Requirement of ryanodine receptors for pacemaker Ca^{2+} activity in ICC and HEK293 cells

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Summary

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations seen in interstitial cells of Cajal (ICCs) are considered to be the primary pacemaker activity in the gut. Here, we show evidence that periodic Ca^{2+} release from intracellular Ca^{2+} stores produces $[Ca^{2+}]_i$ oscillations in ICCs, using cell cluster preparations isolated from mouse ileum. The pacemaker $[Ca^{2+}]_i$ oscillations in ICCs are preserved in the presence of dihydropyridine Ca^{2+} antagonists, which suppress Ca^{2+} activity in smooth muscle cells. However, applications of drugs affecting either ryanodine receptors or inositol 1,4,5-trisphosphate receptors terminated $[Ca^{2+}]_i$ oscillations at relatively low concentrations. RT-PCR analyses revealed a predominant expression of type 3 RyR (RyR3) in isolated c-Kit-immunopositive cells (ICCs). Furthermore, we demonstrate that pacemaker-like global $[Ca^{2+}]_i$ oscillation activity is endowed by introducing RyR3 into HEK293 cells, which originally express only IP_3Rs. The reconstituted $[Ca^{2+}]_i$ oscillations in HEK293 cells possess essentially the same pharmacological characteristics as seen in ICCs. The results support the functional role of RyR3 in ICCs.

Key words: Ryanodine receptor, Pacemaker, Calcium oscillation, c-Kit-immunopositive cells, Interstitial cells of Cajal

Introduction

Many missing links in the pathway that generates spontaneous rhythmicity in the gastro-intestinal tract remain, despite its importance. Like the heart beat, spontaneous movement of the gastro-intestinal tract is essential to mix, digest and transport the luminal contents. Recent studies have suggested that, as in sino-atria node cells in the heart, specialised pacemaker cells exist in the gastro-intestinal wall, and drive smooth muscle cells to achieve their functions (Suzuki, 2000). The pacemaker cells of the gastro-intestinal wall express a specific tyrosine kinase, c-Kit, and are characterised from their histological features as interstitial cells of Cajal (ICCs).

L-type Ca^{2+} channels play a central role in the excitation-contraction coupling of smooth muscle (Nakayama et al., 1996; Beech and McHugh, 1996). Electrical pacemaker activity in the form of slow waves, is, however, hardly affected by dihydropyridine (DHP) L-type Ca^{2+} channel blockers. This fact suggests that other Ca^{2+} sources play important roles in gastro-intestinal pacemaking. Several groups have suggested that Ca^{2+}-activated Cl^- channels in ICCs are responsible for electrical pacemaker potentials (Tokutomi et al., 1995; Edwards et al., 1999; Huizinga et al., 2002). It can therefore be deduced that oscillations of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), presumably through release of Ca^{2+} from intracellular stores, are the primary pacemaker activity. This putative underlying mechanism also accounts for the low voltage-sensitivity of pacemaker frequency (Tomita, 1981).

Two families of intracellular Ca^{2+} release channels, such as inositol 1,4,5-trisphosphate (InsP_3) receptor types 1-3 and ryanodine receptor types 1-3, are known along with several intracellular messengers and modulators (e.g. cyclic adenosine diphosphate ribose and nicotinic acid adenine dinucleotide phosphate) (Galione and Churchill, 2002; Masgrau et al., 2003). Several lines of evidence suggest that the InsP_3 receptor (IP_3R) plays a role in generating spontaneous electrical activity in gastro-intestinal pacemaker cells (Suzuki et al., 2000; Hirst and Edwards, 2001; Malysz et al., 2001; Sergeant et al., 2001), and it has been suggested that RyR is involved in NO-induced Ca^{2+} transients in colonic ICCs (Publicover et al., 1993). However, the contribution of the ryanodine receptor (RyR) family to the pacemaker activity is still unclear. Recently, we have developed a new preparation to study mechanisms underlying gastro-intestinal pacemaker activity: small cell clusters containing c-Kit-immunopositive interstitial cells (ICCs), smooth muscle cells and enteric neurones, which show stable spontaneous rhythmicity in terms of mechanical, electrical and intracellular Ca^{2+} activities (Nakayama and Torihashi, 2002; Torihashi et al., 2002). In the present study, using this preparation, we directly demonstrate that RyR as well as IP_3R contribute to intracellular Ca^{2+} oscillation in ICCs, the putative pacemaker cells.

RT-PCR reveals that ICCs predominantly express ryanodine receptor type 3 (RyR3). Among the three types of RyRs, RyR3 has been proved to be widely expressed in brain, smooth muscles, skeletal muscle and non-excitable cells (Bennett et al., 1996; Ogawa et al., 2002; Sorrentino and Volpe, 1993). Elucidation of the physiological impact of RyR3 on cellular functions has, however, been hampered by lower expression levels of RyR3 in comparison with other isoforms of RyRs co-expressed in many types of cells. The contribution to $[Ca^{2+}]_i$ oscillations in ICCs is, so far as we know, the first evidence for an obligatory role of RyR3. Furthermore, we have reconstituted pacemaker-like $[Ca^{2+}]_i$ oscillations by transfecting an expression vector for RyR3 in HEK293 cells, which originally express IP_3Rs, but not RyRs, and do not display spontaneous $[Ca^{2+}]_i$ oscillation. Interestingly, RyR or IP_3R inhibitors had essentially the same effects on $[Ca^{2+}]_i$ oscillations seen in ICCs and the HEK293 cells. Taken together, these observations suggest that RyR3 can be a key molecule to generate, or at least modulate, pacemaker $[Ca^{2+}]_i$ oscillations in both cell clusters containing ICCs and reconstituted cell systems.

Materials and Methods

Preparation of cell clusters and cell dispersion

The preparation of cell clusters has been described previously (Nakayama and Torihashi, 2002). The mice were treated ethically according to the Guidelines for the Care and Use of Animals approved by the Physiological Society of Japan. BALB/c mice (10-20 d after birth) were killed by cervical dislocation, and small pieces of the small intestine were incubated in Ca^{2+}-free Hanks' solution containing digestive enzymes as previously reported (Nakayama and Torihashi, 2002). After being rinsed with Ca^{2+}-free Hanks' solution, the muscle pieces were triturated with fire-blunted glass pipettes of decreasing tip diameter. The resultant small cell clusters were plated onto a lab-made culture dish (a sterile silicone ring approximately 20 mm in diameter on a pig collagen-coated sterile 25 mm glass cover slip). After 2-3 days of incubation, the cultured cell clusters were used for Ca^{2+} imaging.

The resultant cell suspension was incubated with 'normal' solution containing phycoerythrin-conjugated anti-mouse CD117 (c-Kit) antibody (PE-ACK2, eBioscience, San Diego, CA) in 1/100 v/v for 10 minutes. About 5-10 isolated smooth muscle cells and c-Kit-immunopositive cells were separately collected with glass pipettes of 10-20 μm tip diameter under a fluorescent microscope and kept at –80°C until the use of RT-PCR. Smooth muscle cells and c-Kit-immunopositive cells (ICCs) were judged by their characteristic spindle shape and immunofluorescence.

Cell culture and DNA transfection

HEK293 cells (Japanese Health Science Research Resources Bank, Tokyo, Japan) were maintained as previously reported (Imaizumi et al., 2002). Cells were transfected with DNA encoding RyR2 or RyR3, using the Ca_2PO_4 co-precipitation method. Cells were plated on cover slips about 24 hours before transfection and were transfected with 2-4 μg of DNA per dish. Control cells were treated in the same way with no DNA.

Ca^{2+} imaging

The cultured cell clusters were incubated for 3-4 hours in normal solution containing 8 μM fluo-3/AM and detergents (0.02% Pluronic F-127, Dojindo, Kumamoto, Japan; 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 monitor continuously digital images of fluo-3 emission light. The cell clusters were illuminated at 488 nm, and emission light of 515-565 nm was detected. Digital images were normally collected at 300 millisecond intervals. Changes in fluorescence emission intensity (F) were expressed as F_t/F_0, where F_0 is the basal fluorescence intensity obtained at the start of the experiment. During Ca^{2+} imaging, the temperature of the recording chamber was kept at 35°C using a micro-warm plate system (DC-MP10DM, Kitazato Supply, Fujinomiya, Japan). When the amplitude of $[Ca^{2+}]_i$ oscillations fell below 10% of the control value or within the noise level, we judged it to cease.

Average of $[Ca^{2+}]_i$ in transfected and non-transfected HEK293 cells was measured with fura-2/AM. About 48 hours after transfection, cells were loaded with 10 μM fura-2/AM for 20 minutes. $[Ca^{2+}]_i$ was measured with a Ca^{2+} imaging system (ARGUS-50/CA, Hamamatsu, Japan) under constant flow of KRH solution at room temperature. The intensity of emission fluorescence at 500 nm was measured synchronously to the alternate excitation (F340 and F380). The ratio of fluorescence emitted at 340 nm and 380 nm was converted to Ca^{2+} concentration using methods introduced previously (Grynkiewicz et al., 1985).

