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ORIGINAL PAPER

Role of PGE_2 in the colonic motility: PGE_2 generates and enhances spontaneous contractions of longitudinal smooth muscle in the rat colon

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Abstract The aim of this study was to determine which PGE_2 receptors ($EP_{1–4}$ receptors) influence colonic motility. Mucosa-free longitudinal smooth muscle strips of the rat middle colon spontaneously induced frequent phasic contractions (giant contractions, GCs) in vitro, and the GCs were almost completely abolished by a cyclooxygenase inhibitor, piroxicam, and by an EP_3 receptor antagonist, ONO-AE3-240, but enhanced by tetrodotoxin (TTX). In the presence of piroxicam, exogenous PGE_2, both ONO-AE-248 (EP_3 agonist), and ONO-DI-004 (EP_1 agonist) induced GC-like contractions, and increased the frequency and amplitude. These effects of EP receptor agonists were insensitive to TTX and ω-conotoxins. In immunohistochemistry, the EP_1 and EP_3 receptors were expressed in the longitudinal smooth muscle cells. These results suggest that the endogenous PGE_2 spontaneously generates and enhances the frequent phasic contractions directly activating the EP_1 and EP_3 receptors expressed on longitudinal smooth muscle cells in the rat middle colon.

Keywords Colonic motility · Giant migrating contractions · Prostaglandins · EP receptors

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Introduction

Appropriate regulation of colonic motor activity and water absorption is required to form adequate feces and propel them through the colon. Three distinct types of colonic contractions have been recorded in vivo: rhythmic phasic contractions (RPCs), giant migrating contractions (GMCs), and tonic contractions (TCs) [1, 2]. These colonic motor activities are considered to be induced and regulated by myogenic and neurogenic mechanisms, as well as a mechanism involving the interstitial cells of Cajal (ICC), which act as pacemaker cells in intestinal motility [3, 4]. It is considered that these mechanisms interact with each other through a variety of chemical mediators, including prostaglandins (PGs) [3].

PGs are ubiquitously produced in many organs by the metabolism of arachidonic acid by cyclooxygenase (COX). They are involved in a variety of physiological functions, such as vasoconstriction, reproduction, and gastric cytoprotection [5, 6]. In general, COX-1 is thought to be constitutively expressed in physiological conditions, whereas COX-2 is inducible and increases the PG production in inflammatory conditions [7]. However, in the GI tract, it has been reported that both COX-1 and COX-2 are constitutively expressed [8]. One reason for this is the GI mucosa is continually exposed to the intestinal contents that include nutrients, as well as pathogens and toxins, which induce "physiologic inflammation" in the GI mucosa [9].

It has been reported that PGs are produced in the mucosa and in the muscle layers [10]. Most functional studies of PGs on GI motility were performed in the 1960s, before the PG receptors had been identified. These early studies showed that PGE_2 enhances longitudinal muscle (LM) motility [11–13], but reduces circular muscle (CM) motility [12, 13].

In the 1990s, PGE_2 receptors were cloned and subdivided into four subtypes, EP_1, EP_2, EP_3, and EP_4, in humans [14–16], mice [14, 17–20], and rats [21]. These EP receptor subtypes are coupled to G proteins and trigger different signaling pathways. EP_1 is coupled to $G_{\alpha q}$ and increases intercellular Ca^{2+} concentrations. EP_2 and EP_4 are coupled to $G_{\alpha s}$ and activate adenylyl cyclase to produce cAMP. EP_3 is coupled to $G_{\alpha i}$ and reduces intracellular cAMP levels [22]. These differences in signaling pathways among the EP receptor subtypes play a critical role in determining how PGE_2 controls its physiologic functions.

Although the molecular biological properties of EP receptors have been unraveled, few reports have examined the role of each EP receptor in the control of GI motility. Grasa et al. [23] reported the cellular distribution of EP receptors in the rabbit small intestine, and reported that PGE_2 induced LM contraction via the EP_1 and EP_3 receptors. More recently, Fairbrother et al. [24] reported that PGE_2 enhances GI motility via EP_1 receptors in the human colon, and via both EP_1 and EP_3 receptors in mouse ileum and colonic segments. However, there are no reports describing the role of PGs in controlling colonic spontaneous contractions, namely RPCs, GMCs, and TCs. Therefore, the aim of the present study was to elucidate the roles of PGE_2 in controlling these spontaneous contractions, and to identify the involved EP receptor subtypes in LM strips isolated from the rat middle colon.

Materials and methods

Tissue preparation

Male Wistar rats (263.5 ± 2.1 g; Japan SLC, Hamamatsu, Japan) were used in all experiments. The rats were anesthetized with ether and decapitated with a guillotine. Animal handling and euthanization were conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka*, and the study was approved by the University of Shizuoka Animal Use Ethics Committee. Segments of the middle colon were removed. We chose the middle colon to investigate the generation of GCs, because it is considered that the luminal contents change from semi-solid feces to solid feces there. Then the tissues were cut along the mesenteric border, and placed in Krebs–Ringer solution containing (in mM) 117 NaCl, 4.7 KCl. 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, and 11 glucose. The tissues were pinned flat to the bottom of a dish coated with silicone rubber. Then, the mucosa and submucosa were removed from the tissues with sharp forceps under a stereomicroscope, and the tissue was cut parallel to the long axis of LM to obtain muscle strip preparations (approximately 2 mm wide and 8–10 mm long). All preparations were connected to an isotonic transducer (type 45196A; NEC San-ei Instruments, Tokyo, Japan) under a constant load (0.5 g). The loaded tension was as much as previous studies [25–29]. An amplifier (transducer amplifier N6682; NEC San-ei Instruments) and a PowerLab system (ML846; ADInstruments, Bella Vista, NSW, Australia) were used to record LM activity. The preparations were suspended in 15-ml tissue baths containing Krebs–Ringer solution at 37 °C, and the solution was continuously bubbled with 95 % O_2 and 5 % CO_2. After setting up the tissue strips, they were equilibrated for about 1 h. At the end of the experiments, 10 μM carbachol (CCh) was added, and the amplitude of CCh-induced contraction was used as an internal control of the contraction amplitude (%CCh).

Experimental protocol

In earlier studies, three distinct types of colonic contractions, RPCs, GMCs, and TCs, were recorded in vivo in the canine colon by strain-gauge transducers [1, 2]. By in vitro isometric recording, Gonzalez and Sarna [30] demonstrated that isolated CM strips from the rat middle colon also generate three distinct contractions in an organ bath: giant contractions (GCs) similar to GMCs, RPCs, and TCs. Meanwhile, Ono et al. [26] and Powell et al. [31] showed that the LM from the rat distal colon exhibits GC-like spontaneous contractions, but not RPCs. Plujà et al. [32] also reported the absence of RPC-like contractions in rat colonic LM strips. Therefore, in the present study, the spontaneous contractions in the LM that mimic GCs in the CM are called GCs.

