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Research Article

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Stimulation by Leukotriene D_4 of Increases in the Cytosolic Concentration of Calcium in Dimethylsulfoxide-differentiated HL-60 Cells

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Abstract

The C6-sulfidopeptide leukotrienes C_4 (LTC_4) and D_4 (LTD_4) evoked increases in the cytosolic concentration of intracellular calcium ($[Ca^{+2}]_i$) in dimethylsulfoxide-differentiated HL-60 cells, as assessed by the fluorescence of quin-2. The increases in $[Ca^{+2}]_i$ reached a peak within 15–90 s, attained 50% of the maximum level at 1.2 nM LTD_4 and 60 nM LTC_4, were greater in maximal magnitude for LTD_4 than LTC_4, and subsided in 5–7 min. Flow cytometric evaluation of the LTD_4-induced increases in $[Ca^{+2}]_i$, reflected in increases in the fluorescence of intracellular indo-1, revealed that a mean of 77% of differentiated HL-60 cells responded, as contrasted with lesser increases in only 50% of undifferentiated HL-60 cells. The capacity of pretreatment of HL-60 cells with LTD_4 to prevent subsequent responses of $[Ca^{+2}]_i$ to LTC_4 and LTD_4, and the finding that the serine–borate inhibitor of conversion of LTC_4 to LTD_4 suppressed concurrently both LTC_4-induced rises in $[Ca^{+2}]_i$ and increases in adherence to Sephadex G-25 indicated that the responses of HL-60 cells to LTC_4 required conversion to LTD_4. That pertussis toxin and a chemical antagonist of LTD_4 reduced the $[Ca^{+2}]_i$ response suggested a dependence on LTD_4 receptors. The LTD_4-induced increases in $[Ca^{+2}]_i$ were dependent on extracellular calcium and diminished by lanthanum, but not affected by nifedipine nor associated with changes in membrane potential, as measured with the fluorescent probe 3,3′-dipentyloxacarbocyanine. Thus, the increase in $[Ca^{+2}]_i$ in HL-60 cells, which is coupled to an increase in adherence, appears to involve LTD_4 receptor-specific and voltage-independent calcium channels in the plasma membrane.

Introduction

Structurally diverse factors exhibiting chemotatic activity for polymorphonuclear (PMN)[1] leukocytes, including *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (1, 2) and leukotriene B_4 (LTB_4) (3–5), evoke rapid increases in the cytosolic concentration of intracellular calcium ($[Ca^{+2}]_i$) in PMN leukocytes (1–5) and myelocytes derived from HL-60 cells (5, 6), which may be a critical event in the mediation of functional responses to the factors (7). The C6-sulfidopeptide leukotriene constituents of the slow-reacting substance of anaphylaxis, leukotrienes C_4 (LTC_4) and D_4 (LTD_4), lack leukocyte chemotactic activity, but enhance the adherence of PMN leukocytes to synthetic surfaces **in vitro** (8) and to vascular endothelium **in vivo** (9). Whereas the activation of PMN leukocytes by LTC_4 and LTD_4 is dependent in part on stimulation of the cyclooxygenation of arachidonic acid in target cells, the major biochemical prerequisites for this selective recruitment of function have not been elucidated definitively. The present study was designed to evaluate the effects of the C6-sulfidopeptide leukotrienes on $[Ca^{+2}]_i$ in myelocytic leukocytes derived from HL-60 cells by incubation with dimethylsulfoxide (DMSO), and to examine the dependence of such effects on specific calcium channels and the metabolism of leukotrienes by the leukocytes.

Methods

Hanks' balanced salt solution without phenol red (HBSS), RPMI 1640 medium, glutamine, a mixture of penicillin G and streptomycin, and fetal bovine serum (FBS) were obtained from University of California Cell Culture Facility, San Francisco, CA; sodium dodecyl sulfate (SDS), fMLP, DMSO, lanthanum, nifedipine (NIF), and gramicidin D, from Sigma Chemical Co., St. Louis, MO; 14,15(*N*)[^{3}H]LTC_4 ([^{3}H]LTC_4) (39.0 Ci/mmol), from New England Nuclear, Boston, MA; quin-2 AM, from Amersham Corp., Arlington Heights, IL; and indo-1 AM and 3,3′-dipentyloxacarbocyanine (di-O-C_5(3)), from Molecular Probes Inc., Junction City, OR. Synthetic LTB_4, LTC_4, LTD_4, and LTE_4 were supplied by Dr. J. Rokach of Merck Frosst Canada, Ltd. (Dorval, Canada). The dithioacetal LTD_4 receptor antagonist SKF 102922 was a gift of Dr. J. G. Gleason (Smith Kline & French Laboratories, Philadelphia, PA). Purified pertussis toxin was a gift by Dr. R. D. Selcura of the National Institute of Child Health and Human Development, Bethesda, MD.

Culture and differentiation of HL-60 cells. HL-60 cells were supplied by Dr. Z. Bar-Shavit (Washington University Medical Center, St. Louis, MO) (10), and suspensions were cultured in 75-ml plastic flasks in RPMI 1640 medium supplemented with 10% FBS (vol/vol), 1 g/100 ml of glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The density of HL-60 cells was maintained between 5×10^5 and 2×10^6/ml. The HL-60 cells were cultured in the same medium containing 1% (vol/vol) DMSO for 4 d to achieve differentiation into myeloid cells resembling PMN leukocytes (11).

Quantification of $[Ca^{+2}]_i$ by quin-2 fluorescence. Cultured HL-60 cells were loaded with quin-2 by a modification of the method of Tsien et al. (5, 12). Suspensions of 1.2×10^7 HL-60 cells per ml of HBSS containing 0.1 g/100 ml of ovalbumin and 10 mM HEPES at pH 7.4 were incubated with 20 μM diacetomethoxy ester of quin-2 in the dark at 37°C for 10 min and at room temperature for 20 min. The quin-2–

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1. *Abbreviations used in this paper:* $[Ca^{+2}]_i$, cytosolic concentration of intracellular calcium; di-O-C_5(3), 3,3′-dipentyloxacarbocyanine; DMSO, dimethylsulfoxide; EGTA, ethylene glycol-bis (β aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution without phenol red; LTB_4, LTC_4, LTD_4, LTE_4, leukotrienes B_4, C_4, D_4, E_4; [^{3}H]-LTC_4, [^{3}H]LTD_4, 14,15(*N*)[^{3}H]leukotrienes C_4, D_4; NIF, nifedipine.

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loaded HL-60 cells were washed three times with HBSS, resuspended at 6×10^6 per ml of HBSS containing 0.1 g/100 ml of ovalbumin and 10 mM HEPES, and kept on ice until used for the fluorescence measurements.

