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Original Article

Distribution and Function of Platelet-derived Growth Factor Receptor Alpha-positive Cells and Purinergic Neurotransmission in the Human Colon: Is It Different Between the Right and Left Colon?

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Background/Aims
Platelet-derived growth factor receptor alpha-positive (PDGFRα^+) cells function in the purinergic regulation of gastrointestinal motility, and purines are reportedly inhibitory neurotransmitters in the enteric nervous system. We explore the distribution and function of PDGFRα^+ cells related to purinergic inhibitory neurotransmission in human right and left colons.

Methods
Human colonic segments were prepared with mucosa and submucosa intact, and the circular muscle tension and longitudinal muscle tension were recorded. Purinergic neurotransmitters were administered after recording the regular contractions. Immunohistochemistry was performed on the circular muscle layers. Intracellular recording was performed on the colonic muscular layer. *SK3*, *P2RY1*, and *PDGFR-α* mRNA expression was tested by quantitative real-time polymerase chain reaction (qPCR).

Results
Adenosine triphosphate (ATP) treatment significantly decreased the frequency and area under the curve (AUC) of the segmental contraction in right and left colons. Beta-nicotinamide adenine dinucleotide (β-NAD) decreased the frequency in the right colon and the amplitude, frequency and AUC in the left colon. Apamin significantly increased frequency and AUC in the left colon, and after apamin pretreatment, ATP and β-NAD did not change segmental contractility. Through intracellular recordings, a resting membrane potential decrease occurred after ATP administration; however, the degree of decrease between the right and left colon was not different. PDGFRα^+ cells were distributed evenly in the circular muscle layers of right and left colons. *SK3*, *P2RY1*, and *PDGFRα* expression was not different between the right and left colon.

Conclusion
Purines reduce right and left colon contractility similarly, and purinergic inhibitory neurotransmission can be regulated by PDGFRα^+ cells in the human colon.
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Key Words
Colon; Humans; Interstitial cells of Cajal; Motility; Purines

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Introduction

Platelet-derived growth factor receptor alpha-positive (PDGFRα+) cells are a type of interstitial cell in the gastrointestinal (GI) smooth muscles that have also been called fibroblast-like cells.[1,2] They are distinguished from the interstitial cells of Cajal (ICCs) and are characterized by a well-developed rough endoplasmic reticulum with dilated cisterns. Mitochondria and the Golgi apparatus are mainly located in the perinuclear region.[3,4] Smooth muscle cells (SMCs) closely contact ICCs and PDGFRα+ cells and form a syncytium (SIP syncytium). The enteric nerve terminals from the myenteric plexus innervate these cells, and the SMCs are electrically coupled with the ICCs and PDGFRα+ cells through gap junctions.[5,6]

PDGFRα+ cells are considered to play a role in the purinergic inhibitory regulation of GI motility based on numerous studies using animal models. Purines, such as adenosine triphosphate (ATP) and beta-nicotinamide adenine dinucleotide (β-NAD), have been reported to be inhibitory neurotransmitters in the enteric nervous system. They act as ligands for G-protein coupled P2Y1 receptors on the membranes of PDGFRα+ cells and can activate small-conductance Ca^{2+}-activated K^+ (SK3) channels. The fast inhibitory junction potential (IJP) evoked by electric field stimulation (EFS) is also considered to be related to purinergic inhibitory responses.[7,8] Recently, a functional role for PDGFRα+ cells in murine colonic smooth muscles has been revealed,[9] and one study using transgenic mice constitutively expressing enhanced green fluorescent protein in PDGFRα+ cells showed that purines induce high-amplitude, apamin-sensitive outward K^+ currents through SK3 channels and that the P2Y1 receptor antagonist MRS2500 could block the K^+ current.[10]

The function of the human colon is the absorption of water, fermentation of carbohydrates from microbiota, and propulsion of feces to the rectum. It was revealed that colonic smooth muscle motility is optimized for these functions, which presents as less frequent and larger amplitude contractions than those in the small intestine.[11,12] The right proximal colon may function in mixing and absorption, whereas the left distal colon functions in the propagation of stool to the rectum.[13] However, there have been few studies verifying the mechanistic differences between right and left colonic motility or associated purinergic neurotransmission. We performed this study to explore the distribution and function of PDGFRα+ cells in the human colon using immunohistochemistry and in vitro experiments involving mechanical recordings of human colonic segments treated with inhibitory purines.

Materials and Methods

Tissue Acquisition

Human colon samples were obtained immediately from operations for non-obstructive colon diseases. No greater colon resections were performed than were medically necessary, because the amount of tissue needed for the experiments was very small. This study was approved by the Institutional Review Board (IRB) of the Clinical Research Institute of the Seoul National University Hospital (IRB approval No. H-0603-071-170). The study protocol was performed in accordance with the guidelines and regulation of the Seoul National University Hospital IRB. Written informed consent was obtained from all patients before operations.

After resection of the colon, 4 × 2 cm colonic segments were removed from the resected sections. The specimens were immediately placed into oxygenated Krebs-Ringer Bicarbonate solution (KRB). The KRB contained (in mM) 120.4 NaCl, 5.9 KCl, 15.5 $NaHCO_3$, 11.5 glucose, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, and 2.5 $CaCl_2$ and had a pH of 7.3-7.4 at 37.5°C when bubbled to equilibrium with 97% O_2 and 3% CO_2. All in vitro techniques were similar to

those in our previous studies.[12,14]

Tissue Preparations for Isometric Tension Recordings of Colonic Segments

Colonic segments with intact mucosa and submucosa were prepared by cutting the whole layer of a segment parallel to the longitudinal muscle (LM); the segments were 4 cm in length and 2 cm in width. The colonic segments were suspended in a tissue chamber and perfused with pre-warmed, preoxygenated KRB solution. A stainless steel rod (10 cm long, 3 mm in diameter) was placed parallel to the LM in the organ bath. Circular muscle (CM) tension was recorded at 3 sites, namely proximal, middle, and distal sites, each located 1 cm apart, and the LM tension was recorded via perpendicular traction using sutures placed at each site and forming a small tube from the colonic segment (Fig. 1). Stainless steel spring clips (7 mm × 18 mm) were attached to both ends of the sutured muscle, and each clip was attached to an isometric force transducer. Another clip was attached to the distal end of the colon flap, which effectively recorded the distal LM tension.[11] The temperature was maintained at 37.5 ± 0.5℃. The muscles were equilibrated for at least 2 hours before experiments began. A resting force of 98 mN (10 g) was applied to each measurement site, and the tension of each site was measured using an isometric strain gauge. The frequency (/min), amplitude (mN), and area under the curve (AUC, sec × mN) for the contraction waves were measured as contractility with AcqKnowledge software (Biopac Systems, Inc, Goleta, CA, USA). The AUC was defined as the integrated area under the curve over a period of 10 minutes. The contractility before drug administration was used as a control, and the changes in contractility after the administration of drugs were calculated as relative values for comparisons. All in vitro techniques were similar to those in our previous studies.[12,14]

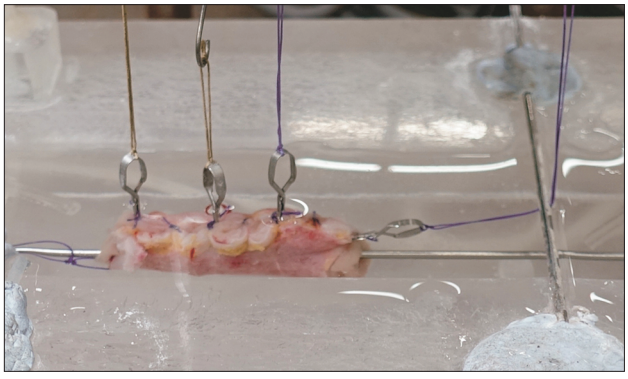

Figure 1. Setup for recording the tension from a colonic segment.

Tissue Preparations for Isometric Tension Recordings of Colonic Smooth Muscle Strips and Electric Field Stimulation

Tissues were pinned down on a Petri dish coated with Sylgard with the mucosa side facing upward. The mucosal and submucosal layers were gently removed with a pair of scissors. CM bundles were obtained by sharp dissection. The size of the muscle strips was 2 mm in width and 1 cm in length. To record contractile activities, the muscle strips were attached to an isometric strain gauge (World precision Instruments, Sarasota, FL, USA) with a suture in a tissue chamber perfused with pre-warmed, pre-oxygenated KRB solution. The temperature was maintained at 36.5 ± 0.5℃. The muscle strips were equilibrated for at least 1 hour before beginning the experiments, and a resting force of 9.8 mN (1 g) was applied. After contraction was stable, EFS was performed. Parallel platinum electrodes were placed on either side of the muscle strips to elicit neural responses with square pulses of EFS (0.3 milliseconds pulse duration, 10 Hz, train durations of 10 seconds, 150 V) delivered by a Grass S48 stimulator (Grass Instrument Company, Quincy, MA, USA). Isometric force measurements were performed as previously described for EFS. The procedure was repeated after drug treatment. The mechanical signals were digitized and recorded using Acknowledge software (Biopac Systems, Inc) for data analysis. The frequency (/min), amplitude (mN), and AUC (sec × mN) of the contractions were analyzed. AUC was defined as the integrated area under a single wave. All in vitro techniques were similar to those in our previous studies.[12,14]

Drug Administration

After regular waves were detected, drugs were added to the tissue chamber, and changes in the variables were measured. ATP (300 μM) and β-NAD (1.5 mM) were used as purines. Apamin (500 nM) was administered to block SK3 channels, and MRS2500 (1 μM) was used to inhibit P2Y1 receptors in PDGFRα$^+$ cells. All drugs were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). For the analysis of frequency, amplitude, and AUC of the tension recording, recordings were analyzed for approximately 30 minutes each before and after drug administration.