After equilibration, the frequency and mean amplitude of the spontaneous GCs were measured for 20 min, after which the bath solution was replaced with fresh Krebs solution. After 30 min, piroxicam (10^{-5} M) was added to the bath, and the frequency and mean amplitude of GCs was measured for 20 min before and 10 min after the addition of piroxicam. To determine whether the piroxicam-sensitive spontaneous GCs were induced by PGE_2 and which EP receptors were involved, ONO-8713 (EP_1 antagonist; 10^{-8} or 10^{-7} M), AH6809 (EP_1/EP_2 antagonist; 10^{-7} M), ONO-AE3-240 (EP_3 antagonist; 10^{-8} or 10^{-7} M), or ONO-AE3-208 (EP_4 antagonist; 10^{-7} M) were added to the bath after equilibration without washing and before the addition of piroxicam.

To confirm the role of PGE_2 in the generation and regulation of spontaneous GCs, PGE_2 was cumulatively added to the tissue bath every 30 min after the wash and in the presence of piroxicam (10^{-5} M). Furthermore, to confirm which of the EP receptor subtypes were involved in PGE_2-induced GC-like contractions (GCLC) and tonus, ONO-DI-004 (EP_1 agonist), ONO-AE1-259 (EP_2 agonist),

ONO-AE-248 (EP_3 agonist), or ONO-AE1-329 (EP_4 agonist) were cumulatively added to the organ bath.

To examine the neural components involved in PGE_2- and EP agonist-induced contractions, the muscle preparations were pretreated with tetrodotoxin (TTX; 1 μM) after washing and the addition of piroxicam. After 30 min of equilibration (as described below), PGE_2 (10^{-6} M) was added to the tissue bath and incubated for over 30 min. ONO-DI-004 or ONO-AE-248 (10^{-5} M) were added to the tissue bath 30 min after the addition of TTX. To examine the effects of neurotransmitter exocytosis from the nerve endings and varicosities on PGE_2-induced GCLCs, preparations were pretreated with a selective voltage-gated calcium channel (VGCC) blocker, ω-conotoxin (MVIIC and GVIA; both, 100 nM) followed 30 min later by 1 μM PGE_2. The additional concentration of these regents was selected based on those used by Karaki and Kuwahara [33].

Data analysis

If there were no GCs or GCLCs, the frequency was defined as 0, but was not counted in the assessment of amplitude. The GCs/GCLCs that were <50 % of the maximum values for 20 min were not counted. All data are expressed as mean ± SEM. The Tukey–Kramer test was used for multiple comparisons. Values of $P < 0.05$ were considered statistically significant. The n values represent the number of strips.

The effects of the EP_3 antagonist on spontaneous contractions were calculated based on the change in the frequency and mean amplitude of phasic contractions and defining these values for contractions before the addition of antagonists as 100 %.

Concentration-dependent response curves of the frequency (Eq. 1) and amplitude (Eq. 2) of GCLCs were fitted using the following equations:

$$V = \frac{V_{\max} \cdot X}{X + \mathrm{EC}_{50}} \tag{1}$$

$$V = \frac{V_{\max} \cdot X}{X + \mathrm{EC}_{50}} + V_0 \tag{2}$$

where V is the response (%CCh), $V_{\max}$ is the maximum response (%CCh), V_0 is the initial minimum amplitude (%CCh), EC_{50} is the 50 % effective concentration, and X is the concentration of the agonist. All plots were fit using Marquardt's method [34]. Kyplot software v.5.0 (KyensLab, Tokyo, Japan) was used for all data analysis.

Chemicals

Carbachol chloride and piroxicam were purchased from Sigma (St. Louis, MO, USA). TTX, ω-conotoxin MVIIC, and ω-conotoxin GVIA were from Tocris Bioscience (Ellisville, MO, USA). PGE_2 was from Cayman Chemical (Ann Arbor, MI, USA). EP agonists and antagonists were a kind gift from Ono Pharmaceutical. (Osaka, Japan). PGE_2, piroxicam, EP agonists, and EP antagonists were dissolved in dimethyl sulfoxide while the other chemicals were dissolved in distilled water. The volumes of dissolved drugs in distilled water or dimethyl sulfoxide added to the bath solutions did not exceed 150 and 15 μL, respectively.

Immunohistochemical analysis

The middle colonic tissues removed as described above were immediately frozen with optimal cutting temperature compound (TissuTek; Sacra Finetechnical, Tokyo, Japan) in liquid nitrogen. The tissue was then cut into 10-μm-thick sections on a cryostat (CM1100; Leica Microsystems, Weltzlar, Germany). The resulting sections were placed on glass slides, dried, and fixed for 10 min in 100 % methanol (Kantokagaku, Tokyo, Japan) at −20 °C or for 30 min in Zamboni's fixative [2 % formaldehyde and 0.2 % picric acid in 0.1 M phosphate buffer (pH 7.4)] at 4 °C. Then, the tissue was washed in PBS (3 × 10 min) and incubated with 10 % normal donkey serum in PBS at room temperature for 30 min to block non-specific binding of antibodies. Then, the sections were incubated with the primary antibodies (Table 1) in PBS at 4 °C overnight. After washing in PBS (3 × 10 min), the sections were incubated with secondary antibodies conjugated with fluorescent dyes (Table 1) and 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/mL; Dojindo Molecular Technologies, Kumamoto, Japan) in PBS for 1 h at room temperature. After another wash in PBS (3 × 10 min), coverslips were mounted on the glass slides with mounting medium (DakoCytomation, Glostrup, Denmark). Immunoreactivity and DAPI-stained nuclei were visualized using a fluorescence microscope (Axio Observer

Table 1 Antibodies

	Host/label	Dilution	Source
Primary antibody			
Anti-EP_1 (C-terminal)	Rabbit	1:400	Cayman (101740)
Anti-EP_3 (C-terminal)	Rabbit	1:400	Cayman (101760)
Anti-α-smooth muscle actin	Mouse	1:20,000	Sigma-Aldrich (A5238)
Anti-c-Kit	Goat	1:2,000	Santa Cruz (SC-1494)
Secondary antibody			
Donkey anti-rabbit IgG	Alexa594	1:400	Molecular probes (A21207)
Goat anti-mouse IgG	Alexa488	1:400	Molecular probes (A11001)
Donkey anti-goat IgG	Alexa488	1:400	Molecular probes (A11055)

Z1; Carl Zeiss, Oberkochen, Germany), and the images were captured using a cooled charge-coupled device digital camera system (AxioVision 135; Zeiss, Munich-Halbergmoos, Germany).

