

The possible roles of hyperpolarization-activated cyclic nucleotide channels in regulating pacemaker activity in colonic interstitial cells of Cajal

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Abstract

Background Hyperpolarization-activated cyclic nucleotide (HCN) channels are pacemaker channels that regulate heart rate and neuronal rhythm in spontaneously active cardiac and neuronal cells. Interstitial cells of Cajal (ICCs) are also spontaneously active pacemaker cells in the gastrointestinal tract. Here, we investigated the existence of HCN channel and its role on pacemaker activity in colonic ICCs.

Methods We performed whole-cell patch clamp, RT-PCR, and Ca^{2+} -imaging in cultured ICCs from mouse mid colon.

Results SQ-22536 and dideoxyadenosine (adenylate cyclase inhibitors) decreased the frequency of pacemaker potentials, whereas both rolipram (cAMP-specific phosphodiesterase inhibitor) and cell-permeable 8-bromo-cAMP increased the frequency of pacemaker potentials. CsCl, ZD7288, zatebradine, clonidine (HCN channel blockers), and genistein (a tyrosine kinase inhibitor)

suppressed the pacemaker activity. RT-PCR revealed expression of HCN1 and HCN3 channels in c-kit and Ano1 positive colonic ICCs. In recordings of spontaneous intracellular Ca^{2+} $[\text{Ca}^{2+}]_i$ oscillations, rolipram and 8-bromo-cAMP increased $[\text{Ca}^{2+}]_i$ oscillations, whereas SQ-22536, CsCl, ZD7288, and genistein decreased $[\text{Ca}^{2+}]_i$ oscillations.

Conclusions HCN channels in colonic ICCs are tonically activated by basal cAMP production and participate in regulation of pacemaking activity.

Keywords Hyperpolarization-activated cyclic nucleotide channels · Interstitial cells of Cajal · Pacemaker activity · cAMP · Colon

Introduction

Interstitial cells of Cajal (ICCs) are pacemaker cells and play an important role in the regulation of smooth muscle activity by producing and propagating slow waves, transducing neural input to smooth muscles, and acting as stretch receptors in the gastrointestinal (GI) tract [1–3]. Many neurotransmitters and hormones affect the frequency and configuration of slow waves, thus regulating GI motility [4]. ICCs generate spontaneous pacemaker potentials that correlate with peristaltic contractions in the small intestine [5]. The pacemaking mechanism has been well studied in cultured ICCs and intact mouse small intestine [6, 7]. The coupling between IP_3 -gated Ca^{2+} release from endoplasmic reticulum and reuptake of Ca^{2+} into mitochondria is linked to generation of pacemaker activity by periodically activating Ca^{2+} -dependent membrane pacemaker ion channels [8]. Transient receptor potential (TRP) channels or Ca^{2+} -activated Cl^- channels

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were candidates as pacemaker channels in ICCs [9–12]. Recently it was found that Ano1, which encodes Ca^{2+} -activated Cl^- channels and was known as a selective marker of ICCs, plays an important role in pacemaking due to loss of slow waves by the knockout of Ano1 [13, 14]. However, Ano1 is also expressed in non-active secretory epithelial cells [15–17] of the lung, kidney and salivary glands. It was also reported that Ano1 acts as a regulator for proliferation of ICCs [18].

The frequency and configuration of GI pacemaker activity is affected by temperature and energy metabolism [19, 20]. Moreover, slow waves were found to be sensitive to intracellular cAMP level in the canine colon [21, 22]. Therefore, it was suggested that an intracellular metabolic process involving Ca^{2+} release from endoplasmic reticulum that is sensitive to cAMP levels triggers the colonic slow waves. cAMP is a second messenger and acts as an inhibitory agent in regulating GI motility [23]. However, in spontaneously active cardiac and neuronal cells, cAMP mediates excitatory responses that increase heart rates and neuronal firing rates. Hyperpolarization-activated cyclic nucleotide (HCN) channels are pacemaker channels that present in a variety of spontaneously active cells such as heart and neurons and play an important role in regulation of cell excitability [24, 25]. These channels are activated directly by cAMP [26]. ICCs are spontaneously active pacemaker cells and are rich in mitochondria, suggesting that HCN channels may exist in these cells. Thus, in this present study, we performed a series of functional and molecular experiments to investigate whether HCN channels exist in colonic ICCs and what physiological roles they have in the pacemaking activity of the mouse mid-colon.

Methods

Preparation of cells and tissues

The protocols and animal care used in these experiments were in accordance with the guiding principles approved by the ethics committee in Chosun University and the National Institutes of Health Guide, South Korea for the Care and Use of Laboratory Animals. Mice had free access to water and a standard mouse diet until the day of experimentation. Balb/C mice (5–8 days old) of either sex were anesthetized with ether and sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. The colon from below cecum to rectum were removed and mid region of the colon was used. Colon were opened along the mesenteric border. The luminal contents were washed away with Krebs–Ringer

bicarbonate solution. The tissues were pinned to the base of a Sylgard dish and the mucosa was removed by sharp dissection. Small strips of intestinal muscle or colonic muscle were equilibrated in Ca^{2+} -free Hank's solution for 30 min, and the cells were dispersed with an enzyme solution containing 1.3 mg/ml collagenase (Worthington Biochemical Co., Lakewood, NJ, USA), 2 mg/ml bovine serum albumin (Sigma, St. Louis, MO, USA), 2 mg/ml trypsin inhibitor (Sigma), and 0.27 mg/ml ATP. Cells were plated onto sterile glass coverslips coated with poly L-lysine (2.5 $\mu\text{g}/\text{ml}$, Sigma) in 35 mm culture dishes. The cells were then cultured at 37 °C in a 95 % $\text{O}_2/5$ % CO_2 incubator in smooth muscle growth medium (SMGM, Clonetics Corp., San Diego, CA, USA), supplemented with 1 % antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and 5 ng/ml murine stem cell factor (SCF, Sigma).

