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Protein Kinase C-Dependent Regulation of Sulfidopeptide Leukotriene Biosynthesis and Leukotriene C_4 Synthase in Neutrophilic HL-60 Cells

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SUMMARY

In response to calcium ionophore (A23187) stimulation, human granulocyte/macrophage colony-stimulating factor-primed, dimethylsulfoxide-differentiated HL-60 cells (which resemble mature granulocytes) synthesized leukotrienes (LTs) LTA_4, LTB_4, LTC_4, and LTD_4. The synthesis of the sulfidopeptide LTs, LTC_4 and LTD_4, was specifically inhibited in cells incubated in the presence of both A23187 and phorbol-12-myristate-13-acetate (PMA), an activator of protein kinase C (PKC). In contrast, neither the synthesis of LTB_4, a product of the nonpeptide branch of the LT pathway, nor the formation of LTA_4, the precursor for both branches of the LT biosynthetic pathway, was significantly affected by the presence of PMA during A23187 stimulation. The inhibition by PMA of LTC_4 production in A23187-stimulated HL-60 cells was dose dependent, with an IC_{50} value of approximately 3.5 nM. The PKC inhibitor staurosporine completely reversed the inhibition by PMA of LTC_4 production in A23187-stimulated cells, in a dose-dependent fashion, with an IC_{50} value of approximately 30 nM. Bisindolylmaleimide, another PKC inhibitor, was also able to prevent PMA-mediated inhibition of LTC_4 formation, whereas inhibitors of protein kinase A, tyrosine kinases, or the respiratory-burst oxidase were not. Measurement of LTC_4 synthase enzymatic activity in cells challenged with A23187 and PMA in the presence or absence of staurosporine demonstrated that the activity of the LTC_4 synthase enzyme was inhibited in cells co-stimulated with A23187 and PMA and that inhibition could also be completely prevented by the presence of staurosporine. Because PMA is known to activate PKC, and staurosporine and bisindolylmaleimide are inhibitors of PKC, these results suggest that LTC_4 synthase in HL-60 cells may be phosphoregulated.

LTs are produced by a variety of inflammatory cells and have biological effects including bronchoconstriction, vasoconstriction, leukocyte chemotaxis, and increased vascular permeability (1). Because these potent mediators may play an important role in hypersensitivity and in asthmatic and inflammatory diseases, the regulation of LT biosynthesis is of considerable pathological importance.

LTs are synthesized in inflammatory cells (for review, see Refs. 2–5) after the liberation of arachidonic acid from the *sn*-2 position of membrane phospholipids, probably by an arachidonate-selective phospholipase A_2 (6, 7). Free arachidonic acid is converted to LTA_4 by 5-lipoxygenase, in association with the membrane-bound 5-lipoxygenase-activating protein, and is subsequently metabolized by one of two pathways. It may be either stereoselectively hydrolyzed to form LTB_4 by the enzyme LTA_4 hydrolase or conjugated with glutathione to form the sulfidopeptide LTC_4 in a reaction catalyzed by the enzyme LTC_4 synthase. LTC_4 is converted to LTD_4 and then LTE_4 by successive removal of L-glutamate and then glycine residues. Collectively, LTC_4, LTD_4, and LTE_4 (the cysteinyl LTs) comprise the slow-reacting substance of anaphylaxis (8).

LTC_4 synthase catalyzes the first committed step in the biosynthesis of the cysteinyl LTs. It is a membrane-bound glutathione *S*-transferase activity that is distinct from α, μ, π, θ, and microsomal glutathione *S*-transferases and appears to be specifically dedicated to LTC_4 formation (9). LTC_4 synthase has recently been characterized and purified from human promyelocytic leukemia cell lines (9–11). The amino terminus of purified human LTC_4 synthase contains the PKC consensus sequence -Ser-Ala-Arg- (11), suggesting that phosphoregulation may govern the biosynthetic activity of this enzyme. We therefore investigated this possibility in a neutrophil-like cell line

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ABBREVIATIONS: LT, leukotriene; Me_2SO, dimethylsulfoxide; LTA_4, 5,6-oxido-7,9,11,14-eicosatetraenoic acid; LTB_4, 5(*S*),12(*R*)-dihydroxy-6,8,10,14-eicosatetraenoic acid; LTC_4, 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTD_4, 5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; GMCSF, granulocyte/macrophage colony-stimulating factor; PGB_2, prostaglandin B_2; PMA, phorbol-12-myristate-13-acetate; PKC, protein kinase C; HPLC, high performance liquid chromatography.

capable of complete *de novo* biosynthesis of both the cysteinyl and noncysteinyl LTs.

The human HL-60 cell line is of promyelocytic lineage and can be differentiated in culture, by growth in the presence of Me_2SO, into cells that morphologically resemble mature granulocytes (12–14). Me_2SO-differentiated cells contain the complete complement of enzymes necessary for the formation of LTA_4, LTB_4, LTC_4, and LTD_4 (9, 15–18). Furthermore, the overall LT biosynthetic capacity of differentiated HL-60 cells has been shown to be enhanced by priming with the cytokine GMCSF, which appears to elevate both 5-lipoxygenase and 5-lipoxygenase-activating protein levels (19, 20) but does not affect LTC_4 synthase.[1] Me_2SO-differentiated HL-60 cells synthesize LTs in response to a variety of stimuli, including the calcium ionophore A23187, and via receptor-mediated mechanisms such as those stimulated by *N*-formyl-methionyl-leucyl-phenylalanine (15, 16, 21). Specifically, in response to stimulation by these activators, Me_2SO-differentiated HL-60 cells synthesize LTA_4, which is converted enzymatically to LTB_4, LTC_4, and LTD_4 and nonenzymatically to 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4. Because Me_2SO-differentiated HL-60 cells are capable of forming all of the major LTs (whereas other myelocytic leukemia cell lines are not), they are a useful model for studying the regulation of specific enzymes in the LT biosynthetic pathway.

