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(−)-Epigallocatechin Gallate Inhibits the Pacemaker Activity of Interstitial Cells of Cajal of Mouse Small Intestine

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The effects of (−)-epigallocatechin gallate (EGCG) on pacemaker activities of cultured interstitial cells of Cajal (ICC) from murine small intestine were investigated using whole-cell patch-clamp technique at 30°C and Ca^{2+} image analysis. ICC generated spontaneous pacemaker currents at a holding potential of −70 mV. The treatment of ICC with EGCG resulted in a dose-dependent decrease in the frequency and amplitude of pacemaker currents. SQ-22536, an adenylate cyclase inhibitor, and ODQ, a guanylate cyclase inhibitor, did not inhibit the effects of EGCG. EGCG-induced effects on pacemaker currents were not inhibited by glibenclamide, an ATP-sensitive K^+ channel blocker and TEA, a Ca^{2+}-activated K^+ channel blocker. Also, we found that EGCG inhibited the spontaneous $[Ca^{2+}]_i$ oscillations in cultured ICC. In conclusion, EGCG inhibited the pacemaker activity of ICC and reduced $[Ca^{2+}]_i$ oscillations by cAMP-, cGMP-, ATP-sensitive K+ channel-independent manner.

Key Words: (−)-epigallocatechin gallate (EGCG), Interstitial cells of Cajal (ICC), Pacemaker currents, Intestinal motility

INTRODUCTION

The major components of green tea include (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC). These components have various biological effects, including sedative, hyptensive, lipid-lowering and tumorigenesis effects (Hara, 2001). EGCG is known to be the largest component of catechins (Matsuzaki and Hara, 1998).

Catechins have diverse functions on gastrointestinal tract, especially gastrointestinal motility and transit. For examples, gossypin, epicatechin and hydroxyethyl rutosides delay intestinal transit in a dose-dependent manner in mice, while naloxone, yohimbine, and phentolamine antagonize the effects of these compounds. Prazosin, propranolol, atropine, physostigmine, hexamethonium, pheniramine, and metiamide have no effect.

Opiate and alpha-2 adrenergic receptors have earlier been suggested to mediate the flavonoid-induced delay in the transit in the small intestine (Viswanathan et al, 1984). and polyphenolic compounds have been shown to have a strong, dose-dependent inhibitory effect on gastrointestinal transit in mice, whereas catechin dose not show a significant effect (Di et al, 1993). Furthermore, EGCG has been reported to have direct depolarizing effects on a population of myenteric neurons in the guinea-pig small intestine in vitro and facilitates cholinergic ganglion transmission in enteric neurons (Homma et al, 2001; Katayama et al, 2002).

There has been increasing evidences to indicate that the interstitial cells of Cajal (ICC) are the pacemaker cells of spontaneous motility in the gastrointestinal tract (Huizinga et al, 1995; Kobayashi et al, 1996; Sanders et al, 1999). ICC are small spindle-shaped or stellate cells with numerous mitochondria and long processes that form networks between and within smooth muscle layers by forming gap junctions in the GI tract (Thuneberg, 1982; Sanders, 1996). Many studies showed that ICC generate pacemaker potentials which mediate the spontaneous contraction of smooth muscle and this generation of potentials is due to the activation of spontaneous inward currents (Ward et al, 1994; Tokutomi et al, 1995; Thomsen et al, 1998; Koh et al, 1998). since these cells play an important role as basic regulators of gastrointestinal motility, therefore many hormones, neurotransmitter, and various substances are likely able to modulate GI tract motility by influencing ICC.

There are many reports to indicate that catechins play function in intestinal motility, however, no studies have so far determined the effects of catechins on the electrical events in mouse ICC. Therefore, the purpose of our study was to investigate the effects of catechins on pacemaker activity in cultured ICC.

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ABBREVIATIONS: ICC, interstitial cells of Cajal; EGCG, (−)-epigallocatechin gallate; TEA, tetraethylammonium chloride.

