Original Paper

Quercetin Inhibits Pacemaker Potentials via Nitric Oxide/cGMP-Dependent Activation and TRPM7/ANO1 Channels in Cultured Interstitial Cells of Cajal from Mouse Small Intestine

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Key Words

Interstitial Cells of Cajal • Quercetin • Gastrointestinal tract • TRPM7 channel • ANO1

Abstract

Background: Quercetin regulates gastrointestinal (GI) motor activity but the molecular mechanism involved has not been determined. The authors investigated the effects of quercetin, a flavonoid present in various foods, on the pacemaker activities of interstitial cells of Cajal (ICCs) in murine small intestine *in vitro* and on GI motility *in vivo*. ***Materials and Methods:*** Enzymatic digestion was used to dissociate ICCs from mouse small intestines. The whole-cell patch-clamp configuration was used to record pacemaker potentials in cultured ICCs in the absence or presence of quercetin and to record membrane currents of transient receptor potential melastatin (TRPM) 7 or transmembrane protein 16A (Tmem16A, anoctamin1 (ANO1)) overexpressed in human embryonic kidney (HEK) 293 cells. The *in vivo* effects of quercetin on GI motility were investigated by measuring the intestinal transit rates (ITRs) of Evans blue in normal mice. ***Results:*** Quercetin (100–200 µM) decreased the amplitudes and frequencies of pacemaker activity in a concentration-dependent manner in current clamp mode, but this action was blocked by naloxone (a pan-opioid receptor antagonist) and by GDPβS (a GTP-binding protein inhibitor). However, potassium channels were not involved in

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these inhibitory effects of quercetin. To study the quercetin signaling pathway, we examined the effects of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an inhibitor of guanylate cyclase, and of RP-8-CPT-cGMPS, an inhibitor of protein kinase G (PKG). These inhibitors blocked the inhibitory effects of quercetin on pacemaker activities. Also, L-NAME (100 µM), a non-selective NO synthase (NOS) inhibitor, blocked the effects of quercetin on pacemaker activity and quercetin stimulated cGMP production. Furthermore, quercetin inhibited both Ca^{2+}-activated Cl^- channels (TMEM16A, ANO1) and TRPM7 channels. *In vivo*, quercetin (10-100 mg/kg, *p.o.*) decreased ITRs in normal mice in a dose-dependent manner. **Conclusions:** Quercetin inhibited ICC pacemaker activities by inhibiting TRPM7 and ANO1 via opioid receptor signaling pathways in cultured murine ICCs. The study shows quercetin attenuates GI tract motility, and suggests quercetin be considered the basis for the development of novel spasmolytic agents for the prevention or alleviation of GI motility dysfunctions.

Introduction

Quercetin (3,3',4',5,7-pentahydroxyl-flavone) is one of the most common flavonoids and is present in various foods, such as, onions, apples, broccoli, tea, and red wine [1-4]. Quercetin has a variety of activities in man. For example, it has been reported to have antioxidant [5-7], anti-inflammatory [8], anti-anaphylactic [9], anti-aging [10], antiplatelet aggregatory [11], radioprotective [12], immunomodulatory [13], antidiabetic [14], and anticancer [15] effects, and to improve lipid metabolism [16, 17] and endurance exercise capacity [18]. Quercetin also reduces gastrointestinal (GI) intestinal transit in mice [19], induces intestinal smooth muscle relaxation [20], and prevents castor oil-induced diarrhea [21]. These properties are consistent with the use of flavonoids as antidiarrheal drugs in traditional medicine. However, the cellular and molecular mechanisms underlying the GI motility effects of quercetin are not well understood.

Interstitial cells of Cajal (ICCs) are the GI pacemaker cells that generate rhythmic oscillations in membrane potentials known as slow waves [22, 23], and ICC loss has been implicated in several motility disorders, which suggests that they play an important role in the regulation of GI motility [24]. In addition, ICCs mediate inhibitory and excitatory signals from the enteric nervous system to smooth muscle [25-27], and evidence indicates that endogenous agents, such as, neurotransmitters, hormones, and paracrine substances, modulate GI tract motility by influencing ICCs [28-31]. Furthermore, it has been shown that the pacemaker activities of ICCs in the murine small intestine are mainly due to periodic activations of nonselective cation channels (NSCCs) [32, 33] or Cl^- channels [34-36]. Kim et al. [24] suggested transient receptor potential melastatin (TRPM) 7 is required for ICC pacemaker activity in the murine small intestine, and that a Ca^{2+}-activated Cl^- channel (CaCC) is involved in the slow waves generated by ICCs; this Cl^- channel was later identified as transmembrane protein 16A (Tmem16A; anoctamin1 (ANO1)) [35]. The role played by ANO1 in ICC pacemaker activity was identified in ANO1 knockout mice [37], and several other authors have since shown that ANO1 participates in pacemaker activity [38, 39]. Kim et al. [24, 40] showed that TRPM7 mRNA and TRPM7 currents are expressed in cultured ICCs, and Shahi et al. [40, 41] showed that ANO1 mRNA and ANO1 currents are also expressed in cultured ICCs. Accordingly, it has been proposed that TRPM7 and ANO1 be considered potential targets for the pharmacological treatment of GI motility disorders.

Quercetin plays a vital role in the GI system [42-46], but relatively little is known about its effects on the pacemaker activities of ICCs in the GI tract or on GI motility-related ion channel activities. In the present study, we investigated the inhibitory effects of quercetin on the pacemaker potentials of cultured ICCs, and characterized quercetin-mediated signaling pathways. In addition, we investigated the inhibitory effects of quercetin on GI motor functions by measuring the intestinal transit rates (ITR) of Evans blue in normal mice.

