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Effect of adenosine-1 receptor activation on pacemaker activity of interstitial cells of Cajal from mouse colon

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ABSTRACT

Adenosine plays an important role on gastrointestinal (GI) motility through adenosine receptors. Interstitial cells of Cajal (ICC) are pacemaker cells that regulate GI smooth muscle activity. The functional role and its signal mechanism of adenosine on the pacemaker activity were investigated using whole-cell patch clamp, RT-PCR, and intracellular Ca^{2+}-imaging with ICC from mouse colon. Adenosine depolarized the membrane potentials and increased the pacemaker potential frequency, which was blocked by a selective A1-receptor antagonist, but not A2a-, A2b, or A3-receptor antagonist. A selective A1 receptor agonist represented similar effects as those of adenosine and mRNA transcript of A1-receptor was expressed in ICC. The adenosine-induced effects were blocked by phospholipase C (PLC) and a Ca^{2+}-ATPase inhibitor. Adenosine increased spontaneous intracellular Ca^{2+} oscillations, as seen fluo4/AM. Both hyperpolarization-activated cyclic nucleotide (HCN) channel inhibitors and adenylate cyclase inhibitors blocked the adenosine-induced effects. And adenosine increased the basal cellular adenylate cyclase activity in colonic ICC. However, adenosine and adenylate cyclase inhibitors did not show any influence on pacemaker activity in small intestinal ICC for a comparison with that of the small intestine. These results suggest adenosine modulates the pacemaker potentials by acting HCN channels- and intracellular Ca^{2+}- dependent mechanisms through A1-receptor. Therefore, adenosine may be a therapeutic target in colonic motility disorders.

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Introduction

Adenosine is one of the body's more useful molecules and is linked to the digestion process. As such, adenosine is present in all tissues and plays an important functions in most body systems (1-3). Adenosine effects are exerted via G-protein-coupled adenosine receptors that are comprised via four subtypes: the A1-, A2a-, A2b-, and A3-receptors (4). In GI tract, adenosine receptors are distributed throughout enteric neuronal cells, smooth muscle cells, and epithelial cells, indicating that adenosine receptor activation is involved in regulating GI motility, blood flow, and secretion (5). Generally, exogenous adenosine relaxes smooth muscles and inhibits GI motility through the inhibition of the release of excitatory enteric neurotransmitters via activation of adenosine receptors in smooth muscles (6-8). However, some articles have reported on the contrary that adenosine contracts smooth muscles, with adenosine receptor activation having contracted isolated circular muscles of rat ileum, rat colons, and cat esophageal smooth muscles (9-11). This difference in adenosine-mediated effects may be due to receptor subtypes, species, or regional differences.

ICC is pacemaker cells which regulates smooth muscle motility by generating spontaneous electrical slow waves (12). ICC form the network in a GI tract and connects with smooth muscles through gap junctions, mediating enteric neuronal signals to said smooth muscles (13). ICC is highly metabolic cell, with numerous mitochondria and purinergic receptors expressed there within (14-16). This indicates the possibility that adenosine can regulate GI motility through the modulation of pacemaker activity of ICC.

Although the effects and signals of adenosine on GI motility have been investigated in enteric neurons and GI smooth muscles, adenosine pathways may contribute to the control of pacemaker activity in ICC. Thus, in this study, we detail the effects of exogenous adenosine on the generation of pacemaker potentials in colonic ICC, including the receptor types and their signal mechanism.

Materials and Methods

Preparation of cells and patch-clamp experiment

BALB/c mice (3–7 days old) of either sex were anaesthetized with ether and killed by cervical dislocation. The colon from below the cecum to the rectum was removed and the mid-region of the colon was used. Luminal contents were rinsed with Krebs–Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish and the mucosae were removed by sharp dissection. Small tissue strips of colonic muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca^{2+}-free Hank's solution containing (in mM): KCl 5.36, NaCl 125, NaOH 0.34, Na_2HCO_3 0.44, glucose 10, sucrose 2.9, and HEPES 11, for 30 min. Then, the cells were

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dispersed with an enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, USA) 1.3 mg/ml, bovine serum albumin (Sigma Chemical Co., St Louis, MO, USA) 2 mg/ml, trypsin inhibitor (Sigma) 2 mg/ml, and ATP 0.27 mg/ml. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/ml, Falcon/Bectin Dickinson, Franklin Lakes, NJ, USA) in a 35-mm culture dish. The cells were then cultured at 37°C in a 5% CO_2 incubator in a smooth muscle growth medium (SMGM, Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF, 5 ng/ml, Sigma).