Intracellular Recording

Colonic tissues were prepared in a Petri dish coated with Sylgard (Dow corning Co, Midland, MI, USA). Colonic muscles were cut parallel to the LM fibers between taenia, with a knife

consisting of a pair of parallel scalpel blades set 1.5 mm apart, and turned on their side to expose a cross-section of the entire muscle layer in the electrophysiologic chamber. The chamber was constantly perfused with prewarmed, pre-oxygenated KRB solution. Temperature was maintained at 37.5 ± 0.5°C. The muscles were equilibrated for at least 2 hours before experiments began. Conventional microelectrode recordings were performed using a sharp microelectrode filled with 3 mol/L KCl, as previously described.[11] Membrane potentials were measured with a high-input resistance electrometer, and outputs were displayed on an oscilloscope. Resting membrane potentials were measured using pClamp softwareR (version 9.0; Axon Instruments, Foster City, CA, USA) and Origin software (MicroCal Software, Northampton, MA, USA). Resting membrane potentials (mV), amplitudes (mV), and frequencies (/min) of the slow waves were analyzed before and after the administration of ATP at 300 μM. For the analysis of resting membrane potentials, amplitudes, and frequencies of the recording, recordings were analyzed for approximately 10 minutes each before and after drug administration. All in vitro techniques were similar to those in our previous studies.[12,14]

Whole-mount Immunofluorescence

Colon tissues were cut into 4 × 4 cm sections and pinned flat on a SYLGARD-coated dish with phosphate-buffered saline (PBS). The PBS was discarded, and the tissues were fixed with 4% paraformaldehyde in saline (Biosesang, Seongnam-si, Korea) overnight at 4°C. The tissues were then removed from the fixative and washed with PBS 3 times for 30 minutes per wash. The tissues were then dehydrated in 5%, 10%, and 15% sucrose in PBS (weight/volume) for 1 hour each and then in 20% sucrose overnight at 4°C. The fixed colon samples were cut into 0.5 × 0.5 cm pieces and embedded flat into Tissue-Tek Cryomold with optimum cutting temperature compound (OCT; Sakura Finetek, Torrance, CA, USA) with the mucosa facing upward before being snap frozen in liquid nitrogen. The frozen blocks were stored at −80°C.

The frozen blocks were sectioned on a Leica CM3050 S cryostat (Leica Biosystems, Wetzlar, Germany) at 100 μm thickness, and the sections were collected in PBS. The sectioned specimens were washed 3 times in PBS for 30 minutes per wash to remove the OCT; nonspecific binding in the tissues was blocked with 10% normal goat serum (Sigma-Aldrich) for 1 hour, and then, the samples were incubated with either a human anti-PDGFRα antibody (AF-307; R&D Systems, Minneapolis, MN, USA) at a dilution of 1:100 in 2% Triton-X 100 (Sigma-Aldrich) or a human anti-cKit antibody (SC-168; Santa Cruz Biotechnology, Dallas, TX, USA) at a dilution of 1:100 in 2% Triton-X 100 for 48 hours at 4°C. After incubation, the tissues were washed 3 times in PBS for 30 minutes per wash to remove the excess unbound antibodies. Donkey anti-goat Alexa Fluor 488 or donkey anti-rabbit Alexa Fluor 594 (1:1000 in PBS; Thermo Scientific, Carlsbad, CA, USA) was used to detect each primary antibody, with an 1 hour incubation followed by washing 3 times with PBS for 30 minutes per wash. For double-labeling studies, anti-PDGFRα was labeled first with Alexa Fluor 488, followed by anti-cKit labeling and detection with donkey anti-goat Alexa Fluor 594. The tissues were placed flat on a microscope slide, mounted with Aqua-Mount (Thermo Scientific), and stored at 4°C until imaging. Images were acquired using an Olympus FV1000 confocal microscope (Olympus America, Pennsylvania, PA, USA) with Z stacks between 10 μM and 70 μM. The acquired images were exported using Olympus FluoView and were then composed and organized in Adobe Photoshop version 12. We followed procedures similar to those in a previous report.[15]

Quantitative Real-time Polymerase Chain Reaction for *PDGFRα*, *SK3*, *P2RY1*, *Kit*, *ANO1*, *NOS1*, and *MYH11* Messenger RNA

The mucosa and submucosa were removed by sharp dissection, and the remaining smooth muscle tissue was used for quantitative real-time polymerase chain reaction (qPCR) for *PDGFRα*, *SK3*, and *P2RY1* as molecular markers for PDGFRα+ cells, *Kit* and *ANO1* as molecular markers for ICCs, *NOS1* as a molecular marker for neuronal cells, and *MYH11* as a molecular marker for muscle cells. Total RNA was extracted using the RNeasy Protect Mini Kit (Qiagen, Hiden, Germany). Complementary DNA was synthesized with 1 μg total RNA as the template using the SuperScript III First-Strand synthesis system according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Real-time PCR was conducted using the StepOne Real-Time PCR system (Thermo Fisher Scientific Inc, MA, USA). Specific primers for *PDGFRα*, *SK3*, *P2RY1*, *Kit*, *ANO1*, *NOS1*, *MYH11*, and 18S ribosomal RNA[16] were used. The primer sequences used for qPCR were as follows: *PDGFRα* forward, 5'-GAGATCACCACTGATGTGGAA-3' and reverse, 5'-CTTCTT CCTTAGCACGGATCA-3'; *SK3* forward, 5'-GACCATGCCAAAGTGAGGAA-3' and reverse, 5'-ACTCAGCTTCCTCTGTTCCA-3'; *P2RY1* forward, 5'-CCTCTTCTACTCAGGTACCGG-3' and reverse, 5'-ATCCGTAACAGCCCAGAATCAGC-3'; *Kit* forward, 5'-GGATTCCCAGAGCCCACAA-3' and reverse, 5'-ACATCCACTGGCAGTACAGAA-3'; *ANO1* forward, 5'-GACGTGTACAAAGGCCAAGTAC-3' and

reverse, 5'-ATCGTGCAGTGGGTATGCA3'; *NOS1* forward, 5'-ACCCTCCACCTTAAGAGCAC-3' and reverse, 5'-AAGAGCTGTCCTTTTGTGCG-3'; *MYH11* forward, 5'-GTGAAGAACGACAACTCCTCAC-3' and reverse, 5'-TGTTGGCTCCCACGATGTAA-3'. mRNA expression of each gene was normalized against 18S mRNA levels. Results were calculated as ΔCt values. The relative expression levels of *PDGFRα, SK3, P2RY1, Kit, ANO1, NOS1,* and *MYH11* mRNA were examined in the smooth muscle layer and compared between the right and left colon.

Statistical Methods

All data are expressed as the mean ± standard error of the mean, and statistical analysis was conducted using SPSS version 21.0 for Windows (IBM Corp, Armonk, NY, USA). The Mann–Whitney *U* test and Wilcoxon signed-rank test were used for comparisons of values, and statistical significance was defined as a *P*-value < 0.05.