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Materials and Methods

Preparation of cells and cell cultures

Animal care and experiments were conducted in accordance with the guidelines issued by the ethics committee of Pusan National University (Republic of Korea). Balb/c mice were used throughout the study. Small intestines (from 1 cm below the pyloric ring to the cecum) were removed and opened along the mesenteric border. Luminal contents were removed using Krebs-Ringer bicarbonate solution, and the tissues obtained were pinned to the base of Sylgard dishes. Mucosae were removed by sharp dissection and small tissue strips of intestine muscle (consisting of circular and longitudinal muscles) were equilibrated for 30 min 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; pH 7.4). Cells were then dispersed in an enzyme solution containing collagenase (Worthington Biochemical, Lakewood, NJ, USA; 1.3 mg ml^{-1}), bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO; 2 mg ml^{-1}), trypsin inhibitor (Sigma-Aldrich; 2 mg ml^{-1}), and ATP (0.27 mg ml^{-1}), and plated onto sterile glass coverslips coated with murine collagen (2.5 µg ml^{-1}; Falcon/BD, Franklin Lakes, NJ, USA) in 35 mm culture dishes. Cells were then cultured at 37°C in a 95% O_2-5% CO_2 incubator in smooth muscle growth medium (SMGM; Clonetics, San Diego, CA, USA) supplemented with 2% antibiotics/ antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF; 5 ng ml^{-1}; Sigma-Aldrich). All experiments on ICC clusters were performed after culture for 12 hrs. ICCs were identified immunologically using an anti-c-kit antibody (phycoerythrin (PE)-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, San Diego, CA) at a dilution of 1:50 for 20 min (Fig. 1). ICCs were morphologically distinct from other cell types in the culture and thus it was possible to identify the cells by phase contrast microscopy once they had been verified with anti c-kit antibody.
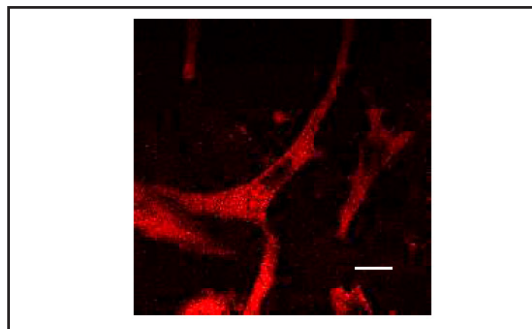
Patch-clamp experiments

The physiological salt solution used to bathe cultured ICC clusters (Na^+-Tyrode) contained (in mM): KCl 5, NaCl 135, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2, and HEPES 10 (adjusted to pH 7.4 with NaOH). The pipette solution used to examine pacemaker activity contained (in mM): KCl 140, $MgCl_2$ 5, K_2ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, HEPES 5, and EGTA 0.1 (adjusted to pH 7.2 with KOH). Patch-clamp techniques were conducted in whole-cell configuration to record membrane currents (voltage clamp mode) and potentials (current clamp mode) from cultured ICCs using Axopatch I-D and Axopatch 200B amplifiers (Axon Instruments, Foster, CA). Command pulses were applied using an IBM-compatible personal computer and pClamp software (version 6.1 and version 10.0; Axon Instruments). Data were filtered at 5kHz and displayed on an oscilloscope, a computer monitor, and/or a pen recorder (Gould 2200; Gould, Valley View, OH, USA). Results were analyzed using pClamp and Origin software (version 6.0, Microcal, USA). All experiments were performed at 30–33°C.

Ca^{2+} activated Cl^- channel expression in human embryonic kidney (HEK)-293T cells

A mammalian expressible plasmid for mouse ANO1/TMEM16A (pEGFP-N1-mANO1, ac isoform) was generously provided by Dr. Min Goo Lee (Yonsei University, Korea). Plasmids were transiently transfected into human embryonic kidney (HEK) 293T cells using Lipofectamine Plus Reagent (Life Technologies, USA), according to the manufacturer's instructions. Briefly, HEK293T cells were cultured in DMEM supplemented

Fig. 1. Cultured ICCs from murine small intestine. Tunica muscularis of small bowel was digested with collagenase, and dispersed cells were cultured for 12 hours. This confocal microscope image shows Kit immunopositive ICCs in culture. Scale bar: 10 µm.

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with 10% fetal bovine serum and 0.5% penicillin-streptomycin at 37°C in a 90% O_2-10% CO_2 incubator. One day before transfection, cells were transferred on glass coverslips. For electrophysiological experiments, HEK293T cells were transfected with 0.9 μg of mANO1 plasmid and 0.1 μg of a plasmid expressing green fluorescent protein (pEGFP-N1). Cells transfected with 1 μg of pEGFP-N1 were used as negative controls. HEK-293T cells were routinely studied at 21-25°C, 24 h after transfection. The bath solution contained 146 mM HCl, 10 mM HEPES, 10 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 150 mM N-methyl-D-glucamine (NMDG), adjusted to pH 7.4. The pipette solution contained 150 mM NMDG-Cl, 1 mM $MgCl_2$, 3 mM MgATP, 10 mM EGTA, 5 mM $CaCl_2$ and 5 mM HEPES at pH 7.2 (titrated with NMDG). The free calcium concentration was fixed at 200 nM using WEBMAX-C software (C. Patton, Stanford University, www.stanford. edu/~cpatton/maxc.html).

TRPM7 Expression in HEK-293T cells

HEK-293T cells transfected with the Flag-murine LTRPC7/pCDNA4-TO construct were grown on glass coverslips in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, blasticidin (5 μg/ml), and zeocin (0.4 mg/ml). TRPM7 (LTRPC7) expression was induced by adding 1 μg/ml tetracycline to culture medium. Whole-cell patch-clamp experiments were performed at 21-25°C 24 h after induction using cells grown on glass coverslips. The internal pipette solution for whole cell current recordings contained (in mM) 145 Cs-methanesulfonate, 8 NaCl, 10 Cs-2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, and 10 HEPES-CsOH adjusted to pH 7.2 with CsOH. The standard extracellular Tyrode's solution contained (in mM) 145 NaCl, 2.8 KCl, 2 CaCl2, 1.2 MgCl2, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH. All experiments were performed at 21–25°C.

cAMP and cGMP assay

ICCs were preincubated with 100 μM IBMX for 30 min at 37°C to inhibit cAMP and cGMP degradation and then incubated with quercetin (200 μM) for 10 min. Cells were then homogenized in buffer containing 4 mM EDTA to prevent the degradations of enzymatic cAMP or cGMP and homogenates were then heated for 5 min in a boiling water bath to coagulate protein. After centrifugation at 3,000 rpm for 5 min, supernatants were transferred to new tubes and stored at 4°C. Samples were assayed for cAMP or cGMP using cAMP or cGMP ELISA kits (Enzo Life Science, Farmingdale, NY, USA).

