Regular Article*Highlighted Paper selected by Editor-in-Chief***Docosahexaenoic Acid and Eicosapentaenoic Acid Inhibit the Contractile Responses of the Guinea Pig Lower Gastrointestinal Tract**

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Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are n-3 polyunsaturated fatty acids (PUFAs), and are abundant in fish oil. These n-3 PUFAs have been reported to improve the lower gastrointestinal (LGI) disorders such as ulcerative colitis and Crohn's disease through their anti-inflammatory effects. However, there are few studies on the effect of n-3 PUFAs on motility of the LGI tract, such as the ileum and colon, the parts frequently affected by these inflammatory disorders. To elucidate the effects of DHA and EPA on the LGI tract motility, we performed comparative evaluation of their effects and linoleic acid (LA), an n-6 PUFA, on contractions in the ileal and colonic longitudinal smooth muscles (LSMs) isolated from guinea pigs. In the ileal and colonic LSMs, DHA and EPA (3×10^{-5} M each) significantly inhibited contractions induced by acetylcholine (ACh), histamine, and prostaglandin (PG) $F_{2\alpha}$ (vs. control), and these effects are stronger than that of LA (3×10^{-5} M). In the colonic LSMs, DHA and EPA also significantly inhibited contractions induced by PGD₂ (vs. control). In addition, DHA and EPA significantly inhibited CaCl₂-induced ileal and colonic LSM contractions in Ca²⁺-free 80 mM-KCl solution (vs. control). Any ileal and colonic LSM contractions induced by ACh, histamine, PGF_{2 α} , and CaCl₂ were completely suppressed by verapamil (10^{-5} M), a voltage-gated/dependent Ca²⁺ channel (VGCC/VDCC) inhibitor. These findings suggest that DHA and EPA could improve the abnormal contractile functions of the LGI tract associated with inflammatory diseases, partly through inhibition of VGCC/VDCC-dependent ileal and colonic LSM contractions.

Key words docosahexaenoic acid; eicosapentaenoic acid; linoleic acid; ileum; colon; voltage-dependent Ca²⁺ channel

INTRODUCTION

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are n-3 polyunsaturated fatty acids (PUFAs), and are abundant in fish oil. DHA and EPA are scarcely synthesized in the human body, and the level of these fatty acids in the human body depends on the intake of foods containing them. In the 1970s, an epidemiological study by Bang *et al.* demonstrated that intake of a diet rich in n-3 PUFAs may reduce the incidence of cardiovascular diseases.¹⁾ Later, observational and interventional studies conducted in the 1980s and the early 2000s showed that n-3 PUFAs are effective against ischemic heart disease, atrial and ventricular arrhythmia, hypertension, peripheral arterial disease, and heart failure.²⁾ In addition, drugs containing DHA/EPA or their ethyl esters have been approved for the patients with dyslipidemia because they significantly reduce serum triglycerides.³⁾ Furthermore, in recent years, n-3 PUFAs have been shown to have beneficial effects against many other diseases, such as diabetes, neurodegenerative diseases, autoimmune diseases, inflammatory diseases, and malignant tumors.^{4,5)}

In addition, n-3 PUFAs have been reported to exhibit preventive and therapeutic effects on the conditions associated with lower gastrointestinal (LGI) tract. For example, n-3 PUFAs have been reported to suppress the activity of inflammatory bowel disease (IBD) such as ulcerative colitis

and Crohn's disease, and reduce the risk of onset of IBD.^{6–8)} The inhibitory effect of n-3 PUFAs on IBD activity is attributable, to a limited extent, to their anti-inflammatory property.⁹⁾ Although many LGI disorders, including IBD, show abnormal motor function of the LGI tract, such as the ileum and colon,^{10,11)} there are only few studies available on the effect of n-3 PUFAs on motility of the LGI tract.

To elucidate the effects of DHA and EPA on the LGI tract motility, we performed comparative evaluation between these fatty acids and linoleic acid (LA), an n-6 PUFA, based on their effect on the contractions in ileal and colonic longitudinal smooth muscles (LSMs) isolated from guinea pig. We also investigated whether these fatty acids had any effect on the contraction *via* the activation of voltage-gated/dependent Ca²⁺ channels (VGCCs/VDCCs), as anti-spasmodics such as VGCC/VDCC inhibitors have been used as first-line treatment against pain-predominant irritable bowel syndrome (IBS).¹²⁾

MATERIALS AND METHODS

Animals Male Hartley guinea pigs (5–9 weeks old; weight 283–500 g, Kyudo, Co., Ltd., Saga, Japan) were housed under controlled conditions (21–22°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 User Committee (Ap-

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proval Nos. 18-54-294, 19-55-294, 20-51-444, 21-52-444) and was conducted in accordance with the user's guideline of the Laboratory Animal Center of the Faculty of Pharmaceutical Sciences, Toho University.

Ileal and Colonic LSM Preparation The guinea pigs were anesthetized with isoflurane (inhalation) and exsanguinated from the carotid artery. The ileum and ascending colon were immediately removed and placed in Locke–Ringer solution comprising (in mM) NaCl, 154; KCl, 5.6; CaCl_2 , 2.2; MgCl_2 , 2.1; NaHCO_3 , 5.9; and glucose, 2.8. After irrigating the contents of the ileum and ascending colon with Locke–Ringer solution, they were cut to a length of 3–5 cm and 4–6 cm, respectively. These preparations were passed through an acrylic rod, and the adipose tissues were removed. The ileal and colonic LSMs were then isolated from these preparations using tweezers and cotton swabs in Locke–Ringer solution. The ileal and colonic LSM strips 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 $32 \pm 1^\circ\text{C}$. These strips were subjected to a resting tension (0.5 g) and allowed to equilibrate for 20 min. The resting tension was determined according to methods previously described.^{13,14} Changes in the mechanical activity of the strips were recorded isotonicity. All experiments were carried out in presence of indomethacin ($3 \times 10^{-6}\text{M}$) to inhibit any plausible influence of endogenous prostaglandins.

