




# 7,8-Dihydroxyflavone Enhanced Colonic Cholinergic Contraction and Relieved Loperamide-Induced Constipation in Rats

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## Abstract

**Background** Whether 7,8-dihydroxyflavone (7,8-DHF), a tyrosine kinase receptor B (TrkB) agonist, modulates colonic smooth muscle motility and/or alleviates constipation has not yet been studied.

**Aims** Here, we aimed to determine how 7,8-DHF influences carbachol (CCh)-stimulated contraction of colonic strips and the in vivo effect of 7,8-DHF on constipation.

**Methods** Muscle strips were isolated from rat colons for recording contractile tension and performing western blotting. Constipation was induced in rats with loperamide.

**Results** Although it specifically activated TrkB, 7,8-DHF applied alone neither activated PLC $\gamma$ 1 in the colonic strips nor induced colonic strip contraction. However, 7,8-DHF enhanced CCh-stimulated PLC $\gamma$ 1 activation and strip contraction. The PLC $\gamma$ 1 antagonist U73122 suppressed both CCh-stimulated and 7,8-DHF-enhanced/CCh-stimulated contraction. While clarifying the underlying mechanism, we revealed that 7,8-DHF augmented muscarinic M3 receptor expression in the colonic strips. The M3-selective antagonist tarafenacin specifically inhibited the 7,8-DHF-enhanced/CCh-stimulated contraction of the colonic strips. Since 7,8-DHF increased Akt phosphorylation, and LY294002 (an antagonist of PI3K upstream of Akt) dramatically inhibited both 7,8-DHF-augmented M3 expression and 7,8-DHF-enhanced/CCh-stimulated contractions, we assumed that 7,8-DHF/TrkB/Akt was associated with the modulation of M3 expression in the colonic strips. ANA-12, a specific TrkB antagonist, not only inhibited TrkB activation by 7,8-DHF but also suppressed 7,8-DHF-enhanced cholinergic contraction, 7,8-DHF/CCh-mediated activation of PLC $\gamma$ 1/Akt, and M3 overexpression in colonic strips. In vivo 7,8-DHF, also by promoting intestinal motility and M3 expression, significantly alleviated loperamide-induced functional constipation in rats.

**Conclusions** Our results suggest that 7,8-DHF regulates colonic motility possibly via a TrkB/Akt/M3 pathway and may be applicable for alleviating constipation.

**Keywords** 7,8-dihydroxyflavone · Colonic strip · Colonic motility · Muscarinic M3 receptor · Constipation

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## Introduction

Functional constipation is a common abnormality of humans caused by the dysfunction of colonic dynamics [1]. Low colonic dynamics usually constitute an initial step in the development of constipation. Therefore, medicine that increases colonic motility is clinically beneficial for constipation treatment. However, therapeutics with satisfactory effects on the abnormalities remain limited.

Brain-derived neurotrophic factor (BDNF) is a natural agonist for tyrosine kinase receptor B (TrkB). BDNF was initially discovered in the nervous system, and when TrkB was activated by BDNF, three intracellular signaling factors were subsequently activated: PLC (phospholipase C), ERK1/2 (Ras/extracellular signal regulated kinase 1 and 2), and PI3K (phosphatidylinositol-3-kinase)/Akt [2]. The signals cause a functional increase in the intracellular  $\text{Ca}^{2+}$  concentration and promote excitatory transduction at synapses and neuromuscular junctions [3]. Some studies have reported that TrkB and BDNF exist in the digestive system as well and that they enhance the gut motility of animals with slow transit constipation (STC) and peristalsis of the intestinal tract in humans [4,5]. In this study, we speculated that BDNF/TrkB in colonic tracts plays a role in promoting colonic motility.

A synthetic TrkB agonist, 7,8-dihydroxyflavone (7,8-DHF) is a flavonoid that is widely distributed in many plants and plays various important roles in neuroprotection and antioxidation; has anti-diabetes, anticancer, anti-hypertension, pro-gastrointestinal dynamics, and anti-cardiotoxicity effects; and ameliorates intestinal ischemia–reperfusion injury, etc. [4–13]. It was the first drug found to have biological functions similar to those of BDNF [14,15]. Similarly, the interaction of 7,8-DHF with TrkB in the neural system activates PLC $\gamma$ 1, Akt, and ERK1/2 signaling pathways that function in neural protection and lead to behavioral changes [14,15]. Since TrkB was found in colonic tracts, it has been assumed that 7,8-DHF is probably able to activate TrkB in colonic smooth muscles, elevate intracellular  $\text{Ca}^{2+}$  concentration, and enhance colonic dynamics. It was reported that BDNF can promote cholinergic intestinal motility [4,5]. However, 7,8-DHF and BDNF seemingly play conflicting roles in the intestine. Al-Qudah et al. stated that both BDNF and 7,8-DHF increased the carbachol (CCh)-stimulated contraction of rabbit intestines, but the molecular mechanism was not identified [16]. In contrast, Chen et al. reported that exogenously applied BDNF did not alter the cholinergic contraction in murine intestines [17].

Based on the fact that 7,8-DHF specifically activates TrkB and its downstream targets PLC $\gamma$ 1, PI3K/Akt, and ERK1/2 [14,15], we recently revealed that 7,8-DHF

enhances rat gastric cholinergic contraction [12]. Here, we hypothesized that 7,8-DHF might exhibit a similar enhancement effect in colonic cholinergic contraction and aimed to discover its alleviating effect in a rat constipation model. Through the use of selective antagonists of these signaling molecules and contractile tension measurements of ex vivo rat colonic strips, we not only observed the effect of in vitro 7,8-DHF on rat colonic motility but also identified its underlying molecular mechanism and assessed the in vivo effect on the STC of a rat model.

## Methods

### Chemicals

Carbachol and loperamide were purchased from Sigma, St. Louis, MO, USA; 7,8-dihydroxyflavone was obtained from TCI laboratories, Tokyo, Japan; ANA-12 was acquired from Fisher Scientific, Hampton, NH, USA; PD98059, U73122, and LY294002 were obtained from Enzo Life Sciences, Farmingdale, NY, USA; anti-GAPDH, anti-TrkB, anti-ERK1/2, anti-p-PLC $\gamma$ 1 (Y783), and anti-PLC $\gamma$ 1 antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti-p-TrkB (Y516), anti-p-Akt (S473), anti-Akt, and anti-p-ERK1/2 (T202/Y204) antibodies were obtained from Cell Signaling, Boston, USA; anti-M2 and anti-M3 antibodies were obtained from Abcam, Cambridge, UK. VU0255035 and tarafenacin from APEX BIO Technology, Boston, USA; 3-quinuclidinyl benzilate from BOC Sciences, New York, USA.

### Animals

Male rats ( $300 \pm 20$  g,  $n = 238$ ) were provided by the Center for Experimental Animals, Institute of Drug Examination, Qingdao, China. Typically, one rat was sacrificed to obtain 4 colonic strips each day. At the end of the daily experiments, the strips were frozen in a  $-80^\circ\text{C}$  freezer for use in western blotting. Eight to ten rats were used for each experimental group in all figures.

In the experiments, the animals were treated following the Guide for the Care and Use of Laboratory Animals (the National Institutes of Health, United States). The Ethics Committee of the Affiliated Hospital, Qingdao University, approved the protocol for animal treatment in the experiments.

