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ORIGINAL ARTICLE

Docosahexaenoic acid and eicosapentaenoic acid strongly inhibit prostanoid TP receptor-dependent contractions of guinea pig gastric fundus smooth muscle

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Abstract

The inhibitory effects of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and linoleic acid (LA) on the contractions induced by five prostanoids and U46619 (a TP receptor agonist) were examined in guinea pig gastric fundus smooth muscle (GFSM). Tension changes were isometrically measured, and the mRNA expression of prostanoid receptors was measured by RT-qPCR. DHA and EPA significantly inhibited contractions induced by the prostanoids and U46619, whereas LA inhibited those induced by prostaglandin D_2 and U46619. The mRNA expression levels of the prostanoid receptors were TP ≈ EP_3 >> FP > EP_1. The inhibition by DHA, EPA, and LA was positively correlated with that by SQ 29,548 (a TP receptor antagonist) but not with that by L-798,106 (an EP_3 receptor antagonist). DHA and EPA suppressed high KCl-induced contractions by 35% and 25%, respectively, and the contractions induced by the prostanoids and U46619 were suppressed by verapamil, a voltage-dependent Ca^{2+} channel (VDCC) inhibitor, by 40%–85%. Although LA did not suppress high KCl-induced contractions, it suppressed U46619-induced contractions in the presence of verapamil. However, LA did not show significant inhibitory effects on U46619-induced Ca^{2+} increases in TP receptor-expressing cells. In contrast, LA inhibited U46619-induced contractions in the presence of verapamil, which was also suppressed by SKF-96365 (a store-operated Ca^{2+} channel [SOCC] inhibitor). These findings suggest that the TP receptor and VDCC are targets of DHA and EPA to inhibit prostanoid-induced contractions of guinea pig GFSM, and SOCCs play a significant role in LA-induced inhibition of U46619-induced contractions.

KEYWORDS
docosahexaenoic acid, eicosapentaenoic acid, gastric fundus smooth muscle, *n*–3 polyunsaturated fatty acids, prostanoid TP receptor, prostanoids

Abbreviations: AUC, area under the curve; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; EPA, eicosapentaenoic acid; GFSM, gastric fundus smooth muscle; LA, linoleic acid; *n*–3 PUFA, *n*–3 polyunsaturated fatty acid; PGA_2, prostaglandin A_2; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGI_2, prostaglandin I_2; ROCC, receptor-operated Ca^{2+} channel; SOCC, store-operated Ca^{2+} channel; TXA_2, thromboxane A_2; VDCC, voltage-dependent Ca^{2+} channel.

1 | INTRODUCTION

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are *n*−3 polyunsaturated fatty acids (*n*−3 PUFAs) that are abundant in fish oil. DHA and EPA have been shown to be effective in preventing various cardiovascular diseases (e.g., ischemic heart disease, ventricular arrhythmias, hypertension, atherosclerosis, and heart failure).[1] These *n*−3 PUFAs have also been reported to exert preventive effects on many non-cardiovascular diseases such as dyslipidemia, diabetes, neurodegenerative diseases, autoimmune diseases, inflammatory diseases, and malignant tumors.[2–6] Although the mechanisms by which DHA and EPA exert preventive effects on these diseases have not been fully elucidated, the suppression of contractile prostanoid production resulting from long-term ingestion of these *n*−3 PUFAs is suggested to play an important role in their cardiovascular protection.[7] In contrast, we found that DHA and EPA selectively suppressed blood vessel contractions induced by U46619 (a thromboxane A_2 [TXA_2] mimetic) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which suggests that direct and immediate inhibition by DHA and EPA against prostanoid-induced vascular contractions partly accounts for their protective effects.[8–11]

Prostanoids play important roles in the regulation of contractile responses not only in tonic muscles, such as blood vessels, but also in phasic muscles, including the gastrointestinal tract. For example, various prostanoids and a prostanoid mimetic (U46619) have been reported to produce gastric fundus smooth muscle (GFSM) contractions in experimental animals and humans, namely prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, prostaglandin I_2 (PGI_2),[12] and U46619[13] in mice; PGE_2,[14] $PGF_{2\alpha}$,[15] PGI_2,[16] and U46619[17] in guinea pigs; prostaglandin A_2 (PGA_2),[18] PGD_2,[19] PGE_2, $PGF_{2\alpha}$, PGI_2,[20] and U46619[21] in rats; and PGD_2, PGE_2, $PGF_{2\alpha}$, and U46619[22] in humans. Furthermore, prostaglandin overproduction has been suggested to cause gastric dyskinesia.[23] The long-term intake of fish oil including DHA and EPA has been reported to significantly reduce the production of prostanoids (thromboxane B_2 (TXB_2) [a metabolite of TXA_2], PGE_2, and $PGF_{2\alpha}$) in rat stomach.[24] Therefore, the long-term intake of these *n*−3 PUFAs is expected to improve gastric dyskinesia induced by overproduced prostanoids. In addition to the suppression of contractile prostanoid production, if DHA and EPA selectively suppress prostanoid-induced contractions in GFSM, these immediate effects could be involved in improving gastric dyskinesia induced by overproduced prostanoids. However, the immediate effects of DHA and EPA on the prostanoid-induced contractile responses of GFSM have not been examined.

Regarding the mechanisms of the immediate effects of DHA and EPA on the prostanoid-induced contractile responses of GFSM, prostanoids have been reported previously to bind multiple prostanoid receptors,[25] and multiple prostanoid receptors were shown to mediate the stomach contractions induced by PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2 in knockout mice.[12] In addition, we have reported that DHA and EPA could affect verapamil-sensitive Ca^{2+} signaling pathways in the guinea pig's lower gastrointestinal tract, suggesting the involvement of L-type Ca^{2+} channels.[26] However, to the best of our knowledge, the prostanoid receptor subtypes and putative Ca^{2+} signaling pathways in GFSM contractions have not been examined in guinea pigs to date.

In this study, in order to clarify the immediate effects of *n*−3 PUFAs on the promotion of gastric motility by prostanoids, DHA and EPA were examined for their ability to inhibit guinea pig GFSM contractions induced by various prostanoids and U46619. These inhibitory activities were compared with those of linoleic acid (LA), a representative *n*−6 PUFA. In addition, to identify potential mechanisms involved in the inhibitory effects of these PUFAs, the prostanoid receptor subtypes and putative Ca^{2+} signaling pathways responsible for the contractions induced by prostanoids and U46619 were investigated pharmacologically.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Hartley guinea pigs (4–16 weeks old; weight 283–670 g, Kyudo Co. Ltd.) were housed under controlled conditions (21°C–22°C, relative air humidity 50% ± 5%) and a fixed 12-h light-dark cycle (08:00–20:00) and provided with food and water ad libitum. This study was approved by the Toho University Animal Care and Use Committee (approval numbers: 18–54–294, 19–55–294, 20–51–444, 21–52–444) and was conducted in accordance with the guidelines of the Laboratory Animal Center of the Faculty of Pharmaceutical Sciences, Toho University.

2.2 | GFSM preparation

The guinea pigs were anesthetized with isoflurane (inhalation) and exsanguinated from the carotid artery. The stomach was immediately removed and placed in Locke–Ringer solution containing (in mM) NaCl, 154; KCl, 5.6; $CaCl_2$, 2.2; $MgCl_2$, 2.1; $NaHCO_3$, 5.9; and glucose, 2.8. After removing the adipose and connective tissues in Locke–Ringer solution, the stomach was separated into the gastric fundus and gastric body. After irrigating its interior with Locke–Ringer solution, the gastric fundus was cut longitudinally, and the epithelium was removed using cotton swabs, tweezers, and dissecting scissors to prepare GFSM (approximately 5–20 mm in length and 2–3 mm in width).

2.3 | Tension changes

The GFSM preparations were suspended in a 20-ml organ bath containing Locke–Ringer solution, which was oxygenated with 95% O_2 and 5% CO_2 and maintained at 32°C ± 1°C. These strips were subjected to a constant resting tension (1.0 g) and allowed

to equilibrate for 60 min while exchanging the bath solution. Muscle tension changes were isometrically recorded with a force-displacement transducer (FORT 25, World Precision Instruments; TB-612T, Nihon Kohden) connected to a carrier amplifier (TBM4 M, World Precision Instruments; AP-621G, Nihon Kohden; signal conditioner MSC-2, Labo Support Co.) and recorded using PowerLab™ and LabChart™ (Version 7) software (ADInstruments). After 60-min incubation, the GFSM preparations were contracted using carbachol (10^{-5} M) at least three times with an interval of 10 min. All experiments were carried out in the presence of indomethacin (3×10^{-6} M) to inhibit any potential influence of endogenous prostaglandins.

2.4 | Effects of DHA, EPA, LA, and verapamil on the GFSM contractions induced by prostanoids and U46619

After carrying out the procedures described in Section 2.3, the GFSM preparations were contracted using PGA_2 (3×10^{-6} M), PGD_2 (3×10^{-6} M), PGE_2 (10^{-7} M), $PGF_{2\alpha}$ (10^{-6} M), PGI_2 (10^{-6} M), or U46619 (3×10^{-6} M) for 10 min at least twice with an interval of 30 min. After stable contractions were obtained, ethanol (0.1%), DHA (3×10^{-5} M), EPA (3×10^{-5} M), LA (3×10^{-5} M), or verapamil (10^{-5} M, a voltage-dependent Ca^{2+} channel (VDCC) inhibitor) was added to the bath solution. After an equilibration period of 30 min, the GFSM preparations were contracted using the tested prostanoid/U46619 for 10 min.