With the exception of recent studies describing the possible role of phosphorylation in the agonist-induced activation of cytosolic phospholipase A_2 (22, 23), little is known about potential regulatory mechanisms that might affect LT production or profiles in inflammatory cells. Because LTC_4 synthase contains a putative PKC phosphorylation site, we have examined the effect of stimulating HL-60 cells with A23187 in the presence of the tumor promoter PMA, a known activator of PKC (24). When differentiated HL-60 cells were challenged with A23187 in the presence of PMA, the biosynthesis of LTC_4 and LTD_4, but not LTA_4 or LTB_4, was specifically inhibited. Inhibitors of PKC prevented the PMA-mediated inhibition of sulfidopeptide LT formation, whereas inhibitors of other kinases did not. The enzymatic activity of LTC_4 synthase in PMA-treated cells was decreased, and this loss of activity was completely prevented by the presence of staurosporine. These results suggest that cysteinyl LT formation may be controlled by phosphoregulation of LTC_4 synthase in a PKC-dependent manner.

Experimental Procedures

Cell growth and differentiation. HL-60 cells (CCL 240; obtained from the American Type Culture Collection, Rockville, MD) were grown in continuous suspension culture in Iscove's modified Dulbecco's medium, supplemented with 20% (v/v) heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (Flow Laboratories, McLean, VA), at 37° in a humidified atmosphere with 7% CO_2. Cells were induced to differentiate towards granulocytes by exposure to 1.2% (v/v) Me_2SO for 4–6 days, as described previously (12–14). When cells were differentiated in spinner flasks, an additional 0.6% (v/v) Me_2SO was added to cells after 2–3 days in culture.

LT formation in intact cells. Before stimulation, cells were pelleted by centrifugation at 500 × *g* for 10 min, dispersed in Dulbecco's phosphate-buffered saline, pelleted again, and then resuspended at 2×10^7 cells/ml in Dulbecco's phosphate-buffered saline containing 1 mM EDTA and 1 mg/ml D-glucose. Cells were primed by incubation with 200 pM recombinant human GMCSF (Genzyme, Cambridge, MA) for 30 min at 37°, to increase LT biosynthetic capacity (25). After priming, calcium (as $CaCl_2$) was added to the cells to a final concentration of 2.1 mM and the cells were incubated for 8 min at 37° with gentle mixing. Stock solutions of 1 mM A23187, 1.6 mM PMA, and 2 mM staurosporine (all in Me_2SO) were prepared and added to cells, as indicated, for a final concentration of 1 μM A23187, 50 or 100 nM PMA, and 1 μM staurosporine, unless indicated otherwise. (No PMA-dependent cytotoxicity was observed with up to 1.6 μM PMA, as determined by trypan blue exclusion.) Activators and staurosporine were mixed together and then added to cells when appropriate. The activated cells (4×10^7 cells, in a final volume of 2 ml) were incubated for 10 min at 37°, and then the reaction was terminated by addition of 2 ml of ice-cold methanol containing 0.2 nmol/ml PGB_2. Cell viability was determined by trypan blue exclusion before termination of the reaction. LTs were extracted from the samples and analyzed by reverse phase HPLC on a Novapak C_{18} column (3.9 × 150 mm; Waters Associates, Milford, MA) as described previously (26), except that the mobile phase was acetonitrile/methanol/water/acetic acid (28:18:54:1, v/v), pH 5.6, at a flow rate of 1.2 ml/min. The identified products, monitored by on-line measurement of the absorbance at 270 nm, were LTC_4, LTD_4, 6-*trans*-LTB_4, 6-*trans*-12-*epi*-LTB_4, and LTB_4, with approximate retention times of 7, 13, 16, 18, and 22 min, respectively. Under these conditions, the internal standard PGB_2 eluted at approximately 11 min. LT production was quantitated by comparison with the PGB_2 internal standard, correcting for the relative extinction coefficient of the products. LTA_4 was determined by adding the values for the peaks attributable to 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4, the nonenzymatic hydrolytic products of LTA_4.

Measurement of LTC_4 synthase enzymatic activity. LTC_4 synthase enzymatic activity was measured in 150-μl incubation mixtures containing 0.1 M potassium phosphate buffer, pH 7.4, 0.2 mg/ml L-α-phosphatidylcholine, 50 mM serine-borate complex or 2.5 mM acivicin (to block metabolism of LTC_4 to LTD_4), 10 mM reduced glutathione, and 40 μM LTA_4 (free acid), essentially as described previously (10). The mixtures, containing up to 1×10^7 cells, were incubated for 15 min at 25° and then reactions were terminated by the addition of 150 μl of cold acetonitrile/methanol/acetic acid (50:50:1, v/v). Precipitated proteins were removed by centrifugation for 15 min at 16,000 × *g* and LTs in the resulting supernatant were resolved by isocratic reverse phase HPLC on a Novapak C_{18} column (3.9 × 150 mm; Waters Associates) with a mobile phase composed of acetonitrile/methanol/water/acetic acid (28:18:54:1, v/v), pH 5.6, at a flow rate of 1.0 ml/min. LTC_4 (retention time, 9.5 min) was monitored by on-line measurement of the effluent at 280 nm. In whole-cell experiments (e.g., Table 3), pretreatment with kinase activators or inhibitors was performed with intact cells, followed by measurement of LTC_4 synthase activity immediately after disruption of the cells by sonication (Kontes micro-tip probe sonifier, 20 kHz, 25 W, for 60 sec on ice).

