Phorbol ester-induced suppression of leukotriene C_4 synthase activity in human granulocytes

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Abstract The effect of the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), on the metabolism of exogenous leukotriene (LT)A_4 in human granulocytes was investigated. After incubation with LTA_4 decreased levels of LTC_4 but not LTB_4 were observed in granulocyte suspensions pretreated with PMA. This finding could in part be ascribed to oxidative metabolism of LTC_4, since PMA induced a rapid degradation of exogenously added LTC_4. After blocking of LTC_4 metabolism with the H_2O_2 scavenger catalase, a PMA-provoked suppression of the conversion of LTA_4 to LTC_4 was observed, indicating PKC-dependent regulation of LTC_4 synthase activity. This effect, as well as PMA-induced degradation of LTC_4 was prevented by specific protein kinase C inhibitors.

Key words: Protein kinase C; Leukotriene C_4 synthase; Phorbol ester; Human granulocyte

1. Introduction

During cell activation leukotriene (LT) biosynthesis is initiated by liberation of arachidonic acid from membrane phospholipids through the action of a specific high molecular weight cytosolic phospholipase A_2 ($cPLA_2$) [1]. Subsequently, the fatty acid is transformed to the unstable epoxide LTA_4. The latter reaction is catalyzed by the 5-lipoxygenase (5-LO), an enzyme operating in concert with a 5-lipoxygenase activating protein (FLAP), which makes arachidonic acid available for enzymatic conversion [2].

The capacity to produce LTA_4 from endogenous substrate is a common feature among cells of myeloid origin. In contrast, the expression of enzymes capable of metabolizing this intermediate differs between neutrophils, which utilise LTA_4 hydrolase to produce the leukocyte activator LTB_4 [3], and eosinophils/basophils whitch make use of LTC_4 synthase, conjugating LTA_4 with reduced glutathione, to synthesise LTC_4 [4,5]. In accordance, northern blot analysis demonstrated mRNA coding for LTC_4 synthase in human eosinophils, but not in neutrophils [6]. Furthermore, monocytes/macrophages express both LTA_4 hydrolase and LTC_4 synthase activities [7,8]. Human platelets are devoid of 5-LO activity, but possess LTC_4 synthase activity [9,10], which enables them to produce LTC_4 when supplied with LTA_4 from surrounding cells, e.g. activated granulocytes or monocytes [11].

Leukotriene C_4 may be enzymatically transformed to the likewise biologically active metabolites LTD_4 and LTE_4 [12]. These three cysteinyl leukotrienes are powerful inflammatory mediators involved in allergy and asthma. Alternatively, LTC_4 may be rapidly inactivated by myeloperoxidase-dependent oxidative metabolism [13,14].

Leukotriene C_4 synthase has been purified from guinea pig and human lung tissue [8,15] as well as from various cell lines, including established myeloid cells [16–18] and human erythroleukemia cells [19], and was found to be an 18 kDa polypeptide active as a homodimer. The enzyme was recently cloned and the deduced amino acid sequence demonstrated to be similar to FLAP, but not to any known cytosolic or microsomal glutathione S-transferase [6,20]. The presence of LTC_4 synthase in human lung tissue, alveolar macrophages, eosinophils and platelets was recently demonstrated, using a polyclonal antibody against the enzyme [8].

It has recently been reported that the activity of LTC_4 synthase in myeloid cells can be controlled via phosphoregulatory mechanisms. Thus, phorbol 12-myristate 13-acetate (PMA) provoked suppression of ionophore A23187- or LTA_4-induced LTC_4 formation in established human pre-leukemic cell lines [21,22]. Similarly, LTC_4 synthase activity in human platelets has been demonstrated to be phosphoregulated via both PKC-dependent and receptor-mediated, PKC-independent mechanisms [23]. It was therefore of interest to investigate whether LTC_4 formation in normal human granulocytes is governed via similar mechanisms. The present findings demonstrate that activation of PKC in granulocytes lowers the levels of LTC_4, not only via stimulation of oxidative LTC_4 degradation, but also via inhibition of LTC_4 synthase activity.

