



Western Michigan University
ScholarWorks at WMU

Master's Theses

Graduate College

12-1995

Regulation of Leukotriene C4 Biosynthesis in Calcium Ionophore A23187-Challenged August Rat Peritoneal Leukocytes

James M. Huebner

Follow this and additional works at: https://scholarworks.wmich.edu/masters_theses



Part of the Biology Commons

Recommended Citation

Huebner, James M., "Regulation of Leukotriene C4 Biosynthesis in Calcium Ionophore A23187-Challenged August Rat Peritoneal Leukocytes" (1995). *Master's Theses*. 4503.

https://scholarworks.wmich.edu/masters_theses/4503

This Masters Thesis-Open Access is brought to you for free and open access by the Graduate College at ScholarWorks at WMU. It has been accepted for inclusion in Master's Theses by an authorized administrator of ScholarWorks at WMU. For more information, please contact wmu-scholarworks@wmich.edu.



REGULATION OF LEUKOTRIENE C₄ BIOSYNTHESIS IN CALCIUM
IONOPHORE A23187-CHALLENGED AUGUST RAT
PERITONEAL LEUKOCYTES

by

James M. Huebner

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Biological Sciences

Western Michigan University
Kalamazoo, Michigan
December 1995

ACKNOWLEDGMENTS

There are many people without whose assistance this paper would not be possible. First, I would like to thank my graduate advisor, committee chairperson and friend, Dr. Leonard J. Beuving for his guidance and encouragement both on a professional and personal level. I am also particularly grateful to the remainder of my committee, Dr. William F. Jackson, Dr. Charles D. Mackenzie, and Robert R. Eversole for their counsel, insight and invaluable recommendations concerning various aspects of this project.

The following people generously contributed time, laboratory space, equipment and/or supplies: Dr. Kevin L. Blair, Randy Enciso, Dr. Karim Essani, Ginny Garlock, Dr. Leonard C. Ginsberg, B. Will Green, Marcia Hurley, Mark Lombard, Dr. Stephen B. Malcom, John Phipps, Dr. David S. Reinhold, Dr. Donald R. Schreiber, Celene Spangler, and Dr. Frank F. Sun and Bruce Taylor of The Upjohn Company. A special thanks goes to Dr. Susan R. Stapleton who donated several hours of her own time, discussing much of the work contained herein.

I am grateful for the financial support provided by the Biological Imaging Center, the Department of Biological Sciences, The Graduate College, and the Center for Research in Environmental Signal Transduction at Western Michigan University.

Acknowledgments-Continued

Finally, I wish to dedicate this thesis to my wife Linda for her unflagging belief in me and her selfless emotional and financial support throughout my college career. She wanted this for me more than anyone.

James M. Huebner

REGULATION OF LEUKOTRIENE C₄ BIOSYNTHESIS IN CALCIUM
IONOPHORE A23187-CHALLENGED AUGUST RAT
PERITONEAL LEUKOCYTES

James M. Huebner, M.S.

Western Michigan University, 1995

Leukotriene (LT) C₄ is a potent myotropic product of arachidonic acid 5-lipoxygenation that is thought to play a role in the pathophysiology of immediate hypersensitivity reactions and inflammation. We designed the present study to evaluate the capacity of August (AUG) rat mixed peritoneal leukocytes to generate LTC₄ when challenged with different concentrations of the Ca²⁺ ionophore A23187 and to test the hypothesis that LTC₄ biosynthesis is regulated via a protein kinase C (PKC)-dependent mechanism. The AUG rat, which has a spontaneously high number of peritoneal eosinophils that can be harvested by a simple lavage procedure, is a potentially important animal model of eosinophilia that has not been well characterized. Our findings indicate that A23187-stimulated LTC₄ biosynthesis is 5-lipoxygenase activating protein (FLAP) inhibitor-sensitive and is both Ca²⁺/Mg²⁺- and concentration-dependent. Furthermore, LTC₄ production appears to be regulated antagonistically via serine/threonine- and tyrosine-specific phosphorylation mechanisms.

TABLE OF CONTENTS

ACKNOWLEDGMENTS.....	ii
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
INTRODUCTION.....	1
Cellular Formation of the Leukotrienes.....	2
Is There a Role for Protein Kinase C Activation in the Regulation of Cysteinyl Leukotriene Biosynthesis?.....	7
MATERIALS AND METHODS.....	11
Animals.....	11
Reagents.....	11
Peritoneal Leukocyte Isolations.....	12
Cell Counts.....	13
Incubation Conditions.....	13
Assay of LTC ₄ Activity.....	14
Statistics.....	15
RESULTS.....	17
DISCUSSION.....	23
APPENDICES	
A. IACUC Approval Form.....	30

Table of Contents-Continued

BIBLIOGRAPHY.....	32
-------------------	----

LIST OF TABLES

1. Ca^{2+} , Mg^{2+} and 5-Lipoxygenase Activating Protein (FLAP) Activity Are Essential for Ca^{2+} Ionophore A23187-Stimulated LTC_4 Synthesis..... 19

LIST OF FIGURES

1. 5-Lipoxygenase Pathway of Leukotriene Biosynthesis.....	3
2. The Classical Pathway of PKC Activation.....	8
3. Calcium Ionophore A23187 Stimulates LTC ₄ Synthesis in a Dose-Dependent Manner.....	18
4. LTC ₄ Biosynthesis is Attenuated by PKC Activation and PTK Inhibition in A23187-Challenged AUG Rat Mixed Peritoneal Leukocytes.....	20

INTRODUCTION

The functional repertoire of both infiltrating and *in situ* inflammatory cells includes the biosynthesis and release of leukotrienes (LTs) derived from the 5-lipoxygenation of arachidonic acid (AA; 20:4). The pharmacological activities of these compounds, their relative potencies, and their prominence in biological fluids and inflammatory exudates suggest a role in the pathophysiology of human bronchial asthma and other immediate hypersensitivity reactions (Lam & Austen, 1992). Functionally, the LTs can be divided into two classes: (1) the dihydroxy acid LTB₄, which is a powerful chemotactic, aggregative, and chemokinetic agent (Ford-Hutchinson, Bray, Doig, Shipley, & Smith, 1980); and (2) the cysteinyl-containing LTs C₄, D₄, and E₄; collectively termed the "slow-reacting substance of anaphylaxis" or SRS-A (Samuelsson, 1983). Whereas LTB₄ has relatively few myotropic activities (Piper, 1984), the C₆ amino acid-substituted cysteinyl LTs are potent contractors of both vascular and non-vascular smooth muscle. Specifically, these compounds can evoke bronchospasm and mucus secretion in human airway, increase both arteriolar constriction and microvascular permeability, contract gastrointestinal smooth muscle, reduce myocardial contractility and coronary blood flow, and can induce prolonged hypotension and shock when injected systematically (see reviews; Hammarström, 1983; Samuelsson, 1983; Lewis & Austen, 1984; Piper, 1984; Samuelsson, Dahlén, Lindgren, Rouzer, & Serhan, 1987; Lam & Austen, 1992).

Cellular Formation of the Cysteinyl Leukotrienes

LT biosynthesis first requires the phospholipase-catalyzed liberation of AA from esterified stores in membrane glycerophospholipids (Irvine, 1982). Free AA is enzymatically transformed to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) via the stereospecific insertion of molecular oxygen at C5 (Panossian, Hamberg, & Samuelsson, 1982), and subsequently dehydrated to the epoxide 5,6-oxido-7,9,11,14-eicosatetraenoic acid (LTA₄) (Maas, Ingram, Taber, Oates, & Brash, 1982) (Figure 1). Both reactions proceed in intact cells through the catalytic action of 5-lipoxygenase (5-LO; EC 1.13.11.34) (Rouzer, Matsumoto, & Samuelsson, 1986), a single, iron-containing protein (Rådmark et al., 1995), which translocates from the cytosol to the nuclear membrane upon activation (Woods et al., 1993). In human leukocytes, optimal enzymatic activity requires a complex series of stimulatory factors including Ca²⁺, ATP, phospholipid and lipid hydroperoxide (reviewed in DeWolf, 1991; Rouzer, 1991; Wong & Crooke, 1991); unfortunately, the specific properties contributed by these agents remain largely undetermined. Another cofactor has been identified from neutrophil cell extracts by photoaffinity labelling and affinity chromatography with MK-886 {3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid} (Miller et al., 1990; see also Gillard et al., 1989), a selective inhibitor of its activity. Current evidence suggests that this novel 18 kDa polypeptide, termed 5-LO-activating protein (FLAP) (Dixon et al.,

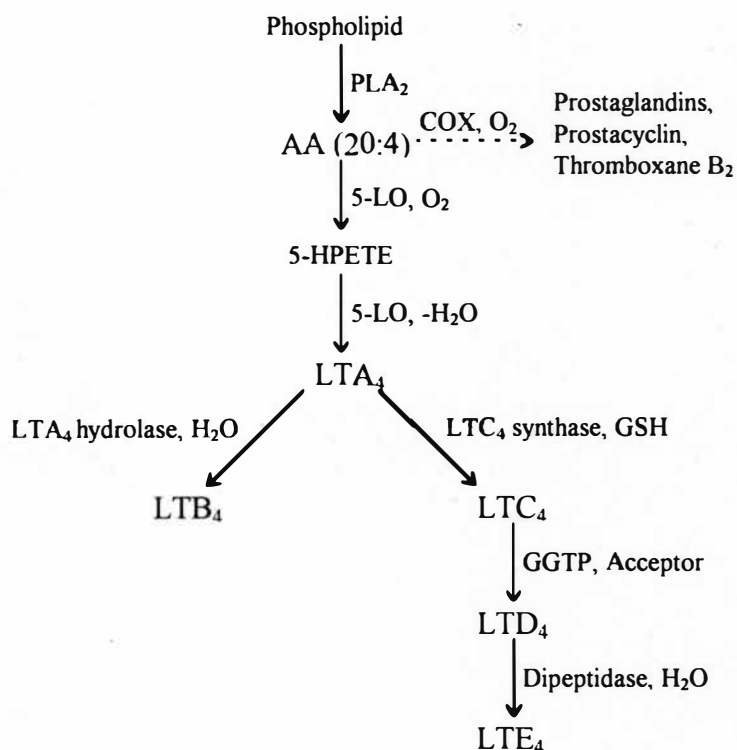


Figure 1. 5-Lipoxygenase Pathway of Leukotriene Biosynthesis.