2.5 | Effect of SQ 29,548 on the GFSM contractions induced by prostanoids and U46619

After carrying out the procedures described in Section 2.3, the GFSM preparations were contracted using PGA_2 (3×10^{-6} M), PGD_2 (3×10^{-6} M), PGE_2 (10^{-7} M), $PGF_{2\alpha}$ (10^{-6} M), PGI_2 (10^{-6} M), or U46619 (3×10^{-6} M) for 10 min at least twice with an interval of 30 min. Subsequently, ethanol (1.5%), which was the solvent for SQ 29,548 (a selective TP receptor antagonist), was added to the bath solution. After an equilibration period of 30 min, the GFSM preparations were contracted using the tested prostanoid/U46619 for 10 min. Next, SQ 29,548 (3×10^{-5} M) was added to the bath solution, and after an equilibration period of 30 min, the GFSM preparations were contracted by the tested prostanoid/U46619 for 10 min.

2.6 | Effect of L-798,106 on the GFSM contractions induced by prostanoids and U46619 in the presence of SQ 29,548

After carrying out the procedures described in Section 2.3, the GFSM preparations were contracted using PGA_2 (3×10^{-6} M), PGD_2 (3×10^{-6} M), PGE_2 (10^{-7} M), $PGF_{2\alpha}$ (10^{-6} M), or PGI_2 (10^{-6} M) for 10 min at least twice with an interval of 30 min. Ethanol (1.5%) and dimethyl sulfoxide (DMSO, 0.015%), which were the solvents for SQ 29,548 and L-798,106 (a selective EP_3 receptor antagonist), respectively, were then added to the bath solution. After an equilibration period of 30 min, the GFSM preparations were contracted using the tested prostanoid for 10 min. Next, SQ 29,548 (3×10^{-5} M) and DMSO (0.015%) were added to the bath solution, and after an equilibration period of 30 min, the GFSM preparations were contracted by the tested prostanoid for 10 min. SQ 29,548 (3×10^{-5} M) and L-798,106 (3×10^{-7} M) were then added to the bath solution, and after an equilibration period of 30 min, the GFSM preparations were contracted by the tested prostanoid for 10 min.

2.7 | Effects of DHA and EPA on the concentration-response curves (CRCs) of U46619

The strips were subjected to a constant resting tension (1.0 g) in a 10–20-ml organ bath and allowed to equilibrate for 60 min while exchanging the bath solution. After the 60-min incubation, the GFSM preparations were contracted using carbachol (10^{-5} M) at least twice with an interval of 10 min. After these procedures, the GFSM preparations were contracted using U46619 (3×10^{-6} M) for 10 min at least twice with an interval of 30 min. After 30 min of incubation, U46619 (10^{-9}–3×10^{-6} M) was cumulatively added to the bath medium at least once with an interval of 30 min. Afterward, DHA or EPA (10^{-5} M or 3×10^{-5} M) was added to the bath medium. After 30 min of incubation, U46619 (10^{-9}–3×10^{-6} M) was cumulatively added to the bath medium.

2.8 | Effects of DHA, EPA, LA, and verapamil on the GFSM contractions induced by 80 mM KCl

After carrying out the procedures described in Section 2.3, the bath solution was changed to 80 mM KCl solution containing (in mM) NaCl, 79.6; KCl, 80.0; $CaCl_2$, 2.2; $MgCl_2$, 2.1; $NaHCO_3$, 5.9; and glucose, 2.8 and incubated for 10 min. After at least two cycles of 80 mM KCl-induced contractions were obtained with an interval of 30 min, ethanol (0.1%), DHA (3×10^{-5} M), EPA (3×10^{-5} M), LA (3×10^{-5} M), or verapamil (10^{-5} M) was added to the bath solution. After an equilibration period of 30 min, the bath solution was changed to 80 mM KCl solution containing the tested drug (ethanol, DHA, EPA, LA, or verapamil) and incubated for 10 min.

2.9 | Effect of LOE 908, SKF-96365, and LA on the GFSM contractions induced by U46619 in the presence of verapamil

After carrying out the procedures described in Section 2.3, the GFSM preparations were contracted using U46619 (3×10^{-6} M)

for 10 min at least twice with an interval of 30 min. Subsequently, verapamil (10^{-5} M), verapamil (10^{-5} M) plus DMSO (0.05%, the solvent for LOE 908), or verapamil (10^{-5} M) plus ethanol (0.1%, the solvent for LA) was added to the bath solution. After an equilibration period of 30 min, the GFSM preparations were contracted with U46619 for 10 min. Next, verapamil (10^{-5} M) plus SKF-96365 (3×10^{-5} M, a store-operated Ca^{2+} channel [SOCC] and receptor-operated Ca^{2+} channel [ROCC] inhibitor), verapamil (10^{-5} M) plus LOE 908 (3×10^{-5} M, an ROCC inhibitor), or verapamil (10^{-5} M) plus LA (3×10^{-5} M) was added to the bath solution. After an equilibration period of 30 min, the GFSM preparations were contracted with U46619 for 10 min.

2.10 | Quantitative reverse transcription PCR (RT-qPCR) of mRNA expression of prostanoid receptors in GFSM

Total RNA was extracted from isolated guinea pig GFSM using the acid guanidinium thiocyanate-phenol-chloroform extraction method.[27] The extracted total RNA was treated with deoxyribonuclease (Nippon Gene Co. Ltd.) at 37°C for 30 min. Phenol-chloroform extraction was performed after the removal of contaminating DNA, followed by ethanol precipitation. The RNA pellets were dissolved in diethyl pyrocarbonate-treated water. First-strand cDNA was synthesized by reverse transcription with 1 μg total RNA per 20 μl reaction mixture using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO Co. Ltd.) according to the manufacturer's protocol.

RT-qPCR was performed using the THUNDERBIRD® Next SYBR® qPCR Mix (TOYOBO Co. Ltd.) according to the manufacturer's protocol. The primers used in this study are listed in Table S1. PCR and DNA amplification (fluorescence intensity) measurements were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). The thermal cycler parameters were set at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 35 s. The amplified DNA fragment (fluorescence intensity) was measured at each elongation step. After PCR completion, the melting curve of each PCR product was measured from 60°C–95°C. The data were analyzed using Sequence Detection Software Version 1.4 (Applied Biosystems). Samples that did not reach the fluorescence intensity threshold after 40 cycles of amplification were judged to have no detectable mRNA expression. The mRNA expression level of each gene was calculated as a relative value, normalized to the mRNA expression level of the *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) gene, which was set to 1.

2.11 | Drugs

The following drugs were used in this study: PGA_2; PGE_2; PGI_2; U46619; EPA; SQ 29,548; L-798,106; and SKF-96365 (Cayman Chemical Co.); DHA (Cayman Chemical Co., or Tokyo Chemical Industry Co., Ltd.); LA (Cayman Chemical Co., or Nacalai Tesque, Inc.); carbamoylcholine chloride; indomethacin; and (±)-verapamil hydrochloride (Sigma-Aldrich Co.); PGD_2 (Cayman Chemical Co., or FUJIFILM Wako Pure Chemical Co.); $PGF_{2\alpha}$, (Fuji Pharma Co. Ltd.); and LOE 908 (Nippon Boehringer Ingelheim Co. Ltd.).

DHA, EPA, and LA were dissolved in ethanol to prepare stock solutions of 3×10^{-2} M. PGA_2, PGD_2, PGE_2, PGI_2, and U46619 were dissolved in ethanol to prepare stock solutions of 2×10^{-2} M. SQ 29,548 was dissolved in ethanol to prepare a stock solution of 2×10^{-3} M. Indomethacin was dissolved in ethanol to prepare a stock solution of 10^{-2} M. LOE 908 was dissolved in DMSO to prepare a stock solution of 6×10^{-2} M. L-798,106 was dissolved in DMSO to prepare a stock solution of 2×10^{-3} M. All other drugs were dissolved in and diluted with distilled water.

2.12 | Data analysis

The area under the curve (AUC) and contractions were analyzed using LabChart™. AUC was analyzed for 10 min after the administration of prostanoid/U46619/80 mM KCl. Contractions induced by prostanoid/U46619 were analyzed at the maximum contraction for 10 min. Contractions induced by 80 mM KCl were analyzed 10 min after KCl administration. The AUC and contractions in the presence of the tested drugs are shown as relative values, with the corresponding value in the absence of tested drugs set as 100%.

To construct the U46619 CRCs, the tension level before cumulative application of U46619 was defined as 0% contraction, and the maximum contractions of U46619 (3×10^{-6} M) before administrations of DHA/EPA were designated as 100%. The data were plotted as a function of agonist concentration and fitted using GraphPad Prism™ (Version 6) (GraphPad Software Inc.). The pA_2 value of DHA/EPA versus U46619 was calculated from a Schild plot analysis of DHA/EPA versus U46619.

Data are expressed as the means ± SEM or the means with 95% confidence intervals, where n refers to the number of experiments. Statistical analyses were carried out using paired t-tests, multiple t-tests, or one-way ANOVA, followed by *post hoc* Dunnett's test, as appropriate, using GraphPad Prism™. All statistical analyses were conducted with a significance level of $\alpha = 0.05$ ($p < .05$).

