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Leukotriene C_4 enhances the contraction of porcine tracheal smooth muscle through the activation of Y-27632, a rho kinase inhibitor, sensitive pathway

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1 An unsaturated fatty acid, leukotriene C_4 (LTC_4), has a potent contractile effect on human airway smooth muscle, and has been implicated in the pathogenesis of human asthma. Using front-surface fluorometry with fura-PE3, the effect of LTC_4 on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension were investigated in porcine tracheal smooth muscle strips.

2 The application of LTC_4 induced little or no contraction despite a small and transient increase in $[Ca^{2+}]_i$. In the presence of LTC_4, however, the contractions evoked by high K^+ depolarization or a low concentration of carbachol (CCh) were markedly enhanced without inducing any changes in the $[Ca^{2+}]_i$ levels, thus indicating that LTC_4 increases the Ca^{2+} responsiveness of the contractile apparatus. This LTC_4-induced increase in Ca^{2+} responsiveness could partly be reproduced in the permeabilized preparation of tracheal smooth muscle strips.

3 The LTC_4-induced enhancement of contraction was accompanied by an increase in myosin light chain (MLC) phosphorylation and was blocked by a rho kinase inhibitor (Y-27632), but not by either a PKC inhibitor (calphostin C) or a tyrosine kinase inhibitor (genistein).

4 These results indicated that, in porcine tracheal smooth muscle, LTC_4 enhances the contraction by increasing the Ca^{2+} responsiveness of the contractile apparatus in a MLC phosphorylation dependent manner, possibly through the activation of the rho-rho kinase pathway.
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Keywords: Leukotriene C_4; porcine tracheal smooth muscle; intracellular Ca^{2+} concentration; Ca^{2+} sensitivity; rho-kinase; Y-27632; myosin light chain phosphorylation

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CCh, carbachol; CSS, cytosolic substitution solution; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGTA, ethylene glycol-bis (beta-aminoethyl ether) N,N,N′,N-tetra-acetic acid; fura-PE3/AM, fura-PE3 acetoxymethyl ester; LT, leukotriene; MBS, myosin-binding subunit; MLC, myosin light chain; PSS, physiological salt solution; SRS-A, slow-reacting substance of anaphylaxis

Introduction

Cysteinyl-leukotrienes (LTs) are a family of biologically active compounds derived from arachidonic acid *via* the 5-lipoxygenase pathway (Murphy *et al*., 1979). It is now generally accepted that a mixture of LTC_4 and LTD_4 makes up the material originally known as the 'slow-reacting substance of anaphylaxis' (SRS-A), first described by Feldberg & Kellaway (1938). LTs have been shown to be potent bronchoconstrictors (Dahlen *et al*., 1980) in both normal humans and asthma patients (Barnes *et al*., 1984), thus indicating that the main pathophysiological role of cysteinyl-LTs lies in bronchial asthma (Piper *et al*., 1991). It is thus expected that LTs might have a potent broncoconstricting effect. Indeed, it has been reported that LTs are thousands of times more active than histamine in the airway smooth muscle in humans and guinea-pigs (Dahlen *et al*., 1983). In addition to the direct bronchoconstricting effect, LTs might also be related to the airway hyper-responsiveness in the late asthmatic reaction (Henderson *et al*., 1996; O'Hickey *et al*., 1991). However, LTs have also been reported to be less active than histamine in the monkey trachea (Smedegard *et al*., 1982) and have little or no effect on airway smooth muscle in the rat, cat or dog (Krell *et al*., 1981). It is thus suggested that considerable variation exist among species in the responses of airway smooth muscle to LTs.

It is generally accepted that smooth muscle contraction is primarily regulated by the increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and subsequent phosphorylation of myosin light chain (MLC) by Ca^{2+}-calmodulin dependent MLC kinase (Kamm & Stull, 1985). However, it has also been recognized that the Ca^{2+} sensitivity of the contractile apparatus changes during stimulation. It has been reported that L-type Ca^{2+} channel antagonists inhibited the effect of LTC_4 on muscle tension and Ca^{2+} influx (Saad & Burka, 1984; Zschauer *et al*., 1988). However, the relationship between LTC_4-induced tension and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) remains controversial and the effects of LTC_4 on the Ca^{2+} sensitivity of the contractile apparatus and MLC phosphorylation are unknown. Furthermore, the

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mechanism of LTs-induced airway hyper-responsiveness remains unclear.

The aim of the present study was to investigate the effect of LTC_4 on the contraction of porcine tracheal smooth muscle. We found that LTC_4 enhanced tension development, although it had no direct contractile effect on porcine tracheal smooth muscle in normal physiological salt solution (PSS). Therefore, we performed experiments using simultaneous measurements of $[Ca^{2+}]_i$ and tension in fura-PE3 loaded intact muscle strips and receptor coupled permeabilized preparations, in order to explore the mechanism underlying the LTC_4-induced enhancement of contraction. We further investigated the pathway for the signals elicited by LTC_4, using inhibitors for several kinases and measured the extent of MLC phosphorylation. The results obtained indicated that LTC_4 enhanced the contraction induced by high K^+ depolarization or carbachol (CCh) without changing the $[Ca^{2+}]_i$ level and this was accompanied by an increase in the MLC phosphorylation possibly mediated by the rho-rho kinase pathway.

Methods

Tissue preparation

The tracheas were dissected from adult pigs at a local slaughterhouse using a protocol approved by the Animal Research Committee of the Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University. The tracheas were placed in ice-cold PSS. The lower end of the trachea, just above the first bronchus branching, three tracheal rings in length, were used for the experiments. The posterior portion of the trachea was excised longitudinally, and all cartilage was detached. Both the mucosa and adventitial tissue were carefully removed under microscopic observation. The muscle sheets were transversely cut into rectangular strips measuring approximately 3 mm in length and 1 mm in width (Kai *et al*., 1993).