### Isolation of Colonic Muscular Strips

Fasted 12 h before experiments but with water freely accessible, the rats were anesthetized with 10% thiobutabarbital (100 mg/kg body weight, i.p.). An abdominal incision

was made through which a segment of colon (2 cm long and ~5 cm from the ileocecal junction) was rapidly removed and immersed in Krebs solution (in mM, 4.75 KCl, 118 NaCl, 1.19 KH<sub>2</sub>PO<sub>4</sub>, 2.54 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 0.5 EDTA-Na<sub>2</sub>, 11 glucose, pH 7.4) at 37 °C. A mix of 5% CO<sub>2</sub>/95% O<sub>2</sub> was continuously bubbled through the solution. In solution, the colon segment was longitudinally cut, and the luminal content was cleared. Under a dissecting microscope, colonic mucosa and submucosa were removed gently. Longitudinal muscular strips (~10 mm × ~5 mm) were prepared with one end of surgery silk thread tied to the end of a strip and the other end tied to a chamber that contained Krebs solution (6 ml), where they remained hung at 37 °C and with mixed 5% CO<sub>2</sub>/95% O<sub>2</sub> constantly bubbling through the solution. The strips were subjected to 0.5 g tension (basal tension) and rinsed twice with 37 °C Krebs for 15 min each time to stabilize spontaneous contraction of strips. Before the application or substitution of a drug, the strips were washed with Krebs three times for ~10 min each time [12,18].

### Recording Colonic Strip Tension

The tension recording apparatus (Chengdu Instrument and Equipment Factory, Chengdu, China) consisted of a four-chamber system for organ perfusion, multiple channels for physiological signal collection, and tension signal collecting software (RM6240 series). The strips were treated consecutively with 10<sup>-2</sup>, 10<sup>-1</sup>, 1, 10, 20, 100, and 1000 μM CCh or with 10<sup>-1</sup>, 1, 10, 30, and 100 μM 7,8-DHF. The concentration of these drugs was cumulatively increased. Generally, the strip contraction to reach maximum tension within 3 min, recorded as raw data for analysis. The tension unit was in grams (g). Whenever the CCh drug concentrations were changed, the strips were washed with Krebs to ensure its return to the basal tension, which was used as the control for the CCh experiments. The control tension for the experiments used to test the effects of 7,8-DHF or antagonists was the contraction obtained with 10 μM CCh stimulation [12]. Each strip served as its own control when the contraction amplitudes were compared between treatment conditions and controls.

To confirm the appropriate length of 7,8-DHF treatment for modulating the strip contraction induced by CCh, we evaluated the lengths after various duration times of 7,8-DHF incubation. Considering 7,8-DHF efficiency and the time need for a strip to recover to the basal tension level, we ultimately decided that a 30 min incubation was optimal for allowing 7,8-DHF effects. Therefore, the following experimental procedure was used: CCh tension was used as a control when a strip was stabilized and stimulated by CCh (10 μM) for 3 min. After the CCh tension was recorded, the strip was rinsed until the basal tension was recovered, and

then, the strip was incubated with serial concentrations of 7,8-DHF. For each measurement, 7,8-DHF was applied for 30 min prior to a 3 min treatment with CCh (10 μM). When contracting tension reached a plateau, the tension value was recorded as 7,8-DHF + CCh tension. All antagonists used in the study were added to chambers 15 min before the addition of 7,8-DHF. The contraction percentage was obtained based on the following calculation:

$$\text{Contraction (\%)} = \frac{\text{CCh}_{\text{tension}} \text{ or } (\text{DHF} + \text{CCh})_{\text{tension}}}{\text{CCh}_{\text{tension}}} \times 100\%.$$

### Induction of Rat Constipation with Loperamide

A rat model of functional constipation was treated with loperamide [19]. The model was generated to replicate a type of STC. Prior to STC induction, the rats were allowed to adapt to the ambient environment for one week, and then, fecal pellets were collected, counted, and weighed from ten rats for seven consecutive days. The daily average weight of the feces was calculated and taken as the basal fecal weight (gram). The basal content (%) of water in the feces was obtained as follows by calculating the wet weight and the weight of dried feces:

$$\text{Fecal water content (\%)} = \frac{(\text{wet weight} - \text{dry weight})}{\text{wet weight}} \times 100\%.$$

To induce constipation, 20 rats were injected intraperitoneally with loperamide (5 mg/kg body weight) twice a day (9:00 a.m. and 18:00 p.m.) for five days. Then, the basal fecal pellet output/90 min, fecal weight, and fecal water percentage for each rat were recorded for 3 days without stopping the loperamide treatment. When the fecal pellet output was reduced from ~3.5 to ~1.5/90 min, the fecal weight was reduced from ~7 to ~3 g, and the water content was reduced from ~50 to ~20% per rat, indicating that constipation was successfully induced with the loperamide treatment, which was then used for the subsequent experiments. The model rats were randomly allocated to two groups: the dissolvent Dimethyl sulfoxide (DMSO)/natural saline (NS) control and 7,8-DHF treatment. To observe whether 7,8-DHF alleviated STC, DMSO/NS or 7,8-DHF (1 mg/kg body weight [12,13]) was administered intragastrically to model rats every day for 7 days. During the final 3 days of 7,8-DHF treatment, the fecal weight and water content were measured.

### Rate of Intestinal Carbon Propulsion

STC model rats treated with DMSO/NS or 7,8-DHF as described above were fasted for 12 h but allowed ad libitum to drink water containing 1 mM 7,8-DHF or corresponding

volume of DMSO/NS. Then, the rats were fed an active charcoal suspension (10 mg/kg body weight) by gavage and housed in a single cage for 30 min. The active charcoal suspension (1 g/ml) was prepared with a 10% charcoal suspension including 5% gum arabic. Thirty minutes later, the rats were anesthetized with thiobutabarbital (100 mg/kg, i.p.) and the entire small intestines were obtained by surgery. The length of the whole small intestine and the small intestine stained with charcoal was measured, and the propulsion rates of charcoal in the intestine were calculated as follows [13]:

Propulsion rate of charcoal (%) = length of the small intestine with charcoal/whole length of the small intestine  $\times$  100%.

Colonic strip tissues in SDS sample buffer (Beyotime, Haimen, China) were minced completely. The minced tissues were homogenized on ice and then sonicated for 5 min. The fragmented tissue was subjected to 12,000 $\times$ g centrifugation for 30 min after 45 min of static incubation on ice. The supernatant (whole cellular proteins) was removed by pipetting to confirm the concentration of protein with a BCA protein assay kit (Pierce Biotechnology, USA). SDS-PAGE was performed to separate the proteins in the gel. The gel-separated proteins (50  $\mu$ g/lane) were electrophoretically transferred to PVDF membranes (0.45  $\mu$ m) (Millipore, USA), which were incubated overnight with the primary antibodies at 4 °C. The primary antibodies were diluted to 1:500 (anti-p-TrkB), 1:300 (anti-TrkB), 1:500 (anti-p-PLC $\gamma$ 1), 1:300 (anti-PLC $\gamma$ 1), 1:300 (anti-p-Akt), 1:300 (anti-Akt), 1:500 (anti-p-ERK1/2), 1:300 (anti-ERK1/2), 1:500 (anti-M3), or 1:1000 (anti-M1 or anti-M2). The antibody-stained membranes were washed and incubated with the secondary antibody, anti-GAPDH (1/10,000) conjugated to horseradish peroxidase, for 60 min at 25 °C.

The fluorescent signals emitted from the antibody-stained protein bands on the PVDF membrane were detected with a fusion-enhanced chemiluminescence imager (Vilber, France). The density of the protein bands was analyzed and measured by ImageJ software. The brightness values of the target protein bands were normalized to the values of GAPDH.