3 | RESULTS

3.1 | Effects of DHA, EPA, and LA on prostanoid- and U46619-induced contractions of GFSM

Figure 1 shows representative experimental traces of the effects of DHA (3×10^{-5} M, a), EPA (3×10^{-5} M, b), and LA (3×10^{-5} M, c) on the GFSM contractions induced by five prostanoids (PGA_2 (3×10^{-6} M, A), PGD_2 (3×10^{-6} M, B), PGE_2 (10^{-7} M, C), $PGF_{2\alpha}$ (10^{-6} M, D), PGI_2 (10^{-6} M, E)), and U46619 (3×10^{-6} M, F). Figure 2

shows the quantitative analyses of the results of the experiments shown in Figure 1. DHA and EPA significantly suppressed all GFSM contractions by the examined prostanoids and U46619. Particularly, DHA and EPA strongly suppressed the contractions induced by PGD_2 (Figures 1B and 2B) and U46619 (Figures 1F and 2F); the inhibition by DHA (3×10^{-5} M) at the AUC level was 76.9% ± 6.0% for PGD_2 ($n = 5$) and 64.2% ± 5.3% for U46619 ($n = 5$), and the inhibition by EPA (3×10^{-5} M) at the AUC level was 52.3% ± 7.0% for

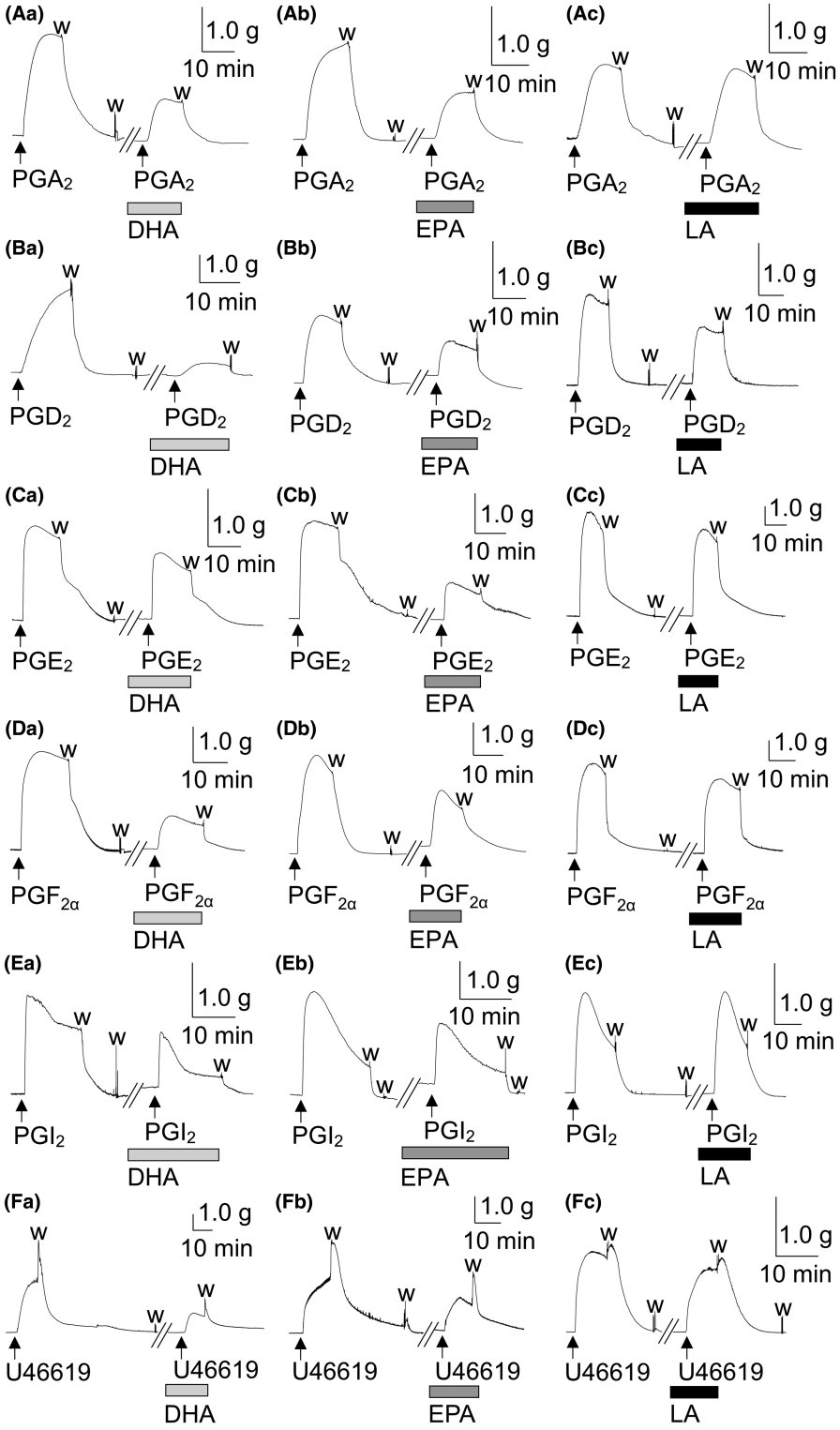

FIGURE 1 Representative traces showing the effects of docosahexaenoic acid (DHA, a), eicosapentaenoic acid (EPA, b), and linoleic acid (LA, c) (each 3×10^{-5} M) on guinea pig gastric fundus smooth muscle contractions induced by prostaglandin (PG) A_2 (3×10^{-6} M, A), PGD_2 (3×10^{-6} M, B), PGE_2 (10^{-7} M, C), $PGF_{2\alpha}$ (10^{-6} M, D), PGI_2 (10^{-6} M, E), and U46619 (3×10^{-6} M, F). w, wash out

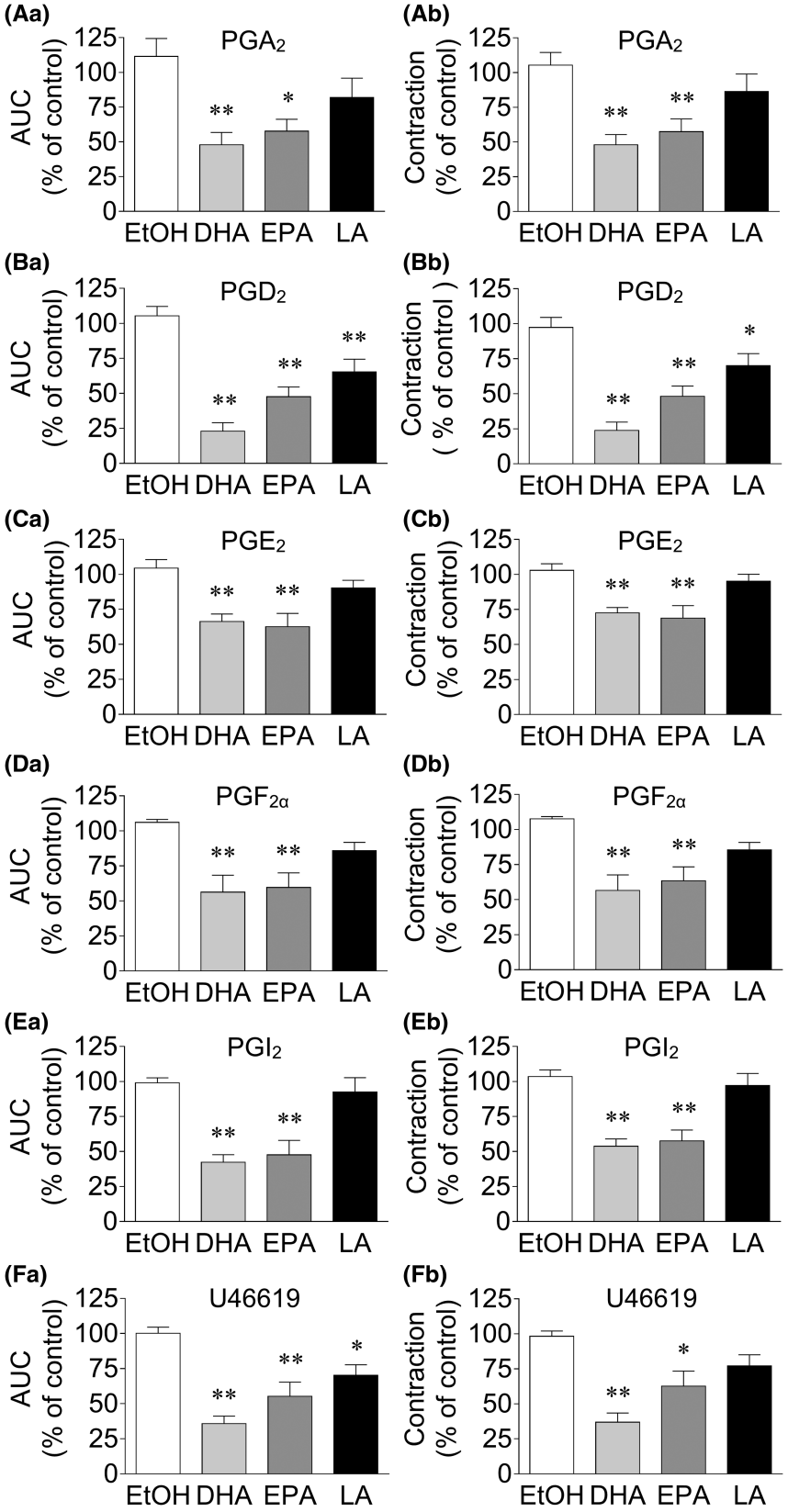

FIGURE 2 Quantified data of the effect of ethanol (EtOH, 0.1%), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and linoleic acid (LA) (each 3×10^{-5} M) on the area under the curve for 10 min (AUC, a) and maximum contractions (b) of guinea pig gastric fundus smooth muscle responses induced by prostaglandin (PG) A_2 (3×10^{-6} M, A), PGD_2 (3×10^{-6} M, B), PGE_2 (10^{-7} M, C), $PGF_{2\alpha}$ (10^{-6} M, D), PGI_2 (10^{-6} M, E), and U46619 (3×10^{-6} M, F) shown in Figure 1. Data are expressed as the means ± SEM. ($n = 13$ (LA in F), $n = 7$ (EPA in F), $n = 6$ (LA in B), and $n = 5$ (all others)). *$p < .05$, **$p < .01$ versus EtOH (*post hoc* Dunnett's test after one-way ANOVA)

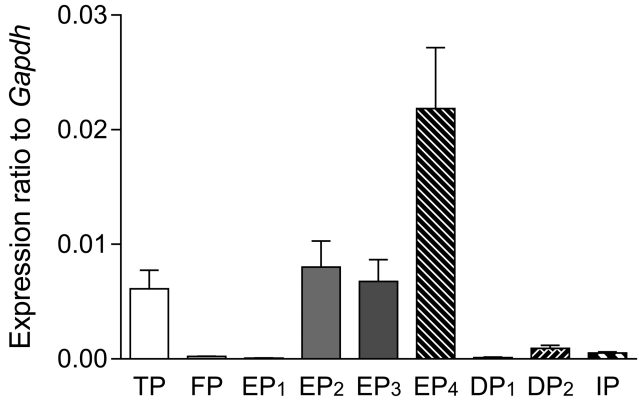

FIGURE 3 Comparison of prostanoid receptor mRNA expression levels in guinea pig gastric fundus smooth muscle. The mRNA expression levels of TP (*Tbxa2r*), FP (*Ptgfr*), EP_1 (*Ptger1*), EP_2 (*Ptger2*), EP_3 (*Ptger3*), EP_4 (*Ptger4*), DP_1 (*Ptgdr*), DP_2 (*Ptgdr2*), and IP (*Ptgir*) receptors were determined using RT-qPCR. The expression level of each mRNA is shown relative to the mRNA expression level of *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*), which is set as 1. Data are expressed as the means ± SEM ($n = 5$ each)

PGD_2 ($n = 5$) and 44.7% ± 10.1% for U46619 ($n = 5$). LA (3×10^{-5} M, Figure 1c) did not significantly suppress the contractions induced by PGA_2, PGE_2, $PGF_{2\alpha}$, and PGI_2, but significantly suppressed the contractions induced by PGD_2 and U46619.

