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ORIGINAL ARTICLE

Basic Study

Inhibitory effects of patchouli alcohol on stress-induced diarrhea-predominant irritable bowel syndrome

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Abstract

AIM

To elucidate the mechanism of patchouli alcohol (PA) in treatment of rat models of diarrhea-predominant irritable bowel syndrome (IBS-D).

METHODS

We studied the effects of PA on colonic spontaneous motility using its cumulative log concentration (3×10^{-7} mol/L to 1×10^{-4} mol/L). We then determined the responses of the proximal and distal colon segments of rats to the following stimuli: (1) carbachol (1×10^{-9} mol/L to 1×10^{-5} mol/L); (2) neurotransmitter antagonists including N^{ω}-nitro-L-arginine methyl ester hydrochloride (10

μmol/L) and (1R*, 2S*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (1 μmol/L); (3) agonist α,β-methyleneadenosine 5′-triphosphate trisodium salt (100 μmol/L); and (4) single KCl doses (120 mmol/L). The effects of blockers against antagonist responses were also assessed by pretreatment with PA (100 μmol/L) for 1 min. Electrical-field stimulation (40 V, 2-30 Hz, 0.5 ms pulse duration, and 10 s) was performed to observe nonadrenergic, noncholinergic neurotransmitter release in IBS-D rat colon. The ATP level of Kreb's solution was also determined.

RESULTS

PA exerted a concentration-dependent inhibitory effect on the spontaneous contraction of the colonic longitudinal smooth muscle, and the half maximal effective concentration (EC_{50}) was 41.9 μmol/L. In comparison with the KCl-treated IBS-D group, the contractile response (mg contractions) in the PA + KCl-treated IBS-D group (11.87 ± 3.34) was significantly decreased in the peak tension ($P < 0.01$). Compared with CCh-treated IBS-D rat colon, the cholinergic contractile response of IBS-D rat colonic smooth muscle (EC_{50} = 0.94 μmol/L) was significantly decreased by PA (EC_{50} = 37.43 μmol/L) ($P < 0.05$). Lack of nitrergic neurotransmitter release in stress-induced IBS-D rats showed contraction effects on colonic smooth muscle. Pretreatment with PA resulted in inhibitory effect on L-NAME-induced (10 μmol/L) contraction ($P < 0.05$). ATP might not be the main neurotransmitter involved in inhibitory effects of PA in the colonic relaxation of stress-induced IBS-D rats.

CONCLUSION

PA application may serve as a new therapeutic approach for IBS-D.

Key words: Patchouli alcohol; Colonic longitudinal smooth muscles; Diarrhea-predominant irritable bowel syndrome; Enteric nervous system; Cholinergic nerves; Non-adrenergic non-cholinergic; Potassium channel

Core tip: We reported the results from an isolated colonic smooth muscle experiment in a chronic wrap-restraint stress-induced rat model of diarrhea-predominant irritable bowel syndrome (IBS-D). The model enabled us to study the possible mechanisms underlying IBS-D and the inhibitory effects of patchouli alcohol (PA) on an isolated IBS-D rat colon. This study demonstrated for the first time that the PA was involved in cholinergic and nonadrenergic, noncholinergic neurotransmitter regulation in the enteric nervous system (ENS) *in vitro*. PA acts as a neurotransmitter agent in ENS. The results suggest that PA is a new treatment option for IBS-D.

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INTRODUCTION

Pogostemonis Herba is the dry aerial part of *Pogostemon cablin* (Blanco) Benth, which is a well-known medicinal herb in Asian countries and has been widely used to treat functional gastrointestinal disorders[1]. Patchouli alcohol (PA; its structure is shown in Figure 1) is a tricyclic sesquiterpene extracted from Pogostemonis Herba; this compound is also the major active ingredient of Pogostemonis Herba. In European countries, PA is widely used in products for daily use and cosmetics[2]. Previous studies on PA have shown its component's pharmacological effects, including immunomodulatory, anti-inflammatory, antioxidative, antimicrobial, and antitumor activities; however, the use of PA in the medical field has not been reported[3]. In two previous works[4,5], PA affects the calcium ion antagonism and exerts antiemetic properties by ameliorating the excessive contraction in the digestive organ smooth muscle. These results implied that PA might play a role in neurotransmission regulation of the digestive system smooth muscle. However, no further research on the pharmacological effects of PA in neurotransmission regulation has been reported.

Irritable bowel syndrome (IBS) is a prevalent functional bowel disorder, and diarrhea-predominant IBS (IBS-D) is a major subtype of this disease. This subtype not only inflicts a significant socioeconomic burden[6], but also severely decreases the quality of life in patients with this condition[7]. At present, the precise pathophysiological mechanism of IBS-D remains unclear. Psychosocial stress, neuroendocrine abnormality, and disturbed gastrointestinal motility are potential modes of pathogenesis[8]. Recent studies[9,10] have shown that the abnormal changes in peripheral nerve factors can directly affect the normal movement and secretion of the gastrointestinal tract and sensitivity of gastrointestinal wall to mechanical or chemical stimuli. The myenteric plexus in the intestinal tract regulates the intestinal motility. Neuropsychological factors have been given serious attention in clinical and basic medical studies on IBS-D. Several drugs that target the neurotransmitter receptors, such as loperamide, eluxadoline, alosetron, and some antidepressants, are considered as the treatment options for patients with IBS-D[11]. Additionally, medical food, medications, and psychological therapies can alleviate the symptoms of IBS-D to a certain extent[12]. However, a standard treatment algorithm has not

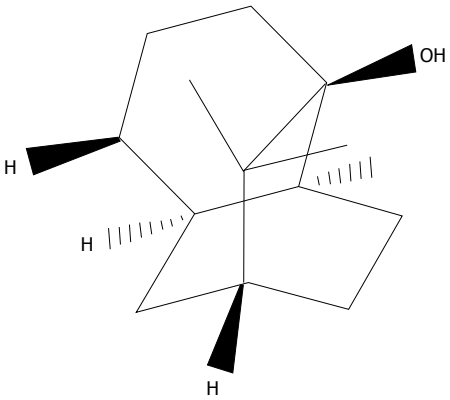


Figure 1 Structure of patchouli alcohol.

been established for IBS-D[13]. The present *in vitro* study was based on our *in vivo* model. Our *in vivo* study showed that compared with the control group, significant visceral hypersensitivity in rat model rats was observed by the abdominal withdrawal reflex score on the 21st day to the 28th day of the model establishment. The frequency of defecation in model rats increased significantly from the 14th day to the 28th day of model establishment. The stool of the model rats was mostly mushy. HE staining indicated that the intestine tissue samples in model rats showed no evident pathological changes compared with rats in the control group.

Therefore, based on the literature and our previous work, we hypothesize that the action mechanism of PA to treat IBS-D is related to the drug regulation of the neural pathways in the enteric nervous system (ENS) *via* its influence on neurotransmitter release. Therefore, the present study aims to further explore the effects of PA on isolated IBS-D rat colon *in vitro*. Our results also indicated that PA plays a specific role in neurotransmitter release regulation in ENS.

MATERIALS AND METHODS

Animal preparation

Male Sprague-Dawley (SD) rats aged four weeks and weighing 100 ± 10 g were purchased and cared for in strict compliance with the Guide to Animal Use and Care published by the Research Center for Laboratory Animals (Guangzhou University of Chinese Medicine, China). Rats were maintained under a constant 12 h/12 h light/dark cycle at an environmental temperature of 20 ℃ to 25 ℃ and humidity of 50% to 70%. The Animal Care and Use Committee of the Guangzhou University of Chinese Medicine approved all procedures used in this study.