Assessment of the Effects of DHA, EPA, LA, and Verapamil on Acetylcholine (ACh)/Histamine/Prostaglandin (PG) $\text{F}_{2\alpha}$ /PGD₂-Induced Ileal and Colonic LSM Contractions First, the ileal and colonic LSM preparations were contracted using 10^{-6}M -ACh at least three times with an interval of 10 min. Some colonic LSM preparations were further contracted using $3 \times 10^{-5}\text{M}$ -ACh. After an equilibration period of 20–30 min, ACh/histamine/PGF_{2 α} /PGD₂ was incrementally added to the bath medium until a maximum response was obtained. For PGF_{2 α} /PGD₂, the first concentration–response curve was considered as the control response. For ACh/histamine, this procedure was repeated, and the second concentration–response curve was considered as the control response. Next, ethanol (EtOH, 0.1%), or the test drug (DHA, EPA, LA: $10^{-5}/3 \times 10^{-5}\text{M}$, verapamil: 10^{-5}M , or L-798,106, MK-0524: $3 \times 10^{-7}\text{M}$) was added to the bath solution, and after an equilibration period of 30 min, the concentration–response curve of ACh/histamine/PGF_{2 α} /PGD₂ was obtained. Verapamil (10^{-5}M) completely inhibited the binding of [³H]D888 (desmethoxyverapamil, 4.2 nM) to rat cardiac membranes.¹⁵ In addition, verapamil (10^{-5}M) completely inhibited the depolarizing contraction, induced by 80 mM KCl, in both ileal and colonic LSM. Thus, a concentration of 10^{-5}M verapamil was considered sufficient to exhibit functional inhibition of VGCCs/VDCCs.

Assessment of the Effects of DHA, EPA, LA, and Verapamil on the Ileal and Colonic LSM Contractions Induced by 80 mM-KCl The ileal and colonic LSM preparations were contracted using 10^{-6}M -ACh at least three times with an interval of 10 min, and further contracted using $3 \times 10^{-5}\text{M}$ -ACh. After an equilibration period of 30-min, the LSM preparations were contracted at least twice with an interval of 30 min using 80 mM-KCl Locke–Ringer solution comprising (in mM) NaCl, 79.6; KCl, 80; CaCl_2 , 2.2; MgCl_2 , 2.1; NaHCO_3 , 5.9; and glucose, 2.8. Subsequently, 80 mM-KCl-induced contrac-

tions were obtained in the presence of EtOH (0.1%), or the test drug (DHA, EPA, or LA: $3 \times 10^{-5}\text{M}$) with a recording time of 20 min. After that, the preparations were washed with fresh Locke–Ringer solution and incubated for 30 min. Next, 80 mM-KCl-induced contractions were obtained in the presence of verapamil (10^{-5}M). For these series of experiments, the test drugs (DHA, EPA, LA, and verapamil) were added to the bath solution 30 min before application of 80 mM-KCl and were also present in the 80 mM KCl solution.

Assessment of the Effects of DHA, EPA, LA, and Verapamil on CaCl_2 -Induced Ileal and Colonic LSM Contractions in Ca^{2+} -Free High-KCl Solution The ileal and colonic LSM preparations were suspended in a 20-mL organ bath containing Ca^{2+} -free high (80 mM)-KCl Locke–Ringer solution comprising (in mM) NaCl, 79.6; KCl, 80; MgCl_2 , 2.1; NaHCO_3 , 5.9; and glucose, 2.8. These preparations were subjected to a resting tension (0.5 g) and allowed to equilibrate for 20 min. The ileal and colonic LSM preparations were contracted using 10^{-2}M - CaCl_2 at least three times with an interval of 10 min. After washing with fresh medium and a 30-min equilibration period, CaCl_2 was incrementally added to the bath medium to obtain the concentration–response curve for CaCl_2 in the presence of EtOH (0.1%) or the test drug (DHA, EPA, or LA: $10^{-5}/3 \times 10^{-5}\text{M}$), or verapamil (10^{-5}M). In this series of experiments, each test drug was added to the bath medium 30 min before the cumulative application of CaCl_2 .

Drugs DHA (Catalog No. 90310), EPA (Catalog No. 90110), LA (Catalog No. 90150), L-798,106 (Catalog No. 11129), and MK-0524 (Catalog No. 10009835) were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). PGD₂ (Catalog No. 12010/161-14391) were purchased from Cayman Chemical Co./FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). ACh chloride was purchased from Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Dinoprost (PGF_{2 α}) was purchased from Fuji Pharma Co., Ltd. (Tokyo, Japan). Indomethacin (Catalog No. I7378), histamine dihydrochloride (Catalog No. H7250), and ($\pm$)-verapamil hydrochloride (Catalog No. V4629) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.).

DHA, EPA, and LA were dissolved in ethanol to prepare $3 \times 10^{-2}\text{M}$ -stock solutions, and diluted further with distilled water to the desired concentrations. PGD₂ and MK-0524 were dissolved in ethanol to prepare $2 \times 10^{-2}\text{M}$ -stock solution, and diluted further with distilled water to the desired concentrations. L-798,106 was dissolved in dimethyl sulfoxide to prepare $2 \times 10^{-3}\text{M}$ -stock solution, and diluted further with distilled water to the desired concentrations.

Indomethacin was dissolved in ethanol to prepare 10^{-2}M -stock solution. All other drugs were prepared as aqueous stock solutions and diluted with distilled water.