The mean forces induced by the prostanoids and U46619 before ethanol treatment (control) shown in Figure 2 were as follows: PGA_2, 10.4 mN; PGD_2, 18.3 mN; PGE_2, 18.3 mN; $PGF_{2\alpha}$, 18.2 mN; PGI_2, 19.6 mN; and U46619, 23.6 mN.

3.2 | Expression of mRNA of various prostanoid receptors in GFSM tissues

Figure 3 shows the relative mRNA expression levels of various prostanoid receptors in GFSM tissues, which were determined by RT-qPCR. The most abundant prostanoid receptor mRNA examined was EP_4 (*Ptger4*), followed by EP_2 (*Ptger2*), EP_3 (*Ptger3*), and TP (*Tbxa2r*), the expression levels of which were almost comparable. When the expression level of EP_4 was regarded as 100%, the expression levels of EP_2, TP, and EP_3 were 36.7%, 31.0%, and 28.0%, respectively. The mRNA expression levels of the other prostanoid receptors were less than 5% of the EP_4 receptor mRNA level: DP_2 (*Ptgdr2*, 4.2%), IP (*Ptgir*, 2.4%), FP (*Ptgfr*, 1.0%), DP_1 (*Ptgdr*, 0.6%), and EP_1 (*Ptger1*, 0.3%). The mRNA expression levels of the contractile prostanoid receptors[12,28] in guinea pig GFSM were in the order of TP ≈ EP_3 >> FP > EP_1.

3.3 | Effect of SQ 29,548 on the contractions induced by prostanoids and U46619

Figure 4A shows representative traces of the effects of SQ 29,548 (3×10^{-5} M) on the contractions produced by PGA_2 (3×10^{-6} M, a), PGD_2 (3×10^{-6} M, b), PGE_2 (10^{-7} M, c), $PGF_{2\alpha}$ (10^{-6} M, d), PGI_2 (10^{-6} M, e), and U46619 (3×10^{-6} M, f). Figure 4B shows the quantitative analyses of the results obtained from the experiments shown in Figure 4A. The contractions induced by PGD_2 and U46619 were strongly suppressed by SQ 29,548 (3×10^{-5} M) at both the AUC and contraction levels. For example, the inhibition by SQ 29,548 was 62.9% (PGD_2) and 72.8% (U46619) at the AUC level. These contractions by PGD_2 and U46619 that were highly inhibited by SQ 29,548 were also largely inhibited by DHA and EPA (Figures 1 and 2). Regarding PGA_2 and PGI_2, their contractions were partly inhibited by SQ 29,548 (3×10^{-5} M), 24.8% and 36.6%, respectively, at the AUC level. In contrast to the above four agonists, the contractions by PGE_2 and $PGF_{2\alpha}$ were not substantially inhibited by SQ 29,548; the inhibition by SQ 29,548 (3×10^{-5} M) at the AUC level was 4.5% (PGE_2) and 4.0% ($PGF_{2\alpha}$).

3.4 | Effect of L-798,106 on the contractions induced by PGA_2, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2 in the presence of SQ 29,548

Figure 5A shows representative experimental traces of the effects of L-798,106 (3×10^{-7} M) on the contractions induced by PGA_2 (3×10^{-6} M, a), PGD_2 (3×10^{-6} M, b), PGE_2 (10^{-7} M, c), $PGF_{2\alpha}$ (10^{-6} M, d), and PGI_2 (10^{-6} M, e) in the presence of SQ 29,548 (3×10^{-5} M). Figure 5B shows the quantified results of the experiments shown in Figure 5A. The most prominent inhibitory effect of L-798,106 was observed for PGA_2. The contraction by PGA_2 in the presence of SQ 29,548 (3×10^{-5} M) was strongly suppressed by 51.9% with L-798,106 (3×10^{-7} M), from 71.6% to 19.7% at the AUC level (100% is the contraction by PGA_2 in the absence of both SQ 29,548 and L-798,106) (Figure 5Aa and Ba).

The next strongest inhibitory effect of L-798,106 was shown for PGE_2 (Figure 5Ac) and PGI_2 (Figure 5Ae); the L-798,106-inhibitable components of PGE_2- and PGI_2-induced total contractions (100% for each) were estimated to be ~35%. The contractions induced by PGE_2 and PGI_2 in the presence of SQ 29,548 (3×10^{-5} M) were suppressed with L-798,106 (3×10^{-7} M) at the AUC level by 35.1% for PGE_2 (from 87.9% to 52.8%) and by 37.2% for PGI_2 (from 83.4% to 46.2%).

The least inhibitory effects of L-798,106 were observed for PGD_2 (Figure 5Ab) and $PGF_{2\alpha}$ (Figure 5Ad); the L-798,106-inhibitable components of PGD_2- and $PGF_{2\alpha}$-induced total contractions (100% for each) were estimated to be ~20%. The contractions induced by PGD_2 and $PGF_{2\alpha}$ in the presence of SQ 29,548 (3×10^{-5} M) were suppressed with L-798,106 (3×10^{-7} M) at the AUC level by 16.9% for PGD_2 (from 25.7% to 8.8%) and by 21.6% for $PGF_{2\alpha}$ (from 86.5% to 64.9%).

3.5 | Relationships between the inhibitory effects of DHA/EPA/LA and the inhibitory effects of SQ 29,548 and L-798,106

Figure 6a shows the relationships between the inhibitory effects of DHA (A)/EPA (B)/LA (C) on the contractions induced by prostanoids/U46619 (Figure 2Aa–Fa) and the inhibitory effects of SQ 29,548

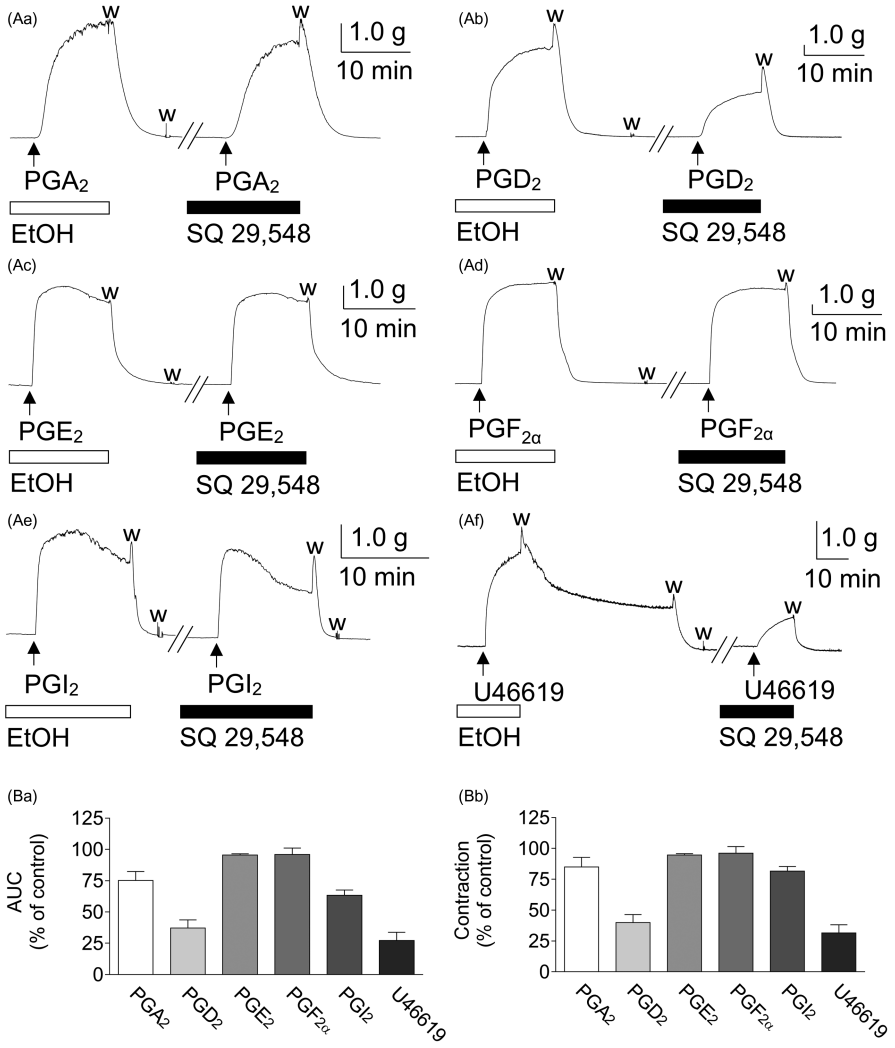

FIGURE 4 Representative traces (A) and quantified data (B) showing the effects of SQ 29,548 (3×10^{-5} M) on the area under the curve (AUC, Ba) and maximum contractions (Bb) of guinea pig gastric fundus smooth muscle responses induced by prostaglandin (PG) A_2 (3×10^{-6} M, Aa), PGD_2 (3×10^{-6} M, Ab), PGE_2 (10^{-7} M, Ac), $PGF_{2\alpha}$ (10^{-6} M, Ad), PGI_2 (10^{-6} M, Ae), and U46619 (3×10^{-6} M, Af). Data are expressed as the means ± SEM ($n = 11$ ($PGF_{2\alpha}$), $n = 10$ (PGD_2 and PGI_2), and $n = 5$ (all others)). EtOH, ethanol (1.5%); w, wash out

(Figure 4Ba). The relationships between the inhibitory effects of DHA (A)/EPA (B)/LA (C) and L-798,106 (Figure 5Ba) are also shown in Figure 6b. A positive correlation was found between the inhibitory effects of DHA (Figure 6Aa)/EPA (Figure 6Ba)/LA (Figure 6Ca) and the inhibitory effect of SQ 29,548 (Figure 6Aa–Ca). In contrast, no correlation was found between the inhibitory effects of DHA (Figure 6Ab)/EPA (Figure 6Bb)/LA (Figure 6Cb) and the inhibitory effect of L-798,106 (Figure 6Ab–Cb).