Patch-clamp experiments

The patch-clamp technique was tested using ICCs that showed the network-like structures in culture (2–3 days). The whole-cell configuration of the patch clamp technique was used to record membrane potentials (current clamp). Membrane potentials were amplified by Axopatch 200B (Axon Instruments, Foster, CA, USA). Command pulse was applied using an IBM-compatible personal computer and pClamp software (version 9.2, Axon Instruments). The data were filtered at 5 kHz and displayed on an oscilloscope and a computer monitor. Results were analyzed using Clampfit program (Axon Instruments) and GraphPad Prism software (version 2.01, GraphPad Software Inc., San Diego, CA, USA). All experiments pertaining to patch clamp were performed at 30 °C.

Collection of ICCs and RT-PCR

Cells which had the morphology of ICCs, triangular or spindle shapes with multiple branches, were collected (about 2–5 cells) by applying negative pressure to a cell in contact with a recording pipette, lifting the cell out of the bath, and immediately expelling the cell from the pipette by applying positive pressure into a 1.5 ml tube which contained Phosphate-buffered saline. The cells were spun down at 3000 rpm for 5 min and then lysis buffer was added. Total RNA was isolated using TRIzol reagent according to the manufacturer specifications (Invitrogen, CA, USA). cDNAs were produced from the total RNA using Superscript One-Step RT-PCR with Platinum Taq (Invitrogen). The primers used were as follows: HCN1 (Accession number NM_010408), forward 5'-GCCATGC TGAGCAAGCTGAGATTT-3', reverse 5'-TCCGATCG AGTCGGTCAATAGCAA-3' (with a product size of 325 bp); HCN2 (Accession number NM_008226), forward

5'-CACAGCCATGCTGACAAAGCTCAA-3', reverse 5'-ATCTAGCCGGTCAATAGCCACAGT-3' (with a product size of 325 bp); HCN3 (Accession number NM_008227), forward 5'-TCTGCAGTTTCTGGTCCCTATGCT-3', reverse 5'-ACTGCTCCACCTGCTTGTACTTCT-3' (with a product size of 308 bp); HCN4 (Accession number NM_001081192), forward 5'-ATCGTGGTGGAGGACAA CACAGAAT-3', reverse 5'-GACACAGCAGAAGCATC ATGCCAA-3' (with a product size of 337 bp); c-Kit (Accession number Y00864), forward 5'-GAGCCTTCCT GTGACAGTTCAAAT-3', reverse 5'-TCTATTCTTGCG GATCTCCTCTTG-3' (with a product size of 101 bp); Ano1 (Accession number NM_178642), forward 5'-AGGC CAAGTACAGCATGGGTATCA-3', reverse 5'-AGTACA GGCCAACCTTCTCACCAA-3' (with a product size of 213 bp); Myosin (Accession number NM_013607.2), forward 5'-GAGAAAGGAAACACCAAGGTCAAGC-3', reverse 5'-AACAAATGAAGCCTCGTTTCCTCTC-3' (with a product size of 233 bp); and PGP9.5 (Accession number NM_011670), forward 5'-GCCAACAACCAAGACAAGC TGGAA-3', reverse 5'-GCCGTCCACGTTGTTGAACAG AAT-3' (with a product size of 213 bp). The thermal cycler was programmed such that cDNA synthesis was followed immediately by PCR amplification. The cDNA synthesis was carried out at 45 °C for 30 min for the reverse transcription reaction followed by 94 °C for 5 min for the denaturation of the cDNA hybrid. The three-step cycling process was carried out as follows for 38 cycles: 94 °C for 30 s for denaturation, 60 °C for 30 s for annealing, and 72 °C for 30 s for the extension. The PCR products were visualized using 2 % agarose gel electrophoresis followed by ethidium bromide staining.

Measurement of intracellular Ca^{2+} concentration

Fluo-3/AM, which was initially dissolved in dimethyl sulfoxide and stored at -20 °C, was used for monitoring changes in the intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) concentration. Cultured ICCs on coverslips (25 mm) were rinsed twice using bath solution (recipe below), followed by incubation in bath solution containing 1 μM fluo-3/AM with 5 % CO_2 at 37 °C for 5 min, and were then rinsed 2 more times with bath solution. They were then mounted on a perfusion chamber and scanned every 0.4 s using a Nikon Eclipse TE200 inverted microscope equipped with a Perkin-Elmer Ultraview confocal scanner and a Hamamatsu Orca ER 12-bit CCD camera (200 \times). Fluorescence excitation occurred at a wavelength of 488 nm and the emitted light was observed at 515 nm. The temperature of the perfusion chamber containing the cultured ICC was maintained at 30 °C during the scanning of Ca^{2+} imaging. Variations of

$[\text{Ca}^{2+}]_i$ fluorescence emission intensity were expressed as $F1/F0$ where $F0$ is the intensity of the first imaging.

Reagents

The cells were bathed in bath solution containing (mM): KCl 5, NaCl 135, CaCl_2 2, glucose 10, MgCl_2 1.2 and HEPES 10 and was adjusted to pH 7.4 with Tris. The pipette solution contained 140 mM KCl, 5 mM MgCl_2 , 2.7 mM K_2ATP , 0.1 mM Na_2GTP , 2.5 mM creatine phosphate disodium, 5 mM HEPES, and 0.1 mM EGTA, adjusted to pH 7.2 with Tris. The drugs used were: tetrodotoxin, nifedipine, SQ-22536, dideoxyadenosine, rolipram, 8-bromo-cAMP, CsCl, ZD7288, zatebradine, clonidine, genistein, *L-cis*-diltiazem, KT-5720, and 8-pCPT-2'-*O*-Me-cAMP. ZD7288 and zatebradine were purchased from Calbiochem (San Diego, CA, USA) and the other compounds were purchased from Sigma.