PLA₂, phospholipase A₂; AA, arachidonic acid; COX, cyclooxygenase; 5-LO, 5-lipoxygenase; GSH, glutathione; GGTP; γ -glutamyl transpeptidase.

1990; Miller et al., 1990), facilitates the presentation of free AA to 5-LO (Ford-Hutchinson, 1994; Miller et al., 1990), thereby increasing the efficiency of epoxide formation while having no direct effect on subcellular 5-LO activity or AA release.

The labile epoxide LTA₄ can be hydrolyzed both enzymatically to 5-S-12R-dihydroxy-6,14,8,10-eicosatetraenoic acid (LTB₄) (Borgeat & Samuelsson, 1979) and non-enzymatically to biologically inactive 6-*trans*-LTB₄ diastereoisomers and small quantities of 5,6-dihydroxy-eicosatetraenoic acids (5,6-DHETEs) (Rådmark, Malmsten, Samuelsson, Goto, Marfat, & Corey, 1980). Alternatively, LTA₄ can be conjugated to 5S-12R-dihydroxy-6R-S-glutathionyl-7,9,11,14-eicosatetraenoic acid (LTC₄) via the enzymatic adduction of a reduced glutathione (GSH) to the epoxide C6 (Rådmark, Malmsten, & Samuelsson, 1980). This reaction is catalyzed by a distinctive membrane-localized GSH-S-transferase (LTC₄ synthase; EC 2.5.1.37), specifically dedicated to the conversion of LTA₄ *in vivo* (Nicholson, Ali, Klemba, Munday, Zamboni, & Ford-Hutchinson, 1992). Human LTC₄ synthase, a homodimer composed of two identical 18 kDa subunits, has recently been purified to homogeneity from the monocytic leukemia cell line THP-1 (Nicholson et al., 1993) and its cDNA cloned from THP-1 and human myelocytic KG-1 cell cDNA expression libraries (Lam, Penrose, & Austen, 1995; Welsch, Creely, Mathis, Hauser, & Isakson, 1995). Interestingly, analysis of the predicted amino acid sequence indicate two putative Ser-Ala-Arg consensus sequences for protein kinase C phosphorylation (Lam et al., 1995; Welsch et al., 1995; Nicholson et al., 1993), suggesting that the enzyme may be phosphoregulated *in vivo* (see below). This hypothesis may be of

considerable significance, given that the conjugation of LTA_4 is the first committed step in the biosynthesis of all cysteinyl LTs, and thus in determining the extracellular profile of these mediators in the acute and chronic inflammatory responses with which they are associated.

However, not all cells that have the capability to synthesize LTC_4 necessarily modify the tripeptide side chain to form the lytic derivatives of this parent compound (Piper, 1984). For example, human eosinophils stimulated with Ca^{2+} ionophore A23187 generate LTC_4 as their sole cysteinyl LT (Weller, Lee, Foster, Corey, Lewis, & Austen, 1983). A23187-stimulated murine mastocytoma cells (Murphy, Hammarström, & Samuelsson, 1979), differentiated murine bone-marrow cells (Razin, Mencia-Huerta, Lewis, Corey & Austen, 1983), *N*-formyl-Met-Leu-Phe (FMLP)-stimulated guinea pig lung parenchymal strips (Shore, Stimler-Gerard, Smith, & Drazen, 1987) and zymosan-stimulated murine peritoneal and pulmonary macrophages (Rouzer, Scott, Hamill, & Cohn, 1982; Rouzer, Scott, Cohn, Blackburn, & Manning, 1980) also do not appear to significantly convert LTC_4 *in vitro*. In other cells and tissues, particularly those with secretory and absorptive functions (Meister & Anderson, 1983), LTC_4 undergoes further processing by γ -glutamyl transpeptidase (GGTP; EC 2.3.2.2), yielding its cysteinylglycyl derivative, 5S-hydroxy-6R-S-cysteinylglycyl-7,9,11,14-eicosatetraenoic acid (LTD_4) (Örming, Hammarström, & Samuelsson, 1980). GGTP is a widely-distributed membrane-bound ectoenzyme which catalyzes the transfer of a γ -glutamyl moiety to amino acids, dipeptides, GSH

(i.e., autotranspeptidation) or water (Meister & Anderson, 1983; Allison, 1985; Tate & Meister, 1985; Hammarström, Örning, & Bernström, 1986). In the bioconversion of LTC₄ to LTD₄, amino acids are the preferred γ -glutamyl acceptors (Hammarström et al., 1986). The reaction is also readily reversible; using GSH as a substrate, GGTP can catalyze the addition of a γ -glutamyl residue to the amino group of LTD₄, thus driving the formation of LTC₄ (Hammarström, 1981; 1983).

Hydrolysis of the remaining peptide bond in LTD₄ results in elimination of the C-terminal glycine residue and yields the cysteinyl derivative, 5S-hydroxy-6R-S-cysteinyl-7,9,11,14-eicosatetraenoic acid (LTE₄) (Bernström & Hammarström, 1981). This reaction is catalyzed by a ubiquitous membrane-associated dipeptidase (E.C. 3.4.13.-) (Bernström & Hammarström, 1981; Sok, Pai, Atrache, Kang, & Sih, 1981; Anderson, Allison, & Meister, 1982), an activity which cleaves several cysteinylglycine and S-substituted cysteinylglycine substrates into their component amino acids (Anderson et al., 1982; Tate, 1985). A further γ -glutamyl derivative, 5S-hydroxy-6R-S- γ -glutamylcysteine-7,9,11,15-eicosatetraenoic acid (LTF₄), has also been generated by incubating LTE₄ with GGTP and GSH (Anderson et al., 1982; Bernström & Hammarström, 1982) and, more recently, by incubating LTC₄ with bovine pancreas carboxypeptidase A (Reddenna, Whelan, & Reddy, 1988). However, LTE₄ is probably a more significant metabolite *in vivo*; LTE₄, *N*-acetyl LTE₄ and *N*-acetyl-11-*trans*-LTE₄ have long been recognized as biliary/fecal or urine products of

endogenous cysteinyl LT metabolism in rats and primates respectively (reviewed in Hammarström, Örning, & Keppler, 1988; Hammarström et al., 1986).

Is There a Role for Protein Kinase C Activation in the Regulation of Cysteinyl Leukotriene Biosynthesis?

In the now classical pathway for transducing extracellular signals into cells, agonist-induced hydrolysis of membrane inositol phospholipids results in the transient formation of 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) (Nishizuka, 1986). Under normal conditions, DAG can induce the activation of Ca²⁺/phospholipid-dependent protein kinase C (PKC; E.C. 2.7.1.-) (Figure 2), which in turn leads to the phosphorylation of specific target substrates (i.e., serine and threonine residues), including cytoskeletal and contractile proteins, ion channels and transporters, cell surface receptors, and a variety of soluble proteins (Nishizuka, 1986). Tumor-promoting phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), can also substitute for DAG, activating PKC directly without initiating the hydrolysis of phosphatidylinositol (Castagna, Takai, Kaibuchi, Sano, Kikkawa, & Nishizuka, 1982).

Under the appropriate conditions, it now appears that PKC activation frequently synergizes with Ca²⁺ mobilization to enhance certain cellular events (Nishizuka, 1984; 1986; 1992), a phenomenon that can be demonstrated *in vitro* by the selective application of a permeable DAG or phorbol diester and a ligand capable of circumventing Ca²⁺ gating mechanisms (Reed & Lardy, 1972), such as the Ca²⁺

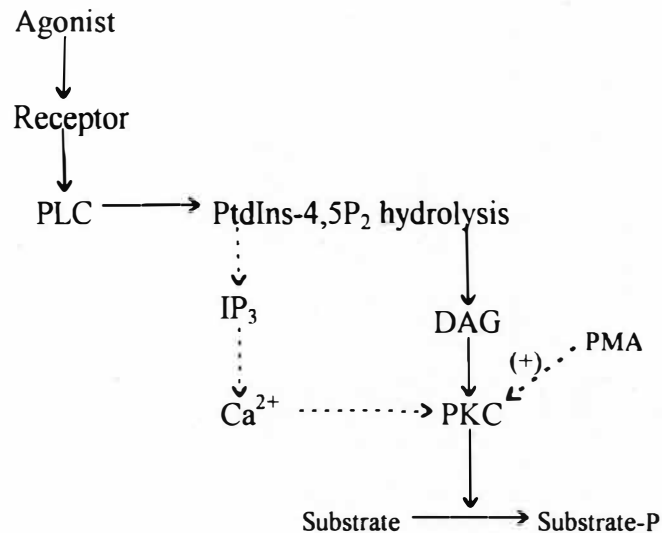


Figure 2. The Classical Pathway of PKC Activation.

