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HUMAN LEUKOTRIENE C_4 SYNTHASE: A UNIQUE HOMODIMERIC AND PHOSPHOREGULATED GLUTATHIONE S-TRANSFERASE

BY

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Human LTC_4 Synthase: a phosphoregulated homodimeric GST

ABSTRACT

Human Leukotriene C_4 synthase is a membrane-bound glutathione S-transferase that catalyzes the first committed step leading to the biosynthesis of the pro-inflammatory mediators, the cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4). Using chromatographic separation and photoaffinity labelling techniques, LTC_4 synthase was found to be a unique enzyme distinct from all other known glutathione S-transferases. An 18 kDa membrane polypeptide was specifically labelled in the microsomal membranes of myelocytic cell lines containing LTC_4 synthase activity (U937 and THP-1), with a photoaffinity derivative of LTC_4 (azido [I^{125}]-LTC_4) and was identified as a candidate for being LTC_4 synthase or a subunit thereof. LTC_4 synthase was subsequently purified to homogeneity from THP-1 cells and the purified preparation contained only one polypeptide which had a molecular mass of 18 kDa. By gel filtration chromatography, the native molecular mass of LTC_4 synthase was determined to be approximately 39 ± 3 kDa. It was therefore concluded that LTC_4 synthase is enzymatically active as a homodimer. The sequence of the N-terminal 35 amino acids of purified human LTC_4 synthase was determined and was found to be unique, composed primarily of hydrophobic amino acids and containing a protein kinase C (PKC) consensus sequence. Further analysis of the potential phosphoregulation of LTC_4 synthase in neutrophilic and eosinophilic HL-60 cells demonstrated that cysteinyl leukotriene biosynthesis, but not non-cysteinyl leukotriene biosynthesis, was specifically attenuated by phorbol ester-mediated activation of PKC. In eosinophilic HL-60 and THP-1 cells this decrease in cysteinyl leukotriene production was demonstrated to be due to non-competitive inhibition of LTC_4 synthase activity. Concomitant with the PKC-mediated decrease in cysteinyl leukotriene biosynthesis, an increase in prostanoid biosynthesis occurred. Based on these observations, a model for the regulation of eicosanoid biosynthesis in eosinophils is proposed whereby the activation of PKC shifts the profile of lipid mediators synthesized by inflammatory cells from leukotrienes to prostanoids.

RÉSUMÉ

La synthase humaine de leucotriène C_4 est une glutathion S-transférase membranaire qui catalyse la première étape de la biosynthèse des médiateurs pro-inflammatoires, les peptido-leucotriènes (LTC_4, LTD_4 et LTE_4). L'utilisation de la chromatographie et du marquage photosensible a permis d'établir que la LTC_4 synthase est une enzyme unique distincte de toutes les autres glutathion S-transférases connues. Un polypeptide membranaire de 18 kDa a été marqué de façon spécifique dans les membranes microsomiques des lignées cellulaires myélocytiques contenant l'activité LTC_4 synthase (U937 et THP-1), avec un dérivé photosensible du LTC_4 (azido (I^{125})-LTC_4) et a été identifié comme étant un candidat à la LTC_4 synthase ou une sous-unité de celle-ci. La LTC_4 synthase a par la suite été purifiée jusqu'à l'homogénéité à partir de cellules THP-1, et la préparation purifiée ne contenait qu'un polypeptide de 18 kDa. La filtration sur gel a permis d'établir à environ 39 ± 3 kDa le poids moléculaire de la LTC_4 synthase à l'état natif. Cela a mené à la conclusion que la LTC_4 synthase exerce son activité enzymatique sous la forme d'un dimère. La séquence des 35 acides aminés N-terminal de la LTC_4 synthase humaine purifiée a été déterminée et s'est révélée être unique, composée principalement d'acides aminés hydrophobiques et présentant un site de phosphorylation pour la protéine kinase C (PKC). L'analyse de la phosphorégulation potentielle de la LTC_4 synthase dans les cellules neutrophiles et éosinophiles HL-60 a démontré que la biosynthèse des peptido-leucotriènes, mais non la biosynthèse des non-peptido-leucotriènes, est atténuée de façon spécifique par l'activation de la PKC provoquée par les esters de phorbol. Dans le cas des cellules éosinophiles HL-60 et THP-1, cette diminution de la production des peptido-leucotriènes a été attribuée à l'inhibition non-compétitive de l'activité LTC_4 synthase. Cette réduction, liée à l'action de la PKC, de la biosynthèse des peptido-leucotriènes survient en concomitance avec une augmentation de la biosynthèse des prostanoïdes. À la lumière de ces observations, on a pu proposer un modèle de régulation de la biosynthèse des

eicosanoïdes dans les cellules éosinophiles, par lequel l'activation des PKC change le profil des médiateurs lipidiques synthétisés par les cellules inflammatoires en les faisant passer des leucotriènes aux prostanoïdes.

Dedicated to my parents, sisters and husband for their support and love

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ABBREVIATIONS

PBS	phosphate-buffered saline
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
KPi	potassium phosphate
Me_2SO	dimethylsulfoxide
LT	leukotriene
PG	prostaglandin
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
LTC_2	5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9,trans-eicosadinoic acid
LTD_4	5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LTE_4	5(*S*)-hydroxy-6(*R*)-*S*-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
PMA	phorbol 12-myristate 13-acetate
PKC	protein kinase C
PKA	protein kinase A
MPK	MAP kinase
TK	tyrosine kinase
SS	staurosporine
rhGM-CSF	recombinant human granulocyte-macrophage colony stimulating factor
IL	interleukin
TXB_2	thromboxane B_2
PLA_2	phospholipase A_2
COX	cyclooxygenase
5-LO	5-lipoxygenase
12-LO	12-lipoxygenase
15-LO	15-lipoxygenase
FLAP	five-lipoxygenase activating protein
GST	glutathione S-transferase
GSH	reduced glutathione
EDTA	ethylenediamine-tetraacetic acid
DTNB	3-carboxy-4-nitrophenyl disulfide
PAF	platelet activating factor
SRS-A	slow reacting substance of anaphylaxis
FEV	forced expiratory volume

FEV_1	forced expiratory volume (in 1 second)
PMN	polymorphonuclear cells
RBL	rat basophilic leukemia cells
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine

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1. INTRODUCTION

1.1 The eicosanoids

The family of chemical mediators derived from arachidonic acid are termed eicosanoids. The eicosanoids include products of several distinct pathways including, the cyclooxygenase pathway, the 5-lipoxygenase pathway, and the 12 or 15-lipoxygenase pathways which synthesize prostanoids, leukotrienes, and hydroxyeicosatetraenoic acids (HETEs), respectively (1). The prostaglandins and leukotrienes are potent chemical mediators whose chemical structures, biosynthesis, mechanisms of action, and roles in pathophysiological states have been extensively studied.

The leukotrienes and prostaglandins are derived from 20-carbon essential fatty acids that contain three, four or five sites of unsaturation: dihomo-γ-linolenic acid, arachidonic acid and eicospentaenoic acid. These fatty acids are the precursors of the 3, 4 and 5 series of leukotrienes or the 1, 2 and 3 series of prostaglandins, respectively. Since arachidonic acid is the most abundant of the polyunsaturated fatty acids in the membranes of mammalian cells, the 4-series of leukotrienes and the 2-series of prostaglandins are predominantly synthesized.

1.2 Phospholipases and the release of arachidonic acid

Cellular concentrations of arachidonic acid are quite low (2) thus the biosynthesis of eicosanoids depends predominantly on the release of arachidonic acid from membrane phospholipids (3). Arachidonic acid can be liberated by the action of several enzymes including, phospholipase A_2, both phospholipase C and diacylglycerol lipase (2-5), phospholipase A_1 and phospholipase D (6). These enzymes can be activated directly by the actions of hormones, autocoids or other chemical mediators through their interaction with G-protein coupled receptors or via an increase in the cytosolic concentration of free calcium. Once liberated, arachidonic acid, if not metabolized into the

eicosanoids, is rapidly re-esterified by acyltransferase enzymes (2). In most cells, phospholipase A_2 is the primary enzymatic activity which regulates arachidonic acid release for eicosanoid biosynthesis.

Phospholipase A_2 (PLA_2) catalyzes the release of arachidonic acid from the *sn*-2 position of membrane phospholipids (mainly phosphatidylcholine). PLA_2 is the rate limiting enzyme for the biosynthesis of prostanoids and leukotrienes (2,7). Concomitant with the release of arachidonic acid, PLA_2 also releases lyso-phospholipid, the precursor of the inflammatory mediator, platelet-activating factor (PAF)(8).

Several forms of PLA_2 have now been identified which include the secretory PLA_2 (or $sPLA_2$) and the recently described arachidonate-selective cytosolic PLA_2 (or $cPLA_2$). Most studies have focused on the secretory forms of PLA_2 derived from snake venoms and mammalian pancreas (9-13). Large amounts of related enzymes, with low molecular masses of 13-15 kDa, have also been detected in inflammatory exudates, gastric mucosa, and spleen (for review see 14). A 'non-pancreatic' $sPLA_2$ has also been isolated from human platelets and rheumatoid synovial fluid (15). As such, $sPLA_2$, has been postulated to have a role in digestion, cell membrane homeostasis, and inflammation (11,14,16,17). The secretory forms of $^{D}LA_2$ do not demonstrate any selectivity among the *sn*-2 position fatty acids in membrane phospholipids (16) and they require the presence of 7 disulfide bridges within the polypeptide structure for activity and are thus sensitive to disulfide reducing agents (18).

The cytosolic form of PLA_2 has been characterized in various tissues and cell types, including the kidney (19), platelets (20), the human pro-monocytic cell line, U937 (21), and the rat macrophage cell line, RAW 264.7 (22). All of the above mentioned sources contain a calcium-dependent, arachidonate-selective, $cPLA_2$ with an apparent molecular mass in the range of 85-110 kDa. A 85 kDa $cPLA_2$ has been purified and cloned from U937 cells (21-23). There is also increasing evidence that $cPLA_2$ is coupled to receptors via G-proteins (14). $cPLA_2$ has also most recently been identified as a substrate for MAP kinase which phosphorylates $cPLA_2$ at serine-505 and stimulates its catalytic activity *in vitro* (17).

A sequence of approximately 50 amino acids has been identified in $cPLA_2$ as being homologous to the calcium-binding regulatory domain of protein kinase C (PKC) (C_2 region) (22,23). Thus it has been postulated that this domain may render $cPLA_2$ calcium-dependent (22). Recent studies have demonstrated that in response to an increase in intracellular calcium concentration to micromolar levels, $cPLA_2$ translocates from the cytosol to the membrane where its substrate phospholipids are located (22,25). It has been postulated that agonist-stimulated phosphorylation of $cPLA_2$ in combination with increased intracellular Ca^{2+} levels leads to the full activation of $cPLA_2$ (26). Recently, it has been demonstrated that the pro-inflammatory cytokine, interleukin-1(IL-1), stimulates the production of prostaglandin E_2 which is mediated in part by increased expression of $cPLA_2$ (27). Furthermore, pre-treatment with the anti-inflammatory agent dexamethasone suppressed the IL-1 mediated increase in $cPLA_2$ expression (27). Thus, due to its preference for arachidonate-containing phospholipids and stimulation by the inflammatory cytokine, IL-1, $cPLA_2$ has been postulated to have a key role in the inflammatory response (17).

Calcium-independent forms of PLA_2 have also been identified in the cytosol of a number of organs including brain, lung, liver, spleen and heart but have not yet been extensively characterized or cloned (28,29).

1.3 Prostanoids: biological actions and receptors

The prostanoids are the products of the cyclooxygenase (prostaglandin G/H synthase) pathway, the first of the arachidonic acid metabolic pathways to be elucidated (for review see 1,30). Two forms of the cyclooxygenase (COX) enzyme have now been identified; a constitutively expressed enzyme, COX-1, and the recently cloned inducible or glucocorticoid-regulated form, COX-2 (31). The prostanoids include the prostaglandins (PGs) and thromboxanes (TXs). Prostaglandins are a group of polyunsaturated oxygenated C_{20} fatty acids classified into 3 series based on the substitution of the cyclopentane ring, the number of double bonds in the side chains and the lipid precursor from which

they are derived. The 2-series of prostaglandins are derived from arachidonic acid (the most abundant of the prostanoid precursors) and thus constitute the most abundant naturally occurring prostanoids (32). The primary prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2, act on specific receptors on cell membranes. Using pharmacological (33,34) and ligand binding techniques (34) distinct receptors were identified for each of the naturally occurring prostanoids and a comprehensive classification of the receptors was developed (35). The receptors have been classified according to the relative potencies of a series of agonists (natural PGs) and antagonists. According to the relative affinities of the natural PGs for specific receptors, the receptors with highest affinities for PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 were designated as the DP, EP, FP, IP, and TP receptors, respectively. Several subtypes of these receptors have also been identified.

Once released, prostaglandins have been reported to have a diverse spectrum of biological activities in a variety of cell and tissue types (for review see 30). Of the various biological activities associated with prostaglandins, the most well characterized are their contractile and relaxant effects on smooth muscle. Four of the prostanoid receptor subtypes (EP_1, EP_3, TP, and FP) mediate contraction of smooth muscle via increased intracellular calcium and three (EP_2, DP, IP) mediate relaxation via increased intracellular cAMP (30). Depending on the type of receptor present, prostaglandin-mediated contraction or relaxation has been detected in smooth muscle from respiratory, gastro-intestinal, vascular, reproductive and urinary systems as well as in the eye. Recently, several of the prostanoid receptors and their isoforms have been cloned from mouse, bovine and human cDNA libraries and include the EP_1 (36,37), EP_2 (38), EP_3 (which exist as a number of alternatively spliced forms (39,40,41)), FP(42) and TP(43,44) receptors. The four isoforms of the EP_3 receptor (A-D) recently isolated (41) differ only in their C-terminal tails and have been shown to couple to different G-proteins to activate different second messenger systems. The identification of such isoforms for the prostaglandin receptors may help explain the diversity of prostaglandin functions.

1.4 Leukotrienes: identification as potent inflammatory mediators

The leukotrienes are potent lipid mediators that have been implicated in the pathophysiology of several inflammatory diseases. The biological action of a subgroup of leukotrienes, termed the cysteinyl leukotrienes, was previously ascribed to the *slow reacting substance of anaphylaxis* or *SRS-A*. The *slow reacting substance* was first identified by Feldberg and Kellaway in 1938 as a smooth-muscle contracting substance that was produced by guinea-pig lung perfused with a cobra venom (45). Subsequently, an immunologically generated SRS (referred to as SRS-A) was identified and found to be released with other inflammatory mediators (histamine, chemotactic factors) after the interaction of IgE with receptor and antigen (46,47). Although SRS-A was extensively studied, its structural characterization was hindered due to the lack of a pure preparation. In the late 1970's, with the identification of murine mastocytoma cells as high yield producers of SRS-A, the constituents of SRS-A were identified and characterized as being 20-carbon fatty acids conjugated with glutathione, presently known as leukotriene C_4 (LTC_4), LTD_4 and LTE_4 or collectively as the cysteinyl or sulfidopeptide leukotrienes (48,49). The term *leukotriene* was used to describe these potent chemical mediators since they were initially identified as products of leukocytes containing three conjugated double bonds in the lipid domain of the molecule (50). Following the chemical synthesis of the leukotrienes (49), their presence and biological actions were identified in various inflammatory cells and rat, guinea-pig, mouse and human tissues (51). LTD_4 was determined to be the major constituent of highly purified guinea-pig SRS-A (52) and caused vasoconstriction and increased permeability in guinea-pig skin (53). LTC_4 was also shown to be a vasoconstrictor (54). LTB_4, a non-cysteinyl leukotriene, first identified as a compound formed after the treatment of human neutrophils with the calcium ionophore, A23187 (55), was found to be a potent chemoattractant for neutrophils *in vitro* (56) and an inducer of neutrophil-dependent increased microvascular permeability *in vivo* (57).

1.5 Leukotriene Biosynthesis

All leukotrienes are formed through the action of 5-lipoxygenase which catalyzes the conversion of released arachidonate in a two-step mechanism to the unstable epoxide intermediate, LTA_4. LTA_4 is the labile precursor of both LTB_4 and the cysteinyl leukotrienes (figure 1.1). LTA_4 can then either be stereoselectively hydrolysed to LTB_4 by the action of LTA_4 hydrolase or it can be conjugated with reduced glutathione to form LTC_4 by the action of LTC_4 synthase. LTC_4 is then further metabolized to LTD_4 with the loss of a γ-glutamyl residue and then to LTE_4 with the loss of a glycine residue by the actions of γ-glutamyl transpeptidase and cysteinylglycine dipeptidase, respectively .

1.5.1 Biosynthesis of Leukotriene A_4: 5-lipoxygenase and the 5-lipoxygenase activating protein

5-Lipoxygenase (5-LO) acts on arachidonic acid to produce 5-hydroperoxyeicosatetraenoic acid (5-HPETE), a short-lived intermediate that is then either degraded by a peroxidase to the corresponding alcohol 5-hydroxy-eicosatetraenoic acid (5-HETE) or converted to the epoxide intermediate LTA_4 by 5-LO. 5-HETE and 5-HPETE stimulate superoxide generation in guinea-pig and human neutrophils. They increase intracellular calcium levels and PKC-mediated activation of the respiratory burst mechanism in neutrophils (59,60). Many cells also contain 12 and 15-lipoxygenases which catalyze the abstraction of hydrogen at the delta-11 and 15 positions and the insertion of oxygen at the delta-12 or 15 positions of arachidonic acid, respectively. These enzymes also lead to the formation of the HPETEs and HETEs. These mediators may have immunoregulatory functions such as activation of T-cell function and proliferation and enhancement of human natural killer cell activity by 15-HPETE (61-63). 12-HETE has been shown to be weakly chemotactic for neutrophils *in vitro* (64). Specific receptors for the HETEs have not been identified and thus it has been postulated that they may mediate their effects via incorporation into cellular

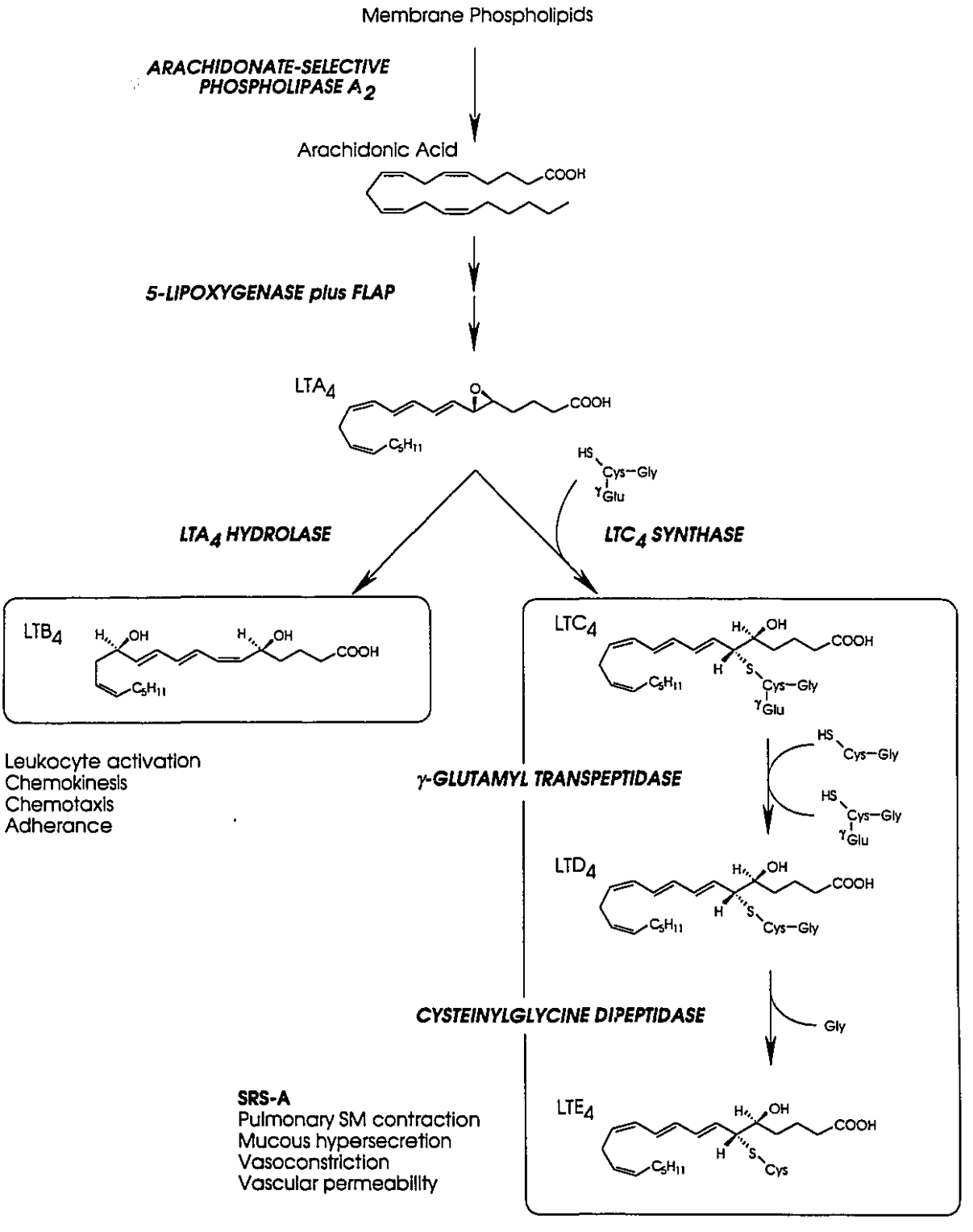

Figure 1.1. Leukotriene Biosynthesis (adapted from Nicholson, D.W., 1993, (ref. 58))

phospholipids and in turn modify membrane and cellular activity (65). All three forms of lipoxygenases have now been cloned (66-75).

The conversion of arachidonic acid to 5-HETE was first detected in rabbit polymorphonuclear (PMN) cells and was the first reported observation supporting the existence of the 5-lipoxygenase enzyme (76). Later, with the first purification of 5-LO from potato tubers (77), it was discovered that not only did 5-LO catalyze the conversion of arachidonic acid to 5-HPETE but it also catalyzed the formation of LTA_4 from 5-HPETE (78-81). 5-LO has since then been purified from murine mastocytoma cells (80), rat basophilic leukemia (RBL) cells (81.82), porcine leukocytes (79), and human sources (78,83). 5-LO is a soluble enzyme with a molecular mass of 78 kDa whose activity is dependent on calcium and ATP (78-81,83). It has also been demonstrated in a cell-free system that in the presence of calcium 5-LO associates with the membrane fraction and that upon the removal of calcium from the system, the enzyme dissociates (84). These studies have shown that 5-LO activity is also dependent on the presence of hydroperoxy fatty acids and either phosphatidylcholine micelles or cell membrane preparations (79-83). Activation of 5-LO by ionophore challenge, for example, has been shown to cause translocation of the enzyme from the cytosol to the membrane of human peripheral blood leukocytes (85-87). This translocation is calcium-dependent and is required for the activation of the 5-LO enzyme and the subsequent burst in leukotriene synthesis (85-87). After leukotriene biosynthesis is triggered by the activation of 5-LO, the enzyme appears to be irreversibly inactivated (85). Recently, a potent inhibitor of leukotriene biosynthesis, MK-886, has been discovered. This compound inhibited leukotriene biosynthesis in whole cells, including neutrophils, eosinophils, mast cells, and monocytes, activated by various stimuli (88). However, MK-886 had no effect on the biosynthetic activity of 5-LO or the availability of arachidonic acid (88). The mechanism of action of this compound was partially elucidated by Rouzer and co-workers who determined that in intact human neutrophils MK-886 dose-dependently inhibited the translocation to the membrane and subsequent activation of 5-LO (89). With the use of a novel photoaffinity probe and affinity

chromatography based on the structure of MK-886, a novel protein, called five lipoxygenase activating protein (FLAP), was identified and purified from leukocyte membranes (90). Using co-transfection experiments of FLAP and 5-LO in osteosarcoma cells, it was demonstrated that FLAP was required for cellular leukotriene biosynthesis (91). A model was proposed in which activation of 5-LO causes the enzyme to translocate to the membrane where it interacts with FLAP and its substrate, arachidonic acid, resulting in leukotriene biosynthesis. Binding of MK-886 to FLAP would prevent this association and thus inhibit leukotriene biosynthesis (88,90).

Human 5-LO was cloned from the cDNA library of the human promyelocytic cell line, HL-60 (75), and from human placental and lung expression libraries (74). The cDNA has been successfully expressed in both a baculovirus-insect cell system (92) and mammalian osteosarcoma cells (93). The recombinant protein was found to have identical characteristics (molecular weight, requirement for stimulatory factors and antigenicity) when compared with human peripheral blood leukocyte 5-LO (92,93).

Since 5-LO catalyses the first two steps of leukotriene biosynthesis, cellular regulation of this enzyme has been a key area of investigation. The induction of 5-LO mRNA and activity with differentiation of HL-60 cells into neutrophil-like cells by growth in the presence of dimethylsulfoxide indicates that this enzyme may be transcriptionally regulated (85). The gene structure for the 5-LO enzyme has recently been characterized (94). The 5-LO gene spans 82 kilobases of DNA and contains 14 exons and 13 introns. The promoter region lacks TATA and CCAAT boxes near the transcription start site, has multiple Sp1 transcription factor binding sites and is GC-rich. Further characterization of the 5' region, by ligation to the chloramphenicol acetyl transferase reporter gene, has revealed the presence of several positive and negative regulatory regions, including binding sequences for the Myb product, AP-2, which mediates the effects of phorbol myristate acetate (PMA) during cellular differentiation (95), and NF-κB.

1.5.2 Metabolism of LTA_4

The product of 5-LO, LTA_4 was first discovered in 1979 in rabbit peritoneal leukocytes by Borgeat and Samuelsson who were able to detect two diastereomers of 5(S)-hydroxy-12(R,S)-methoxy-eicosatetraenoic acid by trapping these products with acidic methanol (96). LTA_4, is an unstable epoxide intermediate which can be stabilized by serum proteins (97) and can follow one of several pathways of metabolism. First, LTA_4 can undergo non-enzymatic hydrolysis in the cell to form the inactive diastereomers: 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4. Secondly, LTA_4 can be enzymatically hydrolysed by LTA_4 hydrolase to form the potent chemoattractant, LTB_4. Thirdly, LTA_4 can be conjugated with reduced glutathione by the action of LTC_4 synthase to form the potent bronchoconstrictive agent, LTC_4.

1.5.3 Biosynthesis of LTB_4: LTA_4 Hydrolase

LTA_4 hydrolase is the rate limiting enzyme for the formation of the potent chemoattractant leukotriene B_4 (LTB_4). LTA_4 hydrolase has been purified from rat (98), guinea-pig (99,100) and human (101-103) sources, and has recently been cloned and expressed in *E. coli* as an active fusion protein (104-106). However, the gene as yet has not been characterized. LTA_4 hydrolase is a cytosolic, monomeric enzyme with a molecular mass of 69 kDa distinct from other known epoxide hydrolases (107) involved in the detoxification of xenobiotics. Unlike 5-LO, LTA_4 hydrolase does not require cofactors for enzymic activity (107), although suicide inactivation of the enzyme by its substrate, LTA_4, has been described (108).

Upon examination of the amino acid sequence of LTA_4 hydrolase, the enzyme was identified as a possible member of the Zn^{2+}-containing metallohydrolase family (109-111). Functional analysis of the enzyme revealed that in addition to its hydrolase activity, LTA_4 hydrolase also possessed intrinsic peptidase activity(112,113) which was stimulated by certain halides and monovalent anions (114) and inhibited by the aminopeptidase inhibitor, bestatin, and the angiotensin converting enzyme inhibitor, captopril (115). Atomic

absorbtion spectrometry detected the presence of one molecule of zinc per molecule of enzyme which is believed to be present primarily for catalytic function or for stabilization of the protein structure (112,116). Unlike 5-LO, LTA_4 hydrolase is a widely distributed enzyme. Its activity has been detected in several rat (117), guinea-pig (118) and human (119) tissues, including those that lack expression of the 5-LO enzyme (120,121). Thus it has been postulated that transcellular metabolism of LTA_4 as reported in several cell types (122-125), may be of significance for the further metabolism of LTA_4 (126).

1.5.4 Biosynthesis of LTC_4: Glutathione S-transferases and LTC_4 synthase

Glutathione S-transferases (GSTs) catalyze the conjugation of reduced glutathione to various hydrophobic and electrophilic substances (127,128) such as heme, bilirubin and polycyclic hydrocarbons (129-132). GSTs are composed of homo or heterodimeric proteins formed from a permutation of at least 7 isozymes (133) and have been shown to play a role in xenobiotic metabolism, drug biotransformation and protection against peroxidative damage. An exception to these detoxification enzymes is LTC_4 synthase which catalyzes a GST-like activity, but one which is dedicated to the biosynthetic formation of LTC_4. GSTs are expressed in a tissue-specific manner and developmental changes in isoenzyme composition in the liver have been described (for review see 129).

Although a microsomal form of GST has been identified and cloned, most forms of this enzyme are cytosolic (129,133). Several of the cytosolic GSTs have been purified by S-hexylglutathione (134) or glutathione-bound sepharose affinity chromatography (135) followed by chromatofocusing to separate individual forms. Based on the nomenclature proposed by Mannervik and co-workers, human GSTs have been divided into 5 groups called the α, μ, π, θ and microsomal classes (136) (table 1.1). Within each class there may be several isoforms composed of different subunit permutations. The class is named by capital letter while the subunits are numbered within the class. To date, 5 and 11 isoforms have been identified in the α and μ classes, respectively, while only one form has been identified in the π, θ and microsomal classes. Subunit molecular

CLASS	FORM	SUBUNIT Mr	NEW NAME
Alpha	B_1B_1 B_1B_2 B_2B_2 9.9 ω	26,000 26,000 28,500 26,000	A1-1 A1-2 A2-2 A3-3
Mu	μ 1-type 2-1 ψ 4 5 M_1M_2 M_2N_1 M_3N_2 5.2 6 N_2N_3	27,000 27,000 26,500 27,000 27,000/26,500 27,000/26,500	Ma1-1a M1a-1b M1b-1b M2-2 M3-3 M1a-2
Pi	π	24,500	P1-1
Theta	θ		T1-1
Microsomal	Microsomal	17,300	MIC

Table 1.1. Molecular Forms of Human Glutathione S-Transferases
(adapted from Tsuchida, S. and Sato, K., 1992 (ref. 129))

masses for most soluble forms of the GSTs range from 25 to 27 kDa and 18 kDa for the microsomal form. Alpha and μ forms generally function as homo- or heterodimers, while microsomal GST most likely functions as a homotrimer. GST-π was purified from human placenta and the most recently described θ form was isolated from rat and human liver cytosol. The rat equivalent of the human GST-π (GST-P), has been identified as a marker for rat hepatic pre-neoplastic and neoplastic lesions (135). Human GST-π has also been demonstrated to be increased in pre-neoplastic and neoplastic lesions in a variety of organs. Thus this GST isoform may serve as a tumor marker in human tissues (129).

GSTs are encoded by five different gene families (4 cytosolic and 1 microsomal) (129-132) which have been extensively characterized to reveal several regulatory elements. Specifically the rat GST subunit 1 (Ya) gene (equivalent to the human B1 subunit) is transcriptionally activated by xenobiotics and with the use of deletion constructs five distinct 5' regulatory elements were identified including: XRE (xenobiotic response element), GRE (glucocorticoid response element), ARE (antioxidant response element), a hepatocyte-specific enhancer and an element required for basal expression of the gene. GSTs from the rat have been the most extensively studied.

LTC_4 synthase is a novel GST that catalyzes the first committed step leading to the generation of the pro-inflammatory mediators, the cysteinyl leukotrienes. As such, a focus of several studies has been the purification and characterization of LTC_4 synthase, a potential therapeutic target for the treatment of various inflammatory diseases.

After the first identification of LTC_4 in mouse mastocytoma cells (138) different groups of investigators began to study the enzyme catalyzing the biosynthesis of this mediator. Since LTC_4 biosynthesis involves the conjugation of reduced GSH to LTA_4, initial studies focused on the LTC_4 synthesizing abilities of the various known glutathione S-transferases. Of the cytosolic GST isozymes, those with the most efficient LTC_4 biosynthetic activity were : GST-Yn_1 Yn_1 (GST 6-6 isolated from rat brain but immunologically related to GST-6-6 from rat testes (139)), GST-4-4 (rat liver (140)), and GST-μ (human liver (141)). However, several

studies in RBL cells (142,143), PMN cells (144), mouse peritoneal macrophages (145), mouse mastocytoma cells (146), U937 cells and THP-1 cells (147,148) subsequently demonstrated that the enzymes responsible for the formation of cysteinyl leukotrienes were particulate. Therefore, to distinguish LTC_4 biosynthetic activity from microsomal GST activity, detailed characterization of the two enzyme activities was done in rat basophilic leukemia cells (143). This initial study demonstrated that LTC_4 biosynthetic activity could be assigned to a new and distinct GST, which, unlike microsomal GST, did not react with chromophoric substrates (eg. 1-chloro-2,4-dinitrobenzene) and was not solubilized by Triton X-100, a non-ionic detergent which solubilized and potentiated microsomal GST activity (143). Triton X-100 in fact inhibited LTC_4 synthase activity in the same concentration range as it potentiated microsomal GST activity. Diethylcarbamazine, a known inhibitor of SRS-A (149), was also shown to selectively inhibit LTC_4 synthase but not microsomal GST in rat basophilic leukemia cells (150). Due to the differences in substrate specificity and inhibition profile, LTC_4 synthase was designated as a distinct enzyme in rat basophilic leukemia cells (143,151).

A similar study in mouse mastocytoma cells also demonstrated that LTC_4 synthase was distinct from cytosolic and microsomal GSTs (146). Söderström and co-workers revealed a large difference in the sensitivity to Rose Bengal between LTC_4 synthase (IC_{50} value = 50 μM) and cytosolic GSTs (IC_{50} values = 500 μM and 1000 μM). Although the cytosolic GSTs were able to catalyze the conversion of LTA_4 to LTC_4, their concentration in the cell was too low to account for the high level of LTC_4 biosynthesis in this cell type. The microsomal GST in mouse mastocytoma cells had no activity with LTA_4 or LTA_4 methyl ester as an electrophilic substrate, therefore it was concluded that this form of the GSTs was not responsible for LTC_4 biosynthesis.

The first reported partial purification (3-fold) of the LTC_4 synthase enzyme was described in rat basophilic leukemia cells by Yoshimoto and co-workers in 1985 (152). Subsequently, several other groups improved on this partial purification (91-fold, guinea-pig lung (153); 21-fold, guinea-pig lung (154); 10-fold,

rat basophilic leukemia cells (155); 4-fold, mouse mastocytoma cells (156)). Attempts to further purify LTC_4 synthase were hindered by its hydrophobic properties, low abundance and apparent instability in the semi-purified state.

Recently, with the construction of an affinity column based on a product analogue (LTC_2), and with the identification of several critical parameters that influence not only stability of LTC_4 synthase but also the measurement of its activity in the semi-purified state, Nicholson and co-workers were able to achieve a 1000-fold purification of LTC_4 synthase from the dimethylsulfoxide-differentiated human monoblast cell line, U937 (157). Measurement of the activity of greater than 500-fold purified LTC_4 synthase was absolutely dependent on the presence of divalent cations and phospholipids. Specifically, Mg^{2+} and Ca^{2+} stimulated enzymic activity by 3-fold ($Ca^{2+} > Mg^{2+}$) . Of the various phospholipids tested, phosphatidylcholine stimulated LTC_4 synthase activity the most, increasing activity by 2-3 fold with concentrations ranging from 0.2 to 1 mg/ml. Furthermore, LTC_4 synthase was found to be stabilized by its substrate, reduced glutathione (GSH). The presence of GSH (2-4 mM) was required during storage although GSH concentrations greater than 5 mM irreversibly inactivated human LTC_4 synthase when present during freeze-thaw cycles. The identification of these parameters was found to be critical to obtain highly purified LTC_4 synthase.

1.5.5 Metabolism of LTC_4

LTC_4 is metabolised into LTD_4 with the loss of an L-glutamate residue by the action of γ-glutamyl transpeptidase. Recently, a specific γ-glutamyl transpeptidase dedicated to the biosynthesis of LTD_4 has been cloned (158). LTD_4 is then further metabolized by the action of cysteinylglycine dipeptidase which catalyzes the removal of glycine to form LTE_4, the final excreted metabolite of leukotriene biosynthesis in humans. To date, a specific cysteinylglycine dipeptidase dedicated solely to the production of LTE_4 has not been identified. Inactivation of the cysteinyl leukotrienes can occur through oxidative metabolism in the extracellular space of neutrophils or eosinophils engaged in a respiratory burst. The sulfur moiety of these cysteinyl leukotrienes is attacked by

hypochlorous (HOCl) acid which converts them to the corresponding sulfoxide and the inactive 6-*trans*-diastereomers of LTB_4 (159).

1.6 Biological actions and receptors of the leukotrienes

1.6.1 Biological actions of LTB_4

The chemical structure of LTB_4 was determined in 1979 by Borgeat and Samuelsson (160). It was shown to have pro-inflammatory activities in many tissues, organs and species including the induction of the chemotaxis and chemokinesis of leukocytes, increases in vascular permeability, modulation of pain responses as well as effects on lymphocyte function (for review see 161, 162). The effects of LTB_4 are highly stereoselective and specific and its biological activity can be measured *in vitro* by monitoring polymorphonuclear cell (PMN) chemotaxis, chemokinesis and lysosomal enzyme release induced by LTB_4 (163-165). LTB_4 has also been shown to enhance the movement of eosinophils and macrophages *in vitro* (166). LTB_4-mediated chemotaxis *in vivo* has been demonstrated by studies showing leukocyte accumulation following LTB_4 injection or application to human skin (167), the guinea-pig peritoneal cavity (166), rabbit skin and the eye (167,168).

LTB_4 also increases vascular permeability *in vivo* as demonstrated by the induction of edema formation after intradermal injection of LTB_4 in rabbit skin (169). The induction of edema by LTB_4 was found to be inhibited by leukocyte depletion and was potentiated by the vasodilator, PGE_2 (169). It has therefore been postulated that the change in vascular permeability induced by LTB_4 is dependent on PMN accumulation. In response to LTB_4, neutrophils adhere to the vascular endothelium and release granules containing chemical mediators that increase vascular permeability in the presence of vasodilators such as PGE_2. Similar results have been obtained in guinea pig (170), hamster (171) and pig (172). After intradermal injection of LTB_4, plasma leakage, PMN adherence to venular endothelial cells (hamster cheek pouch, 171,173) and PMN emigration into the vascular tissues (167,168,170,174) has been observed .

LTB_4 has also been identified as a potential modulator of the immune response. Regulation of lymphocyte activation by LTB_4, specifically B-lymphocytes and T-lymphocytes, has been demonstrated in several *in vitro* studies. First, LTB_4 enhances CD23 expression (a marker of B-cell activation) on resting B-cells in synergy with B-cell stimulating factors such as interleukin-2 and interleukin-4. In the presence of lymphokines, LTB_4 also stimulated DNA synthesis and cellular replication in intermediate density B-cells and increased by 2-fold, the production of immunoglobulins by low and intermediate density B-cells. In the absence of lymphokines, however, LTB_4 had no effect, and thus it has been described as a 'fine tuning modulator' of certain B-cell functions (175,176).

Certain T-lymphocytes have been shown to have receptors for LTB_4 (177). *In vitro*, LTB_4 inhibits the proliferation of mitogen-stimulated helper T-lymphocytes and induces T-lymphocyte suppressor cells and has thus been postulated to cause immunosuppression (178,179). LTB_4 also augments the activity of human natural cytotoxic cells (180). LTB_4 can replace the helper T-cell or interleukin-2 requirement by murine Lyt-1-,2+ cells for the production of γ-interferon (181).

A role for LTB_4 in the induction of pain has been defined based on the ability of LTB_4 to cause slight hyperalgesia upon injection and to reduce the latency period of hyperalgesia and prevent the initial hypoalgesia induced by yeast in the rat paw (182). As described for the LTB_4-induced increase in vascular permeability, induction of hyperalgesia was found to require the presence of PMNs (183) and may also involve the release of a nociceptor-sensitizing mediator from the PMNs.

1.6.2 Leukotriene B_4 receptor

LTB_4 is a potent inflammatory mediator active at nanomolar concentrations. Its high potency and stereoselective action suggested that a specific receptor exists to mediate its actions (184). [^{3}H]LTB_4 binding sites have been identified in cells and membranes isolated from human PMN (185), macrophages (186), U937 cells (187), differentiated HL-60 cells (188), guinea-pig eosinophils (189), guinea-pig lung (190), guinea-pig spleen (191), porcine spleen

(192) and the human monocytic cell line, THP-1(193). Two hypotheses have been proposed for the LTB_4 receptor. First, Goldman and Goetzl proposed that two different receptor subtypes existed for LTB_4 in human PMNs (185) based on their affinity for LTB_4. The high affinity receptor mediates chemotaxis and chemokinesis (185), promotes adherence of neutrophils to the endothelial walls (194) and increases cytosolic calcium (194). The low affinity receptor mediates lysosomal enzyme release (185) and also triggers the increase in cytosolic calcium (194). [^{3}H]LTB_4 specific binding sites are selectively inhibited by non-hydrolysable guanine nucleotides (190,195,196) demonstrating that the LTB_4 receptor is a G-protein coupled receptor. A second hypothesis for the LTB_4 receptor was proposed (197) where only one receptor existed but had interconvertible affinity states. The high-affinity state receptor would be coupled to the G-protein and the low-affinity state receptor would be uncoupled; however, the receptor density would not be affected in either state (190). LTB_4 actions are regulated by ω-oxidation by cytochrome P450 to the inactive metabolites 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 (198). LTB_4 and its structural analogues, 20-hydroxy-LTB_4, 20-carboxy-LTB_4, and 6-*trans*-LTB_4 compete with [^{3}H]LTB_4 in decreasing order of potency for binding to membrane sites and with the rank order observed for LTB_4 induced functional responses (chemotaxis and chemokinesis) (190,195,196). LTB_4 binding sites appear to be highly stereoselective, saturable, specific and low density and thus represent pharmacologically important membrane receptors.

The LTB_4 signal transduction mechanism was first elucidated in PMN membranes. LTB_4 stimulated phosphoinositol hydrolysis leading to the formation of inositol-1,4,5-trisphosphate and calcium mobilization in rat (199) and human (187,194) PMNs and in U937 (196) and HL-60 (194,200) cells. The LTB_4 receptor was found to be coupled to the pertussis toxin-sensitive inhibitory G_i protein. This G_i protein triggered the subsequent formation of inositol phosphates through the activation of phosphoinositide phospholipase C (PI-PLC) which mediates LTB_4 signal transduction (185,196,199). Treatment with pertussis toxin abolished LTB_4 induced chemotaxis, chemokinesis, aggregation and enzyme secretion in rabbit and human PMNs (201-203). Thus phosphoinositol hydrolysis was determined to

be the signal transduction mechanism eliciting the functional responses of LTB_4.

1.6.3 Biological actions of the cysteinyl leukotrienes

The bronchoconstrictive properties of the cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4) have been extensively studied in many species (for review see 204). LTC_4, LTD_4 and LTE_4 constitute and are collectively responsible for the biological actions of the *slow reacting substance of anaphylaxis*. The cysteinyl leukotrienes most potently stimulate the contraction of isolated airway preparations from guinea pig (205-207), man (208-210) and monkey (211,212). *In vivo*, the guinea pig is the best studied model where LTC_4 and LTD_4 demonstrate bronchoconstriction at picomolar concentrations (204).

Following aerosol and intravenous administration, the cysteinyl leukotrienes and LTB_4 have produced bronchoconstriction in the guinea pig *in vivo*. However, the effect of LTB_4 is transient and inhibitable by cyclooxygenase inhibitors, and thus appears to be mediated by prostanoids. Two mechanisms by which the cysteinyl leukotrienes induce bronchconstriction *in vivo* and *in vitro* in the guinea pig have been described (205). The first (indirect) mechanism is through the release of the endogenous prostanoid, thromboxane A_2, and the second is by the direct action of the leukotrienes on specific receptors. In the guinea pig, the route of adminstration of the cysteinyl leukotrienes determines the role of cyclooxygenase products (205,213). When administered by aerosol, LTC_4, LTD_4 and LTE_4 induce bronchoconstriction with greater than 1000-fold higher potency than histamine (205,213-215) and this effect is enhanced by the COX inhibitor, indomethacin (215). However, upon intravenous administration, the cysteinyl leukotrienes induce bronchoconstriction that is inhibited by COX inhibitors (205,213,216), indicating that the intravenous route of administration may preferentially stimulate the release of COX products *in vivo* in the guinea pig. In man (217-219) and the hyperreactive rat (220) only the direct mechanism has been demonstrated to mediate bronchoconstriction indicating that the leukotrienes act via specific receptors in these species. In man aerosol-administered LTC_4, LTD_4 and to a lesser extent LTE_4 produce potent

bronchoconstriction (6000-fold more potent than histamine) (217,221,222) that is not affected by aspirin or indomethacin, suggesting that the prostanoids do not play a role in leukotriene-mediated bronchoconstriction in man *in vivo* (217,218,219).

In addition to their bronchoconstrictive properties, the cysteinyl leukotrienes also stimulate mucus production. This has been demonstrated *in vitro* and *in vivo*. Leukotriene-induced mucus production has been observed in isolated human airways (223,224) and canine trachea (225,226). Also, the LTD_4 or SRS-A receptor antagonist, FPL 55712 has been shown to inhibit LTD_4-induced mucus release (223). The leukotrienes have been detected in the sputum of patients suffering from obstructive lung disease and asthma (227) and increased mucin production with impaired mucociliary transport has also been observed in patients with asthma (228,229). Thus the cysteinyl leukotrienes have been proposed to be one of the important mediators causing impaired mucociliary function (230,231).

The cysteinyl leukotrienes also mediate vasoconstriction. In 1981, Kito and co-workers, demonstrated contraction of the isolated rabbit coronary artery induced by LTD_4 and LTE_4 at nanomolar concentrations (232). The vasoconstriction induced by the LTC_4 and LTD_4 could not be modulated by COX inhibitors but could be inhibited by FPL 55712 supporting the direct effect of leukotrienes via specific receptors (232). Although there are considerable differences between species and different vascular beds, the cysteinyl leukotrienes appear to be potent constrictors of the coronary vasculature in several species including the guinea pig, rat, rabbit, and cat (233-236). The cysteinyl leukotrienes are also vasoconstrictive in humans. LTC_4 and LTD_4 (and LTE_4 but less potently) but not LTB_4 contract the human pulmonary artery and this effect was completely inhibited by FPL 55712 (237).

In vitro, in isolated cardiac myocyte preparations from the guinea pig and human (233) the cysteinyl leukotrienes have negative inotropic actions that are specifically antagonized by FPL 55712. *In vivo*, intravenous injection of LTC_4 (2 nmol) in humans caused an increase in coronary vasculature resistance,

decrease in coronary blood flow and a fall in blood pressure (arterial) (238). Intracoronary LTD_4 also caused a decrease in arterial blood pressure and increased vasculature resistance, thus cysteinyl leukotrienes were designated as vasoconstrictive mediators *in vivo* in the human species (238).

1.6.4 Leukotriene C_4 receptor

LTC_4 stereoselectively and potently induces contractions in guinea-pig lung and trachea (50,138,239,240). Since LTC_4 and its metabolites LTD_4 and LTE_4 have identical biological actions but with differing levels of potency, the existence specific receptors for each of these cysteinyl leukotrienes was examined in several investigations. The existence of an LTC_4 receptor that was distinct from LTD_4 was postulated based on radioligand binding studies and smooth muscle contraction assays. It was observed in guinea-pig trachea that the SRS-A antagonist FPL 55712 was not as effective at blocking LTC_4 induced contraction as it was at blocking LTD_4 and LTE_4 induced contractions (200,241). Furthermore, in the presence of serine-borate, an inhibitor of γ-glutamyl transpeptidase that prevents the conversion of LTC_4 to LTD_4 (242), FPL 55712 was even less effective at inhibiting LTC_4-induced tracheal contractions. Radioligand binding assays, also done in the presence of serine-borate (243), detected the presence of specific $[^3H]LTC_4$ binding sites in rat and guinea-pig lung and ileal smooth muscle membrane preparations plus other tissues and cells in culture (243-249). The identified binding sites were high in density (B_{max} = 2-50 pmol/mg), saturable (K_d = 1-50 nM) and selective for LTC_4, but did not bind LTD_4 or FPL 55712 and thus these properties were used to support the concept of a distinct LTC_4 receptor.

Recently, with the identification of two highly selective and potent LTD_4 receptor antagonists, MK-571 and its R-enantiomer, MK-679, pharmacological studies have provided additional functional evidence for the presence of distinct LTC_4 receptors (250,251). These antagonists are approximately 100-fold more potent than FPL 55712 at inhibiting $[^3H]LTD_4$ binding and LTD_4-induced contractions in guinea-pig and human airways (250,251). In guinea-pig trachea, contractions induced by LTC_4, LTD_4 and LTE_4 were antagonized by MK-571 and

MK-679, however LTC_4-induced contractions were only inhibited in the absence but not in the presence of serine-borate (which blocks metabolic conversion of LTC_4 to LTD_4), thus demonstrating the specificity of these antagonists and LTC_4-mediated contractions (250,251).