Fura-PE3 loading

The tracheal strips were loaded with the Ca^{2+} indicator dye, fura-PE3, in the form of acetoxymethyl ester (fura-PE3/AM), as previously described (Kai *et al*., 1993). The strips were incubated in 1 ml aerated (95% O_2: 5% CO_2) Dulbecco's modified Eagle's medium (DMEM) containing 50 μM fura-PE3/AM and 5% foetal bovine serum for 4 h at 37°C. After loading with fura-PE3, the strips were washed with normal PSS to remove the dye in the extracellular space, and then were equilibrated in normal PSS for at least 1 h before the measurements.

Measurement of tension development

Each strip was mounted vertically in a 6-ml quartz organ bath, which was maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. The lower end of the strip was fixed, and the upper end of the strip was connected to a force transducer (TB-612T, Nihon Koden, Japan) to record the isometric tension. During the 1 h post-fura-PE3 loading equilibration period, the strips were stimulated with 40 mM K^+ PSS at 5–10 min intervals, and the muscle length was increased in a stepwise manner after each stimulation until the developed tension reached a maximum. When exposed to 40 mM K^+ PSS, most strips produced a stable tension within 15 min, with or without an initial transient force response (Kai *et al*., 1993; 1996; Yoshimura *et al*., 1995). Any strips showing instability in tension, as induced by 40 mM K^+ PSS, were excluded from the study. The responsiveness of each strip to 40 mM K^+ PSS was then recorded before starting the experimental protocol, because almost all the maximum reproducible responses of tension to high K^+ depolarization were obtained at this concentration of K^+. The developed tension was expressed as a percentage, while assigning the values in normal (5.9 mM K^+) PSS and a steady state of the contraction induced by 40 mM K^+ PSS to be 0 and 100%, respectively. Except for the experiment with CCh, 1 μM atropine was added to block any response to cholinergic nerves within the preparation.

Measurements of fura-PE3 fluorescence

Changes in the fluorescence intensity of the fura-PE3-Ca^{2+} complex were monitored using a front surface fura-PE3 fluorometer (model CAM-OF). The details of our front-surface fluorometry system have been described elsewhere (Hirano *et al*., 1990; Kai *et al*., 1993; Kanaide, 1999). In brief, two wavelengths of excitation light (340 and 380 nm) were obtained spectroscopically from a Xenon light source. The strips were illuminated by guiding the two alternating (400 Hz) wavelengths of excitation light through quartz optic fibres. The surface fluorescence of the strip was collected by glass optic fibres and introduced through a 500 nm band pass filter into a photomultiplier. We thus measured the fura-PE3 fluorescence intensity of 500 nm emission light, which was induced by alternating two wavelengths of excitation light (340 and 380 nm).

The ratio of the fluorescence intensities (fluorescence ratio) at 340 nm excitation to that at 380 nm excitation was monitored to estimate the changes in $[Ca^{2+}]_i$ and was expressed as a percentage, while assigning the values in normal PSS (5.9 mM K^+) and a steady state of the contraction induced by 40 mM K^+ PSS to be 0 and 100%, respectively.

Tension measurement of the α-toxin permeabilized tracheal strips

The permeabilization of the tracheal strips by α-toxin was performed according to the methods described by Nishimura *et al*. (1988) with minor modifications. In brief, small strips (about 0.5 mm in width and 2 mm in length) of the porcine tracheal smooth muscle were mounted between two tungsten wires, one of which was fixed and the other one was attached to a force transducer (UL2; Minebea Co., Japan). Permeabilization was carried out in the Ca^{2+}-free cytosolic substitution solution (CSS; in mM: potassium methanesulphonate 100, Na_2ATP 2.2, $MgCl_2$ 3.38, EGTA 10, creatine phosphate 10, Tris-maleate 20 (pH 6.8)) with 5000 units ml^{-1} *Staphylococcus aureus* α-toxin for 30 min. The composition of Ca^{2+} solution (activating solution) was the same as the CSS described above, except that it contained the indicated concentration of free Ca^{2+} buffered by 10 mM EGTA. All experiments using permeabilized tissue were performed at

room temperature. The resting tension in the relaxing solution and the maximal tension induced by 10 μM Ca^{2+} were taken as 0 and 100%, respectively.

Measurement of MLC phosphorylation

The extent of MLC phosphorylation in the tracheal strips was determined using the urea-glycerol gel electrophoresis technique (Persechini *et al*., 1986), followed by immunoblot detection with a specific mouse monoclonal anti-MLC antibody. Tracheal strips were obtained and treated in a similar way as that described in Tissue preparation. At the indicated times, the tracheal strips were transferred into 90% acetone, 10% trichloroacetic acid and 10 mM dithiothreitol (DTT) pre-chilled at -80°C to stop the reaction. All tissue specimens were then washed extensively and stored in acetone containing 10 mM DTT at -80°C. After the tissue was dried to remove acetone, it was extracted in the sample buffer (8 M urea, 20 mM Tris-base, 23 mM glycine, 0.004% bromophenol blue and 10 mM DTT) at room temperature for 1.5 h. The supernatant was subjected to electrophoresis on 10% polyacrylamide, 40% glycerol, followed by transfer onto polyvinylidene difluoride membrane (BioRad, Hercules, CA, U.S.A.) in 10 mM Na_2HPO_4 (pH 7.6). The 20 kDa MLC, both unphosphorylated and phosphorylated, was detected by the specific antibody ($\times 200$ dilution), and a horse radish peroxidase-conjugated secondary antibody ($\times 1000$ dilution). The immune complex was detected using enhanced chemiluminescence technique (ECL plus kit; Amersham, Buckinghamshire, U.K.). X-OMAT AR Film (Kodak, Rochester, NY, U.S.A.) was used to detect light emission. After scanning the X-ray film on an ATTO ImageSaver AE-6905C, the density of unphosphorylated and phosphorylated MLCs was determined by Gel Plotting Macros of the NIH image ver. 1.61 (National Institute of Health, U.S.A.). The percentage of the phosphorylated form in total MLC (sum of unphosphorylated and phosphorylated forms) was calculated to indicate the extent of MLC phosphorylation.

