Sulphonylurea receptors differently modulate ICC pacemaker Ca^{2+} activity and smooth muscle contractility

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Summary

Appropriate gastrointestinal motility is essential to properly control the body energy level. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations in interstitial cells of Cajal (ICCs; identified with c-Kit immunoreactivity) are considered to be the primary mechanism for the pacemaker activity in gastrointestinal motility. In the present study, RT-PCR examinations revealed predominant expression of the type 1 isoform of sulphonylurea receptors (SUR1) in ICCs of the mouse ileum, but expression of SUR2 was predominant in smooth muscle. In cell clusters prepared from the same tissue, smooth muscle contractility and pacemaker $[Ca^{2+}]_i$ activity in ICCs were found to be differentially modulated by K_{ATP} channel openers and sulphonylurea compounds, in accordance with the expression of SUR isoforms. 1 μM cromakalim nearly fully suppressed the mechanical activity in smooth muscle, whereas ICC pacemaker $[Ca^{2+}]_i$ oscillations persisted. Greater concentrations (~10 μM) of cromakalim attenuated pacemaker $[Ca^{2+}]_i$ oscillations. This effect was not reversed by changing the reversal potential of K^+, but was prevented by glibenclamide. Diazoxide at 30 μM terminated ICC pacemaker $[Ca^{2+}]_i$ oscillations, but again treatment with high extracellular K^+ did not restore them. These results suggest that SUR can modulate pacemaker $[Ca^{2+}]_i$ oscillations via voltage-independent mechanism(s), and also that intestinal pacemaking and glucose control are closely associated with SUR.

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Key words: Gastrointestinal motility, Pacemaker, Intracellular Ca^{2+} oscillations, Sulphonylurea receptors, c-Kit immunoreactivity, K^+ channel openers

Introduction

Interstitial cells of Cajal (ICCs), identified using c-Kit immunoreactivity, are distributed throughout the gastrointestinal (GI) tracts. These cells are believed to play an essential role in GI motility, such as pacemaking (e.g. Thuneberg, 1982; Suzuki, 2000; Hirst and Ward, 2003; Takaki, 2003). There is now an accumulating body of evidence that some gastroenteropathies involve impairment of ICCs. For instance, it has been shown that the number of ICCs decreases in patients with diabetes mellitus, which is frequently complicated by impaired motility of the GI, and consequently making it more difficult to control the postprandial blood-glucose concentration (Koch, 2001; Camilleri, 2002).

Pacemaker potentials underlie spontaneous mechanical activity in the GI tract. There are several studies reporting that Ca^{2+}-dependent plasmalemmal ion channels are periodically activated in ICCs (Tokutomi et al., 1995; Huizinga et al., 2002; Walker et al., 2002). It is therefore deduced that oscillations of the intracellular (cytosolic) Ca^{2+} concentration ($[Ca^{2+}]_i$) in these cells are the primary mechanism generating pacemaker potentials. Indeed, we have previously demonstrated that spontaneous electrical activity occurs in synchrony with $[Ca^{2+}]_i$ oscillations in c-Kit-immunopositive-cell-rich regions (Torihashi et al., 2002).

It has been extensively shown in numerous regions of the GI tract that spontaneous mechanical and electrical activities are highly temperature-dependent and sensitive to metabolic inhibitors (e.g. Tomita, 1981; Conner et al., 1974; Nakayama et al., 1997). If pacemaker $[Ca^{2+}]_i$ oscillations in ICCs are generated through mechanisms involving and/or affected by energy-level-relating signals, such mechanisms could be responsible for these characteristic spontaneous rhythmicity of the gut.

From the muscle layers of the gastrointestinal tract, we have

recently developed a cultured cell cluster preparation containing essential minimum cell members to investigate gastrointestinal motility: smooth muscle, enteric neurons and ICCs (c-Kit-immunopositive interstitial cells). This preparation shows spontaneous contractions, and preserves several characteristic features seen in tissue-level experiments (Nakayama and Torihashi, 2002; Torihashi et al., 2002). It is hypothesized that the link between gut pacemaker activity and energy metabolism could be mediated by K_{ATP} channels and sulphonylurea receptors (SURs). In the present study, we thus examined the effects of K_{ATP} channel openers on pacemaker $[Ca^{2+}]_i$ oscillations in ICCs and smooth muscle contractions, using cell cluster preparations from the mouse ileum. We also carried our RT-PCR and immunostaining examinations, and found that SUR2 occurs in smooth muscle cells, while surprisingly, SUR1 is predominant in ICCs. Accordingly, we observed modulations of pacemaker $[Ca^{2+}]_i$ activity and contraction, reflecting these distinct SUR isoforms in ICCs and smooth muscle. Our findings may provide a new insight into the mechanisms of blood glucose control, and also a link between gastrointestinal motility and metabolic diseases.

Materials and Methods

Cell clusters

The preparation of cell clusters used in the present study has been described previously (Nakayama and Torihashi, 2002; Torihashi et al., 2002). The mice used 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 days after birth) were killed by cervical dislocation, after being anaesthetized with diethyl ether. The smooth muscle layers (both circular and longitudinal) along with the myenteric plexus were carefully dissected from the ileum (5 cm in length, from 1 cm below the pyloric ring to the caecum), and incubated in Ca^{2+}-free Hanks' solution containing collagenase (1 mg/ml; Wako Chemical, Osaka, Japan), trypsin inhibitor (2 mg/ml; type I-S, Sigma, St Louis, MO, USA), ATP (0.3 mg/ml; Seikagakukogyo, Tokyo, Japan) and bovine serum albumin (2 mg/ml; Sigma) for 40 minutes at 37°C. The muscle preparation was then, triturated with fire-blunted glass pipettes. The resultant cell clusters were plated onto a lab-made culture dish, and kept in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% foetal bovine serum (Sigma) and antibiotics (streptomycin 30 μg/ml and penicillin 30 units/ml; Sigma) at 37°C. After 2-3 days of incubation, the cultured cell clusters were used for Ca^{2+} imaging.

The cultured cell clusters have several advantages to investigate mechanisms underlying spontaneous rhythmicity and its modulations (Nakayama and Torihashi, 2002; Torihashi et al., 2002). (1) This preparation contains essential members, i.e. smooth muscle, enteric neurones and c-Kit-immunopositive interstitial cells (equivalent to ICCs). (2) Many cell clusters show spontaneous contraction, when enzymatic treatment is appropriate. (3) The origins of spontaneous rhythmicity are all present in a small, limited space for ease of examination under a microscope. (4) It is relatively easy to load intracellular Ca^{2+} indicators.

Ca^{2+} imaging and evaluation of mechanical activity

The cultured cell cluster preparations were incubated in 'normal' solution containing approximately 8 μM Fluo3-AM (acetoxymethyl ester of Fluo-3; Dojindo, Kumamoto, Japan) and detergents (0.02% Pluronic F-127; Dojindo; 0.02% cremophor EL; Sigma) for 3-4 hours at room temperature. A CCD camera system (Argus HiSCA, Hamamatsu Photonics, Hamamatsu, Japan) was used to continuously monitor Fluo-3 light emission by digital imaging. The cell clusters were illuminated at 488 nm, and emission light of 515-565 nm was detected. The temperature of the recording chamber was kept at 35°C using a micro-warm plate system (MP10DM, Kitazato Supply, Fujinomiya, Japan). Digital images (0.963 μm/pixel) were normally collected at approximately 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. Ratio-images were constructed by dividing each Ca^{2+} image with a Ca^{2+} image acquired at a basal $[Ca^{2+}]_i$ time after subtracting background fluorescence, and by applying a smoothing filter of a 5×5 matrix.

