Regulation of Murine Myometrial Contraction by Ginger Extract Via Activation of Voltage Dependent Ca²⁺ Channels

Seung Hwa Hong[1], Kyu-Sang Kyeong[2], Il Woon Ji[1], Eun-Hwan Jeong[1], Hak Soon Kim[1], Bang Yeon Hwang[3], Chul Lee[3], Seung Myeung Son[4], Young Chul Kim[5]*, Ra Young You[5], Sang Jin Lee[5], Kyuen Na[6], Soyoung Ahn[6], Ji Young Park[7], Chan Hyung Kim[8], Hun Sik Kim[8], Woong Choi[8], Yeon Jin Park[9] and Wen-Xie Xu[10]

[1]Department of Obstetrics and Gynecology, College of Medicine, Chungbuk National University(CBNU), Chungdae-ro 1, Seowon-gu, Cheongju, Chungbuk, 28644, Korea

[2]Department of Obstetrics and Gynecology, Hallym Univ, Dongtam, Sared Heart Hospital, Heaseong, Korea

[3]Department of Pharmacy, CBNU, Cheongju, Chungbuk, 28644, Korea

[4]Department of Pathology, CBNU, Cheongju, Chungbuk, 28644, Korea

[5]Department. of Physiology, College of Medicine, CBNU, Cheongju, Chungbuk, 28644, Korea

[6]A Medical student, College of Medicine, CBNU, Cheongju, Chungbuk, 28644, Korea

[7]Deptartment of Psychiatry, CBNU, Cheongju, Chungbuk, 28644, Korea

[8]Dept. of Pharmacology, College of Medicine, CBNU, Cheongju, Chungbuk, 28644, Korea

[9]Department of Obstetrics and Gynecology, Chengju ST. Mari's Hospital, Cheonwon-Gu, Jusung-Ro, Cheongju, Chungbuk, 173-19, Korea

[10]Department of Physiology, College of Medcine, Shanghai Jiaotong University, 800 Dongchun Rd. Shanghai, 200240, P.R. China

*Corresponding author: Young Chul Kim, Department of Physiology, Chungbuk National University, College of Medicine, Chungdae-ro 1, Seowon-Gu, Cheongju 28644, Korea

Co-Corresponding author: Woong Choi, Department of Pharmacology, Chungbuk National University, College of Medicine, Chungdae-ro 1, Seowon-Gu, Cheongju 28644, Korea

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Abstract

Voltage-dependent L-type Ca²⁺ channel ($VDCC_L$) and T-type Ca²⁺ channel ($VDCC_T$) in murine myometrium was identified in murine myometrium. Its regulatory functions were characterized by using extracts of ginger. Methanol extract of ginger was used to obtain dichloromethane fraction (Gin C). Spontaneous uterine contractions were enhanced by BayK 8644, a $VDCC_L$ activator. However, such effects were inhibited by nifedipine (a $VDCC_L$ blocker) and mibefradil (a $VDCC_T$ blocker). Mibefradil also inhibited oxytocin (OXT), prostaglandins $F_{2\alpha}$ ($PGF_{2\alpha}$), and prostaglandins E_2 (PGE_2)-induced contractions. However, application of BayK 8644 in the presence of mibefradil recovered those contractions in a nifedipine-sensitive manner. These results suggest that both $VDCC_L$ and $VDCC_T$ are important in the regulation of murine myometrial contractions. Gin C (200 mg/mL) completely inhibited spontaneous contractions of murine uterus reversibly. The inhibition by Gin C on spontaneous contractions independent of L-NAME, K⁺ channel blockers, and nerve blockers. High K⁺ (50 mM)-induced contraction in the presence and absence of cyclopizonic acid (CPA) was also completely inhibited by Gin C, respectively. In addition, Gin C inhibited oxytocin (OXT; 10 nM)-induced contraction independent of L-NAME and blockers of protein kinases. Prostaglandin F_{2a} (PGF_{2a})- and acetylcholine (ACh) produced contractions were also inhibited by Gin C. These results raise the possibility that Ginger extracts C inhibits spontaneous, high K⁺-, OXT-, PGF_{2a}- and ACh-induced contractions by inhibition of $VDCC_L$ in mouse uterine longitudinal smooth muscle.