## Statistical Analysis

A software package named concise statistics, CS10.34, was used for the data analysis. The analyzed data are presented as the means  $\pm$  SD. To confirm the significance of the differences among groups, Student's *t* test was used to compare a difference between two groups, while one-way ANOVA was used to assess the difference when more than two groups were compared. The effect of 7,8-DHF on fecal pellet output, weight, and water content was analyzed by *t* test based on the dependent

variables with each animal serving as its own control. When the *P* value was less than 0.05, the difference was considered significant. Statistical data points were fitted to a logistic function for curves, EC<sub>50</sub>, and E<sub>max</sub>.

## Results

### CCh Stimulated the Contraction of the Colonic Strips from the Rats in a Dose-Dependent Manner

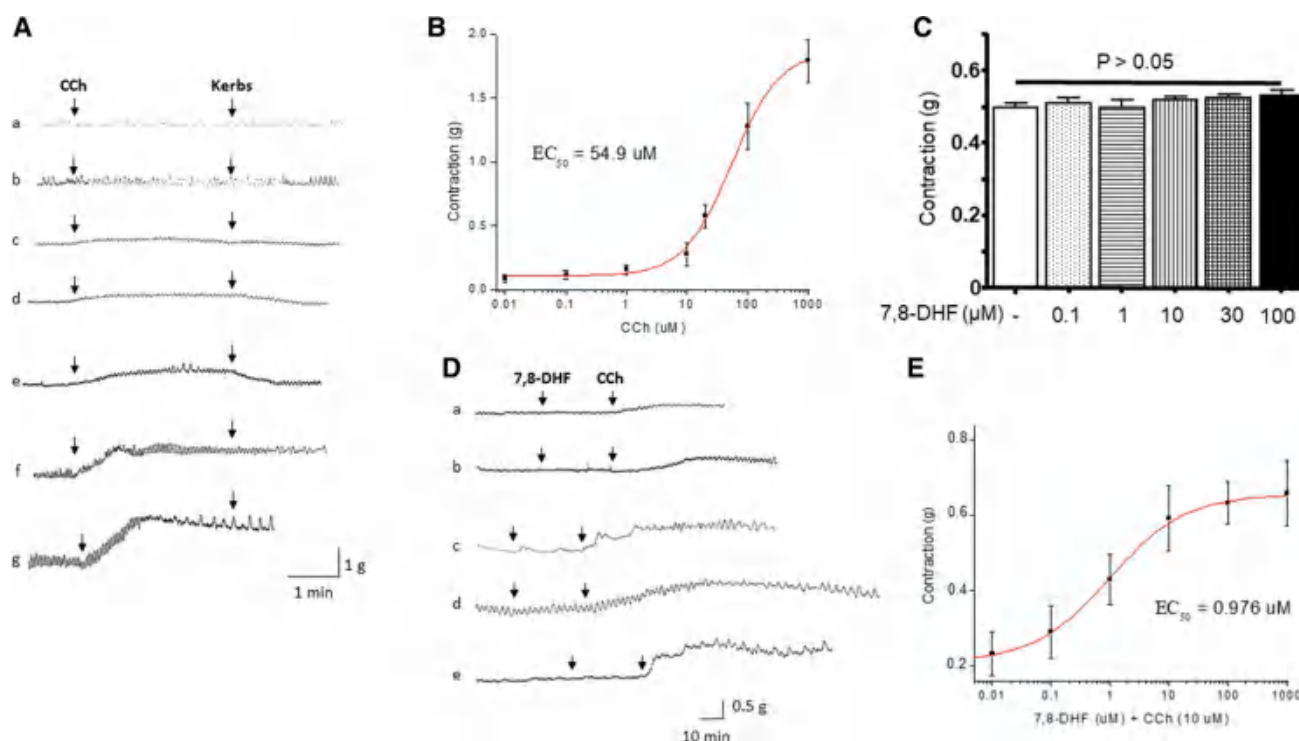
In this study, experiments were first performed with isolated rat colonic muscle strips to find an optimal CCh concentration. Figure 1a shows the contractions of one strip after treatment with various concentrations of CCh. CCh stimulated strip contraction in a dose-dependent manner at concentrations that were increased from 10<sup>-2</sup>, 10<sup>-1</sup>, 1, 10, 20, 10<sup>2</sup> to 10<sup>3</sup>  $\mu$ M. Figure 1b exhibits the statistically analyzed data based on the CCh-stimulated contractions with an EC<sub>50</sub> equal to 54.9  $\mu$ M. We noticed that when CCh concentrations were  $\geq$  20  $\mu$ M, the strip contraction did not recover to the baseline level after washing, indicating that the strips may have been damaged at high concentrations of CCh. Therefore, we selected 10  $\mu$ M as the optimal CCh concentration for use in the subsequent experiments.

### 7,8-DHF Enhanced the CCh-Stimulated Contraction of the Colonic Strips

To test whether 7,8-DHF exerted a direct influence on colonic muscle contraction, the colonic strips were exposed to only 7,8-DHF at concentrations from 0.1, 1, 10, 30, to 100  $\mu$ M. The results showed that 7,8-DHF alone did not significantly change the basal tension of the colonic strips (Fig. 1c).

Next, we tested whether CCh-stimulated colonic strip contraction may be influenced by 7,8-DHF. The muscular strips were treated with 7,8-DHF at 0.1, 1, 10, 30, and 100  $\mu$ M followed by treatment with CCh (10  $\mu$ M) to stimulate contraction. The contractile tension recorded was compared to that of the CCh-alone-stimulated tension. The latter was considered to be 100%. As shown in Fig. 1d and e, 7,8-DHF clearly enhanced CCh-stimulated muscle contraction in a dose-dependent manner with an EC<sub>50</sub> equal to 0.976  $\mu$ M. Since the CCh-stimulated contraction enhanced by 10  $\mu$ M 7,8-DHF neared the maximal level and was easily returned to the baseline level by washing, 10  $\mu$ M 7,8-DHF was selected as the optimal concentration in the subsequent experiments. DMSO was used as a 7,8-DHF dissolvent, but when it was added to the muscular strips, the CCh-stimulated contraction was not significantly changed (data not shown).





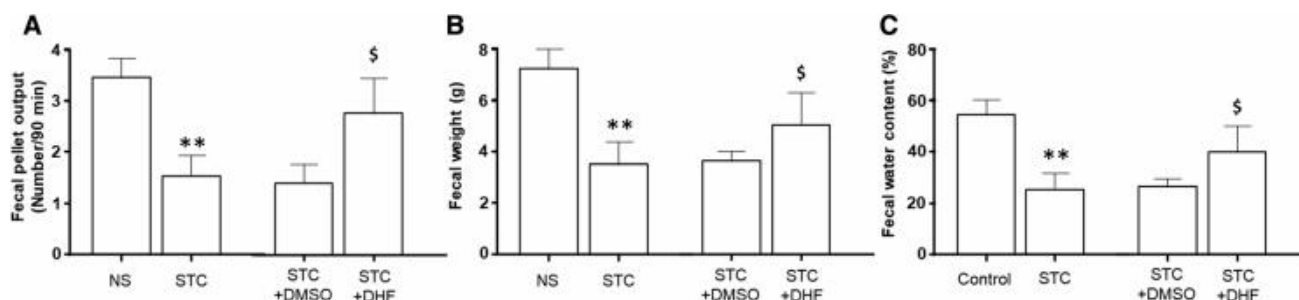
**Fig. 1** Treatment with 7,8-DHF enhanced the carbachol (CCh)-stimulated rat colonic strip contraction. Preparation of colonic strips is described in Methods section. **a** CCh-stimulated contractions of one colonic muscular strip. Contractile tension was recorded in grams (g) after one strip was stimulated with CCh (in  $\mu\text{M}$ ) at  $10^{-2}$  (a),  $10^{-1}$  (b), 1 (c), 10 (d), 20 (e),  $10^2$  (f), or  $10^3$  (g). Curves showing the contractions recorded. **b** Statistically analyzed data showing dose-dependent CCh-stimulated contractions. **c** Treatment with 7,8-DHF alone did not lead to strip contraction. Bars show the contraction of the strips

treated with 7,8-DHF at the indicated concentrations (0.1, 1, 10, 30, or 100  $\mu\text{M}$ ). **d** Contractions of the colonic strips induced by 7,8-DHF-enhanced CCh stimulation. Contraction curves are based on one strip that was treated for 30 min with 0.1 (a), 1 (b), 10 (c), 30 (d), or 100 (e)  $\mu\text{M}$  7,8-DHF followed by CCh (10  $\mu\text{M}$ ). **e** Statistically analyzed data of the 7,8-DHF-enhanced/CCh-stimulated contractions. For each group,  $n=8$  to 10 samples (B, C, and E). Curves were obtained by fitting the points to the logistic function