PLC, phospholipase C; PtdIns-4,5P_2, phosphatidylinositol 4,5 bisphosphate; DAG, diacylglycerol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate. Adapted from Parker, P.J. (1994). Protein kinase C: history and perspectives. In J.F. Kuo (Ed.), Protein Kinase C, (p. 4). New York: Oxford University Press.

ionophore A23187. For example, previous studies have shown that PMA acts synergistically with A23187 to enhance the formation of LTs in human eosinophils (Tamura, Agrawal, & Townley, 1988), human neutrophils (McColl, Hurst, & Cleland, 1986; Liles, Meier, & Henderson, 1987; McIntyre, Reinhold, Prescott, & Zimmerman, 1987) and murine macrophages (Humes, 1988; Tripp, Mahoney, & Needleman, 1985). It has been envisaged that the simultaneous application of these agonists may mimic the cellular events responsible for the release of free AA and/or the activation of 5-LO as induced by physiologic stimuli (Liles et al., 1987). In fact, there is evidence to suggest that the phosphorylation of specific target proteins by PKC may intensify the A23187-stimulated release of AA in several cell types (Halenda, Zavoico, & Feinstein, 1985; Peters-Golden, McNish, Sporn, & Balazovich, 1991) as well as the biosynthetic activity of AA-selective phospholipase A₂ (PLA₂; Lin, Lin, & Knopf, 1992; Lin, Wartmann, Lin, Knopf, Seth, & Davis, 1993).

In contrast, it has recently been demonstrated in human basophils that both PMA and inhibitors of protein tyrosine kinase activity can effectively block the IL-3-dependent formation of LTC₄, indicating that while the IL-3 signaling pathways and LTC₄ production in these cells may depend on tyrosine phosphorylation, it does not require the activation of PKC (Krieger, von Tscherner, & Dahinden, 1992). Similarly, other laboratories have shown that co-stimulation with PMA specifically attenuates LTC₄ biosynthesis in differentiated human promyelocytic HL-60 cells challenged with ionophore or with saturating concentrations of exogenous LTA₄ (Ali, Ford-Hutchinson, & Nicholson, 1994; Kargman, Ali, Vaillancourt, Evans, &

Nicholson, 1994).

We designed the present study to evaluate the capacity of August (AUG) rat mixed peritoneal leukocytes to generate LTC₄ when challenged with different concentrations of A23187 and to test the hypothesis that LTC₄ biosynthesis in this model is regulated via a serine/threonine-specific phosphorylation mechanism. The AUG rat, which has a spontaneously high number of peritoneal eosinophils (Spry, 1988) that can be harvested by a simple lavage procedure (Mackenzie, Jungery, Taylor, & Ogilvie, 1981), is a potentially important animal model of eosinophilia that has not been well characterized. Parasite-independent, non-induced examples of eosinophilia may prove particularly useful since both quantitative and qualitative differences exist between different preparations of eosinophils depending on the method of induction (Cook, Musgrove, & Ashworth, 1987).

MATERIALS AND METHODS

Animals

Eleven healthy adult male AUG rats (Harlan/Olac, UK) aged 27-35 wks were used in this study. Animals were housed at Western Michigan University (Kalamazoo, MI) (68-72°C; 12 h-12 h light-dark cycles) in clear polycarbonate cages and in accordance with the Institutional Animal Care and Use Committee (IACUC) at this institution (Appendix A). Rodent chow #5001 (Purina Mills, St. Louis, MO) and RO-purified water were provided *ad libitum*.

Reagents

Except as noted below, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). MK-886 was a gift from Merck Frosst Canada (Montreal, Canada). H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine] was purchased from Toronto Research Chemicals, Inc. (Ontario, Canada). Chelerythrine chloride and genestein (4',5,7-trihydroxyisoflavone) were from LC Laboratories (Woburn, MA). The cytospin cell preparations were stained using Diff-Quik[®] Stain Sets from Baxter Scientific Products (McGraw Park, IL). Stock solutions of agonists and inhibitors were prepared in dimethyl sulfoxide (DMSO) (stock concentrations in mM: A23187,

10; MK-886, 10; PMA, 8.1; chelerythrine chloride, 25; H-7, 5; genestein, 25) and stored at -20 °C. When necessary, stocks were serially diluted immediately before use with a NaHCO₃-free Hanks' Balanced Salt Solution (HBSS) containing 2.4 mM Ca²⁺ and 1.6 mM Mg²⁺. For Ca²⁺/Mg²⁺-free experiments, the A23187 stock was diluted with a Modified HBSS (without NaHCO₃, CaCl₂, or MgSO₄). The final concentration of DMSO in the reaction mixture was never greater than 0.4 %. Huber, Trautmann, Becker, Schenck, Peskar, & Schepp (1993) found that similar concentrations of DMSO (0.37%) affected neither basal nor A23187-mediated LT release in Wistar rat isolated gastric mucosal cells.

Peritoneal Leukocyte Isolations

Rats were killed by carbon dioxide inhalation and a small ventral midline incision was made exposing the peritoneal cavity. The resident peritoneal cells were harvested by washing the cavity with 50 mL of Modified HBSS buffered with 20 mM HEPES at pH 7.3. The lavage solution was aspirated by syringe and centrifuged at 200 × g for 10 min at 4°C in a Beckman (Fullerton, CA) GS-6R refrigerated centrifuge. The supernatant was discarded and the pelleted cells were resuspended in 5 mL Modified HBSS and kept on ice for subsequent experimental procedures.

Cell Counts

An aliquot of the cell suspension was counted in a hemocytometer (American Optical, Buffalo, NY), excluding red blood cells (RBC's) and cell fragments. The cell density was adjusted to yield 3.0×10^6 cells/mL with Modified HBSS.

Cytocentrifuge slide preparations were used to determine differential cell counts in freshly isolated cell solutions. Briefly, 10 μ l of the cell suspension and 90 μ l 0.1 M phosphate buffer were cytocentrifuged (in duplicate) at 600 rpm for 8 min (Cytospin 3, Shandon Inc., Pittsburgh, PA). Air-dried slides were then fixed and stained with a Diff-Quik[®] Stain Set and counted using a Nikon (Tokyo, Japan) Microphot-FXA microscope.

Incubation Conditions

Counted cell suspensions from 2-3 animals were pooled for all experiments. To elicit LTC₄ biosynthesis, different concentrations of A23187 along with requisite volumes of HBSS (final concentrations of 1.2 mM Ca²⁺ and 0.8 mM Mg²⁺) were added to 0.5 mL aliquots of the pooled cell suspension to equal a final volume of 1 mL (1.5×10^6 cells). In Ca²⁺/Mg²⁺-free treatments, equal volumes of Modified HBSS were substituted for the control HBSS. The resulting reaction mixtures were incubated for 10 min (37°C, with constant agitation). Some preparations were co-incubated with 0.5 μ M A23187 and 0.1 μ M MK-886, a selective antagonist of LT

synthesis (Gillard et al., 1989). In other experiments, cells were stimulated with 0.5 μ M A23187 in the presence of 50 nM PMA and/or each of the following protein kinase inhibitors: (a) chelerythrine chloride (1.8 μ M), a benzophenanthridine alkaloid that is a putative highly specific PKC antagonist (Herbert, Augereau, Gleye, and Maffrand, 1990); (b) H-7 (6.0 μ M), a nonspecific PKC inhibitor; or (c) genestein (100 μ M), an isoflavone compound that is a selective antagonist of tyrosine kinase activity (Akiyama et al., 1987). The concentrations of the agonists and inhibitors used have been previously shown by other investigators to be in the effective range [Ca²⁺ ionophore A23187 (Lindgren, Hammarström, & Goetzl, 1983); MK-886 (Rouzer, Ford-Hutchinson, Morton, Gillard, 1990); PMA and genestein (Ali, Ford-Hutchinson, & Nicholson, 1994); chelerythrine chloride (Herbert et al., 1990); and H-7 (Kawamoto & Hidaka, 1984)]. All reactions were terminated by the addition of 3 mL ice-cold methanol and samples were subsequently stored at -70°C until examination.

Assay of LTC₄ Activity

The cell reaction mixtures were analyzed for LTC₄ content by acetylcholinesterase (AChE) enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI). All assay buffers and lyophilized reagents were reconstituted in ion-exchanged Millipore Milli-Q[®] purified water, 18 M Ω . To prepare for analysis,

samples were thawed and centrifuged at $1500 \times g$ for 10 min at 4°C . The resulting supernatants were then evaporated to dryness in a heated (approximately 50°C) water bath under a stream of nitrogen. Dried samples were reconstituted in 1 mL of assay buffer and stored overnight at 4°C . Each sample was assayed in duplicate. Briefly, assay buffer, LTC_4 standards, LTC_4 AChE tracer, antisera, and the reconstituted samples were aliquoted onto 96 well microtiter plates (pre-coated with mouse monoclonal anti-rabbit IgG) according to the manufacturer's instructions. The plates were covered with plastic film and incubated for 18 hr at room temperature. To measure the bound AChE activity, 200 μL of enzyme substrate (Ellman's reagent) was added to each well and the plates were developed in the dark on an orbital shaker (Orbit Microprocessor Shaker Bath, Lab-Line Instruments, Melrose Park, IL). Absorbance was read at 415 nm (Bio Rad Model 450 microplate reader, Richmond, CA) when the readings of the maximum binding wells (maximum tracer-to-antiserum binding in the absence of free LTC_4) reached at least 0.3 absorbance units (A.U.). Results were calculated from a standard curve plot of % displacement of bound tracer versus LTC_4 concentration (pg/mL).

Statistics

Standard curves were third or fourth-order regression curves ($r^2 \geq 0.95$) generated using Simga Plot[®] software (San Rafael, CA). All data are expressed as mean \pm SEM. Homogeneity of variance was confirmed by Hartley's test and a two-

tailed, unpaired Student's *t*-test or separate-variance *t*-test was used where appropriate (Ott, 1988). A *p* value < 0.05 was considered significant.

