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Effect of phosphodiesterase-4 inhibitor rolipram on colonic hypermotility in water avoidance stress rat model

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Abstract

Background: Phosphodiesterase (PDE) inhibition has been reported to play a role in regulating gut motility, but the evidence is insufficient, and the mechanism remains unknown. The aim of this study was to investigate the possible role of phosphodiesterase-4 (PDE4) inhibitor rolipram in water avoidance stress-induced colonic hypermotility.

Methods: A rat model of irritable bowel syndrome (IBS) with diarrhea (IBS-D) was established by water avoidance stress (WAS). Intestinal motility was assessed by fecal pellets expulsion per hour. The cyclic adenosine monophosphate (cAMP) and nitric oxide (NO) level in colon tissue were detected using ELISA assay and the Griess test, respectively. Western blotting was performed to assess the protein level of PDE, PKA/p-CREB, and neuronal nitric oxide synthase (nNOS) in the colon. To determine the role of rolipram in gut motility, the rats of the WAS + Rolipram and Rolipram group were injected with rolipram intraperitoneally. The colonic contractile activity was recorded with a RM6240 multichannel physiological signal system.

Key Results: WAS-induced gastrointestinal hypermotility and increased defecation in rats. After repeated stress, protein levels of PDE4 in the colon were promoted while PKA/p-CREB and nNOS were highly decreased. cAMP content in colon tissue did not change significantly. However, NO content decreased after WAS, and rolipram partly enhanced NO in WAS-exposed rats. In addition, intraperitoneal injection of rolipram partly inhibited the colonic motility in vivo. Meanwhile, we observed rolipram inhibited the contraction of colonic smooth muscle strips, and this inhibitory effect was abolished by Nω-Nitro-L-arginine (L-NNA), a nitric oxide synthase (NOS) inhibitor, tetrodotoxin (TTX), a blocker of neuronal voltage-dependent Na^+ channels, Rp-Adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt hydrate (Rp-cAMPS), an antagonist of cAMP.

Conclusions and Inferences: Rolipram could relieve stress-induced gastrointestinal hypermotility. This effect may be partly through the cAMP-PKA-p-CREB pathway and NO pathway.

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KEYWORDS
cAMP, chronic stress, gastrointestinal motility, irritable bowel syndrome, nitric oxide, rolipram

1 | INTRODUCTION

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder referred to gastroenterologists with a prevalence of 1.1%–29.2% of the general population.[1] With its variable symptoms of abdominal pain, visceral hypersensitivity, constipation, and diarrhea as well as unobvious pathological manifestation, the mechanisms underlying IBS remain largely unknown.[1] It is said that stress has been reported to play a key role in the pathogenesis of IBS,[2] and IBS is repeatedly reported as a stress-related disorder.[3]

Phosphodiesterase (PDE) is widely expressed in different tissues and cells which could hydrolyze intracellular cAMP and cGMP or both, implicating the diverse biological function of them. The PDE superfamily is large and complex with more than 60 different subtypes, among them, PDE4 specifically hydrolyzes cAMP.[4] Selective inhibition of PDE4 could produce a large number of therapeutic values, such as anti-inflammatory effects,[5] improving memory[6] and cognition,[7] ischemic stroke[8] as well as anti-tumor effects.[9] PDE4 inhibitors have been approved for the treatment of clinical diseases, such as chronic obstructive pulmonary disease (COPD)[10] and psoriasis.[11] Moreover, PDE4 is related to the intestinal diseases, such as inflammatory bowel disease,[12,13] gastroparesis,[14] and colorectal cancer.[15] Interestingly, several lines of evidence suggest PDE inhibitors are related to gut motility. It is reported that PDE5 inhibitor sildenafil (4 mg/kg, i.v.) delayed gastric emptying and gastrointestinal transit in rats.[16] Furthermore, PDE4 inhibitor piclamilast (5 mg/kg, i.p.) triggered a time-dependent accumulation of food in the stomach of mice by increasing gastric volumes. In addition, various types of PDE inhibitors (0.01 μM −300 μM) inhibited the contraction of gastric fundus longitudinal and circular smooth muscle strips in a concentration-dependent manner.[17] Also PDE4 inhibitor roflumilast possessed an antispasmodic effect by inhibiting spontaneous contractions of rabbit jejunum tissues in a dose-dependent manner (0.001–0.1 mg/mL).[18] In parallel, PDE4 inhibitors (0.1 μM −300 μM) concentration-dependently inhibited muscle contraction in the ileal longitudinal smooth muscle strips.[19] Although the actions of PDE inhibitors on gastric and small intestinal motility have been studied, the effect of PDE on colonic motility remains unclear, because only a few studies have been reported the relationship between PDE and colon,[20,21] and the mechanism by which PDE regulates gastrointestinal motility is not yet fully understood. Reports have proved that the effect of PDE on gastrointestinal motility may involve the autonomic nervous system, the cAMP, and NO signaling pathway[14,18,19,22]; however, there is insufficient evidence for that idea of inhibition of PDE.

It is not known which subtype of PDE is involved in the regulation of colonic function, PDE4 might be the subtype of interest. Because lipopolysaccharide (LPS), as the gut microbial product, may play a role in the immune reactivity of IBS-D,[23] and PDE4 is important in LPS-induced cellular signaling.[24]

Here, we investigated the efficacy and underlying mechanisms of PDE4 inhibitor rolipram in attenuating stress-induced IBS-D in rats. As such, we recorded the colonic contractile activity in vitro and assessed the alterations in expression of PDE4 protein and downstream signaling pathways in the rat model to determine the role of PDE4 in colonic motility.

2 | MATERIALS AND METHODS

2.1 | Animals

We used male rats because the female hormones from female rats might not only have an effect on intestinal motility but also female hormone levels could change through the menstrual cycle, which might influence the level of intestinal motility. All adult male Wistar rat (200-250 g) purchased from Vital River (Beijing, China) were housed at optimum temperature (22 ± 1° C), relative humidity 55% ± 5%, and equal exposure to a 12 hour light/dark cycle (07:00–19:00). All animals were provided with a standard pellet diet and water ad libitum, the bedding we provided were corncob balls. There were four rats in each cage, with the size of cage was 460*300*160 mm. All protocols were approved by the Institutional Animal Care and Use Committee of Renmin Hospital of Wuhan University (Approval ID: SYXK 2015–0027, Hubei, China) and adhered to the ethical guidelines of the International Association for the Study of Pain.

2.2 | Chemicals

Rolipram was purchased from Medchem Express. Tetrodotoxin (TTX), Nω-Nitro-L-arginine (L-NNA), Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate (Rp-cAMPS) were all purchased from Sigma-Aldrich. Primary antibodies were used as follows: the rabbit anti-PDE4D(ab171750, Abcam), the rabbit anti-p-CREB (ab32096, Abcam), and the rabbit anti-PKA(ab75991, Abcam), the rabbit anti-nNOS (4231, Cell signaling), BCA protein assay kit (Beyotime).

The Nitric Oxide Assay kit was bought from Beyotime (S0021S, Beyotime), and cAMP ELISA kit was from Elabscience(E-EL-0056c, Elabscience).

