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Luteolin suppresses colonic smooth muscle motility *via* inhibiting L-type calcium channel currents in mice

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Abstract. As a naturally occurring flavone, luteolin has received much attention due to its antioxidant, anti-inflammatory and anticancer functions. In the present study, we investigated the effect of luteolin on colonic motility and its mechanism using isometric muscle recording and the whole-cell patch-clamp technique in mice. Luteolin dose-dependently inhibited colonic smooth muscles motility and CMMC significantly. BayK8644, an L-type Ca^{2+} channel agonist, significantly attenuated the luteolin-induced inhibition. Moreover, the calcium currents recorded in colonic smooth muscle cells were dramatically inhibited by luteolin. However, no significant changes were found in the luteolin-induced inhibitory effect in the presence of TEA, a nonselective K^+ channel blocker, glibenclamide, an ATP-dependent K^+ channel blocker, and apamin, a small-conductance Ca^{2+}-activated K^+ channel blocker. Additionally, luteolin did not affect potassium currents. Furthermore, TTX, a Na^+ channel blocker, L-NAME, an inhibitor of nitric oxide (NO) synthase, ODQ, an inhibitor of NO-sensitive guanylyl cyclase, and Ani9, a specific ANO1 channels blocker, had no effect on the luteolin-induced suppression. These results suggest that luteolin inhibited colonic smooth muscle motility by inhibiting L-type calcium channels in mice but not through potassium channels, the enteric nervous system (ENS), NO signaling pathways or ANO1 channels of interstitial cells of Cajal (ICCs).

Key words: Luteolin — Colonic motility — L-type Ca^{2+} channel

Introduction

To seek new targets for the clinical treatment of refractory diseases, an increasing number of studies have focused on phytochemicals derived from natural sources (Nabavi et al. 2015) , including many Chinese medicine ingredients, such as luteolin, i.e. 2-(3,4-dihydroxyphenyl)-5,7 dihydroxy-4*H*-1-benzopyran-4-one, a naturally occurring flavonoid. As a main ingredient in dandelion flowers, flavonoids have been suggested to play a major role in the pharmacological effects of dandelion (Wu et al. 2005; Gu et al. 2007; Chen et al. 2014); among these, luteolin is the main flavonoid (Yao et al. 2007; González-Castejón et al. 2012). Shi et al. found that dandelion-containing serum can significantly induce colonic smooth muscle cell contraction in rats (Shi et al. 2009). Our previous studies showed that dandelion extract significantly promoted gastric emptying (Wu and Piao 2005; Guo et al. 2009). However, it remains unclear whether the luteolin monomeris involved in the effect. Recent studies have shown that luteolin has antioxidant, anti-inflammatory and anticancer activity (Chen et al. 2014; Li et al. 2015; Lu et al. 2015). However, the effect of luteolin on colonic motility has not been reported. Therefore, the present study was designed to investigate the effect of luteolin on colonic smooth muscle contraction and its mechanism.

The colon is the end of the digestive tract, and its main function is to form and expel stool. The propulsion of colonic transmission comes from the colonic migrating motor

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complex (CMMC) (Heredia et al. 2009), which is mainly regulated by the enteric nerve system (ENS). The ENS regulates smooth muscle contraction through excitatory neurotransmitters, such as acetylcholine and substance P (Iino et al. 2004, 2006; Chen et al. 2007), and inhibitory neurotransmitters, such as nitric oxide (NO), ATP and ADP (Ny et al. 2000; Kurahashi et al. 2014). In addition to the ENS, two types of interstitial cells, interstitial cells of Cajal (ICCs) and platelet-derived growth factor receptor alpha-positive ($PDGFRa^+$) cells, also putatively regulate smooth muscle contraction in the gastrointestinal tract (Burnstock et al. 1981). It has been reported that excitatory neurotransmitters, such as acetylcholine (Ach), may function primarily by binding to the M receptor on the deep muscular plexus ICC membrane to activate calcium-activated chloride channels (ANO1 channels) expressed by ICCs, mediating smooth muscle depolarization (Ny et al. 2000; Zhu et al. 2011). It has also been reported that electrical field stimulation (EFS) can induce changes in the membrane potential mediated by inhibitory neurotransmitters called the inhibitory junction potential (IJP) and subsequent relaxation reactions (Durnin et al. 2017; Hibberd et al. 2017). IJPs are composed of two components: a fast, transient hyperpolarization IJP (fIJP) and a subsequent slow, sustained hyperpolarization IJP (sIJP) (Zhang et al. 2010; Lies et al. 2015). The NO released from nitrergic neurons inhibits ANO1 channel in ICCs to initiate and transmit sIJPs to smooth muscle *via* electrical coupling between ICCs and smooth muscle (Kaji et al. 2016; Smith and Koh 2017). However, the neurotransmitters released from purinergic inhibitory motor neurons bind to the purinergic receptors (P2Y1) on $PDGFR\alpha^+$cells to activate small-conductance calcium-activated potassium (SK3) channels and induce hyperpolarization of the cell membrane. $PDGFR\alpha^+$ cell hyperpolarization is transmitted to smooth muscle *via* electric coupling between $PDGFR\alpha^+$ cells and smooth muscle and then induces smooth muscle relaxation (Sanders et al. 2014; Baker et al. 2015). Thus, the effect of luteolin on colonic smooth muscle may be related to the ENS or interstitial cells.