METHODS

Preparation of cells and tissues

Balb/C mice (3～7 days old) of either gender were anesthetized with ether and sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. The luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish, and the mucosa was removed by sharp dissection. Small stripes of intestinal muscle were equilibrated in Ca^{2+}-free Hank's solution for 30 min and cells were dispersed with an enzyme solution containing 1.3 mg/ml collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 2 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA), 2 mg/ml trypsin inhibitor (Sigma) and 0.27 mg/ml ATP. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 (g/ml, Falcon/BD) in 35 mm culture dishes. The cells were then cultured at 37°C in a 95% O_2-5% CO_2 incubator in SMGM (smooth muscle growth medium, 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). Interstitial cells of Cajal (ICC) were identified immunologically with a monoclonal antibody for Kit protein (ACK_2) labelled with Alexa Fluor 488 (molecular Probe, Eugene, OR, USA).

Patch clamp experiments

The whole-cell configuration of the patch-clamp technique was used to record membrane currents (voltage clamp) and membrane potentials (current clamp) from cultured ICC. Currents or potentials were amplified by the use of an Axopatch 1-D (Axon Instruments, Foster, CA, USA). Command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and a pen recorder (Gould 2200, Gould, Valley view, OH, USA).

Results were analyzed using pClamp and Sigma plot (version 9.0) software. All experiments were performed at 30°C.

Measurement of the intracellular Ca^{2+} concentration

Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored by using fluo-3/AM, which was initially dissolved in dimethyl sulfoxide and stored at −20°C. The cultured ICC on coverslips (25 mm) were rinsed twice with a bath solution (5 mM: KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$ and 10 mM HEPES, adjusted to pH 7.4 with Tris). The coverslips were then incubated in the bath solution containing 5 (M fluo-3 with 5% CO_2 at 37°C for 5 min, rinsed two more times with the bath solution, mounted on a perfusion chamber, and scanned every 0.4 seconds with Nikon Eclipse TE200 inverted microscope equipped with a Perkin-Elmer Ultraview confocal scanner and a Hamamatsu Orca ER 12-bit CCD camera. (×200). Fluorescence was excited at a wavelength of 488 nm, and emitted light was observed at 515 nm. During scanning of the Ca^{2+} imaging, the temperature of the perfusion chamber containing the cultured ICC was kept at 30°C. The variations of intracellular Ca^{2+} fluorescence emission intensity were expressed as F1/F0 where F0 is the intensity of the first imaging.

Solutions and drugs

The cells were bathed in a solution containing (mM) : 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, and 10 mM HEPES, pH adjusted to 7.2 with Tris. The pipette solution contained 140 mM KCl, 5 mM MgCl2, 2.7 mM K_2ATP, 0.1 mM Na_2GTP, 2.5 mM creatine phosphate disodium, 5 mM HEPES, 0.1 mM EGTA, pH adjusted to 7.2 with Tris.

Drugs used were: polyphenon 60, (−)-epigallocatechin gallate, (−)-epicatechin gallate, glibenclamide, tetraethylammonium chloride, and ODQ (1H-[1,2,4]Oxadiazolo[4,3-a] quinoxalin-1-one). ODQ was purchased from Calbiochem Co., and the others were purchased from Sigma Chemical Co.

Statistical analysis

Data are expressed as mean±standard error. Differences in the data were evaluated by Student's t test. A P values less than 0.05 were taken as a statistically significant difference. The n values reported in the text refer to the number of cells used in the patch-clamp experiments.

RESULTS

Effect of catechins on pacemaker currents in cultured ICC

We performed the electrophysiological recording from cultured ICC under voltage clamp mode. Under a voltage clamp at a holding potential of −70 mV, the ICC generated spontaneous inward currents. We examined the effects of