Fluorescence studies were performed as described (5) with a spectrofluorimeter (model 650-40; Perkin-Elmer Corp., Norwalk, CT) equipped with temperature-controlled cuvettes and a magnetic stirrer under the cuvetter holder. 3×10^6 quin-2–loaded HL-60 cells were pelleted at 8,000 *g* for 15 s in a microfuge (model B; Beckman Instruments, Inc., Palo Alto, CA), resuspended in 2 ml of HBSS at 37°C, and transferred to a cuvette held at 37°C. Fluorescence was recorded continuously at excitation and emission wavelengths of 339 and 492 nm, respectively. Maximum and minimum fluorescence were determined sequentially by the addition of 20 μl of 10% (vol/vol) Triton X-100 in distilled water, followed by 40 μl of 0.5 M ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) (pH 10); the nanomolar values of $[Ca^{+2}]_i$ were calculated as before (5, 12). In some experiments, the standardization of quin-2 fluorescence was confirmed by the addition to replicate suspensions of ionomycin and $MnCl_2$ for maximum and minimum fluorescence, respectively (13). In each experiment, the autofluorescence value was determined under the same conditions with HL-60 cells lacking quin-2 and was subtracted from the fluorescence value of quin-2–loaded HL-60 cells.

Flow cytometric assessment of $[Ca^{+2}]_i$ by indo-1 fluorescence. Replicate suspensions of 2×10^7 HL-60 cells per ml of RPMI 1640 were incubated with 3 μM acetoxy-methyl ester of indo-1 for 20 min at 37°C in the dark, diluted 10-fold in RPMI 1640, and incubated for an additional 20 min at 37°C as described (14). The indo-1–loaded HL-60 cells were washed twice and resuspended in RPMI 1640 at 2.5×10^6 per ml and kept on ice. The effects of stimulation on indo-1–loaded HL-60 cells were analyzed in a cell sorter (FACS IV; Beckton Dickinson & Co., Sunnyvale, CA) equipped with an argon ion laser emitting 600 mW at 351–364 nm (Spectra-Physics Inc., Mountain View, CA). Blue (486/4 nm) and violet (404/4 nm) band pass filters were used to collect the fluorescence emitted by indo-1. The ratio of violet-to-blue fluorescence, which is directly related to the $[Ca^{+2}]_i$, was expressed on a logarithmic scale (14).

Flow cytometric evaluation of the membrane potential of HL-60 cells. Replicate suspensions of 10^6 HL-60 cells per ml of RPMI 1640 were preincubated at 37°C with 10 nM di-O-C_5(3) as described (15). The fluorescence of the di-O-C_5(3) in HL-60 cells was analyzed in a cell sorter (FACS IV; Beckton Dickinson & Co.) using an argon ion laser emitting 600 mW at 457 nm. Forward and 90° light scatters were collected for all cells to resolve viable from nonviable cells. Green fluorescence was collected through a band pass 530/30 nm filter for a minimum of 10,000 cells in each specimen and the values were expressed on a logarithmic scale.

Measurement of the metabolism of $[^3H]LTC_4$ and $[^3H]LTD_4$ by HL-60 cells. To assess the extent of conversion of LTC_4 to LTD_4 and LTD_4 to LTE_4 and more polar oxidative products, DMSO-differentiated HL-60 cells were incubated with 4.5 mM $[^3H]LTC_4$ and 100 or 300 nM nonradioactive LTC_4 or with 4.5 nM $[^3H]LTD_4$ and 100 nM nonradioactive LTD_4 under the same conditions as for the measurement of $[Ca^{+2}]_i$ by quin-2 fluorescence. At defined times, 200 μl samples of each suspension were removed and added to 300 μl of methanol and 25 μl of 0.5 N acetic acid at −20°C, followed by centrifugation at 8,000 *g* for 2 min in a microfuge (model B; Beckman Instruments, Inc.). Each supernatant then was dried under a stream of nitrogen; redissolved in 80 μl of methanol and 3.1 mM phosphoric acid in water (65:35 vol/vol, pH 5.5) containing 50 ng each of LTC_4, LTD_4, and LTE_4; and analyzed by high performance liquid chromatography (HPLC) with a dual pump system (Beckman Instruments, Inc.), which utilizes a 4.6-mm by 25-cm Ultrasil 10-μm octadecylsilane particle reverse-phase column (Beckman Instuments, Inc.) that was developed isocratically with methanol and 3.1 mM phosphoric acid (65:35 vol/vol, pH 5.5) at 1 ml/min. Absorbance of the eluate at 280 nm was monitored continuously,and 1 ml fractions were collected and assessed for radioactivity in a liquid scintillation counter (Beckman Instruments, Inc.).

Measurement of adherence of HL-60 cells to Sephadex G-25. DMSO-differentiated HL-60 cells were washed once in HBSS and resuspended in HBSS containing 0.1 g/100 ml of ovalbumin. Replicate 0.2-ml portions of suspensions of 10^7 differentiated HL-60 cells/ml were incubated with stimuli for 2 min at 37°C. The cells then were applied to 0.4-ml columns of Sephadex G-25 that had been prewashed with 3 ml of HBSS containing 0.1 g/100 ml of ovalbumin. The columns were washed twice with 2 ml of HBSS containing 0.1 g/100 ml of ovalbumin, and the effluent and washes containing the nonadherent HL-60 cells were pooled. The pools of nonadherent HL-60 cells were washed twice with 2 ml of HBSS lacking protein and then dissolved in 3 g/100 ml of SDS in distilled water. The absorbancy at 280 nm of each of the dissolved pools and of two washed and dissolved aliquots of the suspension applied to the columns were quantified with a spectrophotometer (model 240; Gilford Instrument Laboratories, Inc., Oberlin, OH). Percentage adherence to Sephadex G-25 was calculated according to the formula: 1 − [(absorbance of nonadherent pool in the presence of stimuli)/(absorbance of nonadherent pool in buffer)] × 100.

Results

LTC_4- and LTD_4-induced changes in the $[Ca^{+2}]_i$ of DMSO-differentiated HL-60 cells. The $[Ca^{+2}]_i$ in unstimulated DMSO-differentiated HL-60 cells was 142.2±12.7 nM (mean±SD, $n = 16$), as assessed by quin-2 fluorescence. LTC_4 increased the $[Ca^{+2}]_i$ in a concentration-dependent manner, with the elicitation of changes that were just reproducibly detectable, half-maximal, and maximal by ~ 12.5, 60, and 200 nM LTC_4, respectively (Fig. 1). At 200 nM, the response of the $[Ca^{+2}]_i$ reached a peak within 15–45 s and thereafter returned to the resting level over a period of 7–10 min (Fig. 2). In contrast, the lowest effective concentration of LTC_4 evoked a response in $[Ca^{+2}]_i$ that developed and subsided more slowly than that attributable to optimal concentrations. LTD_4 also induced rapid and concentration-dependent increases in $[Ca^{+2}]_i$ (Fig. 1). However, the potency of LTD_4 was ~ 50-fold greater than that of LTC_4, with a 50% effective concentration of 1.2 nM, and LTD_4 elicited a maximal increase in $[Ca^{+2}]_i$ nearly twice that attained by the optimal concentration of LTC_4 (Fig. 1). Also, even the lowest effective concentration of LTD_4 achieved the peak response in the $[Ca^{+2}]_i$ within 60 s (Fig. 3). Finally, LTE_4 at concentrations up to 300 nM did not elicit an increase in $[Ca^{+2}]_i$.