RESULTS

Cell preparations used in all experiments contained a mixture of macrophages ($45 \pm 1.0\%$), eosinophils ($36 \pm 0.8\%$), mast cells ($11 \pm 0.7\%$) and small lymphocytes ($8 \pm 0.5\%$) ($38.0 \pm 2.0 \times 10^6$ total cells/harvest, mean \pm SEM; range, 29.5×10^6 - 48.6×10^6).

Figure 3 shows the effect of various concentrations of calcium ionophore A23187 on the cellular generation of LTC₄. Stimulation with 0.5 - 10.0 μ M A23187 for 10 min at 37 °C produced a dose-dependent release with a maximum (5.2 ± 0.3 ng LTC₄/10⁶ cells) occurring at 5.0 μ M. In some preliminary experiments, the smallest detectable quantity of LTC₄ was limited by the conditions under which the cell supernatants were assayed. For example, in the absence of both Ca²⁺ and Mg²⁺ the stimulatory effects of A23187 were completely abolished (Table 1), generating LTC₄ levels that fell below the detection limit (all < approximately 0.03 ng/10⁶ cells; $n = 5$) when assayed at the same dilution as the control group (0.5 μ M A23187). Similarly, LT release was not observed in cells incubated in the absence of the ionophore stimulus ($n = 9$; data not shown).

As an additional test of the integrity of the LT biosynthetic pathway, we assessed the affects of MK-886, a potent FLAP antagonist (Rouzer, Ford-Hutchinson, Morton, & Gillard, 1990; Gillard et al., 1989), on the cellular generation of LTC₄. At

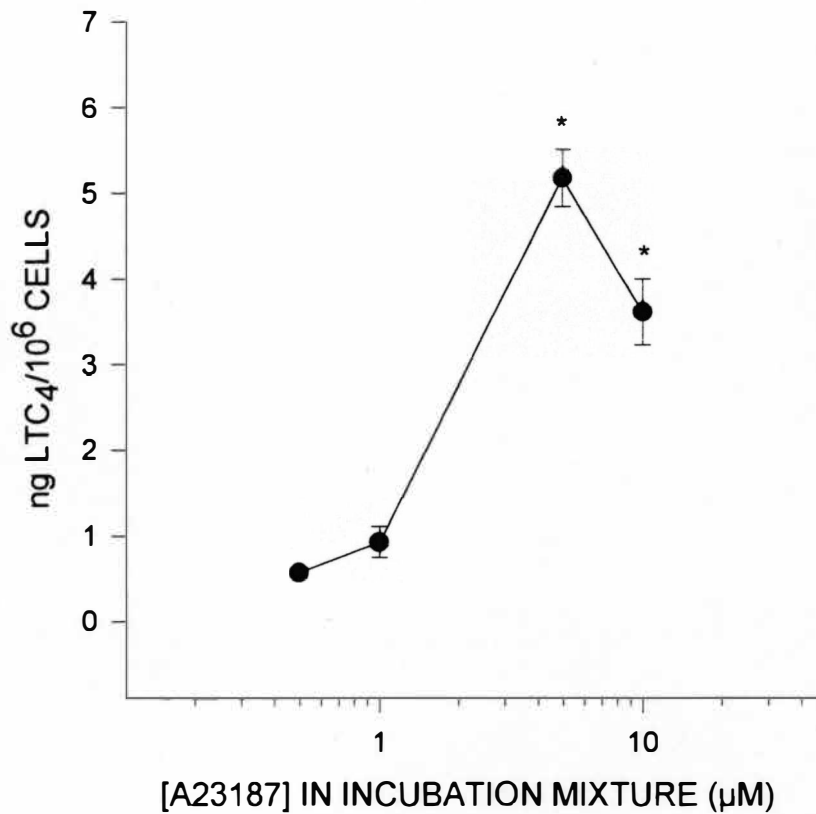


Figure 3. Calcium Ionophore A23187 Stimulates LTC₄ Synthesis in a Dose-Dependent Manner.

Aliquots (1.5×10^6 cells) of AUG rat mixed peritoneal leukocytes in HEPES buffered HBSS (containing 1.2 mM Ca²⁺ and 0.8 mM Mg²⁺, pH 7.3) were incubated with 0.5 - 10.0 μM A23187 in DMSO (maximum vehicle concentration ≤ 0.4 %) for 10 min at 37 °C. Reactions were terminated by the addition of methanol, and the levels of LTC₄ determined by EIA (Cayman Chemical, Ann Arbor, MI). Data are expressed as the mean of 4 - 14 duplicate experiments and are shown ± SEM.

*Significantly different from 0.5 μM A23187 treatment ($p < 0.05$).

0.1 μM , this agent significantly inhibited 0.5 μM A23187-stimulated LT synthesis by approximately 92 % ($p < 0.05$; $n = 6$) (Table 1).

Next, we investigated the possible regulatory role of PKC in the A23187-stimulated synthesis of LTC_4 by co-incubating some isolates with 50 nM PMA, a diterpene phorbol ester capable of directly activating PKC *in vitro* (Castagna et al., 1982). As evidenced in Figure 4 (column 1 vs. column 2), PMA significantly inhibited LTC_4 release in response to 0.5 μM A23187 by approximately 66% ($p < 0.05$; $n = 7$).

To confirm that activation of PKC was indeed responsible for the observed down-regulation of LTC_4 release in PMA-treated cells, we employed two functionally distinct inhibitors of PKC activity (Herbert et al., 1990; Hidaka, Inagaki, Kawamoto, & Sasaki, 1984). However, neither chelerythrine chloride (1.8 μM) nor H-7 (6.0 μM ; $n = 4$ for both groups) were able to abrogate the effects of PMA at the

Table 1

Ca^{2+} , Mg^{2+} and 5-Lipoxygenase Activating Protein (FLAP) Activity Are Essential for Ca^{2+} Ionophore A23187-Stimulated LTC_4 Synthesis

Treatment	1.2 mM Ca^{2+}	0.8 mM Mg^{2+}	pg $\text{LTC}_4/10^6$ Cells ^a
0.5 μM A23187	+	+	577.3 \pm 60.9 (14)
0.5 μM A23187	-	-	ND (5) ^b
0.5 μM A23187 + 0.1 μM MK-886	+	+	46.3 \pm 3.0 (6)*

^a Results expressed as mean \pm SEM with the number of duplicate experiments given in parentheses.

^b ND = Not detectable when assayed at the same dilution as the control group.

* Significantly different from control ($p < 0.05$).

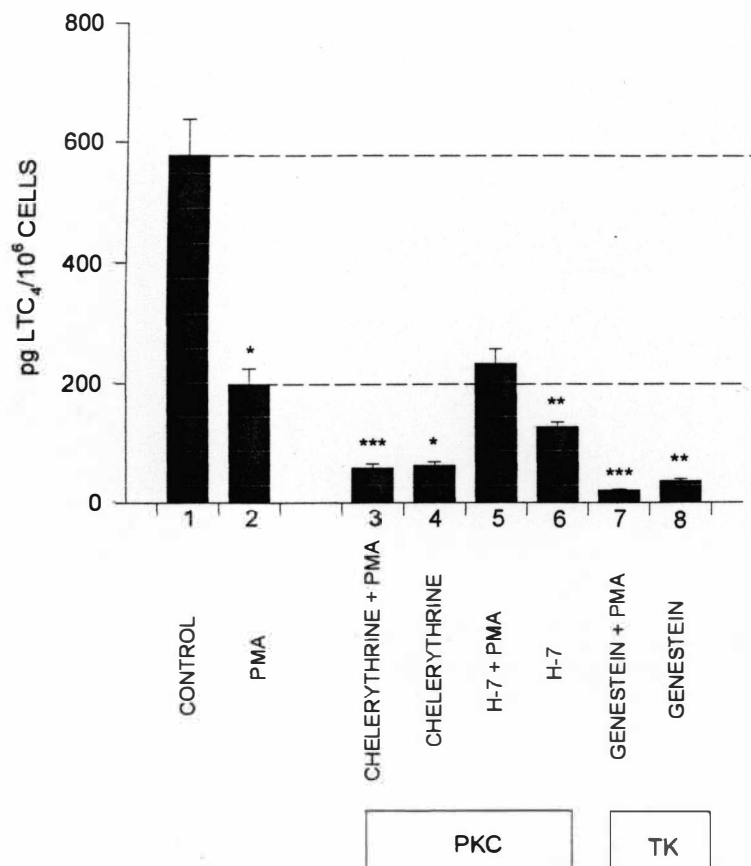


Figure 4. LTC₄ Biosynthesis is Attenuated by PKC Activation and PTK Inhibition in A23187-Challenged AUG Rat Mixed Peritoneal Leukocytes.