TTX, L-NNA, and Rp-cAMPS were dissolved in Tyrode's buffer. In contraction recordings of colonic muscle strips in vitro, the dimethylsulfoxide (DMSO) concentration was <0.01%, and the rest of the diluent for rolipram was Tyrode's buffer. In experiments of intraperitoneal

administration in rats, the diluent for rolipram was DMSO (the added concentration of DMSO was 10%), 70% PEG300, 10% Tween-80, and 10% saline, so the final DMSO concentration was 1%.

2.3 | Experimental Protocol

According to the previous experimental methods, the water avoidance stress (WAS) procedure was performed to induce IBS-D.[25] The WAS procedure was as follows: Briefly, the rats were placed on a platform (10 × 8 × 8 cm; length×width×height) in the center of a water tank (45×25×35 cm) filled with water (25°C) for 1 hour per day for 10 consecutive days. The water level in the tank was maintained at 1 cm below the platform. Rats were randomly divided into three groups: Control group, Water Avoidance Stress (WAS) group, and WAS + Rolipram group.

WAS rats and WAS + Rolipram rats were subjected to the block with water, while the Control rats were not exposed to water in the tank. Each rat in WAS + Rolipram group was intraperitoneally injected with 1 mL rolipram (5mg/kg), 1 hour before WAS for 10 days. The procedures were performed between 7:00 and 10:00 AM to minimize the effects of circadian rhythm. Fecal pellets number in the tank were counted for each rat at the end of each 1-hour session for the three groups.

2.4 | Tissue preparation and contraction recordings

The rats were treated to death with cervical dislocation after 24 hours from the end of the animal model. A 3-cm segment of the proximal colon relatively closed to the cecum, which contained all layers of the colon was removed and cleaned in Ca^{2+}-free physiological saline solution (in mmol/L: 135.0 NaCl, 5.0 KCl, 10.0 Glucose, 1.2 $MgCl_2$, and 10.0 Hepes), the pH of solution was adjusted to 7.35–7.45 with NaOH, and it was bubbled with carbogen (95% O_2 / 5% CO_2). The colon was opened along the edge of the mesentery, washed carefully, and then fixed in a dish. Colonic strips including all layers were cut in the circular muscle or longitudinal muscle orientation, and 3 mm×10 mm (width × length) colonic circular muscle (CM) or longitudinal muscle (LM) strips were prepared for subsequent experiment. Next, the smooth muscle strips were fixed to the organ bath that was connected to the isometric force transducer for recording the contractile activity. The bath filled with Tyrode's buffer containing 147.0 mM NaCl, 4.0 mM KCl, 2.0 mM $CaCl_2$, 0.42 mM NaH_2PO4, 2.0 mM Na_2HPO4, 1.05 mM $MgCl_2$, and 5.5 mM glucose (the pH was adjusted to 7.35–7.45 with NaOH) and bubbled with oxygen, and the temperature was maintained at 37°C. The colonic strips were placed under an initial resting tension (CM strip: 1.0 g, LM strip: 1.5 g) and allowed to equilibrate. The contractile amplitude and frequency of each strip were recorded with a RM6240 multichannel physiological signal system (Cheng Du, China). It should be clearly stated here that a group of 30 normal rats were used in the muscle bath experiment shown in Figure 1, Figure 2, and Figure 3. In addition, Figure 4, Figure 5, Figure 6, and Figure 7 had showed 5 rats in each of Control group, WAS group, and WAS + Rolipram group without including the information about the normal rats.

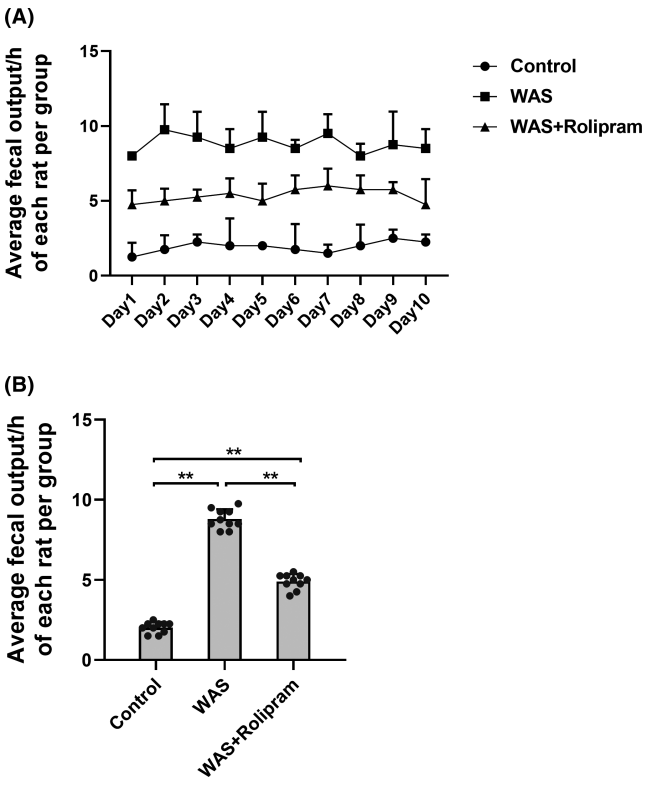

FIGURE 1 Effects of repeated WAS and rolipram on rat defecation. (A) The mean numbers of fecal output per hour per rat across the 10 days. (B) Summarized results of fecal pellet expulsion among Control, WAS, and WAS + Rolipram group. Data are expressed as Mean ± SD and analyzed by one-way ANOVA; $^{**}p < 0.01$; N = 4/group

2.5 | Western Blot

Approximately 100mg of the proximal colon was homogenized in RIPA lysis buffer and subsequently subjected to centrifugation at 12000 RPM/13523 × g RCF, 4°C for 20 min. The supernatant was collected and used for BCA protein assay. The protein samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel and electrophoresed. Then, the proteins were transferred to PVDF membranes, which were blocked with tris-buffered saline Tween (TBST) containing 5% milk to block nonspecific binding (2h, room temperature). The blots were then incubated overnight at 4°C with the primary antibody against PDE, PKA, p-CREB, and nNOS. After washing several times, the membranes were incubated with the appropriate secondary antibody (1 hour at room temperature). Finally, the specific protein bands were visualized using the ECL kit (Beijing Praeli Gene Technology Co., Ltd) and an X-ray film (Ruike, Xiamen, China). The gray scale value of each band was quantified using Image J software 2.0.

