



Original Article

Naringenin inhibits pacemaking activity in interstitial cells of Cajal from murine small intestine

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ABSTRACT

Background: Naringenin (NRG) is a common dietary polyphenolic constituent of fruits. NRG has diverse pharmacological activities, and is used in traditional medicine to treat various diseases including gastrointestinal (GI) disorders. Interstitial cells of Cajal (ICCs) are pacemaker cells of the GI tract. In this study, the authors investigated the effects of NRG on ICCs and on GI motility *in vitro* and *in vivo*.

Methods: ICCs were dissociated from mouse small intestines by enzymatic digestion. The whole-cell patch clamp configuration was used to record pacemaker potentials in cultured ICC clusters. The effects of NRG on GI motility were investigated by calculating percent intestinal transit rates (ITR) using Evans blue in normal mice.

Results: NRG inhibited ICC pacemaker potentials in a dose-dependent manner. In the presence of tetraethylammonium chloride or iberiotoxin, NRG had no effect on pacemaker potentials, but it continued to block pacemaker potentials in the presence of glibenclamide. Preincubation with SQ-22536 had no effect on pacemaker potentials or on their inhibition by NRG. However, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one blocked pacemaker potential inhibition by NRG. In addition, L-NG-nitroarginine methyl ester blocked pacemaker potential inhibition by NRG. Furthermore, NRG significantly suppressed murine ITR enhancement by neostigmine *in vivo*.

Conclusion: This study shows NRG dose-dependently inhibits ICC pacemaker potentials via a cyclic guanosine monophosphate/nitric oxide-dependent pathway and Ca²⁺-activated K⁺ channels *in vitro*. In addition, NRG suppressed neostigmine enhancement of ITR *in vivo*.

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1. Introduction

Natural products derived from foods and traditional herbs have been traditionally used to treat infections, inflammatory and gastrointestinal (GI) diseases, and other disease types.^{1–3} Naringenin [NRG; 5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one] is a type of natural flavonoid, and is found in grapefruit, bitter orange, and other fruits.² NRG has wide range of biological and pharmacological activities, which include anti-inflammatory,⁴ antimutagenic,⁵ antiatherogenic,⁶ antioxidant,⁷ and anticancer⁸ effects, and has a relaxant effect on vascular smooth muscle⁹ and a regulatory effect on GI function.^{10,11}

Interstitial cells of Cajal (ICCs) are the pacemaker cells of GI muscularis propria, and are involved in the generation of primary electrical pacemaker activity, which controls GI motility.^{12–14} These cells also form a bridge between enteric motor nerve terminals and smooth muscle cells.¹⁴ Because of the central role played by ICCs in GI motility, loss of these cells is extremely detrimental to GI functions.¹⁵

As the effects of NRG on ICCs and GI motility have not been previously investigated, we undertook to investigate its effects on the pacemaker potentials of clusters of murine small intestine ICCs *in vitro* and on GI motor functions *in vivo* by measuring intestinal transit rates (ITRs) in Evans blue in mice.

2. Methods

2.1. Ethics

Animal care and experiments were conducted in accordance with the guidelines issued by the ethics committee of Pusan National University (Busan, Korea; Approval no. PNU-2016-1370) and those issued by the National Institute of Health 'Guide for the Care and Use of Laboratory Animals' (2013).

2.2. Preparation of cells and cell cultures

BALB/c mice (aged 2–6 days; weight, 2.1–2.3 g; Samtako Bio Korea Co., Ltd., Osan, Korea) were anesthetized with 0.1% ether (Sigma–Aldrich, St Louis, MO, USA; Merck Millipore, Darmstadt, Germany) and sacrificed by cervical dislocation. Mice were maintained under controlled conditions ($21 \pm 2^\circ\text{C}$; relative humidity $50 \pm 5\%$; 12-h light/dark cycle) and allowed free access to food and water. Small intestines were removed and opened along the mesenteric border, and luminal contents were removed by washing with Krebs–Ringer bicarbonate solution. Small intestine mucosae were removed by sharp dissection and small strips of intestine muscle were equilibrated in Ca^{2+} -free physiological salt solution (125 mM NaCl, 5.36 mM KCl, 0.34 mM NaOH, 0.44 mM Na_2HCO_3 , 10 mM glucose, 2.9 mM sucrose, and 11 mM HEPES buffer) for 20 min and then dispersed using an enzyme solution containing 1.5 mg/mL collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA), 2.5 mg/mL bovine serum albumin (Sigma–Aldrich; Merck Millipore), 2.5 mg/mL trypsin inhibitor (Sigma–Aldrich; Merck Millipore), and 0.5 mg/mL adenosine triphosphate (ATP; Sigma–Aldrich; Merck Millipore). Cells were plated on