Patch-clamp experiments

The patch-clamp technique was performed using ICC that showed network-like structures in culture (2–3 days). The cell culture dishes were mounted on the stage of a model TE-2000s inverted microscope (Nikon, Melville, NY, USA). The bath solution was 3 ml/min, and all experiments were performed at 30°C. The whole-cell configuration of the patch-clamp technique was used to record membrane potentials (I = 0) from cultured ICC. Axopatch 200B (Axon Instruments, Foster, CA, USA) amplified membrane currents and potentials. The command pulse was applied using a personal computer and pClamp software (version 9.2; Axon Instruments). The data were filtered at 5 kHz. Results were analyzed using pClamp and GraphPad Prism (version 5) software.

Solution and drugs

The cells were bathed in a solution containing (measured in mM) KCl 5, NaCl 135, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2, and HEPES 10, and were adjusted to a pH of 7.4 with Tris. The pipette solution contained (measured in mM) KCl 140, $MgCl_2$ 5, K_2ATP 2.7, Na_2GTP 0.1, creatine phosphate disodium 2.5, HEPES 5, and EGTA 0.1, and was adjusted to a pH of 7.2 with Tris.

The drugs used were adenosine, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 8-(3-chlorostyryl) caffeine, 9-chloro-2-(2-furanyl)-5-[(phenylacetyl) amino][1,2,4]-triazolo(1,5-c)quinazoline (MRS 1191), 3,7-dimethyl-1-propargylxanthib (DMPX) N^6-phenylisopropyladenosine (R-PIA), 2,-p-(2-carboxyethyl) phenetylamino-5'-N-ethylcarboxamidoadenosine hydrochloride hydrate (CGS 21680), N^6-(3-iodobenzyl) adenosine-5'-N-methyluronamide1-deoxy-1-[6-[((3-iodophenyl)methyl)amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA), U-73122, thapsigargin, SQ22536, dideoxyadenosine (DDA), $CsCl_2$, and ZD 7288. All drugs and other compounds were purchased from Sigma.

All drugs and other compounds were purchased from Sigma. All drugs were dissolved in DW or DMSO to prepare stock solutions (10 or 100 mM). The final concentration of DMSO registered at less than 0.05%.

Collection of ICC and RT-PCR

Collection of ICC performed as previously described (17). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. cDNA was produced from the total RNA using Superscript One-Step RT-PCR with Platinum Taq (Invitrogen). The primers used are shown in Table 1. 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

Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentrations were monitored using fluo-4/AM pre-dissolved in DMSO and stored at –20 °C. The cells on coverslips were incubated in the bath solution containing 5 μM fluo-4 under 5% CO_2 at 37 °C for 5 min. The cells were then scanned under a confocal microscope every 0.4 seconds (200x; Fluoviews 300, Olympus) at 30 °C. Excitation and emission wavelengths of 488–515 nm were used for fluorescence imaging. The variations of $[Ca^{2+}]_i$ fluorescence emission intensity in the entire cytoplasm was expressed as F1/F0, where F0 corresponds to the intensity of the first imaging process.

Table 1. Nucleotide sequences of the primers used for RT-PCR.

Gene	Sequences	Accession No	Size (bp)
Ano-1	(F) AGGCCAAGTACAGCATGGGTATCA (R) AGTACAGGCCAACCTTCTCACCAA	NM_178642	213
Myosin	(F) GAGAAAGGAAACACCAAGGTCAAGC (R) AACAAATGAAGCCTCGTTTCCTCTC	NM_010860	264
PGP 9.5	(F) GCCAACAACCAAGACAAGCTGGAA (R) GCCGTCCACGTTGTTGAACAGAAT	AF172334	213
Adenosine A1	(F) TGCCCGGAAATGTACTGGTGAT (R) TGGAGCTCTGGGTGAGGATGAG	NM_001008533	213
Adenosine A2a	(F) GCTGCCTTGCCTTGTAGAGCAG (R) GCCAGGGAGGGGAAGTCAATAA	NM_001331095	213
Adenosine A2b	(F) GATGAATGTGGCCATCCTCCTG (R) GCCTTCTTCTCCAAAAGGCCAG	NM_007413	217
Adenosine A3	(F) ACTGGTGCCGTGGCTACTTCAG (R) CCATTGGCCCGGTCTTCTCTAT	NC_000069	215