Results

Tension Recordings of Colonic Segments With Adenosine Triphosphate and Beta-Nicotinamide Adenine Dinucleotide Administration

Contractile waves were detected in colonic segments using ten-

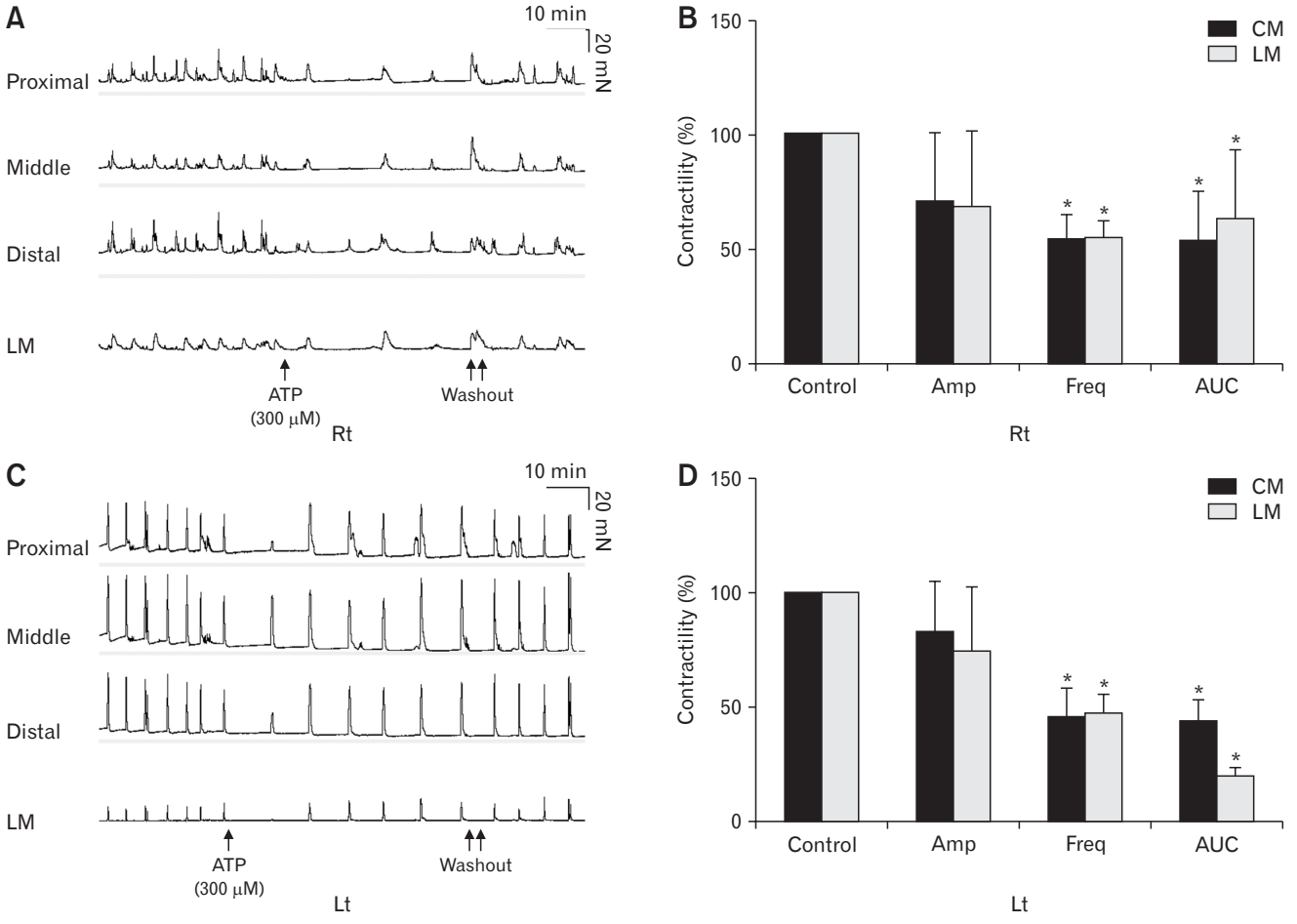

Figure 2. Effects of adenosine triphosphate (ATP) treatment in the human colon segment. The contractility of the colonic segments was reduced after 300 μM ATP administration in both the right (A) and left colon (C). The frequency and area under the curve (AUC) in the right colonic segment significantly decreased after 300 μM ATP administration in the circular muscle (CM) and longitudinal muscle (LM) (B). The frequency and AUC in the left colonic segment significantly decreased after 300 μM ATP administration in the CM and LM (D). Control, before 300 μM ATP; Amp, amplitude after 300 μM ATP; Freq, frequency after 300 μM ATP; AUC, area under the curve after 300 μM ATP; Rt, right; Lt, left. *$P < 0.05$.

sion recordings at proximal, middle, and distal sites of the CM and LM. In the right colon, the amplitudes of the CM (n = 5) and LM (n = 5) contractions before the administration of 300 μM ATP were 13.53 ± 11.37 mN and 6.55 ± 1.02 mN, respectively, and the frequencies were 0.52 ± 0.32/min and 0.57 ± 0.31/min. The AUCs were 1649.03 ± 1070.29 sec × mN and 906.38 ± 419.74 sec × mN. In the left colon, the amplitudes of the CM (n = 5) and LM (n = 5) contractions before the administration of 300 μM ATP were 47.92 ± 55.29 mN and 16.67 ± 21.43 mN, respectively, and the frequencies were 0.25 ± 0.05/min and 0.23 ± 0.06/min. The AUCs were 3337.27 ± 4006.57 sec × mN and 1175.95 ± 1827.87 sec × mN. The contractility of the colonic segments was reduced after 300 μM ATP administration in both the right and left colon (Fig. 2). In the right colonic segment, the amplitude decreased after the administration of 300 μM ATP to 70.40 ± 30.87% in the CM and to 68.16 ± 33.93% in the LM, but these decreases did not reach statistical significance ($P = 0.080$ and 0.138). However, the frequency in the right colonic segment significantly decreased after 300 μM ATP administration to 53.84 ± 11.73% in the CM and 54.60 ± 8.03% in the LM ($P = 0.043$ and 0.043). The AUC also significantly decreased in the right colonic segment after 300 μM ATP administration to 53.28 ± 22.27% in the CM and 62.89 ± 31.04% in the LM ($P = 0.043$ and 0.043). In the left colonic segment, the amplitude decreased after the administration of 300 μM ATP to 83.15 ± 22.63% in the CM and 74.70 ± 28.58% in the LM, but these decreases did not

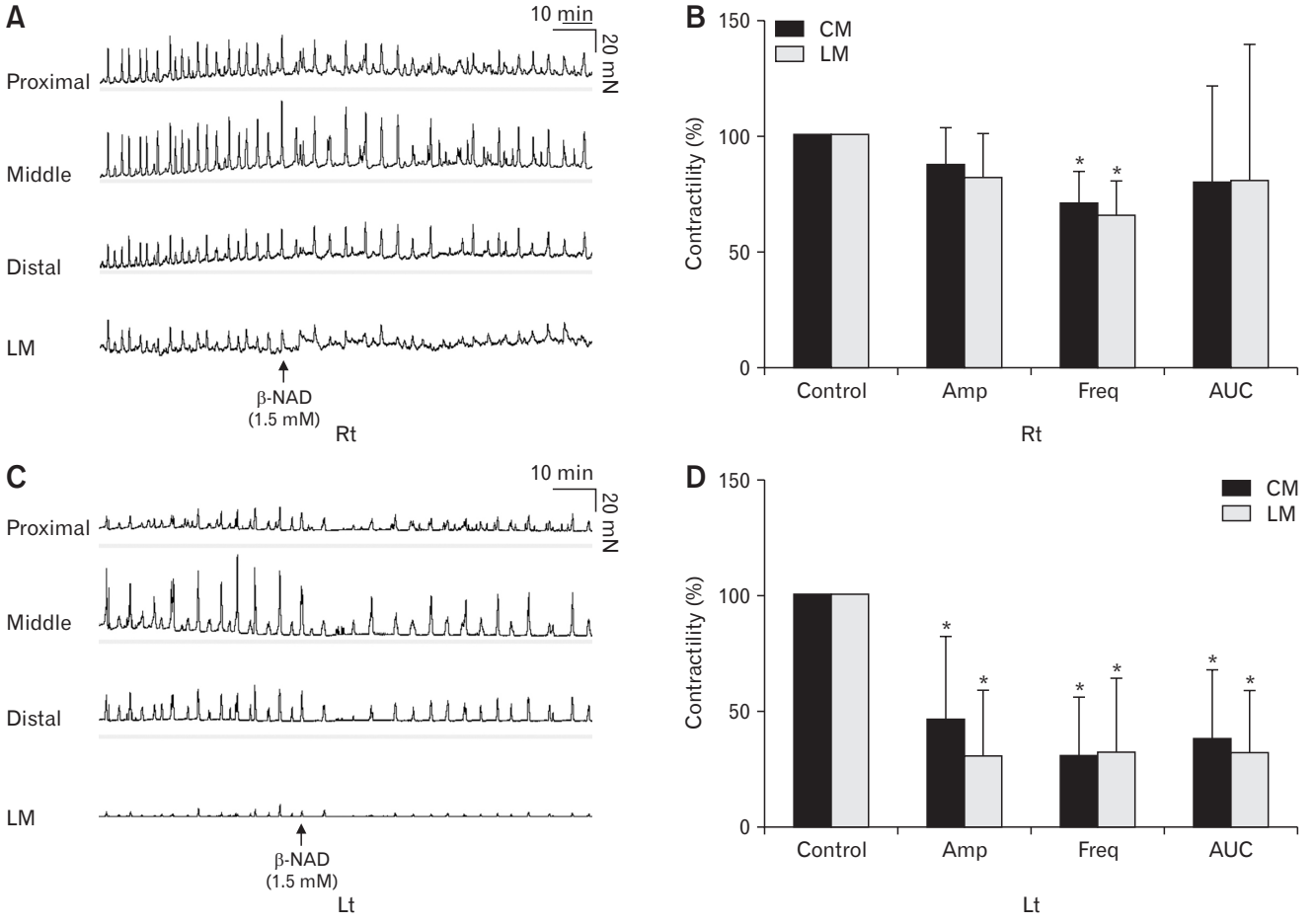

Figure 3. Effects of beta-nicotinamide adenine dinucleotide (β-NAD) treatment in the human colon segment. Similar to the results for adenosine triphosphate (ATP), the contractility of the colonic segments was also reduced after the administration of 1.5 mM β-NAD in both the right (A) and left colon (C). The frequency in the right colonic segment significantly decreased after 1.5 mM β-NAD administration in the circular muscle (CM) and longitudinal muscle (LM) (B). In the left colonic segment, the amplitude, frequency, and area under the curve (AUC) significantly decreased after the administration of 1.5 mM β-NAD (D). Control, before 1.5 mM β-NAD; Amp, amplitude after 1.5 mM β-NAD; Freq, frequency after 1.5 mM β-NAD; AUC, area under the curve after 1.5 mM β-NAD; Rt, right; Lt, left. *$P < 0.05$.