Data Analysis and Statistics To construct concentration–response curves for agonist-induced contractions, the tension level before cumulative application of the agonist was considered as 0% contraction, and the maximum contraction in the control response was considered as 100%. The data were plotted as a function of the agonist concentration and fitted to the following equation:

$$E = E_{\max} \times A^{n_H} / (EC_{50}^{n_H} + A^{n_H})$$

where E is % contraction at a given concentration, $E_{\max}$ is the maximum response, A is agonist concentration, n_H is the

Fig. 1. Effect of Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA), and Linoleic Acid (LA) on Acetylcholine (ACh)-Induced Contractions in the Ileal (A) and Colonic (B) Longitudinal Smooth Muscles (LSMs)

a–g: Effects of EtOH (0.1%, a), DHA (10^{-5} M, b; 3×10^{-5} M, c), EPA (10^{-5} M, d; 3×10^{-5} M, e), and LA (10^{-5} M, f; 3×10^{-5} M, g) on the concentration–response curves for ACh-induced contractions. Data are presented as mean $\pm$ S.E.M. ($n = 7$, Ad; $n = 6$, Ag, Bb; and $n = 5$, others). * $p < 0.05$, ** $p < 0.01$ vs. control response at different concentrations of ACh (two-way-ANOVA followed by Šidák's *post-hoc* test).

Hill coefficient, and EC_{50} is the agonist concentration producing a 50% response. Curve-fitting was performed using the GraphPad Prism™ (version 6.07; GraphPad Software, Inc., San Diego, CA, U.S.A.).

To evaluate 80mM-KCl-induced contractions, the tension level before application of 80mM-KCl was considered as 0% contraction, and the contraction in the control response was designated as 100%. Since the ileum LSM preparations were able to elicit tonic contractions, the contractile response was analyzed 20min after application of 80mM-KCl, whereas maximal contraction was analyzed in the colon LSM that elicited only phasic responses.

All values are presented as the mean $\pm$ standard error of then mean (S.E.M.) of the data obtained from different number (n) of preparations. Statistical analysis was performed using the GraphPad Prism™ software. Differences among concentration-response curves were evaluated using two-way ANOVA followed by the Šidák's *post-hoc* test. Differences among high-KCl-induced contractions were evaluated using one-way ANOVA followed by the Dunnett's *post-hoc* test. Statistical significance was set at $p < 0.05$.

RESULTS

Effects of DHA, EPA, and LA on ACh-Induced Contractions in the Ileal and Colonic LSMs Figure 1 shows the effect of DHA, EPA, and LA ($10^{-5}/3 \times 10^{-5}$ M) on ACh-induced contractions in the ileal (A) and colonic (B) LSMs. As shown in the figure, the vehicle (0.1% EtOH) did not significantly affect ACh-induced contractions in the ileal and colonic LSMs (Figs. 1Aa, Ba, vs. control). In contrast, DHA (10^{-5} M) significantly inhibited ACh-induced contractions in both ileum and colon (Figs. 1Ab, Bb, vs. control) with increased effect at higher concentration (3×10^{-5} M) (Figs. 1Ac, Bc). DHA ($10^{-5}/3 \times 10^{-5}$ M) reduced 10^{-5} M-ACh-induced maximum contraction response to $77.7 \pm 4.5\%$ ($n = 5$)/ $60.9 \pm 7.7\%$ ($n = 5$) in the ileum and $74.3 \pm 7.4\%$ ($n = 6$)/ $60.9 \pm 7.7\%$ ($n = 5$) in the colon.

EPA (10^{-5} M) significantly inhibited the ileal contractions (Fig. 1Ad, vs. control), however, it did not significantly inhibit the contractions in the colon (Fig. 1Bd, vs. control). EPA (3×10^{-5} M) exhibited stronger and significant inhibition of ACh-induced contractions in both ileal and colonic LSMs (Figs. 1Ae, Be). The maximum contraction response induced

Fig. 2. Effect of Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA), and Linoleic Acid (LA) on Histamine-Induced Contractions in the Ileal (A) and Colonic (B) Longitudinal Smooth Muscles (LSMs)

a–g: Effects of EtOH (0.1%, a), DHA (10^{-5} M, b; 3×10^{-5} M, c), EPA (10^{-5} M, d; 3×10^{-5} M, e), and LA (10^{-5} M, f; 3×10^{-5} M, g) on the concentration–response curves for histamine-induced contractions. Data are presented as mean $\pm$ S.E.M. ($n = 5$). * $p < 0.05$, ** $p < 0.01$ vs. control response at different concentrations of histamine (two-way-ANOVA followed by Šidák's *post-hoc* test).

by ACh (10^{-5} M) in the presence of EPA ($10^{-5}/3 \times 10^{-5}$ M) was $92.9 \pm 4.9\%$ ($n = 7$)/ $53.8 \pm 8.8\%$ ($n = 5$) in the ileum and $95.2 \pm 2.7\%$ ($n = 5$)/ $60.5 \pm 10.9\%$ ($n = 5$) in the colon.

LA (10^{-5} M) did not significantly affect ACh-induced contractions in the ileum and colon (Figs. 1Af, Bf, vs. control). However, LA (3×10^{-5} M) significantly inhibited the contraction in the ileum (Fig. 1Ag), although it did not show significant inhibition in the colon (Fig. 1Bg, vs. control). The maximum contraction response induced by ACh (10^{-5} M) in the presence of LA ($10^{-5}/3 \times 10^{-5}$ M) was $102.2 \pm 2.3\%$ ($n = 5$)/ $85.3 \pm 3.4\%$ ($n = 6$) in the ileum and $105.4 \pm 5.2\%$ ($n = 5$)/ $99.6 \pm 6.2\%$ ($n = 5$) in the colon.