Measurement of adenylate cyclase activity

Adenylate cyclase activity was measured in cultured ICC. ICC was rinsed twice with ice-cold PBS and collected in PBS. ICC was homogenized with five strokes of a homogenizer. The homogenate was then centrifuged at 1000 × g for 5 min at 4°C. The supernatant was then transferred into a centrifuge tube and centrifuged at 5000 × g for 10 min. The pellet was suspended in buffer (30 mM Na-HEPES, 5 mM $MgCl_2$, and 2 mM DTT, pH 7.5) to attain a total protein concentration of approximately 1 mg/mL before being added into tubes containing the drug and adenylate cyclase assay buffer (30 mM Na-HEPES, 100 mM NaCl, 1 mM EGTA, 10 mM $MgCl_2$, 1 mM isobutylmethylxanthine, 1 mM ATP, 10 mM phosphocreatine, 5 μM GTP, 60 U/mL creatine phosphokinase, and 0.1% bovine serum albumin, pH 7.5). Adenylate cyclase activity assays were performed using a mouse adenylate cyclase ELISA kit (MyBioSource Company).

Statistical analysis

Data are expressed as means ± standard error (S.E.) or deviation (S.D.). Student t- or one-way ANOVA test was used to test for significance at the level of $p < 0.05$. The n values reported in the text refer to the number of cells used in the patch-clamp experiments.

Results

Adenosine modulates pacemaker potentials in colonic ICC

To determine the functional role of adenosine action on pacemaker activities of colonic ICC, we recorded the pacemaker potentials. Under clamp mode, ICC showed spontaneous pacemaker potentials. The basal membrane potential and frequency were –55.5 ± 1.4 mV and 12.2 ± 1.3 cycles/5 min, respectively (n = 37). The addition of adenosine (1 μM, 5 μM, 10 μM and 50 μM) increased the pacemaker potential frequency and depolarized the membrane (Figure 1A-D). The change in membrane potential and pacemaker potential frequency via adenosine was demonstrated in a dose-dependent manner. The effects of adenosine on pacemaker potential are summarized in Figures 1E and F.

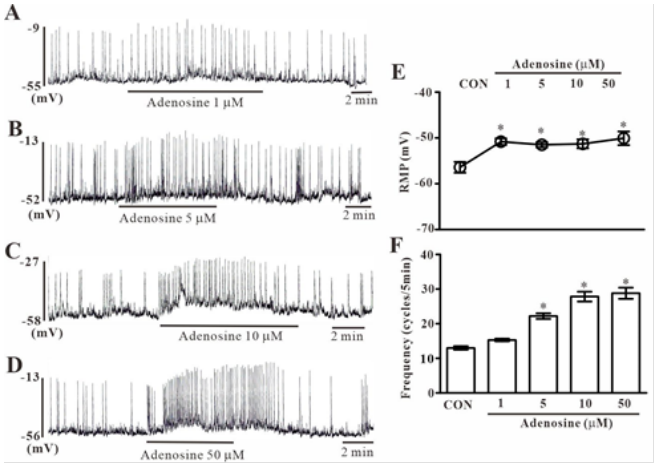

Figure 1. Effects of adenosine on pacemaker potentials of cultured colonic ICC from a mouse colon. (A-D) The pacemaker potentials of ICCs exposed to adenosine from 1 μM to 50 μM in the current clamp mode. Adenosine depolarized the membrane and increased the pacemaker potential frequency, dose-dependently. (E and F) A summary of the facilitatory effects of adenosine on pacemaker potentials. Each column represents the mean ± S.D. Asterisks indicate values that are significantly different compared to the control values ($p < 0.05$). CON: Control, RMP: Resting membrane potentials.

