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ω-3 Polyunsaturated fatty acids—modulation of voltage-dependent L-type Ca^{2+} current in guinea-pig tracheal smooth muscle cells

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Abstract

ω-3 polyunsaturated fatty acids have been reported to be associated with favorable changes in the respiratory system. To determine one of the mechanisms for this effect, membrane currents were recorded in guinea-pig tracheal myocytes by using the whole-cell voltage clamp technique. Without EGTA in the patch pipette containing the Cs-internal solution, command voltage pulses positive to +0 mV from a holding potential of −60 mV elicited a voltage-dependent L-type Ca^{2+} current ($I_{Ca\cdot L}$) and a subsequent outward current. Upon repolarization, slowly decaying inward tail currents were recorded. The outward currents and the inward tail current were enhanced by methyl-1,4,-dihydro-2,6-dimethyl-3-nitro-4-(2-trigluromethylphenyl)-pyridine-5-carboxylate, and blocked by Cd^{2+} or nifedipine. Inclusion of EGTA (5 mM) in the patch pipette also abolished these currents, indicating that they were Ca^{2+}-dependent. When $[Cl^-]_o$ or $[Cl^-]_i$ was changed, the reversal potential of these currents shifted, thus behaving like a Cl^--sensitive ion channel. 4,4′-Diisothiocyanatostilbene-2,2′-disulphonic acid, a Cl^- channel blocker, inhibited the currents. The ω-3 polyunsaturated fatty acids eicosapentaenoic acid (3–30 μM) and docosahexaenoic acid (30 μM) suppressed $I_{Ca\cdot L}$ and then inhibited $I_{Ca\cdot Cl}$ in a reversible manner. Similar inhibitory effects of eicosapentaenoic acid on $I_{Ca\cdot L}$ were observed with 5 mM EGTA in the patch pipette. Neurokinin A (1 μM) and caffeine (10 mM) also transiently activated $I_{Cl\cdot Ca}$, probably due to Ca^{2+} release from Ca^{2+} storage sites. Pretreatment of the cells with eicosapentaenoic acid markedly suppressed the activation of $I_{Cl\cdot Ca}$ by neurokinin A or caffeine. These results suggest that ω-3 polyunsaturated fatty acids inhibit voltage-dependent L-type Ca^{2+} currents and also Ca^{2+}-activated Cl^- currents in tracheal smooth muscle cells from the guinea-pig, which may play a role in modulation of tracheal smooth muscle tone.

Keywords: Smooth muscle cell; Tracheal; Ca^{2+} current; Voltage-dependent; L-type; ω-3 Polyunsaturated fatty acid; Eicosapentaenoic acid; Voltage clamp; Whole-cell; Cl^- current, Ca^{2+}-dependent

1. Introduction

The influx of Ca^{2+} through transmembrane Ca^{2+} channels such as voltage-dependent Ca^{2+} channels plays a fundamental role in excitation–contraction coupling in airway smooth muscle (Middleton, 1984; Godfraind et al., 1986). Voltage-dependent L-type Ca^{2+} channel blockers, i.e., nifedipine and verapamil, inhibit agonist-induced human bronchial smooth muscle contraction in vitro (Cerrina et al., 1983; Drazen et al., 1983) and block allergen-induced bronchoconstriction in asthmatics (Henderson et al., 1983; Miadonna et al., 1983). Thus, these Ca^{2+} channel antagonistic drugs may be beneficial in treating certain patients with respiratory diseases. In fact, they may inhibit exercise-induced asthma and bronchoconstriction induced by agonists or deep inspiration (Barnes et al., 1981; Rolla et al., 1982; Corris et al., 1983).

ω-3 polyunsaturated fatty acids, such as eicosapentaenoic and docosahexaenoic acid, which are major fatty acid components of fish oil, have been shown to cause a number of biochemical and physiological changes that might be beneficial in the treatment of respiratory diseases (Britton, 1995). They inhibit the conversion of arachidonic

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acid via the cycloxygenase pathway to prostanoid metabolites and reduce the production of chemical mediators such as platelet-activating factor (Arm et al., 1994). Eicosapentaenoic acid also reduces the production of leukotriene B_4, a chemical mediator responsible for the recruitment of inflammatory cells (Lee et al., 1985), and also cytokine tumor necrosis factor, which increases airway responsiveness (Endres et al., 1989; Thomas et al., 1995), exerting anti-inflammatory effects as a result (Kremer et al., 1987). Accordingly, one would expect ω-3 polyunsaturated fatty acids to have a beneficial effect in allergic disorders such as bronchial asthma. In fact, fish oil treatment reduces the allergen-induced asthmatic response (Arm et al., 1989) and produces significant increases in forced expiratory volume in 1 s (Dry and Vincent, 1991). A reduced risk of occurrence of asthmatic attacks has also reported to be associated with the consumption of fish oil (Satomi et al., 1994). Polyunsaturated fatty acids such as arachidonic acids are major components of phospholipids, the fundamental structural units of biological membranes, and play a key role in their functions (Merrill and Schroeder, 1993). Dietary fats can modulate the composition of the fatty acid moiety of membrane phospholipids in tissues and may influence the activity of membrane-bound proteins, such as enzyme and ion channels. In various types of cells, including smooth muscle cells, arachidonic acids and other free fatty acids have been reported to inhibit voltage-dependent L-type Ca^{2+} channels (Linden and Routtenberg, 1989; Shimada and Somlyo, 1992; Khurana and Bennett, 1993; Nagano et al., 1995). In contrast, they activate these channels in rat pituitary GH_3/B_6 cells and cardiac ventricular cells (Vacher et al., 1989; Vacher et al., 1992; Huang et al., 1992). In cardiac myocytes, eicosapentaenoic acid and docosahexaenoic acid have been reported to modulate voltage-dependent L-type Ca^{2+} channels (Hallaq et al., 1992; Xiao et al., 1997), but the effects of ω-3 polyunsaturated fatty acids on ionic currents have not been investigated yet in tracheal smooth muscle cells.

To investigate the effects of ω-3 polyunsaturated fatty acids on tracheal smooth muscle cells, we applied the whole-cell voltage clamp technique to single smooth muscle cells from the guinea-pig trachea. Here, we report that ω-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid, inhibit voltage-dependent L-type Ca^{2+} currents in single tracheal smooth muscle cells, which may play a role in modulation of tracheal smooth muscle tone.