Colonic smooth muscle contraction is closely related to potassium channels and calcium channels that are abundantly expressed by smooth muscle cells. Potassium currents, such as delayed rectifier potassium currents (IK_V) and calcium-activated potassium currents (IK_{Ca}), represent the dominant repolarizing conductance within the physiological range of membrane potentials (–50 mV to 0 mV) and play an important role in smooth muscle relaxation. Additionally, potassium channels are critical in maintaining the resting potential and excitability of smooth muscle cells (Wade et al. 1999; Lingle et al. 2002). Furthermore, ATP-dependent K^+ channels activated by abnormal energy metabolism of smooth muscle have also been shown to be involved in the generation and maintenance of slow waves (Koh et al. 2000). The calcium channels associated with smooth muscle contraction are mainly L-type voltage-dependent calcium channels, which are characterized by a high threshold, large conductance, slow inactivation and long opening time. Moreover, the amplitude of L-type calcium currents is increased when barium ions are used instead of extracellular calcium ions, but T-type calcium currents are not affected; additionally, L-type voltage-dependent calcium channels are sensitive to BayK8644, while T-type calcium channels are not (McDonald et al. 1994). Extracellular calcium enters smooth muscle cells mainly through L-type calcium channels during the action potential period, resulting in smooth muscle contraction (Sanders 2008). Therefore, luteolin may act on ion channels and change the contractile function of colonic smooth muscle.

Based on the above research and theoretical basis, this study investigated the effect and mechanism of luteolin on colonic smooth muscle motility and laid a theoretical foundation for the clinical application of luteolin.

Materials and Methods

Animal preparation

Adult male ICR mice aged 4 and 8 weeks were provided by the Experimental Animal Center of Shanghai Jiao Tong University School of Medicine. The mice were housed at 20–25°C under a 12 hour light/dark cycle and with water and food *ad libitum*. The current study strictly complied with the Guide for the Care and Use of Laboratory Animals of the Science and Technology Commission of China (STCC Publication No. 2, revised 1988). The protocol was approved by the Committee on the Ethics of Animal Experiments of Shanghai Jiao tong University School of Medicine (Permit Number: Hu 686-2009). All operations were performed under anesthesia induced using isoflurane to maximally relieve the suffering of the experimental animals.

Tissue preparation and isometric measurements

After anesthesia was induced using isoflurane, the mice were sacrificed by cervical dislocation, and the colon was quickly removed, placed into Krebs solution, opened and cleared of the contents. The colon was then pinned onto a silica gel plate using small needles, the mucosa and submucosa were removed, and the proximal colonic muscle tissue was cut into small strips (2 mm × 8 mm). Tied with silk threads at both ends of the tissue, the strips were hung along the circular axis in an 8 ml organ bath perfused with warm (37°C) oxygenated Krebs solution. Mechanical activity was recorded by an isometric force transducer (RM6240C; Chengdu Instrument Factory, China) connected to an amplifier.

Colonic migrating motor complexes

The mice were sacrificed by cervical dislocation after anesthesia was induced by isoflurane inhalation. Next, the abdomen was opened, and the colon was removed and quickly placed into oxygen saturated Krebs solution at the appropriate temperature. The colon was fixed in a base dish, and the mesentery was excised under a microscope. Fecal pellets were artificially excluded using a 1 ml injector, and a glass capillary linked to an imitation fecal pellet was inserted through the lumen. The capillary was settled to the floor of the silica gel plate at its two ends using U-shaped pins in a rectangular organ bath filled with a volume of 20 ml of warm, oxygen-saturated Krebs solution (36.5 ± 0.5°C); then, the colon was allowed to stabilize for 30–40 min to recover its contractile activity. Silk threads were attached to the proximal and distal colon linked to an amplifier device by an isometric force transducer (RM6240C, Chengdu Instrument Factory, China). A tension of 0.1 g was applied to the colon before measurement.

Isolation of smooth muscle cells

Animals (4–8 weeks postpartum) were anesthetized by isoflurane and sacrificed by cervical dislocation. The colon was removed and placed into Ca^{2+}-free physiological salt solution (Ca^{2+}-free PSS) that was oxygenated. After the mucosal layer was excised, the proximal colon muscle layer was dissected into small segments (1 mm × 4 mm). They were then incubated at 37.5°C for 20–30 min in 1 ml of digestion medium (Ca^{2+}-free PSS) containing 4–5 mg of type II collagenase, 8–9 mg of trypsin inhibitor, 8–9 mg of bovine serum albumin (BSA), 2 mmol dithiothreitol (DTT), and 2 mmol papain. After digestion, the smooth muscle cells were dispersed by gentle agitation with a wide-bore, fire-polished glass pipette. Isolated smooth muscle cells were kept at 4°C until use.

Electrophysiological recordings

The isolated smooth muscle cells were transferred to a small chamber on the stage of an inverted IX-70 Olympus microscope (Japan), where they were allowed to attach for 10–15 min to the bottom of the chamber, followed by perfusion with PSS and other solutions. Experiments were performed at 20–25°C, and the whole-cell configuration of the patch-clamp technique was applied. The resistance of the patch pipette was 3–5 MΩ when filled with pipette solution. The pipette and membrane capacitance and series resistance were electronically compensated, and the whole cell currents were recorded with a patch-clamp amplifier (EPC 10, HEKA Instruments, Germany).