glass coverslips coated with 0.01% poly-L-lysine solution (Sigma–Aldrich; Merck Millipore) and cultured in a 95% O_2 /5% CO_2 atmosphere in smooth muscle basal medium (Clonetics Corp.; Lonza, Walkersville, MA, USA) supplemented with stem cell factor (5 ng/mL; Sigma–Aldrich; Merck Millipore) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C .

2.3. Patch-clamp experiments

The cells were transferred onto a solution chamber on the stage of an invert microscope (IX70; Olympus, Tokyo, Japan). The whole-cell patch-clamp technique was used to record the membrane potentials (current clamp) of cultured ICC as described previously,^{6,7} and an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) was used to amplify membrane potentials. Bath solution (Normal Tyrode) contained 135 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose, and 10 mM HEPES with a pH that was adjusted to 7.4 using NaOH. The internal solution contained 145 mM Cs-glutamate, 8 mM NaCl, 10 mM BAPTA, and 10 mM HEPES-CsOH, with pH adjusted to 7.2 with CsOH. All experiments were performed at 30°C .

2.4. In vivo intestine motility measurements

Mice were randomly allocated into four groups: the normal group (saline controls); neostigmine (0.2 mg/kg) group; neostigmine and low-dose naringenin (10 mg/kg) group; and the neostigmine and high-dose naringenin (20 mg/kg) group. Animals were fasted for 12 h prior to the intraperitoneal administration of drugs or saline solution. Immediately after treatment, 0.5 mL of Evans blue was administered by gavage. Mice were sacrificed 30 min after Evans blue administration and entire intestines (from pylorus to the anus (anal ring?)) were excised. Total intestinal lengths and distances traveled by Evans blue were then measured. Intestine transit rate (ITR) was defined as the distance traveled by Evans blue expressed as a percentage of total intestinal length, as described elsewhere.^{10,16,17}

2.5. Drugs

Drugs were purchased from Sigma–Aldrich. To produce stock solutions, all drugs were dissolved in distilled water or dimethylsulfoxide (DMSO) and stored at -20°C . The final concentration of DMSO in bath solution was always $<0.1\%$, and at this level DMSO did not affect the results.

2.6. Statistics

Results are expressed as mean \pm standard error. The analysis was performed using the Student t test or by analysis of variance (ANOVA) followed by Tukey's multiple comparison test, as appropriate, using GraphPad Prism version 6. Statistical significance was accepted for p values <0.05 . The n values reported in the text refer to the number of cells used in patch-clamp experiments