3.6 | Effects of different concentrations of DHA, EPA, and LA on U46619-induced contractions

Figure 7Aa and Ba show the pretreatment effects of DHA and EPA at two concentrations (10^{-5} M and 3×10^{-5} M) on the CRCs of U46619. Both DHA and EPA substantially suppressed the CRCs of U46619 in a concentration-dependent manner. Figure 7Ab and Bb show the Schild plot analysis carried out for DHA and EPA against U46619 based on the results of Figure 7Aa and Ba. The Schild plot analysis showed that DHA and EPA (10^{-5} M and 3×10^{-5} M) apparently inhibited the U46619-induced contractions in a competitive manner, which was evidenced by the slopes of the regression lines being close to unity (1.26 (0.38–2.14, $n = 12$) for DHA and 1.10 (0.24–1.97, $n = 22$) for EPA). The apparent pA_2 values of DHA and EPA were 5.13 (4.91–6.04, $n = 12$) and 4.92 (4.72–5.66, $n = 22$), which were not significantly different.

Figure S1 shows the pretreatment effects of LA at three concentrations (10^{-5} M, 3×10^{-5} M, and 10^{-4} M) on the contraction induced by U46619 (3×10^{-6} M). The inhibitory effect of LA was larger at 3×10^{-5} M than at 10^{-5} M. However, even at 10^{-4} M, the inhibitory effect of LA was the same as that at 3×10^{-5} M.

Figure S2 shows the effects of LA on U46619-induced Ca^{2+} increases in TP receptor-expressing 293T cells. LA (3×10^{-5} M) did not show sufficient inhibitory effects on the U46619-induced Ca^{2+} increases to explain the U46619-induced inhibition.

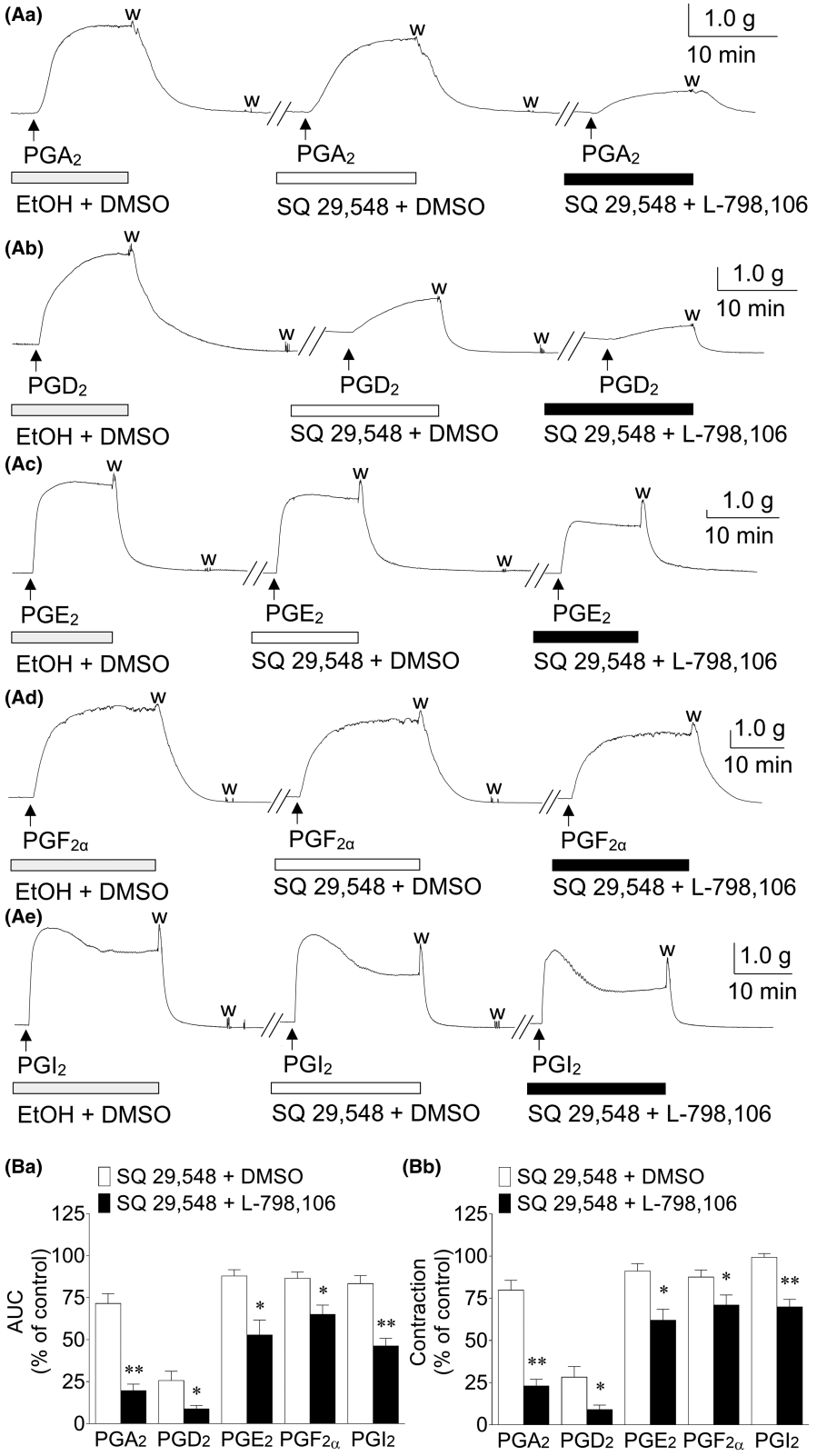

FIGURE 5 Representative traces (A) and quantified data (B) showing the effects of L-798,106 (3×10^{-7} M) on the area under the curve (AUC, Ba) and maximum contractions (Bb) of guinea pig gastric fundus smooth muscle responses induced by prostaglandin (PG) A_2 (3×10^{-6} M, Aa), PGD_2 (3×10^{-6} M, Ab), PGE_2 (10^{-7} M, Ac), $PGF_{2\alpha}$ (10^{-6} M, Ad), and PGI_2 (10^{-6} M, Ae) in the presence of SQ 29,548 (3×10^{-5} M). Data are expressed as the means ± SEM ($n = 12$ (PGD_2 and PGI_2), $n = 10$ ($PGF_{2\alpha}$), and $n = 5$ (all others)). $^{*}p < .05$, $^{**}p < .01$ versus SQ 29,548 + DMSO (multiple *t*-tests). EtOH: ethanol (1.5%); DMSO, dimethyl sulfoxide (0.015%); w, wash out

3.7 | Effects of DHA, EPA, and LA on 80 mM KCl-induced contractions

Figure 8A shows representative experimental traces of the effects of DHA, EPA, and LA (3×10^{-5} M for each) on 80 mM KCl-induced contractions. Figure 8B shows the quantified results. DHA and EPA significantly suppressed the 80 mM KCl-induced contractions; the inhibition by DHA and EPA at the AUC level was 32.9% ± 6.5% ($n = 5$) and 24.4% ± 7.4% ($n = 7$), respectively. In contrast, a substantial inhibitory effect was not observed with LA toward the KCl-induced

