Leukotriene D_4-Induced Activation of Smooth-Muscle Cells From Human Bronchi Is Partly Ca^{2+}-Independent

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Cysteine-containing leukotrienes (cysteinyl-LTs) are potent bronchoconstrictors and play a key role in asthma. We found that histamine and LTD_4 markedly constrict strips of human bronchi (HB) with similar efficacy. However, in human airway smooth-muscle (HASM) cells, LTD_4, at variance with histamine, elicited only a small, transient change in intracellular calcium ion concentration. HASM cells express both Ca^{2+}-dependent and -independent isoforms of protein kinase C (PKC) (i.e., PKC-α and PKC-ε). Western blot analysis showed that PKC-α is activated by histamine and, to a lesser extent, by LTD_4, whereas only LTD_4 translocates PKC-ε. This translocation was specifically inhibited by the LTD_4 antagonist pobilukast. Phorbol-dibutyrate ester (PDBu) (a PKC activator) contracted HB strips to the same extent in the presence as in the absence of extra- and intracellular Ca^{2+}. In the absence of Ca^{2+}, LTD_4 contracted HB strips to the same extent as did PDBu, suggesting the involvement of a Ca^{2+}-independent PKC in LTD_4-mediated signal transduction. PDBu-induced desensitization and the PKC inhibitor H7 abolished the slow and sustained LTD_4-triggered contraction of HB strips in the absence of Ca^{2+}, although H7 did not greatly affect the response in the presence of the ion. Thus, in human airways, we identified a novel LTD_4 transduction mechanism linked to bronchial smooth-muscle contraction, which is partly independent of Ca^{2+} and involves the activation of PKC-ε.

Cysteine-containing leukotrienes (cysteinyl-LTs) are pivotal inflammatory mediators formed through the 5-lipoxygenase pathway of arachidonic acid (1), and may contribute to the pathogenesis of asthma (2–4). In particular, cysteinyl-LTs are very potent constrictors of human bronchi (HB) not only *in vitro* (5), but also *in vivo*, in both normal and asthmatic individuals (6).

Among the cysteinyl-LTs, LTD_4 has been studied most extensively, and is known to act through specific G-protein–coupled receptors (7, 8), inducing phosphoinositol hydrolysis (9, 10) and an increase in the cytosolic Ca^{2+} concentration (intracellular $[Ca^{2+}]$; $[Ca^{2+}]_i$) (11–13). The latter effect in particular occurs in tracheal smooth-muscle cells (14).

Differences exist between cell types with respect both to the role of Ca^{2+} and to the mechanisms of the LTD_4-induced increase in $[Ca^{2+}]_i$ (15). For instance, LTD_4 can induce either Ca^{2+} influx through the plasma membrane without any Ca^{2+} release from intracellular stores (11, 12), or Ca^{2+} release without influx (16), and, in many cell types can induce both Ca^{2+} influx and release (9, 13, 14).

The role of Ca^{2+} in muscle contraction has been investigated for many years: Ca^{2+}-dependent phosphorylation of myosin light chain is a major pathway for the regulation of smooth-muscle contractile force (17). However, the force/Ca^{2+} ratio is variable (18), and the mechanism of action of agonist-induced smooth-muscle contraction (pharmacomechanical coupling) may consist of an alteration of the sensitivity of the contractile apparatus to Ca^{2+} (19). In some instances, agonist-triggered contraction can occur with little or no change in $[Ca^{2+}]_i$ (17, 20). In airway smooth muscle (ASM), including that of the human bronchus, the Ca^{2+}-sensitivity of the contractile apparatus is very high and displays high positive cooperativity (21); quite often, the tonic activation of ASM by a variety of agonists depends on Ca^{2+} influx from the extracellular space (22, 23). However, despite the existence of voltage-operated calcium channels (VOC) in human airways (24), Ca^{2+}-entry blockers are relatively ineffective in inhibiting bronchoconstriction in asthma, indicating that airway narrowing in asthma is mediated mainly by other means than VOCs. Accordingly, it has recently been suggested (25) that LTD_4 contracts human bronchi through a receptor-operated Ca^{2+} channel (ROC).

The aim of the present study was to clarify whether variations in $[Ca^{2+}]_i$ play a major role in LTD_4-induced contraction of HB, or whether other signal-transduction mechanisms are involved. For this purpose, we compared the *in vitro* contraction of isolated HB with increases in $[Ca^{2+}]_i$ and protein kinase C (PKC) activation in isolated smooth-muscle cells obtained from the same tissue.

METHODS

All experiments were performed in the presence of 10 mM cysteine to prevent metabolism of LTD_4 (26).

Cell Isolation Procedure

Smooth-muscle cells from HB were isolated as previously described (27). Briefly, macroscopically normal fragments of lung were obtained at thoracotomy. Third-order bronchi were removed, under sterile conditions, the connective tissue and the epithelium were removed, and the smooth muscle was cut into pieces weighing approximately 10 mg each. The explants were grown at 37° C in a humidified atmosphere of 5% CO_2 in Medium 199, with the addition of 20% (vol/vol) fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in monolayers in minimum essential medium (MEM) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and were used between passages 3 and 8. The cells stained positively with an antibody to smooth-muscle α-actin.