2. Materials and methods

2.1. Cell preparation

Single tracheal smooth muscle cells were isolated from guinea-pigs by enzymatic dispersion using a technique similar to that previously described (Hazama et al., 1994). Briefly, adult guinea-pigs were killed by cervical dislocation under sodium pentobarbitone anesthesia, and the trachea was removed. Then, after removal of surrounding connective tissue, the cartilaginous portion of the trachea was cut open longitudinally, and the mucosa was removed with a scapel. The membranous portion of the trachea was cut into small pieces and incubated in dissociation medium containing 0.5 mg/ml papain (Sigma, St. Louis, MO, USA) and 0.05% bovine serum albumin at 4°C for 14–18 h. The tissue was then incubated with an enzymatic solution containing 3 mg/ml collagenase (Worthington CLS II, Freehold, NJ, USA), 0.5 mg/ml trypsin inhibitor (Sigma Type I-S) and 0.15 mM dithiothreitol (Sigma) at 37°C for 45 min. Subsequently, the tissue was transferred to the enzyme-free dissociation medium and maintained at 4°C. Single tracheal smooth muscle cells were dispersed by trituration in enzyme-free dissociation medium just prior to the experiments. This yielded an acceptable number of viable single smooth muscle cells, which were contracted by acetylcholine or neurokinin A (1 μM) as shown previously (Hisada et al., 1990; Nakajima et al., 1995). The enzymatically dispersed single tracheal smooth muscle cells from guinea-pig were spindle-shaped and had a smooth surface and adhered well to the bottom of the chamber. The mean length and width of tracheal smooth muscle cells used in this study were 51.3 ± 15 μm and 13.5 ± 3.2 μm (mean ± S.D., $n = 124$), respectively. All experiments were performed at 35–37°C. The dissociation medium contained (in mM): NaCl, 110; $NaHCO_3$, 10; KCl, 5; $MgCl_2$, 0.5; $NaHPO_4$, 0.5; $CaCl_2$, 0.16; EDTA, 0.49; taurine, 10; phenol red, 0.02; HEPES–NaOH buffer, 10; glucose, 11 (pH 8.0).

2.2. Solutions and drugs

The composition of the normal Tyrode solution was as follows (in mM): NaCl, 136.5; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.53; glucose, 5.5; HEPES–NaOH buffer, 5 (pH 7.4). When the external Cl^- ($[Cl^-]_o$) or internal Cl^- concentration ($[Cl^-]_i$) was changed, Cl^- was exchanged for aspartate. When the external Na^+ concentration ($[Na^+]_o$) was altered, Na^+ was replaced with equimolar tetraethylammonium while maintaining the Cl^- concentration constant. The patch pipette contained the Cs^+-internal solution to block K^+ currents (in mM): CsCl, 140; EGTA, 0 or 5; $MgCl_2$, 2; Na_2ATP, 1; guanosine-5′-triphosphate (Na^+ salt, Sigma), 0.1; and HEPES–CsOH buffer, 5 (pH 7.2). To record voltage-dependent Ca^{2+} currents, the composition of the bath solution was as follows (in mM): NaCl, 126.5; $CaCl_2$, 5.0; $MgCl_2$, 0.53; TEA, 10; glucose, 5.5; HEPES–CsOH buffer, 5 (pH 7.4). Neurokinin A was purchased from Peptide Ins. (Osaka, Japan). Caffeine and 4,4′-diisothiocyanastilbene-2,2′-disulphonic acid were obtained from Sigma. *Cis*-5,8,11,14,17-eicosapentaenoic acid (Na salt) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (free acid) were purchased from Sigma. To prepare the docosahexaenoic acid salt (Na salt, 30 mM), 3 M NaOH

was added to 3 M docosahexaenoic acid (free acid). ω-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid, Na salt) were dissolved in the control bathing solution. Indomethacin and nordihydroguaiaretic acid were purchased from Sigma and were dissolved in ethanol at a concentration of 10 mM.

2.3. Recording technique and data analysis

Membrane currents were recorded with glass pipettes under whole-cell voltage clamp conditions (Hamill et al., 1981; Nakajima et al., 1989), using a patch clamp amplifier (EPC-7, List Electronics, Darmstadt, Germany). Heat-polished patch pipettes, filled with the artificial internal solution (for composition, see above), had a tip resistance of 3–5 MΩ. The series resistance was compensated. The membrane currents were continuously monitored with a high-gain storage oscilloscope (COS 5020-ST, Kikusui Electronic, Tokyo, Japan). The data were stored on line on videotape by using the PCM converting system (RP-880, NF electronic instrument, Tokyo, Japan). The data were reproduced, low-pass filtered at 2 kHz (−3 dB) with a Bessel filter (FV-665, NF, 48 dB/octave slope attenuation), sampled at 5 kHz and analyzed off-line on a personal computer using p-Clamp software (Axon Instruments, CA). The resting membrane potential of single tracheal smooth muscle cells measured under current clamp conditions was -43 ± 4 mV ($n = 30$). During the voltage clamp experiments to measure voltage-dependent Ca^{2+} currents, cells were stable at least for 10–20 min under our experimental conditions. Data are expressed as means ± S.D. Student's *t*-tests were performed and a value of $P < 0.05$ was considered significant.

3. Results

3.1. ω-3 polyunsaturated fatty acids inhibit voltage-dependent L-type Ca^{2+} currents

Fig. 1A and C show the effects of methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trigluromethylphenyl)-pyri-

