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Involvement of ryanodine receptors in pacemaker Ca^{2+} oscillation in murine gastric ICC

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Abstract

Using a cell cluster preparation from the stomach smooth muscle tissue of mice, we measured intracellular Ca^{2+} oscillations in interstitial cells of Cajal (ICCs) in the presence of nifedipine. Pacemaker $[Ca^{2+}]_i$ activity in ICCs was significantly suppressed by caffeine application and restored after washout. Application of either ryanodine or FK-506 terminated the pacemaker $[Ca^{2+}]_i$ activity irreversibly. Immunostaining of smooth muscle tissue showed that c-Kit-immunopositive cells (that form network-like structure cells in the myenteric plexus, equivalent to ICCs) clearly express ryanodine receptors (RyR). RT-PCR revealed that ICCs (identified with c-Kit-immunoreactivity) predominantly express type 3 RyR (RyR3). Furthermore, the FK-binding proteins 12 and 12.6, both of which would interact with RyR3, were detected. In conclusion, we provide first evidence for the essential contribution of RyR to generating pacemaker activity in gastric motility. Similar mechanisms might account for spontaneous rhythmicity seen in smooth muscle tissues distributed in the autonomic nervous system.

Keywords: Stomach; Pacemaker; Calcium oscillations; c-Kit-immunopositive cells; Interstitial cells of Cajal; Ryanodine receptors; FK506-binding proteins; Caffeine

Calcium release from InsP_3R is known to contribute to many important cellular functions, including generation of spontaneous rhythmicity in gastrointestinal motility [1]. There are several reports of inhibitors for InsP_3R significantly suppressing or terminating spontaneous electrical activity, referred to as slow waves, in the gastrointestinal tract [2–5]. Suzuki et al. [1] have demonstrated that spontaneous electrical activity is not observed in stomach smooth muscle tissue of mice lacking type 1 InsP_3R. We have recently provided evidence using RT-PCR that the interstitial cells of Cajal (ICCs, putative pacemaker cells) express type 1 and 2 InsP_3R (InsP_3R1 and 2), while stomach smooth muscles express all three InsP_3R isoforms [6]. Furthermore, using cell cluster preparations from the stomach, we have shown that InsP_3R inhibitors terminate spontaneous $[Ca^{2+}]_i$ oscillations in ICCs, which are considered to be the primary mechanism in generating pacemaker potentials. Taken together, these results are indicative of an essential role for InsP_3R1 in generating pacemaker activity in gastric motility.

It is well known that properties of spontaneous rhythmicity are different in different regions of gastrointestinal tracts [7]. Generally, spontaneous contractions driven by pacemaker potentials are less frequent in the stomach than in the small intestine. It has been reported that unlike InsP_3R inhibitors, application of ryanodine does not affect spontaneous activity in murine stomach [1,8]. On the other hand, using cell cluster preparations

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from the small intestine, we have previously demonstrated that ryanodine receptors (RyR) co-ordinate with InsP_3R to generate pacemaker $[Ca^{2+}]_i$ oscillations, and that type 3 RyR isoform (RyR3) is predominant in ICCs [9].

In the present study, we assessed whether our hypothesis that RyR and InsP_3R act in tandem to generate spontaneous rhythmicity is acceptable to tissues throughout the gastrointestinal tract. We prepared cell clusters from murine stomach smooth muscle tissue which includes ICCs, and in which the characteristic features of spontaneous rhythmicity (such as lower frequency and requirement of InsP_3R), are maintained. We examined the effects of drugs affecting ryanodine receptors on pacemaker $[Ca^{2+}]_i$ oscillations in ICCs in these cell clusters. Also, we confirmed the expression of RyR in ICCs using RT-PCR and immunostaining.

Materials and methods

Animals and cell cluster preparation. BALB/c mice (~3 weeks after birth) were killed by cervical dislocation after being anaesthetized with diethyl ether. The mice were treated ethically according to the Guidelines for the Care and Use of Animals approved by the Physiological Society of Japan.

The preparation of cell clusters has been described previously [6,10]. The stomach was excised and cut into two parts along the greater and lesser curvature. Small muscle pieces (containing circular and longitudinal smooth muscle with the myenteric plexus) dissected from the antrum and pylorus were incubated in Ca^{2+}-free Hanks' solution containing collagenase (1.5 mg/ml, No. 034-10533; Wako Chemical, Osaka, Japan), trypsin inhibitors (2 mg/ml, type I-S; Sigma, St. Louis, MO, USA), ATP (0.2 mg/ml, Seikagakukogyo, Tokyo, Japan), and bovine serum albumin (2 mg/ml, Sigma) for 60 min (37 °C). After being rinsed with Ca^{2+}-free Hanks' solution, the muscle pieces were triturated with fire-blunted glass pipettes. The resultant small cell clusters were plated onto a lab-made culture dish (a silicone ring approx. 20 mm in diameter on a cover glass of 25 mm in diameter and 0.12–0.17 mm thick, coated with pig collagen (Nitta Gelatin, Osaka, Japan)), and were kept in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (Sigma) and antibiotics [streptomycin (30 μg/ml) and penicillin (30 U/ml), Sigma]. Each culture dish contained 250–300 μl of the culture medium, and cell cluster preparations were incubated at 37 °C.