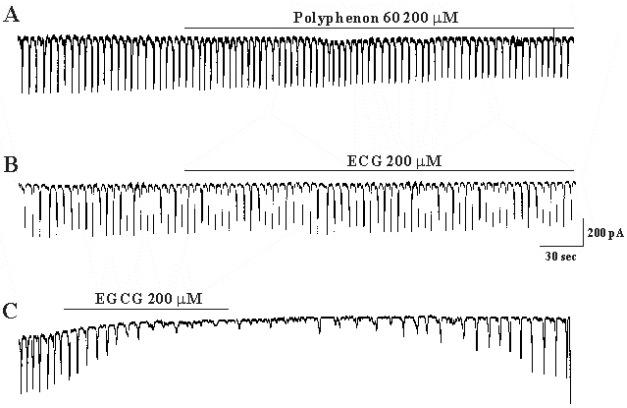

Fig. 1. Effects of catechins on pacemaker currents recorded in cultured ICC from mouse small intestine (A) Pacemaker currents of ICC recorded at a holding potential of −70 mV, when exposed to polyphenon 60 (200 μM). (B) Pacemaker currents of ICC recorded at a holding potential of −70 mV when exposed to epicatechin gallate (200 μM). (C) Pacemaker currents of ICC recorded at a holding potential of −70 mV which were exposed to epigallocatechin gallate (200 μM). Vertical solid line scales amplitude of pacemaker current and horizontal solid line scales duration of recording (s) pacemaker currents. ECG: (−)-epicatechin gallate, EGCG: (−)-epigallocatechin gallate.

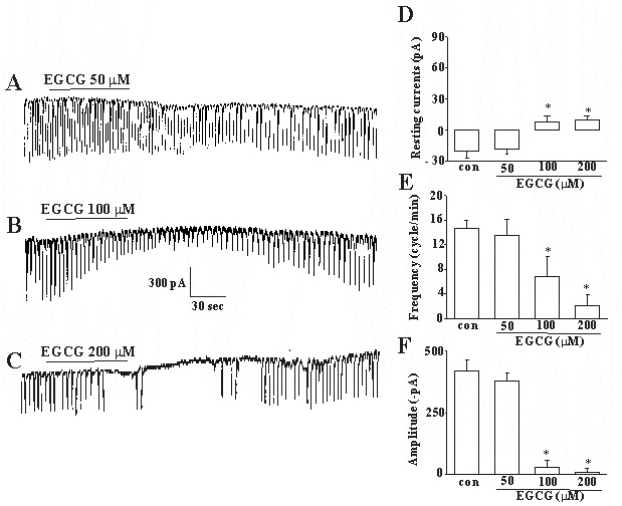

Fig. 2. Dose-dependent effects of (−)-epigallocatechin gallate on pacemaker currents in cultured ICC of mouse small intestine. (A), (B), and (C) show pacemaker currents of ICC exposed to (−)-epigallocatechin gallate (50, 100, or 200 μM respectively) at a holding potential of -70 mV. Vertical solid line scales amplitude of pacemaker current and horizontal solid line scales duration of recording (s) pacemaker currents. (−)-epigallocatechin gallate at 100 and 200 μM concentrations inhibited spontaneous pacemaker currents of ICC. (D), (E), and (F) summarize the inhibitory effects of (−)-epigallocatechin gallate on pacemaker currents in ICC. Bars represents means±SE (n=5/group). *Asterisks mean significantly different from the controls ($p<0.05$). EGCG: (−)-epigallocatechin gallate, CON: control.