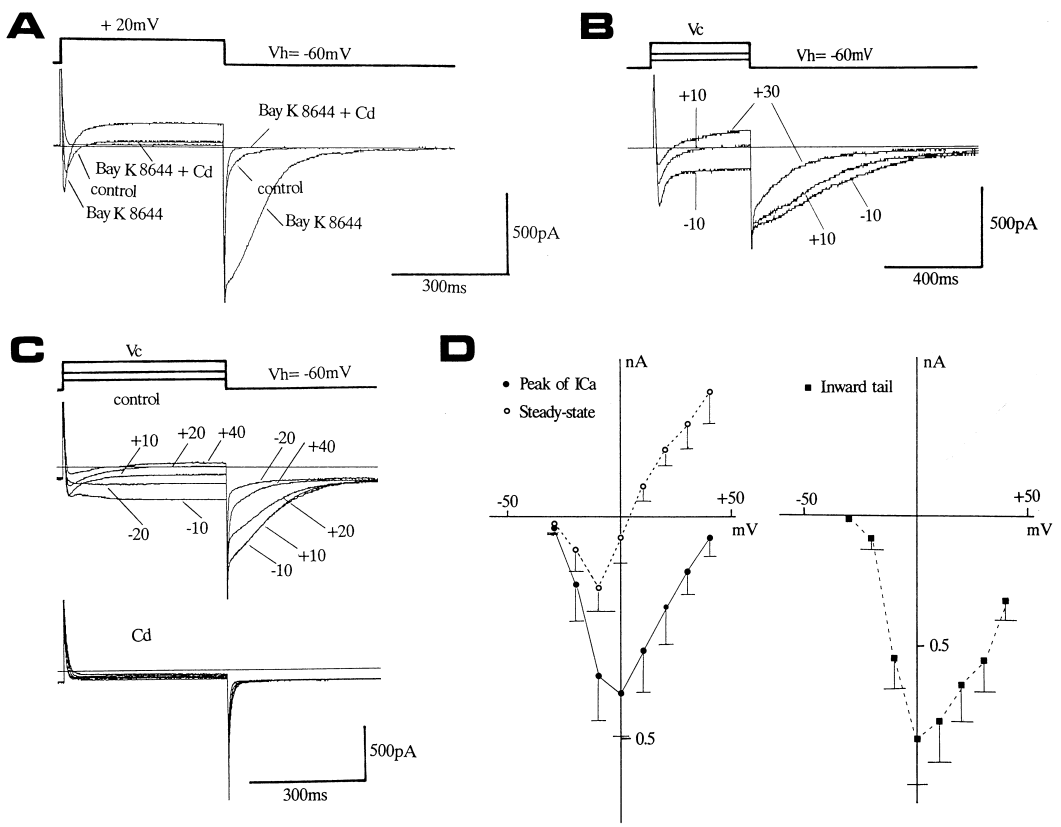

Fig. 1. Effects of Bay K 8644, Cd^{2+}, replacement of Na^+ by tetraethylammonium (TEA) and 4,4′-diisothiocyanastilbene-2,2′-disulphonic acid (DIDS) on membrane currents in single guinea-pig tracheal smooth muscle cells. Without EGTA in the patch pipette, the cells were held at −60 mV and command voltage steps to +20 mV were applied at 0.1 Hz (A). The original current traces in (A) are shown for control conditions and for Bay K 8644 (500 nM) and after additional application of Cd^{2+} (0.3 mM). The zero current level is indicated by the horizontal lines. The current traces in (B) were obtained from a cell when extracellular Na^+ ions were totally replaced by TEA. In (C), the original current traces elicited during various command voltage steps are shown for control conditions and in the presence of Cd^{2+} (0.3 mM). The mean current–voltage relationships at the initial peak of Ca^{2+} current and measured at the end of the pulses (steady-state) are shown for controls in (D) (left part). The amplitude of the tail current is plotted against each command voltage step in (D) (right part). The data were obtained from six different cells, and the mean ± S.D. value is shown.

Table 1
Reversal potential of the Ca^{2+}-activated currents in tracheal myocytes isolated from the guinea-pig

Ionic conditions		Reversal potential (calculated reversal potential)
Bath	Pipette solution	
Control Tyrode solution (136 mM Na^+, 145 mM Cl^-)	CsCl-internal solution (140 mM Cs^+, 145 mM Cl^-)	4 ± 6 mV ($n = 6$) (+0 mV)
Control Tyrode solution (136 mM Na^+, 145 mM Cl^-)	Cs-aspartate-internal solution (140 mM Cs^+, 30 mM Cl^-)	-25 ± 9 mV* ($n = 6$) (−41 mV)
Low Cl^- solution (136 mM Na^+, 70 mM Cl^-)	CsCl-internal solution (140 mM Cs^+, 145 mM Cl^-)	23 ± 8 mV* ($n = 6$) (19 mV)
Low Na^+ solution (140 mM TEA, 145 mM Cl^-)	CsCl-internal solution (140 mM Cs^+, 145 mM Cl^-)	5 ± 6 mV ($n = 5$) (+0 mV)

Extracellular Na^+ was replaced by TEA, and extracellular or intracellular Cl^- was replaced by aspartate. The data represent the mean ± S.D. and the number of cells tested (n) is shown in parenthesis. * $P < 0.05$ when compared with the control (140 mM $[Cl^-]_o$/145 mM $[Cl^-]_i$). The calculated equilibrium potential of Cl^- ions under these conditions is also shown.

dine-5-carboxylate (Bay K 8644) and Cd^{2+} on membrane currents in single tracheal smooth muscle cells. Without EGTA in the patch pipette, the cell was held at −60 mV, and command voltage pulses to +20 mV were applied at 0.1 Hz. Under these conditions, a transient inward current was elicited, followed by an outward current. A slowly decaying inward tail current was observed upon repolarization (Fig. 1A). After application of Bay K 8644 (500 nM), a Ca^{2+} channel agonist, $I_{Ca \cdot L}$ increased. The outward current during the command pulse and the inward tail current were also dramatically enhanced. Cd^{2+} (0.3 mM), an organ Ca^{2+} channel antagonist (Fig. 1A), or nifedipine (1 μM, data not shown) completely abolished the activation of these currents. Tetrodotoxin (50 μM, data not shown) or replacement of extracellular Na^+ ions by TEA^+ (Fig. 1B) did not inhibit the transient inward current, suggesting that the inward current mainly consists of a dihydropyridine-sensitive (L-type) Ca^{2+} channel, but not a voltage-dependent Na^+ channel or T-type Ca^{2+} channel as previously reported (Hisada et al., 1990; Hazama et al., 1996). Fig. 1C shows typical traces obtained from a cell at each command voltage step under control conditions and after application of Cd^{2+}. Fig. 1D (bottom) shows the mean current–voltage (I–V) relationships obtained from