Confocal Ca^{2+} images were obtained using a fast laser scanning confocal microscope (RCM 8000, Nikon, Japan) and Ratio3 software (Nikon), as reported previously (Imaizumi et al., 1998). Cells were loaded with 10 μM fluo-4/AM for 30 minutes. Images were analyzed using Ratio3 software and GLOBAL LAB image (Data Translation, Marlboro, MA).

Immunohistochemistry and immunocytochemistry

Smooth muscle layers (including the myenteric plexus) isolated from mouse small intestine were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 10 minutes. 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) and anti-c-Kit (mouse CD117) antibody for 1.5 hours. This was followed by incubation with secondary antibodies, Alexa-conjugated anti-mouse or rat IgG (Molecular Probes, Eugene, OR) at the concentration of 15 μg/ml for 1 hour. 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).

The level of RyR protein expression in HEK293 transfectants was examined using similar protocols. After the measurements of $[Ca^{2+}]_i$, the coverslips were fixed, permeabilized, and treated with anti-RyR antibody specific for RyR2 or RyR3 for 2 hours. After washing, the cover slips were incubated with Alexa-conjugated anti-rabbit or mouse IgG for 60 minutes. Finally, the cover slips were mounted on slides after washing, and used for the analyses with the confocal microscope (RCM 8000; Nikon, Japan).

Total RNA extraction and RT-PCR

Total RNA extraction and reverse-transcription were performed as reported previously (Ohya et al., 1997). The resultant cDNA products were amplified by PCR with gene-specific primers (Table 1). The amplification profile was as follows: 15 seconds at 95°C and 60 seconds 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). The no template control (NTC: Fig. 5) was an RT-PCR product in which no sample RNA was added in order to monitor non-specific amplification and spurious primer-dimer fragments. Each

Table 1. Primers for PCR

Clones	Primer sequence (+) sense, (–) antisense	Primer site	Product length (bp)	GenBank accession no.
RyR1	(+) 5′-ATTACAGAGCAGCCCGAGGAT-3′	450-470	113	X83932
	(–) 5′-AGAACCTTCCGCTTGACAAACT-3′	541-562		
RyR2	(+) 5′-CTTCGATGTTGGCCTTCAAGAG-3′	432-453	100	NM_023868
	(–) 5′-CCAACACGCACTTTTTCTCCTT-3′	512-533		
RyR3	(+) 5′-GGCCAAGAACATCAGAGTGACTAA-3′	385-408	101	AF111166
	(–) 5′-TCACTTCTGCCCTGTCAGTTTC-3′	464-485		
c-Kit	(+) 5′-CAATGGAAGGTTGTCGAGGA-3′	370-389	101	X58687
	(–) 5′-GCCTGTTTCTGGGAAACTCC-3′	451-470		
FKBP12	(+) 5′-ACTAGGCAAGCAGGAGGTGA-3′	247-266	104	NM_008019
	(–) 5′-CTCCATAGGCATAGTCTGAGGAGAT-3′	326-350		
FKBP12.6	(+) 5′-AGAAGGCACTGCCCAGATGA-3′	287-306	116	NM_016863
	(–) 5′-AAAGATGAGGGTGGCATTGG-3′	385-404		
GAPDH	(+) 5′-CATGGCCTTCCGTGTTCCT-3′	730-749	104	M32599
	(–) 5′-CCTGCTTCACCACCTTCTTGA-3′	814-833		

amplicon was sequenced using a DSQ-1000L sequencer (Shimadzu, Kyoto, Japan).

Solutions and drugs

The composition of the standard solution used for cell clusters was (mM): 125 NaCl; 5.9 KCl; 1.2 $MgCl_2$; 2.4 $CaCl_2$; 11 glucose; 11.8 Tris-HEPES (pH 7.4). The standard solution used for HEK293 cells contained (mM): 125 NaCl, 5 KCl, 1.2 KH_2PO_4, 6 glucose, 1.2 $MgCl_2$, 2 $CaCl_2$ and 25 Hepes; pH 7.4, adjusted with NaOH. Ca^{2+}-free KRH solution was prepared by omitting Ca^{2+} in the preparation of standard KRH solution and the addition of 0.01 mM EGTA.

The source of pharmacological agents were as follows: nifedipine and ryanodine (Sigma); caffeine (anhydrous, Kanto Kagaku, Tokyo, Japan); xestospongin C and 2APB (2-aminoethoxydiphenyl borate) (Calbiochem, San Diego, CA); fura-2/AM, fluo-4/AM, and Alexa™-labelled secondary antidodies (Molecular Probes); fluo-3/AM (Dojindo).

Statistics

Numerical data are expressed as means±s.d. in the text. Statistical significance (P<0.05) was evaluated using ANOVA or Student's t-test.

Results

Intracellular Ca^{2+} oscillations in ICCs

Cultured cell clusters from mouse small intestine were loaded with fluo3-AM, and fluo-3 emission light was monitored as an index of intracellular Ca^{2+} using a CCD camera system. In a 'normal' solution containing 2.4 mM Ca^{2+}, many cell clusters showed spontaneous contractions at a frequency of 8-20 cycles/minute at 35°C (Nakayama and Torihashi, 2002). It is known that DHP L-type Ca^{2+} channel blockers abolish spontaneous contraction in gastro-intestinal smooth muscle preparations, but have little effect on slow waves (the pacemaker potentials) (e.g. Dickens et al., 1999; Huang et al., 1999). In the present study, nifedipine was thus used to distinguish pacemaker Ca^{2+} activity in cultured cell clusters. (We also checked that spontaneous contractions in cell cluster preparations were accompanied by such pacemaker Ca^{2+} activity, and cell clusters contracted like a functional syncytium in the absence of Ca^{2+} channel blockers; S.N., unpublished.) In the presence of 1-2 μM nifedipine, $[Ca^{2+}]_i$ oscillations were observed in one or more regions (e.g. Fig. 1Aa,Ba) of the cell clusters which had shown spontaneous contraction before nifedipine application. In the majority of cell clusters in which $[Ca^{2+}]_i$ oscillations were observed in multiple regions, the periodic increases in $[Ca^{2+}]_i$ were synchronized. The mean frequency of $[Ca^{2+}]_i$ oscillations in the presence of nifedipine was 18.4±5.1 cycles/minute (n=26).

Ryanodine receptors are involved in generation of Ca^{2+} oscillations

Using cell cluster preparations in the presence of a DHP Ca^{2+} channel blocker, we examined drugs that could affect intracellular Ca^{2+} release channels. Fig. 1 shows examples of the effects of ryanodine on cell clusters with single (Fig. 1A) or multiple regions of $[Ca^{2+}]_i$ oscillations (Fig. 1B). The traces in Fig. 1Ab show changes in the fluorescence intensity (F_t/F_0) measured in the square region indicated in Fig. 1Aa, panel 1. The Ca^{2+} images of Fig. 1Aa, panels 1-3 were recorded at the times indicated by lines 1-3 in Fig. 1Ab, respectively. In the presence of 2 μM nifedipine, application of 1 μM ryanodine significantly reduced the amplitude and frequency of $[Ca^{2+}]_i$ oscillations after 5 minutes. An increase in the ryanodine concentration to 10 μM completely terminated $[Ca^{2+}]_i$ oscillations after 5 minutes, and the subsequent washout of ryanodine for 5 minutes did not restore it. After examination of the effects of ryanodine, the cell cluster was stained using ACK2. c-Kit-immunoreactivity was detected in the square region, indicating that $[Ca^{2+}]_i$ oscillations in ICCs were modulated by ryanodine. Similar effects of ryanodine were observed in three other cell clusters.

The cell cluster preparation shown in Fig. 1Ba contained multiple regions of $[Ca^{2+}]_i$ oscillations. The time courses of changes in $[Ca^{2+}]_i$ in Fig. 1Bb were monitored in the boxed regions x (blue line) and y (red line). The $[Ca^{2+}]_i$ oscillations seen in the two regions of the cell cluster were well synchronised (in the presence of nifedipine). Application of 10 μM ryanodine reduced both $[Ca^{2+}]_i$ oscillation amplitude and frequency in a time-dependent manner. As shown in the second and third traces of Fig. 1Bb, $[Ca^{2+}]_i$ in the x and y regions were affected similarly in terms of amplitude and frequency. Fig. 1Bc shows the correlation between F_t/F_0 in regions x and y for