Introduction

Ginger (*Zingiber officinae* Roscoe) is a flowering plant in family Zingiberaceae whose roots is widely used as an ingredient in both cooking and East Asian traditional medicine. Ginger is traditionally used to treat fever, nausea, vomiting, and uterine disorders. Ginger has also been used to treat paralytic ileus in Japan [1-4]. It has

been reported that ginger can regulate contractility of uterine, gastrointestinal (GI) tract, and airway smooth muscle. Rat uterine muscle tone and spontaneous contractions can also be enhanced by ginger extract [5]. Such enhancing effect on the contraction is produced by activation of voltage-dependent L-type Ca^{2+} channels ($VDCC_L$) and release of Ca^{2+} from sarcoplasmic reticulum [5]. Other reports have suggested similar enhancing effects of ginger extract on rabbit uterine smooth muscle tone and its spontaneous contractions [6]. In general, $VDCC_L$ is essential for the regulation of smooth muscle contractility in many species [7-10]. Nifedipine, a $VDCC_L$ blocker, can also block oxytocin (OXT)-induced phasic contraction in murine myometrium (unpublished data). Regulatory effects of ginger on contractility of uterine, gastrointestinal (GI) tract, and airway smooth muscle have been reported [5-6,11].

Ginger also can improve gastric emptying and motility, intestinal contractility, and irritable bowel syndrome (IBS) in the GI tract [12-13]. In addition, ginger extract and its components can also inhibit motility of GI including the lower esophageal sphincter (LES) [14-15]. Other studies have suggested that ginger extract has regulatory effects on airway and vascular smooth muscle [16-17]. However, the mechanism of action involved in the effect of ginger extract on these organs remains unclear. In this study, we found that ginger extract could inhibit uterine smooth muscle contractility by blocking VDCCs. Since ginger is safe for humans, ginger might have potential to be developed as a tocolytic agent to relieve excessive uterine contractions.

Materials and Methods

Tissue Preparation for Isometric Contraction

All experiments were performed in accordance with the guidelines for animal care and use provided by Chungbuk National University (CBNUA-383-11-01; CBNUA-597-13-02; CBNUA-719-14-01; CBNUA-863-15-01; CBNUA-988-16-01; CBNUA-1125-17-02; CBNUA-1162-18-02). All animal experiments were conducted in accordance with the National Institutes of Health (USA) Guidelines for the Care and Use of Laboratory Animals (the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press)) and were approved by the Chungbuk National University Medical School Research Institutional Animal Care and Use Committee (Korea). Female non-pregnant mouse was used in this whole study. Institute of Cancer Research (ICR) mice (age, 10–12 weeks) were anaesthetized with fluoromethyl 2,2,2-trifluoro-1(trifluoromethyl) ethyl ether (Sevoflurane; Maruishi Pharma., Osaka, Japan) and/or chloroform and killed by cervical dislocation. Their uteri were cut open from the neck to the end of uterine horns, rinsed in Krebs-Ringer bicarbonate (KRB) solution, and pinned on a Sylgard plate to maintain their original shape. Connective tissues were removed, and these uteri were cut.

The endometrium was separated from other muscle layers in KRB solution. Longitudinal muscle strips (1×5 mm) were mounted onto vertical chambers (25 and 75 mL) in an isometric contractile measurement system with one end of the tissue tied tightly to a fixed holder while the other end of the tissue was linked to a force transducer (Harvard Instruments, Holliston, MA, USA) by a hook. The force transducer was connected to a Power Lab-Data Acquisition System and a personal computer running Charter v5.5 software (ADinstruments, Boulder, CO, USA) to measure isometric contractions. Each strip was stretched passively to resting tension for 1-2 hours after equilibration for 1.5 hours. Contractile responses of the strip to high K^+ (50 mM) were repeated twice.

Ginger Extraction

Dried ginger powder was purchased from a local company and 300 g was extracted twice with methanol for 2 L 24 hours at room temperature. Four liters of this methanol extract was dried in a rotary evaporator to yield 21 g of precipitate. The precipitate was resuspended in 500 ml of water and mixed with 500 ml of dichloromethane. The mixture was set at room temperature until water and dichloromethane phases separated clearly. Water and dichloromethane fractions were then collected and freeze-dried. Yields of water and dichlromethane fractions were 9.4 g and 11.4 g, respectively. We already got patent in Korea (Patent No: 1018089440000; Title: Composition for preventing and treating dysmenorrhea and premature labor comprising non-polar solvent subfraction from *Zingiber officinale* extract; Web site: http://engportal.kipris.or.kr/engportal/search/total_search.do)