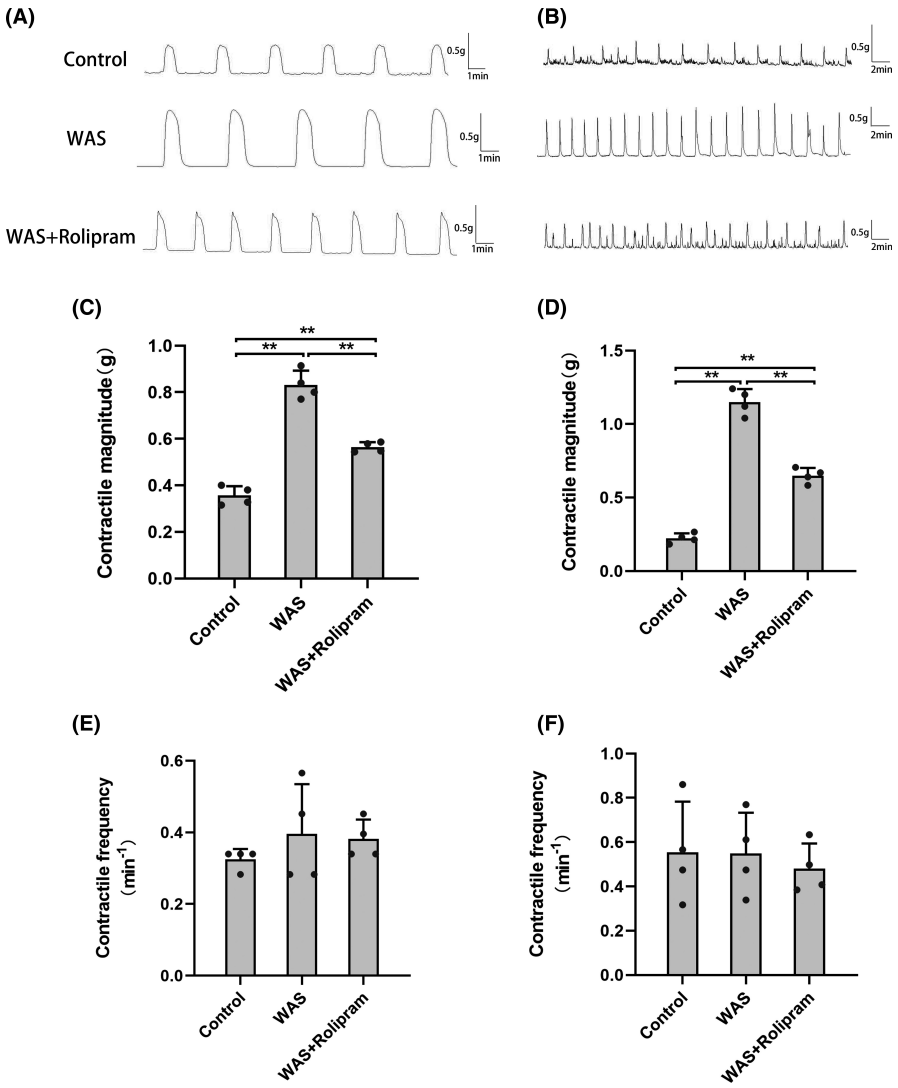

FIGURE 2 Effects of repeated WAS on contractile activities of proximal colonic muscle strips. The spontaneous contraction of LM(A) and CM(B) strips in the Control, WAS, and WAS + Rolipram rats. Summarized results of the LM(C) and CM(D) strips contractile activities in Control, WAS, and WAS + Rolipram rats. Data are expressed as Mean ± SD and analyzed by one-way ANOVA; $^{**}p < 0.01$; N = 4/group

2.6 | cAMP and NO detection

Approximately 100 mg of fresh proximal colon tissue was diluted and homogenized with precooled PBS on ice, then centrifuged at 3500 RPM/1150 × g RFC for 10 min and supernatant was collected for detection. The concentration of cAMP in the colon was measured using ELISA kits with the sensitivity of 0.94 ng/mL specific for rats, according to the manufacturer's instruction, results were expressed as ng/g protein. Colonic tissue was used for the Griess reaction with the sensitivity of 1 μm/L to detect NO content, and results were expressed as μmol/mg protein. The data were measured and averaged from each group of 4 independent data.

2.7 | Statistical Analysis

All data analysis was performed using SPSS version24.0, Image J software 2.0 and GraphPad Prism software 8.0. The data were presented as the Mean ± SD. In all analyses, "n" refers to the sample size of available data.

In this experiment, the data showed in Figure 1, Figure 2, Figure 3, Figure 4, and Figure 5 were partially excluded. The main exclusion criterion was that not every colonic muscle strip could record the contraction waveform in the muscle bath experiments and that a very small number of model rats did not meet the criteria for successful modeling.

In data processing, we used blind method for the result in Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6, and Figure 7. Some people mainly performed the research, some collected the data, some analyzed the data and wrote the paper, and so on. Due to the relatively small sample size, we assumed that these data conform to normal distribution in data processing, so normality was not evaluated.

Differences among the groups for Figure 1 were analyzed using 2-way repeated-measures ANOVA, and the Bonferroni post hoc test was used where appropriate. One-way ANOVA with Bonferroni post hoc test was used for comparisons of more than two groups. Level of $p < 0.05$ was considered statistically significant.