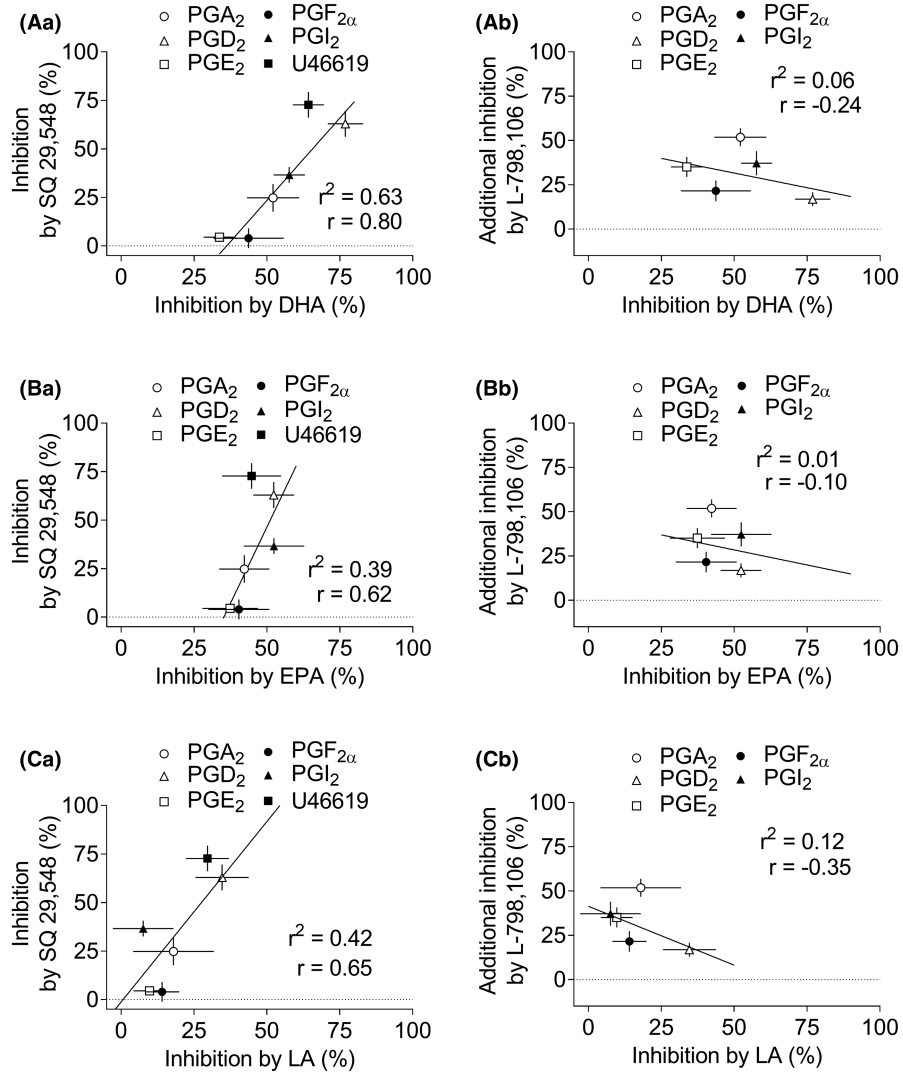

FIGURE 6 Relationships between the inhibitory effects of docosahexaenoic acid (DHA) (A), eicosapentaenoic acid (EPA) (B), and linoleic acid (LA) (C) on the area under the curve (AUC) of the contractions induced by prostanoids and U46619 (shown in Figure 2Aa–Fa) versus the inhibitory effects of SQ 29,548 on those contractions (shown in Figure 4Ba) (a) and versus the additional inhibitory effects of L-798,106 on those contractions in the presence of SQ 29,548 (shown in Figure 5Ba) (b). Data are expressed as the means ± SEM. *r*: correlation coefficient; r^2: coefficient of determination

contractions. Contraction with 80 mM KCl was completely suppressed by verapamil (10^{-5} M) ($n = 5$).

3.8 | Effect of verapamil on the contractions induced by the five prostanoids and U46619

Figure 9A shows representative experimental traces of the effect of verapamil on the contractions induced by PGA_2, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and U46619, and Figure 9B shows plots of the quantified results.

The most prominent inhibitory effect of verapamil (10^{-5} M) was shown against PGA_2 (3×10^{-6} M, Figure 9Aa), an inhibition of 85.2% ± 2.9% ($n = 5$) at the AUC level (Figure 9Ba). Regarding the other prostanoids (PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2) and U46619, verapamil (10^{-5} M) showed inhibitory effects of 40%–60% at the AUC level, specifically 61.1% ± 5.0% for PGD_2, 52.3% ± 6.9% for PGE_2, 44.1% ± 12.0% for $PGF_{2\alpha}$, 46.2% ± 9.8% for PGI_2, and 36.6% ± 3.7% for U46619 ($n = 5$ for all).

3.9 | Effects of LOE 908 and SKF-96365 on the contractions induced by U46619 in the presence of verapamil

Figure 10A,B show representative experimental traces of the effects of LOE 908 (Figure 10A) and SKF-96365 (Figure 10B) on the contractions induced by U46619 in the presence of verapamil. Figure 10C,D show plots of the quantified results.

U46619 (3×10^{-6} M)-induced contractions in the presence of verapamil (10^{-5} M) were not substantially inhibited by LOE 908 (3×10^{-5} M) (Figure 10A,C), but were significantly inhibited by

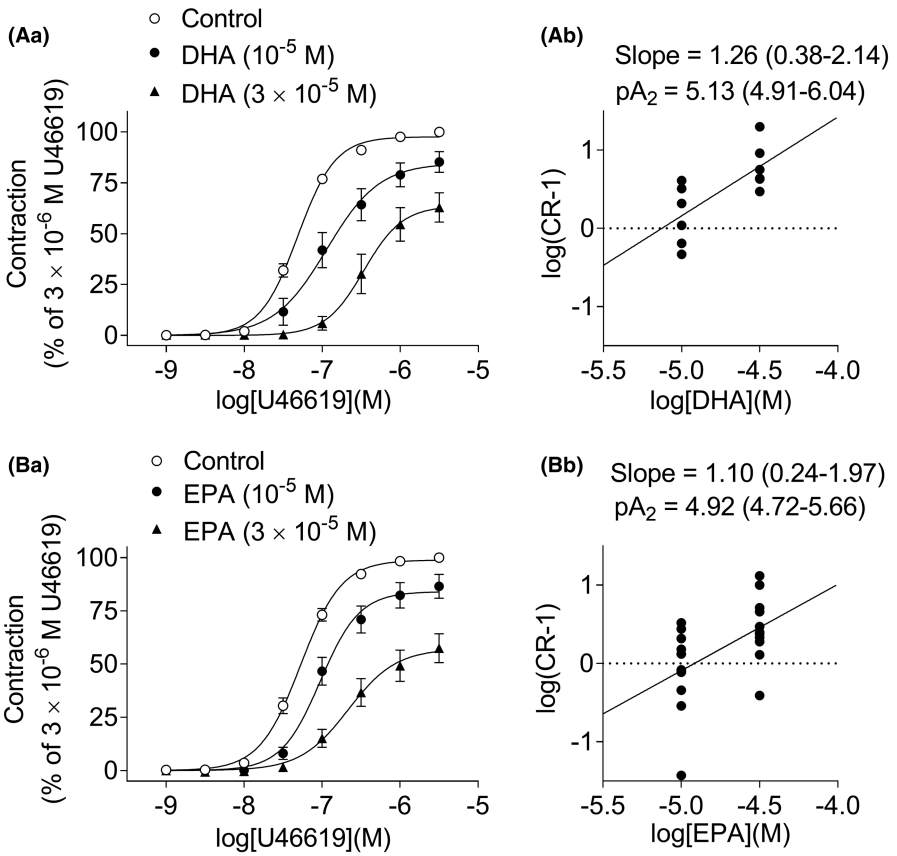

FIGURE 7 Effects of docosahexaenoic acid (DHA) (A) and eicosapentaenoic acid (EPA) (B) on the concentration-response curve of U46619. (a) Summarized data of the effects of DHA (10^{-5} M and 3×10^{-5} M) on the concentration-response curves of U46619. Data are presented as means ± SEM ($n = 12$ for control of Aa, $n = 6$ for 10^{-5} M DHA, $n = 6$ for 3×10^{-5} M DHA, $n = 23$ for control of Ba, $n = 11$ for 10^{-5} M EPA, $n = 12$ for 3×10^{-5} M EPA). (b) Schild plot analysis of DHA (A)/EPA (B) versus U46619. The slope and pA_2 values are presented as means with 95% confidence intervals ($n = 12$ for Ab and $n = 22$ for Bb)

SKF-96365 (3×10^{-5} M) (Figure 10B,D). Specifically, SKF-96365 (3×10^{-5} M) inhibited the contractions from 52.2% ± 7.7% to 16.4% ± 2.5% ($n = 5$) at the AUC level. LA (3×10^{-5} M) also significantly suppressed the contractions induced by U46619 (3×10^{-6} M) in the presence of verapamil (10^{-5} M) from 61.0% ± 28.8%% to 28.8 ± 5.4% ($n = 5$) at the AUC level (Figure S3).

4 | DISCUSSION

In this study, the effects of DHA, EPA, and LA on the contractile responses of isolated guinea pig GFSM to five prostanoids (PGA_2, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2) and a TXA_2 mimetic (U46619) were examined. The results showed that DHA and EPA significantly suppressed all contractions, whereas the inhibitory effects of LA were limited to the contractions induced by PGD_2 and U46619. In addition, the inhibitory effects of DHA and EPA were suggested to involve TP receptor antagonism and VDCC inhibition, whereas the inhibitory effects of LA were suggested to involve SOCC inhibition. DHA and EPA are expected to improve gastric dyskinesia induced by overproduced prostanoids.

Previous reports have shown that PGE_2,[14] $PGF_{2\alpha}$,[15] PGI_2,[16] and U46619[17] cause contractions in guinea pig GFSM. The present study showed that guinea pig GFSM is able to contract strongly in response to PGA_2 and PGD_2 in addition to the abovementioned prostanoids (PGE_2, $PGF_{2\alpha}$, and PGI_2) and U46619. DHA and EPA were also shown to inhibit all contractions induced by the tested prostanoids and U46619 by 40%–80%.

To date, we have reported that DHA and EPA very strongly suppress the contractions induced by $PGF_{2\alpha}$ and U46619 in guinea pig aorta,[8] rat aorta,[9] rat mesenteric arteries,[10] and porcine coronary and basilar arteries.[11] In addition, we found in the present study that DHA and EPA show immediate inhibitory effects against PGA_2, PGD_2, PGE_2, and PGI_2. To the best of our knowledge, we are the first to report the effects of these *n*−3 PUFAs. LA showed very weak inhibitory effects against PGA_2, PGE_2, $PGF_{2\alpha}$, and PGI_2; this degree of inhibition was clearly weaker than that of DHA and EPA and was not statistically significant. In contrast, LA showed significant inhibition against PGD_2 and U46619. In this regard, we previously reported that LA barely suppresses the contraction induced by U46619 in rat aorta[9] and mesenteric arteries.[10] Therefore, our results suggest that LA selectively suppresses TXA_2-induced hypercontraction of stomach smooth muscle without showing an inhibitory effect on vascular smooth muscle.