Drugs and solutions

The following drugs were used in this study: luteolin, BayK8644, tetraethylammonium (TEA), glibenclamide, tetrodotoxin (TTX), apamin, Nω-nitro-L-arginine methyl ester (L-NAME), 1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ) and Ani9. The drugs were all purchased from Sigma (Sigma-Aldrich, St. Louis, MO, United States). All drugs were dissolved in distilled water or dimethyl sulfoxide (DMSO). All drugs were kept at –20°C. The Krebs solution contained the following (mmol/l): glucose, 11.5; $CaCl_2$, 2.5; NaCl, 121.9; $NaHCO_3$, 15.5; KCl, 5.9; $MgSO_4$, 1.2; KH_2PO_4, 1.2. The Ca^{2+}-free solution contained the following (mmol/l): NaCl, 134.8; KCl, 4.5; glucose, 5; $MgCl_2{\cdot}6\ H_2O$ and N-(2-hydroxyethyl) piperazine-N-(2-ethanesulphonic acid) (HEPES), 10, adjusted to pH 7.40 with Tris. The perfusate for recording the barium current contained the following (mmol/l): NaCl, 134.8; KCl, 4.5; glucose, 10; $MgCl_2{\cdot}6H_2O1$ and HEPES, 10; $BaCL_2$, 10, adjusted to pH 7.40 with Tris. The pipette solution for recording the barium current contained the following (mmol/l): CsCl, 125; tetraethylammonium, 20; egtazic acid, 10; HEPES, 10; Na_2ATP, 2; $MgCl_2{\cdot}6\ H_2O$, 4, adjusted to pH 7.35 with Tris.

Statistical analysis

The data were analyzed using Origin 7.5 software and are shown as the mean ± SEM. We used one-way ANOVA with Bonferroni's post hoc test or Student's paired *t*-test to compare groups of data. The value $p < 0.05$ was considered statistically significant.

Results

Effect of luteolin on colonic smooth muscle motility in mice

To study the effect of luteolin on colonic motility, we observed the effect of luteolin on the spontaneous contraction of proximal colonic smooth muscle strips and CMMC of the whole colon *in vitro*. Luteolin significantly suppressed the proximal colonic spontaneous contraction in a dose-dependent manner (Fig. 1A), and the inhibition rate at a concentration of 10, 20 and 30 mM was 51.2 ± 5.5%, 32.6 ± 6.1%, and 20.4 ± 4.1%, respectively ($n = 6$; * $p < 0.05$; # $p = 0.002$ F (9.580); one-way ANOVA; Fig. 1B). To further confirm the effect of luteolin on colonic motility, we observed the effect of luteolin on the CMMC. Similarly, luteolin dramatically inhibited the CMMC from 100% for the control to 56.5 ± 6.6% in the proximal colon and 55.4 ± 6.8% in the distal colon ($n = 8$; * $p < 0.05$; Fig. 1C, D). The results indicate that luteolin significantly suppresses colonic **s**mooth muscle motility.

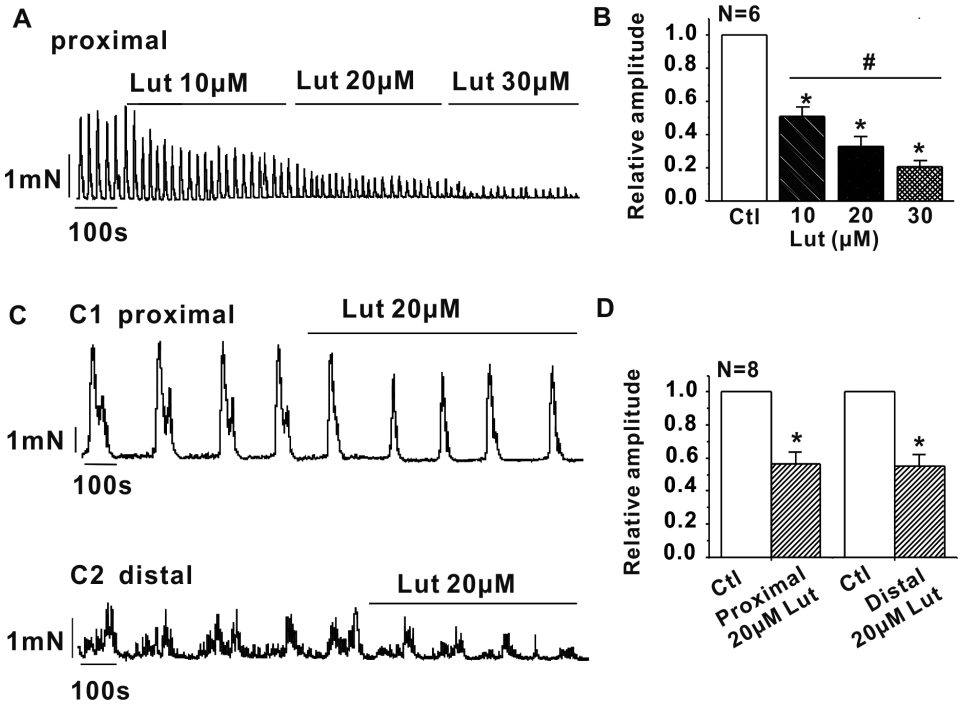

Figure 1. Effect of luteolin on colonic smooth muscle motility in mice. **A, B.** Inhibitory effect of luteolin (Lut, 10 μM, 20 μM, and 30 μM) on spontaneous contraction in a dose-dependent manner and summary data of the contraction amplitude. The data were normalized to the value before the application of luteolin ($n = 6$; * $p < 0.05$ *vs.* control; # $p = 0.002$ F(9.580), one-way ANOVA). **C, D.** Inhibitory effect of luteolin (20 μM) on the CMMC in the proximal (Fig. 1C1) and distal (Fig. 1C2) colon and summary of the area under the curve (AUC) at 400 seconds. The data were normalized to the value before the application of luteolin ($n = 8$; * $p < 0.05$ *vs.* control). Lut, luteolin; Ctl, control.