Animal model of IBS-D

After the 7-day adaptation, the male SD rats were randomly divided into two groups as follows: control and model groups. Model group rats were subjected to a 2 h wrap-restraint period daily for 14 d. Then, these rats underwent a fourteen-day rest period.

Chemicals and reagents

In our previous studies, we confirmed PA (purity > 99.0%) by its melting point, infrared spectrometry, ^{1}H and ^{13}C nuclear magnetic resonance, and mass spectrometry analyses[14,15]. The following drugs were used. Dimethyl sulfoxide (DMSO) was used as medium to dissolve PA (DMSO < 0.2% in all experiments). Carbachol (CCh; Sigma-Aldrich, United States), N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma-Aldrich, United States), α,β-methyleneadenosine 5′-triphosphate trisodium salt (α,β-MeATP; Tocris Bioscience, United Kingdom), (1R*,2S*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS 2500; Tocris Bioscience, United Kingdom), potassium chloride (Sigma-Aldrich, United States), and an ATP determination kit (ThermoFisher, United States) were also utilized. All these reagents were dissolved in distilled water except for KCl, which was distilled in Kreb's solution. All other reagents used were of analytical grade.

Tissue preparation

After euthanasia by CO_2 asphyxiation, the rats were weighed, and colons and jejuna were excised and placed in 37 ℃ Kreb's solution (NaCl, 120 mmol/L; KCl, 5.9 mmol/L; $NaHCO_3$, 25 mmol/L; $Na_2HPO_4{\cdot}12H_2O$, 1.2 mmol/L; $MgCl_2{\cdot}6H_2O$, 1.2 mmol/L; $CaCl_2$, 2.5 mmol/L; and dextrose, 11.5 mmol/L). At the end of the experiments, the weight of each tissue from the colon and jejunum was recorded after blotting on filter paper.

Measurement of the longitudinal smooth muscle contraction

Full-thickness colonic and jejunal segments were isolated from the control and IBS-D model rats. Hung in the direction of the longitudinal muscle, one end of each tissue was attached to a fixed hook, whereas the other end of each strip was attached to a flexible hook with surgical suture. Each surgical suture was affixed in a force transducer that measured the isometric tension (Harvard Apparatus, United States). Afterward, the tissues were transferred to an organ bath containing Kreb's solution, maintained at 37 ℃, and continuously gassed with carbogen (950 mL/L O_2 + 50 mL/L CO_2). After a tension-free adaptive treatment for 0.5 h, the tissues from the colon and jejunum were stretched under 1.4 g of tension and equilibrated for 1 h. Force measurements were displayed on a strip chart recorder (Harvard Apparatus, United States; LabChart, New Zealand) and were digitally acquired by computer (LabChart software).

After equilibration, PA of various concentrations (3×10^{-7} mol/L to 1×10^{-4} mol/L) were added to each bath

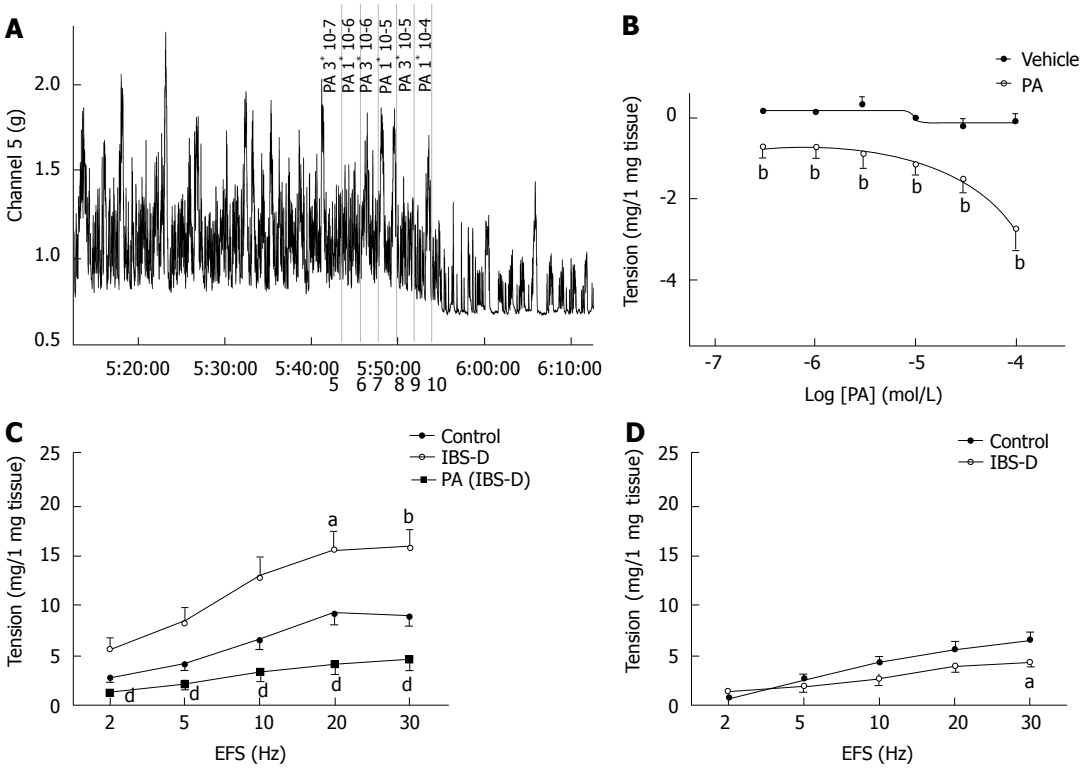


Figure 2 Inhibitory effects of patchouli alcohol on the spontaneous and EFS-induced contractions of the isolated colonic longitudinal smooth muscle. A and B: mechanical recording and linear regression curve of the cumulative log concentration-response of PA-induced (3×10^{-7} mol/L to 1×10^{-4} mol/L) relaxation of the spontaneous contraction in the colonic longitudinal smooth muscles of the control rats (n = 8; $^{b}P < 0.01$ *vs* vehicle DMSO group; unpaired t test). C: Relaxation effect of PA on EFS-induced contraction response of the colonic longitudinal smooth muscle in control, IBS-D, and PA-treated (100 μmol/L) IBS-D rats (control group: n = 38, IBS-D group: n = 31, PA-treated IBS-D group: n = 8. $^{a}P < 0.05$ *vs* control, $^{b}P < 0.01$ *vs* control, $^{d}P < 0.01$ *vs* group IBS-D; ANOVA). D: Contraction response to EFS of the jejunal longitudinal smooth muscle (control group: n = 9, IBS-D group: n = 6. $^{a}P < 0.05$ *vs* control; unpaired t test). Data are expressed as mean ± SE. PA: Patchouli alcohol; EFS: Electrical field stimulation, PA: Patchouli alcohol.

to observe the drug effects on spontaneous contraction of the colonic longitudinal smooth muscle. At the end of each experiment, the colonic contractile responses to KCl (120 mmol/L) in the control and IBS-D model rats were compared.

In this *in vitro* study, CCh (1×10^{-9} mol/L to 1×10^{-5} mol/L) was added to the organ bath, and the contractile responses in the control and IBS-D model rats were compared. After the contractile response of CCh reached a plateau, the strips were washed thrice and equilibrated for 1 h in the next experiment.