Development of responsiveness of $[Ca^{+2}]_i$ to LTC_4 and LTD_4 as a function of the differentiation of HL-60 cells. LTD_4 and LTC_4 elicited increases in $[Ca^{+2}]_i$ of undifferentiated HL-60 cells that were significantly smaller than the corresponding responses observed in HL-60 cells pretreated with DMSO. The $[Ca^{+2}]_i$ of undifferentiated HL-60 cells was 153±25 nM (mean±SD, $n = 7$) and rose to 196±7 and 351±55 nM after the introduction of 100 nM LTC_4 and LTD_4, respectively. After DMSO-induced differentiation was complete, the basal $[Ca^{+2}]_i$ of 143±21 nM (mean±SD, $n = 7$) was elevated to 368±33 and 892±143 nM, respectively, by 100 nM LTC_4 and LTD_4 ($P < 0.001$ and $P < 0.005$ relative to undifferentiated HL-60 cells).

The response of $[Ca^{+2}]_i$ in the undifferentiated HL-60 cells also was dependent on the concentration of LTD_4, but a half-maximal response occurred at 10 nM, as contrasted with 1.2 nM for DMSO-differentiated HL-60 cells. The magnitude of the maximal response of $[Ca^{+2}]_i$ elicited by 100 nM LTD_4 also was significantly lower in undifferentiated than in DMSO-differentiated HL-60 cells.

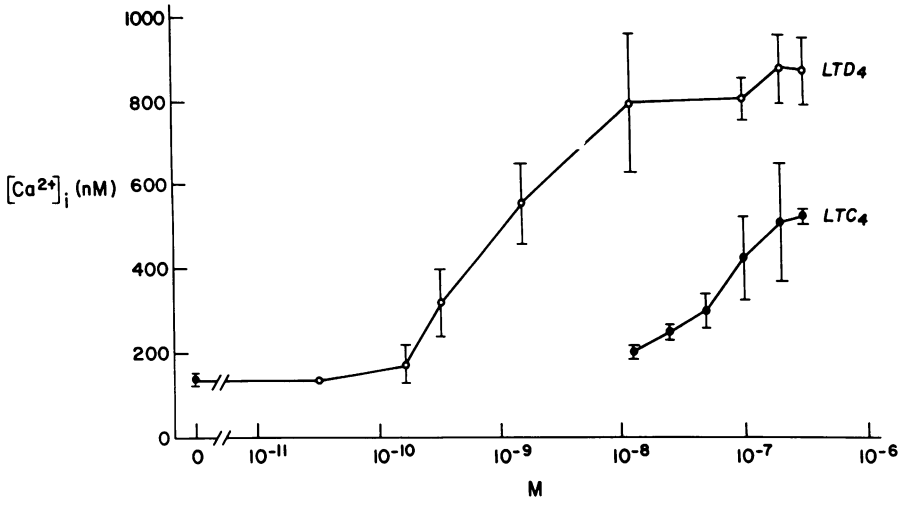

Figure 1. Concentration-dependence of the effects of LTC_4 (●) and LTD_4 (○) on the $[Ca^{+2}]_i$ in DMSO-differentiated HL-60 cells. Results are expressed as the maximal value of the $[Ca^{+2}]_i$ attained after the introduction of a stimulus. Each point is the mean±SD of three to four determinations performed in separate experiments.

The development of greater net responsiveness to the leukotrienes with differentiation might be attributable to the appearance of a higher percentage of responsive HL-60 cells, as contrasted with an increase in the level of responsiveness of all of the HL-60 cells. To distinguish between these possibilities, flow cytometric assays of the responses of individual cells were performed with indo-1 as the fluorescent indicator of $[Ca^{+2}]_i$ (Fig. 4). After exposure to 200 nM LTD_4, DMSO-differentiated HL-60 cells demonstrated typically homogeneous increases in the $[Ca^{+2}]_i$ (Fig. 4 *A*). Maximal fluorescence was detected in 77.1% of all of the HL-60 cells at 15 s and returned to the prechallenge level by 3 min. In contrast, only 50.5% of the undifferentiated HL-60 cells exhibited any increase in fluorescence (Fig. 4 *B*), and the modal level of the increases in fluorescence was lower than that observed in differentiated HL-60 cells. Also, the increases in $[Ca^{+2}]_i$ evoked by LTD_4 in undifferentiated HL-60 cells subsided to prechallenge levels by 30 s. Thus, DMSO-induced differentiation transforms most of

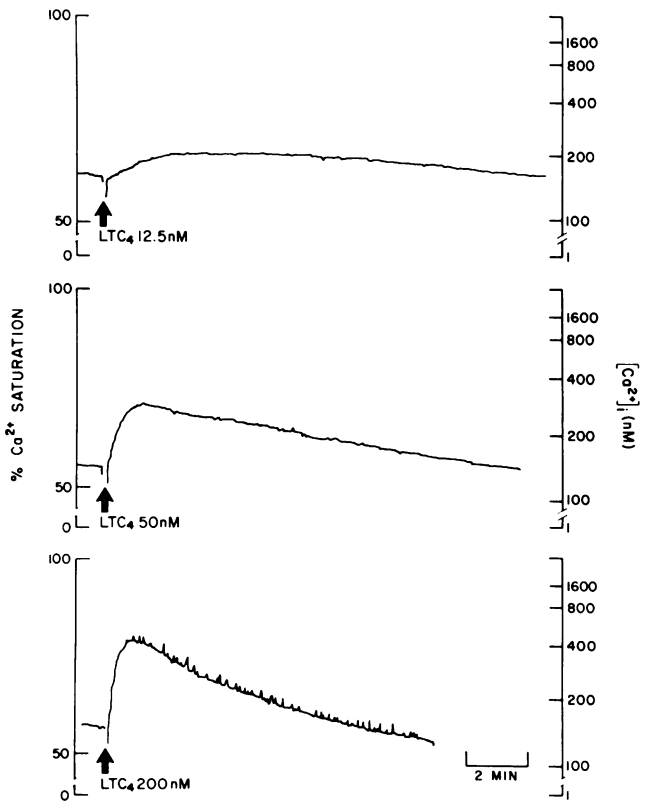

Figure 2. Time courses of the increases in the $[Ca^{+2}]_i$ of DMSO-differentiated HL-60 cells induced by exposure to three different concentrations of LTC_4. Each panel shows a typical tracing of the results of a representative experiment.