Solution and Drugs

KRB solution (CO_2/bicarbonate-buffered Tyrode) contained (in mM) the following: 122 mM NaCl, 4.7 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 15 mM $NaHCO_3$, 0.93 mM KH_2PO_4, and 11 mM glucose (pH 7.3-7.4, bubbled with 5 % CO_2/95 % O_2). Equimolar concentration of Na^+ was replaced with K^+ to make high K^+ (50 mM) solution. The external solution was changed with solution that had been bubbled with 5% CO_2/95% O2, 36 °C) in a water bath before application. Various blockers were applied for 12-15 minutes before application of Gin C. Then a K^+ channel blocker cocktail (KBC) was applied before application of stimulators to block each K^+ channel's responses. KBC contained 4-aminopyridine (4-AP, 2 mM), tetraethylammonium (TEA, 5 mM), apamin (APA, 300 nM), and glibenclamide (Glib, 20 µM). To rule out nerve mediated response, a nerve blocker cocktail (NBC) was used. NBC contained tetrodotoxin (TTX, 0.4 µM), guanethidine (1 µM), and atropine (ATR, 1 µM) [7-9]. All drugs used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistics

Data are expressed as means ± standard errors of the mean (SEM). Statistical significance was measured using analysis of variance (ANOVA) and student's t-test. Any p-value less than 0.05 was regarded as statistically significant.

Results

Isometric Contraction of Mouse Uterine Longitudinal Smooth Muscle

Oxytocin (OXT, 10 nM) produced tri-phasic contractions, showing an initial contraction followed by a tonic contraction overlapped with a phasic contraction (Figure 1). Uterine smooth muscle produced spontaneous contractions of 1.4 ± 0.25 g with a frequency of 0.5 ± 0.05 cycles/min (Figure 1B; n = 43). $VDCC_L$ activator BayK 8644 enhanced the strength and the frequency of uterine spontaneous contractions to 215 ± 49.0 % and 228 ± 29.0 % of the control [n = 4 each, p < 0.05, (Figure 1B)]. These enhanced contractions caused by BayK 8644 were completely inhibited by nifedipine (2 μM, n = 3, p < 0.05). Phasic OXT-induced contractions were completely blocked by 2 μM nifedipine [(Figure 1C), n = 4, p < 0.05]. The strength and frequency of OXT-induced phasic contractions were also enhanced significantly by BayK 8644 compared to those of the control (n = 6, respectively, p < 0.05; data not shown). These enhanced OXT-induced phasic contractions were completely inhibited by nifedipine (2 μM, n = 6, p < 0.05, data not shown).
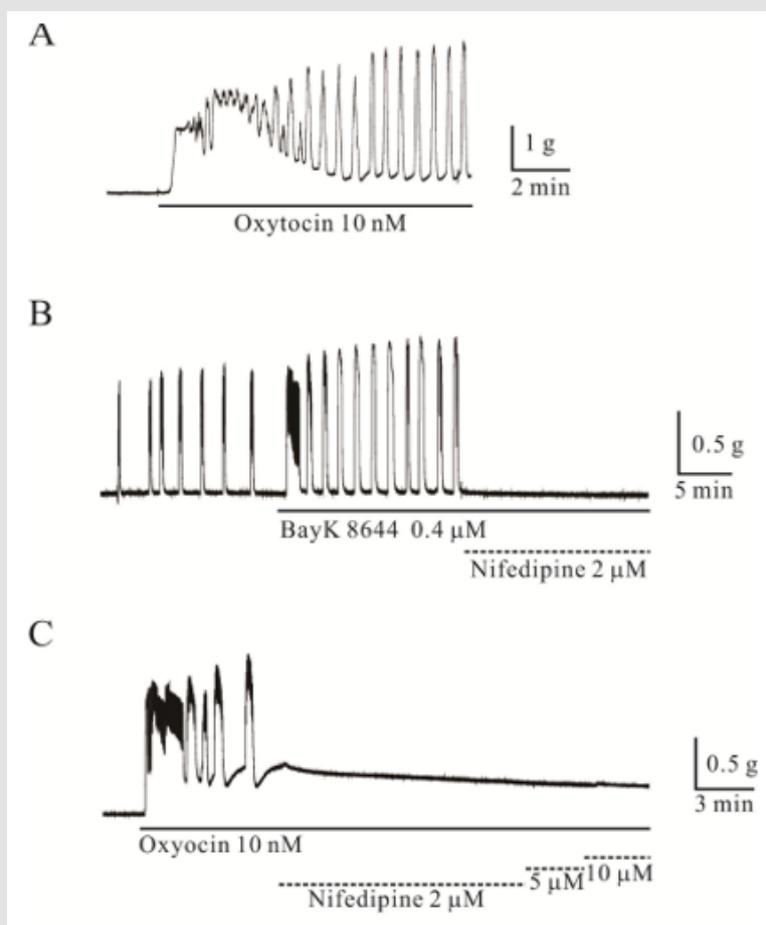

