BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 28(4), 328-336 (2020)

The Altered Signaling on EFS-Induced Colon Contractility in Diabetic Rats

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Abstract

Diabetes mellitus affects the colonic motility developing gastrointestinal symptoms, such as constipation. The aim of the study was to examine the role of intracellular signaling pathways contributing to colonic dysmotility in diabetes mellitus. To generate diabetes mellitus, the rats were injected by a single high dose of streptozotocin (65 mg/kg) intraperitoneally. The proximal colons from both normal and diabetic rats were contracted by applying an electrical field stimulation with pulse voltage of 40 V in amplitude and pulse duration of 1 ms at frequencies of 1, 2, 4, and 6 Hz. The muscle strips from both normal rats and rats with diabetes mellitus were pretreated with different antagonists and inhibitors. Rats with diabetes mellitus had lower motility than the control group. There were significant differences in the percentage of inhibition of contraction between normal rats and rats with diabetes mellitus after the incubation of tetrodotoxin (neuronal blocker), atropine (muscarinic receptor antagonist), prazosin (α_1 adrenergic receptor antagonist), DPCPX (adenosine A1 receptor antagonist), verapamil (L-type Ca^{2+} channel blocker), U73122 (PLC inhibitor), ML-9 (MLCK inhibitor), udenafil (PDE_5 inhibitor), and methylene blue (guanylate cyclase inhibitor). The protein expression of p-MLC and PDE_5 were decreased in the diabetic group compared to the normal group. These results showed that the reduced colonic contractility resulted from the impaired neuronal conduction and decreased muscarinic receptor sensitivity, which resulted in decreased phosphorylation of MLC via MLCK, and cGMP activity through PDE_5.

Key Words: Gastrointestinal motility, Electrical field stimulation, Colon, Diabetes mellitus

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder (non-communicable disease) characterized by increased serum blood glucose level for a prolonged period due to defective insulin secretion. Many diseases, for example cardiovascular disease, diabetic neuropathy, urinary bladder dysfunction, and kidney disease, are associated with DM (Weissman, 2006; Van Den Eeden *et al*., 2009; Tuttle *et al*., 2014; Amaral and Okonko, 2015; Pecoits-Filho *et al*., 2016). Approximately 76% of patients with DM suffer from gastrointestinal (GI) disorders, such as constipation, diarrhea, abdominal pain, or fecal incontinence, due to the uncontrolled glycemic condition (Feldman and Schiller, 1983; Bytzer *et al*., 2001). The pathogenesis of GI dysfunction associated with DM appears to be multifactorial and complex but remains unclear.

Neurotransmitters bind to a specific G-protein coupled receptor, which stimulates phospholipase C (PLC) activity and causes the catalysis of lipid phosphatidylinositol 4, 5-bisphosphate (PIP2). The catalysis of PIP2 leads to the production of inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 binds to the IP3 receptor (IP3R) and causes the release of calcium from the sarcoplasmic reticulum into the cytosol. Intracellular calcium binds to calmodulin and activates myosin light chain kinase (MLCK) (Khromov *et al*., 2006). The phosphorylation of the 20 kDa myosin regulatory light chain (MLC20) by MLCK at a serine residue at position 19 is enough to cause smooth muscle contraction (Kamm and Stull, 2001; Somlyo and Somlyo, 2003). The function of myosin light chain phosphatase (MLCP) is opposite to that of MLCK; MLCP removes the phosphate from serine 19 on MLC20 (Hartshorne *et al*., 2004). It has been shown that MLC20 phosphorylation can be regulated, either directly or through the regulation of MLCP activity and associated proteins, by several kinases. In addition, receptors such as the α_1-adrenergic and adenosine A_1 receptors are involved in the modulation of neurotransmission processes in the gastrointestinal tract (Ruwart *et al*., 1979; Paton, 1981; Broad *et al*., 1992; Christofi and Wood, 1993). The

Open Access **https://doi.org/10.4062/biomolther.2019.181**

Received Oct 30, 2019 **Revised** Dec 24, 2019 **Accepted** Jan 7, 2020
Published Online Mar 4, 2020

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colon response to the electrical field stimulation (EFS) is characterized by simultaneous stimulation of all neurons within myenteric plexus, including excitatory and inhibitory elements sympathetic and parasympathetic nerves, cholinergic, and non-adrenergic and non-cholinergic (NANC) neurotransmitters like adenosine triphosphate (Kilbinger *et al*., 1982). Nitric oxide (NO) released from endothelium activates soluble guanylate cyclase (sGC), producing cyclic guanosine monophosphate (cGMP). cGMP plays a crucial role in smooth muscle relaxation and is important for the optimal function of phasic and tonic contractions. The intracellular cGMP levels are regulated by GC and its hydrolysis through phosphodiesterase 5 (PDE_5) (Beavo, 1995; Murthy, 2001; Rybalkin *et al*., 2003; Conti and Beavo, 2007; Francis *et al*., 2011).

Streptozotocin (STZ) has been used to destroy pancreatic β-cells, resulting in DM in experimental animals. Alterations in colon motility and small intestine smooth muscle were found in rats with STZ-induced DM (Nowak *et al*., 1990; Forrest *et al*., 2008). The decrease in the colonic contractility has various causes. Cholinergic receptor sensitivities and neuronal nitric oxide synthase activity in the colon of diabetic rats appear to be weakened (Kim *et al*., 2011).

The purpose of the present study was to investigate the impairment of colonic motility in DM rats and to understand the underlying mechanism with respect to G-protein coupled receptor (GPCR), the PLC-dependent signaling pathway, and the cGMP signaling pathway, which may affect the changes in colonic contractility.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (SD) rats weighing 210-240 g were purchased from Samtako Bio (Osan, Korea). The animals were housed individually in cages with wire-net floors in a room with controlled temperature (24-25°C), humidity (70-75%) and light cycle (12 h light/12 h dark). Rats were fed a normal laboratory diet (Samtako Bio) and were fasted for over 14 h prior to experiments. However, the rats were allowed free access to tap water throughout. The experiments were performed in accordance with guidelines and approved by the Institutional Animal Care Use Committee of Chung-Ang University (IACUC 2019-00010).

Solutions and drugs

Tissues were maintained in Krebs buffer [NaCl, 116.6 mM; $NaHCO_3$, 21.9 mM; NaH_2PO_4, 1.2 mM; KCl, 3.4 mM; $CaCl_2$, 2.5 mM; glucose, 5.4 mM; and $MgCl_2$, 1.2 mM]. ML-9, chelerythrine chloride, leupeptin, and streptozotocin (STZ) were purchased from Tocris Cookson Ltd (Bristol, UK). Atropine sulfate was obtained from Merck (Whitehouse Station, NL, USA). β-Actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p-MLC, MLC and PDE_5 were obtained from Cell Signaling Technology (Danvers, MA, USA). Tetrodotoxin, U73122, NaHS, verapamil hydrochloride, atropine sulfate, SB212090, PD98059, 4-aminopyridine (4-AP), tetraethylammonium chloride (TEA), prazosin hydrochloride, DPCPX, methylene blue, and other reagents were obtained from Sigma (St. Louis, MO, USA).