Solutions and drugs

Normal PSS was of the following composition (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25, and D-glucose 11.5. High K^+ PSS was identical to normal PSS, except for an equimolar substitution of KCl for NaCl. The Ca^{2+}-free PSS was produced by exclusion of $CaCl_2$ from the composition of normal PSS. PSS was bubbled with 95% O_2 and 5% CO_2, with a resulting pH of 7.4 at 37°C.

LTC_4 was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Fura PE3/AM was from Texas Fluorescence Laboratory (Austin, TX, U.S.A.). DMEM was purchased from Gibco (Grand Island, NY, U.S.A.). The carbachol and α-toxin was from Sigma Chemical (St. Louis, MO, U.S.A.). The atropine sulfate was from Wako (Osaka, Japan). Calphostin C was obtained from Kyowa (Tokyo, Japan). Genistein was obtained from CALBIOCHEM (La Jolla, CA, U.S.A.). Y-27632 ((R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride, monohydrate) was kindly donated by Yoshitomi Pharmaceutical Industries (Osaka, Japan). EGTA was obtained from Dojindo (Kumamoto, Japan).

Data analysis

The measured values were expressed as the mean ± s.e.mean (n = number of observations). Unpaired Student's t-test was used to determine the statistical significance of the LTC_4 effect on the contractions induced by extracellular Ca^{2+} during high K^+ depolarization. Analysis of covariance was used to determine the statistical significance of the shift of the $[Ca^{2+}]_i$-tension relationship. For the rest of the measurements, the paired t-test was used. P values of less than 0.05 were considered to be significant.

Results

Effect of LTC_4 on the changes in $[Ca^{2+}]_i$ and tension in normal PSS

As shown in Figure 1, the application of 10^{-7} M LTC_4 induced little or no contraction (0.32 ± 3.0%, $n=9$) with a small transient increase in $[Ca^{2+}]_i$ (20.6 ± 3.28% $n=9$). At 5 and 10 min after the application of LTC_4, the $[Ca^{2+}]_i$ was 5.62 ± 4.52 and 5.29 ± 5.35%, respectively. No significant contraction could be obtained even when the incubation time was prolonged for up to 1 h of observation (data not shown).

Effect of pretreatment with LTC_4 on the changes in $[Ca^{2+}]_i$ and tension induced by 40 mM K^+ depolarization

Although the application of 10^{-7} M LTC_4 in normal PSS induced little or no contraction, it greatly enhanced the contraction induced by a subsequent application of 40 mM K^+ PSS (from 100% to 182.3 ± 13.2%, $n=9$). It could be postulated that this enhanced contraction might be due to the enhanced increase in $[Ca^{2+}]_i$ during 40 mM K^+ depolarization after exposure to LTC_4, because it is generally accepted that the smooth muscle contraction is primarily regulated by the increase in $[Ca^{2+}]_i$ and the subsequent phosphorylation of MLC by Ca^{2+}-calmodulin dependent MLC kinase (Kamm & Stull, 1985). However, the level of $[Ca^{2+}]_i$ (103.5 ± 5.9%, $n=9$; Figure 1A–C) was not changed by the treatment with LTC_4. The calculated $[Ca^{2+}]_i$ in normal PSS (0%) and at steady state in 40 mM K^+ PSS (100%), determined in separate measurements was 90 ± 14 and 499 ± 54 nM, respectively (Kai *et al*., 1993). For the quantitative analysis of the effect of LTC_4, we chose the pretreatment time of 15 min based on the observation that a pretreatment time of less than 15 min induced a less potent enhancement, while pretreatments of >15 min to 40 min showed the same degree of enhancement (data not shown) as the 15 min pretreatment. When the strip was exposed to LTC_4 for 15 min and then washed out, the observed enhancement of tension development lasted for up to 2 h (data not shown).

Effect of pretreatment with LTC_4 on changes in $[Ca^{2+}]_i$ and tension induced by CCh

Prior to the determination of the effects of LTC_4 on the elevations of $[Ca^{2+}]_i$ and tension induced by CCh, we first recorded the levels of $[Ca^{2+}]_i$ and tension induced by 3×10^{-8} M CCh (control contraction). The strip was then