Effects of TTX, L-NAME, ODQ and Ani9 on luteolin-suppressed colonic spontaneous contraction

To investigate the mechanism underlying the luteolin-induced inhibitory effect, we compared luteolin-induced inhibitory responses before and after pretreatment of the muscle strips with blockers by elution. We first observed the effect of TTX (0.4 μM), a Na^+ channel blocker, on luteolin-induced inhibitory responses. TTX showed no significant effect on the luteolin-induced inhibitory response. Contraction before and after pretreatment with TTX decreased to 23.5 ± 4.0% and 21.8 ± 6.4% after the administration of luteolin, respectively ($n = 5$; * $p < 0.05$; Fig. 2A, B). Subsequently, to examine whether the luteolin-induced inhibitory effect was achieved through NO generation, L-NAME, an inhibitor of NOS, was employed. L-NAME did not affect the luteolin-induced inhibitory response. Contraction before and after pretreatment with L-NAME decreased to 44.0 ± 7.4% and 42.5 ± 7.3% after the administration of luteolin, respectively ($n = 8$; * $p < 0.05$; Fig. 2C, D). We also found that ODQ, an inhibitor of guanylyl cyclase, failed to markedly affect the luteolin-induced inhibitory response. Contraction before and after pretreatment with ODQ were reduced to 7.6 ± 4.8% and 4.1 ± 2.2% after the administration of luteolin, respectively ($n = 4$; * $p < 0.05$, Fig. 2E, F). To determine whether the luteolin-induced inhibitory response was modulated by the ANO1 channels of ICCs, Ani9, an ANO1 channel specific blocker, was tested, and the results showed that Ani9 could not block the luteolin-induced inhibitory response. Contraction before and after pretreatment with Ani9 were reduced to 48.6 ± 10.5% and 41.8 ± 8.8% after the administration of luteolin, respectively ($n = 4$, * $p < 0.05$, Fig. 2G, H). These results suggest that the luteolin-induced inhibitory response is not mediated by the ENS, NO signaling pathways or ICC-ANO1 channels.

Role of potassium channels in luteolin-suppressed colonic spontaneous contraction in mice

To explore whether potassium channels mediate the luteolin-induced inhibitory effect, we observed the luteolin-induced inhibitory response in the presence of several channel blockers. No significant changes were found in the luteolin-induced inhibitory effect in the presence of TEA (10 mM), a nonselective K^+ channel blocker (contraction reduced to 19.0 ± 10.4% and 21.8 ± 11.6% before and after pretreatment with TEA, respectively; $n = 6$; * $p < 0.05$; Fig. 3A, B), glibenclamide, an ATP-dependent potassium channel blocker (contraction reduced to 43.8 ± 6.9% and 40.0 ± 6.7% before and after pretreatment with glibenclamide, respectively; $n = 7$; * $p < 0.05$; Fig. 3C, D), and apamin, a small-conductance calcium-activated potassium channel blocker (contraction reduced to 35.4 ± 11.8% and 35.0 ± 8.1% before and after pretreatment with apamin, respectively; $n = 6$; * $p < 0.05$; Fig. 3E, F). To further confirm the possibility of potassium channel participation, the outward potassium current was detected in freshly dispersed proximal colonic smooth muscle cells using the whole-cell

patch-clamp technique, and the results showed that there were no changes in the potassium current before and after luteolin treatment (Fig. 3G, H). These results indicate that the luteolin-induced inhibitory effect is not related to potassium channels.

Role of L-type calcium channels in luteolin-induced contraction suppression in mice

To investigate whether calcium channels are involved in the luteolin-induced inhibitory response, BayK8644, an L-type calcium channel activator, was applied. Luteolin significantly inhibited spontaneous colonic smooth muscle contraction in the presence of BayK8644 (Fig. 4A). And the luteolin-induced inhibitory response was significantly attenuated by BayK8644 (Fig. 4A). Contraction decreased to 50.3 ± 9.3% and 67.3 ± 9.3% before and after pretreatment with BayK8644, respectively ($n = 5$; * $p < 0.05$; # $p < 0.05$; Fig. 4B).