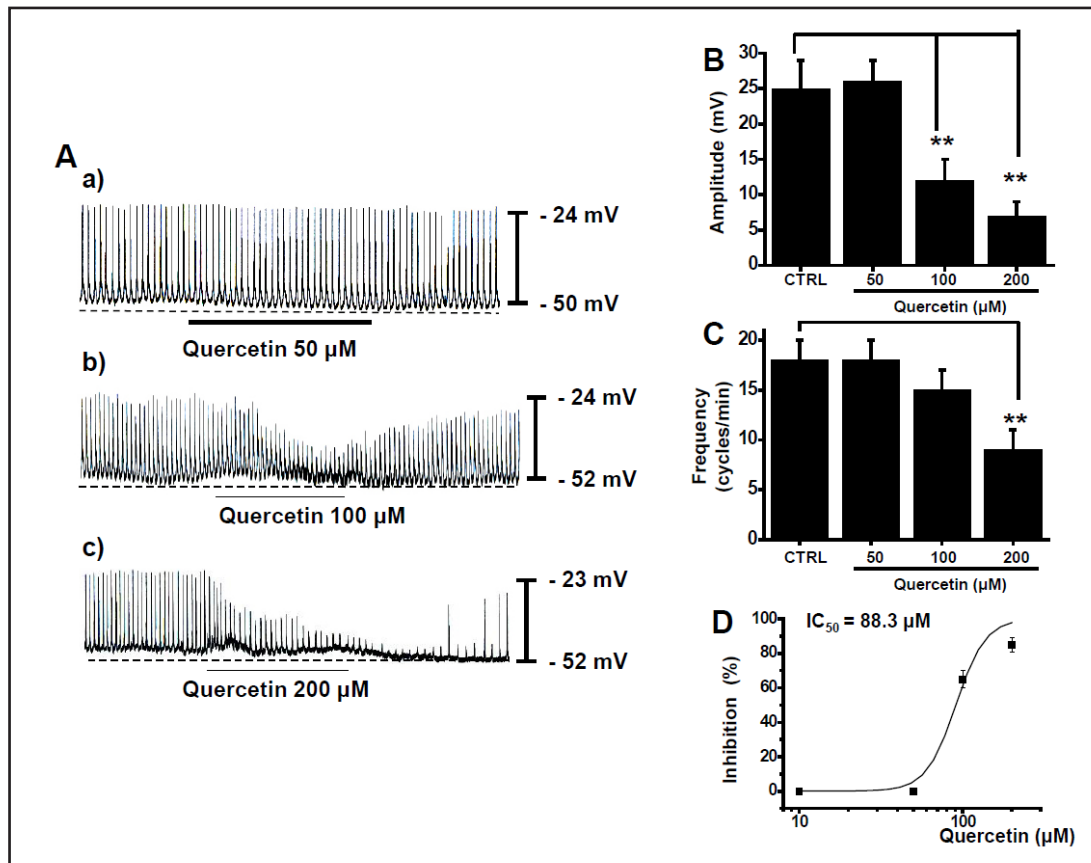### Measurement of Evans blue ITRs

The effects of quercetin on intestinal motility were assessed by measuring the intestinal transit rates (ITRs) of Evans blue solution (5 %, w/v, in DW). Briefly, 30 min after the intragastric (i.g.) administration of quercetin to normal mice, 0.1 ml of Evans blue solution was administered i.g. through an orogastric tube. Animals were sacrificed 30 min after this administration, and intestinal transit was determined by measuring the distance that the Evans blue had migrated in intestines from the pylorus. ITR (%) were calculated by expressing the distance traveled by the dye as a percentage of the total length of small intestine (from the pylorus to the ileal end). To minimize possible inter-day variations, all measurements were performed on same days.

Drugs

Quercetin was purchased from Alexis Biochemicals (Vinci Biochem, Florence, Italy) and naloxone from TOCRIS (USA). All other drugs were obtained from Sigma. Stock solutions were prepared and stored, according to the manufacturer's instructions. Chemicals were dissolved in Na^+-Tyrode solution to their final concentrations immediately before use.

Statistical analysis

Results are expressed as means ± SEMs. Statistical analysis was performed using the student's *t* test or by analysis of variance (ANOVA) followed by Tukey's multiple comparison test, as appropriate. The analysis was performed using GraphPad Prism version 6, and *p* values of <0.05 were considered statistically significant. The *n* values reported in the text refer to the number of cells used in patch-clamp experiments.

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Fig. 2. Effects of quercetin on the pacemaker activity of cultured ICC clusters. (A) Pacemaker activities of ICCs exposed to quercetin (50-200 μM) in current-clamp mode (I=0). Quercetin decreased pacemaker amplitude and frequency in a concentration-dependent manner. Responses to quercetin are summarized in (B and C). (D) Concentration-dependent inhibition of ICC pacemaker activity by quercetin. The estimated median inhibitory concentration value for quercetin was 88.3 μM. Bars represent means±SEs. CTRL: Control. **$P<0.01$.
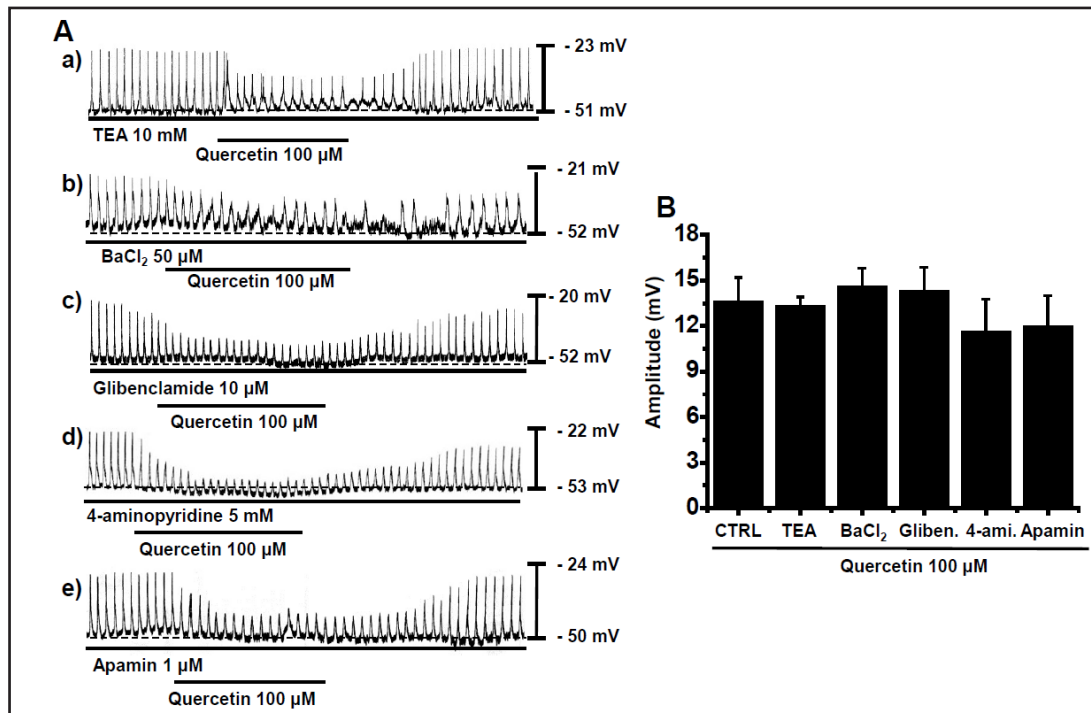
Results

Inhibitory effects of quercetin on the pacemaker activities of cultured ICCs

Cultured ICC clusters had a mean resting membrane potential of -51 ± 5 mV and produced electrical pacemaker activity at a frequency of 17 ± 2 cycles per minute and an amplitude of 25 ± 4 mV (n = 40) at 30°C in current clamp mode. Quercetin at concentrations from 100 to 200 μM decreased pacemaker amplitude and frequency in a concentration-dependent manner with an IC_{50} of ~88.3 μM (Fig. 2).