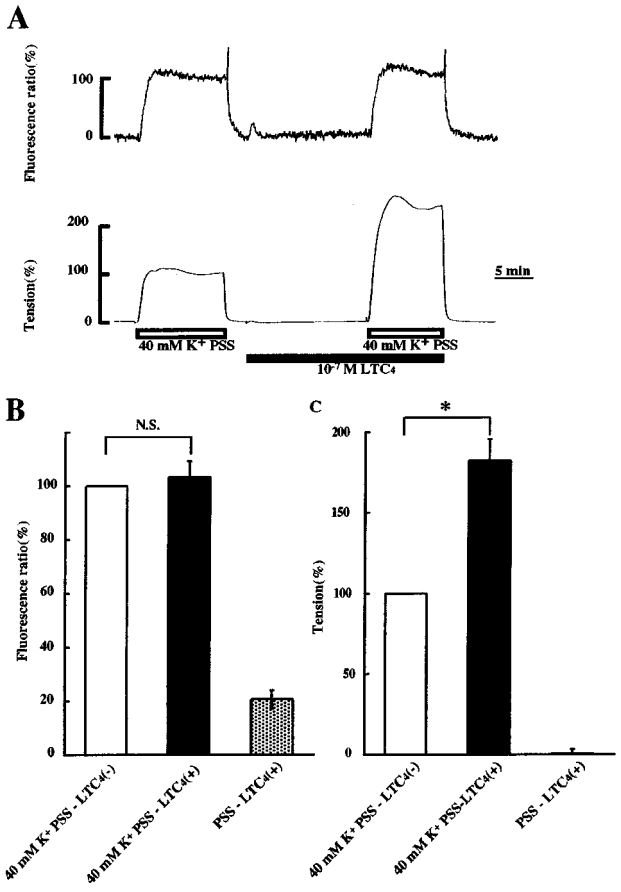

Figure 1 Effect of LTC_4 on the $[Ca^{2+}]_i$ and tension levels of the porcine tracheal smooth muscle. (A) A representative recording showing the effect of LTC_4 (10^{-7} M, 15 min before and during activation) on the increases in $[Ca^{2+}]_i$ and tension induced by 40 mM K^+ depolarization. (B, C) Summary of the results obtained from nine separate measurements performed in a similar manner as that shown in (A). The levels of $[Ca^{2+}]_i$ (B) and tension (C) induced by 40 mM K^+ depolarization, those induced by 40 mM K^+ depolarization in the presence of 10^{-7} M LTC_4 and those induced by 10^{-7} M LTC_4 alone in normal PSS were plotted. The levels of $[Ca^{2+}]_i$ and tension were expressed as a percentage, assigning the values of normal (5.9 mM) and 40 mM K^+ PSS to be 0 and 100%, respectively. Data are the mean + s.e.mean ($n=9$). $^*P<0.05$.

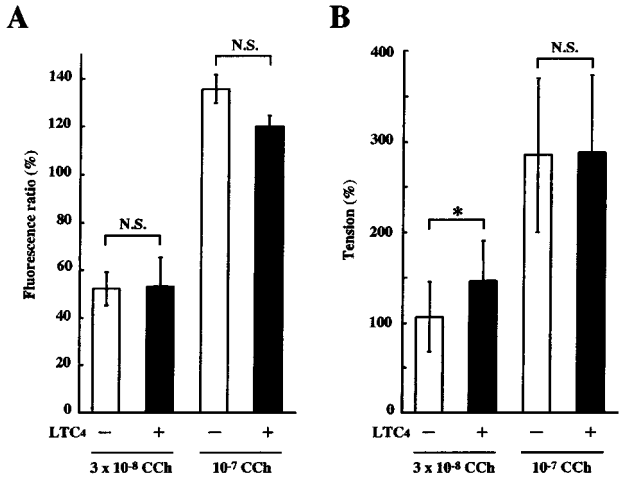

Figure 2 Effect of LTC_4 on the $[Ca^{2+}]_i$ (A) and tension (B) levels induced by two different concentrations of CCh. The levels of $[Ca^{2+}]_i$ and tension induced by the indicated concentration of CCh in the presence or absence of 10^{-7} M LTC_4 were plotted. The levels of $[Ca^{2+}]_i$ and tension were expressed as a percentage, assigning the values of normal (5.9 mM) and 40 mM K^+ PSS to be 0 and 100%, respectively. Data are the mean + s.e.mean ($n=8$). $^*P<0.05$.

washed in order to relax the strip with normal PSS. After 15 min of pretreatment by 10^{-7} M LTC_4, 3×10^{-8} M CCh was again applied in the presence of 10^{-7} M LTC_4 and the levels of $[Ca^{2+}]_i$ and tension were compared with the control CCh-induced contraction. As shown in Figure 2, the contraction in the presence of LTC_4 was significantly enhanced (from 106.5 ± 39.0 to $147.2\pm45.4\%$; $n=8$) with no significant change in $[Ca^{2+}]_i$ (from 52.2 ± 7.1 to $53.1\pm12.5\%$; $n=8$) by 3×10^{-8} M CCh. However, when the concentration of CCh was raised to 10^{-7} M, the enhancement of the contraction by LTC_4 became negligible (Figure 2).

Effect of LTC_4 on the changes in $[Ca^{2+}]_i$ and tension induced by the changes in extracellular Ca^{2+} concentration during 40 mM K^+ depolarization