Absorption tests were performed to verify the specificities for EP_1 and EP_3 receptors. EP_1 or EP_3 receptor antibody (neat) was mixed with each blocking peptide (EP_1 Cat.#301740, EP_3 Cat.#301760) in a 1:1 (v/v) ratio, and was incubated for 1 h at room temperature. Then, the mixture was diluted to the final working antibody concentration (Table 1) and was applied to the tissue sections. Thereafter, the following steps were the same protocol described above.

Results

Effects of basal PG production on spontaneous GCs

Figure 1a shows a representative trace of contractions. After equilibration for about 1 h, spontaneous GCs occurred in 179 of 211 strips. The mean frequency of the GCs was 5.3 ± 0.2 contractions in 20 min ($n = 211$), including the strips with 0 contractions (Fig. 1b). The mean amplitude of GCs was 27.3 ± 1.0 % of the contraction induced by 10^{-5} M CCh ($n = 179$, excluding 32 strips with 0 contractions; Fig. 1c).

Replacing the bath solution with fresh Krebs–Ringer solution abolished the GCs in 36 strips and reduced the frequency of GCs to 2.7 ± 0.2 contractions in 20 min ($n = 211$, including the strips with 0 contractions; $P < 0.001$ vs. the basal level; Fig. 1b). The mean amplitude in the 143 strips with intact GCs was reduced to 23.3 ± 1.0 %CCh ($P < 0.01$ vs. basal level; Fig. 1c).

The addition of piroxicam (10^{-5} M) abolished the spontaneous GCs in 179 of 211 strips (Fig. 1a) and reduced the frequency to 0.4 ± 0.05 contractions in 20 min ($n = 211$; $P < 0.001$ vs. after washing; Fig. 1b). Similarly, piroxicam reduced the mean amplitude of the residual contractions was reduced to 14.5 ± 1.6 %CCh ($n = 32$; $P < 0.001$ vs. after washing; Fig. 1c). These results indicate that endogenous PGs induce and enhance spontaneous GCs in LM strips of the rat middle colon. Therefore, in the following studies, almost all experiments were performed after washing and the addition of piroxicam to remove the effects of endogenous PGs.

Effects of EP antagonists on spontaneous GCs

To identify the EP receptors involved in inducing the basal GCs, ONO-8713 (EP_1 antagonist), AH6809 (EP_1/EP_2 antagonist), ONO-AE3-240 (EP_3 antagonist), or

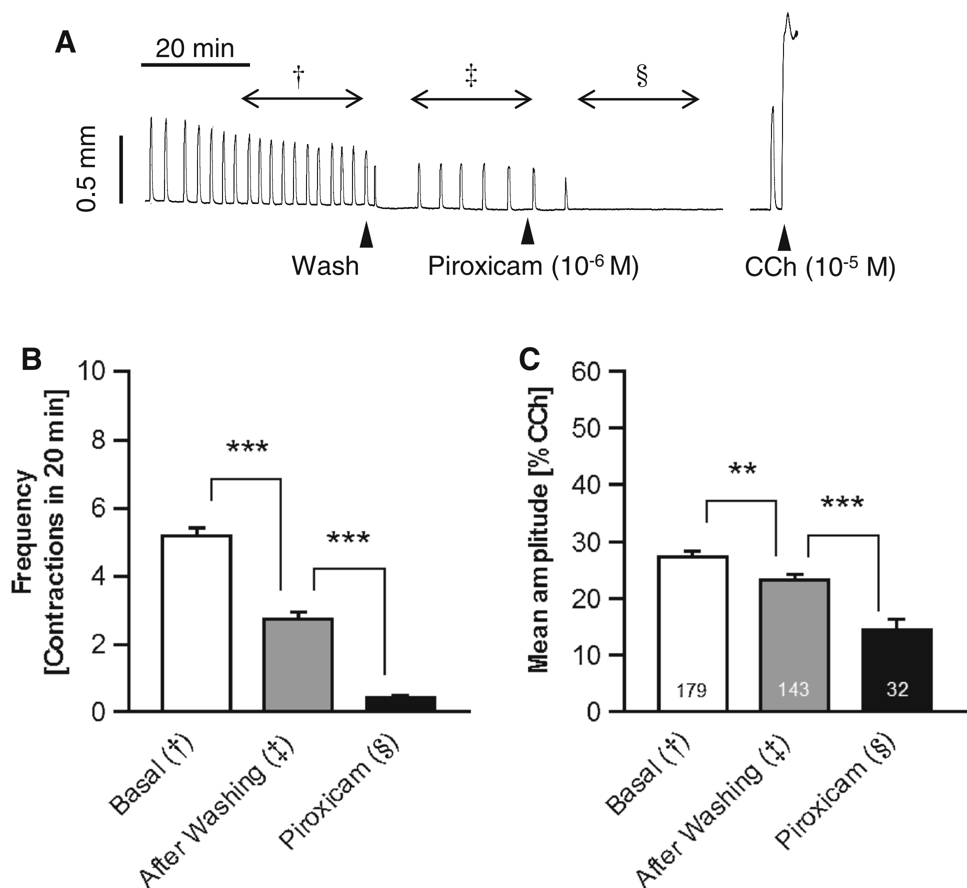

Fig. 1 Effects of basal PG production on the motility of longitudinal muscle (LM). The frequency and amplitude of GCs were measured in LM from the rat middle colon. After measuring spontaneous GCs, the bath solution was replaced, and 30 min later, piroxicam (10^{-5} M) was added. **a** A representative trace of spontaneous GCs (†), GCs after washing (‡), and GCs in the presence of piroxicam (10^{-5} M) (§). **a** and **b** Frequency and mean amplitude (**c**) of spontaneous GCs (†), GCs after washing (‡), and GCs in the presence of piroxicam (10^{-5} M) (§). Values are mean ± SEM ($n = 211$). The *numbers* in each *column* in (**c**) indicate the number of preparations, excluding preparations with an amplitude of 0. ***$P < 0.001$ and **$P < 0.01$ (Tukey–Kramer test)