Effects of potassium channel blockers on pacemaker activity inhibition by quercetin

To investigate which potassium channels mediate quercetin-induced pacemaker activity, we used various potassium channel blocker types. The treatment of ICCs with tetraethylammonium chloride (TEA) (10 mM; a Ca^{2+}-activated K^+ channel blocker) or $BaCl_2$ (50 μM; an inward rectifying K^+ channel blocker) had no effect on pacemaker activities, and in the presence of TEA or $BaCl_2$, quercetin still inhibited pacemaker activities (n = 5; Fig. 3Aa, 3Ab and 3B). In addition, treatments with glibenclamide (10 μM; an ATP-sensitive K^+ channel blocker), 4-aminopyridine (5 mM; a transient voltage-dependent K^+ channel blocker), or apamin (1 μM; a Ca^{2+}-activated K^+ channel blocker) had no effect on pacemaker activity,

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Fig. 3. Effects of TEA, $BaCl_2$, glibenclamide, 4-aminopyridine, and apamin on pacemaker activity inhibition by quercetin in cultured ICC clusters. Pretreatment with (Aa) TEA (10 mM), (Ab) $BaCl_2$ (50 µM), (Ac) gliben-clamide (10 µM), (Ad) 4-aminopyridine (5 mM), or (Ae) apamin (1 µM) did not affect the inhibitory effects of quercetin. Responses to quercetin in the presence of potassium channel blockers are summarized in (B). TEA: tetraethylammonium chloride.

Fig. 4. Effects of naloxone, a pan opioid receptor antago-nist, on pacemaker activity in-hibition by quercetin in cultu-red ICC clusters. (A) Effects of naloxone (a pan opioid recep-tor antagonist) on pacemaker activity inhibition by querce-tin. Naloxone blocked the in-hibitory effects of quercetin. (B) Responses to quercetin in the presence of naloxone are summarized. Bars represent means±SEs. CTRL: Control. **$P<0.01$.
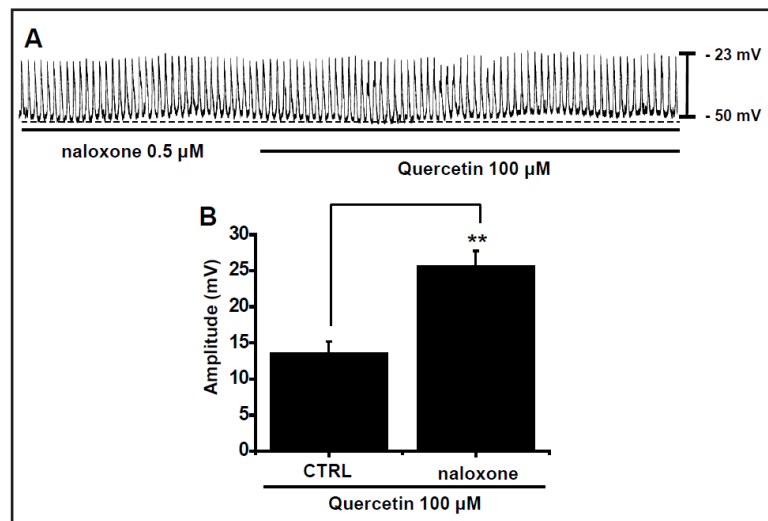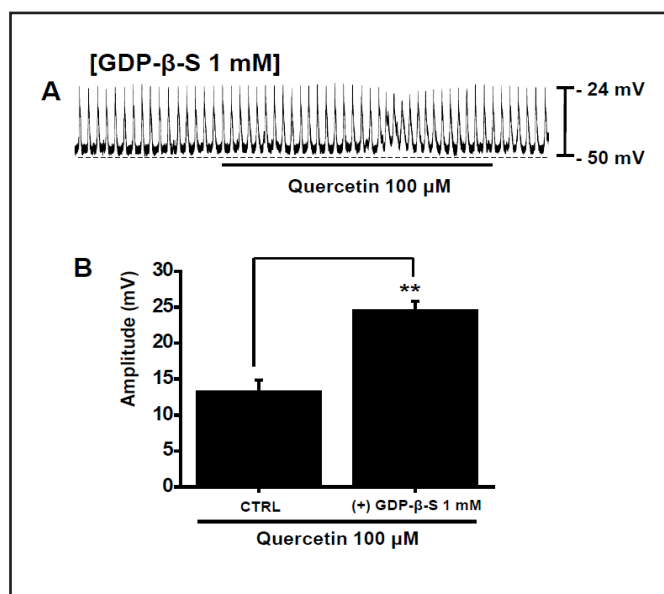

and quercetin still inhibited pacemaker activities when co-treated with glibenclamide, 4-aminopyridine, or apamin (n = 5; Fig. 3Ac, 3Ad, 3Ae and 3B).