To examine the effect of LTC_4 on the Ca^{2+} responsiveness of the contractile apparatus, we examined the $[Ca^{2+}]_i$-tension relationship of the contractions induced by the cumulative application of extracellular Ca^{2+} during 40 mM K^+ depolarization, either with or without LTC_4 treatment. Namely, after a 10 min incubation in Ca^{2+}-free PSS containing 2 mM EGTA, followed by a 5 min incubation in Ca^{2+}-free PSS without EGTA, the tracheal strips were immersed in Ca^{2+}-free 40 mM K^+ solution, and then, the extracellular Ca^{2+} concentration was increased in a stepwise manner by the cumulative addition of $CaCl_2$. Figure 3A shows the representative recordings of changes in the $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ in Ca^{2+}-free 40 mM K^+ solution. In response to the stepwise increment of the extracellular Ca^{2+} concentration (0.05–2.5 mM), $[Ca^{2+}]_i$ and the tension increased in a concentration-dependent manner (Figure 3A,B). The level of $[Ca^{2+}]_i$ increased from $-11.5\pm1.80\%$ at 0.05 mM extracellular Ca^{2+} to $99.7\pm3.7\%$ at 2.5 mM extracellular Ca^{2+}, and the tension increased from -1.3 ± 0.3 to $93.2\pm3.5\%$ ($n=6$). Treatment with 10^{-7} M LTC_4 15 min before and during the cumulative application of extracellular Ca^{2+} significantly enhanced only the increases in tension (Figure 3B). In the LTC_4-treated strips, the tension increased from $-4.0\pm5.2\%$ at 0.05 mM extracellular Ca^{2+} to $176.5\pm22.0\%$ at 2.5 mM extracellular Ca^{2+} ($n=5$). Figure 4 shows the $[Ca^{2+}]_i$-tension relationship obtained from the averaged data illustrated in Figure 3. The $[Ca^{2+}]_i$-tension relationship in the presence of LTC_4 significantly shifted upward and to the left of that in the absence of LTC_4, thus indicating that LTC_4 induced an increase in the Ca^{2+} responsiveness of the contractile apparatus in porcine tracheal smooth muscle.

Effect of LTC_4 on the contraction of the α-toxin permeabilized strips

To further confirm the enhancing effect of LTC_4 on the Ca^{2+} responsiveness, we applied LTC_4 to the α-toxin permeabilized strips of porcine tracheal smooth muscle. As shown in Figure

5A, the application of 3×10^{-7} M LTC_4 to the steady state contraction evoked by 5×10^{-7} M Ca^{2+} CSS containing 10^{-5} M GTP induced a small but significant increase in the tension at a fixed Ca^{2+} concentration. However, the LTC_4-induced enhancement of the Ca^{2+}-induced contraction was much smaller than that induced by 10^{-5} M CCh (Figure 5B).

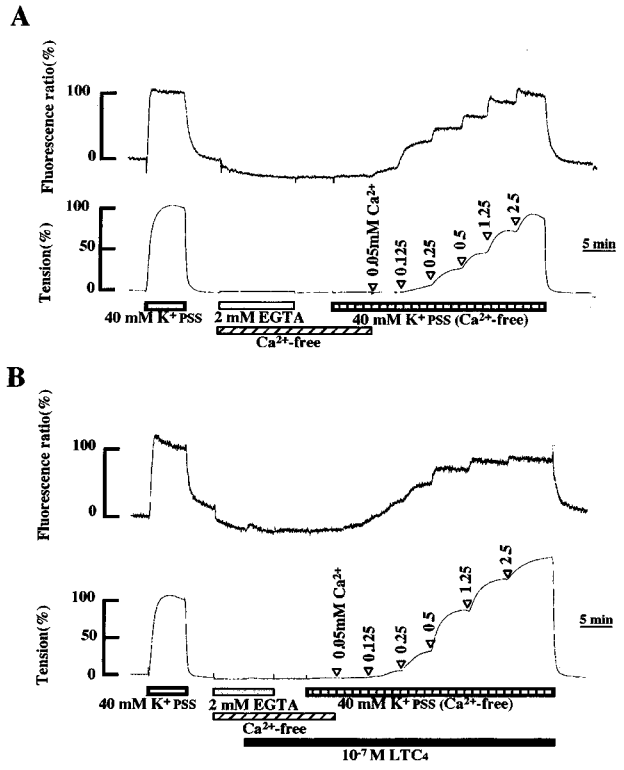

Figure 3 Effect of LTC_4 on $[Ca^{2+}]_i$ and tension induced by the cumulative application of external Ca^{2+} (0.05–2.5 mM) during 40 mM K^+ depolarization. (A) Representative recordings of the changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ in Ca^{2+}-free 40 mM K^+ solution. (B) Representative recordings of the effect of 10^{-7} M LTC_4 on the changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ during 40 mM K^+ depolarization.

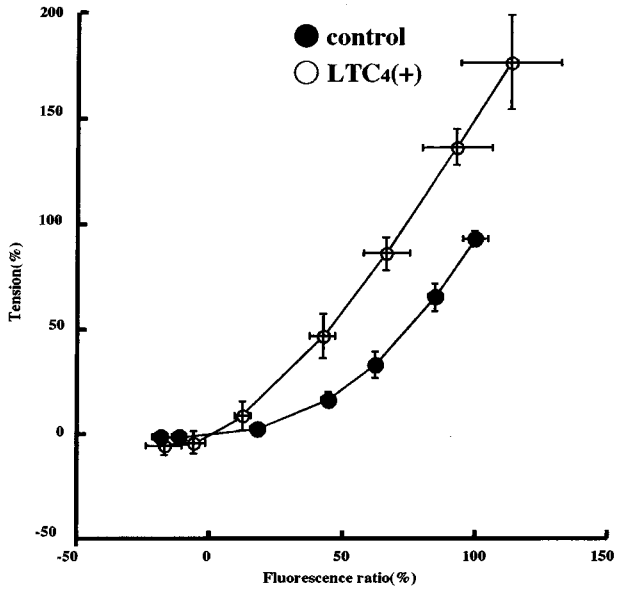

Figure 4 Effect of LTC_4 on the $[Ca^{2+}]_i$ -tension relationship. The $[Ca^{2+}]_i$ -tension curves were constructed using the data shown in Figure 3.