reach statistical significance ($P = 0.138$ and 0.138). The frequency in the left colonic segment significantly decreased after 300 μM ATP administration to 45.64 ± 13.34% in the CM and 47.10 ± 9.46% in the LM ($P = 0.043$ and 0.043). Finally, the AUC in the left colonic segment also significantly decreased after 300 μM ATP administration to 43.66 ± 10.25% in the CM and 19.67 ± 5.09% in the LM ($P = 0.043$ and 0.043).

In the right colon, the amplitudes of the contractions in the CM (n = 5) and LM (n = 5) before the administration of 1.5 mM β-NAD were 32.53 ± 15.91 mN and 17.96 ± 13.55 mN, respectively, and the frequencies were 0.42 ± 0.14/min and 0.44 ± 0.14/min, respectively. The AUCs were 2830.19 ± 1804.24 sec × mN and 2337.02 ± 2033.95 sec × mN. In the left colon, the amplitudes of the contractions in the CM (n = 6) and LM (n = 5) before the administration of 1.5 mM β-NAD were 38.81 ± 19.93 mN and 7.50 ± 3.47 mN, respectively, and the frequencies were 0.54 ± 0.59/min and 0.39 ± 0.13/min, respectively. The AUCs were 1917.45 ± 536.76 sec × mN and 501.56 ± 292.02 sec × mN. Similar to the results for ATP, the contractility of the colonic segments was also reduced after the administration of 1.5 mM β-NAD in both the right and left colon (Fig. 3). In the right colonic segment, the amplitude decreased after the administration of 1.5 mM β-NAD to 87.38 ± 16.94% in the CM and 81.96 ± 19.38% in the LM, but these differences did not reach statistical

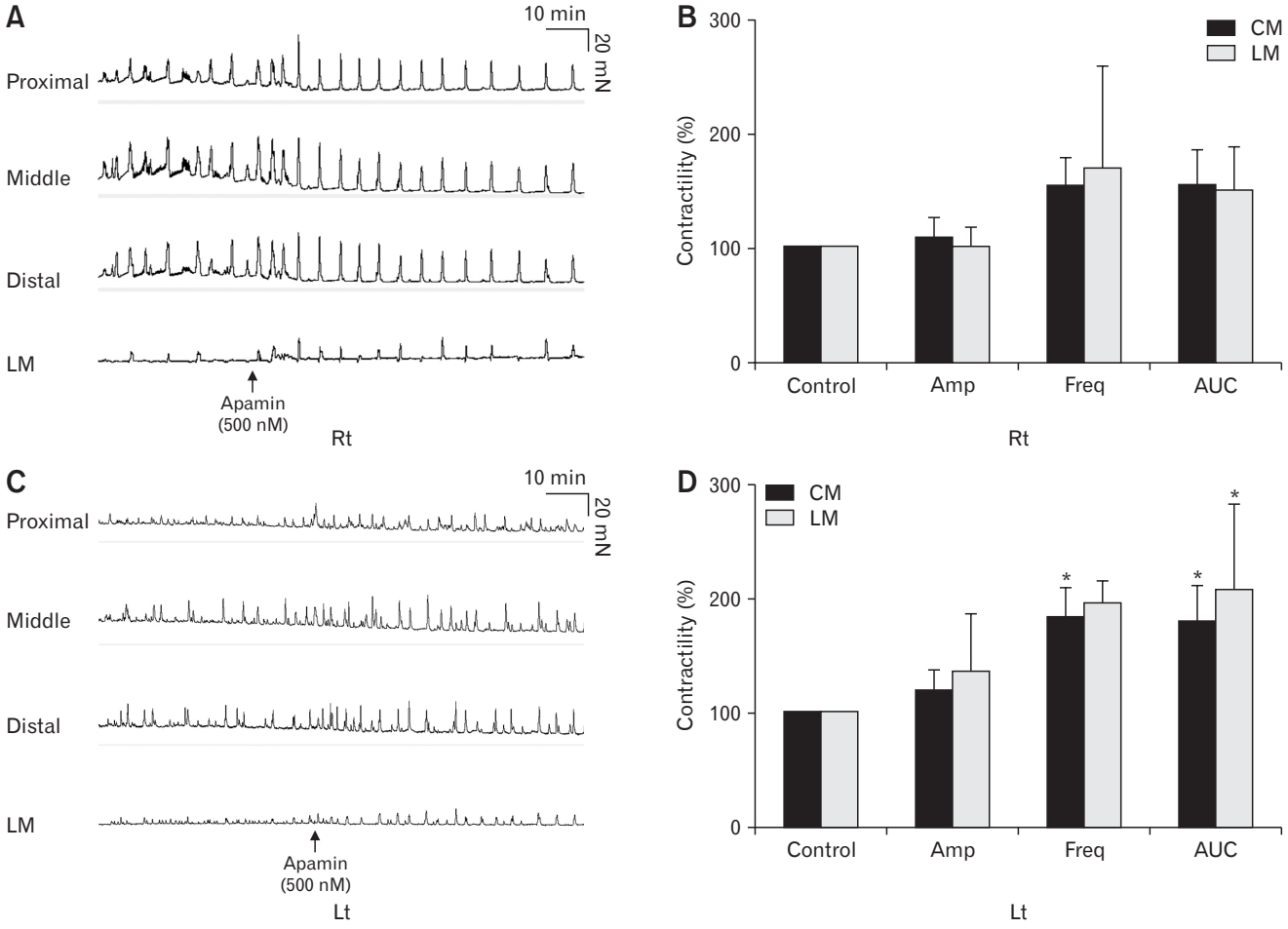

Figure 4. Effects of apamin treatment in the human colon segment. After 500 nM apamin administration, the contractility of the colonic segments increased in both the right (A) and left colon (C). In the right colonic segment, after the administration of 500 nM apamin, the amplitude, frequency, and area under the curve (AUC) increased in the circular muscle (CM) and longitudinal muscle (LM), but the differences did not reach statistical significance (B). In the left colonic segment, the frequency significantly increased after 500 nM apamin administration in the CM but not significantly increased in the LM. The AUC in this segment also significantly increased after 500 nM apamin administration in the CM and LM (D). Control, before 500 nM apamin; Amp, amplitude after 500 nM apamin; Freq, frequency after 500 nM apamin; AUC, area under the curve after 500 nM apamin; Rt, right; Lt, left. *$P < 0.05$.

significance (P = 0.138 and 0.080). The frequency in the right colonic segment significantly decreased after 1.5 mM β-NAD administration to 70.56 ± 14.55% in the CM and 65.28 ± 15.94% in the LM (P = 0.043 and 0.043). The AUC in the right colonic segment decreased after 1.5 mM β-NAD administration to 79.51 ± 42.55% in the CM and 80.26 ± 59.70% in the LM, but these differences did not reach statistical significance (P = 0.225 and 0.345). In the left colonic segment, the amplitude significantly decreased after the administration of 1.5 mM β-NAD to 45.86 ± 36.87% in the CM and 30.15 ± 28.92% in the LM (P = 0.018 and 0.043); the frequency in this segment also significantly decreased after 1.5 mM β-NAD administration to 30.29 ± 26.14% in the CM and 31.55 ± 33.24% in the LM (P = 0.018 and 0.043). Finally, the AUC in the left colonic segment significantly decreased after 1.5 mM β-NAD administration to 37.56 ± 30.78% in the CM and 31.41 ± 27.97% in the LM (P = 0.028 and 0.043).