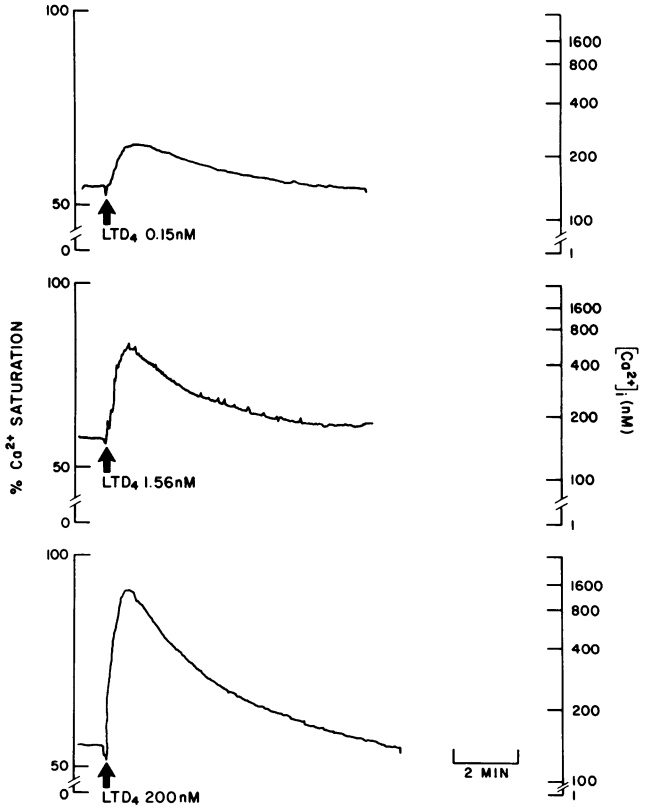

Figure 3. Time courses of the increases in the $[Ca^{+2}]_i$ of DMSO-differentiated HL-60 cells induced by exposure to three different concentrations of LTD_4. Each panel shows a typical tracing of the results of a representative experiment.

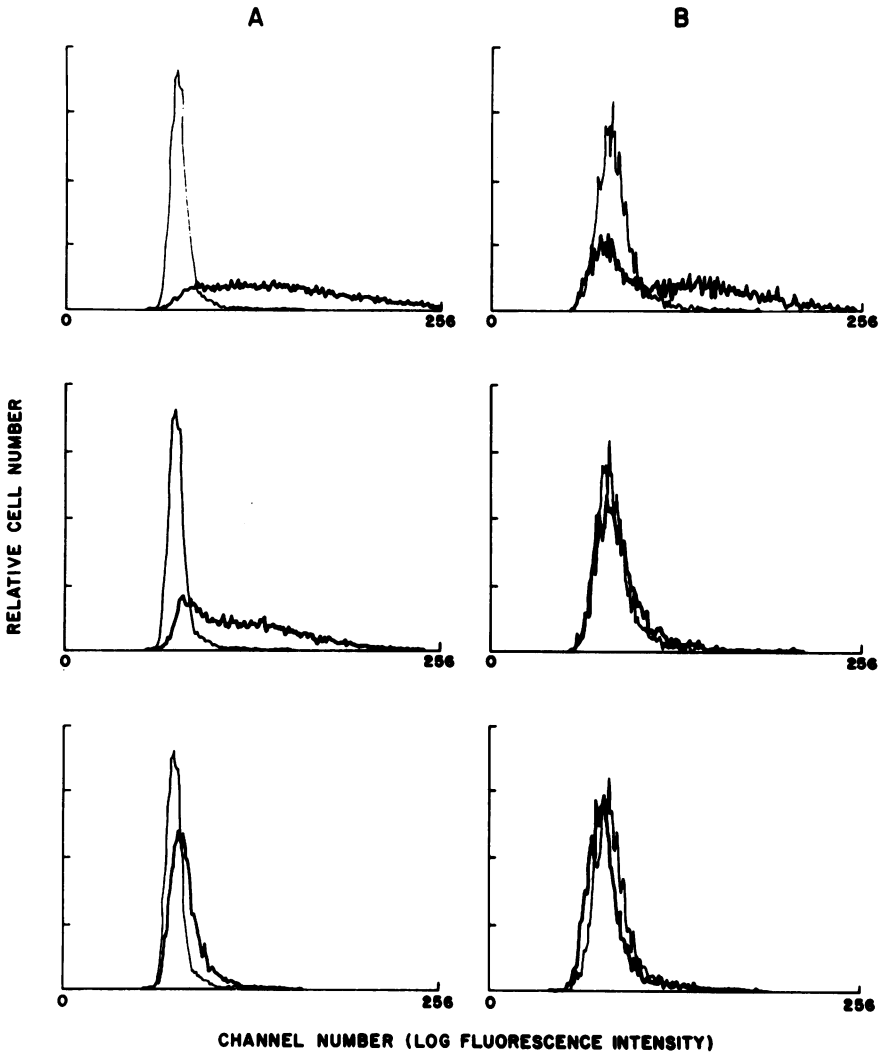

Figure 4. Effect of LTD_4 on the fluorescence histograms of indo-1-loaded HL-60 cells after (*A*) or before (*B*) 4 d of exposure to 1% DMSO. The patterns of fluorescence distribution were obtained before (——) or 15 s (*top*), 30 s (*middle*), and 3 min (*bottom*) after (▬) stimulation by 200 nM LTD_4.

the HL-60 cells into an LTD_4-responsive state, characterized by responses of the $[Ca^{+2}]_i$ to LTD_4 that are longer and greater in magnitude than those of undifferentiated HL-60 cells.

Dependence of the effect of LTC_4 on conversion to LTD_4. The effect of prior exposure to one leukotriene on the response of DMSO-differentiated HL-60 cells to that stimulus and other leukotrienes was examined to further define the pathways of activation (Fig. 5). Prior exposure of the HL-60 cells to an optimally active concentration of LTD_4 eliminated completely any response of the $[Ca^{+2}]_i$ to further challenges with LTD_4 (Fig. 5 *A*) or LTC_4 (Fig. 5 *C*). Pretreatment with a functionally similar concentration of LTC_4 suppressed substantially the response to subsequent challenge with LTD_4 (Fig. 5 *B*). In contrast, pretreatment with a maximally active concentration of fMLP (100 nM) had no apparent effect on the subsequent response of the $[Ca^{+2}]_i$ to LTD_4 (Fig. 5 *D*).