Treatment of cell clusters with fluo-3AM often stained pacemaker-cell-rich regions faster than smooth-muscle-cell-rich regions. In the present study we aimed to investigate $[Ca^{2+}]_i$ oscillations in pacemaker cells (ICCs). As a result, it was often difficult to simultaneously evaluate the changes in $[Ca^{2+}]_i$ in smooth muscle cells corresponding to the contractile activity in normal control solution. In the present study, the frequency of spontaneous $[Ca^{2+}]_i$ oscillations in pacemaker cells did not differ from that of spontaneous oscillatory inward currents previously measured by the patch clamp technique (16.6±3.7 cycles/minute: the range from 9.8 to 27.6 cycles/minute; n=34) (Nakayama and Torihashi, 2002). (The oscillatory inward currents are synchronised with spontaneous contractions of ileal cell clusters.) We thus judged that the procedure for loading fluo-3 was appropriate to measure spontaneous $[Ca^{2+}]_i$ activity in pacemaker cells.

In normal solution, three-dimensional contractions of cell clusters made it difficult to accurately estimate spontaneous $[Ca^{2+}]_i$ activity. Under these conditions, analysis was limited to those cell cluster preparations in which the area showing spontaneous $[Ca^{2+}]_i$ activity was large enough to occlude the mechanical artefact (i.e. the area of $[Ca^{2+}]_i$ activity was greater than the size of spontaneous contraction). However, the spontaneous $[Ca^{2+}]_i$ activity in pacemaker cells was mainly assessed in the presence of nifedipine, a Ca^{2+}-channel antagonist that suppresses contraction and $[Ca^{2+}]_i$ activity in smooth muscle. It is well known that pacemaker electrical activity referred to as 'slow waves' (in isolated gastrointestinal smooth muscle tissues) are preserved in the presence of dihydropyridine Ca^{2+} channel antagonists, despite the suppression of smooth muscle contraction (Dickens et al., 1999; Huang et al., 1999). Indeed, we have previously confirmed that in the presence of 1 μM nifedipine, c-Kit-immunopositive cells (equivalent to ICCs and pacemaker cells) show spontaneous $[Ca^{2+}]_i$ oscillations synchronised with electrical activity in the same type of ileal cell cluster preparations as used in the present study (Torihashi et al., 2002).

In some cell cluster preparations, 'landmark' high-density areas were tracked in order to simultaneously monitor the spontaneous mechanical activity, using the same digital fluo-3 fluorescent images for $[Ca^{2+}]_i$ measurements (Image Tracker PTV software package: DigiMo, Osaka, Japan). The high intensity fluorescence areas (with little change in the fluorescence intensity during experiments) were used as a landmark, and the movement of the centre of gravity of such landmarks in each image was plotted against time. (This was because the shift of the centre of the landmark in the absolute two-dimensional co-ordinates was not negligible over many oscillation cycles.)

In order to show the presence of putative pacemaker cells (ICCs-rich regions), some cell clusters were treated with an anti-c-Kit antibody (ACK2; eBioscience, San Diego, CA, USA) for 1.5 hours after $[Ca^{2+}]_i$ measurements. This was followed by incubation with a secondary antibody and Alexa Fluor 594-conjugated anti-rat IgG (Molecular Probes) at a concentration of 15 μg/ml in PBS for 1 hour at room temperature.

RT-PCR

A similar isolation procedure for cell cluster preparations was used to obtain isolated c-Kit-immunopositive cells and smooth muscle cells.

A longer (50 minutes) enzymatic incubation and more complete trituration with the glass pipettes were performed. 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 1/200–1/100 v/v for 10 minutes. The cell suspension was then centrifuged, and washed with normal solution twice. About 5-10 isolated smooth muscle cells and c-Kit-immunopositive cells were separately collected into sterile tubes, and kept at –20°C until subjected to RT-PCR (reverse transcriptase polymerase chain reaction). The cells were picked up with glass pipettes of 10-20 μm tip diameter under a fluorescence microscope. Smooth muscle cells and c-Kit-immunopositive cells (ICCs) were assessed by their characteristic spindle shape and immunofluorescence (excitation wavelength 520-550 nm; emission wavelength >580 nm).

cDNA was produced by reverse-transcribing RNA samples with Superscript™ II RNase H^- (Invitrogen, Carlsbad, CA, USA) as previously reported (Ohya et al., 2002). The resultant cDNA products were amplified by PCR with gene-specific primers. Genomic DNA was removed by adding 1 U RNase-free DNase (Promega, Madison, WI, USA), followed by incubation for 30 minutes at 37°C. The following PCR primers were used. Kir6.1 (GenBank accession no. NM_008428, 611-713, amplicon = 103 bp): 5′-GGTTCAAGTGA-CCATTGGGTTT-3′ and 5′-GTTGATGATCAGACCCACGATGT-3′; Kir6.2 (AF037313, 773-893, amplicon = 121 bp): 5′-CCGCTTCG-TGTCCAAGAAA-3′ and 5′-GAGCAGAGTGTGTGGCCATTT-3′; SUR1 (AF037313, 72-172, amplicon = 101 bp): 5′-CAAGACGC-CAAGGGAAGTGA-3′ and 5′-ACCAGTAGGTCCCCTTTGATA-GC-3′; SUR2 (AF037274, 3402-3503, amplicon = 102 bp): 5′-CGA-ACATCATCGACCAGCAT-3′ and 5′-CGGGTGTAGCATAGGAG-ATCATC-3′; c-Kit (X58687, 371-470, amplicon = 100 bp): 5′-CAA-TGGAAGGTTGTCGAGGA-3′ and 5′-GCCTGTTTCTGGGAA-ACTCC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (M32599, 730-833, amplicon = 104 bp): 5′-CATGGCCTTC-CGTGTTCCT-3′ and 5′-CCTGCTTCACCACCTTCTTGA-3′. The amplification profile was as follows: 15 seconds at 95°C and 60 seconds at 60°C for 45 cycles. The RT-PCR products were separated by electrophoresis on a 2.5% agarose/1× TAE (Tris, acetic acid, EDTA) gel. The DNA bands were visualized by staining with ethidium bromide, and documented on FluorImager 595 (Amersham Biosciences, Piscataway, NJ, USA). No template control (NTC) was an RT-PCR product in which no sample RNA was added, monitoring non-specific amplification and spurious primer-dimer fragments. Each amplicon was sequenced using a Thermo Sequenase Cycle Sequencing kit (Amersham Biosciences, Piscataway, USA), with a DSQ-1000L sequencer (Shimadzu, Kyoto, Japan).

Immunohistochemistry

Small segments (10×5 mm) of smooth muscle layers (including the myenteric plexus) isolated from mouse ileum, were fixed with 4% paraformaldehyde (4°C), and permeabilized with 0.1% Triton X-100 and 5% BSA (bovine serum albumin, fraction V: Sigma) for 1 hour. The tissue was double stained sequentially with anti-SUR1 antibody (C16: sc-5789, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-c-Kit antibody (ACK2, eBioscience) in 100 mM PBS (phosphate-buffered saline) solution for 1.5 hours. The PBS contained 1% BSA in order to block any non-specific reaction. This was followed by incubation with secondary antibodies, Alexa-conjugated anti-rat or goat IgG (Alexa Fluor 594 or Alexa Fluor 488: Molecular Probes, Eugene, OR, USA) at the concentration of 15 μg/ml for 1 hour. Double-stained small segments were mounted on a glass slide with an anti-fading agent (ProLong: Molecular Probes) and scanned with a confocal microscope (MRC-1024: Bio-Rad, Hercules, CA, USA). Controls were prepared by omitting the primary antibodies. The reactivity was negligible in network-forming cells in the myenteric plexus (i.e. ICCs).