After equilibration, electrical field stimulation (EFS; 40 V, 2 Hz to 30 Hz, 0.5 ms pulse duration, 10 s) was performed to elicit the nerve-mediated contraction of the colonic tissue strips. EFS was achieved through the platinum electrodes connected to a stimulator (Harvard Apparatus, United States). The inhibitory or excitatory neurotransmitter agents, such as L-NAME (10 μmol/L), α,β-MeATP (100 μmol/L), and MRS 2500 (1 μmol/L), were separately treated in each bath before EFS.

Tissues were pretreated at a single PA concentration of 100 μmol/L to observe its drug effects on the functions of the neurotransmitter agents, including CCh (10^{-9} mol/L to 10^{-5} mol/L), L-NAME (10 μmol/L), MRS 2500 (1 μmol/L), and high-extracellular-concentration KCl (120 mmol/L), separately during the spontaneous or EFS-induced contraction of the intestinal segments. In this *in vitro* study, each tissue was subjected to a 1 h equilibration before the next test.

ATP release measurements

After EFS, Kreb's solution in each bath was collected (400 μL), frozen immediately in liquid nitrogen, and then stored at -80 ℃ until assay for ATP. The ATP levels in samples were determined by the luciferin-luciferase ATP bioluminescence assay kit (ATP Determination Kit, Thermo Fisher, United States). To calculate ATP release, we corrected the amounts detected in samples using the standard curve.

Statistical analysis

Data are expressed as means ± SEs around the mean. Non-pairwise comparisons were performed using the Student's t test. ANOVA was used to test three or more variables for statistical significance. Nonlinear and linear regression analyses were also

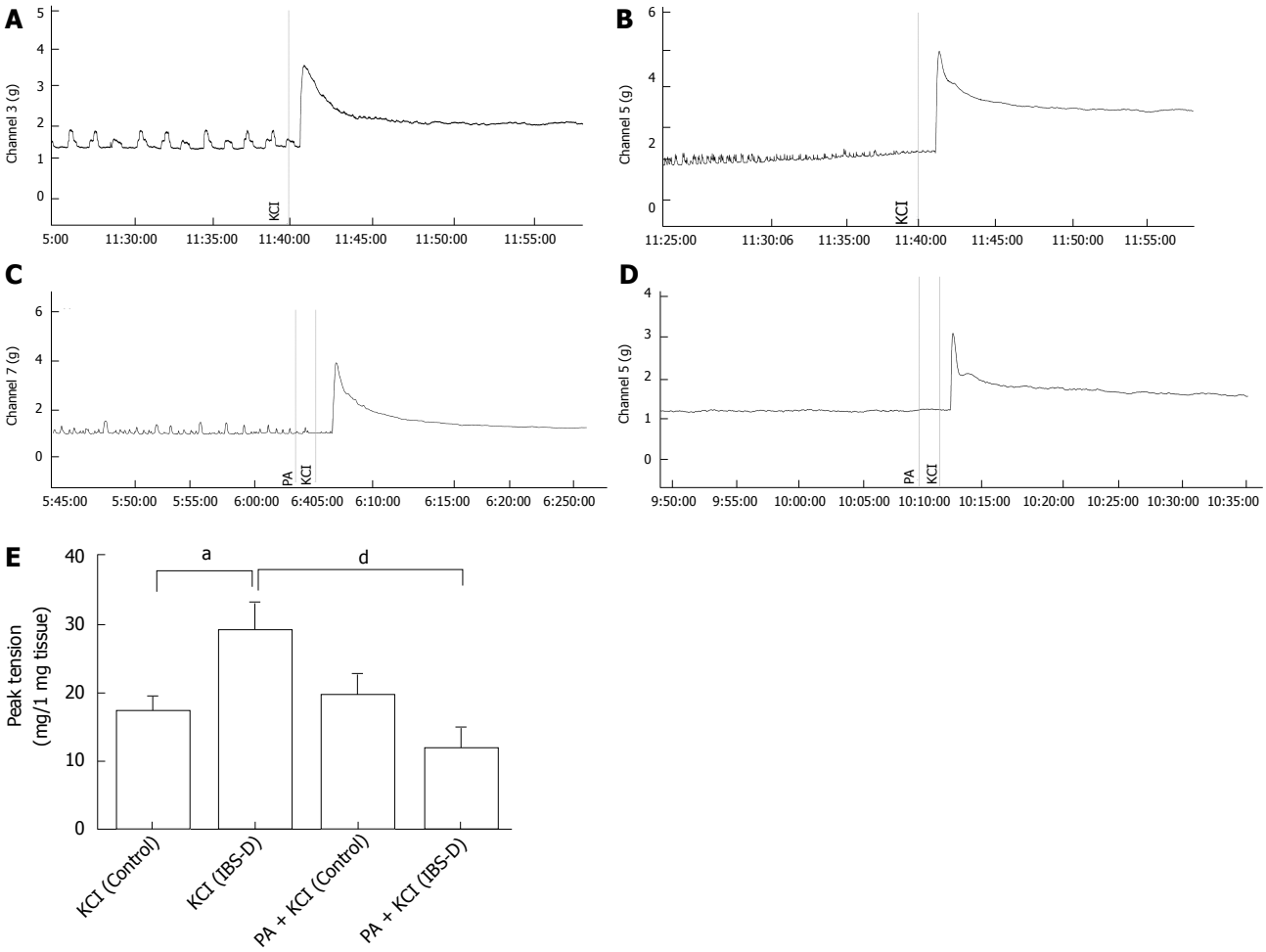


Figure 3 Inhibitory effects of patchouli alcohol on contractions induced by high extracellular KCl levels in isolated colonic longitudinal smooth muscle. Mechanical recording (A to D) and histogram (E) of relaxation effect of PA (100 μmol/L) on colonic contractions induced by high extracellular KCl levels in IBS-D rats [KCl-treated control group A: n = 27 , KCl-treated IBS-D group B: n = 23, PA + KCl-treated control group C: n = 8, PA + KCl-treated IBS-D group D: n = 8. $^{a}P < 0.05$ *vs* KCl-treated control group, $^{d}P < 0.01$ *vs* KCl-treated IBS-D group; unpaired t-test]. Data are expressed as mean ± SE.

utilized as appropriate. Calculations were performed using SPSS 20.0 based on the number of individual tissue segments. A P value of < 0.05 was considered statistically significant.

RESULTS

Inhibitory effects of PA on the spontaneous and EFS-induced contraction of the isolated colonic longitudinal smooth muscle

After equilibration, colonic muscle strips developed spontaneous basal tension for several minutes. A total of 6 cumulative PA concentrations (3×10^{-7} mol/L to 1×10^{-4} mol/L) caused a notable concentration-dependent decrease in the basal tension of the isolated rat colonic longitudinal muscle, and the half maximal effective concentration (EC_{50}) was 41.9 μmol/L (P < 0.01). PA (3×10^{-6} to 1×10^{-4} mol/L) lowered the amplitude of the spontaneous contraction in the longitudinal smooth muscles of the control rats relative to that in the vehicle strips; however, the difference was insignificant (P > 0.05). The effect of PA on the frequency of the spontaneous contraction of the colonic smooth muscle did not show significant differences between groups (P > 0.05). Compared with the control group, the EFS-induced contraction of the colonic longitudinal smooth muscles in the IBS-D group significantly increased at 20 and 30 Hz (P < 0.05). In contrast, the colonic tissues of the PA-pretreated group (100 μmol/L, 1 min) showed a significantly lower EFS-induced contraction than that of the IBS-D group (P < 0.01). Meanwhile, the jejunal tissue tension was significantly lower at 30 Hz in the IBS-D group than in the control group (P < 0.05) (Figure 2).