2. Experimental

2.1. Materials

Leukotrienes B_4 and C_4 and 4β-phorbol 12-myristate 13-acetate (PMA) were obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Ro 31–8220 was a kind gift from Dr. Trevor J Hallam, Roche Research Centre, Herts, UK. Catalase (from bovine liver, 65.000 units/mg) and GF 109203X were obtained from Calbiochem-Boehring (La Jolla, CA, USA). D-Glucose, gluthatione and fatty acid-free human serum albumin were purchased from Sigma (St. Louis, MO, USA). Lymphoprep was from Nycomed Pharma AS (Oslo, Norway). Leukotriene A_4-methyl ester was a generous gift from Dr. Robert Zipkin, Biomol Research Laboratories, and was saponified as described [23].

2.2. Preparation of granulocyte suspensions and sonicates

Peripheral blood from healthy volunteers who had not taken any medication for at least 10 days was collected in EDTA-containing Vacutainer blood collection tubes (Becton Dickinson, Rutherford, NJ, USA). After centrifugation at $200 \times g$ for 15 min, the platelet-rich plasma was removed and the granulocytes were isolated from the remaining lower phase by dextran sedimentation, hypotonic ammonium chloride lysis and Lymphoprep centrifugation [24]. The pellet (containing >98% granulocytes) was resuspended in phosphate-buffered saline (PBS, with 0.9 mM calcium chloride and 5 mM D-glucose, pH 7.4) at a concentration of 15×10^6 cells/ml. Platelet contamination was less than 0.5 platelet per granulocyte, as determined by phase-contrast

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microscopy after lysis of the granulocytes. Granulocyte sonicates were prepared by sonication of cell suspensions supplemented with 3 mM glutathione (using an Ultrasonic disintegrator Mk2, Sussex, UK; power output: 50–150 W) at 0°C for 15 s.

2.3. Incubation procedure

Cell suspensions (0.5–1.0 ml) with or without catalase (60 μg/ml) were equilibrated at 37°C for 5 min in the presence of human serum albumin (0.3 mg/ml). Thereafter the cells were incubated for 2–60 min in the absence or presence of PMA (5–160 nM). In some experiments the cells were incubated for 2 min with or without Ro 31–8220 (3 μM) or GF 109203X (3 μM) prior to PMA treatment. Incubations were followed by determination of leukotriene metabolism as described below.

2.4. Determination of leukotriene metabolism

The metabolism of LTA_4 in human granulocytes was determined by incubation with LTA_4 (10 μM) at 37°C for 5 min (cell suspensions) or 2.5 min (granulocyte sonicates). The ability to metabolize LTC_4 was investigated by incubation of intact cells with LTC_4 (600 nM) for 5 min at 37°C. Reactions were stopped by addition of five volumes of ethanol containing prostaglandin B_2 as internal standard. After centrifugation and evaporation of the samples, leukotrienes were analyzed by reversed phase HPLC, using a Nova-Pak C_{18} column (3.9 × 150 mm, Waters Ass., Milford, MA, USA), eluted with acetonitrile/methanol/water/acetic acid (27:18:54:0.8, v/v, apparent pH 5.6) at a flow rate of 1.0 ml/min, and a variable wavelength UV-detector (LDC Spectromonitor III) connected to an integrator (LDC/Milton Roy CI-4000).

3. Results

3.1. Effect of phorbol ester on LTC_4 metabolism in human granulocyte suspensions

Addition of PMA to human granulocyte suspensions provoked a rapid oxidative metabolism of exogenous LTC_4 in agreement with earlier findings [13,14]. Thus, after 5 min. approximately 40% of added LTC_4 was recovered from granulocyte suspensions which had been preincubated with PMA for 2 or 20 min, respectively (Fig. 1). The disappearance of LTC_4 was accompanied by the appearence of six new HPLC-peaks, including 6-trans-LTB_4 isomers and LTC_4 sulphoxides (results not shown), in correspondence with previously reported oxidative metabolism of LTC_4 [13,14]. The metabolism could be inhibited by the H_2O_2 scavenger catalase (Fig. 1). No degradation of LTC_4 was found in unstimulated control suspensions and conversion of LTC_4 to LTD_4 and LTE_4 was not observed neither in control suspensions nor in the PMA-treated samples (results not shown).