Statistical analysis

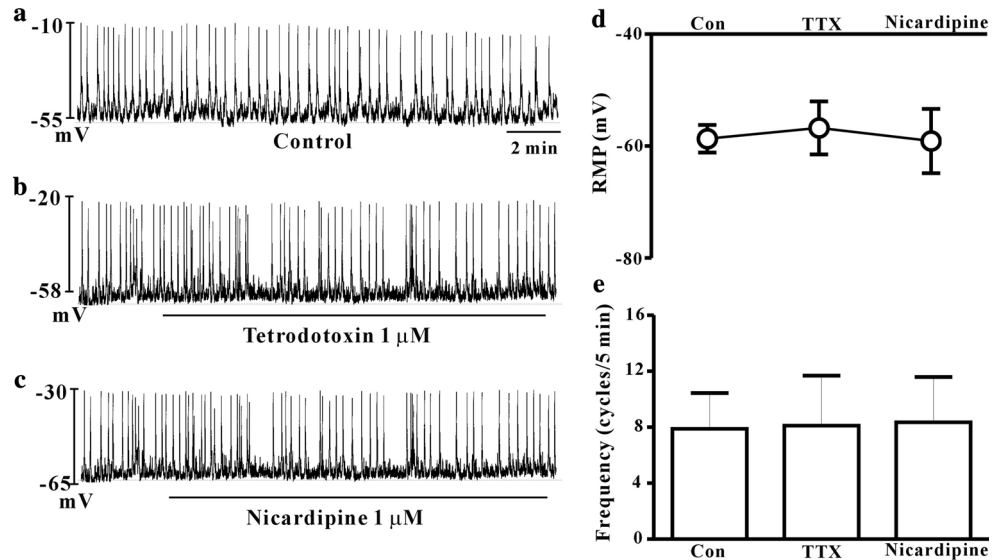
Data are expressed as the means \pm standard errors. Differences in the data were evaluated by the Student's *t* test. A *p* value <0.05 was considered to indicate a statistically significant difference. The *n* values reported in the text refer to the number of cells used in the patch clamp experiments.

Results

Involvement of cAMP production in generating pacemaker potentials in ICCs

The patch clamp technique was tested with ICCs that had network-like structures in culture (2–3 days). In the current clamp mode, the ICCs generated pacemaker potentials (Fig. 1a). Under control conditions in the current clamp mode, the resting membrane potential, frequency and amplitude were -58.7 ± 2.5 mV, 7.9 ± 2.6 cycles/5 min, and 41.4 ± 7.9 mV, respectively ($n = 23$; Fig. 1a, d, e). To evaluate whether voltage-dependent Na^+ and Ca^{2+} channels were involved in generating pacemaker potentials, we first treated with tetrodotoxin (TTX, 1 μM ; $n = 10$) and nifedipine (1 μM ; $n = 9$). We found that the resting membrane potential, frequency, and amplitude of pacemaker potentials were not changed by TTX and nifedipine (Fig. 1b, c), indicating that voltage-dependent Na^+ and L-type Ca^{2+} channels were not involved in generating pacemaker potentials. Since HCN channels were activated directly by cAMP, we next examined the involvement of cAMP using SQ-22536 (100 μM ; $n = 6$),

Fig. 1 Effects of voltage-dependent Na^+ or L-type Ca^{2+} channel blocker on the spontaneous pacemaker potentials in cultured ICCs of the mouse colon. Control (a), tetrodotoxin (TTX; $1 \mu\text{M}$) (b) and nicardipine ($1 \mu\text{M}$) (c) had no effect on the spontaneous pacemaker potentials in current clamping mode. The effects of TTX and nicardipine on pacemaker potentials are summarized in d and e. Bars represent mean \pm SE values ($p < 0.05$) *con* control, *TTX* tetrodotoxin



dideoxyadenosine ($100 \mu\text{M}$; $n = 6$) (both adenylate cyclase inhibitors), rolipram (a cAMP-specific phosphodiesterase inhibitor, $100 \mu\text{M}$; $n = 7$), and cell-permeable 8-bromo-cAMP ($100 \mu\text{M}$; $n = 8$). Both SQ-22536 and dideoxyadenosine decreased the frequency of pacemaker potentials and hyperpolarized the membrane. In contrast, rolipram and cell-permeable 8-bromo-cAMP increased the frequency of pacemaker potentials and depolarized the membrane (Fig. 2a–d). The values of the frequency induced by above drugs were significantly different from the control values (Fig. 2f). However, the values of the resting membrane potential induced by these drugs were not significantly different from control values even though they changed membrane potentials (Fig. 2e). These results suggest that basal intracellular cAMP plays an important role in regulating the pacemaker frequency in colonic ICCs.

The cellular action of cAMP is mediated mainly by protein kinase A (PKA) or cAMP-regulated guanine nucleotide exchange factor (Epac) [27]. Thus, to assess whether basal cAMP action was mediated by either PKA or Epac, we examined the effect of KT-5720 (a PKA inhibitor, $10 \mu\text{M}$; $n = 7$) or 8-pCPT-2'-O-Me-cAMP (an Epac agonist, $100 \mu\text{M}$; $n = 7$), and found that both KT-5720 and Epac agonist had no effects on pacemaker activity (Fig. 3a, b). Since cyclic nucleotide gated (CNG) channel is another downstream target of cAMP [28], we treated with *L-cis*-diltiazem, a CNG channel blocker ($10 \mu\text{M}$; $n = 7$). We found that *L-cis*-diltiazem also had no effect on pacemaker activity (Fig. 3c). The values of the resting membrane potential and frequency induced by the above drugs were not significantly different from control values (Fig. 3d, e). These results suggest that cAMP-dependent regulation of pacemaker frequency was not mediated by PKA, Epac or CNG channels.