Ca^{2+} imaging. After 2–4 days of incubation, the cultured cell clusters were used for Ca^{2+} imaging. This preparation was suitable to investigate mechanisms underlying $[Ca^{2+}]_i$ oscillations in ICCs, because of advantages in loading intracellular Ca^{2+} indicators (compared to isolated smooth muscle tissues) and in occurrence of spontaneous $[Ca^{2+}]_i$ oscillations (compared to isolated ICCs). When enzymatic isolation was appropriate, spontaneous $[Ca^{2+}]_i$ oscillations were measured in 50–80% cell clusters.

The methods used for Ca^{2+} imaging were essentially the same as previously described [6,9]. In order to load Ca^{2+} indicator fluo-3, the cultured cell clusters were incubated for 3–4 h in 'normal' solution containing 8 μM fluo-3/AM and detergents (0.02% Pluronic F-127, Dojindo; 0.02% cremophor EL, Sigma). A CCD camera system (Argus/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan) combined with an inverted microscope (Axiovert S100TV, Zeiss, Germany) was used to acquire Ca^{2+} images. The cell clusters were illuminated at 488 nm, and emission light of 515–565 nm was detected. Digital images were normally collected at 300 ms intervals. Changes in fluorescence emission intensity (F) were expressed as F_t/F_0, where F_0 is the basal fluorescence intensity at around the middle of the recording. During Ca^{2+} imaging, the temperature of the recording chamber was kept at 35 °C using a micro-warm plate system (DC-MP10, Kitazato Supply, Fujinomiya, Japan). When the amplitude of $[Ca^{2+}]_i$ transients fell within the noise level (normally ~10% of the control value), the oscillation was judged to have ceased. We also confirmed that $[Ca^{2+}]_i$ transients did not appear during next 30 s.

It is known that DHP L-type Ca^{2+} channel blockers abolish spontaneous contraction in gastrointestinal smooth muscle preparations, but have little effect on electrical pacemaker activity [11,12]. In the present experiments, pacemaker $[Ca^{2+}]_i$ oscillations in ICCs were thus isolated by use of nifedipine (1 μM). Furthermore, the inhibitory effect of nifedipine on cell cluster contractions enabled us to more reliably estimate pacemaker $[Ca^{2+}]_i$ oscillations by fixing a region of interest.

NA extraction and RT-PCR. Methods used for mRNA extraction and reverse transcription polymerase chain reaction (RT-PCR) were essentially the same as reported previously [13]. A similar digestive procedure used for preparing cell clusters was carried out to obtain isolated ICCs from stomach. Longer (50 min) enzymatic incubation and more complete trituration with fire-blunted glass pipettes were applied. The resultant cell suspension was incubated with 'normal' solution containing phycoerythrin-conjugated anti-mouse CD117 (c-Kit) antibody (PE-ACK2, eBioscience, San Diego, CA, USA) in 1/100 v/v for 10 min. And then the cell suspension was centrifuged and washout with 'normal' solution. Using glass pipette of 10–20 μm diameter, about 15–20 isolated c-Kit-immunopositive cells were separately collected in RNA stabilization solution (RNAlater, Qiagen, Hilden, Germany), and kept in a deep freezer until the use of RT-PCR.

cDNA was produced by reverse-transcribing RNA samples with Superscript II RNase H^- (Invitrogen) and 500 μg/ml oligo(dT), following digestion with RNase-free DNase. The resultant cDNA products were amplified by PCR with gene-specific primers (Table 1) according to a protocol recommended by Applied Biosystems. The amplification profile was as follows: a 15 s at 95 °C and a 60 s at 60 °C. In the tissue and cell-based RT-PCR, the amplification was performed for 35 and 45 cycles, respectively. The RT-PCR products were separated by electrophoresis on a 2% agarose gel and documented on a FluorImager 595 (Amersham Biosciences, Piscataway, NJ, USA). In order to rule out genomic contamination, negative controls of PCR were carried out using the RNA samples without RT procedure. Also, the no primer control shown in Fig. 3 was an RT-PCR product in which no PCR primer was added. Amplicons were sequenced using a DSQ-1000 L sequencer (Shimadzu, Kyoto, Japan) Table 2.

Immunostaining. Smooth muscle layers (including the myenteric plexus) isolated from mouse stomach were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 10 min. The tissue was cut into small segments (~10 mm), and was double stained sequentially with anti-RyR antibody (clone 34C produced in mouse, Sigma, St. Louis, MO, USA) and anti-c-Kit (mouse CD117) antibody for 1.5 h. This was followed by incubation with secondary antibodies, Alexa-conjugated anti-mouse or rat IgG (Molecular Probes), at the concentration of 15 μg/ml for 1 h. Controls were prepared by omitting the primary antibodies. Double-stained small segments were mounted on a slide glass with an anti-fading agent (ProLong: Molecular Probes) and scanned using a confocal microscope (MRC-1024: Bio-Rad, Hercules, CA, USA).

Solutions and drugs. The composition of 'normal' extracellular medium used for $[Ca^{2+}]_i$ imaging experiments was (mM): NaCl, 125; KCl, 5.9; $MgCl_2$, 1.2; $CaCl_2$, 2.4; glucose, 11; and Tris–Hepes, 11.8 (pH 7.4).

The sources of pharmacological agents were as follows: nifedipine and ryanodine (Sigma, St. Louis, MO, USA); FK506 (Calbiochem, San Diego, CA, USA); caffeine (anhydrous, Kanto Kagaku, Tokyo, Japan); and fluo-3/AM (Dojindo, Kumamoto, Japan). Stock solutions of nifedipine were prepared by dissolving this drug with ethanol, and