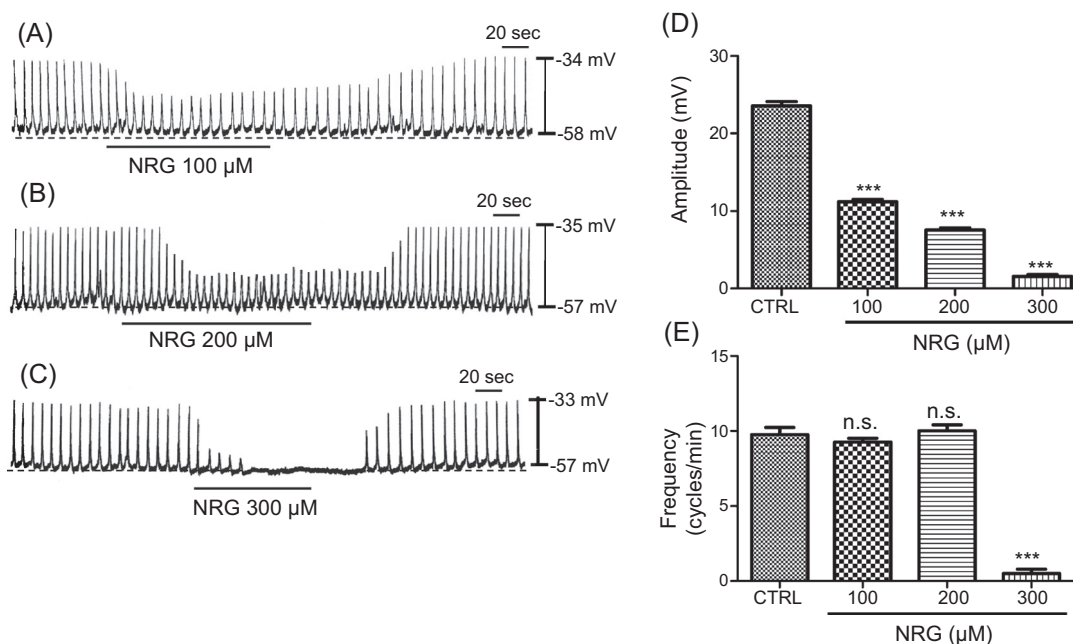


Fig. 1 – The effect of NRG on the pacemaker potentials of cultured interstitial cells of Cajal. (A–C) Pacemaker activities of ICCs exposed to NRG (100–300 μM) in current-clamp mode ($I=0$). NRG inhibited PP amplitudes in a concentration-dependent manner. (D, E) The graphs summarize responses to NRG. Bars represent mean \pm standard error. * $p < 0.001$. CTRL, control; NRG, naringenin; n.s., not significant.**

3. Results

3.1. Effects of NRG on ICC pacemaker potentials

Spontaneous pacemaker potentials (PPs) were generated by cultured ICCs in current clamp mode ($I=0$). Under current clamp conditions, NRG (100–300 μM) inhibited spontaneous PPs (Fig. 1A–C). The mean amplitude or frequency of ICC PPs in the absence of NRG was 23.5 ± 1.7 mV or 9.8 ± 0.9 cycles/min ($n=8$), but after treatment with NRG, mean PP amplitude or frequency changed to 11.2 ± 0.9 mV or 9.2 ± 0.5 cycles/min at 100 μM , 7.5 ± 0.7 mV or 10.1 ± 0.8 cycles/min at 200 μM , and 1.6 ± 0.8 mV or 0.5 ± 0.6 cycles/min at 300 μM ($n=7-9$, Fig. 1D, E). These results suggest that NRG dose-dependently inhibits ICC PPs.

3.2. The effect of potassium channel blockers on PP inhibition by NRG

Various types of potassium channel blockers were used to identify the potassium channels responsible for NRG-induced PP inhibition. Treatment of ICC with 1 mM tetraethylammonium chloride (TEA, a Ca^{2+} -activated K^+ channel blocker) or iberiotoxin (100 μM) had no effect on the pacemaker activities of treatment naïve ICCs. In the presence of TEA or iberiotoxin, NRG had no effects on PPs ($n=5$; Fig. 2B, C). Furthermore, glibenclamide (10 μM ; an ATP-sensitive K^+ channel blocker), did not affect the PPs of treatment naïve ICCs or PP inhibition by NRG ($n=6$, Fig. 2D). In the presence of NRG, PP amplitudes and frequency were: 22.3 ± 1.1 mV and 10.1 ± 0.8 cycles/min for TEA; 22.3 ± 1.0 mV and 9.6 ± 0.8 cycles/min for iberiotoxin;

and 1.5 ± 0.6 mV and 0.3 ± 0.5 cycles/min for glibenclamide ($n=6-8$; Fig. 2E, F). These results suggest that NRG activates Ca^{2+} -activated K^+ channel in ICCs.