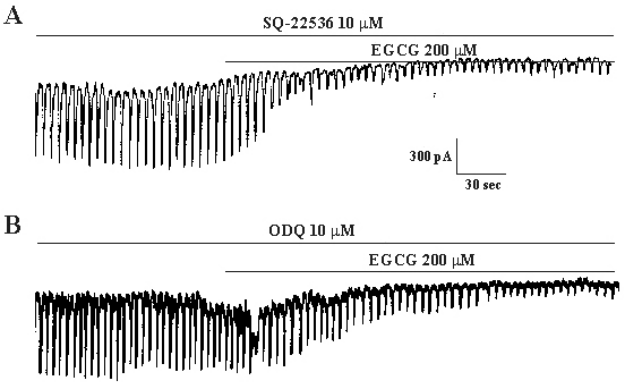

Fig. 3. Effects of SQ-22536, an inhibitor of adenylate cyclase, and ODQ, an inhibitor of guanylate cyclase, on (−)-epigallocatechin gallate-induced response in cultured ICC from mouse small intestine. (A) Pretreatment with SQ-22536 (10 μM) did not affect the inhibitory effects of (−)-epigallocatechin gallate (200 μM) on spontaneous inward currents. (B) Pretreatment with ODQ (10 μM) did not affect the inhibitory effects of (−)-epigallocatechin gallate (200 μM) on spontaneous inward currents. Vertical solid line scales amplitude of pacemaker current and horizontal solid line scales duration of recording (s) pacemaker currents. EGCG: (−)-epigallocatechin gallate.

polyphenon and catechins on pacemaker currents. Treatment of cultured ICC with polyphenon 60 (200 μM) and ECG (200 μM) did not show any influence on pacemaker current of ICC (Fig. 1A and B). However, when EGCG (200 μM) was applied in ICC, both the frequency and the amplitude of pacemaker currents were decreased (Fig. 1C). Next, we tested whether EGCG has a dose-dependent inhibitory effect on pacemaker currents in cultured ICC. Under a voltage clamp at a holding potential of −70 mV, ICC generated spontaneous inward currents. The addition of 50 μM EGCG had no effect on pacemaker currents (Fig. 2A), however 100 μM EGCG reduced the amplitude and frequency of these pacemaker currents (Fig. 2B). In the presence of 200 μM EGCG, the pacemaker currents were largely inhibited (Fig. 2C). The values of frequency and amplitude by EGCG (100 and 200 μM) were significantly different from those of control (n=4, Fig. 2D∼F), suggesting that only EGCG inhibits pacemaker currents in cultured ICC in a dose-dependent manner.

Effects of adenylate and guanylate cyclase inhibitor on EGCG-induced responses in cultured ICC

The effects of SQ-22536, an inhibitor of adenylate cyclase, and ODQ, an inhibitor of guanylate cyclase, were examined for possible regulation of pacemaker currents by cyclic adenosine (cAMP) and guanosine monophosphate (cGMP)-dependent pathway. Thus, cultured ICC were pretreated with SQ-22536 and ODQ for 10 min before the application of EGCG. In the presence of SQ-22536 (10 μM) and ODQ (10 μM), the effects of EGCG on pacemaker currents were not inhibited (n=5, Fig. 3A and B). These findings indicate that cyclic AMP and GMP do not mediate the EGCG-induced effects.

Effects of potassium channels blockers on EGCG-induced responses in cultured ICC

To investigate which potassium channels mediate EGCG action on pacemaker currents, two kinds of potassium channel blockers were tested. The application of tetraethylammonium chloride (TEA) (2 mM), a Ca^{2+}-activated K^+ channel blocker, and glibenclamide (10 μM), an ATP-sensitive K^+ channel blocker, to ICC itself had no effect on the pacemaker currents. Furthermore in the presence of TEA or glibenclamide, EGCG (200 μM) still inhibited the pacemaker currents in ICC (n=5; Fig. 4A and B).

Involvement of $[Ca^{2+}]_i$ in PGE_2-induced action in ICC

Many reports suggested $[Ca^{2+}]_i$ oscillations in ICC as the primary mechanism for the pacemaker activity in gastrointestinal activity, therefore we examined the effect of EGCG on $[Ca^{2+}]_i$ oscillations in ICC. In this study, we measured spontaneous $[Ca^{2+}]_i$ oscillations of ICC which were connected with cell clusters, and spontaneous $[Ca^{2+}]_i$ oscillations were observed after loading the cells with fluo3-AM (Fig. 5A). In the presence of 200 μM EGCG, $[Ca^{2+}]_i$ oscillations in ICC declined rapidly (Fig. 5B). Also, spontaneous $[Ca^{2+}]_i$ oscillations inhibited by EGCG were recovered to it's control condition. The temporal data are summarized in Fig. 5C). These results suggest that the action of EGCG on ICC may involve regulation of spontaneous $[Ca^{2+}]_i$ oscillations.