Results

PMA specifically inhibits sulfidopeptide LT biosynthesis in A23187-stimulated HL-60 cells, without affecting LTA_4 or LTB_4 formation. Me_2SO-differentiated, GMCSF-primed HL-60 cells synthesized LTA_4 (measured as 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4), LTB_4, LTC_4, and LTD_4 in response to A23187 stimulation. To examine the effect of PMA on LT biosynthesis, cells were stimulated with A23187 in the absence or presence of the phorbol ester PMA. Analysis by reverse phase HPLC of the products formed (Fig. 1) revealed that the production of LTC_4 and LTD_4 was specifically reduced in A23187- and PMA-treated HL-60 cells (Table 1). In contrast, LTB_4 and the nonenzymatic hydrolysis products of LTA_4 (6-*trans*-LTB_4 and 6-*trans*-12-epi-LTB_4) did not appear to be

[1] K. Scoggan and D. W. Nicholson, unpublished observations.

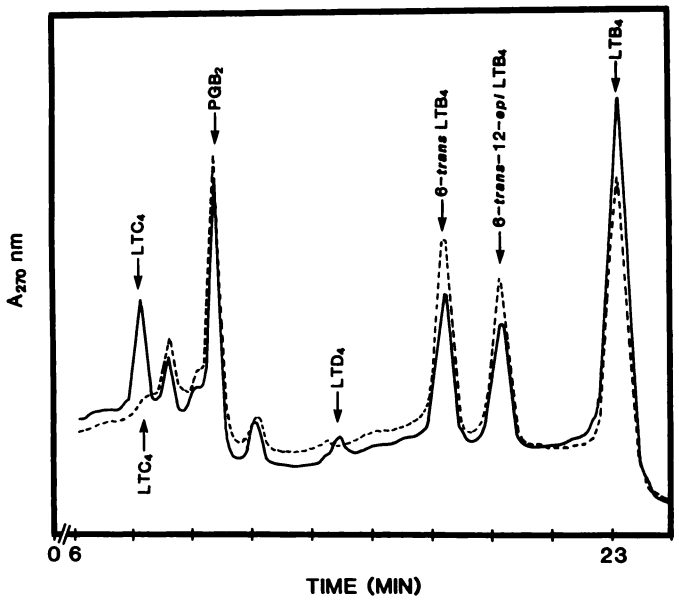

Fig. 1. LTs formed by A23187-stimulated, Me_2SO-differentiated HL-60 cells incubated in the absence or presence of PMA. Me_2SO-differentiated HL-60 cells were primed for 30 min at 37° in the presence of 200 pM GMCSF and then stimulated with 1 μM A23187, in the presence or absence of 100 nM PMA, for 10 min at 37°. Samples were mixed with an equal volume of ice-cold methanol containing PGB_2 and extracted, and LTs were analyzed by reverse phase HPLC. The elution positions of LT standards are indicated *above the peaks*. Chromatographs from samples incubated in the absence (——) or presence (– – –) of PMA are superimposed. Chromatograms from an experiment that is representative of about 20 others are shown.

TABLE 1

Preferential inhibition by PMA of sulfidopeptide LT biosynthesis in Me_2SO-differentiated HL-60 cells stimulated with A23187

Me_2SO-differentiated HL-60 cells were primed for 30 min at 37° in the presence of 200 pM GMCSF and then stimulated with 1 μM A23187, in the absence or presence of 1.6 μM PMA, for 10 min at 37°. Samples were mixed with an equal volume of ice-cold methanol containing PGB_2 and extracted, and LTs were analyzed by reverse phase HPLC. A representative experiment is shown, where the values represent the mean ± range of duplicate determinations. Values for each LT produced in the presence of PMA are also expressed as a percentage of the same LT produced in the absence of PMA (percentage of control).

LT	LT formed		
	−PMA	+PMA	+PMA
	nmol/10^6 cells		*% of control*
LTC_4	0.45 ± 0.04	0.06 ± 0.02	13.3
LTD_4	0.03 ± 0.01	0 ± 0	0
6-*trans*-LTB_4	1.55 ± 0.23	1.36 ± 0.15	87.7
6-*trans*-12-*epi*-LTB_4	0.99 ± 0.03	1.00 ± 0.03	101
LTB_4	3.29 ± 0.33	2.84 ± 0.19	86.3
Total LT	6.31 ± 0.78	5.26 ± 0.47	83.4

significantly affected by this treatment. There was a small reduction in the overall production of LTs by these cells, corresponding to the loss of sulfidopeptide LT levels. Determination of the amount of each LT produced in response to A23187 in the absence or presence of PMA demonstrated that there was an approximately 90% reduction in LTC_4 formation and abolition of LTD_4 formation but only a 0–15% reduction in LTB_4 production in A23187- and PMA-treated HL-60 cells, in comparison with cells stimulated by A23187 alone.