To further confirm the role of L-type calcium channels in luteolin-induced colonic inhibition, L-type calcium currents were recorded using the whole-cell patch-clamp technique in freshly dispersed proximal colonic smooth muscle cells. Barium ions are good carriers of L-type calcium channel currents and have many advantages, such as amplifying L-type calcium channel current, preventing cell shrinkage, and minimizing current run-down. So barium currents (IBa)

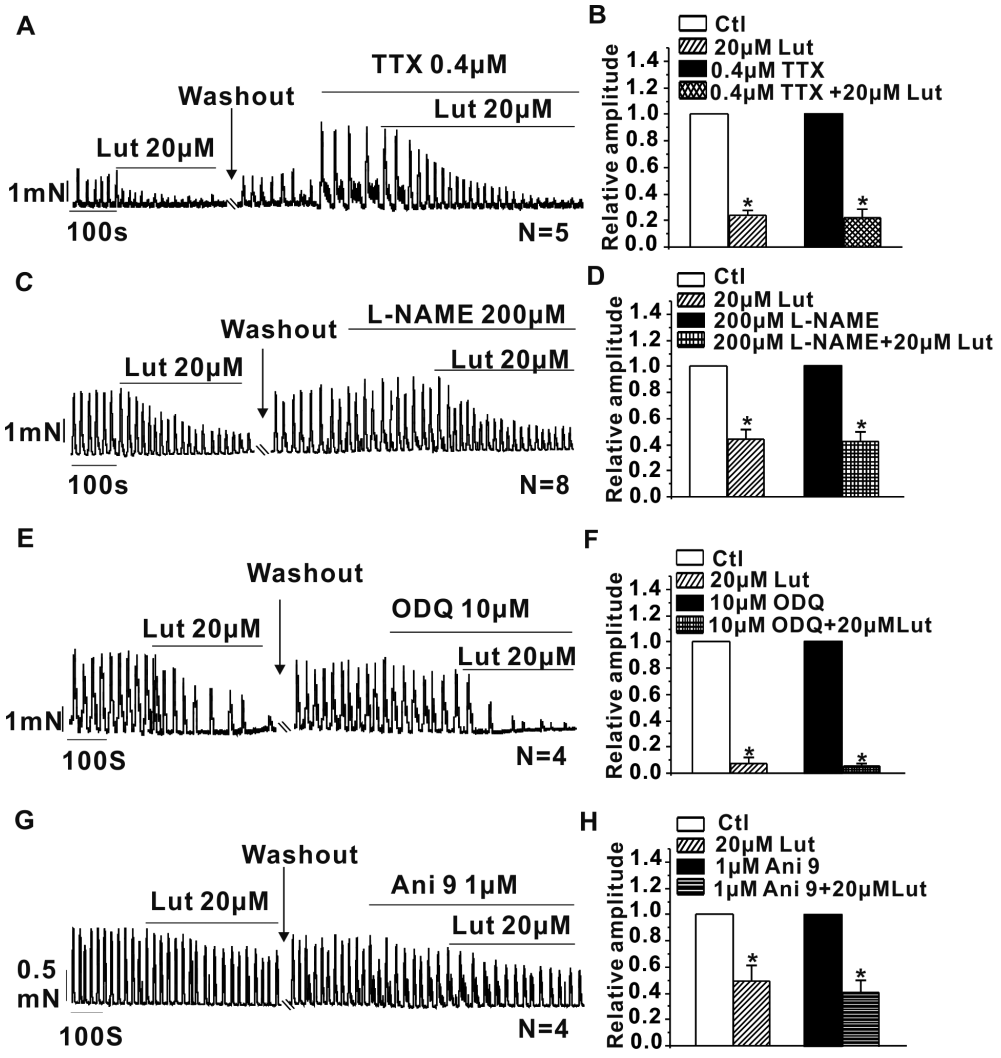

Figure 2. Effects of TTX, L-NAME, ODQ and Ani9 on luteolin-suppressed proximal colonic spontaneous contraction in mice. **A, B.** Effect of TTX on luteolin-suppressed proximal colonic spontaneous contraction and comparison of luteolin-induced inhibitory effect before and after pretreatment with 0.4 mM TTX by elution. The data were normalized to the value before the application of 20 mM luteolin ($n = 5$; * $p < 0.05$ vs before the administration of luteolin). **C, D.** Effect of L-NAME on luteolin-suppressed proximal colonic spontaneous contraction and comparison of luteolin-induced inhibitory effect before and after pretreatment with 200 mM L-NAME by elution. The data were normalized to the value before the application of 20 mM luteolin ($n = 8$; * $p < 0.05$ *vs.* before the administration of luteolin). **E, F.** Effect of ODQ on luteolin-suppressed proximal colonic spontaneous contraction and comparison of luteolin-induced inhibitory effect before and after pretreatment with 10 mM ODQ by elution. The data were normalized to the value before the application of 20 mM luteolin ($n = 4$; * $p < 0.05$ *vs.* before the administration of luteolin). **G, H.** Effect of Ani9 on luteolin-suppressed proximal colonic spontaneous contraction and comparison of luteolin-induced inhibitory effect before and after pretreatment with 1 mM Ani9 by elution. The data were normalized to the value before the application of 20 mM luteolin ($n = 4$; * $p < 0.05$ *vs.* before the administration of luteolin). Lut, luteolin; Ctl, control; TTX, tetrodotoxin; L-NAME, Nω-nitro-L-arginine methyl ester; ODQ, 1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one.

were recorded instead of calcium currents in our experiments. IBa were elicited by a step voltage command pulse from −40 mV to +70 mV for 440 ms with a 10 mV increment at a 10 s interval and a holding potential of −80 mV (Figure 4C); 20 mM luteolin significantly decreased IBa from 100% for the control to 69.6 ± 2.8% at 0 mV (n = 7; * p < 0.05; Fig. 4D). The peak values of IBa at 0 mV were significantly inhibited in a dose-dependent manner, and the inhibition rate of luteolin on IBa at a concentration of 10, 20 and 30 mM was 79.7 ± 2.0%, 70.7 ± 2.4%, and 52.4 ± 6.8%, respectively (n = 4; # p = 0.005 F(10.345); one-way ANOVA; Fig. 4E). These results show that luteolin-induced inhibitory effect of colonic smooth muscle contraction may be related L-type calcium channels.