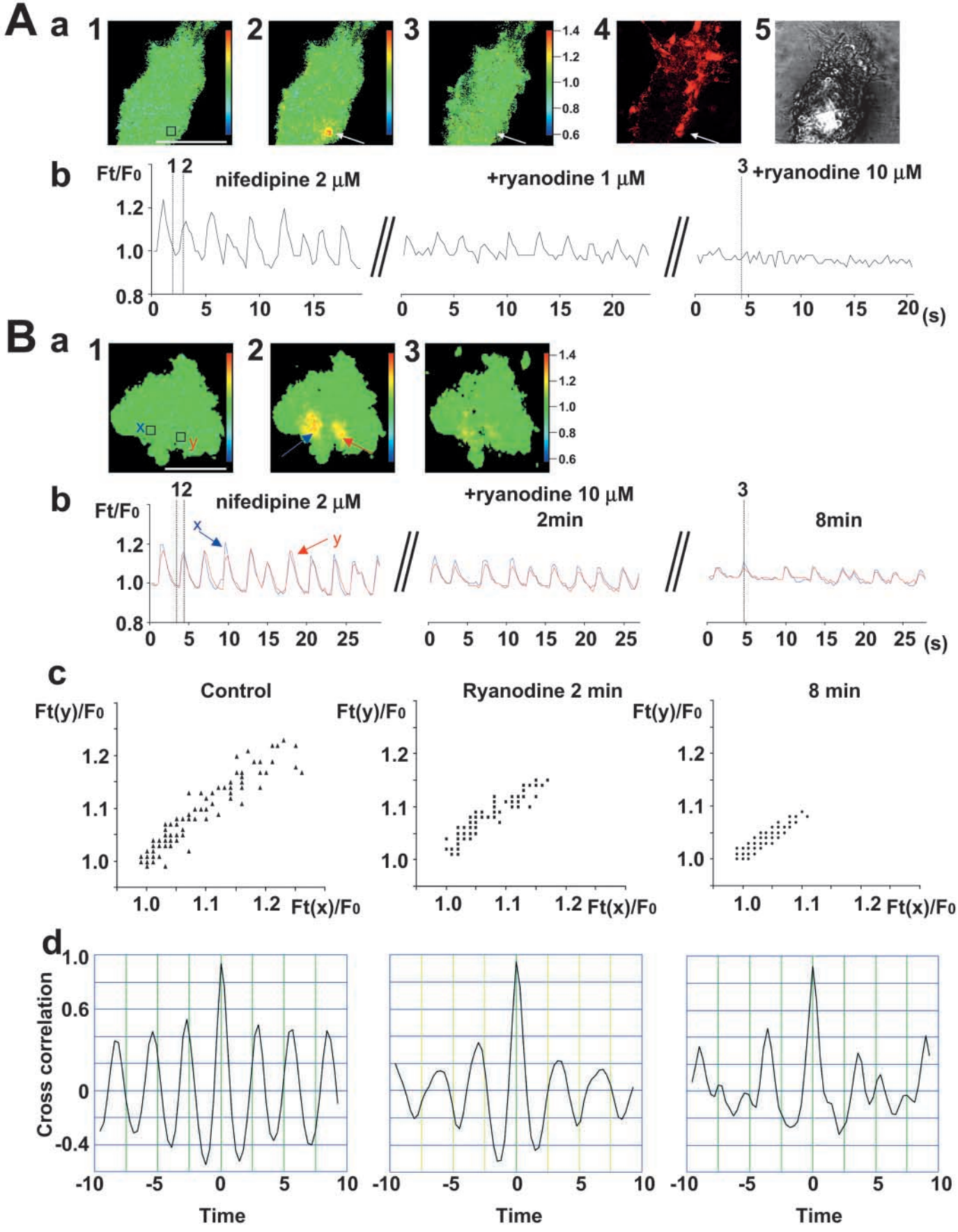
A
a
1
2
3
4
5
1.4
1.2
1.0
0.8
0.6
b
Ft/F0
1 2
nifedipine 2 μM
+ryanodine 1 μM
3
+ryanodine 10 μM
1.2
1.0
0.8
0 5 10 15
0 5 10 15 20
0 5 10 15 20 (s)
B
a
1
2
3
x
y
1.4
1.2
1.0
0.8
0.6
b
12
nifedipine 2 μM
+ryanodine 10 μM
2min
3
8min
Ft/F0
x
y
1.2
1.0
0.8
0 5 10 15 20 25
0 5 10 15 20 25
0 5 10 15 20 25 (s)
c
Control
Ryanodine 2 min
8 min
Ft(y)/F0
Ft(x)/F0
1.0 1.1 1.2
d
Cross correlation
1.0
0.6
0
-0.4
-10 -5 0 5 10
Time

Fig. 1. Effects of ryanodine on $[Ca^{2+}]_i$ oscillations in the presence of nifedipine. Single or multiple $[Ca^{2+}]_i$ oscillation sites are seen in A or B, respectively. Aa1 to 3 show pseudo-colour ratio images of the fluo-3 fluorescent intensity recorded at lines 1 to 3 (indicated in Ab) from a cultured cell cluster preparation in the presence of 2 μM nifedipine. The fluo-3 fluorescence was used as an index of $[Ca^{2+}]_i$ activity. Each image was normalised by the fluorescent intensity of the initial image (acquired at t=0 in the first or third trace of Ab). The panels Aa4 and Aa5 show c-Kit-immunostaining and transmission images, respectively, obtained from the same cell cluster. Bar, 100 μm. Note that the square region in Aa1 showing $[Ca^{2+}]_i$ oscillations indicates c-Kit-immunopositivity. The traces in Ab show changes in $[Ca^{2+}]_i$ (fluo-3 intensity: F_t/F_0) in the square region. After observing control $[Ca^{2+}]_i$ oscillations in the presence of 2 μM nifedipine (left trace), 1 μM ryanodine was applied. The second (middle) trace of $[Ca^{2+}]_i$ oscillations was obtained 5 minutes after 1 μM ryanodine application. Subsequently, the ryanodine concentration was increased to 10 μM. The third (right) trace in Ab was obtained 5 minutes after increasing the ryanodine concentration. The three panels in Ba are pseudo-colour ratio images of $[Ca^{2+}]_i$ activity obtained from another cell cluster preparation showing multiple oscillation sites. Images Ba1 to Ba3 were acquired at lines 1 to 3 in Bb. In Bb the blue and red traces indicate changes in $[Ca^{2+}]_i$ in the square regions of x [$=F_t(x)/F_0$] and y [$=F_t(y)/F_0$] in panel Ba1. After observing control $[Ca^{2+}]_i$ oscillation in the presence of nifedipine (2 μM) (left trace in Bb), 10 μM ryanodine was applied to the extracellular solution. The second (middle) and third (right) traces were obtained 2 and 8 minutes after the application of ryanodine, respectively. In Bc, $F_t(y)/F_0$ (y-axis) is plotted against $F_t(x)/F_0$ (x-axis). Each graph Bd indicates the cross-correlation function between $F_t(x)/F_0$ and $F_t(y)/F_0$. Note, $[Ca^{2+}]_i$ oscillations in x and y are well correlated even when significantly impaired.

the three traces shown in Fig. 1Bb. For each pair of F_t/F_0, the cross correlation function was maximal (0.93-0.95) at t=0 (Fig. 1Bd). When the $[Ca^{2+}]_i$ oscillation amplitude was reduced significantly, the frequency also tended to decrease. The time for $[Ca^{2+}]_i$ oscillations to cease ranged from 3 to 18 minutes after 10 μM ryanodine application (6.6±4.9 minutes). In the F_t/F_0 trace recorded before cessation, the mean amplitude and frequency were 54±32% and 71±21% of the control, respectively.

It is well known that caffeine modulates the properties of ryanodine receptors and amplifies Ca^{2+}-induced Ca^{2+} release (Endo, 1977; Ogawa et al., 2002). In Fig. 2A, panels 1 and 2 the Ca^{2+} images were obtained at resting and peak times of $[Ca^{2+}]_i$ oscillations, respectively (indicated by lines 1 and 2 in Fig. 2Ba). In the presence of nifedipine (2 μM), application of 10 mM caffeine significantly suppressed $[Ca^{2+}]_i$ oscillations. In all clusters examined (n=5), $[Ca^{2+}]_i$ oscillations ceased within 5 minutes, while in four out of the five clusters, as the amplitude decreased, the frequency also decreased. The amplitude and frequency of $[Ca^{2+}]_i$ oscillations in the traces before cessation were 42±23% and 40±24% of the control, respectively (n=4). As shown in Fig. 2Bb, caffeine prominently reduced the $[Ca^{2+}]_i$ oscillation frequency, but the $[Ca^{2+}]_i$ oscillations seen over the pacemaker area were well synchronised (blue line from region x; red line from region y). The trace Fig. 2Bc was recorded 3 minutes after 10 mM caffeine application, and the Ca^{2+} image in Fig. 2A, panel 3 was obtained at the time indicated by line 3 in Fig. 2Bc. The inhibitory effect of caffeine on $[Ca^{2+}]_i$ oscillations was reversible as shown in Fig. 2Bd. Lower concentrations (~1 mM) of caffeine produced similar inhibitory effects, but $[Ca^{2+}]_i$ oscillations ceased within 5 minutes in only two out of five preparations.

In order to further confirm the involvement of RyR, we examined effects of other drugs affecting RyR, (because caffeine is also known to interact with type 1 IP_3R) (Maes et al., 1999; Maes et al., 2000; Bezprozvanny et al., 1994). Tetracaine and procine are known RyR blockers (e.g. Lukyanenko et al., 2001; Kimball et al., 1996). In the presence of nifedipine (1 μM), application of 30 μM tetracaine terminated $[Ca^{2+}]_i$ oscillations within 3-5 minutes (n=5) (Fig. 2C). Also, 30 μM procaine terminated it within 5 minutes (n=4, not shown). By contrast, FK506 is known to modulate the RyR channel function by binding to the 12 kDa FK506-binding proteins (FKBP12 and FKBP12.6) (Masumiya et al., 2003; Bultynck et al., 2001). As shown in Fig. 2D, $[Ca^{2+}]_i$ oscillations very rapidly ceased (1-2 minutes) after application of 10 μM FK506 (n=5).