### Treatment with 7,8-DHF Enhanced the Defecation of the Rats with Loperamide-Induced Constipation

These results led us to hypothesize that 7,8-DHF may be able to alleviate constipation. To test this hypothesis, we induced slow transit constipation (STC) in rats with

loperamide [19]. When the model rats suffered constipation, they showed an evident reduction in fecal pellet output, fecal weight, and fecal water content (Fig. 2a–c). Oral administration of 7,8-DHF (1 mg/kg body weight), however, significantly corrected the abnormalities in fecal pellet output, fecal weight, and fecal water content



**Fig. 2** In vivo 7,8-DHF treatment alleviated slow transit constipation (STC) in rats. The rat STC model was induced with intraperitoneal injection of loperamide (see Methods section for details). Treatment with 7,8-DHF (1 mg/kg body weight) administered orally reduced

the fecal pellet output (pellets/90 min) (a), weight (g) (b), and water content (%) (c) of the STC rats. \*\* Indicates  $P < 0.01$  versus the NS control; \$  $P < 0.05$  versus STC+DMSO. For each group,  $n=8$  to 20 samples (A–C)

(Fig. 2a–c). Apparently, 7,8-DHF has the potential to relieve constipation.

### TrkB Antagonist ANA-12 Inhibited the Enhancing Effect of 7,8-DHF on the Cholinergic Contraction of the Colonic Strips

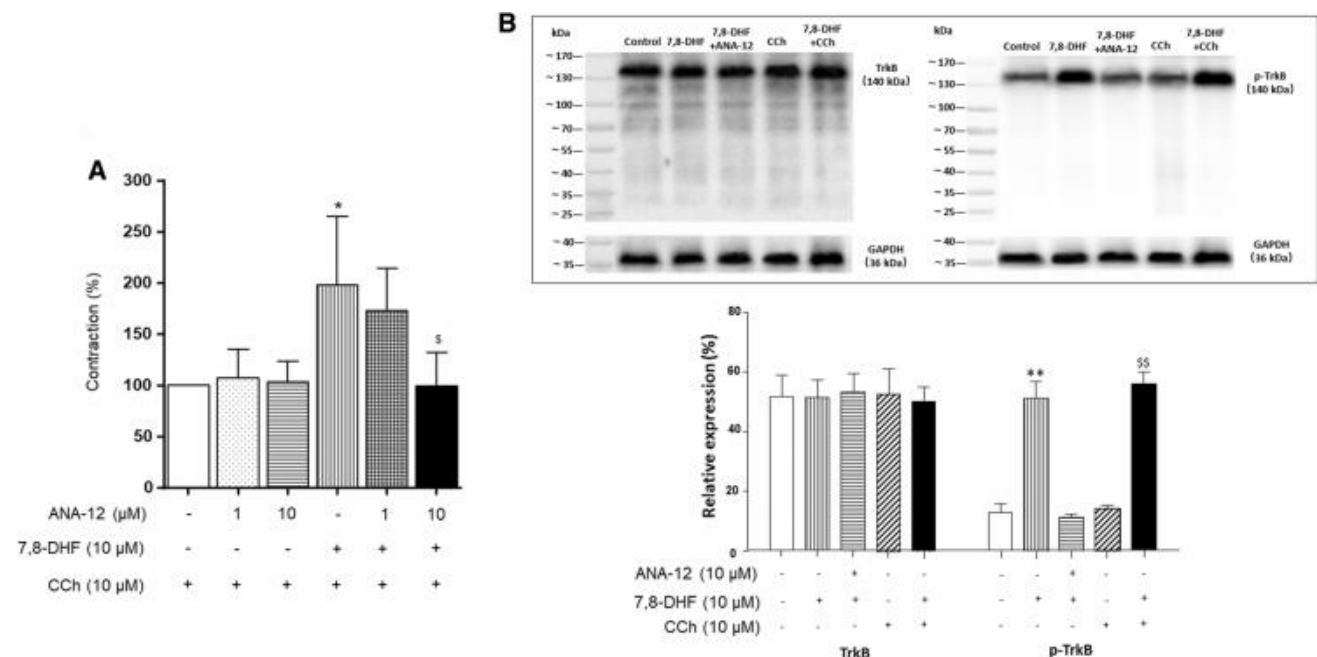
The colonic strips were treated with ANA-12, an antagonist specific for TrkB, in an attempt to study the working mechanism for 7,8-DHF. As shown in Fig. 3a, ANA-12 (10  $\mu$ M) dramatically suppressed the CCh-stimulated contraction that had been enhanced by 7,8-DHF, while at 1  $\mu$ M, it slightly suppressed this contraction, but not to a significant level. The combination of CCh (10  $\mu$ M) with ANA-12 did not affect the CCh-stimulated contraction (Fig. 3a), implying that TrkB may not participate alone in CCh-stimulated contraction. ANA-12 (10  $\mu$ M) alone did not influence the tension of the colonic strips (data not shown). The result indicates that 7,8-DHF plays an enhancing role by interacting with TrkB in the colonic muscles.

The biological activity of a receptor is usually indicated by self-phosphorylation. Therefore, the extent of TrkB

phosphorylation (p-TrkB) likely indicates the extent of its activation. According to the western blot analysis, while TrkB protein expression was not changed by CCh (10  $\mu$ M) or 7,8-DHF (10  $\mu$ M) (Fig. 3b, left), the intensity of p-TrkB was significantly increased upon treatment with 7,8-DHF or the 7,8-DHF + CCh combination, while ANA-12 (10  $\mu$ M) almost completely inhibited the activating effect of 7,8-DHF on TrkB phosphorylation (Fig. 3b, right). CCh did not significantly alter the level of p-TrkB, indicating that it did not participate in the TrkB activation. The results further supported the assumption that TrkB in the colonic strips was activated by 7,8-DHF.