Measurements of $[Ca^{2+}]_i$

Human bronchial cells were seeded onto coverslips and used when they reached 100% confluence. The cells were incubated for 45 min at 30° C in the dark, with 5 μM Fluo3 acetoxymethyl ester (Fluo3/AM) (28) in MEM plus 0.03% Pluronic F-127, 2.5 mM probenecid, and 10 mM 4-(2-hydroxyethyl)-1-piperazine-*N*′-2-ethanesulfonic acid (HEPES). After loading, cells were washed twice with saline solution (NaCl 145 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM, HEPES 10 mM,

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glucose 10 mM; pH 7.4) plus 2.5 mM probenecid, and were kept at room temperature for 15 min to complete hydrolysis of the Fluo3/AM. The coverslips were then transferred to a spectrofluorometer (Perkin Elmer LS5; Perkin Elmer Italia, Monza, Italy) cuvette, and the fluorescence of the cell preparations was monitored at 30° C (506-nm excitation, 530-nm emission). In order to evaluate $[Ca^{2+}]_i$ from fluorescence readings, F_{max} (maximal fluorescence of the system) was obtained by adding 2.7 μM ionomycin and 100 μM digitonin and F_{min} was obtained by measuring the autofluorescence of cells that were not exposed to Fluo3/AM (29). $[Ca^{2+}]_i$ was determined according to the method of Tsien and colleagues (30), with K_d = 646 nM, which was calculated by assuming a linear dependence on temperature (29). Increases in $[Ca^{2+}]_i$ were expressed as the ratio of the stimulated over the basal level (s/b).

Preparation of Isolated HB

Macroscopically normal HB (2- to 3-mm diameter) were obtained from patients undergoing thoracic surgery for pulmonary carcinoma, and were prepared as previously described (31). Briefly, the bronchi were dissected free of parenchyma and blood vessels, and were cut helically into strips 2 to 3 mm wide and about 10 mm long. The strips were suspended in organ baths containing Tyrode's solution (composition in mM: NaCl, 140; KCl, 3; $CaCl_2$, 1; $MgCl_2$, 0.05; NaH_2PO_4, 0.5; glucose, 8.4; $NaHCO_3$, 12; pH 7.4) at 37° C, which was bubbled with 95% O_2–5% CO_2. Contractions were measured with a Basile 7004 isometric force transducer (Ugo Basile, Comerio, Italy) and recorded on a Basile Gemini 7070 polygraph. Bronchial strips were set at an initial tension of 1 g and repeatedly washed over a 60-min equilibration period. Contractions are expressed as the percent of the contraction induced by 300 μM acetylcholine (ACh). When necessary, the Tyrode's solution was replaced after ACh administration, with a solution lacking Ca^{2+} and containing 1 mM ethylene glycol-*bis*-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 1 μM thapsigargin to eliminate intra- and extracellular calcium. At the end of experiments with calcium- depleted strips, contractility was verified by addition of 30 mM $BaCl_2$.

Cell Contraction

Cells were grown in monolayers in Petri dishes as described earlier, and were used at submaximal (95%) confluence. Cell contraction was recorded by measuring the increase in intercellular spaces by means of a TV camera connected to an inverted light microscope (Axiovert; ×20 lens; Zeiss, Jena, Germany) coupled to an image analyzer (a Macintosh computer [Apple Computer, Inc., Cupertino, CA] running NIH Image v.1.62, developed by Dr. Wayne Rasband, of the National Institutes of Health, Bethesda, MD) through a Data Translation Inc. (Marlboro, MA) QuickCapture frame grabber card. Images were collected for 15 min (one frame every 30 s). After 5 min of recording of the control state, either histamine or LTD_4 was added. When necessary, intra- and extracellular Ca^{2+} was removed as described earlier.

Image intensity is reported on a scale of 255 Gray Units with 0 for white and 255 for black. The background intensity level (empty Petri dishes) was determined to be in the range of 0 to 50 of Gray Units, and all Gray-level readings of 50 or lower were converted to a red color to represent intercellular spaces and to compute the percent increase in intensity over the basal state.

Laser Scanning Confocal Fluorescence Microscopy

Cells were grown in monolayers on chamber slides and loaded with Fluo3/AM as described earlier. The chamber slide was placed on a thermostatically controlled copper plate (at 30° C) on the stage of a confocal microscope (MRC-600; Bio-Rad, Microsciences Division, Hemel Hempstead, UK) coupled to an upright epifluorescence microscope (Optiphot-2; Nikon, Tokyo, Japan). The indicated concentrations of histamine or LTD_4 were added to the cells with a microsyringe. Intracellular Fluo3 fluorescence in single living cells was measured by confocal microscopy. Argon ion laser excitation was used at 488 nm, and the power of the laser was kept at low values (< 0.1 mW) to preserve cell viability and to avoid possible saturation of the fluorescence signal. Fluorescence emission was detected in the photon-counting mode with a photomultiplier (S20 Thorn-EMI-9890; Bio-Rad Microsciences Division, Hemel, Hempstead, UK) through a long pass filter at wavelengths above 515 nm. The indicator fluorescence was followed in a field of view of about 400 × 300 μ^2, in which about 10 cells were inspected at the same time through a ×20 air objective. The scan speed of the laser excitation was set to 0.25 s for a frame of 384 × 256 pixels. Under these conditions, a single scan of the field of view enabled us to obtain an excellent fluorescence image at a confocal aperture of about 2 mm and a numerical aperture of 0.4. A temporal resolution of 2 s was used. The time course of the indicator fluorescence was followed by using the time series option of the MRC-600 COMOS software.