Induction of diabetes mellitus

A rat model of DM in adulthood was produced as previously described (Wu and Huan, 2008). Specifically, experimental diabetes was induced in rats fasted overnight by a single dose of 65 mg/kg STZ. STZ was dissolved in 50 mM citrate buffer, pH 4.5, and administered intraperitoneally to rats; these rats were considered the DM rats. The control group received an intraperitoneal injection of equal volume of citrate buffer. Diabetes was confirmed by an increase in the non-fasting serum glucose levels of 300 mg/dL or above, which was checked 2 days after the administration of STZ by using a Care Sens II glucose meter from i-SENS (Seoul, Korea). To counteract initial hypoglycemia due to the insulin release from destroyed beta cells, 10% sucrose water was supplied for the first 24 h after the injection of STZ. The body weight was monitored in normal and DM rats on days 1, 3, and 7 of the experiment. Daily food consumption was calculated from the measurement of the mass of food from day 1 of the experiment to the day 7 of the experiment. The daily water intake was calculated by measuring the volume of liquid remaining on the same days as the food consumption was measured. All measurements were performed at the same time.

Tissue preparation

One week after the administration of STZ, the rats were euthanized by CO_2 asphyxiation. The proximal colon (approximately 1 cm from the cecum) was collected and immediately placed in oxygenated (95% O_2 and 5% CO_2) Krebs solution. Transversely oriented muscle strips were taken from the proximal colon and then cut into 2 smaller strips (2 mm wide by 7 mm long). These strips were tied with silk ligatures at both ends. The muscle strips were mounted vertically between platinum electrodes in separate 1 mL muscle chambers. One edge was fixed to the bottom of the muscle chamber and the other one was attached to a force transducer (FT03, Grass In-

Table 1. The blood glucose level and body weight of normal and STZ-induced diabetic rats

Category	Rats	Day 1	Day 3	Day 7
Blood glucose level (mg/dL)	Normal rats	137 ± 4	149 ± 8	145 ± 6
	DM rats	143 ± 5	440 ± 28***	453 ± 21***
Body weight (g)	Normal rats	241± 6	258 ± 6	293 ± 7
	DM rats	239 ± 5	229 ± 5	245 ± 5**

The rats were fasted overnight and induced diabetes mellitus by a single dose of streptozotocin (65 mg/kg, i.p). The blood glucose level and the body weight were observed on Day 1, 3, 7. Blood glucose level greater than 300 mg/dL was considered as diabetes mellitus. ** p<0.01, *** p<0.001 vs. normal rats by Student t-test, n=13.
Data are expressed as mean ± SEM.

struments Co., Quincy, MA, USA). Changes in isometric force were recorded by using a polygraph (Model 79; Grass Instruments Co.).

They were initially stretched to 1 *g* to bring them to near conditions of optimal force development and were equilibrated for 90 min with continuous perfusion with oxygenated Krebs buffer. At that moment, the tension in the muscle strips decreased rapidly and stabilized at less than 0.5 *g*. The solution was equilibrated with a gas mixture containing 95% O_2 and 5% CO_2 at pH 7.45 and 37°C.

Induction of EFS

The strips were stimulated with pulse trains of 40 V in amplitude and 10 s in train duration, with pulse duration of 1 ms at frequencies of 1, 2, 4, and 6 Hz by using a stimulator (model S 88; Grass Instruments Co.). After a stable resting tone of was obtained in the muscle strips, the frequency-response relationship (1, 2, 4, and 6 Hz) was constructed and the strips were washed five to six times. Subsequently, they were allowed to equilibrate for 30 min after EFS to recover completely from the stimulated responses to EFS.

Assessment of drug responses

The responses to EFS on the resting tension of circular smooth muscle were investigated. To determine whether neuronal origin and cholinergic neurotransmission had different effects on the normal and diabetic muscle strips in response to EFS, the strips were pre-treated with tetrodotoxin (TTX, 10^{-6} M) and atropine sulfate (10^{-6} M) for 30 min. In addition, to confirm that Ca^{2+} influx plays a crucial role in the contraction, the strips were exposed to verapamil (10^{-6} M), an L-type calcium channel blocker, for 15 min. Moreover, the strips were pretreated with prazosin (10^{-6} M) and DPCPX (10^{-6} M), respectively, for 30 min to measure the effects of the α_1-adrenergic and adenosine A1 receptors. Furthermore, 4-AP (10^{-4} M) and TEA (10^{-3} M) were applied to the strips 5 min before EFS to investigate K^+ efflux, and U73122 (10^{-6} M) was applied to the strips to determine the PKC activity in diabetic smooth muscle contraction. In addition, the strips were pre-incubated with SB202190 (10^{-5} M) and PD98059 (10^{-5} M), respectively, for 15 min to investigate the action of MEK and p38 MAPK enzymes. To determine MLCK activity, ML-9 hydrochloride (10^{-5} M) was added 10 min before EFS was applied to the strips, which were exposed to a PKC inhibitor, chelerythrine chloride (10^{-5} M), and incubated for 20 min.

To determine the smooth muscle relaxation activities in diabetic conditions, the strips were exposed to udenafil (10^{-6} M) and methylene blue (10^{-4} M) for 15 min. Between each part of the experiments, the muscle strips were washed in Krebs buffer five to six times and kept at equilibrium for 35 min. All drugs were placed in the organ bath in volumes not exceeding 100 μL (10% of the organ bath volume). The same volume of the Krebs buffer without inhibitor or antagonist was used as control.

Protein sample preparation and western blotting

To measure the phosphorylated form of MLC (p-MLC) and PDE_5 protein expression, the protein sample was prepared as previously described (Ha *et al*., 2017). Briefly, the colon tissues were homogenized in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1% (w/v) Triton X-100, 0.01% (w/v) SDS, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM PMSF, 10 μL/mL phosphatase inhibitor cocktail-3, and 0.7 g/mL β-mercaptoethanol. The lysates were sonicated (30 s 8 times, 8 cycles, power 20%) and centrifuged at 12,000 rpm for 30 min at 4°C to yield the whole tissue extract. The protein concentration of the supernatant was determined by using Bradford reagent in accordance with the manufacturer's instructions (Bio-Rad Chemical Division, Richmond, CA, USA). The absorbance at 595 nm was measured spectrophotometrically.

Immunoblotting analysis of p-MLC, MLC, PDE_5 and β-actin were determined. The supernatants were electrophoresed on an SDS-PAGE and transferred to nitrocellulose membranes for subsequent western blotting. Following incubation with the primary antibodies (1:1,000) overnight at 4°C, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1 h at room temperature. The immunoreactive bands were detected by Quantity One analysis software (Bio-Rad Chemical Division), with β-actin used as the loading control.

Data analysis

The contraction responses (amplitudes) were expressed as actual tone changes (diabetic versus normal group). The data were provided explicitly as the mean ± SEM. Statistical differences among the groups were analyzed by using two-tailed Student's *t*-test and two-way repeated measures ANOVA (analysis of variances) test. A *p*-value less than 0.05 was onsidered to be statistically significant.