(A) Oxytocin (OXT, 10 nM) produced tri-phasic contractions, such as initial contraction, followed by tonic contraction overlapped with phasic contraction. (B) Uterine smooth muscle produced spontaneous contractions of 1.4 g with a frequency of 0.5 cycles/min. BayK 8644 (voltage-dependent L-type Ca^{2+} channel [$VDCC_L$] activator) enhanced uterine spontaneous contraction and frequency to 215 % and 228 % of the control. The spontaneous and enhanced contractions by BayK 8644 were completely inhibited by nifedipine (2 μM). (C) OXT-induced phasic contractions was blocked by 2 μM nifedipine.

Figure 1: Isometric contractions of mouse uterine longitudinal smooth muscle.

Inhibitory Effect of Ginger Extract (Gin C) on High K⁺-Induced Contraction

As shown in Figure 2, high K⁺ (50 mM) produced tonic contraction in uterine smooth muscle (1.8 ± 0.43 g, n=20). In the presence of cyclopiazonic acid (CPA), high K⁺ (50 mM)-induced contraction of 1.4 ± 0.75 g (n=7) and was completely blocked by application of nifedipine [2-5 μM, n=7; (Figure 2B)]. Gin C at 40-400 mg/ mL inhibited high K⁺-induced contractions (Figure 2C). Gin C at 100, 200, and 400 mg/ mL inhibited high K+-induced contractions to 46 ± 10.2%, 15 ± 4.9%, and 3.4 ± 1.4%, respectively, of the control (p < 0.05; n = 5, data not shown). Gin C also inhibited high K⁺ (50 mM)-induced contraction in the presence of CPA completely (400 mg/ mL, n=5; data not shown).
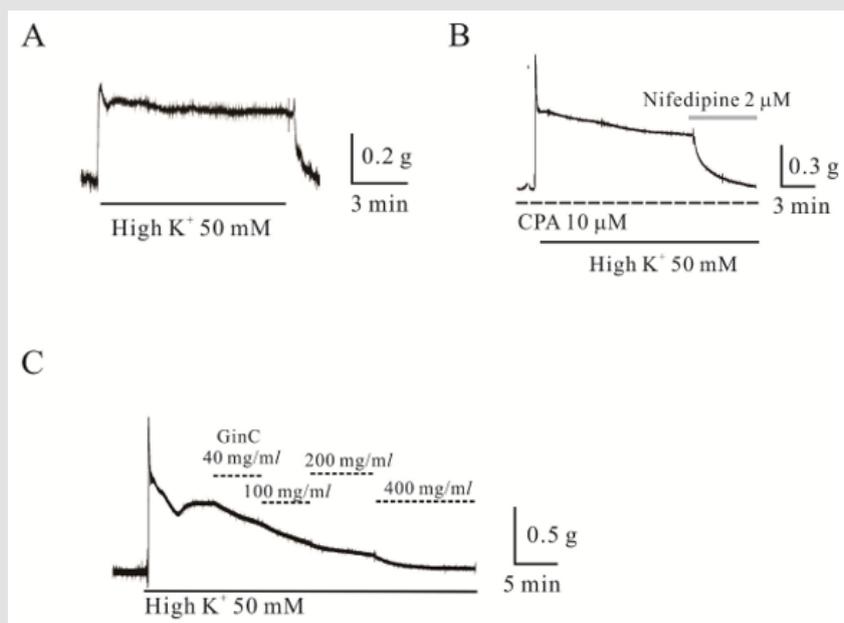
High K⁺ (50 mM) produced tonic contraction of longitudinal smooth muscle in the absence (A) and presence (B) of cyclopiazonic acid (CPA). C: Gin C (40-400 mg/mL) inhibited high K⁺ (50 mM)-induced contractions of mouse uterine longitudinal smooth muscle.

Figure 2: Inhibitory effect of ginger extract (Gin C) on high K⁺ (50 mM)-induced contractions of mouse uterine longitudinal smooth muscle