Although there is considerable functional evidence for the presence of an LTC_4 receptor, several recent studies have indicated that $[^3H]LTC_4$ binding sites do not represent LTC_4 receptors. Thus radioligand binding studies may not identify the LTC_4 receptor. The first, was that these sites were often found in membranes of tissues, cells and organelles (252) that lacked pharmacological responses to leukotrienes. Secondly, several glutathione derivatives bound to these sites in guinea-pig lung but showed no pharmacological effect (246). Thirdly, the affinity of structural analogues of LTC_4 for these sites did not correlate with their ability to induce contractions in guinea-pig trachea or lung smooth muscle preparations (246). Fourthly, in rat liver and in dimethylsulfoxide-differentiated U937 cells (dU937 cells) the primary LTC_4 binding site was determined to be a subunit of glutathione S-transferase (253) or microsomal glutathione S-transferase (254), respectively. The K_d values obtained for the specific binding of $[^3H]LTC_4$ to pure microsomal GST or to dU937 cell membranes were determined to be 6.5 nM and 25 nM, respectively. These values were well within the reported K_d values for the putative LTC_4 receptor in other studies, thus radioligand binding assays cannot be used to identify the LTC_4 receptor site responsible for the pharmacological actions of LTC_4 in various tissues unless a method to block the binding of LTC_4 to glutathione S-transferases is developed.

1.6.5 Leukotriene D_4 receptor

Unlike LTC_4, *in vitro* radioligand binding as well as tissue and cell-based functional assays and *in vivo* studies in conscious or anaesthetized animals have clearly presented evidence supporting the existence of specific LTD_4 receptors. Stereoselective, specific, and saturable binding sites have been identified in membrane preparations of smooth muscle cells isolated from several tissues and cell types including sheep trachea (255), guinea-pig lung (243-247,256-258),

guinea-pig myocardium (259), human lung (260), U937 (187) and RBL cells (261). Functionally, LTD_4 can induce smooth muscle contraction in a highly stereoselective manner (138,205,239,262,263-266) in tissues from several species including the guinea pig, primates, sheep, and man. The response to LTD_4 and LTE_4 is highly potent (active in nanomolar concentration) and can be blocked by specific receptor antagonists (eg. FPL 55712, MK-571, MK-679) *in vitro* and *in vivo* (208,210,250,251,267). In addition, the correlation between the specific binding of several LTD_4 and LTE_4-like agonists and functional antagonists (268,269) and their ability to mediate smooth muscle contraction in the guinea pig (270) has further defined the presence of a biochemically and physiologically relevant LTD_4 receptor.

Regulation of [^{3}H]LTD_4 binding in guinea-pig lung membrane preparations by the non-hydrolysable GTP analog, GTPγS, sodium ions, and divalent cations (271,272) has identified the LTD_4 receptor as a member of the G-protein coupled receptor family. Recently, a 45 kDa protein was specifically photolabelled with a novel photoaffinity probe, ^{125}I-azido-LTD_4, in guinea-pig lung membranes and was identified as the LTD_4 receptor. As demonstrated for ^{125}I-azido-LTD_4 specific binding, the photolabeling of this polypeptide was saturable, modulated by cations and inhibited by non-hydrolysable nucleotide analogues (271). Only one class of LTD_4 receptor has been defined in guinea-pig lung membrane preparations whose affinity state is modulated by the presence of guanine nucleotides, divalent cations and sodium ions. In the presence of GTPγS and sodium ions and the absence of divalent cations, the receptor converts from the high to the low affinity state, however, in the presence of divalent cations, the proportions of the high affinity receptor increases. Based on the mechanism of action of other G-protein linked receptors, as explained by the ternary compiex hypothesis (273), the following model has been proposed for the function of the LTD_4 receptor (272). Normally cells have a high GTP concentration and a low divalent ion concentration, thus the low affinity state of the receptor dominates. Upon binding of LTD_4 to its receptor, G-proteins will be recruited from within the target cell to transiently form the high affinity LTD_4-receptor-G-protein complex.

Subsequently, GTP binds and activates the G-protein and results in dissociation of the ternary complex. This dissociated complex then activates the PI-PLC cascade to form the intracellular messengers, diacylglycerol and inositol triphosphate.

The signal transduction mechanism for the LTD_4 receptor was elucidated based on the results of several studies which demonstrated the involvement of phosphatidylinositol (PI) hydrolysis with subsequent mobilization of internal calcium stores and protein kinase C activation. First, LTD_4 and its agonist analogues induced the rapid accumulation of IP_1, IP_2, and IP_3 (255,274-276). Secondly this PI hydrolysis was stereoselectively inhibited by the receptor antagonist, SK&F 104353 (275,276) and thirdly, PI hydrolysis correlated directly with smooth muscle contraction in guinea-pig lung (277).

1.6.6 Leukotriene E_4 interactions with the LTD_4 receptor

LTE_4 functions as a partial agonist to the LTD_4 receptor. LTE_4 binds to the LTD_4 receptor in membranes isolated from the guinea-pig lung, human lung and other cells and tissues (244,244,256-258,260,261). LTE_4 induces smooth muscle contractions in guinea-pig tracheal preparations (205,265) and also serves as a partial agonist in sheep (255) and guinea-pig (278) tracheal smooth muscle. FPL 55712 was the first LTD_4 receptor antagonist that demonstrated inhibition of LTE_4-mediated contraction in guinea-pig tracheal preparations (200,241). Subsequently, MK-571 and MK-679 also demonstrated similar activity (250,251). LTE_4 has also been shown to bind to LTD_4 receptors in membrane preparations, can also induce cellular signalling, including PI hydrolysis and calcium mobilization (261), and this response can be specifically blocked by LTD_4 receptor antagonists (272). Furthermore, a recent study characterizing the leukotriene receptors and their signalling mechanisms in guinea-pig gastric smooth muscle cells has presented evidence using receptor protection assays that supports the existence of two distinct cysteinyl leukotriene receptors, one that mediates the actions of LTC_4 and the other that mediates the actions of both LTD_4 and LTE_4 (279). All three leukotrienes were equipotent contractile agents in these gastric

smooth muscle cells, and both the LTC_4 and the LTD_4/LTE_4 receptors stimulated PI hydrolysis, protein kinase C activation, and calcium mobilization (279). Thus cysteinyl leukotrienes appear to mediate their cellular actions through two distinct receptors, an LTC_4 and an LTD_4/LTE_4 receptor, and their intracellular signalling mechanism is mediated through PI-hydrolysis and the subsequent cascade of physiological events.

1.7 The role of cysteinyl leukotrienes in human bronchial asthma

Asthma is a complex respiratory disease characterized by airway inflammation, reversible airway obstruction and bronchial hyperresponsiveness to a variety of stimuli (280,281). The importance of the cysteinyl leukotrienes in asthma was first highlighted by Kellaway and Trethewie more than 50 years ago (46). Since then, extensive investigations into the functions of these mediators in inflammation have been carried out. Although several lipid mediators and cytokines produced by a variety of inflammatory cell types have been implicated in the pathology of asthma, the development of specific leukotriene biosynthesis inhibitors (MK-886, MK-0591, zileuton) and receptor antagonists (MK-571, MK-679, ICI-204219) have established a definitive role for the leukotrienes in this disease.

Asthma is characterized by two responses: an early asthmatic response (EAR) followed by a late asthmatic response (LAR). EAR occurs just after allergen challenge and is most likely due to the release of bronchoconstrictive mediators (LTC_4, PGD_2, histamine) released by IgE-activated mast cells and the LAR occurs several hours later and involves the infiltration of inflammatory cells which release various arachidonate metabolites, including cysteinyl leukotrienes (282).

Cysteinyl leukotrienes have been shown to cause bronchoconstriction in both healthy (282,283,284) and asthmatic patients (285,286), although the response is somewhat exaggerated in asthmatic individuals. The role of the cysteinyl leukotrienes in bronchial asthma was postulated not only based on their ability to elicit bronchoconstriction and the inflammatory response observed in asthmatic patients, but also due to their detection in the sputum (287), urine (288),

bronchoalveolar lavage fluid (289), plasma (290), and nasal secretions (291) of asthmatic patients.

The effects of leukotrienes mimic the pathological changes observed in asthma both *in vitro* and *in vivo*. The chronic inflammation observed in asthma is associated with widespread airway obstruction (292) that can be caused by four distinct mechanisms: i) bronchoconstriction, ii) mucosal edema, iii) increased mucus secretion, and iv) an eosinophil-rich inflammatory cell infiltrate (281). There is now evidence that leukotrienes can elicit the above-mentioned pathology.

The ability of cysteinyl leukotrienes to cause bronchoconstriction both *in vitro* and *in vivo* has been extensively discussed previously (cf. section 1.6.3). MK-571 and MK-679, both potent and selective antagonists of the LTD_4 receptor, antagonized the bronchoconstrictive effects of LTD_4 in a number of *in vitro* pharmacological and biochemical studies and were also able to block exogenous or endogenous leukotriene-induced bronchoconstriction, *in vivo*, in three animal species (250,251). Further clinical studies have shown the ability of these drugs to attenuate the EAR and LAR to inhaled antigen (293) as well as inhibit exercise-induced bronchoconstriction in human subjects (294). The 5-LO inhibitor, MK-886, inhibits the production of all leukotrienes *in vivo* and *in vitro*. *In vivo*, MK-886 has demonstrated inhibition of antigen-induced bronchoconstriction (early and late response) in rats, squirrel monkeys, and sheep (295). In clinical trials, MK-886, also blocked EAR and LAR to inhaled antigen in man (296,297). Thus, these studies have provided significant evidence for a role of leukotrienes in the bronchoconstriction observed in human bronchial asthma.

Increased vascular permeability at post-capillary venules which results in the extravasation of proteins and water is the main cause of mucosal edema in human bronchial asthma. In response to the topical application of cysteinyl leukotrienes extravasation of Evans Blue dye, radiolabelled albumin or fluorescein-conjugated macromolecules in guinea-pig and hamster cheek pouches and in the bronchial tree have been observed. In humans, intradermal injections of the cysteinyl leukotrienes can produce flare and wheal reactions (298,299).

Increased mucus secretion is one of the main causes of airway obstruction in asthmatics. *In vitro*, cysteinyl leukotrienes have been shown to be potent secretagogues in human airways as measured by the incorporation of radiolabelled amino-sugars into mucus glycoproteins (300,301).

Infiltration of inflammatory cells is a common feature of the asthmatic airway. Although there is little evidence that cysteinyl leukotrienes mediate chemotaxis, it is a key function of LTB_4 which predominantly attracts neutrophils and to a lesser extent eosinophils (56,302).

In clinical asthma, several reports have demonstrated the efficacy of both leukotriene receptor antagonists and 5-LO inhibitors. Short-term studies with the leukotriene receptor antagonists, FPL 55712 and the more potent, ICI-204219, have shown significant improvements in FEV_1 in asthmatic patients (303,304). Later studies with more prolonged treatments (4-6 weeks) with various leukotriene receptor antagonists have also generated interesting results. Treatment with ICI-204219 orally for 6 weeks (305) has demonstrated significant improvements in morning flow rates, night-time wakening and daytime symptom scores versus placebo. Oral MK-571 or MK-679 have also been effective at reducing symptom scores, β_2-agonist use and spirometry with 4 week treatment (AWFH, personal communication). In patients with mild chronic asthma, oral tomelukast (LTD_4 receptor antagonist) over a 6-week period has also demonstrated an improvement in FEV_1 versus placebo (306).

5-Lipoxygenase inhibitors consist of drugs that can act via one of two mechanisms: either directly on the 5-LO enzyme or by binding to and inactivating FLAP. The direct 5-LO inhibitor, zileuton has been shown to attenuate the asthmatic response to cold air (307). In clinical asthma, after a 4 week treatment period, this inhibitor improved FEV_1, forced vital capacity, and peak expiratory flow rates, symptom scores and β_2-agonist use. It has been noted that the beneficial effects of this drug were both acute and cumulative and thus it has been postulated that it may be affecting the underlying inflammatory process (308). MK-886 has also been demonstrated to be effective in attenuating EAR and LAR to inhaled allergen (296).

Due to their potent pathophysiological effects and the demonstrable efficacy of leukotriene receptor antagonists and biosynthesis inhibitors in human bronchial asthma, the leukotrienes are likely to be important mediators of chronic inflammation and widespread airway obstruction that define this respiratory disease and leukotriene receptor antagonists and biosynthesis inhibitors should therefore have a role in the management of the clinical condition.

Since LTC_4 synthase catalyzes the first committed step leading to the production of the pro-inflammatory cysteinyl leukotrienes, inhibition of its biosynthetic activity is one potential therapeutic approach for the management of inflammatory diseases. However, the purification and characterization of this enzyme is required before strategies to attenuate LTC_4 synthase activity can be elaborated. Thus, this objective was the focus of the following study.

2. RESEARCH OBJECTIVES

LTC_4 synthase is a glutathione S-transferase that catalyzes the conjugation of reduced glutathione to LTA_4 leading to the production of LTC_4. LTC_4 can then be further metabolized into LTD_4 and LTE_4 by the actions of γ-glutamyl transpeptidase and cysteinylglycine dipeptidase, respectively. LTC_4, LTD_4 and LTE_4, collectively known as the cysteinyl leukotrienes, are responsible for the actions of the slow reacting substance of anaphylaxis (SRS-A). The cysteinyl leukotrienes are formed in response to a variety of inflammatory stimuli and have potent biological actions including pulmonary smooth muscle contraction, vasoconstriction, mucus secretion and changes in vascular permeability. As such, the cysteinyl leukotrienes have been implicated in the pathology of human bronchial asthma and related inflammatory conditions. Since LTC_4 synthase catalyzes the first committed step leading to the production of these inflammatory mediators it is a potential target for therapeutic intervention. At the outset of these studies, LTC_4 synthase had not been purified to homogeneity or fully characterized from any source. Thus, the objectives of this study were:

1. To determine if LTC_4 synthase is a unique enzyme distinct from all known glutathione S-transferases in tissues of human origin.

2. To identify potential polypeptide candidates for LTC_4 synthase in the microsomal membranes of various human myelocytic leukemia cell lines known to have LTC_4 synthase activity using photoaffinity labelling techniques.

3. To purify human LTC_4 synthase to homogeneity and determine its amino acid sequence.

4. To elucidate the regulatory mechanisms governing cysteinyl leukotriene biosynthesis in inflammatory cells.

3. STATEMENT OF CONTRIBUTIONS

*Manuscripts and Authorship:

Candidates have the option, subject to the approval of their Department, of including as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.
- If this option is chosen, connecting texts, providing logical bridges between the different papers are mandatory.
- The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.
- Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
- In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers.

*reproduced from *Guidelines Concerning Thesis Preparation*, Faculty of Graduate Studies and Research, McGill University

This thesis is composed of 5 manuscripts and the contributions of co-authors and myself are described below:

A. Nicholson, D.W., Ali, A., Klemba, M.W., Munday, N.A., Zamboni, R.J. and Ford-Hutchinson, A.W. (1992) *J. Biol. Chem.* 267: 17849-17857.

This manuscript describes the ongoing project in the laboratory of Dr. D.W. Nicholson when I joined the laboratory. My contribution to this paper was the identification of LTC_4 synthase as a unique enzyme distinct from the known glutathione S-transferases (figure 5) and the identification of a potential polypeptide candidate for being LTC_4 synthase using photoaffinity labelling

techniques (figure 7). M.W. Klemba and N.A. Munday were students in the laboratory that contributed to the remaining work. Dr. R.J. Zamboni synthesized the photoaffinity ligand described in figure 6A. The work was supervised by Drs. D.W. Nicholson and A.W. Ford-Hutchinson.

B. Ali, A., Zamboni, R.J., Ford-Hutchinson, A.W. and Nicholson, D.W. (1993) *FEBS Lett.* 317: 195-201.

All of the experiments described in this paper were planned and performed by myself. This manuscript was also written by myself. Dr. R.J. Zamboni synthesized the photoaffinity ligand described in figure 1A. The work was supervised by Drs. D.W. Nicholson and A.W. Ford-Hutchinson.

C. Nicholson, D.W., Ali, A., Vaillancourt, J.P., Calaycay, J.R., Mumford, R.A., Zamboni, R.J. and Ford-Hutchinson, A.W. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90: 2015-2019.

The purification of LTC_4 synthase to homogeneity was done through the collaborative efforts of all authors. Dr. D.W. Nicholson constructed the affinity column and designed the initial strategy for the partial purification of the enzyme as described in reference 159 which was then modified by myself to purify the enzyme to homogeneity. Specifically, I developed the gel filtration chromatography step (final purification step, figure 3) which purified the protein to homogeneity (figure 4). I also determined that the enzyme functions as a homodimeric protein (figure 3) and successfully labelled an 18 kDa polypeptide as a putative candidate for the enzyme (figure 5). The N-terminal sequence was determined by R.A. Mumford and J.R. Calaycay. J. P. Vaillancourt was involved in performing the labor-intensive chromatographic steps of the purification. The work was supervised by Drs. D.W. Nicholson and A.W. Ford-Hutchinson.

D. Kargman, S., Ali, A., Vaillancourt, J.P., Evans, J.F. and Nicholson, D.W. (1994) *Mol. Pharmacol.* In press.

This manuscript describes the first identification of the phosphoregulatory mechanism governing cysteinyl leukotriene biosynthesis. I determined that the attenuation of cysteinyl leukotriene biosynthesis by PMA-mediated phosphorylation was due to the inhibition of LTC_4 synthase activity (table 3). I also determined that PMA and staurosporine did not have any non-specific effects on LTC_4 synthase (figure 5) and that this phosphoregulatory mechanism did not involve the activation of any other kinases (table 2). S. Kargman and J.P. Vaillancourt did the remaining work. This work was done under the supervision of Drs. J.F. Evans and D.W. Nicholson.

E. Ali, A., Ford-Hutchinson, A.W. and Nicholson, D.W. (1994) *J. Immunol.* In press.

All of the experiments described in this paper were planned and performed by myself. This manuscript was also written by myself. The work was supervised by Drs. D.W. Nicholson and A.W. Ford-Hutchinson.

4. MANUSCRIPT A

Human Leukotriene C_4 Synthase Expression in Dimethylsulfoxide-Differentiated U937 Cells

J. Biol. Chem. 267: 17849-17857 (1992)

This paper presents evidence that human LTC_4 synthase is an enzyme distinct from other known glutathione S-transferases. It also identifies dimethylsulfoxide-differentiated U937 cells as an abundant source of human LTC_4 synthase. Furthermore, this paper describes the first identification of a putative polypeptide candidate for LTC_4 synthase using photoaffinity labelling techniques.

Human Leukotriene C_4 Synthase Expression in Dimethylsulfoxide-Differentiated U937 Cells

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SUMMARY

Leukotriene C_4 (LTC_4) synthase was highly expressed in the human U937 monoblast leukemia cell line when differentiated into monocyte/macrophage-like cells by growth in the presence of dimethylsulfoxide. The specific activity of LTC_4 synthase in differentiated cells (399.0 ± 84.1 pmol LTC_4 formed•min^{-1}•mg^{-1}) was markedly higher (10-fold; $p<0.001$) than in undifferentiated U937 cells (39.9 ± 16.7 pmol LTC_4 formed•min^{-1}•mg^{-1}) or freshly-isolated blood monocytes (21.5 ± 4.8 pmol LTC_4 formed•min^{-1}•mg^{-1}). The increase in LTC_4 synthase activity following dimethylsulfoxide-induced differentiation was substantially higher than the increase observed for other proteins involved in leukotriene biosynthesis. LTC_4 synthase activity was unaffected in U937 cells differentiated by growth in the presence of phorbol-12-myristate-13-acetate. The HL-60 myeloblast leukemia cell line expressed higher LTC_4 synthase levels when differentiated into either neutrophil-like or macrophage-like cells by growth in the presence of dimethylsulfoxide or phorbol-12-myristate-13-acetate (respectively), but reached a specific activity comparable only to undifferentiated U937 cells. Human LTC_4 synthase was found to be a unique membrane-bound enzymatic activity completely distinct from α, μ, π, θ and microsomal glutathione S-transferases, as determined by differential detergent solubilization, chromatographic separation, substrate specificity and Western blot analysis. An 18 kDa polypeptide was specifically labelled in membranes from dimethylsulfoxide-differentiated U937 cells using azido ^{125}I-LTC_4, a photoaffinity probe based on the product of the LTC_4 synthase-catalyzed reaction. Photolabelling of the 18 kDa polypeptide was specifically competed for by LTC_4 (>50% at 0.1μM) but not by 100,000-fold higher concentrations of reduced glutathione (10mM). Elevation of both the level of the specifically photolabelled 18 kDa polypeptide and of LTC_4 synthase specific activity occurred concomitantly with dimethylsulfoxide differentiation of U937 cells.

We conclude that differentiation of U937 cells into monocyte/macrophage-like cells by growth in the presence of dimethylsulfoxide

results in high levels of expression of LTC_4 synthase activity. Human LTC_4 synthase is a unique enzyme with a high degree of specificity for LTA_4 and may therefore be dedicated exclusively to the formation of LTC_4 *in vivo*. An 18 kDa membrane polypeptide, specifically labelled by a photoaffinity derivative of LTC_4, is a candidate for being either LTC_4 synthase or a subunit thereof.

INTRODUCTION

Leukotrienes are potent biological mediators that are formed in response to a variety of immunologic and inflammatory stimuli. Their predominant effects include leukocyte chemotaxis, pulmonary smooth muscle contraction, vasoconstriction, increased vascular permeability and mucous hypersecretion. Consequently, leukotrienes have been implicated as potential mediators of immediate hypersensitivity and inflammatory conditions (for review see Samuelsson, 1983; Piper, 1984; Ford-Hutchinson, 1990; Lewis et al, 1990).

Like the prostaglandins and thromboxanes, leukotrienes are derived from arachidonic acid which is released from the *sn-2* position of membrane phospholipids. A recently identified arachidonate-selective cytosolic phospholipase A_2 is probably responsible for this activity (Clark et al, 1991; Sharp et al, 1991). Newly liberated arachidonic acid is converted to the unstable epoxide leukotriene A_4 (LTA_4)‡ in a two step reaction that is catalyzed by the cytosolic enzyme 5-lipoxygenase following its translocation to cellular membranes in a process that is dependent on the membrane protein FLAP (5-lipoxygenase-activating protein)(Rouzer et al, 1990; Miller et al, 1990; Dixon et al, 1990; Ford-Hutchinson, 1991). Leukotriene A_4 is further metabolized down one of two pathways to form biologically active products (for review see Samuelsson, 1985; Shimizu, 1988; Lewis et al, 1990). It is either stereospecifically hydrated by the cytosolic enzyme LTA_4 hydrolase, producing LTB_4, or it is conjugated with reduced glutathione by membrane-bound LTC_4 synthase to form the sulfidopeptide leukotriene C_4. LTC_4 is further metabolized by removal of the L-glutamate residue to form LTD_4 followed by subsequent removal of the glycine residue to form LTE_4 in reactions catalyzed by γ-glutamyl transpeptidase and cysteinylglycine dipeptidase, respectively. Collectively, the sulfidopeptide leukotrienes, LTC_4, LTD_4 and LTE_4, comprise the slow reacting substance of anaphylaxis (SRS-A).

Leukotrienes are predominantly formed in circulating cells of myeloid lineage, although the exchange of leukotriene and other eicosanoid precursors between various cell types has been demonstrated (Marcus, 1986; Maclouf et al, 1989; Lagarde, 1989). LTB_4 biosynthesis occurs mainly in polymorphonuclear granulocytes, particularly neutrophils, whereas peptide leukotriene biosynthesis has been demonstrated principally in mononuclear phagocytes, mast cells and eosinophils (see reviews by Samuelsson, 1983; Piper, 1984; Ford-Hutchinson, 1990). The human promyelocytic leukemia cell line HL60 (Collins et al, 1977; Gallagher et al, 1979) and the promonocytic leukemia cell line U937 (Sundström & Nilsson, 1976) can be selectively differentiated into cells having morphological and functional characteristics resembling either neutrophils, eosinophils, monocytes or macrophages by growth in culture in the presence of various agents, such as dimethylsulfoxide or phorbol esters and to a lesser degree with lymphokines or retinoic acid (for overview see Harris & Ralph, 1985). As a consequence, these cell lines are becoming useful for examining the role that various myeloid cell types play in leukotriene biosynthesis and responses.

The enzyme that catalyzes the first committed step in the biosynthesis of the sulfidopeptide leukotrienes, namely LTC_4 synthase, is a glutathione S-transferase which appears to be specific for LTC_4 formation. The enzyme has been characterized and partially purified from mouse mastocytoma cells (4-fold; Söderström et al, 1990), RBL cells (10-fold; Yoshimoto et al, 1985) and guinea pig lung (91-fold; Yoshimoto et al, 1988; Izumi et al, 1988; Izumi et al, 1989). Attempts to purify human LTC_4 synthase have not been reported, owing principally to the low specific activity of the enzyme in available human tissues and cell lines. In this report we have examined LTC_4 synthase activity in human cells including granulocytes and monocytes isolated from fresh blood, and in the promyelocytic leukemia cell lines U937 and HL-60, each differentiated by growth in the presence of dimethylsulfoxide or phorbol ester. We demonstrate that differentiation of U937 cells by growth in the presence of dimethylsulfoxide results in a marked (10-fold) increase in LTC_4 synthase activity. We show that human LTC_4 synthase is a

unique membrane-bound enzyme, specific for LTC_4 biosynthesis, which is distinct from α, μ, π, θ and microsomal glutathione S-transferases. Finally, we have developed a radioiodinated photoaffinity ligand based on the LTC_4 synthase enzymatic product (LTC_4) that specifically labels two membrane polypeptides, one with high specificity for LTC_4.

MATERIALS AND METHODS

Materials

U937 cells (CRL 1593) and HL-60 cells (CCL 240) were obtained from the American Type Culture Collection (Rockville, Maryland). Cell culture media, antibiotics and fetal bovine serum were purchased from the Sigma Chemical Co. (St. Louis, Missouri). LTA_4-methyl ester, LTB_4, LTC_4, LTC_2, LTD_4 and LTE_4 were synthesized by the Department of Medicinal Chemistry at the Merck Frosst Centre for Therapeutic Research. [14,15-^{3}H(N)]leukotriene A_4-methyl ester (42 Ci/mmol), [^{35}S]glutathione (145 Ci/mmol), [^{125}I]Protein A (80 μCi/mg) and Na[^{125}I] were from New England Nuclear (Mississauga, Ontario). Taurocholate (Ultrol grade), CHAPS, CHAPSO and glycodeoxycholate were from Calbiochem (La Jolla, California). Dimethylsulfoxide (Me_2SO) was from J.T. Baker (Phillipsburg, New Jersey). HPLC solvents were purchased from BDH (Toronto, Ontario). MonoQ HR5/5 columns were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Specific polyclonal antisera raised against purified human α, μ, π and microsomal glutathione S-transferases were the kind gift of Dr. John D. Hayes, University Department of Clinical Chemistry, The Royal Infirmary, Edinburgh, Scotland. Other reagents were of analytical grade and were purchased from Sigma (St. Louis, Missouri).

Cell Growth and Isolation

U937 Cell Growth and Differentiation - Cells from the human leukemic monoblast cell line U937 (American Type Culture Collection CRL1593; Sundström and Nilsson 1976) were cultured in sterile RPMI-1640 medium (supplemented with 0.2% (w/v) $NaHCO_3$ and 0.03% (w/v) L-glutamine) containing 50 U penicillin/ml, 50 μg streptomycin/ml and 10% (v/v) fetal bovine serum (Sigma Hybri-Max, not heat inactivated). Unless otherwise indicated, all cultures were grown at 37°C in a

humidified atmosphere containing 6% CO_2 in either 175 cm^2 culture flasks or spinner flasks (25 rpm). U937 cell stocks were maintained by subculturing cells every fourth or fifth day in fresh medium at a seed density of 0.5 x 10^5 cells/ml (subculturing was performed earlier if the cell density exceeded 1.5 x 10^6 cells/ml). For differentiation, cells were seeded in fresh medium at an initial density of 0.1 to 0.2 x 10^6 cells/ml and supplemented with 1.3% (v/v) Me_2SO (or 10 nM phorbol-12-myristate-13-acetate, as indicated) and grown for 4 to 5 days before isolation.

HL-60 Cell Growth and Differentiation - The human leukemic myeloblast cell line HL-60 (American Type Culture Collection CCL240; Collins et al, 1977) was grown and differentiated in a similar manner except that the culture medium was Iscove's modified Dulbecco's medium (with 0.058% (w/v) L-glutamine, 25 mM Hepes, supplemented with 0.30% (w/v) $NaHCO_3$) instead of RPMI-1640.

Isolation of U937 and HL-60 Cells - Cells were harvested by centrifugation at 600 xg for 20 min at 4°C. The resulting cell pellet was washed by resuspending the cells in the original culture volume in cold (4°C) phosphate-buffered saline (pH 7.4) containing 2 mM EDTA (PBS (pH 7.4), 2 mM EDTA) and re-sedimenting them at 1200 x g for 15 min. The washed cells were resuspended in PBS (pH 7.4), 2 mM EDTA with light Dounce homogenization ('B' clearance pestle), adjusted to a final density of 1 x 10^8 cells/ml and stored in aliquots at -80°C after freezing in liquid nitrogen.

Separation of Cells from Whole Blood - Venous blood was collected from healthy human volunteers and cells were separated by discontinuous gradient centrifugation on Histopaque 1077/1119 (Sigma). The gradient steps were formed by layering 12 ml Histopaque 1077 over 12 ml Histopaque 1119. The freshly-isolated, heparin-treated blood was diluted with an equal volume of phosphate-buffered saline (pH 7.2) and 24 ml of the mixture was layered over the Histopaque 1077. The tubes were centrifuged at ambient temperature for 30 min at 700 x g (swing out rotor). The monocyte/platelet fraction (at the upper surface of the Histopaque 1077 phase) and the granulocyte fraction (at the Histopaque 1077/1119 interface) were retrieved with pasteur pipets and transferred to new

tubes where they were washed by diluting the cells with 5 volumes of PBS (pH 7.2) followed by centrifugation for 20 min at 2000 x g. The cell pellet was resuspended in PBS (pH 7.2) and the density was adjusted to 1 x 10^8 cells/ml. Aliquots were stored at -80°C after freezing in liquid nitrogen.

Nitrogen Cavitation and Subcellular Fractionation - Differentiated U937 cells were thawed and supplemented with 2 mM phenylmethylsulfonylfluoride (added from a fresh 200 mM stock in ethanol). The cells were dispersed with 10 strokes in a Dounce homogenizer (with a tight fitting "A" pestle) and then transferred into a nitrogen cavitation cell (Kontes). For each cycle (30 ml/cycle) the cavitation unit was pressurized with nitrogen for 15 min at 800 psi on ice. Following rapid decompression, the cell lysate was collected and spun at 1000 xg for 15 min. The pellet was discarded (unless otherwise indicated) and the resulting supernatant was collected and spun at 10,000 xg for 20 min. Following centrifugation, the supernatant was retained and re-spun at 100,000 xg for 30 min. The resulting microsomal pellet (100,000 xg pellet) was retained, washed and dispersed at a density equivalent to 10^9 original cells/ml in PBS (pH 7.4), 2 mM EDTA using a Dounce homogenizer (10 strokes, tight "A" pestle). Aliquots were frozen in liquid nitrogen and stored at -80°C.

Solubilization of LTC_4 Synthase with Detergent - Microsomal membrane suspensions (typically 15-20 mg protein/ml) were thawed and combined with an equal volume of two-fold concentrated detergent as indicated (usually 4% (w/v) taurocholate) in PBS (pH 7.4), 2 mM EDTA. The mixture was vigorously shaken for 30 min at 4°C then spun at 200,000 x g for 60 min at 4°C. The upper 3/4 of the resulting supernatant was retained. Aliquots of the extracts were frozen in liquid nitrogen and stored at -80°C.

Measurement of LTC_4 Synthase Activity

LTC_4 synthase activity was measured by the formation of LTC_4 in incubation mixtures containing reduced glutathione and LTA_4 (free acid) as determined by reverse-phase HPLC following termination of reactions.

Hydrolysis of LTA_4 Methyl Ester - The methyl ester of leukotriene A_4 was hydrolyzed to the free acid essentially as described previously (Carrier *et al.* 1988). The hydrolysis was monitored by determination of LTA_4 by reverse-phase HPLC at pH 10 (Wynalda et al, 1982) except that LTA_4 and LTA_4-methyl ester were eluted by a gradient of 20 to 70% (v/v) acetonitrile in borate buffer instead of isocratically as described. LTA_4-methyl ester (1.0 mg in hexane) was dried under a stream of nitrogen and then dissolved in 4 ml of 0.25 M NaOH:acetone (2:8, v/v). Following incubation for 60 min at 25°C, the hydrolysis mixture was portioned into aliquots that were stored at -80°C (up to 1 month). (Following this procedure, the recovery of LTA_4 free acid was >95% as confirmed by analysis of the hydrolysis products by reverse-phase HPLC under alkaline conditions (as above). The longevity of the free acid of LTA_4 in incubation mixtures under aqueous condition (see below) was substantially improved by: i) the inclusion of 0.05% (w/v) bovine serum albumin in the incubation mixtures and ii) the use of potassium phosphate as the buffering agent. In their presence, the free acid of LTA_4 had a half-life of approximately 30 min in mock incubation mixtures.) Immediately prior to being used for LTC_4 synthase activity measurement (up to 1 h preceding the assay), an aliquot of the hydrolysis mixture was thawed, dried under a stream of nitrogen and dissolved in absolute ethanol to yield a stock concentration of 4 mM LTA_4 (100-fold the final concentration in LTC_4 synthase incubation mixtures).

Preparation of Serine-Borate Complex - Serine-borate complex was used to inhibit γ-glutamyl transpeptidase activity in order to prevent the conversion of newly-formed LTC_4 to LTD_4 and then to other leukotrienes (Tate and Meister 1978). Separate solutions of 1 M L-serine and 1 M boric acid were prepared in 10 mM Hepes/KOH (pH 7.4). KOH (from a 10 N stock) was added to re-adjust the pH to 7.4 and improve solubility as necessary (heating was also used when required). Equal volumes of the 1 M L-serine and 1 M boric acid solutions (each at pH 7.4) were then combined (producing a 500 mM stock of serine-borate complex; 10-fold the final concentration in LTC_4 synthase incubation mixtures) and stored in aliquots at -20°C. Prior to use, thawed aliquots required warming to 37°C to redissolve the serine-borate complex.

LTC_4 Synthase Incubation Mixtures - Unless otherwise indicated, LTC_4 synthase activity was measured in 0.1 M potassium phosphate (KPi) pH 7.4 buffer (150 µl final volume) in the presence of 50 mM serine-borate complex, 10 mM reduced glutathione and 40 µM LTA_4 (free acid, prepared immediately before used by diluting the 4 mM ethanolic stock described above to 0.4 mM with 0.1 M KPi (pH 7.4) containing 5 mg bovine serum albumin/ml, then further diluting this 10-fold directly in the incubation mixtures (giving a final concentration of 40 µM)). The mixtures were incubated for 15 min at 25°C and reactions were terminated by the addition of an equal volume (150 µl) of cold (4°C) acetonitrile:methanol:acetic acid (at 50:50:1, v/v/v). The mixtures were allowed to stand for a minimum of 30 min at 4°C (or overnight). Precipitated proteins were removed by centrifugation at 16,000 xg for 15 min. The bulk of the resulting supernatant (250 µl) was then transferred to sample vials for reverse-phase HPLC of which 200 µl was injected for analysis.

Analysis of LTC_4 Formation by Reverse-Phase HPLC - The reaction products formed in the incubation mixtures described above were resolved by isocratic reverse-phase HPLC on a Waters Associates Novapak C_{18} column (3.9 x 150 mm, 4 µ particle size). The mobile phase was acetonitrile:methanol:water:acetic acid at 54:14:28:1 adjusted to pH 5.6 with 10 NaOH and was pumped at a flow rate of 1.0 ml/min. LTC_4 was quantified by on-line measurement of the optical density at 280 nm. The LTC_4 peak was identified by its retention time compared to synthetic standard (normally 10 min). In initial experiments to establish the human LTC_4 synthase assay, the identity of the LTC_4 peak was confirmed by i) retention time similarity with synthetic LTC_4, ii) leukotriene spectrum determined with an on-time diode array detector, (iii) the presence of ^3H radioactivity when incubation mixtures contained [14,15-^{3}H(N)]leukotriene A_4 instead of unlabelled LTA_4, iv) the presence of ^{35}S radioactivity when glutathione was replaced with [^{35}S]glutathione, and v) radioimmunoassay (not shown).

Under these assay conditions, the formation of LTC_4 by human LTC_4 synthase was linear up to approximately 100 pmol LTC_4/min in the 150 µl incubation mixtures, corresponding to approximately 800 µg of monocyte protein

or 400 μg of U937 cell protein.

Measurement of Glutathione-S-Transferase Activity

Glutathione-S-transferase activity was measured spectrophotometrically essentially as described previously (Habig *et al.*, 1974). Incubation mixtures were prepared in 0.1 M KPi buffer (pH 6.5). Substrate combinations were either 1 mM 1-chloro-2,4-dinitrobenzene plus 1 mM reduced glutathione (product formation monitored by $\Delta A_{340\ nm}$, $\varepsilon_{340\ nm}$ ($mM^{-1}cm^{-1}$)=9.6) or 5 mM 1,2-epoxy-3-(4-nitrophenoxy) propane plus 5 mM reduced glutathione (product formation monitored by $\Delta A_{360\ nm}$; $\varepsilon_{360\ nm}$ ($mM^{-1}cm^{-1}$)=0.5) or 1 mM 4-nitrobenzyl chloride plus 5 mM reduced glutathione (product formation monitored by $\Delta A_{310\ nm}$, $\varepsilon_{310\ nm}$ ($mM^{-1}cm^{-1}$)=1.9). Microsomal glutathione S-transferase activity was measured in the presence of Triton X-100 following N-ethylmaleimide pretreatment of samples as described previously (McLellan et al, 1989; Mosialou & Morgenstern, 1990)

Photoaffinity Labelling

Synthesis of Azido^{125}I-LTC$_4$ - To a solution of ^{125}I-NHS-ASC (Ji & Ji, 1982) in 200 μl dioxane was added LTC_4 (2 mg in 200 μl phosphate buffer pH 7.4). The mixture was stirred at room temperature overnight. Reverse-phase HPLC of the reaction mixture (on a Waters Associates μ-Bondapak C18 column, 3.8 x 300 mm, using a mobile phase comprised of methanol:H_2O:acetic acid:2-mercaptoethanol (75:25:0.1:0.01, v/v) containing 0.5 mM EDTA) afforded the partially purified azido^{125}I-LTC_4. Repurification using the same solvent conditions (twice) afforded the pure photoaffinity ligand.

Photoaffinity Labelling of Differentiated U937 Cell Membranes - Incubation mixtures (1.0 ml each) were prepared in a buffer comprised of 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol plus 50 mM serine-borate, containing (unless otherwise indicated) 0.3 mg of U937 microsomal membrane protein, 20 pM azido^{125}I-LTC_4 (introduced in ethanol) plus varying concentrations of competing

ligands (either 0.1 to 10 μM LTC4 or 0.1 to 10 mM reduced glutathione). The mixtures were incubated in 1.5 ml microcentrifuge tubes for 30 min at 25°C then transferred to 35 mm diameter cluster plate wells for photolysis. The samples in cluster plates were cooled for 5 min at 4°C then illuminated from above with a 40 W ultraviolet light source (Phillips, λ_{max}=350 nm) at a distance of 5 cm for 2 min at 4°C. The samples were then transferred back into 1.5 ml microcentrifuge tubes and the membranes were re-isolated by centrifugation for 15 min at 200,000 xg. The resulting pellets were dissociated in SDS-containing sample buffer, denatured at 95°C for 5 min and proteins were resolved by SDS-gel electrophoresis (Laemmli, 1970). Radioactive bands in the dried gels were visualized by autoradiography and quantified by laser densitometry.

Miscellaneous

Protein was determined by the method of Bradford (Bradford, 1976) using bovine gamma globulin as standard. SDS/polyacrylamide gel electrophoresis was performed using standard techniques (Laemmli 1970). Western blotting, probing with specific antisera and [^{125}I]protein A labelling was performed essentially according to Burnette (1981).

RESULTS

Measurement of Human LTC_4 Synthase Activity

In order to develop a reliable, quantitative assay for the measurement of LTC_4 synthase activity in tissues of human origin, human whole blood was first fractionated by discontinuous gradient centrifugation through Histopaque (Sigma). Of the four resulting fractions (erythrocytes, granulocytes, mononucleocyte/platelets and plasma), the formation of LTC_4 from added LTA_4 plus reduced glutathione was highest in the presence of the mononuclear cell/platelet fraction (21.5 ± 4.8 pmol•min^{-1}•mg^{-1}, n = 5) (hereafter called monocytes). Taking into account the observations from other studies, optimal assay conditions for human LTC_4 synthase were established using intact monocytes as a source of enzymatic activity (not shown).

Monocytes incubated with LTA_4 and reduced glutathione produced LTC_4, LTD_4 and LTE_4. Therefore, in order to use the formation of LTC_4 to specifically measure LTC_4 synthase activity, it was necessary to block the conversion, by the enzyme γ-glutamyl transpeptidase (EC 2.3.2.2), of newly-formed LTC_4 to LTD_4 with serine-borate complex, a transition-state inhibitor of γ-glutamyl transpeptidase (Tate & Meister 1978). In the absence of serine-borate, the reaction products were predominantly LTE_4 (51.9 pmol•min^{-1}•mg^{-1}) and LTC_4 (27.1 pmol•min^{-1}•mg^{-1}) whereas in the presence of 50 mM serine-borate, the incubation product was exclusively LTC_4 (82.8 pmol•min^{-1}•mg^{-1})(Figure 1). The low amount of detectable LTD_4 was presumably due to the rapid conversion of LTD_4, once formed from LTC_4, into LTE_4 by the enzyme cysteinylglycine dipeptidase (EC 3.4.13.6). The appearance of LTE_4 at the expense of LTC_4 could, however, be blocked entirely by the inclusion of 50 mM serine-borate in incubation mixtures. LTC_4 formation itself was not affected by the presence of serine-borate since total peptide leukotriene formation (LTC_4 plus LTD_4 plus LTE_4) was constant (80 to 84 pmol•min-

^{1}•mg-^{1}) at all serine-borate concentrations. Identical results were observed if 2.5 mM acivicin (Upjohn) was used to inhibit γ-glutamyl transpeptidase activity instead of serine-borate (not shown).

LTC_4 formation in the presence of monocytes was highest at alkaline pH (Figure 2). In the absence of monocytes, the non-enzymatic conjugation of reduced glutathione with LTA_4 became significant at > pH 7.5 (reaching 3 pmol/min in the150 µl incubation mixtures at pH 8.5) contributing, in part, to the high levels of LTC_4 formation observed at alkaline pH in the presence of monocytes. In addition to non-enzymatic LTC_4 formation, the enhanced stability of the LTA_4 substrate under alkaline conditions (not shown) probably contributed to higher levels of LTC_4 formation in the presence of monocytes at high pH. In order to assay LTC_4 synthase under physiologically relevant conditions and to minimize non-enzymatic LTC_4 formation, all subsequent incubations were performed at pH 7.4.

In summary, an assay was established and optimized for the specific measurement of human LTC_4 synthase activity using monocytes isolated from peripheral blood. The presence of 0.1 M potassium phosphate and 0.05% (w/v) bovine serum albumin markedly improved the stability of the substrate LTA_4 in LTC_4 synthase incubation mixtures such that the formation of LTC_4 was linear for 20 min at 25°C (the optimal temperature) with up to 800 µg monocyte protein (not shown). The presence of 50 mM serine-borate in incubation mixtures prevented the underestimation of LTC_4 formation by inhibiting γ-glutamyl transpeptidase and thereby preventing the further metabolism of newly formed LTC_4 (a precaution not taken in other studies of LTC_4 synthase). Contrary to that demonstrated with guinea-pig lung microsomes (Yoshimoto *et al.* 1988), the preferred substrate for human LTC_4 synthase was the free acid of LTA_4 and not LTA_4-methyl ester (Figure 2, open versus closed squares). The Km for LTA_4 was 5.6 µM and for reduced glutathione was 1.2 mM (not shown; 40 µM LTA_4 and 10 mM reduced glutathione were routinely used in incubation mixtures). LTC_4 synthase

activity was inhibited by N-ethylmaleimide (IC_{50} value = 20 μM) and by diethylcarbamazine (IC_{50} value = 50 μM) but was not inhibited by up to 50 μM S-hexyl glutathione (not shown).

LTC_4 Synthase Activity is Highly Expressed in Dimethylsulfoxide-Differentiated U937 Cells

The specific activity of LTC_4 synthase measured in the promonocytic leukemia cell line U937 (39.9 ± 16.7 pmol•min^{-1}•mg^{-1}, n=8) was marginally higher ($p < 0.5$) than that in freshly isolated blood monocyte fraction (21.5 ± 4.8 pmol•min^{-1}•mg^{-1}, n=5). When differentiated by growth in the presence of 1.3% (v/v) Me_2SO, U937 cells expressed approximately 10-fold higher levels of LTC_4 synthase activity compared to undifferentiated U937 cells (Figure 3). No increase in LTC_4 synthase activity was seen when macrophage-like cells were generated by growth of U937 cells in the presence of 10 nM phorbol-12-myristate-13-acetate. Expression of LTC_4 synthase in the promyelocytic cell line HL-60 occurred when they were grown in the presence of either Me_2SO (producing neutrophil-like cells) or phorbol-12-myristate-13-acetate (producing macrophage-like cells). The highest LTC_4 synthase specific activity observed in differentiated HL-60 cells, however, was approximately the same as untreated U937 cells (inset of Figure 3).

Expression of LTC_4 synthase activity in U937 cells was dependent on the concentration of Me_2SO in the culture medium (Figure 4). The highest LTC_4 synthase specific activities were present in cells grown in the presence of 1.5 to 1.6% (v/v) Me_2SO, but at the expense of cell growth and viability. Overall, the activity of LTC_4 synthase in differentiated U937 cells grown in the presence of 1.3% Me_2SO was 10-fold higher than in undifferentiated U937 cells (399.0 ± 84.1 pmol•min^{-1}•mg^{-1} (n=47) versus 39.9 ± 16.7 pmol•min^{-1}•mg^{-1} (n=8), respectively; $p< 0.001$). U937 cells that were maintained in culture over long periods of time (e.g. > 35 population doublings) had reduced responsiveness to added Me_2SO,

although levels of LTC_4 synthase activity were still 6 to 7-fold higher in these older cultures than in undifferentiated U937 cells (not shown).

Human LTC_4 Synthase is a Unique Enzyme, Distinct from Other Glutathione S-Transferases

Glutathione S-transferases are ubiquitous in most cell types and catalyze the conjugation of glutathione with a broad variety of substrates. We therefore wanted to determine whether LTC_4 formation in Me_2SO-differentiated U937 cells was due to the activity of previously characterized glutathione S-transferases or was the product of an independent LTC_4 synthase enzyme. To address this question, Me_2SO-differentiated U937 cells were first ruptured by nitrogen cavitation and fractionated by differential centrifugation (Table 1). LTC_4 synthase activity was predominantly located in the 100,000 xg microsomal pellet (61%) and the specific activity was enriched 3.8-fold. The plasma membrane marker enzymes γ-glutamyl transpeptidase and leucine aminopeptidase were also enriched in the microsomal fraction. Similar results were obtained when peripheral blood monocytes were fractionated by the same procedure (not shown).

LTC_4 synthase activity could be quantitatively solubilized by mixing microsomal membranes from Me_2SO-differentiated U937 cells with the anionic detergent taurocholate (2% (w/v) final concentration) followed by centrifugation at 200,000 xg (not shown). Taurocholate-solubilized microsomal membranes were then applied to an anion-exchange column which was subsequently developed with a linear NaCl gradient (Figure 5A). LTC_4 synthase activity eluted as a single peak (centered in fraction 25) at 0.75 M NaCl. Two peaks of glutathione-S-transferase activity (fractions 3 and 20) were detected using 1-chloro-2,4-dinitrobenzene as co-substrate in the assay. Neither of the two glutathione S-transferase activities was stimulated, however, by pretreatment with N-ethylmaleimide (a feature characteristic of the microsomal glutathione S-

transferase). Fractions containing glutathione S-transferase activity using 1-chloro-2,4-dinitrobenzene as a co-substrate (fractions 3 and 20) did not conjugate glutathione with LTA_4 to form LTC_4. The opposite was also true; namely, the LTC_4 synthase activity peak (fraction 25) showed no detectable glutathione-S-transferase activity when measured in the presence of 1-chloro-2,4-dinitrobenzene (Figure 5A) or in the presence of 4-nitrobenzyl-chloride or 1,2-epoxy-3-(p-nitrophenoxy)-propane (not shown).

Western blot analysis of fraction 3, 20 and 25 was performed using polyclonal antisera raised against purified human α, μ, π and microsomal glutathione S-transferases (Figure 5B). Fractions 3 and 20 were found to contain π and μ class glutathione S-transferase, respectively (normally cytosolic, they were probably contaminants carried over during membrane preparation). The microsomal glutathione S-transferase, present in the membrane fraction prior to detergent solubilization, was not extracted by taurocholate and was therefore not part of the sample applied to the anion exchange column.