The inhibition of LTC_4 production by PMA in A23187-stimulated HL-60 cells was dose dependent, with an IC_{50} value of approximately 3.5 nM (Fig. 2A). Examination of the effect

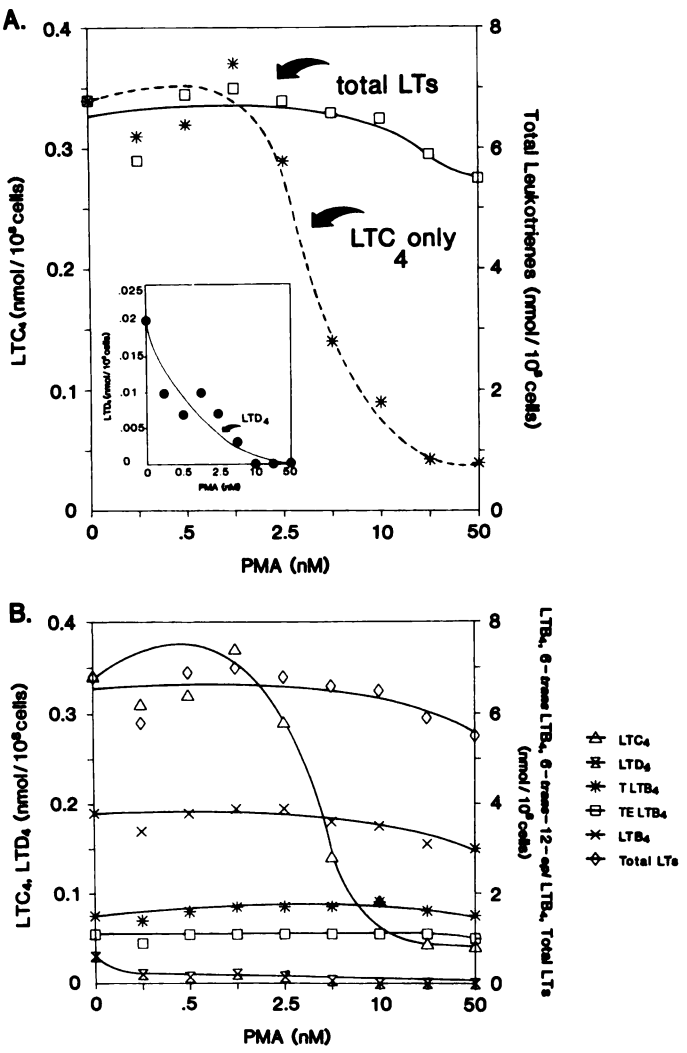

Fig. 2. Inhibition by PMA of LT production in Me_2SO-differentiated HL-60 cells. Me_2SO-differentiated HL-60 cells were primed for 30 min at 37° in the presence of 200 pM GMCSF and then stimulated with 1 μM A23187, in the absence or presence of varying concentrations of PMA, for 10 min at 37°. Samples were mixed with an equal volume of ice-cold methanol containing PGB_2 and extracted, and LTs were analyzed by reverse phase HPLC. A, PMA dose-response curve for total LTs, LTC_4, and LTD_4 (*inset*). B, PMA dose-response curve for LTC_4, LTD_4, 6-*trans*-LTB_4 (*T* LTB_4), 6-*trans*-12-*epi*-LTB_4 (*TE* LTB_4), LTB_4, and total LTs. Values are the average of duplicate determinations of a representative experiment.

of PMA on each LT separately confirmed that, with the exception of LTD_4, only LTC_4 was inhibited by these concentrations of PMA (Fig. 2B). LTD_4 production would be expected to be inhibited by PMA in these cells, because LTC_4 is the precursor from which LTD_4 is formed. In fact, although the quantity of LTD_4 synthesized by HL-60 cells was extremely low, the IC_{50} value of PMA for inhibition of LTD_4 formation was determined to be approximately 2.5 nM (Fig. 2A, *inset*), nearly identical to that for inhibition of the formation of the LTD_4 precursor LTC_4. Accumulation of LTA_4 or LTB_4 in PMA-treated cells would be expected to occur in the absence of LTC_4 formation but was not statistically measurable above the high levels of LTA_4 and LTB_4 produced by these cells. Because PMA did not substantially affect the biosynthesis of the LTB_4 branch of the LT biosynthetic pathway or the formation of the common

precursor LTA_4, it appears to act at a site affecting sulfidopeptide LT formation only.

GMCSF priming has previously been shown to elevate LT formation in response to various challenges (19, 20, 25, 27–30). Whether GMCSF priming was required in order for Me_2SO-differentiated HL-60 cells to respond to PMA was therefore tested by performing A23187 challenges, in the presence or absence of PMA, in cells that either had been primed or not by preincubation with 200 pM GMCSF. In both GMCSF-primed and nonprimed cells, PMA specifically abolished sulfidopeptide LT formation in response to A23187 challenge (data not shown). The effects of GMCSF, which elevated overall LT formation, did not include any modulation of the PMA-dependent reduction in sulfidopeptide LT formation.