Solutions and drugs

The composition of the 'normal' solution used in $[Ca^{2+}]_i$ measurements was (mM): NaCl, 125; KCl, 5.9; $MgCl_2$ 1.2; $CaCl_2$ 2.4; glucose 11; Tris-Hepes 11.8 (pH 7.4). Nifedipine, glibenclamide and were purchased from Sigma (St Louis, MO, USA); diazoxide from BIOMOL (Plymouth Meeting, PA, USA). Cromakalim was a generous gift from SmithKline Beecham Laboratories. Stock solutions of nifedipine were prepared by dissolving these drugs in ethanol, and cromakalim, diazoxide and glybenclamide were dissolved in dimethyl sulphoxide (DMSO). The working concentrations of the solvents were less than 1%. In preliminary experiments, we observed that application of this concentration of either solvent alone had little effect on $[Ca^{2+}]_i$ oscillation in cell cluster preparations. DMEM and other reagents for cell culture were purchased from Sigma.

Statistics

Numerical data are expressed as mean±s.d. Differences between means were evaluated by paired or unpaired *t*-tests. A probability (P) <0.05 was taken as a statistically significant difference.

Results

Intracellular Ca^{2+} oscillations in cell cluster preparations

Cultured cell clusters were prepared from mouse ileum. It has been shown that spontaneous electrical activity originating from ICCs persists in the presence of a dihydropyridine (DHP) Ca^{2+} channel antagonist that abolishes GI spontaneous mechanical activity (Dickens et al., 1999; Huang et al., 1999). Using this selective antagonistic effect on smooth muscle activity, we have previously demonstrated that DHP Ca^{2+} channel antagonists can isolate pacemaker $[Ca^{2+}]_i$ oscillations in c-Kit-immunopositive cells (predominantly ICC-MY) in the same cell cluster preparations as used in the present study (Torihashi et al., 2002). When multiple regions show spontaneous $[Ca^{2+}]_i$ oscillations in the presence of Ca^{2+} antagonists (e.g. 1 μM nifedipine), these oscillations are well synchronised (Fig. S1 in supplementary material), consistent with these preparations operating as a functional syncytium.

Effects of cromakalim on spontaneous $[Ca^{2+}]_i$ and mechanical activities

Cromakalim is known to activate K_{ATP} channels in smooth muscle (Beech et al., 1993; Edwards and Weston, 1993). At relatively low concentrations this compound suppresses the mechanical activity of stomach smooth muscle accompanied by hyperpolarization of the cell's membrane potential, but has little effect on the frequency of spontaneous electrical activity (Katayama et al., 1993). In order to elucidate the relationship between ICC pacemaking and gut smooth muscle contractility, we first measured pacemaker $[Ca^{2+}]_i$ activity in ICCs in the absence and presence of cromakalim.

Fig. 1 shows an ileal cell cluster preparation treated with 3 μM cromakalim. This treatment suppressed mechanical activity, but pacemaker $[Ca^{2+}]_i$ oscillations persisted. Fig. 1a,b shows images of basal (F_0) and peak Ca^{2+} (F_{peak}/F_0), respectively, of an oscillation cycle, revealing that $[Ca^{2+}]_i$ oscillations occurred in the lower region of this cell cluster preparation (Fig. 1b). Fig. 1c shows a transmission image

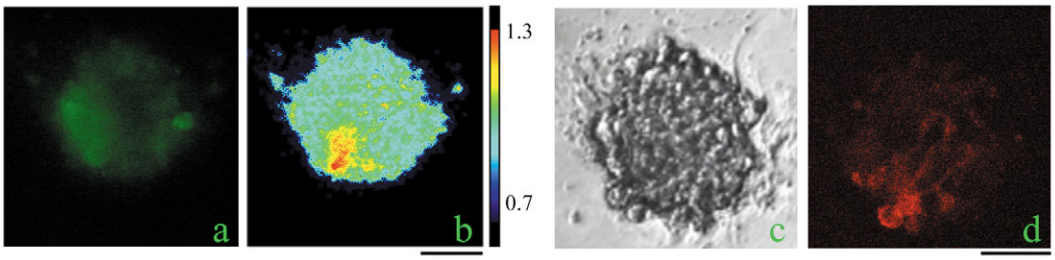

Fig. 1. Spontaneous $[Ca^{2+}]_i$ oscillation seen in a c-Kit-immunopositive region of a cell cluster preparation in the presence of cromakalim. (a,b) Fluo-3 Ca^{2+} images acquired from a cell cluster preparation at basal and peak times of an oscillation cycle, respectively. The Ca^{2+} image in b was normalized to a [(a)=F_0, (b)=F_{peak}/F_0]. This cell cluster preparation was treated with 3 μM cromakalim. (c,d) A transmission image of the same cell cluster and an immunostaining with an anti-c-Kit antibody, ACK2, respectively. This immunostaining was done after the $[Ca^{2+}]_i$ measurement. Scale bars: (below b for a and b, and below d for c and d) 50 μm.

obtained from the same cell cluster, Fig. 1d shows immunostaining with anti-c-Kit antibody (ACK2). Taken together, Fig. 1 indicates that the region showing spontaneous $[Ca^{2+}]_i$ oscillations had high c-Kit immunoreactivity. This suggests that cromakalim, a K_{ATP} channel opener, at relatively low concentrations can be used to isolate ICC pacemaker $[Ca^{2+}]_i$ oscillations, in a similar manner to DHP Ca^{2+} channel antagonists.

The relationship between mechanical activity and spontaneous $[Ca^{2+}]_i$ oscillations was further analysed. Fig. 2 shows the effect of cromakalim (1 μM) in a cell cluster preparation that contained a discrete region of high-intensity fluorescence indicated by an arrow in the panel Aa. The fluorescence intensity of this region was stable over time, and could therefore be used to monitor the contractility, using image tracker software. In the upper region of this cell cluster