The effects of HCN channel blockers on pacemaker activity in cultured colonic ICCs

To evaluate whether the cAMP-dependent regulation of pacemaker activity was mediated through HCN channels, we treated ICCs with HCN channel blockers such as CsCl (5 mM ; $n = 6$), ZD7288 ($10 \mu\text{M}$; $n = 8$), zatebradine ($10 \mu\text{M}$; $n = 8$), and clonidine ($100 \mu\text{M}$; $n = 6$) [29]. In addition, there is a report that Src tyrosine kinase inhibitor, genistein, modulates HCN channels [30], we also treated genistein ($10 \mu\text{M}$; $n = 5$) to pacemaker potential generating colonic ICCs. We found that CsCl, ZD7288, zatebradine, clonidine, and genistein all decreased the frequency of pacemaker potentials (Fig. 4a–e). The responses to the above drugs on the pacemaker potentials in ICCs are summarized in Fig. 4f and g. The values of the frequency induced by the above drugs were significantly different from control values. These results suggest that the cAMP-dependent regulation of pacemaker activity may be mediated through HCN channels.

The expression of HCN1 and HCN3 channels in colonic ICCs

To support the above functional data, we performed RT-PCR to detect expression of HCN channels. There are four subtypes of the HCN channels: HCN1–HCN4. In this study, we used mouse heart and HEK-293 cells as positive and negative controls for HCN channels ($n = 4$; Fig. 5a). RT-PCR analysis revealed the mRNA transcripts for all four HCN channel subtypes in whole mount cultured colonic cells ($n = 12$; Fig. 5b). However, the mRNA transcripts for only HCN1 and HCN3 channel were detected in cultured c-kit and Anol1 positive isolated ICCs ($n = 14$; Fig. 5c).

Fig. 2 Effects of cAMP-related drugs on the spontaneous pacemaker potentials in cultured ICCs of the mouse colon. SQ-22536 (100 μ M) (a) and dideoxyadenosine (100 μ M) (b), both adenylate cyclase inhibitors, decreased the frequency of pacemaker potentials. Whereas rolipram (100 μ M) (c), a cAMP specific PDE 4 inhibitor, and cell permeable 8-bromo-cAMP (100 μ M) (d) increased the frequency of pacemaker potentials. The effects of these drugs on pacemaker potentials are summarized in e and f. Bars represent mean \pm SE values ($p < 0.05$) con control, dda dideoxyadenosine

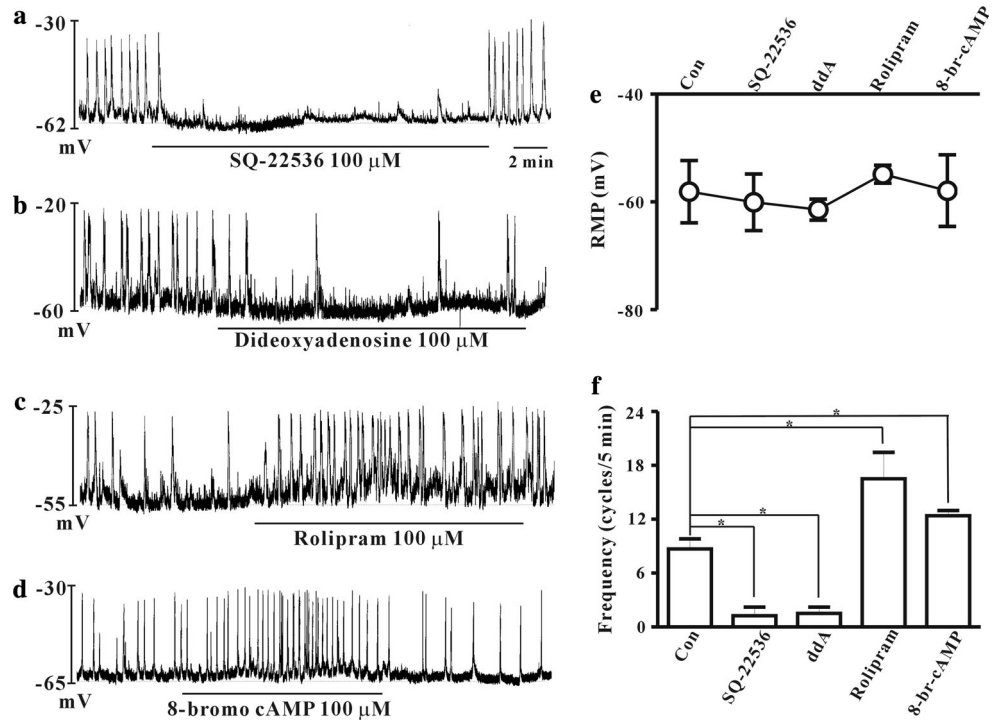
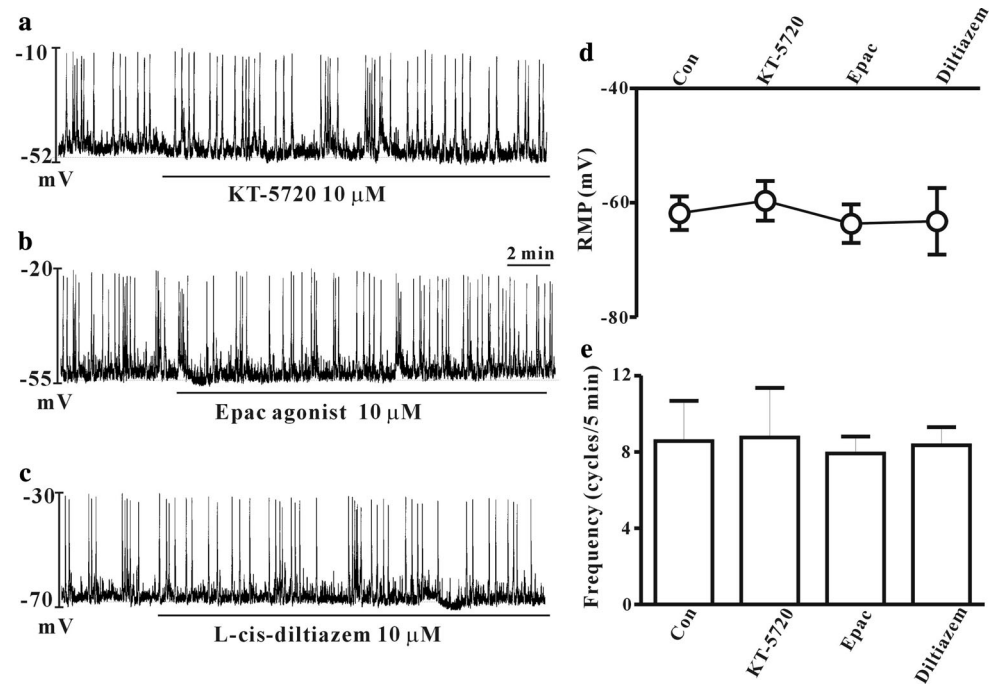