Image fluorescence intensities were recorded on a scale of 256 Gray Units (from 0 for black to 255 for peak white). False colors were assigned to four ranges of Gray-Unit values, corresponding to four ranges of Ca^{2+} concentrations, as shown in Figure 7. The time dependences of fluorescence intensities were studied in selected areas inside single cells (about 40 to 80 μ^2 wide), in which the mean Ca^{2+} concentrations were evaluated.

Evaluation of PKC Translocation

Smooth-muscle cells were incubated for 18 h in culture medium without FCS, and were then treated with the agents being investigated at 37° C for 5 min, unless otherwise indicated. The incubation was stopped at 4° C and the cells were washed twice in cold phosphate-buffered saline (PBS). Cells were then lysed at 4° C for 30 min in 20 mM Tris-HCl, pH 7.4, containing 2 mM EGTA, 20 mM ethylenediamine tetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and a set of protease inhibitors (Complete; Boehringer Mannheim, Mannheim, Germany). The lysed suspension was centrifuged at 100,000 × *g* for 60 min, with the supernatant comprising the cytosolic fraction. The pellet was resuspended in lysis buffer plus 0.1% Triton X-110 at 4° C for 45 min, and centrifugation was then repeated, with the resulting supernatants representing the membrane fraction. The proteins in the cytosolic and membrane fractions were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (running gel: acrylamide 11%), and were electroblotted onto nitrocellulose paper. PKC isozymes were evaluated by Western blot analysis, utilizing polyclonal antibodies raised against PKC-α (at a dilution of 1:1,500) and PKC-ε (at a dilution of 1:1,000) for 1 h at 25° C. After washing with Tris-buffered saline (TBS) containing 0.1% Tween-20, the sheets were incubated (1 h, 25° C) with peroxidase-conjugated antirabbit IgG (1:3,000) and washed as described previously. Antigen–antibody complexes were detected by enhanced chemiluminescence. Results were analyzed with a computer-assisted image analysis program (NIH Image v.1.62), and were expressed as percents of PKC translocation versus basal conditions.

Statistical Analysis

Statistical analysis of the concentration–response curves for $[Ca^{2+}]_i$ versus histamine or LTD_4 was done with the ALLFIT computer program (32), which calculates lower and upper plateaus, slope, and EC_{50} values, and allows the comparison of two or more curves. Selection of the best fitting model and evaluation of the statistical significance of parameter differences was based on the F test for the extra-sum-of-squares principle (33). All curves shown in the illustrations were computer generated.

Statistical comparison of multiple groups was done through one-way analysis of variance followed by Bonferroni's *post hoc* test. A statistical level of significance of $p < 0.05$ was accepted. Data are expressed as mean ± SE. Each experiment was performed at least three times and, when possible, in triplicate.

Materials

TBS, PBS, Hanks' balanced salt solution (HBSS), Medium 199, Eagle's MEM, trypsin-EDTA, EGTA, penicillin (10,000 U/ml), streptomycin (10 mg/ml), L-glutamine (200 mM), H7, dimethylsulfoxide, ACh, histamine, probenecid, atropine, pyrilamine, L-cysteine, thapsigargin, phorbol-dibutyrate ester (PDBu), anti–smooth-muscle α-actin antibody, peroxidase-conjugated antirabbit IgG, PMSF, and DTT were from Sigma Chemical Co., St. Louis, MO. Antibodies to the PKC-α and PKC-ε isoforms, were from Boheringer Mannheim. LTD_4 was from Cayman Chemical Co., Ann Arbor, MI. All salts for Tyrode's solution were from Merck, Darmstadt, Germany. FCS was from PBI International, Milan, Italy. Disposable culture flasks, Petri dishes, and filters were from Corning Glassworks, Corning, NY. Fluo3/AM and