Aliquots (1.5×10^6 cells) of AUG rat mixed peritoneal leukocytes in HEPES buffered HBSS (containing 1.2 mM Ca²⁺ and 0.8 mM Mg²⁺, pH 7.3) were incubated with 0.5 μ M A23187 in DMSO (maximum vehicle concentration ≤ 0.4 %) in the presence of the indicated agent(s) (PMA, column 2; chelerythrine chloride + PMA, column 3; chelerythrine chloride, column 4; H-7 + PMA, column 5; H-7, column 6; genestein + PMA, column 7; genestein, column 8) for 10 min at 37 °C. Reactions were terminated by the addition of methanol, and the levels of LTC₄ determined by EIA (Cayman Chemical, Ann Arbor, MI). Data are expressed as mean \pm SEM of 4 - 14 duplicate experiments (see text). *Significantly different from control group ($p < 0.05$). **Significantly different from control group and significantly different from same inhibitor in the presence of PMA ($p < 0.05$). ***Significantly different from PMA treatment group ($p < 0.05$).

concentrations tested (columns 3 and 5 vs. column 2); in fact, chelerythrine further inhibited LT synthesis by another 24% ($p < 0.05$; H-7 plus PMA vs. A23187 plus PMA not significant, $p > 0.05$). Consequently, we next tested the independent ability of each inhibitor to modulate LT production in the absence of PMA. As shown in Figure 4 (columns 4 and 6 vs. column 1), both agents significantly down-regulated the synthesis of LTC₄ when compared to cells treated with ionophore alone ($p < 0.05$, $n = 4$ for both groups). Interestingly, the inhibitory response to chelerythrine did not differ appreciably between A23187 or A23187 plus PMA treatments (columns 3 and 4; $p > 0.05$), suggesting that chelerythrine may be operating in a fashion that is independent of PKC activation. On the other hand, the response to H-7 was significantly more pronounced in the absence of PMA (column 6 vs. column 5; $p < 0.05$). Although the reasons for this discrepancy remain unclear, the problems involved with the specificity of H-7 for PKC have been considered previously (Garland et al., 1987).

Gilmore & Martin (1983) demonstrated that PMA can effect tyrosine-specific as well as serine/threonine-specific phosphorylation events, suggesting that PMA may interact with PTK as well as PKC. To study this possibility, we measured the level of LTC₄ production in A23187- and A23187 plus PMA-challenged cells treated with 100 μ M genestein, a selective inhibitor of PTK activity (Akiyama et al., 1987). As shown in Figure 4 (column 7 vs. column 2), PTK inhibition did not prevent the antagonism of PMA on LTC₄ synthesis, but rather enhanced this effect by another 31% ($p < 0.05$, $n = 5$). Since genestein also inhibited mediator formation in cells

challenged with 0.5 μ M ionophore alone ($p < 0.05$, $n = 3$; column 8 vs. column 1), we suggest that LTC₄ biosynthesis in these cells may be regulated via serine/threonine- and tyrosine-specific phosphorylation mechanisms in an antagonistic fashion; a phenomenon previously demonstrated, following IL-3 preincubation, in C5a-stimulated human basophils (Krieger et al., 1992).

DISCUSSION

Eosinophils may play an important role in such diverse pathologies as bronchial asthma (Butterfield & Leiferman, 1993; Kay, 1991) and helminth parasitic infestations (Butterworth & Thorne, 1993). Thus, a clear understanding of the processes involved in activation and secretion by these cells is of obvious value. However, the study of eosinophil function has been difficult, due to the inherent problems involved in isolating homogeneous populations of viable cells, particularly since the method used in recruitment may have a marked effect on their responses *in vitro* (Cook et al., 1987). Recent work in our laboratory (see also Orcutt, 1994) has utilized the syngeneic AUG rat strain, which has a high spontaneous number of peritoneal eosinophils (Spry, 1988), to provide a consistent and reliable source of these cells.

As part of a larger cooperative effort to understand the biology of the peritoneal cell population in this animal, the present *in vitro* study demonstrates the calcium/magnesium ion and dose-dependent stimulation of LTC₄ biosynthesis by the calcium ionophore A23187, and further, the inhibition of this response by MK-886 (Gillard et al., 1989), a specific antagonist of FLAP activity (Rouzer, Ford-Hutchinson, Morton, & Gillard, 1990). Because MK-886 reportedly binds FLAP at a site corresponding to its putative AA binding domain (reviewed in Ford-Hutchinson, 1994), thereby preventing the efficient transfer of AA to 5-LO (Ford-Hutchinson,

1994; Miller et al., 1990), the findings presented here thus suggest that the incipient complex cellular events leading to the *de novo* formation of LTC₄ are intact in isolated AUG rat mixed peritoneal cells.

Control of LT biosynthesis lies, at least in part, with the remarkably cell-specific expression of the terminal enzymes. Depending on cell phenotype and the species or tissue source, leukocytes preferentially express either LTA₄ hydrolase or LTC₄ synthase and thus are capable of synthesizing their respective products with singular specificity. For example, human alveolar macrophages (AMs) are capable of secreting only LTB₄ (Martin, Altman, Albert, & Henderson, 1984; Fels, Pawlowski, Cramer, King, Cohn, & Scott, 1984), while both murine and rat AMs are able to conjugate LTA₄ to LTC₄ (Rouzer et al., 1982; Rankin et al., 1984). Although we did not directly identify the cellular source of LTC₄ production in our model, peritoneal macrophages (Wenzel, Trudeau, Riches, Westcott, & Henson, 1993), eosinophils (reviewed in Gleich et al., 1992), and some populations of mast cells (reviewed in Raible & Lichtenstein, 1988) have all been implicated as important sources of this lipid mediator in other systems. Moreover, in the tissue reactions associated with several inflammatory disease processes, such bronchial asthma, it is unlikely that any one cell phenotype or secreted mediator is responsible for all of the clinical manifestations accompanying pathogenesis (Kay, 1991). Nevertheless, current investigations in our laboratory are quantifying peritoneal leukocyte LTC₄ release in AUG rats infected with the nematode *Nippostrongylus brasiliensis* (R. Eversole,

unpublished data), a technique previously shown to stimulate blood eosinophilia and basophilia in this animal (Ogilvie, Hesketh, & Rose, 1978; Ogilvie, Askenase, & Rose, 1980). Along with novel cell separation or depletion tools, such as Dynabeads[®] M-450 CD14 (DYNAL, Inc., Lake Success, NY) or discontinuous metrizamide gradient centrifugation (Mackenzie et al., 1981) for suspended macrophages and eosinophils respectively, these studies should allow us to determine the relative contributions made by specific cell types to the total eicosanoid production in both infected and uninfected states.

In view of the broad spectrum of agonists that can trigger the breakdown of inositol phospholipids (reviewed in Meldrum, Parker, & Carozzi, 1991), it is not surprising that PKC has been implicated in a multitude of short-term and long-term cellular responses. For example, previous studies have shown that PMA and suboptimal or threshold concentrations of A23187 synergistically potentiate the synthesis of LTC₄ in human eosinophils (Tamura et al., 1988) and murine macrophages (Humes, 1988; Tripp et al., 1985). However, consistent with recent findings in human basophils (Krieger et al., 1992), human platelets (Edinius et al., 1995), and differentiated substrains of the human promyelocytic HL-60 cell line (Ali et al., 1994; Kargman et al., 1994), our data suggest that PMA-mediated activation of PKC negatively regulates cysteinyl LT biosynthesis in AUG rat mixed peritoneal leukocytes. Since it can also be demonstrated in other non-immunologic cell types that activation of PKC does not effectively modulate LTC₄ release in response to

A23187 (Huber et al., 1993), it thus appears likely that the regulation of this biosynthetic pathway may differ substantially across species, compartments or cell phenotypes. This is almost certainly the case in the cell-cell cooperation (i.e., transcellular biosynthesis) where LTA_4 is transferred from donor neutrophils to 5-LO-deficient acceptor cells, such as platelets (MacIouf & Murphy, 1988), endothelial cells (Feinmark & Cannon, 1986), and vascular smooth muscle cells (Feinmark & Cannon 1987; Feinmark, 1988), and is subsequently conjugated with GSH via LTC_4 synthase.

On the other hand, this interpretation does not fully explain the observations of other PMA-mediated effects, such as the potentiation of A23187-stimulated AA release (Halenda et al., 1985; Peters-Golden et al., 1991), the activation of AA-selective PLA_2 (Lin, et al., 1992; 1993), or the stimulation of prostanoid formation via the transcriptional activation of the cyclooxygenase-2 gene (Gilbert & Herschman, 1993; Kujubu, Fletcher, Varnum, Lim, & Herschman, 1991). In fact, PKC is known to stimulate or enhance prostanoid biosynthesis in several cell types (Ali et al., 1994; Humes et al., 1982; Ota et al., 1993; Peters-Golden, Coburn, & Chauncey, 1992; Simonson, Wolfe, Konieczkowski, Sedor, & Dunn, 1991; Thore, Nam, & Busija, 1994; Yokota, 1991). One plausible interpretation of these findings is the mechanism proposed by Ali et al. (1994; 1995) whereby the phosphorylation of LTC_4 synthase, or a putative modulator of its activity, non-competitively inhibits LTC_4 biosynthesis, and the profile of eicosanoid mediators shifts from cysteinyl LTs

towards the cyclooxygenase products of AA metabolism. The rapid accumulation of pharmacologically inactive LTA₄ would not necessarily be reflected in higher levels of LTB₄ production, since LTA₄ hydrolase is saturated at 40 μ M LTA₄ (Ali et al., 1994). If true, this hypothesis suggests a novel negative feedback mechanism whereby potent mediators such as LTD₄ can both attenuate their own production through binding receptors that trigger the activation of phosphatidylinositol-specific phospholipase C (PtdIns-PLC; Crooke, Mong, Sarau, Winkler, & Vegesna, 1988) (refer to Figure 2) and concomitantly stimulate the synthesis of other mediators (i.e., prostaglandins) some of which may counteract their physiological actions in tissues (Ali et al., 1994).