Fig. 3 Effects of protein kinase A (PKA) inhibitor, cAMP-regulated guanine nucleotide exchange factor (Epac) agonist, and cyclic nucleotide gated (CNG) channel blocker on the spontaneous pacemaker potentials in cultured ICCs of the mouse colon. KT-5720, a PKA inhibitor (10 μ M) (a), 8-pCPT-2'-O-Me-cAMP, an Epac agonist (10 μ M) (b), and L-cis-diltiazem, a CNG channel blocker (10 μ M) (c) had no effect on the spontaneous pacemaker potentials. The effects of KT-5720, 8-pCPT-2'-O-Me-cAMP and L-cis-diltiazem on pacemaker potentials are summarized in d and e. Bars represent mean \pm SE values ($p < 0.05$)



The effects of cAMP-related drugs and HCN channel blockers on $[Ca^{2+}]_i$ oscillations of ICCs

In cultured intestinal ICCs, the activity of pacemaker channels was found to be closely coupled with spontaneous $[Ca^{2+}]_i$ oscillations [31]. Thus, to evaluate whether HCN channels activation is coupled with spontaneous $[Ca^{2+}]_i$ oscillations, we measured spontaneous $[Ca^{2+}]_i$ oscillations

in cultured colonic ICCs that are connected with cell cluster and treated with 8-bromo-cAMP (100 μ M; $n = 6$), rolipram (100 μ M; $n = 6$), SQ-22536 (100 μ M; $n = 6$), CsCl (5 mM; $n = 6$), ZD7288 (10 μ M; $n = 6$), and genistein (10 μ M; $n = 4$). ICCs were loaded with fluo3-AM and spontaneous $[Ca^{2+}]_i$ oscillations were observed in a time series. The frequency of $[Ca^{2+}]_i$ oscillations was 7.3 ± 1.6 cycles/5 min in the control ($n = 34$). Both

Fig. 4 Effects of hyperpolarization-activated cyclic nucleotide (HCN) channel blockers on the spontaneous pacemaker potentials in cultured ICCs of the mouse colon. CsCl (5 mM) (a), ZD7288 (10 μ M) (b), zatebradine (10 μ M) (c), clonidine (100 μ M) (d), and genistein (10 μ M) (e). All traces showed the reduced frequency of pacemaker potentials. The effects of these drugs on pacemaker potentials are summarized in f and g. Bars represent mean \pm SE values ($p < 0.05$)

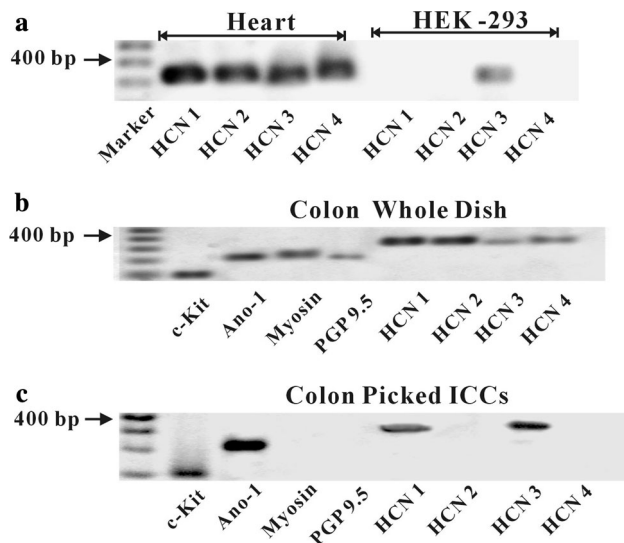
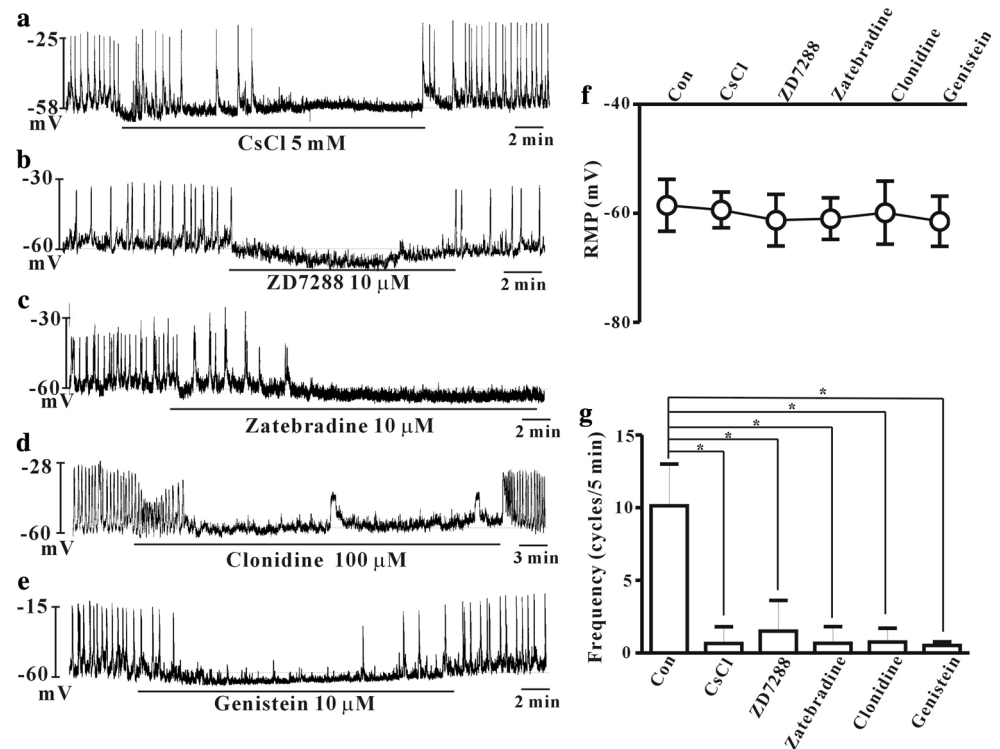