RESULTS

DM rats have lower body weight and greater water and food intake

The non-fasting blood glucose levels of normal and DM rats are shown in Table 1. After 2 days of injection, the rats rapidly developed diabetes and maintained the diabetic state up to 7 days, with a blood glucose level greater than 300 mg/dL (145 ± 25 mg/dL vs. 453 ± 22 mg/dL, $p<0.001$). The body weight in DM rats was significantly lower than in normal rats (293 ± 7 g vs. 245 ± 5 g) (Table 1). Daily food and water consumption are presented in (Fig. 1). The daily food consumption in

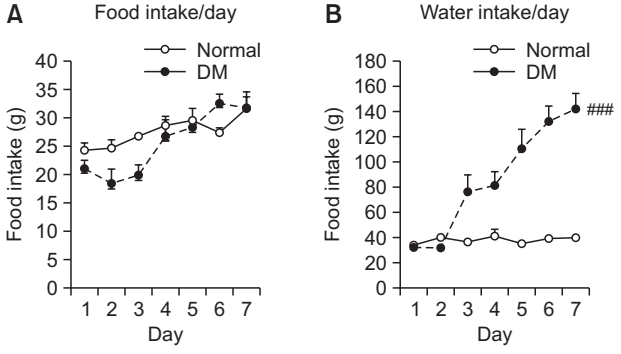

Fig. 1. Daily food and water intake in normal and STZ-induced diabetic rats. The average food and water intakes were calculated from the measurement of the intake on each day at the same time. The data are expressed as the mean ± SEM (n=13). $^{###}p<0.001$ compared with normal rats by two-way ANOVA test.

normal and DM rats was not significantly different throughout the whole experiment. Regarding the liquid intake, the diabetic rats consumed significantly more water than the normal rats (p<0.001).

Contractile force of the colon was reduced in DM rats

In the present study, the colonic tissues from normal and DM rats showed spontaneous contractility. However, the amplitudes produced by EFS were significantly lower in tissues from the DM rats (Fig. 2A vs. Fig. 2B) (p<0.001). The average contractility in the colon smooth muscle is presented in Fig. 2C. Contractility of the colon was expressed as tone changes (Δg). The contraction response was obtained by the induction of EFS (1, 2, 4, and 6 Hz). The average contractility of EFS-induced contraction was analyzed by using a two-way ANOVA test to compare the two curves.

Impaired neuronal conduction and sensitivities of muscarinic, α_1-adrenergic and adenosine A_1 receptors in muscle tissue from DM rats

The contraction-induced by EFS was inhibited by TTX (10^{-6} M, a blocker of voltage-gated sodium channel), atropine (10^{-6} M, muscarinic receptor antagonist), prazosin (10^{-6} M, α_1 adrenergic receptor antagonist) and DPCPX (10^{-6} M, adenosine A_1 receptor antagonist) in both normal and DM rat. The percentages of the contraction inhibition in DM rats were significantly lower than those in normal rats (Fig. 3; p<0.001, p<0.001, p<0.01 and p<0.01 respectively).

Alteration of Ca^{2+} and K^+ channels in tissue from DM rats

The effects of the Ca^{2+} and K^+ channel blockers are shown in (Fig. 4). The muscle strips were pre-incubated with 10^{-3} M TEA (tetraethylammonium; non-specific K^+ channel blocker) and 10^{-4} M 4-AP (4-aminopyridine; voltage-gated potassium channel blocker) for 10 min before the application of EFS. The contraction induced by EFS was facilitated in the presence of TEA and 4-AP. The percentage of contraction by 4-AP (Fig. 4B) in the DM colon strips was significantly lower than that in normal colon strips (p<0.05). However, there was no significant difference between normal and DM rats in TEA-treated tissues (Fig. 4A). Verapamil, a calcium channel blocker (10^{-6} M) blocked the EFS-induced contraction in the colon of both normal and DM rats. Consequently, the inhibitory percentage on contraction by verapamil in the colon of DM rats was sig-

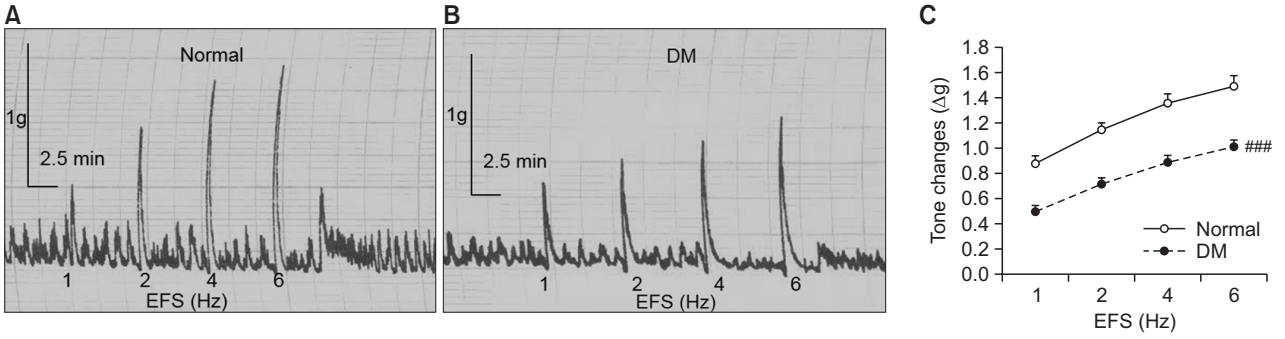

Fig. 2. Representative traces and tension comparison on EFS-induced contraction of colon in normal and DM rats. EFS-induced contraction in normal rats (A); EFS-induced contraction in DM rats (B); and the average tension of EFS-induced contraction in normal and diabetic colons (C). The contractile response was obtained from the application of EFS at 1 Hz, 2 Hz, 4 Hz, and 6 Hz, for 5 min intervals. The data are expressed as the mean ± SEM (n=13). ###p<0.001 compared with normal rats by two-way ANOVA test. DM, diabetes mellitus.

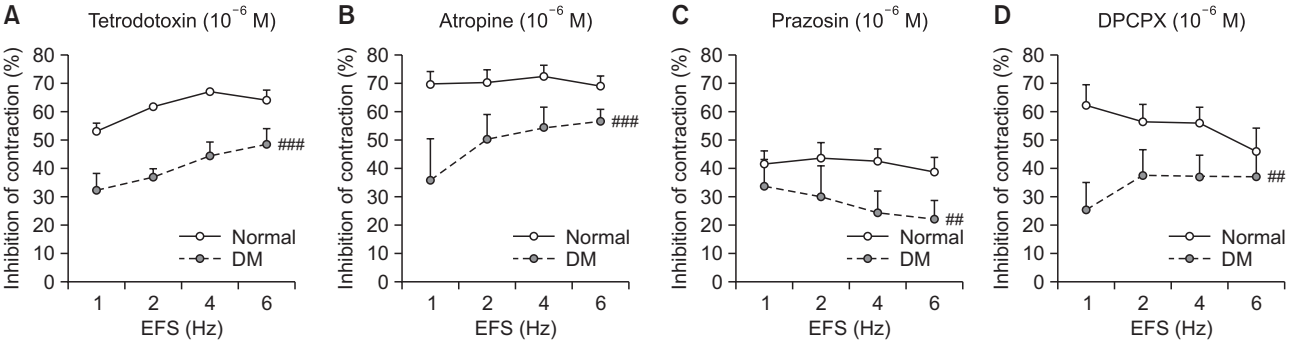

Fig. 3. Percentage of inhibition of contraction in the colon induced by various receptor antagonists between normal and DM rats. EFS-induced contraction of the normal and DM colon in the presence of tetrodotoxin (A), atropine (B), prazosin (C), and DPCPX (D). The contractile response was obtained by the application of EFS at 1, 2, 4, and 6 Hz, for 5 min intervals. Tetrodotoxin (10^{-6} M), atropine (10^{-6} M), prazosin (10^{-6} M), and DPCPX (10^{-6} M) were added to the muscle strips for 30 min before the EFS was applied. The data are expressed as the mean ± SEM (n=6). ##p<0.01 and ###p<0.001 compared with normal rats by two-way ANOVA test. DM, diabetic mellitus; tetrodotoxin, neuronal blocker/sodium channel blocker; atropine, muscarinic receptor antagonist; prazosin, α_1-adrenergic receptor antagonist; DPCPX, adenosine A1 receptor antagonist.