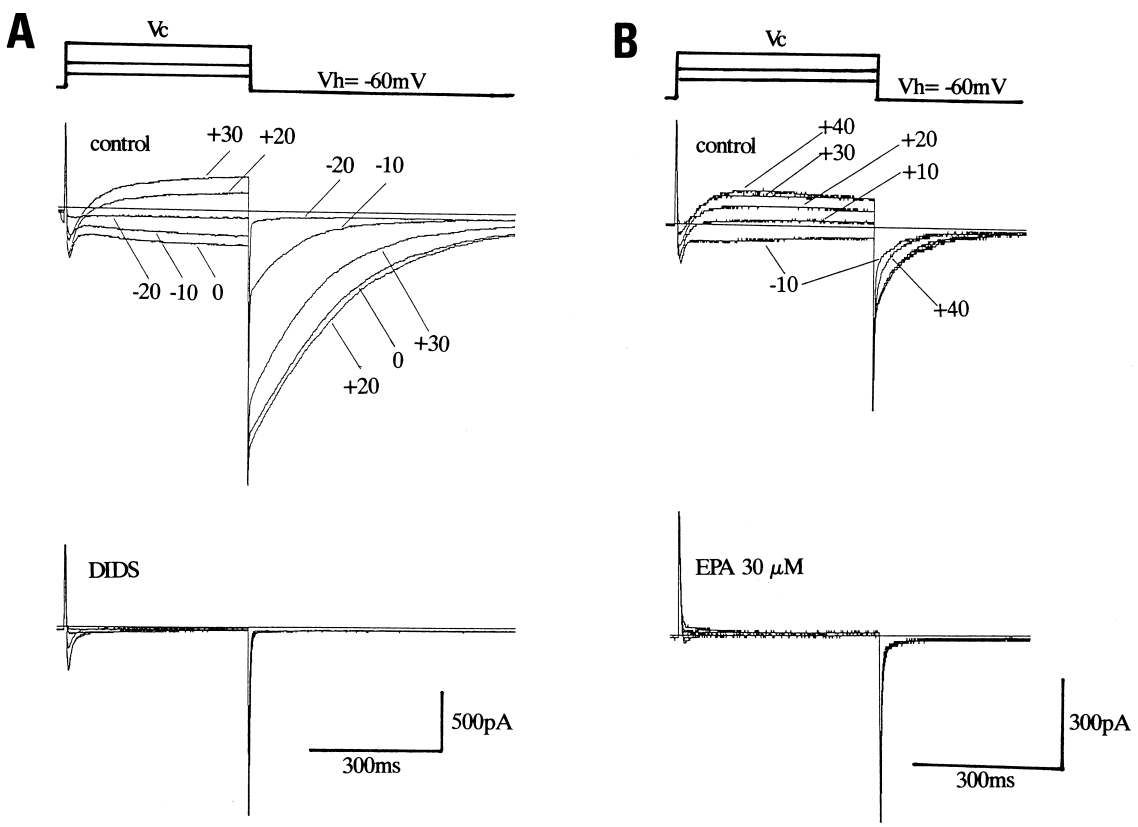

Fig. 2. Effects of 4,4′-diisothiocyanastilbene-2,2′-disulphonic acid (DIDS) and eicosapentaenoic acid on membrane currents in guinea-pig tracheal myocytes. Effects of DIDS (1 mM, A) and eicosapentaenoic acid (30 μM, B) on membrane currents. The cells were held at −60 mV, and command voltage pulses to various membrane potentials were applied at 0.1 Hz. The original current traces are shown for control conditions and in the presence of DIDS (1 mM, A) and eicosapentaenoic acid (30 μM, B).

six different cells. Under control conditions, $I_{Ca \cdot L}$ was followed by a steady inward current at potentials negative to +0 mV, and the outward current was elicited during the command pulses at potentials positive to +0 mV. Upon repolarization to the holding potential, an inward tail current was observed. The *I–V* relationship at the steady state under control conditions crossed zero current level at approximately +6 mV, which was near the equilibrium potential of Cl^- ions under these conditions (145 mM $[Cl^-]_o$/145 mM $[Cl^-]_i$). The reversal potential (E_{rev}) of the current was unaffected when $[Na^+]_o$ was replaced by TEA^+ (Table 1). When $[Cl^-]_o$ or $[Cl^-]_i$ was replaced by aspartate, the E_{rev} shifted in a near Nernstian manner as shown in Table 1. The amplitude of the inward tail current plotted against each command pulse reached a peak value at +0 mV, where $I_{Ca \cdot L}$ was maximal, and the relationship was U-shaped (Fig. 1D). These findings suggest that $I_{Ca \cdot L}$ may be involved in the activation of the late currents during the command pulse and the inward tail current. Actually, after $I_{Ca \cdot L}$ was blocked by Cd^{2+} (0.3 mM), these currents were totally abolished. 4,4′-diisothiocyanastilbene-2,2′-disulphonic acid (1 mM), a Cl^- channel blocker, only slightly depressed $I_{Ca \cdot L}$ from −400 to −300 pA in this cell, but completely abolished the following currents during the command pulse and the inward tail current (Fig. 2A). Similar results were obtained from five different cells. Furthermore, under the conditions with high EGTA (5 mM) in the patch pipette, these currents were not elicited as shown in Fig. 4A. These results suggest that these currents were Ca^{2+}-activated Cl^- currents ($I_{Cl \cdot Ca}$),