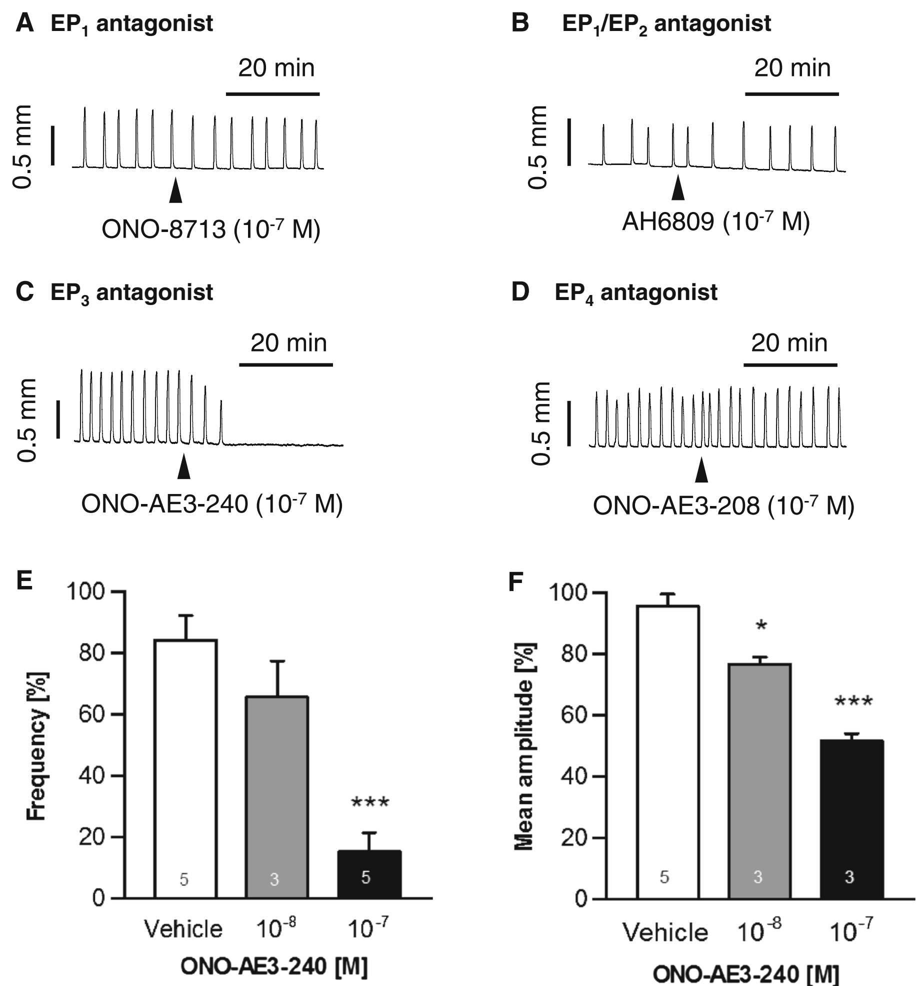

Fig. 2 Effects of ONO-AE3-248 (EP_3 antagonist) on spontaneous GCs. The frequency and amplitude of GCs after the addition of ONO-8713 (EP_1 receptor antagonist; 10^{-7} M), AH6809 (EP_1/EP_2 antagonist 10^{-7} M), ONO-AE3-240 (EP_3 receptor antagonist; 10^{-8} and 10^{-7} M), or ONO-AE3-208 (EP_4 antagonist 10^{-7} M) was measured in the presence of piroxicam. Representative traces of spontaneous GCs in the presence of ONO-8713 (**a**), GCs in the presence of AH6809 (**b**), GCs in the presence of ONO-AE3-248 (**c**), and GCs in the presence of ONO-AE3-208 (**d**). Effects of ONO-AE3-204 on the frequency (**e**) and mean amplitude (**f**) as a percentage of the control values. Values are mean ± SEM ($n = 5$). ***$P < 0.001$ and *$P < 0.05$ (Dunnett's test). The *numbers* in each *column* in (**e**, **f**) indicate the number of preparations

ONO-AE3-208 (EP_4 antagonist) were administered to the tissues during spontaneous GCs. Representative traces showing the timing of administration are shown in Fig. 2a–d. Only ONO-AE3-240 significantly reduced the frequency and amplitude of spontaneous GCs in a concentration-dependent manner (Fig. 2e, f). ONO-AE3-240 at 10^{-7} M reduced the frequency of spontaneous GCs to 15 ± 6.3 %CCh (vs. 84.2 ± 8.2 % for vehicle; $P < 0.001$; Fig. 2e). ONO-AE3-240 at 10^{-7} M reduced the amplitude to 51.5 ± 2.5 % (vs. 95.6 ± 4.0 % for vehicle, $P < 0.001$; $n = 3$; Fig. 2f). The other EP receptor antagonists, ONO-8713, AH6809, and ONO-AE3-208 did not significantly affect the amplitude or frequency of spontaneous GCs (Fig. 2a, b, d).

Effects of EP agonists in the presence of piroxicam

In the presence of piroxicam, the cumulative addition of PGE_2 (10^{-9} to 10^{-5} M) induced frequent contractions mimicking spontaneous GCs, as shown in Fig. 3a. The frequency and amplitude of the PGE_2-induced GC-like contractions (GCLCs) increased in a concentration-dependent manner. To identify which EP receptor subtype mediated these PGE_2-induced contractions, the EP-specific agonists were cumulatively added to the organ bath. ONO-DI-004 (EP_1) and ONO-AE-248 (EP_3), but not ONO-AE1-259 (EP_2) or ONO-AE1-329 (EP_4), concentration-dependently induced GCLCs (Fig. 3). The resulting data were fitted to normal and modified Michaels–Menten equations, as described in "Materials and methods", and the calculated parameters, including EC_{50} values, are summarized in Table 2. The rank order of potency was PGE_2 > ONO-AE3-240 > ONO-DI-004 (Fig. 3f) for frequency and PGE_2 > ONO-DI-004 > ONO-AE-240 (Fig. 3g) for mean amplitude.

Effects of neural factors on EP agonist-induced contractions

To confirm the influence of neural factors on the EP agonist-induced GCLCs, TTX (10^{-6} M) was added before the addition of PGE_2 (10^{-6} M), ONO-DI-004 (10^{-5} M), or ONO-AE-248 (10^{-5} M). TTX itself induced GCLCs in 5 of 17 strips (frequency 2.6 ± 0.5 contractions in 20 min, amplitude 8.7 ± 2.3 %CCh, $n = 5$). However, TTX did