Inhibitory Effect of Ginger Extract (Gin C) on Spontaneous Contraction of Mouse Uterine Longitudinal Smooth Muscle
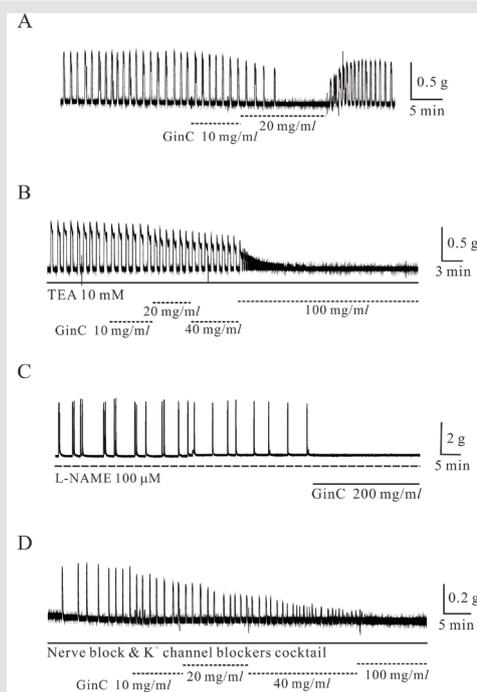
A: Spontaneous contractions of longitudinal smooth muscle were reversibly inhibited by Gin C (10 and 20 mg/mL).

B: Gin C produced relaxation in the presence of tetraethylammonium (TEA, 10 mM), which block Ca²⁺-activated K⁺ (Kᶜᵃ) channels.

C: The inhibitory effect of Gin C on spontaneous contractions was not mediated by nitric oxide (NO). Gin C (200 mg/mL) inhibited spontaneous contractions in the presence of NG-nitro-L-arginine methyl ester (L-NAME; 100 μM, an NO synthesis inhibitor).

D: The effect of Gin C on the uterine smooth muscle contractions in the presence of the K⁺ channel blocker cocktail (KBC) and nerve blockers was also observed.

Figure 3: Inhibitory effect of ginger extract (Gin C) on spontaneous contractions of mouse uterine longitudinal smooth muscle.

Spontaneous contractions of longitudinal smooth muscle were inhibited by Gin C (10–200 mg/mL) in a reversible manner (Figure 3). Gin C at 10, 20, 100, and 200 mg/mL inhibited spontaneous contractions to 46 ± 14.7%, 40 ± 15.5%, 26 ± 19.3 %, and 0 %, of the control, respectively (p < 0.05; n = 5, 7, 5, and 3, respectively). Gin C at 100 and 200 mg/mL also inhibited basal tone slightly to −0.03 ± 0.01 g and −0.05 ± 0.01 g, respectively (n = 5 and n = 3, respectively). However, the inhibitory effect of Gin C on spontaneous contractions was not mediated by nitric oxide (NO): Gin C at 100 mg/mL inhibited spontaneous contractions completely in the presence of NO synthesis inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 100 μM; n = 9; Figure 3C).

Effects Of K⁺ Channel and Nerve Blockers on Gin C-Induced Relaxation in Mouse Uterine Smooth Muscle

We studied the effect of Gin C on uterine smooth muscle contractions in the presence of KBC and NBC to investigate whether K⁺ channels and nerves were activated during Gin C-induced relaxation. Gin C produced relaxation (n = 2) in the presence of tetraethylammonium (TEA, 10 mM) which blocked Ca^{2+}-activated K⁺ (K^{ca}) channels (Figure 3B). Gin C-induced relaxation was also observed in the presence of KBC and NBC (Figure 3D). Gin C at 100 and 200 mg/l produced relaxation up to 14 ± 7.3 % and 0 ± 0 %, respectively, of the control (n = 3 and n = 2, respectively).

Inhibitory Effects of Gin C on Oxt- And High K⁺-Induced Mouse Uterine Longitudinal Smooth Muscle Contractions