Adenosine A1-receptor mediates adenosine-induced effects in colonic ICC

To identify the adenosine receptor subtypes, we used adenosine receptor antagonists and agonists. Pretreatment with DPCPX (10 μM), a selective A1-receptor antagonist, blocked the adenosine (50 μM)-induced effects on pacemaker potentials (n = 9, Figure 2A). However, 8-(3-chlorostyryl) caffeine (10 μM, n = 7), DMPX (10 μM, n = 7), and MRS 1191 (1 μM, n = 7), A2a-, A2b, and A3-receptor antagonist, did not block the adenosine-induced effects (Figure 2B-D). These are summarized in Figure 2E. In the adenosine agonist test, R-PIA (an A1-receptor agonist, 10 μM, n = 9) represents the mimicked effects of adenosine (Figure 3A); however, CGS 21680 (an A2-receptor agonist, 10 μM, n = 7) and IB-MECA (an A3-receptor agonist, 10 μM, n = 7) had no effects on pacemaker potentials (Figure 3B and C). These are summarized in Figures 3D and E. To confirm the existence of the A1-receptor subtype, an RT-PCR was performed. Anoctamin 1 (ANO1) primer was used for ICC detection because ANO1 is a selective marker of ICC. From whole cells, all types of adenosine receptors were detected, while only A1-receptors were detected from ANO1-positive cells (Figure 3F).

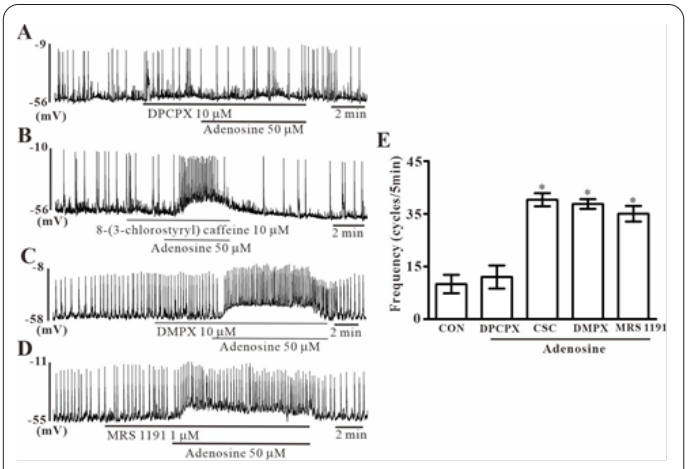

Figure 2. Effects of adenosine receptor antagonists on adenosine-induced action of pacemaker potentials. (A) DPCPX (10 μM, $n = 9$), an A1-receptor antagonist, blocked the adenosine (50 μM)-induced action of pacemaker potentials. (B-D) 8-(3-chlorostyryl) caffeine (10 μM, $n = 7$), DMPX (10 μM, $n = 7$) and MRS 1191 (1 μM, $n = 7$), selective A2a-, A2b-, and A3-receptor antagonist, respectively, did not block the adenosine-induced action of pacemaker potentials. (E) The summarized effects of adenosine in the presence of adenosine receptor antagonists on the pacemaker potential frequency in colonic ICC. Each column represents the mean ± S.D. Asterisks indicate values that are significantly different compared to the control values ($p < 0.05$). CON: Control.

Involvement of PLC-dependent $[Ca^{2+}]_i$ release on adenosine effects

To determine whether adenosine-induced effects were mediated through PLC-dependent $[Ca^{2+}]_i$ release pathway, we tested with U-73122 (a PLC inhibitor) and thapsigargin (a Ca^{2+}-ATPase inhibitor from the endoplasmic reticulum).