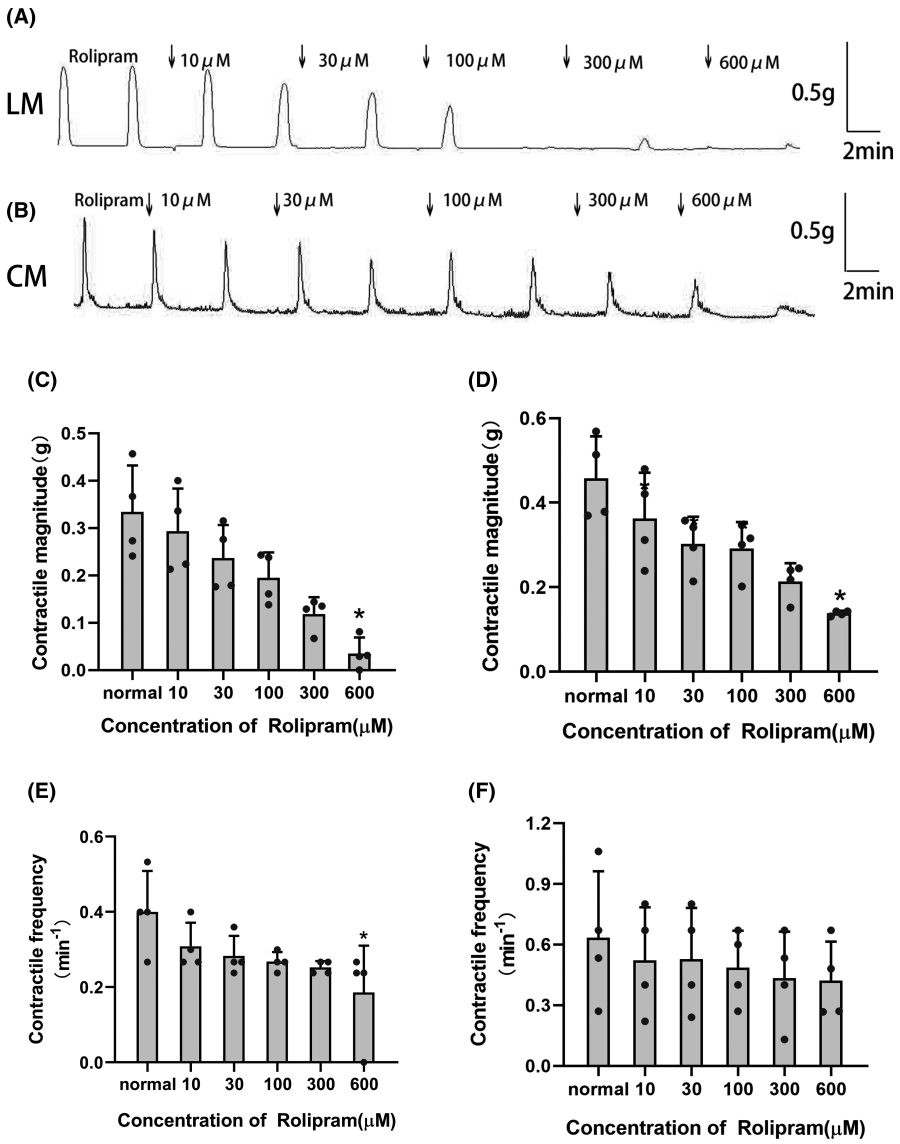

FIGURE 3 Effects of rolipram on spontaneous contraction of colonic muscle strips Rolipram inhibited the spontaneous contractions of LM(A) strips and CM(B) strips in a concentration-dependent manner. Bar graph showed that summarized results of contractile amplitude of LM(C) and CM(D) strips. Bar graph showed that summarized results of contractile frequency of LM(E) and CM(F) strips. Data are expressed as Mean ± SD and analyzed by one-way ANOVA; $*p < 0.05$ vs normal; $**p < 0.01$ vs normal; N = 4/group

3 | RESULTS

3.1 | Evaluation of the rat IBS-D model induced by WAS

As shown in Figure 1, water avoidance stress induced a significant increase on fecal pellet expulsion. Figure 1A showed the average fecal pellet expulsion per hour for each group over a 10-day period in Control group, WAS groups, and WAS + Rolipram group. Figure 1B is a histogram drawn for Figure 1A. The results for this part were analyzed using two-way repeated-measures ANOVA, and then followed by a Bonferroni post hoc test. The main effect of group was F(1.899,56.96) = 408.1, $p = 0.000$, main effect of day was F(9,30) = 0.5789, $p = 0.8036$, and the interaction effect was F(18,60)=0.7922,$p = 0.7009$. During the session, the fecal pellets expulsion per hour per rat of WAS group were higher than that of the Control group (WAS 8.8 ± 0.61 vs Control 2.03 ± 0.34, $p = 0.000$).

As shown in Figure 2A, B, WAS significantly increased the contractile activity of the proximal colonic strips. In Figure 2C, the average contraction amplitude of the LM from the WAS rats was higher than that from the Control rats (0.83 ± 0.06g vs 0.36 ± 0.04g, F(2,9) = 113.856, $p = 0.000$). In Figure 2D, the average contractile amplitude of the CM from the WAS rats was significantly higher than that from the Control rats (1.15 ± 0.08g vs 0.22 ± 0.03g, F(2,9) = 220.100, $p = 0.000$). However, there was no significant difference in frequency among these three groups, as shown in Figure 2E and Figure 2F.

3.2 | Rolipram alleviated colonic hypermotility in vivo and in vitro

Rolipram, a selective PDE4 inhibitor, was administered to explore the role of PDE in intestinal hypermotility of stress-exposed rats. The fecal pellet expulsion can be used to evaluate intestinal motor function. In Figure 1, interestingly, we observed that increased fecal defecation in WAS rats was inhibited by rolipram (WAS 8.8±0.61 vs WAS+Rolipram 4.9 ± 0.47, $p = 0.000$), but it was still higher than

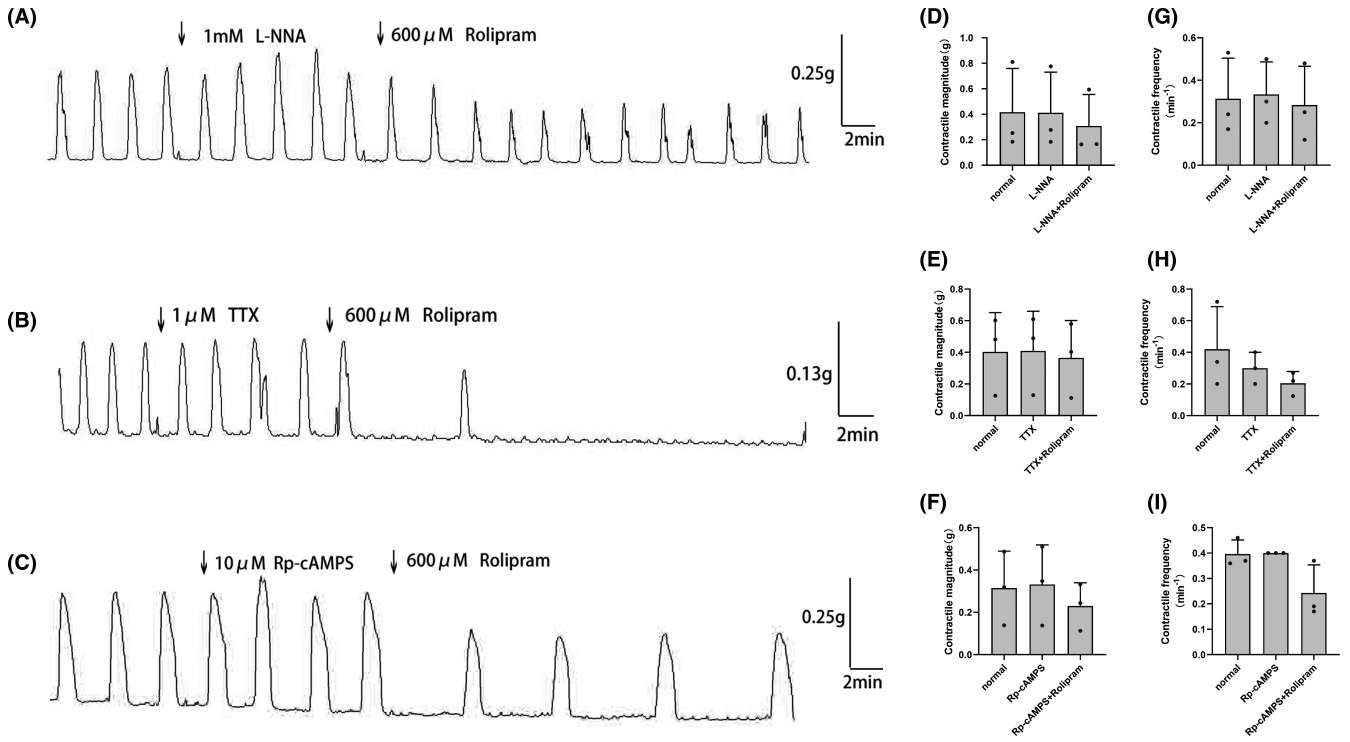

FIGURE 4 Effects of rolipram on colonic LM strips in the presence of L-NNA, TTX, or Rp-cAMPS. The representative image showing spontaneous contractions of colonic LM strips in vitro induced by 600 μM rolipram in the presence of L-NNA(A), TTX(B), or Rp-cAMPS(C). Bar graph showed that summarized results of contractile amplitude of LM in the presence of L-NNA(D), TTX(E), or Rp-cAMPS(F). Bar graph showed that summarized results of contractile frequency of LM in the presence of L-NNA(G), TTX(H), or Rp-cAMPS(I). Data are expressed as Mean ± SD and analyzed by one-way ANOVA; N = 3 or 4 /group

that of the Control group (WAS + Rolipram 4.9 ± 0.47 vs Control 2.03 ± 0.34, $p = 0.000$).