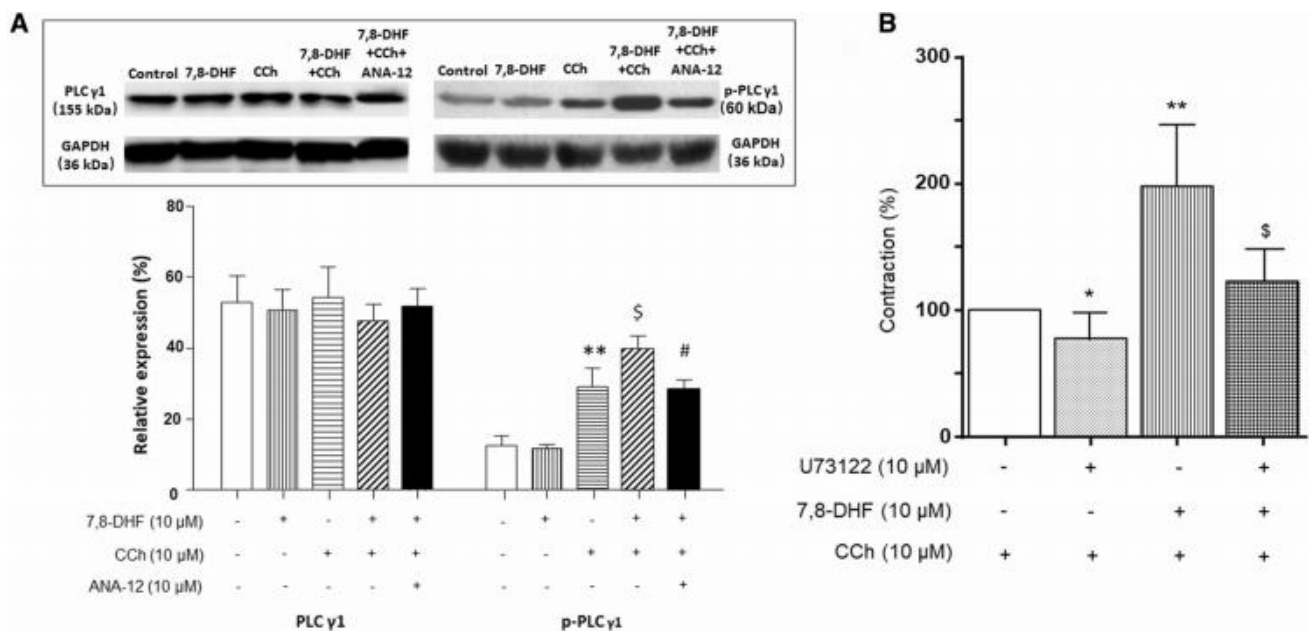
### 7,8-DHF-Enhanced/CCh-Stimulated Contraction of the Colonic Strips Was Mediated by Activated PLC $\gamma$ 1

Three cellular factors are usually induced downstream of activated TrkB. We first observed a change in PLC $\gamma$ 1 phosphorylation. PLC $\gamma$ 1 protein expression in the colonic strips was not significantly altered by CCh (10  $\mu$ M), 7,8-DHF (10  $\mu$ M) or the combined treatment (Fig. 4a, left). Treatment



**Fig. 3** Treatment with 7,8-DHF activated TrkB in the colonic strips. See Fig. 1 legend for the details of strip preparation. **a** Statistical analysis of the results of ANA-12 blocking the 7,8-DHF-enhanced/CCh-stimulated contraction of colonic strips. Strips were incubated with CCh (10  $\mu$ M) for the induction of contraction to serve as controls; after washing, the same strips were treated with ANA-12 (1 or 10  $\mu$ M) and then 7,8-DHF (10  $\mu$ M) before CCh stimulation. \* Indicates  $P < 0.05$  versus CCh alone; \$  $P < 0.05$  versus 7,8-DHF + CCh. **b** Treatment with 7,8-DHF further phosphorylated TrkB in the colonic strips. After the strips were treated with 7,8-DHF (10  $\mu$ M),

CCh (10  $\mu$ M), or ANA-12 (10  $\mu$ M), the proteins in the strips were extracted for use in western blotting (see Methods section for details). For immunoblotting, anti-TrkB (left) or anti-phosphorylated-TrkB (p-TrkB, right) antibodies were applied to stain the western blot membranes. The upper panel shows representative immunoblots. The lower panel shows the statistically analyzed relative expression (%) of the target bands with densities normalized to the corresponding GAPDH bands. \*\*Indicates  $P < 0.01$  versus the control; \$\$  $P < 0.01$  versus CCh alone. For each group,  $n = 8$  to 10 samples (A and B)



**Fig. 4** PLCγ1 was activated in the colonic strips treated with 7,8-DHF/CCh. See Fig. 1 legend for the details of strip preparation. **a** CCh but not 7,8-DHF activated PLCγ1 in the colonic strips. See Fig. 3B legend for western blotting details. For immunoblotting, anti-PLCγ1 (left) or anti-phosphorylated-PLCγ1 (p-PLCγ1, right) antibodies were applied to stain the western blot membranes. \*\* Indicates  $P < 0.01$  versus the control; \$  $P < 0.05$  versus CCh alone;

#  $P < 0.05$  versus 7,8-DHF + CCh. **b** U73122 (10 μM) blocked 7,8-DHF-enhanced/CCh-stimulated contraction of the colonic strips. See Fig. 3a legend for details of antagonist application. \* Indicates  $P < 0.05$  versus CCh alone; \*\* $P < 0.01$  versus CCh alone; \$ $P < 0.05$  versus 7,8-DHF + CCh. For each group,  $n = 8$  to 10 samples (A and B)

with 7,8-DHF alone did not significantly alter PLCγ1 phosphorylation in the strips (Fig. 4a, right). This indicated that TrkB activation did not trigger PLCγ1 phosphorylation in the colonic muscle strips, which explained why 7,8-DHF failed to stimulate their contraction (Fig. 1c). CCh activated PLCγ1 by increasing its phosphorylation, and interestingly, the treatment of CCh combined with 7,8-DHF evidently strengthened PLCγ1 phosphorylation, while ANA-12 blocked the PLCγ1 phosphorylation (Fig. 4a, right). These data suggested that CCh activated PLCγ1 and that 7,8-DHF somehow promoted the CCh-mediated PLCγ1 activation via TrkB.

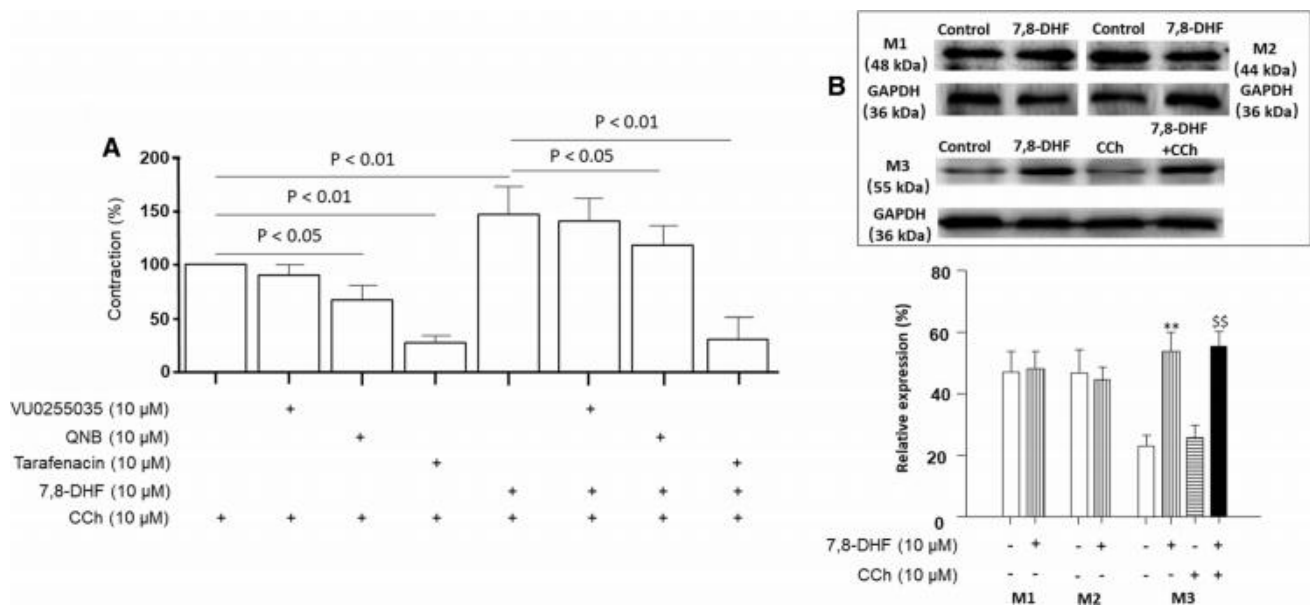
We found that U73122 (10 μM), a PLCγ1 antagonist, reduced the strip contraction stimulated by CCh or CCh combined with 7,8-DHF by ~25% and ~40%, respectively (Fig. 4b). These results suggested that PLCγ1 mediated both the CCh-stimulated and 7,8-DHF-enhanced/CCh-stimulated contraction of the colonic strips.