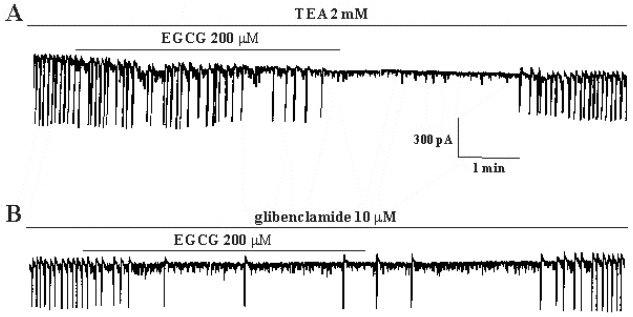

Fig. 4. Effects of tetraethylammonium chloride, a Ca^{2+}-activated K^+ channel blocker, and glibenclamide, an ATP-sensitive K^+ channel blocker, on (−)-epigallocatechin gallate-induced response in cultured ICC from mouse small intestine. (A) Pretreatment with tetraethylammonium chloride (2 mM) did not affect the inhibitory effects of (−)-epigallocatechin gallate (200 μM) on spontaneous inward currents. (B) Pretreatment with glibenclamide (10 μM) did not affect the inhibitory effects of (−)-epigallocatechin gallate (200 μM) on spontaneous inward currents. Vertical solid line scales amplitude of pacemaker current and horizontal solid line scales duration of recording (s) pacemaker currents. EGCG: (−)-epigallocatechin gallate, TEA: tetraethylammonium chloride.

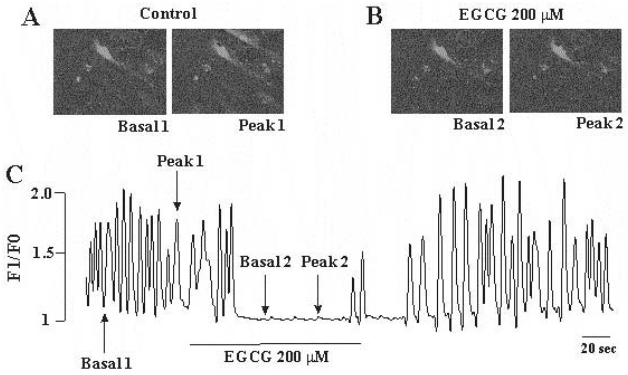

Fig. 5. Effects of (−)-epigallocatechin gallate on intrcellular Ca^{2+} oscillation in cultured ICC from mouse small intestine. (A) Sequential fluorescence intensity images of fluo-3-loaded cultured ICC in control condition. (B) Sequential fluorescence intensity images of fluo-3-loaded cultured ICC in the presence of (−)-epigallocatechin gallate (200 μM). The images of basal (1 and 2) and peak (1 and 2) in (A) and (B) acquired from indicators in (B). (C) Fluorescence intensity change plotted in (A) and (B) red marker.

DISCUSSION

Catechins perform a variety of biological actions, including antitumor and gastrointestinal functions. Actually, there are many reports to indicate that catechins influence gastrointestinal functions other than motility. A synthetic flavonoid protects rats from ulcerogens and does not affect either gastric acid secretion or pepsin output in rats (Konturek et al, 1986; Jayaraj et al, 1988). On the other hand, catechins inhibit gastric H^+, $K(^+)$-ATPase activity, and are the most potent inhibitor. These findings suggest that catechins usually show anti-secretory and anti-ulcerogenic effects in gastrointestinal tract. However, recent study showed that catechins can also alter spontaneous activity of small intestine (Ceregrzyn and Kuwahara, 2003). Furthermore, The present study demonstrated that EGCG regulates intestinal motility by modulating the pacemaker currents of ICC, and that this modulation is mediated via acting on intracellular Ca^{2+} mobilization in cAMP and cGMP-independent manner.