Inhibition by PMA of LTC_4 formation is prevented by PKC inhibitors but not by other kinase inhibitors. To investigate whether phorbol ester activation of PKC may occur in cells stimulated by A23187 in the presence of PMA, we utilized a PKC inhibitor, staurosporine (31), to try to prevent the inhibition of LTC_4 production by PMA. An HPLC profile of LTs produced in differentiated HL-60 cells stimulated by A23187 in the absence or presence of PMA (Fig. 3A) shows specific abolition of LTC_4 and LTD_4 formation. When cells were incubated in the presence of staurosporine in addition to PMA and A23187, the inhibition of LTC_4 and LTD_4 production was almost completely prevented (Fig. 3B; these results were quantified and are shown in Fig. 3, C and D, for LTC_4 and LTD_4, respectively). Staurosporine was able to reverse the PMA-mediated inhibition of LTC_4 and LTD_4 production in a dose-dependent fashion, with an IC_{50} value of approximately 25 nM for each LT (Fig. 4A). As seen in Fig. 4B, only the formation of LTC_4 and LTD_4 was affected at these concentrations of staurosporine.

LTC_4 formation in response to ionophore challenge was inhibited by treatment with PMA (an activator of PKC) but not by dibutyryl-cAMP (an activator of protein kinase A) (data not shown). The inhibitory effects of PMA on LTC_4 production were prevented by the presence of the PKC inhibitors stauro-

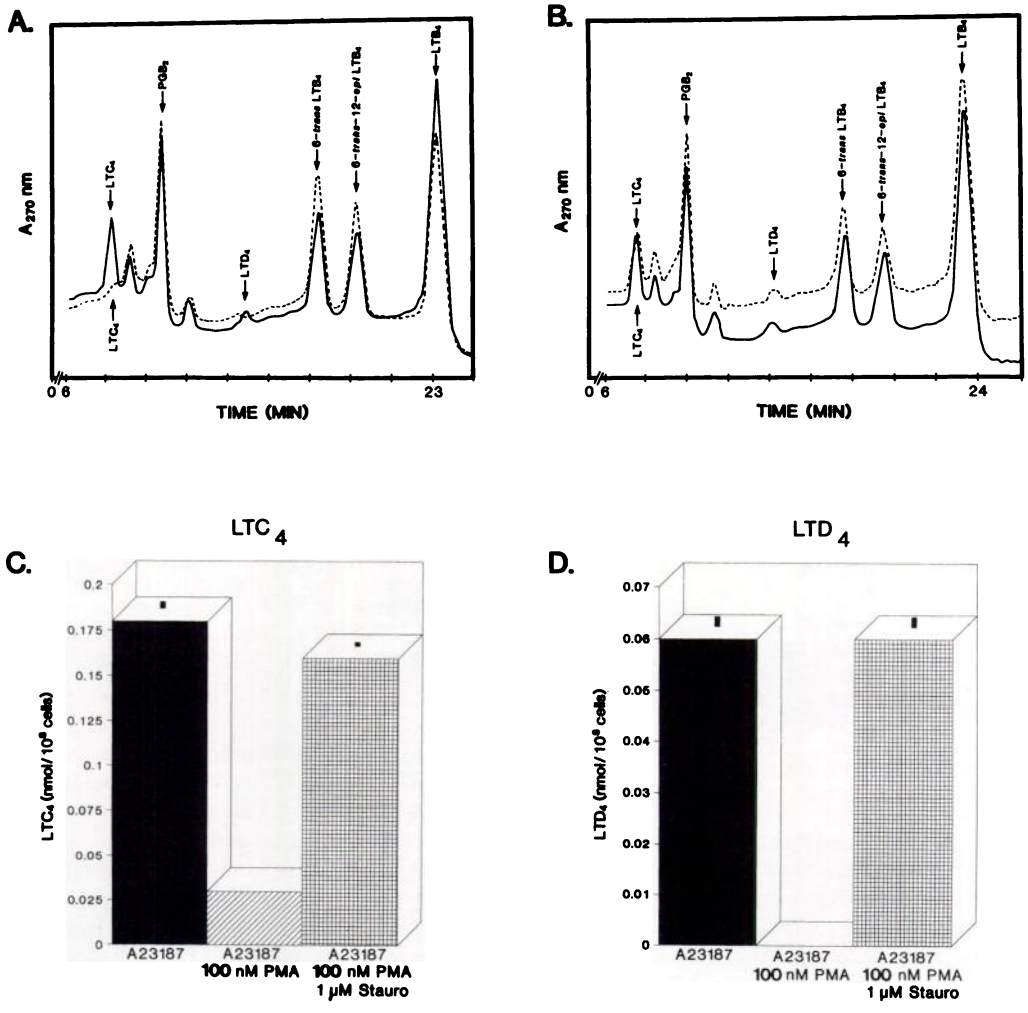

Fig. 3. Reversal by staurosporine of PMA-induced inhibition of LTC_4 and LTD_4 production. Me_2SO-differentiated HL-60 cells were primed for 30 min at 37° in the presence of 200 pM GMCSF and then incubated with 1 μM A23187, in the absence or presence of 100 nM PMA and 1 μM staurosporine, as indicated, for an additional 10 min at 37°. Samples were mixed with an equal volume of ice-cold methanol containing PGB_2 and extracted, and LTs were analyzed by reverse phase HPLC. A, HPLC profile of LTs from A23187-stimulated cells incubated in the absence (——) or presence (– – –) of PMA. B, HPLC profile of LTs from A23187-stimulated cells incubated in the absence (——) or presence (– – –) of PMA and staurosporine. C, Quantitation of the inhibition by PMA of LTC_4 biosynthesis and the reversal by staurosporine (*Stauro*) of the PMA-induced inhibition of LTC_4 formation. D, Quantitation of the inhibition by PMA of LTD_4 biosynthesis and the reversal by staurosporine of the PMA-induced inhibition of LTD_4 formation. For C and D, values are the mean ± range of duplicate determinations of a representative experiment.