The fraction from anion exchange chromatography containing LTC_4 synthase activity (fraction 25) did not contain any polypeptides specifically recognized by the anti-α, anti-μ, anti-π or anti-microsomal glutathione S-transferase antiserums. A specific antibody for the recently identified θ glutathione S-transferase (Meyer *et al.* 1991) was not available for these studies; however, since no activity was detectable using 1,2-epoxy-3-(p-nitrophenoxy)-propane in any of the fractions we conclude that θ class glutathione S-transferase was not present in these preparations (not shown).

The biosynthesis of LTC_4 in human cells is therefore catalyzed by a unique LTC_4 synthase enzyme that is distinct from other known glutathione S-transferases.

A Radioactive LTC_4 Photoaffinity Ligand Specifically Labels an 18 kDa Polypeptide in Microsomal Membranes from Me_2SO-Differentiated U937 Cells

In order to probe U937 microsomal membranes for LTC_4 synthase candidate polypeptides or subunits of LTC_4 synthase, an iodinated photoaffinity ligand based on the product of the LTC_4 synthase catalyzed reaction (LTC_4) was synthesized. LTC_4 was chosen as the affinity ligand over the two LTC_4 synthase substrates (LTA_4 and reduced glutathione) owing in the case of LTA_4 to the disadvantages of the labile epoxide bond and high hydrophobicity, and in the case of reduced glutathione to the predicted low affinity of glutathione-based photoaffinity ligands for LTC_4 synthase, as indicated by the relatively high Km for glutathione (1.2 mM), and the liklihood that they would also label glutathione S-transferases. In contrast, a photoaffinity ligand based on LTC_4 would not have the same solubility and stability disadvantages. In addition, it would be a specific probe for proteins that recognize both the arachidonic acid backbone plus the glutathione moiety of LTC_4.

A radioiodinated, photoreactive derivative of LTC_4 having high specific activity (ca. 2200 Ci/mmol) was therefore synthesized (azido^{125}I-LTC_4) (Figure 6A). Its relative affinity for LTC_4 synthase was tested by the ability of a non-radioactive, but otherwise identical, LTC_4 photoaffinity ligand (azido^{127}I-LTC_4) to inhibit LTC_4 synthase catalyzed formation of LTC_4 in standard incubation mixtures containing 40μM LTA_4 and 10 mM glutathione (Figure 6B). LTC_4 synthase biosynthetic activity was specifically inhibited by LTC_2 (IC_{50} value = 2.6 μM) and by azido^{127}I-LTC_4 (IC_{50} value = 7.0 μM) but not by LTB_4. (LTC_2 was used instead of LTC_4 as a positive control for the competitive inhibition of LTC_4 synthase since its retention time on reverse-phase HPLC was markedly different than LTC_4, making enzymatically-formed LTC_4 distinguishable from LTC_2 that was added to the incubation mixtures for inhibition. LTC_2 was a competitive inhibitor of LTC_4 synthase as determined by kinetic analysis in which the effect of varying substrate concentrations on the initial rate of LTC_4 formation was tested in the presence of fixed concentrations of

LTC_2 (0, 0.75 or 2.0 μM). Double-reciprocal (Lineweaver-Burk) plots of 1/(nmol LTC_4 formed•min^{-1}•mg^{-1}) versus 1/[substrate] showed that LTC_2 was competitive with respect to both reduced glutathione and LTA_4, although in the latter case interpretation of data was complicated by the lability of LTA_4, particularly at low concentrations, and the presence of BSA with which LTA_4 was also in equilibrium. (Data not shown) The comparable inhibition profiles of azido ^{127}I-LTC_4 and LTC_2 therefore indicated that the photoaffinity ligand was specifically recognized by (and therefore inhibited) LTC_4 synthase.

The equivalent radioactive form of the LTC_4 photoaffinity probe (azido ^{125}I-LTC_4) was incubated with microsomal membranes from Me_2SO-differentiated U937 cells (Figure 7A) in the presence of varying concentrations of either LTC_4 (0.1 - 10 μM) or reduced glutathione (0.1 - 10 mM) as competing ligands. Following photolysis, the photoaffinity ligand specifically labelled an 18 kDa and a 27 kDa polypeptide. Photolabelling of the 18 kDa polypeptide was specifically competed for by low concentrations of LTC_4 (0.1 μM LTC_4 reduced specific labelling by half, compare lane 7 versus 3) but was not affected by the presence of even 10 mM reduced glutathione (compare lane 8 versus 3). The opposite was true for the specific labelling of the 27 kDa band which was nearly abolished by the lowest concentration of reduced glutathione tested (0.1 mM, compare lane 10 versus 3) and was incompletely competed for by up to 10 μM LTC_4 in the incubation mixtures. The photolabelling of a 50 kDa polypeptide was partially competed for by LTC_4; however, this was not consistently the case in all experiments and was therefore not considered further. The level of the 18 kDa polypeptide that could be specifically photolabelled by azido ^{125}I-LTC_4 was elevated in Me_2SO-differentiated U937 cells versus undifferentiated U937 cells (Figure 7B). Specific (LTC_4 competed) photolabelling of the 18 kDa polypeptide was approximately 5-fold higher in the membranes from differentiated cells, paralleling the increase in LTC_4 synthase biosynthetic activity in these cells. (Note: the elevation in the photolabelable 18 kDa polypeptide resulting from Me_2SO differentiation (5-fold) did not exactly match the increase in LTC_4 synthase

specific activity (10-fold). This discrepancy appeared to be a result of a substantial increase in total non-specific (not LTC_4 competed) binding of azido^{125}I-LTC_4 to membrane polypeptides accompanying differentiation, thereby reducing the amount of azido^{125}I-LTC_4 free to associate with the 18 kDa polypeptide; not shown.) The level of the photolabelled 27 kDa polypeptide was reduced following Me_2SO differentiation (not shown).

We conclude that the azido^{125}I-LTC_4 specifically bound to and subsequently photolabelled two polypeptides in microsomal membranes from Me_2SO-differentiated U937 cells (18 and 27 kDa). The 27 kDa protein may have recognized and bound the glutathione moiety of the photoaffinity ligand, and therefore photolabelling could be specifically competed for by both reduced glutathione and by LTC_4. Photolabelling of the 18 kDa polypeptide by azido^{125} I-LTC_4, on the other hand, was specifically competed for by LTC_4 at 0.1 μM but not by even a 100,000-fold higher concentration of reduced glutathione (10 mM). The 18 kDa protein therefore recognizes the arachidonic acid backbone of the azido^{125} I-LTC_4 photoaffinity ligand and as a consequence is a strong candidate for being either LTC_4 synthase or a subunit of LTC_4 synthase. In support of this, the level of the specifically photolabelled 18 kDa polypeptide was elevated following Me_2SO differentiation of U937 cells (5-fold) as was LTC_4 synthase biosynthetic activity (10-fold).

DISCUSSION

The human leukemia cell lines HL-60 and U937 have the histochemical and morphological characteristics of immature cells of myelomonocytic lineage. When differentiated by growth in culture in the presence of various agents, they can be converted to the neoplastic equivalents of polymorphonuclear granulocytes (HL-60) or mononuclear phagocytes (HL-60 and U937) depending on growth conditions (for review see Harris and Ralph, 1985). Since HL-60 and U937 cells can be selectively grown to resemble differentiated myeloid cells having properties similar to those found in human blood, they have been used to examine the mechanisms and mediators involved in inflammatory processes. These cells lines are furthermore proving to be suitably abundant sources of human tissue for enzyme and receptor characterization as well as purification, such as has been recently reported for a U937 cell arachidonate-selective phospholipase A_2 (Clark *et al.*, 1991; Kramer *et al.*, 1991).

We have examined the biosynthesis of peptidoleukotrienes, specifically the activity of LTC_4 synthase which catalyses the first committed step in peptide leukotriene formation, in freshly isolated human blood cells and then in both HL-60 and U937 cells. LTC_4 synthase specific activity in undifferentiated U937 cells was comparable to that found in density gradient-purified monocytes from freshly drawn human blood. When converted to monocyte/macrophage-like cells by growth in the presence of Me_2SO, however, U937 cells expressed 10-fold higher LTC_4 synthase activity (Figures 3 and 4). The concentration of Me_2SO and time required in culture to provoke this response was nearly identical to that reported for full expression of the U937 cell monocyte/macrophage phenotype (Harris and Ralph, 1985). The average specific activity of LTC_4 synthase in Me_2SO differentiated U937 cells (399 pmol•min^{-1}•mg^{-1}) was similar to that reported in guinea pig lung (386 pmol•min^{-1}•mg^{-1}; Izumi *et al.* 1988) but markedly higher than that reported in human lung (90 pmol•min^{-1}•mg^{-1}; Izumi *et al,* 1988) human

monocyte/platelet fraction (21.5 pmol•min^{-1}•mg^{-1}; $p < 0.001$; this study), human granulocytes (2.3 pmol•min^{-1}•mg^{-1}; $p<0.001$; this study), mixed human peritoneal cells (8.1 pmol•min^{-1}•mg^{-1}; Taylor *et al.* 1988) and rat basophilic leukemia cells (13 pmol•min^{-1}•mg^{-1}; Yoshimoto *et al.* 1985). The expression of LTC_4 synthase activity following growth of U937 cells in the presence of Me_2SO was not paralleled by substantially increased levels of either γ-glutamyl transpeptidase or cysteinylglycine dipeptidase activities, both of which were present in undifferentiated U937 cells and were elevated 2.2-fold in Me_2SO-differentiated cells (Rasper and Nicholson, unpublished).

Previous reports have described the increased expression of leukotriene receptors in Me_2SO-differentiated U937 cells, including a 3-fold increase in LTD_4 receptor binding sites (Sarau and Mong, 1989; Frey, Nicholson & Metters, submitted) and 30-fold higher levels of LTB_4 receptor binding sites (Sarau and Mong, 1989). Other activities relating to leukotriene formation were also increased to varying degrees during differentiation. For example, arachidonate-specific phospholipase A_2 activity was only marginally elevated (1.5 to 2-fold) in Me_2SO-differentiated U937 cells (Weech and Nicholson, unpublished). Interestingly, neither undifferentiated nor Me_2SO-differentiated U937 cells contained detectable 5-lipoxygenase and were therefore incapable of making leukotrienes in response to ionophore or peptide challenge. The expression of 5-lipoxygenase-activating protein (FLAP), however, was increased 4-fold in U937 cells differentiated by growth in the presence of Me_2SO. Consequently, U937 cells that were transfected with 5-lipoxygenase cDNA and then differentiated by growth in the presence of Me_2SO were capable of making peptide leukotrienes in response to ionophore stimulation (Mancini *et al.* 1991; Evans *et al.* in preparation). Taken together, these studies demonstrate that the entire biosynthetic pathway for peptide leukotriene biosynthesis (and at least two specific leukotriene receptors) is present in Me_2SO-differentiated monocyte/macrophage-like U937 cells, with the exception of 5-lipoxygenase. As a consequence, Me_2SO-differentiated U937 cells may be a useful defined human

cell line for examining the mechanism of transcellular leukotriene biosynthesis in co-culture with cells having 5-lipoxygenase activity and competence for LTA_4 biosynthesis.

In summary, LTC_4 synthase levels in Me_2SO-differentiated U937 cells were 10-fold higher than those in undifferentiated monoblast progenitor cells. The increase in LTC_4 synthase activity observed following Me_2SO-induced differentiation cells was substantially higher than that observed for other enzymes and proteins involved in peptide leukotriene formation. The high specific activity of LTC_4 synthase in these cells (399 ± 84 pmol LTC_4 formed•min^{-1}•mg^{-1}) was higher than that in tissues from all other sources reported and therefore makes Me_2SO-differentiated U937 cells a suitable source for the further characterization, purification and cloning of the human enzyme. In this study, LTC_4 synthase specific activity was enriched 20-fold by anion-exchange chromatography following detergent solubilization of microsomal membranes from Me_2SO-differentiated U937 cells.

LTC_4 is formed by the conjugation of glutathione to the unstable epoxide intermediate LTA_4. As such, the enzyme that catalyzes the biosynthesis of LTC_4 is a member of the glutathione S-transferase family of enzymes. The majority of glutathione S-transferases are soluble dimeric enzymes belonging to one of four multi-gene families (α, μ, π and θ). In addition, an N-ethylmaleimide-activatable membrane-bound microsomal glutathione S-transferase exists in some cell types (McLellan *et al.*, 1989). An important consideration in these studies, therefore, was to determine whether LTC_4 formation in human cells was catalyzed by a distinct LTC_4 synthase enzyme, or was a product of the enzymatic activity of a previously identified glutathione S-transferase. Evidence in both rat (Yoshimoto *et al.* 1985) and guinea-pig (Yoshimoto *et al.* 1988) indicates that rodent LTC_4 synthase activity is distinguishable from other glutathione S-transferase activities, although neither microsomal nor θ-class glutathione S-transferase activities were specifically tested for in these studies.

Our studies show that human LTC_4 synthase from Me_2SO-differentiated U937 cells is a unique enzyme, distinguishable from other known glutathione S-transferases by the following criteria: 1) LTC_4 biosynthesis occurs exclusively in the membrane fraction of differentiated U937 cells, making it unlikely that any of the soluble glutathione S-transferases account for LTC_4 formation. 2) LTC_4 synthase activity was chromatographically separated from two peaks of glutathione S-transferase activity (using 1-chloro-2,4-dinitrobenzene as co-substrate); one of which was identified by Western blotting as π-class glutathione S-transferase and one as μ -class glutathione S-transferase (presumably cytosolic contaminants carried over during membrane preparation)(Figure 5). 3) The chromatographic fraction having LTC_4 synthase activity was devoid of glutathione S-transferase activity towards 1-chloro-2,4-dinitrobenzene in both the absence of N-ethylmaleimide (which can be used to detect most glutathione S-transferase activities) and presence of N-ethylmaleimide pretreatment (which can be used to specifically measure microsomal glutathione S-transferase). Similarly, the fraction was not active using either 4-nitrobenzyl chloride or 1,2-epoxy-3-(p-nitrophenoxy)-propane (the latter of which can be used to detect θ-class glutathione S-transferase activity) as co-substrates with glutathione. 4) LTC_4 biosynthetic activity could not be due to microsomal glutathione S-transferase since microsomal glutathione S-transferase was not solubilized from membranes by taurocholate (as determined by both N-ethylmaleimide-insensitive enzymatic activity (not shown) and by Western blot analysis (Figure 5)) whereas > 85% of membrane-bound LTC_4 synthase was solubilized by taurocholate. 5) Antisera raised against purified human α, μ, π and microsomal glutathione S-transferases did not specifically recognize polypeptides in the fraction containing LTC_4 biosynthetic activity (as determined by Western blot analysis in Figure 5).

In order to identify putative polypeptides that could be LTC_4 synthase, or subunits of LTC_4 synthase, we designed a specific radio-iodinated photoaffinity probe based on LTC_4, the product of the LTC_4 synthase catalyzed reaction.

Human LTC_4 synthase is subject to end product inhibition as indicated by the ability of the LTC_4 analogue LTC_2 (chosen because of its difference in HPLC retention time compared to LTC_4) to inhibit LTC_4 formation from LTA_4 and reduced glutathione (Figure 6). (The Ki for LTC_2 was 0.32μM, whereas the Km for LTA_4 was 5.6μM and for reduced glutathione was 1.2 mM.) As such, a photoaffinity probe based on the reaction product was predicted to be able to specifically bind to and subsequently label LTC_4 synthase. Azido^{125}I-LTC_4 specifically labelled two polypeptides in Me_2SO-differentiated U937 cell membranes. Photolabelling of the 18 kDa polypeptide was specifically competed for by 100,000-fold lower concentrations of LTC_4 than reduced glutathione, and it is therefore a strong candidate for being LTC_4 synthase, although it is not entirely possible to exclude other LTC_4 or glutathione binding proteins such as microsomal glutathione S-transferase, γ-glutamyl transpeptidase, putative LTC_4 receptor sites or transport proteins (Lam *et al.* 1989; Ishikawa *et al.* 1990). Nevertheless, owing to the high degree of specificity with which LTC_4 displaced azido^{125}I LTC_4 in competitive photolabelling experiments and the concomitant elevation of both the specifically labelled 18 kDa polypeptide and LTC_4 synthase activity accompanying the differentiation of U937 cells with Me_2SO, the Mr of human LTC_4 synthase or one of its subunits may be 18,000.

The present data strongly indicate that LTC_4 synthase is a unique enzyme specifically dedicated to the formation of LTC_4 from LTA_4 and distinct from the more generalized glutathione S-transferases. Thus, LTC_4 synthase is a potentially important controlling enzyme whose activity could substantially affect the profile of leukotriene release in pathological situations.

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FIGURE LEGENDS

Figure 1. **Serine-borate (an inhibitor of γ-glutamyl transpeptidase) increases detectable LTC_4.** Human monocytes (500 μg protein) were incubated in standard mixtures in the presence of varying concentrations of serine-borate complex for 15 min at 25°C as described in Methods. The reactions were terminated and the amounts of LTC_4 (○), LTD_4 (●) and LTE_4 (❑) formed were determined by reverse-phase HPLC. (Note. The LTE_4 peak on HPLC was partly obscured by the 6-trans LTB_4 breakdown product of LTA_4. LTE_4 was therefore quantified by subtracting the area attributable to 6-trans LTB_4 (determined in controls to which no cells were added) from the area of the combined LTE_4 plus 6-trans LTB_4 peak.) Total peptide leukotriene formation (■) is the sum of LTC_4 + LTD_4 + LTE_4.

Figure 2. **Effect of pH on LTC_4 biosynthesis.** Standard incubation mixtures were prepared as described in Methods except that i) the buffer used was comprised of 25 mM PIPES (pKa=6.8), 25 mM Tricine (pKa=8.1), 25 mM CHES (pKa=9.3), adjusted to the indicated pH with KOH, and ii) either the free acid of LTA_4 (○,●)or LTA_4-methyl ester (■) was used. Except for where the non-enzymatic formation of LTC_4 was tested (●), the mixtures contained human monocytes (500 μg protein)(○,●,■). All samples were incubated for 15 min at 25°C after which LTC_4 (○,●) or LTC_4-methyl ester (■) formation was determined by reverse-phase HPLC. Enzymatic (protein dependent) LTC_4 formation (❑) was calculated by subtracting the amount of non-enzymatic LTC_4 formed in the absence of monocytes (●) from total LTC_4 formed in the presence of monocytes (○).

Figure 3. **Time course of expression of LTC_4 synthase activity in differentiated U937 cells grown in culture.** U937 cells were seeded at a density of 0.2×10^6 cells/ml of complete RPMI-1640 medium supplemented with either 1.3% (v/v)

dimethylsulfoxide (Me_2SO;○) or 10 nM phorbol-12-myristate-13-acetate (PMA;●) and were grown in culture at 37°C as described in Methods. At 24 h intervals, aliquots were withdrawn, cells were harvested by centrifugation and LTC_4 synthase activity was determined. The data presented are the average of two experiments (values were within 10% of each other). An identical experiment was performed using HL-60 cells in IMDM medium supplemented with either 1.3% (v/v) dimethylsulfoxide (❑) or 10 nM phorbol-12-myristate-13-acetate (■) (inset).

Figure 4. **Effect of dimethylsulfoxide concentration of LTC_4 synthase expression in U937 cells.** Forty ml aliquots of U937 cells were seeded in 175 cm^2 culture flasks at an initial density of $0.12x10^6$ cells/ml medium. Triplicate samples were supplemented with varying concentrations of dimethylsulfoxide as indicated. Following 5 days in culture, the cells were counted and the number of viable cells determined by Trypan Blue exclusion (○). The cells were harvested by centrifugation at 600 xg for 15 min, washed in PBS(pH 7.4), 2 mM EDTA and resuspended at $1x10^8$ viable cells/ml in PBS(pH 7.4), 2 mM EDTA. LTC_4 synthase specific activity (●) was then determined as described in Methods. Data are expressed as the average ± s.d.

Figure 5. **Human LTC_4 synthase is distinct from other glutathione S-transferase activities.** *A. Chromatographic separation of LTC_4 synthase activity from glutathione S-transferase activities by anion exchange.* The 100,000 xg membrane fraction from Me_2SO-differentiated U937 cells was solubilized with 2% (w/v) taurocholate as described in Methods. A portion of the taurocholate extract (2 ml) was applied to an anion exchange column (MonoQ HR5/5, 0.5 x 5 cm, Pharmacia) that had been pre-equilibrated in 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 4 mM reduced glutathione, 1 mM dithiothreitol, 0.1% (w/v) taurocholate (buffer A) at a flow rate of 1.0 ml/min. After further equilibration in buffer A, proteins were eluted by a linear gradient from 0 to 1.0 M NaCl in buffer A as

indicated (dashed line). Fractions (1.0 ml) were collected throughout and were subsequently assayed for LTC_4 synthase activity (●) and for glutathione S-transferase activity (using 1-chloro-2,4-dinitrobenzene as co-substrate) in the absence (❑) or presence (○) of N-ethylmaleimide preactivation as described in Methods. *B. Western blot analysis.* The indicated samples were resolved by SDS gel electrophoresis on 8-16% gradient gels and then transferred to nitrocellulose by Western blotting. The nitrocellulose membranes were probed with antisera raised against purified human α, μ, π or microsomal glutathione S-transferases, as indicated. The nitrocellulose membranes were then decorated with [^{125}I]Protein A and exposed by autoradiography. The relevant area of the resulting autoradiographs is shown. The positions of molecular weight standards (x10-3) are indicated on the right for each blot. Samples were 100,000 xg microsomal membranes (Memb), the 200,000 xg supernatant following taurocholate solubilization (TCsup), the 200,000 xg pellet following taurocholate solubilization (TCpellet), and fractions 3 (f3), 20 (f20) and 25 (f25) derived from chromatographic separation of the taurocholate supernatant described in panel A.

Figure 6. **Inhibition of LTC_4 synthase activity by Azido^{127}I-LTC_4.** *A. Structure of the LTC_4 photoaffinity ligand azido^{125}I-LTC_4.* Azido^{125}I-LTC_4 was synthesized as described in Methods. It is comprised of a photoreactive, radioiodinated aryl azide group attached via a spacer arm to the α-amino group of the γ-glutamate residue of LTC_4. *B. The non-radioactive (^{127}I) but otherwise identical form of the LTC_4 photoaffinity inhibits LTC_4 synthase activity.* Standard LTC_4 synthase incubation mixtures containing 100,000 xg microsomal membranes (75 μg protein) from Me_2SO-differentiated U937 cells, plus varying concentrations of either LTC_2 (○), LTB_4 (❑) or azido^{127}I-LTC_4 (●) were incubated for 15 min at 25°C. The LTC_4 formed was then determined by reverse-phase HPLC as described in Methods. Activity is expressed as a percentage of the control to which no competing ligand was added.

Figure 7. **Photoaffinity labelling of microsomal membrane proteins by azido^{125}I-LTC$_4$.** *A. Photolabelling of Me$_2$SO-differentiated U937 cell microsomal membranes.* Incubation mixtures (1 ml each) were prepared containing 0.3 mg of microsomal membrane protein from Me$_2$SO-differentiated U937 cells (except lanes 1-4 as indicated), 20 pM azido^{125}I-LTC$_4$, plus either 10, 1.0, 0.1μM LTC$_4$ (lanes 5-7) or 10, 1.0, 0.1 mM reduced glutathione (lanes 8-10). The mixtures were incubated for 30 min at 25°C, cooled for 5 min at 4°C then photolyzed for 2 min as described in Methods. The membranes were re-isolated by centrifugation for 15 min at 200,000 xg, dissociated in SDS-containing sample buffer and resolved on an SDS polyacrylamide gel. An autoradiograph of the resulting dried gel of a representative experiment (n>5) is shown. The migration of molecular weight standards (x10^{-3}) are indicated on the left. The position of two specifically-labelled polypeptides are indicated by arrowheads on the right. Lane 3 is the control sample (no competing ligand, but otherwise identical) for lanes 5-10. Photolabelling was quantified by laser densitometry. *B. Photolabelling of an 18 kDa polypeptide in undifferentiated versus Me$_2$SO-differentiated U937 cell microsomal membranes.* Incubation mixtures (1 ml each) were prepared containing 0.3 mg of microsomal membrane protein from either undifferentiated U937 cells (lanes 1 & 2) or Me$_2$SO-differentiated U937 cells (lanes 3 & 4) and 20 pM azido^{125}I-LTC$_4$. The mixtures were incubated for 30 min at 25°C in the absence (lanes 1 & 3) or presence (lanes 2 & 4) of 10 μM LTC$_4$ then photolyzed and processed as described for panel A. Photolabelling of an 18 kDa protein is shown.

Table 1. **Subcellular localization of human LTC_4 synthase activity in Me_2SO-differentiated U937 cells.** Me_2SO-differentiated U937 cells were ruptured by nitrogen cavitation (15 min at 800 psi) and then fractionated by successive centrifugation runs at 1000 xg (15 min), 10,000 xg (20 min) then 100,000 xg (60 min). The resulting pellets from each centrifugation run were resuspended in PBS (pH7.4), 2 mM EDTA. LTC_4 synthase specific activity was then determined in each fraction as described in Methods. The relative specific activity is expressed with respect to the lysate following nitrogen cavitation, which was set at 1. Recovery in each fraction following differential centrifugation is expressed as a percentage of the total activity in the nitrogen cavitation lysate (which itself was >90% of the activity in the intact cells). The specific activities of both γ-glutamyl transpeptidase and leucine aminopeptidase (plasma membrane marker enzymes) were highest in the 100,000xg pellet (not shown). Results from a typical experiment are shown (similar results using monocytes isolated from fresh blood were also observed; not shown).

Fraction	Specific Activity (pmol $LTC_4 \cdot min^{-1} \cdot mg^{-1}$)	Relative Specific Activity	% of Total Activity
Intact dU937 Cells	266		
N_2 Cavitation Lysate	241	1.00 (set)	100 (set)
1000 xg Pellet	270	1.12	17.5
10,000 xg Pellet	658	2.73	21.5
100,000 xg Pellet	922	3.84	61.0
100,000 xg Supernatant	0	0	0

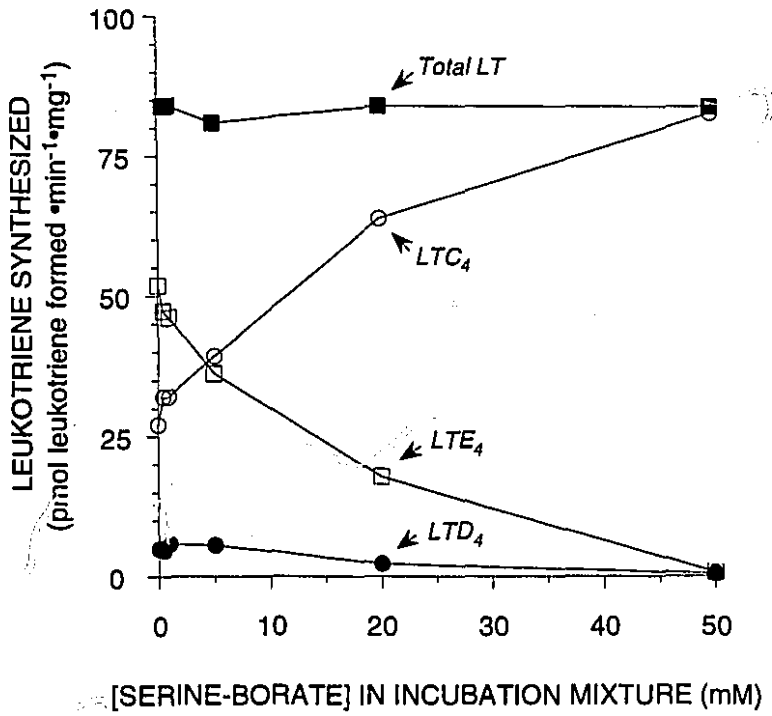
100
75
50
25
0
0
10
20
30
40
50
Total LT
LTC_4
LTE_4
LTD_4
LEUKOTRIENE SYNTHESIZED
(pmol leukotriene formed •min^{-1}•mg^{-1})
[SERINE-BORATE] IN INCUBATION MIXTURE (mM)

Figure 1

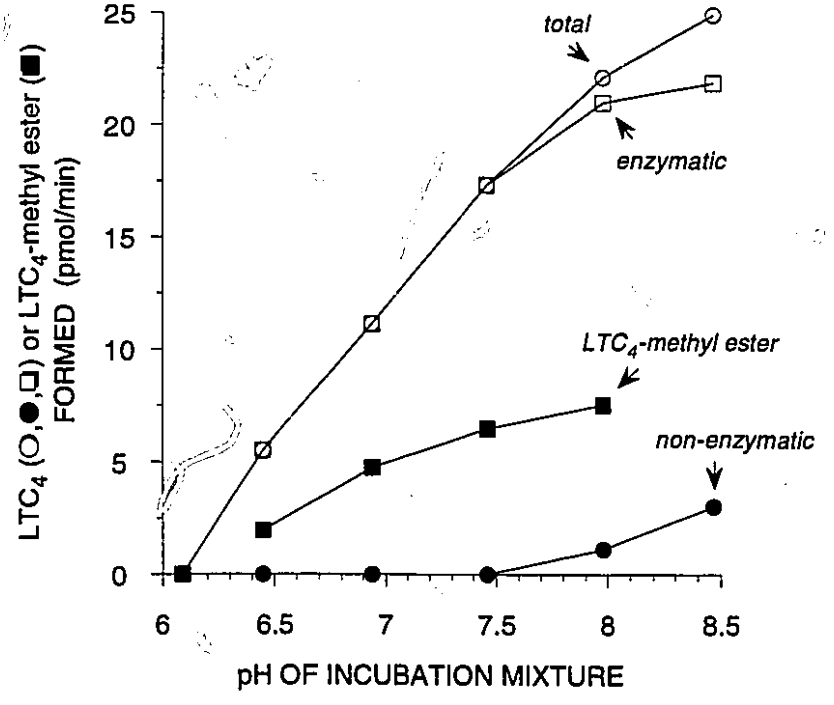
25
20
15
10
5
0
6
6.5
7
7.5
8
8.5
total
enzymatic
LTC4-methyl ester
non-enzymatic
LTC4 (O,●,□) or LTC4-methyl ester (■) FORMED (pmol/min)
pH OF INCUBATION MIXTURE

Figure 2

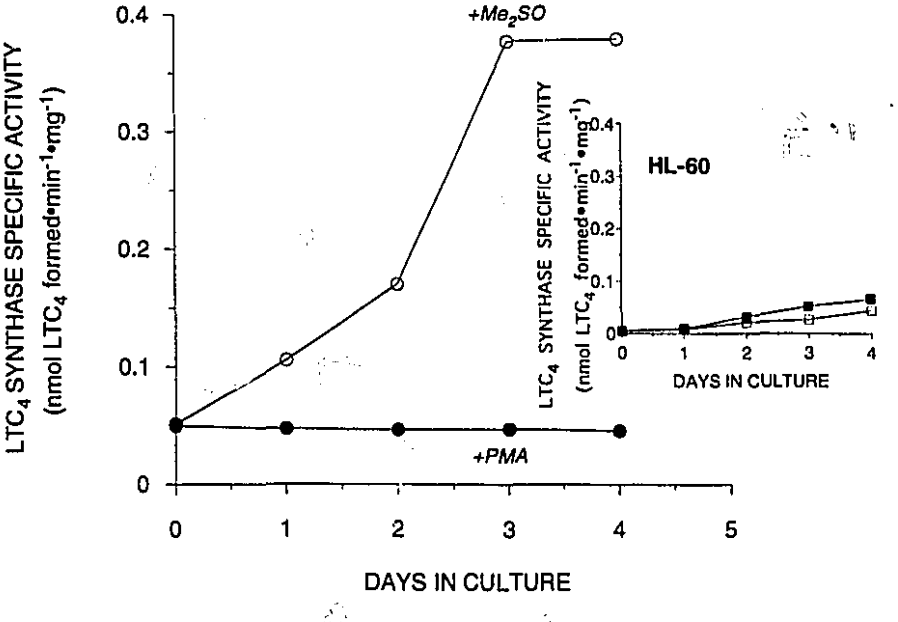
LTC4 SYNTHASE SPECIFIC ACTIVITY
(nmol LTC4 formed•min-1•mg-1)
+Me2SO
+PMA
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
DAYS IN CULTURE
HL-60
LTC4 SYNTHASE SPECIFIC ACTIVITY
(nmol LTC4 formed•min-1•mg-1)
0.4
0.3
0.2
0.1
0
0
1
2
3
4
DAYS IN CULTURE

Figure 3

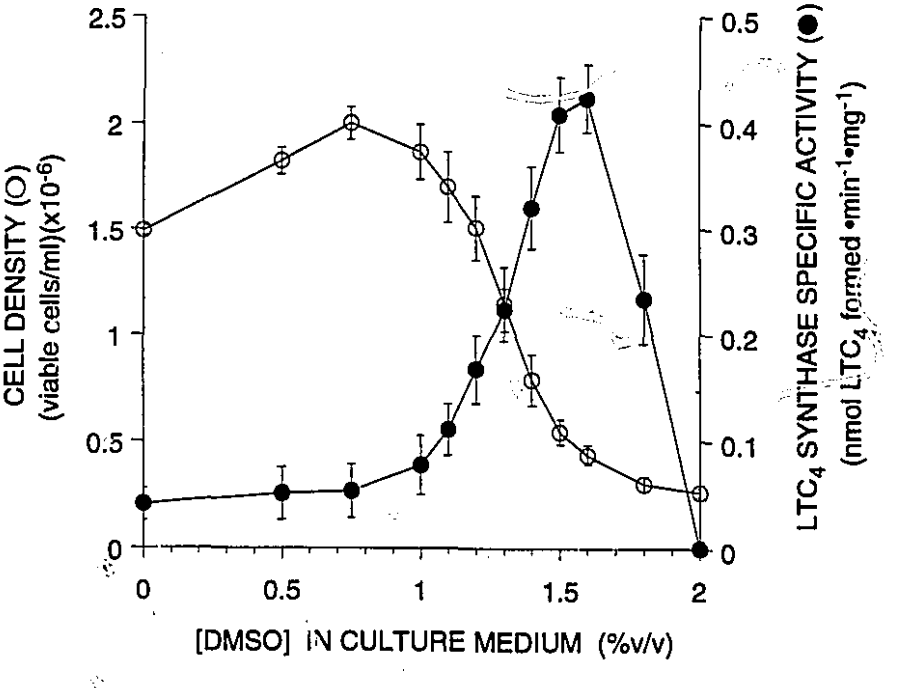
CELL DENSITY (O)
(viable cells/ml)(x10-6)
LTC4 SYNTHASE SPECIFIC ACTIVITY (●)
(nmol LTC4 formed •min-1•mg-1)
[DMSO] IN CULTURE MEDIUM (%v/v)
0
0.5
1
1.5
2
2.5
0.1
0.2
0.3
0.4
0.5

Figure 4

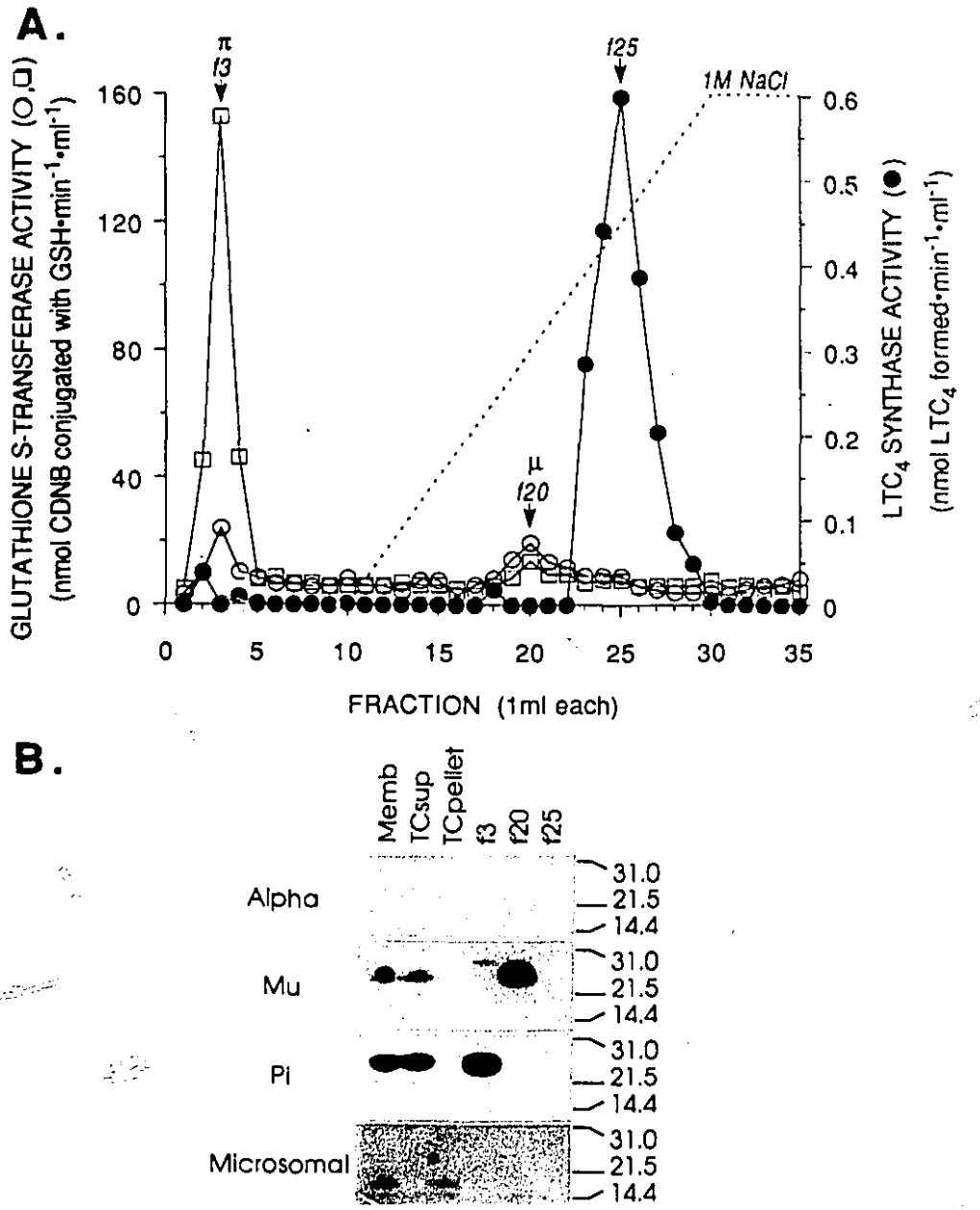
A.
GLUTATHIONE S-TRANSFERASE ACTIVITY (O,□)
(nmol CDNB conjugated with GSH•min-1•ml-1)
LTC4 SYNTHASE ACTIVITY (●)
(nmol LTC4 formed•min-1•ml-1)
π
f3
μ
f20
f25
1M NaCl
FRACTION (1ml each)
B.
Memb
TCsup
TCpellet
f3
f20
f25
Alpha
Mu
Pi
Microsomal
31.0
21.5
14.4

Figure 5

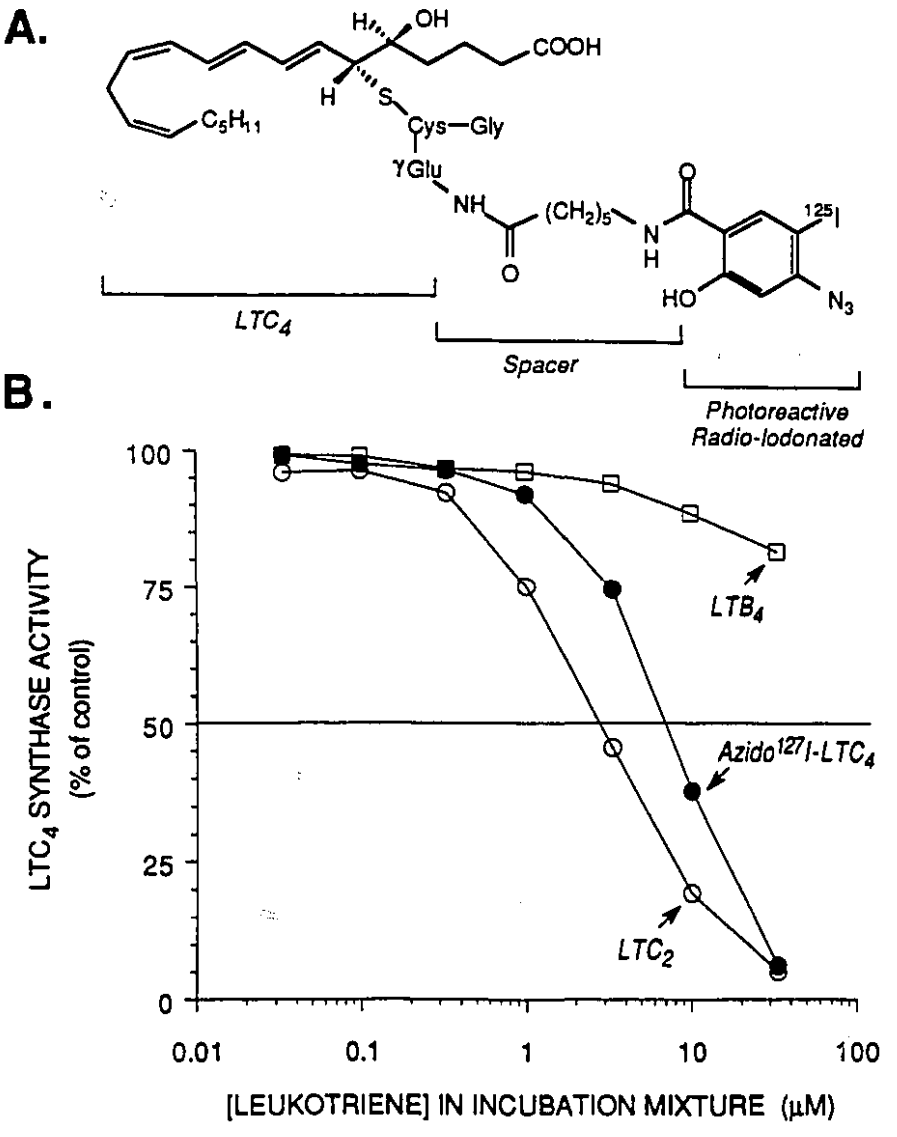
A.
H
OH
COOH
H
S
Cys—Gly
C_5H_{11}
γGlu
NH
$(CH_2)_5$
N
H
O
O
^{125}I
HO
N_3
LTC_4
Spacer
Photoreactive
Radio-Iodonated
B.
100
75
50
25
0
LTC_4 SYNTHASE ACTIVITY
(% of control)
LTB_4
Azido^{127}I-LTC_4
LTC_2
0.01
0.1
1
10
100
[LEUKOTRIENE] IN INCUBATION MIXTURE (μM)

Figure 6

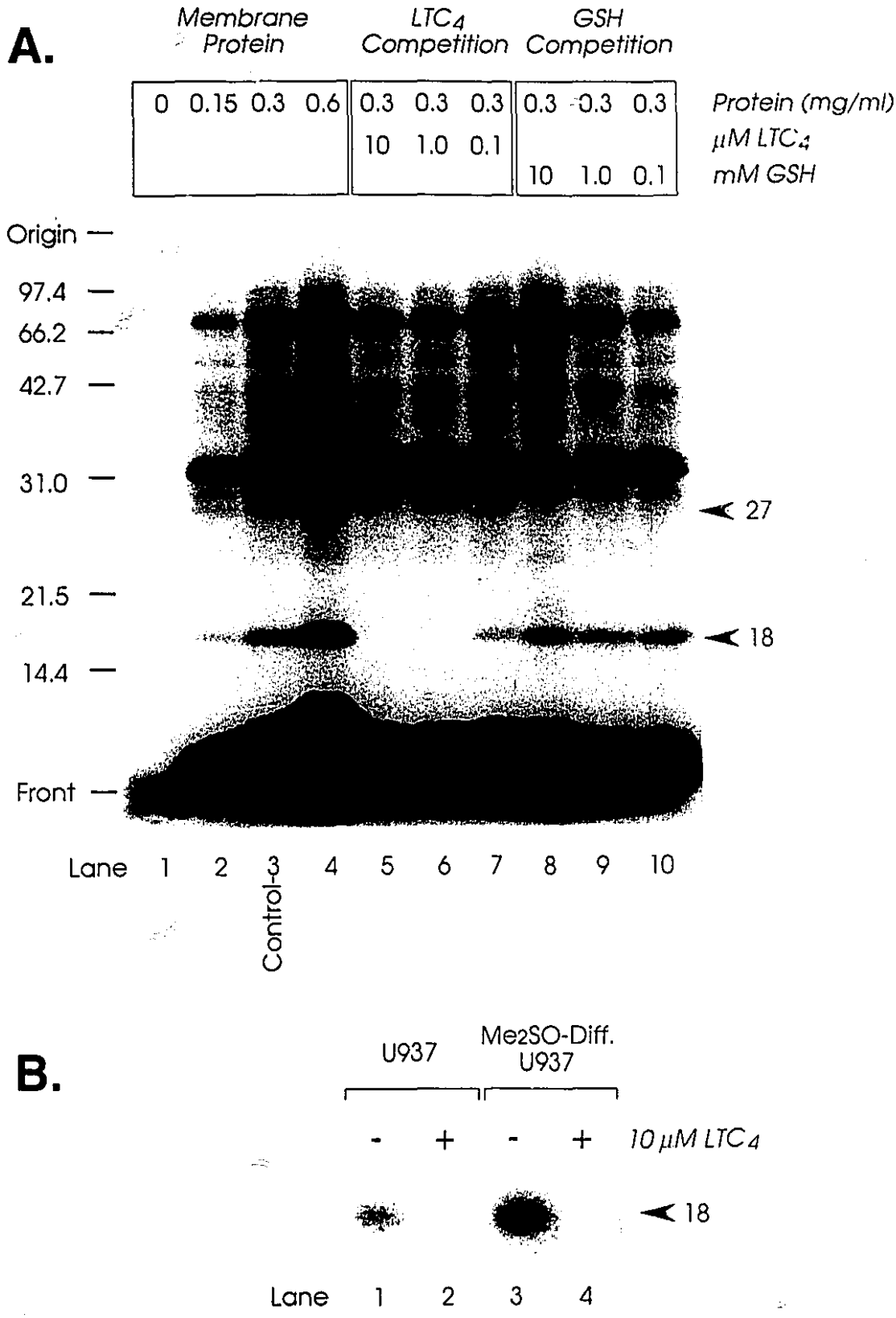
A.
Membrane Protein
LTC4 Competition
GSH Competition
0 0.15 0.3 0.6
0.3 0.3 0.3
10 1.0 0.1
0.3 0.3 0.3
10 1.0 0.1
Protein (mg/ml)
μM LTC4
mM GSH
Origin
97.4
66.2
42.7
31.0
21.5
14.4
Front
27
18
Lane 1 2 3 4 5 6 7 8 9 10
Control-3
B.
U937
Me2SO-Diff. U937
- + - +
10 μM LTC4
18
Lane 1 2 3 4

Figure 7

5. MANUSCRIPT B

Photoaffinity Labelling of Human Leukotriene C_4 Synthase in THP-1 Cell Membranes

FEBS Lett. 317: 195-201 (1993)

Having identified a putative polypeptide (18 kDa) candidate for LTC_4 synthase, a comprehensive study of the photolabelling was performed to demonstrate that the polypeptide labelled was in fact LTC_4 synthase and not microsomal GST which has a similar molecular mass. This study also identified the human monocytic cell line, THP-1, as another abundant source of LTC_4 synthase which had activity equivalent to U937 cells but did not require differentiation.

Photoaffinity Labelling of Human Leukotriene C_4 Synthase in THP-1 Cell Membranes

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ABSTRACT

Human leukotriene C_4 synthase specific activity in the human monocytic leukemia cell line THP-1 (0.302 ± 0.062 nmol LTC_4 formed•min^{-1}•mg^{-1}) was 7.6-fold higher than in U937 cells (0.040 ± 0.017 nmol LTC_4 formed•min^{-1}•mg^{-1}) and comparable to dimethylsulfoxide-differentiated U937 cells (0.399 ± 0.084 nmol LTC_4 formed•min^{-1}•mg^{-1}). Using azido^{125}I-LTC_4, a radioiodinated photoaffinity probe whose structure is based on the product of the LTC_4 synthase catalyzed reaction, a single polypeptide with a molecular mass of 18 kDa was specifically labelled in THP-1 cell microsomal membranes. The rank order of potencies for competition of azido^{125}I-LTC_4 photolabelling of the 18 kDa protein by glutathione, leukotrienes and their analogs was found to be LTC_2 > (azido^{127}I-LTC_4 ≈ LTC_4) > (LTD_4 ≈ LTE_4) > (LTA_4 ≈ LTB_4) > S-hexyl glutathione > glutathione. This rank order corresponded exactly with the rank order of potencies for inhibition of LTC_4 synthase biosynthetic activity but not for inhibition of microsomal glutathione S-transferase activity. Furthermore, both the specifically-photolabelled 18 kDa polypeptide and LTC_4 synthase activity, but not microsomal glutathione S-transferase activity, were selectively solubilized by the anionic detergent taurocholate.