Gin C inhibited OXT-induced uterine smooth muscle contractions. Gin C at 40, 100, 200, and 400 mg/mL inhibited OXT-induced phasic contractions to 56 ± 12.2%, 54 ± 11.6%, 2 ± 2%, and 0% of the control, respectively (Figure 4A),(p < 0.05; n = 16, 14, 12, and 2, respectively). In the presence of L-NAME, Gin C at 100 and 200 mg/mL also inhibited OXT-induced phasic contractions to 20 ± 7.0% and 0 %, respectively, of the control (p < 0.05; n = 10 and n = 6, respectively; data not shown). In addition, Gin C at 100, 200, and 400 mg/mL inhibited high K⁺-induced contractions to 46 ± 10.2%, 15 ± 4.9%, and 3.4 ± 1.4%, respectively, of the control (p < 0.05; n = 5, data not shown; Figure 4).
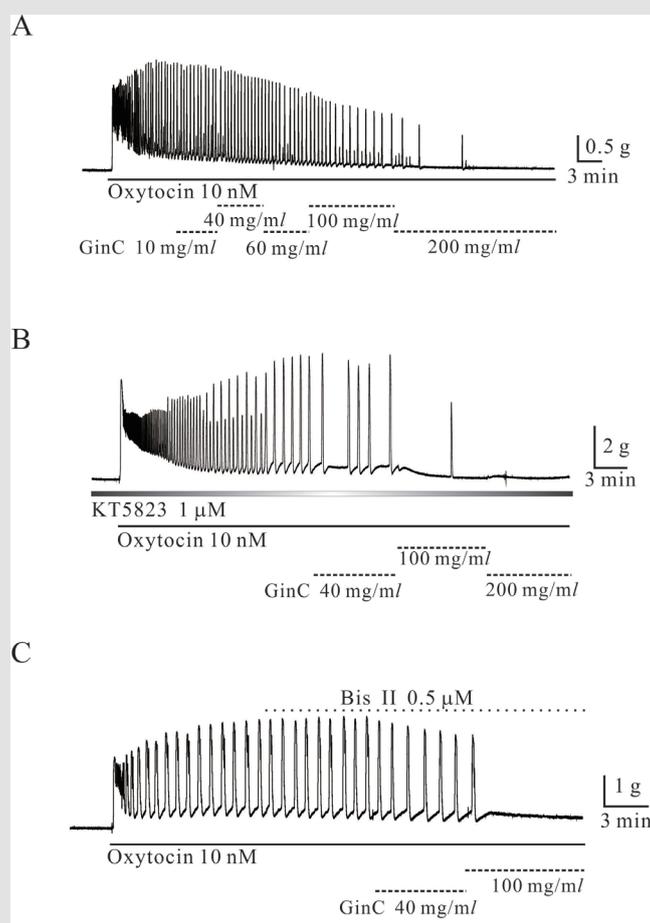

Gin C inhibited OXT-induced uterine smooth muscle contractions. A: OXT-induced contractions of uterine smooth muscle were also inhibited by Gin C. Gin C (10 - 200 mg/mL) inhibited OXT-induced phasic contractions to 56 %, 54 %, 2 %, and 0 % of the control. B: Inhibitory effects of Gin C on OXT-induced contractions were studied in the presence of KT 5823, which inhibit PKG. OXT-induced contractions was inhibited by Gin C in the presence of KT 5823 to 50 %, 0 %, and 0 % of the control. C: Inhibitory effect of Gin C (40, 100, and 200 mg/mL) on OXT-induced contractions was not affected by bisindolylmaleimie II (Bis II), which is a PKC inhibitor. In the presence of Bis II (0.5 μM), Gin C (40 and 100 mg/mL) inhibited OXT-induced contractions to 36 % and 1 % of the control.

Figure 4: Inhibitory effects of ginger extract (Gin C) on Oxytocin (OXT)-induced contractions of mouse uterine smooth muscle.

Inhibitory Effects of Gin C on Oxt-Induced Mouse Uterine Longitudinal Smooth Muscle Contractions in The Presence of Protein Kinase Inhibitors

Inhibitory effects of Gin C on OXT-induced contractions were studied in the presence of KT 5823 and KT 5720 known to inhibit protein kinase G (PKG) and PKA, respectively. OXT-induced contraction was inhibited by Gin C at 40, 100 and 200 mg/l in the presence of KT 5823 to 50 ± 20.8%, 0 %, and 0 % of the control, respectively (n = 4, 3, and 2, respectively, p < 0.05; Figure 4C). OXT-