We recently found that DHA strongly suppresses U46619-/$PGF_{2\alpha}$-induced contractions in pig coronary and basilar arteries and U46619-/$PGF_{2\alpha}$-induced increases in Ca^{2+} concentrations in TP receptor-expressing cells, suggesting that the TP receptor is a primary target for DHA.[11] Therefore, in this study, to clarify the degree to which TP receptors contribute to the contractions induced by the five tested prostanoids (PGA_2, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2) and U46619, the inhibitory effects of SQ 29,548 (a TP receptor antagonist) against these contractions were examined and compared

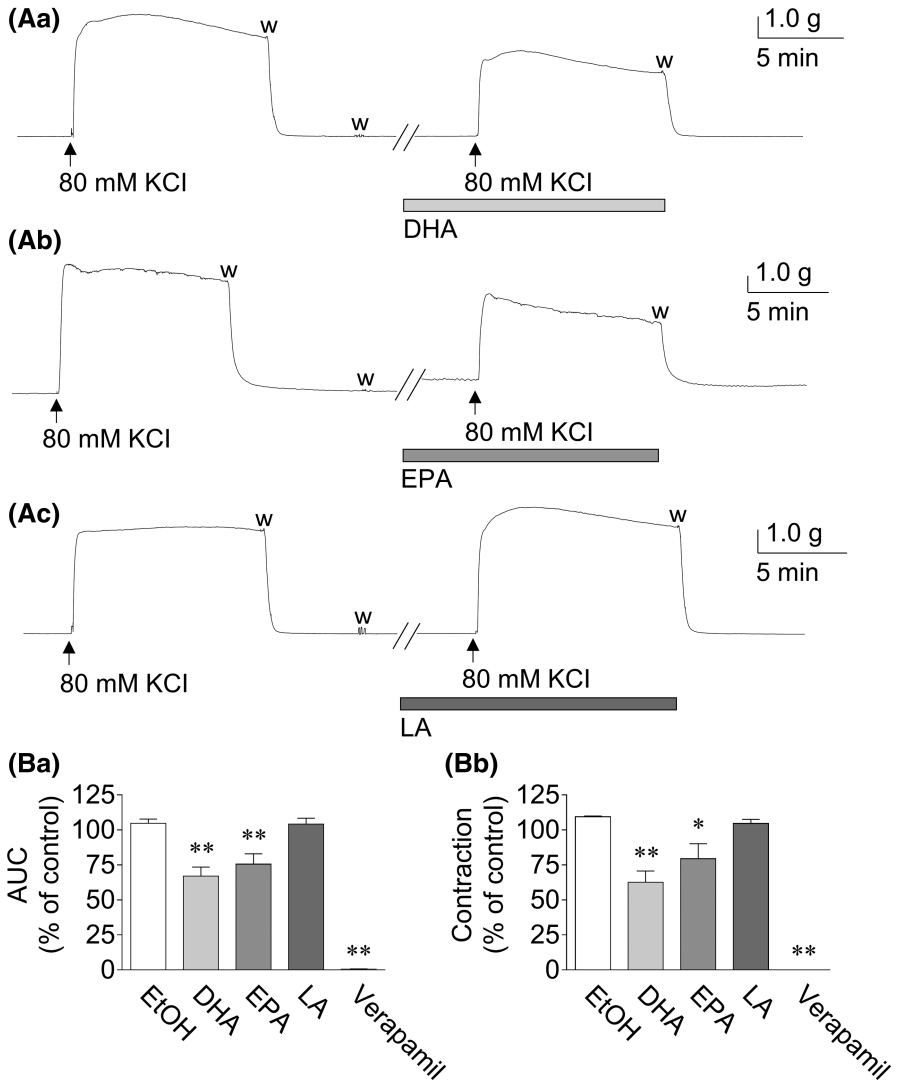

FIGURE 8 Representative traces (A) and quantified data (B) showing the effects of docosahexaenoic acid (DHA) (Aa), eicosapentaenoic acid (EPA) (Ab), and linoleic acid (LA) (Ac) (each 3×10^{-5} M) on the area under the curve (AUC) (Ba) and contractions (Bb) of guinea pig gastric fundus smooth muscle responses induced by 80 mM KCl. The quantified data of the effects of ethanol (EtOH, 0.1%) and verapamil (10^{-5} M) on those responses are also shown in Ba and Bb. Data are expressed as the means ± SEM ($n = 7$ (EPA) and $n = 5$ (all others)). $^{*}p < .05$, $^{**}p < .01$ versus EtOH (*post hoc* Dunnett's test after one-way ANOVA). w, wash out

with the suppression exhibited by DHA, EPA, and LA (Figure 6). The results suggest that the DHA-induced inhibitory effects against the GFSM contractions induced by the five tested prostanoids and U46619 were partly but substantially correlated with the inhibitory effect of SQ 29,548 ($r = .08$, $r^2 = .63$) (Figure 6Aa). The inhibitory effects of EPA were also partly correlated with the inhibitory effects of SQ 29,548 ($r = .62$, $r^2 = .39$) (Figure 6Ba), although the coefficient of determination (r^2) was smaller than that for DHA. SQ 29,548 is a specific TP receptor antagonist that does not show antagonistic effects at the concentration used in the present study (3×10^{-5} M).[25] Based on these findings, the five prostanoids and U46619 were divided into three categories, focusing on the degree of contribution of TP receptor antagonism to the inhibitory effects of DHA/EPA. For this categorization, we assumed that the SQ 29,548-inhibitable components were totally reflected in the DHA/EPA-induced inhibition. The three categories are as follows: (1) PGD_2 and U44619 (strongly inhibited by SQ 29,548). A large portion of the inhibitory effects of DHA/EPA was due to TP receptor antagonism; the inhibition by SQ 29,548/DHA was 62.9/76.9% versus PGD_2 and 72.8/64.2% versus U46619, and the inhibition by SQ 29,548/EPA was 62.9/52.3% versus PGD_2 and 72.8/44.7% versus U46619. 2) PGA_2 and PGI_2 (partly inhibited by SQ 29,548). Fifty to seventy percent of the inhibitory effects of DHA/EPA were due to TP receptor antagonism; the inhibition by SQ 29,548/DHA was 24.8/52.1% versus PGA_2 and 36.6/57.6% versus PGI_2, and the inhibition by SQ 29,548/EPA was 24.8/42.2% versus PGA_2 and 36.6/52.4% versus PGI_2. (3) PGE_2 and $PGF_{2\alpha}$ (almost no inhibition by SQ 29,548). The contribution of TP receptor antagonism was almost negligible. The significant role of the TP receptor in guinea pig GFSM contractions induced by various prostanoids and U46619 and this receptor being the primary target for DHA and EPA were also supported by the finding that the mRNA expression levels of the contractile prostanoid receptors in guinea pig GFSM were in the order of TP ≈ EP_3 >> FP > EP_1.

The examination of prostanoid receptors at the mRNA level showed that the EP_3 receptor was expressed to the same extent as the TP receptor. Therefore, to estimate the involvement of EP_3 receptors in contractions induced by the five prostanoids (PGA_2, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2), the inhibitory effects of L-798,106, an EP_3 receptor antagonist, on the prostanoid-induced contractions in the presence of SQ 29,548 were investigated. The effects of L-798,106 were evaluated for contractile components not suppressed by SQ 29,548 to eliminate the possibility that L-798,106 suppressed the TP receptor.

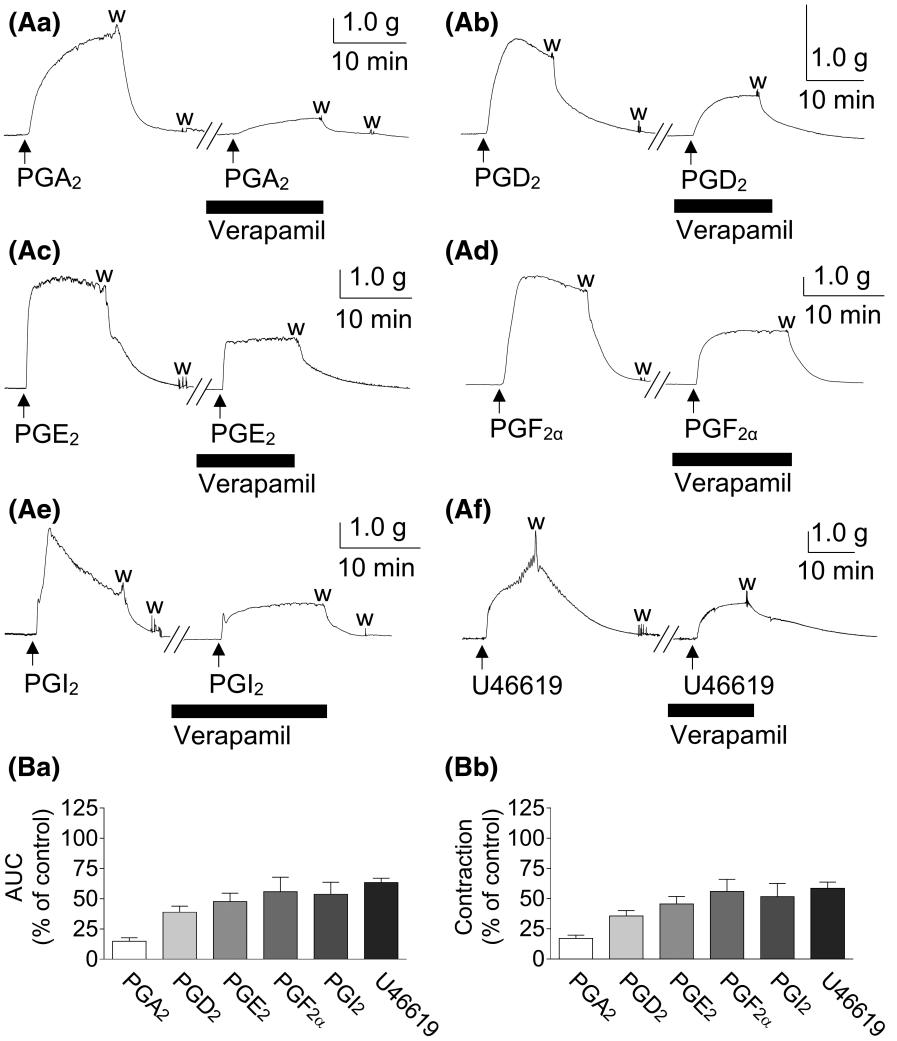

FIGURE 9 Representative traces (A) and quantified data (B) showing the effects of verapamil (10^{-5} M) on the area under the curve (AUC) (Ba) and maximum contractions (Bb) of guinea pig gastric fundus smooth muscle contractions induced by prostaglandin (PG) A_2 (3×10^{-6} M, Aa), PGD_2 (3×10^{-6} M, Ab), PGE_2 (10^{-7} M, Ac), $PGF_{2\alpha}$ (10^{-6} M, Ad), PGI_2 (10^{-6} M, Ae), and U46619 (3×10^{-6} M, Af). Data are expressed as the means ± SEM (each $n = 5$). w, wash out