The 18 kDa protein specifically labelled by azido^{125}I-LTC_4 had high specificity for LTC_4 and closely related leukotrienes and was distinct from microsomal glutathione S-transferase. We conclude that azido^{125}I-LTC_4 specifically photolabels LTC_4 synthase which is an 18 kDa polypeptide or contains an 18 kDa subunit.

1. INTRODUCTION

The leukotrienes are a group of arachidonic acid metabolites with important pro-inflammatory effects. They are formed through the oxygenation of free arachidonic acid at the C5 position and subsequent dehydration to form an unstable epoxide intermediate, leukotriene A_4 (LTA_4) through the action of 5-lipoxygenase. Further metabolism of LTA_4 follows two distinct biochemical pathways. LTA_4 can either be hydrolyzed by the enzyme LTA_4 hydrolase to form the potent leukocyte activator LTB_4, or LTA_4 can be conjugated with reduced glutathione by LTC_4 synthase to form the sulfidopeptide leukotriene, LTC_4. With the sequential removal of a glutamic acid residue then a glycine residue, LTC_4 can be further metabolized to LTD_4 and then to LTE_4 by γ-glutamyl transpeptidase and cysteinylglycine dipeptidase, respectively. LTC_4, LTD_4 and LTE_4 collectively are responsible for the biological activity of the slow reacting substance of anaphylaxis (SRS-A). The effects of these bioactive mediators include vasoconstriction, smooth muscle contraction and mucous hypersecretion which implicate the sulfidopeptide leukotrienes in diseases such as bronchial asthma and other allergic reactions (for reviews see (1-4)).

Sulfidopeptide leukotriene biosynthesis, which predominantly occurs in mast cells and cells of myeloid lineage (particularly eosinophils and macrophages), is initiated by the membrane-bound enzyme LTC_4 synthase. This enzyme is distinct from other known glutathione S-transferases being responsible specifically for the biosynthesis of LTC_4 (5). The complete purification and characterization of LTC_4 synthase has been hindered owing to the apparent instability of the enzyme in the semi-purified state (6), and the lack of an abundant source of the enzyme. However, partial purification and characterization of LTC_4 synthase from guinea pig lung (91-fold; (6-8)), rat basophilic leukemia cells (10-fold; (9)), mouse mastocytoma cells (4-fold; (10)) and human dimethylsulfoxide-differentiated U937 cells (10,000-fold; (11)) has been reported.

We have recently described that differentiation of the human promonocytic leukemia cell line U937 into monocyte-like cells by growth in the

presence of dimethylsulfoxide results in a 10-fold increase in LTC_4 synthase specific activity (5). In initial experiments, two polypeptides (18 kDa and 27 kDa) were specifically photolabelled in differentiated U937 cell microsomal membranes with the use of a novel radioiodinated photoaffinity ligand based on the structure of LTC_4, the product of the LTC_4 synthase-catalyzed reaction. The 18 kDa polypeptide in particular was identified as a candidate for being LTC_4 synthase, although it was not possible to exclude other proteins that might bind LTC_4, especially microsomal glutathione S-transferase. The intrinsic LTC_4 synthase specific activity in the related human monocytic cell line, THP-1, is equivalent to dimethylsulfoxide-differentiated U937 cells. We now demonstrate the specific labelling of a single polypeptide with a molecular mass of 18 kDa in the microsomal membranes of THP-1 cells. This 18 kDa polypeptide has high specificity for LTC_4 and closely related leukotrienes and is distinct from microsomal glutathione S-transferase.

2. MATERIALS AND METHODS

2.1 *Materials*

THP-1 cells (TIB 202) were obtained from the American Type Culture Collection (Rockville, Maryland). Cell culture media, antibiotics and fetal bovine serum were purchased from the Sigma Chemical Co. (St. Louis, Missouri). LTA_4-methyl ester, LTB_4, LTC_4, LTC_2, LTD_4, and LTE_4 were synthesized by the Department of Medicinal Chemistry at the Merck Frosst Centre for Therapeutic Research. Taurocholate was obtained from Calbiochem (La Jolla, California). HPLC solvents were from BDH (Toronto, Ontario). S-Hexyl glutathione and all other reagents were of analytical grade and were purchased from Sigma (St. Louis, Missouri).

2.2 *Cell Growth and Subcellular Fractionation*

THP-1 Cell Growth - Cells from the human monocytic leukemic cell line THP-1 (12) were cultured in sterile RPMI-1640 medium (supplemented with 0.2% (w/v) $NaHCO_3$, 0.05 mM 2-mercaptoethanol and 0.03% (w/v) L-glutamine) containing

50 U penicillin/ml, 50 µg streptomycin/ml and 10% (v/v) fetal bovine serum (Sigma Hybri-Max, not heat-inactivated) at 37°C in a humidified atmosphere containing 6% CO_2. Cells were isolated by continuous-flow centrifugation, ruptured by nitrogen cavitation and the microsomal-membrane fraction was isolated as described previously for U937 cells (11).

Taurocholate solubilization - THP-1 cell microsomal membranes (100,000 x g pellet) at a protein concentration of 15 mg/ml were combined with an equal volume of 4% (w/v) taurocholate in PBS (pH 7.4), 2 mM EDTA. The mixture was shaken at 4°C for 30 min and subsequently spun at 200,000 x g for 60 min. The resulting supernatant (post-taurocholate supernatant) was recovered and the remaining pellet (post-taurocholate pellet) was resuspended in PBS (pH 7.4), 2 mM EDTA in the same volume as the original microsomal membranes by Dounce homogenization ('A' clearance pestle, 10 strokes).

2.3 *Measurement of LTC_4 Synthase and Glutathione S-Transferase Activities*

LTC_4 synthase activity was measured by monitoring the formation of LTC_4 (as determined by reverse-phase HPLC) in the presence of reduced glutathione and LTA_4 (free acid) essentially as described before (5,11). Microsomal glutathione S-transferase activity was measured following pretreatment of Triton X-100 lysed samples by N-ethylmaleimide (13,14) then monitoring glutathione conjugation in the presence of 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM reduced glutathione (ΔOD_{340nm}, $\varepsilon_{340\,nm}$ (mM^{-1} cm^{-1}) = 9.6 ; (15)). Protein was determined as described by Bradford (16) using bovine γ-globulin as standard.

2.4 *Photoaffinity Labelling in THP-1 Microsomes*

Azido^{127}I-LTC_4 and azido^{125}I-LTC_4 (2200 Ci/mmol) were synthesized as described previously (5). THP-1 cell microsomes were suspended at a concentration of 0.3 mg/ml (unless otherwise indicated) in 20 mM Tris/HCl (pH 7.4), 1 mM EDTA and 50 mM serine-borate in the presence and absence of varying concentrations of competing ligands as indicated. Azido^{125}I-LTC_4 was then added to a final concentration of 20 pM and the mixture was incubated in the

dark at 25°C for 30 min, followed by incubation at 4°C for 5 min. The samples were transferred to 6-well tissue culture cluster plates, photolysed under a UV light source (Phillips, λ_{max}=350 nm) for 2 min at 4°C at a distance of 14 cm. Membranes were re-harvested by centrifugation at 200,000 x g for 15 min at 4°C, solubilized in SDS-containing sample buffer, and resolved by SDS gel electrophoresis (17). The polyacrylamide gel was dried under vacuum and used to expose X-Omat AR film (Eastman Kodak Co., Rochester, NY) for 2-3 weeks at -80°C. Band intensities were quantified by laser densitometry (Molecular Dynamics Computing Densitometer).

3. RESULTS

We have recently reported that the activity of the microsomal enzyme LTC_4 synthase, which is unique and distinct from microsomal glutathione S-transferase, can be increased 10-fold through the differentiation of a human pro-monocytic cell line, U937, by growth in the presence of dimethylsulfoxide (5). Similar to U937 cells, THP-1 cells are also of human monocytic lineage; however, their intrinsic LTC_4 synthase activity is equivalent to dimethylsulfoxide-differentiated U937 cells (0.302 ± 0.062 (n=9) and 0.399 ± 0.084 (n=47) nmol LTC_4 formed • min^{-1} • mg^{-1}, respectively). Thus, THP-1 cells are also a suitable source for the purification, cloning and characterization of human LTC_4 synthase. We have previously described preliminary experiments in which an 18 kDa polypeptide was specifically labelled in U937 cell microsomal membranes using a radioiodinated, photolabile affinity analog of LTC_4 (azido^{125}I-LTC_4). In order to demonstrate that microsomal membranes from THP-1 cells, which intrinsically express high levels of LTC_4 synthase activity, also possess an 18 kDa polypeptide that can be specifically photolabelled, we probed THP-1 cell microsomal membranes using azido^{125}I-LTC_4 in the absence or presence of competing ligands.

3.1 *Photoaffinity labelling of THP-1 cell microsomal membranes by azido ^{125}I-LTC_4 : competition by LTC_4 and reduced glutathione*

THP-1 cell microsomal membranes were probed with a radioiodinated and photoreactive derivative of LTC_4 (azido ^{125}I-LTC_4; ca. 2200 Ci/mmol) to identify potential LTC_4 synthase candidates or subunits of LTC_4 synthase (Fig.1). Only one polypeptide, with a molecular mass of 18 kDa, was specifically labelled in THP-1 cell microsomal membranes. The 27 kDa polypeptide photolabelled in U937 cell microsomal membranes that was competed by both LTC_4 and glutathione at high concentrations (5) was not observed in these cells and is therefore probably not involved in LTC_4 biosynthesis. Photolabelling of the 18 kDa polypeptide was inhibited by 50% in the presence of 0.1 μM LTC_4 (Fig.1, Lane 4 versus 2) but was not competed for at all by the presence of even 1.0 mM reduced glutathione (Fig.1, Lane 8). Thus, as in U937 cell microsomal membranes, the 18 kDa polypeptide may recognize the arachidonic acid backbone of LTC_4 in addition to the glutathione moiety and is a potential candidate for being either LTC_4 synthase or a subunit of LTC_4 synthase.

3.2 *Competition of azido ^{125}I-LTC_4 photolabelling of an 18 kDa membrane polypeptide corresponds to inhibition of LTC_4 synthase biosynthetic activity*

To assess the relative potencies of leukotrienes structurally related to LTC_4 as competing ligands for photolabelling of the 18 kDa polypeptide, THP-1 cell microsomal membranes were incubated with azido ^{125}I-LTC_4 in the presence of varying concentrations of either LTC_4, LTC_2, LTD_4, LTE_4, LTB_4, LTA_4 , reduced glutathione, S-hexyl glutathione or azido ^{127}I-LTC_4 (Fig.2). Using laser densitometry, the relative potencies of the various competing ligands was quantified. The rank order of potencies for the inhibition of labelling of the 18 kDa polypeptide was LTC_2 > (azido ^{127}I-LTC_4 ≈ LTC_4) > (LTD_4 ≈ LTE_4) > (LTB_4 ≈ LTA_4) > S-hexyl glutathione > glutathione.

Next, to determine if the relative potencies of the various leukotrienes and S-hexyl glutathione as competing ligands for the photoaffinity labelling of the 18 kDa polypeptide was reflected by their ability to inhibit enzymic activity, LTC_2,

LTD_4, LTE_4, LTB_4, azido ^{127}I-LTC_4 and S-hexyl glutathione at varying concentrations were included in LTC_4 synthase enzyme incubation mixtures. (LTC_4 was not tested for its ability to inhibit LTC_4 synthase activity since the retention time on reverse-phase HPLC of enzymatically produced LTC_4 is indistinguishable from LTC_4 added to the incubation mixture. Similarly, neither LTA_4 nor glutathione were tested since they are the substrates of LTC_4 synthase.) The IC_{50} values for LTC_2 and azido ^{127}I-LTC_4 were determined tc be 2.6 μM and 7.0 μM, respectively, indicating that azido ^{127}I-LTC_4 was specifically recognized by and therefore inhibited LTC_4 synthase activity. The rank order of potencies for the inhibition of LTC_4 synthase activity was LTC_2 > azido ^{125}I-LTC_4 > ($LTD_4 \approx LTE_4$) > LTB_4 > S-hexyl glutathione (Fig.3). This rank order of potencies for the inhibition of LTC_4 synthase activity mirrors the rank order of potencies for the inhibition of labelling of the 18 kDa polypeptide, and therefore supports the hypothesis that this specifically labelled polypeptide is LTC_4 synthase. The IC_{50} values for inhibition of labelling of the 18 kDa polypeptide was lower overall than the IC_{50} values for inhibition of LTC_4 synthase biosynthetic activity. This was due to the fact that in the former experiment (photolabelling) the added ligand was competing with 20 pM azido ^{125}I-LTC_4 whereas in the latter experiment (LTC_4 synthase) the added ligand had to compete for binding to LTC_4 synthase in the presence of 40 μM LTA_4 and 10 mM glutathione. Therefore, the rank order of potencies and not the IC_{50} values of the various competitors was compared.

3.3 *Competition of azido ^{125}I-LTC_4 photolabelling of an 18 kDa membrane polypeptide does not correspond to inhibition of microsomal glutathione S-transferase activity*

THP-1 cell microsomal membranes contain an N-ethylmaleimide-insensitive glutathione S-transferase activity (microsomal glutathione S-transferase). This enzyme has a molecular mass of about 17.2 kDa (18). To address the possibility that the 18 kDa polypeptide photolabelled by azido ^{125}I-LTC_4 is microsomal glutathione S-transferase, the inhibition profiles of LTC_4, LTC_2, azido ^{127}I-LTC_4, LTD_4, LTE_4, LTB_4 and S-hexyl glutathione for microsomal glutathione S-transferase activity

were determined. The rank order of potencies was determined to be as follows: LTC_2 > (LTC_4 ≈ azido^{127}I-LTC_4 ≈ S-hexyl glutathione) > LTD_4 > LTE_4 > LTB_4 (Fig.4). This rank order was not reflected by the rank order of potencies of these competing ligands for the inhibition of photolabelling of the 18 kDa polypeptide. In particular, S-hexyl glutathione was as potent as LTC_4 in inhibiting microsomal glutathione S-transferase activity, but was a much less potent competitor than LTC_4 for photolabelling of the 18 kDa polypeptide. In the case of LTC_4 synthase, S-hexyl glutathione was also a much less potent inhibitor (IC_{50} value=10 mM) than the LTC_4 analog, LTC_2 (IC_{50} value= 2.6 μM). This indicates that the recognition of the glutathione moiety alone was sufficient to mediate inhibition of microsomal glutathione S-transferase activity whereas inhibition of LTC_4 synthase activity required a competing ligand with both the glutathione moiety and the arachidonic acid backbone for effective inhibition, as was also the case for competition of specific photolabelling of the 18 kDa polypeptide.

3.4 *LTC_4 synthase activity and a specifically photolabelled 18 kDa polypeptide are both selectively solubilized by the anionic detergent taurocholate, whereas microsomal glutathione S-transferase is not.*

Taurocholate is an anionic detergent that has previously been shown to solubilize approximately 80-100 % of total LTC_4 synthase activity but not microsomal glutathione S-transferase activity from U937 cell microsomal membranes (5). THP-1 cell microsomal membranes were similarly solubilized with 2% (w/v) taurocholate. Following centrifugation at 200,000 x g both the post-taurocholate pellet and the post-taurocholate supernatant were assayed for LTC_4 synthase activity and microsomal glutathione S-transferase activity (Fig.5). Taurocholate effectively solubilized LTC_4 synthase activity from THP-1 cell microsomal membranes but not microsomal glutathione S-transferase activity.

Having differentially separated LTC_4 synthase and microsomal glutathione S-transferase by taurocholate solubilization it was predicted that if the photoaffinity ligand specifically bound to LTC_4 synthase, photolabelling of the 18 kDa polypeptide could occur in the 100,000 x g microsomal membrane fraction

but not in the post-taurocholate pellet. As was also observed in Fig.1, specific labelling of the 18 kDa polypeptide occurred in the 100,000 x g THP-1 microsomal fraction (Fig.6) and 0.1, 1.0 and 10 μM LTC_4 competed for the labelling of the 18 kDa polypeptide (Fig. 6a, lanes 1-4). The specifically-labelled 18 kDa polypeptide, however, was not present in the post-taurocholate pellet (Fig.6a, lanes 5-8). Instead, a 20 kDa polypeptide was labelled in the post-taurocholate pellet but was not completely competed for by up to 10 μM LTC_4. In an alternative approach, THP-1 cell microsomal membranes were first photolabelled by azido^{125}I-LTC_4 then subsequently solubilized with taurocholate (Fig. 6b). The specifically labelled 18 kDa polypeptide was found exclusively in the resulting supernatant fraction, co-fractionating with LTC_4 synthase. In summary, these results support the hypothesis that the 18 kDa polypeptide specifically photolabelled by azido^{125}I-LTC_4 in THP-1 cell microsomal membranes is either LTC_4 synthase or a subunit of LTC_4 synthase but not microsomal glutathione S-transferase.

4. DISCUSSION

Recently, we have presented evidence supporting the hypothesis that LTC_4 synthase, an enzyme specifically dedicated to the production of LTC_4 from LTA_4 and reduced glutathione, is a unique enzyme distinct from other known glutathione S-transferases (5). In an attempt to identify putative polypeptides as candidates for being LTC_4 synthase, a novel radioiodinated photoreactive affinity ligand with a structure based on LTC_4 was synthesized. LTC_4 was chosen as the basis of the photoaffinity ligand since it was likely to have high affinity for LTC_4 synthase, as evidenced by the high potency of LTC_2, a structural analogue of LTC_4 , for inhibiting LTC_4 synthase activity (IC_{50} = 2.6 μM). Photoreactive derivatives of the substrates of LTC_4 synthase, LTA_4 and glutathione were not synthesized due to their high instability and low specificity, respectively.

In an initial experiment, we had demonstrated that the photoaffinity ligand, azido^{125}I-LTC_4, specifically labelled an 18 kDa polypeptide and a 27 kDa polypeptide in U937 cell microsomal membranes (5). Photolabelling of the 27 kDa polypeptide, which was competed for by both LTC_4 and glutathione, was not observed in THP-1 cell microsomal membranes and this polypeptide therefore does not appear to be involved in LTC_4 biosynthesis. Specific labelling an 18 kDa polypeptide, however, did occur in THP-1 cell microsomal membranes and was strongly competed for by LTC_4 (>50% at 0.1 μM) but not at all by up to 1 mM glutathione, indicating that this polypeptide had a high affinity for LTC_4 and thus was a likely candidate for being LTC_4 synthase or a subunit thereof. We were, however, unable to exclude the possibility that this LTC_4-specific polypeptide may be microsomal glutathione S-transferase, a putative LTC_4 receptor or transport protein or γ-glutamyl transpeptidase. To further address the possibility that this polypeptide may be LTC_4 synthase, two experimental approaches were taken.

First, we compared the rank order of potencies of leukotrienes and S-hexyl glutathione at competing for the specific labelling of the 18 kDa polypeptide with their ability to inhibit LTC_4 synthase and microsomal glutathione S-transferase activities. The rank order of potencies of the various competing ligands for

inhibition of the photolabelling of the 18 kDa polypeptide was mirrored exactly by their ability to inhibit LTC_4 synthase activity, but not microsomal glutathione S-transferase, supporting the hypothesis that the 18 kDa polypeptide photolabelled by azido^{125}I-LTC_4 was LTC_4 synthase and not microsomal glutathione S-transferase.

Competition of the photolabelling of the 18 kDa polypeptide and inhibition of LTC_4 synthase biosynthetic activity were dependent on both the presence of an arachidonic acid-like backbone plus the glutathione moiety. For example, LTC_2 and azido^{127}I-LTC_4 were both potent inhibitors of the 18 kDa polypeptide photolabelling and LTC_4 synthase activity whereas the absence of one amino acid (LTD_4), two amino acids (LTE_4) or all amino acids (LTA_4, LTB_4) led to sequentially less potency. Similarly, competition for the 18 kDa polypeptide photolabelling and inhibition of LTC_4 synthase activity was much poorer if a simple hydrophobic chain was substituted for the arachidonic acid backbone (ie. S-hexyl glutathione). In contrast, inhibition of microsomal glutathione S-transferase was not substantially different using S-hexyl glutathione versus LTC_4 or azido^{127}I-LTC_4, indicating that arachidonate specificity does not exist for this enzyme.

To further support the hypothesis that the 18 kDa photolabelled polypeptide is LTC_4 synthase we exploited the ability of the anionic detergent taurocholate to solubilize LTC_4 synthase from THP-1 cell microsomal membranes but not microsomal glutathione S-transferase. As expected, the specific labelling of the 18 kDa polypeptide was observed in the THP-1 cell microsomal membranes before but not after solubilization with taurocholate. Similarly, the photolabelled 18 kDa polypeptide could be solubilized with taurocholate, consistent with the solubility of LTC_4 synthase with this detergent.

We therefore conclude that the 18 kDa polypeptide specifically labelled in THP-1 cell microsomal membranes by azido^{125}I-LTC_4 is LTC_4 synthase or a subunit of LTC_4 synthase and that this polypeptide is distinct from microsomal glutathione S-transferase.

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FIGURE LEGENDS

Figure 1. **Photoaffinity labelling of THP-1 cell microsomal membranes by azido^{125} I-LTC_4.** *(A)* Azido^{125}I-LTC_4 is comprised of LTC_4 coupled to a radio-iodinated, photo-reactive group through the α-amino of the γ-glutamate residue. *(B)* THP-1 cell microsomal membranes (0.3 mg protein, except lanes 1-3 as indicated) were incubated with 20 pM azido^{125}I-LTC_4 in either the absence (lanes 1-3) or presence of 0.1, 1.0, 10 μM LTC_4 (lanes 4-6) or 0.1, 1.0 mM reduced glutathione (GSH; lanes 7-8) for 30 min at 25°C, cooled then photolysed as described under "Methods". Labelled proteins were resolved by SDS gel electrophoresis and visualized by autoradiography. A representative experiment is shown (n=4). The migration of molecular weight standards (x10-3) is indicated on the left. A specifically labelled 18 kDa band is indicated by an arrowhead on the right.

Figure 2. **Competition by leukotrienes and glutathiones for photoaffinity labelling of the 18 kDa microsomal membrane protein by azido^{125}I-LTC_4 is dependent on structural relatedness to LTC_4.** Incubation mixtures (1 ml each) were prepared containing 0.3 mg of microsomal membrane protein from THP-1 cells, 20 pM azido^{125}I-LTC_4 plus varying concentrations of either the indicated leukotrienes, S-hexyl glutathione or glutathione. The mixtures were incubated for 30 min at 25°C, cooled then photolysed as described under "Methods". The membranes were re-isolated by ultracentrifugation, dissociated in SDS-containing sample buffer and resolved by SDS-gel electrophoresis. Radioactive bands were visualized by autoradiography of the dried gels and the intensities of the photolabelled 18 kDa polypeptide were quantified by laser densitometry. Data are expressed as a percentage of the controls to which no competing ligand was added. Each point represents the mean ± SEM of three separate experiments.

Figure 3. **Inhibition of LTC_4 synthase activity by leukotrienes and S-hexyl glutathione.** LTC_4 synthase activity in THP-1 cell microsomal membranes was measured in the presence of varying concentrations of LTC_2(O), azido^{127}I-LTC_4 (●), LTD_4 (❑), LTE_4(■), LTB_4(Δ) or S-hexyl-glutathione (Hex-GSH, ▲) in standard LTC_4 synthase incubation mixtures as described in "Methods". Activity is expressed as a percentage of the control to which no competing ligand was added. Each point represents the mean ± SEM of three separate experiments.

Figure 4. **Inhibition of microsomal glutathione S-transferase activity by leukotrienes and S-hexyl glutathione.** Microsomal glutathione S-transferase activity was measured in incubation mixtures containing 0.75 mg of N-ethylmaleimide-treated THP-1 cell microsomal membranes and varying concentrations of LTC_2 (O),azido^{127}I-LTC_4 (●), LTC_4 (◊), LTD_4 (❑), LTE_4 (■), LTB_4 (Δ) or S-hexyl glutathione (Hex-GSH, ▲) as described under "Methods". Activity is expressed as a percentage of the controls to which no competing ligand was added. Each point represents the mean ± SEM of three separate experiments.

Figure 5. **Differential taurocholate solubilization of microsomal glutathione S-transferase and LTC_4 synthase activities from THP-1 cell microsomal membranes.** THP-1 cell microsomal membranes at a protein concentration of 15 mg protein/ml were solubilized by combining them with an equal volume of 4% (w/v) taurocholate and shaking for 30 min at 4°C. Following centrifugation at 200,000 x g both the post-taurocholate pellet and the post-taurocholate supernatant were assayed for LTC_4 synthase activity and microsomal glutathione S-transferase activity as described under "Methods". Activity is expressed as a percentage of total activity recovered. Each bar represents the mean ± SEM of three separate experiments.

Figure 6. (A) **Photoaffinity labelling of THP-1 cell microsomal membranes and the post-taurocholate pellet fraction with azido^{125}I-LTC_4.** THP-1 cell microsomal membranes (0.3 mg protein/ml; lanes 1-4) and the 200,000 x g post-taurocholate

pellet fraction (an amount equivalent to 0.3 mg/ml of original microsomal-membrane protein; lanes 5-8) were photoaffinity labelled with 20 pM azido^{125}I-LTC_4 in the presence of the indicated concentrations of LTC_4 as described in "Methods". The migration of molecular weight standards (x10^{-3}) are indicated on the left. (B) **Taurocholate solubilization of of a specifically photolabelled 18 kDa membrane polypeptide.** THP-1 cell microsomal membranes were photoaffininity labelled in the absence (lane 1) or presence (lane 2) of 10 μM LTC_4 as described for panel A. The membranes from half the samples (the other halves having been used for lanes 1 and 2) were re-isolated by centrifugation (30 min at 200,000 x g) then solubilized with taurocholate. Following further centrifugation (30 min at 200,000 x g), the resulting pellet (Post-TC Pellet; lanes 3 and 4) and supernatant (Post-TC Supernatant; lanes 5 and 6) were were resolved by SDS gel electrophoresis and visualized by autoradiography (proteins in the supernatant following taurocholate solubilization were precipitated overnight by acetone at-20°C prior to gel electrophoresis). The relevent section of the resulting autoradiograph, containing the 18 kDa polypeptide (arrowhead), is shown.

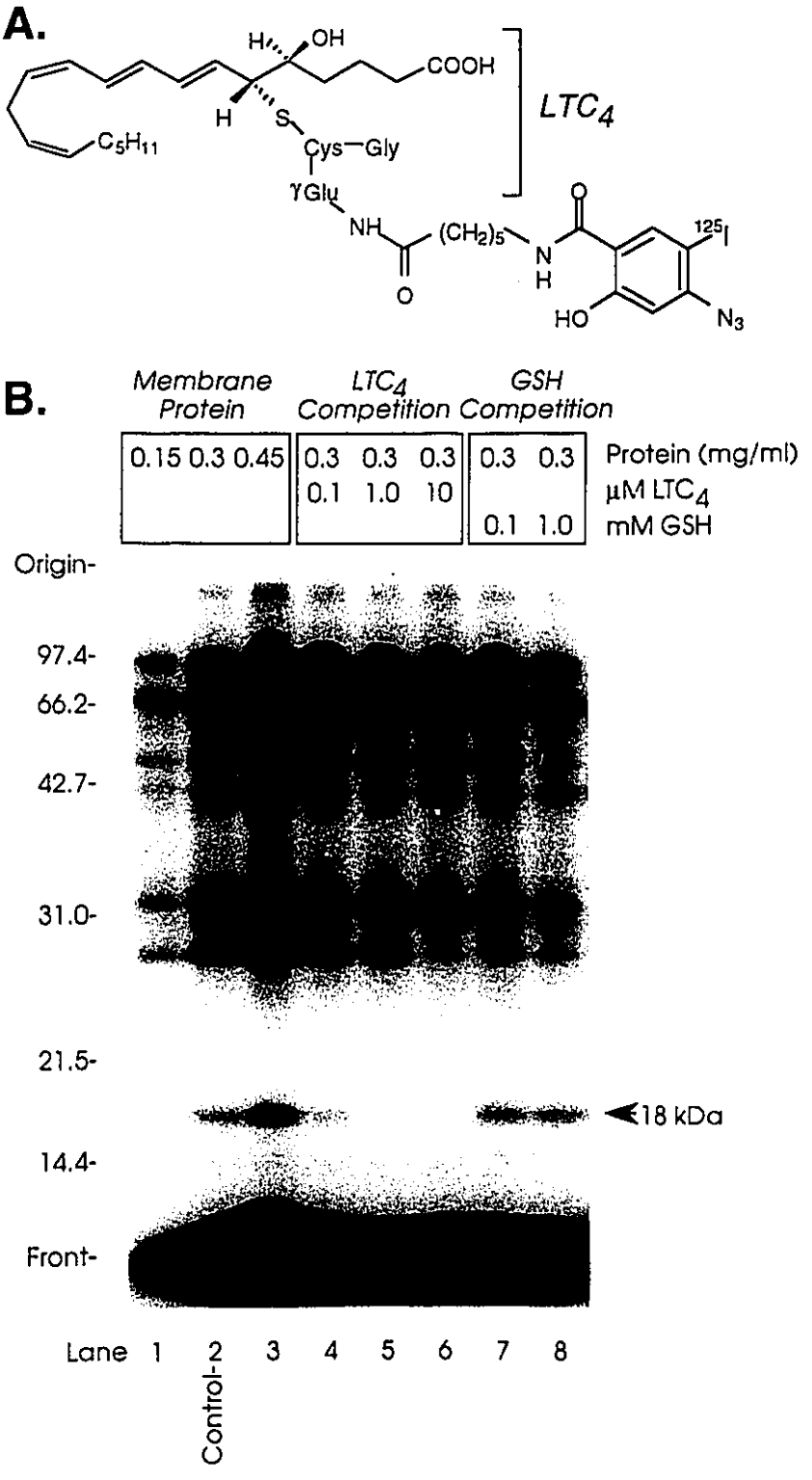
A.
H
OH
COOH
H
S
Cys—Gly
γGlu
C_5H_{11}
LTC_4
NH
$(CH_2)_5$
N
H
O
HO
^{125}I
N_3
B.
Membrane Protein
LTC_4 Competition
GSH Competition
0.15 0.3 0.45
0.3 0.3 0.3
0.1 1.0 10
0.3 0.3
0.1 1.0
Protein (mg/ml)
μM LTC_4
mM GSH
Origin-
97.4-
66.2-
42.7-
31.0-
21.5-
14.4-
Front-
18 kDa
Lane 1 2 3 4 5 6 7 8
Control-

Figure 1

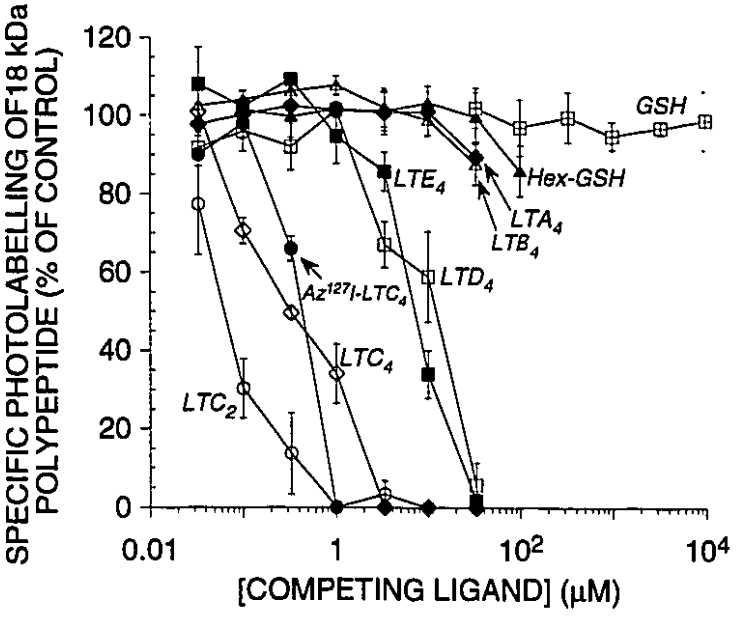
SPECIFIC PHOTOLABELLING OF18 kDa POLYPEPTIDE (% OF CONTROL)
120
100
80
60
40
20
0
0.01
1
10²
10⁴
[COMPETING LIGAND] (μM)
GSH
Hex-GSH
LTE4
LTA4
LTB4
LTD4
Az127I-LTC4
LTC4
LTC2

Figure 2

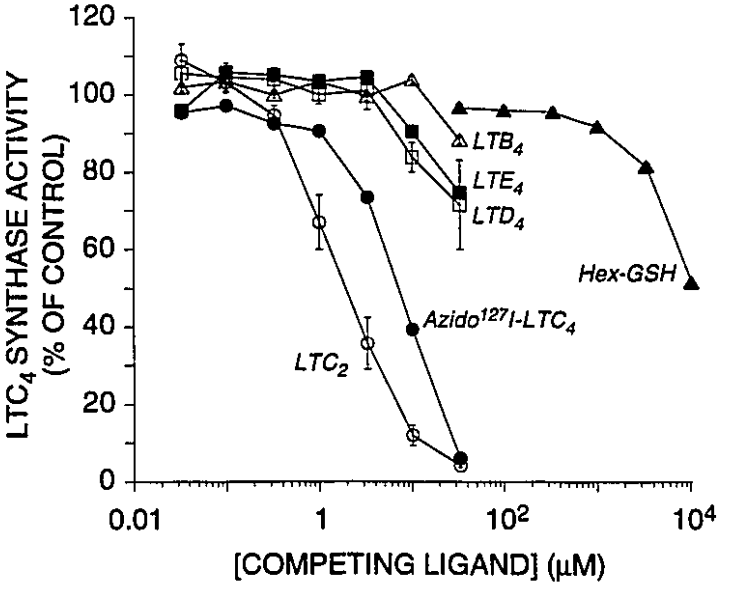
120
100
80
60
40
20
0
0.01
1
10²
10⁴
LTC4 SYNTHASE ACTIVITY (% OF CONTROL)
[COMPETING LIGAND] (μM)
LTB4
LTE4
LTD4
Hex-GSH
Azido127I-LTC4
LTC2

Figure 3

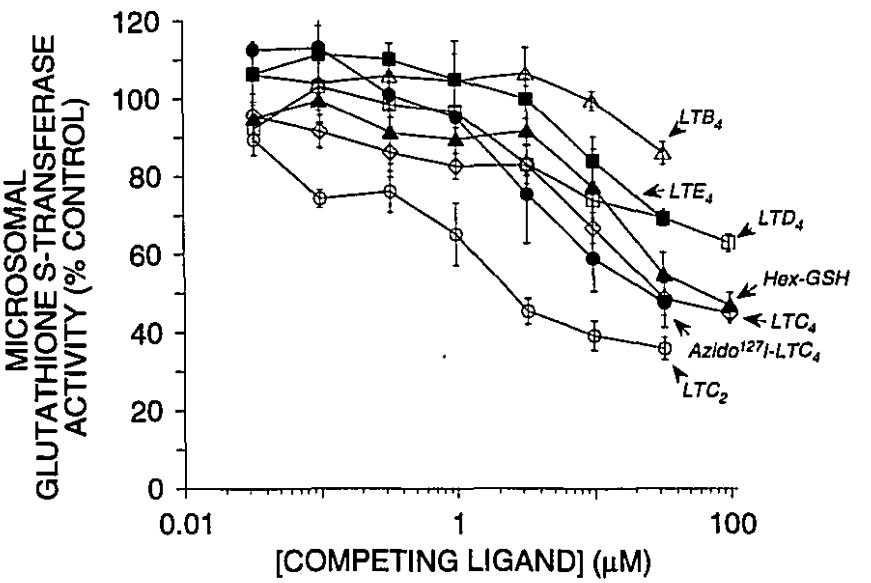
MICROSOMAL
GLUTATHIONE S-TRANSFERASE
ACTIVITY (% CONTROL)
120
100
80
60
40
20
0
0.01
1
100
[COMPETING LIGAND] (μM)
LTB4
LTE4
LTD4
Hex-GSH
LTC4
Azido127I-LTC4
LTC2

Figure 4

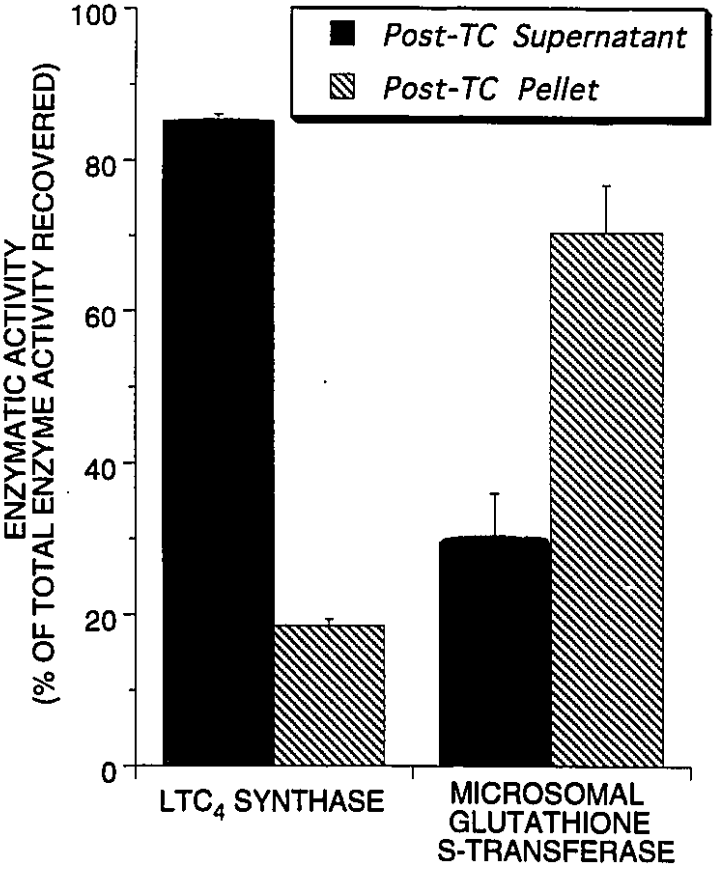
Post-TC Supernatant
Post-TC Pellet
ENZYMATIC ACTIVITY
(% OF TOTAL ENZYME ACTIVITY RECOVERED)
100
80
60
40
20
0
LTC4 SYNTHASE
MICROSOMAL
GLUTATHIONE
S-TRANSFERASE

Figure 5

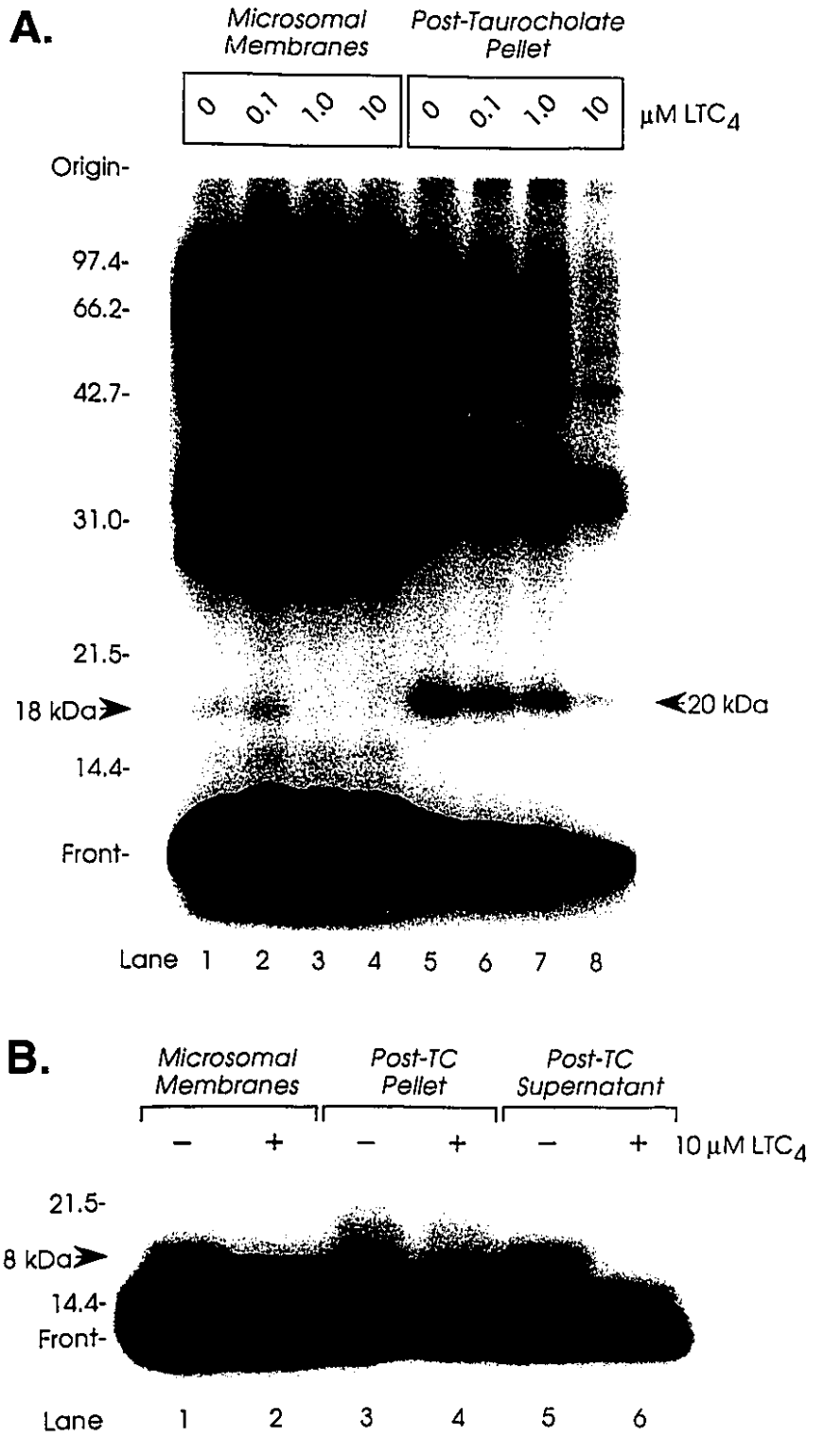
A.
Microsomal Membranes
Post-Taurocholate Pellet
0
0.1
1.0
10
0
0.1
1.0
10
μM LTC4
Origin-
97.4-
66.2-
42.7-
31.0-
21.5-
18 kDa
20 kDa
14.4-
Front-
Lane 1 2 3 4 5 6 7 8
B.
Microsomal Membranes
Post-TC Pellet
Post-TC Supernatant
– + – + – + 10 μM LTC4
21.5-
18 kDa
14.4-
Front-
Lane 1 2 3 4 5 6

Figure 6

6. MANUSCRIPT C

Purification to Homogeneity and Amino-Terminal Sequence of Human Leukotriene C_4 Synthase: A Unique Homodimeric Glutathione S-Transferase Comprised of 18 kDa Subunits

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The next step in the characterization of LTC_4 synthase was its purification from THP-1 cells. This paper describes a 3-step purification scheme to purify the enzyme to homogeneity and demonstrates that LTC_4 synthase is an 18 kDa enzyme that functions as a homodimer. It also describes the N-terminal sequence of human LTC_4 synthase and identifies a protein kinase C consensus sequence.

Purification to Homogeneity and Amino-Terminal Sequence of Human Leukotriene C_4 Synthase: A Unique Homodimeric Glutathione S-Transferase Comprised of 18 kDa Subunits

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SUMMARY

Human leukotriene C_4 synthase was purified > 25,000-fold to homogeneity from the monocytic leukemia cell line THP-1. Beginning with taurocholate-solubilized microsomal membranes, leukotriene C_4 synthase was chromatographically resolved by i) anion exchange, ii) affinity chromatography (through a resin comprised of biotinylated LTC_2 immobilized on streptavidin-agarose), then iii) gel filtration. The final preparation was comprised of a single polypeptide having a molecular mass of 18 kDa. The molecular mass of the pure polypeptide was consistent with an 18 kDa polypeptide from THP-1 cell membranes that was specifically photolabeled by an LTC_4 photoaffinity probe, azido^{125}I-LTC_4. On calibrated gel-filtration columns, purified LTC_4 synthase activity eluted at a volume corresponding to 39.2 ± 3.3 kDa (n=12). The sequence of the N-terminal 35 amino acids was determined and found to be a unique sequence comprised predominantly of hydrophobic amino acids and containing a consensus sequence for protein kinase C phosphorylation. We therefore conclude that human leukotriene C_4 synthase is a unique glutathione S-transferase comprised of an 18 kDa polypeptide that is enzymatically active as a homodimer and which may be phosphoregulated *in vivo*.

INTRODUCTION

Leukotrienes (LTs) are potent lipid-derived mediators that are released in response to a variety of immunologic and inflammatory stimuli (1,2). The enzyme LTC_4 synthase catalyses the first committed step in the biosynthesis of the cysteinyl leukotrienes, LTC_4, LTD_4 and LTE_4, which collectively comprise the slow-reacting substance of anaphylaxis (SRS-A) and appear to play a pivotal role in the pathogenesis of human bronchial asthma (3). 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 exclusively committed to the biosynthesis of LTC_4 (4). Of the known human glutathione S-transferases, it is the only one that has not yet been purified to homogeneity or cloned, owing predominantly to the extreme lability of LTC_4 synthase in partially purified forms and the lack of a suitably abundant source of the enzyme.

Recently, we have described the induction of high levels of LTC_4 synthase activity in the human promonocytic leukemia cell line U937 following differentiation into monocyte/macrophage-like cells by growth in the presence of dimethylsulfoxide (4). From the microsomal membranes of these cells, LTC_4 synthase was purified > 10,000-fold and an 18 kDa membrane polypeptide was identified by photoaffinity labelling as a strong candidate for being LTC_4 synthase or a subunit of the enzyme (5). In this report, we describe the purification to homogeneity of human LTC_4 synthase from the monocytic leukemia cell line THP-1 and show that like other known glutathione S-transferases, which are all low molecular mass dimeric or trimeric enzymes, human LTC_4 synthase is comprised of a single 18 kDa polypeptide which is functionally active as a homodimer.

MATERIALS AND METHODS

Cell Growth and Subcellular Fractionation. U937 and HL-60 cells were grown and differentiated as described previously (4). Cells from the human monocytic leukemia cell line THP-1 (American Type Culture Collection TIB 202; (6)) were propagated in culture in a similar manner except the medium was RPMI-1640 (supplemented with 0.2% (w/v) $NaHCO_3$, 0.03% (w/v) L-glutamine and 50 μM 2-mercaptoethanol) containing 10% (v/v) fetal bovine serum (Sigma Hybri-Max, not heat-inactivated), 50 U penicillin/ml and 50 μg streptomycin/ml. Cells were harvested after 4-7 days in culture by continuous-flow centrifugation, ruptured by nitrogen cavitation (30 min at 800 psi) and the 100,000 x g microsomal membrane fraction isolated as described previously (5).

Preparation of Membranes from Human Lung. Postmortem human lung samples were obtained from the International Institute for the Advancement of Medicine (Essington, Pennsylvania). The 100,000 x g membrane fraction was isolated from homogenized parenchyma essentially as described before (7) except that the homogenization and resuspension buffer was 0.25 M sucrose, 10 mM Mops/KOH (pH 7.4), 2 mM EDTA containing 2 mM phenylmethylsulfonylfluoride (added from a fresh 200 mM stock in ethanol).

Photoaffinity Labelling of THP-1 Cell Membranes. The preparation of azido^{125}I-LTC_4, a radioiodinated photolabile derivative of LTC_4, and photoaffinity labelling of THP-1 cell microsomal membranes was performed essentially as described before for photolabeling of U937-cell membranes (4).

Purification of Human LTC_4 Synthase from THP-1 Cells. LTC_4 synthase activity was measured essentially as described previously (5) by the formation of LTC_4 in incubation mixtures containing reduced glutathione and the free acid of LTA_4. Unless otherwise indicated, all purification procedures were performed either at 4°C or on ice.

Solubilization of LTC_4 Synthase with Taurocholate. Membrane-bound LTC_4 synthase activity was solubilized with 2% (w/v) taurocholate (Calbiochem) by combining 100,000 x g microsomal membrane suspensions (typically 15-20 mg

protein/ml in PBS (pH 7.4), 2 mM EDTA) with an equal volume of a two-fold concentrated detergent solution (4% (w/v) taurocholate in PBS (pH 7.4), 2 mM EDTA). The mixture was vigorously shaken for 30 min at 4°C then spun at 200,000 x g for 60 min at 4°C. The upper 3/4 of the resulting supernatant was retained and clarified by passing it first through a 0.45 μm filter (Millex-HA, Millipore) then a 0.22 μm filter (Millex-G, Millipore).

Step 1. Anion-Exchange Chromatography. The taurocholate extract of THP-1 cell microsomal membranes (50 ml, containing 500 to 800 mg protein) was injected onto a HiLoad Q Sepharose HP 26/10 anion exchange column (Pharmacia, 2.6 x 10 cm) that had been equilibrated in buffer A, which was composed of 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 2 mM reduced glutathione, 1 mM dithiothreitol, 0.1% (w/v) taurocholate, 0.5% (w/v) *n*-octyl glucoside (Boehringer Mannheim) and 0.5% (w/v) CHAPS (3-((3-cholamidopropyl) dimethylammonia)-1-propane sulfonate; Calbiochem) at a flow rate of 10 ml/min. After washing the column with 300 ml buffer A, bound proteins were eluted with a linear gradient of NaCl (0 to 1.0 M, 1200 ml gradient volume) in buffer A.