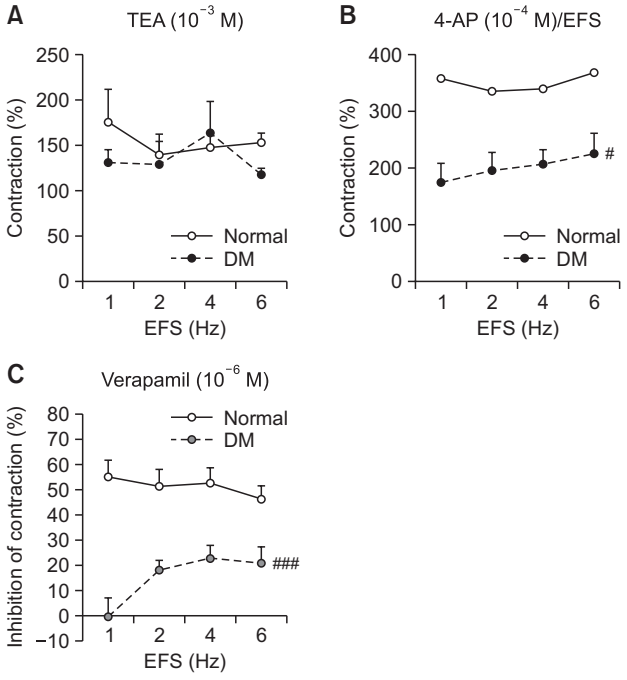

Fig. 4. Percentage of contraction and of inhibition on contraction in the colon induced by channel blockers in normal and DM rats. The EFS-induced contraction of normal and DM colon in the presence of TEA (A), 4-AP (B), and verapamil (C). The contractile response was obtained by the application of EFS at 1 Hz, 2 Hz, 4 Hz, and 6 Hz, for 5 min intervals. TEA (10^{-3} M), 4-AP (10^{-4} M), and verapamil (10^{-6} M) were added to the muscle strips for 15 min before EFS was applied. The data are expressed as the mean ± SEM (n=6). $^{\#}p<0.05$ and $^{\#\#\#}p<0.001$ compared with normal rats by two-way ANOVA test. DM, diabetic mellitus; TEA, tetraethylammonium (non-specific K^+ channel blocker); 4-AP, 4-aminopyridine (voltage-gated K^+ channel blocker); verapamil, L-type calcium channel blocker.

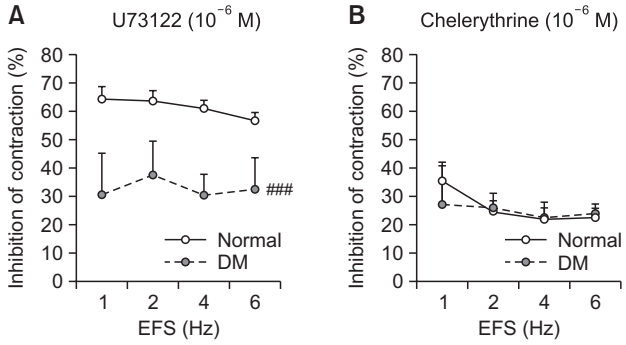

Fig. 5. Percentage of inhibition of contraction in the colon induced by PLC and PKC inhibitors in normal and DM rats. The EFS-induced contractility of normal and DM colon in the presence of U73122 (A) and chelerythrine (B). The contractile response was obtained by the application of EFS at 1, 2, 4, and 6 Hz, for 5 min intervals. U73122 (10^{-6} M) and chelerythrine (10^{-5} M) were added to the muscle strips for 10 min before the EFS was applied. The data were expressed as the mean ± SEM (n=6). $^{\#\#\#}p<0.001$ compared with normal rats by two-way ANOVA test. DM, diabetic mellitus; U73122, phospholipase C inhibitor; chelerythrine, PKC inhibitor.

nificantly lower than that in normal rats ($p<0.001$) (Fig. 4C).

PLC enzyme was altered in the colon of DM rats

The effect of intracellular signaling inhibitors is presented in Fig. 5. To obtain accurate information on the mechanism of the reduced contractions induced by EFS in DM colon strips, 10^{-6} M U73122 and 10^{-5} M chelerythrine chloride were used. U73122 is a PLC inhibitor that inhibits calcium release from the intracellular sarcoplasmic reticulum (Smith *et al*., 1990; Mogami *et al*., 1997). The percentage of inhibition of contraction in DM rats was significantly lower than that in normal rats ($p<0.001$) (Fig. 5A). Chelerythrine chloride is a non-competitive PKC inhibitor with respect to ATP (EcklyMichel *et al*., 1997). However, there was no significant difference when both normal and DM tissues were treated with chelerythrine (Fig. 5B).

p-MLC was reduced in the colon of DM rats

Subsequently, further signaling pathways were examined. The ERK inhibitor (PD98059; 10^{-5} M) and the p38 MAPK inhibitor (SB202190; 10^{-5} M) blocked the contractile responses generated by EFS in both normal and DM rats. The percentage of inhibition of contraction in the tissues from DM rats was not significantly lower than that in normal rats in both SB202190- (Fig. 6A) and PD98059- (Fig. 6B) treated tissues, as well. ML-9 (10^{-5} M; a MLCK inhibitor) inhibited the contractile response induced by EFS in both normal and DM rats. In the tissue samples from DM rats, responses to EFS were significantly smaller than those in control tissues (Fig. 6C). In order to examine whether the p-MLC was changed in the DM state, protein expression of p-MLC and MLC were analyzed by western blotting. p-MLC from MLC is caused by MLCK. Relative expression level of p-MLC to MLC was significantly reduced by diabetes mellitus (Fig. 6D).

cGMP signaling pathway was altered in DM rats

When the tissues were pre-treated with udenafil (10^{-6} M); a PDE_5 inhibitor, contractility was abolished in both groups. The percentage of inhibition of contraction in DM rats was significantly lower than that in normal rats ($p<0.001$) (Fig. 7A). The basal smooth muscle contraction in both normal and DM smooth muscle strips was increased when methylene blue (10^{-4} M), a guanylate cyclase inhibitor, was added. The percentage of contraction in the tissues of DM rats were significantly smaller than those in normal rats ($p<0.001$) (Fig. 7B). The expression of PDE_5 was analyzed by western blotting (Fig. 7C). PDE_5 is an enzyme that hydrolyzes cGMP to 5'-GMP. The density of PDE_5 relative to β-actin in the DM rats was significantly lower than that in normal rats ($p<0.05$).