3.3. Involvements of guanylate cyclase and nitric oxide in pacemaker potential inhibition by NRG

To determine whether PP inhibition by NRG is mediated by a cyclic nucleotide-dependent pathway, we investigated the effects of pretreating ICCs with SQ-22536 (a adenylate cyclase inhibitor) or 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (a guanylate cyclase inhibitor; ODQ). Preincubation with SQ-22536 (10 μM) for 10 min had no effect on PPs, and in the presence of SQ-22536, NRG (300 μM) inhibited PPs (Fig. 3B). However, ODQ (10 μM) blocked PP inhibition by NRG (Fig. 3C). Because nitric oxide (NO) activates soluble guanylyl cyclase, and thus results in the formation of cyclic guanosine monophosphate (cGMP),¹⁸ we treated ICCs with L-NAME (10 μM ; L-NG-nitroarginine methyl ester, a nonselective NO synthase inhibitor) to determine whether PP inhibition by NRG was mediated by NO. It was found that L-NAME blocked PP inhibition by NRG (Fig. 3D). Also, to confirm the involvement of NO, we applied NO donor, sodium nitroprusside (SNP) in PP. It was found SNP inhibited ICC PPs (Fig. 3E). In the presence of NRG, PP amplitudes or frequency were 1.3 ± 0.5 mV or 0.5 ± 0.6 cycles/min for SQ-22536, 22.1 ± 1.3 mV or 9.3 ± 0.5 cycles/min for ODQ, and 22.1 ± 1.2 mV or 9.8 ± 0.6 cycles/min for L-NAME ($n=5-7$; Fig. 3F, G). These results suggest that cGMP and NO participate in PP inhibition by NRG.

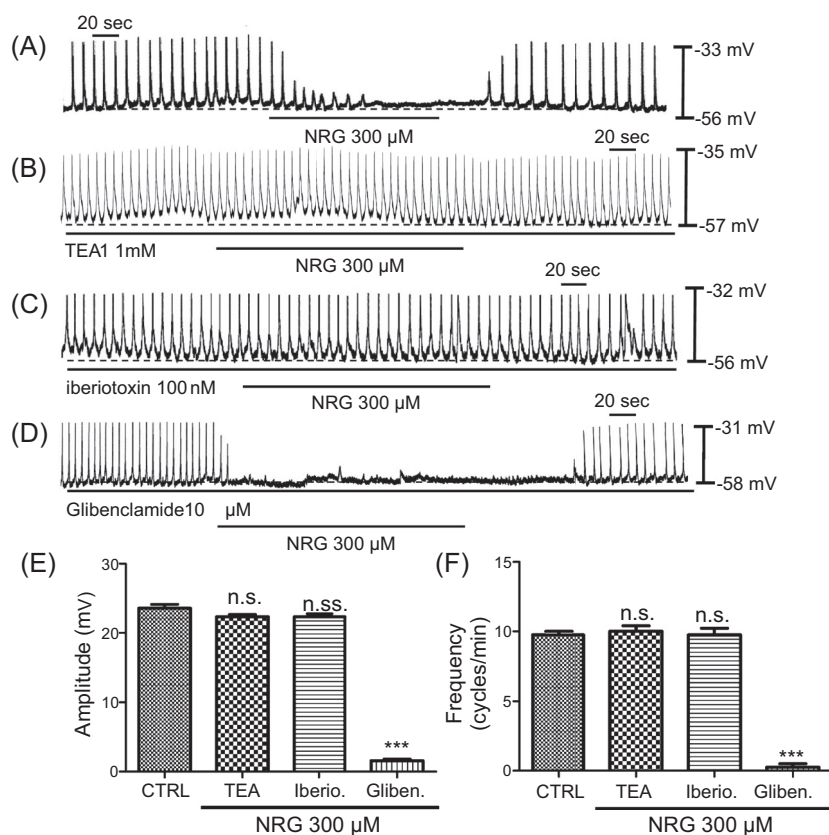


Fig. 2 – The effects of TEA, iberiotoxin, and glibenclamide on pacemaker potential (PP) inhibition by NRG in cultured interstitial cells of Cajal. (A) Pacemaker activities of interstitial cells of Cajal exposed to NRG (300 μM) in current-clamp mode ($I = 0$). Pretreatment with (B) TEA (1 mM), or (C) iberiotoxin (100 nM) blocked PP inhibition by NRG. (D) Pretreatment with glibenclamide (10 μM) did not affect PP inhibition by NRG. (E,F) The graph summarizes responses to NRG in the presence of potassium channel blockers. Bars represent mean \pm standard error. * $p < 0.001$. CTRL, control; NRG, naringenin; TEA, tetraethylammonium chloride; Iberio., iberiotoxin; Gliben., Glibenclamide; n.s., not significant.**

3.4. NRG inhibited neostigmine-induced intestinal hyperactivity

The observed inhibitory effect of NRG on the PP amplitudes of ICCs *in vitro* led us to examine the effect of NRG on ITRs *in vivo*. Previous studies have demonstrated neostigmine increases intestinal motor activity, colonic contractility, and ITRs in man.^{19,20} In neostigmine-administered mice, application of 10 mg/kg or 20 mg/kg NRG significantly reduced neostigmine-induced ITR enhancement (Fig. 4).