Step 2. LTC_2 Affinity Chromatography. The preparation of LTC_2-X-biotin: streptavidin-agarose affinity resins has been recently described (5). LTC_2, a stable analogue of the LTC_4 synthase enzymatic product, LTC_4, was synthesized for use as an affinity ligand essentially as before (8). Biotin was linked to the α-amino group of the γ-glutamate component of the LTC_2 glutathione moiety by incubation with an N-hydroxysuccinimide ester of biotin having a spacer arm length of 2.24 nm (NHS-X-biotin; succinimidyl 6-(biotinamido) hexanoic acid; Molecular Probes) to form LTC_2-X-biotin. LTC_2-X-biotin was then immobilized on streptavidin-agarose at a ligand concentration of 40 μM. The resulting LTC_2-X-biotin:streptavidin agarose affinity resin was packed into a Pharmacia HR10/2 FPLC column (10 mm diameter x 4.5 mm bed height) and was pre-equilibrated at 1.0 ml/min in running buffer B, which was composed of 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 0.1% (w/v) taurocholate, 0.5% (w/v) *n*-octyl glucoside, 0.5% (w/v) CHAPS (similar to running buffer A except without reduced glutathione). The active fractions from the anion-exchange step were pooled

(typically 20 ml in total) and diluted 5-fold into running buffer B to reduce NaCl and glutathione concentrations, both of which inhibited LTC_4 synthase binding to the affinity resin. The preparation was then injected onto the LTC_2 affinity column at a flow rate of 0.2 ml/min. Following sample application, the flow rate was returned to 1.0 ml/min and the column was washed with 10 ml running buffer B. LTC_4 synthase was eluted with a linear NaCl/reduced glutathione co-gradient (from 0 to 1.0 M NaCl and 0 to 4 mM reduced glutathione; 15 ml gradient volume in buffer B).

Step 3. Gel Permeation Chromatography. The active fractions from two LTC_2 affinity column runs were combined (typically 6 ml in total) then concentrated on a YM-10 ultrafiltration membrane (Amicon) to ≤ 0.25 ml and subsequently injected onto a Superdex 75 HR 10/30 column (Pharmacia, 1 x 30 cm) that had been equilibrated in buffer C, which was comprised of 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 2 mM reduced glutathione, 1 mM dithiothreitol, 0.1% (w/v) taurocholate, 0.5% (w/v) n-octyl glucoside, 0.5% (w/v) CHAPS and 0.25 M NaCl (similar to buffer A except also containing NaCl). LTC_4 synthase activity was eluted isocratically at a flow rate of 0.25 ml/min. The Superdex 75 column was calibrated by performing an identical chromatographic separation of LTC_4 synthase co-injected with a mixture of molecular mass standards (0.4 mg each). Fractions were collected throughout the chromatogram and the molecular mass standards were positively identified on silver-stained SDS/polyacrylamide gels. Column calibration was performed by constructing a standard curve of the known molecular mass of the standards versus their respective partition coefficients, K_{av} (where $K_{av}=(V_e-V_o)/(V_t-V_o)$; V_e being the elution volume, V_o the void volume of the column and V_t the total bed volume of the column). The resulting sigmoidal plot of log molecular mass versus K_{av} was used to calculate the native molecular mass of LTC_4 synthase based on the elution volume of enzymatic activity. Calibration and chromatographic separation on Superose 12 HR 10/30 columns (Pharmacia, 1 x 30 cm) was performed in an identical manner.

Amino-Terminal Sequence Determination. Fractions containing the pure 18 kDa LTC_4 synthase polypeptide (approximately 100 pmol) were pooled

together and incubated at room temperature for 48 h with end-over-end mixing in a tube which also contained a 3 x 5 mm sliver of polyvinylidenedifluoride (PVDF) membrane. The sliver was washed several times with water then used directly for sequencing. Conventional Edman degradation microsequencing was performed on a modified Applied Biosystems 477A gas-phase sequenator equipped with a continuous-flow-reactor (9).

Miscellaneous Methods. Human monocytes were isolated from the blood of healthy human volunteers using discontinuous Histopaque gradients as described before (4). Glutathione S-transferase activity was measured spectrophotometrically (10). Microsomal glutathione S-transferase activity was measured in the presence of Triton X-100 following N-ethylmaleimide pretreatment of samples (11,12). SDS/polyacrylamide gel electrophoresis was performed using standard methods (13) and protein bands in gels were visualized by silver staining (14). Silver-staining artefact bands (15) were identified in blank lanes containing SDS/sample buffer only. Protein was determined as described (16) using bovine γ-globulin as standard. In highly purified LTC_4 synthase preparations where protein determination was not possible by this method, protein was estimated by laser densitometry of samples run on SDS/polyacrylamide gels and silver stained. Where indicated, LTC_4 synthase activity is expressed as the mean ± the standard deviation of independent experiments.

RESULTS

THP-1 Cells are an Abundant Source of LTC_4 Synthase Activity. Human lung parenchyma membranes were found to contain a substantial amount of LTC_4 synthase activity (316.5 ± 71.6 pmol of LTC_4 formed • min^{-1} • mg $protein^{-1}$, n = 4; Table 1). We have previously described that the specific activity of LTC_4 synthase in the human promonocytic leukemia cell line U937 (39.9 ± 16.7 pmol of LTC_4 formed • min^{-1} • mg^{-1}; n = 8) was elevated 10-fold (to 399.0 ± 84.1 pmol of LTC_4 formed • min^{-1} • mg^{-1}; n = 47) concomitantly with Me_2SO-induced differentiation into monocyte/macrophage-like cells (4) and was thus comparable to that found in human lung. We therefore tested for LTC_4 synthase activity in related cell lines of monocytic lineage and found endogenously high levels of LTC_4 synthase in the monocytic leukemia cell line THP-1 (302.0 ± 62.0 pmol LTC_4 formed • min^{-1} • mg^{-1}; n=9) without further differentiation.

Purification of Human LTC_4 Synthase. As with Me_2SO-differentiated U937 cells (4,5), LTC_4 synthase activity in THP-1 cells was predominantly localized in the 100,000 x g microsomal membrane fraction and could be solubilized with high recovery (>90%) by the anionic detergent taurocholate (not shown). A permutation of detergents comprised of 0.1% (w/v) taurocholate, 0.5% (w/v) *n*-octyl glucoside plus 0.5% (w/v) CHAPS was found to work well in all chromatographic steps and enabled the purification of LTC_4 synthase from THP-1 cell membranes to homogeneity as described below.

In the first chromatographic step (anion exchange), taurocholate extract from THP-1 cell microsomal membranes (50 ml, containing 500-800 mg protein) was applied to a quaternary aminoethyl anion-exchange column. The column was developed with a linear gradient of NaCl and LTC_4 synthase activity eluted at (c.a.) 0.2 M NaCl (Figure 1). The specific activity of LTC_4 synthase was increased 25 to 35-fold over the taurocholate extract used as starting material and the yield in this step was generally 40-60%.

In a previous report, we described the development of a novel affinity column, LTC_2-X-biotin:streptavidin-agarose, to which LTC_4 synthase could be

bound and eluted in an active form (5). The affinity ligand, LTC_2 (a stable analog of the product of the LTC_4 synthase-catalyzed reaction), was immobilized by first biotinylating it via the primary amino group on the γ-glutamate residue of the LTC_2 glutathione moiety, then immobilizing the biotinylated LTC_2 on streptavidin-agarose at a concentration of 40 μM. The column containing the LTC_2-X-biotin:streptavidin-agarose was equilibrated in a chromatography buffer which was identical to that used for anion exchange except that glutathione was omitted since it competed with the LTC_2 affinity ligand for LTC_4 synthase binding. The active fractions from anion-exchange chromatography were pooled, diluted 5-fold with chromatography buffer (in order to reduce the concentration of NaCl and glutathione, both of which prevented LTC_4 synthase binding to the affinity column) and passed on to the affinity column at a low flow rate (Figure 2). The vast majority of the total protein went through the affinity column whereas all of the LTC_4 synthase activity was bound. The column was developed with a NaCl/reduced glutathione co-gradient and LTC_4 synthase activity eluted at (c.a.) 0.3 M NaCl, 1.2 mM reduced glutathione. LTC_4 synthase activity was enriched 50 to 60-fold by this step with a yield of 5-15%.

The final chromatographic step in the purification of human LTC_4 synthase from THP-1 cell membranes was gel permeation through a Pharmacia Superdex 75 column (exclusion limit = 100,000; selectivity range = 3,000-70,000). The active fractions from LTC_2 affinity chromatography were pooled, concentrated to ≤ 250 μL by ultrafiltration using a YM-10 membrane (Amicon) then injected onto the column (Figure 3). LTC_4 synthase specific activity was enriched a further 3 to 5-fold over the LTC_2 affinity column active fraction used as starting material with a recovery of 20-30%.

Properties of Purified Human LTC_4 Synthase. A representative purification series is summarized in Table 2. Beginning with 2×10^{11} THP-1 cells, LTC_4 synthase was purified 27,384-fold over intact cells. The yield was < 1% owing in part to the intentional use of only the very centre fractions (generally accounting for ≤ two-thirds) of the activity peaks. The final preparation consisted of a single polypeptide having a molecular mass of about 18 kDa (Figure 4). It was judged

to be homogeneous based on the presence of a single protein band on silver-stained SDS-denaturing gels (Figure 4b) and on isoelectric focusing gels (not shown) and by the presence of single PTH amino-acid products following each cycle of sequential Edman degradation (see below). Approximately 3.5 μg of the purified 18 kDa polypeptide (c.a. 200 pmol) was generated by this procedure from 2×10^{11} THP-1 cells. (This was reproducible in other preparations within 25%.) The V_{max} of the final preparation was 4.1 μmol LTC_4 formed • min^{-1} • mg^{-1} (Table 2) and the K_m for LTA_4 was 9.9 μM whereas the K_m for reduced glutathione was 1.7 mM (not shown). This was in close agreement with the K_m values for LTC_4 synthase in Me_2SO-differentiated U937 cells and in human blood monocytes (5). Human LTC_4 synthase did not have detectable glutathione S-transferase activity in the presence of reduced glutathione plus either 1-chloro-2,4-dinitrobenzene, 4-nitrobenzyl chloride or 1,2-epoxy-3-(4-nitrophenoxy)propane (not shown).

LTC_4 Synthase is a Homodimer Comprised of 18 kDa Subunits. As described above and in Figure 4b, the > 25,000-fold purified preparation of LTC_4 synthase was composed of a single polypeptide having a molecular mass of 18 kDa. This polypeptide corresponds to an 18 kDa polypeptide from Me_2SO-differentiated U937 cell membranes that was specifically photolabeled by azido^{125}I-LTC_4, a radioiodinated photoreactive LTC_4 photoaffinity probe (4,5). An apparently identical 18 kDa polypeptide was the only protein specifically labelled by azido^{125}I-LTC_4 in THP-1 cell membranes (Ali *et al.*, in preparation). The ability of various leukotrienes to competitively inhibit LTC_4 synthase activity in THP-1 cell microsomal membranes ($LTC_2 \geq LTC_4 > LTD_4 > LTE_4 > LTB_4$; Ali *et al.*, in preparation) corresponded exactly with their ability to compete for photolabelling of the 18 kDa polypeptide in THP-1 cell membranes (Figure 5) lending further support to the identification of the 18 kDa polypeptide as being LTC_4 synthase. On calibrated gel filtration columns, however, purified LTC_4 synthase activity eluted at a volume corresponding to 39.2 ± 3.3 kDa (n=12), twice the molecular mass of the 18 kDa polypeptide constituent of the active fractions (see Figure 3 inset). This was the case for two different gel filtration media where the native molecular mass was determined to be 37.5 ± 3.1 kDa (Pharmacia Superose 12; n=5) and 40.4 ± 3.2

(Pharmacia Superdex 75; n=7). Although it is possible that the native molecular mass for LTC_4 synthase was overestimated owing to artefacts sometimes associated with performing gel filtration on detergent-solubiiized membrane proteins, it does not appear to be the case in this instance since in some preparations of LTC_4 synthase where activity was lost following LTC_2 affinity chromatography the 18 kDa polypeptide eluted later at a volume corresponding to 15-20 kDa. Enzymatically active LTC_4 synthase therefore appears to be a homodimer comprised of two identical 18 kDa subunits.

Amino-terminal Sequence of Human LTC_4 Synthase. The pure 18 kDa LTC_4 synthase polypeptide (≈ 100 pmol, purified from 2 x 10^{11} THP-1 cells) was adsorbed onto a sliver of polyvinylidenedifluoride (PVDF) membrane and the amino terminal sequence of the first 35 amino acids, representing an estimated 20% of the 18 kDa polypeptide, was determined by automated Edman degradation (Figure 6). The sequence was unique and was not homologous to any known sequences contained in available databases. The protein kinase C consensus phosphorylation sequence Ser-Ala-Arg (17) was present at amino acid positions 28-30. A methionine residue was present in position 1, indicating that LTC_4 synthase is probably not synthesized as a larger precursor polypeptide.

DISCUSSION

LTC_4 synthase is a key enzyme is the synthesis of biologically active leukotrienes. Since it catalyses the first committed enzymatic step in the formation of the cysteinyl leukotrienes, which together comprise the slow-reacting substance of anaphylaxis, its activity may substantially affect the profile of these inflammatory mediators in pathological situations.

LTC_4 synthase catalyses a glutathione S-transferase reaction in which reduced glutathione is conjugated to the precursor leukotriene A_4 to form LTC_4. As such, LTC_4 synthase may be a member of the glutathione S-transferase multi-gene family of enzymes that so far, with the exception of LTC_4 synthase, are involved in cellular detoxification events (for review see (18,19)). Except for LTC_4 synthase, these glutathione S-transferases have been purified and in most cases cloned. We have previously demonstrated that human LTC_4 synthase is a unique membrane-bound enzymatic activity that is completely distinct from α, μ, π, θ and microsomal glutathione S-transferases (4). A partial purification of the enzyme (>10,000-fold) from the microsomal membranes of Me_2SO-differentiated U937 cells was enabled by the finding that enriched LTC_4 synthase preparations (>500-fold) were dependent on the presence in LTC_4 synthase incubation mixtures of Mg^{2+} and phosphatidylcholine for activity (5). In the current study, we have purified LTC_4 synthase >25,000-fold to homogeneity from the human monocytic leukemia cell line THP-1 and found that LTC_4 synthase has some similarities with other members of the glutathione S-transferase family of enzymes. In particular, LTC_4 synthase is enzymatically active as a homodimer of two identical 18 kDa subunits, a common feature of other glutathione S-transferases which are multimeric enzymes comprised of low molecular mass subunits (11,18,19).

That the purified 18 kDa polypeptide is LTC_4 synthase was further supported by photoaffinity labelling studies using azido^{125}I-LTC_4. In crude membrane preparations from both Me_2SO-differentiated U937 cells (4,5) and THP-1 cells (Ali *et al.* in preparation), azido^{125}I-LTC_4 specifically labelled an 18 kDa polypeptide that was elevated during Me_2SO-induced differentiation of U937 cells

concomitantly with LTC_4 synthase activity, and which was constitutively present in THP-1 cells without further differentiation as was LTC_4 synthase activity.

The amino-terminal sequence of human LTC_4 synthase was comprised predominantly of hydrophobic amino acids, consistent with the membrane-bound localization of the enzyme. It contained an amino-terminal methionine residue indicating that human LTC_4 synthase is probably not proteolytically processed during its biogenesis *in vivo*. Most interestingly, a consensus sequence for protein kinase C phosphorylation was present suggesting that human LTC_4 synthase may be a phosphoregulated enzyme. Phosphoregulation of LTC_4 synthase may constitute a potentially important controlling mechanism that may substantially affect cysteinyl leukotriene production in several inflammatory disease states. Preliminary results in our laboratories using HL-60 cells have demonstrated that phorbol-12-myristate-13-acetate, an activator of protein kinase C, abolished cysteinyl leukotriene production in ionophore-challenged HL-60 cells, whereas inhibition could be prevented by the presence of the protein kinase C inhibitor staurosporine (Kargman *et al.*, in preparation).

In summary, human LTC_4 synthase, a homodimeric glutathione S-transferase comprised of two identical 18 kDa subunits, has been purified to homogeneity. LTC_4 synthase is a unique enzyme which is responsible for the production of LTC_4 and may therefore be a key controlling enzyme in the biosynthesis of inflammatory leukotriene mediators.

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FIGURE LEGENDS

Figure 1 Anion-exchange chromatography. A taurocholate extract (50 mL) of the 100,000 x g membrane fraction from THP-1 cells was applied to a High Load 26/60 Q-Sepharose anion-exchange column (see Methods) that had been equilibrated in buffer A. The column was developed with a linear gradient of NaCl in buffer A and was monitored by on-line measurement of the optical density at 280 nm as indicated ([NaCl] and OD_{280nm}, respectively). Fractions were collected throughout the chromatogram and were subsequently assayed for LTC_4 synthase activity in standard incubation mixtures as described in Methods. LTC_4 synthase activity (●) was eluted from the column at about 0.2 M NaCl in the region indicated by the hashed bar (to which the insert corresponds). The relative specific activity of LTC_4 synthase in each fraction (○) was calculated with respect to the LTC_4 synthase specific activity of the taurocholate (TC) extract used as starting material, which was set at 1.

Figure 2 LTC_2 affinity chromatography. The active fractions from anion-exchange chromatography were pooled, diluted 5-fold in buffer B then applied to an LTC_2 affinity column (constructed by immobilizing biotinylated LTC_2 on streptavidin-agarose) that had been equilibrated in buffer B (see Methods). The column was developed with a linear NaCl/reduced glutathione co-gradient in buffer B and monitored by on-line measurement of the optical density at 280 nm as indicated ([NaCl, GSH] and OD_{280nm} respectively; note, the gap indicated in the chromatogram during the loading portion of the run corresponds to 20 mL). Fractions were collected throughout the chromatogram and were subsequently assayed for LTC_4 synthase activity in standard incubation mixtures as described in Methods. LTC_4 synthase activity (inset, ●) was eluted at 0.3 M NaCl, 1.2 mM

reduced glutathione).

Figure 3 Gel filtration chromatography. The active fractions from LTC_2 affinity chromatography (2 runs) were pooled, concentrated by ultrafiltration then applied to a Superdex 75 gel filtration column that had been equilibrated in buffer C (see Methods). Proteins were eluted isocratically with buffer C. Fractions were collected throughout the chromatogram and were subsequently assayed for LTC_4 synthase activity (●) in standard incubation mixtures as described in Methods. *Inset:* The Superdex 75 column was calibrated and the native molecular mass of LTC_4 synthase determined by performing an identical chromatographic separation of LTC_4 synthase co-injected with a mixture (0.4 mg each) of the indicated standards. A calibration curve was constructed by plotting the log of the known molecular mass of the standards (O) versus their respective partition coefficient (K_{av}) on the column (see Methods). LTC_4 synthase activity (●) eluted at a volume corresponding to 38 kDa.

Figure 4 Polypeptide constituents of chromatography fractions. Various fractions from LTC_2 affinity chromatography (panel A) or Superdex 75 gel filtration (panel B) were dissociated in SDS-containing sample buffer then resolved by electrophoresis through SDS/polyacrylamide gels (8-16% polyacrylamide gradient). Protein bands were visualized by silver staining. Each gel included a sample-buffer-only blank (lane 4 panel A, lane 1 panel B) to identify artefact bands resulting from silver staining (see Methods), which are indicated by an asterisk. The migration of molecular mass standards (in kDa), the origin, (Or.) and front (Fr.) are indicated in the lane marked Stds.

A. LTC_2 affinity chromatography. Equal volumes of the anion-exchange pool used as starting material for LTC_2 affinity

chromatography (lane 1), the column pass-through (lane 2) and the fractions from the elution gradient in the region where LTC_4 synthase activity was detected (lanes 5-12) were resolved. LTC_4 synthase activity peaked in the fraction corresponding to lane 7.
B. Gel filtration. Equal volumes of the fractions in the region where LTC_4 synthase activity eluted from the column were resolved. LTC_4 synthase activity peaked in the fraction corresponding to lane 3.

Figure 5 Photoaffinity labelling of an 18 kDa polypeptide in the THP-1 cell 100,000 x g membrane fraction. THP-1 cell microsomal membranes (0.3 mg protein) were incubated with 20 pM azido^{125}I-LTC_4 in either the absence (Control) or presence of 0.33 μM (panel A) or 3.3 μM (panel B) competing LTC_2, LTC_4, LTD_4, LTE_4, LTA_4 or LTB_4, as indicated, for 30 min at 25°C (see Methods). Following photolysis, labelled proteins were resolved on SDS/polyacrylamide gels and visualized by autoradiography. The specifically-labelled 18 kDa polypeptide is shown (molecular mass standards (in kDa) are indicated on the right under Stds). Lanes were organized by the rank order of potency of the indicated leukotrienes to competitively inhibit LTC_4 synthase activity in standard incubation mixtures (which was $LTC_2 \geq LTC_4 > LTD_4 > LTE_4 > LTB_4$; LTA_4 being a co-substrate for LTC_4 synthase could not be tested for inhibition).

Figure 6 Amino-terminal sequence of human LTC_4 synthase. Purified human LTC_4 synthase was sequenced by automated Edman degradation as described in Methods. Beginning with the N-terminal methionine, amino acids are listed by their three-letter abbreviation below their single letter code. The residue at position 31 could not be distinguished between isoleucine or arginine. A potential protein kinase C phosphorylation site at positions 28-30 is underlined.

Table 1. LTC_4 synthase activity in cells of human origin. The specific activity of LTC_4 synthase was determined in human cell lines, cells from peripheral human blood or membranes from human lung parenchyma as described in Methods. For human cell lines (U937, HL-60, THP-1) the prefix 'd' indicates they were differentiated in culture by growth in the presence of either dimethylsulfoxide (Me_2SO) or phorbol-12-myristate-13-acetate (PMA) as indicated. Data are the mean ± the standard deviation of separate experiments. *Data derived from ref 14.

Source	LTC_4 Synthase Specific Activity (pmol LTC_4 formed•min^{-1}•mg^{-1})	
Human Cell Line		
U937	39.9 ± 16.7	(n=8)*
dU937/Me_2SO	399.0 ± 84.1	(n=47)*
dU937/PMA	45.9 ± 8.4	(n=5)
HL-60	6.8 ± 2.3	(n=4)
dHL-60/Me_2SO	30.7 ± 12.7	(n=3)
dHL-60/PMA	50.5 ± 17.3	(n=3)
THP-1	302.0 ± 62.0	(n=9)
Human Tissue		
monocytes	21.5 ± 4.8	(n=5)*
lung membranes	316.5 ± 71.6	(n=4)

Table 2. Summary of LTC_4 synthase purification from THP-1 cells. A representative purification series is shown. LTC_4 synthase was purified beginning with $2x10^{11}$ THP-1 cells. LTC_4 synthase activity was determined in standard incubation mixtures as described in Methods. The yield (% recovery) was calculated based on the total activity in cells which was set at 100%. The relative specific activity was calculated with respect to the LTC_4 synthase specific activity in cells which was set at 1.0.

Fraction	Volume (ml)	Activity ($nmol \cdot min^{-1} \cdot ml^{-1}$)	Total Activity (nmol/min)	Recovery (%)	[Protein] (mg/ml)	Total Protein (mg)	Specific Activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)	Relative Specific Activity
Harvested THP-1 Cells	2000	4.364	8728	100 (set)	28.9	57,800	0.15	1.0 (set)
100,000 xg membranes	150	16.1	2418	27.7	40.0	6000	0.40	2.7
Taurocholate extract	270	8.26	2230	25.5	13.2	3554	0.63	4.2
HiLoad Q Anion Exchange	100	9.83	982.5	11.3	0.49	49.0	20.6	137
LTC_2 Affinity	30	2.50	74.76	0.86	0.0023	0.069	1084	7178
Superdex 75 Gel Filtration	11	1.38	15.16	0.17	0.00033	0.0037	4135	27,384

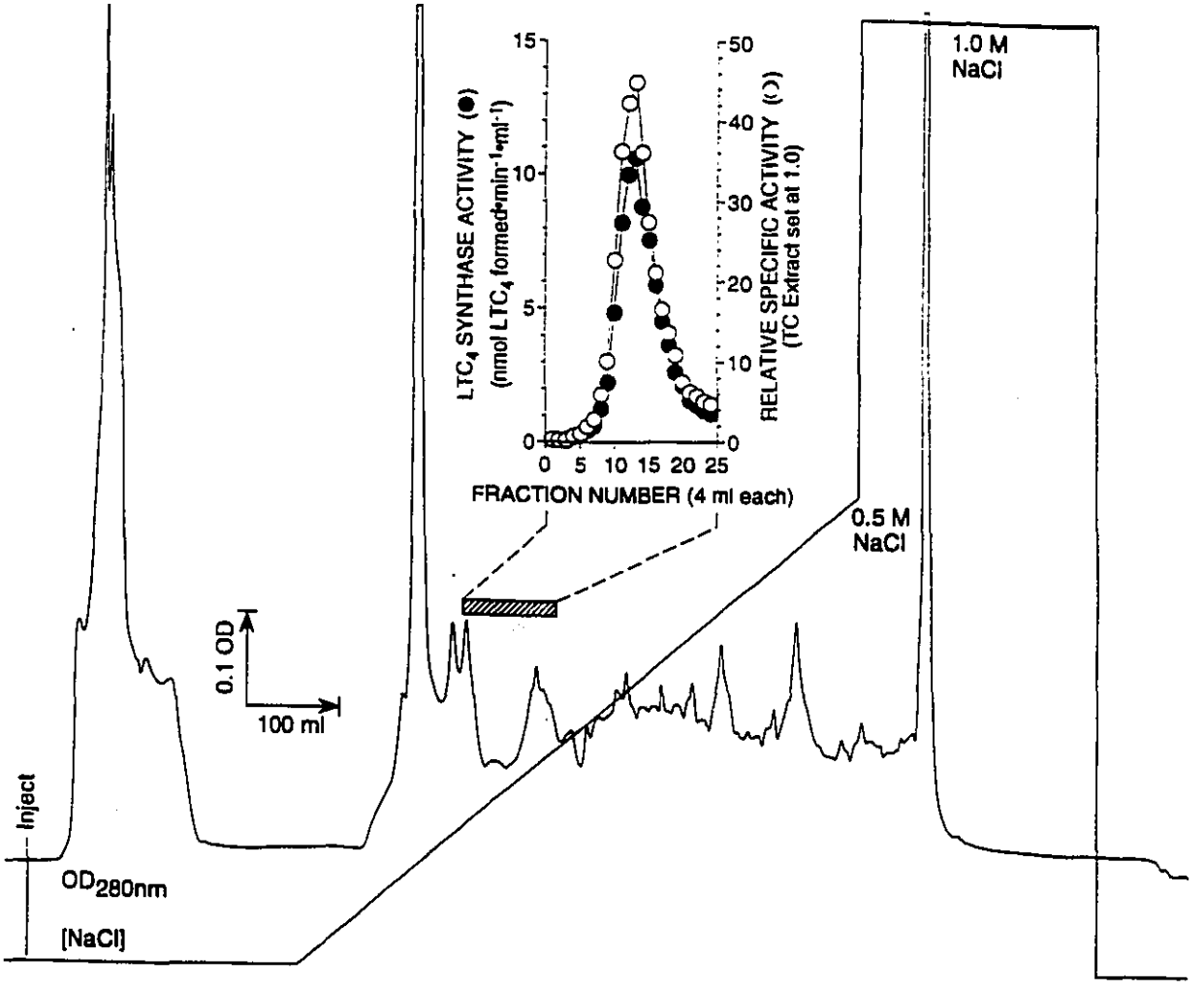
1.0 M
NaCl
0.5 M
NaCl
LTC4 SYNTHASE ACTIVITY (●)
(nmol LTC4 formed•min-1•ml-1)
RELATIVE SPECIFIC ACTIVITY (○)
(TC Extract set at 1.0)
FRACTION NUMBER (4 ml each)
0.1 OD
100 ml
Inject
OD280nm
[NaCl]

Figure 1

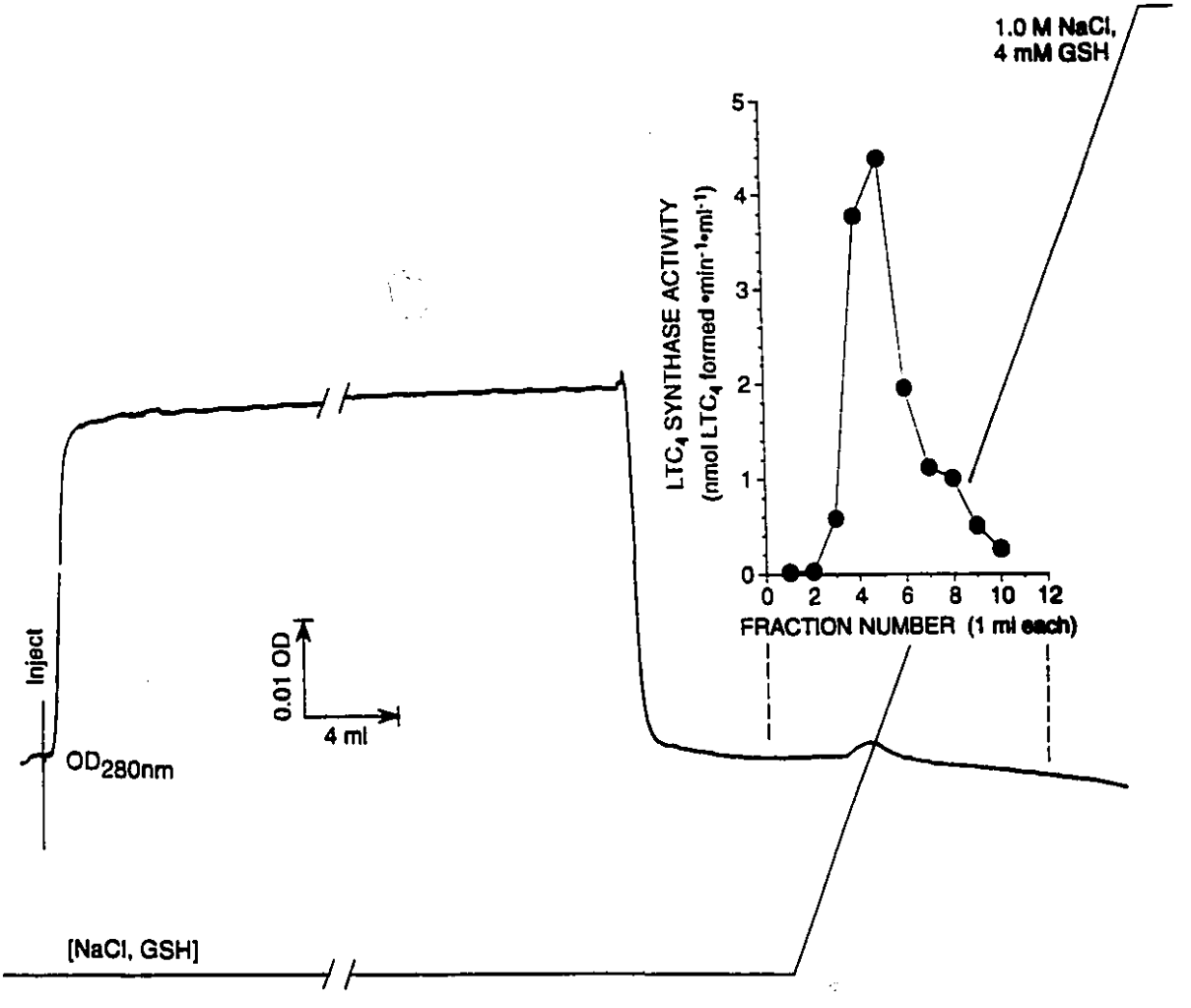
1.0 M NaCl,
4 mM GSH
LTC4 SYNTHASE ACTIVITY
(nmol LTC4 formed •min-1•ml-1)
0
1
2
3
4
5
0 2 4 6 8 10 12
FRACTION NUMBER (1 ml each)
Inject
OD280nm
0.01 OD
4 ml
[NaCl, GSH]

Figure 2

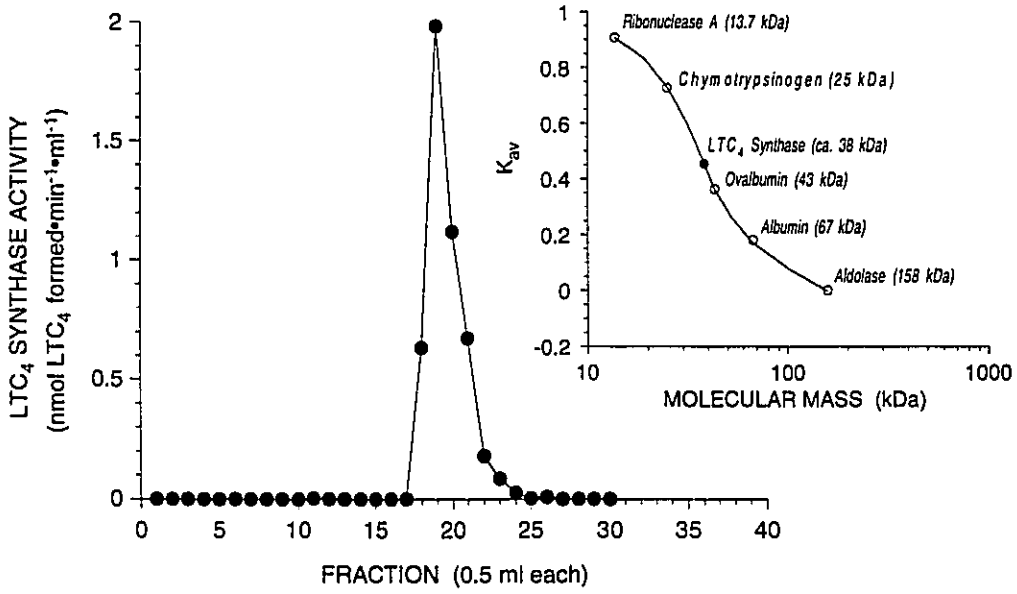
LTC4 SYNTHASE ACTIVITY
(nmol LTC4 formed•min-1•ml-1)
FRACTION (0.5 ml each)
Kav
MOLECULAR MASS (kDa)
Ribonuclease A (13.7 kDa)
Chymotrypsinogen (25 kDa)
LTC4 Synthase (ca. 38 kDa)
Ovalbumin (43 kDa)
Albumin (67 kDa)
Aldolase (158 kDa)

Figure 3

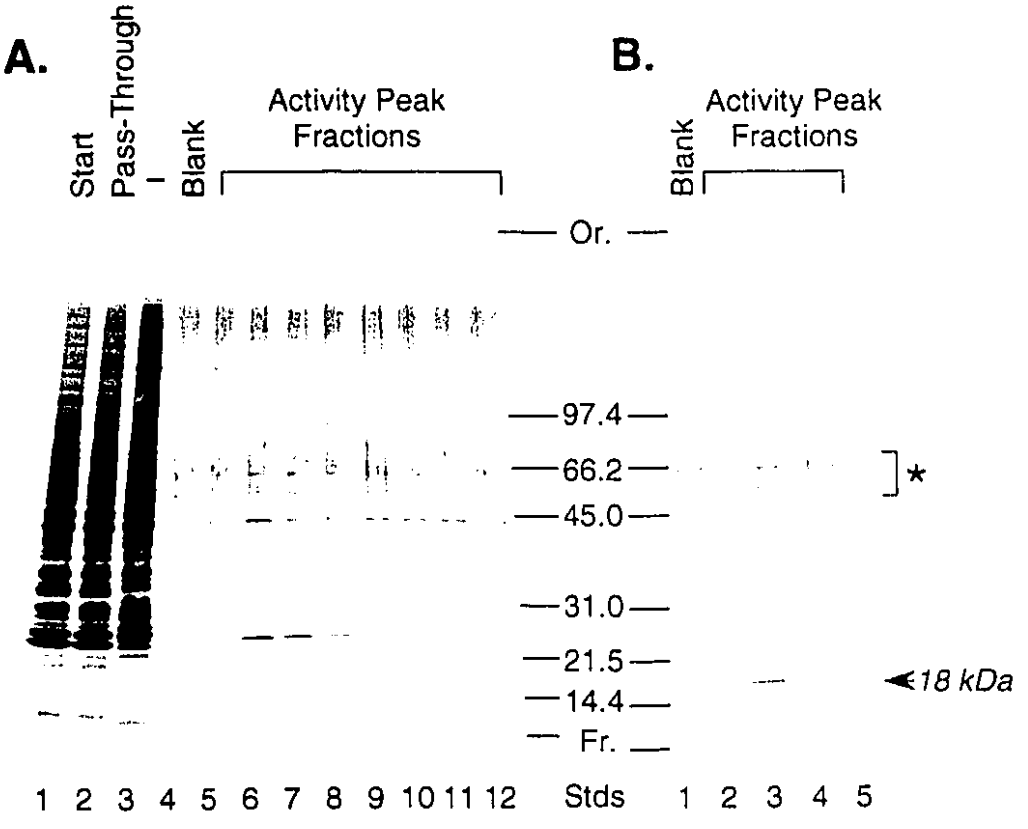
A.
B.
Start
Pass-Through
Blank
Activity Peak Fractions
Blank
Activity Peak Fractions
Or.
97.4
66.2
45.0
31.0
21.5
14.4
Fr.
*
18 kDa
1 2 3 4 5 6 7 8 9 10 11 12 Stds 1 2 3 4 5

Figure 4

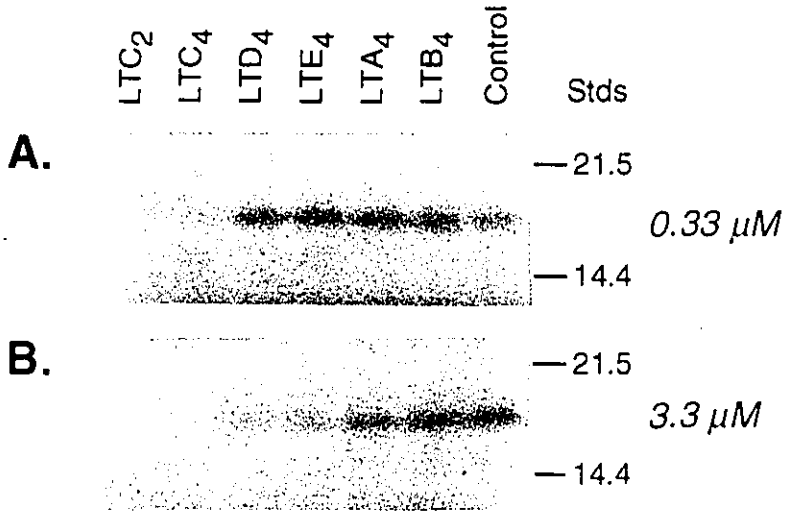
LTC2
LTC4
LTD4
LTE4
LTA4
LTB4
Control
Stds
A.
21.5
0.33 μM
14.4
B.
21.5
3.3 μM
14.4

Figure 5

M K D E V A L L A A V T L L G
Met-Lys-Asp-Glu-Val-Ala-Leu-Leu-Ala-Ala-Val-Thr-Leu-Leu-Gly- 15

V L L Q A G F S L Q V I *S A R*
Val-Leu-Leu-Gln-Ala-Gly-Phe-Ser-Leu-Gln-Val-Iso-*Ser-Ala-Arg*- 30

I A F R V
[Iso]-Ala-Phe-Arg-Val-
[Arg]

Figure 6

7. MANUSCRIPT D

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

Mol. Pharmacol. In press.(1994)

Coincident with the purification of LTC_4 synthase and the identification of protein kinase C consensus sequence in the amino terminus of LTC_4 synthase, a preliminary study demonstrated specific inhibition of cysteinyl leukotriene biosynthesis by phorbol-ester mediated activation of protein kinase C in a neutrophilic HL-60 cell line.

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, GMCSF-primed, dimethylsulfoxide-differentiated HL-60 cells, which resemble mature granulocytes, synthesized leukotrienes LTA_4, LTB_4, LTC_4 and LTD_4. The synthesis of the sulfidopeptide leukotrienes, 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 non-peptide branch of the leukotriene pathway, nor the formation of LTA_4, the precursor for both branches of the leukotriene biosynthetic pathway, was significantly affected by the presence of PMA during A23187 stimulation. 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. The protein kinase C inhibitor staurosporine completely reversed the inhibition of LTC_4 production by PMA 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. Since 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.

Introduction

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

Leukotrienes are synthesized in inflammatory cells (for review see 2-5) following 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 (FLAP), and is subsequently metabolized by one of two pathways. It may either be stereoselectively hydrolysed to form LTB_4 by the enzyme LTA_4 hydrolase, or conjugated with glutathione to form the sulfidopeptide leukotriene 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 leukotrienes) comprise the slow reacting substance of anaphylaxis (8).

LTC_4 synthase catalyses the first committed step in the biosynthesis of the cysteinyl leukotrienes. 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

protein kinase C 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 capable of complete *de novo* biosynthesis of both the cysteinyl and non-cysteinyl leukotrienes.

The human HL-60 cell line is of promyelocytic lineage and can be differentiated in culture by growth in the presence of dimethylsulfoxide (Me_2SO) into cells that morphologically resemble mature granulocytes (12-14). Me_2SO-differentiated cells contain the complete compliment of enzymes necessary for the formation of leukotrienes LTA_4, LTB_4, LTC_4 and LTD_4 (9, 15-18). Furthermore, the overall leukotriene 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 FLAP levels (19,20) but does not affect LTC_4 synthase (K. Scoggan and D. Nicholson, unpublished results). Me_2SO-differentiated HL-60 cells synthesize leukotrienes 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 (fMLP) (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 non-enzymatically to 6-*trans* LTB_4 and 6-*trans*-12-*epi* LTB_4. Since Me_2SO-differentiated HL-60 cells are capable of forming all of the major leukotrienes (whereas other myelocytic leukemia cell lines are not), they are a useful model for studying the regulation of specific enzymes in the leukotriene 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 leukotriene production or profile in inflammatory cells. Since LTC_4 synthase contains a putative protein kinase C 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 protein kinase C (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 protein kinase C prevented the PMA-mediated inhibition of sulfidopeptide leukotriene 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 leukotriene formation may be controlled by phosphoregulation of LTC_4 synthase in a protein kinase C-dependent manner.

Experimental Procedures

Cell Growth and Differentiation - HL-60 cells, obtained from the American Type Culture Collection (CCL 240, 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, St. Louis, MO), 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (Flow Laboratories, McLean, VA) at 37°C 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.

Leukotriene Formation in Intact Cells - Prior to stimulation, cells were pelleted by centrifugation at 500x *g* for 10 min, dispersed in Dulbecco's phosphate buffered saline, pelleted again and then resuspended in Dulbecco's phosphate-buffered saline containing 1 mM EDTA and 1 mg/ml D-glucose at 2 x 10^7 cells/ml. Cells were primed by incubation with 200 pM recombinant human granulocyte macrophage-colony stimulating factor (GMCSF) (Genzyme, Cambridge, MA) for 30 min at 37°C to increase leukotriene 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°C with gentle mixing. Stocks 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 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 x 10^7 cells in a final volume of 2 ml) were incubated for 10 min at 37°C, and then the reaction was terminated by addition of 2 ml ice-cold methanol containing 0.2 nmol/ml of prostaglandin B_2 (PGB_2). Cell viability was determined by trypan-blue exclusion prior to termination of the reaction. Leukotrienes were extracted from the samples and analyzed by reverse-phase HPLC on a Novapak C_{18} column (3.9 x 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. Leukotriene 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 non-enzymatic 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 (pH 7.4) buffer, 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 x 10^7 cells, were incubated for 15 min at 25°C, then reactions were terminated by the addition of 150 µl cold

acetonitrile:methanol:acetic acid (50:50:1, v/v). Precipitated proteins were removed by centrifugation for 15 min at 16,000 x g and leukotrienes in the resulting supernatant were resolved by isocratic reverse-phase HPLC on a Novapak C_{18} column (3.9 x 150 mm; Waters Associates, Milford MA) with a mobile phase comprised 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 (eg. Table 3), pre-treatment with kinase activators or inhibitors was performed on intact cells followed by measurement of LTC_4 synthase activity immediately following 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 Leukotriene 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. In order to examine the effect of PMA on leukotriene biosynthesis, cells were stimulated with A23187 in the absence or presence of the phorbol ester PMA. Analysis of the products formed by reverse-phase HPLC (Figure 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 non-enzymatic hydrolysis products of LTA_4 (6-*trans* LTB_4 and 6-*trans*-12 *epi* LTB_4) did not appear to be significantly affected by this treatment. There was a small reduction in the overall production of leukotrienes by these cells corresponding to the loss of sulfidopeptide leukotriene levels. Determination of the amount of each leukotriene produced in response to A23187 in the absence or presence of PMA demonstrated that there was an approximate 90% reduction in LTC_4 formation, 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 to 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 (Figure 2A). Examination of the effect of PMA on each leukotriene separately confirmed that with the exception of LTD_4, only LTC_4 was inhibited by these concentrations of PMA (Figure 2B). LTD_4 production would be expected to be inhibited by PMA in

these cells since 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 (Figure 2A, inset), nearly identical to that for the inhibition of the formation of its 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. Since PMA did not substantially affect the biosynthesis of the LTB_4 branch of the leukotriene biosynthetic pathway or the formation of the common precursor leukotriene, LTA_4, it appears to act at a site affecting sulfidopeptide leukotriene formation only.

GMCSF priming has previously been shown to elevate leukotriene 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 were either primed or not by pre-incubation with 200 pM GMCSF. In both GMCSF-primed and non-primed cells, PMA specifically abolished sulfidopeptide leukotriene formation in response to A23187 challenge (data not shown). The effects of GMCSF, which elevated overall leukotriene formation, did not include any modulation of the PMA-dependent reduction in sulfidopeptide leukotriene formation.

Inhibition of LTC_4 Formation by PMA is Prevented by Protein Kinase C Inhibitors, but not by Other Kinase Inhibitors - In order to investigate whether phorbol-ester activation of PKC may be occurring 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 leukotrienes produced in differentiated HL-60 cells stimulated by A23187 in the absence or presence of PMA (Figure 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 (Figure 3B; these results were quantified and are shown in Figs. 3C and 3D 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 leukotriene (Figure 4A). As seen in Figure 4B, only the formation of LTC_4 and LTD_4 were affected at these concentrations of staurosporine.

LTC_4 formation in response to ionophore challenge was inhibited by treatment with PMA (an activator of protein kinase C) 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 protein kinase C inhibitors staurosporine 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 Following 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.

In order 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 which was devoid of PKC (ref. 11 active fraction from anion-exchange). Neither PMA nor staurosporine at concentrations from 0.05-10 μM had any effect on the enzymatic activity of purified LTC_4 synthase (Figure 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.

We have combined pure human LTC_4 synthase with pure PKC in the presence of (^{32}P)ATP but despite several permutations have been so far 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 which 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 tumour 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 leukotrienes) was specifically inhibited. Since 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 leukotriene 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 leukotriene pathway; namely, LTC_4 synthase. Since LTC_4 synthase catalyzes the first committed step in the biosynthesis of cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4), modulation of its activity would be expected to affect the production of the subsequently formed peptido-leukotrienes, such as LTD_4, as was in fact observed in these studies.

Since PMA is known to activate PKC, we used a PKC inhibitor, staurosporine (31), to examine the specificity of the PMA-induced inhibition of sulfidopeptide leukotriene formation. Staurosporine was able to reverse the PMA-induced inhibition of LTC_4 and LTD_4 biosynthesis in A23187-stimulated HL-60 cells. In order 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 genistein or

herbimycin A. These results suggest that LTC_4 synthase may be regulated by phosphorylation in a negative fashion 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 (PAF) and PMA have been shown to induce the cellular synthesis of leukotrienes. There are reports of synergy between some of these stimuli resulting in enhanced leukotriene synthesis. It has been demonstrated, for example, that PMA and A23187 synergistically induced LTC_4 synthesis in murine macrophages (32,33) whereas others have shown that A23187 and PMA synergistically induced LTB_4 synthesis in human PMNs (34). These investigations differed from our studies in two ways. Firstly, these investigators used sub-optimal concentrations of PMA and A23187 and secondly, they found that neither stimuli alone induced leukotriene synthesis. In contrast, we observed leukotriene 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 leukotriene 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 abovementioned 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 leukotriene profile.

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 has identified the PKC consensus sequence -Ser-Ala-Arg- within the N-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, further 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 MAP 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 catalyses the first committed step in the formation of the biologically-active sulfidopeptide leukotrienes, appears to be phosphoregulated by a PKC-type activity. Downregulation 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 leukotrienes involved in airway hyperreactivity and related inflammatory conditions.

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Figure Legends

Figure 1: Leukotrienes 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°C 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°C. Samples were mixed with an equal volume of ice-cold methanol containing PGB_2 , extracted and leukotrienes analyzed by reverse-phase HPLC. The elution positions of leukotriene standards is indicated above peaks. Chromatographs from samples incubated in the absence (solid line) or presence (broken line) of PMA are superimposed. Chromatograms from an experiment that is representative of about 20 others are shown.

Figure 2: Inhibition by PMA of Leukotriene Production in Me_2SO-differentiated HL-60 Cells. Me_2SO-differentiated HL-60 cells were primed for 30 min at 37°C 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°C. Samples were mixed with an equal volume of ice-cold methanol containing PGB_2, extracted and leukotrienes analyzed by reverse-phase HPLC. **A.** PMA dose-response curve for total leukotrienes, 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 leukotrienes. Values are the average of duplicate determinations of a representative experiment.