Effects of opioid receptors on pacemaker activity inhibition by quercetin

Opioid receptor antagonists, such as, naloxone, are used to treat bowel diseases [47], and numerous studies have described the pharmacological effects of naloxone on GI motility *in vitro* in guinea pigs, rodents, and humans [48-52]. Therefore, we investigated the relationship between quercetin and opioid receptors by examining the effect of naloxone (a

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Fig. 5. Effects of GDPβS in the pipette on pacemaker activity inhibition by quercetin in cultured ICC clusters. (A) Pacemaker activities of ICCs exposed to quercetin in the presence of GDPβS (1 mM) in the pipette solution. Under these conditions, quercetin had no effect on pacemaker activity. (B) Responses to quercetin in the presence of GDPβS in the pipette are summarized. Bars represent means±SEs. CTRL: Control. **$P<0.01$.
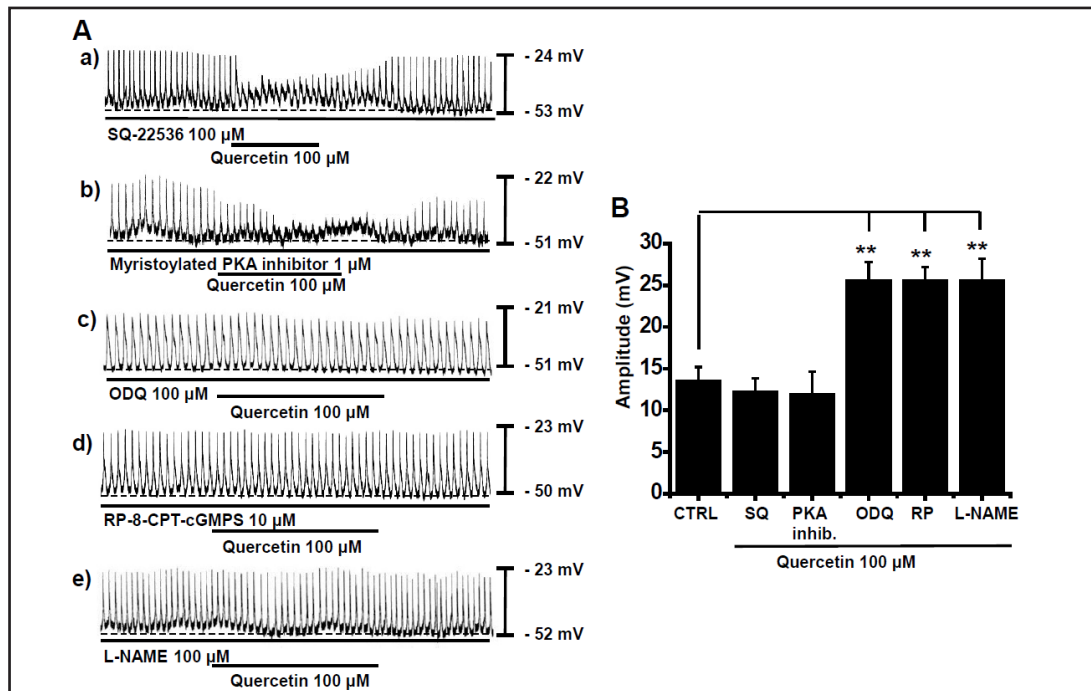

pan-opioid receptor antagonist) on pacemaker activity inhibition by quercetin. In the absence of naloxone, quercetin decreased the pacemaker activity amplitude and frequency (Fig. 2), but naloxone prevented pacemaker activity inhibition by quercetin (Fig. 4A) (pacemaker activity amplitude was 24 ± 4 mV in the presence of naloxone (Fig. 4B)).

*Involvement of G proteins in pacemaker activity inhibition by quercetin*

To investigate the signaling mechanisms involved and the roles played by G proteins during pacemaker activity inhibition by quercetin, we applied GDPβS (1 mM; a non-hydrolysable guanosine 5′-diphosphate analogue that permanently inactivates GTP binding proteins [53, 54]) to the patch pipette solution, and this resulted in the prevention of pacemaker activity inhibition by quercetin (n = 5; Fig. 5A). Mean depolarizations were 24.6 ± 1.2 mV and in the presence and absence of GDPβS (Fig. 5B).

Involvement of guanylate cyclase, protein kinase G and nitric oxide in pacemaker activity inhibition by quercetin

To investigate whether pacemaker activity inhibition by quercetin is mediated by a cyclic nucleotide-dependent pathways, we used SQ-22536, an inhibitor of adenylate cyclase, and 1H-[1, 2, 4]oxadiazolo[4, 3-a]quinoxalin-1-one (ODQ), an inhibitor of guanylate cyclase. Preincubation of ICCs with SQ-22536 (100 μM) for 10 min had no effect on pacemaker activity, and in the presence of SQ-22536, quercetin (100 μM) still inhibited pacemaker activity (n = 6; Fig. 6Aa and 6B). However, ODQ (100 μM) blocked pacemaker activity inhibition by quercetin (n = 6; Fig. 6Ac and 6B). Also, to see whether the pacemaker activity inhibition by quercetin was mediated by cAMP-dependent protein kinase A (PKA) or cGMP-dependent protein kinase G (PKG), we examined the effects of myristoylated PKA inhibitor (an inhibitor of PKA) and RP-8-CPT-cGMPS (an inhibitor of PKG). Preincubation of ICCs with myristoylated PKA inhibitor (1 μM) had no effect on pacemaker activities, and in the presence of myristoylated PKA inhibitor, quercetin inhibited pacemaker activities (n = 5; Fig. 6Ab and 6B). However, in the presence of RP-8-CPT-cGMPS (10 μM), quercetin failed to inhibit pacemaker activities (n = 5; Fig. 6Ad and 6B). Nitric oxide (NO) activates soluble guanylyl cyclase, which results in the formation of cGMP and in the activation of PKG [55]. To investigate whether pacemaker activity inhibition by quercetin is mediated by NO, we added L-NAME (100 μM; a non-selective NO synthases (NOS)) inhibitor, and found that it blocked pacemaker activity inhibition by quercetin (n = 5; Fig. 6Ae and 6B). In addition,