Effects of DHA, EPA, and LA on Histamine-Induced Contractions in the Ileal and Colonic LSMs Figure 2 shows the effect of DHA, EPA, and LA ($10^{-5}/3 \times 10^{-5}$ M) on histamine-induced contractions in the ileal (A) and colonic (B) LSMs. EtOH (0.1%) did not significantly affect histamine-induced ileal and colonic contractions (Figs. 2Aa, Ba, vs. control). However, DHA ($10^{-5}/3 \times 10^{-5}$ M) significantly inhibited histamine-induced contractions with stronger inhibition at 3×10^{-5} M than at 10^{-5} M in the ileum (Figs. 2Ab, Ac, vs. control). DHA ($10^{-5}/3 \times 10^{-5}$ M) inhibited histamine-induced

colonic contractions, although the inhibition was statistically significant only at 3×10^{-5} M (Figs. 2Bb, Bc, vs. control). DHA ($10^{-5}/3 \times 10^{-5}$ M) reduced the maximum contraction response induced by histamine (10^{-5} M) to $90.1 \pm 5.8\%$ ($n = 5$)/ $57.6 \pm 6.2\%$ ($n = 5$) in the ileum and $83.6 \pm 8.7\%$ ($n = 5$)/ $52.0 \pm 10.5\%$ ($n = 5$) in the colon.

EPA (10^{-5} M) did not show significant inhibition of histamine-induced contractions in both the ileal and colonic LSMs (Figs. 2Ad, Bd, vs. control). However, EPA (3×10^{-5} M) significantly inhibited histamine-induced contractions in both LSMs (Figs. 2Ae, Be, vs. control). EPA ($10^{-5}/3 \times 10^{-5}$ M) reduced the maximum contraction response induced by histamine (10^{-5} M) to $98.3 \pm 1.9\%$ ($n = 5$)/ $78.8 \pm 2.0\%$ ($n = 5$) in the ileum and $93.3 \pm 0.7\%$ ($n = 5$)/ $83.5 \pm 3.9\%$ ($n = 5$) in the colon.

LA (10^{-5} M) did not significantly affect histamine-induced contractions in the ileal LSM (Fig. 2Af, vs. control). However, LA (3×10^{-5} M) significantly inhibited histamine-induced contractions (Fig. 2Ag, vs. control). LA ($10^{-5}/3 \times 10^{-5}$ M) changed the maximum contraction response induced by histamine (10^{-5} M) to $101.8 \pm 1.4\%$ ($n = 5$)/ $82.9 \pm 3.1\%$ ($n = 5$) in the ileal LSM. In contrast, LA ($10^{-5}/3 \times 10^{-5}$ M) did not show significant inhibition in the colon, and the maximum contrac-

Fig. 3. Effect of Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA), and Linoleic Acid (LA) on Prostaglandin (PG) $F_{2\alpha}$ -Induced Contractions in the Ileal (A) and Colonic (B) Longitudinal Smooth Muscles (LSMs)

a–g: Effects of EtOH (0.1%, a), DHA (10^{-5} M, b; 3×10^{-5} M, c), EPA (10^{-5} M, d; 3×10^{-5} M, e), and LA (10^{-5} M, f; 3×10^{-5} M, g) on the concentration–response curves for $PGF_{2\alpha}$ -induced contractions. Data are presented as mean $\pm$ S.E.M. ($n = 7$, Aa; $n = 6$, Ba–Bc; and $n = 5$, others). * $p < 0.05$, ** $p < 0.01$ vs. control response at different concentrations of $PGF_{2\alpha}$ (two-way-ANOVA followed by Šidák's *post-hoc* test).

tion response induced by histamine (10^{-5} M) in the presence of LA ($10^{-5}/3 \times 10^{-5}$ M) was $104.8 \pm 5.7\%$ ($n = 5$)/ $100.3 \pm 4.0\%$ ($n = 5$) (Figs. 2Bf, Bg, vs. control).

Effects of DHA, EPA, and LA on $PGF_{2\alpha}$ -Induced Contractions in the Ileal and Colonic LSMs Figure 3 shows the effect of DHA, EPA, and LA ($10^{-5}/3 \times 10^{-5}$ M) on $PGF_{2\alpha}$ -induced contractions in the ileal (A) and colonic (B) LSMs. As shown in Figs. 3Aa and Ba, EtOH (0.1%) did not significantly affect $PGF_{2\alpha}$ -induced contractions in the ileal and colonic LSMs (vs. control).

DHA ($10^{-5}/3 \times 10^{-5}$ M) significantly inhibited $PGF_{2\alpha}$ -induced contractions in the ileal LSM, and the inhibition was stronger at 3×10^{-5} M than at 10^{-5} M; the maximum contraction response induced by $PGF_{2\alpha}$ (10^{-4} M) was reduced to $76.2 \pm 1.6\%$ ($n = 5$)/ $57.9 \pm 7.6\%$ ($n = 5$) in the ileum (Figs. 3Ab, Ac, vs. control). In colonic LSM, DHA (10^{-5} M) did not show significant inhibition, but DHA (3×10^{-5} M) significantly inhibited $PGF_{2\alpha}$ -induced contractions (Figs. 3Bb, Bc, vs. control) with reduction of the maximum contraction response by $PGF_{2\alpha}$ (3×10^{-6} M) to $88.9 \pm 8.3\%$ ($n = 6$)/ $58.5 \pm 8.5\%$ ($n = 6$) in the colon.

EPA (10^{-5} M) did not show significant inhibition of $PGF_{2\alpha}$ -

induced contractions in the ileal and colonic LSMs, but EPA (3×10^{-5} M) significantly inhibited the contractions (Figs. 3Ad, Ae, Bd, Be, vs. control); the maximum response induced by $PGF_{2\alpha}$ (10^{-4} M) was reduced to $98.6 \pm 2.5\%$ ($n = 5$)/ $73.4 \pm 2.9\%$ ($n = 7$) in the ileum and $90.8 \pm 7.3\%$ ($n = 5$)/ $67.5 \pm 6.6\%$ ($n = 5$) in the colon with respect to EPA at 10^{-5} and 3×10^{-5} M, respectively.