The effect of rolipram on spontaneous contractions of colonic strips was presented in Figure 2. As shown above, the average magnitude of WAS + Rolipram group was partly decreased compared with that of WAS group (LM: WAS 0.83 ± 0.06g vs WAS + Rolipram 0.56 ± 0.02g, $F(2,9) = 113.856$, $p = 0.000$; CM: WAS 1.15 ± 0.08g vs WAS + Rolipram 0.65 ± 0.04g, $F(2,9) = 220.100$, $p = 0.000$), but it was still higher than that of the Control group (LM: WAS + Rolipram 0.56 ± 0.02g vs Control 0.36 ± 0.04g, $F(2,9) = 113.856$, $p = 0.000$; CM: WAS + Rolipram 0.65 ± 0.04g vs Control 0.22 ± 0.03g, $F(2,9) = 220.100$, $p = 0.000$).

3.3 | Rolipram inhibited the spontaneous contractile activities of colonic muscle strips in a concentration-dependent manner

As shown in Figure 3A, B, rolipram produced a decrease in the spontaneous contractions of both CM strips and LM strips in a concentration-dependent manner. Before the addition of rolipram, the mean amplitude of contractions in LM strips (Figure 3C) was 0.33 ± 0.10g. After the addition of rolipram at concentrations of 10, 30, 100, 300, and 600 μM, the amplitude was reduced to 0.29 ± 0.11g ($F(5,18) = 10,684$, $p = 0.985$ vs normal), 0.24 ± 0.06g ($F(5,18) = 10,684$, $p = 0.613$ vs normal), and 0.20 ± 0.06 g ($F(5,18) = 10,684$, $p = 0.282$ vs normal), 0.12 ± 0.04 g ($F(5,18) = 10,684$, $p = 0.083$ vs normal), 0.04 ± 0.01 g ($F(5,18) = 10,684$, $p = 0.03$ vs normal), respectively. As for CM strips (Figure 3D), before the addition of rolipram, the mean amplitude of contractions was 0.46 ± 0.10 g, after the addition of rolipram at concentrations of 10, 30, 100, 300, and 600 μM, the amplitude was reduced to 0.36 ± 0.09 g ($F(5,18) = 9.518$, $p = 0.779$ vs normal), 0.30 ± 0.07 g ($F(5,18) = 9.518$, $p = 0.237$ vs normal), and 0.29 ± 0.05 g ($F(5,18) = 9.518$, $p = 0.194$ vs normal), 0.21 ± 0.04 g ($F(5,18) = 9.518$, $p = 0.056$ vs normal), and 0.14 ± 0.03 g ($F(5,18) = 9.518$, $p = 0.035$ vs normal), respectively.

Additionally, rolipram inhibited the frequency of LM strips in a concentration-dependent manner. The average contractile frequency before adding rolipram was 0.33 ± 0.10/min, and it changed to 0.29 ± 0.09/min ($F(5,18) = 3.420$, $P = 1.000$ vs normal), 0.24 ± 0.07/min ($F(5,18) = 3.420$, $p = 0.660$ vs normal), and 0.20 ± 0.05/min ($F(5,18) = 3.420$, $p = 0.376$ vs normal), 0.12 ± 0.04/min ($F(5,18) = 3.420$, $p = 0.206$ vs normal), 0.04 ± 0.03/min ($F(5,18) = 3.420$, $p = 0.014$ vs normal), respectively (Figure 3E), with different concentrations at 10 μM, 30 μM, 100 μM, 300 μM, and 600 μM. However, there was no significant difference in contractile frequency of CM strips (Figure 3F).

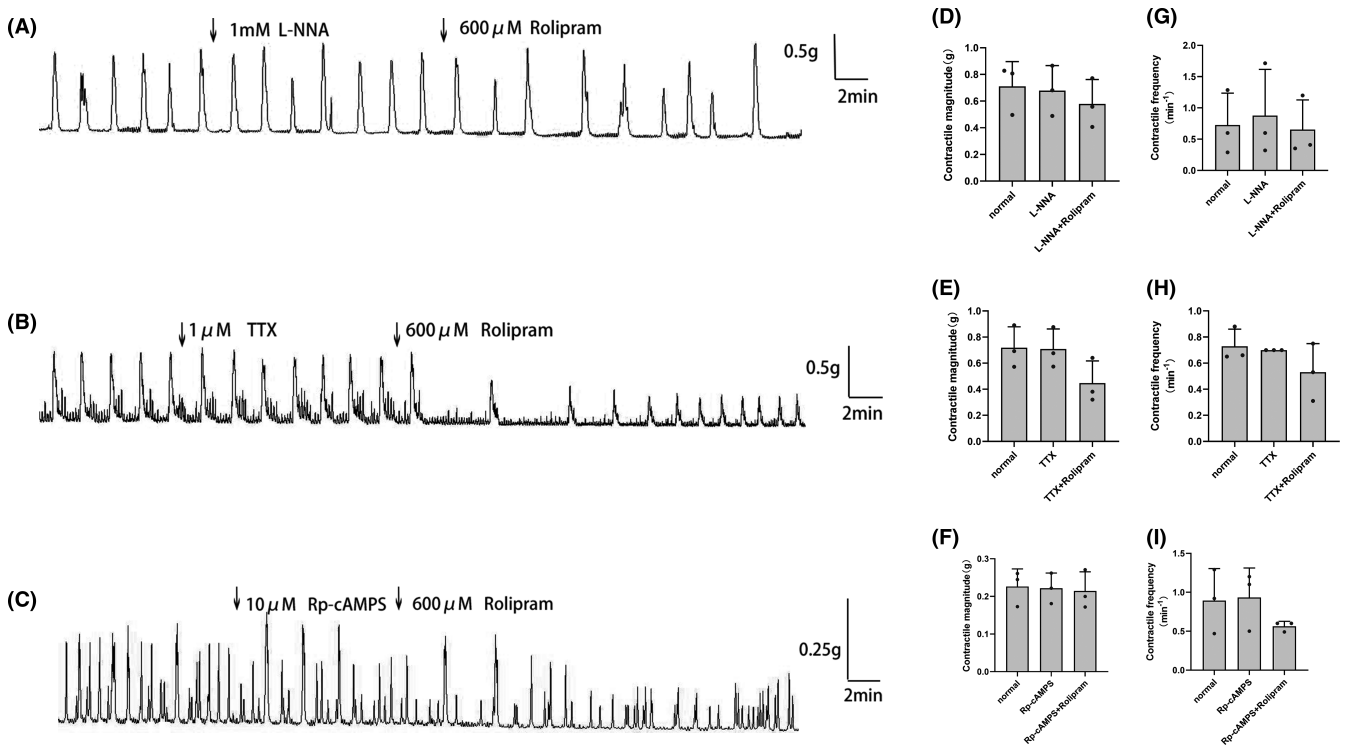

FIGURE 5 Effects of rolipram on colonic CM strips in the presence of L-NNA, TTX, or Rp-cAMPS. The representative image showing spontaneous contractions of colonic CM strips in vitro induced by 600μM rolipram in the presence of L-NNA(A), TTX(B), or Rp-cAMPS(C). Bar graph showed that summarized results of contractile amplitude of CM in the presence of L-NNA(D), TTX(E), or Rp-cAMPS(F). Bar graph showed that summarized results of contractile frequency of CM in the presence of L-NNA(G), TTX(H), or Rp-cAMPS(I). Data are expressed as Mean ± SD and analyzed by one-way ANOVA; N = 3 or 4 /group