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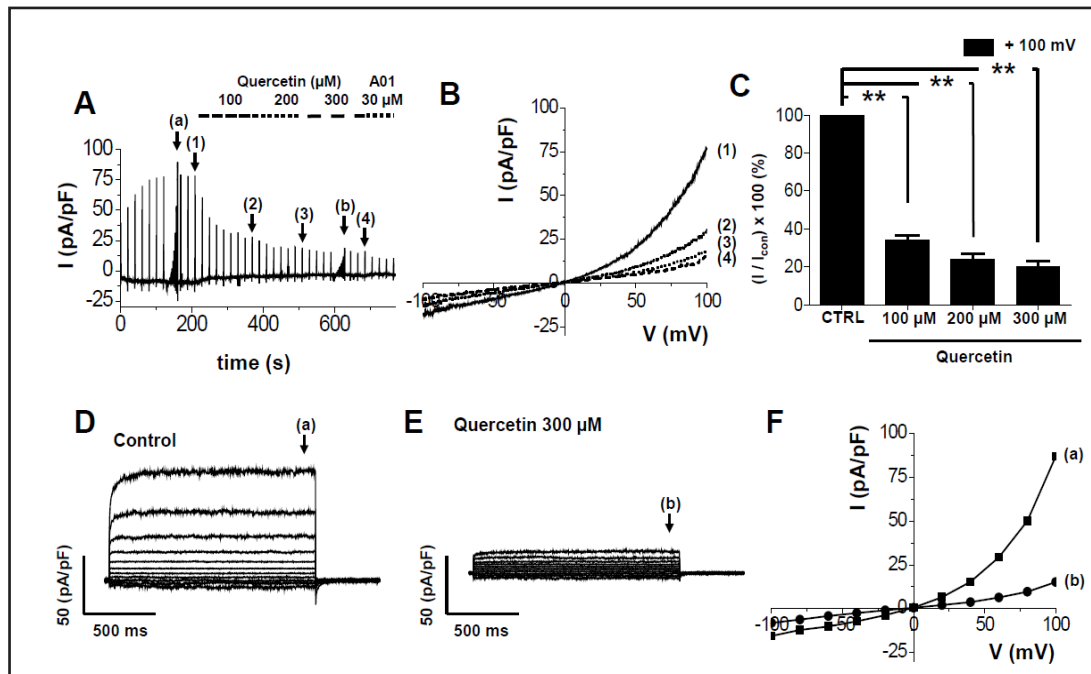Gim et al.: Effects of Quercetin on ICCs

Fig. 6. Effects of SQ-22536 (an inhibitor of adenylate cyclase), ODQ (an inhibitor of guanylate cyclase), myristoylated PKA inhibitor, RP-8-CPT-cGMPS (an inhibitor of PKG) and L-NAME (a non-selective NO synthases (NOS) inhibitor) on pacemaker activity inhibition by quercetin in cultured ICC clusters. (Aa) Pacemaker activities of ICCs exposed to quercetin in the presence of SQ-22536 (100 µM). SQ-22536 had no effects on the inhibitory effects of quercetin on pacemaker activity. (Ab) Treatment with myristoylated PKA inhibitor (1 µM) had no effect on pacemaker activity inhibition by quercetin and under these conditions, this inhibition was not suppressed by a myristoylated PKA inhibitor. Pacemaker activities of ICCs exposed to quercetin in the presence of (Ac) ODQ (100 µM), (Ad) RP-8-CPT-cGMPS (10 µM), or (Ae) L-NAME (100 µM). ODQ, RP-8-CPT-cGMPS, or L-NAME blocked pacemaker activity inhibition by quercetin. (B) Responses to quercetin in the presence of SQ-22536, ODQ, myristoylated PKA inhibitor, RP-8-CPT-cGMPS, and L-NAME are summarized. Bars represent means±SEs. CTRL: Control. **$P<0.01$.
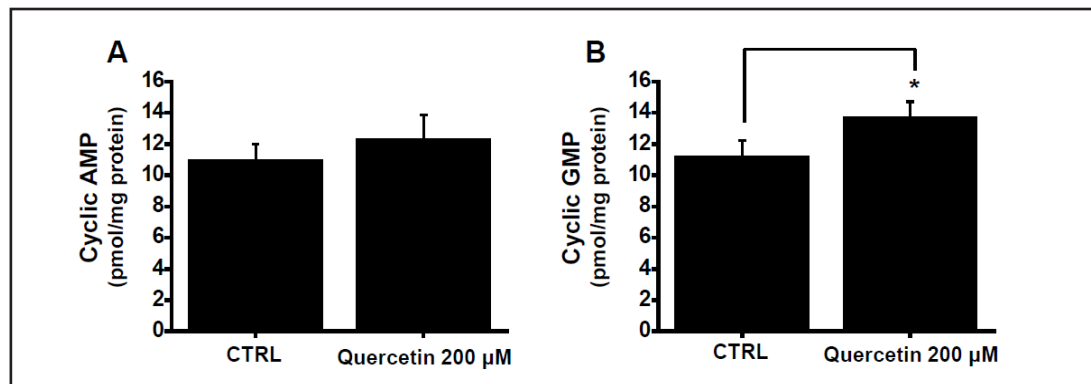
Fig. 7. Effects of cAMP and cGMP production on pacemaker activity inhibition by quercetin in cultured ICC clusters. (A) Preincubation of ICC with quercetin (200 µM) did not significantly stimulate cAMP production. (B) However, preincubation of ICC plus quercetin (200 µM) significantly stimulated cGMP production. Bars represent means ± SEs. CTRL: Control. *$P<0.05$.

to determine whether changes in cAMP and cGMP contents are involved in the inhibitory effect of quercetin on ICCs, intracellular cAMP and cGMP contents were measured under basal and quercetin-stimulated conditions. Quercetin did not stimulate cAMP production

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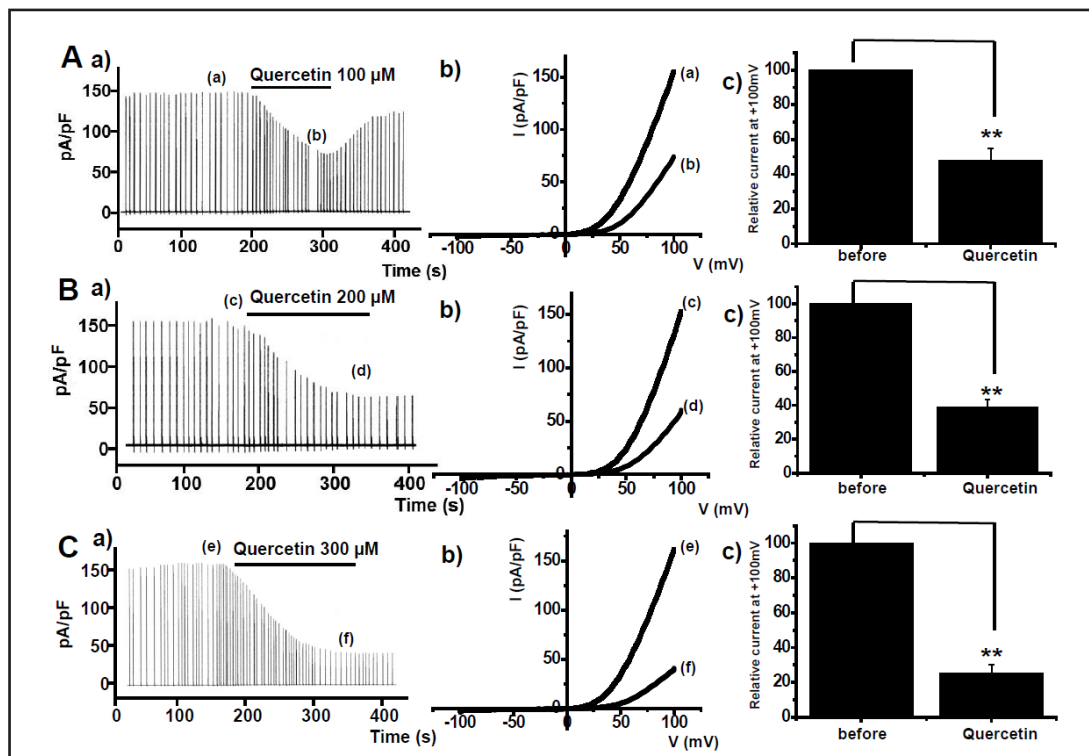Gim et al.: Effects of Quercetin on ICCs