Figure 3: Reversal of PMA-induced Inhibition of LTC_4 and LTD_4 Production by Staurosporine. Me_2SO-differentiated HL-60 cells were primed for 30 min at 37°C 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°C. Samples were mixed with an equal volume of ice-cold methanol containing PGB_2, extracted and leukotrienes analyzed by reverse-

phase HPLC. **A.** HPLC profile of leukotrienes from A23187-stimulated cells incubated in the absence (solid line) or presence (broken line) of PMA. **B.** HPLC profile of leukotrienes from A23187-stimulated cells incubated in the absence (solid line) or in the presence of PMA and staurosporine (broken line). **C.** Quantitation of inhibition of LTC_4 biosynthesis by PMA and the reversal of the PMA-induced inhibition of LTC_4 formation by staurosporine. **D.** Quantitation of inhibition of LTD_4 biosynthesis by PMA and the reversal of the PMA-induced inhibition of LTD_4 formation by staurosporine. For **C** and **D**, values are the mean ± range of duplicate determinations of a representative experiment.

Figure 4: Reversal by Varying Concentrations of Staurosporine 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°C 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°C. Samples were mixed with an equal volume of ice-cold methanol containing PGB_2, extracted and leukotrienes 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 leukotrienes. Values are the average of duplicate determinations of a representative experiment.

Figure 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 $nmol \cdot min^{-1} \cdot mg^{-1}$) which was incubated at 25°C 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 prepared in Me_2SO) in a final volume of 150 μl. The reaction was terminated with the addition of 150 μl cold acetonitrile:methanol:acetic acid (50:50:1, v/v/v). Products were

analyzed by reverse-phase HPLC as described under "Experimental Procedures". Values are the average of duplicate determinations of a representative experiment.

Footnotes

Acknowledgments: We thank Philip Tagari for insightful discussions and Pascale Rousseau for excellent technical assistance. We also thank Carolyn Green for her assistance in the preparation of this manuscript.

Table 1: Preferential Inhibition by PMA of Sulfidopeptide Leukotriene Biosynthesis in Me_2SO-differentiated HL-60 Cells Stimulated with A23187. Me_2SO-differentiated HL-60 cells were primed for 30 min at 37°C 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°C. Samples were mixed with an equal volume of ice-cold methanol containing PGB_2, extracted and leukotrienes analyzed by reverse-phase HPLC. The table is a representative experiment where the values represent the mean ± range of duplicate determinations. Values for each leukotriene produced in the presence of PMA are also expressed as a percentage of the same leukotriene produced in the absence of PMA (% of control).

	nmol leukotriene formed / 10^8 cells		
LEUKOTRIENE	- PMA	+ PMA	% 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 Leukotrienes	6.31 ± 0.78	5.26 ± 0.47	83.4

Table 2: Effect of Various Inhibitors on Total Sulfidopeptide Leukotriene Production in Me_2SO-Differentiated HL-60 Cells Treated with PMA. Me_2SO-differentiated HL-60 cells were primed for 30 min at 37°C with 200 pM GMCSF and then incubated with the indicated inhibitor or vehicle (Me_2SO) for 90 min at 37°C. Following 10 min treatment with 50 nM PMA at 37°C, cells were challenged with 1 μM A23187 for 10 min at 37°C. Reactions were terminated by the addition of an equal volume of ice-cold methanol containing PGB_2. Products were extracted and leukotrienes were analyzed by reverse-phase HPLC. The table is a representative experiment where the values represent the mean ± range of duplicate determinations. Data is also expressed as a percentage of the control to which no PMA was added (% of control).

PMA	INHIBITOR	INHIBITOR TYPE	TOTAL SULFIDOPEPTIDE LEUKOTRIENES	
			pmol/min/10^6 cells	% of control
-			3.64 ± 0.17	100
+			0.55 ± 0.06	15.1
+	staurosporine (1μM)	PKC	4.34 ± 0.12	119
+	bisindolylmaleimide (1μM)	PKC	3.47 ± 0.06	95.3
+	genistein (100μM)	TK	0.63 ± 0.05	17.4
+	herbimycin A (40μM)	TK	0.75 ± 0.13	20.5
+	KT5720 (1μM)	PKA	0.11 ± 0.01	3.1
+	apocynin (50μM)	oxidative burst	0.52 ± 0.12	14.2

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°C 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°C. At the end of the incubation, samples were assayed for LTC_4 synthase activity as described under "Experimental Procedures". The data is a representative experiment performed in triplicate where the values represent the mean ± S.E.M. * $p < 0.05$ versus control (cells treated with A23187 alone)

A23187	PMA	STAURO-SPORINE	LTC_4 SYNTHASE ACTIVITY	
			pmol/min/10^6 cells	% of control
+			4.84 ± 0.48	100
+	+		3.25 ± 0.10*	67.2
+	+	+	4.96 ± 0.18	102

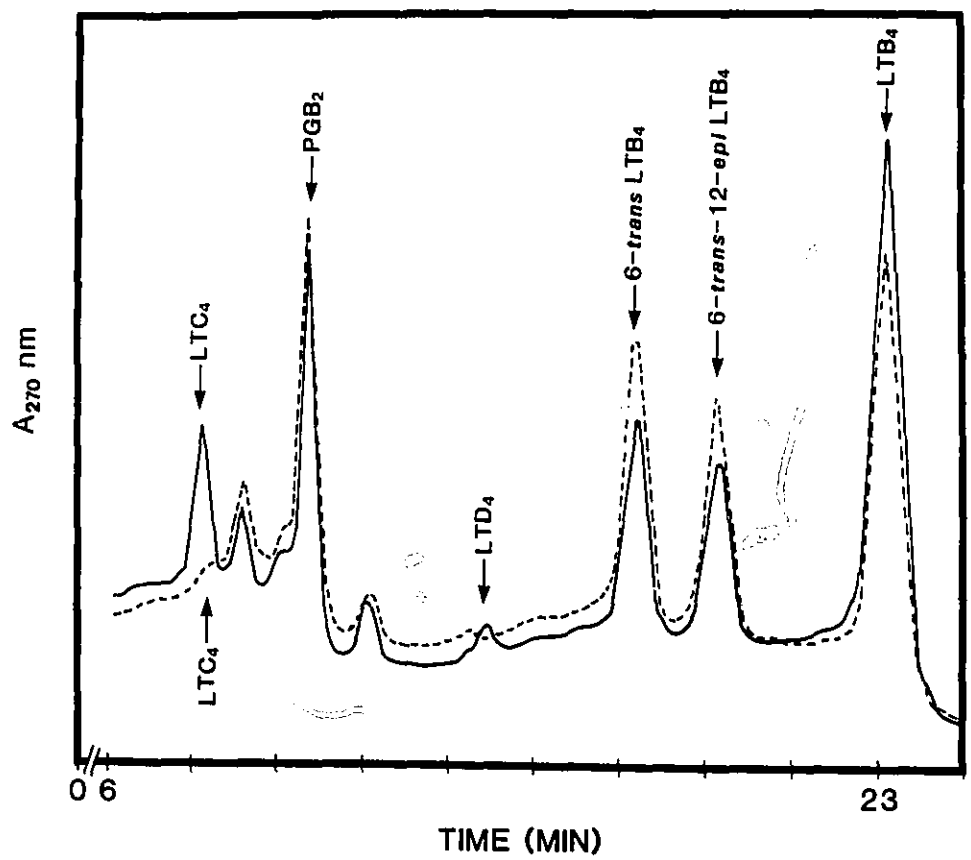

A_{270} nm
LTC_4
PGB_2
LTD_4
6-*trans* LTB_4
6-*trans*-12-*epi* LTB_4
LTB_4
LTC_4
0 6
23
TIME (MIN)

Figure 1

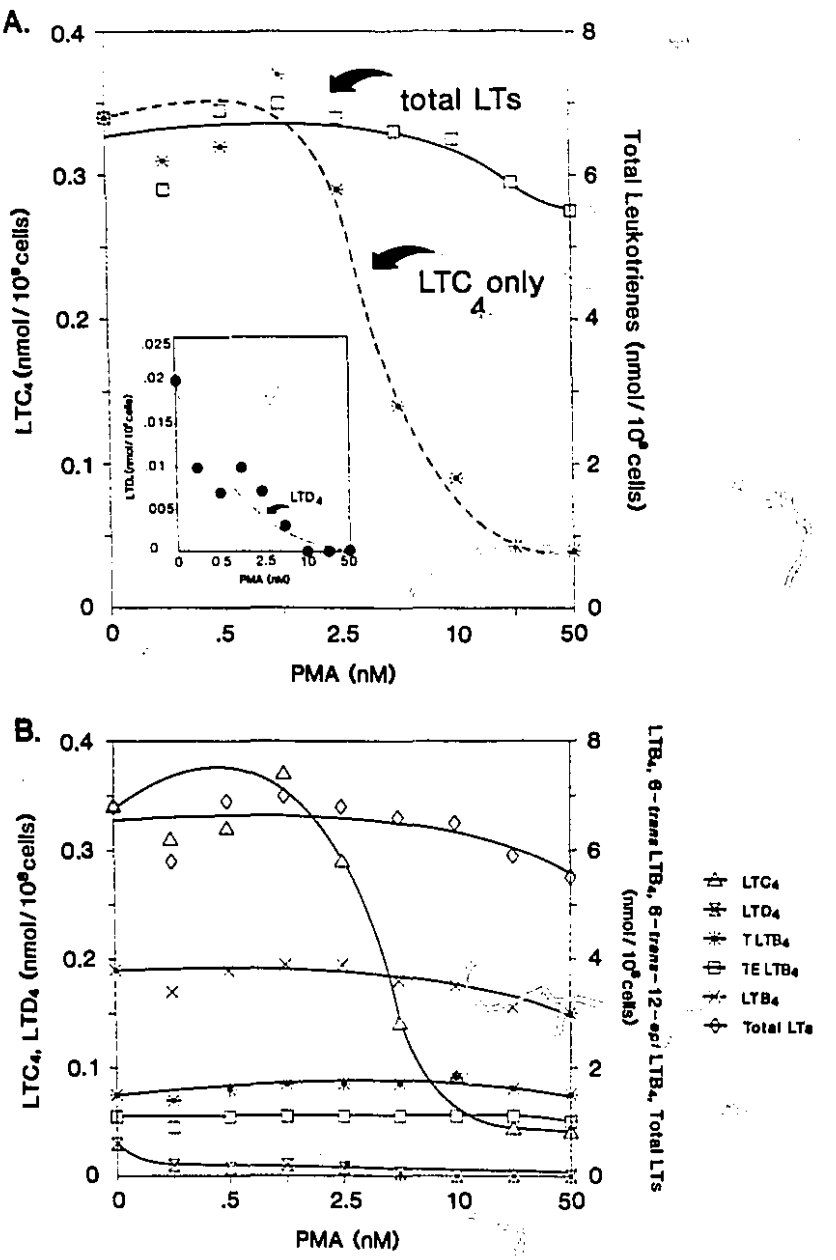
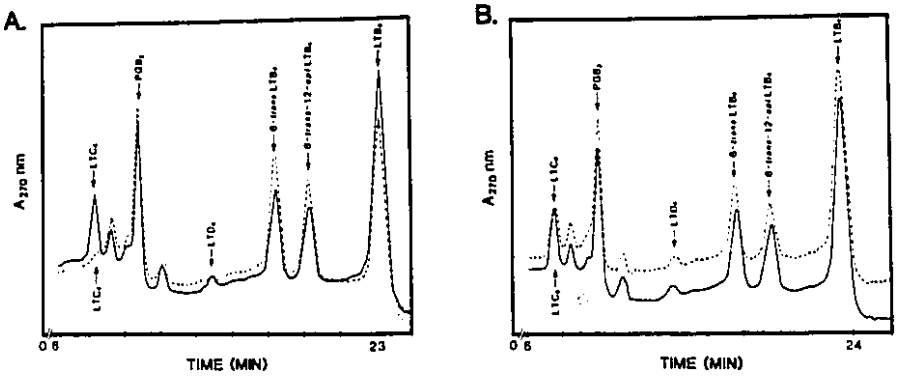

A.
total LTs
LTC4 only
LTC4 (nmol/10^6 cells)
Total Leukotrienes (nmol/10^6 cells)
PMA (nM)
0
.5
2.5
10
50
LTD4
B.
LTC4, LTD4 (nmol/10^6cells)
LTB4, 6-trans LTB4, 6-trans-12-epi LTB4, Total LTs (nmol/10^6 cells)
LTC4
LTD4
T LTB4
TE LTB4
LTB4
Total LTs

Figure 2


A.
B.
TIME (MIN)
TIME (MIN)

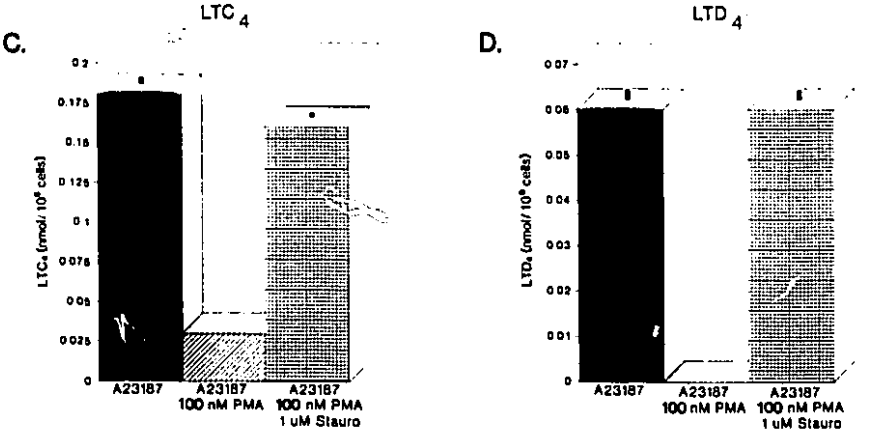

C.
LTC 4
D.
LTD 4
A23187
A23187 100 nM PMA
A23187 100 nM PMA 1 uM Stauro

Figure 3

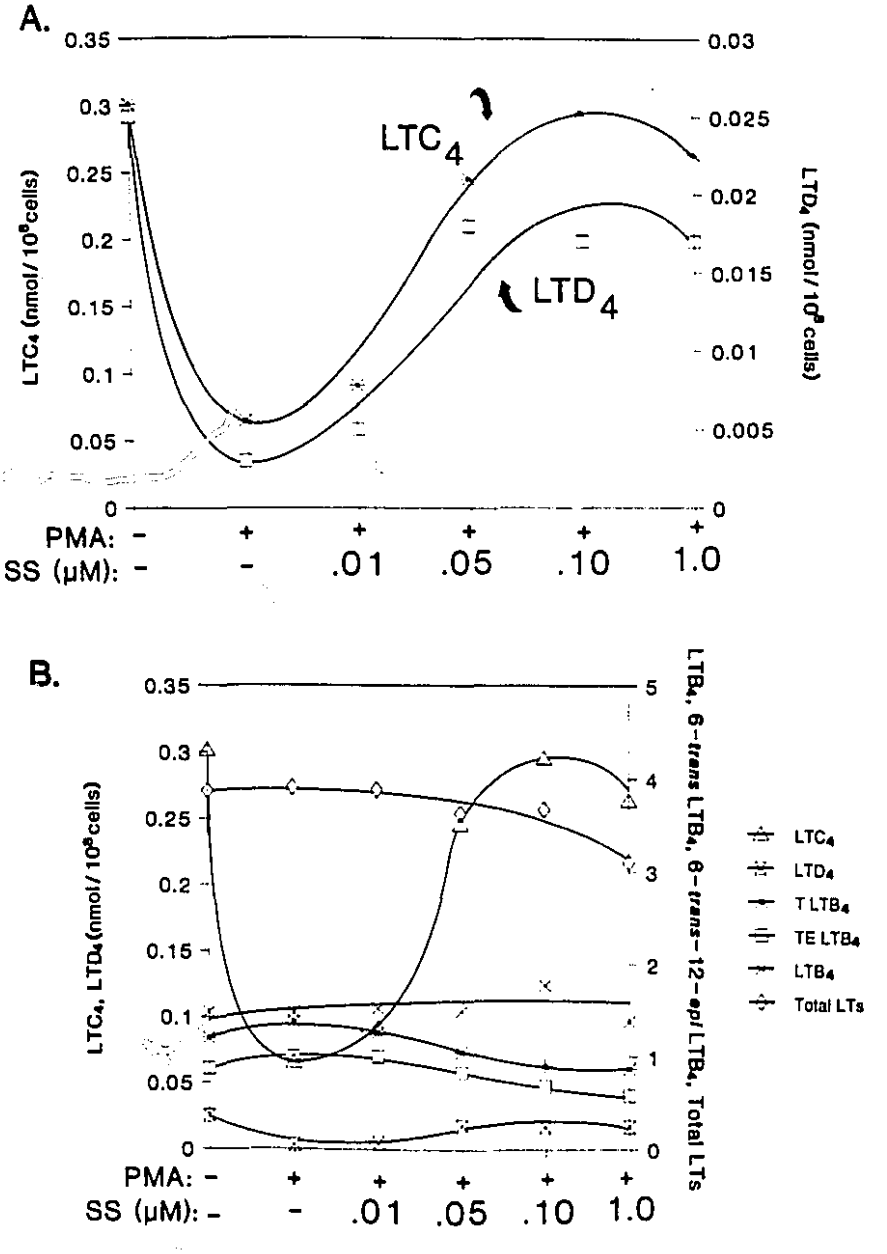

A.
LTC4
LTD4
LTC4 (nmol/10^6 cells)
LTD4 (nmol/10^6 cells)
PMA: - + + + + +
SS (μM): - - .01 .05 .10 1.0
B.
LTC4, LTD4 (nmol/10^6 cells)
LTB4, 6-trans LTB4, 6-trans-12-epi LTB4, Total LTs
LTC4
LTD4
T LTB4
TE LTB4
LTB4
Total LTs
PMA: - + + + + +
SS (μM): - - .01 .05 .10 1.0

Figure 4

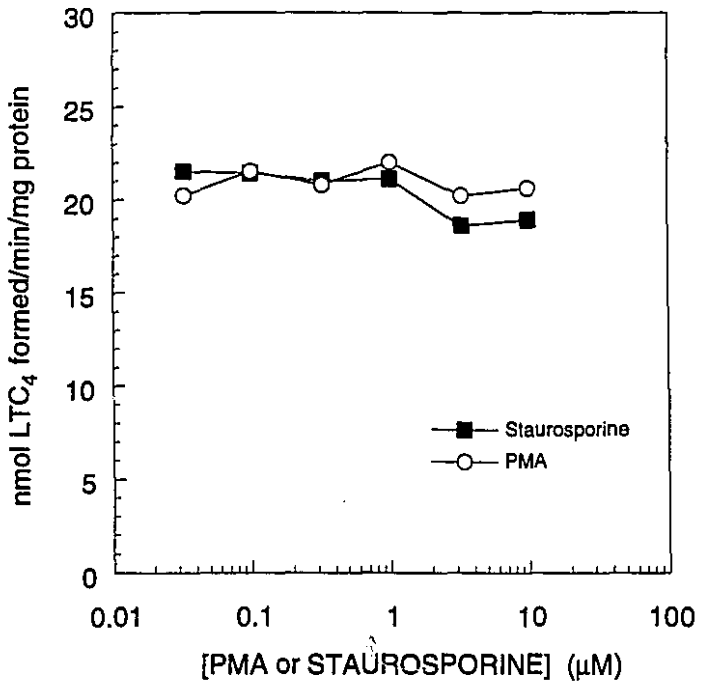

30
25
20
15
10
5
0
nmol LTC_4 formed/min/mg protein
0.01
0.1
1
10
100
[PMA or STAUROSPORINE] (μM)
Staurosporine
PMA

Figure 5

8. MANUSCRIPT E

Activation of Protein Kinase C Down-Regulates Leukotriene C_4 Synthase Activity and Attenuates Cysteinyl Leukotriene Production in an Eosinophilic Substrain of HL-60 cells

J. Immunol. In press (1994)

Since only cysteinyl leukotriene biosynthesis was affected by phorbol ester-mediated activation of protein kinase C in the previous study, the objective of the final paper was to determine the effects of protein kinase C activation on LTC_4 synthase and on eicosanoid biosynthesis in general in an eosinophilic HL-60 cell line.

ACTIVATION OF PROTEIN KINASE C DOWN-REGULATES LEUKOTRIENE C_4 SYNTHASE ACTIVITY AND ATTENUATES CYSTEINYL LEUKOTRIENE PRODUCTION IN AN EOSINOPHILIC SUBSTRAIN OF HL-60 CELLS

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ABSTRACT

An eosinophilic substrain of HL-60 cells (HL-60#7) predominantly synthesized cysteinyl leukotrienes following stimulation with the calcium ionophore A23187. Activation of protein kinase C (PKC) by phorbol myristate acetate (PMA) specifically attenuated cysteinyl leukotriene production without affecting the biosynthesis of non-cysteinyl leukotrienes. The inhibition of cysteinyl leukotriene biosynthesis was prevented only by specific PKC inhibitors (staurosporine and bisindolylmaleimide) but not by inhibitors of tyrosine kinases (genistein, tyrphostin 47, and herbimycin A), protein kinase A (KT5720), or the oxidative burst (apocynin). Similar results were obtained when LTC_4 synthase enzymatic activity was measured directly in the presence of saturating concentrations of exogenously-added substrates. Therefore, the inhibitory effects of PKC activation on cysteinyl leukotriene formation in intact cells was attributable to effects on the LTC_4 synthase enzyme. The mechanism of inhibition of LTC_4 synthase by PKC activation was determined by kinetic analysis to be non-competitive in both eosinophil-like HL-60#7 cells and monocytic THP-1 cells. Contrary to the effect of PKC activation on cysteinyl leukotriene biosynthesis, the formation of prostaglandin E_2 and thromboxane B_2 was elevated 2 to 3-fold following PMA treatment which was prevented by the PKC inhibitor, staurosporine. We propose a regulatory model in which PKC activation shifts the profile of eicosanoid mediators produced by eosinophils from cysteinyl leukotrienes to prostanoids.

INTRODUCTION

Eosinophils have been implicated in the pathogenesis of allergic diseases such as bronchial asthma. They release cytotoxic mediators including major basic protein, eosinophil cationic protein and eosinophil peroxidase which both damage the respiratory epithelium and trigger bronchial hyper-responsiveness. Bioactive lipid mediators, including platelet activating factor (PAF) and products of the cyclooxygenase and 5-lipoxygenase pathways are also released by eosinophils and have been shown to play a role either directly or indirectly in many of the physiological changes detected in asthmatic patients (1).

Leukotrienes (LT) are a group of potent eicosanoid mediators synthesized by many pro-inflammatory cells including eosinophils (2). They have been postulated to play important roles in the pathogenesis of bronchial asthma (3). Leukotriene biosynthesis is initiated by the release of arachidonic acid from membrane phospholipids, probably through by the action of an arachidonate-selective phospholipase A_2 (4,5). Arachidonate metabolism can then follow one of several pathways, one of which includes transformation by 5-lipoxygenase. Upon translocation to cell membranes, 5-lipoxygenase is activated by the 5-lipoxygenase activating protein (FLAP) and in a two-step reaction oxidizes and dehydrates arachidonate to form the unstable epoxide intermediate, leukotriene A_4 (LTA_4). LTA_4 is then either stereoselectively hydrolysed by LTA_4 hydrolase to form LTB_4 or conjugated with reduced glutathione by the action of LTC_4 synthase to form LTC_4. LTC_4 can then be further metabolized to LTD_4 and LTE_4 by γ-glutamyl transpeptidase and cysteinylglycine dipeptidase, respectively. Together, the cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4, comprise the slow reacting substance of anaphylaxis (6,7,8).

Recent reports have demonstrated that leukotriene formation may be metabolically regulated by a mechanism involving protein kinase C (PKC)(9). The biosynthetic activity of the arachidonate-selective PLA_2, for example, is elevated following PKC activation (9,10). On the other hand, preliminary studies from our laboratory in a neutrophil-like cell line indicate that LTC_4 synthase, which catalyzes the first committed step in the formation of the cysteinyl leukotrienes, is down-regulated in response to PKC activation (11). The role of protein kinase C (PKC) in regulation of arachidonate metabolism may therefore be an important controlling mechanism for cells to modulate the formation and profile of eicosanoid mediators. PKC, now recognized as a family of nine isoenzymes. It can be activated by diacylglycerol or tumor promoting agents such as phorbol esters which stimulate PKC both *in vitro* and *in vivo* (12,13). PKC activation is an early event incorporated in many signal transduction cascades. Several growth factors, cytokines and hormones mediate their cellular actions by stimulating the hydrolysis of phosphoinositides and the generation of second messengers (inositol 1,4,5-triphosphate and diacylglycerol) which in turn activate PKC (for review see 14,15,16).

Since eosinophils predominantly produce LTC_4 and have been implicated in the pathology of allergic diseases (17,18), elucidation of the regulatory pathways governing cysteinyl leukotriene biosynthesis, particularly in eosinophils is of substantial importance. In this study, we have investigated the role of protein kinases in the regulation of cysteinyl leukotriene biosynthesis in eosinophil-like HL-60 cells. We show that a similar mode of regulation exists both in eosinophil-like HL-60 and neutrophil-like cells, where LTC_4 biosynthesis is specifically inhibited by PKC activation. In addition, we demonstrate that this loss of LTC_4 is not due to either the depletion of the glutathione pool, activation of the respiratory burst, tyrosine kinases, or protein kinase A. Kinetic analysis of LTC_4 synthase activity

demonstrates that PKC-mediated phosphorylation of LTC_4 synthase, or a putative regulator of its activity, inhibits the enzyme in a non-competitive manner. We propose a regulatory model in which PKC activation shifts the profile of eicosanoid mediators produced by eosinophils from cysteinyl leukotrienes to prostanoids.

MATERIALS AND METHODS

Materials

HL-60 cells (CCL 240) and THP-1 cells (TIB 202) were obtained from the American Type Culture Collection (Rockville, MD). Cell culture media, antibiotics, fetal bovine serum, *n*-butyric acid, staurosporine, phorbol 12-myristate 13-acetate and acivicin were purchased from Sigma Chemical Co. (St. Louis, MO). LTA_4-methyl ester was synthesized by the Department of Medicinal Chemistry at the Merck Frosst Centre for Therapeutic Research. Recombinant human Granulocyte Macrophage Colony Stimulating Factor(rhGM-CSF) was obtained from R & D Systems (Minneapolis, MN). Tyrphostin 47, herbimycin A and genistein were purchased from Biomol (Plymouth Meeting, PA). Apocynin was obtained from ICN Biomedicals (Cleveland, OH). KT5720 and bisindolylmaleimide was from Calbiochem (La Jolla, CA). PGB_2 was purchased from Cayman Chemical Co (Ann Arbor, MI). All other reagents were of analytical grade.

Cell Growth and Differentiation

Eosinophil-like HL-60#7 cells

Cells from the human leukemic myeloblast cell line, HL-60 (19), were cultured in sterile RPMI-1640 medium (supplemented with 0.2% (w/v) $NaHCO_3$ and 0.03% (w/v) L-glutamine) containing 50 U penicillin/ml, 50 μg streptomycin/ml and 10% (v/v) fetal bovine serum (Sigma, Hybri-Max, not heat-inactivated) at 37°C in a humidified atmosphere containing 6% CO_2. A pro-eosinophilic substrain of HL-60 cells (designated HL-60/MF211#7, herein called HL-60#7 cells) was developed at the Merck Frosst Centre for Therapeutic Research (M.P.Scheid and D.W.Nicholson, unpublished). This cell line has a high propensity for conversion into eosinophil-like cells when differentiated by growth in culture in the presence

of *n*-butyric acid. For differentiation into eosinophil-like cells, HL-60#7 cells were subcultured in a 1 l spinner flask at a density of 0.2 x 10^6 cells/ml in the medium described above containing 0.4 mM *n*-butyric acid (added from a 150 mM stock in ethanol). After 7 days of differentiation, cells were split with an equal volume of fresh medium containing 0.4 mM *n*-butyric acid. Cells were harvested following 9 to 11 days of differentiation.

THP-1 cells

Cells from the human monocytic leukemia cell line, THP-1 (20), were cultured at a cell density of 0.2 x 10^6 cells/ml in sterile RPMI-1640 medium (supplemented with 0.2% (w/v) $NaHCO_3$, 0.03% (w/v) L-glutamine and 50 µM 2-mercaptoethanol) containing 50 U penicillin/ml, 50 µg streptomycin/ml and 10% (v/v) fetal bovine serum (Sigma, Hybri-Max, not heat-inactivated) at 37°C in a humidified atmosphere containing 6% CO_2. Cells were harvested after 4-7 days in culture when a cell density of 0.7-1 x 10^6 cells/ml was obtained.

Measurement of Leukotriene Formation in Ionophore-Challenged Whole Cells

Butyric-acid differentiated HL-60#7 cells (eosinophil-like cells) were harvested by centrifugation at 500 *xg* for 15 min at room temperature. Cells were washed by resuspending them in Dulbecco's phosphate buffered saline (PBS) and by repelleting at 500 *xg* for 15 min. Cells were then resuspended in Dulbecco's PBS containing 1 mM EDTA and 1mg/ml D-glucose at a cell density of 2 x 10^6 cells/ml and were primed by incubation with 200 pM rhGM-CSF for 30 min at 37°C.

For experiments testing various inhibitors, stocks of all inhibitors (staurosporine, bisindolylmaleimide, tyrphostin 47, genistein, apocynin, herbimycin A, and KT5720) and PMA were prepared in Me_2SO at 1000-fold the required final

concentration (final concentrations were: staurosporine, 1 μM; bisindolylmaleimide, 1 μM; tyrphostin 47, 100 μM; genistein, 100 μM; apocynin, 50 μM; herbimycin A, 40 μM; KT5720, 1 μM; PMA, 50 nM). Each inhibitor (2 μl) or Me_2SO (control) was added to 4 x 10^7 cells in a final volume of 2 ml and the cells were then incubated for 90 min at 37°C. Calcium was then added to cells to a final concentration of 2.1 mM and the cells were incubated for another 8 min at 37 °C. PMA at a final concentration of 50 nM or Me_2SO (control) was then added to the cells and they were further incubated for 15 min at 37°C. Cells were then challenged with 1 μM A23187 for 10 min at 37°C and the reaction was terminated by the addition of 2 ml of ice-cold methanol containing 75 pmol/ml prostaglandin B_2 (PGB_2, used as an internal HPLC standard). Samples were Sep-Pak purified on Waters C_{18}-light Sep-Pak cartridges as follows: Sep-Pak C_{18}-light cartridges were conditioned sequentially with 10 ml H_2O, 10 ml MeOH, and 10 ml H_2O. Terminated reactions were spun at 150 *xg* for 5 min at 4°C and the supernatant was removed and diluted with 6 ml H_2O to a final volume 10 ml. The 10 ml diluted supernatant was respun at 150 *xg* for 15 min at 4°C and applied to conditioned Sep-Pak C_{18}-light cartridges at a maximum flow rate of 1 ml/min. Cartridges were washed with 20 ml H_2O and leukotrienes were eluted with 1 ml methanol and subsequently dried by vacuum centrifugation. Samples were resuspended in 200 μl of HPLC mobile phase (acetonitrile: methanol: water: acetic acid at 54:14:28:1, pH 5.6), and analyzed by reverse-phase HPLC as described previously (21).

For all other experiments, calcium was first added to cells to a final concentration of 2.1 mM and the cells were incubated for 8 min at 37 °C. Stocks of PMA alone or PMA plus staurosporine or bisindolylmaleimide (activator/inhibitor mix) were prepared in Me_2SO at 1000-fold the final concentration. Each activator/inhibitor mix (2 μl) or Me_2SO (control) was added to 4 x 10^7 cells in a final

volume of 2 ml and the cells were then incubated for 15 min at 37°C. Cells were then challenged with 1 μM A23187 for 10 min at 37°C and the reaction was terminated by the addition of 2 ml of ice-cold methanol containing 75 pmol/ml prostaglandin B_2 (PGB_2). Samples were Sep-Pak purified on Waters C_{18}-light Sep-Pak cartridges as described above and analyzed by reverse-phase HPLC on a Waters Novapak C_{18} column as described previously (21). Essentially, leukotrienes were resolved by isocratic reverse-phase HPLC on a Novapak C_{18} column (3.9 x 150 mm; Waters Associates, Milford MA) with a mobile phase comprised of acetonitrile:methanol:water:acetic acid (28:18:54:1, v/v) pH 5.6, at a flow rate of 1.0 ml/min. Leukotrienes were monitored by on-line measurement of the effluent at 280 nm.

Measurement of Leukotriene C_4 Synthase Activity in Whole Cells

THP-1 cells or butyric-acid differentiated HL-60#7 cells (eosinophil-like cells) were harvested and primed as described for the whole cell ionophore-challenge assay. Cells were suspended at a density of 10^7 cells/ml and divided into 3 ml aliquots.

For experiments testing various inhibitors, concentrated inhibitors (3 μl of 1000-fold concentrated stock in Me_2SO or Me_2SO alone (control) was added and the cells were incubated at 37°C for 90 min. Calcium was added to a final concentration of 2.1 mM and the cells were further incubated for 8 min. Cells were then treated with 50 nM PMA for 15 min at 37°C. After the addition of 2.5 mM acivicin, the cells were divided into 0.48 ml aliquots and then the reaction was started with the addition of 20 μl of 125 μM LTA_4 (final volume = 0.5 ml) unless otherwise indicated. Cells were incubated for 4 min at 37°C with gentle shaking. The reaction was terminated with the addition of 0.5 ml ice-cold methanol containing 0.9 nmol/ml PGB_2. Samples were Sep-Pak purified on Waters C_{18}-light

Sep-Pak cartridges as described above and analyzed by reverse-phase HPLC on a Waters Novapak C_{18} column as described previously (21).

For all other experiments, calcium was first added to cells to a final concentration of 2.1 mM and the cells were incubated for 8 min at 37°C. Stocks of PMA alone or PMA plus staurosporine or bisindolylmaleimide (activator/inhibitor mix) were prepared in Me_2SO at 1000-fold the required final concentration. Each activator/inhibitor mix (3 µl) or Me_2SO (control) was added to cells in a final volume of 3 ml after which the cells were then incubated for 15 min at 37°C. After the addition of 2.5 mM acivicin, the cells were divided into 0.48 ml aliquots and then the reaction was started with the addition of 5 µM LTA_4 (in a final volume of 0.5 ml) unless otherwise indicated. Cells were incubated for 4 min at 37°C with gentle shaking. The reaction was terminated with the addition of 0.5 ml ice-cold methanol containing 0.9 nmol/ml PGB_2. Samples were Sep-Pak purified on Waters C_{18}-light Sep-Pak cartridges as described above and analyzed by reverse-phase HPLC on a Waters Novapak C_{18} column as described previously (21).

Measurement of Purified LTC_4 Synthase Activity

LTC_4 synthase activity was measured essentially as described previously (20). Briefly, incubation mixtures containing partially purified LTC_4 synthase in 0.1 M potassium phosphate buffer (pH 7.4) containing 40 µM LTA_4, 10 mM glutathione, 0.2 mg/ml L-α-phosphatidylcholine, 20 mM $MgCl_2$, 50 mM serine-borate and varying concentrations of the inhibitor being tested or vehicle were incubated at 25°C for 15 min. The reaction was terminated by the addition of 150 µl cold acetonitrile:methanol:acetic acid (50:50:1, v/v/v). Products were analyzed by reverse-phase HPLC as described previously (21).

Measurement of Cellular Glutathione Content

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined as described previously (22). Briefly, 1 ml of control, PMA-treated or PMA and staurosporine-treated cells were pelleted by centrifugation at 10,000 *xg* for 5 min at 4°C. The supernatant was added to 500 µl of cold 10 mM hydrochloric acid and the cells were lysed. After spinning the samples at 10,000 *xg* for 5 min at 4 °C, 200 µl of the resulting supernatant was removed and deproteinized with the addition of 100 µl 5-sulfosalicylic acid (10%, w/v). Precipitated proteins were pelleted by spinning samples at 10 000 xg at 4°C and 100 µl of the supernatant was assayed for glutathione content using the DTNB-GSSG reductase-recycling assay for GSH and GSSG. To assay for total glutathione, 700 µl of 0.3 mM NADPH in stock buffer (125 mM Na phosphate, 6.3 mM Na EDTA pH 7.5), 100 µl of 6 mM DTNB (Ellman's reagent, Sigma) in stock buffer and 100 µl of the supernatant from above were combined to give a final volume of 1 ml then mixed and equilibrated to 30°C for 12-15 minutes. After the addition of 10 µl of glutathione reductase (EC 1.6.4.2; 250 units/ml stock buffer, Sigma) the samples were transferred to a cuvette and the ΔOD_{412nm} was monitored over 3 minutes. The glutathione content of the sample was determined by comparison of the rate observed to a standard curve generated with known amounts of glutathione. To determine GSSG content, the GSH in the sample was first derivatized by the addition of 2 µl of 2-vinyl-pyridine (Aldrich) followed by 6 µl of triethanolamine to the 100 µl supernatant. This sample was vigorously mixed and allowed to stand at 25°C for 60 minutes. The residual glutathione (GSSG) was determined as described above and GSH content was determined by subtracting the determined GSSG content from the total glutathione content.

Measurement of Prostanoid Formation in Ionophore-Challenged Whole Cells

Prostanoid formation was measured in cells treated essentially as described for the measurement of leukotriene formation in ionophore-challenged whole cells (see above) except that reactions were stopped with ice-cold methanol without PGB_2. Terminated reactions were spun at 150 *xg* for 5 min at 4°C and samples of the supernatant were then taken for PGE_2 and TXB_2 measurement.

TXB_2 was measured using an enzyme-linked immunosorbant assay kit (ELISA Technologies, Lexington, KY) according to the manufacturer's instructions. PGE_2 was measured using an [I^{125}] radioimmunoassay kit (Du Pont, Boston, MA) according to the manufacturer's instructions.

RESULTS

Cysteinyl leukotriene biosynthesis in A23187-challenged, eosinophil-like HL-60#7 cells is down-regulated by PMA-mediated activation of protein kinase C

HL-60 cells can be differentiated into neutrophil or eosinophil-like cells by differentiation in the presence of either dimethylsulfoxide (Me_2SO) or butyric acid, respectively. We have recently presented evidence that indicates that LTC_4 biosynthesis in rhGM-CSF-primed, Me_2SO- differentiated neutrophil-like HL-60 cells is negatively regulated by protein kinase C activation (11). Since eosinophils preferentially produce LTC_4 (18) we investigated the regulation of LTC_4 biosynthesis in butyric acid differentiated HL-60#7 cells, a cell line which resembles mature eosinophils.

Consistent with our previous results in neutrophil-like HL-60 cells, cysteinyl leukotriene biosynthesis in response to ionophore (A23187) challenge was down-regulated by phorbol ester-mediated activation of protein kinase C (Fig.1, column 2) and this inhibition was prevented by two protein kinase C inhibitors, staurosporine (Fig.1, column 3) and bisindolylmaleimide (Fig.1, column 4). In other regulatory systems, a possible cascade of events triggered by PKC and leading to the activation of tyrosine kinases (23) or MAP kinase (10) has been postulated. To investigate whether such a cascade of events was triggered in our experimental model, we examined the effects of tyrosine kinase inhibitors (herbimycin A, tyrphostin 47, genistein (Fig.1, columns 5,6,7)), a protein kinase A inhibitor (KT5720 (Fig.1, column 8)), and an oxidative burst inhibitor (apocynin (Fig.1, column 9)) on PMA-mediated inhibition of cysteinyl leukotriene biosynthesis. None of these inhibitors were able to prevent the effects of PMA; in fact, tyrphostin 47 further down-regulated cysteinyl leukotriene biosynthesis. This effect

of tyrphostin 47 was determined to be due to direct non-specific inhibition of the LTC_4 synthase enzyme as described below.

Phosphoregulation of LTC_4 biosynthesis in eosinophil-like HL-60#7 cells occurs at the level of LTC_4 synthase

The calcium ionophore, A23187 has been shown to induce translocation of protein kinase C from the cytosol to the membrane of human neutrophils (24) . Furthermore, several studies have shown that PMA can potentiate the effects of A23187 on human neutrophils and murine macrophage arachidonate metabolism (25-28). In addition, the recently purified and cloned cytosolic phospholipase A_2 (4,5), which appears to be selectively involved in the liberation of arachidonate, the precursor of leukotrienes, may be activated by protein kinase C (9,10), MAP kinase (10) and increases in intracellular calcium(29). To therefore bypass PLA_2 regulation and to examine the direct effects of protein kinase C activation on LTC_4 synthase activity, leukotriene biosynthesis was examined in the presence of saturating concentrations of exogenously-added substrates for the LTC_4 synthase enzyme (10 mM reduced glutathione and 40 μM LTA_4). Also included was 2.5 mM acivicin, a γ-glutamyl transpeptidase inhibitor which was present in the whole cell LTC_4 synthase assay to prevent metabolism of newly formed LTC_4 to LTD_4 and LTE_4 (Fig. 2A). LTC_4 and LTB_4 biosynthesis was examined in control, PMA treated and PMA plus staurosporine-treated eosinophil-like HL-60#7 cells in the presence of exogenous LTA_4 or LTA_4 and reduced glutathione. The phosphoregulatory mechanism of LTC_4 synthase in response to PKC activation was still observable in the presence of exogenously added substrates (Fig. 2B). Treatment with 50 nM PMA caused an approximate 50% decrease in LTC_4 biosynthesis (column 1 versus 2 and 4 versus 5) which was

completely prevented by the presence of staurosporine (columns 3 and 6) whereas there was no effect on the LTB_4 formed from exogenously-added substrates. Since LTA_4 hydrolase is saturated at 40 μM LTA_4, accumulation of LTA_4 resulting from LTC_4 synthase inhibition would not be expected to be reflected in higher levels of LTB_4 in PMA-treated samples. The inhibitory effect of PMA on LTC_4 formation was dose-dependent, with an IC_{50} value of 50 nM (Fig. 3A), and time-dependent reaching maximal inhibition (50% of control) after 15 minutes (Fig.3B). Therefore the inhibitory effects of PKC activation on cysteinyl leukotriene formation is attributable to effects on the LTC_4 synthase enzyme and not upstream enzymes in the leukotriene biosynthetic pathway.

PMA-mediated inhibition of LTC_4 synthase is prevented in a dose-dependent manner by two protein kinase C inhibitors

To determine if PMA-mediated PKC activation was responsible for the observed inhibition of the LTC_4 synthase enzyme, two specific protein kinase C inhibitors were tested for their ability to prevent the effect of PMA. Both staurosporine and bisindolylmaleimide interact with the ATP-binding site of protein kinase C (30,31), although bisindolylmaleimide has been reported to be a more selective inhibitor of protein kinase C with less cytotoxic effects (31). Cells were treated with varying concentrations of staurosporine or bisindolylmaleimide in the presence of 50 nM PMA and were subsequently assayed for LTC_4 synthase activity (Fig.4). Both staurosporine and bisindolylmaleimide were able to completely reverse the effects of PMA in a dose-dependent manner with EC_{50} values of 0.08 μM and 0.65 μM, respectively.

Phosphoregulation of LTC_4 synthase involves protein kinase C but not other kinases

Consistent with results obtained in A23187-challenged eosinophil-like HL-60#7 cells (Fig.1), LTC_4 biosynthesis in the presence of exogenous LTA_4 was inhibited to 50% of control by 15 minute pre-treatment with 50 nM PMA (Fig.5, column 1 versus 2). This inhibition was prevented by staurosporine and bisindolylmaleimide (columns 3 and 4), slightly enhanced by the tyrosine kinase inhibitor, tyrphostin 47 (column 5), and not significantly affected by herbimycin A, genistein, KT5720, or apocynin (columns 6-9). Therefore, activation of protein kinase C by phorbol ester appears to suppress LTC_4 synthase activity, thus accounting for the reduction in cysteinyl leukotriene biosynthesis.

LTC_4 synthase is directly inhibited by tyrphostin 47 but not by PMA

Since tyrphostin 47 further inhibited LTC_4 synthase activity in PMA-treated cells, it was possible that a tyrosine kinase (which would be inhibited by tyrphostin 47) supported LTC_4 synthase activity. However, two other tyrosine kinase inhibitors, herbimycin A and genistein had no effect on LTC_4 synthase activity. To determine if tyrphostin 47 had any direct inhibitory effect on LTC_4 synthase itself, the activity of partially-purified LTC_4 synthase in the presence of varying concentrations of tyrphostin 47 was tested. Tyrphostin 47 inhibited partially-purified LTC_4 synthase activity in a dose-dependent manner with an IC_{50} value of 100 μM (Fig. 6A, solid circles). In the whole cell assay, tyrphostin 47 also inhibited LTC_4 biosynthesis with an IC_{50} value of approximately 150 μM (Fig. 6A, open circles). PMA, on the other hand, had no direct inhibitory effect on partially-

purified LTC_4 synthase activity (Fig. 6B, solid squares) at concentrations up to 10 μM, but did however inhibit LTC_4 synthase in the whole cell assay in a dose-dependent manner with an IC_{50} value of 50 nM (Fig. 6B, open squares). Together these data demonstrate that the effects of PMA require constituents of intact cells (specifically PKC) whereas the effect of tyrphostin 47 was attributable to non-specific inhibition of LTC_4 synthase.

To determine if PMA treatment affected the cellular glutathione levels, which might adversely affect substrate availability for LTC_4 synthase, we measured the level of reduced glutathione (GSH) and oxidized glutathione (GSSG) in control, PMA, and PMA plus staurosporine treated cells. PMA did not, however, reduce cellular glutathione levels (Table I). Therefore the effect of PMA on LTC_4 synthase activity does not occur as a consequence of a reduction in the glutathione pool.

Protein kinase C activation inhibits LTC_4 synthase non-competitively in eosinophil-like HL-60#7 cells and in monocytic THP-1 cells

The mechanism of inhibition of LTC_4 synthase by PMA-mediated activation of PKC was determined by substrate-saturation kinetics which was then analyzed using a double reciprocal (Lineweaver-Burk) transformation. Inhibition of LTC_4 synthase by PKC activation was independent of substrate (LTA_4) concentration (Fig.7A,B). Kinetic analysis of LTC_4 synthase activity after PMA treatment of eosinophil-like HL-60#7 cells (Fig.7A) and THP-1 cells (Fig.7B), which also have high LTC_4 synthase enzyme levels, demonstrated that putative phosphorylation of LTC_4 synthase or a potential regulator of its activity by PKC causes an approximate 50% decrease in V_{max} without any effect on the K_m (Table II). Treatment with staurosporine was able to completely prevent the PMA-mediated reduction of

the V_{max} and restored LTC_4 synthase biosynthetic activity to control levels in both cell lines. Thus, protein kinase C activation inhibits LTC_4 synthase non-competitively in both eosinophil-like HL-60#7 cells and monocytic THP-1 cells.

Prostanoid biosynthesis in A23187-challenged eosinophil-like HL60#7 cells is upregulated by PMA-mediated activation of protein kinase C

Recent evidence indicates that PKC activation stimulates prostanoid biosynthesis in several cell types (32-38). To determine if such a regulatory mechanism was triggered by PMA in the A23187-challenged, eosinophil-like HL-60#7 cells, TXB_2 and PGE_2 levels were quantified in control, PMA-treated and PMA plus staurosporine treated cells. As observed previously, PMA treatment reduced cysteinyl leukotriene biosynthesis by approximately 50% (Fig.8A). In contrast, however, PMA treatment increased TXB_2 (Fig.8B) and PGE_2 (Fig.8C) biosynthesis by approximately 2-3 fold when compared to control cells. The PKC inhibitor, staurosporine, was able to completely prevent this increase in prostanoid biosynthesis and decrease in cysteinyl leukotriene biosynthesis. Therefore, under identical conditions the activation of PKC reduced cysteinyl leukotriene biosynthesis concomitantly with an elevation of prostanoid formation.

DISCUSSION

Regulation of arachidonate metabolism has been an area of intense investigation due to the potent pro-inflammatory actions of arachidonate metabolites in allergic and inflammatory diseases. In this study we have demonstrated that eosinophil-like HL-60#7 cells down-regulate the biosynthesis of LTC_4 but not LTB_4 in a protein kinase C specific manner. This inhibition of LTC_4 biosynthesis can be triggered dose-dependently by PMA and the engagement of this phosphoregulatory mechanism can be prevented by two specific protein kinase C inhibitors, staurosporine and bisindolylmaleimide. The phosphoregulation of LTC_4 biosynthesis was examined in two models. In the first, we examined LTC_4 regulation in A23187-challenged eosinophil-like HL-60#7 cells, which can be paralleled to situations *in vivo* where extracellular signals both stimulate PKC and increase intracellular calcium levels. Previous studies have shown that PMA and A23187 synergistically stimulate LTC_4 biosynthesis in murine macrophages (25,26) and eosinophils (39) and stimulate LTB_4 biosynthesis in human neutrophils (27,28). However all of these studies used suboptimal concentrations of A23187 and did not demonstrate the prevention of the stimulatory effect by specific PKC inhibitors. In contrast to these studies, it has been demonstrated in human basophils that interleukin 3-dependent LTC_4 biosynthesis in response to C5a is abolished by treatment with PMA or tyrosine kinase inhibitors and the effect of PMA can be prevented by staurosporine (40). In our recent study of the phosphoregulation of LTC_4 biosynthesis in neutrophil-like HL-60 cells we have demonstrated that co-stimulation with optimal concentrations of A23187 and PMA specifically inhibits LTC_4 biosynthesis with no effect on LTA_4 or LTB_4

biosynthesis (11). These observations were confirmed in the eosinophil-like HL-60#7 cell line used in the present study.