Discussion

In the present study, we found that luteolin significantly inhibited the spontaneous contraction of colonic smooth muscles and the CMMC (Fig. 1). The luteolin-induced inhibitory effect was not affected by TTX (a Na^+ channel blocker), L-NAME (an inhibitor of NO synthase), ODQ (an inhibitor of guanylyl cyclase) or Ani9 (a specific ANO1 channel blocker). Potassium channels are important for maintaining the smooth muscle cell membrane potential. TEA (a nonselective K^+ channel blocker), glibenclamide (an ATP-dependent potassium channel blocker) and apamin (a small-conductance calcium-activated potassium channel blocker) did not block the luteolin-induced inhibitory

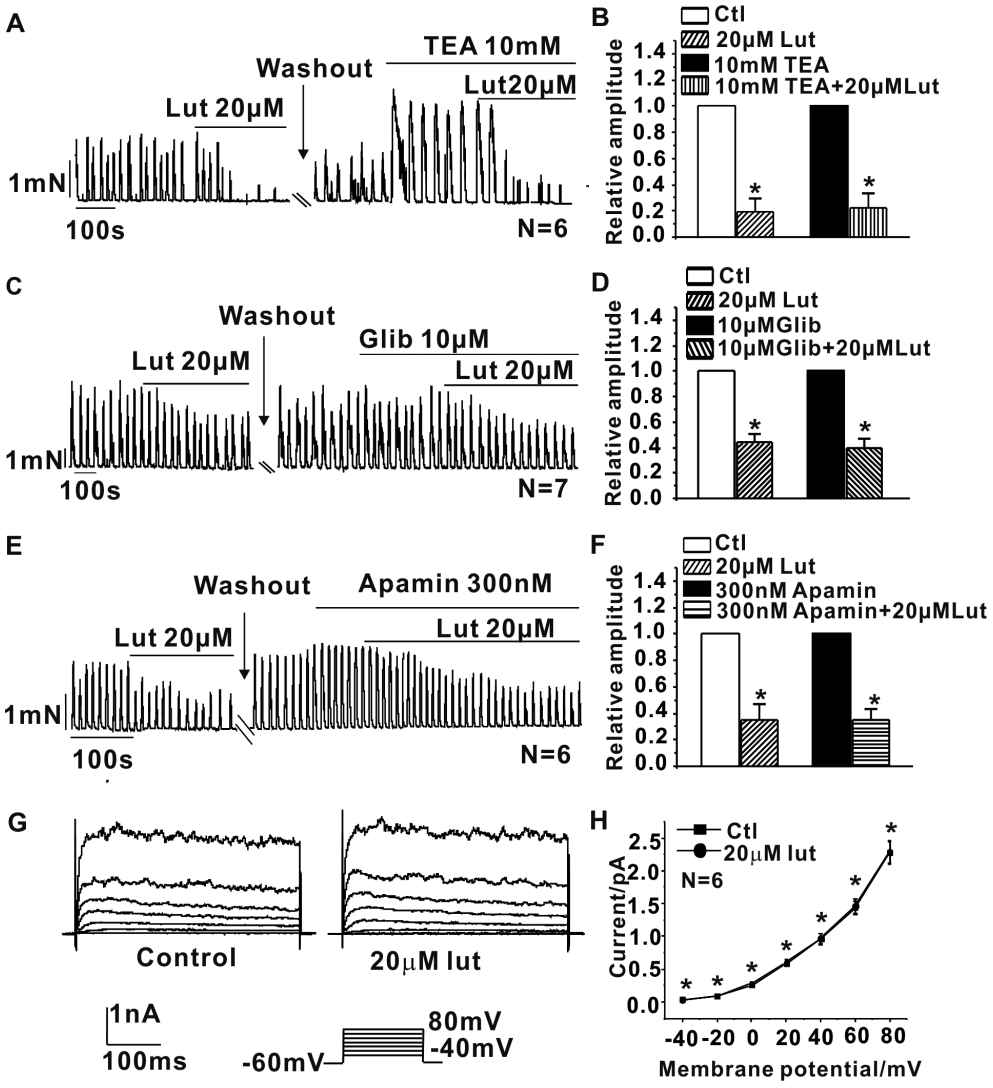

Figure 3. Role of potassium channels inluteolin-suppressed proximal colonic spontaneous contraction in mice. **A, B.** Effect of TEA on luteolin-suppressed proximal colonic spontaneous contraction and comparison of luteolin-induced inhibitory effect before and after pretreatment with 10 mM TEA by elution. The data were normalized to the value before the application of 20 μM luteolin (n = 6; * p < 0.05 *vs.* before the administration of luteolin). **C, D.** Effect of glibenclamide (Glib) on luteolin-suppressed proximal colonic spontaneous contraction and comparison of luteolin-induced inhibitory effect before and after pretreatment with 10 μM Glib by elution. The data were normalized to the value before the application of 20 μM luteolin (n = 7; * p < 0.05 *vs.* before the administration of luteolin). **E, F.** Effect of apamin on luteolin-suppressed proximal colonic spontaneous contraction and comparison of luteolin-induced inhibitory effect before and after pretreatment with 300 nM apamin by elution. The data were normalized to the value before the application of 20 μM luteolin (n = 6; * p < 0.05 *vs.