The implication that LTC_4 stimulated increases in the $[Ca^{+2}]_i$ only after conversion to LTD_4 was supported by the 100-fold greater potency of LTD_4 than LTC_4 (Fig. 1) and the slower rate of action of LTC_4 (Fig. 2). To assess the dependence of the activity of LTC_4 on conversion to LTD_4, the effect of the serine-borate complex inhibitor of γ-glutamyl transpeptidase activity was examined (16). The presence of a mixture of 5 mM L-serine and 10 mM sodium borate did not prevent the induction of a $[Ca^{+2}]_i$ signal by LTD_4, but totally abolished the response of $[Ca^{+2}]_i$ to LTC_4 (Fig. 6). To delineate the role of the target cells in metabolism of LTC_4, DMSO-differentiated HL-60 cells were incubated for 1 or 3 min with 4.5 nM $[^3H]LTC_4$ and 100 or 300 nM nonradioactive LTC_4 in the absence and presence of the serine-borate complex. The radioactivity was extracted from the suspensions and analyzed by HPLC in three different experiments (Table I). In the absence of serine-borate complex, a mean of 1.7% and 9.1% of 300 and 100 nM $[^3H]LTC_4$, respectively, was converted to $[^3H]LTD_4$, without a significant change in the generation of polar impurities and other products. After 3 min of incubation, the concentration of $[^3H]LTC_4$ declined further and the conversion to $[^3H]LTD_4$ reached a mean of 2.9% and 13.7% for 300 and 100 nM $[^3H]LTC_4$, respectively. No LTE_4 was detected. In the experiments conducted in the presence of serine-borate complex, the peptidolysis of 100 and 300 nM $[^3H]LTC_4$ was inhibited by ~ 50% and over 99%, respectively (Table I). The endogenous capacity of DMSO-differentiated HL-60 cells to generate 5–15 nM LTD_4 from LTC_4 in the absence of serine-borate complex is sufficient to evoke the observed increases in the $[Ca^{+2}]_i$ through actions of LTD_4 alone (Fig. 1). The increase in the response of $[Ca^{+2}]_i$ to LTD_4 associated with DMSO-induced differentiation of HL-60 cells was not due to a

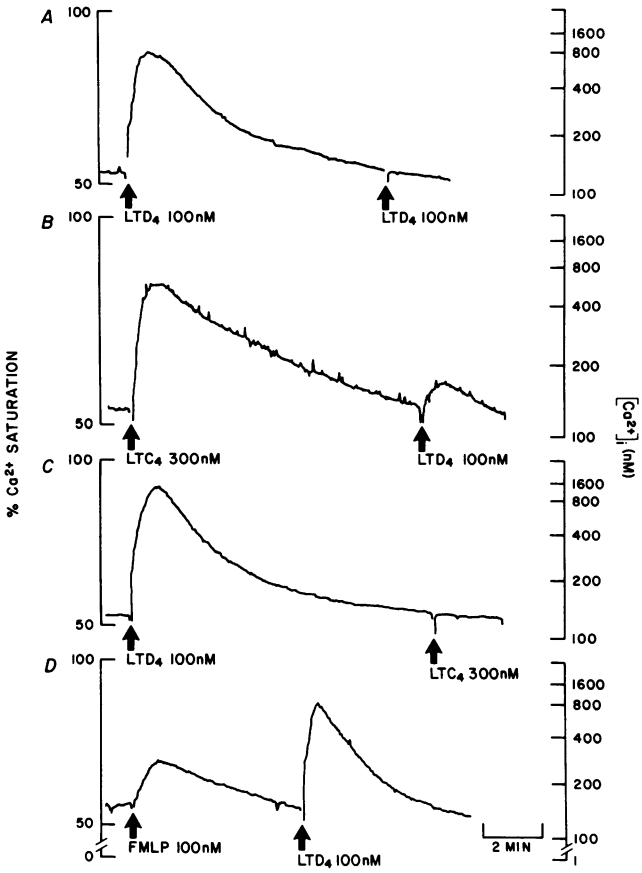

Figure 5. Effect of stimulus pretreatment on the increases in the $[Ca^{+2}]_i$ of DMSO-differentiated HL-60 cells elicited by leukotrienes. HL-60 cells were incubated first with LTD_4 (*A, C*), LTC_4 (*B*), or fMLP (*D*) and subsequently stimulated with maximally effective concentrations of LTD_4 (*A, B, D*) or LTC_4 (*C*). Each panel shows a typical tracing of the results of one representative experiment from a total of four.

modification in the capacity of the HL-60 cells to generate or metabolize LTD_4. There was < 15% change in the rate of conversion of $[^3H]LTC_4$ to $[^3H]LTD_4$ in DMSO-differentiated HL-60 cells, as compared with undifferentiated HL-60 (n = 3). After a 3-min incubation at 37°C with 100 nM nonradioactive LTD_4 and 4.5 nM $[^3H]LTD_4$, the radioactivity recovered was 87.5±4.2% and 85.8±3.7% (mean±SD, n = 4) LTD_4 for undifferentiated and DMSO-differentiated HL-60 cells, respectively.

That the increase in $[Ca^{+2}]_i$ evoked by LTD_4 was a receptor-mediated event was evidenced by significant inhibition of the effect of LTD_4 by the SKF 102922 dithioacetal antagonist of LTD_4 binding to receptors in other tissues (17). SKF 102922 inhibited in a concentration-dependent relationship the increase in $[Ca^{+2}]_i$ elicited by LTD_4 in DMSO-differentiated HL-60 cells (Fig. 7). Complete inhibition was achieved at 1 μM SKF 102922, which by itself demonstrated no detectable agonist activity. The response of $[Ca^{+2}]_i$ to 100 nM fMLP was not modified by 1 μM SKF 102922.

Cellular mechanisms of stimulation of the $[Ca^{+2}]_i$ *by* LTD_4. To investigate a requirement for extracellular Ca^{+2} in the observed increases in the $[Ca^{+2}]_i$, DMSO-differentiated HL-60 cells were incubated in a medium without Ca^{+2} and with 1 mM EGTA (Fig. 8). In the presence of EGTA, LTD_4 did not elicit any rise in the $[Ca^{+2}]_i$ above the baseline concentration of 143.51±2.48 nM (n = 3). Also, a concentration of lanthanum sufficient to block Ca^{+2} channels also prevented almost completely the rise in the $[Ca^{+2}]_i$ recorded in the complete medium (Fig. 8). NIF, a blocker of voltage-dependent calcium channels did not affect the maximal response of the $[Ca^{+2}]_i$ to 100 nM LTD_4 in three different experiments (Fig. 9). To confirm that the Ca^{+2} channels activated by LTD_4 are not of the voltage-dependent type, the membrane potential of the cells was quantified with a membrane potential-sensitive cyanine dye, di-O-C_5(3) (15). The fluorescence of di-O-C_5(3) in unstimulated HL-60 cells, which reflects the resting membrane potential, was not modified by the addition of 100 nM LTD_4, whereas the membrane-depolarizing compound gramidicin at 0.5 μM (18) decreased fluorescence (Fig. 10). Depolarization of the HL-60 cells with 60 mM KCl or 0.5 μM gramicidin did not elicit any change in quin-2 fluorescence.