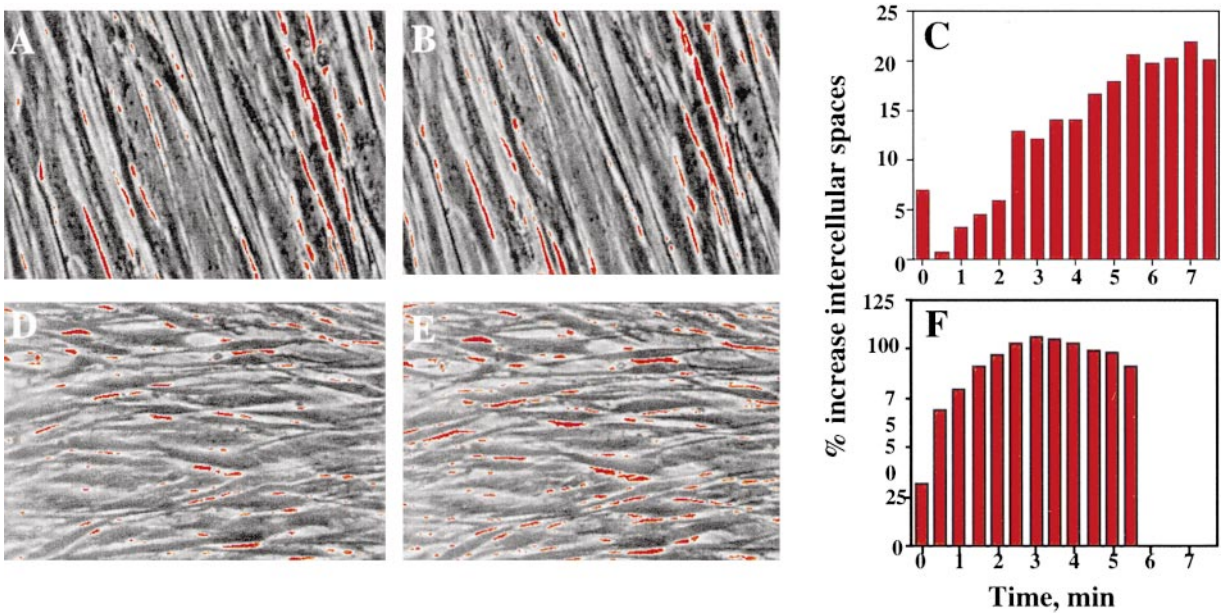

Figure 1. Light microscopic images of HASM cells made with a false-color map. Cell images are given in a scale of 256 Gray-Unit levels (from 0 for white to 255 for black); of *red color* (0 to 50 Gray-Unit levels) represents intercellular spaces. Cells were used at submaximal (95%) confluence. (*A*) Control cells. (*B*) Same field as in *A* after stimulation with 0.3 μM LTD_4 (8 min). (*C*) Time-course of the percent increase in intercellular spaces induced by LTD_4 (data from *A* and *B*); frames were recorded at 30-s intervals. (*D*) Control cells. (*E*) Same field as in *D* after stimulation with 100 μM histamine (5 min). (*F*) Time-course of the percent increase in intercellular spaces induced by histamine (data from *D* and *E*); frames were recorded at 30-s intervals. The experiments were performed in the presence of 1 mM extracellular Ca^{2+}.

pluronic F-127 were purchased from Molecular Probes, Eugene, OR. MK 571 was a generous gift from Dr. A. W. Ford-Hutchinson of Merck Frosst, Point Dorval, PQ, Canada, and SKF 104353 was kindly provided by Dr. A. von Sprecher of the Research Department, Pharmaceutical Division, Novartis Ltd., Basel, Switzerland.

RESULTS

Contraction of Human Airway Smooth-Muscle Cells and of Isolated Strips of HB

Contraction of human airway smooth-muscle (HASM) cells in monolayers was recorded by measuring the increase in intercellular spaces by means of an image analyzer coupled to a light microscope. LTD_4 was able to contract HASM cells to the same extent in the presence of Ca^{2+} (30 ± 8% [mean ± SE] increase in intercellular spaces, n = 6; Figures 1A and 1B) as in its absence (which was effected by pretreatment with 1 mM EGTA and 300 nM thapsigargin, to eliminate extra- and intracellular Ca^{2+}, respectively) (28 ± 3%, n = 3, data not shown). The time-course of the increase in intercellular space in the same field is shown in Figure 1C. On the contrary, the effect of histamine, albeit generally greater than that of LTD_4 (66 ± 9%, n = 6; Figures 1D and 1E), was greatly decreased by the absence of Ca^{2+} (26 ± 9%, n = 3, data not shown). Figure 1F shows the time course of the effect of histamine. The concentration of thapsigargin used and the preincubation time were sufficient to deplete the sensitive cytoplasmic stores of Ca^{2+} as assessed by $[Ca^{2+}]_i$ measurement (data not shown).

Both LTD_4 (Figure 2A) and histamine (Figure 2B) caused marked concentration-dependent bronchoconstriction with comparable efficacy in isolated strips of HB. The calculated EC_{50} values for LTD_4 and histamine are summarized in Table 1.

$[Ca^{2+}]_i$ Increase in a Population of HASM Cells and in Single HASM Cells

In a population of HASM cells, LTD_4 (Figure 2C) was able to elicit only a very modest, if any, transient increase in $[Ca^{2+}]_i$ (maximum $[Ca^{2+}]_i$ increase: s/b = 1.8 ± 0.35). On the contrary, the response to histamine (Figure 2D) was concentration-dependent (EC_{50} = 30 μM), with a marked increase in $[Ca^{2+}]_i$ (maximum increase: s/b = 8.9 ± 0.89). Representative traces showing the $[Ca^{2+}]_i$ increases evoked by 1 μM LTD_4

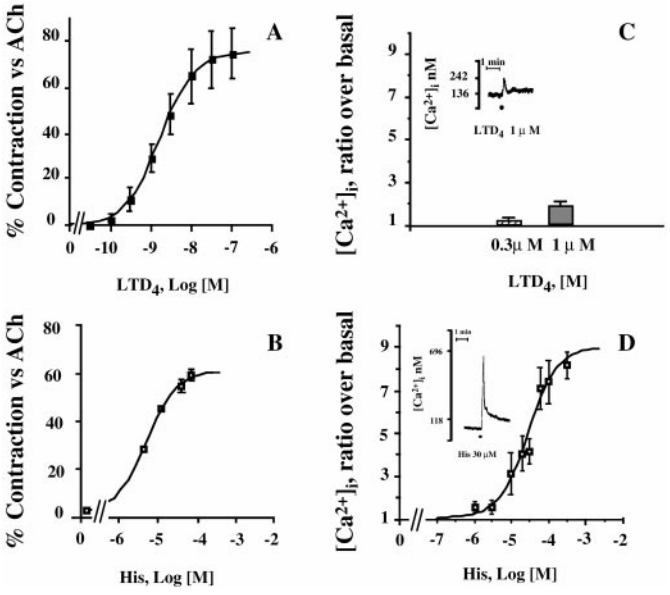

Figure 2. Concentration–response curves of agonist-induced contraction (*A* and *B*) of isolated human bronchi, and $[Ca^{2+}]_i$ increase (*C* and *D*) in HASM cells. (*A* and *C*) LTD_4. (*B* and *D*) Histamine. Insets in *C* and *D* are representative tracings of the individual effect of agonist concentrations in inducing $[Ca^{2+}]_i$ increases. Curves in *A* and *B* were constructed with strips from seven different lung specimens. Curves in *C* and *D* were obtained from four to seven experiments on cells obtained from three to five different lung specimens, respectively.