### Muscarinic M3 Receptor Overexpression in Colonic Muscle Was Associated with 7,8-DHF

Gq11 protein-coupled muscarinic receptors are cholinergic receptors in the gut, and their activation leads to PLCγ1 activation [20]. Since PLCγ1 activation of the strips by CCh was strengthened after the CCh and 7,8-DHF

cotreatment, we assumed that 7,8-DHF possibly indirectly acted on the Gq11 protein-coupled muscarinic receptor or PLCγ1.

To corroborate this assumption, we first focused on the muscarinic receptors M1, M2, and M3 because these three types of receptors are predominant in colonic tissues [21–23]. Since CCh is a nonselective agonist for muscarinic receptors [23], in the following experiments, we used selective antagonists of muscarinic receptors to determine which type of muscarinic receptors might be associated with the effect of 7,8-DHF. VU0255035 is an antagonist specific to the M1 receptor, which at 10 μM slightly inhibited both CCh-stimulated and 7,8-DHF-enhanced/CCh-stimulated contractions, but the difference was not statistical significance (Fig. 5a), indicating that the M1 receptor may not be among the dominant muscarinic receptors in colonic smooth muscle or may not participate in 7,8-DHF-enhanced/CCh-stimulated contraction. While 3-quinuclidinyl-benzilate (QNB, an M2 antagonist) at 10 μM inhibited CCh-stimulated contraction by ~30%, it inhibited 7,8-DHF-enhanced/CCh-stimulated contraction by only ~20%, implying that M2 was probably not involved in the 7,8-DHF effect. However, tarafenacin (an M3 antagonist) at 10 μM inhibited CCh-stimulated contraction by ~70% inhibition, while it inhibited 7,8-DHF-enhanced/CCh-stimulated contraction by ~80%. This result strongly suggested that the M3 receptor was probably a



**Fig. 5** M3 expression in the colonic strips was augmented by 7,8-DHF. See Fig. 1 legend for details of strip preparation. **a** Effect of muscarinic receptor antagonists on the 7,8-DHF-enhanced/CCh-stimulated contraction of the colonic strips. See Fig. 3a legend for details of the antagonist treatments. **b** 7,8-DHF augmented M3 recep-

tor expression in the colonic strips. See Fig. 3b legend for the western blotting details. Anti-M3 antibody was used for immunoblotting. \*\*Indicates  $P < 0.01$  versus the control;  $^{SS}P < 0.01$  versus CCh alone. For each group,  $n = 8$  to 10 samples (A and B)

factor critical for the enhancing role of 7,8-DHF in cholinergic contraction.

Then, we performed western blotting to determine whether the M1, M2, and/or M3 receptor expression was altered by the 7,8-DHF treatment. The results showed that neither M1 nor M2 expression was significantly changed by 7,8-DHF (10  $\mu$ M) (Fig. 5b). However, 7,8-DHF treatment dramatically augmented the expression of the M3 receptor in the colonic strips (Fig. 5b). The combination treatment of 7,8-DHF and CCh (10  $\mu$ M) led to a similar result, which led us to hypothesize that 7,8-DHF was able to activate some intermediating signaling factor of TrkB and that the role of this unknown factor augmented M3 expression. However, in some cases, 7,8-DHF exerted its effect via a non-TrkB pathway [11]. To confirm that 7,8-DHF played a role specifically through TrkB activation in the colonic strips, Akt was subsequently examined because Akt is also a downstream factor specifically activated by TrkB.

### Akt Activation Was Correlated with M3 Expression in the Colonic Strips

The correlation between the augmentation of M3 expression and 7,8-DHF was tested with LY294002, an antagonist of PI3K (a signaling factor upstream of Akt) and western blotting. The results showed that LY294002 (10  $\mu$ M) significantly blocked M3 expression in the colonic strips augmented by 7,8-DHF treatment (10  $\mu$ M) (Fig. 6a). ANA-12 blocked the 7,8-DHF-augmented M3 expression to the

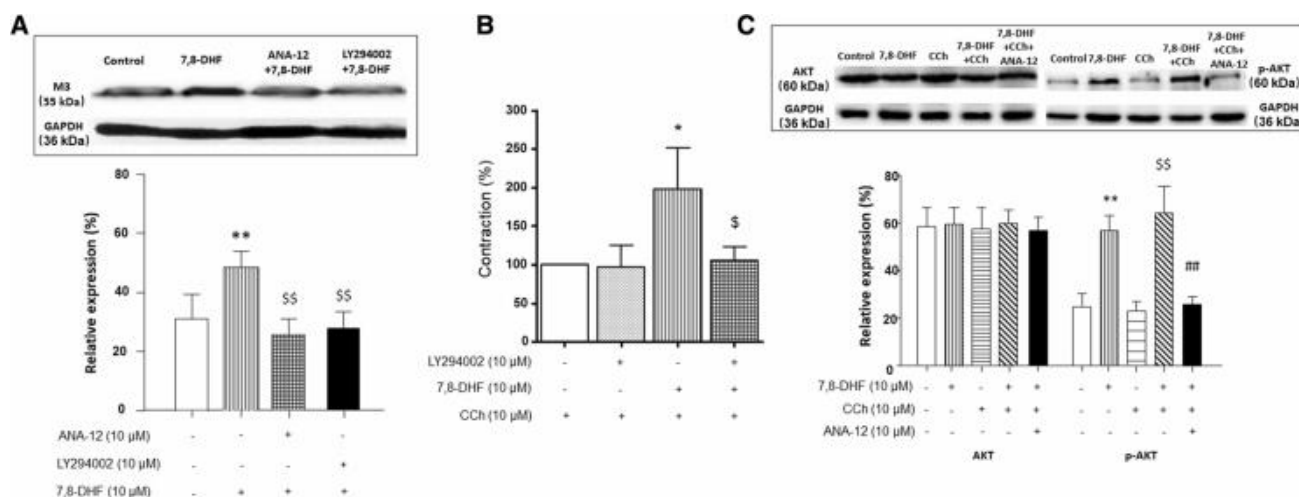
extent similar to LY294002. These further suggest that the 7,8-DHF-augmented M3 expression was probably mediated by TrkB/PI3K/Akt.

In the experiment used to measure the contraction of the colonic strips, LY294002 almost completely suppressed the cholinergic contraction enhanced by 7,8-DHF, while it negligibly suppressed the CCh-stimulated contraction (Fig. 6b). Treatment with 7,8-DHF or CCh did not affect Akt protein expression (Fig. 6c, left), while 7,8-DHF increased Akt phosphorylation (p-Akt) by more than 100% (Fig. 6c, right). Additionally, CCh alone did not increase p-Akt levels, while the combined treatment of 7,8-DHF and CCh increased the amount of p-Akt as much as 7,8-DHF did alone, and ANA-12 significantly blocked 7,8-DHF-stimulated Akt phosphorylation (Fig. 6c, right). Based on these results, we concluded that the activation of TrkB/Akt was correlated with M3 expression in the colonic strips.