In the second model used to study phosphoregulation of LTC_4 biosynthesis we examined LTC_4 biosynthesis initiated by the addition of exogenous LTA_4 to eosinophil-like HL-60#7 cells. This model was the focus of our study and had three advantages. First, we were able to bypass phosphoregulation of PLA_2 (9,10) and the stimulatory effects of A23187 on PKC translocation and activation (24). Secondly, we were able to determine that the inhibitory effects of PKC activation on cysteinyl leukotriene production were due to the PKC-mediated inhibition of the LTC_4 synthase enzyme itself and not upstream enzymes in the leukotriene biosynthetic pathway. Thirdly, this model can be used to study the regulation of transcellular interactions, where cells receive exogenous LTA_4 from other cell types at the site of inflammation for cysteinyl leukotriene biosynthesis. Such transcellular interactions have been reported to occur *in vitro* where human granulocyte-derived LTA_4 is utilized by human endothelial cells (41), human platelets (42) and porcine aortic endothelial and smooth muscle cells (43,44) for the biosynthesis of cysteinyl leukotrienes.

The phosphoregulatory mechanism of leukotriene biosynthesis was observable in the presence of exogenously-added substrates and was specific to LTC_4 biosynthesis only. No significant effect on LTB_4 biosynthesis was observed. It has been postulated that PKC can trigger a cascade of events leading to the activation of both tyrosine kinases (23) and MAP kinase (10). Furthermore, a recent study on the regulation of LTC_4 biosynthesis in human basophils has suggested that IL-3-dependent LTC_4 biosynthesis is regulated by serine/threonine and tyrosine phosphorylation in an antagonistic manner (40). We investigated the involvement of other kinases in the regulation of LTC_4 biosynthesis through the use of specific kinase inhibitors. Only PKC inhibitors were effective in preventing the

effects of PMA as reported for the A23187 challenged eosinophil-like HL-60#7 cells. Only one of the tyrosine kinase inhibitors tested, tyrphostin 47, modulated the effect of PMA. Tyrphostin 47 enhanced the PMA-mediated inhibition of LTC_4 biosynthesis in both the A23187 challenged eosinophil-like HL-60#7 cells and in the cells receiving exogenous LTA_4. However this effect was demonstrated to be attributable to non-specific inhibition of the LTC_4 synthase enzyme by tyrphostin 47. This is consistent with reports that several tyrosine kinase inhibitors, including tyrphostin 47, not only inhibit tyrosine kinases but act also on other enzymes including those involved in fatty acid synthesis, lactate transport, mitochondrial oxidative phosphorylation, and aldehyde dehydrogenase (45). Thus regulation of LTC_4 synthase in human eosinophil-like cells involves PKC and the involvement of tyrosine kinases as reported for human basophils (40) is unlikely.

Having demonstrated an inhibition of LTC_4 synthase activity upon treatment of eosinophil-like HL-60#7 cells with PMA we examined the effects of PKC activation on the kinetic parameters of LTC_4 synthase. Our results indicate that phosphorylation of LTC_4 synthase or a putative regulator of its activity causes a decrease in the V_{max} of LTC_4 synthase without any effect on its K_m, and thus demonstrates non-competitive inhibition which is typical for a phosphoregulatory mechanism. Although the presence of a PKC consensus sequence in the amino terminus of the LTC_4 synthase polypeptide supports a direct role for PKC in the modulation of its activity (46), the specific mechanism by which this occurs will require recombinant LTC_4 synthase or specific antibodies; neither of which are available yet.

The significance of the downregulation of LTC_4 biosynthesis in eosinophil-like HL-60#7 cells by PKC activation can be seen in light of actions of PKC on the regulation of arachidonate metabolism in eosinophils as well as other cell types. Activation of MAP kinase by PKC has now been shown to activate phospholipase

A_2, the enzyme that catalyzes the formation of arachidonic acid and the precursor of PAF, lyso-PAF. In addition, arachidonic acid itself has been shown to activate PKC (47,48). In human eosinophils obtained from asthmatic patients, PAF, LTB_4, PMA and calcimycin potently activate the cell to generate and release cyclooxygenase metabolites, with TXB_2 being the most abundant (49). Together with data presented here, these studies support a mechanism for the regulation of these eicosanoids in eosinophils (Fig.9). First, through the activation of PKC and the subsequent decrease in cysteinyl leukotriene biosynthesis, availability of arachidonic acid for the synthesis of the cyclooxygenase products may increase. Recently, it has been reported activation of PKC stimulates transcription of the cyclooxygenase-2 gene (33,34). This transcriptional activation of the cyclooxygenase-2 gene no doubt further increases prostanoid synthesis. In several other cell types, PKC activation has been linked to increased prostanoid biosynthesis as well (35-38). The results of this study demonstrate that concomitant with a specific decrease in cysteinyl leukotriene biosynthesis there is an increase in prostanoid biosynthesis upon PKC activation, and as reported for human eosinophils, TXB_2 was the most abundant prostanoid formed (49). Thus, evidence to date and the results of our study indicate that activation of PKC down-regulates cysteinyl leukotriene biosynthesis and stimulates prostanoid biosynthesis. This inhibition of LTC_4 synthase may be through direct phosphorylation of LTC_4 synthase or through phosphorylation of a LTC_4 synthase regulatory protein. Events triggered by PMA not only inhibit LTC_4 biosynthesis selectively and rapidly, but may also inhibit the actions of this mediator by blocking the signal transduction pathway of its metabolite, LTD_4 (50). PMA mediated activation of PKC stimulates the cyclooxygenase pathway (32), the respiratory burst (51), and the release of cytotoxic granules in inflammatory cells (49,51,52). Thus, PKC activation changes the profile of mediators synthesized by

the eosinophil from leukotrienes to prostanoids and causes eosinophils to assume a physiologically activated state.

The change in the ratio of prostaglandins and leukotrienes synthesized may be of clinical significance in asthma, gastric ulcerations, and psoriasis where decreased prostaglandin/leukotriene ratios have been reported. Platelets and macrophages from asthmatic subjects show a decrease in COX metabolites and an increase in lipoxygenase products when compared with platelets and macrophages of normal subjects (53-54). In gastric ulcers, prostanoids, in particular, PGE_2, are cytoprotective while leukotrienes may be pro-ulcerogenic (55,56). Similarly, decreased prostanoid and increased leukotriene production has also been reported in psoriatic lesions (57,58). Thus, the identification of factors or mechanisms which physiologically regulate the balance of COX and lipoxygenase products may be of particular relevance in these pathologies. Furthermore, it has been suggested that PGE_2 and PGI_2 may serve as physiological antagonists of bronchoconstrictive mediators such as histamine and leukotrienes since their constrictive effects are potentiated if the synthesis of prostaglandins is inhibited (59-61). Based on these observations it has been hypothesized that bronchoconstrictive mediators may promote the release of PGE_2 and PGI_2 in a kind of homeostasis mechanism such that if prostanoid biosynthesis is inhibited their effects are unmasked or potentiated (59). The observation that LTD_4 can stimulate the release of prostaglandins, *in vivo*, is of particular significance with respect to the model proposed here for the regulation of eicosanoid biosynthesis. The mechanism by which LTD_4 stimulates prostaglandin biosynthesis occurs via stimulation of PLC and the subsequent production of IP_3 and diacylglycerol upon interaction of LTD_4 with its receptor. These mediators in turn activate PKC which, according to the mechanism proposed here, would stimulate prostanoid biosynthesis and inhibit cysteinyl

leukotriene biosynthesis. Thus this model may be the basis of a negative feedback mechanism by which potent bronchoconstrictive mediators such as LTD_4 can downregulate not only their own production but can concomitantly stimulate the production of bronchodilatory mediators to counteract their physiological actions.

The leukotrienes and prostaglandins are potent inflammatory mediators whose actions contribute to both physiological and pathophysiological conditions. Taken together, these studies demonstrate that the elucidation of mechanisms regulating not only their production but also the balance of prostanoids and leukotrienes formed is important. A defect in this mechanism may in part be responsible for the pathophysiological changes observed in diseases such as bronchial asthma.

FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Leukotriene C_4 biosynthesis is down-regulated by PKC activation in A23187 challenged eosinophil-like HL-60#7 cells. Eosinophil-like HL-60#7 cells were primed for 30 minutes at 37°C with 200pM rhGM-CSF and then incubated with the indicated inhibitor (staurosporine (column 3), bisindolylmaleimide (column 4), tyrphostin 47 (column 5), herbimycin A (column 6), genistein (column 7), KT5720 (column 8), apocynin (column 9) or vehicle (Me_2SO, (columns 1,2)) for 90 minutes at 37°C. Calcium was added to a final concentration of 2.1 mM and the cells were incubated for 8 minutes at 37°C. Following 15 minute treatment with 50 nM PMA at 37°C, cells were challenged with 1μM A23187 for 10 minutes at 37°C. Reactions were terminated by the addition of an equal volume of 75 pmol/ml PGB_2 in ice-cold methanol. Leukotrienes were purified by solid phase extraction with *Sep-Pak* C_{18} cartridges and quantified by reverse-phase HPLC as described in "Materials and Methods". Data is expressed as the mean of triplicate samples +/- S.D. (n=3).

FIGURE 2. Phosphoregulation of LTC_4 biosynthesis occurs at the level of LTC_4 synthase. Eosinophil-like HL-60#7 cells were primed for 30 minutes at 37°C with 200 pM rhGM-CSF and then incubated with 2.1 mM calcium for 8 minutes at 37°C. A, Cells were incubated with the vehicle (Me_2SO at 1/1000 of final volume) for 10 minutes at 37°C. Following the addition of varying concentrations of acivicin the reaction was started by the addition of LTA_4 (40μM) and cells were incubated for 10 minutes at 37°C. Acivicin inhibited the conversion of LTC_4 to LTD_4 in a dose-dependent manner with an IC_{50} value of 0.25 mM. B, Cells were incubated with vehicle (columns 1,4), 50 nM PMA (columns 2,5), or 50 nM PMA plus 1 μM

staurosporine (columns 3,6) for 15 minutes at 37°C. Following the addition of 2.5 mM acivicin the reaction was started with the addition of 5 μM LTA_4 (columns 1,2,3) or 5 μM LTA_4 and 10 mM glutathione (columns 4,5,6). (The availability of exogenously added GSH to LTC_4 synthase in intact cells was confirmed by the ability of (^{35}S) GSH to be incorporated into LTC_4. Data is not presented.) Cells were incubated at 37°C for 4 minutes. All reactions were terminated by the addition of an equal volume of 0.9 nmol/ml PGB_2 in cold methanol. Leukotrienes were purified by solid phase extraction with *Sep-Pak* C_{18} cartridges and quantified by reverse-phase HPLC as described in "Materials and Methods". Data is expressed as the mean of triplicate samples +/- S.D. (n=3).

FIGURE 3. PMA inhibits LTC_4 biosynthesis in a dose-dependent and time-dependent manner. Eosinophil-like HL-60#7 cells were primed for 30 minutes at 37°C with 200 pM rhGM-CSF and then incubated with 2.1 mM calcium for 8 minutes at 37°C. A, Cells were incubated with varying concentrations of PMA for 15 minutes at 37°C and after the addition of 2.5 mM acivicin, the reaction was started with the addition of 5 μM LTA_4. Cells were incubated for 4 minutes at 37°C with gentle shaking. Data is expressed as the mean of triplicate samples +/- S.D. A representative experiment of two is shown. B, Cells were incubated with the vehicle, 50 nM PMA (O), or 50 nM PMA and 1 μM staurosporine (●) for the indicated time, and after the addition of 2.5 mM acivicin, the reaction was started with the addition of 5 μM LTA_4. Cells were incubated for 4 minutes at 37°C. Data is expressed as the mean of duplicate samples. A representative experiment of two is shown. All reactions were terminated by the addition of an equal volume of 0.9 nmol/ml PGB_2 in cold methanol. Leukotrienes were purified by solid phase extraction with *Sep-Pak* C_{18} cartridges and quantified by reverse-phase HPLC as described in "Materials and Methods".

FIGURE 4. Protein kinase C inhibitors prevent PMA-mediated inhibition of LTC_4 synthesis. Eosinophil-like HL-60#7 cells were primed for 30 minutes at 37°C with 200 pM rhGM-CSF and then incubated with 2.1 mM calcium for 8 minutes at 37°C. Cells were incubated with 50 pM PMA and varying concentrations of staurosporine (●) or bisindolylmaleimide (O), as indicated, for 15 minutes at 37°C. Following the addition of 2.5 mM acivicin the reaction was started with the addition of 5 μM LTA_4. Cells were incubated for 4 minutes at 37°C and the reaction was terminated by the addition of an equal volume of 0.9 nmol/ml PGB_2 in cold methanol. Leukotrienes were purified by solid phase extraction with *Sep-Pak* C_{18} cartridges and quantified by reverse-phase HPLC as described in "Materials and Methods". Activity is expressed as a percentage of the control to which only the vehicle was added. Data is expressed as the mean of triplicate samples (n=3).

FIGURE 5. Phosphoregulation of LTC_4 synthesis is protein kinase C-specific in the whole cell LTC_4 synthase assay. Eosinophil-like HL-60#7 cells were primed for 30 minutes at 37°C with 200 pM rhGM-CSF. Cells were incubated with vehicle (control) or the indicated inhibitor for 90 minutes at 37°C and were then incubated with 2.1 mM calcium for 8 minutes at 37°C. Following 15 minute treatment with 50 nM PMA and the subsequent addition of 2.5 mM acivicin, the reaction was started with the addition of 5 μM LTA_4 . Cells were incubated for 4 minutes at 37°C and the reaction was terminated by the addition of an equal volume of 0.9 nmol/ml PGB_2 in cold methanol. Leukotrienes were purified by solid phase extraction with *Sep-Pak* C_{18} cartridges and quantified by reverse-phase HPLC as described in "Materials and Methods". Data is expressed as the mean of triplicate samples +/- S.D (n=3).

FIGURE 6. Tyrphostin 47 but not PMA directly inhibits LTC_4 synthase activity. *Partially purified LTC_4 synthase assay:* LTC_4 synthase activity was measured using 4.5 μg of partially purified LTC_4 synthase (Hiload Q fraction at a specific activity of 16.1 nmol•min^{-1}•mg^{-1}) which was incubated at 25°C for 15 min in 0.1 M potassium phosphate buffer in the presence of 40 μM LTA_4, 10 mM glutathione, 0.2 mM $MgCl_2$, 50 mM serine-borate and varying concentrations of tyrphostin 47 (●,A) or PMA (■,B) as indicated (0.5 μl of stock prepared in Me_2SO) in a final volume of 150 μl. The reaction was terminated with the addition of an equal volume of cold acetonitrile:methanol:acetic acid (50:50:1, v/v/v). Products were analyzed by reverse-phase HPLC as described in "Materials and Methods" Data is expressed as the mean of duplicate samples. *Whole cell LTC_4 synthase assay:* Eosinophil-like HL-60#7 cells were primed for 30 minutes at 37°C with 200 pM rhGM-CSF and then incubated with vehicle (❑,B), or varying concentrations of tyrphostin 47 (○,A) for 90 minutes at 37°C. Cells were incubated with 2.1 mM calcium for 8 min at 37°C and then the vehicle (○,A) or varying concentrations of PMA (❑,B) was added for 15 minutes at 37°C. Following the addition of 2.5 mM acivicin the reaction was started with the addition of 5 μM LTA_4. Cells were incubated for 4 minutes at 37°C and the reaction was terminated by the addition of an equal volume of 0.9 nmol/ml PGB_2 in cold methanol. Leukotrienes were purified by solid phase extraction with *Sep-Pak* C_{18} cartridges and quantified by reverse-phase HPLC as described in "Materials and Methods". Activity is expressed as a percentage of control to which only the vehicle was added. Data is expressed as the mean of triplicate samples. For each of panels A and B, a representative experiment of two is shown.

FIGURE 7. Protein kinase C activation inhibits LTC_4 synthase non-competitively in eosinophil-like HL-60#7 cells and in THP-1 cells. Eosinophil-like HL-60#7 cells(A) or THP-1 cells(B) were primed for 30 minutes at 37°C with 200 pM rhGM-CSF, incubated for 8 minutes with 2.1 mM calcium at 37°C and then incubated with vehicle(▲) or 50 nM PMA(O) or 50 nM PMA and 1 μM staurosporine(●) for 15 minutes at 37°C. Following the addition of 2.5 mM acivicin the reaction was started by the addition of varying concentrations of LTA_4 . Cells were incubated for 4 minutes at 37°C and the reaction was terminated by the addition of an equal volume of 0.9 nmol/ml PGB_2 in cold methanol. Leukotrienes were purified by solid phase extraction with *Sep-Pak* C_{18} cartridges and quantified by reverse-phase HPLC as described in "Materials and Methods". FIG. 7 is a representative experiment of four. K_m and V_{max} values were calculated based on the Lineweaver-Burk (double-reciprocal) plot of the data (inset). The K_m was unchanged whereas the V_{max} was reduced by approximately 50% (see table 2).

FIGURE 8. Prostanoid biosynthesis in A23187-challenged, eosinophil-like HL-60#7 cells is upregulated by PMA-mediated activation of protein kinase C. Eosinophil-like HL-60#7 cells were primed for 30 minutes at 37°C with 200 pM rhGM-CSF and then incubated with 2.1 mM calcium for 8 minutes at 37°C. Cells were incubated with vehicle, 50 nM PMA or 50 nM PMA plus 1 μM staurosporine for 15 min at 37°C. Cells were then challenged with 1μM A23187 for 3 minutes at 37°C. Reactions were terminated by the addition of an equal volume of ice-cold methanol. Aliquots of the terminated reactions were removed for TXB_2 (B) and PGE_2 (C) quantification by ELISA and RIA, respectively. Leukotrienes (A) were purified from the remaining terminated reactions by solid phase extraction with *Sep-Pak* C_{18} cartridges and quantified by reverse-phase HPLC as described in "Materials and

Methods". Data is expressed as the mean +/- S.D. of three experiments each performed with triplicate samples.

FIGURE 9. Proposed model for the regulation of eicosanoid biosynthesis in eosinophils by protein kinase C. Protein kinase C (PKC) is activated by receptor-mediated events or by phorbol esters (PMA). Activated PKC translocates to cellular membranes whereupon it initiates a myriad of cellular events which attenuate cysteinyl leukotriene production while concomitantly elevating prostanoid formation: a) Activated PKC increases the pool of free arachidonate by stimulating cytosolic phospholipase A_2 ($cPLA_2$) either directly or via MAP kinase (MPK); B) Transcription of the gene for the inducible isoform of cyclooxygenase (cyclooxygenase-2; COX-2) is activated by a PKC-dependent mechanism resulting in higher levels of the COX-2 enzyme to accommodate the enlarged pool of free arachidonate; c) Meanwhile, cysteinyl leukotriene formation is reduced by PKC-dependent inhibition of the LTC_4 synthase enzyme (represented by the solid black bar). PM, plasma membrane; M, cellular membranes; N, nucleus; PMA, phorbol 12-myristate 13-acetate; L, ligand; R, receptor; PKC, protein kinase C; MPK, MAP kinase; $cPLA_2$, cytosolic phospholipase A_2 (the phosphorylated/active forms of the latter three enzymes is indicated by -P); PL, membrane phospholipid; lyso-PL, lyso-phospholipid; PG, prostaglandin; TX, thromboxane; LT, leukotriene; COX-2, cyclooxygenase-2; 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein. Leukotrienes and prostanoids not normally synthesized in eosinophils are indicated by brackets.

TABLE I.

PMA treatment does not alter cellular glutathione levels in eosinophil-like HL-60#7 cells.

TREATMENT	*LTC_4 BIOSYNTHESIS* (pmol LTC_4 formed $\cdot min^{-1} \cdot 10^6$ $cells^{-1}$)	*CELLULAR GSH* (mM)	*CELLULAR GSSG* (mM)
Control	24.2 ± 3.0	0.595 ± 0.033	N.D.
PMA	14.7 ± 0.51	0.516 ± 0.031	N.D.
PMA & Staurosporine	24.9 ± 4.1	0.531 ± 0.033	N.D.

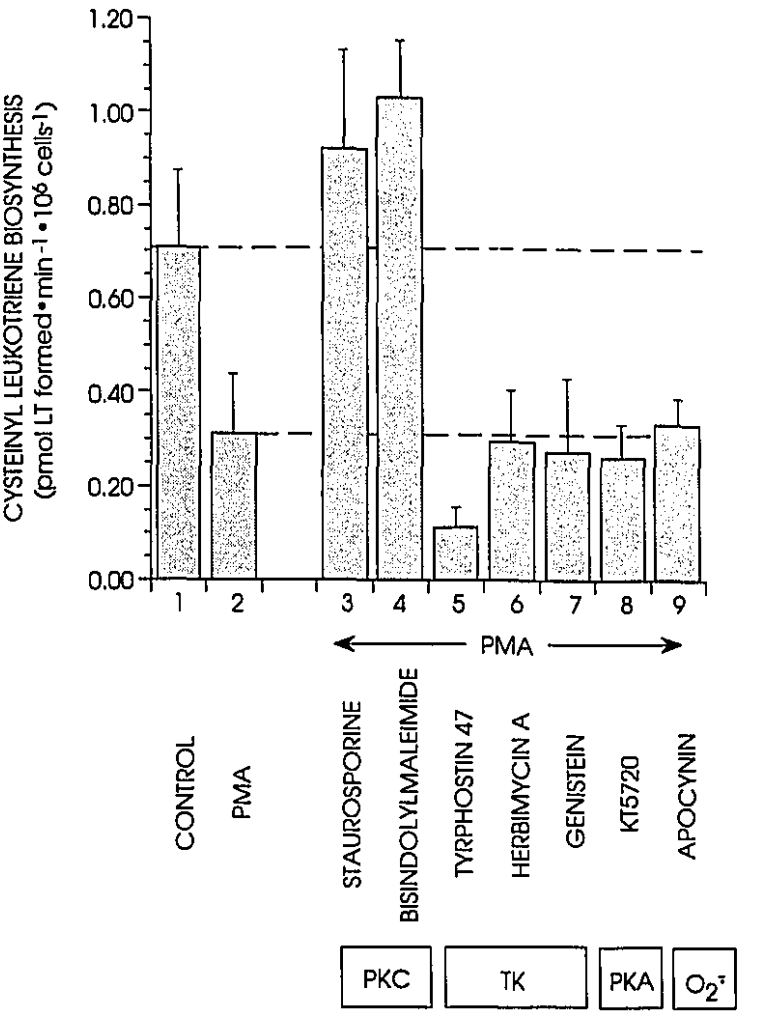
Eosinophil-like cells were primed for 30 min at 37°C with 200 pM rhGM-CSF, incubated with 2.1 mM calcium for 8 min at 37°C,and then incubated with vehicle (control), 50 nM PMA, or 50 nM PMA and 1 μM staurosporine for 15 min at 37°C. Triplicate samples of treated cells were taken for determination of glutathione content as described in "Materials and Methods". Following the addition of 2.5 mM acivicin to remaining cells the reaction was started with the addition of 5 μM LTA_4. Cells were incubated at 37°C for 4 min. Reactions were terminated by the addition of an equal volume of 0.9 nmol/ml PGB_2 in cold methanol. Leukotrienes were purified by solid phase extraction with Waters Sep-Pak C_{18}-light cartridges and quantified by reverse-phase HPLC as described in "Materials and Methods". Data is expressed as the mean of triplicate samples ± S.D. (n=2). (N.D. = Not detectable)

Table II.

Protein kinase C activation inhibits LTC_4 synthase non-competitively in butyric-acid differentiated HL-60 cells and in THP-1 cells.

	EOSINOPHIL-LIKE HL-60#7 CELLS		MONOCYTIC THP-1 CELLS	
TREATMENT	K_m (μM)	V_{max} (pmol LTC_4 formed $\cdot min^{-1} \cdot 10^6$ cells)	K_m (μM)	V_{max} (pmol LTC_4 formed $\cdot min^{-1} \cdot 10^6$ cells)
Control	0.927	24.6	7.14	110.6
PMA	0.896	15.0	6.25	37.1
PMA & Staurosporine	0.825	22.5	6.66	74.0

Kinetic analysis of LTC_4 synthase activity in eosinophil-like cells or THP-1 cells was performed as described in figure 7. K_m and V_{max} values were calculated based on the Lineweaver-Burk (double-reciprocal) plot of the data. With PMA treatment, the K_m was unchanged whereas the V_{max} was reduced by approximately 50%. Co-treatment with PMA and staurosporine returned the values of the kinetic parameters to control levels.


CYSTEINYL LEUKOTRIENE BIOSYNTHESIS
(pmol LT formed • min-1 • 10⁶ cells-1)
1.20
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1
2
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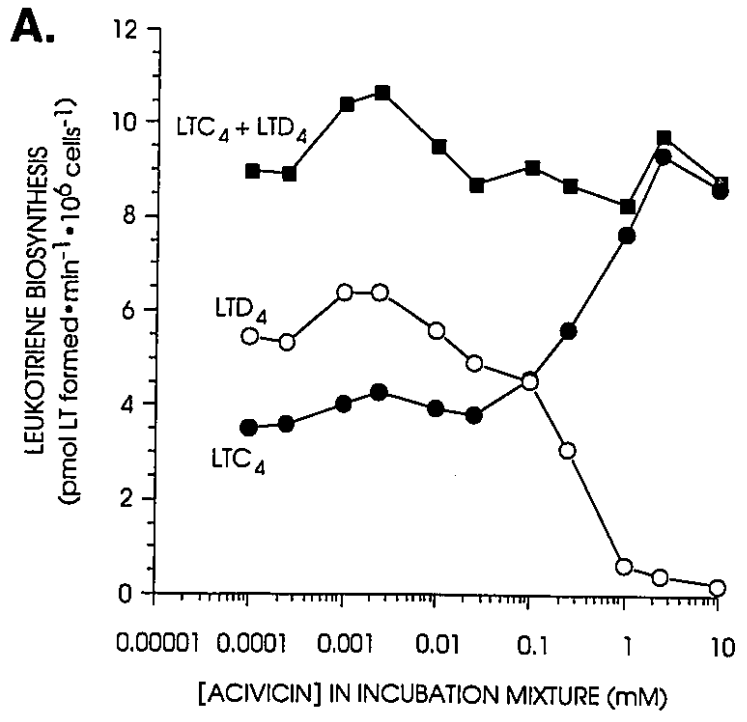
PMA
CONTROL
PMA
STAUROSPORINE
BISINDOLYLMALEIMIDE
TYRPHOSTIN 47
HERBIMYCIN A
GENISTEIN
KT5720
APOCYNIN
PKC
TK
PKA
O2-

Figure 1


A.
LEUKOTRIENE BIOSYNTHESIS
(pmol LT formed•min-1•10^6 cells-1)
LTC4 + LTD4
LTD4
LTC4
0
2
4
6
8
10
12
0.00001
0.0001
0.001
0.01
0.1
1
10
[ACIVICIN] IN INCUBATION MIXTURE (mM)

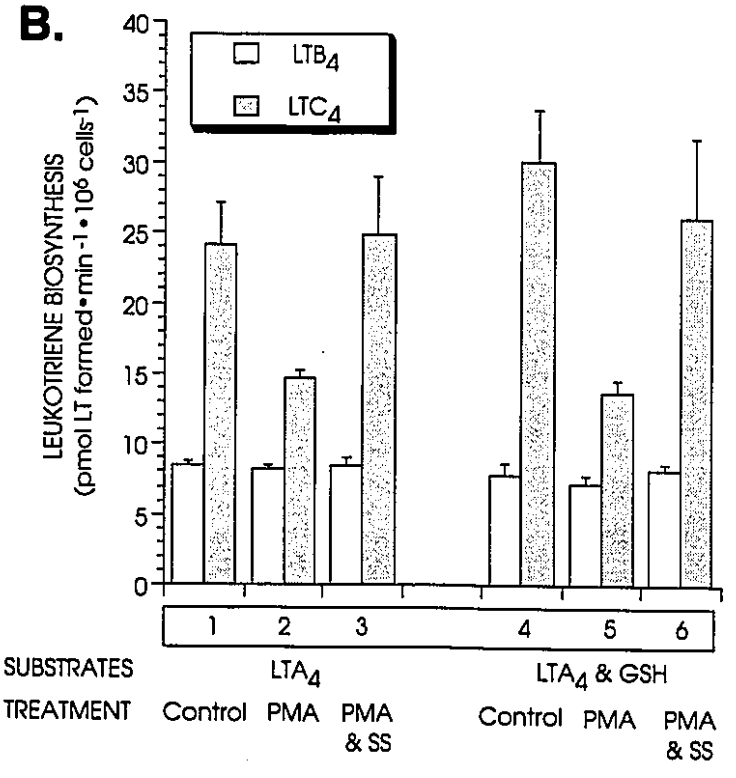
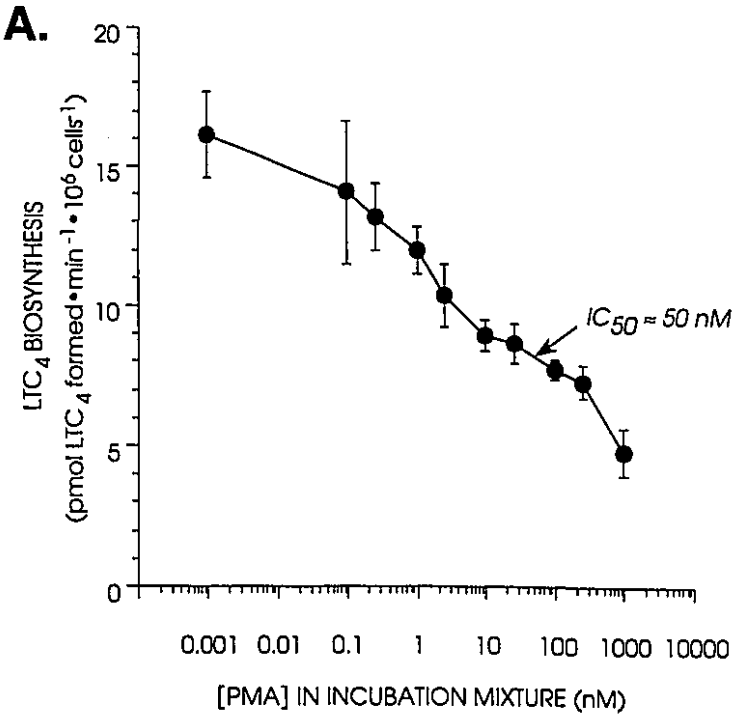

B.
LEUKOTRIENE BIOSYNTHESIS
(pmol LT formed•min-1•10^6 cells-1)
LTB4
LTC4
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
SUBSTRATES
LTA4
LTA4 & GSH
TREATMENT
Control
PMA
PMA & SS
Control
PMA
PMA & SS

Figure 2


A.
20
15
10
5
0
LTC4 BIOSYNTHESIS
(pmol LTC4 formed•min-1•10^6 cells-1)
IC50 ≈ 50 nM
0.001 0.01 0.1 1 10 100 1000 10000
[PMA] IN INCUBATION MIXTURE (nM)

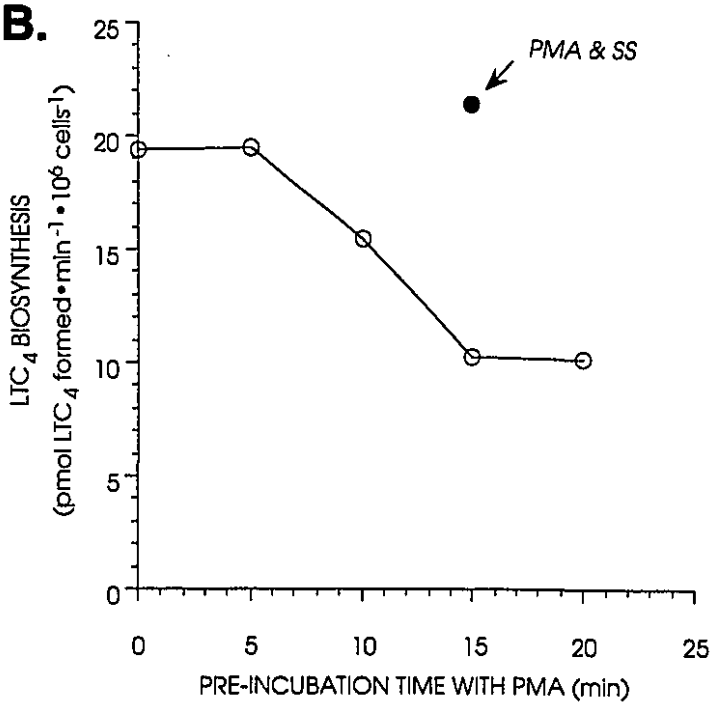

B.
25
20
15
10
5
0
LTC4 BIOSYNTHESIS
(pmol LTC4 formed•min-1•10^6 cells-1)
PMA & SS
0 5 10 15 20 25
PRE-INCUBATION TIME WITH PMA (min)

Figure 3

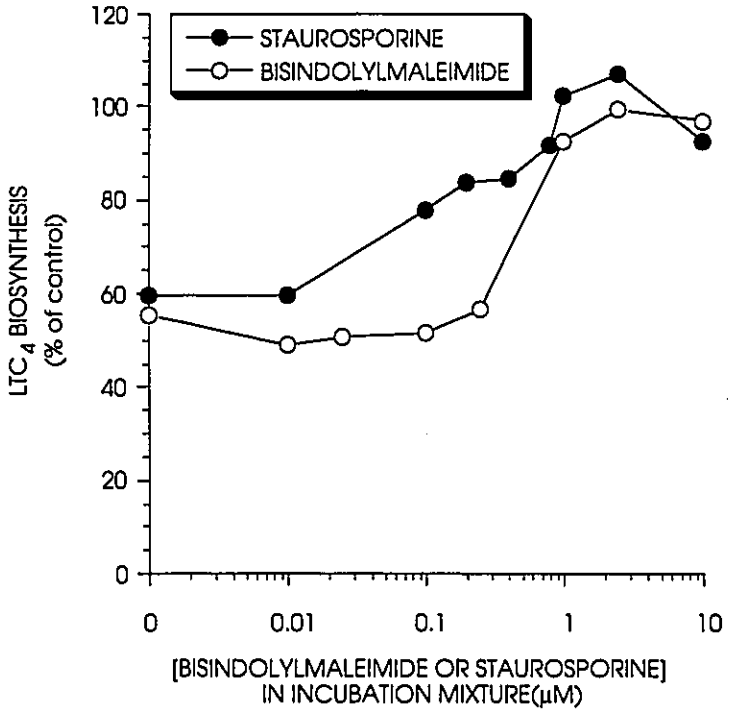

STAUROSPORINE
BISINDOLYLMALEIMIDE
120
100
80
60
40
20
0
LTC4 BIOSYNTHESIS
(% of control)
0
0.01
0.1
1
10
[BISINDOLYLMALEIMIDE OR STAUROSPORINE]
IN INCUBATION MIXTURE(μM)

Figure 4

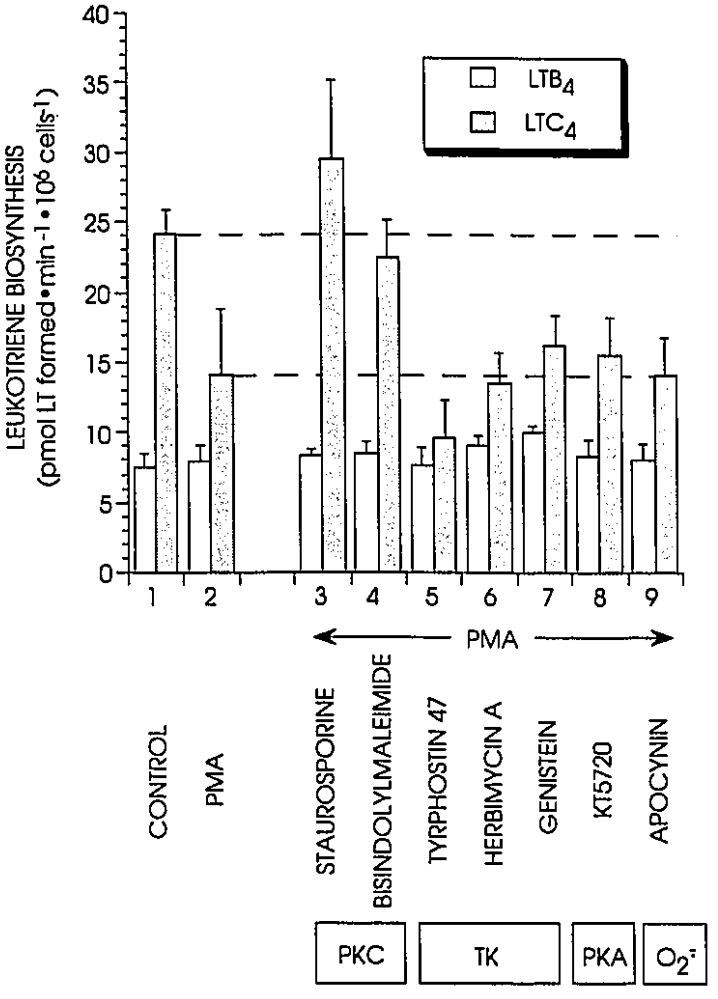

LEUKOTRIENE BIOSYNTHESIS
(pmol LT formed • min -1 • 10^6 cells-1)
40
35
30
25
20
15
10
5
0
LTB4
LTC4
1
2
3
4
5
6
7
8
9
PMA
CONTROL
PMA
STAUROSPORINE
BISINDOLYLMALEIMIDE
TYRPHOSTIN 47
HERBIMYCIN A
GENISTEIN
KT5720
APOCYNIN
PKC
TK
PKA
O2-

Figure 5

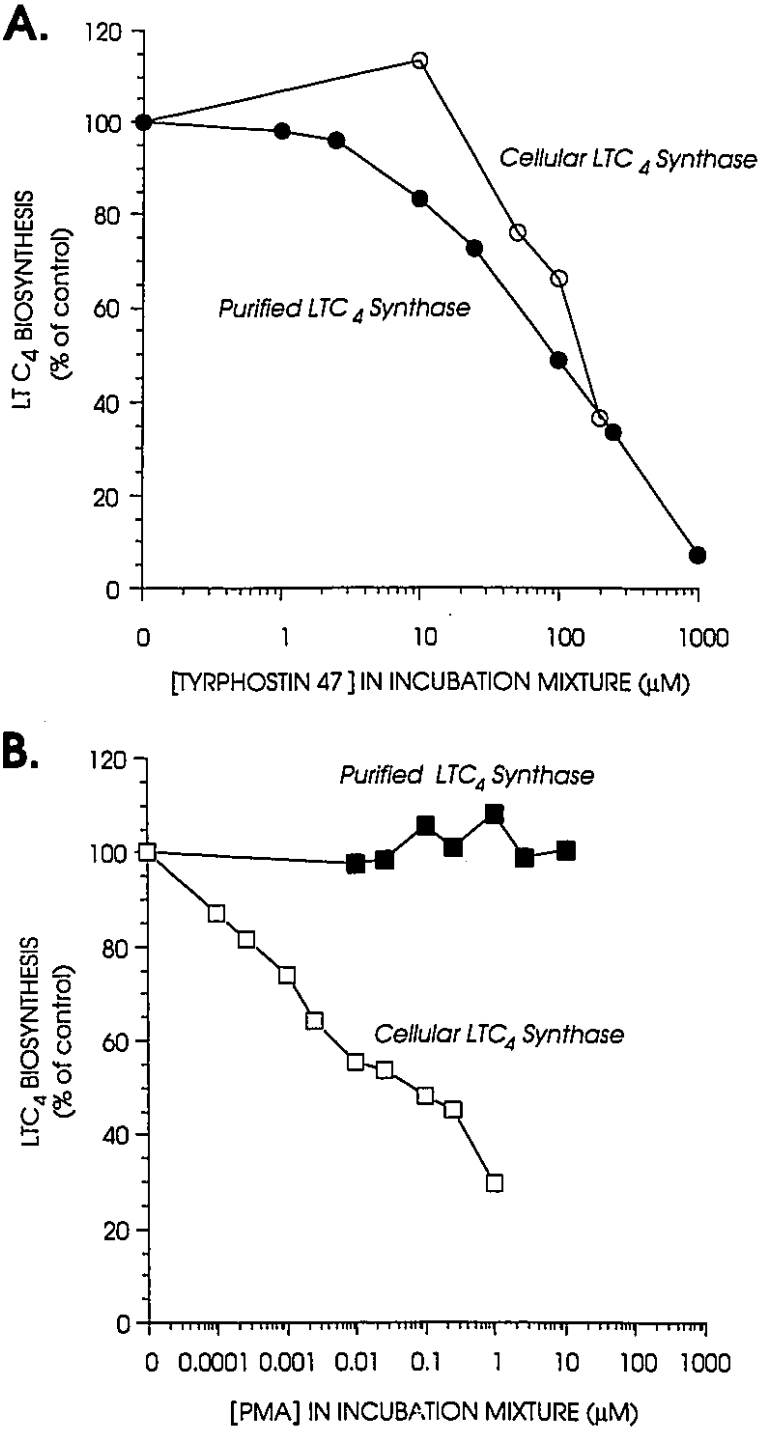

A.
120
100
80
60
40
20
0
LT C4 BIOSYNTHESIS
(% of control)
Cellular LTC 4 Synthase
Purified LTC 4 Synthase
0
1
10
100
1000
[TYRPHOSTIN 47] IN INCUBATION MIXTURE (μM)
B.
120
100
80
60
40
20
0
LTC4 BIOSYNTHESIS
(% of control)
Purified LTC4 Synthase
Cellular LTC4 Synthase
0 0.0001 0.001 0.01 0.1 1 10 100 1000
[PMA] IN INCUBATION MIXTURE (μM)

Figure 6

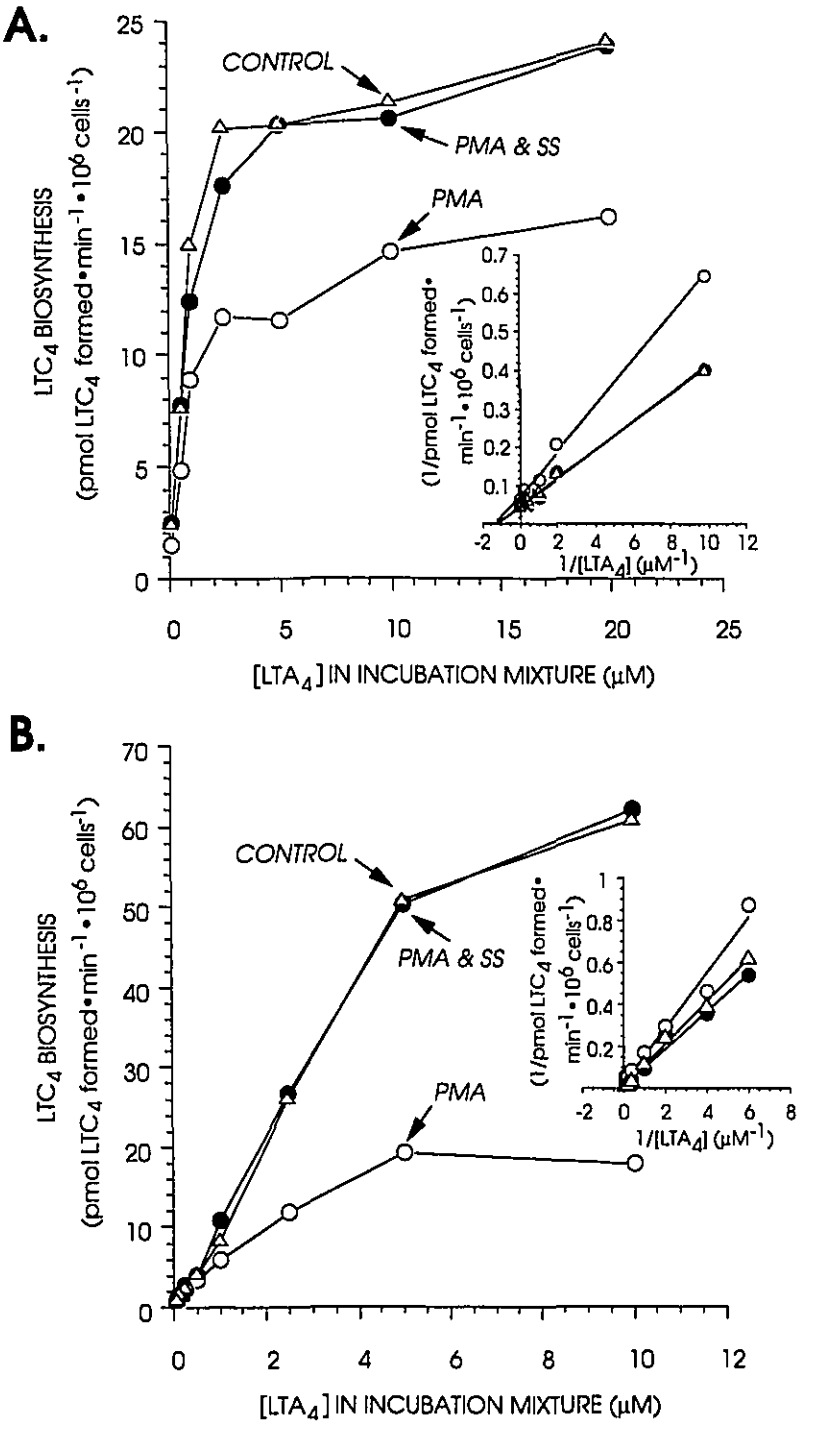

A.
CONTROL
PMA & SS
PMA
LTC4 BIOSYNTHESIS
(pmol LTC4 formed•min-1•10^6 cells-1)
[LTA4] IN INCUBATION MIXTURE (μM)
(1/pmol LTC4 formed• min-1•10^6 cells-1)
1/[LTA4] (μM-1)
B.
CONTROL
PMA & SS
PMA
LTC4 BIOSYNTHESIS
(pmol LTC4 formed•min-1•10^6 cells-1)
[LTA4] IN INCUBATION MIXTURE (μM)
(1/pmol LTC4 formed• min-1•10^6 cells-1)
1/[LTA4] (μM-1)

Figure 7

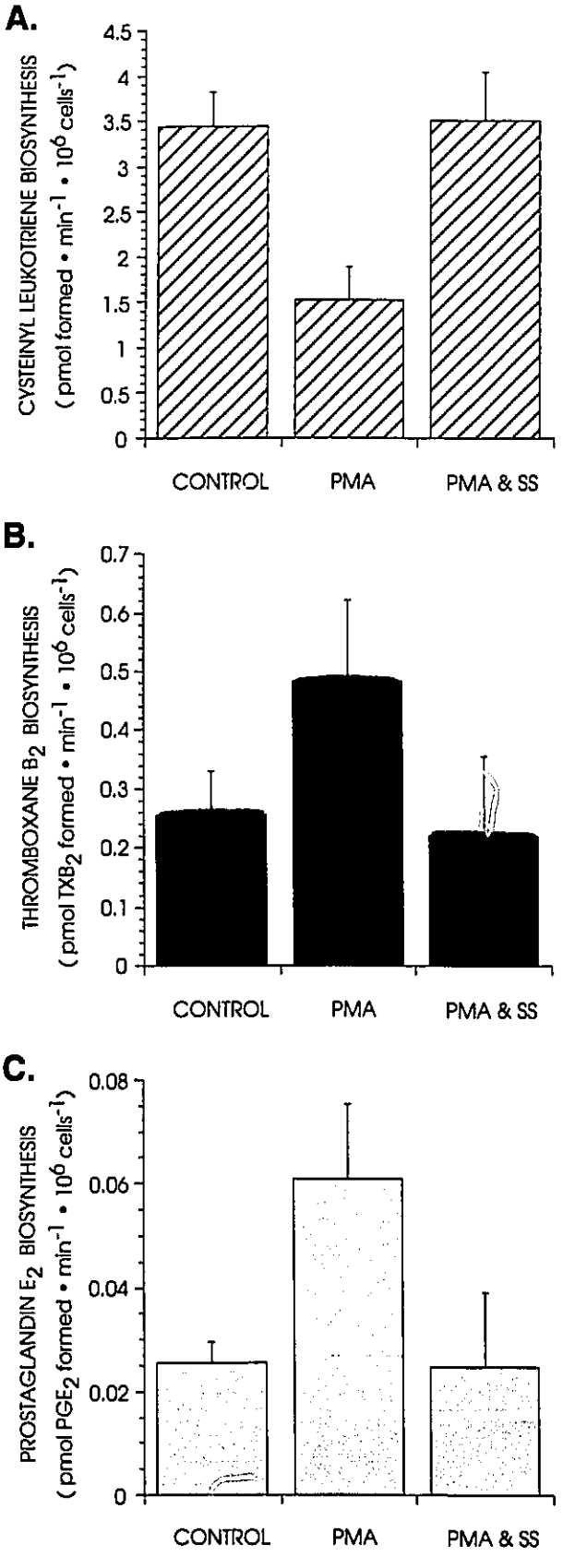

A.
CYSTEINYL LEUKOTRIENE BIOSYNTHESIS
(pmol formed • min-1 • 10^6 cells-1)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
CONTROL
PMA
PMA & SS
B.
THROMBOXANE B2 BIOSYNTHESIS
(pmol TXB2 formed • min-1 • 10^6 cells-1)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CONTROL
PMA
PMA & SS
C.
PROSTAGLANDIN E2 BIOSYNTHESIS
(pmol PGE2 formed • min-1 • 10^6 cells-1)
0.08
0.06
0.04
0.02
0
CONTROL
PMA
PMA & SS

Figure 8

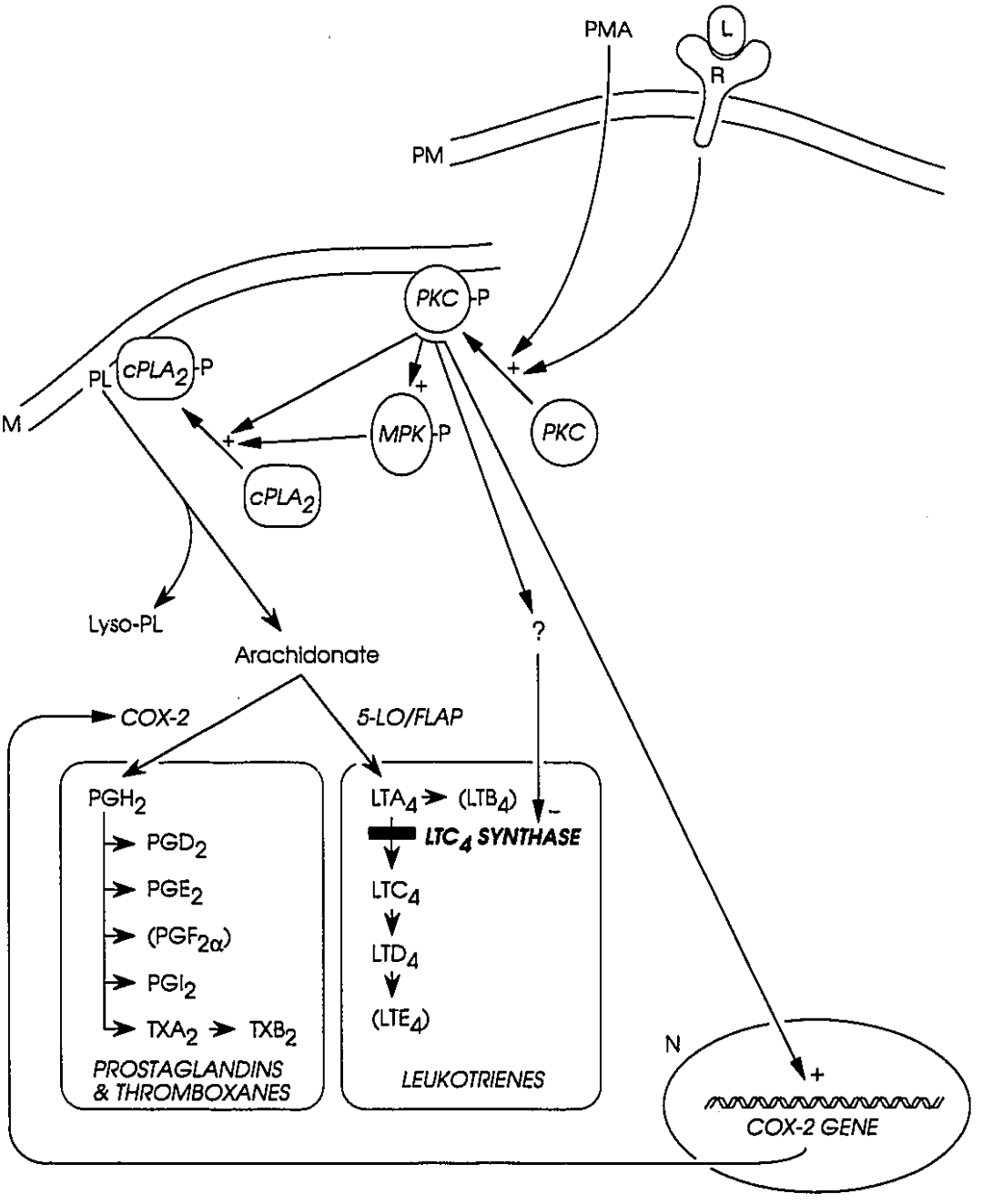

PMA
L
R
PM
PKC -P
PL
cPLA2 -P
M
+
MPK -P
PKC
cPLA2
Lyso-PL
Arachidonate
?
COX-2
5-LO/FLAP
PGH2
PGD2
PGE2
(PGF2α)
PGI2
TXA2 → TXB2
PROSTAGLANDINS & THROMBOXANES
LTA4 → (LTB4)
LTC4 SYNTHASE
LTC4
LTD4
(LTE4)
LEUKOTRIENES
N
COX-2 GENE

Figure 9

9. DISCUSSION

LTC_4 synthase catalyzes the conjugation of the unstable epoxide, LTA_4, with reduced glutathione. This biosynthetic activity had been previously attributed to the known glutathione S-transferase enzymes. The first pieces of evidence that indicated that LTC_4 biosynthesis might be catalyzed by a unique enzyme, distinct from the other known forms of the glutathione S-transferases, was the identification of differences in the substrate specificity and inhibitor profiles of the two enzymes. LTC_4 synthase catalyzes the rate-limiting step leading to the production of the potent inflammatory mediators, the cysteinyl leukotrienes. Thus the characterization and purification of this enzyme are critical for the elaboration of strategies and the development of drugs to attenuate its activity.