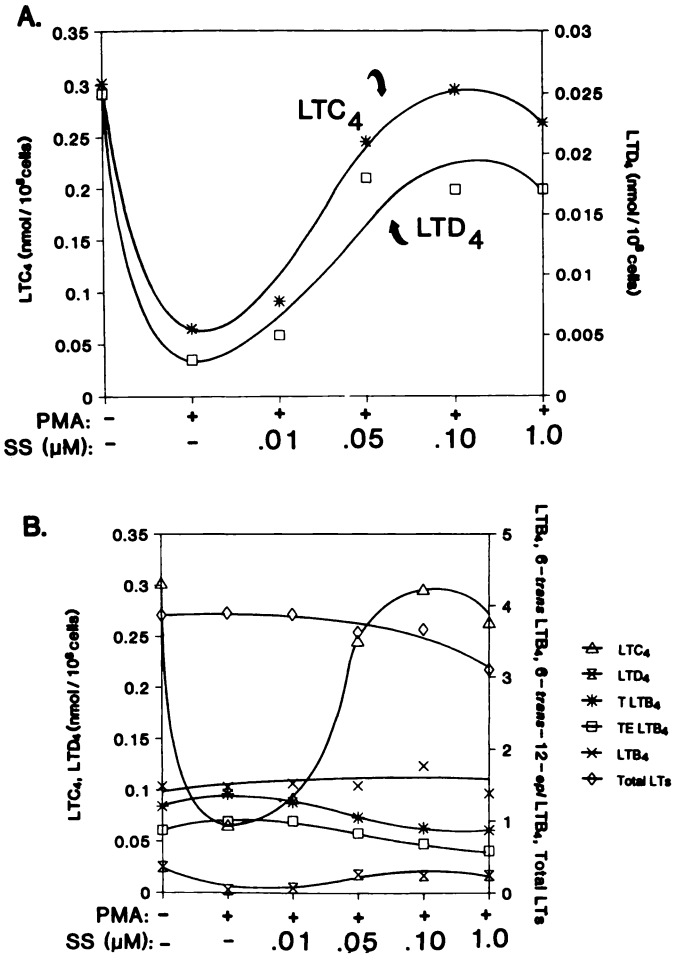

Fig. 4. Reversal by varying concentrations of staurosporine (*SS*) of the PMA-induced inhibition of LTC_4 and LTD_4 production in Me_2SO-differentiated HL-60 cells. Me_2SO-differentiated HL-60 cells were primed for 30 min at 37° in the presence of 200 pM GMCSF and then stimulated with 1 μM A23187, in the absence or presence of 50 nM PMA and varying concentrations of staurosporine, for 10 min at 37°. Samples were mixed with an equal volume of ice-cold methanol containing PGB_2 and extracted, and LTs were analyzed by reverse phase HPLC. A, Staurosporine dose-response curve for LTC_4 and LTD_4 only. B, Staurosporine dose-response curves for LTC_4, LTD_4, 6-*trans*-LTB_4 (*T LTB*$_4$), 6-*trans*-12-*epi*-LTB_4 (*TE LTB*$_4$), LTB_4, and total LTs. Values are the average of duplicate determinations of a representative experiment.

TABLE 2

Effect of various inhibitors on total sulfidopeptide LT production in Me_2SO-differentiated HL-60 cells treated with PMA

Me_2SO-differentiated HL-60 cells were primed for 30 min at 37° with 200 pM GMCSF and then incubated with the indicated inhibitor or vehicle (Me_2SO) for 90 min at 37°. After a 10-min treatment with 50 nM PMA at 37°, cells were challenged with 1 μM A23187 for 10 min at 37°. Reactions were terminated by the addition of an equal volume of ice-cold methanol containing PGB_2. Products were extracted and LTs were analyzed by reverse phase HPLC. A representative experiment is shown, where the values represent the mean ± range of duplicate determinations. Data are also expressed as a percentage of the control to which no PMA was added (percentage of control).

PMA	Inhibitor	Inhibitor type	Total sulfidopeptide LTs	
			pmol/min/10⁶ cells	*% of control*
–			3.64 ± 0.17	100
+			0.55 ± 0.06	15.1
+	Staurosporine (1 μM)	PKC	4.34 ± 0.12	119
+	Bisindolylmaleimide (1 μM)	PKC	3.47 ± 0.06	95.3
+	Genistein (100 μM)	TK[a]	0.63 ± 0.05	17.4
+	Herbimycin A (40 μM)	TK	0.75 ± 0.13	20.5
+	KT5720 (1 μM)	PKA	0.11 ± 0.01	3.1
+	Apocynin (50 μM)	Oxidative burst	0.52 ± 0.12	14.2

[a] TK, tyrosine kinase; PKA, protein kinase A.