3.4 | 4 Effects of L-NNA, TTX, and Rp-cAMPS on the spontaneous contractile activities of colonic muscle strips

To investigate the possible mechanism of the inhibitory effect of rolipram on smooth muscle contraction, muscle strips were incubated with 1 mM L-NNA, 1 μM TTX, and 10 μM Rp-cAMPS for 10 min. The results for this part were analyzed one-way ANOVA.

As shown in Figure 4A-C, we observed that the inhibitory effect of rolipram on LM strips was reversed by the L-NNA, TTX, and Rp-cAMPS treatment. Figure 4D-F showed that L-NNA, TTX, and Rp-cAMPS reversed the inhibitory effect on amplitude of LM strips by rolipram. The baseline of contractile amplitude in LM strips was 0.42 ± 0.34 g, 0.40 ± 0.25 g, and 0.32 ± 0.17 g, respectively, before L-NNA, TTX, and Rp-cAMPS treatment, after incubation with L-NNA, TTX, and Rp-cAMPS, the amplitudes of LM strips were 0.41 ± 0.32 g ($F(2,6) = 0.121$, $P = 1.000$ VS normal), 0.41 ± 0.25g ($F(2,6) = 0.029$, $P = 1.000$ VS normal), 0.33 ± 0.19g ($F(2,6) = 0.35$, $P = 1.000$ VS normal). When 600 μM rolipram was added, the amplitudes of LM strips were changed to 0.31 ± 0.25 g ($F(2,6) = 0.121$, $P = 1.000$ VS normal), 0.37 ± 0.24 g ($F(2,6) = 0.029$, $P = 1.000$ VS normal), 0.23 ± 0.11 g ($F(2,6) = 0.35$, $P = 1.000$ VS normal), respectively.

Similarly, we observed that the inhibitory effect of rolipram on CM strips was reversed by the L-NNA, TTX, and Rp-cAMPS treatment in Figure 5A-C. Figure 5D-F showed that L-NNA, TTX, and Rp-cAMPS reversed the inhibitory effect on amplitude of CM strips by rolipram. The baseline of contractile amplitude in CM strips was 0.71 ± 0.19 g, 0.72 ± 0.16 g, and 0.23 ± 0.05 g, respectively, before L-NNA, TTX, and Rp-cAMPS treatment, after incubation with L-NNA, TTX, and Rp-cAMPS, the amplitudes of CM strips were 0.68 ± 0.19 g ($F(2,6) = 0.412$, $P = 1.000$ VS normal), 0.71 ± 0.15 g ($F(2,6) = 0.051$, $P = 1.000$ VS normal), and 0.22 ± 0.04g ($F(2,6) = 0.412$, $P = 1.000$ VS normal). When 600μM rolipram was added, the amplitudes of CM strips were changed to 0.58 ± 0.19g ($F(2,6) = 0.412$, $P = 1.000$ VS normal), 0.45 ± 0.17 g ($F(2,6) = 2.722$, $p = 0.257$ VS normal), 0.21 ± 0.05 g ($F(2,6) = 0.051$, $P = 1.000$ VS normal), respectively.

Besides, Figure 4G-I showed that L-NNA, TTX, and Rp-cAMPS reversed the inhibitory effect of rolipram on frequency of LM strips. The baseline of contractile frequency in LM strips was 0.31 ± 0.19/min, 0.42 ± 0.27/min, and 0.40 ± 0.06/min, respectively, before L-NNA, TTX, and Rp-cAMPS treatment, after incubation with L-NNA, TTX, and Rp-cAMPS, the frequency of LM strips was 0.33 ± 0.15/min ($F(2,6) = 0.061$, $P = 1.000$ VS normal), 0.30 ± 0.10/min ($F(2,6) = 0.099$, $P = 1.000$ VS normal), and 0.4 ± 0.00/min ($F(2,6) = 4.754$, $p = 0.994$ VS normal). When 600 μM rolipram was added, the frequency of LM strips was changed to 0.28 ± 0.18/min ($F(2,6) = 0.061$, $P = 1.000$ VS normal), 0.20 ± 0.07/min ($F(2,6) = 0.099$, $p = 0.523$ VS normal), and 0.24 ± 0.11/min ($F(2,6) = 4.754$, $p = 0.115$ VS normal), respectively. In Figure 5G-I,