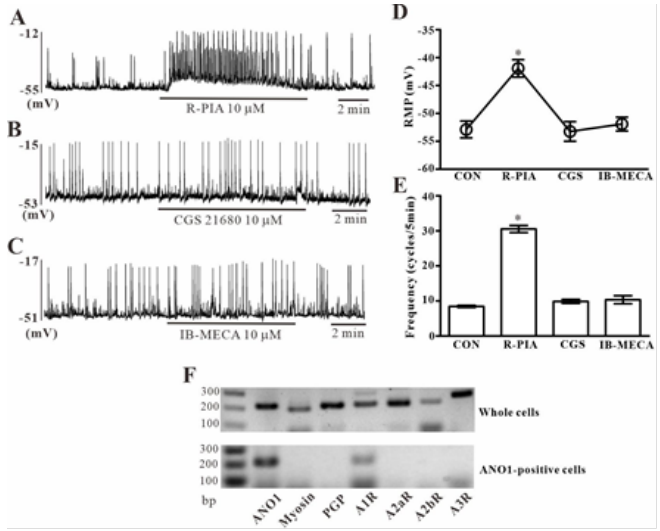

Figure 3. Effects of adenosine receptor agonists on pacemaker potentials of cultured colonic ICC. (A) R-PIA (10 μM, *n* = 9), an A1-receptor agonist, represented the mimicked effects that those of adenosine. (B and C) CGS 21680 (10 μM, *n* = 7), an A2-receptor agonist and IB-MECA (10 μM, *n* = 7), an A3-receptor agonist, had no effects on pacemaker potentials. (D and E) The summarized effects of adenosine receptor agonists on the membrane potential and pacemaker potential frequency in colonic ICC. Each column represents the mean ± S.D. Asterisks indicate values that are significantly different compared to the control values ($p < 0.05$). (F) Expression of adenosine receptors in whole cells or ICC of a mouse colon. RT-PCR only detected the mRNA transcripts for A-1 receptors in ANO1-positive cultured colonic ICC. CON: Control, RMP: Resting membrane potentials.

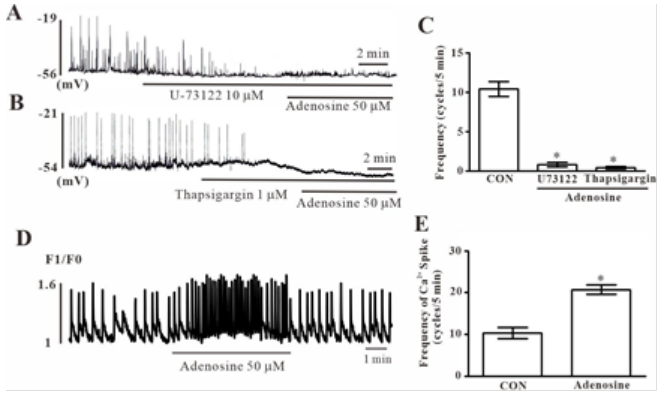

Figure 4. Effects of a PLC inhibitor or Ca^{2+}-ATPase inhibitor on adenosine-induced effects on pacemaker potentials and the effects of adenosine action on $[Ca^{2+}]_i$ oscillations in cultured colonic ICC. (A and B) U-73122 (10 μM, *n* = 7), a PLC inhibitor and thapsigargin (10 μM, *n* = 8), a Ca^{2+}-ATPase inhibitor from the endoplasmic reticulum blocked the adenosine (50 μM)-induced effects on pacemaker potentials. (C) The summarized effects of adenosine in the presence of U-73122 and thapsigargin on the pacemaker potential frequency in colonic ICC. (D) ICC was loaded with fluo4-AM and spontaneous $[Ca^{2+}]_i$ oscillations were observed across a time series. Adenosine (50 μM, *n* = 7) increased spontaneous $[Ca^{2+}]_i$ oscillations. (E) The summarized effects of adenosine on the $[Ca^{2+}]_i$. Each column represents the mean ± S.E. or S.D. Asterisks indicate values that are significantly different compared to the control values ($p < 0.05$). RMP: Resting membrane potentials.