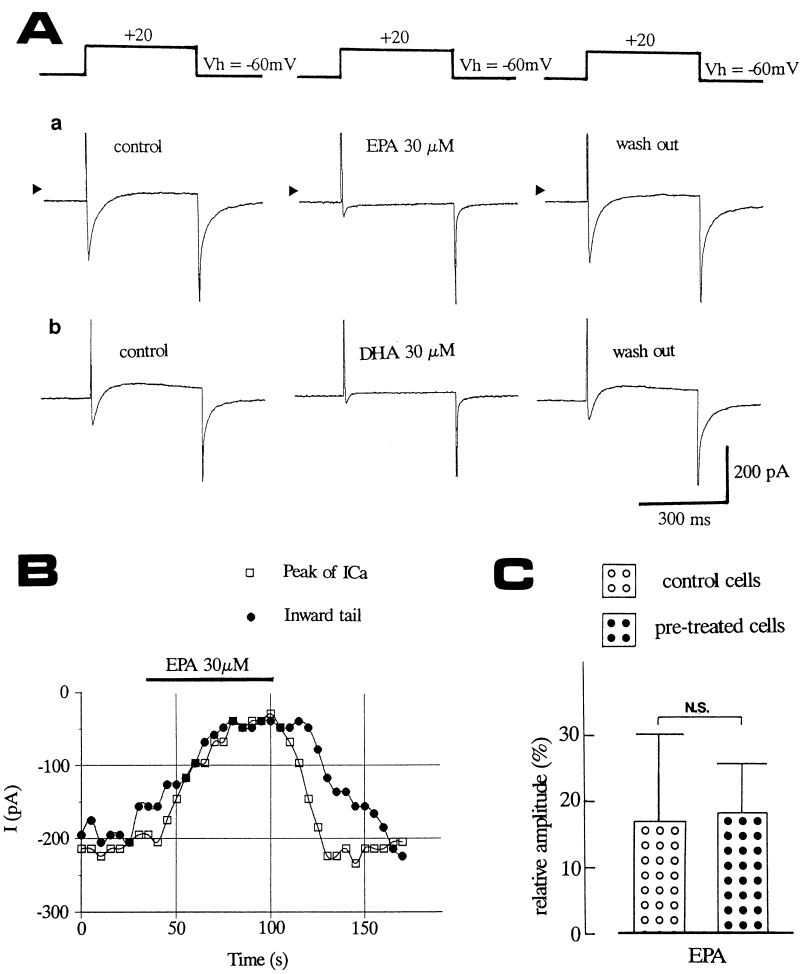

Fig. 3. Effects of eicosapentaenoic acid and docosahexaenoic acid on membrane currents in single tracheal smooth muscle cells. The patch pipette contained Cs^+-containing solution without EGTA. The cell was held at −60 mV, and the command voltage steps to +20 mV were applied at 0.1 Hz. The zero current level is indicated by the arrowheads. In (A), the current traces are shown for the control conditions, in the presence of eicosapentaenoic acid (30 μM, a) and docosahexaenoic acid (30 μM, b) and after washout with bathing solution containing albumin (0.1%). The time courses of the changes in the amplitude of the voltage-dependent Ca^{2+} current (measured from the zero current level) and the inward tail current (measured from the holding current) are plotted in (B). (C) Effects of eicosapentaenoic acid on $I_{Ca \cdot L}$ in the presence of both indomethacin and nordihydroguaiaretic acid. The cells were pre-treated with indomethacin (10 μM) and nordihydroguaiaretic acid (10 μM) for approximately 30 min. The percent inhibition by eicosapentaenoic acid of the voltage dependent L-type Ca^{2+} currents was compared in non-treated cells and treated cells. The mean ± S.D. value is shown, and the data were obtained from six different cells.

and that the rise in $[Ca^{2+}]_i$ produced by the Ca^{2+} influx due to $I_{Ca \cdot L}$ was involved in evoking these currents as previously described (Hazama et al., 1996).

Fig. 2B and Fig. 3A show the effects of eicosapentaenoic acid on membrane currents. The cell was held at −60 mV and command voltage pulses to +20 mV were applied at 0.1 Hz. Under control conditions (Fig. 3Aa), $I_{Ca \cdot L}$ was followed by $I_{Cl \cdot Ca}$. The large inward tail current for $I_{Cl \cdot Ca}$ was also observed. After eicosapentaenoic acid (30 μM) was added to the bathing solution, the amplitude of $I_{Ca \cdot L}$ dramatically depressed, and the amplitude of $I_{Cl \cdot Ca}$ decreased (Fig. 3Aa). After eicosapentaenoic acid washout with 0.1% albumin, the depressed currents immediately recovered to the control level in this cell. The time courses of the effects of eicosapentaenoic acid on the amplitude of $I_{Ca \cdot L}$ and $I_{Cl \cdot Ca}$ are shown in Fig. 3B. Fig. 2B shows the effects of eicosapentaenoic acid on $I_{Ca \cdot L}$ and $I_{Cl \cdot Ca}$ at various command voltage steps in the absence of EGTA. Eicosapentaenoic acid (30 μM) decreased the peak of $I_{Ca \cdot L}$ from −160 pA to −20 pA and reduced the amplitude of $I_{Ca \cdot L}$ at all command voltage pulses. Then, $I_{Cl \cdot Ca}$ was totally abolished. Similar results were obtained when docosahexaenoic acid (30 μM) was applied (Fig. 3Ab). To confirm the effects of eicosapentaenoic acid on $I_{Ca \cdot L}$, the patch pipettes were filled with 5 mM EGTA to block $I_{Cl \cdot Ca}$ (Fig. 4A). The cell was held at −60 mV and command voltage steps to various membrane potentials were applied at 0.1 Hz. Eicosapentaenoic acid (30 μM) decreased the peak of $I_{Ca \cdot L}$ at all command potentials (Fig. 4A and B). After eicosapentaenoic acid washout with 0.1% albumin, the depressed current returned near to the control level. The mean peak current–voltage relationships obtained from six different cells in the absence or presence of eicosapentaenoic acid (30 μM) are shown in Fig. 4B. Fig. 4C and Table 2 show the effects of various concentrations of eicosapentaenoic acid on $I_{Ca \cdot L}$. The cell was held at −60 mV and command pulses to +20 mV were applied under control conditions and after addition of eicosapentaenoic acid (3–30 μM). Eicosapentaenoic acid decreased $I_{Ca \cdot L}$ in a concentration-dependent manner (Fig. 4C). The inhibitory effects of eicosapentaenoic acid on $I_{Ca \cdot L}$ were observed at concentrations greater than 3 μM,