Recent findings support an association of LTD_4 receptors in guinea pig lung with a guanine nucleotide-binding regulatory protein, N_i. Pretreatment of guinea pig lung tissue with

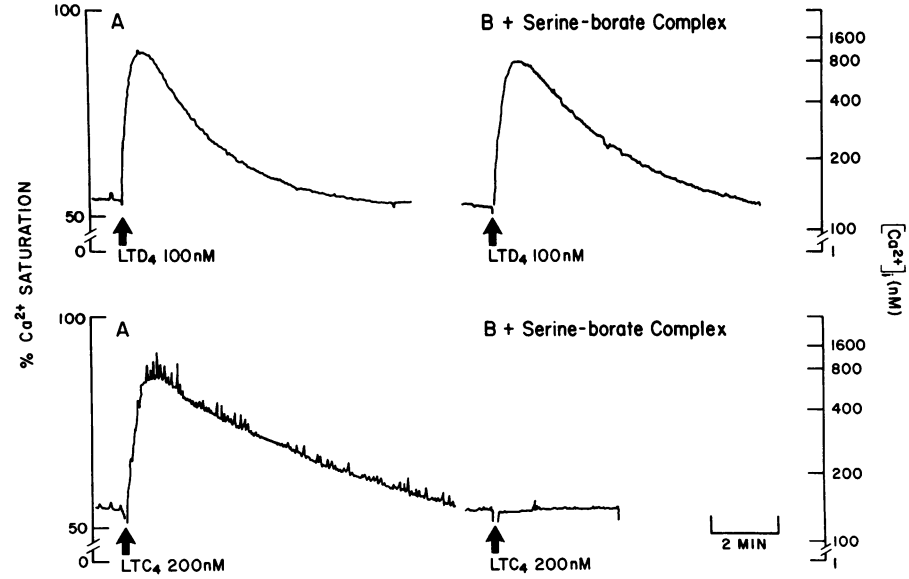

Figure 6. Effect of serine–borate complex on the response of the $[Ca^{+2}]_i$ of DMSO-differentiated HL-60 cells to maximal concentrations of LTD_4 (*top*) or LTC_4 (*bottom*). HL-60 cells were preincubated without (*A*) or with (*B*) a mixture of 5 mM L-serine and 10 mM sodium borate for 10 min at 37°C before the addition of the leukotrienes. Each panel shows a tracing of the results obtained in the same experiment.

Table I. Metabolism of [^{3}H]LTC$_4$ by DMSO-differentiated HL-60 Cells

Time of incubation	Concentration of nonradioactive LTC$_4$	Percentage of original [^{3}H]LTC$_4$					
		Without serine–borate complex			With serine–borate complex		
		LTC$_4$	LTD$_4$	Polar products	LTC$_4$	LTD$_4$	Polar products
min	*nM*	%	%	%	%	%	%
1	300	86.5±1.9	1.7±0.4	11.8±2.2	91.4±3.4	0	8.6±3.4
3	300	84.3±1.1	2.9±0.8	12.7±1.2	85.7±10.8	0	14.3±10.8
1	100	77.0±3.0	9.1±3.8	14.2±1.0	82.3±1.5	4.7±2.1	13.2±0.8
3	100	69.3±3.1	13.7±1.5	17.0±1.7	77.3±2.5	7.2±3.3	15.7±2.3

DMSO-differentiated HL-60 cells were incubated at 37°C in the absence or presence of a mixture of 5 mM L-serine and 10 mM sodium borate and with 4.5 nM [^{3}H]LTC$_4$ and 100 or 300 nM unlabeled LTC$_4$. Each value represents the mean±SD of the quantities of each factor recovered in three different experiments.

pertussis toxin at concentrations that inactivate N_i partially suppressed the LTD$_4$-induced synthesis and release of prostanoids (17). To investigate a possible requirement for the guanine nucleotide–binding regulatory protein in the observed increases in $[Ca^{+2}]_i$, DMSO-differentiated HL-60 cells were preincubated for 2 h with 100 ng/ml of pertussis toxin. At this concentration, pertussis toxin inhibited LTD$_4$-induced increases in $[Ca^{+2}]_i$ by 85.5±6.5% (mean±SD, n = 3) (Fig. 11).

The induction by DMSO of differentiation of HL-60 cells into mature granulocytes with receptors for fMLP and LTB$_4$ has been well-documented (6, 11, 19–21). LTC$_4$ and LTD$_4$ have been shown to enhance human PMN leukocyte adherence to synthetic surfaces in vitro (8) and to microvascular endothelium in vivo (9). To evaluate a possible functional response of DMSO-differentiated HL-60 cells to LTC$_4$ and LTD$_4$, the effects of the leukotrienes on the adherence of the cells to Sephadex G-25 was examined with a previously standardized technique (Table II). Both LTC$_4$ and LTD$_4$ enhanced significantly the adherence of DMSO-differentiated HL-60 cells to Sephadex G-25. The preincubation of the cells with serine–borate complex partially inhibited the effect of LTC$_4$, but not LTD$_4$, LTB$_4$, or fMLP on adherence.

Discussion

The C6-sulfidopeptide leukotrienes LTC$_4$ and LTD$_4$ have the capacity to activate broadly or with functional selectivity smooth muscle cells (22, 23), endothelial cells (24), glomerular epithelial (25) and mesangial cells (26), keratinocytes (27), neutrophils (8), and monocytes (28). As the pattern of functional responses of human leukocytes to LTC$_4$ and LTD$_4$ is substantially more restricted than that elicited by other stimuli, it was anticipated that the receptors for leukotrienes and the transductional sequence of biochemical events might also have unique characteristics. DMSO-differentiated HL-60 cells provide a convenient model for studies of such cellular events since the parent line is stable and differentiates homogeneously into myelocytic leukocytes bearing receptors for LTB$_4$ and peptide mediators, which are coupled to increases in $[Ca^{+2}]_i$ and other biochemical and functional concomitants of activation (5, 6). Also, the continuous suspension cultures of differentiated HL-60 cells permit more reliable application of biochemical techniques than monolayer cultures (13).