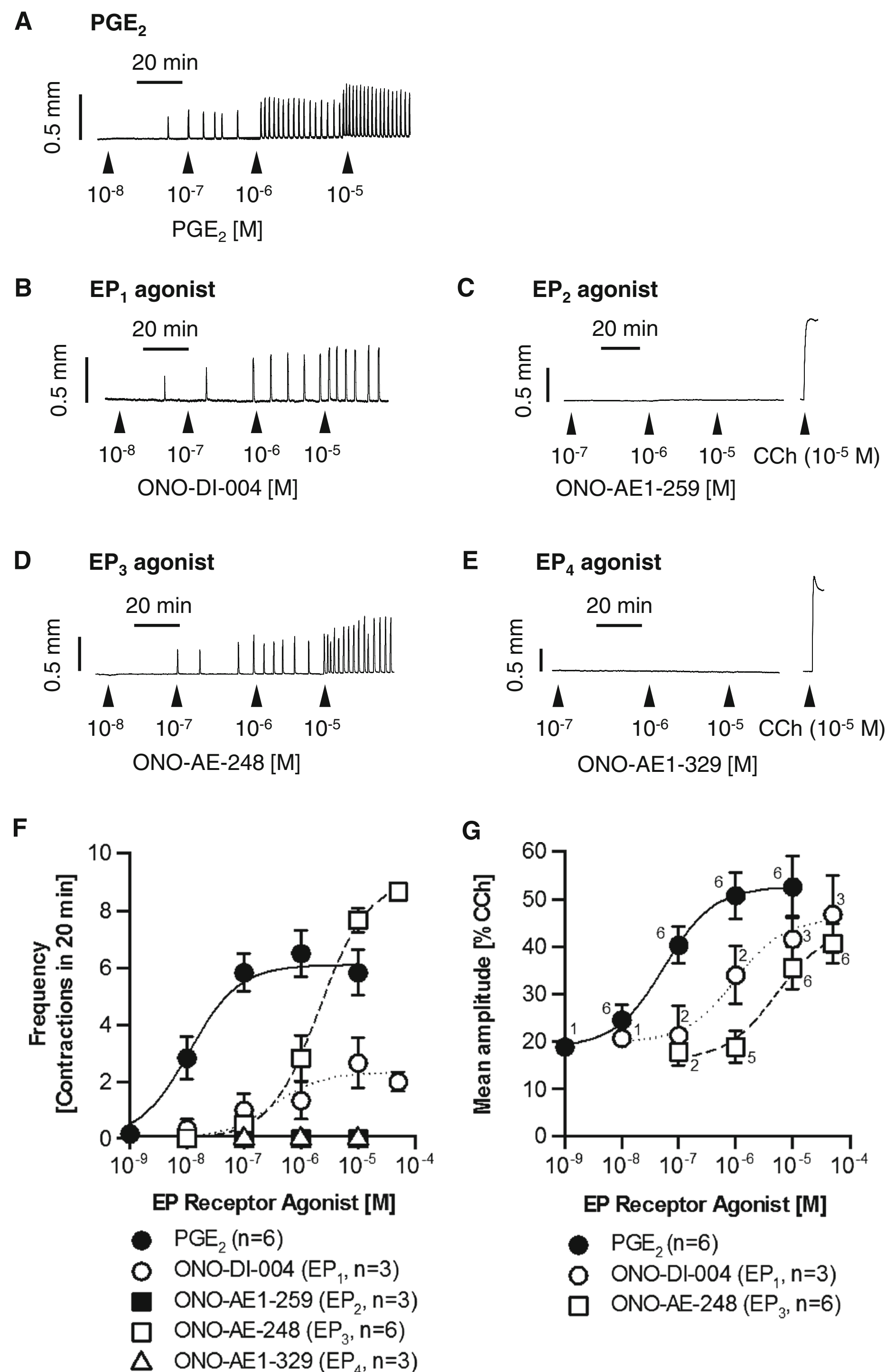

Fig. 3 Concentration-dependent effects of PGE_2 and EP agonists on the induction of GCLCs. PGE_2 and EP_1 (ONO-DI-004), EP_2 (ONO-AE1-259), EP_3 (ONO-AE-248), and EP_4 (ONO-AE1-329) agonists were cumulatively added to the bath solution of tissues pretreated with piroxicam. Representative traces showing the cumulative addition of PGE_2 (**a**), ONO-DI-004 (EP_1 agonist; **b**), ONO-AE1-259 (EP_2 agonit, **c**), ONO-AE-248 (EP_3 agonist; **d**), and ONO-AE1-329(EP_4 agonist, **e**). Concentration–response curves for the effects of PGE_2 and EP agonists on the frequency (**f**) and mean amplitude (**g**) of GCLCs. Values are mean ± SEM. The numbers in (**g**) indicate the number of preparations, excluding preparations with a mean amplitude of 0. The mean amplitude of tissue preparations treated with the EP_2 and EP_4 agonists is not shown

not affect the contractions induced by PGE_2, ONO-DI-004, or ONO-AE-248 (data not shown). To examine whether exocytosis of neurotransmitters from nerve ending was involved, ω-conotoxins (MVIIC and GVIA; both, 10^{-7} M) were added before the addition of PGE_2. ω-conotoxins itself did not affect GCLCs nor did they affect PGE_2-induced GCLCs (frequency 8.3 ± 1.5 contractions in 20 min, amplitude 43.1 ± 8.5 %CCh) compared with the control level (frequency 10.3 ± 1.3 contractions in 20 min, amplitude 47.9 ± 4.2 %CCh).

In addition, we checked the effect of TTX on the GCs before washing and the addition of piroxicam. As a result, TTX itself significantly increased the frequency of GCs 164.7 ± 11.6 % of the frequency before the addition of

Table 2 Concentration-dependent effects of PGE_2, ONO-DI-004, or ONO-AE-248 on longitudinal muscle

	Frequency[a]			Mean amplitude[b]			
	V_{max} (/20 min)	EC_{50} (M)	R^2	V_{max} (%CCh)	EC_{50} (M)	V_0 (M)	R^2
PGE_2	6.1	1.1×10^{-8}	0.98	33.8	5.6×10^{-8}	18.8	0.99
ONO-DI-004	2.3	3.2×10^{-7}	0.77	26.1	9.7×10^{-7}	19.8	0.99
ONO-AE-248	9.1	2.1×10^{-6}	0.99	27.7	5.1×10^{-6}	16.1	0.96

Values were derived from the Michaelis–Menten plots

V_{max} maximum response, EC_{50} 50 % effective concentration, V_0 initial minimum value, R^2 relative contribution to the plot fit

[a] Calculated using Eq. (1)

[b] Calculated using Eq. (2)

TTX ($P < 0.01$ vs vehicle control), but did not affect the amplitude of GCs ($n = 5$).

Distribution of EP_1 and EP_3 receptors in the muscle layer of the rat middle colon

The distribution of EP_1 and EP_3 receptors in the muscle layer of the rat middle colon was determined by immunohistochemistry. In the rat colon, α-actin-positive smooth muscle cells were observed in the LM and CM layers (Figs. 4b, 5b). EP_1 immunoreactivity (IR) was detected in the nuclei of smooth muscle cells (Fig. 4d, arrows) EP_3 IR was found in the perinuclear site of α-actin-positive smooth muscle cells (Fig. 5d, arrows). These immunoreactivities of both EP_1 and EP_3 disappeared in absorption tests. EP_1 and EP_3 coexpression was detected in myenteric neurons (Fig. 6c″, d″, mg), but not in c-Kit-positive ICC cells located around the myenteric ganglion. c-Kit expression was not colocalized with the EP_1 or EP_3 receptors in the LM (Fig. 6a″, b″).

Discussion

This study has shown for the first time that the generation of GCs in the LM of the rat middle colon required PGs, and was predominantly mediated via EP_3 receptors expressed on smooth muscle cells.