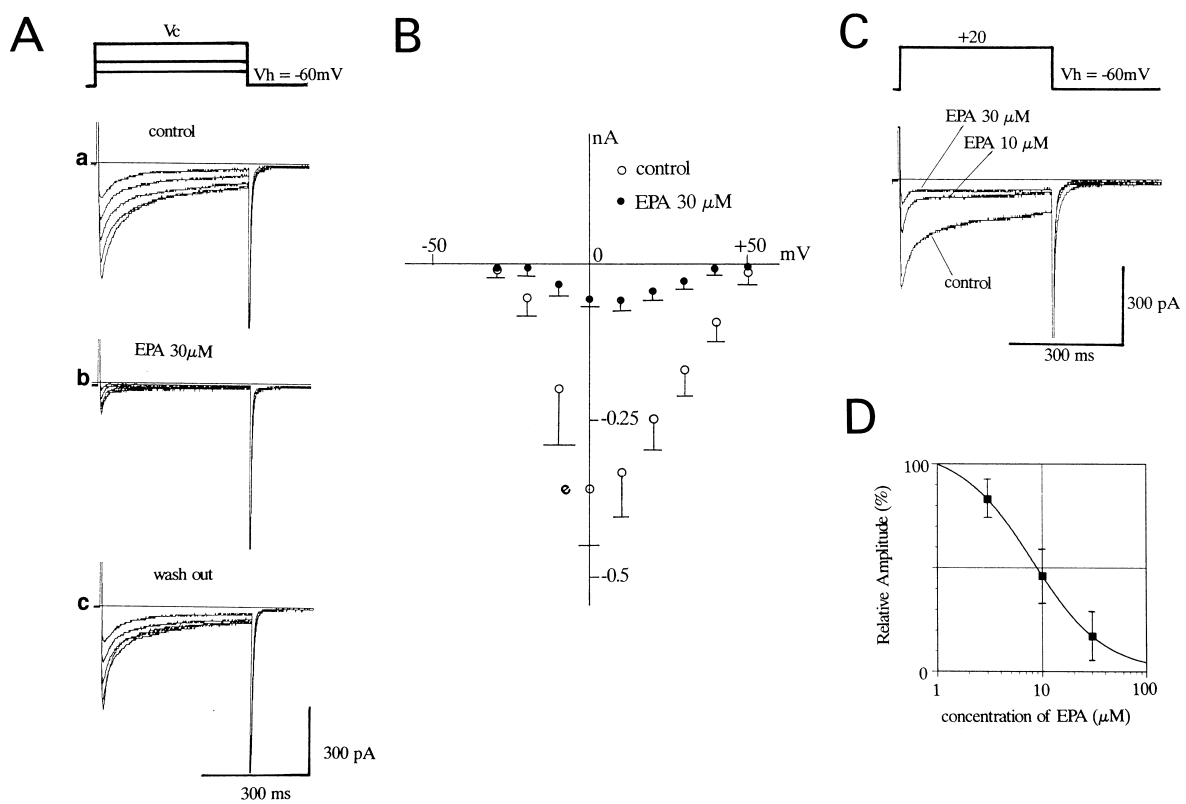

Fig. 4. Effects of eicosapentaenoic acid on the voltage-dependent L-type Ca^{2+} currents in single tracheal smooth muscle cells. The patch pipette contained Cs^+-internal solution with EGTA. The cell was held at −60 mV, and command voltage pulses to various membrane potentials were applied at 0.1 Hz. In (A), the original current traces are shown for control conditions (a), in the presence of eicosapentaenoic acid (30 μM, b) and after washout with 0.1% albumin (c). The mean current–voltage relationships at the peak $I_{Ca \cdot L}$ are plotted in (B). The data were obtained from six different cells, and the mean ± S.D. value is shown. (C) and (D) Concentration-dependent inhibition of $I_{Ca \cdot L}$ by eicosapentaenoic acid. Various concentrations of eicosapentaenoic acid (3–30 μM) were tested. The typical current tracings are shown in (C). The amplitude of the peak for $I_{Ca \cdot L}$ during application of eicosapentaenoic acid was compared with the control value. The percent inhibition by eicosapentaenoic acid of $I_{Ca \cdot L}$ (the mean ± S.D. value) is shown. The data were obtained from six different cells.

Table 2
Effect of ω-3 polyunsaturated fatty acids (eicosapentaenoic acid and docosahexaenoic acid) on the voltage-dependent L-type Ca^{2+} currents and Ca^{2+}-dependent Cl^- currents activated by caffeine and neurokinin A in tracheal myocytes isolated from the guinea-pig

ω-3 polyunsaturated fatty acids (concentration)	Percent inhibition
A: Effect of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the voltage-dependent L-type Ca^{2+} currents	
EPA (3 μM)	17 ± 10% (n = 6)
EPA (10 μM)	54 ± 14% (n = 6)
EPA (30 μM)	83 ± 8% (n = 6)
EPA (30 μM) in the presence of NDGA and indomethacin	81 ± 8% (n = 6)
DHA (30 μM)	79 ± 10% (n = 6)
B: Effect of eicosapentaenoic acid (EPA) on Ca^{2+}-dependent Cl^- currents elicited by caffeine and neurokinin A	
EPA (30 μM) (caffeine-evoked Cl^- current)	85 ± 10% (n = 6)
EPA (30 μM) (neurokinin A-induced Cl^- current)	82 ± 8% (n = 6)

EPA (eicosapentaenoic acid); DHA (docosahexaenoic acid); NDGA (nordihydroguaiaretic acid). The data represent the mean ± S.D. and the number of cells tested (n) is shown in parenthesis.

and the half-maximal inhibitory concentration of eicosapentaenoic acid for $I_{Ca \cdot L}$ was approximately 8 μM (Fig. 4D). These findings suggest that the ω-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid, inhibit voltage-dependent L-type Ca^{2+} currents in single tracheal smooth muscle cells.

To exclude the possible involvement of cyclo-oxygenase and lipoxygenase pathways, the effects of eicosapentaenoic acid on $I_{Ca \cdot L}$ were investigated in the presence of indomethacin and nordihydroguaiaretic acid (Fig. 3C and Table 2). As shown in Fig. 3C, the inhibitory effects of eicosapentaenoic acid (30 μM) on $I_{Ca \cdot L}$ were observed even in the presence of both indomethacin (10 μM), a cyclo-oxygenase inhibitor, and nordihydroguaiaretic acid (10 μM), a lipoxygenase inhibitor. The responses were not statistically different from those of the cells under control conditions.