4. Discussion

In this study, we investigated the effect of NRG on GI motility by examining its influence on the pacemaker activities of ICCs of the murine small intestine *in vitro* and on intestinal transit rates *in vivo*. NRG inhibited ICC PPs via Ca^{2+} -sensitive K^+ channel and a cGMP/NO-dependent pathway, which suggests that NRG might have potential use for the regulation of GI motility.

Flavonoids are important natural compounds as they possess diverse biologic activities. NRG is a well-known flavonoid and a member of the flavanones. It is particularly abundant in citrus fruits, especially the grapefruit.²¹ NRG has been

shown to have therapeutic effects in various diseases, such as, metabolic syndrome.²² Furthermore, NRG affects the GI tract, and has relaxing effects on contractility of vascular smooth muscle⁹ and inhibits rat and rabbit colon smooth muscle contraction.^{23,24} NRG has also been reported to reduce gastric tone²⁵ and intestinal peristalsis,²⁶ to alter gut microecology, and have positive effects on gut health.²⁷ Accordingly, it was suggested that NRG might be a useful dietary adjunct for the treatment of various human ailments.²⁸

GI diseases importantly affect quality of life. However, the management of GI disorders is now problematic as several drugs, like cisapride and tegaserod (both now withdrawn), that ameliorate symptoms have significant adverse events.^{29,30} In complementary and alternative medicine, herbal products are usually used to treat GI disorders. In fact, up to 40% of patients with irritable bowel syndrome were found to be taking some form of herbal medication.^{31,32} ICCs act as pacemaker cells of the gastrointestinal tract and control GI motility,^{12–14} and many GI motility disorders, such as achalasia of cardia, slow transit constipation, and inflammatory bowel disease, have been associated with ICC abnormalities.³³ Thus, understanding of the mechanisms underlying pacemaker activity and ICC excitability is of crucial importance.