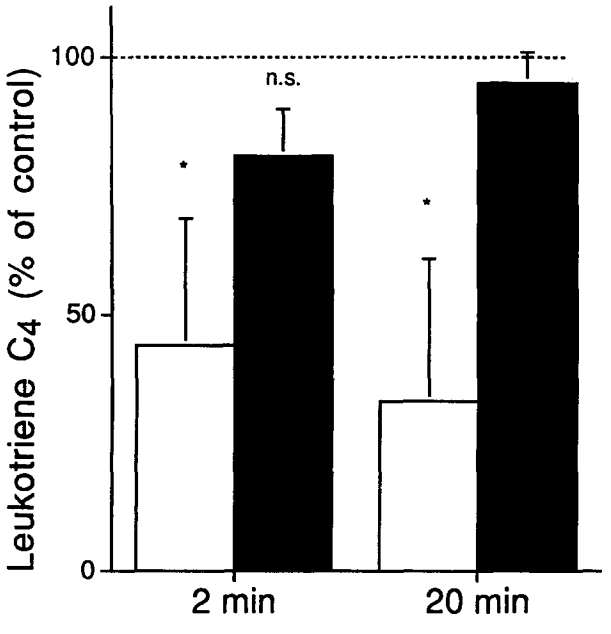

Fig. 1. Effect of PMA on the metabolism of exogenous LTC_4 in human granulocytes. Human granulocyte suspensions (15×10^6 cells/ml) containing human serum albumin (0.3 mg/ml) were equilibrated with (black bars) or without (white bars) catalase (60 μg/ml) at 37°C for 5 min and preincubated in the absence (control) or presence of PMA (80 nM) for another 2 min or 20 min. Thereafter the cells were incubated with LTC_4 (600 nM) for 5 min. Each value represent the mean ± S.D. of 3–5 experiments performed in duplicate. Statistical analysis was performed using Student's *t*-test for paired samples. n.s.: not significant; $^*P < 0.05$

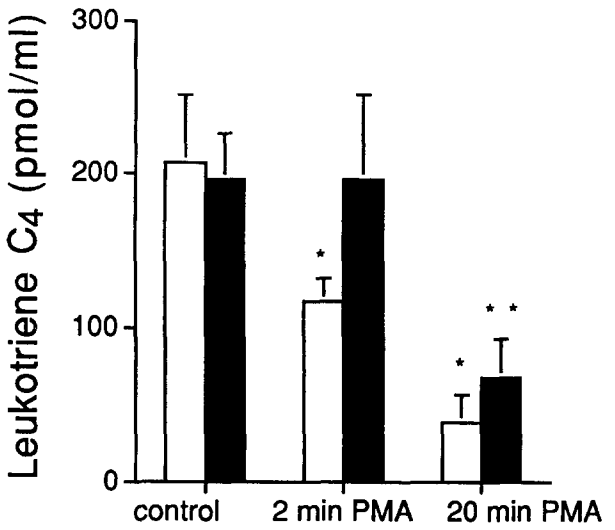

Fig. 2. Effect of PMA on LTC_4 levels in human granulocytes after addition of exogenous LTA_4. Human granulocyte suspensions (15×10^6 cells/ml) containing human serum albumin (0.3 mg/ml) were equilibrated with (black bars) or without (white bars) catalase (60 μg/ml) at 37°C for 5 min and preincubated in the absence (control) or presence of PMA (80 nM) for another 2 min or 20 min. Thereafter the cells were incubated with LTA_4 (10 μM) for 5 min. Each value represent the mean ± S.D. of 3–4 experiments performed in duplicate. Statistical analysis was performed using Student's *t*-test for paired samples (PMA with or without catalase versus control with or without catalase respectively). n.s.: not significant; $^*P < 0.05$; $^{**}P < 0.01$.

3.2. Effect of phorbol ester pretreatment on the levels of LTC_4 and LTB_4 after incubation of human granulocyte suspensions with exogenous LTA_4