induced contractions in the presence of KT 5720 were inhibited by Gin C at 20, 40, 100 and 200 mg/l to 78 ± 0.2 %, 82 ± 0.1 %, 31 ± 0.2 %, and 0 % of the control, respectively (p < 0.05, n = 5, 5, 5, and 0, respectively; data now shown). To evaluate the involvement of PKC in Gin C-induced inhibition of OXT-induced contractions, Gin C was applied in the presence of a PKC inhibitor. As shown in Figure 4C, in the presence of PKC inhibitor bisindolylmaleimie II (Bis II, 0.5 μM), Gin C at 40 and 100 mg/l inhibited OXT-induced contractions to 36 ± 20.9 % and 1 ± 0.8 % of the control, respectively (p < 0.05; n = 4 and n = 2, respectively).
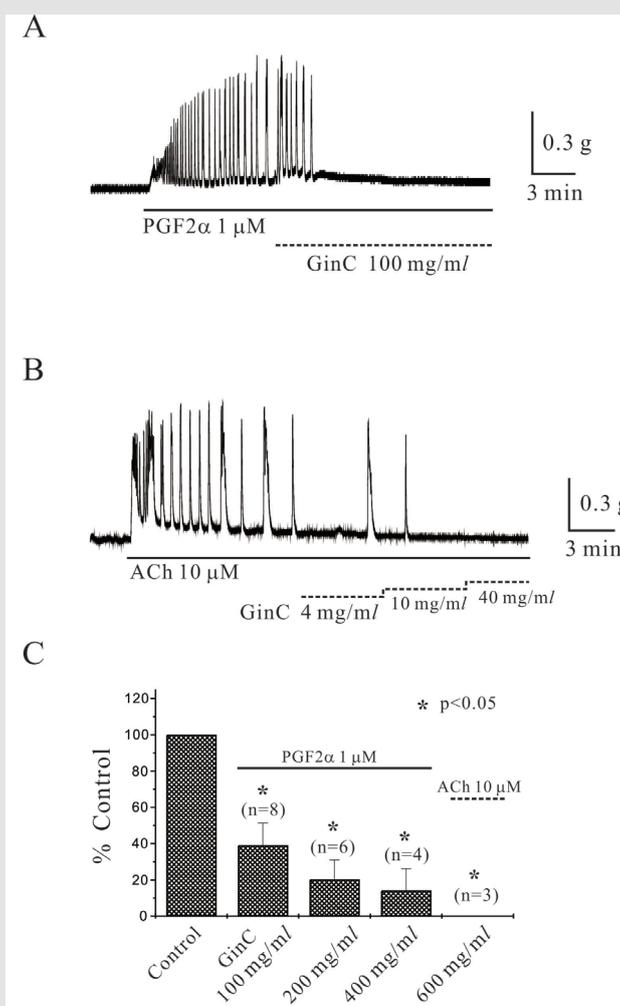
A and C: $PGF_{2\alpha}$ produced tri-phasic contractions, such as an initial contraction, followed by a tonic contraction overlapped with a phasic contraction. $PGF_{2\alpha}$-induced phasic contractions were inhibited by Gin C (100, 200, and 400 mg/mL) to 39 %, 20 %, and 14 % of the control. B: ACh-induced phasic contraction was completely inhibited by Gin C and data as averaged in C.

Figure 5: Inhibitory effects of ginger extract (Gin C) on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)- and induced mouse uterine smooth muscle contractions.

Inhibitory Effects of Gin C on Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)- And Acetylcholine (ACh)-Induced Mouse Uterine Smooth Muscle Contractions

$PGF_{2\alpha}$ produced tri-phasic contractions in murine uterine smooth muscle, showing an initial contraction followed by a tonic contraction overlapped with a phasic contraction (Figure 5). $PGF_{2\alpha}$-

induced phasic contractions were inhibited by Gin C at 100, 200, and 400 mg/l to 39 ± 12.3 %, 20 ± 10.8 %, and 14 ± 12.0 % of the control (n = 8, 8, and 6, respectively, p<0.05; Figure 4B). As shown in Figure 5B, Gin C also inhibited ACh-induced phasic contractions. In particular, Gin C at 200 mg/l completely inhibited ACh-induced phasic contraction (n = 3; Figure 5C).

Discussion

It is well known that regulation of myometrial contractility is tightly linked to Ca^{2+} influx and Ca^{2+} signaling at cellular level [18]. In this study, we found that Gin C inhibited contractions of murine uterus longitudinal smooth muscle by inhibition of $VDCC_L$. As shown in (Figures 1 & 2B), murine myometrial contraction was tightly related to the activation of VDCC. Well-known pharmacological blockers such as nifedipine inhibited myometrial contractions. This implies that both $VDCC_L$ is important for the regulation of myometrial contraction. In this study, Gin C inhibited high K^+-, OXT-, ACh-, and $PGF_{2\alpha}$- induced phasic contractions. Its effect was independent of NO synthesis, protein kinases (PKA, PKG and PKC), K^+ channel, or nerve blockers (Figures 3 & 4). This finding suggests that Gin C may inhibit mouse uterine smooth muscle contractions by inhibiting $VDCC_L$ and/or $VDCC_T$ strongly. It has been reported that ginger can enhance and inhibit uterine, GI tract, and airway smooth muscle contractions by activating $VDCC_L$ [4,14,19]. In the present study, we found that murine uterine spontaneous contractions and OXT-induced phasic contractions were sensitive to BayK 8644 and nifedipine (Figures 1B & 1C).