Tension Recordings of Colonic Segments With Apamin Administration

In the right colon, the amplitudes of the contractions in the CM (n = 4) and LM (n = 4) before 500 nM apamin administration were 9.73 ± 2.64 mN and 4.57 ± 3.15 mN, respectively, and the frequencies were 0.38 ± 0.11/min and 0.46 ± 0.23/min. The AUCs were 748.69 ± 165.97 sec × mN and 440.07 ± 495.63 sec × mN. In the left colon, the amplitudes of the contractions in the CM (n = 5) and LM (n = 5) before 500 nM apamin administra-

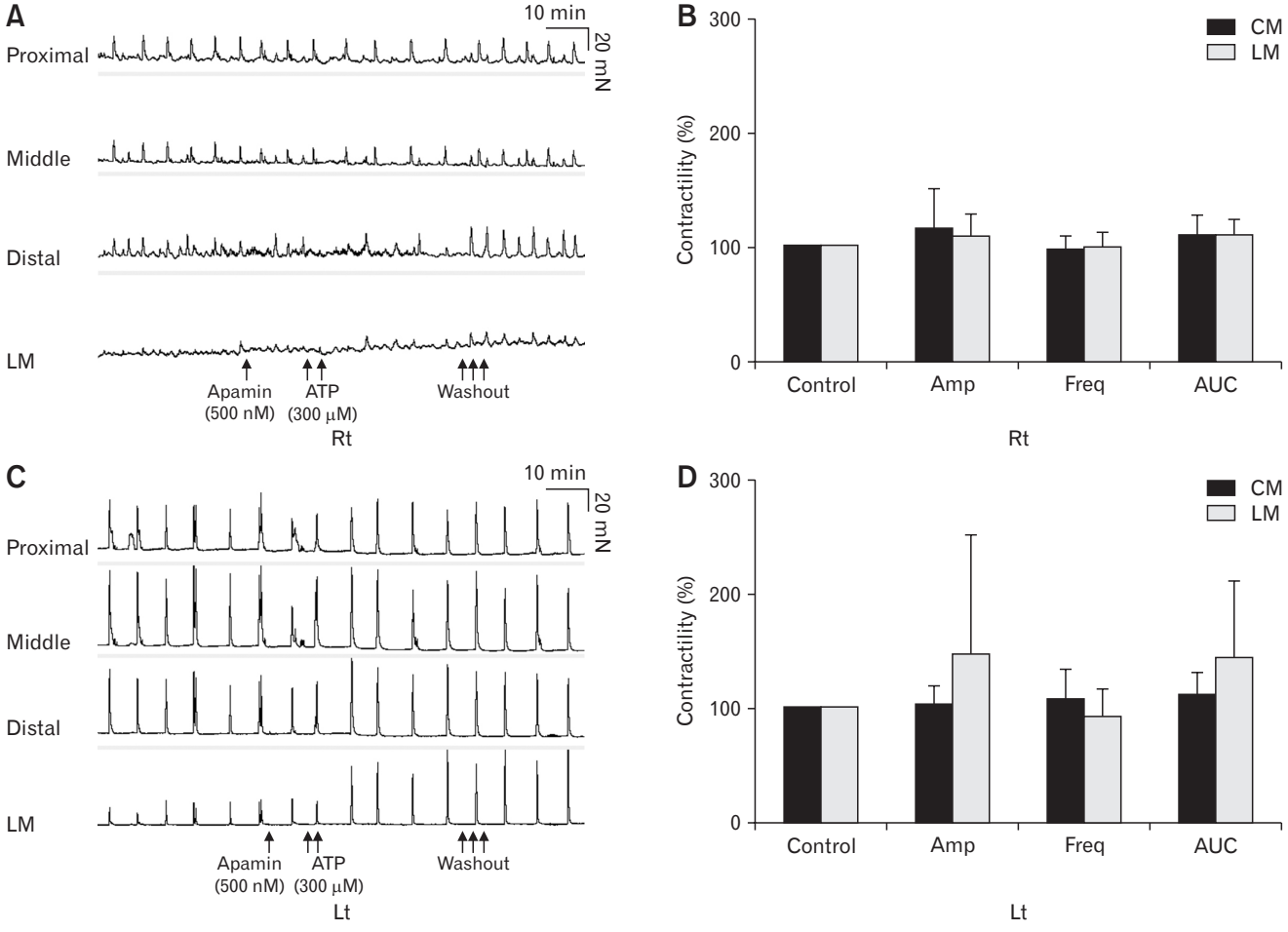

Figure 5. Effects of adenosine triphosphate (ATP) treatment with apamin pretreatment in the human colon segment. The contractility of the colonic segments did not decrease after 300 μM ATP administration with 500 nM apamin pretreatment in the right (A) or left colon (C). The amplitude, frequency, and area under the curve (AUC) did not statistically decrease after the administration of 300 μM ATP with 500 nM apamin pretreatment in the circular muscle (CM) or longitudinal muscle (LM) of the right colonic segment (B) and left colonic segment (D). Control, before 500 nM apamin; Amp, amplitude after 300 μM ATP; Freq, frequency after 300 μM ATP; AUC, area under the curve after 300 μM ATP; Rt, right; Lt, left.

tion were 35.56 ± 15.17 mN and 5.05 ± 2.07 mN, respectively, and the frequencies were 0.27 ± 0.09/min and 0.25 ± 0.10/min. The AUCs were 2030.39 ± 1015.68 sec × mN and 335.13 ± 330.24 sec × mN. After 500 nM apamin administration, the contractility of the colonic segments increased in both the right and left colon (Fig. 4). After the administration of 500 nM apamin, the amplitude in the right colonic segment increased to 108.34 ± 19.25% in the CM and 101.11 ± 17.57% in the LM, but the differences did not reach statistical significance (P = 0.465 and > 0.999). The frequency in this segment also increased after 500 nM apamin administration to 154.99 ± 24.82% in the CM and 169.73 ± 91.51% in the LM, but again, the differences were not significant (P = 0.068 and 0.068). Similarly, the AUC in the right colonic segment increased after 500 nM apamin administration to 154.15 ± 32.32% in the CM and 149.86 ± 39.87% in the LM, but the differences did not reach statistical significance (P = 0.068 and 0.068). In the left colonic segment, the amplitude increased after the administration of 500 nM apamin to 118.51 ± 36.87% in the CM and 135.35 ± 51.84% in the LM, but these differences did not reach statistical significance (P = 0.138 and 0.225). The frequency in this segment significantly increased after 500 nM apamin administration to 182.84 ± 27.70% in the CM (P = 0.043) but did not significantly increase in the LM (195.14 ± 20.98%, P = 0.225). The AUC in this segment also significantly increased after 500 nM apamin administration to 179.84 ± 31.82% in the CM and 207.46 ± 76.04% in the LM (P = 0.043 and 0.043).