LA ($10^{-5}/3 \times 10^{-5}$ M) did not significantly affect $PGF_{2\alpha}$ -induced contractions in the ileal and colonic LSMs (Figs. 3Af, Ag, Bf, Bg, vs. control). The maximum contraction response induced by $PGF_{2\alpha}$ (10^{-4} M) was changed by LA ($10^{-5}/3 \times 10^{-5}$ M) to $97.8 \pm 5.9\%$ ($n = 5$)/ $97.7 \pm 12.5\%$ ($n = 5$) in the ileum, and that induced by $PGF_{2\alpha}$ (3×10^{-6} M) to $106.3 \pm 3.3\%$ ($n = 5$)/ $101.4 \pm 6.4\%$ ($n = 5$) by LA ($10^{-5}/3 \times 10^{-5}$ M) in the colon.

Effects of DHA, EPA and LA on PGD_2 -Induced Contractions in the Colonic LSM PGD_2 (10^{-5} M) induced contractions in the colonic LSM correspond to approximately 45% of that produced by ACh (3×10^{-5} M). In contrast, comparatively smaller contraction was detected (equivalent to approximately 30% of that by 10^{-6} M-ACh) in the ileal LSM using a comparatively higher concentration (3×10^{-5} M) of PGD_2 (data

Fig. 4. Effect of Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA), and Linoleic Acid (LA) on PGD_2 -Induced Contractions in the Colonic Longitudinal Smooth Muscles (LSMs)

A–G: Effects of EtOH (0.1%, A), DHA (10^{-5} M, B; 3×10^{-5} M, C), EPA (10^{-5} M, D; 3×10^{-5} M, E), and LA (10^{-5} M, F; 3×10^{-5} M, G) on the concentration–response curves for PGD_2 -induced contractions. Data are presented as mean $\pm$ S.E.M. ($n = 7$, A; $n = 6$, B–C; and $n = 5$, others). * $p < 0.05$, ** $p < 0.01$ vs. control response at different concentrations of PGD_2 (two-way-ANOVA followed by Šidák's *post-hoc* test).

Fig. 5. Effect of Verapamil (10^{-5} M) on the Ileal (A) and Colonic (B) Longitudinal Smooth Muscle (LSM) Contractions Induced by Acetylcholine (ACh) (a), Histamine (b), Prostaglandin (PG) $\text{F}_{2\alpha}$ (c), and PGD_2 (d)

Data are presented as mean $\pm$ S.E.M. ($n = 5$). ** $p < 0.01$ vs. control response at different concentrations of agonists (two-way-ANOVA followed by Šidák's *post-hoc* test).

not shown). Therefore, the effects of PUFAs on PGD_2 -induced contractions were examined only in the colonic LSM.

Figure 4 shows the effects of DHA, EPA, and LA ($10^{-5}/3 \times 10^{-5}$ M) on PGD_2 -induced contractions in the colonic LSM. EtOH (0.1%) did not significantly affect PGD_2 -induced contractions (Fig. 4A, vs. control). In contrast, DHA ($10^{-5}/3 \times 10^{-5}$ M) significantly inhibited the contraction induced by PGD_2 , and stronger inhibition was observed at higher DHA concentration (3×10^{-5} M) than at 10^{-5} M (Figs. 4B, 4C, vs. control). This was evidenced by the findings that DHA ($10^{-5}/3 \times 10^{-5}$ M) reduced the maximum contraction response induced by PGD_2 (10^{-5} M) to $74.0 \pm 6.0\%$ ($n = 7$)/ $56.1 \pm 5.3\%$ ($n = 5$).

EPA ($10^{-5}/3 \times 10^{-5}$ M) inhibited PGD_2 -induced contractions, although the results were statistically significant only at 3×10^{-5} M and in limited number of experiments (Figs. 4D, 4E, vs. control). The maximum contraction response by PGD_2 (10^{-5} M) in the presence of EPA ($10^{-5}/3 \times 10^{-5}$ M) was

$80.6 \pm 5.5\%$ ($n = 5$)/ $58.4 \pm 11.1\%$ ($n = 5$).

LA (10^{-5} M) did not significantly affect PGD_2 -induced contractions (Fig. 4F, vs. control). However, LA (3×10^{-5} M) significantly inhibited the contractions (Fig. 4G, vs. control) with altered maximum contraction response induced by PGD_2 (10^{-5} M) in the presence of LA ($10^{-5}/3 \times 10^{-5}$ M) to $98.2 \pm 4.4\%$ ($n = 5$)/ $66.5 \pm 8.2\%$ ($n = 5$).

U46619, a proteinoid TP receptor agonist, elicited only marginal contractions in the ileal and colonic LSMs (data not shown). This was supported by a previous study reporting that U46619 and PGD_2 were inactive in the guinea pig ileum.¹⁶⁾ Moreover, PGA_2 and PGE_2 elicited contractions in the ileal and colonic LSMs, but these contractions were attenuated by repeated administration (data not shown). Therefore, the present study did not include U46619, PGA_2 , and PGE_2 .

Effect of Verapamil on ACh/Histamine/ $\text{PGF}_{2\alpha}$ / PGD_2 -Induced Contractions in the Ileal and Colonic LSMs Figure 5 shows the effect of verapamil (10^{-5} M), a VGCC/VDCC

Fig. 6. Effect of Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA), Linoleic Acid (LA) (3×10^{-5} M Each), and Verapamil (Ver, 10^{-5} M) on the Ileal Longitudinal Smooth Muscle (LSM) Tonic Contractions (A) and Colonic LSM Phasic Contractions (B) Induced by 80 mM-KCl

Data are presented as mean $\pm$ S.E.M. for $n=21$ (Ver in A), $n=20$ (Ver in B), $n=6$ (DHA in A), $n=5$ (others). ** $p < 0.01$ vs. 80 mM-KCl-induced contractions in the presence of 0.1% EtOH (one-way-ANOVA followed by Dunnett's *post-hoc* test).

inhibitor, on the contractions induced by ACh (Figs. 5Aa, Ba), histamine (Figs. 5Ab, Bb), $\text{PGF}_{2\alpha}$ (Figs. 5Ac, Bc), and PGD_2 (Fig. 5Bd) in the ileal and colonic LSMs. All contractions in both LSMs were completely abolished by verapamil (10^{-5} M).