Effects of $InsP_3$ receptor blockers

Spontaneous electrical activity, i.e. slow waves, is observed at irregular intervals, or not at all in the gastric antral smooth muscle of mice lacking type 1 IP_3R (Suzuki et al., 2000), suggesting that IP_3Rs play an important role. Accordingly, we have investigated the possible contribution of IP_3R Ca^{2+} release channels to $[Ca^{2+}]_i$ oscillations seen in ICCs in small intestine, by examining the effects of 2APB and xestospongin C, both of which have been reported to diffuse across the cell membrane to block IP_3R (Maruyama et al., 1997; Gafni et al., 1997). Fig. 3A shows an example of the effects of 2APB on nifedipine-resistant $[Ca^{2+}]_i$ oscillations (pacemaker $[Ca^{2+}]_i$ oscillations). The blue and red lines indicate changes in $[Ca^{2+}]_i$ in two pacemaker regions ~50 μm apart. Application of 10 μM 2APB completely abolished $[Ca^{2+}]_i$ oscillations after 5 minutes (Ac). In all four preparations used for 10 μM 2APB experiments, $[Ca^{2+}]_i$ oscillations ceased in 5 minutes. By contrast, application of 1 μM 2APB abolished $[Ca^{2+}]_i$ oscillation within 5 minutes in only two out of six preparations. 2APB tended to reduce the amplitude of $[Ca^{2+}]_i$ oscillations, without a large change in frequency. In the F_t/F_0 traces recorded before cessation of $[Ca^{2+}]_i$ oscillations, the average amplitude and frequency were 61±32% and 98±11% of the control, respectively (n=5). The traces in Fig. 3B show an example of the effect of xestospongin C. At 10 μM this drug also completely abolished pacemaker $[Ca^{2+}]_i$ oscillation in 2 minutes (n=3). For both drugs, subsequent washout did not restore $[Ca^{2+}]_i$ oscillation within 5 minutes. Taken together, these results indicate that IP_3Rs are also involved in $[Ca^{2+}]_i$ oscillations in ICCs.

Immunohistochemistry and mRNA expression

In order to confirm the existence of the ryanodine receptors (RyR) in ICCs of mouse small intestine, we stained the isolated smooth muscle (including the myenteric plexus) with an anti-c-Kit antibody (ACK2) and an anti-RyR antibody (clone 34C) simultaneously (Fig. 4). Fig. 4A (red) shows that the c-Kit-immunopositive cells are distributed in a network-like configuration within the myenteric plexus. In Fig. 4B, the

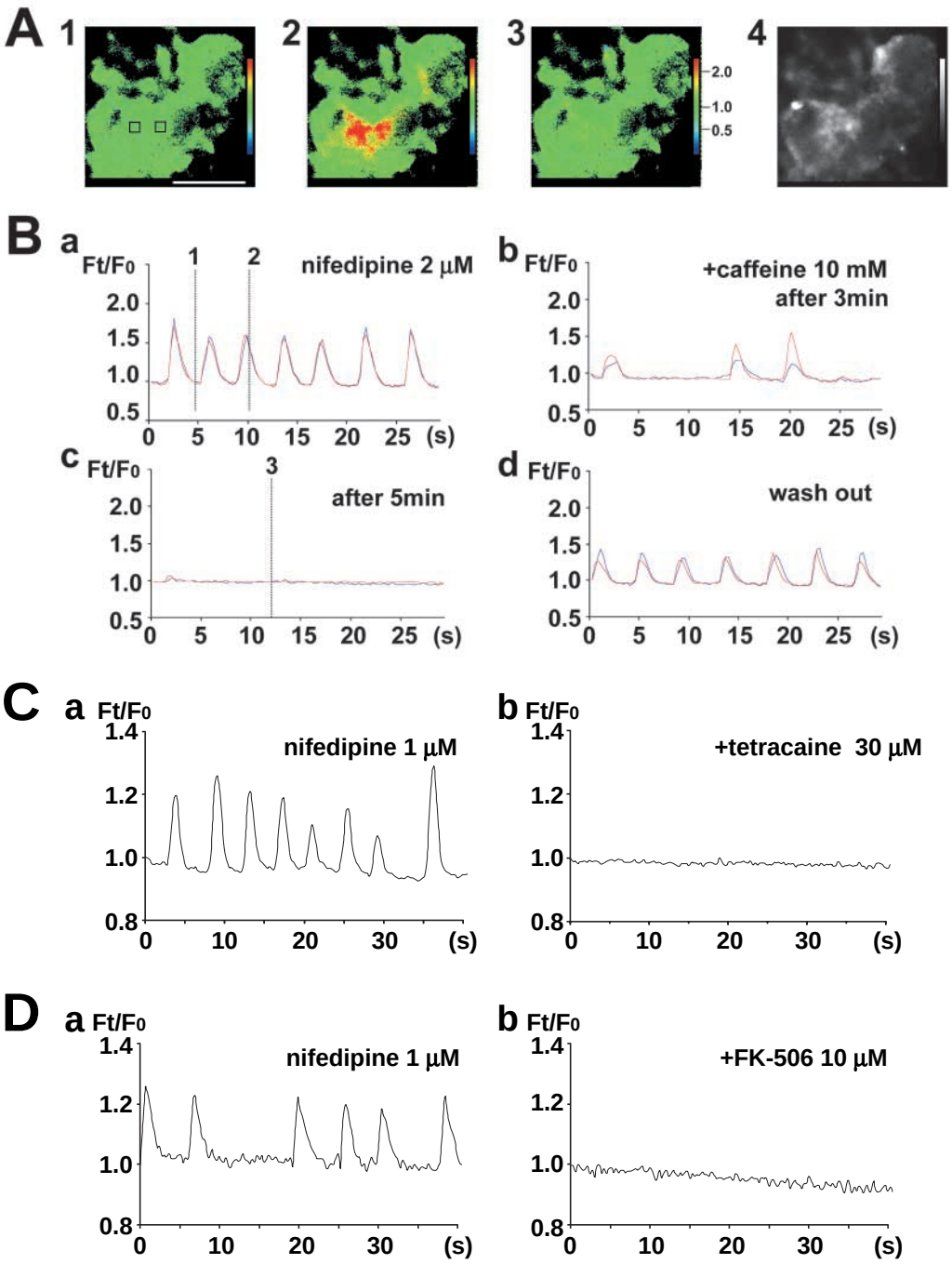

Fig. 2. Caffeine reversibly suppresses $[Ca^{2+}]_i$ oscillations in ICCs. Panels A1 to 3 show $[Ca^{2+}]_i$ images recorded at lines (1) to (3) in B, respectively. Horizontal bar=100 μm. Traces in B indicate the time course of the changes in $[Ca^{2+}]_i$ (F_t/F_0) in the square regions indicated in A(1): blue line for (x) and red line for (y). After observing control $[Ca^{2+}]_i$ oscillations in the presence of 2 μM nifedipine (Ba), 10 mM caffeine was applied. Bb and Bc were recorded after 3 minutes and 5 minutes, respectively. Bd shows recovery of $[Ca^{2+}]_i$ oscillations 5 minutes after washout of caffeine in the presence of nifedipine. In C and D, after observing control $[Ca^{2+}]_i$ oscillations (a), effects of 30 μM tetracaine and 10 μM FK506 were examined, respectively. The trace Cb was recorded 5 minutes after tetracaine application, while Db was 2 minutes after FK506 application.

fluorescence of the anti-RyR antibody (green) was detected in both the c-Kit immunopositive cells (network-like) and smooth muscle cells (running perpendicularly). The merged image (Fig. 4C) clearly indicates that most of the c-Kit-immunopositive cells also reacted to the anti-ryanodine receptor antibody (yellow).