### ERK1/2 Signaling Did Not Participate in the 7,8-DHF Enhancement of Cholinergic Contraction of the Colonic Strips

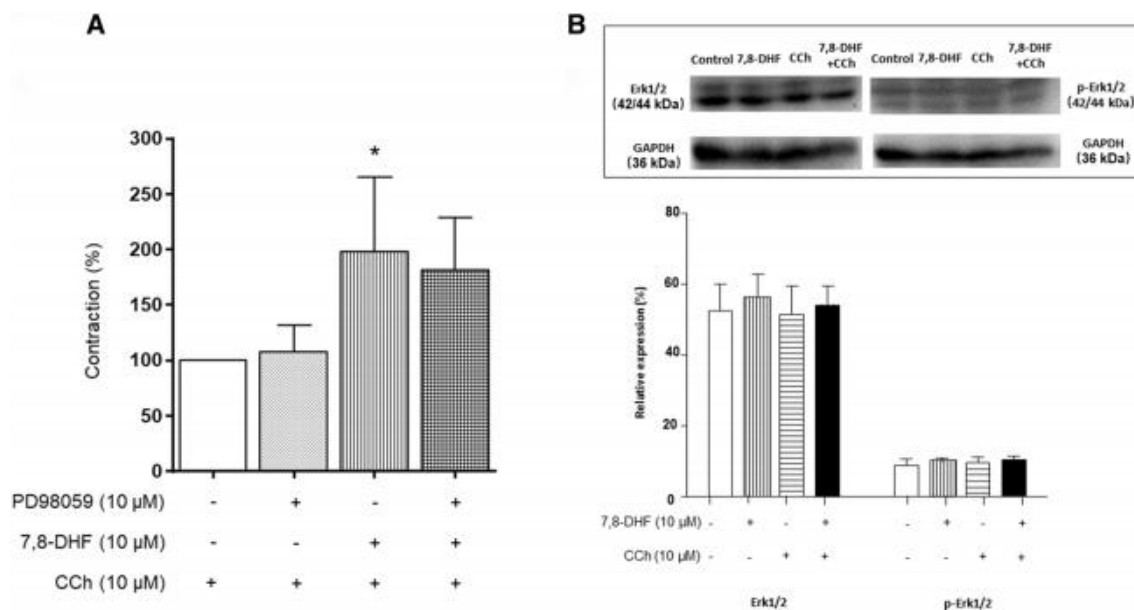
ERK1/2 were activated by TrkB [2]. The ERK1/2 antagonist PD98059 was used to determine whether ERK1/2 had a role in 7,8-DHF-enhanced cholinergic contraction. However, no effect was produced by PD98059 (10  $\mu$ M) in the CCh-stimulated or 7,8-DHF-enhanced/CCh-stimulated contraction of the colonic strips (Fig. 7a). Treatment with CCh





**Fig. 6** Akt activation was correlated with 7,8-DHF-augmented M3 expression in the colonic strips. Refer to Fig. 3 legend for details of western blotting and antagonist application. **a** Antagonist LY294002 (10 μM) suppressed the M3 expression augmented by 7,8-DHF. \*\*Indicates  $P < 0.01$  versus the control; \$\$  $P < 0.01$  versus 7,8-DHF. **b** LY294002 (10 μM) suppressed the 7,8-DHF-enhanced/CCh-stimulated contraction of the colonic strips. \*Indicates  $P < 0.05$

versus CCh; \$ $P < 0.05$  versus 7,8-DHF+CCh. **c** Treatment with 7,8-DHF activated Akt in the colonic strips. For immunoblotting, anti-Akt (left) or anti-phosphorylated-Akt (p-Akt, right) antibodies were applied for western membrane staining. \*\*Indicates  $P < 0.01$  versus the control; \$\$ $P < 0.01$  versus CCh alone; ## $P < 0.01$  versus DHF+CCh. Each group has  $n = 8$  to 10 samples (A–C)



**Fig. 7** ERK1/2 was not activated in the 7,8-DHF-enhanced/CCh-stimulated contraction of the colonic strips. **a** Antagonist PD98059 (10 μM) for ERK1/2 did not affect the 7,8-DHF/CCh-stimulated contraction of the colonic strips. See Fig. 3a legend for details on the antagonist treatment. \*Indicates  $P < 0.05$  versus CCh alone. **b**

CCh and 7,8-DHF did not phosphorylate ERK1/2 in the colonic strips. See Fig. 3b legend for details of the western blot protocol. For immunoblotting, anti-ERK1/2 (left) or anti-phosphorylated-ERK1/2 (p-ERK1/2, right) antibodies were applied to stain the western membranes. Each group has  $n = 8$  to 10 samples (A and B)

(10  $\mu$ M) alone or in combination with 7,8-DHF (10  $\mu$ M) did not change the expression or phosphorylation of ERK1/2 in the colonic strips (Fig. 7B), implying that ERK1/2 did not participate in 7,8-DHF to enhance the cholinergic contraction of the colonic strips.

### 7,8-DHF Increased the Rate of Intestinal Charcoal Propulsion and Intestinal M3 Expression in STC Rats

To further test the in vivo effect of 7,8-DHF on the intestinal motility of STC rats, the propulsion rate of the contents of their small intestines was examined, although fecal pellet output indicated that intestinal motility was improved to some extent by 7,8-DHF (Fig. 2a). 7,8-DHF-treated STC model rats were fed carbon powder and observed to determine the intestinal propulsion rate indicated by the position of the carbon powder (see Methods). Compared to that of the control (NS) rats, the rate of intestinal carbon propulsion of the STC rats was dramatically decreased, while 7,8-DHF (1 mg/kg body weight) feeding elevated the rate by ~70% (Fig. 8a, STC + DHF). The rate was slightly but significantly increased in the normal rats fed 7,8-DHF compared with the DMSO control rats.

Consistent with the in vitro results, 7,8-DHF feeding significantly stimulated M3 expression in the intestinal and colonic smooth muscles of STC rats (STC + DHF) and normal rats (DHF), as detected by western blotting while loperamide treatment (STC) did not appear to influence M3 expression (Fig. 8b, c).

## Discussion

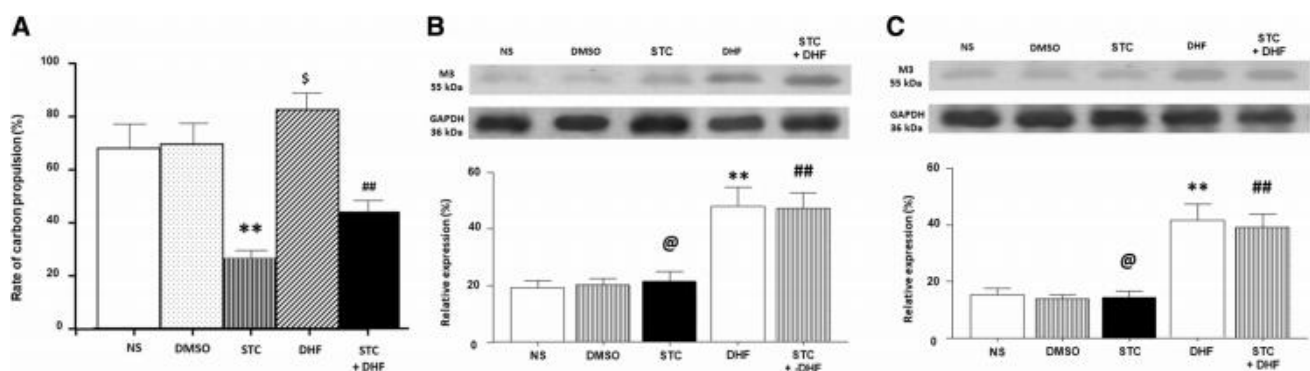
Here, we studied the role of 7,8-DHF in relieving the constipation of an STC rat model and showed that 7,8-DHF enhanced the CCh-stimulated contraction of colonic strips. We found that

7,8-DHF specifically activated TrkB and Akt in the muscular strips and it somehow augmented M3 receptor expression in the jejunal and gastric strips [12,13]. We think that when M3 expression was augmented by 7,8-DHF, the efficiency of CCh-stimulating colonic contraction was subsequently increased, such that the same dose of CCh stimulated a greater degree of contraction. Importantly, in vivo 7,8-DHF alleviated the STC in the loperamide-induced rat model.