Fig. 5 RT-PCR detection and expression of hyperpolarization-activated cyclic nucleotide (HCN) channels protein in cultured ICCs of mouse mid colon. Mouse heart and HEK-293 cells were used as positive and negative controls (a). Four HCN channel subtypes (HCN1–HCN4) were amplified in whole mounted cultured cells from mouse colon (b). However, only the HCN1 and HCN3 channel subtypes were amplified in c-kit and Ano1 positive cultured colonic ICCs (c)

8-bromo-cAMP and rolipram increase spontaneous $[Ca^{2+}]_i$ oscillations (Fig. 6a, b). In contrast, SQ-22536, CsCl, ZD7288, and genistein all decreased spontaneous $[Ca^{2+}]_i$ oscillations (Fig. 6c–f). The values of the frequency

induced by the above drugs were significantly different from control values. These results suggested that the basal activation of HCN channels may be coupled with spontaneous $[Ca^{2+}]_i$ oscillations (Fig. 6g).

HCN channels were not involved in the generation of pacemaker potentials in intestinal ICCs

To evaluate whether HCN channels also involve in intestinal pacemaking, we treated intestinal ICCs with SQ-22536 (100 μ M; $n = 5$), rolipram (100 μ M; $n = 5$), 8-bromo-cAMP (100 μ M; $n = 9$), CsCl (5 mM; $n = 6$), ZD7288 (10 μ M; $n = 5$), zatebradine (10 μ M; $n = 5$), clonidine (100 μ M; $n = 5$), and genistein (10 μ M; $n = 6$) and performed RT-PCR. We found that all the drugs had no effect on pacemaker potentials of intestinal ICCs (Fig. 7a–h). In RT-PCR analysis, the mRNA transcripts for four HCN channel subtypes were detected in whole-mount cultured intestinal cells ($n = 6$; Fig. 8a). However, none of the HCN channel subtypes were expressed in cultured c-kit and Ano1 positive intestinal ICCs ($n = 10$; Fig. 8b).

Discussion

In the present study, our data suggest that tonically activated HCN channels participate in regulating pacemaker activity in colonic ICCs. The major findings of this study are that basal intracellular cAMP production maintains the

Fig. 6 The effects of cAMP-related drugs and hyperpolarization-activated cyclic nucleotide (HCN) channel blockers on spontaneous intracellular Ca^{2+} oscillations in cultured colonic ICCs. Cell permeable 8-bromo-cAMP (100 μ M) (a) and rolipram (100 μ M) (b) increased the frequency of intracellular Ca^{2+} oscillations. In contrast SQ-22536 (100 μ M) (c), dideoxyadenosine (100 μ M) (d), ZD7288 (10 μ M) (e) and genistein (10 μ M) (f) decreased the frequency of intracellular Ca^{2+} oscillations