In the present study, we were unable to prevent the effects of PMA by the application of two structurally distinct PKC antagonists, chelerythrine chloride (IC₅₀ = 0.7 μ M) (Herbert et al., 1990) and the isoquinolinesulfonamide H-7 (K_i for PKC = 6.0 μ M) (Hidaka, Inagaki, Kawamoto, & Sasaki, 1984). Given that the PMA-mediated activation of PKC is both widely accepted and well documented at the concentrations used in our experiments, we suggest that these data indicate probable non-specific or toxic drug effects relating to the inhibitors themselves, rather than any PKC-independent effects of the phorbol ester. This interpretation is facilitated by the facts that (a) both chelerythrine chloride and H-7 were potent inhibitors of LTC₄ biosynthesis in their own right and, in the case of H-7, these effects may actually be partially averted by PMA or PMA-induced PKC activation; (b) both agents were only

tested during short-term incubations at a single dose, raising the possibility that the drugs may, in fact, be sufficiently effective against PKC at alternate concentrations and/or longer exposure periods; (c) H-7 inhibits PKC by competing with ATP for the catalytic site (Hidaka et al., 1984) and hence is non-specific, since most protein kinases, as well as several enzymes, utilize ATP as a substrate (Mahoney & Huang, 1994; see also Garland et al., 1987); (d) the chelerythrine-induced antagonism of LT synthesis described here was not significantly different in either the presence or absence of phorbol ester, suggesting that the inhibitory effects of the compound are independent of, or superlative to those mitigated by PMA; and finally, (e) at least one other whole-cell mediator release study (von Stebut, Amon, Herbert, & Wolff, 1994), encountered conflicting results between chelerythrine and other specific PKC-inhibitors, suggesting that chelerythrine may be less selective for PKC. Thus, a conservative interpretation of the data presented here would be that chelerythrine chloride and H-7 may have potent effects either wholly or partially unrelated to the inhibition PKC activity. In view of the scope of our objectives, however, any putative non-specific properties relating to these compounds remain unclear.

Recently, a number of investigators have shown a PTK inhibitor-dependent attenuation of LT biosynthesis (Atluru, Jackson, & Atluru, 1991; Krieger et al., 1992; Atluru & Gudapaty, 1993; Hagman, 1994), suggesting that tyrosine phosphorylation may play a role in the short-term responses associated with the formation of these lipid mediators. Consistent with the recent demonstration of antagonism between serine/threonine and tyrosine-specific phosphorylation mechanism in the IL-3-

dependent formation of LTC₄ by human basophils (Krieger et al., 1992), our study shows that A23187-stimulated LTC₄ synthesis in AUG rat peritoneal leukocytes can be blocked by the application of a selective PTK antagonist. Simultaneous treatment with both genestein and PMA also significantly enhances the inhibitory properties of either agent alone, suggesting that the effects of PKC activation and PTK-antagonism may be additive. However, these data need to be interpreted with caution, since non-specific drug effects cannot be entirely ruled out, especially as genestein, like H-7, is a competitive inhibitor with respect to ATP (Akiyama et al., 1987). Moreover, two recent studies have shown several unrelated antagonists of PTK activity to be ineffective in down-regulating cysteinyl LT biosynthesis in other cell types (Ali et al., 1994; Kargman et al., 1994). Nevertheless, genestein only weakly inhibits the activity of serine/threonine specific kinases *in vivo* (Akiyama et al., 1987; Akiyama and Ogawara, 1991) indicating that non-specific activation of PKC is probably very unlikely. Further studies with mechanistically dissimilar PTK antagonists (see review, Levitzki & Gazit, 1995) are needed to interpret the role of tyrosine phosphorylation in the synthesis of this bioactive lipid mediator. Inasmuch as the pharmacological activities of the LTs suggest a role in the pathophysiology of immediate hypersensitivity diseases (Lam & Austen, 1992), these findings could ultimately provide a useful basis for drug development targeted at lessening the severity of tissue reactions associated with inflammation.

Appendix A
IACUC Approval Form



WESTERN MICHIGAN UNIVERSITY

Date: December 16, 1993

To: James M. Huebner
Department of Biological Sciences

From: Alex Ojerio, D.V.M.
Acting Chair, IACUC

Re: Protocol Approval

Enclosed is a copy of your approved application IACUC #93-12-01, "Measurement of Arachidonic Acid Metabolites and Degranulation Products from August Rat Peritoneal Eosinophils," for your use and file. Please note the provision of approval on page four. Please feel free to photocopy this document for any required verification of approval.

c: Leonard Beuving (w/enc.)

BIBLIOGRAPHY

- Akiyama, T., Ishidá, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., & Fukami, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. Journal of Biological Chemistry, 262, 5592-5595.
- Akiyama, T., & Ogawara, H. (1991). Use and specificity of genestein as inhibitor of protein-tyrosine kinases. Methods in Enzymology, 201, 362-370.
- Ali, A., Ford-Hutchinson, A.W., & Nicholson, D.W. (1994). Activation of protein kinase C down-regulates leukotriene C₄ synthase activity and attenuates cysteinyl leukotriene production in an eosinophilic substrain of HL-60 cells. Journal of Immunology, 153, 776-788.
- Ali, A., Nicholson, D.W., & Ford-Hutchinson, A.W. (1995). Characterization of human LTC₄ synthase. In B. Samuelsson, P.W. Ramwell, R. Paoletti, G. Folco, E. Granström, & S. Nicosia (Eds.), Advances in Prostaglandin, Thromboxane, and Leukotriene Research, Volume 23, (pp. 171-173). New York: Raven Press.
- Allison, R.D. (1985). γ -Glutamyl transpeptidase: kinetics and mechanism. Methods in Enzymology, 113, 419-437.
- Anderson, M.E., Allison, R.D., & Meister, A. (1982). Interconversion of leukotrienes catalyzed by purified γ -glutamyltranspeptidase: concomitant formation of leukotriene D₄ and γ -glutamyl amino acids. Proceedings of the National Academy of Science, U.S.A., 79, 1088-1091.
- Atulru, D., & Gudapaty, S. (1993). Inhibition of bovine mononuclear cell proliferation, interleukin-2 synthesis, protein-tyrosine kinase and leukotriene B₄ production by a protein-tyrosine kinase inhibitor, genestein. Veterinary Immunology and Immunopathology, 38, 113-122.
- Atluru, D., Jackson, T.M., & Atluru, S. (1991). Genestein, a selective protein tyrosine kinase inhibitor, inhibits interleukin-2 and leukotriene B₄ production from human mononuclear cells. Clinical Immunology and Immunopathology, 59, 379-387.
- Bernström, K., & Hammarström, S. (1981). Metabolism of leukotriene D by porcine kidney. Journal of Biological Chemistry, 256, 9579-9582.

- Bernström, K., & Hammarström, S. (1982). A novel leukotriene formed by transeptidation of leukotriene E. Biochemical and Biophysical Research Communications, 109, 800-804.
- Borgeat, P., and Samuelsson, B. (1979). Arachidonic-acid metabolism in polymorphonuclear leukocytes. 5. Unstable intermediate in formation of dihydroxy acids. Proceedings of the National Academy of Science, U.S.A., 76, 3213-3217.
- Butterfield, J.H., & Leiferman, K.M. (1993). Eosinophil-associated diseases. In H. Smith & R.M. Cook (Eds.), Immunopharmacology of Eosinophils, (pp. 151-192). New York: Academic Press.
- Butterworth, A.E., & Thorne, K.J.I. (1993). Eosinophils and parasitic diseases. In H. Smith & R.M. Cook (Eds.), Immunopharmacology of Eosinophils, (pp. 120-150). New York: Academic Press.
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., & Naishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. Journal of Biological Chemistry, 257, 7847-7851.
- Cook, R.M., Musgrove, N.R.J., & Ashworth, R.F. (1987). Activity of rat peritoneal eosinophils following induction by different methods. International Archives of Allergy and Applied Immunology, 83, 423-427.
- Crooke, S.T., Mong, S., Sarau, H.M., Winkler, J.D., & Vegesna, V.K. (1988). Mechanisms of regulation of receptors and signal transduction pathways for the peptidyl leukotrienes. Annals of the New York Academy of Sciences, 524, 153-161.
- DeWolf, W.E., Jr. (1991). Enzymatic studies on purified 5-lipoxygenase. In S.T. Crooke & A. Wong (Eds.), Lipoxygenases and Their Products, (pp. 105-135). San Diego: Academic Press, Inc.
- Dixon, R.A.F., Diehl, R.E., Opas, E., Rands, E., Vickers, P.J., Evans, J.F., Gillard, J.W., & Miller, D.K. (1990). Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. Nature, 343, 282-284.
- Edenius, C., Tornhamre, S., Näsman-Glaser, B., Eriksson, I., Björkholm, S., & Lindgren, J.Å. (1995). Regulation of leukotriene C₄ synthase in human platelets via receptor-mediated mechanisms. In B. Samuelsson, P.W. Ramwell, R. Paoletti, G. Folco, E. Granström, & S. Nicosia (Ed.s), Advances in

- Prostaglandin, Thromboxane, and Leukotriene Research, Vol. 23, (pp. 333-335). New York: Raven Press.
- Feinmark, S.J. (1988). Cooperative synthesis of leukotrienes by leukocytes and vascular cells. Annals of the New York Academy of Sciences, 524, 122-132.
- Feinmark, S.J., & Cannon, P.J. (1986). Endothelial cell leukotriene C₄ synthesis results from transfer of leukotriene A₄ synthesized by polymorphonuclear leukocytes. Journal of Biological Chemistry, 261, 16466-16472.
- Feinmark, S.J., & Cannon, P.J. (1987). Vascular smooth muscle cell leukotriene C₄ synthesis: requirement for transcellular leukotriene A₄ metabolism. Biochimica et Biophysica Acta, 922, 125-135.
- Fels, A.O.S., Pawlowski, N.A., Cramer, E.B., King, T.K.C., Cohn, Z.A., & Scott, W.A. (1982). Human alveolar macrophages produce leukotriene B₄. Proceedings of the National Academy of Science, U.S.A., 79, 7866-7870.
- Ford-Hutchinson, A.W. (1994). Leukotriene C₄ synthase and 5-lipoxygenase activating protein: regulators of the biosynthesis of sulfido-leukotrienes. Annals of the New York Academy of Sciences, 744, 78-83.
- Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, M.E., & Smith, M.J. (1980). Leukotriene B₄, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. Nature, 286, 264-265.
- Garland, L.G., Bonser, R.W., & Thompson, N.T. (1987). Protein kinase C inhibitors are not selective. Trends in Pharmacological Sciences (letters), 8, 334.
- Gilbert, R.S., & Herschman, H.R. (1993). Macrophage nitric oxide synthase is a glucocorticoid-inhibitable primary response gene in 3T3 cells. Journal of Cell Physiology, 157, 128-132.
- Gillard, J., Ford-Hutchinson, A.W., Chan, C., Charleson, S., Denis, D., Foster, A., Fortin, R., Leger, S., McFarlane, C.S., Morton, H., Piechuta, H., Riendeau, D., Rouzer, C.A., Rokach, J., Young, R., MacIntyre, D.E., Peterson, L., Bach, T., Eiermann, G., Hopple, S., Humes, J., Hupe, L., Luell, S., Metzger, J., Meurer, R., Miller, D.K., Opas, E., & Pacholok, S. (1989) L-663,5396 (MK-886) (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid), a novel, orally active leukotriene biosynthesis inhibitor. Canadian Journal of Physiology and Pharmacology, 67, 456-464.