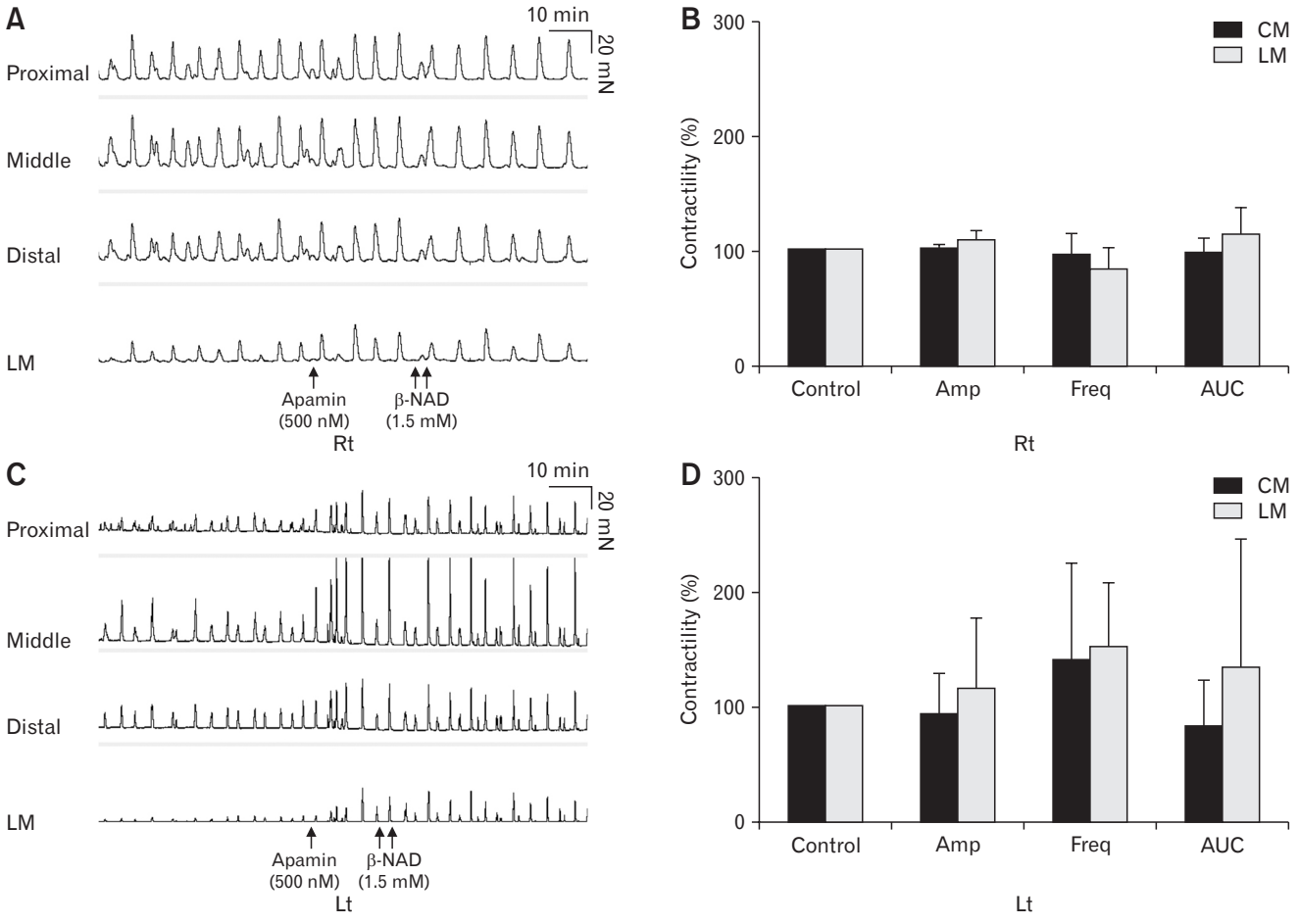

Figure 6. Effects of beta-nicotinamide adenine dinucleotide (β-NAD) treatment with apamin pretreatment in the human colon segment. The contractility of the colonic segments did not decrease after the administration of 1.5 mM β-NAD with 500 nM apamin treatment in the right (A) or left colon (C). Similar to the results of adenosine triphosphate (ATP) administration, the amplitude, frequency, or area under the curve (AUC) did not statistically decrease after the administration of 1.5 mM β-NAD with 500 nM apamin pretreatment in the circular muscle (CM) or longitudinal muscle (LM) of the right colonic segment (B) and left colonic segment (D). Control, before 500 nM apamin; Amp, amplitude after 1.5 mM β-NAD; Freq, frequency after 1.5 mM β-NAD; AUC, area under the curve after 1.5 mM β-NAD; Rt, right; Lt, left.

Tension Recordings of Colonic Segments with Adenosine Triphosphate and Beta-Nicotinamide Adenine Dinucleotide Administration and Apamin Pretreatment

In the right colon, the amplitudes of the contractions in the CM (n = 4) and LM (n = 4) before 500 nM apamin and 300 μM ATP administration were 12.93 ± 9.22 mN and 6.40 ± 1.01 mN, respectively, and the frequencies were 0.45 ± 0.34/min and 0.62 ± 0.31/min, respectively. The AUCs were 1489.14 ± 888.30 sec × mN and 1077.05 ± 671.10 sec × mN. In the left colon, the amplitudes of the contractions in the CM (n = 5) and LM (n = 3) before 500 nM apamin and 300 μM ATP administration were 33.65 ± 19.62 mN and 12.55 ± 6.18 mN, respectively, and the frequencies were 0.25 ± 0.10/min and 0.31 ± 0.13/min. The AUCs were 2309.60 ± 1666.97 sec × mN and 559.00 ± 201.23 sec × mN. The contractility of the colonic segments did not decrease after 300 μM ATP administration with 500 nM apamin pretreatment in the right or left colon (Fig. 5). The amplitude did not decrease after the administration of 300 μM ATP with 500 nM apamin pretreatment in the CM (116.34 ± 36.44%, P = 0.715) or LM (109.84 ± 20.56%, P = 0.465) of the right colonic segment. The frequency slightly, but not significantly, decreased after 300 μM ATP administration following 500 nM apamin pretreatment in the CM (97.66 ± 13.54%, P = 0.144) and LM (99.97 ± 14.27%, P = 0.715) of the right colonic segment. The AUC did not decrease after 300 μM ATP administration following 500 nM apamin pretreatment in the CM (110.35 ± 19.30%, P = 0.273) or LM (110.74 ± 15.19%, P = 0.273) of the right colonic segment. In the left colonic segment, the amplitude did not decrease after the administration of 300 μM ATP following 500 nM apamin pretreatment in the CM (102.56 ± 18.48%, P = 0.686) or LM (146.74 ± 106.66%, P > 0.999). The frequency in this segment did not decrease after 300 μM ATP administration with 500 nM apamin pretreatment in the CM (107.28 ± 27.82%, P = 0.686); the frequency slightly decreased in the LM, but the difference was not significant (92.60 ± 25.80%, P = 0.593). Furthermore, the AUC in this segment did not decrease after 300 μM ATP administration with 500 nM apamin pretreatment in the CM (111.06 ± 21.26%, P = 0.345) or LM (144.72 ± 68.26%, P = 0.285).

In the right colon, the amplitudes of the contractions of the CM (n = 3) and LM (n = 3) before 500 nM apamin and 1.5 mM β-NAD administration were 30.38 ± 13.89 mN and 22.73 ± 15.37 mN, respectively, and the frequencies were 0.40 ± 0.12/min and 0.44 ± 0.09/min. The AUCs were 2658.06 ± 1387.29

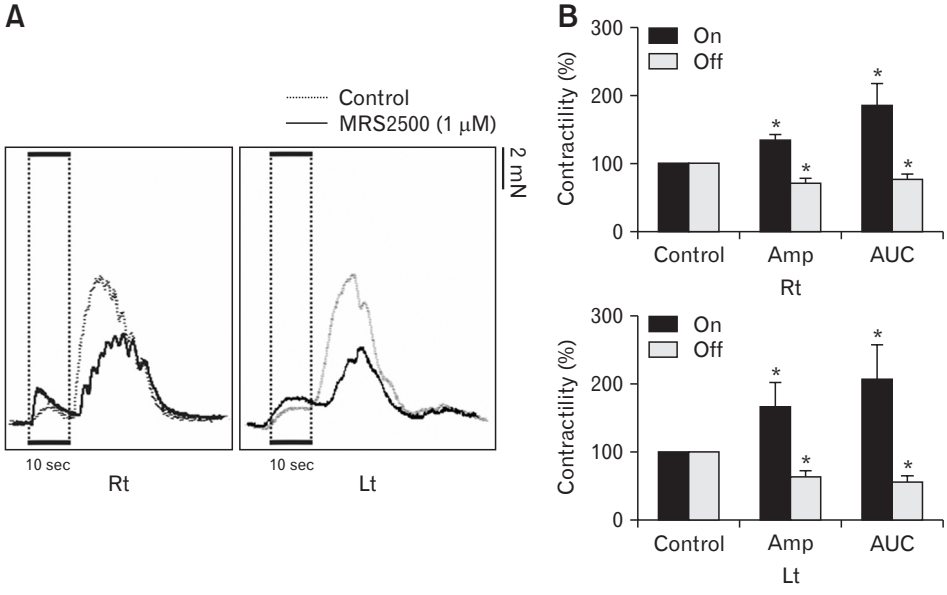

Figure 7. Tension recordings in human colon smooth muscle strip and electric field stimulation after MRS2500 treatment in the human colon. Electric field stimulation of 10 Hz for 10 seconds induced on- and off-contractions in smooth muscle strips of right and left colons before and after 1 μM MRS 2500 administration (A). In the right colon and left colon, the amplitude and area under the curve (AUC) of the on-contraction were significantly elevated after the administration of 1 μM MRS2500 (B). In contrast, the amplitude (Amp) and AUC of the off-contraction in the right and left colon significantly decreased after 1 μM MRS 2500 administration (B). Rt, right; Lt, left; Control, before MRS 2500.*P < 0.05.

sec × mN and 2897.78 ± 1281.46 sec × mN. In the left colon, the amplitudes of the contractions of the CM (n = 4) and LM (n = 4) before 500 nM apamin and 1.5 mM β-NAD administration were 27.94 ± 18.68 mN and 6.82 ± 3.02 mN, respectively, and the frequencies were 0.75 ± 0.68/min and 0.43 ± 0.11/min. The AUCs were 2534.89 ± 988.32 sec × mN and 535.77 ± 255.54 sec × mN. The contractility of the colonic segments did not decrease after the administration of 1.5 mM β-NAD with 500 nM apamin treatment in the right or left colon (Fig. 6). The amplitude did not decrease after the administration of 1.5 mM β-NAD following 500 nM apamin pretreatment in the CM (101.25 ± 5.12%, $P > 0.999$) or LM (107.51 ± 10.84%, $P = 0.285$) of the right colonic segment. The frequency in the right segment slightly decreased after 1.5 mM β-NAD administration following 500 nM apamin pretreatment in the CM (96.00 ± 19.69%, $P = 0.593$) and in the LM (83.15 ± 19.76%, $P = 0.285$), but the differences were not significant. The AUC in this segment decreased after 1.5 mM β-NAD administration following 500 nM apamin pretreatment in the CM (97.56 ± 16.53%, $P > 0.999$) and increased after treatment in the LM (113.28 ± 25.42%, $P = 0.593$), but these differences were not significant. In the right colonic segment, the amplitude slightly, but not significantly, decreased after the administration of 1.5 mM β-NAD following 500 nM apamin pretreatment in the CM (92.64 ± 38.03%, $P > 0.999$), and there was no significant change in the LM (115.59 ± 62.54%, $P > 0.999$). The frequency increased, though not significantly, after 1.5 mM β-NAD administration following 500 nM apamin pretreatment in the CM (140.96 ± 85.58%, $P = 0.715$) and LM (151.76 ± 57.40%, $P = 0.144$) of the right colonic segment. The AUC in the right segment decreased after 1.5 mM β-NAD administration following 500 nM apamin pretreatment in the CM (83.52 ± 40.55%, $P = 0.465$) and increased after treatment in the LM (133.93 ± 113.15%, $P = 0.715$), but neither of these differences was significant.