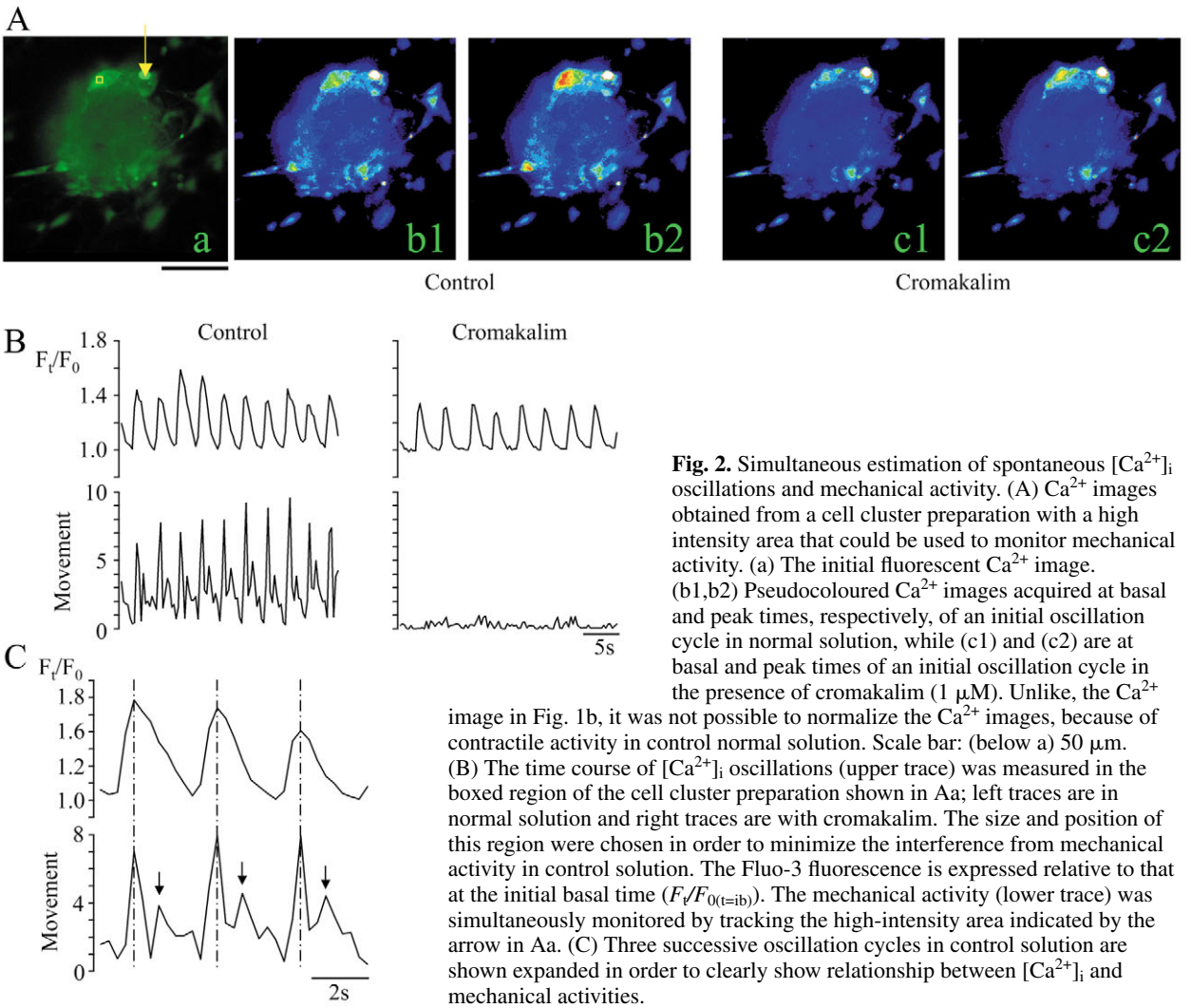

Fig. 2. Simultaneous estimation of spontaneous $[Ca^{2+}]_i$ oscillations and mechanical activity. (A) Ca^{2+} images obtained from a cell cluster preparation with a high intensity area that could be used to monitor mechanical activity. (a) The initial fluorescent Ca^{2+} image. (b1,b2) Pseudocoloured Ca^{2+} images acquired at basal and peak times, respectively, of an initial oscillation cycle in normal solution, while (c1) and (c2) are at basal and peak times of an initial oscillation cycle in the presence of cromakalim (1 μM). Unlike, the Ca^{2+} image in Fig. 1b, it was not possible to normalize the Ca^{2+} images, because of contractile activity in control normal solution. Scale bar: (below a) 50 μm. (B) The time course of $[Ca^{2+}]_i$ oscillations (upper trace) was measured in the boxed region of the cell cluster preparation shown in Aa; left traces are in normal solution and right traces are with cromakalim. The size and position of this region were chosen in order to minimize the interference from mechanical activity in control solution. The Fluo-3 fluorescence is expressed relative to that at the initial basal time ($F_t/F_{0(t=ib)}$). The mechanical activity (lower trace) was simultaneously monitored by tracking the high-intensity area indicated by the arrow in Aa. (C) Three successive oscillation cycles in control solution are shown expanded in order to clearly show relationship between $[Ca^{2+}]_i$ and mechanical activities.

preparation, $[Ca^{2+}]_i$ oscillations were clearly observed (Ab: control; Ac: cromakalim). As shown in Fig. 2B, in 'normal' solution (control), $[Ca^{2+}]_i$ oscillations in the upper regions of the cluster (upper traces) led mechanical activity (lower traces). In contrast, application of cromakalim nearly completely suppressed the mechanical activity of the cluster preparation, while $[Ca^{2+}]_i$ oscillations remained, with small reductions of the peak amplitude (~15%) and frequency (~12%) (right upper and lower traces in Fig. 2B). Qualitatively, the same results were obtained in more than ten cell cluster preparations. With respect to the temporal relationship between changes in $[Ca^{2+}]_i$ and mechanical activity in normal solution, it was noted that the largest contractile movement in each cycle corresponds to the peak amplitude of $[Ca^{2+}]_i$ oscillations (dotted lines in Fig. 2C showing expanded successive oscillation cycles). These results suggest that spontaneous elevations of $[Ca^{2+}]_i$ in the pacemaker cells trigger contractile activity in smooth muscle, presumably via pacemaker potentials, and that cromakalim-induced hyperpolarization prevents activation of (DHP-sensitive) L-type Ca^{2+} channels in smooth muscle.

Frequency and amplitude of pacemaker $[Ca^{2+}]_i$ oscillations

Fig. 3 shows effects of various concentrations of cromakalim on the frequency of $[Ca^{2+}]_i$ oscillations in cell cluster preparations. 1 μM (Fig. 3A, closed circles) or 3 μM cromakalim (Fig. 3A, open circles) was applied in extracellular solution ('normal' solution), and changes in the frequency are shown in individual cell clusters. As described above, the regions showing spontaneous $[Ca^{2+}]_i$ oscillations in the presence of the K_{ATP} channel opener are considered as pacemaker-rich regions. Therefore, after observing $[Ca^{2+}]_i$ oscillations in the presence of cromakalim, the effects of cromakalim on $[Ca^{2+}]_i$ oscillations in the same regions were retrospectively assessed. The frequencies of $[Ca^{2+}]_i$ oscillations

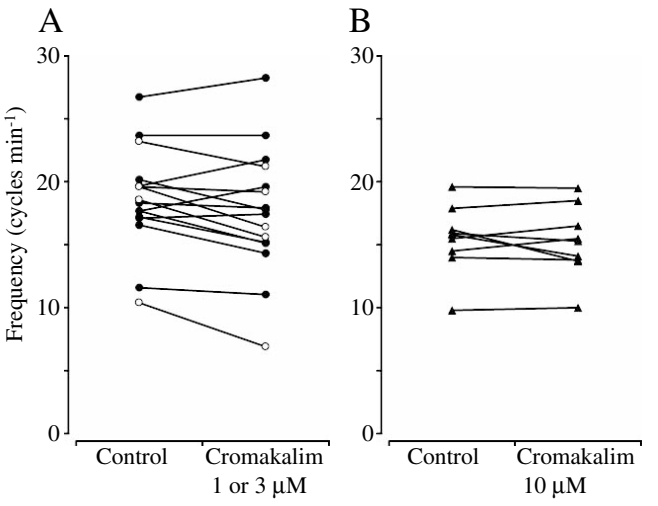

Fig. 3. Effects of various concentrations of cromakalim on the $[Ca^{2+}]_i$ oscillation frequency. Changes in the frequency are plotted for individual cell cluster preparations. The graph in A shows the effects of 1 (filled circles) or 3 μM cromakalim (open circles) applied in normal extracellular medium. In B, 10 μM cromakalim (filled triangles) was used in the presence of 1 μM nifedipine.