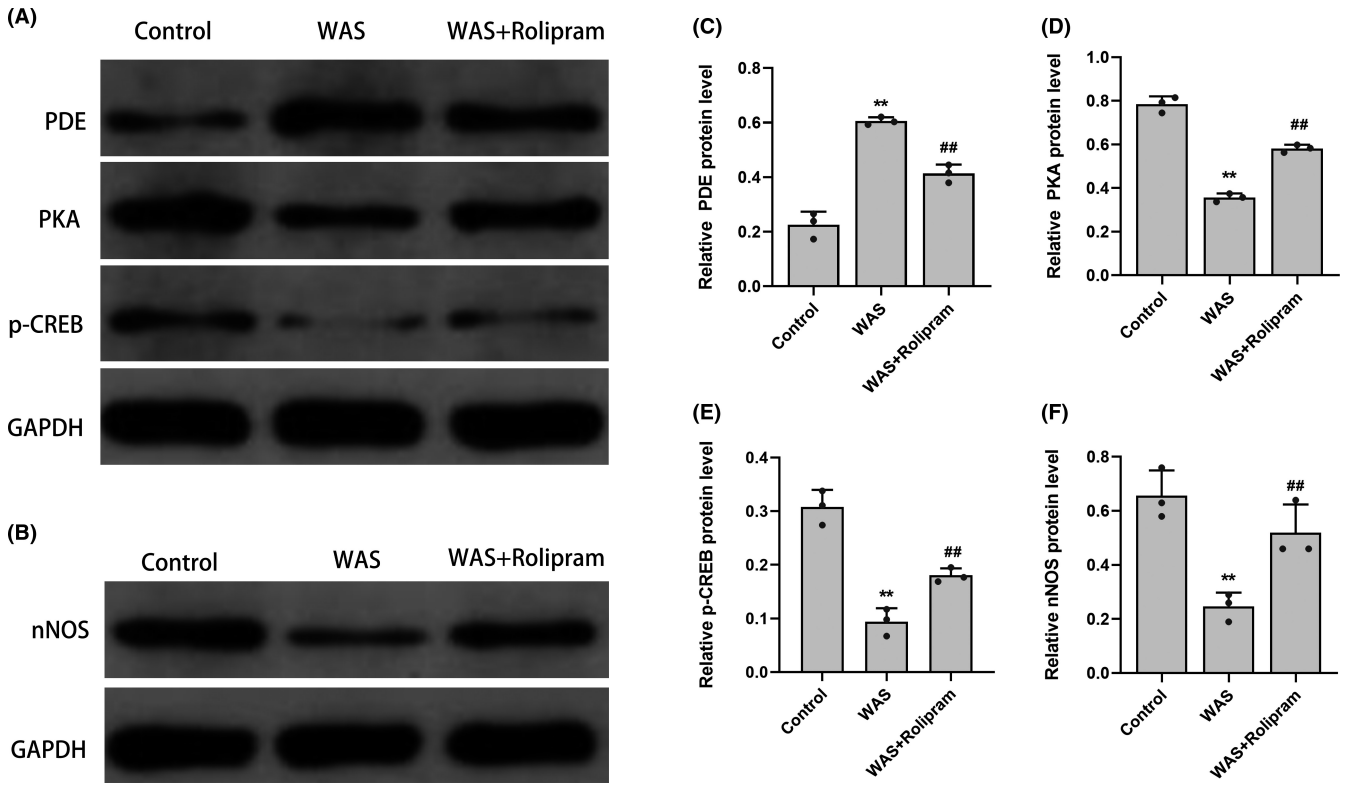

FIGURE 6 Protein expression of PDE, PKA/p-CREB, and nNOS in the colon. Expression levels of PDE, PKA/p-CREB, and nNOS (A and B) protein were detected by Western blot. Summarized results (C, D, E, F) show that the effect of WAS and rolipram on the protein expression level in the proximal colon. Data are expressed as Mean±SD and analyzed by one-way ANOVA; $^{\#\#}p < 0.01$ vs WAS; $^{**}p < 0.01$ vs Control; N = 3/group

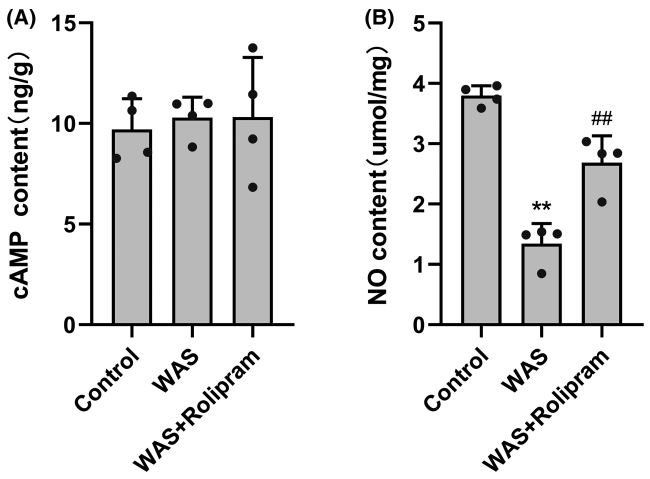

FIGURE 7 Effect of rolipram on production of cAMP (A) and NO (B) in the colon.
Data are expressed as Mean ± SD and analyzed by one-way ANOVA; $^{**}p < 0.01$ vs Control; $^{\#\#}p < 0.01$ vs WAS; N = 4/group

there was no significant change in the contractile frequency of smooth muscle for CM strips after 1 mM L-NNA, 1 μM TTX, and 10 μM Rp-cAMPS incubation.

3.5 | 5 The protein expression of PDE, PKA/p-CREB, and nNOS in the colon

We next investigated whether rolipram alters the cAMP and nNOS signaling induced by WAS exposure. Western blot results showed the changed protein levels in the colon after 10 days of WAS exposure (Figure 6A,B). In Figure 6C, the protein level of PDE in WAS rats was higher than that in Control rats (WAS: 0.61 ± 0.01 vs Control: 0.23 ± 0.05, F(2,6) = 91.347, $p = 0.000$). While WAS significantly decreased the protein expression of PKA (WAS: 0.36 ± 0.02 vs Control: 0.78 ± 0.04, F(2,6) = 211.444, $p = 0.000$), p-CREB (WAS: 0.10 ± 0.03 vs Control: 0.31 ± 0.03, F(2,6) = 56.307, $p = 0.000$), and nNOS (WAS: 0.25 ± 0.05 vs Control: 0.66 ± 0.09, F(2,6) = 17.775, $p = 0.003$), as shown in Figure 6D–F. With the pretreatment of rolipram (5mg/kg) intraperitoneally during session, we observed that protein levels of PKA (WAS + Rolipram: 0.58 ± 0.02 vs WAS: 0.36 ± 0.02, F(2,6) = 211.444, $p = 0.000$), p-CREB (WAS + Rolipram: 0.18 ± 0.01 vs WAS: 0.10 ± 0.03, F(2,6) = 56.307, $p = 0.016$) and nNOS (WAS + Rolipram: 0.52 ± 0.10 vs WAS: 0.25 ± 0.05, F(2,6) = 17.775, $p = 0.024$) were significantly increased and level of PDE was reduced in WAS + Rolipram group (WAS + Rolipram: 0.41 ± 0.03 vs WAS: 0.61 ± 0.01, F(2,6) = 91.347, $p = 0.001$).

3.6 | cAMP and NO content in the colon

In Figure 7A, cAMP content in the colon did not differ significantly among Control, WAS, and WAS + Rolipram groups. Figure 7B showed that NO content in the colon of WAS-exposed rats is considerably decreased compared with Control (WAS: 1.35 ± 0.33μmol/mg vs Control: 3.8 ± 0.17μmol/mg, $F(2,9) = 53.87$, $p = 0.000$). Notably, the reduced NO content in WAS rats is elevated after treatment with rolipram (WAS + Rolipram: 2.69 ± 0.44μmol/mg vs WAS: 1.35 ± 0.33μmol/mg, $F(2,9) = 53.87$, $p = 0.001$).

4 | DISCUSSION

Although PDE inhibitors are a class of agents acting on specific phosphodiesterase enzymes in target cells,[26] an increasing number of studies have shown that PDE inhibitors play important roles in regulating gastrointestinal motor function including increasing gastric volumes, delaying gastric emptying, suppressing gastric fundus contractility, inhibiting small bowel motility, and affecting intestinal transit.[14,16-19] These data suggested that the evidence of PDE4 on intestinal motility has not been fully elucidated. Moreover, the mechanism of PDE4 regulation of intestinal smooth muscle contractility under chronic stress has not been fully explored. Our results demonstrated that administration of rolipram reversed the WAS-induced hypermotility in the rat colon. These data provide the first evidence of the effects of rolipram on IBS in vivo and in vitro using a rat model of IBS.