DISCUSSION

It has been reported that reduced colonic contraction in DM rats is due to the decreased expression of the M_2 and M_3 muscarinic receptors (Kim *et al*., 2011). In contrast, it has been stated that the responsiveness of STZ-induced DM rat ileum longitudinal muscle to acetylcholine, carbachol, and bethanechol is increased (Carrier and Aronstam, 1990). In this study, the results suggested that the decreased colonic contraction in DM was due to the alterations in the muscarinic receptor. This is because the inhibitory on contraction by atropine was less affected in DM colon when compared to the nor-

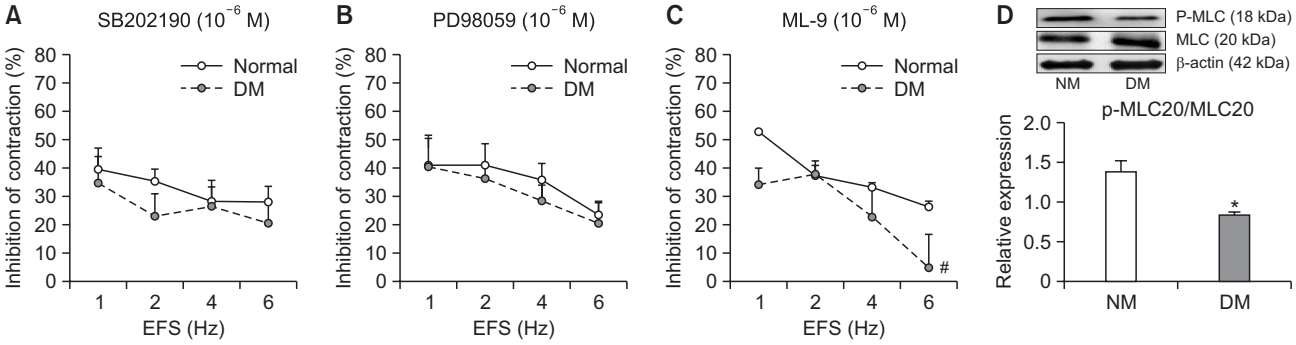

Fig. 6. Percentage of inhibition in the colon induced by various inhibitors and expression of p-MLC and MLC in the colon in normal and DM rats. EFS-induced contractility of the normal and DM colon in the presence of SB 2022190 (A), PD 98059 (B) and ML-9 (C). The contractile response was obtained by the application of EFS at 1 Hz, 2 Hz, 4 Hz, and 6 Hz, for 5 min intervals. SB202190 (10^{-5} M), PD 98059 (10^{-5}M), and ML-9 (10^{-5} M) were added to the muscle strips for 10 min before the EFS was applied. p-MLC was detected by western blotting. The colon was lysed in buffer and subjected to SDS-PAGE electrophoresis, with actin used as a loading control. The expression of p-MLC was displayed as relative expression (D). The data were expressed as the mean ± SEM (n=6). $^{\#}p<0.05$ compared with normal rats by two-way ANOVA test, $^{*}p<0.05$ compared with normal rats by Student's *t*-test. DM, diabetic mellitus; SB 202190, ERK inhibitor; PD 98059, p38 MAPK inhibitor; ML-9, MLCK inhibitor.

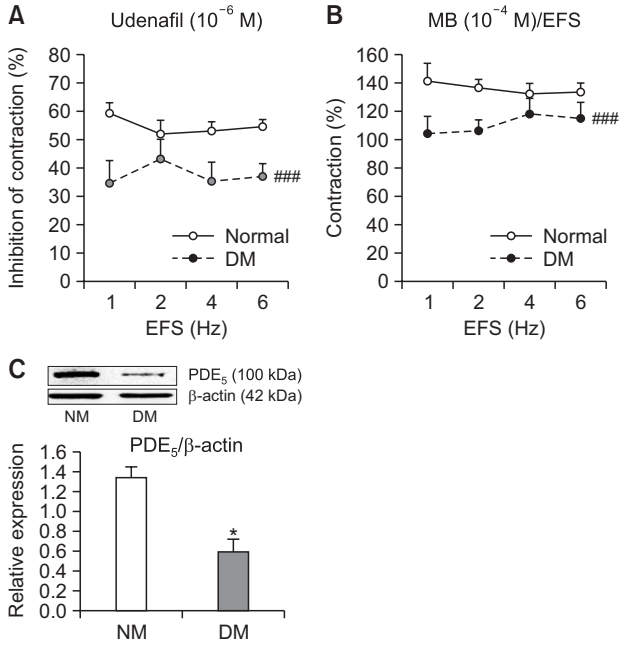

Fig. 7. Percentage of contraction and of inhibition on contraction in the colon induced by enzyme inhibitors and expression of PDE_5 in the colon of normal and DM rats. EFS-induced contractility in normal and DM colon in the presence of udenafil (A) and methylene blue (B) and expression of PDE_5 in colon tissue (C). The contractile response was obtained by the application of EFS at 1 Hz, 2 Hz, 4 Hz, and 6 Hz, for 5 min intervals. Udenafil (10^{-6} M) and methylene blue (10^{-4} M) were added to the strips for 10 min before the EFS was applied. PDE_5 was detected by western blotting. The colon was lysed in buffer and subjected to SDS-PAGE electrophoresis, with actin used as a loading control. The expression of PDE_5 was displayed as relative expression. The data are expressed as the mean ± SEM (n=6). $^{\#\#\#}p<0.001$ compared with normal rats by two-way ANOVA test, $^{*}p<0.05$ (n=3) compared with normal by Student's *t*-test. DM, diabetic mellitus; Udenafil, PDE_5 inhibitor; MB, methylene blue=soluble guanylate cyclase inhibitor.

mal colon. In addition, sympathetic nerve-induced excitation in the longitudinal and circular smooth muscle in all parts of the colon is believed to be mediated through α_1-adrenergic receptors (Luckensmeyer and Keast, 1998). Prazosin led to significant differences in the percentage of inhibition on contraction induced by EFS between the normal and DM rat bladder (Han *et al*., 2018). The present data showed that the percentage of inhibition in DM tissues in the presence of prazosin against EFS was due to the decreased α_1-adrenergic receptor activity.

Consequently, DPCPX resulted in a significant difference in the percentage of inhibition between the colon of normal and DM rats. DPCPX (8-cyclopentyl-1, 3-dipropylxanthine) is a drug that acts on the adenosine A_1 receptor as a selective antagonist (Lohse *et al*., 1987). The contraction in rat colonic muscularis mucosae (RCMM) was mediated through the adenosine A_1 receptors (Reeves *et al*., 1993). There is no specific role of adenosine A_1 receptor in the colon of DM rats. Nonetheless, in our study, DPCPX was shown to result in a significantly lower percentage of inhibition in the colon of DM rats than normal rats. These data showed that the activity of the adenosine A_1 receptor was decreased in DM.

It is reported that hyperglycemia could inhibit the short and long neural reflexes to modulate colonic motility, which may lead to constipation in patients with DM (Sims *et al*., 1995). Impaired contraction and relaxation in colonic circular muscle strips from rats with DM may have resulted from the loss of enteric neurons in the colon due to increased oxidative stress and apoptosis (Chandrasekharan *et al*., 2011). Previous studies have demonstrated that DM following exposure to STZ for 7 days affects GI motility through a neuronal defect in the duodenum and colon (Furlan *et al*., 1999, 2002). In this study, it was shown that the reduced colonic contraction in EFS was due to the impaired neuronal function because blockade of neural activity by TTX was less affected in DM rats when compared to the normal rats.