- Gilmore, T., & G.S. Martin. (1983). Phorbol ester and diacylglycerol induce protein phosphorylation at tyrosine. Nature, 306, 487-490.
- Gleich, G.J., Adolphson, C.R., & Leiferman, K.M. 1992. Eosinophils. In J.I. Gallin, I.M. Goldstein & R. Snyderman (Ed.s), Inflammation: Basic Principles and Clinical Correlates, Second Edition, (pp.663-700). New York: Raven Press.
- Hagman, W. (1994). Cell proliferation status, cytokine action and protein phosphorylation modulate leukotriene biosynthesis in a basophil leukaemia and a mastocytoma cell line. Biochemical Journal, 299, 467-472.
- Halenda, S.P., Zavoico, G.B., & Feinstein, M.B. (1985). Phorbol esters and oleoyl acetoxy glycerol enhance release of arachidonic acid in platelets stimulated by Ca^{2+} ionophore A23187. Journal of Biological Chemistry, 260, 12484-12491.
- Hammarström, S. (1981). Metabolism of leukotriene C_3 in the guinea pig. Journal of Biological Chemistry, 256, 9573-9578.
- Hammarström, S. (1983). Leukotrienes. Annual Review of Biochemistry, 52, 355-377.
- Hammarström, S., Örning, L., & Bernström, K. (1986). Metabolism and excretion of cysteinyl-leukotrienes. In U. Zor, Z. Naor, & F. Kohen (Eds.), Advances in Prostaglandin, Thromboxane, and Leukotriene Research, Volume 16, (pp. 383-396). New York: Raven Press.
- Hammarström, S., Örning, L., & Keppler, A. (1988). Metabolism of cysteinyl leukotrienes to novel polar metabolites in rat and endogenous formation of leukotriene D_4 during systemic anaphylaxis in the guinea pig. Annals of the New York Academy of Sciences, 524, 43-67.
- Herbert, J.M., Augereau, J.M., Gleye, J., & Maffrand, J.P. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochemical and Biophysical Research Communications, 172, 993-999.
- Hidaka, H., Inagaki, M., Kawamoto, S., & Sasaki, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry, 23, 5036-5041.
- Huber, W., Trautmann, M., Becker, I., Schenck, U., Peskar, B.M., & Schepp, W. (1993). Leukotriene B_4 and C_4 production in isolated gastric mucosal cells. American Journal of Physiology, 265, G1021-G1028.

- Humes, J.L. (1988). Regulation of leukotriene formation in inflammatory cells. Annals of the New York Academy of Sciences, 524, 252-259.
- Humes, J.L., Sadowski, S., Galavage, M., Goldenberg, M., Subers, E., Bonney, R.J., & Kuehl, F.A., Jr. (1982). Evidence for two sources of arachidonic acid for oxidative metabolism by mouse peritoneal macrophages. Journal of Biological Chemistry, 257, 1591-1594.
- Irvine, R.F. (1982). How is the free level of arachidonic acid controlled in mammalian cells? Biochemical Journal, 204, 3-16.
- Kargman, S., Ali, A., Vaillancourt, J.P., Evans, J.F., & Nicholson, D.W. (1994). Protein kinase C-dependent regulation of sulfidopeptide leukotriene biosynthesis and leukotriene C₄ synthase in neutrophilic HL-60 cells. Molecular Pharmacology, 45, 1043-1049.
- Kawamoto, S., & Hidaka, H. (1984). 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. Biochemical and Biophysical Research Communications, 125, 258-264.
- Kay, A.B. (1991). Asthma and inflammation. Journal of Allergy and Clinical Immunology, 87, 893-910.
- Krieger, M., von Tscharner, V., & Dahinden, C.A. (1992). Signal transduction for interleukin-3-dependent leukotriene synthesis in normal human basophils: opposing role of tyrosine kinase and protein kinase C. European Journal of Immunology, 22, 2907-2913.
- Kujubu, D.A., Fletcher, B.S., Varnum, B.C., Lim, R.W., & Herschman, H.R. (1991). TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. Journal of Biological Chemistry, 266, 12866-12872.
- Lam, B.K., & Austen, K.F. (1992). Leukotrienes: biosynthesis, release, and actions. In J.I. Gallin, I.M. Goldstein, & R. Snyderman (Eds.), Inflammation: Basic Principles and Clinical Correlates, Second Edition, (pp. 139-147), New York: Raven Press.
- Lam, B.K., Penrose, J.F., & Austen, K.F. (1995). Expression cloning of human LTC₄ synthase. In B. Samuelsson, P.W. Ramwell, R. Paoletti, G. Folco, E. Granström, & S. Nicosia (Eds.), Advances in Prostaglandin, Thromboxane, and Leukotriene Research, Volume 23, (pp. 35-39). New York: Raven Press

- Levitzki, A., & Gazit, A. (1995). Tyrosine kinase inhibition: an approach to drug development. Science, 267, 1782-1787.
- Lewis, R.A., & Austen, K.F. (1984). The biologically active leukotrienes. Journal of Clinical Investigation, 73, 889-887.
- Liles, W.C., Meier, K.E., & Henderson, W.R. (1987). Phorbol myristate acetate and the calcium ionophore A23187 synergistically induce release of LTB₄ by human neutrophils: involvement of protein kinase C activation in regulation of the 5-lipoxygenase pathway. Journal of Immunology, 138, 3396-3402.
- Lin, L.-L., Lin, A.Y., & Knopf, J.L. (1992). Cytosolic phospholipase A₂ is coupled to hormonally regulated release of arachidonic acid. Proceedings of the National Academy of Sciences, U.S.A., 89, 6147-6151.
- Lin, L.-L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A., & Davis, R.J. (1993). cPLA₂ is phosphorylated and activated by MAP kinase. Cell, 72, 269-278.
- Lindgren, J.Å., Hammarström, S., & Goetzl, E.J. (1983). A sensitive and specific radioimmunoassay for leukotriene C₄. FEBS Letters, 152, 83-88.
- Maas, R.L., Ingram, C.D., Taber, D.F., Oates, J.A., & Brash, A.R. (1982). Stereospecific removal of the D_R hydrogen atom at the 10-carbon of arachidonic acid in the biosynthesis of leukotriene A₄ in human leukocytes. Journal of Biological Chemistry, 257, 13515-13519.
- Mackenzie, C.D., Jungery, M., Taylor, P.M., & Ogilvie, B.M. (1981). The in-vitro interaction of eosinophils, neutrophils, macrophages and mast cells with nematode surfaces in the presence of complement or antibodies. Journal of Pathology, 133, 161-175.
- Maclof, J.A., & Murphy, R.C. (1988). Transcellular metabolism of neutrophil-derived leukotriene A₄ by human platelets: a potential cellular source of leukotriene C₄. Journal of Biological Chemistry, 263, 174-181.
- Mahoney, C.W., & Huang, K.-P. (1994). Molecular and catalytic properties of protein kinase C. In Kuo, J.F. (Ed.), Protein Kinase C, (pp.16-63), New York, Oxford University Press.
- Martin, T.R., Altman, L.C., Albert, R.K., & Henderson, W.R. (1984). Leukotriene B₄ production by the human alveolar macrophage: a potential mechanism for amplifying inflammation in the lung. American Review of Respiratory Disease, 129, 106-111.