Tension Recordings of Colonic Smooth Muscle Strips and Electric Field Stimulation Before and After MRS2500 Administration

EFS of 10 Hz for 10 seconds could induce on- and off-contractions in smooth muscle strips of right (n = 6) and left (n = 5) colons (Fig. 7). The amplitude and AUC of the on-contraction were 1.15 ± 0.42 mN and 3.68 ± 1.61 sec × mN in the CM of the right colon. Those values were significantly elevated to 1.52 ± 0.52 mN and 5.42 ± 1.95 sec × mN after the administration of 1 μM MRS2500 ($P = 0.028$ and 0.028). The amplitude and AUC of the off-contraction were 8.76 ± 3.12 mN and 110.67 ± 45.31 sec × mN in the CM of the right colon. Those values were significantly decreased to 5.59 ± 1.63 mN and 71.73 ± 24.37 sec × mN after the administration of 1 μM MRS2500 ($P = 0.028$ and 0.046). The amplitude and AUC of the on-contraction were 1.35 ± 0.37 mN and 4.66 ± 1.78 sec × mN in the CM of the left colon. Those values were significantly elevated to 2.37 ± 0.96 mN and 10.75 ± 5.26 sec × mN after 1 μM MRS2500 admin-

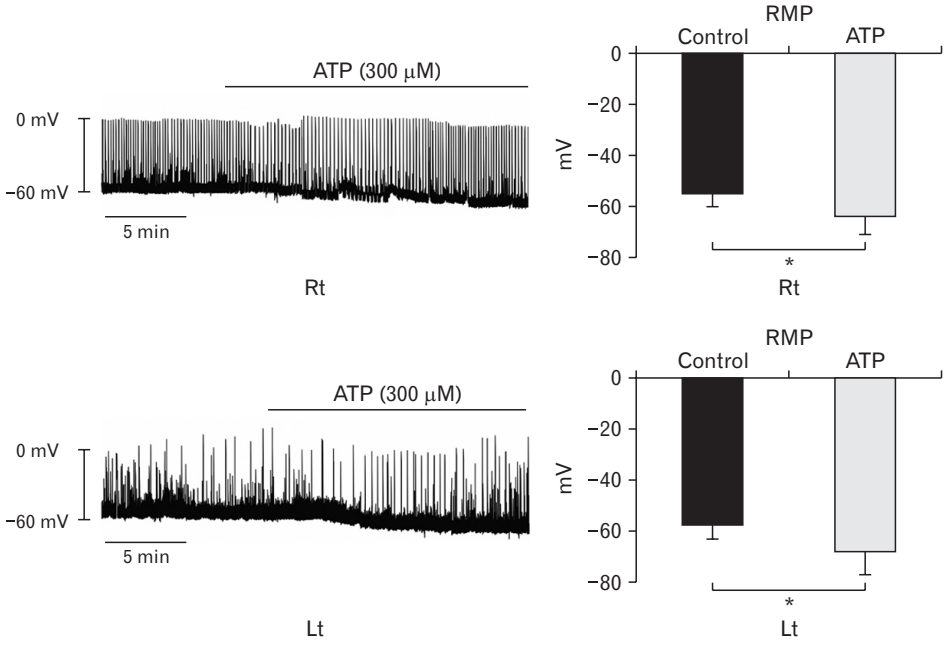

Figure 8. Intracellular recordings in the human colon. Effects of 300 μM adenosine triphosphate (ATP) treatment in the right and left colon are shown. Resting membrane potential (RMP) was hyperpolarized after ATP treatment in both right and left colon. Rt, right; Lt, left. *$P < 0.05$.

istration (P = 0.043 and 0.043). The amplitude and AUC of the off-contraction were 15.45 ± 6.57 mN and 237.01 ± 119.44 sec × mN in the CM of the left colon, and these values significantly decreased to 9.28 ± 4.13 mN and 127.73 ± 74.11 sec × mN after the administration of 1 μM MRS2500 (P = 0.043 and 0.043).

Intracellular Recording Before and After Adenosine Triphosphate Administration

The resting membrane potentials of the right and left colon before the administration of 300 μM ATP were −54.91 ± 5.31 and −57.67 ± 5.76, respectively (P = 0.691; Fig. 8). The frequency in the right and left colon was 103.2 ± 84.44 and 12.50 ± 5.97, and the amplitude was 30.93 ± 7.45 (P = 0.198) and 38.57 ± 7.19 (P = 0.432). After the administration of 300 μM ATP, the resting membrane potentials of the right and left colon were −64.39 ± 7.00 and −68.72 ± 8.77, respectively (Fig. 8). The frequency was 60.80 ± 43.89 and 10.00 ± 3.86, and the amplitude was 33.89 ± 9.07 and 43.23 ± 8.54. In the right colon, there was no difference in frequency (P = 0.188) and amplitude (P = 0.250) before and after the administration of 300 μM ATP. Similarly, in the left colon, there was no difference in frequency (P = 0.341) and amplitude (P = 0.063). However, the resting membrane potential was statistically different and changed to −9.44 ± 2.16 (P = 0.031) and −11.05 ± 3.29 (P = 0.031) in right and left, respectively (Fig. 8).

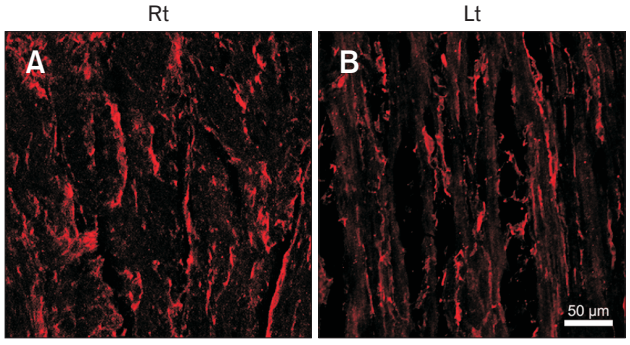

Figure 9. Distribution of platelet-derived growth factor receptor α-positive (PDGFRα+) cells in the human colon. The PDGFRα+ cells were multipolar, spindle-shaped cells parallel to the smooth muscle cells. (A) Immunolabeling of PDGFRα (red) in the circular muscle of the right colon. (B) Immunolabeling of PDGFRα (red) in the circular muscle of the left colon. Rt, right; Lt, left.

Distribution of Platelet-derived Growth Factor Receptor Alpha-positive Cells and Quantitative Real-time Polymerase Chain Reaction for *PDGFRα*, *SK3*, *P2RY1*, *Kit*, *ANO1*, *NOS1*, and *MYH11* Messenger RNA in the Human Colon

Immunohistochemistry revealed that PDGFRα+ cells were evenly distributed in the CM layer in the right and left colon (Fig. 9). The relative expression of molecular markers for PDGFRα+ cells, such as *PDGFRα* (1.659 ± 0.073 vs 1.630 ± 0.034, P = 0.548), *SK3* (1.723 ± 0.118 vs 1.748 ± 0.051, P = 0.691), and *P2RY1* (1.393 ± 0.038 vs 1.329 ± 0.053, P = 0.429) mRNA, was not different between the right and left colon, respectively (Fig. 10). Moreover, there was no difference in *Kit* (P = 0.200), *ANO1* (P = 0.571), *NOS1* (P = 0.100), and *MYH11* (P = 0.548) mRNA between the right and left colon (Fig. 10).