TABLE 1

POTENCY OF HISTAMINE AND LTD_4 IN INDUCING CONTRACTION OF HUMAN BRONCHIAL STRIPS AND Ca^{2+} INCREASE IN CELLS

	Contraction		$[Ca^{2+}]_i$ Increase	
Agonist	EC_{50}	CV (%)	EC_{50}	CV (%)
Histamine	3.7 μM	11	30 μM	29
LTD_4	1.6 nM	5	ND	ND

Definition of abbreviations: $[Ca^{2+}]_i$ = intracellular calcium-ion concentration; CV = coefficient of variation; EC_{50} = effective concentration causing 50% of maximal effect; LTD_4 = leukotriene D_4; ND = not determined.

and 30 μM histamine, respectively, are shown as insets in Figures 2C and 2D. The response was completely abolished by 5 min of preincubation with specific antagonists (i.e., 1 μM MK 571 and 30 μM mepyramine) (data not shown).

$[Ca^{2+}]_i$ transients in single HASM cells loaded with Fluo3 were studied with confocal fluorescence microscopy. Figure 3 shows the false-color images of cells under basal conditions (Figure 3A) and at 40 s (Figure 3B), corresponding to the peak $[Ca^{2+}]_i$ value, after stimulation with 100 μM histamine. Figures 3C and 3D (48 s) show the results obtained with 1 μM LTD_4. Figures 4A and 4B show the mean $[Ca^{2+}]_i$ increase in response to histamine and LTD_4, respectively, as a function of time, measured in the cells indicated in Figure 3, as well as the effect elicited by the vehicle alone (0.1% EtOH; Figure 3C). It is clear that the effect of LTD_4 was not much greater than that of the appropriate control, whereas histamine elicited a much greater increase in $[Ca^{2+}]_i$. Furthermore, the specific antagonist SKF 104353 (10 μM) decreased the LTD_4 response by only a slight degree (data not shown).

Role of PKC in Contraction

Figures 5A through 5D show the contraction tracings for isolated strips of HB prepared from adjacent segments challenged with LTD_4 (0.3 μM). Compound H7 (50 μM), an inhibitor of PKC, did not affect LTD_4-induced contraction in the presence of Ca^{2+} (Figure 5B), whereas in the absence of Ca^{2+} it abolished the residual contraction usually present (compare Figures 5C and 5D). Moreover, in the absence of Ca^{2+}, repeated *in vitro* administration of the PKC activator PDBu (1 μM) completely inhibited LTD_4-induced contraction of bronchial strips (Figure 5E).

When the strips were stimulated with 0.3 μM LTD_4, the removal of extra- and intracellular Ca^{2+} markedly decreased, but did not abolish, the contraction (Figures 6 and 5C). On the contrary, the effect of PDBu, which induced a slowly developing contraction (Figure 5E), was not affected by removal of Ca^{2+} (Figure 6). In the case of histamine, no contraction was detectable in the absence of Ca^{2+} (data not shown).

Translocation of Different Isoforms of PKC

Figure 7 shows a representative Western blot analysis performed with polyclonal antibodies to PKC-α, a calcium-dependent isoform of PKC, and to PKC-ε, a calcium-independent isoform of the enzyme, on membrane proteins of HASM cells incubated with either LTD_4 or histamine. LTD_4 at 0.3 μM induced marked activation of the calcium-independent PKC-ε (170 ± 15%; Figure 7B) and, to a lesser extent, of the calcium-dependent PKC-α isoform (116 ± 17%; Figure 7A), whereas incubation with 100 μM histamine produced marked translocation only of the calcium-dependent PKC-α isoform (514 ± 181%; Figure 7A). The effect of LTD_4 on PKC-ε was time-dependent, being maximal after 5 min of incubation and progressively decreasing at 15 and 30 min (Figure 7C), thus suggesting

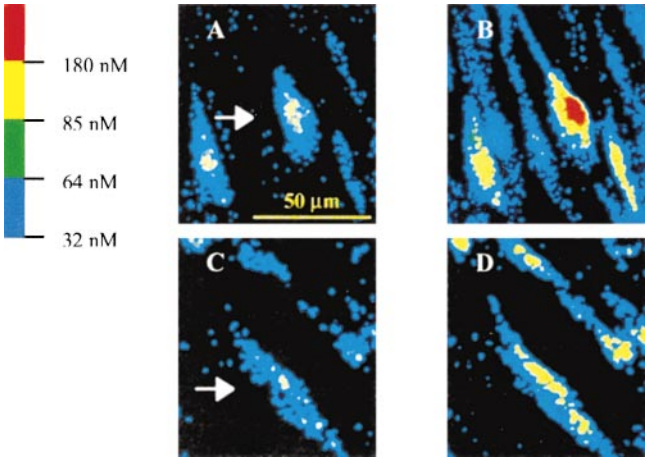

Figure 3. False-color confocal fluorescence microscopic images of HASM cells loaded with the fluorescent Ca^{2+} probe Fluo3. (*A*) Resting cells. (*B*) Appearance at 40 s after the addition of 100 μM histamine. (*C*) Resting cells. (*D*) Appearance at 48 s after the addition of 1 μM LTD_4. Fluorescence intensities were recorded on a scale of 256 Gray-Unit levels and divided into four ranges, corresponding to four ranges of $[Ca^{2+}]_i$ concentrations, to which pseudocolors were assigned. Images shown are representative of seven or eight other experiments (for LTD_4 and histamine, respectively) on cells obtained from three to five different lung specimens.