Effect of LTC_4 on the level of MLC phosphorylation

To determine whether or not the LTC_4-induced enhancement of the contraction is accompanied by increases in MLC phosphorylation, a protocol similar to that shown in Figure 1 was employed and the level of MLC phosphorylation was measured. One group of strips was activated by 40 mM K^+ depolarization in the organ bath while the developed tension was recorded in the same manner as that in Figure 1. After 3 min exposure to 40 mM K^+ PSS, the strips were transferred into 90% acetone, 10% trichloroacetic acid and 10 mM DTT pre-chilled at -80°C to stop the reaction. Another group of strips was activated by 10^{-7} M LTC_4 15 min before and during activation with 40 mM K^+ depolarization, and the reaction was stopped 3 min after the application of 40 mM K^+ PSS. We also determined the MLC phosphorylation at a resting condition in normal PSS. The exposure of the strips to 40 mM K^+ PSS significantly increased the MLC phosphorylation from $13.4\pm8.0\%$ ($n=6$) to $32.1\pm9.3\%$ ($n=6$). The pretreatment by 10^{-7} M LTC_4 and the exposure of the strips to 40 mM K^+ PSS further increased the MLC phosphorylation to $47.1\pm8.5\%$ ($n=6$).

Effect of kinase inhibitors on the LTC_4-induced enhancement in tension development

To explore the signal transduction pathway for the LTC_4-induced enhancement of contraction, we used the inhibitors for three types of kinases that had been previously reported to be involved in Ca^{2+} sensitization. Y-27632, a rho kinase inhibitor (Uehata *et al*., 1997), was applied 10 min before measuring the control contraction and added to all solutions during the protocol. Calphostin C, a protein kinase C inhibitor (Kobayashi *et al*., 1989), was loaded for 1 h under white light after fura-PE3 was loaded. Genistein, a tyrosine

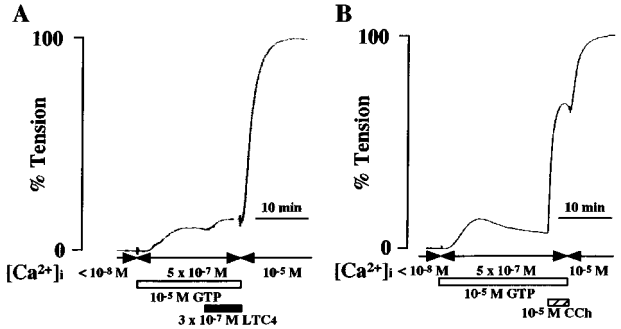

Figure 5 Effects of LTC_4 (A) and CCh (B) on the Ca^{2+}-induced contraction of the α-toxin permeabilized porcine tracheal smooth muscle. After the permeabilization with α-toxin, the strips were contracted by 5×10^{-7} M Ca^{2+} in the presence of 10^{-5} M GTP. When the tension reached a steady state, 3×10^{-7} M LTC_4 or 10^{-5} M CCh was added as indicated under the trace. Maximal tension was obtained in the presence of 10^{-5} M Ca^{2+}. The developed tension was thus expressed as a percentage, while assigning the values in the relaxing solution ($[Ca^{2+}]_i < 10^{-8}$ M) and in the activating solution ($[Ca^{2+}]_i = 10^{-5}$ M) to be 0 and 100%.

kinase inhibitor (Akiyama *et al*., 1987), was applied 30 min before measuring control contraction and that was added to all solutions during the protocol. The application of 3×10^{-7} M Y-27632 completely abolished the enhancing effect of LTC_4 on the tension development induced by 40 mM K^+ depolarization (Figure 6A,E). Neither 10^{-6} M calphostin C (Figure 6B,E) nor 10^{-5} M genistein (Figure 6C,E) had any effect on the LTC_4-induced enhancement of contraction.

Discussion

This is the first report of the enhancement of the contraction induced by LTC_4 and its underlying mechanism in porcine tracheal smooth muscle. The major findings are as follows: (1) LTC_4 greatly enhanced the contraction induced by either a high K^+ depolarization or a low concentration of CCh without changing the $[Ca^{2+}]_i$ level, although the application of LTC_4 alone in the normal PSS induced little or no contraction; (2) LTC_4 induced an increase in the Ca^{2+} responsiveness of the contractile apparatus in the tracheal smooth muscle; (3) the LTC_4-induced enhancement of contraction was accompanied by an increase in MLC phosphorylation; and (4) the LTC_4-induced enhancement of contraction was blocked by a rho kinase inhibitor (Y-27632), but not by either a PKC inhibitor (calphostin C) or a tyrosine kinase inhibitor (genistein). These results indicated that LTC_4 thus enhanced the contraction of the tracheal smooth muscle during activation with other stimulations by increasing the Ca^{2+} responsiveness of the contractile apparatus in a MLC phosphorylation dependent manner, possibly through the activation of the rho-rho kinase pathway.

In normal PSS, LTC_4 had almost no direct contractile effect on the porcine tracheal smooth muscle (Figure 1A). In agreement with this finding, LTs have been reported to have little or no direct effect on the airway smooth muscle contraction in the rat, cat or dog (Krell *et al*., 1981). On the other hand, they have been reported to be very potent bronchoconstrictors in humans and guinea-pigs (Dahlen *et al*., 1980; 1983). However, we found that the contraction induced by 40 mM K^+ increased by almost twice as much after being treated with LTC_4 (Figure 1A lower trace and 1B). In addition, this enhancement continued for a long time (up to 2 h of observation) even after the washout of LTC_4. It could be postulated that this enhanced contraction might be due to the enhanced increase in $[Ca^{2+}]_i$ during 40 mM K^+ depolarization after exposure to LTC_4, because it is generally accepted that the smooth muscle contraction is primarily regulated by the increase in $[Ca^{2+}]_i$ and the subsequent phosphorylation of MLC by Ca^{2+}-calmodulin dependent MLC kinase (Kamm & Stull, 1985). However, this was not the case. The measurement of $[Ca^{2+}]_i$ by fura-PE3 (Figure 1A upper trace and 1C) demonstrated that the increase in $[Ca^{2+}]_i$ during 40 mM K^+ depolarization was not enhanced after the exposure to LTC_4. A similar result could be obtained when we used a low concentration of CCh for the activation (Figure 2). It was thus concluded that the LTC_4-induced enhancement of the contraction of the porcine tracheal smooth muscle is not due to the LTC_4-induced change in Ca^{2+} mobilization.