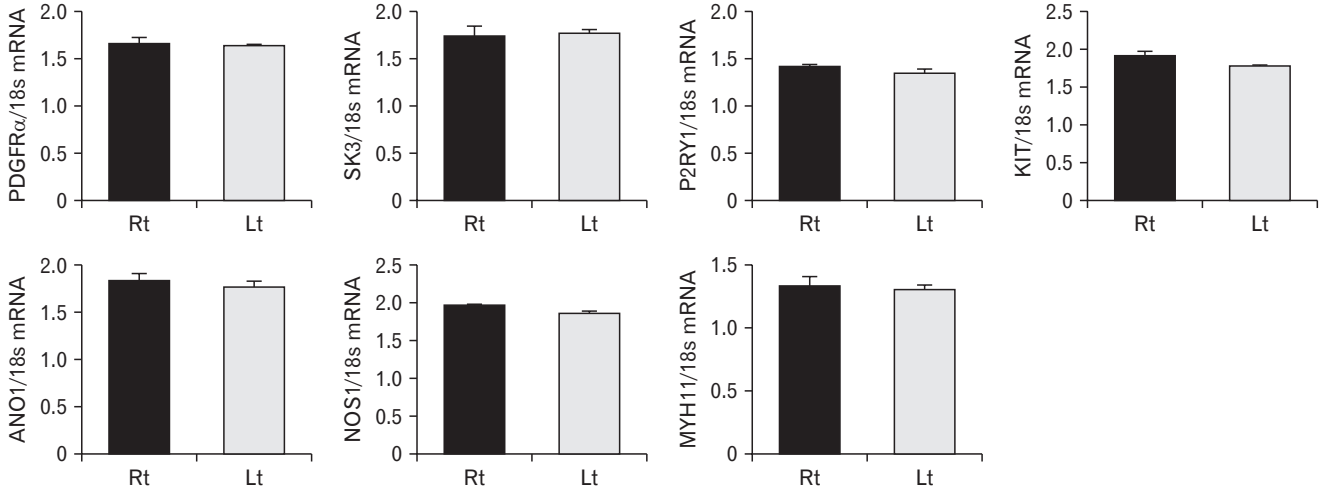

Figure 10. Quantitative real-time polymerase chain reaction for *PDGFRα*, *SK3*, *P2RY1*, *Kit*, *ANO1*, *NOS1*, and *MYH11* mRNA levels in the human colon. Relative levels are shown in the right and left colon. Rt, right; Lt, left.

Discussion

This study revealed that the purinergic inhibition of human colon contractility could be regulated by PDGFRα$^+$ cells. Purines, especially ATP and β-NAD, inhibited segmental contractility, and the frequency and AUC of the contractions significantly decreased in both the right and left colon. When apamin was used to block the SK3 channels of PDGFRα$^+$ cells, the contractility significantly increased in the left colonic segment. Purinergic inhibition with ATP or β-NAD did not occur in the right or left colonic segment after apamin pretreatment. EFS could evoke on- and off-contractions in the right and left colon, and those were changed after inhibiting the P2Y1 receptor with MRS2500. We also verified the distribution of PDGFRα$^+$ cells in the CM of the human right and left colon. Furthermore, we confirmed that there was no difference in molecular markers for PDGFRα$^+$ cells between the human right and left colon using qPCR.

Purinergic neurotransmission in the enteric nervous system plays an important role in GI motility. Numerous studies have revealed that ATP or a related nucleotide can be released by inhibitory nerves and that such release is associated with fast IJPs;[17,18] furthermore, purinergic signaling can cause nonadrenergic, noncholinergic inhibition in the gut.[19,20] Many purinergic receptors have been identified in the gut, such as P1, P2X, and P2Y[21]; ATP and β-NAD mainly act on the P2Y1 receptor and can mediate fast IJPs.[22,23] The role of purinergic neurotransmission in human GI motility has been studied with smooth muscle strips and through intracellular recordings with EFS and was found to be related to inhibitory motor control.[24,25] Our experiments on human colonic segmental contractions also showed purinergic inhibition in both the right and left colon. Although amplitude decreased, representing the strength of the contractions, after ATP administration, this did not reach statistical significance, but the frequency and AUC of the contractions decreased significantly. In addition, our results showed that ATP administration reduced the resting membrane potential of human colon smooth muscle cells based on intracellular recordings. Since purines hyperpolarize the resting membrane potential,[26] as confirmed here, ATP may have caused the slow-wave potentials to be below the threshold, decreasing the contractility and the frequency of contractions.

PDGFRα$^+$ cells are the dominant sites of P2Y1 receptors in the GI tract and play a major role in purinergic inhibition of GI motility.[27,28] Fast IJPs are absent and inhibitory responses from exogenous purines are completely abolished in P2Y1-knockout mice.[8,29] P2Y1 receptors activated by purines are coupled to $G_{q/11}$ proteins, and the downstream phospholipase C pathway is activated. Activation of phospholipase C can increase inositol 1,4,5-triphosphate (IP_3) production and the release of intracellular Ca^{2+} from IP_3 receptor-operated stores, such as the endoplasmic reticulum, and the resulting outward Ca^{2+}-dependent K^+ currents through SK channels can lead to hyperpolarization.[7,30] SK3 channels are predominantly expressed in PDGFRα$^+$ cells in the GI tract,[15,31] and the activation of P2Y1 and SK channels in PDGFRα$^+$ cells comprises the main response to purinergic inhibitory neurotransmission in the GI tract.[6,7] In this study, when SK3 channels were blocked with apamin before ATP or β-NAD administration, the purines could not reduce the segmental contractility. Although P2Y1 receptors and SK channels are expressed in smooth muscle cells and ICC, these present much higher in PDGFRα$^+$ cells.[5,8] Therefore, we suggest that purinergic inhibitory neurotransmission is mainly mediated by PDGFRα$^+$ cells in the human colon.

Our results revealed that β-NAD significantly decreased not only the frequency but also the amplitude and AUC of the segmental contractions only in the left colon, and that apamin significantly increased the frequency and AUC of these contractions. To our best knowledge, there have been no studies on differences in the distribution or function of PDGFRα$^+$ cells between the right and left colon in humans. In our previous studies on the electromechanical characteristics of the human colon, we reported that low-amplitude contractions and retrograde propagations are more common in the right colon than in the left colon but that high-amplitude constrictions were similar between the right and left colon.[11] Low-amplitude contractions are believed to mix stool in the intestinal lumen;[12] thus, it can be assumed that purinergic inhibition might be more dominant in the left colon than in the right colon to suppress small mixing movements and that the left colon could have a role in the propagation of stool from the proximal colon to the distal rectum. However, we could not verify the differences in purinergic inhibition between the right and left colon based on the isometric tension recordings of smooth muscle strips, which were evoked by EFS. Immunohistochemistry and qPCR results for *PDGFRα*, *P2RY1*, and *SK3* mRNA also did not differ in the smooth muscle between the right and left colon. This suggests that the distribution and number of PDGFRα$^+$ cells may not be different; however, the basic actions of the PDGFRα$^+$ cells in the colonic migrating motor complexes could be different between the right and left colon. In a study on PDGFRα$^+$ cells in mice, the protein expression levels of PDGFRα and SK3, assessed by western blotting, indicated that the distal colon had much higher levels than the proximal colon.[32]

However, the results for PDGFRα+ cells in that study were not quantitative values, such as those produced by qPCR. Thus, they may be different from our study results. Therefore, further studies should be performed to verify the different actions of purinergic inhibition and the function of PDGFRα+ cells in the human right and left colon.

Abnormal purinergic neurotransmission has been suggested to play a role in GI motility disorders, and inflammation can alter purinergic signaling.[21,33] Recently, protease-activated receptors, which are associated with the inflammatory response in the GI tract, were found to be expressed predominantly in PDGFRα+ cells and to affect GI motility by mediating PDGFRα+ cell activation.[34] As the importance of PDGFRα+ cells in GI motility has been elucidated, finding a way to control these cells will enable more effective treatments of specific motility disorders related to abnormal purinergic signaling in the future.

The limitation of this study was the small sample size for the in vitro experiments, which prevented statistical significance from being reached in some experiments. In addition, in the experiments to compare the right and the left colon, we did not perform a paired test on the entire colon; instead, we made comparisons with the right and left colonic tissues derived from each other. However, studies on the function of PDGFRα+ cells in the human colon are rare. Furthermore, mechanical recordings of contractions in the colonic segment are important, because the intrinsic structures related to motility are damaged less compared to that in other types of experiments. Although studies using electrophysiology or microscopy with Ca^{2+} imaging have reported the detailed functions of PDGFRα+ cells in animal models, further experiments are necessary to determine their functions in human GI motility.

In conclusion, purines inhibit segmental contractility in the right and left colons of humans similarly, and apamin increases contractility in the left colon. Pretreatment with apamin was found to prevent changes in contractility from occurring after purine treatment. The contractions of the smooth muscle strip significantly changed after MRS2500 treatment in the right and left colon. Further, the distribution and number of PDGFRα+ cells may not be different in the smooth muscle layer between the right and left colon. Overall, the results show that purinergic inhibitory neurotransmission can be regulated by PDGFRα+ cells in the human colon.

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Conflicts of interest: None.

Author contributions: Kil-yong Lee, Seung-Bum Ryoo, Tae Sik Sung, and Byoung H Koh performed the mechanical and electrophysiological recordings and immunohistochemistry experiments; Jung Nyeo Chun performed qPCR; Kil-yong Lee, Seung-Bum Ryoo, Tae Sik Sung, and Byoung H Koh drafted the manuscript and contributed equally to this study; Kyu Joo Park and Insuk So discussed and designed the study; and Shin-Hye Kim performed the experiments and collected the data. All authors reviewed and modified the manuscript.

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