Under normal conditions, the release of acetylcholine from the parasympathetic nerve inactivates K^+ channels, resulting in depolarization and increase in Ca^{2+} influx into the cytoplasm, thus leading to the circular smooth muscle contraction (Somlyo and Somlyo, 2003). Different potassium channel blockers

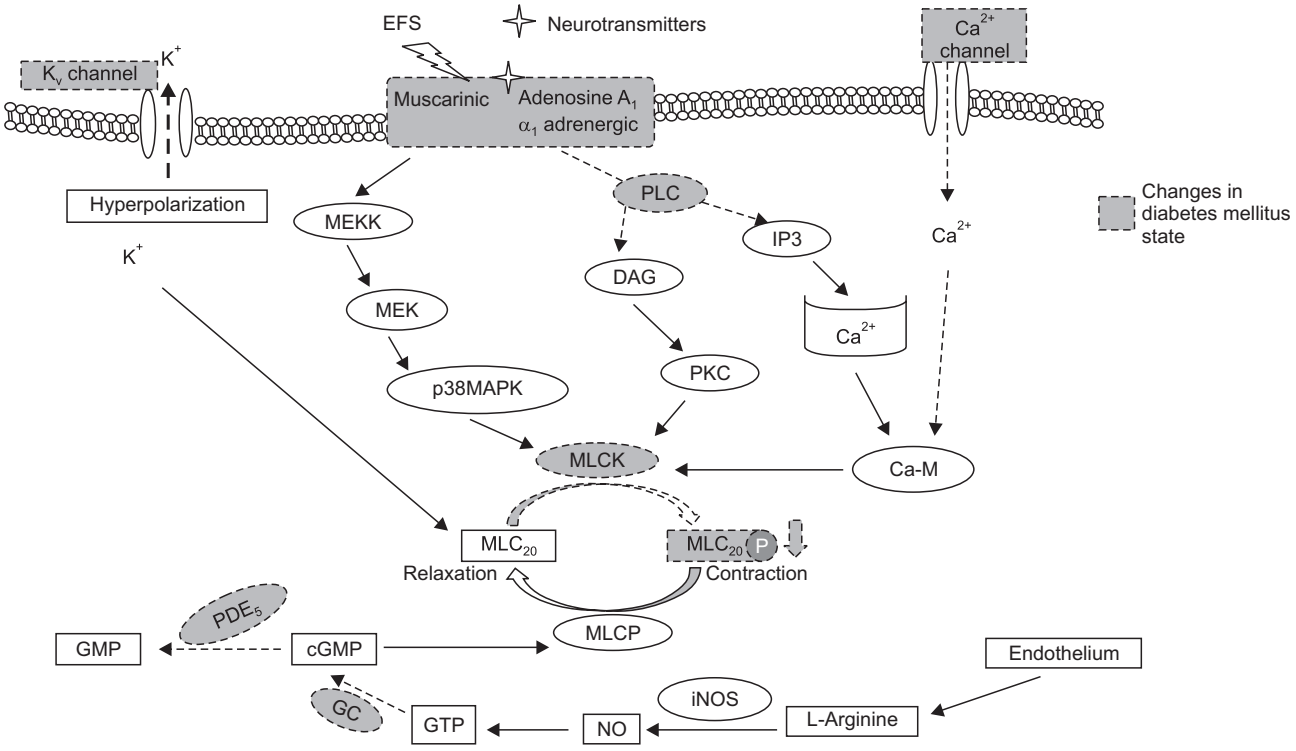

Fig. 8. Summary of the signaling changes in colon motility in rats with diabetes mellitus. Muscarinic, α_1-adrenergic, adenosine A_1 receptor and NO-mediated intracellular signaling pathways were involved in changes of contraction and relaxation in colon smooth muscle. In general, PLC generated intracellular second messenger IP3 and DAG from membrane PIP2. PLC induced Ca^{2+} release and Ca^{2+} bound to calmodulin. This binding activated MLCK that phosphorylates the MLC20. MLCK activation was also driven by PKC via DAG. MLC phosphorylation drove the contraction. Relaxation was caused by MLCP that dephosphorylates MLC20. MLCP activity was regulated by cGMP from NO through GC and PDE_5. In diabetes mellitus state, the impaired muscarinic, α_1-adrenergic and adenosine A_1 receptor-mediated signaling decreased the activity of IP3-dependent Ca^{2+} release which further attenuates the MLCK activity, and reduced PDE_5 activity that led to increase cGMP, and thus increasing MLCP activity, may result in a decrease of colon smooth muscle contractility in rats with diabetes mellitus. EFS, electrical field stimulation; PLC, phospholipase C; DAG, diacylglycerol; IP3, inositol triphosphate; PKC, protein kinase C; Ca-M, calcium-calmodulin complex; MEKK, mitogen-activated protein kinase/ERK kinase kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; iNOS, inducible nitric oxide synthase; NO, nitric oxide; GTP, guanosine triphosphate; GC, Guanylate cyclase; cGMP, Cyclic guanosine monophosphate; PDE_5, phosphodiesterase 5; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase. The dotted lines and grey-shaded shapes indicate the changes in signaling molecules between normal and diabetic rats.

(TEA and 4-AP) were used to examine the role of potassium channels in DM colon. The DM colon had a significantly lower contractile effect than the normal colon in 4-AP treated muscle strips. This finding suggested that the activity of the voltage-sensitive K^+ channel could be decreased in DM colon. Also, this may have a potential therapeutic effect to improve constipation in DM states.

Consequently, impaired colonic contractility in DM mice is associated with impaired Ca^{2+} handling (Forrest *et al*., 2005). Diabetic mice had a significantly lower calcium transient generated from potassium chloride stimulation in the colon than the normal mice, which led to reduced MLC phosphorylation. (Touw *et al*., 2012). Increased global *O*-glycosylation in cardiomyocytes has been shown to decrease Ca2_ sensitivity in these tissues and attenuate contractility (Clark *et al*., 2003). The decreased contractility in the bladder detrusor muscle resulted from the alteration of PLC activity, which mediates Ca^{2+} release (Han *et al*., 2018). In our experiments, the results are in agreements with the fact that the reduced contraction was due to an impaired voltage-sensitive Ca^{2+} channel via PLC signaling.

Subsequently, changes in Ca^{2+} release lead to the alteration of the signaling pathway. The variation in MLC phosphorylation may be influenced through MLCK and MLC phosphatase. MLCK activity was significantly reduced in the colonic smooth muscle of the DM rats. The expression levels of MLCK in the pylorus and ileum of the diabetic group were both significantly reduced compared with the control group (Hu and Feng, 2012). This further decreased the expression level of MLC phosphorylation. In diabetic rabbits compared with normal rabbits, urinary bladder contraction was decreased with the increased level of MLC phosphorylation (Su *et al*., 2004). Phosphorylation of MLC in the coronary microvessels was significantly increased in the diabetic Yucatan miniswine compared with the controls (Clements *et al*., 2009). Another alternative pathway that can directly affect the MLC phosphorylation is through PKC. We did not find any significant differences between normal and DM rats when using a PKC inhibitor (chelerythrine). It can be suggested that decreased colonic motility was due to the reduced MLC phosphorylation through attenuation of MLCK activity.In intestinal smooth muscle, MLCP activity was regulated by CPI-17, MYPT-1, and cGMP (Lincoln, 2007). Re-

cent studies have indicated that MAPK has an important role in the phasic intestinal smooth muscle (Ihara *et al*., 2009). We did not observe any differences in the ERK and MAPK pathways, which inhibit MLCP activity (Ihara *et al*., 2015), between normal and DM rats with the use of appropriate inhibitors (Fig. 5A, 5B). It is therefore suggested that the MAPK signaling pathway was not altered in DM colonic dysfunction.