Functionally relevant concentrations of the sulfidopeptide leukotrienes elicted a transient rise in $[Ca^{+2}]_i$ that attained a

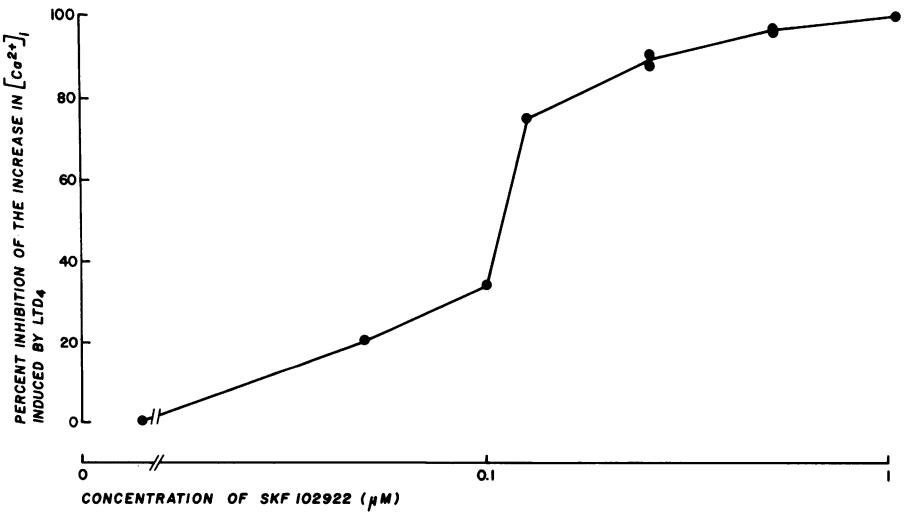

Figure 7. Inhibition of the increases in $[Ca^{+2}]_i$ induced by LTD$_4$ in DMSO-differentiated HL-60 cells by the receptor antagonist SKF 102922. Results are expressed as mean percentage inhibition of the maximal increase in $[Ca^{+2}]_i$ observed after the addition of 100 nM LTD$_4$ (n = 3).

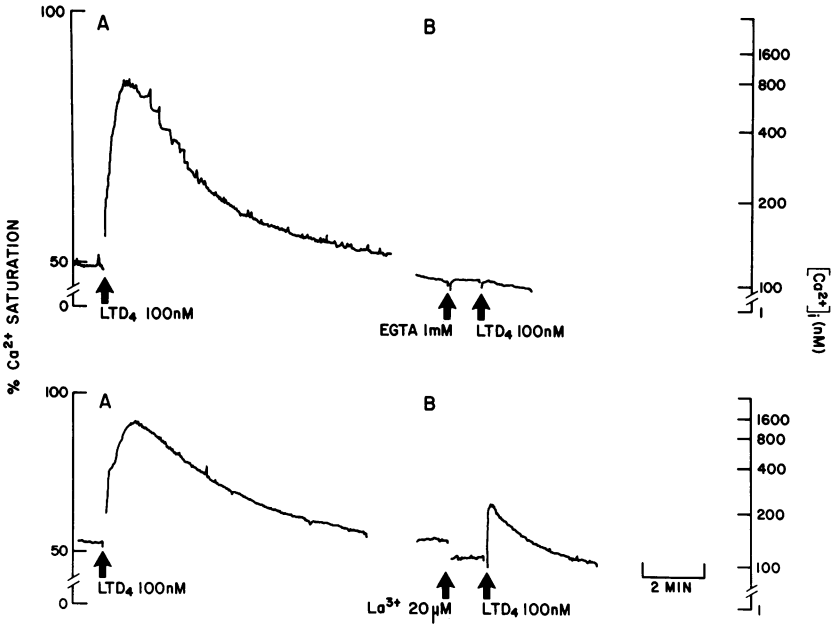

Figure 8. Dependence of the responses of the $[Ca^{+2}]_i$ on extracellular $[Ca^{+2}]$. DMSO-differentiated HL-60 cells were incubated in buffer with (*A*) or without (*B*) 1 mM $CaCL_2$ (*top*). Other suspensions of HL-60 cells were maintained in a medium containing 1 mM $CaCl_2$, but no phosphate salts, without (*A*) or with (*B*) 20 μM lanthanum (*bottom*). Each panel shows the tracing of the results of one representative experiment from a total of three.

peak more rapidly for LTD_4 than for LTC_4 (Figs. 1–3). That the DMSO-induced differentiation of HL-60 cells was accompanied by an increase in the sensitivity and maximal response of the $[Ca^{+2}]_i$ to LTD_4, relative to that of undifferentiated HL-60 cells, linked the alterations in cellular responsiveness to differentiation-associated changes in cellular receptors for LTD_4 or postreceptor biochemical events. The results of flow cytometric assays of $[Ca^{+2}]_i$ indicated that a subset of undifferentiated HL-60 cells react suboptimally to LTD_4 and that DMSO recruits the majority of the cells into an optimally LTD_4-responsive state (Fig. 4). DMSO-induced differentiation did not alter the rate of degradation of LTD_4 by HL-60 cells. Thus, the results of analyses of the specificity and mechanisms of the increases in $[Ca^{+2}]_i$ elicited by LTC_4 and LTD_4 are referable to a relatively homogeneous population of myelocytic leukocytes.

Although the response of $[Ca^{+2}]_i$ to LTC_4 attained one-half of the maximum level at ~ 60 nM, a concentration that correlates with the equilibrium dissociation constant of 50 nM found for the binding of $[^3H]LTC_4$ to DMSO-differentiated HL-60 cells and of 34 nM for human PMN leukocytes (29); LTC_4 was not a direct agonist. The binding sites on human PMN leukocytes were specific for the sulfido-peptide leukotrienes since only C6-peptide leukotrienes and their structural analogs exhibit binding activity (29). The longer time required to reach a maximal $[Ca^{+2}]_i$ with LTC_4 than LTD_4 suggested initially the LTC_4 was not acting directly through binding to a separate set of receptors, but rather was being converted to an active metabolite. The observation that high concentrations of LTD_4 were able to deactivate HL-60 cells to the subsequent effects of LTC_4, as well as LTD_4, on the $[Ca^{+2}]_i$ implied that the two leukotrienes shared a common pathway of activation (Fig. 5). The hypothesis that LTC_4 acted only after conversion to LTD_4 was confirmed by the findings that the serine–borate inhibitor of leukocyte γ-glutamyl transpeptidase suppressed the response of $[Ca^{+2}]_i$ to LTC_4, but not to LTD_4, and reduced concomitantly the LTC_4-induced increases in adherence of HL-60 cells to Sephadex G-25 (Fig. 6, Table II). Under the conditions used for determination of $[Ca^{+2}]_i$ by quin-2 fluorescence, the serine–borate complex inhibited the metabolic conversion of LTC_4 to LTD_4 by 50–100% (Table I). This rate of conversion of LTC_4 to LTD_4 is sufficient to explain the increases in $[Ca^{+2}]_i$ and adherence to surfaces documented for LTC_4 in terms of the LTD_4 generated from LTC_4. In contrast to these results, previous studies have shown that the spasmogenic activity of LTC_4 in guinea pig ileum does not depend on the bioconversion of LTC_4 to LTD_4 (30), and that serine–borate complex enhances the contractile activity of LTC_4 on guinea pig trachea (31).