EGCG represents the major compound of catechin derivatives (Graham, 1992). In vascular system, EGCG showed seemingly contradictory actions on vascular tissues, such as a vasorelaxant activity (Chen et al, 2000; Alvarez et al, 2006) and a contractile behavior (Alvarez-Castro et al, 2004), that could be completely opposite reaction. And in small intestine, EGCG inhibits the motility of smooth muscle cells where a cGMP-dependent mechanism may partly be involved (Huang et al, 2006). Namely, EGCG exhibits more dominant action on vascular or intestinal motility compared with other catechin derivatives. In the present study, ECG and polyphenon did not show any influence on pacemaker activity in ICC, but only EGCG showed inhibitory action. Therefore, EGCG can induce a significant effect of spontaneous activity in the small intestine by acting on smooth muscle, and also the action of EGCG on intestinal motility can influence ICC.

The effect of EGCG seems to be mediated by an increase of cytosolic cAMP and cGMP concentrations: This is via inhibition of various cyclic nucleotide phosphodiesterase (PDE) isoforms (Alvarez et al, 2006). Also, many authors suggested that cGMP plays important roles in mouse intestinal ICC. For example, significant increase of cGMP in the colon was reported in response to exogenous NO (Shuttleworth et al, 1993), and cell-permeable 8-bromo-cyclic GMP itself showed the inhibitory action in ICC (Park et al, 2007). However, the present study showed that the action of EGCG on ICC was not mediated by cAMP and cGMP signaling pathway.

ATP-sensitive K^+ channels regulate membrane electrical potential, thereby determining cell excitability. Activation of ATP-sensitive K^+ channels produces hyperpolarization of cell membrane, thus decreasing Ca^{2+} influx and inhibiting cell excitability (Rodrigo and Standen, 2005). We also reported that ATP-sensitive K^+ channels exist in murine intestinal ICC (Choi et al., 2006). Furthermore EGCG has been reported to inhibit ATP-sensitive K^+ channel which is widely distributed in human tissues, and also all subtypes of ATP-sensitive K^+ channel (Baek et al, 2005). In this study, EGCG showed the mimicked action with pinacidil, an opener of ATP-sensitive K+channels, however the effect of EGCG on ICC was not antagonized by glibenclamide and also TEA. It suggests that EGCG inhibits pacemaker activities of intestinal ICC ,but ATP-sensitive K^+ and Ca^{2+}-activated K^+ channel do not mediate the action of EGCG.

Recent studies suggest that pacemaker activity depends on a link between Ca^{2+} release from cellular stores, oxidative metabolism, and the pacemaker conductance in the plasma membrane (Ward et al, 2000). Furthermore the inositol 1,4,5-triphosphate receptor plays a role in generating spontaneous electrical activity in gastro-intestinal pacemaker cells (Suzuki et al, 2000) .using cell cluster preparations isolated from mouse ileum, suggested that periodic Ca^{2+} release from intracellular Ca^{2+} stores produces $[Ca^{2+}]_i$ oscillations in ICC (Aoyama et al, 2004). These above mentioned actions seen in ICC are considered to be the primary pacemaker activity in the gut. Furthermore, many authors

related the effect of EGCG to the inhibition of Ca^{2+} influx in cellular level. For example, EGCG has vasorelaxant effect on vascular smooth muscle cells, possibly by phosphorylation of transmembrane Ca^{2+} channels or associated regulatory proteins (Orlov et al, 1996; Minowa et al, 1997) and inhibits the release of Ca^{2+} from intracellular stores (Orallo, 1996). In the present study, we examined spontaneous $[Ca^{2+}]_i$ oscillations in ICC and found that EGCG inhibited the $[Ca^{2+}]_i$ oscillations. Therefore, the mechanism of EGCG action on ICC appears to involve the release of $[Ca^{2+}]_i$, and EGCG can have inhibitory effect on pacemaker activity of ICC, when the $[Ca^{2+}]_i$ from intracellular Ca^{2+} storage.

In conclusion, we described herein the effects of EGCG on ICC in the mouse small intestine. EGCG inhibited the pacemaker activity of ICC and internal Ca^{2+} oscillations in a cAMP and cGMP-independent manner. Thus, the effect of EGCG on ICC could be due to it's inhibitory action on GI motility.

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