Spontaneous GCs were induced by endogenous PGE_2 via the EP_3 receptor

Spontaneous GCs in the LM strips of the rat colon lacking mucosa were reduced by replacing the bath solution and were almost completely abolished by piroxicam (Fig. 1). These treatments appear to deplete PGs from the tissue surface by washing away the accumulated PGs and by blocking PG synthesis. Therefore, it seems feasible that PGs are produced and accumulate in the rat colonic smooth muscle layer in basal conditions and spontaneously induce phasic contractions (i.e., GCs) in LM strips. Figure 2a–d shows that the spontaneous GCs were significantly reduced by ONO-AE3-240 (selective EP_3 antagonist; Fig. 2c), but not by ONO-8713 (selective EP_1 antagonist, Fig. 2a), AH6809 (EP_1/EP_2 antagonist; Fig. 2b), or ONO-AE3-208 (selective EP_4 antagonist; Fig. 2d). These results suggest that the GCs are induced by endogenous PGE_2, and their frequency and amplitude are controlled by EP_3 receptors.

Exogenous PGE_2 generates and concentration-dependently enhances the frequency and amplitude of GC-like contractions predominantly via EP_3 and partially via EP_1 receptors

In the presence of piroxicam, the cumulative addition of PGE_2, ONO-DI-004 (EP_1 agonist); and ONO-AE-248 (EP_3 agonist), but not ONO-AE1-259 (EP_2 agonist) or ONO-AE1-329 (EP_4 agonist), induced the frequent phasic contractions mimicking spontaneous GCs. The frequency and amplitude of the GC-like contractions (GCLCs) were also enhanced by PGE_2 in a concentration-dependent manner (Fig. 3).

In terms of the frequency of GCLCs, the EC_{50} for ONO-DI-004 and ONO-AE-248 were about 100 times higher than that of PGE_2, but the V_{max} for ONO-AE-248 was only 1.5 times higher than that of PGE_2, and the V_{max} for ONO-DI-004 was half that of PGE_2 and one-third that of ONO-AE-248 (Table 2). These findings suggest that the PGE_2-induced GCLCs are predominantly mediated by the EP_3 receptor and partially by the EP_1 receptor. Although the K_i for PGE_2 and ONO-AE-248 for the EP_3 receptor in mice was reported to be 5×10^{-9} M and 7.5×10^{-9} M, respectively [6], Kobayashi et al. [35] reported that the EC_{50} for ONO-AE-248 was 6.7×10^{-6} M in rat mesenteric arterial contraction. This value is similar to that in the present study (2.1×10^{-6} M; Table 2). Similarly, the K_i for ONO-DI-004 for the mouse EP_1 receptor was reported to be 0.15×10^{-6} M [6], which is about 2.2 times greater than that in the current study (3.3×10^{-7} M; Table 2).

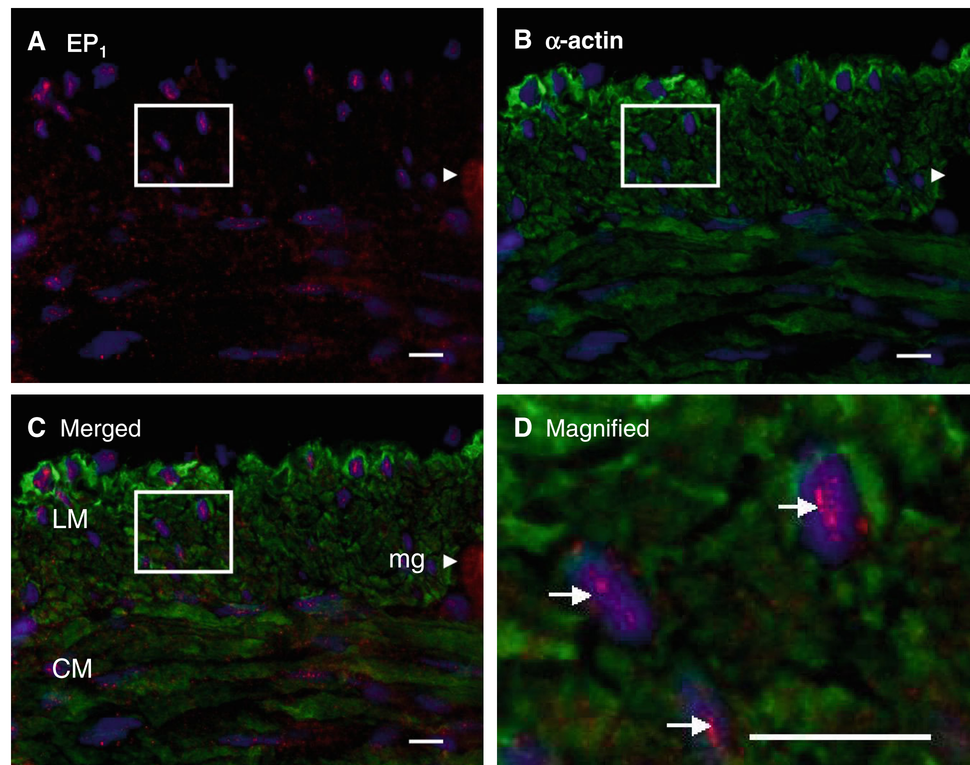

Fig. 4 Immunohistochemistry for the EP_1 receptor in the colonic muscle layer. Cryostat sections (10 μm thick) of the rat middle colon were stained with rabbit anti-EP_1 receptor and mouse anti-α-smooth muscle actin primary antibodies. EP_1 and α-smooth muscle actin IR were visualized by secondary antibodies conjugated with Alexa594 (**a**, *red*) and Alexa488 (**b**, *green*), respectively. The nuclei were stained with DAPI (*blue*). α-smooth muscle actin was used a marker for intestinal smooth muscle. **c** Merged image of (**a**) and (**b**). **d** Magnified image of the longitudinal muscle in (**c**). *Arrows* in (**d**) indicate EP_1 IR on the nuclei of smooth muscle cells. *Bars* 10 μm. *LM* longitudinal muscle, *CM* circular muscle, *mg* myenteric ganglion (*arrowheads*)