Inhibitory effects of PA on the isolated colonic longitudinal smooth muscle contraction induced by high extracellular KCl concentration

Contractile responses to KCl (120 mmol/L) were tested at the end of each experiment; the results showed significant differences between the control and IBS-D groups. The KCl peak contractile responses (mg contractions) reached 17.05 ± 2.47 and 29.00 ± 4.38 in the control and IBS-D rat colons, respectively (P <

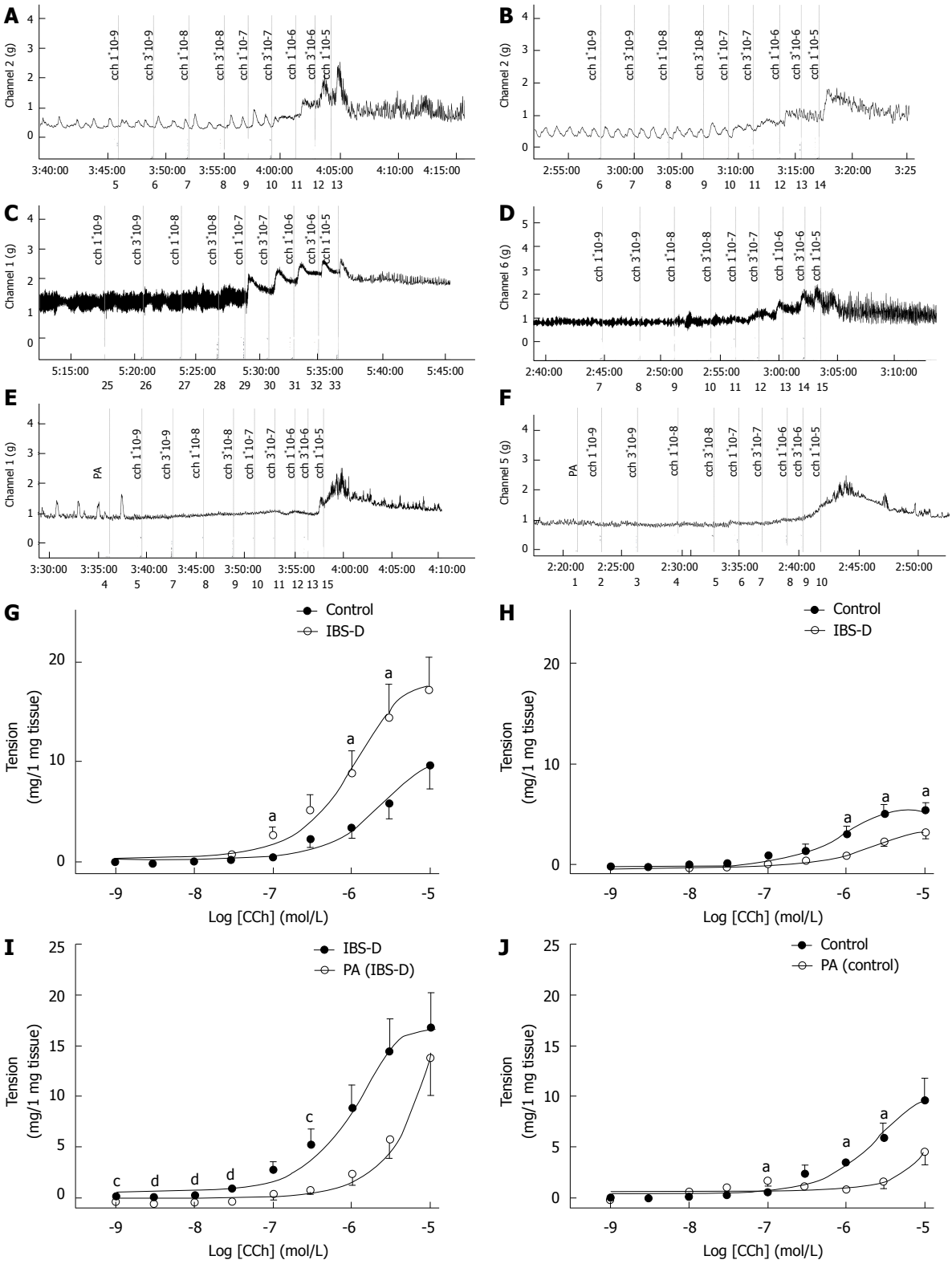


Figure 4 Inhibitory effects of PA on CCh-induced contraction in the isolated colonic longitudinal smooth muscle. Mechanical recording (A to F) and cumulative log concentration-response curve (G) of CCh-induced contraction of the smooth muscle in IBS-D rats. Colonic tissues (control group [A]: n = 14, IBS-D group [B]: n = 16; $^{a}P < 0.05$ *vs* control group, unpaired t test). Jejunal tissues (H) (control group [C]: n = 14, IBS-D group [D]: n = 11; $^{a}P < 0.05$ *vs* control group, unpaired t test). Cumulative log concentration-response curve (I) of the effect of PA (100 μmol/L) on CCh-induced contraction of smooth muscle in IBS-D rats. Colonic tissues (IBS-D group: n = 16, PA-treated IBS-D group [F]: n = 8; $^{c}P < 0.05$ *vs* group IBS-D, $^{d}P < 0.01$ *vs* group IBS-D, unpaired t test). Cumulative log concentration-response curve (J) of the effect of PA (100 μmol/L) on CCh-induced contraction of the smooth muscle in control rats. Colonic tissues (control group: n = 14, PA-treated control group [E]: n = 8. $^{a}P < 0.05$ *vs* control group, $^{b}P < 0.01$ *vs* control group, unpaired t test). Data are expressed as mean ± SE. CCh: Carbachol, IBS-D: Irritable bowel syndrome; PA: Patchouli alcohol.