In isolated ICCs and smooth muscle cells, we examined mRNA expression of ryanodine receptor isoforms using RT-PCR. Fig. 5A shows control amplicons of ryanodine receptor type 1 to 3 (RyR1 to 3) along with GAPDH complementary DNA obtained from brain, heart and skeletal muscles. As shown in Fig. 5B, RT-PCR examination of isolated cells revealed that RyR3 was predominantly expressed in ICCs (c-Kit-immunopositive cells), while RyR2 was the major RyR isoform in smooth muscle cells. The DNA band for *c-kit* was detected only in ICCs. Another 5 cycles of PCR produced no additional DNA band in either ICCs or smooth muscle cells. Furthermore, mRNA for ~12 kDa FKBP was expressed in ICCs as well as smooth muscle cells, as suggested by pharmacological examinations. FKBP12.6 was predominant in smooth muscle, while both isoforms (FKBP12 and FKBP12.6) were detected in ICCs (Fig. 5C), being consistent with previous studies on the interaction between RyR and FKBP isoforms (Timerman et al., 1996; Bultynck et al., 2001). Also, we carried out RT-PCR to check for the expression of InsP_3 receptors. The results of this experiment suggested that the type 3 and type 2

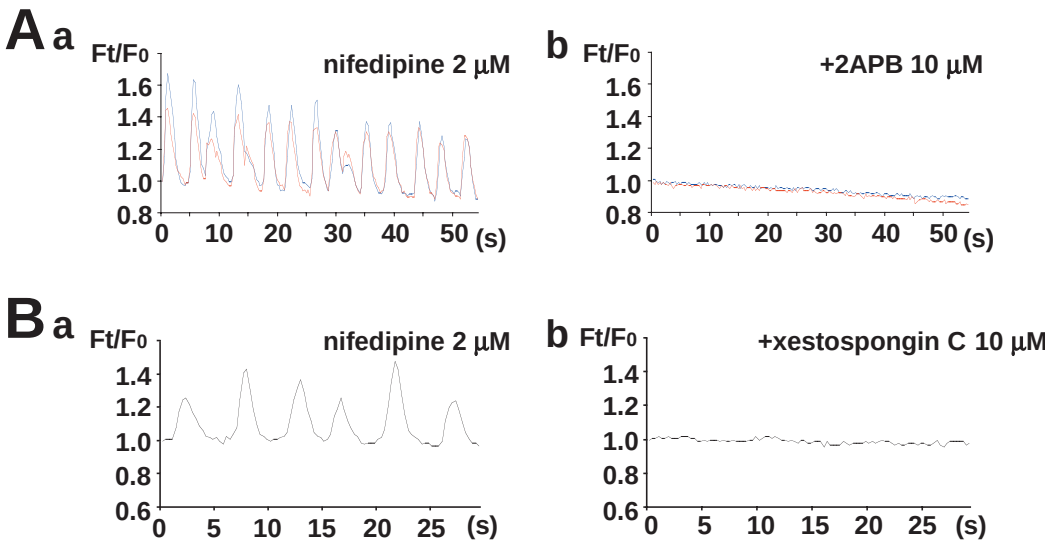

Fig. 3. Examples of the effects of InsP_3 receptor blockers. The traces in A and B were obtained from regions showing $[Ca^{2+}]_i$ oscillations in different cell cluster preparations. In A, after observing control $[Ca^{2+}]_i$ oscillations in the presence of nifedipine (2 μM) (Aa), 10 μM 2APB was applied. The trace Ab was recorded 3 minutes after 10 μM 2APB application. Blue and red lines indicate changes in $[Ca^{2+}]_i$ measured in two different regions at a distance of ~50 μm. This distance is comparable to that for regions x and y shown in Fig. 1Ba1 and Fig. 2A1. In B, after observing a control in the presence of nifedipine (Ba), 10 μM xestospongin C was applied. The trace Bb was obtained 2 minutes after the application of xestospongin C.

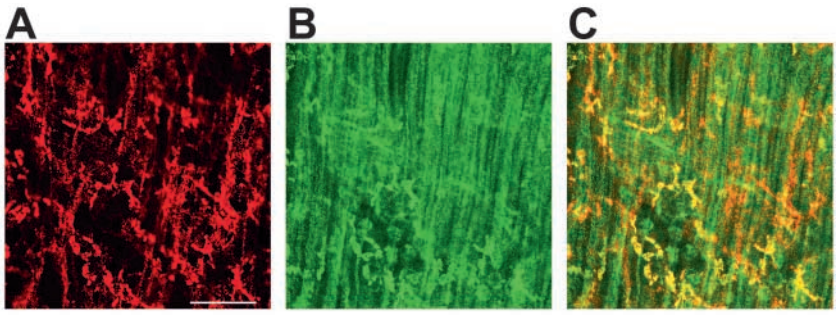

Fig. 4. Immunohistochemistry for c-Kit and ryanodine receptors. Smooth muscle (including the myenteric plexus) of mouse small intestine was double-labelled with anti-c-Kit antibody (A: ACK2 with Alexa 488, red) and anti-ryanodine receptor antibody (B: C34 with Alexa 594, green). C shows a merged image. Note that network-like cells were mostly stained in yellow, indicating that ICCs contain ryanodine receptors.

isoforms of IP_3Rs were the major components in smooth muscle and ICCs, respectively (data not shown).

Reconstituted pacemaker-like $[Ca^{2+}]_i$ oscillations in HEK293 cells

In order to confirm the contribution of RyR3 to $[Ca^{2+}]_i$ oscillations seen in ICCs, we examined the reconstitution of functional RyR3 in HEK293 cells, which originally only express IP_3R. According to RT-PCR results, mRNAs of all three IP_3R types were expressed in native HEK293 cells in the order of IP_3R3 > IP_3R2 > IP_3R1, but mRNAs of RyR subtypes were not detected after 45 cycles of amplification (data not shown).

$[Ca^{2+}]_i$ was measured with a fluorescent Ca^{2+} indicator (Fura-2) 2 days after the transfection. Approximately 10% of HEK293 cells showed spontaneous global $[Ca^{2+}]_i$ oscillations. Fig. 6Aa (black line) demonstrates spontaneous oscillations of $[Ca^{2+}]_i$ over the whole cell area and the elevation of $[Ca^{2+}]_i$ in response to 1 mM caffeine. Further addition of caffeine did not induce marked response. Approximately 30% of HEK293 cells responded to 1 mM caffeine, suggesting that the efficacy for the functional expression of RyR3 was 30%. As shown by the red line in Fig. 6Aa, the remaining HEK cells (~70%) did not respond to caffeine in the range of 1 and 10 mM, but did respond to 1 μM ACh. Fig. 6Ab shows Ca^{2+} images acquired under resting conditions and in the presence of 1 mM caffeine or caffeine plus 1 μM ACh and an image following immunocytochemical staining with anti-RyR antibody. Application of 1 mM caffeine increased $[Ca^{2+}]_i$ in two cells indicated by white arrowheads, but not in other cells. Substantial expression of RyR3 proteins was identified in cells sensitive to caffeine without exception (n>200), indicating a direct correlation between high sensitivity to caffeine and the successful expression of RyR3 proteins in transfected cells. Application of 1 μM ACh in the presence of caffeine evoked $[Ca^{2+}]_i$ transients in caffeine-insensitive cells. The results suggest that RyR3 expressed in the HEK293 cells shares the Ca^{2+} storage sites with IP_3R.

Fig. 6B demonstrates typical spontaneous $[Ca^{2+}]_i$ oscillations in HEK293 cells transfected with RyR3. Two cells (1 and 2) in the confocal Ca^{2+} images exhibited Ca^{2+} oscillations, which occurred as Ca^{2+} waves in each cell. Ca^{2+} waves started from a particular initiation point in each cell, as indicated by white arrowheads in cell 1. The frequency of Ca^{2+} oscillations was 1.3±0.6 cycles/minute (n=39 from separate 8 groups of transfection). The average value of the peak $[Ca^{2+}]_i$ was 1.6±1.4 μM (n=24). In some experiments, the relation between RyR3 protein expression and the generation of Ca^{2+} oscillation was also examined using BODIPY FL-X ryanodine

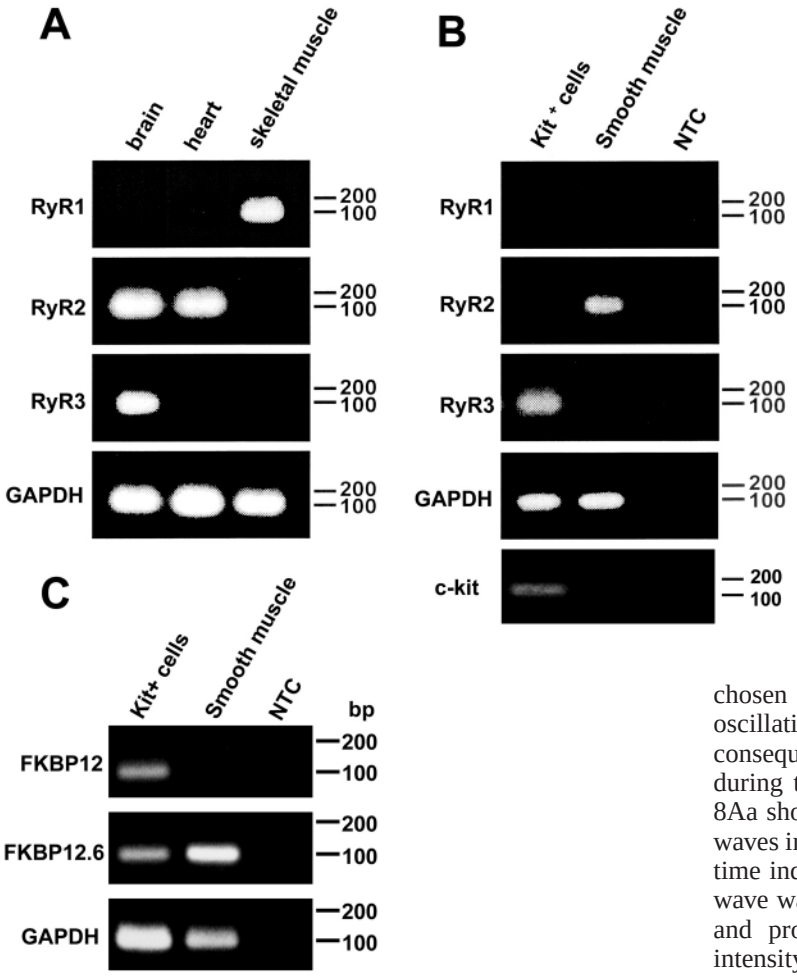

Fig. 5. RT-PCR examination for RyR1 to 3. (A) Control amplicons for RyR1 to 3 and GAPDH extracted from brain, heart and skeletal muscle. Amplification, 35 cycles. (B) RT-PCR examinations of NTC (no template control) and RNA samples obtained from c-Kit-immunopositive cells and smooth muscle cells. Amplification, 45 cycles. (C) RT-PCR examinations for FKBP12 and FKBP12.6. Amplification, 45 cycles. In B, expression of c-kit was also examined. The numbers in the right of each gel indicate the size marker (bp).