Effects of DHA, EPA, and LA on 80 mM-KCl-Induced Contractions in the Ileal and Colonic LSMs Figure 6 shows the effects of DHA, EPA, and LA (3×10^{-5} M) on 80 mM-KCl-induced contractions in the ileal (A) and colonic (B) LSMs. DHA (3×10^{-5} M) showed significant inhibitory effect against 80 mM-KCl-induced tonic contractions as compared to the vehicle (0.1% EtOH) in the ileal LSM (Fig. 6A). In contrast, EPA and LA (3×10^{-5} M) did not produce significant inhibitory effects.

In the colonic LSM, DHA and EPA (3×10^{-5} M) significantly inhibited 80 mM-KCl-induced phasic contractions as compared to the vehicle (0.1% EtOH), whereas LA (3×10^{-5} M) did not show any inhibition (Fig. 6B).

However, verapamil (10^{-5} M) abolished 80 mM-KCl-induced phasic contractions in the ileal and colonic LSMs.

Effects of DHA, EPA, and LA on CaCl_2 -Induced Contractions in Ca^{2+} -Free 80 mM-KCl Solution in the Ileal and Colonic LSMs Figure 7 shows the effects of DHA, EPA, and LA ($10^{-5}/3 \times 10^{-5}$ M) on CaCl_2 -induced contractions in Ca^{2+} -free 80 mM-KCl solution in the ileal and colonic LSMs. CaCl_2 -induced contractions were not affected by the vehicle (0.1% EtOH) (Figs. 7Aa, Ba), and on the contrary, were abolished by verapamil (10^{-5} M) (Figs. 7Ab, Bb) in both LSMs.

The inhibitory effect of DHA (10^{-5} M) was not statistically significant against the ileal and colonic contractions (Figs. 7Ac, Bc, vs. control), whereas the inhibition was significant with DHA (3×10^{-5} M) (Figs. 7Ad, Bd, vs. control). DHA ($10^{-5}/3 \times 10^{-5}$ M) reduced the maximum contraction response induced by CaCl_2 (3×10^{-2} M) to $79.7 \pm 4.8\%$ ($n=7$)/ $54.1 \pm 6.5\%$ ($n=5$) in the ileum and $88.0 \pm 3.9\%$

($n=5$)/ $51.3 \pm 10.2\%$ ($n=5$) in the colon.

EPA (10^{-5} M) did not inhibit the ileal contractions, but slightly inhibited the colonic contractions, although statistical significance was not detected (Figs. 7Ae, Be, vs. control). Inhibitory effect of EPA (3×10^{-5} M) against CaCl_2 -induced contractions was significant in both LSMs (Figs. 7Af, Bf, vs. control). EPA ($10^{-5}/3 \times 10^{-5}$ M) altered the maximum contraction response of CaCl_2 (3×10^{-2} M) to $103.4 \pm 4.2\%$ ($n=5$)/ $76.4 \pm 7.4\%$ ($n=7$) in the ileum and $83.6 \pm 6.3\%$ ($n=5$)/ $55.0 \pm 10.0\%$ ($n=7$) in the colon.

LA ($10^{-5}/3 \times 10^{-5}$ M) did not show significant inhibition in both LSMs (Figs. 7Ag, Ah, Bg, Bh, vs. control), although a slight inhibition was detected at 3×10^{-5} M in the ileal LSM (Fig. 7Ah). The maximum contraction response induced by CaCl_2 (3×10^{-2} M) in the presence of LA ($10^{-5}/3 \times 10^{-5}$ M) was $110.8 \pm 5.9\%$ ($n=5$)/ $99.4 \pm 1.7\%$ ($n=5$) in the ileum and $105.0 \pm 3.9\%$ ($n=5$)/ $100.9 \pm 7.0\%$ ($n=5$) in the colon.

Effects of L-798,106 and MK-0524 on PGD_2 -Induced Contractions in the Colonic LSM Figure 8 shows the effects of L-798,106 (an EP_3 receptor antagonist, 3×10^{-7} M) and MK-0524 (a DP_1 receptor antagonist, 3×10^{-7} M) on PGD_2 -induced contractions in the colonic LSM. L-798,106 (3×10^{-7} M) significantly inhibited the contraction induced by PGD_2 (Fig. 8A, vs. control), whereas MK-0524 (3×10^{-7} M) did not affect the contraction (Fig. 8B).

DISCUSSION

In this study, we examined the effects of DHA and EPA on the contractions mediated through chemical receptors and depolarization stimulation in the ileal and colonic LSMs to investigate whether these n-3 PUFAs could inhibit abnormal motility of the LGI tract. We demonstrated that these PUFAs suppressed various verapamil-sensitive contractions in the colonic and ileal LSMs. These findings suggest that DHA and EPA could ameliorate abnormal contractions of the LGI tract associated with inflammatory diseases, partly through inhibition of VGCC/VDCC-dependent ileal and colonic contractions.