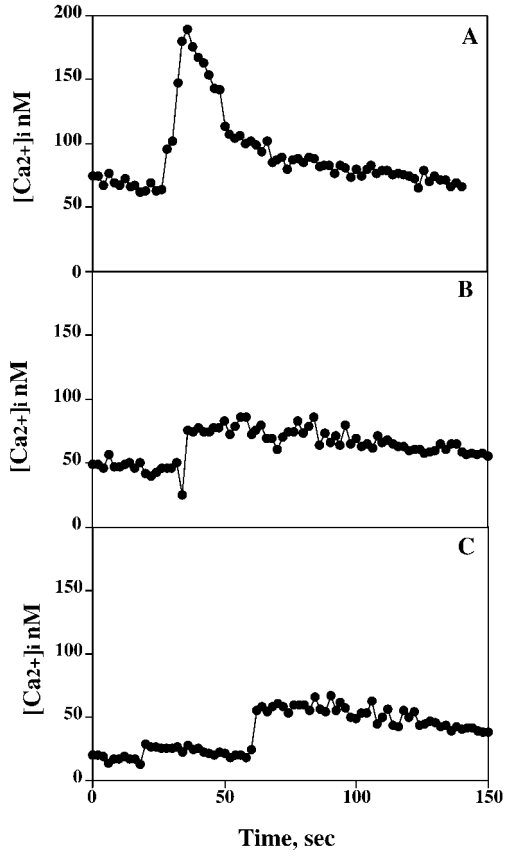

Figure 4. Mean $[Ca^{2+}]_i$ as a function of time with (*A*) 100 μM histamine and (*B*) 1 μM LTD_4. Measurements were made in the cells indicated in Figure 3. (*C*) Effect of vehicle (0.1% EtOH) for LTD_4 alone.

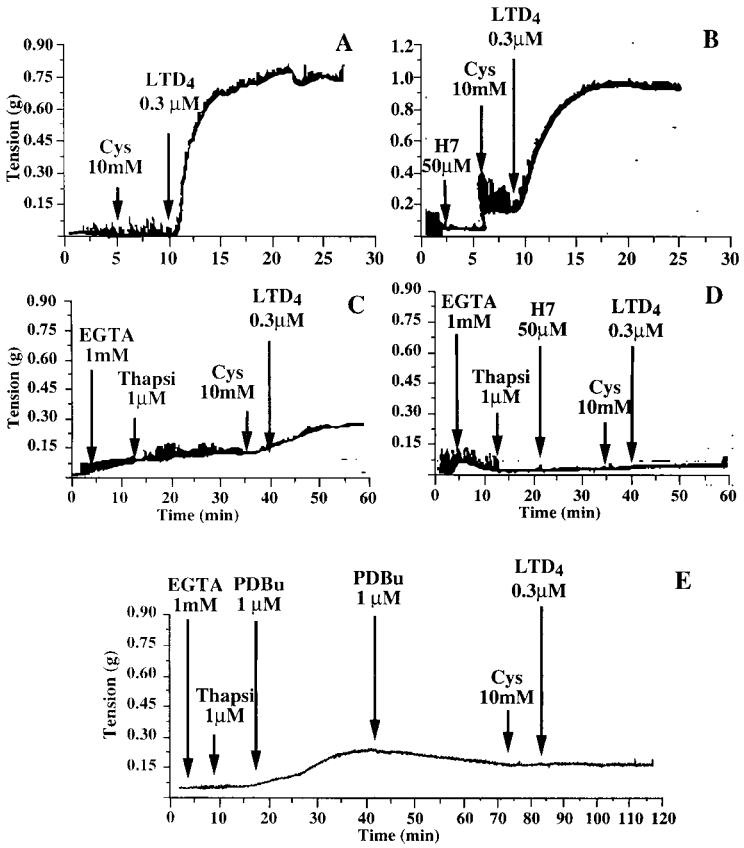

Figure 5. Representative tracings of contraction of isolated strips of HB in response to 0.3 μM LTD_4. (*A* and *B*) Tracings in the presence of Ca^{2+} in the absence (*A*) and in the presence (*B*) of 50 μM H7. (*C* through *E*) Tracings in the absence of Ca^{2+} in the absence (*C*) and in the presence (*D*) of 50 μM H7. (*E*) Tracing made after repeated administration of 1 μM PDBu. The tracings are representative of two or three other tracings.

that prolonged incubation induces downregulation of this isoform of the kinase. Figure 7D shows a Western blot analysis of membrane proteins of cells incubated with increasing concentrations of LTD_4. LTD_4 (5 nM and 0.3 μM) increased the translocation of PKC-ε by 143% ± 21 and 105 ± 16, respectively. In addition, 10 μM of SKF 104353 was able to counteract the effect of the highest concentration of LTD_4.