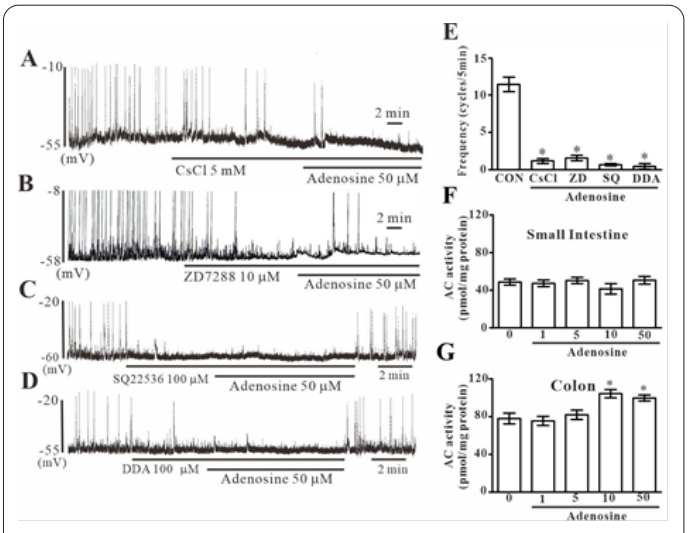

Figure 5. Effects of HCN channel blockers and adenylate cyclase inhibitors on adenosine-induced effects on pacemaker potentials in cultured colonic ICC and effect of adenosine on basal adenylate cyclase activity. (A–D) Pretreatment of CsCl (5 mM, *n* = 8), ZD7288 (10 μM, *n* = 9), SQ22536 (100 μM, *n* = 8), and DDA (100 μM, *n* = 8) blocked the adenosine (50 μM)-induced increase pacemaker potential frequency. (E) The summarized effects of adenosine in the presence of CsCl, ZD7288, SQ22536, and DDA on the pacemaker potential frequency in ICC. (F and G) Basal adenylate cyclase activity was measured in small intestinal and colonic ICC with various doses of adenosine. In colonic ICC, 10 and 50 μM adenosine increased basal AC activity but not small intestinal ICC when compared to the control. Each column represents the mean ± S.E. Asterisks indicate values that are significantly different compared to the control values ($p < 0.05$). CON: Control, ZD: ZD7288. SQ: SQ22536.

Pretreatment with U-73122 (10 μM, n = 7) or thapsigargin (10 μM, n = 8) itself inhibited the generation of pacemaker potentials and blocked adenosine-induced actions (Figure 4A and B). These are summarized in Figure 4C. In $[Ca^{2+}]_i$ analysis, colonic ICC showed rhythmical $[Ca^{2+}]_i$ oscillations and adenosine (50 μM, n = 7) increased spontaneous $[Ca^{2+}]_i$ oscillations (Figure 4D and E).

HCN channels involved in adenosine-induced effects

To examine whether adenosine-induced effects on ICC was mediated through HCN channels, we used $CsCl_2$ and ZD 7288 (HCN channel inhibitors). $CsCl_2$ (5 mM, n = 8), or ZD 7288 (10 μM, n = 9) alone inhibited the generation of pacemaker potential. Under these conditions, adenosine did not increase the pacemaker potential frequency (Figures 5A and B). Given that the activation of the HCN channel was mediated by cAMP, we tested SQ22536 and dideoxyadenosine (DDA) adenylate cyclase inhibitors. Both drugs abolished the generation of pacemaker potentials. Under these conditions, both SQ22536 (100 μM, n = 8) and DDA (100 μM, n = 8) blocked the adenosine-induced effects (Figure 5C and D). To support this, we tested adenylate cyclase activity with various doses of adenosine in small intestinal or colonic ICC and found a high dose of adenosine increased adenylate cyclase activity only in the colon (Figure 5F and G). The effects of HCN channel inhibitors and adenylate cyclase inhibitors are summarized in Figure 5E.

Effects of adenosine on pacemaker activity in small intestinal ICC

To compare with small intestinal ICC on adenosine-induced effects, we recorded with small intestinal ICC. Under current clamp mode, small intestinal ICC showed

spontaneous pacemaker potentials and adenosine (50 μM) had no effect on this (Figure 6A, n = 5). Next, we checked the cAMP regulation on small intestine ICC. Unlike colonic ICC, neither SQ22536 nor DDA adenylate cyclase inhibitors showed any influence on pacemaker activity of small intestine ICC (Figure 6B and C, n = 5-6). Furthermore, 8-bromo-cAMP which is a membrane-permeable cAMP did not show any effect (Figure 6D, n = 7). To find the existence of adenosine receptor subtypes, RT-PCR was performed and only A2a-receptors were detected (Figure 6E). Adenosine, adenylate cyclase inhibitors or cAMP-induced effects are summarized in Figure 6F and G.