DHA and EPA (3×10^{-5} M) significantly suppressed the contractions mediated through all tested chemicals (ACh, histamine, $\text{PGF}_{2\alpha}$, and PGD_2 , vs. control). The findings of this study demonstrated stronger and significant inhibitory effect of DHA than that of EPA even at lower concentration of 10^{-5} M against contractions induced by ACh (Figs. 1Ab vs. Ad, Figs. 1Bb vs. Bd), histamine (Figs. 2Ab vs. Ad), $\text{PGF}_{2\alpha}$ (Figs. 3Ab vs. Ad), and PGD_2 (Figs. 4B vs. D). Since both DHA and EPA (3×10^{-5} M) non-selectively inhibited these ileal/colonic contractions, they were unlikely to directly inhibit a specific chemical receptor; we speculated that they impeded few intracellular signal transduction steps that were common to all these chemical receptors. Possible receptors to mediate LGI tract contractions induced by the tested chemicals were reported as follows: 1) ACh and synthetic muscarinic receptor agonists produce contractions in the guinea pig ileum/colon and mouse ileum *via* muscarinic M_3 receptor¹⁷⁻¹⁹; 2) Histamine produces contractions in the guinea pig ileum/colon *via* histamine H_1 receptor²⁰; and 3) $\text{PGF}_{2\alpha}$ and PGD_2 produce contractions in the mouse ileum mainly *via* prostanoïd FP and EP_3 receptors.²¹ Among these receptors, M_3 , H_1 , and FP receptors are Gq-protein-coupled receptors,^{22,23}

Fig. 7. Effect of Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA), Linoleic Acid (LA), and Verapamil on CaCl_2 -Induced Ileal (A) and Colonic (B) Longitudinal Smooth Muscle (LSM) Contractions in Ca^{2+} -Free 80mM-KCl Solution

a–g: Effects of EtOH (0.1%, a), verapamil (10^{-5} M, b), DHA (10^{-5} M, c; 3×10^{-5} M, d), EPA (10^{-5} M, e; 3×10^{-5} M, f), and LA (10^{-5} M, g; 3×10^{-5} M, h) on the concentration–response curves for CaCl_2 -induced contractions. Data are presented as mean $\pm$ S.E.M. ($n=7$, Ac, Bf; and $n=5$, others). * $p < 0.05$, ** $p < 0.01$ vs. control response at different concentrations of CaCl_2 (two-way-ANOVA followed by Šidák's *post-hoc* test).

Fig. 8. Effect of L-768106 (3×10^{-7} M, A) and MK-0524 (3×10^{-7} M, B) on PGD_2 -Induced Contractions in the Colonic Longitudinal Smooth Muscles (LSMs)

Data are presented as mean $\pm$ S.E.M. ($n=5$, A; and $n=6$, B). * $p < 0.05$, ** $p < 0.01$ vs. control response at different concentrations of PGD_2 (two-way-ANOVA followed by Šidák's *post-hoc* test).

and EP_3 receptor is predominantly Gi-protein-coupled receptor; however, it can also be coupled with Gq-protein.²⁴ Stimulation of Gq-protein-coupled receptors by chemical agonists induce smooth muscle (SM) contractions through activation of VGCCs/VDCCs.²⁵ Considering that the contractions induced by all tested chemicals (ACh, histamine, $\text{PGF}_{2\alpha}$, and PGD_2) were completely abolished by verapamil (10^{-5} M) (Fig. 5), it

was speculated that VGCCs/VDCCs contributed significantly to these contractions. In contrast, DHA and EPA (3×10^{-5} M) partly but significantly inhibited 80mM-KCl-induced contractions (Fig. 6, vs. 0.1% EtOH) and CaCl_2 -induced contractions in Ca^{2+} -free 80mM-KCl solution (Fig. 7, vs. control), which were abolished by verapamil (10^{-5} M). Therefore, it is plausible that inhibitory mechanism of DHA/EPA (3×10^{-5} M) on the ileal and colonic LSMs contractions induced by the tested chemicals (ACh, histamine, $\text{PGF}_{2\alpha}$, and PGD_2) partly involves inhibition of VGCCs/VDCCs or their downstream signaling steps.

Significant contribution of inhibitory effect of DHA/EPA (3×10^{-5} M) on VGCCs/VDCCs or their downstream signaling steps can be supported by the previous findings as follows: 1) DHA ($5 \mu\text{M}$) attenuated the inhibitory effect of nitrendipine (0.5 nM), a VGCC/VDCC inhibitor, on rat myocyte contraction, and DHA/EPA non-competitively inhibited the specific binding of [^3H]nitrendipine to rat myocytes²⁶; 2) EPA ($3\text{--}30 \mu\text{M}$) and DHA ($30 \mu\text{M}$) suppressed the voltage-dependent L-type Ca^{2+} current (I_{Ca}) in guinea pig tracheal SM cells²⁷; 3) EPA and DHA ($5 \mu\text{M}$) suppressed the I_{Ca} in rat ventricular myocytes²⁸; and 4) DHA ($10 \mu\text{M}$) did not affect

the guinea pig ventricular myocyte contraction *via* voltage-sensitive release mechanism, but inhibited the contractions and inward currents *via* Ca^{2+} -induced Ca^{2+} release elicited by the I_{Ca} .²⁹⁾ Therefore, although the possibility that DHA/EPA inhibit the downstream signaling of VGCCs/VDCCs could not be precluded, it is plausible that DHA/EPA (3×10^{-5} M) directly inhibit I_{Ca} , and thus show inhibition of the ileal and colonic LSM contractions induced by contractile chemicals (ACh, histamine, $\text{PGF}_{2\alpha}$, and PGD_2).

Free fatty acid receptors (FFARs), such as GPR40 and GPR120, can be stimulated by DHA and EPA.³⁰⁾ Although GPR120 is expressed in human and mouse intestinal tissues,³⁰⁾ the expression levels of GPR40 and GPR120 have not been investigated in guinea pig intestinal tissues. However, since both GPR40 and GPR120 are Gq protein-coupled receptors³⁰⁾ and the stimulation of Gq protein-coupled receptors results in the activation of VGCCs/VDCCs, these FFARs can be ruled out as the primary targets of DHA and EPA to produce inhibition of VGCC/VDCC-mediated contractions. Therefore, the primary targets of DHA and EPA in the ileal and colonic LSM were considered to be VGCCs/VDCCs (or their downstream signaling steps) rather than FFARs.