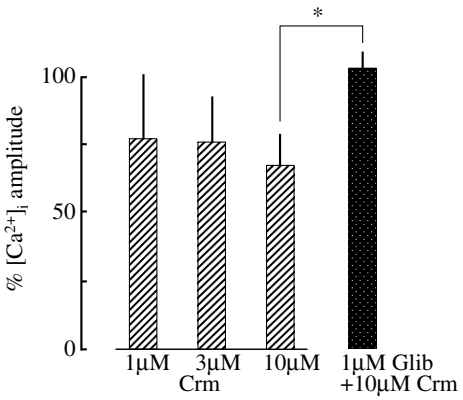

Fig. 4. Effects of cromakalim on the $[Ca^{2+}]_i$ oscillation amplitude. The amplitude of $[Ca^{2+}]_i$ oscillation is expressed relative to that before application of cromakalim. The experiments for 10 μM cromakalim (Crm) were carried out in the presence of nifedipine (1 μM). The number of experiments is shown in brackets. The effect of 10 μM cromakalim was significantly inhibited by a prior application of 1 μM glybenclamide (Glib) ($P<0.05$, indicated by an asterisk).

before and during application of 1 μM cromakalim were 18.8±3.9 and 18.4±4.8 cycles/minute, respectively ($n=11$), while those in the case of 3 μM were 18.3±4.7 and 15.9±5.5 cycles/minute, respectively ($n=5$). The amplitude of $[Ca^{2+}]_i$ was decreased to 76.7±23.7% with 1 μM ($n=11$), and to 75.6±16.8% with 3 μM cromakalim ($P<0.05$, $n=5$) (Fig. 4). In two cell clusters, the amplitude of $[Ca^{2+}]_i$ oscillations fell below a detectable level following application of 3 μM cromakalim (not included in the statistics and figures). Therefore despite considerable variability, 3 μM cromakalim appeared to depress the amplitude of $[Ca^{2+}]_i$ oscillations.

As no DHP Ca^{2+} antagonists were included in the extracellular solution, $[Ca^{2+}]_i$ signals from smooth muscle cells near ICCs may have contributed to the $[Ca^{2+}]_i$ oscillations recorded in pacemaker-rich regions before application of cromakalim. Such interference would over-estimate the inhibitory effect of cromakalim on the magnitude of $[Ca^{2+}]_i$ oscillations in pacemaker cells. Thus, we next examined the effects of 10 μM cromakalim in the presence of nifedipine (1 μM), which would nearly completely block smooth muscle L-type Ca^{2+} channels, while having little effect on the spontaneous electrical activities (Nakayama and Torihashi, 2002; Aoyama et al., 2003). Fig. 3B shows the $[Ca^{2+}]_i$ oscillation frequency before and during application of 10 μM cromakalim in nine individual cell clusters. The averages were 15.5±2.7 and 15.2±2.8 cycles/minute, respectively ($n=8$). However, the amplitude of spontaneous $[Ca^{2+}]_i$ oscillations was significantly decreased to 67.0±11.6% (<0.05, $n=8$) by application of 10 μM cromakalim (Fig. 4). In one cell cluster examined in the presence of 10 μM cromakalim, $[Ca^{2+}]_i$ oscillations fell below the detectable level, and this result was not included in the statistics.

Attenuation of $[Ca^{2+}]_i$ oscillation amplitude via SUR

In order to confirm the involvement of SUR in the cromakalim-induced attenuation of $[Ca^{2+}]_i$ oscillation

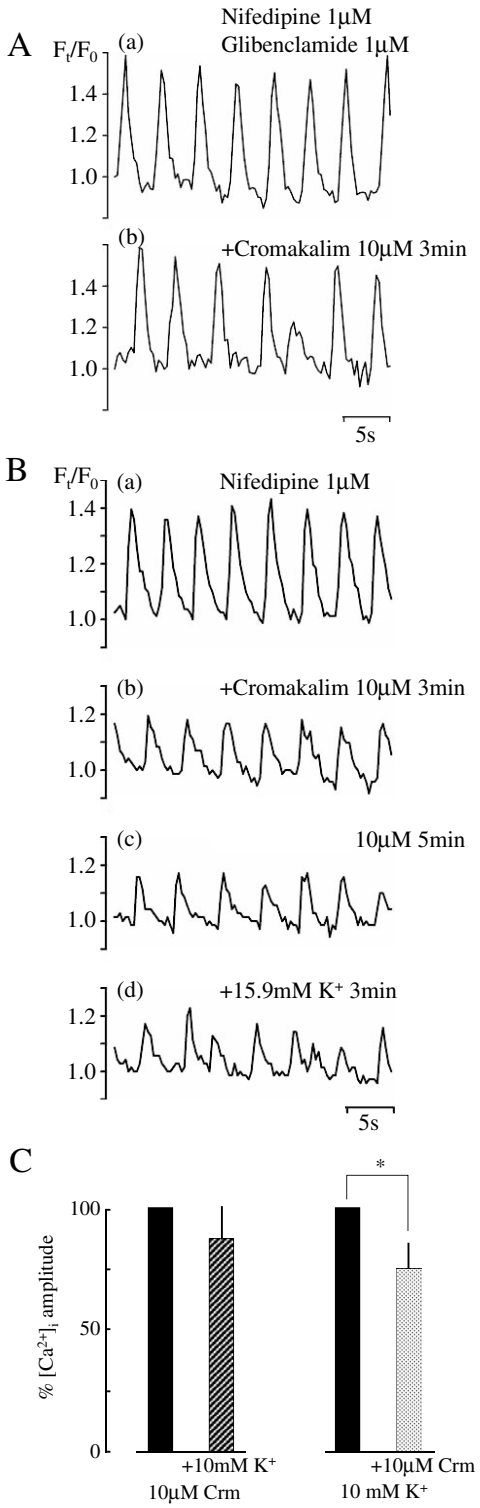

Fig. 5. Interaction of cromakalin, glibenclamide and high-K^+ treatments. In A, $[Ca^{2+}]_i$ oscillations ($F_t/F_{0(t=ib)}$) in a pacemaker region was measured in the presence of nifedipine (1 μM) and glibenclamide (1 μM) (a). Subsequently, 10 μM cromakalim was added (b: after 3 minutes). In B, after observing a control spontaneous $[Ca^{2+}]_i$ oscillations in the presence of 1 μM nifedipine (a), $[Ca^{2+}]_i$ oscillation traces (b) and (c) were obtained 3 and 5 minutes after application of cromakalim (10 μM), respectively. Subsequently, the extracellular K^+ concentration was increased to 15.9 mM (from 5.9 mM) in the presence of cromakalim, and the trace (d) was obtained after 3 minutes.

amplitude in ICCs, we next examined whether this effect could be blocked by 1 μM glibenclamide. In order to isolate the $[Ca^{2+}]_i$ oscillations in ICCs, these experiments were performed in the continued presence of 1 μM nifedipine. It was found that in the presence of 1 μM glibenclamide, cromakalim (10 μM) affected neither the amplitude nor frequency of spontaneous $[Ca^{2+}]_i$ oscillations (Fig. 5A). The amplitude of pacemaker $[Ca^{2+}]_i$ oscillations was 102.1±6.1% (*n*=5) after cromakalim application (Fig. 4), while the frequencies before and during cromakalim application were 14.4±2.6 and 14.9±1.4 cycles/minute (*n*=5).