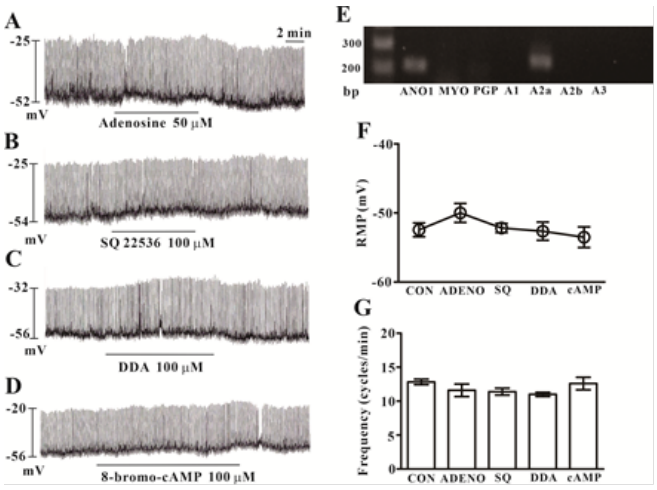

Figure 6. Effects of adenosine, adenylate cyclase inhibitors (SQ22536 and dideoxyadenosine) and 8-bromo-cAMP on pacemaker activity of ICC from the small intestine of a mouse. (A) Adenosine (50 μM) could not show any influence on pacemaker potentials in small intestine ICC. (B and C) Adenylate cyclase inhibitors (SQ22536 100 μM or DDA 100 μM) showed no changes of pacemaker potentials. (D) 8-bromo-cAMP (100 μM), a cell-permeable cAMP, did not show any influence on pacemaker potentials in small intestine ICC. (E) Expressions of adenosine receptors in small intestine ICC. RT-PCR only detected the mRNA transcripts for A2a receptors in ANO1-positive ICC in the small intestine. (F and G) The summarized effects of adenosine in the presence of adenosine, SQ22536, DDA or 8-bromo-cAMP on the pacemaker potential frequency in ICC. Each column represents the mean ± S.E. CON: Control, RMP: Resting membrane potentials, MYO: Myosin, PGP: PGP9.5, ADENO: Adenosine, SQ: SQ22536, cAMP: 8-bromo-cAMP.

Discussion

GI smooth muscle contractions are initiated by electrical spontaneous slow waves, which are caused by spontaneous pacemaker potentials from ICC. Many neurotransmitters and endogenous substances affect smooth muscle contractions by changing the slow wave frequency and configuration (18). In this study, we found exogenous adenosine increased the pacemaker potential frequencies via A1-receptor in colonic ICC through PLC-dependent signal mechanisms and the HCN channel is involved in adenosine-induced regulation of pacemaker activity.

Exogenous adenosine regulates GI smooth muscle contractions via adenosine receptors composed of A1-, A2a-, A2b-, and A3-receptor subtypes, with all subtypes of adenosine receptors, detected in the GI tract (5). In this study, adenosine depolarized the membrane potentials of ICC and increased the pacemaker potential frequency. This was blocked by an A1-receptor antagonist but neither by an A2-receptor antagonist nor an A3-receptor antagonist. In addition, only A1-receptor agonists mimicked the actions produced by adenosine. Furthermore, RT-PCR analysis revealed that only an mRNA transcript of A1 adenosine receptors was detected in ANO1-positive colonic ICC. These results indicate that A1-receptors are a target for adenosine action on pacemaker activity in colonic ICC.

It has been known that A1-receptor activation is linked to the increase of IP_3 and DAG through the activation of PLC (8, 19-21). The pacemaking mechanisms of ICC are coupled with an IP_3-mediated $[Ca^{2+}]_i$ release from the endoplasmic reticulum and subsequent $[Ca^{2+}]_i$ entry into mitochondria. And this leads to the periodic activation of pacemaker channels (22). In this study, PLC and Ca^{2+}-ATPase inhibitors blocked the adenosine-induced effects on pacemaker potentials. In addition, adenosine increased the frequency of $[Ca^{2+}]_i$ oscillations. These results indicate that the adenosine-induced effect is mediated by PLC-dependent IP_3-mediating $[Ca^{2+}]_i$ release in colonic ICC. This suggestion can be supported by previous studies which report adenosine A1-receptors mobilized $[Ca^{2+}]_i$ from the endoplasmic reticulum through a PLC-dependent pathway in human bronchial smooth muscle cells (23).