3.2. Inhibitory effects of ω-3 polyunsaturated fatty acids on Ca^{2+}-dependent Cl^- currents evoked by caffeine or neurokinin A

The above results suggest that ω-3 polyunsaturated fatty acids modulate $I_{Ca \cdot L}$, and then $I_{Cl \cdot Ca}$ evoked by Ca^{2+} influx through voltage-dependent Ca^{2+} channels. To investigate whether ω-3 polyunsaturated fatty acids affect $I_{Cl \cdot Ca}$ channels independently of $I_{Ca \cdot L}$, the effects of eicosapentaenoic acid on $I_{Cl \cdot Ca}$ elicited by caffeine or neurokinin A were investigated. As described previously (Nakajima et al., 1995; Hazama et al., 1996), both caffeine

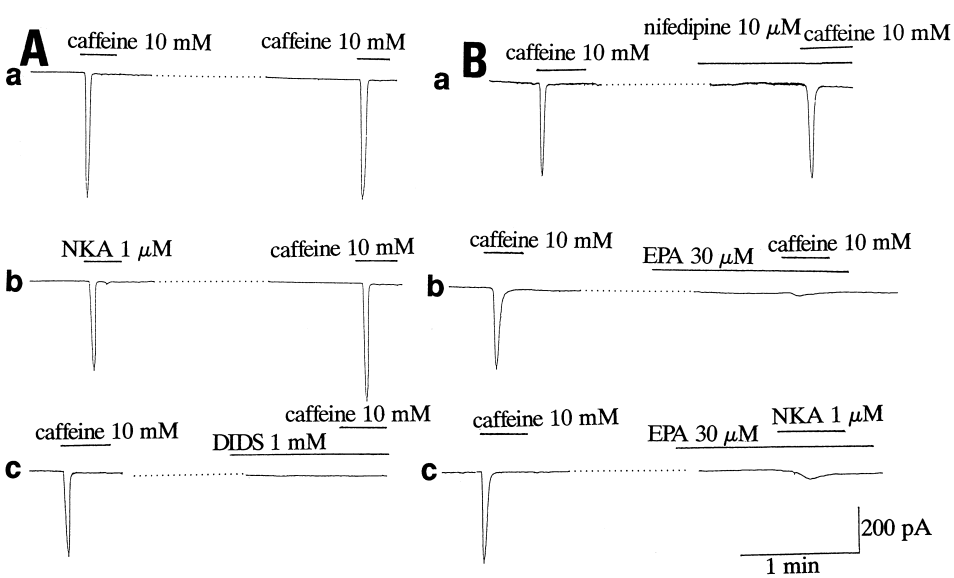

Fig. 5. Effects of eicosapentaenoic acid and nifedipine on Ca^{2+}-dependent Cl^- current ($I_{Ca \cdot Cl}$) evoked by caffeine or neurokinin A in single tracheal smooth muscle cells. The cells were held at −60 mV, and the patch pipette contained Cs^+-internal solution without EGTA. The drug sequence is shown at the top of the original current trace. In each case, the holding current was continuously monitored. The zero current levels are shown as arrowheads.

and neurokinin A transiently activated $I_{Cl \cdot Ca}$ as a result of the release of Ca^{2+} from the sarcoplasmic reticulum induced by neurokinin A or caffeine. Fig. 5A shows the effects of caffeine or neurokinin A on membrane currents. The patch pipette was filled with Cs-internal solution without EGTA, and the cells were held at −40 mV. Both caffeine (Fig. 5Aa, 10 mM) and neurokinin A (Fig. 5Ab, 1 μM) elicited a transient inward current. The second application of caffeine also elicited the same amplitude inward current. However, neither neurokinin A nor caffeine elicited the inward current in the presence of 4,4′-diisothiocyanastilbene-2,2′-disulphonic acid (1 mM) (Fig. 5Ac), suggesting that both agents activate $I_{Cl \cdot Ca}$ in single tracheal smooth muscle cells as previously described (Nakajima et al., 1995; Hazama et al., 1996). Fig. 5Ba, Bb and Bc show the effects of nifedipine (10 μM) and eicosapentaenoic acid on $I_{Cl \cdot Ca}$ evoked by caffeine or neurokinin A. Since the activation of $I_{Cl \cdot Ca}$ by neurokinin A and caffeine was transient, the cells were pretreated with nifedipine or eicosapentaenoic acid. Nifedipine (10 μM) did not significantly inhibit the activation of $I_{Cl \cdot Ca}$ elicited by caffeine. Eicosapentaenoic acid (30 μM) by itself did not evoke current at a holding potential of −40 mV, but it inhibited the amplitude of $I_{Cl \cdot Ca}$ induced by caffeine (10 mM, Fig. 5Bb) or neurokinin A (1 μM, Fig. 5Bc, Table 2).

4. Discussion

The major findings of the present study are as follows. (1) ω-3 polyunsaturated fatty acids, eicosapentaenoic and docosahexaenoic acids, modulated voltage-dependent L-type Ca^{2+} currents in single tracheal smooth muscle cells. (2) ω-3 polyunsaturated fatty acid (eicosapentaenoic acid) also modulated the Ca^{2+}-dependent Cl^- current evoked by caffeine and neurokinin A as a result of Ca^{2+} release from the sarcoplasmic reticulum and Ca^{2+} influx through voltage-dependent L-type Ca^{2+} currents. These effects of ω-3 polyunsaturated fatty acids may modulate tracheal smooth muscle tone, which may contribute to their beneficial effects in pathophysiological conditions such as bronchial asthma.

The present study provides evidence that ω-3 polyunsaturated fatty acids, eicosapentaenoic acid and docosahexaenoic acid, inhibited the voltage-dependent L-type Ca^{2+} current ($I_{Ca \cdot L}$) in single tracheal smooth muscle cells from the guinea-pig. The inhibitory effect of eicosapentaenoic acid on $I_{Ca \cdot L}$ was observed at concentrations greater than 3 μM, and the half maximal inhibitory concentration of eicosapentaenoic acid was approximately 8 μM. Since the plasma concentration of free eicosapentaenoic acid measured by gas chromatography was about 1–6 μM (Asano, Nakajima and Okuda, unpublished result) and can be increased after ingestion of cod liver oil or after treatment with eicosapentaenoic acid–ester (Okuda et al., 1996), the inhibitory action of eicosapentaenoic acid on $I_{Ca \cdot L}$ may contribute to its relaxant action on tracheal smooth muscle cells. Several possible mechanisms to explain the inhibitory effects of ω-3 polyunsaturated fatty acids on $I_{Ca \cdot L}$ can be proposed. In various kinds of cells, unsaturated free fatty acids such as eicosapentaenoic acid and arachidonic acid evoke Ca^{2+} release from Ca^{2+} storage sites (Force et al., 1990; Ling et al., 1992; Maruyama, 1993). These findings suggest that eicosapentaenoic acid may decrease $I_{Ca \cdot L}$ by promoting Ca^{2+}-dependent inactivation of $I_{Ca \cdot L}$ (Ohya et al., 1988). However, this mechanism may be not involved in the inhibitory effects of ω-3 polyunsaturated fatty acids on $I_{Ca \cdot L}$. The effects of eicosapentaenoic acid on $I_{Ca \cdot L}$ were still observed even when the patch pipette contained a high concentration (5 mM) of EGTA. Furthermore, eicosapentaenoic acid (3–30 μM) by itself did not activate Ca^{2+}-dependent Cl^- currents ($I_{Cl \cdot Ca}$), as shown in Fig. 5, whereas caffeine and neurokinin A evoked a huge $I_{Cl \cdot Ca}$ due to Ca^{2+} release from Ca^{2+} storage sites. Thus, eicosapentaenoic acid at concentrations lower than 30 μM may not evoke significant Ca^{2+} release in tracheal smooth muscle cells. Lipoxygenase-dependent metabolites of arachidonic acid regulate several types of ion channel (Piomelli et al., 1987; Kim et al., 1989; Kurachi et al., 1989; Cantiello et al., 1990). In the present study, neither indomethacin, a cyclo-oxygenase inhibitor, nor nordihydroguaiaretic acid, a lipoxygenase inhibitor, prevented the inhibitory effects of ω-3 fatty acids on $I_{Ca \cdot L}$, suggesting that the metabolites of these pathways are not involved in the action of ω-3 fatty acids either. *Cis*-fatty acids have been reported to inhibit voltage-dependent Ca^{2+} channels in mouse neuroblastoma cells due to activation of protein kinase C (Linden and Routtenberg, 1989), and fatty acids, including eicosapentaenoic acid, have been also reported to modulate the activity of protein kinase C (Shearman et al., 1989; Holian and Nelson, 1992). However, protein kinase C may not be involved in the inhibitory effects of ω-3 polyunsaturated fatty acids on $I_{Ca \cdot L}$ because the inhibitory effect of eicosapentaenoic acid on $I_{Ca \cdot L}$ was not blocked by the internal application of 100 μM H-7, a specific inhibitor of protein kinase C (data not shown).