As expected, removal of Ca^{2+} abolished LTD_4-induced translocation of PKC-α, but it did not affect the translocation of PKC-ε (Figure 8).

DISCUSSION

LTD_4 is a potent constrictor of smooth muscle, including bronchial smooth muscle. In most tissues and cells, this effect has been shown to be receptor-mediated and coupled to an increase in $[Ca^{2+}]_i$ (7, 15). Smooth-muscle contraction has been directly correlated with either an increase in $[Ca^{2+}]_i$ (in the guinea pig ileal longitudinal muscle [12]) or with an increased hydrolysis of phosphatydyl inositol in lung [8]). On the contrary, we found that a discrepancy exists between contraction and increased $[Ca^{2+}]_i$ in bronchial tissue. In fact, LTD_4 was able to contract strips of human bronchus *in vitro* to an extent comparable with that elicited by histamine, albeit with much higher potency, but was much less efficient in inducing an increase in $[Ca^{2+}]_i$ in isolated smooth-muscle cells obtained from the same organ.

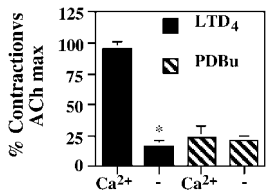

Figure 6. Contraction of isolated strips of HB in the presence or absence of intracellular and extracellular Ca^{2+}. *Bars* represent the mean ± SE. *Closed bars*: LTD_4 at 0.3 μM; *hatched bars*: PDBu at 1 μM. *$p < 0.05$ versus addition of Ca^{2+} (one-way analysis of variance, $n = 4$).

One possible explanation for this discrepancy would be the loss during cell isolation and culture of the LTD_4 receptor that was responsible for contraction. However, this hypothesis can be ruled out, because the cultured cells actually responded to LTD_4 with measurable contractions that were inhibited by MK-571, a specific LTD_4 receptor antagonist. Another possible explanation would be a heterogeneity in the $[Ca^{2+}]_i$ response in the cell population examined, with the cells either not being synchronized in their Ca^{2+} response or with few cells expressing the receptor and therefore responding with an increase in $[Ca^{2+}]_i$. Laser scanning confocal fluorescence microscopy allowed us to measure $[Ca^{2+}]_i$ variations in single HASM cells, and demonstrated that the percentage of cells responding to LTD_4 with increases in $[Ca^{2+}]_i$ was not lower than that responding to histamine. Furthermore, the time-course of Ca^{2+} variation in response to LTD_4 did not vary significantly from cell to cell. The data obtained at the single-cell level therefore confirmed those obtained in the overall cell population, showing a marked difference in the peak $[Ca^{2+}]_i$ level with histamine as opposed to LTD_4, and excluding a heterogeneous cell response as the cause of the discrepant effect of LTD_4.

Thus, our data demonstrate a dissociation between LTD_4-induced contraction and increased $[Ca^{2+}]_i$ elevation and suggest that Ca^{2+}-independent mechanisms contribute to LTD_4-triggered bronchoconstriction in human airways. Indeed, mea-

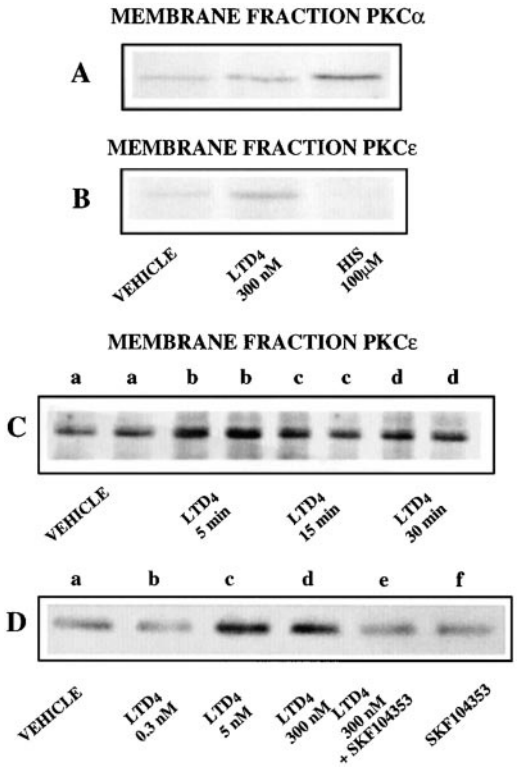

Figure 7. Western blot analysis done with antibodies to PKC-α and PKC-ε on membrane proteins from HASM cells incubated with vehicle, LTD_4, or histamine. (*A*) Effect of LTD_4 (0.3 μM) or histamine (100 μM) on translocation of PKC-α to the membrane. (*B*) Effect of LTD_4 (0.3 μM) or histamine (100 μM) on translocation of PKC-ε. (*C*) Time-course of PKC-ε translocation with 0.3 μM LTD_4 (duplicate lanes are shown in this case). (*D*) Effect of increasing concentrations of LTD_4 on translocation of PKC-ε. The last two lanes represent the effect of 10 μM SKF 104353 on translocation of PKC-ε in cells stimulated with 0.3 μM LTD_4 or in resting cells, respectively. Vehicle was MEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM cysteine. In (*D*) vehicle contained also 0.7% DMSO.