The effect of PMA on the conversion of exogenous LTA_4 in human granulocyte suspensions was studied. Catalase was used in these experiments, in order to avoid the influence of oxidative LTC_4 metabolism. The capacity to transform LTA_4 to LTC_4 and LTB_4 in untreated control suspensions was reflected by the production of 212 ± 56 pmol LTC_4/ml (mean ± S.D.; $n = 6$) and 451 ± 103 pmol LTB_4/ml (mean ± S.D.; $n = 6$), together with minor amounts of the LTB_4 metabolites, 20-OH LTB_4 and 20-COOH LTB_4 (results not shown). Measurable amounts of LTD_4 or LTE_4 were not detected. The basal conversion of LTA_4 to LTC_4 and LTB_4 was not affected by addition of catalase. Treatment of the cells with PMA (80 nM) in the absence of catalase for 2 or 20 min before addition of LTA_4 led to decreased levels of LTC_4, as compared to those obtained in control suspensions (Fig. 2). Addition of catalase totally reversed the short-time, but not the long-time inhibitory effect of

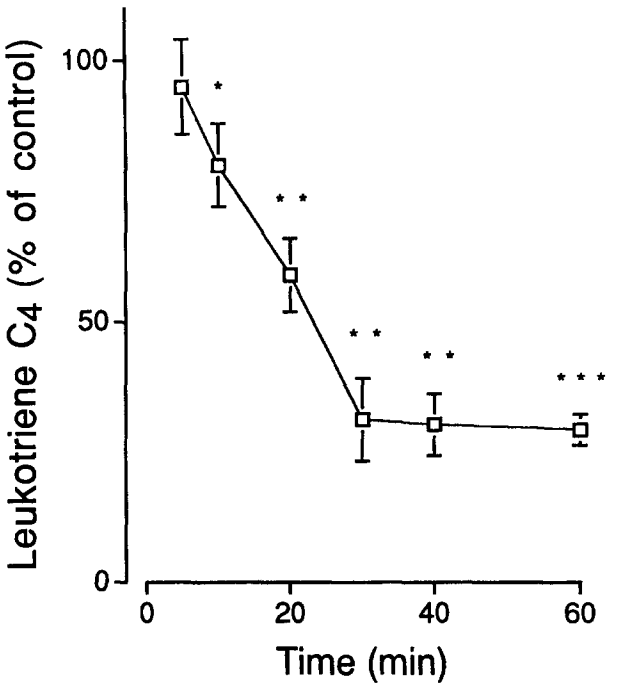

Fig. 3. Time course of the effect of PMA on LTC_4 synthase activity in human granulocytes. Human granulocyte suspensions (15×10^6 cells/ml) containing catalase (60 μg/ml) and human serum albumin (0.3 mg/ml) were equilibrated at 37°C for 5 min and preincubated in the absence (control) or presence of PMA (80 nM) for another 5–60 min prior to incubation with LTA_4 (10 μM) for 5 min. Each value represent the mean ± S.D. of 3–4 experiments performed in duplicate. Statistical analysis was performed using Student's *t*-test for paired samples. n.s.: not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

PMA on LTC_4 levels. The levels of LTB_4 was unaffected by the phorbol ester (results not shown). Measurable amounts of leukotrienes could not be detected after treatment of granulocyte suspensions with PMA (160 nM) in the absence of LTA_4 (results not shown).

3.3. Characterization of PMA-induced supression of LTC_4 synthase activity in human granulocytes

The effect of PMA on LTC_4 synthase activity in granulocyte suspensions was characterized by determination of LTC_4 formation from exogenous LTA_4 in the presence of catalase. Time-course studies demonstrated a significant inhibitory effect of PMA (80 nM) on LTC_4 synthase activity after 10 min, with maximal suppression after 30 min (Fig. 3). In dose-response studies granulocyte suspensions were pretreated with PMA (5–160 nM) for 30 min (Fig. 4). A siginificant inhibitory effect was observed already at 10 nM and maximal inhibition was achieved at about 40–100 nM.

Table 1
Effect of PMA on the formation of LTC_4 and LTB_4 from exogenous LTA_4 in human granulocyte sonicates

	LTC_4 (pmol/ml)	LTB_4 (pmol/ml)
Control	684 ± 228	729 ± 150
PMA	394 ± 221*	654 ± 117

Human granulocyte suspensions (15×10^6 cells/ml) containing catalase (60 μg/ml) and human serum albumin (0.3 mg/ml) were equilibrated at 37°C for 5 min and preincubated in the absence (control) or presence of PMA (80 nM) for 30 min. Thereafter glutathione (3 mM) was added and the cells were disrupted by sonication at 0°C for 15 s and immediately preincubated at 37°C for 30 s prior to incubation with LTA_4 (10 μM) for 2.5 min. Each value represent the mean ± S.D. of 3 separate experiments performed in duplicate. Statistical analysis was performed using Student's *t*-test for paired samples. $*P < 0.05$.