(Fig. 6C). Among 19 cells in Fig. 6C, Ca^{2+} oscillations were recorded in 12 cells, which were well stained with BODIPY FL-X ryanodine, and 4 of which are indicated (1-4).

Fig. 7 shows pharmacological suppressions of spontaneous $[Ca^{2+}]_i$ oscillations in HEK293 cells transfected with RyR3. Application of ryanodine (10 μM) terminated $[Ca^{2+}]_i$ oscillations within 3 minutes (n=3) (Fig. 7A). Furthermore, applications of either tetracaine (Fig. 7B: 50 μM, n=3), procaine (10 μM, n=3, not shown) or FK506 (Fig. 7C: 20 μM, n=4), known modulators for RyR, also terminated it. By contrast, applications of either 2APB (100 μM) (D, n=3) or xestospongin C (20 μM, n=3, data not shown), both IP_3R blockers, abolished $[Ca^{2+}]_i$ oscillations. These results agree well with the notion that both RyR and IP_3R are essential in generating $[Ca^{2+}]_i$ oscillations in ICCs, the putative intestinal pacemaker cells. In Fig. 7E and F, HEK293 cells showing $[Ca^{2+}]_i$ oscillations were treated with a Ca^{2+}-free solution or SK&F96365, a known general channel blocker which blocks Ca^{2+} influx through the transient receptor potential (TRP) family of channels in the plasma membrane (e.g. Zhu et al., 1998). Either treatment completely abolished $[Ca^{2+}]_i$ oscillations, suggesting that Ca^{2+} influx from the extracellular space is required in pacemaking (Torihashi et al., 2002). Addition of 0.1 mM Ni^{2+} or Cd^{2+}, natural Ca^{2+} blockers, also suppressed $[Ca^{2+}]_i$ oscillations (not shown).

Possible underlying mechanisms for $[Ca^{2+}]_i$ oscillation

Fast Ca^{2+} imaging was performed in order to analyse the local Ca^{2+} mobilization underlying spontaneous $[Ca^{2+}]_i$ oscillations. Confocal Ca^{2+} images were acquired every 33 milliseconds from HEK293 cells (1) transfected with RyR3 and (2) highly sensitive to caffeine. As shown in Fig. 7, the HEK293 cells under the two conditions are considered to be a good model for $[Ca^{2+}]_i$ oscillations in ICC. The cell shown in Fig. 8 was chosen because this cell showed spontaneous $[Ca^{2+}]_i$ oscillations (Ca^{2+} waves) with relatively low frequency, and consequently it was easy to observe elementary Ca^{2+} events during the interval between Ca^{2+} waves. The images in Fig. 8Aa show the initiation and propagation of spontaneous Ca^{2+} waves in the cell. The images were sequentially obtained at the time indicated by dashed vertical lines in Fig. 8Ab. The Ca^{2+} wave was initiated at the lower part of the cell indicated by x and propagated in the up-direction. Average fluorescence intensity was measured in the small circles (2 μm in diameter) indicated by x, y and z in Fig. 8Aa. Fig. 8Ab shows the high-time resolution changes in the fluorescent intensity (F_t/F_0) upon initiation of a Ca^{2+} wave. The $[Ca^{2+}]_i$ rise ($\Delta F/F_0 \approx 0.4$) in the region x (red line in Fig. 8Ab) clearly preceded the local rises in $[Ca^{2+}]_i$ in the regions y and z.

Fig. 8Ba shows time courses of F_t/F_0 in the three circles, x, y and z, during the interval of Ca^{2+} waves. The Ca^{2+} image in Bb was acquired at the time indicated by a line in Ba. Transient small $[Ca^{2+}]_i$ rises were recorded only in the region x. It should be noted that abrupt $[Ca^{2+}]_i$ rises are observed upon generation of a Ca^{2+} wave in region x. It is deduced that propagating Ca^{2+} waves observed in this HEK293 cell transfected with RyR3 were triggered by subcellular Ca^{2+} release events, such as Ca^{2+} sparks, in an initiating site (x), as has been reported in cardiac myocytes and cardiac trabeculae (Cheng et al., 1996; Wier et al., 1997). Essentially the same results were obtained in all HEK293 cells analysed for a relationship between local Ca^{2+} events and global $[Ca^{2+}]_i$ oscillations (n=5). The elementary Ca^{2+} events reached the peak within 20 milliseconds (time to peak: 17.3±5.3 milliseconds, n=12). The duration at 50% of the maximal F/F_0 was 38.3±7.6 milliseconds (n=11). These characteristics are consistent with previous reconstitution studies dealing with subcellular Ca^{2+} release mechanisms (Ward et al., 2000; Rossi et al., 2002), suggesting that the subcellular Ca^{2+} events seen in the present study are to be referred to as Ca^{2+} sparks, although IP_3R along with RyR might also have some contribution to the elementary Ca^{2+} events. In this study, 32 sites for generating spontaneous small