High extracellular K^+ does not restore $[Ca^{2+}]_i$ oscillation amplitude

Does cromakalim reduce the amplitude of $[Ca^{2+}]_i$ oscillations through the membrane hyperpolarization caused by activation of K_{ATP} channels? In order to assess this issue, we examined the effect of increasing the extracellular K^+ concentration (Fig. 5B). In the presence of nifedipine (1 μM), application of 10 μM cromakalim significantly reduced the amplitude of the $[Ca^{2+}]_i$ oscillations, despite little effect on the frequency (consistent with the findings in Figs 3 and 4). The traces b and c in Fig. 5B were obtained 3 and 5 minutes after application of cromakalim. Subsequently, the extracellular K^+ concentration was increased (from 5.9) to 15.9 mM. This treatment will shift E_K (the reversal potential for K^+) by ~26 mV in the depolarizing direction (assuming $[K^+]_i$=150 mM), and cancel the hyperpolarizing effect of cromakalim (Huang et al., 1999). Nevertheless, no recovery was observed: rather, the amplitude of $[Ca^{2+}]_i$ oscillations decreased to 87.4±13.7% (*n*=5) (Fig. 5C) of that before the high-K^+ treatment, while the average frequency was not significantly changed (to 100.1±11.3%, *n*=5). These results suggest that mechanisms other than hyperpolarization of the membrane potential underlie the attenuation of $[Ca^{2+}]_i$ oscillations via SUR.

The effect of increasing the extracellular K^+ concentration to 15.9 mM was also examined without prior applications of cromakalim (in the presence of 1 μM nifedipine). This high-K^+ treatment decreased the amplitude of pacemaker $[Ca^{2+}]_i$ oscillations to 74.6±10.7% (P<0.05, *n*=4) (Fig. 5C), while the frequencies before and during the high-K^+ treatment were 15.1±3.8 and 15.1±5.1 cycles/minute, respectively.

Effects of diazoxide on pacemaker $[Ca^{2+}]_i$ oscillations

A K_{ATP} channel opener, cromakalim, appeared to differentially modulate smooth muscle contractility and ICC pacemaker activity. As described later in the RT-PCR section, SUR1 was detected in ICCs. We thus examined the effect of diazoxide, frequently used to activate pancreatic K_{ATP} channels, in which SUR1 forms a subunit (Mannhold, 2004). Fig. 6 exemplifies such an experiment. After observing control $[Ca^{2+}]_i$ oscillations in the presence of nifedipine (1 μM) (a), diazoxide was cumulatively applied. Diazoxide at 10 μM slightly reduced the amplitude of $[Ca^{2+}]_i$ oscillation (70.5±28.9%, *n*=4), but this drug at 30 μM completely abolished spontaneous

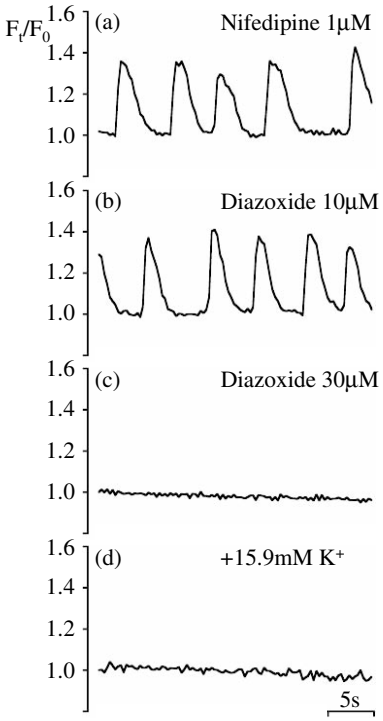

Fig. 6. Effects of diazoxide. After observing control $[Ca^{2+}]_i$ oscillations in the presence of nifedipine (1 μM), diazoxide was cumulatively added. Trace (b) was recorded 3 minutes after application of 10 μM diazoxide, and (c) was 1 min after application of 30 μM diazoxide. Subsequently, the extracellular K^+ concentration was increased to 15.9 mM. The trace (d) was obtained after 3 minutes.

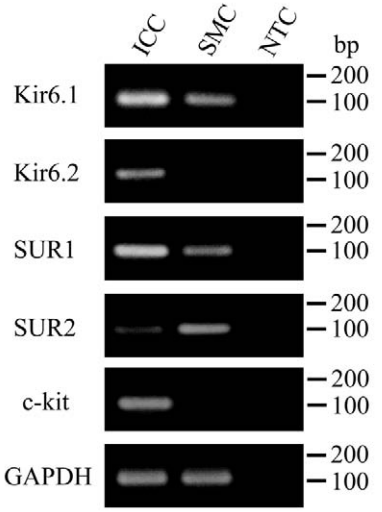

Fig. 7. RT-PCR examinations of effectors for K^+ channel openers. RNA samples were obtained from isolated c-Kit-immunopositive cells (ICCs) or smooth muscle cells (SMCs). NTC represents 'no template control'. RT-PCR was performed with six-pair primers for 45 (Kir6.1, Kir6.2, SUR1. SUR2, and *c-kit*) or 40 (GAPDH) cycles. Amplified products were separated on 2.5% agarose gels and analyzed by ethidium bromide staining. The numbers in the right of each gel indicate the size marker (bp). GAPDH was amplified as an index of proper amplification, and *c-kit* was examined in order to confirm the c-Kit-immunoreactivity under the fluorescent microscope.

$[Ca^{2+}]_i$ oscillations in all four experiments ($P<0.05$). The subsequent elevation of the extracellular K^+ concentration (to 15.9 mM) never restored them. The results are consistent with the cromakalim-induced attenuation, although the inhibitory effect of diazoxide was more potent.

Expression of a family of inward rectifier K^+ channels (Kir6) and SURs

RT-PCR examination was performed in order to assess mechanisms underlying different response to cromakalim on smooth muscle contraction and ICC pacemaker $[Ca^{2+}]_i$ activity. After enzymatic dispersion, isolated pacemaker cells and smooth muscle cells were collected with patch pipettes under a fluorescence microscope. The c-Kit immunoreactivity was used as a marker for ICCs. Since K_{ATP} channel openers, such as cromakalim, are known to activate K^+ channels (Okabe et al., 1990; Beech et al., 1993) of an inward rectifier family (Kir6) via SUR (Inagaki and Seino, 1998), we examined expression of Kir6.1 and Kir6.2 along with SUR1 and SUR2. As shown in Fig. 7, both Kir6.1 and Kir6.2 transcripts were expressed in ICCs, while the Kir6.1 transcript was predominant in smooth muscle cells. With respect to SUR, SUR1 transcript was predominant in ICCs, while both SUR1 and SUR2 transcripts were detected in smooth muscle cells. The primers for SUR2 were designed to amplify both SUR2A and SUR2B. Since SUR2B is known to be abundant in smooth muscle cells (Isomoto et al., 1996; Sim et al., 2002), the band detected in SUR2 in the present study is considered to represent SUR2B. In order to confirm the source cells for the mRNA, we also checked the expression of c-Kit. The amplicon for c-Kit was detectable only in ICCs (c-Kit-immunopositive cells). Essentially similar results were obtained in four pairs of samples for ICCs and smooth muscle cells.

Like ICCs shown in the present study, it is known that some enteric neurones in guinea-pig ileum express both SUR1 and Kir6.2 (Liu et al., 1999), although in ICCs Kir6.2 was a relatively minor component compared to Kir6.1. We thus performed RT-PCR examinations using $P2X_3$ and $P2X_7$ as marker molecules for enteric neurones (van Nassauw et al., 2002; Hu et al., 2001). Neither ionotropic purinoceptor was expressed (data not shown), indicating negligible contamination from enteric neurones in our ICC samples.

By use of immunostaining, we further examined expression of SUR1. Segments of ileal smooth muscle (containing myenteric plexus) were stained with an anti-c-Kit antibody (ACK2 indicated in red in Fig. 8A) and an anti-SUR1 antibody (C-16 indicated in green, in Fig. 8B). The merged image (yellow, Fig. 8C) clearly shows that network-forming cells in the myenteric plexus possess c-Kit and SUR1 immunoreactivity. Similar results were obtained in seven other preparations. In Fig. 8D a single cell with both c-Kit and SUR1 immunoreactivity is shown at higher magnification. The results are compatible with the results of the RT-PCR, showing that ICCs predominantly express SUR1, while smooth muscle cells express other types of SUR.