In smooth muscle, Ca^{2+}-induced Ca^{2+} release (CICR) mechanisms also known to be important to regulate smooth muscle contraction [20-22]. As shown in Figure 2B, effect of Gin C was studied in the presence of CPA too. Therefore, inhibition of murine myometrial by Gin C might be responsible for the inhibition of $VDCC_L$. However, in fact, effects of constituents of ginger extract on smooth muscle contractions were reported differently depending on the diverse extract. Therefore, we will also try to do some more supplementary experiments delicately by using other extracts from ginger in the future. Primary dysmenorrhea may be caused by an increase in $PGF_{2\alpha}$ produced in the uterus that can hyper contract the uterine smooth muscle and/or locally contracts blood vessels [23-24]. However, the exact mechanism of uterine spasms is currently unclear. However, phasic contraction was also sensitive to nifedipine in murine myometrium (unpublished data), Gin C inhibited $PGF_{2\alpha}$-induced contractions by inhibition of $VDCC_L$. Therefore, inhibition of $PGF_{2\alpha}$-induced contractions by Gin C might suggests it could reduce symptoms of dysmenorrhea.

It is well known that $VDCC_L$ performs a key role in the regulation of smooth muscles [7-9]. However, the role of $VDCC_T$ in such regulation is relatively unclear. In myometrium, $VDCC_T$ in rat and human pregnant samples has been reported. In rat pregnant myometrium, spontaneous phasic contraction has been found to be sensitive to micromolar ranges of mibefradil [11]. Meanwhile, specific subtype of $VDCC_T$ ($CaV_{3.1}$) and role of $VDCC_T$ in the regulation of contraction in human pregnant myometrium has been identified [25-26]. Whether the exact subtype of $VDCC_L$ and/or $VDCC_T$ might be related to various conditions of myometrial contractility is not fully understood yet. Furthermore, the role of $VDCC_T$ compared to that of $VDCC_L$ in myometrial contraction is not well studied yet. In fact, even the regulation of rat pulmonary arterial proliferation is regulated by $VDCC_T$ through activation of $CaV_{3.1}$ channel [27]. We identified functional expression of $VDCC_L$ ($CaV_{1.2}$) in murine myometrium by performing mechanics and immunohistochemistry (data not shown in here). However, further study is needed to identify T-type Ca^{2+} channel and more functions of it in murine and human myometria.

Ginger extracts are known to have effects on the GI tract. For example, they can inhibit LES motility [14,19]. In these cases, serotonergic receptors and/or cholinergic M receptors are involved in inhibiting smooth muscle contraction [15,28]. Ginger extract can also improve gastric emptying and IBS for gastric and intestinal motility, respectively [12-13]. That implies ginger extract might produce increasing and decreasing functions of smooth muscle via affecting receptor levels too. However, we found Gin C specifically inhibited murine myometrial contraction by inhibition of VDCCL. Ginger plant has been used to treat inflammation, rheumatic disorders, and diarrhea in traditional medicine [2,4,29]. Zingerone is thought to be the active antidiarrheal component responsible for limiting endotoxin-induced diarrhea [30-31]. Therefore, some effects of ginger extracts are not direct on smooth muscle. Meanwhile, gingerol produces dual effects (enhancing and inhibitory) on ileal contractions in guinea-pig [12-13] through capsaicin-sensitive neurons [32]. Shogaol- [6] from ginger can also inhibit vascular smooth muscle proliferation by activating specific signaling pathways [16].

A cyclooxygenase-related system in vascular smooth muscle may be involved in regulating eicosanoid-induced contraction [16]. Additionally, gingerols exhibit various effects on the cardiovascular system [16] while zingerone may activate the same capsaicin receptors and/or a common pathway in trigeminal ganglion neurons [32]. Some other Gin C components such as ginerols and/or shogaol could also inhibit uterine contractility. Therefore, extracts from ginger produces diverse effects on different organs via various actions. From these results, we tried to exclude involvement of nerves and other K^+ channels by other ginger extracts [33]. As shown in Figure 3B & 3D, the relaxing effect of ginger extract was studied in the presence of TEA, nerve blocker cocktail. In addition, involvement of nitric oxide (NO) in the action of antinociceptive activity by ginger extract was also reported [34]. Therefore, we also studied and found inhibitory effects of Gin C in the presence of and L-NAME (100 μM) on murine uterine smooth muscle (Figure 3C). Our results suggest that Gin C may produce murine uterine relaxation by inhibiting $VDCC_L$. This is first report showing that inhibition of uterine smooth muscle contractility by ginger extract obtained by dichloromethane fraction (Gin C). Our results revealed the possibility that Gin C inhibited High K^+-, OXT-, $PGF_{2\alpha}$-, and ACh-induced uterine contraction and spontaneous contraction of uterine longitudinal smooth muscle of mouse by inhibiting $VDCC_L$.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgement

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