICC_{SM} in the proximal colon of Ws/Ws mutant rats.

In Ws/Ws mutant rats, c-kit positive ICCs are not detectable in the stomach, but they are in the ileum where their number is greatly reduced. Bile reflux is significantly increased and motor activity is apparently impaired in these rats [6]. Thus c-kit positive ICCs are considered to be necessary for the normal function of the pyloric sphincter and the ileum.

On the other hand, W/WV mouse has a point mutation in c-kit that reduces but does not abolish tyrosine kinase activity [3]. In the stomach of W/WV mouse, ICC_{MY} can be identified, but ICCs distributed within the smooth muscle layer (ICC_{IM}) are not detected within the smooth muscle bundles. These mice lack the secondary regenerative component of a slow wave, which consisted of initial and second components [16]. Thus these results indicate that the secondary regenerative component of a slow wave is generated by ICC_{IM}.

In the small intestine of W/WV mouse, where ICC_{MY} is lacking but where ICC_{IM} is normally present, slow waves are not generated, but a wide variety of electrical activities, ranging from total quiescence to generation of action potentials, are observed [2, 3, 17]. Thus ICC_{MY} seems to play a critical element in the generation of pacemaker activity in the mouse small intestine. There are various kinds of ICCs in various species and tissues; thus the functions of ICCs are different, associated with the difference in localizations.

A blockade of the SR Ca^{2+}-ATPase increased the frequency of the RSCs in the rat proximal colon. CPA
increased both the frequency and the amplitude of RSCs, whereas TG increased the frequency without increasing the amplitude. Although we must consider the possibility that CPA depolarized the membrane and increased the frequency, TG increased the frequency without increasing the basal tone. These results indicate that increase in frequency of RSCs in rat proximal colon is caused by a blockade of SR Ca$^{2+}$-ATPase. The effects of a blockade of SR Ca$^{2+}$-ATPase on the smooth muscle or ICCs of the gastrointestinal tract remains controversial. In the guinea pig antral circular muscle, the frequency of slow waves tends to increase with TG and CPA [18]. In the cultured ICC of the murine small intestine, the amplitude and frequency of slow waves are increased with CPA [19]. Our results are consistent with these reports. Furthermore, our results revealed that the blockade of SR Ca$^{2+}$-ATPase by CPA and TG increased the baseline free Ca$^{2+}$ level measured by fura-PE3/AM. All these results suggest that the CPA- and TG-sensitive SR Ca$^{2+}$-ATPase does not contribute to the generation of the slow waves but that the CPA- and TG-sensitive SR Ca$^{2+}$-ATPase does contribute to the decrease of the cytosolic Ca$^{2+}$ concentration in our preparations. We therefore suggest the possibility that the increase of cytosolic Ca$^{2+}$ concentration because of the blockade of SR Ca$^{2+}$-ATPase enhances the frequency of RSCs.

The increasing effects of CPA on the frequency of RSCs were more prominent in Ws/Ws mutant rats. Although we could not elucidate the function of ICCs, these results suggest the possibility that the functional coordination between c-kit negative ICC$_{SM}$ and other mutationally impaired c-kit positive ICC$_{MY}$ and ICC$_{SM}$ are required for moderate regulation in the frequency of spontaneous activity.

In contrast to our present results, CPA reduces the pacemaker frequency in the canine colon [20]. TG blocks pacemaker currents in cultured ICCs from murine jejunum [21], and CPA reduces pacemaker frequency in the small intestine of newborn mice [22]. All these data suggest that the CPA- and TG-sensitive SR Ca$^{2+}$-ATPase is involved in the generation of the slow wave in these preparations. The discrepancies from our results may be due to differences in species and tissues and in experimental methods.

We then revealed the contribution of the extracellular Ca$^{2+}$ entry mechanisms to the effects of CPA on RSCs. Nicardipine, an L-type Ca$^{2+}$ channel blocker, inhibited the CPA-induced increase in the amplitude of RSCs without inhibiting the increase in the frequency. Therefore these results suggest the possibility that voltage-dependent L-type Ca$^{2+}$ channels contribute to the CPA-induced increase in the amplitude.

In the present study, TG did not increase the amplitude, but it increased the frequency of the RSCs. This effect mimics the CPA-induced action in the presence of nicardipine on the RSCs. It is reported that TG at micromolar concentrations can directly block the L-type Ca$^{2+}$ channel in smooth muscles [23, 24], but CPA cannot [23]. Therefore the discrepancy between the effects of TG and CPA may be due to their different actions on the L-type Ca$^{2+}$ channel.

Recently, a phenomenon called capacitative Ca$^{2+}$ entry, which is regulated by Ca$^{2+}$ concentration in intracellular stores, was observed in some nonexcitable cells and smooth muscle tissues [9, 11, 25, 26]. SK&F 96365 is among the most widely used inhibitors of capacitative Ca$^{2+}$ entry [27]. SK&F 96365 antagonized the CPA-induced increase in the amplitude of RSCs, but it did not antagonize the increase in the frequency. SK&F 96365 is believed to inhibit specifically capacitative Ca$^{2+}$ entry at 10$^{-4}$ mol/l, though at higher concentrations it could exert multiple and complex effects on Ca$^{2+}$ signaling [28]. Therefore the present result suggests the possibility that capacitative Ca$^{2+}$ entry mechanisms contribute to the CPA-induced increase in the amplitude of RSCs.

We found that the rhythmic spontaneous contractions are present in the proximal colon of Ws/Ws mutant rats. This finding suggests that c-kit negative ICCs along the submucosal surface of the circular muscle bundles may generate the rhythmic contractions. The blockade of SR Ca$^{2+}$-ATPase increased the frequency of the rhythmic spontaneous contractions in the proximal colon probably because of the increase in intracellular Ca$^{2+}$ concentration. The increasing effect on the frequency of the rhythmic spontaneous contractions by the blockade of SR Ca$^{2+}$-ATPase was more prominent in Ws/Ws mutant rats than in +/+ rats. We conclude that the functional coordination between c-kit negative ICCs along the submucosal surface of the circular muscle bundles and other mutationally impaired c-kit positive ICCs along the submucosal surface of the circular muscle and myenteric plexus may be required for moderate regulation in the frequency of spontaneous activity.

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