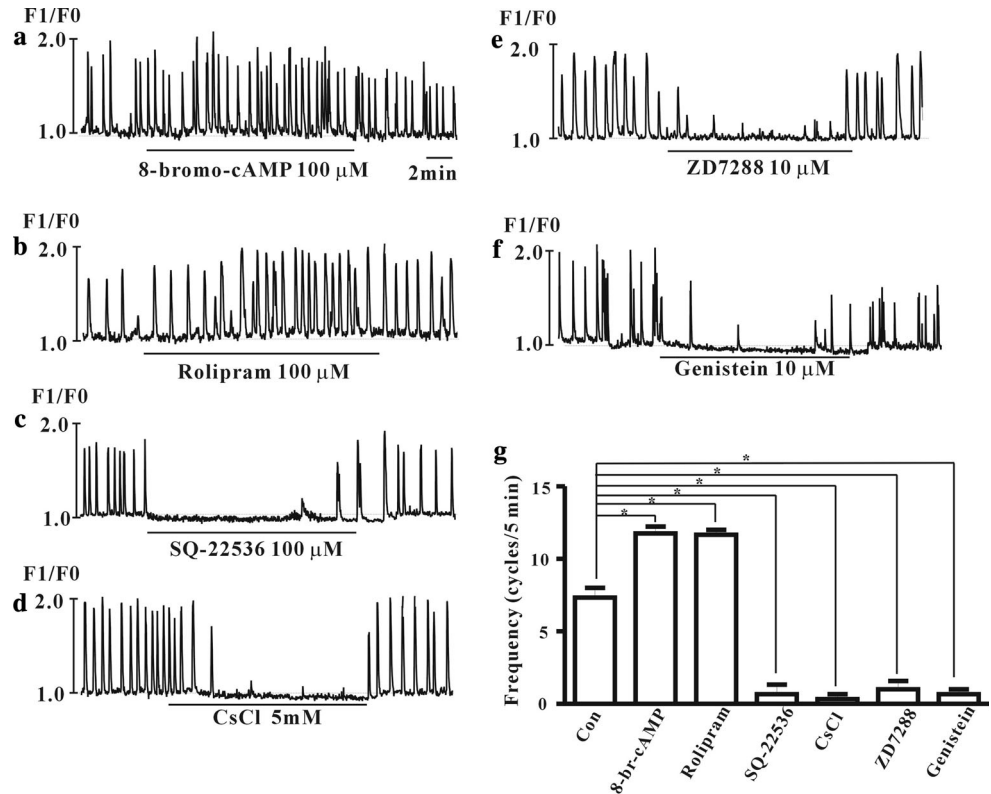
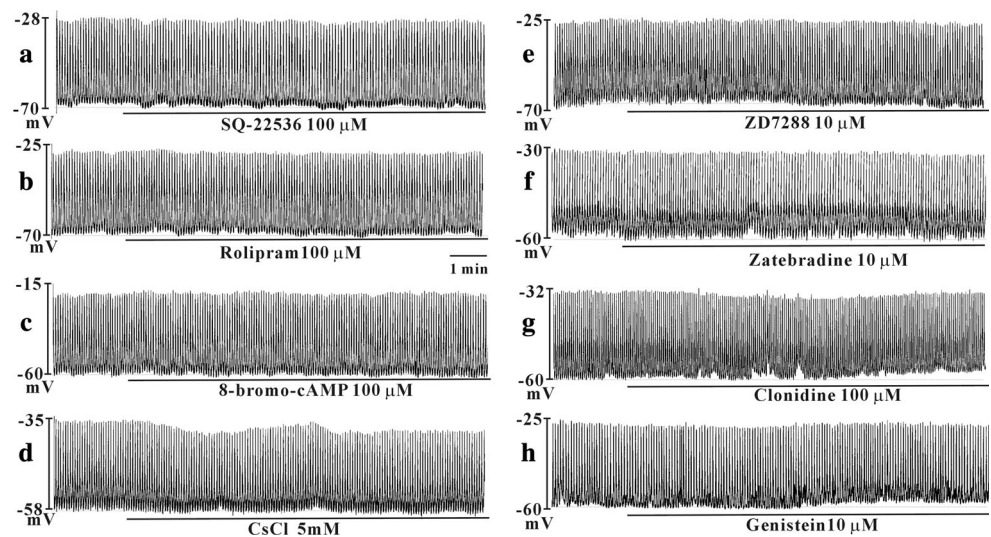


Fig. 7 Effects of cAMP-related drugs and hyperpolarization-activated cyclic nucleotide (HCN) channel blockers on the spontaneous pacemaker potentials in cultured ICCs from mouse small intestine. SQ-22536 (100 μ M) (a), rolipram (100 μ M) (b), 8-bromo-cAMP (100 μ M) (c), CsCl (5 mM) (d), ZD7288 (10 μ M) (e), zatebradine (10 μ M) (f), clonidine (100 μ M) (g), and genistein (10 μ M) (h) all had no effect on pacemaker potentials



resting pacemaker activity in colonic ICCs and that HCN channel blockers inhibited the pacemaker activity and decrease intracellular Ca^{2+} oscillations. In addition, we observed that the expression of HCN channels by RT-PCR in colonic ICCs. To our knowledge, the findings of this study are the first to suggest that HCN channels may be present in colonic ICCs and regulate pacemaker activity.

HCN channels are a family of non-selective cationic channels that conduct Na^+ and K^+ . These channels are comprised of four subtypes (HCN1–HCN4) and each

subtype has six transmembrane domains containing intracellular N- and C-terminus. HCN channels are activated by hyperpolarization of the membrane and by direct binding of intracellular cAMP to a cyclic nucleotide-binding domain (CNBD) on the C-terminus [32]. Neurotransmitters can influence firing rates of active spontaneous cells by either increasing or decreasing cAMP levels through HCN channels. Cardiac sinoatrial cells have I_f (funny)-currents that regulate heart rate mediated by HCN channels. Sympathetic β -adrenoceptor stimulation increases intracellular

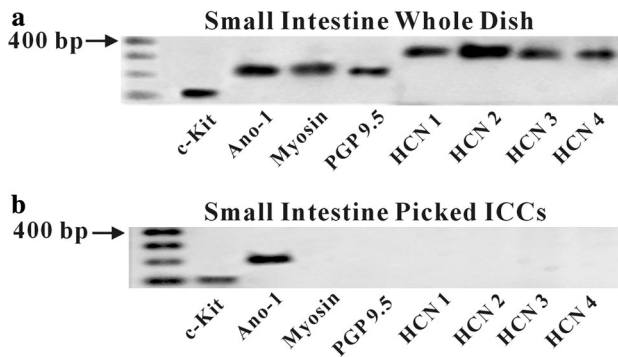


Fig. 8 RT-PCR detection and expression of hyperpolarization-activated cyclic nucleotide (HCN) channel proteins in cultured ICCs of mouse small intestine. Four HCN channel subtypes (HCN1–HCN4) were amplified in whole mount cultured cells from mouse small intestine (a). In contrast, none of the subtypes of HCN channels were detected in c-kit and Ano1 positive cultured intestinal ICCs (b)

cAMP level and leads to increased f-channel activity followed by increased heart rate. Whereas parasympathetic muscarinic receptor stimulation decreases intracellular cAMP level and leads to a decrease in f-channel activity followed by decreased heart rate [33].