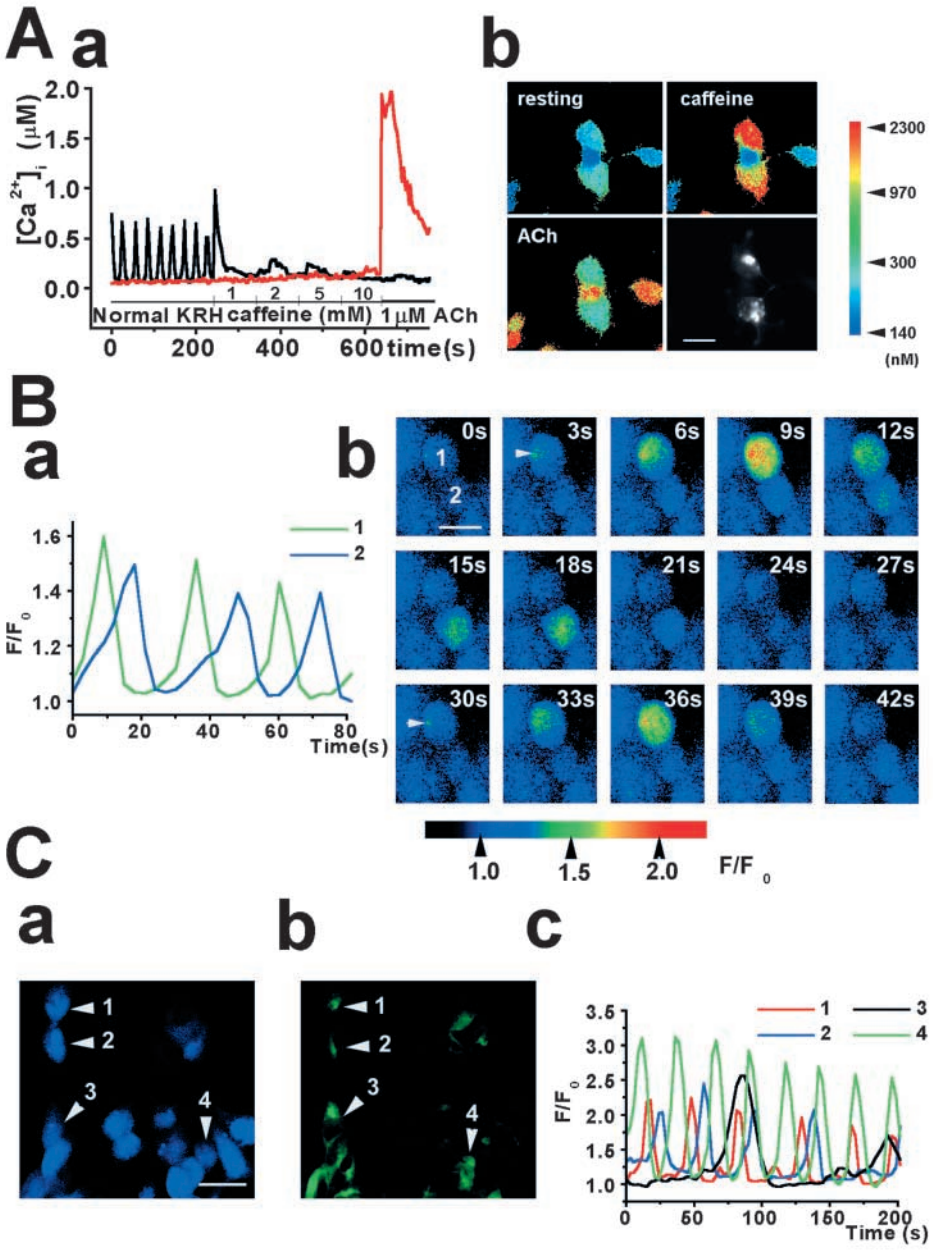

Fig. 6. Spontaneous Ca^{2+} oscillations were detected in HEK293 cells transfected with RyR3. (Aa) The time-course of $[Ca^{2+}]_i$ changes in HEK293 cell expressing RyR3 (black line) and a non-expressing cell (red line). Cells were sequentially treated with 1, 2, 5 and 10 mM caffeine and 1 μM ACh. (Ab) Ca^{2+} images in cells loaded with fura-2AM were obtained during resting conditions and in the presence of 1 mM caffeine or 1 μM ACh. The bottom-right panel in Ab indicates the immunofluorescence staining of RyR. It is noted that cells responding to 1 mM caffeine were stained with immunofluorescent RyR3 antibody. Bars in Ab, 20 μm. (B) The time courses of spontaneous $[Ca^{2+}]_i$ oscillations (a) were obtained from cells (1 and 2) as indicated in a series of confocal images (b). The Ca^{2+} images were obtained every 3 seconds, and cells were loaded with fluo-4 for 15 minutes. (C) Confocal images following fluo-4 loading (a) and staining with BODIPY FL-X ryanodine (b) in HEK293 cells transfected with RyR3. (a) Cells were loaded with fluo-4. $[Ca^{2+}]_i$ oscillations were then recorded in four indicated cells (1-4) among the 19 in this frame. (b) After recording $[Ca^{2+}]_i$ oscillation, cells were stained with BODIPY FL-X ryanodine for 5 minutes and washed. Among the 19 cells in the frame, 12 cells, including all four cells exhibiting $[Ca^{2+}]_i$ oscillations were well stained with BODIPY FL-X ryanodine. (c) The time courses of $[Ca^{2+}]_i$ oscillations of four cells in the frame.

subcelluar $[Ca^{2+}]_i$ rises were observed in 29 cells prepared by 9 separate transfections, and all of these sites tested (n=20) showed RyR3 immunofluorescence reactivity. The size of elementary $[Ca^{2+}]_i$ rises at the peak amplitude was similar to that of punctuated staining patterns of RyR3 protein (2.1±0.3 μm vs. 1.9±0.3 μm, n=13). Small $[Ca^{2+}]_i$ rises occurred repetitively in the same spatial location of a cell with a mean frequency of 2.9±1.2 events/second (n=18). These results strongly suggest that the sites initiating $[Ca^{2+}]_i$ oscillations correspond to regions where RyR3 are expressed in a clustered fashion.

Discussion

The cultured cell cluster preparation that we have recently developed (Nakayama and Torihashi, 2002) contains smooth muscle, enteric neurones and c-Kit-immunopositive interstitial cells (ICCs). The last cell member is the putative pacemaker of the gastro-intestinal tract (Suzuki, 2000; Thuneberg, 1982; Torihashi et al., 1995; Komuro, 1999). In addition, neurotransmission is well known to modulate the spontaneous rhythmicity and contractility in gastro-intestinal smooth muscle tissues (although nervous activity did not seem to contribute basal $[Ca^{2+}]_i$ oscillations in our preparation because tetrodotoxin had no effect). This cell cluster preparation is therefore considered to consist of the essential minimum cell members necessary to investigate mechanisms underlying gastro-intestinal motility and pacemaker function.

In the present study, we demonstrate that reasonably low concentrations (1-10 μM) of ryanodine suppressed and eventually terminated $[Ca^{2+}]_i$ oscillations in ICCs. This is probably because the small size of the cell cluster preparation enables a sufficient amount of ryanodine molecules to enter ICCs relatively rapidly. Further evidence is provided by the

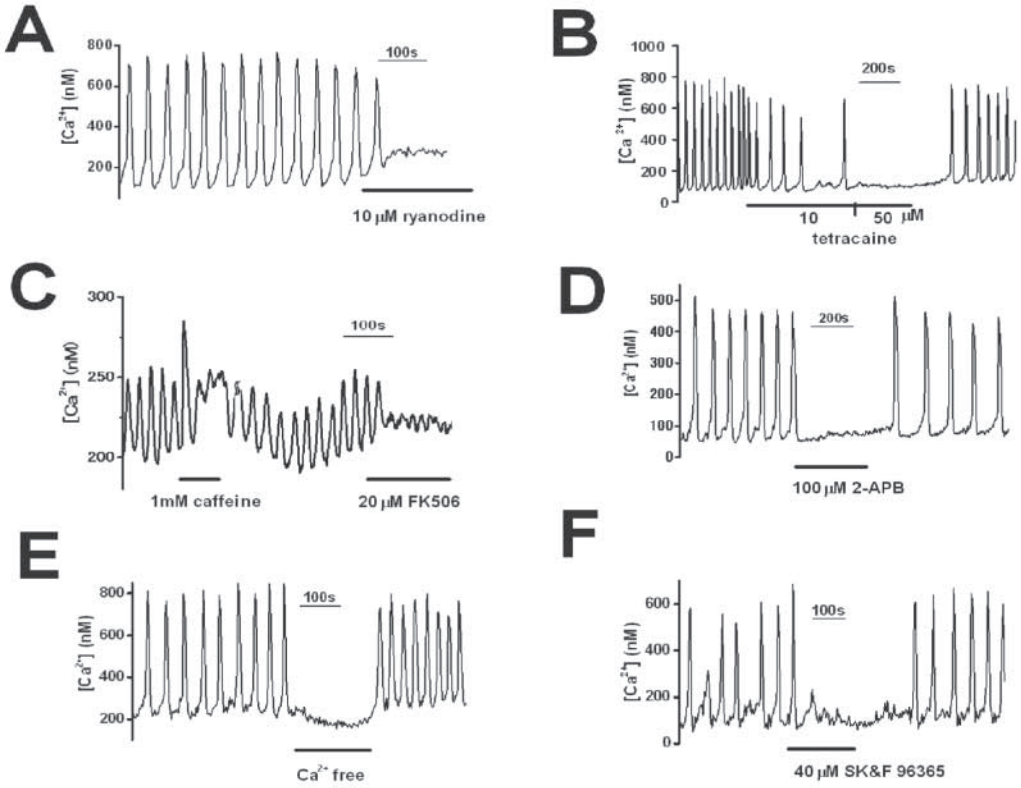

Fig. 7. Pharmacological characterisation of spontaneous Ca^{2+} oscillations in HEK293 cells transfected with RyR3. In A-F, effects of the following drugs or treatments were examined: (A) 10 μM ryanodine; (B) 10-50 μM tetracaine; (C) 20 μM FK506 after transient application of caffeine; (D) 100 μM 2APB; (E) removal of external Ca^{2+}; (F) application of 40 μM SK&F96365.

effects of other drugs affecting RyRs, intracellular Ca^{2+} release channels. As shown in Fig. 2C,D and Fig. 3, application of these drugs (i.e. tetracaine, FK506) completely abolished $[Ca^{2+}]_i$ oscillations at relatively low concentrations (~10 μM). By contrast, IP_3R blockers also eliminated $[Ca^{2+}]_i$ oscillations in ICCs (Fig. 3), as described below. Taken together, the results are the first pharmacological evidence for involvement of both RyR3 and IP_3R in terms of pacemaker $[Ca^{2+}]_i$ oscillations in ICCs. The fact that many drugs, including ryanodine, are effective suggests that use of this preparation is advantageous in pharmacological investigations of $[Ca^{2+}]_i$ oscillations. [In previous reports, greater concentrations (>50 μM) of ryanodine for longer exposure times compared with the present experiments failed to terminate pacemaker potentials in the gastro-intestinal smooth muscle tissues (e.g. Malysz et al., 2001).]

Periodic Ca^{2+} release from the intracellular Ca^{2+} store is considered to be the fundamental event in ICC pacemaking, because Ca^{2+}-dependent ion permeability, i.e. Ca^{2+}-activated Cl^- channels, has been suggested to underlie electrical oscillation (Tokutomi et al., 1995; Edwards et al., 1999; Huizinga et al., 2002). The present experiments indicate that both RyR and IP_3R occur in ICCs. It has been proposed that some smooth muscles have distinct types of ER (endoplasmic reticulum) in terms of the distribution of RyR and IP_3R (Iino, 1991; Yamazawa et al., 1992), but that other smooth muscles do not. Since application of either drugs related to RyR or IP_3R terminated $[Ca^{2+}]_i$ oscillations, both receptors appear to exist in the ER responsible for the periodic Ca^{2+} release in ICCs.