The results showed that the estimated contributions of the EP_3 receptor were 20%–50% for the five prostanoids: PGA_2, 52%; PGD_2, 17%; PGE_2, 35%; $PGF_{2\alpha}$, 22%; and PGI_2, 37%. For PGA_2 in particular, the contribution of the EP_3 receptor was estimated to be >50% at the AUC level. However, the inhibitory effects of DHA or EPA against the five prostanoids (PGA_2, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2) were not correlated with the inhibitory effects of L-798,106 (Figure 6Ab and Bb). Therefore, no evidence was obtained indicating that the inhibitory effect of EP_3 receptors contributes to the inhibition by DHA or EPA. At present, we cannot reach any clear conclusion regarding the role of the EP_3 receptor in the DHA/EPA-induced inhibition of prostanoid-induced guinea pig GFSM contractions. To determine the role of the EP_3 receptor in the inhibitory effects of DHA/EPA, further studies are needed using EP_3 receptor-expressing cells.

DHA and EPA suppressed high KCl-induced contractions by 35% and 25%, respectively (Figure 8), suggesting that DHA and EPA inhibit VDCCs in guinea pig GFSM. The contractions induced by the five prostanoids and U46619 were inhibited by more than 40% (40%–85%) with verapamil, which almost completely suppressed high KCl-induced contractions. Therefore, the direct inhibitory effects on VDCCs were suggested to be involved in the inhibitory effects of DHA/EPA on the contractions induced by the five prostanoids and U46619. In fact, DHA and EPA have been reported to noncompetitively suppress [^{3}H] nitrendipine binding to VDCCs[29] and inhibit Ca^{2+} currents (Ca^{2+} currents recorded in guinea pig tracheal smooth muscle).[30]

LA inhibited contractions by the five prostanoids and U46619; in particular, the inhibitions versus U46619 and PGD_2 were approximately 30%–35%. In addition, a positive correlation was found between the inhibitory effects of LA and those of SQ 29,548 on the contractions induced by the five prostanoids and U46619 (Figure 6Ca). However, in TP receptor-expressing cells, LA did not show sufficient inhibitory effects on U46619-induced Ca^{2+} increases to support the U46619-induced inhibition (Figure S2). Therefore, the possibility that LA targets the TP receptor can be excluded. Since LA did not affect high-KCl-induced contractions, VDCC can also be excluded as a target for LA. Interestingly, LA suppressed U46619-induced contractions in the presence of verapamil by 55%. In contrast, the U46619-induced contractions in the presence of verapamil were not suppressed by LOE 908 (an ROCC inhibitor) but were strongly (~70%) suppressed by SKF-96365 (an SOCC inhibitor), strongly suggesting that SOCC is involved in the contractions caused by U46619. Therefore, we speculated that LA suppressed U46619-induced contractions by suppressing SOCCs. However, this possibility should be examined in detail in the future.

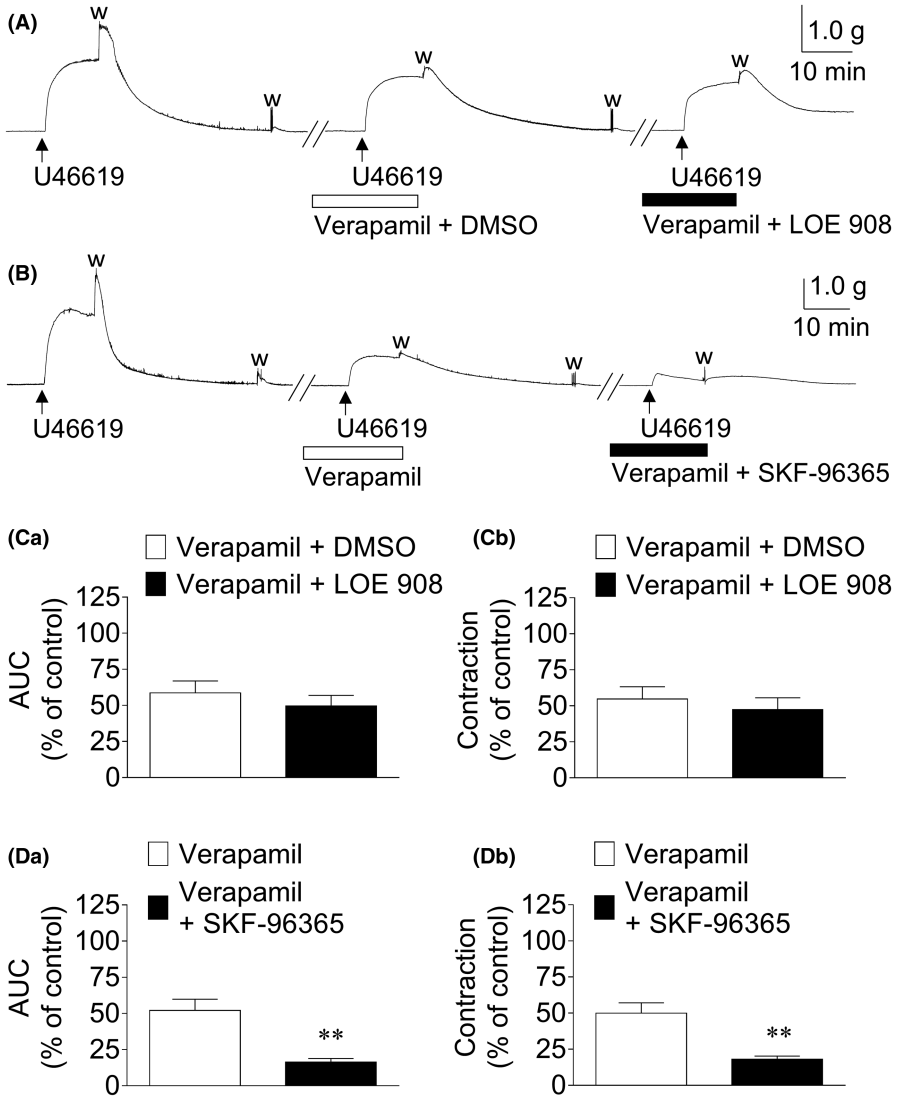

FIGURE 10 Representative traces (A, B) and quantified data (C, D) showing the effects of LOE 908 (3×10^{-5} M, A, C) and SKF-96365 (3×10^{-5} M, B, D) on the area under the curve (AUC) (a) and maximum contractions (b) of guinea pig gastric fundus smooth muscle contractions induced by U46619 (3×10^{-6} M) in the presence of verapamil (10^{-5} M). Data are expressed as the means ± SEM (each $n = 5$). $^{**}p < .01$ versus verapamil/verapamil + DMSO (paired *t*-tests). DMSO, dimethyl sulfoxide (0.015%); w, wash out

Finally, this study had some limitations. Potential immediate effects of DHA and EPA on the GFSM contractions induced by prostanoids and U46619 were studied. However, DHA and EPA can inhibit the production of prostanoids with long-term administration. In rats administered fish oil for 2 weeks, the stomach production of prostanoids (TXB_2, PGE_2, and $PGF_{2\alpha}$) was reported to significantly decrease compared to those administered standard diet.[24] Therefore, when DHA and/or EPA is administered in food or supplements, inhibition of stomach motility is expected to be caused by the immediate direct effects on GFSMs that were observed in this study in addition to the suppression of prostanoid production. This issue should be examined in the future using animal models receiving long-term administration of *n*−3 PUFAs.

ACKNOWLEDGMENTS

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DISCLOSURE

No conflicts of interest.

ETHICAL APPROVAL

This study was approved by the Toho University Animal Care and Use Committee (approval numbers: 18–54–294, 19–55–294, 20–51–444, 21–52–444) and was conducted in accordance with the guidelines of the Laboratory Animal Center of the Faculty of Pharmaceutical Sciences, Toho University.

AUTHOR CONTRIBUTIONS

Participated in research design: Xu, Yoshioka, Obara, Tanaka. Conducted experiments: Xu, Shimizu, Murai, Fujisawa, Ito, Saitoh, Nakagome, Yamashita, Murata, Oikawa, Ou, Yoshioka. Performed data analysis: Xu, Shimizu, Murai, Fujisawa, Ito, Saitoh, Nakagome, Oikawa, Ou, Yoshioka, Obara. Wrote or contributed to writing of the manuscript: Xu, Yoshioka, Obara, Tanaka.

DATA AVAILABILITY STATEMENT

The data and materials that support the findings of this work are available from the corresponding author upon reasonable request.

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