Phorbol ester-induced inhibition of LTC_4 synthase activity was observed also in experiments where the granulocytes were disrupted by sonication in the presence of 3 mM glutathione, subsequent to incubation with or without PMA for 30 min. Thus, pretreatment with PMA provoked approximately 40% attenuation of the conversion of LTA_4 to LTC_4 in granulocyte sonicates (Table 1). In contrast, the production of LTB_4 was unaffected by PMA.

3.4. Reversal of PMA-induced effects on leukotriene metabolism by specific PKC inhibitors

Preincubation of granulocyte suspensions with the specific protein kinase C inhibitors, Ro 31-8220 [25] or GF 109203X [26], reversed the inhibitory effect of PMA on granulocyte LTC_4 synthase activity and abolished PMA-induced oxidative LTC_4 metabolism (Table 2).

4. Discussion

Direct activation of protein kinase C with nanomolar concentrations of the phorbol ester PMA lowered the levels of LTC_4 after incubation of human granulocyte suspensions with exogenous LTA_4. This decrease was due to two distinct effects on leukotriene metabolism. Thus, PMA induced a rapid catalase-inhibitable degradation of exogenously added LTC_4, in agreement with previous reports demonstrating myeloperoxi-

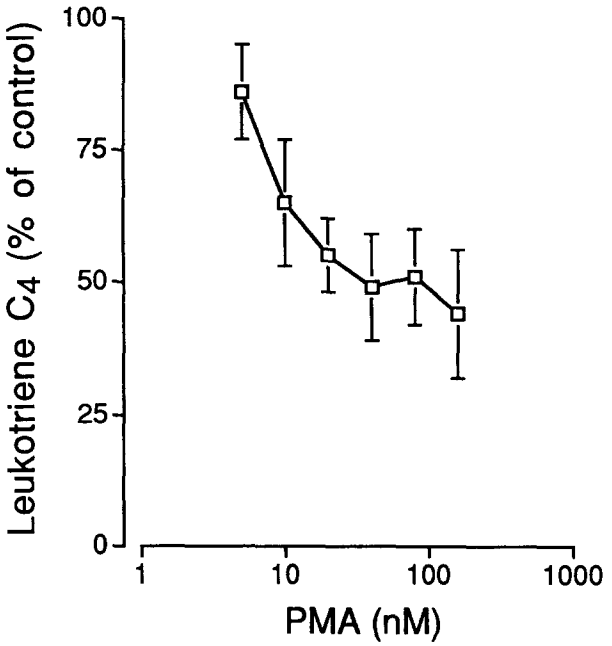

Fig. 4. Dose–response curve of the effect of PMA on LTC_4 synthase activity in human granulocytes. Human granulocyte suspensions (15×10^6 cells/ml) containing catalase (60 μg/ml) and human serum albumin (0.3 mg/ml) were equilibrated at 37°C for 5 min and preincubated in the absence (control) or presence of PMA (5–160 nM) for another 30 min prior to incubation with LTA_4 (10 μM) for 5 min. Each value represent the mean ± S.D. of 3 experiments performed in duplicate.

Table 2
Reversal of PMA-induced effects on leuktriene metabolism in human granulocytes by PKC inhibitors

	A: LTC_4 degradation LTC_4 (% of control)	B: LTC_4 synthase activity LTC_4 (% of control)
PMA	16	51
PMA + Ro 31-8220	97	105
PMA + GF 109203X	99	95

Human granulocyte suspensions (15×10^6 cells/ml) containing human serum albumin (0.3 mg/ml) were equilibrated without (for determination of LTC_4 degradation) or with (for determination of LTC_4 synthase activity) catalase (60 μg/ml) at 37°C for 5 min and preincubated in the presence or absence of PKC inhibitors (3 μM) for another 2 min, prior to treatment with PMA (80 nM, 30 min). Thereafter the cells were incubated with 600 nM LTC_4 (A) or 10 μM LTA_4 (B) for 5 min. Control incubations were performed in the absence of PKC inhibitors and PMA. Each value represents the mean of two independent experiments performed in duplicate.