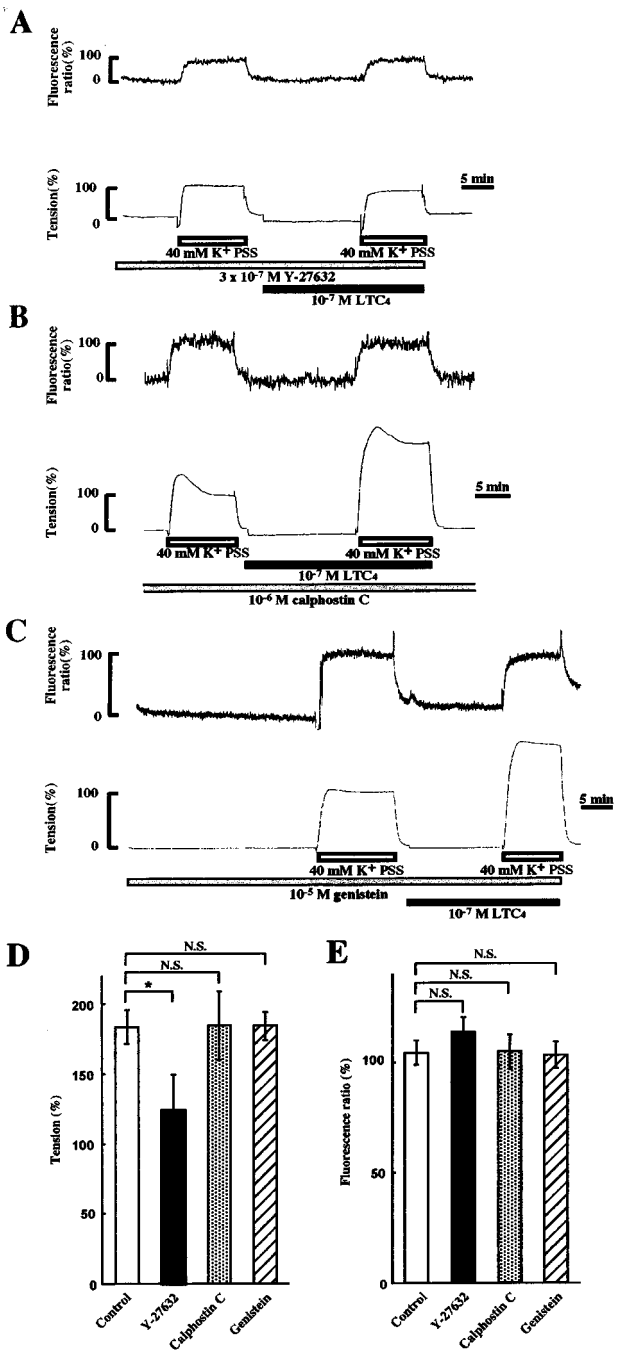

Figure 6 Effects of various kinase inhibitors on LTC_4-induced changes in $[Ca^{2+}]_i$ and the tension induced by 40 mM K^+ PSS. (A) Representative recordings showing the effect of 3×10^{-7} M Y-27632 (a rho-associated kinase inhibitor) on the LTC_4-induced changes in $[Ca^{2+}]_i$ and the tension induced by 40 mM K^+ PSS. (B) Representative recordings showing the effect of 10^{-6} M calphostin C (protein kinase C inhibitor) on the LTC_4-induced changes in $[Ca^{2+}]_i$ and the tension induced by 40 mM K^+ PSS. (C) Representative recordings showing the effect of 10^{-5} M genistein (tyrosine kinase inhibitor) on the LTC_4-induced changes in $[Ca^{2+}]_i$ and the tension induced by 40 mM K^+ PSS. (D, E) Summaries of the results shown in (A–C). The levels of $[Ca^{2+}]_i$ (D) and tension (E) after pretreatment by 10^{-7} M LTC_4 in the presence of indicated kinase inhibitors (the second increase in each panel) were plotted, while assigning the values of the responses to 40 mM K^+ PSS (prior to the application of LTC_4) to be 100%. Vertical bars represent s.e.mean of 3–4 measurements. $*P<0.05$ as compared with control. N.S.; not significant.

Since the LTC_4-induced enhancement of the contraction was not accompanied by the enhanced increase in $[Ca^{2+}]_i$ as mentioned above, we consider the LTC_4-induced enhancement of the contraction to be possibly due to the LTC_4-induced enhancement of the Ca^{2+} responsiveness. This idea was tested by the experiments shown in Figures 3 and 4. As shown in Figure 4, the $[Ca^{2+}]_i$ -tension relationship in the presence of LTC_4 significantly shifted upward from that in the absence of LTC_4. This result clearly demonstrated that LTC_4 does induce an increase in the Ca^{2+} responsiveness of the contractile apparatus of the porcine tracheal smooth muscle.