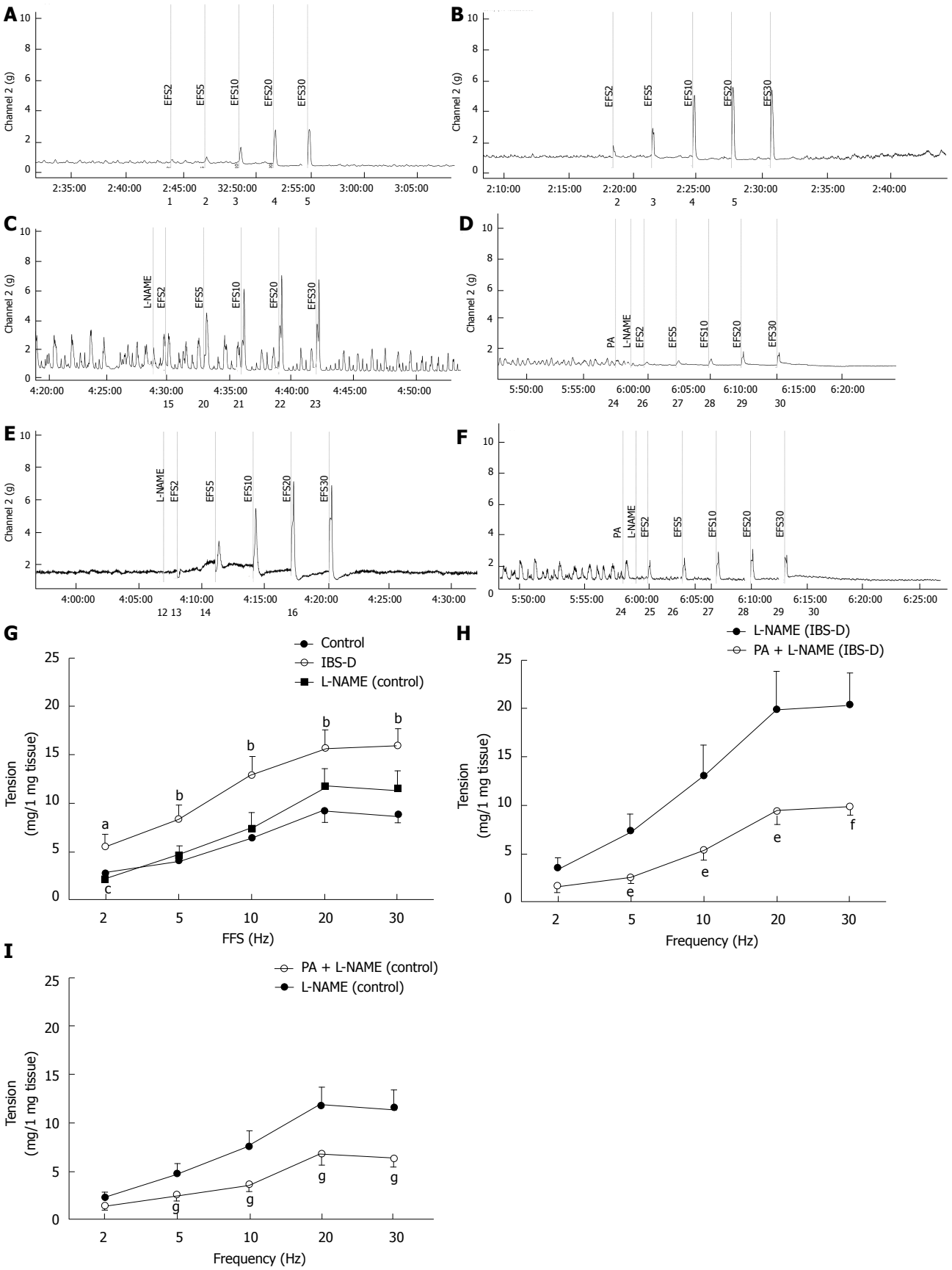
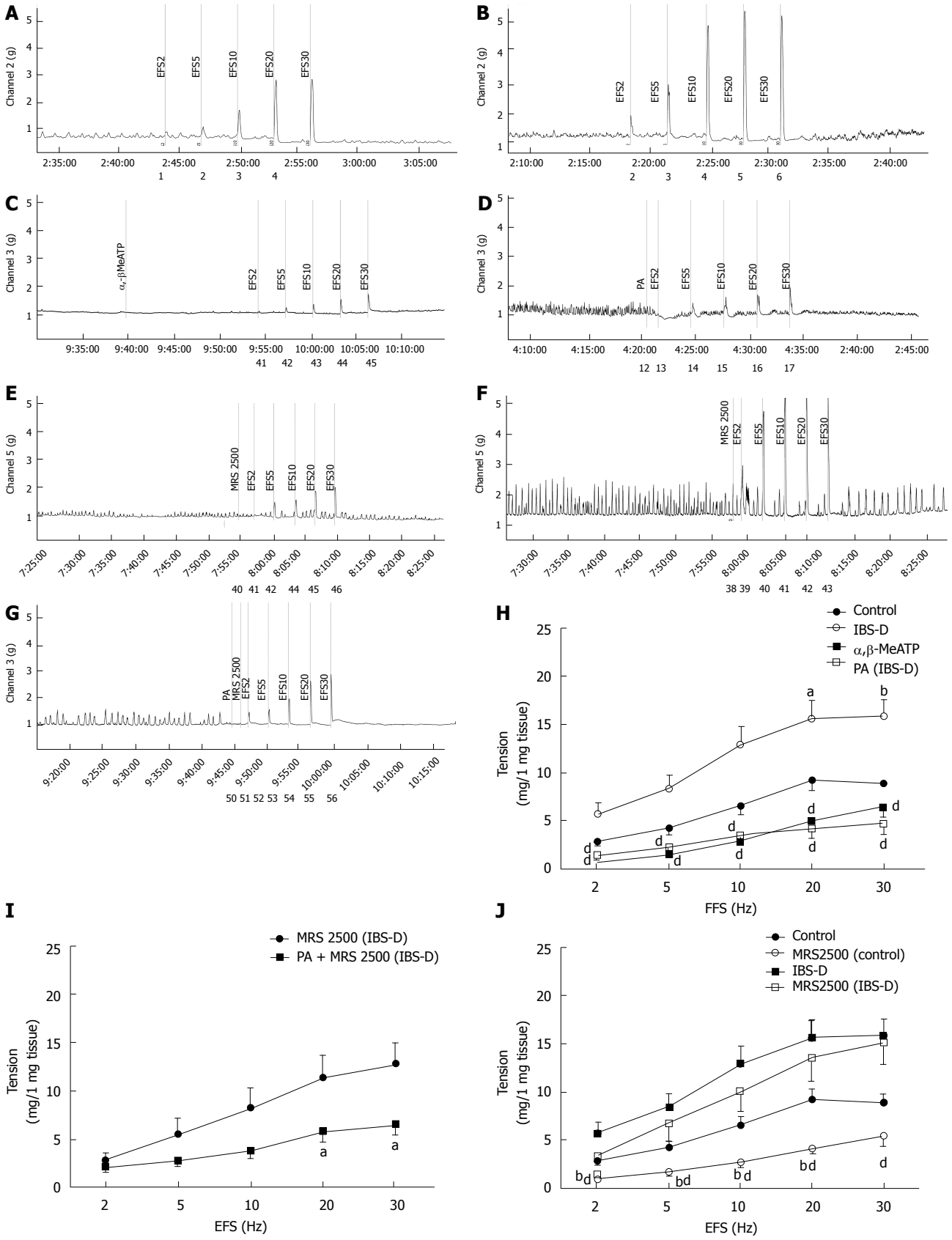


Figure 5 Inhibitory effects of patchouli alcohol on EFS-induced nitrergic contractile responses of isolated IBS-D rat colonic longitudinal smooth muscle. A-F: Mechanical recording and line chart of the mechanism and inhibitory effect of PA (100 μmol/L) on nerve-mediated contraction of stress-induced IBS-D rat colon; G: Effect of L-NAME on EFS-induced contraction of control rat colon [control group (A): n = 32, IBS-D group (B): n = 31, L-NAME-treated control group (C): n = 12; ^{a}P < 0.05 *vs* control group, ^{b}P < 0.01 *vs* control group, ^{c}P < 0.05 *vs* group IBS-D; ANOVA]; H: Effect of PA on L-NAME-induced contraction of IBS-D rat colon [L-NAME-treated IBS-D group (E): n = 14, PA + L-NAME-treated IBS-D group (F): n = 15; ^{e}P < 0.05 *vs* L-NAME-treated IBS-D group, ^{f}P < 0.01 *vs* L-NAME-treated IBS-D group, unpaired t-test]; I: Effect of PA on L-NAME-induced contraction of control rat colon [L-NAME-treated control group: n = 12, PA + L-NAME-treated control group (D): n = 16. ^{g}P < 0.05 *vs* L-NAME-treated control group; unpaired t-test]. Data are expressed as mean ± SE. L-NAME: N^{ω}-nitro-L-arginine methyl ester hydrochloride, EFS: Electrical field stimulation; IBS-D: Irritable bowel syndrome; PA: Patchouli alcohol.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MRS 2500 (IBS-D)
PA + MRS 2500 (IBS-D)
Tension (mg/1 mg tissue)
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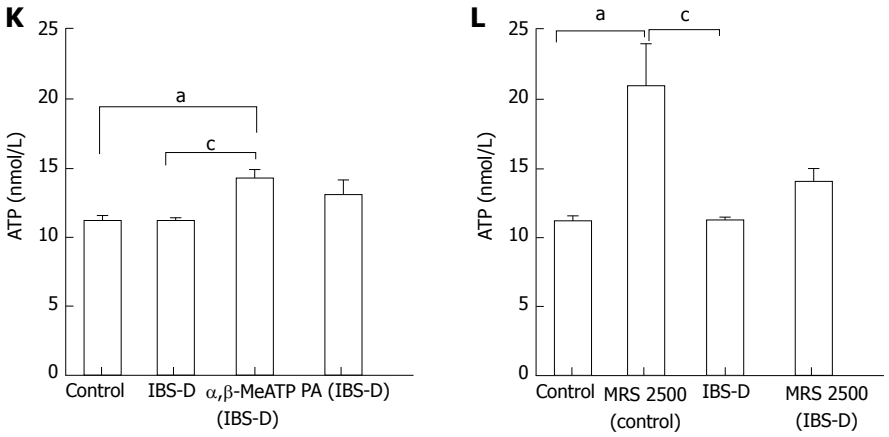