We thought the possibilities that HCN channels can exist in ICCs because ICCs are also active pacemaker cells. Thus, we were curious whether the pacemaker activity in colonic ICCs is regulated by intracellular cAMP. The intracellular cAMP level depends on the activity of two groups of enzyme: the adenylate cyclase that produces cAMP and the phosphodiesterase (PDE) that hydrolyzes cAMP [34, 35]. In this study, SQ-22536 and dideoxyadenosine, both adenylate cyclase inhibitors, decreased the frequency of pacemaker potentials in colonic ICCs. In contrast, rolipram, a specific cAMP-dependent PDE 4 inhibitor, and a cell-permeable 8-bromo-cAMP both increased the frequency of pacemaker potentials. These results strongly suggest that basal intracellular cAMP production maintains the resting pacemaker frequency in colonic ICCs. cAMP is a key second messenger that transmits information to many different effector proteins within the cell. The cellular action of cAMP is mediated by PKA or Epac. However, KT-5720, a PKA inhibitor, or 8-pCPT-2'-O-Me-cAMP, an Epac agonist had no effects on pacemaker activity, indicating that the regulatory effect of cAMP on pacemaker frequency in colonic ICCs is direct rather than being mediated by protein phosphorylation. This finding suggests that another pathway of cAMP-mediated regulation of pacemaker activity exist in colonic ICCs. CNG and HCN channels are other downstream target proteins of cAMP [36]. However, *L-cis*-diltiazem, a CNG channel blocker, did not change the pacemaker potentials. Thus, we postulated that HCN channels may be a target protein of cAMP for regulation of pacemaker activity in

colonic ICCs. To support this hypothesis, we performed pharmacological studies. Pharmacologically, Cs⁺, ZD7288 and zatebradine are HCN channel blockers that are commonly used in identification of HCN channels in various cells [37]. It has also been reported that clonidine, a α_2 -adrenoceptor agonist, directly blocks the HCN channels activity in cardiac sinoatrial cells [38]. Regulation of HCN channel by tyrosine kinases of the Src family has been also reported in heart and neuron [39]. In this study, HCN channel blockers and the tyrosine kinase inhibitor, genistein suppressed pacemaker frequency in colonic ICCs. These above results suggest that HCN channels are under tonically activated by basal cAMP in colonic ICCs. The similar results have been reported in spontaneous firing GH₃ cells where basal cAMP tonically activates HCN channels [40].

To confirm and support the functional data, we performed RT-PCR in colonic ICCs in cultured cells. The distribution of HCN channel subunits was both regional- and species-dependent. In the central nervous system, HCN1 channel was found to be expressed mainly in the photoreceptors, neocortex, and cerebellar cortex. HCN2 channel was expressed in nearly all brain tissues. HCN3 channel was found in the olfactory bulb and HCN4 channel was found in the thalamus and olfactory bulb. In the heart, all four HCN channel subunits are detected [41]. In sinoatrial nodes, the HCN4 channel is the major subunit in all species and the remaining small fraction of these channels is species dependent. HCN1 channel is dominant in rabbit, whereas the HCN2 channel is dominant in mouse and human [42]. In the GI tract of mice, enteric neurons expressed HCN1, HCN2, and HCN4 channels but not HCN3 channels [43]. Recently, Yang et al. [44] investigated the role HCN2 channels on spontaneous rhythmic activity of GI tract in mice. However, immunoreactivity of the anti-HCN2 antibody was only detected in myenteric neurons and not in ICCs. In our study, RT-PCR analysis revealed the presence of mRNA transcripts for all four HCN channels subtypes in the whole mount cultured colonic cells. However, in c-kit and Ano1 positive colonic ICCs, only mRNA transcripts for the HCN1 channel and HCN3 channel were expressed. Taken together these results suggest that ICCs also have HCN channels and have regional differences in distribution of HCN channels in mouse.

The pacemaking mechanism in intestinal ICCs is related to IP₃-dependent intracellular Ca²⁺ oscillations that are coupled with the periodic activation of pacemaker channels. In this study, intracellular Ca²⁺ oscillations are increased by rolipram or 8-bromo-cAMP and decreased by SQ-22536 or dideoxyadenosine in colonic ICCs. In addition, HCN channel blockers suppressed spontaneous intracellular Ca²⁺ oscillations. This suggests the activation

of HCN channels and intracellular Ca^{2+} oscillations are also closely coupled to generate pacemaker activity.

Finally we also performed pharmacological experiments and RT-PCR on intestinal ICCs to compare with the results from colonic ICCs. In contrast to colonic ICCs, all cAMP-related drugs and HCN channel blockers had no effect on the intestinal pacemaker frequency. HCN channels were not detected in the intestinal ICCs by RT-PCR. These results strongly suggest that there are regional differences in HCN channels and that different pacemaking mechanisms may exist between the small intestine and the colon.

In summary, periodically activated HCN channels by basal intracellular cAMP production are present in colonic ICCs and they regulate pacemaker activity. The different regional distribution in HCN channels between the intestine and colon suggest a cause for differential regulation of GI motility. Our results suggest that HCN channel may participate in regulation of pacemaking activity and may be an effective therapeutic target for abnormal colonic motility disorders. However, because our study was performed in cultured cells, further studies are required to confirm the existence of HCN channels and elucidate the fine precise mechanisms in native tissues.

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Conflict of interest The authors disclose no conflicts.

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