The IBS-D rat model induced by WAS is characterized by increased defecation and increased spontaneous contraction of smooth muscle strips. Here, we observed that WAS increased the fecal output and colonic motility, indicating stress-induced hyperactivity was successfully constructed in our study. The previous study showed PDE4 inhibitors rolipram and roflumilast significantly reduced the stress-induced fecal output.[27] In one study, PDE5 inhibitor tadalafil reduced intestinal transit time and increased fecal pellets and fecal water content in IBS-C rat model which was characterized by increased intestinal transit time, with reduced fecal pellets and fecal water content.[28] In our work, we proved that application of PDE4 inhibitor rolipram to IBS-D rats undergoing stress alleviated the colonic hypermotility in vitro and reduced defecation in vivo.

Several lines of studies indicated that altered expression of PDE4 protein could be related to stress,[29] neurological disorder,[30] and heart failure.[31,32] However, only a few studies reported the altered expression of PDE4 protein in the gastrointestinal tract.[13] In our animal model, we detected changes in the expression of PDE4 in the rat colon of the three groups, which has not been explored before, indicating that PDE4 play a role in stress-induced colonic dysmotility. PDE4 is the main family of PDE enzymes expressed in immune cells and inflammatory cells,[5] and IBS patients are reported to have mild immune activation,[33,34] while chronic stress can lead to the imbalance of immune response,[35,36] perhaps this is why the expression of PDE4D protein has been downregulated after repeated stress.

As we know, intracellular Ca^{2+} controls the contraction and relaxation of smooth muscle of the gut.[37] In addition, the effect of PDE4 on calcium ion channel has been reported in a study, showing that PDE4 inhibitor roflumilast could deflect Ca^{2+} concentration-response curves (CRCs) to the right with suppression of the maximum peak similar to verapamil, a Ca^{2+} channel blocker.[18] In our work, we demonstrated that PDE4 inhibitor rolipram decreased the amplitude of spontaneous contractions of colonic smooth muscle strips in a concentration-dependent manner (10μM-600μM). Our observations appear to be consistent with those two studies, showing that PDE inhibitors could block a carbachol-induced contraction of isolated circular colonic muscle strips, with rolipram the most potent inhibitory effect.[21] Besides, PDE inhibitors could produce a concentration-dependent attenuated response to antigen-induced colonic contraction in guinea pigs (1μM–100μM).[20]

Colonic smooth muscle contains a variety of PDE isozymes, and the selective inhibition of PDE isozymes can increase cyclic nucleotide content and thereby antagonize smooth muscle contraction.[21] Intracellular cAMP acts as second messengers between cells by stimulating the actions of many hormones, neurotransmitters, and other cellular effectors.[38] The intracellular concentration of these second messengers is determined by a balance between their synthesis and metabolism. PDE4 is responsible for the breakdown of cAMP and regulates the cellular concentration of cAMP, clearly demonstrating a broad, critical role of PDE4 in cellular and physiological functions.[39] Previously, it has been reported that inhibition of PDE4 can lead to increased intracellular cAMP levels, thereby initiating various signaling pathways, for example, rolipram-mediated cAMP signaling effectively alleviated stress-induced depressive responses in mice in a chronic mild stress test.[40] Besides, intracellular cyclic GMP levels are also regulated by a balance between its rate of synthesis and hydrolysis by PDE5.[41] PDE5 inhibitors sildenafil may induce smooth muscle relaxation by inhibiting cGMP degradation.[42,43] In our study, we tried to explore whether cAMP signaling involved was in PDE4 mediated regulation of intestinal motility in stress-induced IBS-D.

We observed the relaxant effect of rolipram was also significantly reduced by Rp-cAMPS, which has been used as an antagonist of cAMP to block the cAMP-PKA signal pathway,[44] indicating that the effect was mediated by cAMP release. However, our research found that there was no significant difference in the content of cAMP in the rat colon. Although changes of PDE4 protein levels and the expression of downstream signaling proteins PKA and p-CREB in colonic tissues were determined using Western blotting, other compensatory mechanisms typically alter downstream cAMP signaling effectors or alter cAMP production. Our research seems to be contradictory from previous studies, showing that the PDE inhibitors inhibited smooth muscle contraction by increasing cAMP levels.[18,19] The discrepancy among studies may be related to used species (mice, humans, and rats), different drug concentrations, and experimental conditions.

Nitric oxide (NO) is a major inhibitory neurotransmitter that mediates nonadrenergic noncholinergic (NANC) signaling, which plays a negative regulatory role in gastrointestinal motility.[45] It is

reported that altered nNOS-mediated endogenous NO levels in the gut were related to IBS in an animal models.[46] Kato et al[22] found that sildenafil could prevent indomethacin-induced small intestinal hypermotility, and the mechanism of this action was related to endogenous NO. Indeed, there were several studies showing that the effects of PDE inhibitor involved the NO pathway. For instance, in a randomized controlled trial, sildenafil treatment improved vascular endothelial function in patients with cystic fibrosis by increasing NOS3 phosphorylation.[47] PDE1 or PDE5 inhibition could enhance the NO-dependent hypoxic vasoconstriction of coronary artery.[48] Upregulation of PDE5 expression failed to reverse the depletion of neuronal NO and to impaired nNOS activity under sustained high blood pressure.[49] However, researchers have not yet determined whether NO is involved in rolipram-induced colonic relaxation in an IBS model. Our work proved that the relaxant effect of rolipram on colon was also significantly reduced by L-NNA, an inhibitor of NO synthesis, indicating that the effect was mediated by neural NO release. Similarly, the Griess test revealed that NO content in the colon of WAS rats was significantly increased after treatment with rolipram. The altered protein levels were further confirmed that PDE inhibited colonic smooth muscle contraction through the NO pathway.

In addition, rolipram-induced relaxation was significantly abolished by TTX, a blocker of neuronal voltage-dependent Na^+ channels, indicating that neurons within the intramural plexuses are responsible for the action of rolipram. It has been reported that inhibitory effect of PDE4 on gastric emptying may partly be achieved through the autonomic nervous system.[14] What is more, inhibition of PDE4 (but not PDE1, PDE3, or PDE5) produced a depression of neural transmission within the enteric nervous system.[50]

5 | CONCLUSION

Our research proved for the first time that the administration PDE4 inhibitor rolipram could restore chronic stress-induced colonic dysfunction. A reduction in fecal pellets and in colonic motility was observed with rolipram in an IBS-D rat model. The downstream pathway of PDE is complex and shows significant differences among different tissues. Our data suggested the involvement of cAMP signaling pathway and NO signaling pathway. However, more research is warranted to elucidate the exact relationship of gut dysmotility and PDE inhibitors.

6 | COMPETING INTERESTS

The authors have no competing interests.

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AUTHOR CONTRIBUTION

FangTing Yuan and HaiXia Ren conceived and designed the experiments. FangTing Yuan conducted the experiments and wrote the paper. HaiXia Ren and Wei Tan analyzed the data; Ying Wang contributed essential reagents or tools; HeSheng Luo revised of the manuscript and approved the final version to be published.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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