Fig. 8. Effect of quercetin on I_{ANO1} currents in ANO1 overexpressing HEK 293 cells and on the current-voltage (I-V) relationship using the whole cell patch-clamp technique. (A) Representative trace of I_{ANO1} inhibition by quercetin. After confirming I_{ANO1} activation, quercetin was applied at 100, 200, or 300 μM to hANO1-overexpressing HEK293T cells. (B) Representative I-V relationship curve at peak I_{ANO1} current (1), and after treatment with 100 (2), 200 (3), or 300 (4) μM of quercetin. (C) Summary of the inhibition rates of I_{ANO1} at +100 mV. **$P < 0.01$ *vs.* the CTRL (control). (D) Representative raw current traces obtained from step-pulses at point (a) in Fig. 7A, which is the steady-state peak current of I_{ANO1}. Voltage steps (1500 ms) were used from a holding potential of -60 mV to voltages between -100 and +100 mV in 20 mV increments, followed by a step to -60 mV. (E) Representative raw current traces obtained from step pulses at point (b) in Fig. 7A, which is the I_{ANO1} steady-state current obtained in the presence of 300 μM quercetin. The same step pulse protocol is shown in Fig. 7D. (F) Steady-state current-voltage relationships for the control and treatment with 300 μM quercetin. I-V relationships were constructed from currents recorded at the points shown in Fig. 7D (a) and Fig. 7E (b).

(Fig. 7A; control: 11.1 ± 1.2 vs. quercetin: 12.3 ± 1.4 pmol/mg-1 protein), but stimulated cGMP production (Fig. 7B; control: 11.3 ± 1.1 vs. quercetin: 13.8 ± 1.3 pmol/mg-1 protein).

Involvements of ANO1 and TRPM7 channels during pacemaker activity inhibition by quercetin

It has been reported ANO1 and TRPM7 are involved in slow wave generation and that they are required for ICC pacemaker activity in the murine small intestine [24, 35]. Furthermore, TRPM7 and ANO1 mRNA have been reported in ICCs and TRPM7 and ANO1 currents have been described [24, 35, 40, 41]. Thus, we examined the effects of quercetin on the expressions of ANO1 and TRPM7 channels in HEK 293 cells, which overexpress both channels. To investigate whether ANO1 channels are involved in the pacemaker activity of ICCs, we performed whole-cell patch clamp recordings on hANO1-transfected HEK293T cells using a 200 nM free Ca^{2+} pipette solution to activate ANO1 currents (I_{ANO1}). Fig. 8A shows representative I_{ANO1} recordings and its inhibition by different concentrations of quercetin. After membrane break-in, an outward rectifying Cl^- current was immediately activated by diffusion of the pipette solution. After confirming a steady-state current level, we applied depolarizing step pulses from -100 mV to + 100 mV (Fig. 8A (a)). Using step depolarization, delayed activation of outward currents and a deactivation tail current were observed, which

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Fig. 9. Effects of quercetin on TRPM7 currents in TRPM7 overexpressing HEK 293 cells and current-voltage (I-V) relationship obtained using the whole cell patch-clamp technique. (Aa, Ba and Ca) Quercetin inhibited TRPM7 currents in TRPM7 overexpressing HEK 293 cells. Slow ramp depolarizations from +100 to -100 mV were applied from a holding potential of -60 mV before (a) and during (b) treatment with quercetin. (Ab, Bb and Cb) I-V relationships were determined before and during treatment. (Ac, Bc and Cc) Responses to quercetin during TRPM7 currents are summarized. Bars represent means±SEs. **$P<0.01$.

are typical characteristics of I_{ANO1} (Fig. 8D). Fig. 8F (a) shows the steady-state I-V relationship from step depolarization of the untreated control (black arrow). Next, we serially perfused cells with quercetin-containing bath solution (100, 200, or 300 μM). Quercetin significantly inhibited I_{ANO1} currents. The associated I-V relationship curves at peak I_{ANO1} (1) and at quercetin concentrations of 100 (2), 200 (3), or 300 μM (4) were obtained using a ramp-like pulse protocol from -100 mV to + 100 mV (Fig. 8B). After treatment with 300 μM quercetin, we again applied the step-pulse, and found that 300 μM quercetin almost completely inhibited I_{ANO1} current (Fig. 8E). Relative steady-state I-V relationships are presented in Fig. 8F (b). To compare inhibitory effects on ANO1, we treated cells with 30 μM T16Ainh-AO1 (a selective ANO1 inhibitor) at the end of the experiment as a positive control. To determine inhibitory percentages at each concentration, the normalized amplitudes of the currents ($I_{ext}/I_{con} \times 100\%$) of quercetin-treated cells were measured at +100 mV. As shown in Fig. 8C, quercetin significantly inhibited I_{ANO1} in a dose-dependent manner. TRPM7 currents were elicited by voltage ramps ranging from −100 to +100 mV from a holding potential of −60 mV. After break-in, 2–3 min was allowed for current amplitudes to reach the steady state. The currents displayed a profound outwardly rectifying I–V relationship in Ca^{2+}-containing normal bath solution (normal bath), presumably due to the relatively small inward current carried by Ca^{2+} compared to the large outward current carried by Cs^+ [56, 57]. In order to investigate the regulatory effects of quercetin on TRPM7 currents, we applied quercetin at different concentrations. Application of quercetin (100-300 μM) to HEK cells transfected with TRPM7 elicited a rapid decrease in outward and inward whole-cell currents (Figs. 9Aa, 9Ba and 9Ca). The time course of outward current is plotted at +100 mV and inward current at −100 mV for respective cells (n = 7; Figs. 9Ab, 9Bb and 9Cb). These effects were