TABLE 3

Effect of PMA and staurosporine on LTC_4 synthase enzymatic activity in Me_2SO-differentiated HL-60 cells

Me_2SO-differentiated HL-60 cells were primed for 30 min at 37° in the presence of 200 pM GMCSF and then stimulated with 1 μM A23187, in the absence or presence of 50 nM PMA and in the absence or presence of 1 μM staurosporine, for 10 min at 37°. At the end of the incubation, samples were assayed for LTC_4 synthase activity as described in Experimental Procedures. The data are from a representative experiment performed in triplicate, where the values represent the mean ± standard error.

A23187	PMA	Staurosporine	LTC_4 synthase activity	
			pmol/min/10⁶ cells	*% of control*
+	–	–	4.84 ± 0.48	100
+	+	–	3.25 ± 0.10[a]	67.2
+	+	+	4.96 ± 0.18	102

[a] $p < 0.05$ versus control (cells treated with A23187 alone).

sporine or bisindolylmaleimide but not by the tyrosine kinase inhibitors genestein or herbimycin A or the protein kinase A inhibitor KT5720 (Table 2). Similarly, an inhibitor of the respiratory burst oxidase (apocynin, which was tested to exclude the possibility that superoxide anion that would be generated by stimulation of the respiratory burst oxidase with PMA might account for inactivation of LTC_4 synthase) did not reverse or prevent PMA-dependent inhibition of LTC_4 formation. None of these inhibitors affected the production of LTB_4 or its all-*trans* isomers (data not shown). Together, these data demonstrate that the modulation of LTC_4 formation involves a PKC but does not appear to involve other kinases.

Reduction in the enzymatic activity of LTC_4 synthase in cells occurs after PMA treatment and is preventable by staurosporine. Measurement of LTC_4 synthase enzymatic activity in GMCSF-primed, Me_2SO-differentiated HL-60 cells challenged with A23187 and PMA in the absence or presence of staurosporine demonstrated that LTC_4 synthase was inhibited in cells incubated in the presence of PMA (Table 3). Furthermore, staurosporine was able to prevent the PMA-induced inhibition of LTC_4 synthase activity. Although LTC_4 synthase enzymatic activity measured *in vitro* at saturating substrate concentrations was not entirely abolished by PMA treatment, the >30% inhibition of activity by PMA was completely prevented by the presence of staurosporine, exactly as was observed in intact cells.

To exclude the possibility that PMA and staurosporine had direct inhibitory or stimulatory effects on the LTC_4 synthase enzyme, thereby accounting for the modulation of LTC_4 biosynthetic activity seen in whole cells, their effects were tested on a partially purified preparation of the LTC_4 synthase enzyme that was devoid of PKC (active fraction from anion exchange chromatography) (11). Neither PMA nor staurosporine at concentrations of 0.05–10 μM had any effect on the enzymatic activity of purified LTC_4 synthase (Fig. 5), indicating that the abolition of LTC_4 formation in intact cells in the presence of PMA was not due to a direct inhibitory effect on the LTC_4 synthase enzyme itself.

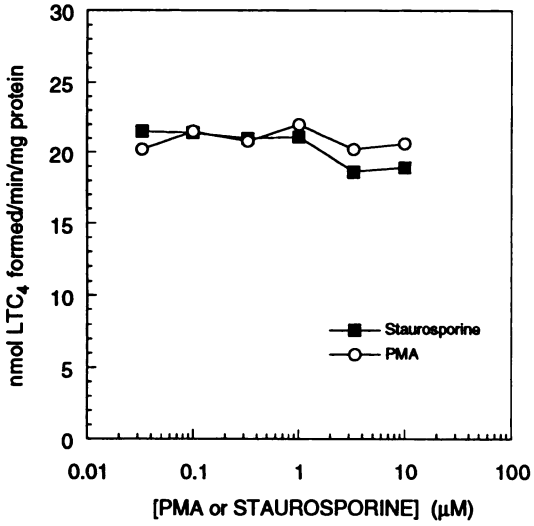

Fig. 5. Effect of PMA and staurosporine on the enzymatic activity of partially purified LTC_4 synthase. LTC_4 synthase activity was measured using 4.5 μg of partially purified LTC_4 synthase (Hi-Load Q fraction, at a specific activity of 21 nmol/min/mg), which was incubated at 25° for 15 min in 0.1 M potassium phosphate buffer containing 40 μM LTA_4, 10 mM glutathione, 0.2 mg/ml phosphatidylcholine, 20 mM $MgCl_2$, 50 mM serine-borate, and varying concentrations of PMA or staurosporine as indicated (0.5 μl of stock solution prepared in Me_2SO), in a final volume of 150 μl. The reaction was terminated by the addition of 150 μl of cold acetonitrile/methanol/acetic acid (50:50:1, v/v). Products were analyzed by reverse phase HPLC, as described in Experimental Procedures. Values are the average of duplicate determinations of a representative experiment.

We have combined pure human LTC_4 synthase with pure PKC in the presence of [^{32}P]ATP but, despite several permutations, have so far been unable to demonstrate direct phosphorylation of the LTC_4 synthase polypeptide or PKC-dependent inhibition of biosynthetic activity. It is possible, however, that the presence of high detergent concentrations (>1%) required for LTC_4 synthase activity, or the absence of a native membrane environment, may interfere with phosphorylation *in vitro*. The system in intact cells may be more complicated, involving subcellular localization or intermediary proteins that link PKC activity to the modulation of LTC_4 synthase. Nevertheless, the modulation of LTC_4 formation in intact cells by PKC activators and inhibitors supports a role for PKC in the phosphoregulation of LTC_4 synthase.