* before the administration of luteolin).**G, H.** Effects of luteolin on outward potassium currents and the I-V relationship when cells were exposed to 20 μM luteolin (n = 6; * p > 0.05 *vs.* control). Lut, luteolin; Ctl, control; TEA, tetraethylammonium; Gli, glibenclamide.

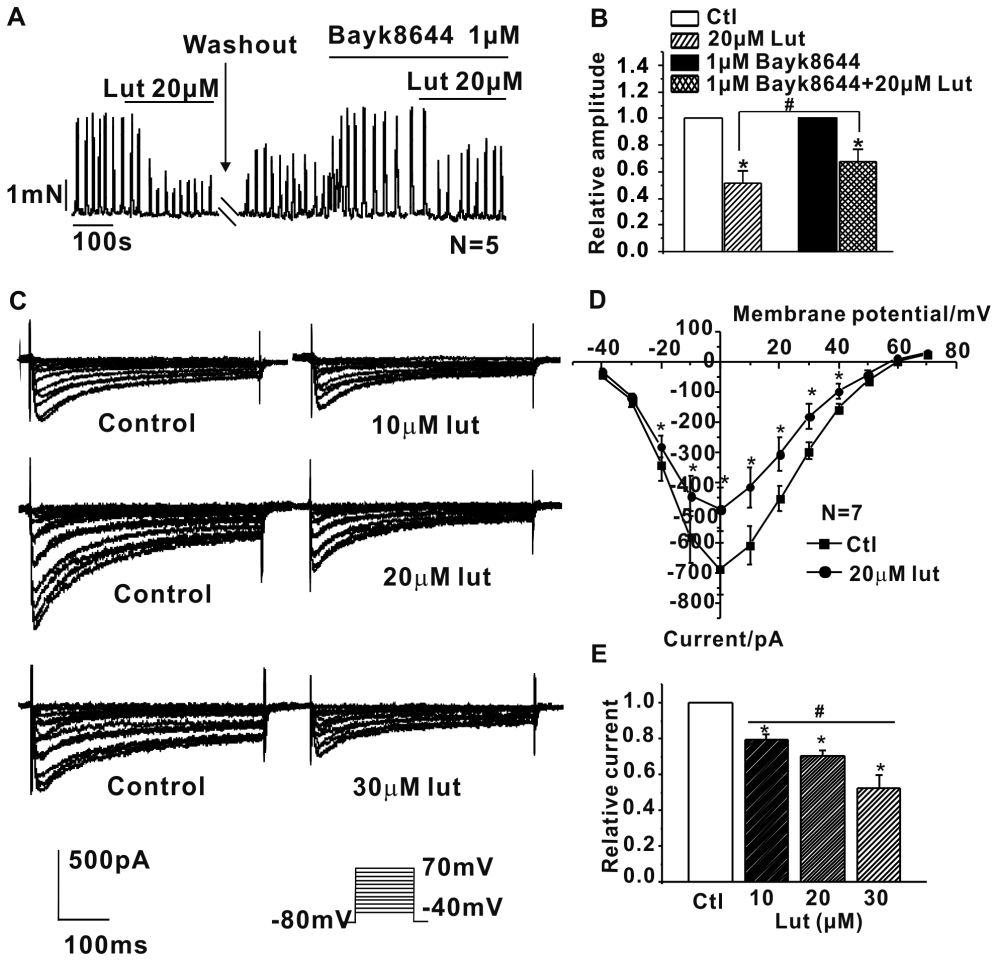

Figure 4. Role of L-type calcium channels in luteolin-induced suppression of colonic motility in mice. **A, B.** Effect of luteolin on BayK8644-induced increase and effect of BayK8644 on luteolin-induced inhibition of proximal colonic spontaneous contraction and comparison of the luteolin-induced inhibitory effect before and after pretreatment with BayK8644 by elution. The data were normalized to the value before the application of 20 μM luteolin (n = 5; * p < 0.05 *vs.* before the administration of luteolin; # p < 0.05 *vs.* before elution). **C.** Representative traces of IBa elicited by a step pulse in proximal colonic smooth muscle cells. **D.** I-V relationship of IBa when cells were exposed to 20 μM luteolin (n = 7; * p < 0.05 *vs.* control). **E.** Peak values of IBa at 0 mV normalized and averaged for cells exposed to different doses of luteolin (n = 4; * p < 0.05 *vs.* control; # p = 0.005 F(10.345), one-way ANOVA). Lut, luteolin; Ctl, control.

effect. Additionally, luteolin did not affect outward potassium currents in freshly isolated colonic smooth muscle cells. However, luteolin directly inhibited L-type calcium channel currents in freshly isolated colonic smooth muscle cells. Moreover, the luteolin-induced inhibitory response was significantly attenuated by BayK8644. The results suggest that the inhibitory effect of luteolin on colonic smooth muscle motility is mediated by the inhibition of L-type calcium channels in mice.