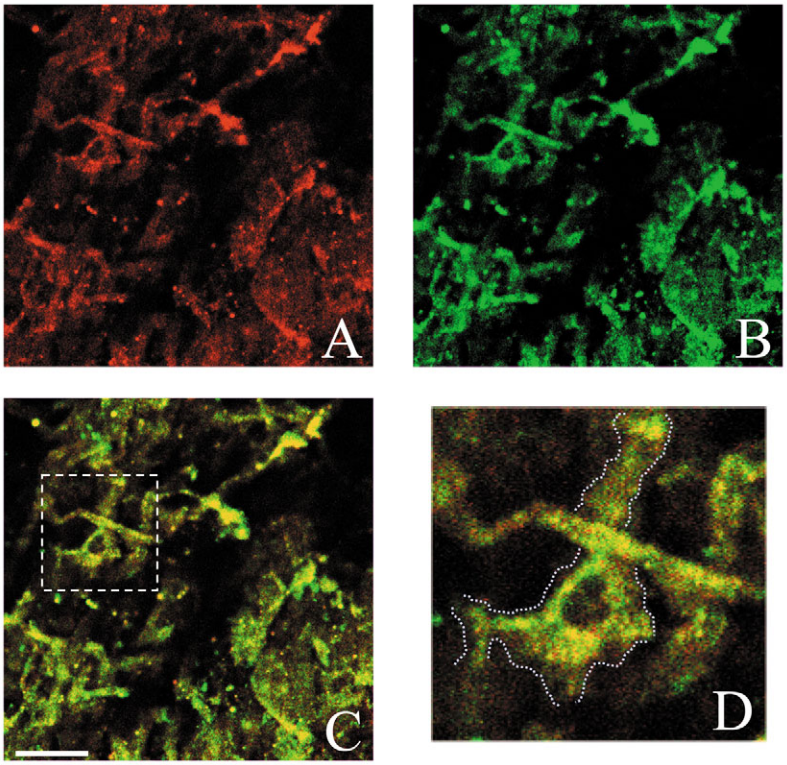

Fig. 8. Immunohistochemistry for c-Kit and SUR. Smooth muscle layer (including the myenteric plexus) of ileum was double-labelled with anti-c-Kit antibody (A: ACK2 with Alexa Fluor 488, red) and anti-SUR1 antibody (B: C16 with Alexa Fluor 594, green); C shows a merged image. Note that network-forming cells were stained yellow, indicating that ICCs in the ileum express SUR1. Scale bar: 20 μm. (D) An enlarged view of the boxed region in C. A single cell with both anti-c-Kit and anti-SUR1 immunoreactivity is indicated by a dotted lines.

Discussion

Spontaneous contractions in the gastrointestinal tracts involve at least two types of cells. It has been shown that pacemaker potentials originate from ICCs (equivalent to c-Kit-immunopositive interstitial cells in the myenteric plexus), and drive smooth muscle cells to contract through activation of voltage-dependent Ca^{2+} channels in these cells (Suzuki, 2000; Hirst and Ward, 2003; Takaki, 2003) (see schema in Fig. 9). The roles of K_{ATP} channels in the modulations of smooth muscle contractility have been studied extensively. However, an additional regulation of gastrointestinal motility via K_{ATP} channels or SUR expressed in pacemaker cells has not yet been explored.

K_{ATP} channel openers and sulphonylurea compounds variably modulate the activity of K_{ATP} channels distributed in numerous cells and tissues, depending upon their associated SUR isoforms (Inagaki and Seino, 1998; Ashcroft and Gribble, 2000; Mannhold, 2004). RT-PCR examinations in the present study revealed predominant expression of SUR2B in smooth muscle, with predominant expression of SUR1 in ICCs. Also, experiments using cell cluster preparations clearly demonstrated K_{ATP} channel openers differentially affect smooth muscle contraction and ICC pacemaker $[Ca^{2+}]_i$ oscillations. Namely, a benzopyran, cromakalim, at 1-3 μM preferably suppressed smooth muscle contraction, presumably by preventing activation of voltage-dependent Ca^{2+} channels in smooth muscle plasma membrane. Greater concentrations of this drug additionally attenuated $[Ca^{2+}]_i$ oscillations in ICCs, and glibenclamide antagonized this effect. In contrast, diazoxide, a prototype compound of thiadiazine, effectively terminated ICC pacemaker $[Ca^{2+}]_i$ activity. These results are consistent with the finding that different SUR subtypes are expressed in smooth muscle and ICCs (Inagaki and Seino, 1998; Ashcroft and Gribble, 2000; Mannhold, 2004).

How does SUR interact with pacemaker Ca^{2+} activity in ICCs? Previously, we have shown co-ordinate actions of intracellular Ca^{2+} release channels, such as ryanodine receptors and $InsP_3$ receptors, yielding pacemaker $[Ca^{2+}]_i$ oscillations (Aoyama et al., 2004; Liu et al., 2005a; Liu et al., 2005b). It is possible that SUR could interact with these intracellular processes indirectly through changes in membrane potential, and it is well known that cromakalim hyperpolarizes the membrane potential of smooth muscle cells (Edwards and Weston, 1993). Pacemaker potentials are thought to conduct from ICCs to smooth muscle via gap junction channels (Nakamura et al., 1998; Nakamura and Shibata, 1999; Daniel and Wang, 1999; Nakayama and Torihashi, 2002). Conversely, cromakalim-induced hyperpolarization in smooth muscle would also conduct to ICCs via gap junctions. Indeed, using conventional microelectrodes, it has been very recently shown that pinacidil, a K^+ channel opener similarly interacting with SUR2 (Shindo et al., 1998), hyperpolarizes the resting membrane potential of ICCs in isolated ileum (Kito et al., 2005).

As shown in the present study, a low concentration range of cromakalim that suppressed smooth muscle contractile activity did not significantly affect ICC pacemaker $[Ca^{2+}]_i$ activity, especially in frequency (Fig. 2). Furthermore, both cromakalim and diazoxide at 10-30 μM significantly suppressed pacemaker $[Ca^{2+}]_i$ activity in ICCs, but it was not restored by high extracellular K^+ treatments, which would cancel hyperpolarizing effects of K_{ATP} channel openers by shifting E_K (Figs 5 and 6). From these results, it is deduced that SUR and pacemaker activities in ICCs are coupled indirectly via membrane potential. Interestingly, Kito and Suzuki (Kito and Suzuki, 2003) have previously reported similar observations on pacemaker potentials recorded directly from stomach ICCs: K_{ATP} channel openers (pinacidil and nicorandil) suppress pacemaker potentials accompanied by hyperpolarization; subsequent application of glibenclamide completely antagonises the effects of K_{ATP} channel openers, but high K^+ treatment causes only partial recovery. SUR1 is known to interact with proteins other than inward recitifier K^+ channels, e.g. cAMP-GEFII (Eliasson et al., 2003), non-selective cation channels (Chen et al., 2003) and syntaxin-1A (Pasyk et al., 2004). Analogous proteins might be involved in the regulation of ICC pacemaker $[Ca^{2+}]_i$ oscillations.