EFS-induced relaxation was enhanced in both amplitude and duration under PDE_5 inhibition in the anococcygeus (O'Kane and Gibson, 1999). In a previous study, PDE_5 signaling was altered in diabetic bladder dysfunction (Han *et al*., 2018). In the present study, PDE_5 inhibition and expression of PDE_5 were reduced in DM. Subsequently, sGC activity in colonic relaxation was changed by diabetes. This study supports the involvement of the cGMP pathway through nitric oxide and PDE_5 in the impaired relaxation of colonic smooth muscle in DM. Although this study has explored the signaling changes in colonic dysfunction that occurs in DM, there are several limitations, such as the type of diabetes, the duration of diabetes and the strains of animal used.

In conclusion, we found that the contractility induced by EFS in the colon was decreased in DM. Tetrodotoxin, atropine, prazosin, DPCPX, 4-AP, verapamil, ML-9, udenafil, and methylene blue had significant lower effects on DM colon when compared to the normal colon. This reduced contractility resulted from the impaired neuronal conduction and decreased muscarinic, α_1-adrenergic and adenosine A_1 receptor activities, which resulted in decreased p-MLC expression through decreased MLCK activity, and changes in MLCP activity via PDE_5 and the cGMP pathway. The activities of the voltage-gated potassium and calcium channels were reduced in DM state. The summary of the signaling changes in colon motility in rats with DM was provided in Fig. 8.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

ACKNOWLEDGMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology [Grant NRF-2019R1F1A1062070].

REFERENCES

Amaral, N. and Okonko, D. O. (2015) Metabolic abnormalities of the heart in type II diabetes. *Diab. Vasc. Dis. Res.* **12**, 239-248.

Beavo, J. A. (1995) Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol. Rev.* **75**, 725-748.

Broad, R. M., McDonald, T. J., Brodin, E. and Cook, M. A. (1992) Adenosine A1 receptors mediate inhibition of tachykinin release from perifused enteric nerve endings. *Am. J. Physiol.* **262**, G525-G531.

Bytzer, P., Talley, N. J., Leemon, M., Young, L. J., Jones, M. P. and Horowitz, M. (2001) Prevalence of gastrointestinal symptoms associated with diabetes mellitus: a population-based survey of 15,000 adults. *Arch. Intern. Med.* **161**, 1989-1996.

Carrier, G. O. and Aronstam, R. S. (1990) Increased muscarinic responsiveness and decreased muscarinic receptor content in ileal smooth muscle in diabetes. *J. Pharmacol. Exp. Ther.* **254**, 445-449.

Chandrasekharan, B., Anitha, M., Blatt, R., Shahnavaz, N., Kooby, D., Staley, C., Mwangi, S., Jones, D. P., Sitaraman, S. V. and Srinivasan, S. (2011) Colonic motor dysfunction in human diabetes is associated with enteric neuronal loss and increased oxidative stress. *Neurogastroenterol. Motil.* **23**, 131-138, e126.

Christofi, F. L. and Wood, J. D. (1993) Presynaptic inhibition by adenosine A1 receptors on guinea pig small intestinal myenteric neurons. *Gastroenterology* **104**, 1420-1429.

Clark, R. J., McDonough, P. M., Swanson, E., Trost, S. U., Suzuki, M., Fukuda, M. and Dillmann, W. H. (2003) Diabetes and the accompanying hyperglycemia impairs cardiomyocyte calcium cycling through increased nuclear O-GlcNAcylation. *J. Biol. Chem.* **278**, 44230-44237.

Clements, R. T., Sodha, N. R., Feng, J., Boodhwani, M., Liu, Y., Mieno, S., Khabbaz, K. R., Bianchi, C. and Sellke, F. W. (2009) Impaired coronary microvascular dilation correlates with enhanced vascular smooth muscle MLC phosphorylation in diabetes. *Microcirculation* **16**, 193-206.

Conti, M. and Beavo, J. (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu. Rev. Biochem.* **76**, 481-511.

EcklyMichel, A. E., LeBec, A. and Lugnier, C. (1997) Chelerythrine, a protein kinase C inhibitor, interacts with cyclic nucleotide phosphodiesterases. *Eur. J. Pharmacol.* **324**, 85-88.

Feldman, M. and Schiller, L. R. (1983) Disorders of gastrointestinal motility associated with diabetes mellitus. *Ann. Intern. Med.* **98**, 378-384.

Forrest, A., Huizinga, J. D., Wang, X. Y., Liu, L. W. and Parsons, M. (2008) Increase in stretch-induced rhythmic motor activity in the diabetic rat colon is associated with loss of ICC of the submuscular plexus. *Am. J. Physiol. Gastrointest. Liver Physiol.* **294**, G315-G326.

Forrest, A., Molleman, A. and Parsons, M. (2005) The responses to manipulation of extracellular and intracellular calcium are altered in the streptozotocin-diabetic rat colon and ileum. *Eur. J. Pharmacol.* **509**, 77-83.

Francis, S. H., Blount, M. A. and Corbin, J. D. (2011) Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions. *Physiol. Rev.* **91**, 651-690.

Furlan, M. M., de Miranda Neto, M. H., Sant'ana Dde, M. and Molinari, S. L. (1999) Number and size of myenteric neurons of the duodenum of adult rats with acute diabetes. *Arq. Neuropsiquiatr.* **57**, 740-745.

Furlan, M. M., Molinari, S. L. and Miranda Neto, M. H. (2002) Morphoquantitative effects of acute diabetes on the myenteric neurons of the proximal colon of adult rats. *Arq. Neuropsiquiatr.* **60**, 576-581.

Ha, H. S., Lee, S. E., Lee, H. S., Kim, G. H., Yoon, C. J., Han, J. S., Lee, J. Y. and Sohn, U. D. (2017) The signaling of protease-activated receptor-2 activating peptide-induced contraction in cat esophageal smooth muscle cells. *Arch. Pharm. Res.* **40**, 1443-1454.

Han, J. S., Min, Y. S., Kim, G. H., Chae, S. H., Nam, Y., Lee, J., Lee, S. Y. and Sohn, U. D. (2018) The change of signaling pathway on the electrical stimulated contraction in streptozotocin-induced bladder dysfunction of rats. *Korean J. Physiol. Pharmacol.* **22**, 577-584.

Hartshorne, D. J., Ito, M. and Erdodi, F. (2004) Role of protein phosphatase type 1 in contractile functions: myosin phosphatase. *J. Biol. Chem.* **279**, 37211-37214.

Hu, W. and Feng, P. (2012) Myosin light chain kinase is involved in the mechanism of gastrointestinal dysfunction in diabetic rats. *Dig. Dis. Sci.* **57**, 1197-1202.