LA (3×10^{-5} M) exhibited significant inhibition of ACh- and histamine-induced contractions in the ileum and PGD_2 -induced contractions in the colon. In contrast, LA (3×10^{-5} M) did not inhibit $\text{PGF}_{2\alpha}$ -induced contractions in the ileum, and ACh-, histamine-, and $\text{PGF}_{2\alpha}$ -induced contractions in the colon. Contrarily, our previous studies on rat thoracic aorta and mesenteric artery showed that LA (3×10^{-5} M) did not affect the contractions elicited by U46619, $\text{PGF}_{2\alpha}$, phenylephrine, and 80 mM-KCl solution.^{31,32)} At present, we do not have a rationale to explain the discrepant results between blood vessels (rat aorta and mesenteric artery) and LGI tract LSMs (guinea pig ileum and colon). However, the following explanations might be possible: 1) Inhibition of VGCCs/VDCCs: LA ($5 \mu\text{M}$) was reported to inhibit the I_{Ca} in rat ventricular myocytes and LA ($10\text{--}30 \mu\text{M}$) was reported to inhibit the I_{Ca} in rabbit distal ileal LSMs.^{28,33)} Therefore, inhibition of VGCCs/VDCCs might be partly involved as a mechanism for LA (3×10^{-5} M)-induced inhibition of the ileal contractions by ACh/histamine, and colonic contractions by PGD_2 . However, inhibition by LA (3×10^{-5} M) against 80 mM-KCl-induced contractions (Fig. 6) and CaCl_2 -induced contractions (Fig. 7) was not substantial; thus, the role of VGCCs/VDCCs seems to be insignificant. 2) Contribution of Na^+/K^+ -ATPase: LA has been reported to induce pig coronary artery relaxation and hyperpolarization through activation of Na^+/K^+ -ATPase.³⁴⁾ Moreover, Na^+/K^+ -ATPase has been reported to regulate intracellular Ca^{2+} concentration in SM cells.³⁵⁾ Therefore, Na^+/K^+ -ATPase activation by LA (3×10^{-5} M) might partly account for its inhibitory effects on the ileal and colonic LSM contractions in guinea pig. 3) Role of LA metabolites: In addition to acting as a substrate for arachidonic acid cascades, LA is a substrate for CYP (CYP1A2, CYP2C, CYP2E1, and CYP3A4) and lipoxygenase, resulting in the production of its relaxant metabolites.^{36,37)} For instance, leukotoxin (9,10-epoxy-12-octadecenoate), a CYP metabolite of LA, has been reported to induce relaxation in rat pulmonary arteries.³⁸⁾ Therefore, some LA metabolites are likely to be involved in the inhibitory effect of LA on the ileal and colonic LSM contractions in guinea pig. The metabolic activity of CYP is high in the

human small intestine, and electrical field stimulation-induced ACh release was shown to be inhibited by a lipoxygenase inhibitor in the guinea pig ileum.^{39,40)} 4) Other possibilities: Regarding PGD_2 -induced contractions in the colonic LSM, it is likely that non-Gq-protein-coupled receptors are involved as reported by PGD_2 -induced relaxation of mouse fundal SMs through the DP receptor (Gs-protein-coupled receptor); however, contraction was induced through stimulation of the EP receptor (Gq-protein-coupled receptor).²¹⁾ Therefore, LA might induce the colonic relaxation through activation of Gs-proteins *via* DP receptors, and might apparently suppress the colonic contraction mediated through Gq-protein-coupled receptors. To verify this assumption, we examined the effects of an EP_3 receptor antagonist (L-798,106) and a DP_1 receptor antagonist (MK-0524) on PGD_2 -induced contractions in the colonic LSM. PGD_2 -induced contractions were not affected by the DP_1 receptor antagonist, but were suppressed by the EP_3 receptor antagonist. Therefore, this assumption (DP_1 receptor role) was not appropriate, and it was concluded that LA may inhibit the EP_3 receptor or their downstream signaling steps.

Finally, we discuss the clinical significance of this study. The present results suggest that DHA/EPA reduces the motility of LGI tract. The total plasma DHA concentration in healthy person is approximately $32 \mu\text{g/mL}$ ($97 \mu\text{M}$), which is increased to approximately $102 \mu\text{g/mL}$ ($311 \mu\text{M}$) after one week of DHA supplementation (1076 mg/d), and reaches a steady state of approximately $120 \mu\text{g/mL}$ or $365 \mu\text{M}$.⁴¹⁾ The total plasma EPA concentration in healthy person is approximately $15 \mu\text{g/mL}$ ($47 \mu\text{M}$), which is increased to approximately $27 \mu\text{g/mL}$ ($89 \mu\text{M}$) after three weeks of DHA supplementation (1076 mg/d).⁴¹⁾ Thus, the concentrations of DHA and EPA in the serum/LGI tract can easily reach $30 \mu\text{M}$, which showed substantial inhibitory effect on the LGI tract contractions in guinea pigs in the present study. Epadel (ethyl ester of EPA) and Lotriga® (ethyl ester of EPA and ethyl ester of DHA) cause gastrointestinal side effects such as abdominal discomfort and constipation.^{42,43)} Therefore, DHA and EPA may improve the LGI hyper-motility in diarrhea associated with IBD and diarrhea-predominant IBS. In contrast, DHA and EPA may worsen the symptoms of constipation-predominant IBS by further reducing the LGI motility.

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Conflict of Interest The authors declare no conflict of interest.

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