Normal colonic motility is regulated by the ENS, in which the main excitatory enteric nerves are the cholinergic nerves (Zhu et al. 2011) and the main inhibitory nerves are the nitrergic and purinergic nerves (Ny et al. 2000; Sanders et al. 2014). It was reported that quercetin, a naturally occurring flavone, inhibited contraction of the smooth muscle in the small intestine of rabbits in association with an increased NO concentration (Zhang et al. 2013). A recent study indicated that NO inhibited the ANO1 channels of intramuscular ICCs and then inhibited the voltage-dependent calcium channels of smooth muscle cells through gap junctions, leading to hyperpolarization and eventually inducing relaxation of the smooth muscle (Lies et al. 2015). Therefore, in the present study, we first investigated whether the ENS was involved in the luteolin-induced inhibitory effect. We used TTX to block the ENS, L-NAME (NO synthase inhibitor) to block the NO synthesis of smooth muscle, and ODQ (NO-sensitive guanylyl cyclase inhibitor) to block cGMP production. We found that the inhibitory effect of luteolin was not affected by these drugs. We also tested Ani9, a specific ANO1 channel blocker, and the luteolin-inhibitory effect was not blocked by Ani9 (Fig. 2). These results suggest that the inhibitory effect of luteolin on colonic motility is not mediated by the ENS or the ANO1 channels of intramuscular ICCs.

The contractility of smooth muscle is closely related to the ion channels of the smooth muscle cell membrane. Potassium channels play an important role in maintaining the resting potential and smooth muscle relaxation (Wade et al. 1999; Lingle et al. 2002). Three types of outward potassium currents are found in smooth muscle cells: delayed IK_V, IK_{Ca} and transient potassium currents (I_{to}) (Kuriyama et al. 1998). The functions of the different types of potassium

currents in smooth muscles vary among different animals, as well as among different organs. Wade et al. demonstrated that IK_V played a dominant role in the regulation of the resting tension of esophageal muscle, whereas IK_{Ca} largely limited contraction associated with excitation (Wade et al. 1999). It has also been reported that ATP-dependent potassium channels are involved in the formation of slow waves and participate in the inhibition of gastrointestinal and vascular smooth muscle activity *via* hydrogen sulfide (Koh et al. 2000; Zhao et al. 2009). Thus, in the present experiment, we employed TEA (a nonselective potassium channel blocker) to block voltage-dependent potassium channels and large-conductance calcium-activated potassium channels and apamin to block SK3 channels. We found that the luteolin-induced inhibitory effect was not affected by these drugs, and luteolin did not affect the outward potassium currents in freshly dispersed colonic smooth muscle cells. Additionally, glibenclamide, an ATP-dependent potassium channel blocker, did not block the luteolin-induced inhibitory effect (Fig. 3). The above results suggest that the inhibitory effect of luteolin is not related to the potassium channels of the smooth muscle cell membrane.

Membrane potential is coupled to the opening of voltage-dependent calcium channels, and the depolarization of gastrointestinal tract smooth muscle cells leads to calcium influx and contraction initiation (Sanders 2008). The voltage-dependent calcium channels of smooth muscle cells are divided into L-type and N-type channel, and L-type channels are the main voltage-dependent calcium channels of the membrane of smooth muscle cells in the gastrointestinal tract (McDonald et al. 1994) that participate in the initiation of action potentials and affect muscle contraction (Sanders 2008). It was reported that the inhibitory effect of luteolin on other smooth muscles was related to the intracellular calcium level or to calcium channels. For example, the luteolin-induced relaxation effect observed in isolated guinea pig tracheas may be due to its inhibitory effect on phosphodiesterase activity and its reduction of $[Ca^{2+}]_i$ (Ko et al. 2005), and luteolin-induced relaxation in the rat thoracic aorta involves the inhibition of Ca^{2+} channels and the activation of K^+ channels (Jiang et al. 2005). In the present experiment, BayK8644, an L-type calcium channel activator, significantly attenuated the inhibitory effect of luteolin. Additionally, the BayK8644-induced increase in colonic smooth muscle contraction was significantly suppressed by luteolin (Fig. 4). To further confirm the involvement of L-type calcium channels in the luteolin-induced inhibitory effect, we observed the effect of luteolin on L-type calcium currents in freshly isolated colon smooth muscle cells using the whole-cell patch-clamp technique. Luteolin significantly decreased IBa at every depolarized command step potential from −20 mV to +40 mV in the I-V relation curve (Fig. 4). These results suggest that luteolin suppresses colon motility by directly inhibiting L-type calcium channel currents.

In summary, as an extract in traditional Chinese medicine, luteolin has a strong inhibitory effect on colonic smooth muscle motility. The luteolin-induced inhibitory effect is not mediated through the ENS or ANO1 channels of intramuscular ICCs but instead through L-type calcium channels in smooth muscle. The effect of luteolin on calcium channels may be direct because TEA (a nonselective potassium channel blocker), apamin (a SK3 blocker), glibenclamide (K_{ATP} channel blocker) and Ani9, a specific ANO1 channel blocker are not affect luteolin-induced inhibition. The pharmacological effect of luteolin can be used in the prevention and treatment of gastrointestinal smooth muscle motility disorder.

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