Arachidonic acid and other fatty acids have been also reported to modulate $I_{Ca \cdot L}$. Arachidonic acid increases $I_{Ca \cdot L}$ in rat pituitary GH_3/B_6 cells (Vacher et al., 1989; Vacher et al., 1992) and guinea-pig ventricular myocytes (Huang et al., 1992), and inhibits $I_{Ca \cdot L}$ in single smooth muscle cells from vas deferens of the guinea-pig and rabbit intestinal smooth muscle cells (Shimada and Somlyo, 1992; Nagano et al., 1995). In our experiments with tracheal smooth muscle cells, arachidonic acid had similar effects on $I_{Ca \cdot L}$ as eicosapentaenoic acid and docosahexaenoic acid, while the saturated stearic acid and the monounsaturated oleic acid had no significant effect (data not shown). These results, including those for eicosapentaenoic acid and docosahexaenoic acid are somewhat similar to those reported for other smooth muscle cells (Shimada and

Somlyo, 1992; Nagano et al., 1995) and rat ventricular myocytes (Xiao et al., 1997). However, our results were different from the results for cardiac myocytes, where eicosapentaenoic acid and docosahexaenoic acid failed to inhibit $I_{Ca \cdot L}$ (Huang et al., 1992). Thus, the effects of ω-3 fatty acids on $I_{Ca \cdot L}$ may vary depending upon the cell type or experimental conditions, which makes it unlikely that their effects are due to a general physicochemical change, such as an increase in membrane lipid fluidity. It is more likely that these ω-3 fatty acids modulate the channels by an interaction with the ion channel protein itself or by acting at lipids sites near the channels after partition into lipid bilayer.

The present results also suggest that ω-3 polyunsaturated fatty acids inhibit $I_{Ca \cdot L}$ and the subsequent Ca^{2+}-dependent Cl^- currents ($I_{Cl \cdot Ca}$) in single tracheal smooth muscle cells. Eicosapentaenoic acid also suppressed the activation of $I_{Cl \cdot Ca}$ by caffeine and neurokinin A, due to Ca^{2+} release from the Ca^{2+} storage sites. Since the Ca^{2+}-induced Ca^{2+} release mechanism of the sarcoplasmic reticulum is also partly involved in the activation of $I_{Cl \cdot Ca}$ by Ca^{2+}-influx through $I_{Ca \cdot L}$ (Hazama et al., 1996), the inhibitory effects of ω-3 polyunsaturated fatty acids may be caused by the inhibition of Ca^{2+} release from storage sites. However, after washout with the bathing solution containing albumin, the inhibitory effects of ω-3 polyunsaturated fatty acids on $I_{Cl \cdot Ca}$ as well as $I_{Ca \cdot L}$ were rapidly reversible Taking these considerations into account, it is likely that the inhibitory effects of ω-3 polyunsaturated fatty acids on $I_{Cl \cdot Ca}$ are not caused by the inhibition of intracellular Ca^{2+} release, but are rather due to a direct interaction with an external domain of the ion channel. These results are compatible with previous findings showing that cis-unsaturated fatty acids directly inhibit apical membrane Cl^- channels in airway epithelial cells (Anderson and Welsh, 1990; Hwang et al., 1990). The activation of $I_{Cl \cdot Ca}$ may depolarize the membrane potential in tracheal smooth muscle cells, resulting in indirect activation of voltage-dependent Ca^{2+} currents and enhancement of the tone of tracheal smooth muscle (Hazama et al., 1996). Therefore, the alterations in airway electrical activity caused by the inhibition of $I_{Cl \cdot Ca}$ by ω-3 polyunsaturated fatty acids may also play a role in regulating tracheal smooth muscle tone. However, since K^+ channel activity also plays an important role in regulating smooth muscle tone, and fatty acids such as arachidonic acid have been reported to modulate a K^+ channel in various types of smooth muscle cells (Ordway et al., 1989; Kirber et al., 1992; Xu and Lee, 1996), further studies are needed to clarify the effects of ω-3 polyunsaturated fatty acids on K^+ channels in tracheal smooth muscle cells.

The present study shows that ω-3 polyunsaturated fatty acids, eicosapentaenoic acid and docosahexaenoic acid, modulate voltage-dependent L-type Ca^{2+} currents and Ca^{2+}-dependent Cl^- currents in single tracheal smooth muscle cells. The activation of voltage-dependent Ca^{2+} channels is considered to play an important role in the regulation of smooth muscle tone, and thus ω-3 polyunsaturated fatty acids, eicosapentaenoic acid and docosahexaenoic acid, may constitute a treatment for pathological conditions such as bronchial asthma.

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