It is well known that the NSC channel and Ca^{2+}-activated Cl^- channel are pacemaker channels found in ICC. In particular, the ANO1 channel (Ca^{2+}-activated Cl^- channels) was a strong candidate for pacemaker channels (24, 25). Furthermore, we reported that HCN channels participate in generating pacemaker activity in colonic ICC but not in small intestinal ICC (26). Thus, HCN channels are suggested as a possible pacemaker channel in colonic ICC. We postulate that basal activation of HCN channels in colonic ICC may be one cause for the differentiation of patterns of pacemaker activity via ICC between the small intestine and the colon (17). And, this indicates one possibility that the adenosine-induced effect on pacemaker activity may act on the HCN channel of colonic ICC. Therefore in this study, in order to determine whether the increase in pacemaker potential frequency by A1-receptor activation was related to HCN channel activation, we tested adenosine action on pacemaker activity in presence of HCN channel blockers in colonic ICC. Both HCN channel blockers inhibited the adenosine-induced effects on pacemaker activity. In addition, because intracellular cAMP gates HCN channels directly, we performed the experiments with cAMP-related drugs in our previous study. In colonic ICC, adenylate cyclase inhibitors decreased the pacemaker potential frequency, whereas cAMP-specific phosphodiesterase inhibitors increased, indicating that basal cAMP participates in generating pacemaker potentials in colonic ICC (17). In our previous study, we showed the existence of adenylate cyclase type 1 that is activated by $[Ca^{2+}]_i$ in colonic ICC (26). This can prove our suggestion that the increased cAMP, via the Ca^{2+} released from the endoplasmic reticulum by adenosine administration, activates HCN channels. It has been reported that Ca^{2+}-stimulated adenylate cyclase regulates the L-type Ca^{2+} channels in atrial myocytes (27, 28). The report that the gene of Ca^{2+}-stimulated adenylate cyclase was detected in ICC also supports our suggestion (29). In figure 6, we showed that adenosine had no effects on pacemaker potentials in small intestine ICC despite A2a-receptor presence in ANO1-positive cells

in itself. Therefore, the difference in adenosine effects on pacemaker potentials in ICC between the small intestine and the colon may due to receptor subtype differences. Furthermore, in our previous study, we reported that HCN channels were not expressed in small intestine ICC (17). And our result showed that adenylated cyclase inhibitors and 8-bromo-cAMP had no effects on pacemaker potentials in small intestinal ICC. These results indicate that the increased pacemaker potential frequency induced by adenosine in colonic ICC can be determined via the HCN channel. The report that adenosine activates HCN channels via the activation of adenosine receptors in neuronal cells supports our suggestion (30).

In conclusion, Adenosine A1-receptor activation depolarized the membrane and increased the pacemaker potential frequency by activating HCN channels in colonic ICC through PLC-dependent $[Ca^{2+}]_i$ release from the endoplasmic reticulum. Therefore, adenosine A1-receptor activation can modulate colonic motility and may be an effective therapeutic target in colonic motility disorders (Figure 7).

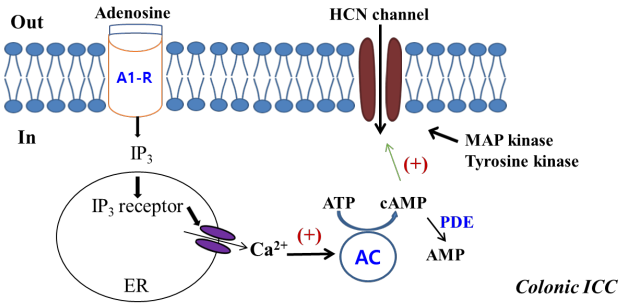

Figure 7. Schematic summary of adenosine actions on pacemaker potentials in colonic ICC. Adenosine binding with A1-receptors leads to Ca^{2+} release from endoplasmic reticulum via IP_3 production. The released Ca^{2+} increases cAMP levels through Ca^{2+}-sensitive adenylate cyclase stimulation; cAMP subsequently activates HCN channels.

Interest conflict

The authors declare that they do not have any conflict of interest.

Consent for publication

The author read and proved the final manuscript for publication

Availability of data and material

All data generated during this study are included in this published article.

Author contributions

S Choi and JH Lee contributed equally to this work. JY Jun: conception and design, acquisition of data, the analysis and interpretation of the data, and manuscript writing; S Choi, JH Lee, C Hong: conception and design, the analysis and interpretation of the data, and the drafting of the manuscripts; DH Shin, W Wu, X Huang: perform experiments, interpretation of the data, and statistical analysis. All authors read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Ethical approval and consent to participate

The experimental and animal care protocols used were in accordance with the guiding principles approved by the ethics committee of Chosun University and the South Korea National Institutes of Health Guide for the Care and Use of Laboratory Animals (Approval no. CIACUC2021-A0019).

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