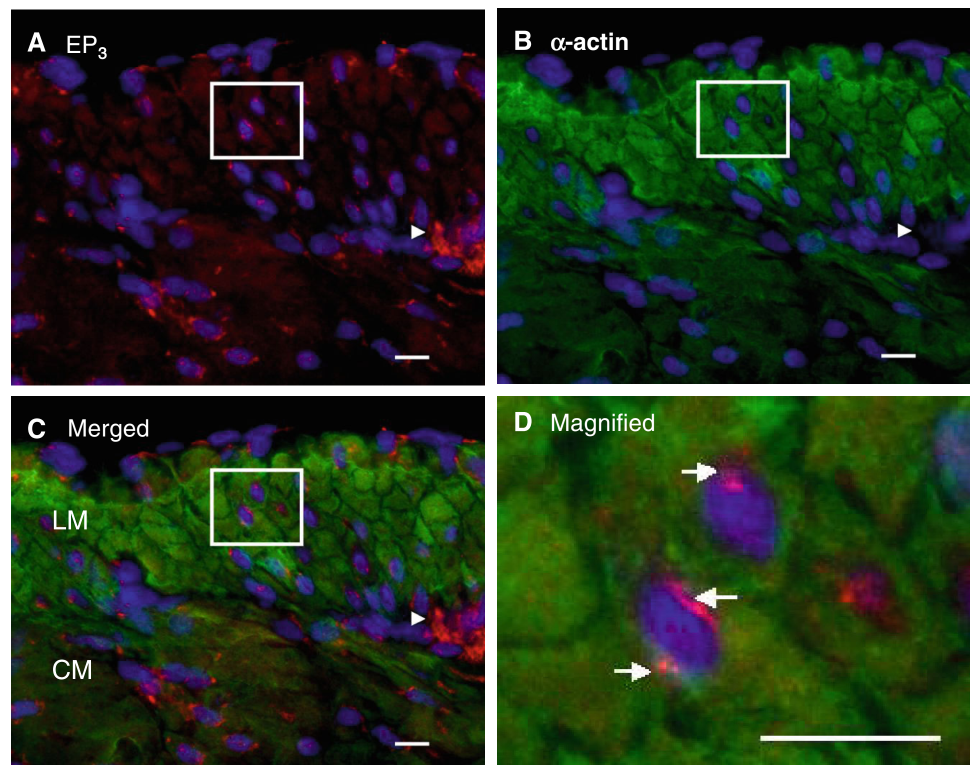

Fig. 5 Immunohistochemistry for the EP_3 receptor in the colonic muscle layer. EP_3 and α-smooth muscle actin IR were visualized using secondary antibodies conjugated with Alexa594 (**a**, *red*) and Alexa488 (**b**, *green*), respectively. The nuclei were stained with DAPI (*blue*). α-smooth muscle actin was used a marker for intestinal smooth muscle. **c** Merged image of **a** and **b**. **d** Magnified image of the longitudinal muscle in **c**. *Arrows* in **d** indicate EP_3 IR around the nuclei of smooth muscle cells. *Bars* 10 μm. *LM* longitudinal muscle, *CM* circular muscle, *mg* myenteric ganglion (*arrowheads*)

Therefore, ONO-AE-248 and ONO-DI-004 may have weaker potency on rat EP_3 and EP_1 receptors, respectively, than on the corresponding receptors in mice.

The amplitude of GCLCs was enhanced by PGE_2, ONO-AE-248, and ONO-DI-004 in concentration-dependent manners (Fig. 3f, g). The data showed that there was a

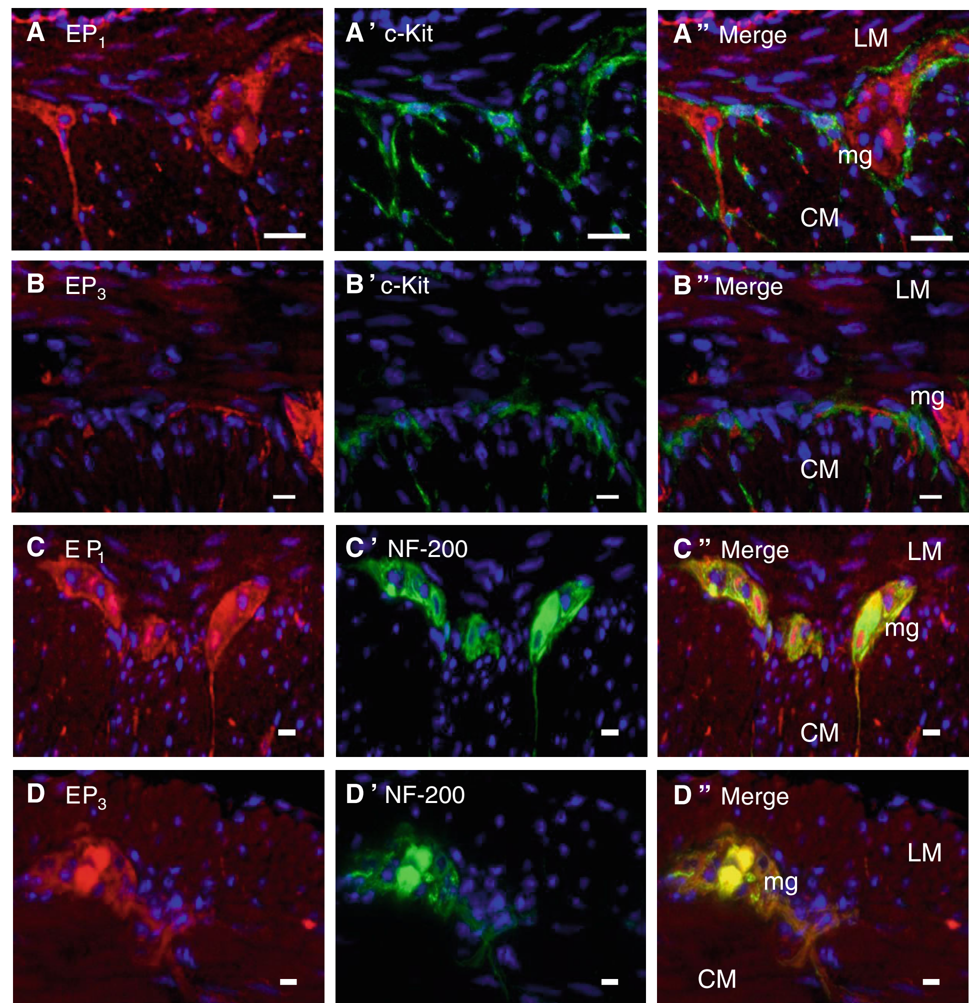

Fig. 6 Double-immunostaining for EP_1/EP_3 receptors and c-Kit or neurofilament-200 (NF-200). EP_1/EP_3 and c-Kit IR were visualized using secondary antibodies conjugated with Alexa594 (**a**, **b**, *red*) and Alexa488 (**a′**, **b′**, *green*), respectively. The nuclei were stained with DAPI (*blue*). c-Kit was used a marker for ICCs. **a″**, **b″** are the merged images of EP_1 with c-Kit and EP_3 with c-Kit, respectively. EP_1/EP_3 and NF-200 IR were visualized using secondary antibodies conjugated with Alexa594 (**c**, **d**, *red*) and Alexa488 (**c′**, **d′**, *green*), respectively. **a″**, **b″** are the merged images of EP_1 with NF-200 and EP_3 with NF-200, respectively. The nuclei were stained with DAPI (*blue*). NF-200 was used as a marker for neurons. *Bars* 10 µm. *LM* longitudinal muscle, *CM* circular muscle, *mg* myenteric ganglion