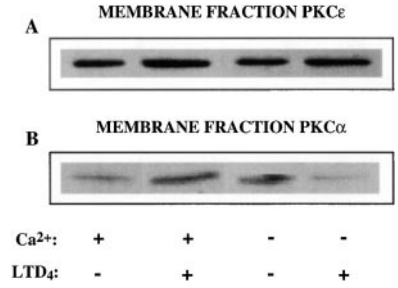

Figure 8. Western blot analysis done with antibodies to PKC-α and PKC-ε on membrane proteins from HASM cells incubated with 0.3 μM LTD_4 in the presence or absence of extracellular Ca^{2+}. Effect of LTD_4 on translocation of PKC-ε (*A*) and PKC-α (*B*) in the presence (first two lanes) and absence (last two lanes) of Ca^{2+}. Control samples contained MEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM cysteine.

surement of single-cell contraction by light microscopy shows that the removal of extra- and intracellular Ca^{2+} elicits only a minor reduction in cell contraction induced by LTD_4 from that observed in the presence of Ca^{2+}. On the contrary, histamine-induced contraction was substantially reduced (by more than 50%) in the absence of Ca^{2+}. This indicates that in isolated HASM cells, the histamine response depends largely on the presence of Ca^{2+}, whereas the LTD_4 response is largely Ca^{2+}-independent.

In seeking a Ca^{2+}-independent mechanism for LTD_4-mediated bronchoconstriction, we postulated an involvement of PKC in LTD_4-mediated signal transduction. Indeed, it is known that: (*1*) LTD_4 can activate PKC (7, 34); (*2*) LTD_4-triggered contraction in HB is rather slow, but is sustained in time; (*3*) PKC is involved in the sustained phase of smooth-muscle contraction in general (35) and in HASM cells in particular (36, 37); and (*4*) HASM cells express different isoforms of PKC (38), some of which (δ, θ, ε, and η) are Ca^{2+} independent.

Thus, using polyclonal antibodies against specific PKC isoforms (i.e., PKC-α) as representatives of the classical Ca^{2+}-dependent isoforms of PKC, and against PKC-ε as a representative of the Ca^{2+}-independent isoforms, we investigated their activation. LTD_4 was able to translocate PKC-ε and, to a lesser extent, PKC-α, whereas histamine was able to translocate only PKC-α. The activation of PKC-ε by LTD_4 was concentration- and time-dependent. The decrease in PKC-ε translocation at 15 and 30 min, with respect to 5 min, might suggest a desensitization of the enzyme. This result is in agreement with the tachyphylaxis displayed by LTD_4 as a contractile agent (39). Furthermore, inhibition of LTD_4-induced translocation of PKC by SKF 104353, a cysteinyl-LT_1 antagonist, showed that the activation was receptor-mediated.

To further link PKC activation and contraction of HB, we evaluated the effect of PDBu, a PKC activator, on strips of HB. PDBu was able to contract HB to the same extent in the presence as in the absence of extra- and intracellular Ca^{2+}. These data are apparently at variance with the previously reported (37, 40) finding that removal of extracellular Ca^{2+} abolished PDBu-induced HB contraction; however, the investigators who reported this generated cumulative concentration–response curves for PDBu, in which desensitization of PKC yields a response lower than the one obtained by single-dose administration, as also confirmed by Yang and Black (40). In our hands, the slowly developing and sustained contraction of HB seemed to be Ca^{2+}-independent.

Interestingly, in the absence of Ca^{2+}, LTD_4 was able to contract HB to the same extent as was PDBu, thus suggesting the involvement of a Ca^{2+}-independent PKC isoform in LTD_4-mediated signal transduction. This is confirmed by the complete inhibition, in the absence of Ca^{2+}, of the LTD_4 response caused by PDBu-induced desensitization of PKC. In addition, the PKC inhibitor H7 abolished the slow and sustained contraction triggered by LTD_4 in the absence of Ca^{2+}. The relatively minor effect of H7 on the response to LTD_4 in the presence of Ca^{2+} suggests the involvement of mechanisms other than PKC activation (possibly activation of myosin light-chain kinase [41]) in the rapid phase of LTD_4 contraction. Thus, the LTD_4-induced response in HB consists of both a Ca^{2+}-dependent phase (responsible for the onset of contraction) and a slower, Ca^{2+}-independent phase (responsible for the maintenance of contractile tone). On the contrary, histamine-induced contraction of HB is completely Ca^{2+}-dependent.

In conclusion, our data, taken collectively, indicate that in human airways, LTD_4-induced contraction is at least in part independent of increases in Ca^{2+}, and this might represent an extreme form of the increase in Ca^{2+} sensitivity induced by agonists (19). Such Ca^{2+}-independent contraction involves the activation of one of the novel isoforms of PKC (i.e., PKC-ε), and indeed, PKC activation has been proposed as a mechanism of increased force development at constant $[Ca^{2+}]_i$ (42). The nature of the transduction pathway involved will be the subject of future research. However, one can speculate that

the LTD_4 receptor is coupled to phospholipase D activation, yielding diacylglycerol formation (and thus PKC activation) without concomitant formation of Ca^{2+}. Additionally, our data support the existence of a link between LTD_4-induced activation of a Ca^{2+}-independent PKC and bronchial smooth-muscle contraction. This pathway, which is specific for LTD_4 (being abolished by two different receptor antagonists and not shared by histamine), represents a novel transduction mechanism for cysteinyl-LTs.

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