### Potential Mechanisms by Which 7,8-DHF Enhanced the Cholinergic Contraction of the Colonic Strips

The evaluation of TrkB roles in gastrointestinal cholinergic contraction through the use of agonists has led to conflicting results [16,17]. Here, we found that 7,8-DHF treatment alone neither changed PLC $\gamma$ 1 phosphorylation nor stimulated the contraction of the muscular rat colon strips, but it specifically activated TrkB and augmented M3 expression, thereby enhancing CCh-stimulated strip contraction. Our results showed that Akt was correlated with an increase in M3 expression following 7,8-DHF treatment in the colonic strips, but the mechanism remains elusive.

We believe that the regulation of M3 expression by 7,8-DHF must involve more factors than Akt alone. Expression of membrane proteins such as M3 receptors includes biological syntheses from DNA to mRNA to protein, i.e., gene transcription, mRNA translation, protein trafficking, and membrane protein turnover. Our experimental duration with the colonic strips was approximately one hour. Therefore, the experiment was too short to presume that augmentation of M3 receptor expression by 7,8-DHF could be due to changes in its gene transcription or mRNA translation. We presume that the regulation of M3 protein trafficking and membrane localization could be involved. However, although trafficking and recycling of M receptors from M1 to M5 in neurons, cardiac and smooth muscle, and glands have been studied for more than three decades, much about



**Fig. 8** Effect of in vivo 7,8-DHF on rate of intestinal carbon propulsion and M3 expression of STC rats. **a** Rate of carbon propulsion. See details for carbon administration and calculation for rate of intestinal carbon propulsion in Methods. \*\*Indicates  $P < 0.01$  versus the NS control; \$  $P < 0.05$  versus the DMSO control; ##  $P < 0.01$  versus

STC. **b, c** Intestinal M3 expression. See Fig. 3B legend for the western blotting details. Anti-M3 antibody was used for immunoblotting. @indicates  $P > 0.05$  versus the NS control; \*\* $P < 0.01$  versus the DMSO control; ## $P < 0.01$  versus STC. For each group,  $n = 8$  to 10 samples (A–C)

these processes remains elusive [24]. Therefore, knowledge of the mechanism responsible for regulating the trafficking and recycling of intestinal M3 receptors is limited. Currently, it is difficult for us to interpret how 7,8-DHF/Akt selectively augments the expression of M3 receptors in intestines. Because of the mechanistic uncertainty, we therefore cannot exclude the possibility that the relationship between 7,8-DHF and colonic motility is unrelated and casual rather than the molecular mechanism, as suggested above. Nonetheless, this is a subject of further research interest for our group.

It is thought that extracellular  $\text{Ca}^{2+}$  influx via voltage-dependent  $\text{Ca}^{2+}$  channels (VDCCs) is the predominant factor in the contraction of gastrointestinal smooth muscle [20]. Muscarinic receptors in gastrointestinal tracts are Gq11-coupled proteins [21]. Activation of M receptors by ACh leads to the activation of the PLC/IP3/  $\text{Ca}^{2+}$  pathway to release  $\text{Ca}^{2+}$  from the endoplasmic reticulum. Intracellular  $\text{Ca}^{2+}$  opens  $\text{Ca}^{2+}$ -dependent cation channels and  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels, e.g., TRP and ANO1 [20,25], which depolarize the sarcolemmal membrane and open VDCCs, causing  $\text{Ca}^{2+}$  influx and muscle contractions.

ANA-12, a specific TrkB antagonist, not only suppressed TrkB activation by 7,8-DHF but also blocked 7,8-DHF-enhanced cholinergic contraction, 7,8-DHF/CCh-mediated activation of PLC $\gamma$ 1/Akt, and M3 overexpression in colonic strips. These results imply that 7,8-DHF-induced M3 augmentation was triggered by TrkB activation.

The high selectivity of the M1, M2, and M3 receptor antagonists found to date [26] made our experiments in this study possible. Since the three M receptors are predominant in colonic tissues [21–23], VU0255035 [27], QNB [28,29], and tarafenacin [30] were selected for blocking the M1, M2, and M3 receptors, respectively, in the colonic contraction experiments. We found that only tarafenacin significantly inhibited the colonic strip contraction induced by CCh or 7,8-DHF/CCh. Tarafenacin inhibited CCh-stimulated contraction by ~70%, while it inhibited 7,8-DHF-enhanced/CCh-stimulated contraction by ~80%. This result supported the assumption that M3 receptor expression was augmented by 7,8-DHF.

### Effect of 7,8-DHF on Loperamide-Induced Functional Constipation

Loperamide suppresses intestinal motility through the opioid receptors of myenteric neurons, diminishing the release of ACh and prostaglandin and disinhibiting the inhibited myenteric neurons [31]. The application of 7,8-DHF to the animal model somehow increased M3 receptor expression in intestinal smooth muscle through TrkB activation, increasing the responsiveness of smooth muscle to muscarinic agonists. In addition, we think that 7,8-DHF activating TrkB on myenteric neurons may have stimulated the release of ACh or prostaglandin from neurons via downstream PLC, Akt, or ERK1/2 signaling or

reversed the inhibition of myenteric neurons, which eventually enhanced enteric smooth muscle contraction. Nonetheless, other 7,8-DHF-mediated mechanisms (e.g., non-TrkB pathway) may have also contributed to the increased motility. Since our research interest is focused on 7,8-DHF, we are currently looking into the mediating mechanisms.

Based on the alleviation of functional constipation in the STC rat model, 7,8-DHF may be a good candidate for treating colonic disorders. BDNF has been demonstrated to enhance gastrointestinal dynamics via the increased transportation of stool removal [4,16,32]. 7,8-DHF was able to simulate the roles of BDNF in the nervous system [33]. Here, we found that 7,8-DHF applied to the colon exerted effects similar to those of BDNF. Our experiments provided preclinical evidence supporting the use of 7,8-DHF to treat disorders derived from abnormal colonic motility. Additionally, instead of assessing colonic propulsive motility, the fecal pellet weight and water content were measurements that reflect the prosecretory/anti-absorptive action of the colon. Nonetheless, prosecretory/anti-absorptive and procontractile actions are probably synergistic in the alleviation of constipation.

Constipation is a very complex pathological process with a complex etiology and mechanism involving abnormal intestinal motility, abnormal secretion/absorption, and other processes [1]. Therefore, the conclusion that 7,8-DHF improves constipation requires further in-depth research work with various constipation models and rigorous clinical observation. These are our future research interests.

In summary, experimental evidence in this study demonstrated some of the molecular mechanisms of 7,8-DHF action in contractile enhancement. The application of 7,8-DHF activated the TrkB/Akt pathway and augmented M3 expression. When CCh was bound with increasingly expressed M3, more p-PLC $\gamma$ 1 was produced, and the extent of the CCh-stimulated colonic contractions was increased. Since 7,8-DHF alleviated rat constipation, we suggest the possibility of 7,8-DHF development and use for correcting constipation in the future.

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### Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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