Figure 6 Effect of patchouli alcohol on a $P2Y_1$ receptor antagonist in EFS-induced contraction and ATP level of isolated colonic longitudinal smooth muscle. A-G: Mechanical recording, line chart, and histogram of the mechanism and effect of PA (100 μmol/L) on nerve-mediated rat colon contraction; H: Relaxation effect of α,β-MeATP and PA on EFS-induced contraction in IBS-D rats [control group (A): n = 32, IBS-D group (B): n = 31, α,β-MeATP-treated IBS-D group (C): n = 16, PA-treated IBS-D group (D): n = 8. $^aP < 0.05$ *vs* control group, $^bP < 0.01$ *vs* control group, $^dP < 0.01$ *vs* group IBS-D; ANOVA]; I: Relaxation response of PA to MRS 2500-treated IBS-D colon [MRS 2500-treated IBS-D group (F): n = 13, PA + MRS 2500-treated IBS-D group (G), n = 15. $^aP < 0.05$ *vs* MRS 2500-treated IBS-D group; unpaired t-test]; J: Effect of MRS 2500 on control and IBS-D rat colons [control group: n = 32, IBS-D group: n = 31, MRS 2500-treated control group (E): n = 13, MRS 2500-treated IBS-D group: n = 13. $^bP < 0.01$ *vs* control group, $^dP < 0.01$ *vs* group IBS-D; ANOVA]; K: ATP level of Kreb's solution [control group: n = 23, IBS-D group: n = 17, α,β-MeATP-treated IBS-D group: n = 21, PA-treated IBS-D group: n = 8. $^aP < 0.05$ *vs* control group, $^cP < 0.05$ *vs* group IBS-D; ANOVA]; L: [Control group: n = 23, MRS 2500 treated control group: n = 12, IBS-D group: n = 17, MRS 2500-treated IBS-D group: n = 16. $^aP < 0.05$ *vs* control group, $^cP < 0.05$ *vs* group IBS-D; ANOVA], EFS: Electrical field stimulation; IBS-D: Irritable bowel syndrome; PA: Patchouli alcohol.

0.05). To observe the effect of PA on the isolated colonic segment contraction induced by high extracellular KCl level, we tested the peak contractile responses of the tissues that were pretreated with PA (100 μmol/L, 1 min) before KCl administration (120 mmol/L) at the end of each experiment. KCl- and PA + KCl-treated control groups showed no significant differences ($P > 0.05$). However, in comparison with the KCl-treated IBS-D group, the contractile responses (mg contractions) of the PA + KCl-treated IBS-D group (11.87 ± 3.34) showed significant decrease in the peak tension ($P < 0.01$) (Figure 3).

Inhibitory effects of PA on cholinergic contractile responses of the isolated IBS-D rat colonic longitudinal Smooth muscle

Cholinergic neurotransmission produced an inverse alteration in the colon and jejunum of IBS-D rats. The contractile force of the colon smooth muscle was significantly higher in the IBS-D group than in the control group ($P < 0.05$). EC_{50} in the IBS-D group (EC_{50} = 0.94 μmol/L) that was obtained in the presence of CCh was lower than that in the control group (EC_{50} = 2.1 μmol/L). Compared with the control group, the contractile force of the jejunal smooth muscle was significantly lower in the IBS-D group ($P < 0.05$). EC_{50} in the IBS-D group (EC_{50} = 1.82 μmol/L) that was obtained in the presence of CCh was higher than that in the control group (EC_{50} = 0.78 μmol/L). The CCh-induced contraction in the colon was abolished by the pretreatment with a single concentration of PA (100 μmol/L). The IBS-D colon pretreated at a single PA concentration of 100 μmol/L significantly decreased the tension of CCh-induced (1×10^{-9} mol/L to 3×10^{-7} mol/L) contraction ($P < 0.01$) than that in the IBS-D group. Compared with the control group, the PA-pretreated control colon presented significantly decreased tension during the CCh-induced contraction (1×10^{-7} mol/L to 3×10^{-6} mol/L) ($P < 0.01$). However, at higher CCh concentrations, PA (100 μmol/L) treatment exhibited nonsignificant blocking effect on the colonic CCh-induced contractions, compared with the control group ($P > 0.05$). Similarly, compared with the control group, the pretreatment of IBS-D colon in 100 μmol/L of PA significantly decreased the tension of the CCh-induced contraction (1×10^{-9} mol/L to 3×10^{-8} mol/L) ($P < 0.05$); it also showed no significant difference at higher CCh concentrations of 1×10^{-7} mol/L to 1×10^{-5} mol/L ($P > 0.05$) (Figure 4).

Inhibitory effects of PA on the EFS-induced nitrergic contractile responses of the isolated colonic longitudinal smooth muscle in IBS-D rats

Compared with the control group, the EFS-induced contraction significantly increased in the colonic longitudinal smooth muscle in the IBS-D group at 2 Hz to 30 Hz ($P < 0.05$). Compared with IBS-D group, the EFS-induced contraction in the L-NAME-treated (10 μmol/L) control group presented no significant difference ($P > 0.05$). This result indicated higher tone in the L-NAME-induced contraction of IBS-D rat colon than in the untreated colon. Moreover, the L-NAME-mediated effect on EFS was abolished by the pretreatment with PA in both the control and IBS-D colon specimens. The EFS-induced contractions were significantly decreased by the coapplication of PA

(100 μmol/L) and L-NAME in the IBS-D colon at 5 Hz to 30 Hz, compared with that in the L-NAME-treated IBS-D group ($P < 0.05$). Similarly, the EFS-induced contractions were significantly decreased by the co-application of PA (100 μmol/L) and L-NAME in the control colon at 5 Hz to 30 Hz, compared with that in the L-NAME-treated control group ($P < 0.05$) (Figure 5).