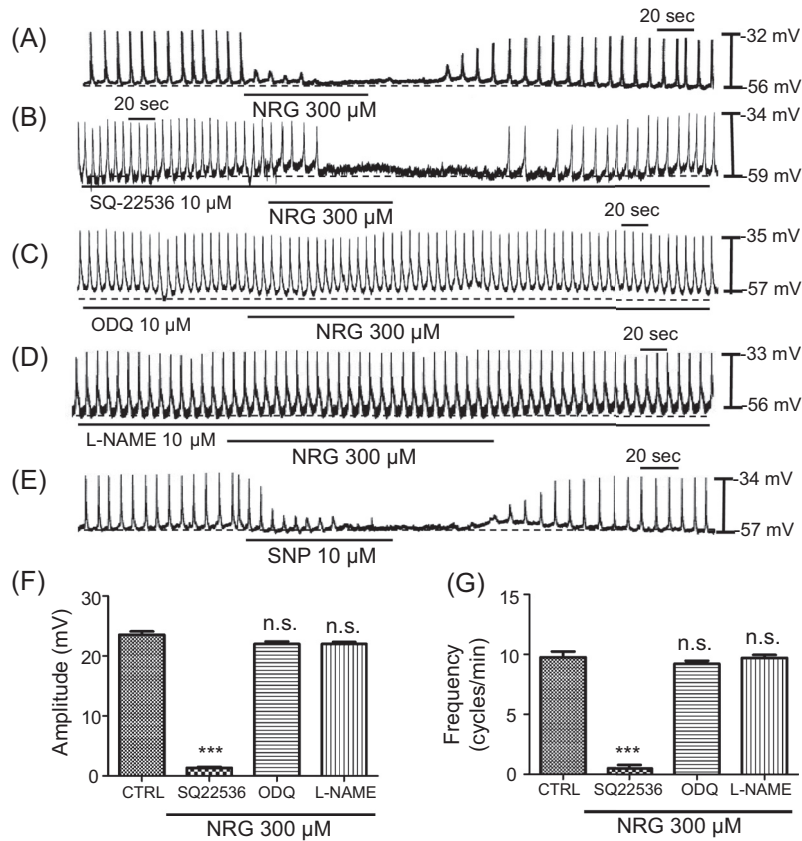


Fig. 3 – The effects of SQ-22536 (an adenylate cyclase inhibitor), ODQ (a guanylate cyclase inhibitor), and L-NAME (a nonselective nitric oxide synthase inhibitor) on pacemaker potential (PP) inhibition by NRG in cultured ICCs. (A) Pacemaker activities of interstitial cells of Cajal exposed to NRG (300 μM) in current-clamp mode ($I = 0$). (B) Pacemaker activities of a single interstitial cell of Cajal exposed to NRG in the presence of SQ-22536 (10 μM). SQ-22536 had no effect on PP inhibition by NRG. Pacemaker activities of ICCs exposed to NRG in the presence of (C) ODQ (10 μM), or (D) L-NAME (10 μM). Both inhibitors blocked PP inhibition by NRG. (E) NO donor, SNP inhibited ICC PPs. (F,G) The graph summarizes responses to NRG in the presence of SQ-22536, ODQ, or L-NAME. Bars represent mean ± standard error. * $p < 0.001$. CTRL, control; NRG, naringenin; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; L-NAME, L-NG-nitroarginine methyl ester; n.s., not significant.**

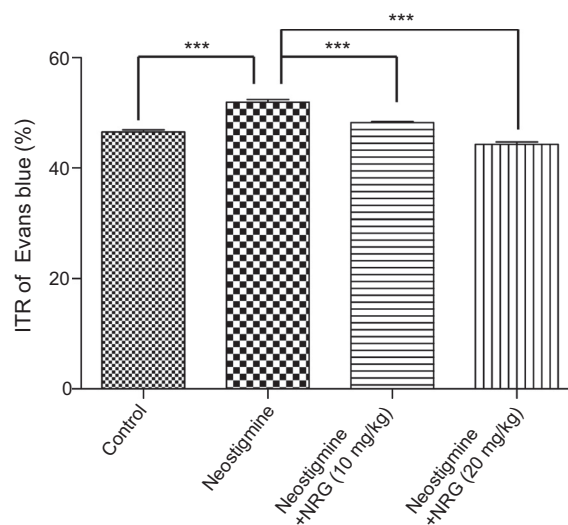


Fig. 4 – The effect of NRG on neostigmine-administered mice in vivo. NRG at 10 mg/kg or 20 mg/kg inhibited intestinal transit rate increases evoked by neostigmine. Bars represent mean ± standard error. * $p < 0.001$. NRG, naringenin.**

In the present study, we examined for the first time, the effects of NRG on the PPs of ICCs of the murine small intestine. These PPs primarily occur because of periodic activations of TRPM7³⁴ or ANO1 channels.³⁵ However, in the case of NRG, it has not been determined which ion channels regulate PPs. In the present study, NRG was found to activate Ca²⁺-activated K⁺ channel in ICCs (Fig. 2). These channels are broadly expressed in GI smooth muscle cells,^{36,37} and play a key role in controlling the excitability of smooth muscle cells.^{38,39} Also, in ICC, the presence of Ca²⁺-activated K⁺ channels in single ICC was found in guinea pig,^{40,41} and many other K⁺ channels were involved in regulation of PPs in ICC. Voltage gated K⁺ 1.1 channels,⁴² small-conductance Ca²⁺-activated K⁺ channels,⁴³ Ether-à-go-go-related gene K⁺ channels⁴⁴ are expressed in ICCs and may play an important role in generating a rhythmic pacemaker current in the GI tract.

Taken together, we found NRG reduced the amplitudes of the PPs of ICCs in a cGMP/NO-dependent manner by activating Ca²⁺-sensitive K⁺ channel. Furthermore, NRG inhibited neostigmine enhancement of ITR *in vivo*.

Conflicts of interest

The authors have no conflict of interest.

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