The complete biochemical characterization and purification of human LTC_4 synthase was dependent on: first, the establishment that the enzyme was distinct from known human GSTs; secondly, the identification of abundant sources of the enzyme; thirdly, the identification of potential polypeptide candidates for this enzyme in cells expressing high levels of LTC_4 synthase activity with the use of a photoaffinity probe; fourthly, the establishment of conditions required to stabilize and or sustain LTC_4 synthase activity and finally the development of chromatographic steps required to purify the enzyme to homogeneity.

9.1 Identification and induction of leukotriene biosynthetic enzymes in inflammatory cells

Leukotriene biosynthesis occurs mainly in cells of myeloid lineage which include the polymorphonuclear granulocytes (eosinophils, neutrophils, basophils) and the mononuclear phagocytes (monocytes and macrophages). Cysteinyl

leukotriene biosynthesis has been reported to predominantly occur in mast cells, eosinophils, monocytes, and through transcellular metabolism in platelets (309,310), while LTB_4 biosynthesis mainly occurs in neutrophils. Although high LTC_4 synthase biosynthetic activity has been detected in the monocytic fraction of cells isolated from human peripheral blood, these cells were not a suitable source of LTC_4 synthase for its purification since LTC_4 synthase itself is a low abundance enzyme that would require isolation of large amounts of monocytic cells from human blood. Therefore, human leukemia cell lines, which can be selectively differentiated in culture in the presence of specific chemical agents (eg. phorbol esters, dimethylsulfoxide (Me_2SO), butyric acid) into cells resembling the various myelocytic cells found *in vivo* and can be grown in large quantities, were tested for LTC_4 synthase biosynthetic activity (figure 9.1). Three human leukemia cell lines, the pro-myelocytic HL-60 cell line, the pro-monocytic U937 cell line, and the monocytic THP-1 cell line, demonstrated relatively high levels of LTC_4 synthase activity, particularly after differentiation. A 10-fold increase in LTC_4 synthase activity was detected upon differentiation of U937 cells into monocytic cells in the presence of Me_2SO. Being monocytic cells, THP-1 cells are mature cells intrinsically expressing high levels of LTC_4 synthase activity relative to U937 and HL-60 cells and thus cannot be differentiated any further. Thus THP-1 and Me_2SO-differentiated U937 (dU937) cells, which had the highest LTC_4 synthase specific activities, were the predominant source of LTC_4 synthase for these studies.

Me_2SO-mediated differentiation of U937 has previously been reported to increase the activity of other leukotriene biosynthetic enzymes and expression of the 5-lipoxygenase activating protein (FLAP) and leukotriene receptors. Me_2SO-differentiated U937 cells demonstrate a 3-fold and 30-fold increase in LTD_4 and LTB_4 receptor binding sites (311), respectively. A doubling in the expression of phospholipase A_2 (PLA_2), γ-glutamyl transpeptidase (γ-GT) and cysteinylglycine

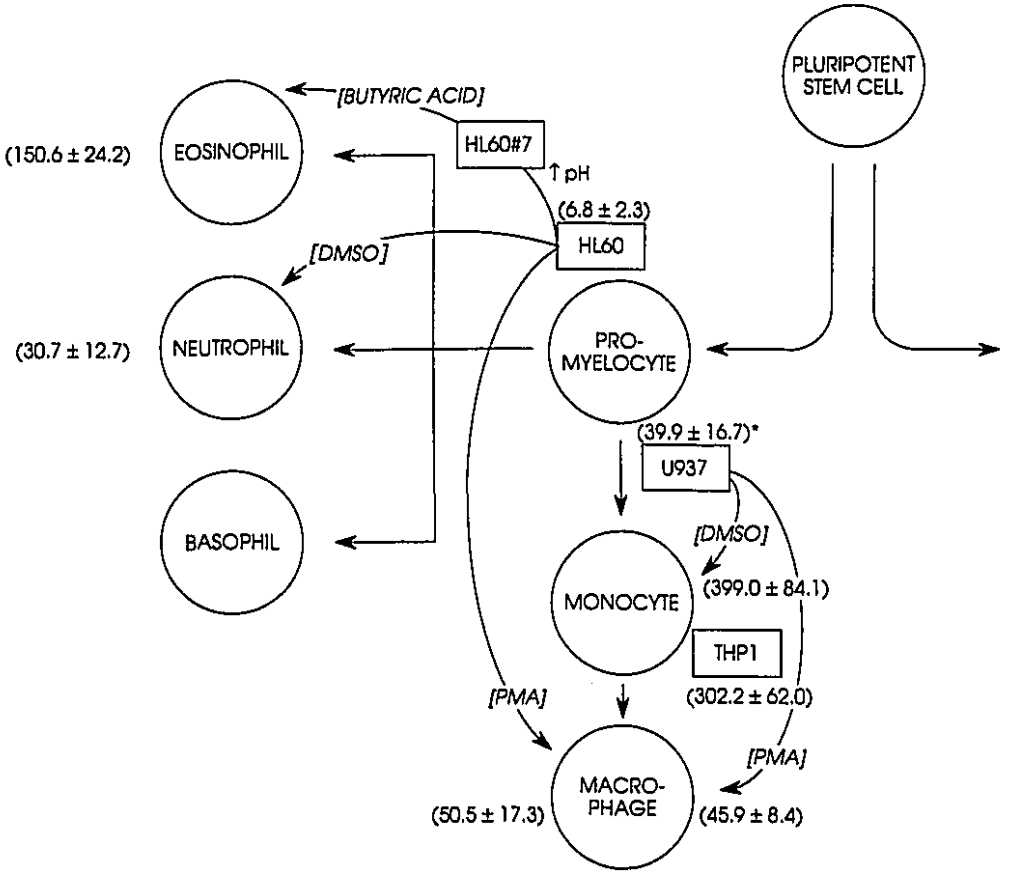


Figure. 9.1. LTC_4 synthase activity in human myeloid cells

* LTC_4 synthase specific activity: pmol LTC_4 formed • min^{-1} • mg^{-1}
(adapted from Nicholson, D.W., 1993, (ref. 58))

dipeptidase has also been detected in these cells (P.K. Weech and D.W. Nicholson, unpublished; D.M. Rasper and D.W. Nicholson, unpublished). Interestingly, although THP-1 and U937 cells both express FLAP they lack expression of the 5-LO enzyme and thus cannot synthesize leukotrienes *de novo*. However, in the case of the U937 cells, expression of FLAP can be increased by 4-fold with Me_2SO-differentiation and with the transfection of 5-LO cDNA these cells become competent to produce leukotrienes upon ionophore challenge (315). HL-60 cells, on the other hand, express all the leukotriene biosynthetic enzymes and thus are capable of synthesizing leukotrienes *de novo*.

9.2 LTC_4 synthase is a unique enzyme distinct from all known glutathione S-transferases

Having identified abundant human sources for the characterization and isolation of LTC_4 synthase, the next objective was to demonstrate that the enzyme was distinct from all known forms of the GSTs. This objective was met by several strategies. The first was the identification of LTC_4 synthase as a microsomal enzyme. Since most forms of the GSTs are soluble enzymes, they were separable from LTC_4 synthase by differential centrifugation following cell rupture (chapter 4 (ref.147)). In THP-1 cells and dU937 cells, LTC_4 synthase activity was predominantly located in the 100 000 xg microsomal pellet fraction (chapter 5 (ref.313),chapter 4 (ref.147),chapter 6 (ref.148)). Previous to these studies, LTC_4 synthase had also been identified as a particulate enzyme in RBL cells (142,143), PMN cells (144), mouse peritoneal macrophages (145), and mouse mastocytoma cells (146).

Secondly, chromatographic separation of LTC_4 synthase activity from GST activity was demonstrated (chapter 4 (ref.147),chapter 6 (ref.148),157). LTC_4

synthase and microsomal GST activity demonstrate differences in their detergent solubility properties. LTC_4 synthase is quantitatively solubilized from microsomal membranes with the anionic detergent, taurocholate, whereas microsomal GST was not. Conversely, microsomal GST activity but not LTC_4 synthase activity was not only solubilized but potentiated by the non-ionic detergent, Triton X-100 (143). It has been demonstrated that LTC_4 synthase can be separated from microsomal GST by taurocholate solubilization (chapter 4 (ref.147), chapter 5 (ref.313)). Subsequently, the detergent-solubilized proteins (containing LTC_4 synthase activity plus any contaminating soluble GSTs) were separated by anion-exchange chromatography and immunoblot analysis of the purified fractions identified two peaks of GST activity attributable to the π and μ isoforms. Furthermore the polypeptide constituents of the peak fractions containing LTC_4 synthase activity showed no immunoreactivity with anti-α, anti-μ, anti-π or anti-microsomal GST antisera (chapter 4 (ref.147)). Although antiserum for the recently identified θ class GST (314) was not available, this class of GST was probably not present in the preparations since there was no detectable activity with 1,2-epoxy-3-(p-nitrophenoxy) propane, the preferred substrate for θ-class GST, in any fraction. Thus LTC_4 synthase was separable from all known forms of the GSTs by differential detergent solublization and chromatographic procedures.

LTC_4 synthase and GSTs also demonstrate differences in substrate specificity and inhibition profile. LTC_4 synthase demonstrates high specific activity for the conjugation of glutathione to LTA_4 but not to any of the electrophilic GST substrates such as 1-chloro-2,4-dinitrobenzene (CDNB), 4-nitrobenzyl chloride or 1,2-epoxy-3-(4-nitrophenoxy) propane (substrate for the θ class of GST) (58). Although cytosolic GSTs from mouse mastocytoma cells catalyzed the conjugation of LTA_4 with GSH, the microsomal GST in this cell type demonstrated no activity with LTA_4 or LTA_4 methyl ester as a co-substrate (146). Microsomal GST

activity was also activated by N-ethylmaleimide, a sulfhydryl blocking reagent, whereas LTC_4 synthase activity was not (chapter 4 (ref.147), 315). LTC_4 synthase and cytosolic GSTs also demonstrated large differences in sensitivity to inhibitors such as Rose Bengal (10-20-fold difference in IC_{50} values). Diethylcarbamazine, a known SRS-A inhibitor, selectively inhibited LTC_4 synthase but not microsomal GST in RBL cells and, as mentioned previously, Triton X-100 selectively solubilizes and potentiates microsomal GST activity but inhibits LTC_4 synthase activity (143).

Finally, the last and most conclusive evidence supporting the distinction of LTC_4 synthase from other GSTs was obtained from the purification of LTC_4 synthase to homogeneity. The N-terminal sequence of LTC_4 synthase was not homologous to any sequence available in the databases including the GST sequences (chapter 6 (ref.148)).

The identification of LTC_4 synthase as a unique GST responsible solely for the production of the cysteinyl leukotrienes is not surprising since there is now considerable evidence indicating that several other biosynthetic steps of the leukotriene cascade are catalyzed by specific enzymes distinct from other more general isoforms. An arachidonate-selective PLA_2 has been identified that appears to be responsible specifically for the release of arachidonic acid from membrane phospholipids (19-23). In addition, LTA_4 hydrolase which catalyzes the hydrolysis of LTA_4 to form LTB_4, was shown to be distinct from other cellular epoxide hydrolases primarily associated with the detoxification of xenobiotics (107). Most recently, a specific γ-glutamyl transpeptidase responsible specifically for the conversion of LTC_4 to LTD_4 was cloned and was found to be distinct from previously identified γ-glutamyl transpeptidase (158). The advantage of having distinct enzymes responsible solely for the biosynthesis of leukotrienes in the cell is quite evident. For example, the cell can specifically regulate the activity of the GSTs and epoxide hydrolases involved in cellular detoxification mechanisms

without affecting leukotriene biosynthesis, and *vice versa*. Furthermore, the specificity of the leukotriene biosynthetic enzymes also has therapeutic implications, whereby inhibitors of leukotriene biosynthesis can be designed to attenuate the activity of these leukotriene-specific enzymes only. Such inhibitors would then have fewer non-specific effects and would also define conclusively the role of these mediators in disease processes.

9.3 Identification of potential polypeptide candidates for LTC_4 synthase

Photoaffinity labelling is a biochemical technique often used to identify receptors or proteins with affinity for the particular ligand in question. This technique is particularly useful for the identification of receptors and has been successfully used to identify polypeptide candidates for the LTB_4 and the LTD_4 receptors (193,271).

In order to identify potential polypeptide candidates for LTC_4 synthase, a novel photoaffinity ligand was constructed based on the product of the enzyme reaction, LTC_4. Although LTA_4 has relatively high affinity for LTC_4 synthase as judged by its K_m value of 5.6 μM (157), it was not chosen as the affinity ligand due to its labile epoxide bond and high hydrophobicity. Glutathione was also not suitable due to its predicted low affinity (K_m= 1.2 mM; (157)) and also because it was likely to be highly non-specific and would thus label the various GSTs. An LTC_4-based photoaffinity ligand was predicted to have high affinity for LTC_4 synthase since its structural analogue, LTC_2, potently inhibits LTC_4 synthase activity (IC_{50} value = 2.6 μM; (157)). This inhibition by LTC_2 also suggests that LTC_4 synthase may be subject to end-product inhibition. LTC_4 itself could not be used to demonstrate this phenomenon since added LTC_4 cannot be distinguished from enzymatically-formed LTC_4 by the reverse-phase high pressure liquid

chromatography (HPLC) technique used to measure LTC_4 synthase activity. LTC_2 however, has a different retention time from LTC_4 and was thus more suitable. The high affinity of the LTC_4-based photoaffinity ligand was then confirmed by the ability of the non-radioactive analogue (azido ^{127}I-LTC_4) to inhibit LTC_4 synthase activity (IC_{50} value = 7.0 μM; chapter 4 (ref.147)).

The LTC_4-based photoaffinity ligand was used to probe U937, dU937 and THP-1 cell microsomal membranes. Two polypeptides (18 kDa and 27 kDa) were specifically labelled in dU937 microsomal membranes (chapter 4 (ref.147)) and only one polypeptide (18 kDa) was specifically labelled in THP-1 cells (chapter 5 (ref.313)). However, the possibility that this 18 kDa polypeptide was microsomal GST, an enzyme with a molecular mass of 17.2 kDa, had to be addressed. The 18 kDa polypeptide was determined to be the most probable candidate for being LTC_4 synthase or a subunit of this enzyme based on several observations.

First, photolabelling of the 18 kDa polypeptide was specifically competed for by 100 000-fold lower concentrations of LTC_4 than reduced glutathione, whereas the opposite was observed for the specific labelling of the 27 kDa polypeptide which was strongly competed for by the lowest concentration of reduced glutathione tested (0.1 mM) but was only slightly competed for by up to 10 μM LTC_4. This 27 kDa polypeptide was most likely a contaminating cytosolic GST most of which have molecular masses in the 25-27 kDa range (chapter 4 (ref.147),chapter 5 (ref.313)).

Secondly, with the differentiation of U937 cells in the presence of Me_2SO there was a concomitant increase in the specific photolabelling of the 18 kDa polypeptide and LTC_4 synthase biosynthetic activity in the microsomal membranes of these cells (chapter 4 (ref.147)).

Thirdly, a comprehensive study of the specific photolabelling of this polypeptide in THP-1 cell microsomal membranes demonstrated that this

polypeptide is unlikely to be microsomal GST (chapter 5 (ref.313)). The specifically labelled 18 kDa polypeptide in THP-1 cell microsomal membranes was strongly competed for by LTC_4 but not at all by up to 1 mM GSH, indicating that this polypeptide had high affinity for the arachidonate backbone and not the glutathione moiety of LTC_4. Furthermore, the rank order of potencies of the structurally related leukotrienes and S-hexyl glutathione for competing for the specific labelling of the 18 kDa polypeptide corresponded exactly with their ability to attenuate LTC_4 synthase activity but not microsomal GST activity. Moreover, the specifically labelled 18 kDa polypeptide could be selectively solubilized by taurocholate consistent with the solubilization of LTC_4 synthase but not microsomal GST with this detergent.

Fourthly, in a highly purified preparation of LTC_4 synthase (10 000-fold) from dU937 cells which contained three major polypeptide constituents with molecular masses of 18, 25 and 37 kDa, only the 18 kDa polypeptide was specifically photolabelled by azido ^{125}I-LTC_4 (157).

These experimental approaches established that the photoaffinity ligand specifically labelled LTC_4 synthase or a subunit of this enzyme and not microsomal GST.

Due to the presence of conjugated double bonds, leukotrienes themselves are photolabile and have been used directly for the photoaffinity labelling of leukotriene binding proteins (316). In an attempt to identify polypeptide candidates for a putative cysteinyl leukotriene transporter in rat liver canalicular membranes, Ishikawa and co-workers have utilized this direct photolabelling technique and have found that LTC_4 preferentially labelled two proteins with molecular masses of 17 kDa and 25 kDa. The 25 kDa polypeptide was identified as subunit 1 (Ya) of GST. Although they did not rule out the possibility that the 17

kDa polypeptide was microsomal GST, this polypeptide may be identical to the 18 kDa polypeptide labelled in the studies described above.

9.4 LTC_4 synthase requires cofactors for stability

Although several investigators have attempted to purify LTC_4 synthase from a variety of sources, the purification of this enzyme has been hampered by many factors. LTC_4 synthase is a membrane-bound enzyme that is consequently quite hydrophobic and thus loses activity when removed from its native membrane environment. One of its substrates, LTA_4, contains an epoxide bond that renders it quite unstable in an aqueous medium. LTC_4 synthase was also found to be very low in abundance in the human cells and leukemia cell lines tested. Furthermore, accurate measurement of LTC_4 synthase activity and storage of the enzyme required the presence of several cofactors including reduced glutathione (substrate stabilization during storage at -70°C), detergents, and lipids, particularly when the enzyme was in a highly purified state (157). A comprehensive analysis of LTC_4 synthase in U937 cells defined the several critical parameters that modulated the activity of the purified enzyme. LTC_4 synthase biosynthetic activity was stimulated by the presence of divalent cations, specifically, Mg^{2+} or Ca^{2+} ($Mg^{2+}>Ca^{2+}$) even in crude preparations (157). Once the enzyme was purified greater than 500-fold detection of its activity became dependent on both the presence of Mg^{2+} and phosphatidylcholine (157). The substrate, reduced glutathione (2-4 mM) stabilized LTC_4 synthase activity, particularly during storage, however at concentrations greater than 5 mM, the presence of this substrate was toxic and irreversibly inactivated the enzyme (157). Substrate stabilization has also been reported for guinea-pig lung LTC_4 synthase (154).

Since LTC_4 synthase is a membrane-bound enzyme, its solubility and stability were affected by the presence of detergents. LTC_4 synthase activity could be solubilized quantitatively by anionic or zwitterionic detergents (eg. CHAPS (3-[(3-cholamindopropyl)-dimethylammonio]-1-propanesulfonate), taurocholate) but not non-ionic detergents (157). Furthermore, the combination of detergents used in the anion-exchange chromatography of LTC_4 synthase substantially affected the resolution of this enzyme from contaminant proteins. The presence of CHAPS in particular was essential for the stability of the enzyme. When this detergent was excluded from the buffers for the first step of the purification (anion-exchange chromatography) the resulting purified fractions lost approximately 50% of the LTC_4 synthase specific activity upon storage at -70°C overnight. Thus CHAPS may have dissociated destabilizing proteins that interact unfavourably with LTC_4 synthase (unpublished observations). Of the various detergents tested, the combination of 0.1% taurocholate, 0.5% *n*-octyl glucoside and 0.5% CHAPS was found to be the ideal detergent permutation required for the effective resolution of LTC_4 synthase from contaminant proteins (chapter 6 (ref.148)).

9.5 LTC_4 synthase is an 18 kDa homodimeric microsomal enzyme

The identification of the conditions required to stabilize LTC_4 synthase activity during the purification and storage and to stimulate its activity *in vitro* were essential for the eventual purification of the enzyme. A partial 3-step purification (10 000-fold) from U937 cell microsomal membranes has been described by Nicholson and co-workers. The polypeptide components of the final preparation had molecular masses of 18 kDa, 25 kDa and 37 kDa (157). The purification procedure was composed of two successive anion-exchange steps followed by affinity chromatography. The purification of LTC_4 synthase to

homogeneity was dependent on several factors. The first was the identification of THP-1 cells which intrinsically have high levels of LTC_4 synthase activity and due to their monocytic lineage require no further differentiation (chapter 6 (ref.148),chapter 5 (ref.313)). The LTC_4 synthase activity in THP-1 cells was comparable to the activity detected in differentiated U937 cells. The second was the addition of the CHAPS detergent to the purification buffers which not only improved the resolution of LTC_4 synthase but also stabilized the enzyme during storage. With the added resolving capacity of CHAPS, one of the anion-exchange steps used previously for the purification of LTC_4 synthase from U937 cell microsomal membranes (157) was eliminated from the purification scheme (chapter 6 (ref.148)). Thirdly, the addition of the LTC_2 affinity chromatography step resolved LTC_4 synthase from many contaminant proteins as judged by SDS-PAGE. This affinity column was constructed on the basis of LTC_2, a structural analogue of LTC_4, and not on the LTC_4 synthase substrates, LTA_4 or reduced GSH, due to reasons described previously for the construction of the LTC_4 based photoaffinity ligand. Briefly, LTA_4 was unsuitable due to its instability and GSH due to its non-specificity. LTC_4 synthase did not bind to glutathione affinity resins that had glutathione immobilized through the α-amino group on the γ-glutamyl residue, but bound to resins where GSH was immobilized via the sulfhydryl group, although with low efficiency and insufficient specificity (157). Thus, LTC_2, which is chemically more stable than LTC_4, was conjugated with biotin and then immobilized on streptavidin-agarose to construct the affinity column. LTC_2 and its biotinylated derivatives had high affinity for LTC_4 synthase as judged by their ability to inhibit LTC_4 synthase activity *in vitro* with relatively high potency (IC_{50} value for LTC_2 = 1.1 μM; IC_{50} value for LTC_2-XX-biotin = 10μM, where XX represents a 3.13 nm spacer) (figure 9.2).

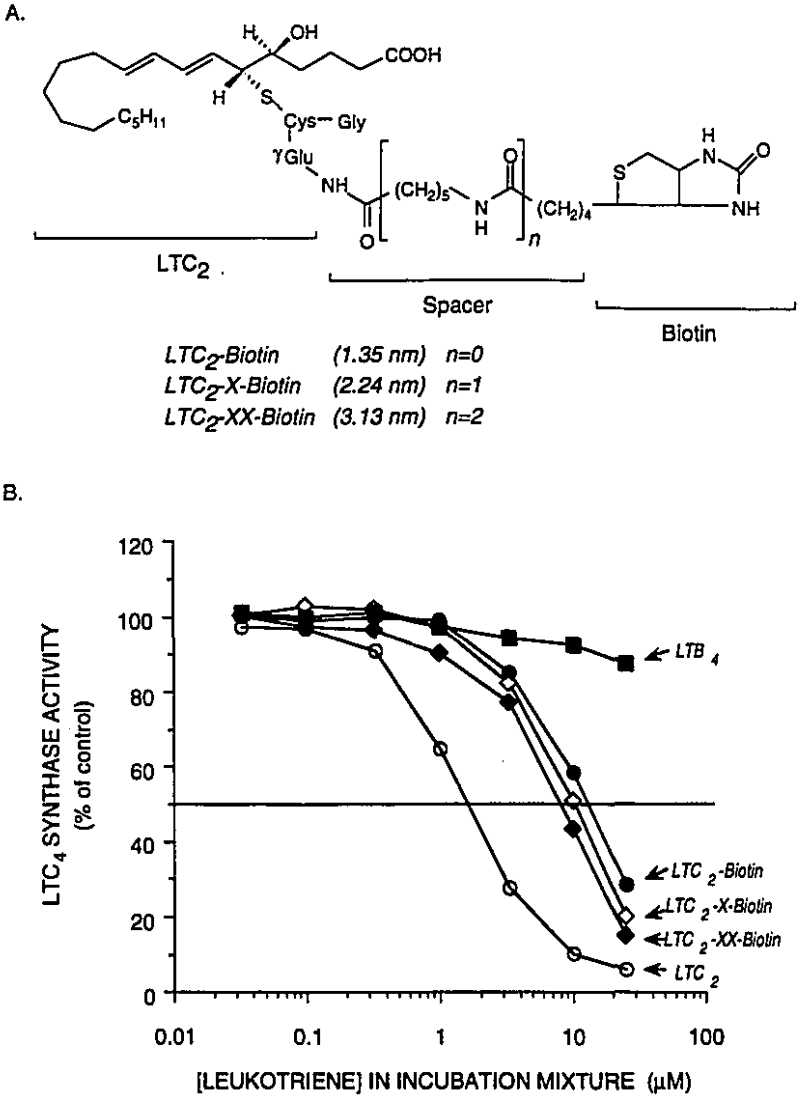


Figure 9.2. A. Structure of biotinylated LTC_2 derivatives. B. Inhibition of LTC_4 synthase activity by biotinylated LTC_2 (adapted from Nicholson, D.W. et al., 1992 (ref. 157))

Gel filtration of the affinity-purified LTC_4 synthase not only purified the enzyme to homogeneity but also determined a native molecular mass of approximately 39 kDa (chapter 6 (ref.148)). Since active fractions only contained a single 18 kDa polypeptide it was concluded that LTC_4 synthase most likely functions as a homodimer composed of two identical subunits. Although membrane proteins in the presence of detergents can form micelles that can migrate anomalously during gel filtration, a homodimeric structure for LTC_4 synthase was most likely accurate for several reasons. First, in inactive preparations of LTC_4 synthase the 18 kDa polypeptide eluted at a volume corresponding to 15-20 kDa suggesting that the enzyme was only active in its dimeric form. Secondly, on two different gel filtration media LTC_4 synthase activity eluted at a volume corresponding to similar molecular weights. Thirdly, multimeric enzyme composition is a common feature among other known GSTs.

9.6 The N-terminal sequence of LTC_4 synthase

The N-terminal sequence of LTC_4 synthase was determined by Edman-degradation microsequencing and had four notable features (chapter 6 (ref.148)). First, LTC_4 synthase contains an N-terminal methionine residue indicating that the enzyme is most likely not proteolytically processed *in vivo*. Secondly, most of the 35 amino acids identified were hydrophobic and this finding was consistent with the membrane-bound localization of the enzyme. Thirdly, a consensus sequence for PKC-mediated phosphorylation was present in the N-terminal sequence (Ser-Ala-Arg) at position 28-30 suggesting that the activity of the enzyme may be phosphoregulated *in vivo*. Finally, the identified sequence was unique and not homologous to any known sequences contained in available databases. If 100% of LTC_4 synthase activity was recovered from the THP-1cells it

is estimated that this enzyme accounts for approximately 0.003% of the total cell protein, thus LTC_4 synthase is also a very low abundance enzyme (chapter 6 (ref.148)).

9.7 Phosphoregulation of eicosanoid biosynthesis

Little is known about the regulatory mechanisms governing eicosanoid biosynthesis in cells. Recent investigations suggest that phosphoregulation may be an important mechanism controlling arachidonate metabolism since both phospholipase A_2 and cyclooxygenase-2, rate-limiting enzymes for the biosynthesis of arachidonic acid and prostaglandins are positively regulated by MAP kinase (MPK) (17) and protein kinase C (PKC) (317), respectively. Certain studies that have examined the regulation of leukotriene biosynthesis demonstrate that phorbol-12-myristate-13-acetate (PMA) and the calcium ionophore, A23187, synergistically stimulate LTC_4 biosynthesis in murine macrophages (318,319) and eosinophils (320) and stimulate LTB_4 biosynthesis in human neutrophils (321,322). This stimulatory effect could be attributed to phosphorylation and activation of PLA_2 resulting in increased arachidonic release and subsequent leukotriene biosynthesis. However, these studies used suboptimal concentrations of ionophore and have not demonstrated the ability of specific PKC inhibitors to reverse the effects of PMA. PMA has been shown to double LTB_4 biosynthesis in fMLP-stimulated human neutrophils and this observation has suggested that LTA_4 hydrolase may be activated by phosphorylation. But this hypothesis has not been proven *in vitro*, since LTA_4 hydrolase has been shown to be a poor substrate for protein kinase C (Haeggstorm, J. and Hansson, A. , unpublished data). The studies described here

indicate that LTC_4 synthase, the rate-limiting enzyme for the production of cysteinyl leukotrienes is also regulated by PKC, but in a negative manner.

Leukotriene biosynthesis occurs predominantly in cells of myeloid lineage, with LTB_4 biosynthesis mainly occurring in neutrophils and LTC_4 biosynthesis in eosinophils (323-326). HL-60 cells are capable of *de novo* leukotriene biosynthesis and when differentiated in the presence of dimethylsulfoxide or butyric acid they assume the morphological and functional characteristics of mature neutrophils or eosinophils, respectively (327-329). Furthermore, leukotriene biosynthesis can be enhanced in these cells by priming with GM-CSF (granulocyte macrophage colony-stimulating factor), a cytokine that elevates both the levels of 5-lipoxygenase and FLAP (330,331) but not LTC_4 synthase (K. Scoggan and D.W. Nicholson, unpublished results). Thus these cell lines were judged to be suitable models to study the regulation of cysteinyl leukotriene biosynthesis.

The identification of a putative PKC consensus sequence in the N-terminal amino acid residues of LTC_4 synthase prompted this study which demonstrated that when neutrophilic or eosinophilic HL-60 cells were challenged with A23187 in the presence of PMA, the biosynthesis of LTC_4 and its metabolite LTD_4 where specifically attenuated whereas biosynthesis of the non-cysteinyl leukotrienes, LTA_4 and LTB_4, were not affected (chapter 7 (ref.332),chapter 8 (ref.333)). There has been recent interest in the communication between tyrosine kinases and serine/threonine kinases in signal transduction pathways (334,335). It has been postulated that PKC can trigger a cascade of events involving the activation of both tyrosine kinases (336) and MPK (17,335). Furthermore, in human basophils, IL-1-dependent LTC_4 biosynthesis has been suggested to be regulated in an antagonistic manner by tyrosine kinases (TK) and PKC. This study demonstrated that IL-3-dependent LTC_4 biosynthesis was activated by TK and inhibited by PKC. These observations were further supported by the ability of staurosporine, a

specific PKC inhibitor, to reverse the effects of PMA-mediated activation of PKC (337). Thus, we investigated the involvement of other kinases in the regulation of LTC_4 biosynthesis with the use of selective inhibitors. The PMA-mediated inhibition of cysteinyl leukotriene biosynthesis was prevented only by PKC-specific inhibitors but not by any other kinase inhibitors (PKA, TK) excluding their involvement in this regulatory mechanism.

Eosinophils preferentially metabolize arachidonic acid to LTC_4 (323-326), and have been implicated to have a role in the pathogenesis of allergic diseases such as bronchial asthma. They release cytotoxic mediators including major basic protein, eosinophil cationic protein and eosinophil peroxidase which both damage the respiratory epithelium and trigger bronchial hyper-responsiveness. Lipid mediators including platelet activating factor, prostaglandins and leukotrienes are released by eosinophils and have been shown to involved in the physiological changes detected in asthmatic patients (for review see 338). Furthermore, hypodense eosinophils, which produce greater quantities of LTC_4 than normodense cells, are found in increased numbers in asthmatic patients (339,340). Moreover, since only cysteinyl leukotriene biosynthesis was affected by PMA-mediated activation of PKC in this study, LTC_4 synthase which is the rate-limiting enzyme for the biosynthesis of these potent mediators and which contains a potential PKC consensus phosphorylation site, was the most likely candidate for being the target of PKC. Thus an eosinophilic HL-60 cell line which has high levels of LTC_4 synthase specific activity (Nicholson, D.W. et al., unpublished data) was determined to be the ideal model cell line to elucidate this regulatory mechanism.

Although the ionophore-challenged whole cell study did demonstrate specific inhibition of cysteinyl leukotriene biosynthesis, these results were complicated by the fact that it has clearly been demonstrated that PLA_2 is

phosphorylated and activated by MPK (17,26). Since PKC can activate MPK, a model to study the phosphoregulation of cysteinyl leukotriene biosynthesis had to be designed such that the phosphoregulation of PLA_2 could be bypassed. This was achieved by providing eosinophilic HL-60 cells with exogenous LTC_4 synthase substrates (LTA_4 and reduced GSH). These studies clearly demonstrated that the inhibitory effects of PKC activation were due to inhibition of the LTC_4 synthase enzyme itself and not upstream enzymes in the biosynthetic pathway (chapter 8 (ref.333)). Kinetic analysis of LTC_4 synthase biosynthetic activity indicated that phosphorylation of LTC_4 synthase or a putative regulator of its activity causes a specific decrease in the V_{max} of the enzyme without any effect on the K_m, and thus demonstrates non-competitive inhibition which is typical for a phosphoregulatory mechanism (chapter 8 (ref.333)). The whole-cell model has certain disadvantages for studying phosphoregulatory mechanisms since we cannot assess whether PKC directly phosphorylates LTC_4 synthase or whether this inhibition is mediated via an intermediate protein. Although the presence of a PKC consensus sequence in the amino terminus of the LTC_4 synthase polypeptide supports a direct role for PKC in the modulation of its activity (chapter 6 (ref.148)), the specific mechanism by which this occurs will require recombinant LTC_4 synthase or specific antibodies neither of which are available as yet.

Contrary to the effect on cysteinyl leukotriene biosynthesis, the formation of PGE_2 and TXB_2 was elevated 2 to 3-fold following PMA treatment and this too was prevented by staurosporine (chapter 8 (ref.333)). Recent evidence in the literature examining the effects of PKC activation on prostanoid biosynthesis in several cell types supports this observation (317, 341-344). Based on these studies, a model was proposed for the regulation of eicosanoid biosynthesis in eosinophils (chapter 8 (ref.333)). Protein kinase C can be activated by receptor-mediated events or by phorbol esters (for review see 345-347). The activated

form of PKC then translocates to the membrane where it can trigger several events that result in the inhibition of cysteinyl leukotriene biosynthesis but the elevation of prostanoid biosynthesis. Activated PKC can stimulate the release of arachidonic acid by stimulating $cPLA_2$ either directly or via MPK (17,26,27). Transcription of the inducible form of the cyclooxygenase gene (COX-2) can also be stimulated by a PKC-dependent mechanism (315). The higher levels of COX-2 will accommodate the enlarged pool of arachidonic acid. Simultaneously, there is an attenuation of cysteinyl leukotriene biosynthesis (chapter 8 (ref.333)), thus the net effect of PKC activation would be a shift in the profile of mediators synthesized by the eosinophil from leukotrienes to prostanoids.

The physiological significance of this regulatory mechanism, particularly with respect to the pathophysiological changes observed in bronchial asthma, can be seen in light of the often opposing actions of leukotrienes and prostaglandins *in vivo*. As described previously (cf. section 1.7) the leukotrienes are important mediators of human bronchial asthma. The role of prostaglandins, on the other hand, has been questioned since non-steroidal anti-inflammatory drugs (NSAIDS) offer little benefit to most asthmatic patients, and moreover, 5-10% of asthmatics actually demonstrate exacerbation of their symptoms after the administration of aspirin or related NSAIDS (348). However, there has been considerable interest in the potential beneficial effects of the bronchodilator prostaglandins (PGE_2 and PGI_2) in asthma. Asthmatic subjects demonstrate exercise-refractoriness (a reduced bronchoconstrictor response to repeated exercise challenge) that can be prevented by treatment with indomethacin (349,350). This observation supported the possible release of inhibitory or bronchodilatory prostaglandins as a possible mechanism of this refractoriness. The release of PGE from human lung (351,352) and from human and guinea-pig airway smooth muscle (355,154) has been reported and thus may be important in the local modulation of bronchial

tone. Inhaled PGE_1 and PGE_2 can cause bronchodilation in humans (355,356) and oral PGE_1 has been shown to reduce airway responsiveness to inhaled histamine and cholinergic agonists in mild asthmatic patients (357).

With respect to the production of leukotrienes, there is some evidence that demonstrates that prostaglandins, particularly of the E type, potently inhibit histamine and leukotriene release from human leukocytes and sensitized lung tissue (358,359) and this effect has been correlated with their ability to increase cAMP levels (360). Furthermore, these effects were not mediated via β-receptor activation since propanolol did not inhibit these effects. The effects of catecholamines on eicosanoid biosynthesis has also been investigated (for review see 361) and data has suggested that catecholamines can increase prostanoid formation while they decrease leukotriene biosynthesis in human polymorphonuclear cells and whole blood (362,363). However, these effects are not receptor-mediated but are due to the phenolic structure of catecholamines which stimulates COX by functioning as a peroxide co-substrate and which inhibits lipoxygenases by reducing the enzyme iron (361).

The change in the ratio of prostaglandins and leukotrienes synthesized may be of clinical significance in asthma, gastric ulcerations, and psoriasis where decreased prostaglandin/leukotriene ratios have been reported. Platelets and macrophages from asthmatic subjects show a decrease in COX metabolites and an increase in lipoxygenase products when compared with platelets and macrophages of normal subjects (365-366). In gastric ulcers, prostanoids, in particular, PGE_2, are cytoprotective while leukotrienes may be pro-ulcerogenic (367,368). Similarly, decreased prostanoid and increased leukotriene production has also been reported in psoriatic lesions (369,370). Thus, the identification of factors or mechanisms which physiologically regulate the balance of COX and lipoxygenase products is of importance.

Airway hyper-responsiveness, is one of the key features of bronchial asthma and it has been postulated, based on evidence from a canine model of ozone-induced responsiveness, that this phenomenon can be caused by inhibition of PGE_2 and PGI_2 production and stimulation of TXA_2 or leukotriene production (371). This study demonstrated that PGE_2 and PGI_2, which they believe are quantitatively the most important COX products in inflammation, reduce or abolish acetylcholine-mediated excitatory junction potentials and contraction to nerve stimulation by inhibiting the release of acetylcholine in canine trachea and bronchi (372,373). Furthermore, it has been suggested that PGE_2 and PGI_2 may serve as physiological antagonists of bronchoconstrictive mediators such as histamine and leukotrienes since their constrictive effects are potentiated if the synthesis of prostaglandins is inhibited (371,374,375). Based on these observations it has been hypothesized that bronchoconstrictive mediators may promote the release of PGE_2 and PGI_2 in a kind of homeostatic mechanism such that if prostanoid biosynthesis is inhibited their effects are unmasked or potentiated (371).

Exercise, inhaled methacholine, and inhaled histamine are potent airway constrictor stimuli in asthmatic subjects and it is believed that the release of inhibitory prostaglandins are responsible for exercise-refractoriness and methacholine and histamine tachyphylaxis (376-378). Methacholine inhalation tests in particular are often used to monitor airway responsiveness in the investigation of asthma (379). Exercise-refractoriness has been demonstrated in asthmatic subjects and can be inhibited by pretreatment with indomethacin (349,350) suggesting a role for inhibitory prostaglandins in this mechanism. The observation that LTD_4 receptor antagonists inhibit exercise-induced bronchoconstriction in asthmatic subjects has implicated LTD_4 as a mediator responsible in part for exercise-induced bronchoconstriction (293). Recently it

has been demonstrated that exercise refractoriness is also caused by LTD_4 (378). Manning and co-workers demonstrated that exercise refractoriness and LTD_4 tachyphylaxis exist in the same subjects, and that the magnitude of protection afforded by exercise correlates with that afforded by LTD_4 (378). In addition, cross refractoriness between the two stimuli was also demonstrated and all these effects were attenuated by flurbiprofen, a COX inhibitor (378). These results demonstrated that the release of inhibitory prostaglandins is in fact the protective mechanism (378). The observation that LTD_4 can stimulate the release of prostaglandins, *in vivo*, is of particular significance with respect to the model proposed for the regulation of eicosanoid biosynthesis (chapter 8 (ref.333)). The mechanism by which LTD_4 stimulates prostaglandin biosynthesis can be elucidated based on the fact that LTD_4, as do all leukotrienes, stimulates PLC and the subsequent production of IP_3 and diacylglycerol upon interaction with its receptor (cf. sections 1.6.2, 1.6.5 and 1.6.6). These mediators in turn activate PKC which, according to the mechanism proposed here, would stimulate prostanoid biosynthesis and inhibit cysteinyl leukotriene biosynthesis. Thus this model may be the basis of a negative feedback mechanism by which potent bronchoconstrictive mediators such as LTD_4 can downregulate not only their own production but can concomitantly stimulate the production of bronchodilatory mediators to counteract their physiological actions.

Furthermore, it has also been demonstrated that the initial response to exercise or inhaled methacholine or histamine is not influenced by pretreatment with indomethacin or flurbiprofen. This observation suggests that inhibitory prostaglandins are not continuously present in human airways and that a bronchoconstrictive stimulus is required before inhibitory prostaglandins are released (376,377,380). Thus it has been postulated that human airways have the

capacity to protect against repeated bronchoconstrictive stimuli through the release of inhibitory prostaglandins (377).

The leukotrienes and prostaglandins are potent inflammatory mediators whose actions are clearly important in both physiological and pathophysiological conditions. Taken together, these studies demonstrate that the elucidation of mechanisms regulating not only their production but also the balance of prostanoids and leukotrienes formed is of great importance. A defect in this mechanism may in part be responsible for the pathophysiological changes observed in diseases such as bronchial asthma.

Further investigations of the mechanisms regulating leukotriene biosynthesis, in particular, LTC_4 synthase activity, are necessary. These studies are largely dependent on the availability of a specific antibody and a cDNA clone for the enzyme and attempts to obtain these tools are in progress. Future work will entail defining several aspects of LTC_4 synthase including 1) definition of its gene structure and 5' regulatory region; 2) identification of post-translational modifications; 3) identification of factors that modulate transcription or translation of the enzyme; 4) expression of the recombinant enzyme in the various available eukaryotic expression systems, which will provide large quantities of the enzyme that can then be used to identify specific inhibitors for therapeutic purposes; 5) further analysis of the phosphoregulatory mechanism with the use of specific antibodies to identify the putative phosphorylated enzyme or regulatory protein; in addition, if LTC_4 synthase is directly phosphorylated, mutational analysis of the enzyme can also be used to identify potential phosphorylation sites.

The purification and characterization of LTC_4 synthase is not only an important advancement for the development of novel therapeutic drugs to attenuate cysteinyl leukotriene biosynthesis but will also further our understanding of both the physiological and pathophysiological role of these inflammatory

mediators *in vivo*. Furthermore the mechanisms regulating the profile of eicosanoids formed under inflammatory conditions may be key to elucidating the biochemical basis of various disease states.

10. ORIGINAL CONTRIBUTIONS TO THE LITERATURE

This thesis demonstrates for the first time that:

1. Human leukotriene C_4 synthase is a unique enzyme, distinct from all known glutathione S-transferases.

2. An 18 kDa microsomal membrane polypeptide that can be specifically photolabelled in human myelocytic cell lines (U937 and THP-1) is a likely candidate for being LTC_4 synthase.

3. Human LTC_4 synthase, which was purified to homogeneity from THP-1 cells, is a novel homodimeric GST composed of 18 kDa subunits. The N-terminal 35 amino acid sequence determined for LTC_4 synthase is unique and contains a consensus sequence for protein kinase C phosphorylation.

4. Cysteinyl leukotriene biosynthesis can be specifically inhibited by phorbol ester-mediated activation of protein kinase C in neutrophilic and eosinophilic HL-60 cells.

5. The effect of phorbol ester-mediated activation of protein kinase C on cysteinyl leukotriene in eosinophilic HL-60 and THP-1 cells is due to non-competitive inhibition of LTC_4 synthase activity and does not involve any other kinases including protein kinase A or tyrosine kinase.

6. Concomitant with attenuation of cysteinyl leukotriene biosynthesis there is an upregulation of prostanoid biosynthesis. Eicosanoid biosynthesis is regulated by protein kinase C such that the activation of this kinase shifts the profile of lipid mediators formed by inflammatory cells from leukotrienes to prostanoids.

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