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Fig. 10. Effects of quercetin on intestinal transit rate (ITR) in normal mice. The ITR (%) values of Evans blue were measured 30 min after the *p.o.* administration of an Evans blue solution 30 min administering quercetin to normal mice. CTRL: Control. PF: *Poncirus trifoliata* Raf. i.g.: intragastrically. **$P<0.01$.
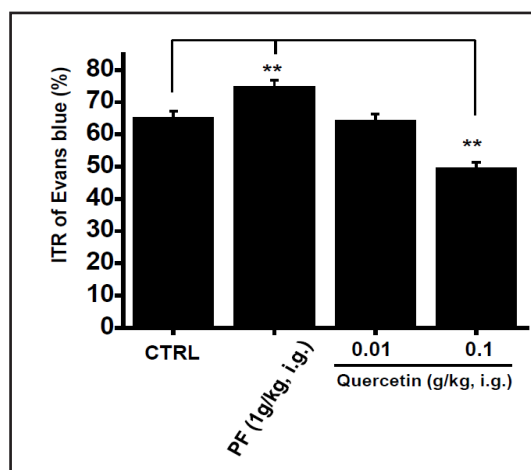
reproducible and fully reversible, and repeated application of quercetin yielded similar reductions in current amplitude. Mean peak inward and outward current densities before and after quercetin application were −2.5 ± 0.5 pA/pF and −1.0 ± 0.3 pA/pF at −100 mV and +155.2 ± 3.2 pA/pF and +74.5 ± 2.1 pA/pF at +100 mV for quercetin at 100 μM, −4.1 ± 0.3 pA/pF and −1.7 ± 0.2 pA/pF at −100 mV and +153.4 ± 1.5 pA/pF and +60.3 ± 2.5 pA/pF at +100 mV for quercetin at 200 μM, and −2.6 ± 0.5 pA/pF and −1.0 ± 0.5 pA/pF at −100 mV and +161.4 ± 2.3 pA/pF and +41.4 ± 2.2 pA/pF at +100 mV for quercetin at 300 μM, respectively (n = 8, mean ± SEM; Fig. 9).

Effects of quercetin on ITR in normal mice

The ITRs (%) of Evans blue over 30 min in normal mice are shown in Fig. 10. The ITR for untreated normal mice (control, n = 25) was 65.3 ± 2.1 %. To examine GI motility regulation by quercetin, the aqueous extract of the dried immature fruits of Poncirus trifoliata Raf. (PF) was used. PF is one of the most popular traditional folk medicines in Korea as is derived from the Rutaceae fruits. It has been shown to have unique and potent prokinetic activities in normal rodents and in rodent models of GI motility dysfunction (GMD) [58, 59]. PF significantly increased ITR (%) at a dose of 1 g/kg (by 75.1 ± 1.8 %, p<0.01), which is consistent with previous reports [60, 61]. However, the ITR values for quercetin administered at 10 and 100 mg/kg doses were 64.5 ± 1.9 and 49.8 ± 1.7 %, respectively (n = 10; Fig. 10), that is, quercetin reduced the ITR.

Discussion

In the present study, we found that quercetin decreased the amplitude and frequency of ICC pacemaker activity via a series of opioid receptor signaling pathways, including G protein-cGMP-PKG-NO activation. As ICC pacemaker activities are mainly due to periodic activations of TRPM7 channels [24, 33] or ANO1 channels [35, 37, 38], TRPM7 and ANO1 modulators are potential targets for the treatment of GI motility disorders. In the present study, quercetin inhibited TRPM7 and ANO1 currents, indicating that the TRPM7 and ANO1 channels are involved in the quercetin-mediated inhibition of pacemaker activity in ICCs. In addition, *in vivo*, quercetin (10-100 mg/kg, *p.o.*) dose-dependently decreased ITR in normal mice.

With regard to intestinal motility, quercetin has been reported to inhibit the peristalsis of guinea pig ileum *in vitro* [62] and to depress intestinal motility in a concentration-dependent manner by facilitating inhibitory enteric pathways [63]. Furthermore, quercetin was found to be associated with decreases in some inflammation markers and changes in gut microbiota when administered to healthy mice [64]. In the present study, quercetin inhibited ICCs pacemaker activity and suppressed ITR in mice.

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In a previous study, we found that ANO1 was well expressed in ICCs within 12 hours of culture, but subsequently decreased with time [40]. Therefore, in the present study, we used ICCs that had been cultured for least than 12 hours (Fig. 1). Previous reports have shown TRPM7 and ANO1 mRNA are present and TRPM7 and ANO1 currents are expressed in cultured ICCs [24, 35, 40, 41], and thus, we examined the effects of quercetin on ANO1 and TRPM7 channels in HEK 293 cells, which overexpress both channels. Quercetin is the most abundant flavonoid and decreases the ICC pacemaker activity (Fig. 2) and induces G protein-cGMP-PKG-NO sensitive pacemaker potentials (Fig, 5 and 6). We are of the opinion that quercetin might decrease GI motility at the tissue level based on the observations that; 1) quercetin inhibited TRPM7 and ANO1 channels and 2) decreased the pacemaker activities of cluster type ICCs.

We believe that TRPM7 and ANO1 are involved in the upstroke of pacemaker potentials. In ICCs, inhibitory neurotransmitters induce hyperpolarization and reduce the amplitude and frequency of pacemaker activity. In the present study, quercetin also reduced the amplitude and frequency of pacemaker activity. Therefore, we believe that quercetin may have the same functions as inhibitory neurotransmitter. Specific details of ion channel mechanisms during the upstroke and plateau phase of pacemaker potentials have not been elucidated. Therefore, it is difficult to determine with precision the effects of quercetin on pacemaker potentials, additional studies are required to identify the ion channels involved. In addition, we cannot explain the exact mechanism underlying the reduction of motility rate induced by quercetin. However, we suppose because quercetin inhibited TRPM7 and ANO1 channels and decreased pacemaker activity in cluster type ICCs that this inhibition might be due to inhibition of smooth muscle or the enteric nervous system.

In conclusion, we found that quercetin reduced the amplitude and frequency of ICC pacemaker activity in a G protein-, cGMP-, PKG-, and NO-dependent manner via opioid receptor activation. Furthermore, TRPM7 and ANO1 were found to be involved in the quercetin-induced regulation of pacemaker activity in cultured murine ICCs. Finally, our findings suggest that quercetin should be viewed as a novel drug development candidate for the treatment of GI spasm, pain, transit disturbances, and other symptoms related to GI motility disorders.

Acknowledgements

Disclosure Statement

The authors have no potential conflict of interest to declare.

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