The LTC_4-induced increase in Ca^{2+} responsiveness could be reproduced in the permeabilized preparation as shown in Figure 5A. However, this increase in Ca^{2+} responsiveness detected in the permeabilized preparation was relatively smaller than that expected from the intact experiments, thus indicating that LTC_4-induced enhancement of tension seen in the intact preparation cannot be fully reproduced in the permeabilized preparations. This discrepancy could partly be explained by the difference in the experimental protocol, namely, pretreatment in the intact tissue and aftertreatment in the permeabilized preparation. When we added LTC_4 during the maintained contraction induced by 40 mM K^+, we could obtain only a small enhancement of contraction similar to that obtained in the permeabilized preparations (data not shown). In addition, we could not obtain any remarkable enhancing effect by the pretreatment protocol in the permeabilized preparations (data not shown). Hirshman & Emala (1999) recently reported that the activation of rho through G_q and G_{i-2} induces actin reorganization. Although it is speculative, if actin reorganization contributes to a part of the LTC_4-induced enhancement of tension, our data could be explained as follows: (1) The actin reorganization cannot be completely achieved during the depolarization in the intact preparation, because the effect of LTC_4 in the aftertreatment protocol of the intact preparation was much smaller than that in the pretreatment protocol; (2) the actin reorganization cannot be completely achieved in the permeabilized preparations, because we could not get a remarkable enhancing effect by the pretreatment protocol in the permeabilized preparations; and (3) the actin reorganization induced by the rho activation needs rho kinase, because Y27632 inhibited the LTC_4-induced enhancement of contraction.

It is now generally accepted that, although smooth muscle contraction is primarily regulated by $[Ca^{2+}]_i$ (Kamm & Stull, 1985), the modulation of Ca^{2+} sensitivity also plays an important role (Kanaide, 1999; Nishimura *et al*., 1988; Somlyo & Somlyo 1994). The mechanism for the increased Ca^{2+} sensitivity (Ca^{2+} sensitization) is still not fully understood. However, this mechanism can be classified into two groups: One is an increased Ca^{2+} sensitivity with an increased MLC phosphorylation (MLC phosphorylation-dependent Ca^{2+} sensitization: (Somlyo & Somlyo, 1994)) while another is that without an increased MLC phosphorylation (MLC phosphorylation-independent Ca^{2+} sensitization (Kodama *et al*., 1994; Moreland *et al*., 1992; Van Eyk *et al*., 1998)). We therefore determined the extent of MLC phosphorylation. The LTC_4-induced increase in the Ca^{2+} responsiveness was accompanied by the concomitant increase in MLC phosphorylation. This result indicated that the LTC_4-induced enhancement of contraction is due to the MLC phosphorylation-dependent Ca^{2+} sensitization.

The signal transduction pathway for MLC phosphorylation-dependent Ca^{2+} sensitization has recently been revealed. The small guanosine triphosphatase rho is implicated in this type of Ca^{2+}-sensitization of smooth muscle contraction. The GTP-bound active form of rhoA activates a downstream kinase, rho kinase, which phosphorylates myosin-binding subunit (MBS) of myosin phosphatase to inhibit the myosin phosphatase activity (Kimura *et al*., 1996). Another possible pathway for MLC phosphorylation-dependent Ca^{2+} sensitization involves PKC and PKC-dependent inhibitor of protein phosphatase 1 (CPI17). This novel protein has been reported to inhibit MLC phosphatase when phosphorylated by PKC (Eto *et al*., 1997). In addition, tyrosine kinase has also been implicated in the smooth muscle contraction (Di Salvo *et al*., 1994). Based on these considerations, we determined the effect of rho kinase inhibitor (Y-27632) (Uehata *et al*., 1997), PKC inhibitor (calphostin C) (Kobayashi *et al*., 1989) and tyrosine kinase inhibitor (genistein) (Akiyama *et al*., 1987) on the enhancement of contraction induced by LTC_4. As shown in Figure 6, the LTC_4-induced enhancement of contraction was inhibited by Y-27632, but not by calphostin C or genistein. These results indicated that the LTC_4-induced enhancement of contraction might thus involve the rho-rho kinase pathway. This idea might also help explain the inability for LTC_4 to enhance contraction when we used the high concentration of CCh (Figure 2) because of the following reasons. As we have reported previously, CCh increases the Ca^{2+} sensitivity of the contractile apparatus in the porcine tracheal smooth muscle (Kai *et al*., 1993) (Figure 5B), which could possibly involve the rho-rho kinase pathway (Otto *et al*., 1996). It is thus possible to speculate that the rho-rho kinase system could have already been fully activated by the high concentration of CCh. This could be the reason why LTC_4-induced enhancement of contraction could no longer be detectable when a high concentration of CCh was used.

The pathophysiological implication of the present study is unclear, especially due to the existence of the considerable species differences in the action of LTC_4. LTC_4 is a potent bronchoconstrictor in humans, while LTC_4 does not induce a significant contraction of the porcine tracheal smooth muscle in the normal PSS. However, it is possible that LTC_4 induces an enhancement of the contraction induced by other agonists as described in the present study and might thus contribute to the pathophysiology of human bronchial asthma.

In summary, the present study described the mechanisms underlying the LTC_4-induced enhancement of the contraction in tracheal smooth muscle, which has not been previously reported as a mode of action for LTC_4. The obtained results indicated that LTC_4 induces an enhancement of the tracheal contraction during activation with other stimulations by increasing the Ca^{2+} responsiveness of the contractile apparatus in a MLC phosphorylation dependent manner, possibly through the activation of the rho-rho kinase pathway.

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