Ihara, E., Beck, P. L., Chappellaz, M., Wong, J., Medlicott, S. A. and MacDonald, J. A. (2009) Mitogen-activated protein kinase pathways contribute to hypercontractility and increased Ca2+ sensitization in murine experimental colitis. *Mol. Pharmacol.* **75**, 1031-1041.

Ihara, E., Yu, Q., Chappellaz, M. and MacDonald, J. A. (2015) ERK and p38MAPK pathways regulate myosin light chain phosphatase and contribute to Ca2+ sensitization of intestinal smooth muscle contraction. *Neurogastroenterol. Motil.* **27**, 135-146.

Kamm, K. E. and Stull, J. T. (2001) Dedicated myosin light chain kinases with diverse cellular functions. *J. Biol. Chem.* **276**, 4527-4530.

Khromov, A. S., Wang, H., Choudhury, N., McDuffie, M., Herring, B.

P., Nakamoto, R., Owens, G. K., Somlyo, A. P. and Somlyo, A. V. (2006) Smooth muscle of telokin-deficient mice exhibits increased sensitivity to Ca2+ and decreased cGMP-induced relaxation. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 2440-2445.

Kilbinger, H., Kruel, R., Pfeuffer-Friederich, I. and Wessler, I. (1982) The effects of metoclopramide on acetylcholine release and on smooth muscle response in the isolated guinea-pig ileum. *Naunyn Schmiedebergs Arch. Pharmacol.* **319**, 231-238.

Kim, S. J., Park, J. H., Song, D. K., Park, K. S., Lee, J. E., Kim, E. S., Cho, K. B., Jang, B. K., Chung, W. J., Hwang, J. S., Kwon, J. G. and Kim, T. W. (2011) Alterations of colonic contractility in long-term diabetic rat model. *J. Neurogastroenterol. Motil.* **17**, 372-380.

Lincoln, T. M. (2007) Myosin phosphatase regulatory pathways: different functions or redundant functions? *Circ. Res.* **100**, 10-12.

Lohse, M. J., Klotz, K. N., Lindenborn-Fotinos, J., Reddington, M., Schwabe, U. and Olsson, R. A. (1987) 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX)--a selective high affinity antagonist radioligand for A1 adenosine receptors. *Naunyn Schmiedebergs Arch. Pharmacol.* **336**, 204-210.

Luckensmeyer, G. B. and Keast, J. R. (1998) Activation of alpha- and beta-adrenoceptors by sympathetic nerve stimulation in the large intestine of the rat. *J. Physiol.* **510**, 549-561.

Mogami, H., Lloyd Mills, C. and Gallacher, D. V. (1997) Phospholipase C inhibitor, U73122, releases intracellular Ca2+, potentiates Ins(1,4,5)P3-mediated Ca2+ release and directly activates ion channels in mouse pancreatic acinar cells. *Biochem. J.* **324**, 645-651.

Murthy, K. S. (2001) Activation of phosphodiesterase 5 and inhibition of guanylate cyclase by cGMP-dependent protein kinase in smooth muscle. *Biochem. J.* **360**, 199-208.

Nowak, T. V., Harrington, B., Weisbruch, J. P. and Kalbfleisch, J. H. (1990) Structural and functional characteristics of muscle from diabetic rodent small intestine. *Am. J. Physiol.* **258**, G690-G698.

O'Kane, K. and Gibson, A. (1999) Characterisation of nitrergic transmission in the isolated anococcygeus muscle of the female mouse. *Eur. J. Pharmacol.* **377**, 69-74.

Paton, D. M. (1981) Structure-activity relations for presynaptic inhibition of noradrenergic and cholinergic transmission by adenosine: evidence for action on A1 receptors. *J. Auton. Pharmacol.* **1**, 287-290.

Pecoits-Filho, R., Abensur, H., Betonico, C. C., Machado, A. D., Parente, E. B., Queiroz, M., Salles, J. E., Titan, S. and Vencio, S. (2016) Interactions between kidney disease and diabetes: dangerous liaisons. *Diabetol. Metab. Syndr.* **8**, 50.

Reeves, J. J., Coates, J., Jarvis, J. E., Sheehan, M. J. and Strong, P. (1993) Characterization of the adenosine receptor mediating contraction in rat colonic muscularis mucosae. *Br. J. Pharmacol.* **110**, 1255-1259.

Ruwart, M. J., Klepper, M. S. and Rush, B. D. (1979) Carbachol stimulation of gastrointestinal transit in the postoperative Ileus rat. *J. Surg. Res.* **26**, 18-26.

Rybalkin, S. D., Yan, C., Bornfeldt, K. E. and Beavo, J. A. (2003) Cyclic GMP phosphodiesterases and regulation of smooth muscle function. *Circ. Res.* **93**, 280-291.

Sims, M. A., Hasler, W. L., Chey, W. D., Kim, M. S. and Owyang, C. (1995) Hyperglycemia inhibits mechanoreceptor-mediated gastrocolonic responses and colonic peristaltic reflexes in healthy humans. *Gastroenterology* **108**, 350-359.

Smith, R. J., Sam, L. M., Justen, J. M., Bundy, G. L., Bala, G. A. and Bleasdale, J. E. (1990) Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. *J. Pharmacol. Exp. Ther.* **253**, 688-697.

Somlyo, A. P. and Somlyo, A. V. (2003) Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol. Rev.* **83**, 1325-1358.

Su, X., Changolkar, A., Chacko, S. and Moreland, R. S. (2004) Diabetes decreases rabbit bladder smooth muscle contraction while increasing levels of myosin light chain phosphorylation. *Am. J. Physiol. Renal Physiol.* **287**, F690-F699.

Touw, K., Chakraborty, S., Zhang, W., Obukhov, A. G., Tune, J. D., Gunst, S. J. and Herring, B. P. (2012) Altered calcium signaling in colonic smooth muscle of type 1 diabetic mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **302**, G66-G76.

Tuttle, K. R., Bakris, G. L., Bilous, R. W., Chiang, J. L., de Boer, I. H., Goldstein-Fuchs, J., Hirsch, I. B., Kalantar-Zadeh, K., Narva, A. S., Navaneethan, S. D., Neumiller, J. J., Patel, U. D., Ratner, R. E., Whaley-Connell, A. T. and Molitch, M. E. (2014) Diabetic kidney disease: a report from an ADA Consensus Conference. *Am. J. Kidney Dis.* **64**, 510-533.

Van Den Eeden, S. K., Sarma, A. V., Rutledge, B. N., Cleary, P. A., Kusek, J. W., Nyberg, L. M., McVary, K. T. and Wessells, H.; Diabetes Control and Complications Trial/Epidemiology of Diabetes Research Group (2009) Effect of intensive glycemic control and diabetes complications on lower urinary tract symptoms in men with type 1 diabetes: Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) study. *Diabetes Care* **32**, 664-670.

Weissman, A. J. (2006) Intensive diabetes treatment and cardiovascular disease. *N. Engl. J. Med.* **354**, 1751-1752; author reply 1751-1752.

Wu, K. K. and Huan, Y. (2008) Streptozotocin-induced diabetic models in mice and rats. *Curr. Protoc. Pharmacol.* **Chapter 5**, Unit 5.47.