Among SUR isoforms SUR1 is most sensitive to ATP, but

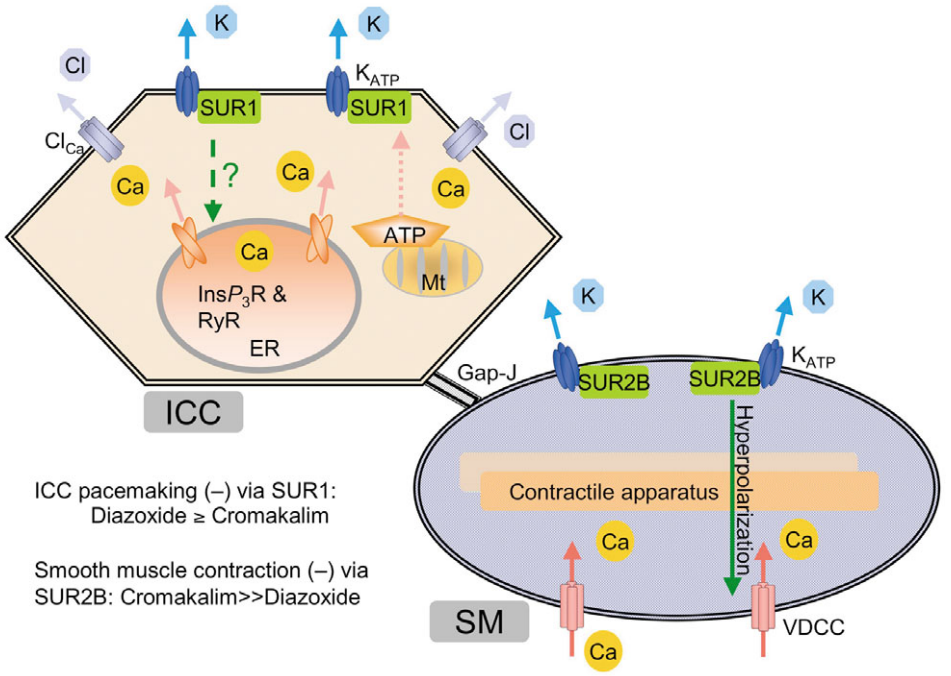

Fig. 9. Schematic diagram showing possible modulation mechanisms on smooth muscle contractility and ICC pacemaker $[Ca^{2+}]_i$ oscillations via SUR. Both smooth muscle and ICCs express K_{ATP} channels, but different SUR isoforms are associated: SUR1 in ICCs and SUR2B in smooth muscle. Under normal conditions, ryanodine receptors (RyR) and $InsP_3$ receptors ($InsP_3R$) in the endoplasmic reticulum (ER) co-ordinately produce pacemaker $[Ca^{2+}]_i$ oscillations in ICCs, thereby pacemaker potentials are generated by periodic activation of plasmalemmal Ca^{2+}-activated Cl^- channels. Pacemaker potentials are conducted toward smooth muscle cells via gap junction channels. Consequently, voltage-dependent Ca^{2+} channels (VDCC) (mainly DHP-sensitive, L-type) are periodically activated to cause spontaneous phasic contractions in the gut. Applications of potent SUR2B activators, such as cromakalim, activate K_{ATP} channels in smooth muscle. Resultant hyperpolarization in the smooth muscle cell membrane prevents the periodic activation of VDCC, and suppresses spontaneous contractions, whereas pacemaker $[Ca^{2+}]_i$ oscillations remain in ICCs. When K_{ATP} channel openers that act on SUR1, such as diazoxide, are applied, ICC pacemaker $[Ca^{2+}]_i$ oscillations are suppressed in a voltage-independent manner. Unknown intracellular signals may link between SUR1 and activity of intracellular Ca^{2+} release channels. Mitochondrial signals might be also involved. The predominant expression of SUR1 in ICCs can account for the fact that spontaneous rhythmicity (electrical activity) in the gut smooth muscle tissues is highly dependent upon energy metabolism (e.g. Tomita, 1981; Nakayama et al., 1997).

SUR2B is under a control of nucleoside diphosphates (Kajioka et al., 1991; Inagaki and Seino, 1998). Therefore, like pancreatic β-cells, the activity of (plasmalemmal) K_{ATP} channels in ICCs are thought to be significantly affected by ATP production and energy levels (Fig. 9). This notion is consistent with previous observations that spontaneous electrical and mechanical activities are highly temperature dependent, and easily suppressed by metabolic inhibitors (Tomita, 1981). Provided that SUR1 interacts with factors involved in pacemaker $[Ca^{2+}]_i$ oscillations, metabolic inhibitors may not always require hyperpolarization. Indeed, we have previously shown that application of iodoacetic acid preferably attenuates the first component of spontaneous electrical activity, which is now considered to reflect ICC pacemaker potentials recorded in smooth muscle, and eventually terminates spontaneous electrical activity itself, and that this effect is not accompanied by membrane hyperpolarization (Nakayama et al., 1997; Clark et al., 2000).

It has recently been pointed out that gastrointestinal dismotility in diabetes mellitus is partly due to impairment of ICCs, pacemaker cells of gastrointestinal tracts (Koch, 2001; Camilleri, 2002). Pancreatic β-cells and ICCs might be similarly impaired during the course of this disease because of common apoptotic signals relating to SUR (e.g. Maedler et al., 2004). As mentioned earlier, we have previously shown that ryanodine and $InsP_3$ receptors co-ordinate to produce periodic rises of $[Ca^{2+}]_i$ in ICCs, which is thought to be the primary pacemaking mechanism (Aoyama et al., 2004). However, there is an increasing body of evidence that Ca^{2+} release from intracellular Ca^{2+} stores plays an important role in insulin secretion (e.g. Masgrau et al., 2003), in addition to the well-known regulation through the membrane potential (reflecting the K_{ATP} channel activity). Therefore, ICCs and pancreatic β-cells might also share common underlying mechanisms in terms of $[Ca^{2+}]_i$ regulation. [In addition, we have previously suggested the essential role of ryanodine receptors in generating spontaneous rhythmicity (ICC pacemaker $[Ca^{2+}]_i$ activity) in a gut-like organ formed from mouse embryonic stem cells (Ishikawa et al., 2004). Interestingly, pancreatic β-cell-like tissues are distributed nearby in this preparation (personal communication from Miyako Takaki), suggesting some developmental association between ICCs and β-cells.]

Many tissues and organs in peripheral autonomic nervous system show spontaneous rhythmicity. ICC-like cells have been found in many places, including urethra, urinary bladder and lymph tracts (Klemm et al., 1999; Sergeant et al., 2000; Sui et al., 1999; McCloskey et al., 2002). These cells may play

an essential role in generating spontaneous rhythmicity, in a similar manner to ICCs in the gastrointestinal tract. Provided analogous mechanisms underlying spontaneous $[Ca^{2+}]_i$ oscillations and pacemaker potentials, the type of SUR isoform expressed in these cells must be important in choosing appropriate pharmaceutical treatments. Furthermore, ICC-like cells might be impaired during the course of diabetes, as appears to be the case for ICCs in the gastrointestinal tracts. The possible impairment of putative pacemaker cells, which are widely distributed in peripheral autonomic nervous system, merits further investigation.

In conclusion, we have demonstrated that K_{ATP} channel openers differentially modulate smooth muscle contractility and ICC pacemaker activity through the different subtypes of SURs expressed in these cells, i.e. predominant expression of SUR2B in smooth muscle and of SUR1 in ICCs. In particular, low concentrations of cromakalim, a SUR2B-specific K_{ATP} channel opener, enabled us to identify pacemaker $[Ca^{2+}]_i$ oscillations. Furthermore, SURs seem to play an important role in the functional association of intestinal pacemaking and blood glucose control. Systematic studies of SURs for ICCs and ICC-like putative pacemaker cells, reported to be distributed in numerous tissues and organs, may provide us with a new insight to understand the pathophysiology of important metabolic diseases and their complications.

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