Discussion

Phorbol esters, a class of tumor promoter, are known to stimulate PKC (24). In this study, we examined the effect of co-stimulating Me_2SO-differentiated HL-60 cells with calcium ionophore (A23187) in the presence of PMA. Our results demonstrate that, in cells stimulated by A23187 and PMA, the biosynthesis of LTC_4 and LTD_4 (sulfidopeptide LTs) was specifically inhibited. Because neither the formation of LTA_4 (the precursor of LTC_4) nor the formation of LTB_4 (the product of the alternate branch of the LT biosynthetic pathway) was affected by PMA treatment, the site of PMA action was assignable to the first committed step in the cysteinyl branch of the LT pathway, namely, LTC_4 synthase. Because LTC_4 synthase catalyzes the first committed step in the biosynthesis of cysteinyl LTs (LTC_4, LTD_4, and LTE_4), modulation of its activity would be expected to affect the production of the subsequently formed peptide LTs, such as LTD_4, as was in fact observed in these studies.

Because PMA is known to activate PKC, we used a PKC inhibitor, staurosporine (31), to examine the specificity of the PMA-induced inhibition of sulfidopeptide LT formation. Staurosporine was able to reverse the PMA-induced inhibition of LTC_4 and LTD_4 biosynthesis in A23187-stimulated HL-60 cells. To examine the protein kinase(s) involved, we examined LTC_4 formation in A23187-stimulated cells treated with PMA in the absence or presence of various protein kinase inhibitors. Our results demonstrate that the formation of LTC_4 in A23187-stimulated HL-60 cells was inhibited by PMA and this inhibition was prevented by the PKC inhibitors staurosporine and bisindolylmaleimide but not by the protein kinase A inhibitor KT5720 or by the tyrosine kinase inhibitors genestein or herbimycin A. These results suggest that LTC_4 synthase may be regulated in a negative fashion by phosphorylation in these cells by a mechanism involving PKC but probably not in concert with other types of kinases.

A variety of cellular stimuli, including *N*-formyl-methionyl-leucyl-phenylalanine, zymosan, A23187, platelet-activating factor, and PMA, have been shown to induce the cellular synthesis of LTs. There are reports of synergy between some of these stimuli, resulting in enhanced LT synthesis. It was demonstrated, for example, that PMA and A23187 synergistically induced LTC_4 synthesis in murine macrophages (32, 33), whereas others showed that A23187 and PMA synergistically induced LTB_4 synthesis in human polymorphonuclear granulocytes (34). Those investigations differed from our studies in two ways. Firstly, those investigators used suboptimal concentrations of PMA and A23187 and, secondly, they found that neither stimuli alone induced LT synthesis. In contrast, we observed LT synthesis when cells were optimally stimulated by A23187 alone. Furthermore, our studies in GMCSF-primed, Me_2SO-differentiated HL-60 cells failed to demonstrate a synergistic increase in LT biosynthesis by A23187 and PMA. In our system, the co-stimulation of HL-60 cells with A23187 and PMA inhibited LTC_4 synthase activity without significantly influencing LTA_4 or LTB_4 biosynthesis. Furthermore, the inhibitory effects of PMA were entirely reversible with two PKC inhibitors (which was not demonstrated in the aforementioned studies), supporting a role for PKC in LTC_4 synthase regulation.

Whether the observed LTC_4 synthase regulation in these cells will be observed in other cell types, or *in vivo*, is under investigation, but it is likely to be the case. It was recently reported, for example, that interleukin-3-dependent LTC_4 synthesis in human basophils was blocked by PMA treatment (35). Phosphoregulation of LTC_4 synthase may therefore be a common mechanism by which several inflammatory cell types control LT profiles.

The exact mechanism by which PMA inhibits LTC_4 biosynthesis in A23187-stimulated neutrophilic HL-60 cells remains unknown. Recent results from our laboratory on purified human LTC_4 synthase have identified the PKC consensus sequence -Ser-Ala-Arg- within the amino-terminal amino acid sequence as a potential site for PKC phosphorylation (11). The presence of this consensus sequence within the LTC_4 synthase polypeptide increases the likelihood that the enzyme is directly phosphorylated by PKC or a PKC-like activity; however, additional experiments are underway to clarify whether LTC_4

synthase itself or an LTC_4 synthase regulatory protein is phosphorylated. Other PKC-dependent regulatory mechanisms might also be possible. For example, PKC activates the cytosolic phospholipase A_2 by phosphorylation via mitogen-activated protein kinase (22, 23) but it activates prostanoid formation by transcriptional activation of the cyclooxygenase-2 gene (36). Analagous mechanisms might play a role in the PKC-dependent modulation of LTC_4 synthase activity.

In summary, LTC_4 synthase, which catalyzes the first committed step in the formation of the biologically active sulfidopeptide LTs, appears to be phosphoregulated by a PKC-type activity. Down-regulation of LTC_4 synthase by phosphorylation would be a rapid and effective way for the cell to tightly control the formation of the potent bronchoconstrictive peptide LTs involved in airway hyperreactivity and related inflammatory conditions.

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