Effects of PA on the P2Y$_1$ receptor antagonist during the EFS-induced contraction and ATP level of the isolated colonic longitudinal smooth muscle from IBS-D rats

EFS-induced contractions of the colonic longitudinal smooth muscle were significantly decreased by the P2-purinoceptor agonist α,β-MeATP (100 μmol/L) in the IBS-D colon at 2 Hz to 30 Hz compared with that in the untreated IBS-D group ($P < 0.01$). Meanwhile, no significant difference was observed in the EFS-induced contractions between the PA-treated (100 μmol/L) IBS-D and α,β-MeATP-treated IBS-D groups ($P > 0.05$). The EFS-induced contractions were significantly decreased by the coapplication of PA (100 μmol/L) and P2Y$_1$ receptor antagonist MRS 2500 in the IBS-D colon at 20 and 30 Hz compared with that in the IBS-D group treated with MRS 2500 only (1 μmol/L) ($P < 0.05$). The EFS-induced contraction was significantly decreased by MRS 2500 relative to that in IBS-D group (1 μmol/L) ($P < 0.01$). The ATP assay yielded the following results. No significant difference was detected in the ATP levels between the IBS-D and control groups ($P > 0.05$). However, α,β-MeATP significantly increased the ATP level of the IBS-D rat colon compared with those of the control ($P < 0.05$) and IBS-D groups ($P < 0.05$). MRS 2500 significantly increased the ATP level of the control rat colon compared with that in the control ($P < 0.05$) and IBS-D groups ($P < 0.05$). The ATP level did not show significant difference between the MRS 2500-treated and untreated IBS-D groups ($P > 0.05$) (Figure 6).

DISCUSSION

Due to the lack of standard treatment algorithm for IBS-D and the limited drugs used to alleviate the symptoms instead of the treatment of disease, alternative therapeutic medications for patients with IBS-D are urgently needed. Pogostemonis Herba is a vital aromatic damp-resolving agent that is often prescribed to treat vomiting and diarrhea. Previous studies[16,17] have demonstrated the antidiarrheal effects of Pogostemonis Herba. PA is a major active ingredient of this herbal medicine, which exhibits notable bioactivities and involves wide applications. The present study aimed to investigate the neurogenic response of PA on an isolated stress-induced IBS-D rat model to identity the potential mechanisms of PA in IBS-D treatment. Given that IBS-D is closely related to motility disorders of the colon rather than that of the small intestine[18,19], The present study focused on the colonic contractile responses.

ENS coordinates the movement patterns in the gastrointestinal tract[20]. The receptors on the neurons and neuroglia in ENS mediate the essential gastrointestinal functions[21,22], including gastrointestinal muscle control. Our study clearly demonstrated that PA (3×10^{-7} mol/L to 1×10^{-4} mol/L) causes concentration-dependent relaxation of the spontaneous contraction of the colonic longitudinal smooth muscles *in vitro* at EC_{50} of 41.9 μmol/L. This study is the first to directly demonstrate the inhibition of PA in rat colon motility. However, the mechanism of this acute response to PA remains unknown. Thus, we further investigated whether this acute response to PA was due to the alteration of the neurotransmissions in ENS using the neuropharmacological and electrophysiological technologies to contract the isolated longitudinal smooth muscles of rat colons. We also determined whether this property of PA could be applied to regulate the neurotransmission disorders in IBS-D rats.

The voltage-gated potassium channel (VGKC) function is determined by the membrane potential. The increased extracellular potassium levels result in the depolarization of the intestinal smooth muscle cells through decreasing the resting membrane potential, followed by muscle contractions at the suprathreshold stimulus. VGKCs are primarily regulated by extracellular drugs and neurotransmitters[23]. Previous studies have shown that the release of neurotransmitters, such as nitric oxide (NO) or acetylcholine, may be induced by KCl at concentrations higher than 60 mmol/L[24]. High sensitivity to KCl-induced contractions was noted in our IBS-D rat colons, which is consistent with a previous study[25]. In the present study, the contractile responses induced by high extracellular KCl levels (120 mmol/L) were abolished by PA in the IBS-D rat colon segments. This result suggests that PA may have functions similar to potassium channel blockers. Although further study is required to confirm this assumption, the involvement of the PA-mediated hyperpolarization on the colonic relaxation of IBS-D rat colons has been shown.

Under normal conditions, the enteric smooth muscle, which is innervated by the autonomic nervous system, contracts and relaxes rhythmically[26]. Previous studies confirmed that the colon motility dysfunction and abnormal neurotransmission changes[27] are important mechanisms underlying the IBS-D development. Neuropathic changes in ENS most possibly generated the symptomatic bowel diseases, including IBS-D[28]. In ENS, the cholinergic excitatory motor neurons exert excitatory effects on the gastrointestinal tract. PA inhibited the CCh-induced contractions; thus, PA may regulate acetylcholine transmission by antagonizing the muscarinic receptor or nicotinic acetylcholine receptor. Cholinergic nerves were excited in colons of stress-induced IBS-D rats, which is consistent with the published data[29]. Pretreatment with a single PA concentration strongly inhibited the CCh-induced (1 ×

10^{-9} mol/L to 3×10^{-7} mol/L) contractions in the IBS-D rat colon *in vitro*. Our study is the first to demonstrate that PA exerts an inhibitory effect on the cholinergic nerves of the IBS-D rat colons. However, whether this inhibitory effect against the acetylcholine operates on the muscarinic receptors, nicotinic acetylcholine receptors, or both, needs further research. In contrast, the isolated jejunum from IBS-D rats exhibited inhibitory effect on the cholinergic nerves. The wrap-restraint stress results in decreased small intestinal transit, whereas it resulted in increased large intestinal transit in rats[30]. Thus, these acetylcholine disorders may result from the wrap-restraint stress. However, further results are needed to confirm this finding. IBS-D treatment may also contribute to the cholinergic receptor-blocking effect of PA on the isolated colon but not the jejunum.

NO is a vital non-adrenergic, non-cholinergic inhibitory transmitter in the gastrointestinal tract. NO is involved in both the colonic relaxation and contractions[31]; however, it typically participates in the nitrergic relaxation. Subsequently, mechanisms of the nitrergic nerves in the gut remain unclear, and the modulation of the nitrergic and cholinergic transmission in the nerve-muscle pathway[32,33] requires further study. NO synthases utilize L-arginine and molecular oxygen to generate NO and L-citrulline. NO synthase inhibitor L-NAME significantly increases the basal tone and enhances the phasic contractions in the rat proximal colon[34]. Our results showed that the increased tone in the IBS-D rat colonic longitudinal smooth muscle in response to L-NAME during the EFS-induced contraction could be inhibited by PA. This observation suggests the lack of NO in the stress-induced IBS-D rat colon. In EFS, we observed that PA contributed to inhibit the contractions induced by the NO synthase inhibitor L-NAME (10 μmol/L) in the isolated rat colon tissues. This result indicates that PA may increase the synthesis of NO in rat colon. In the present study, we found that PA exerts a relaxation effect on the nitrergic nerves of the colonic longitudinal smooth muscle of IBS-D rats *in vitro*. In addition, PA may be a NO donor.