dase-dependent metabolism of cysteinyl leukotrienes in human granulocytes [13,14]. In addition, the phorbol ester provoked inhibition of LTC_4 synthase activity. The latter effect was demonstrated by a PMA-induced suppression of the conversion of LTA_4 to LTC_4 in granulocyte suspensions, which had been pretreated with catalase. Both effects were prevented by specific PKC inhibitors, suggesting that these actions were mediated via PKC activation. Moreover, the suppression of LTC_4 synthase activity could be observed also in experiments where the cells were disrupted by sonication before incubation with LTA_4, in agreement with a phosphoregulation of the enzyme. This finding also excludes the possibility that the PMA-induced attenuation of LTC_4 formation was secondary to interference with cellular leukotriene transport.

The present results confirm recently reported PMA-induced down-regulation of LTC_4 synthase activity in established human pre-leukemic cell lines [21,22] and demonstrate that similar phosphoregulatory mechanisms control LTC_4 synthase activity also in physiologically relevant normal granulocytes. It should be emphasized that granulocyte suspensions contain predominantly neutrophils, which lack LTC_4 synthase, whereas LTC_4-producing eosinophils constitute a minor fraction. Nevertheless, the amounts of LTC_4 produced from LTA_4 in the presence of catalase were almost 50% of LTB_4 levels in intact cells and similar to those of LTB_4 in disrupted granulocytes, demonstrating considerable LTC_4-producing capacity within the granulocyte preparation. The possibility that contaminating platelets contributed substantially to this capacity can be excluded, since the conversion of LTA_4 to LTC_4 in granulocyte suspensions was unaffected by the thromboxane analogue U-46619 (Sjölinder et al., unpublished observation), which induce down-regulation of LTC_4 synthase activity in platelets [23]. Furthermore, the number of remaining platelets in the granulocyte suspensions was always low (see section 2). The finding that neutrophil LTB_4 production was unaffected by treatment with phorbol ester excludes more unspecific effects on leukotriene formation, such as interference with cellular LTA_4 uptake.

Exogenously added LTA_4, rather than agents provoking leukotriene synthesis from endogenous substrate, e.g. calcium ionophore A23187 or fMLP, was used in the present investigation. This was mainly because both A23187 and fMLP has been demonstrated to activate PKC [27,28]. Furthermore, PKC has been reported to increase the release of endogenous arachidonic acid via stimulation of $cPLA_2$ [29]. Therefore, it was of importance to bypass PKC-dependent regulation of arachidonic acid liberation, by supplying the cells with the immediate substrate for LTC_4 synthase.

Interestingly, pronounced differences in time dependence between the two effects of PMA on leukotriene metabolism were observed. Thus, whereas LTC_4 degradation was rapidly induced, the suppression of LTC_4 synthase was preceded by a lag phase and developed slowly. The reason for this discrepancy is presently unknown, but may indicate differences in the signal transduction pathways leading to the various effects. Since direct phosphoregulation of enzyme activity is usually essentially instant, it may be speculated that the LTC_4 synthase is not the primary target for the phosphorylation involved in the control of this enzyme. Thus, phosphorylation of regulatory protein or PKC-dependent induction of other mechanisms leading to down-regulated enzyme activity may be alternative possibilities. On the other hand, LTC_4 synthase has been demonstrated to contain two consensus sequences for PKC phosphorylation, indicating that the activity of the enzyme may be controlled by direct phosphoregulation [6,18,20]. The finding that PMA treatment provoked down-regulation of LTC_4 synthase activity also in the presence of catalase contradicts the possibility that the enzyme inhibition is secondary to the induction of oxidative metabolism. The precise mechanisms behind the phosphoregulatory control of LTC_4 synthase needs to be further clarified.

In summary, the present findings indicate that phosphoregulatory, PKC-dependent mechanisms are involved in the control of LTC_4 synthesis in normal human granulocytes, as earlier reported in established myeloid cell lines [21,22] and human platelets [23]. However, although direct activation of PKC induced attenuation of platelet LTC_4 synthase activity, receptor-mediated regulation of the enzyme appeared to be primarily dependent on the function of another protein kinase, possibly a tyrosine specific kinase [23]. Therefore, further investigation of possible receptor-mediated LTC_4 synthase regulation in myeloid cells is warrented.

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