2APB and xestospongin C are known to affect other mechanisms for $[Ca^{2+}]_i$ regulation (Bootman et al., 2002). For example, 100 μM xestospongin C largely inhibits Ca^{2+} influx through endoplasmic reticulum Ca^{2+} pumps (SERCA). At the concentration (10 μM) used in the present study, this drug, however, has little effect on SERCA (De Smet et al., 1999). As described above, lines of evidence have been shown for the involvement of IP_3R in spontaneous rhythmicity in ICCs (e.g. Suzuki et al., 2000). The present results with 2APB and xestospongin C appear to support the notion for the importance of IP_3R. However, we have previously shown that Ca^{2+} influx from the extracellular space (presumably via TRP homologue channels) is also required to maintain pacemaker activity in ICCs (Nakayama and Torihashi, 2002; Torihashi et al., 2002). In the light of the essential similarity between 2APB and xestospongin C (although the evidence for inhibition of store-operated Ca^{2+} entry has not yet been reported for xestospongin C) (Bootman et al., 2002), we can also speculate that IP_3R might additionally contribute to $[Ca^{2+}]_i$ oscillations in ICCs via store-operated types of Ca^{2+} entry processes.

RT-PCR examinations in the present study revealed that ICCs in murine small intestine predominantly express RyR3, while the major RyR isoform in smooth muscle is RyR2. RyR3 is known as a brain type isoform, because of its characteristic, but faint, expression in several brain regions, i.e. the corpus striatum, hippocampus and thalamus (Ogawa et al., 2002). RyR3 is also expressed in skeletal and smooth muscles, albeit at low levels. RyR3 in skeletal muscle may amplify Ca^{2+} release originally elicited by activation of RyR1 (Ogawa et al., 2002; Rossi and Sorrentino, 2002; Yang et al., 2001). Although the presence of the RyR3 isoform is recognised in numerous tissues and organs, predominant expression over RyR1 or RyR2 has been shown only in non-pregnant uterus, where RyR3 may contribute Ca^{2+} signalling only when SR is overloaded with Ca^{2+} (Mironneau et al., 2002). In arterial

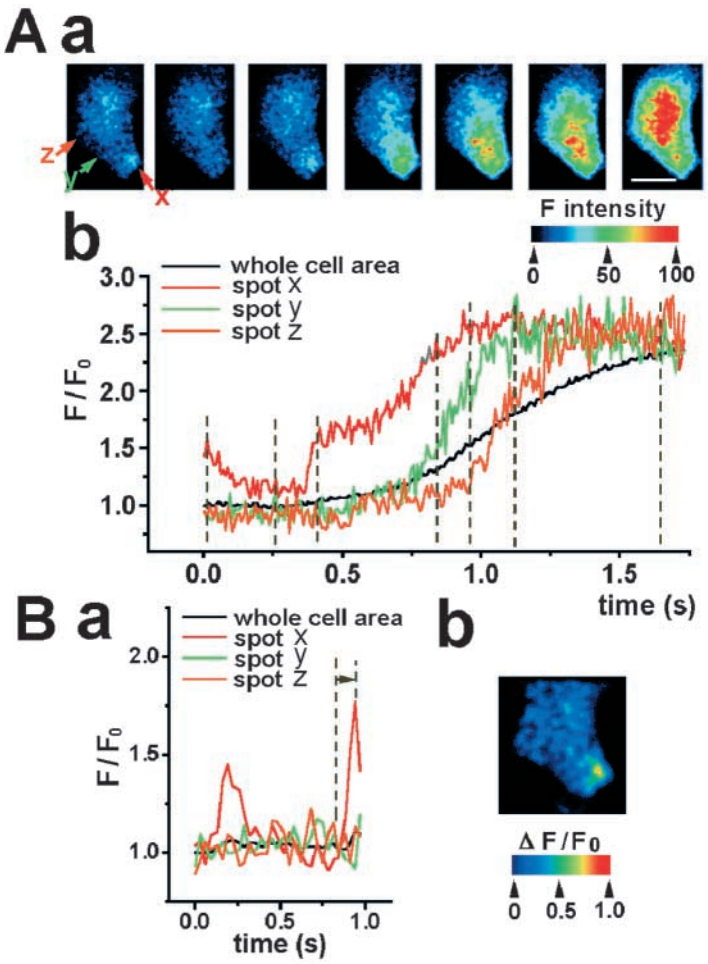

Fig. 8. Images of spontaneous Ca^{2+} release in HEK293 cells expressing RyR3. (Aa) Sequence of seven confocal Ca^{2+} images of fluo-4 fluorescence in HEK293 cells expressing RyR3. The images in Aa were obtained at the time indicated by dashed vertical lines in Ab. (Ab) F and F_0 are fluorescence intensity at a certain time and at resting conditions, respectively. F/F_0 in circular spots (2 μm in diameter) at the point indicated by x, y and z, and that over the whole image of the part of the cell were measured and plotted against time. The black line indicates the change in F/F_0 over the whole area of the cell. Bar in panel Aa, 10 μm. (B) Images of Ca^{2+} sparks (a) and F/F_0 in the areas x, y and z in the same cell.

vascular smooth muscle cells, a contribution by RyR3, in addition to RyR1 and RyR2, to Ca^{2+} spark generation has been suggested based on results from RyR3-deficient mice (Lohn et al., 2001). A similar contribution of RyR3 to cellular Ca^{2+} signalling has been suggested in Ca^{2+}-overloaded cultured rat portal vein smooth muscle cells (Mironneau et al., 2001). It has been found, however, that the RyR3 splice variant predominantly expressed in smooth muscle is not a functional Ca^{2+} release channel, but it could function in a dominant negative fashion (Jiang et al., 2003). There is no report, so far, that RyR3 subtype contributes to generating global $[Ca^{2+}]_i$ oscillation.

The predominant expression of RyR3 in ICCs contributed to pacemaker function, and could be a new molecular marker for pacemaker cells in tissues and organs which possess spontaneous rhythmicity in peripheral systems. Indeed, like the double staining of murine small intestine with anti-ryanodine and anti-c-Kit antibodies in the present study (Fig. 4), significant expression of RyR (stained with 34C) in c-Kit-immunopositive cells has been observed in several other gastro-intestinal tissues (e.g. guinea-pig stomach and small intestine; J.W., unpublished).

Pacemaker-like global $[Ca^{2+}]_i$ oscillations were observed in approximately 10% of HEK293 cells transfected with RyR3 (Fig. 6). Judging from high responsiveness to 1 mM caffeine, the efficacy of transient RyR3 expression may be ~30%. It is first shown in this study that heterologous expression of RyR3 induced periodical Ca^{2+} oscillations. The $[Ca^{2+}]_i$ oscillations seen in the HEK293 cells and ICCs share the following features. (1) Applications of drugs affecting either RyR (ryanodine, caffeine, tetracaine, procaine, FK506) or IP_3R (2APB, xestospongin C) modulate and/or terminate $[Ca^{2+}]_i$ oscillations. In both cases, the contribution of voltage-dependent Ca^{2+} channels to $[Ca^{2+}]_i$ oscillation can be ruled out. (2) Although Ca^{2+} release from the internal stores is the fundamental event in spontaneous $[Ca^{2+}]_i$ oscillations, Ca^{2+} influx from the extracellular space is also essential. Namely, $[Ca^{2+}]_i$ oscillations are suppressed upon Ca^{2+} removal or application of SK&F96365, a TRP homologue channel blocker. Furthermore, applications of inorganic Ca^{2+} blockers, which would totally suppress Ca^{2+} influx, terminate the spontaneous electrical activity corresponding to $[Ca^{2+}]_i$ oscillation [A.Y., unpublished for HEK293 cells; for ICCs, see Torihashi et al. (Torihashi et al., 2002) and Nakayama and Torihashi (Nakayama and Torihashi, 2002)].

Ca^{2+} spark is the landmark event for RyRs (Cheng et al., 1993; Bootman et al., 1995; Imaizumi et al., 1998). From their characteristics in the duration, amplitude and width, the elementary Ca^{2+} events observed in the present study are to be referred to Ca^{2+} sparks, although co-contribution of IP_3R in the frequent 'spark' sites, cannot be ruled out. Ca^{2+} sparks have been identified in cardiac, skeletal and smooth muscle cells (Bootman et al., 1995), where RyR2, RyR1 and RyR2/3 are predominantly expressed, respectively (Ogawa et al., 2002). In oligodendrocyte progenitors, Ca^{2+} sparks and puffs have been recorded and cross-talk between RyR3 and IP_3R2 is speculated (Haak et al., 2001). It has been suggested that IP_3R2 is essential to induced Ca^{2+} puffs and waves, while RyR3 generates the Ca^{2+} sparks. It is noteworthy that 'Ca^{2+} spark' reconstituted in HEK293 cells by expression of RyR3, triggers Ca^{2+} waves repeatedly (i.e.=$[Ca^{2+}]_i$ oscillations) in association with IP_3R. As mentioned above, the $[Ca^{2+}]_i$ oscillations seen in ICCs and reconstituted HEK293 share many characteristic features. Taken together, the results of the present study imply a possibility for reconstruction of the intestinal pacemaker: spontaneous rhythmic $[Ca^{2+}]_i$ oscillations presumably generated by the co-operative activation of RyR3 and IP_3R.

In conclusion, ryanodine receptors are involved in the generation, or at least modulation, of pacemaker $[Ca^{2+}]_i$ oscillations in ICCs. The present study provides the first evidence for an obligatory role of RyR3, and is further

supported by the global $[Ca^{2+}]_i$ oscillations reconstituted with RyR3 in HEK293 cells.

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