ATP is a purine inhibitory neurotransmitter that mediates the non-adrenergic, non-cholinergic relaxation in the gastrointestinal smooth muscles[35,36]. Studies showed that in wrap-restraint stress rat model, ATP participates in the intestinal motility[37]. However, the effect and mechanism of ATP in the IBS-D rat colon still need clarification. On the other hand, α,β-MeATP is a P2-purinoreceptor agonist. Our results demonstrated that excess contraction of the IBS-D rat colon can be inhibited by α,β-MeATP. Under EFS conditions, PA produced an inhibitory effect on the coadministration of MRS 2500 (1 μmol/L) in the IBS-D rat colon. PA affected the muscle tone and caused ATP-induced relaxation, indicating that PA may be involved in the activating effect of ATP on the IBS-D rat colon. However, this observation requires further investigation. $P2Y_1$ receptor is involved in ENS control and coordination of intestinal motility[38]. $P2Y_1$ receptor functions as a receptor for extracellular ATP, which can be activated by the endogenous ligands of ATP and ADP. Recent studies suggested that highly potent and selective $P2Y_1$ receptor antagonist MRS 2500 inhibited the ATP-induced relaxation. However, when we further tested the effect of MRS 2500 on the rat longitudinal smooth muscle, 1 μmol/L of MRS 2500 produced a significant relaxation effect in the control rats *in vitro* but not in the stress-induced IBS-D rats. A previous work[39] reported an unexplained inhibitory effect of MRS 2500 (1 μmol/L) on spontaneous motility *in vitro*. Thus, ATP may play a complementary role in regulating colonic motility of IBS-D rats. In our continuing study, ATP assay results agreed with the conclusions mentioned above. Subsequently, we cannot explain the MRS 2500-induced relaxation of the nerve-mediated contraction elicited by EFS *in vitro*. Thus, additional *in vivo* and *in vitro* studies are necessary to clarify this phenomenon.

Electrophysiological methods were used for our neuropharmacological study. This method shows the effects of PA on the structural and physiological components of the biopsychosocial model of IBS-D; the latter may enable the development for clinical research and application. However, the following limitations must be considered. First, we cannot identify the pathway dominating the IBS-D colon response to PA treatment. These inhibitory effects by PA may be related to the changes in one upstream switch. Nevertheless, our results strongly confirmed the inhibitory effects of PA on the spontaneous, CCh-induced, and EFS-induced colonic contractions. Second, animal models and mechanisms of different IBS-D models vary. The wrap-restraint stress model mimics but cannot fully represent the physiological and pathological conditions of patients with IBS-D. Thus, whether the PA treatment is appropriate for other IBS-D models or patients requires further study. Third, the role of ATP in the wrap-restraint stress IBS-D model or the involvement of ATP in mechanism of PA treatment of IBS-D rats is unclear. Fourth, the nature of our study limits our conclusion because a direct correlation between colonic contractions and electrical signaling cannot be completely confirmed. However, the present study provides evidence regarding the involvement of PA in neuromediated relaxation of the longitudinal smooth muscle in rat colon.

In conclusion, PA exerts inhibitory effects on the IBS-D rat colon, which supports our hypothesis. In addition, related responses possibly involve cholinergic, nitrergic, and K^+ channel pathways. ATP may not be the dominant pathway for participation of PA in the colonic relaxation of the stress-induced IBS-D rats. PA is a potential new candidate to effectively treat IBS-D. The findings in this study may help extend the pharmacological applications of PA. PA may be responsible for the antidiarrheal effect of Pogostemonis

Herba. Additional *in vivo* and *in vitro* investigations on the effect of PA are needed, and potential pharmacological target protein for PA in treatment of IBS-D rat colon needs to be studied.

ARTICLE HIGHLIGHTS

Research background

Irritable bowel syndrome (IBS) is a prevalent functional bowel disorder that inflicts a significant socioeconomic burden and decreases the patient quality of life. In China, the percentage of patients with diarrhea-predominant IBS (IBS-D) is 74.1%. Therefore, the neuropsychological factors have gained much attention in the clinical and basic studies on IBS-D. However, a standard treatment algorithm has not been established for this condition. Pogostemonis Herba is used in Asian countries to treat functional gastrointestinal disorders; patchouli alcohol is the major active ingredient of Pogostemonis Herba. Previous studies have indicated that patchouli alcohol (PA) may participate in the neurotransmission regulation of the smooth muscles in the digestive system. However, no research on the pharmacological effects of PA in the neurotransmission regulation has been published.

Research motivation

This study aimed to investigate the effects of PA on the isolated IBS-D rat colon and its related mechanisms. The findings in this work can help extend the pharmacological applications of PA.

Research objectives

The main objective of this study was to test our hypothesis that the mechanism of PA in treatment of IBS-D is related to the drug regulation of the neural pathways in the enteric nervous system *via* its influence on the neurotransmitter release with focus on PA research. Additional *in vivo* and *in vitro* investigations on the effect of PA and the identification of the potential pharmacological target protein in PA to treat IBS-D rat colon are needed.

Research methods

In this *in vitro* study, the effect of PA on colonic spontaneous motility was studied using the cumulative log concentration (3×10^{-7} mol/L to 1×10^{-4} mol/L). Responses to CCh (10^{-9} mol/L to 10^{-5} mol/L) and neurotransmitter antagonists, including L-NAME (10 μmol/L), MRS 2500 (1 μmol/L), agonist α,β-methyleneadenosine 5'-triphosphate trisodium salt (100 μmol/L), and single KCl doses (120 mmol/L), were obtained from the proximal and distal colon segments of rats. Effects of blockers against the antagonistic responses by pretreatment with PA of 100 μmol/L for 1 min were also assessed. enteric nervous system (40 V, 2 Hz to 30 Hz, 0.5 ms pulse duration, and 10 s) was performed to observe the nonadrenergic, noncholinergic neurotransmitter release in the IBS-D rat colon. Moreover, the ATP level of Kreb's solution was also determined.

Research results

In this study, PA exerted a concentration-dependent inhibitory effect on the spontaneous contraction of the colonic longitudinal smooth muscle. Pretreatment of PA could inhibit the peak tension of high extracellular concentration of the KCl-induced contraction of the IBS-D rat colon. The cholinergic contractile response in the colonic smooth muscle of IBS-D rat, which was induced by CCh, was reduced by the pretreatment of PA. Lack of nitrergic neurotransmitter, which was released in the stress-induced IBS-D rat, showed contraction effects on the colonic smooth muscle. Pretreatment of PA resulted in the relaxant effects on the L-NAME-induced contraction. Thus, ATP may not be the main neurotransmitter involved in the inhibitory effects of PA in the colonic relaxation of the stress-induced IBS-D rats. Therefore, these results indicated that PA played a role in the neurotransmission in ENS of colon, which may help extend the pharmacological applications of PA.

Research conclusions

PA exerts inhibitory effects on the IBS-D rat colon, which supports our hypothesis. In addition, related responses possibly involve cholinergic, nitrergic, and K^+ channel pathways. ATP may not be the dominant pathway for participation of PA in the colonic relaxation of the stress-induced IBS-D rats. PA is a potential new candidate to effectively treat IBS-D. The findings in this study may help extend the pharmacological applications of PA. PA may be responsible for the antidiarrheal effect of Pogostemonis Herba. Additional *in vivo* and *in vitro* investigations on the effect of PA are needed, and potential pharmacological target protein for PA in treatment of IBS-D rat colon needs to be studied.

Research perspectives

Our results strongly confirmed the inhibitory effects of PA on the spontaneous, CCh-induced, and EFS-induced colonic *in vitro* contractions. PA acts as a neurotransmitter agent in ENS, and is thus considered to be a new treatment option for IBS-D. However, more questions will be addressed in the future studies. For instance, the pathways dominating the IBS-D colon in response to PA treatment; and the structural and functional changes in the potential target proteins under the effect of PA. The relaxation effects of PA may be related to changes in one upstream switch. Our further studies will focus on the effect of PA on the potential target proteins in IBS-D rats both *in vivo* and *in vitro*. Patch-clamp methods will be used to measure the K^+ current, and immunofluorescence will be used to investigate the expression and colocalization of the target proteins. Furthermore, Western blot and qPCR will be performed to evaluate the expression of the target proteins.

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