minimum amplitude, requiring the introduction of V_0 into the Michaelis–Menten equation (“Materials and methods”, Eq. 2). The V_0 for PGE_2, ONO-DI-004, and ONO-AE-248 were similar to each other, approximately 19 %CCh (Table 2; Fig. 3g). Therefore, the amplitude of about 19 %CCh amplitude might be the minimum limit of GCLCs. The EC_{50} for PGE_2-, ONO-DI-004-, and ONO-AE-248-induced GCLCs were the same values with the values in the amplitude, respectively. However, the V_{max} were very similar for these treatments, unlike those for frequency. Therefore, it seems that the activation of EP_1 and EP_3 receptors provide equal contributions to the enhanced amplitude. These results are supported by the observation that PGE_2 induces LM tonus via post-junctional EP_1 and EP_3 receptors in the mouse ileum and proximal colon [24]. However, an EP_1 antagonist (ONO-8713) hardly affected spontaneous GCs even though an EP_1 agonist (ONO-DI-004) induced GCLCs. A possible reason for this is that, in rats, the affinity of PGE_2 for the EP_1 receptor ($K_i = 2 \times 10^{-8}$ M) is weaker than that for the EP_3 receptor ($K_i = 10^{-9}$ M; [21]). Therefore, spontaneous GCs occurred at PGE_2 concentrations $<10^{-8}$ M, and EP_3 receptor was more strongly activated than the EP_1 receptor in these conditions.

Mechanism of EP_3 receptor-induced GCLCs

In the present study, neither TTX nor ω-conotoxin inhibited spontaneous GCs or PGE_2-induced GCLCs, indicating that the GCs/GCLCs induced via the EP_3 receptor are not or are only weakly controlled by the enteric nerves. These results suggest that PGE_2-induced GCLCs are induced via direct activation of EP_3 receptors expressed on smooth muscle cells. However, some previous studies have reported that PGE_2 depolarizes enteric neurons [36, 37], and induces LM contraction via lidocaine-sensitive, TTX-insensitive nerves in the human and mouse colon [24]. EP_3 receptors are reported to be expressed by submucosal neurons in rat colon [38]. Our immunohistochemical studies revealed that the EP_1 and EP_3 receptors were also expressed on myenteric neurons (Fig. 6c, d, mg, arrowheads). However, ω-conotoxins—exocytosis inhibiter—did

not inhibit spontaneous GCs or PGE_2-induced GCLCs. Sarna et al. [30] suggested that the GMCs, in vivo, reduced by atropine or hexamethonium, whereas, in vitro, the GCs were not affected by cholinergic and nicotinic antagonist, indicating GCs in vitro induced by myogenic. Furthermore, according to Huizinga et al. [39], spontaneous GCs of strip in in vitro may be induced by stretch which open Ca^{2+} channel. Thus, it seems that PGE_2 must activate EP_3 receptors expressed on enteric neurons, but does not contribute to the induction of GCs.

Our immunohistochemical studies showed the presence of EP_1 and EP_3 IR on neurons and on smooth muscle cells (Figs. 4, 5). However, they were not expressed on c-Kit-positive ICCs (Fig. 6a″, b″). In smooth muscle cells, the IR was localized around the nuclei, but not around the plasma membrane (Figs. 4d, 5d, arrows). Although there are no reports on EP_1 or EP_3 expression in intestinal smooth muscle, it has been reported that EP_3 receptors are localized on the perinuclear region in LM cells of the ovine cervix uteri [40]. Perinuclear localization of EP receptors has also been reported in other tissues/species, including human myometrium [41] and porcine cerebral endothelial cells [42]. Zhu et al. [43] also reported that EP_3 is localized on the nuclear membrane and the plasma membrane, and works via distinct signaling pathways as nuclear EP_3 regulates gene expression and plasma membrane EP_3 regulates the immediate physiologic effects. Therefore, it can be hypothesized that exogenous PGE_2 and EP agonists enter smooth muscle cells and activate EP_3 receptors expressed on the nuclear membrane and EP_1 receptors in the nucleus. This might help explain why higher concentrations of PGE_2 and EP receptor agonists are necessary to induce responses in isolated smooth muscle from the rat colon than in receptor-transfected cell lines. However, it is unclear how extracellular PGE_2 and EP agonists enter the smooth muscle cells, and how the activations of perinuclear EP_3 and nuclear EP_1 receptor lead to enhance the motility. Thus, it is necessary to perform further study about the cellular mechanism in future. Incidentally, we found that the EP_1 and EP_3 receptors are also expressed on neurons (Figs. 4, 5). However, these receptors on neurons are not or are only weakly involved in the GCs/GCLCs because of the pharmacological results mentioned above.

Physiological role of PGE_2-regulated spontaneous GCs

The actual concentration of PGs in the extracellular solution of the tissue is difficult to determine. Therefore, we estimated the concentration of PGE_2 during spontaneous GCs in LM based on the results for spontaneous GCs (Fig. 1b, c) and the concentration-dependent plots of PGE_2 (Fig. 3f, g; Table 2). In the present study, the concentration of PGE_2 was estimated to be 1.9×10^{-8} M and 7.3×10^{-8} M when we substituted the frequency and mean amplitude of spontaneous GCs into the curve-fit equations of the PGE_2-induced GC-like response. These values are consistent with the PGE_2 concentration of $\sim 2.6 \times 10^{-8}$ M that was estimated by Kaji et al. [44]. Although the PGE_2 concentrations might increase as a result of tissue preparation, PGE_2 concentrations in the range of 10^{-9} to 10^{-8} are considered to be physiologic in the rat middle colon. In the present study, 10^{-8} M PGE_2 was a threshold concentration for GC generation (Fig. 3g). PGE_2 levels were reported to be increased by mechanical stimuli [45] and by inflammatory injury [e.g. 46, 47], and concentrations $>10^{-7}$ M are considered to be pathophysiologic [7]. At pathophysiologic concentrations of PGE_2, LM activity is potently enhanced as is mucosal secretion [48]. Hence, in the present study, we hypothesized that the PGE_2-induced GCs are regulated in two ways—switching of contractions and regulation of volume—to control the frequency and amplitude of GCs. Thus, PGE_2 might play a role in triggering LM motility at a threshold concentration of 10^{-8} M. In this way, LM motility is directly enhanced via EP_3 receptors on smooth muscle in response to increased PGE_2 concentrations occurring during inflammation to flush out the GI contents.

Conclusion

In conclusion, the present study suggests that PGE_2 at physiologic concentrations ($<10^{-7}$ M) regulates colonic LM motility by switching GCs on or off. This is achieved by directly activating EP_3 receptors and, to a lesser extent EP_1 receptors, expressed in the perinuclear region of the colonic LM cells.

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Conflict of interest No conflicts of interest, financial or otherwise, declared by the authors.

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