That the effect of LTD_4 on the $[Ca^{+2}]_i$ of HL-60 cells is receptor-mediated was suggested by the potency and susceptibility to stimulus-specific deactivation of LTD_4 activity. This possibility is supported by two other independent lines of evidence. First, the stimulation of increases in $[Ca^{+2}]_i$ by LTD_4

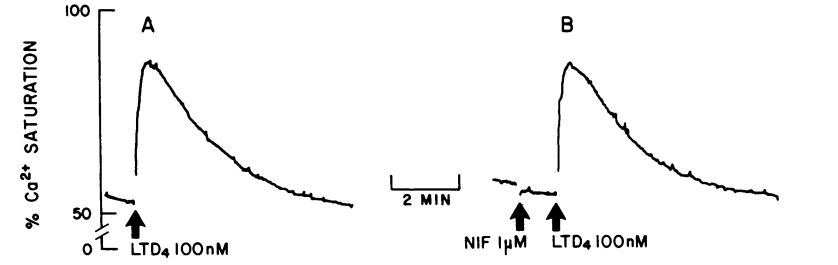

Figure 9. Dependence of LTD_4-induced increases in the $[Ca^{+2}]_i$ on the integrity of the voltage-dependent calcium channels of DMSO-differentiated HL-60 cells. The HL-60 cells were challenged with 100 nM LTD_4 without (*A*) and with (*B*) 1 μM NIF. The results are representative of a total of four identical experiments.

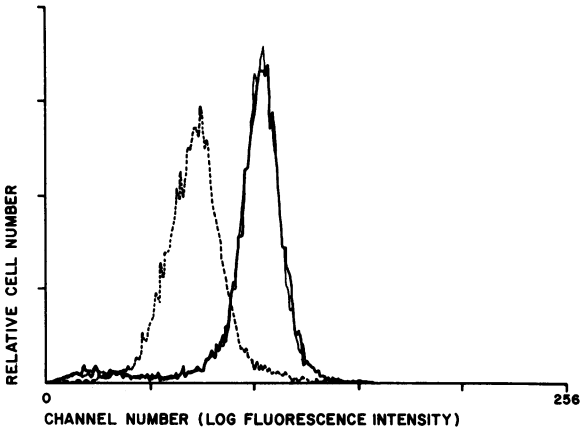

Figure 10. Effect of LTD_4 on the fluorescence histograms of di-O-$C_5(3)$-loaded HL-60 cells. The patterns of distribution of fluorescence were obtained before any challenge (——), 1 min after stimulation by 200 nM LTD_4 (▬▬), and 7 min after addition of 0.5 μM gramicidin (- - -).

was prevented by equimolar concentrations of a specific LTD_4 receptor antagonist (Fig. 7). Second, pretreatment with concentrations of pertussis toxin that inactivate *N* protein, inhibited the LTD_4-induced increases in $[Ca^{+2}]_i$ in HL-60 cells (Fig. 11), as has been demonstrated recently for receptor-mediated effects of LTD_4 in other systems (17, 32).

The responses of the $[Ca^{+2}]_i$ of DMSO-differentiated HL-60 cells to LTD_4 depend on extracellular calcium, as evidenced by the finding that the addition of either EGTA or the calcium entry blocker lanthanum abolishes almost completely the $[Ca^{+2}]_i$ rises elicited by LTD_4 (Fig. 7). It is also apparent that LTD_4 stimulates calcium entry through a voltage-independent calcium channel in the plasma membrane. First, the specific calcium channel blocker NIF does not alter the response of the $[Ca^{+2}]_i$ to LTD_4 (Fig. 8). Second, the LTD_4-induced increase in the $[Ca^{+2}]_i$ is not accompanied by changes in membrane potential (Fig. 9). In guinea pig trachea and lung parenchyma, LTD_4 also triggers cellular responses through a mechanism that is inhibited by a lack of extracellular calcium and only slightly affected by NIF (33). LTD_4 in both cells and tissues thus seems to open a voltage-independent calcium channel similar to that described for the effect of angiotensin II on smooth muscle cells (13) and of the epidermal growth factor on A431 cells (34). These changes in $[Ca^{+2}]_i$ also are associated with a functional response in HL-60 cells, where LTC_4 and LTD_4 enhanced their adherence to the surface of Sephadex G-25 particles (Table II). It is clear from these studies that the increased adherence of HL-60 cells in response to LTC_4 stimulation is primarily due to the generation of LTD_4. Since both LTC_4 and LTD_4 are able to compete with $[^3H]LTC_4$ for binding to human PMN leukocytes (29), the enhanced adherence could possibly be mediated by this population of receptors. In vitro studies of the responses to sulfidopeptide leukotrienes have been limited previously to those of tissues and cellular monolayers. The results of the present studies demonstrate for the first time in suspensions of individual cells increases in the $[Ca^{+2}]_i$, which may represent a critical early biochemical event in the selective activation of leukocyte adherence to surfaces.

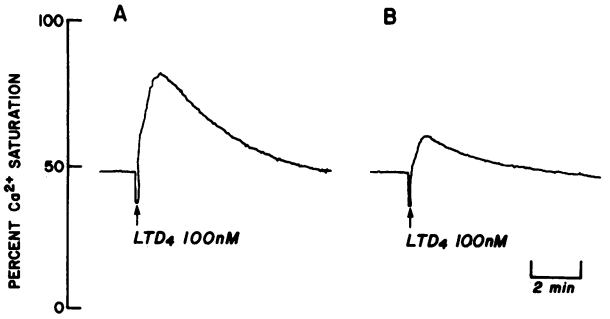

Figure 11. Prevention by pertussis toxin of the increase in $[Ca^{+2}]_i$ induced by LTD_4. HL-60 cells were incubated without (*A*) and with (*B*) 100 ng/ml of pertussis toxin for 2 h at 37°C before the addition of 100 nM LTD_4. The tracings in *A* and *B* are from a single illustrative experiment from a total of three.

Table II. Effect of LTC_4 and LTD_4 on the Adherence of DMSO-differentiated HL-60 Cells to Sephadex G-25

Stimulus‡	Percentage increase in adherence to Sephadex G-25*	
	Without serine-borate	With serine-borate
Buffer	0	
fMLP (10^{-6} M)	42.1	43.4
LTB_4 (10^{-7} M)	24.0	22.9
LTC_4 (10^{-9} M)	1.6	
LTC_4 (10^{-8} M)	11.3	5.4
LTC_4 (10^{-7} M)	20.5	12.1
LTC_4 (10^{-6} M)	26.7	
LTD_4 (10^{-9} M)	1.4	
LTD_4 (10^{-8} M)	7.5	8.2
LTD_4 (10^{-7} M)	20.4	21.8
LTD_4 (10^{-6} M)	41.2	

* The results presented are the mean percentage increase of HL-60 cells adhering to Sephadex G-25 in two experiments performed in duplicate, except for LTD_4 in the presence of serine-borate and LTB_4.
‡ The HL-60 cells were preincubated for 5 min at 37°C without or with 5 mM serine–10 mM sodium borate and then for 2 min with the stimulus or buffer alone.

Acknowledgments

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