- McColl, S.R., Hurst, N.P., & Cleland, L.G. (1986). Modulation by phorbol myristate acetate of arachidonic acid release and leukotriene synthesis by human polymorphonuclear leukocytes stimulated with A23187. Biochemical and Biophysical Research Communications, 141, 399-404.
- McIntyre, T.M., Reinhold, S.L., Prescott, S.M., & Zimmerman, G.A. (1987). Protein kinase C activity appears to be required for the synthesis of platelet-activating factor and leukotriene B₄ by human neutrophils. Journal of Biological Chemistry, 262, 15370-15376.
- Meister, A., & Anderson, M.E. (1983). Glutathione. Annual Review of Biochemistry, 52, 711-760.
- Meldrum, E., Parker, P.J., & Carozzi, A. (1991). The PtdIns-PLC superfamily and signal transduction. Biochimica et Biophysica Acta, 1092, 49-71.
- Miller, D.K., Gillard, J.W., Vickers, P.J., Sadowski, S., Léveillé, C., Mancini, J.A., Charleson, P., Dixon, R.A.F., Ford-Hutchinson, A.W., Fortin, R., Gauthier, J.Y., Rodkey, J., Rosen, R., Rouzer, C., Sigal, I.S., Strader, C.D., & Evans, J.F. (1990). Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. Nature, 343, 278-281.
- Murphy, R.C., Hammarström, S., & Samuelsson, B. (1979). Leukotriene C: a slow-reacting substance from murine mastocytoma cells. Proceedings of the National Academy of Sciences, U.S.A., 76, 4275-4279.
- Nicholson, D.W., Ali, A., Klemba, M.W., Munday, N.A., Zamboni, R.J., & Ford-Hutchinson, A.W. (1992). Human leukotriene C₄ synthase expression in dimethyl sulfoxide-differentiated U937 cells. Journal of Biological Chemistry, 267, 17849-17857.
- Nicholson, D.W., Ali, A., Vaillancourt, J.P., Calaycay, J.R., Mumford, R.A., Zamboni, R.J., & Ford-Hutchinson, A.W. (1993). Purification to homogeneity and the N-terminal sequence of human leukotriene C₄ synthase: a homodimeric glutathione S-transferase composed of 18 kDa subunits. Proceedings of the National Academy of Sciences, U.S.A., 90, 2015-2019.
- Nishizuka, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature, 308, 693-698.
- Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. Science, 233, 305-312.

- Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science, 258, 607-614.
- Ogilvie, B.M., Askenase, P.W., & Rose, M.E. (1980). Basophils and eosinophils in three strains of rats and in athymic (nude) rats following infection with the nematodes *Nippostrongylus brasiliensis* or *Trichinella spiralis*. Immunology, 39, 385-389.
- Ogilvie, B.M., Hesketh, P.M., & Rose, M.E. (1978). *Nippostrongylus brasiliensis*: peripheral blood leucocyte response of rats, with special reference to basophils. Experimental Parasitology, 46, 20-30.
- Orcutt, D.R. (1994). Estrogen induced eosinophil migration into the august rat uterus is associated with intercellular adhesion molecule expression. M.S. thesis, Western Michigan University, Kalamazoo.
- Orning, L., Hammarström, S., & Samuelson, B. (1980). Leukotriene D: a slow reacting substance from rat basophilic leukemia cells. Proceedings of the National Academy of Science, U.S.A., 77, 2017-2017.
- Ota, S., Hata, Y., Terano, A., Yoshiura, K., Hiraishi, H., Kawabe, T., Mutoh, H., Shiina, S., Sugimoto, T. (1993). Roles of Ca^{2+} and protein kinase C in regulation of prostaglandin E_2 release by cultured rabbit gastric epithelial cells. Digestive Diseases and Sciences, 38, 1426-1434.
- Ott, L. (1988). An Introduction to Statistical Methods and Data Analysis. (3rd ed.). Boston: PWS-Kent.
- Panossian, A., Hamberg, M., & Samuelsson, B. (1982). On the mechanism of biosynthesis of leukotrienes and related compounds. FEBS Letters, 150, 511-513.
- Parker, P.J. (1994). Protein kinase C: history and perspectives. In J.F. Kuo (Ed.), Protein Kinase C, (pp. 3-15). New York: Oxford University Press.
- Peters-Golden, M., Coburn, K., & Chauncey, J.B. (1992). Protein kinase C activation modulates arachidonic acid metabolism in cultured alveolar epithelial cells. Experimental Lung Research, 18, 535-551.
- Peters-Golden, M., McNish, R.W., Sporn, P.H., & Balazovich, K. (1991). Basal activation of protein kinase C in rat alveolar macrophages: implications for arachidonate metabolism. American Journal of Physiology, 261, L462-L471.

- Piper, P.J. Formation and actions of leukotrienes. Physiological Reviews, **64**, 744-761.
- Rådmark, O., Malmsten, C., & Samuelsson, B. (1980). Leukotriene A₄: Enzymatic conversion to leukotriene C₄. Biochemical and Biophysical Research Communications, **96**, 1679-1687.
- Rådmark, O., Malmsten, C., Samuelsson, B., Goto, G., Marfat, A., & Corey, E.J.. (1980). Leukotriene A: Isolation from human polymorphonuclear leukocytes. Journal of Biological Chemistry, **255**, 11828-11831.
- Rådmark, O., Zhang, Y.-Y., Hammarberg, T., Lind, B., Hamberg, M., Brungs, M., Steinhilber, D., & Samuelsson, B. (1995). 5-Lipoxygenase: structure and stability of recombinant enzyme, regulation in Mono Mac 6 cells. In B. Samuelsson, P.W. Ramwell, R. Paoletti, G. Folco, E. Granström, & S. Nicosia (Eds.), Advances in Prostaglandin, Thromboxane, and Leukotriene Research, Volume 23, (pp. 1-9). New York: Raven Press.
- Raible, D.G., & Lichtenstein, L.M. (1988). The role of leukotrienes in human pathophysiology. Annals of the New York Academy of Sciences, **524**, 334-344.
- Rankin, J.A., Hitchcock, M., Merrill, W.W., Huang, S.S., Brashler, J.R., Bach, M.K., & Askensase, P.W. (1984). IgE immune complexes induce immediate and prolonged release of leukotriene C₄ (LTC₄) from rat alveolar macrophages. Journal of Immunology, **132**, 1993-1999.
- Razin, E., Mencia-Huerta, J.M., Lewis, R.A., Corey, E.J., & Austen, K.F. (1983). Generation of leukotriene C₄ from a subclass of mast cells differentiated *in vitro* from mouse bone marrow. Proceedings of the National Academy of Sciences, U.S.A., **79**, 4665-4667.
- Reddanna, P., Whelan, J., & Reddy, C.C. (1988). A new pathway for the biosynthesis of leukotriene F₄. Annals of the New York Academy of Sciences, **525**, 391-392.
- Reed, P.W., Lardy, H.A. (1972). A23187: a divalent cation ionophore. Journal of Biological Chemistry, **247**, 6970-6977.
- Rouzer, C.A. (1991). Regulation and structure of human leukocyte 5-lipoxygenase. In S.T. Crooke & A. Wong (Eds.), Lipoxygenases and Their Products, (pp. 51-65). San Diego: Academic Press, Inc.

- Rouzer, C.A., Ford-Hutchinson, A.W., Morton, H.E., & Gillard, J.W. (1990). MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. Journal of Biological Chemistry, 265, 1436-1442.
- Rouzer, C.A., & Kargman, S. (1988). Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. Journal of Biological Chemistry, 263, 10980-10988.
- Rouzer, C.A., Matsumoto, T., & Samuelsson, B. (1986). Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A4 synthase activities. Proceedings of the National Academy of Sciences, U.S.A., 83, 857-861.
- Rouzer, C.A., Scott, W.A., Cohn, Z.A., Blackburn, P., & Manning, J.M. (1980). Mouse peritoneal macrophages release leukotriene C in response to a phagocytic stimulus. Proceedings of the National Academy of Sciences, U.S.A., 77, 4928-4932.
- Rouzer, C.A., Scott, W.A., Hamill, A.L., & Cohn, Z.A. (1982). Synthesis of leukotriene C and other arachidonic acid metabolites by mouse pulmonary macrophages. Journal of Experimental Medicine, 155, 720-733.
- Samuelsson, B. (1983). Leukotrienes: mediators of hypersensitivity reactions and inflammation. Science, 220, 568-575.
- Samuelsson, B., Dahlén, S.-E., Lindgren, J.Å., Rouzer, C.A., & Serhan, C.N. (1987). Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. Science, 237, 1171-1176.
- Shore, S.A., Stimler-Gerard, N.P., Smith, E., & Drazen, J.M. (1987). *N*-Formyl-methionyl-leucyl-phenylalanine induced contractions of guinea pig lung: role of arachidonic acid metabolites. Journal of Applied Physiology, 62: 2450-2459.
- Simonson, M.S., Wolfe, J.A., Konieczkowski, M., Sedor, J.R., & Dunn, M.J. (1991). Regulation of prostaglandin endoperoxide synthase gene expression in cultured rat mesangial cells: induction by serum via a protein kinase-C-dependent mechanism. Molecular Endocrinology, 5, 441-451.
- Sok, D.-E., Pai, J.-K., Atrache, V., Kang, Y.-C., & Sih, C.J. (1981). Enzymatic inactivation of SRS-CYS-GLY (leukotriene D). Biochemical and Biophysical Research Communications, 101, 222-229.

- Spry, C.J.F. (1988). Eosinophils. New York: Oxford University Press.
- Tamura, N., Agrawal, D.K., & Townley, R.G. (1988). Leukotriene C₄ production from human eosinophils in vitro: role of eosinophil chemotactic factors on eosinophil activation. Journal of Immunology, 141, 4291-4297.
- Tate, S.S. (1985). Microvillus membrane peptidases that catalyze hydrolysis of cysteinylglycine and its derivatives. Methods in Enzymology, 113, 471-484.
- Tate, S.S., & Meister, A. (1985). γ -Glutamyl transpeptidase from kidney. Methods in Enzymology, 113, 400-419.
- Thore, C.R., Nam, M., & Busija, D. (1994). Phorbol ester-induced prostaglandin production in piglet cortical astroglia. American Journal of Physiology, 267, R34-R37.
- Tripp, C.S., Mahoney, M., & Needleman, P. (1985). Calcium ionophore enables soluble agonists to stimulate macrophage 5-lipoxygenase. Journal of Biological Chemistry, 260, 5895-5898.
- von Stebut, E., Amon, U., Herbert, J.M., & Wolff, H.H. (1994). Investigations with the selective PKC inhibitor chelerythrine on human basophils. Agents and Actions, 41: C56-C57.
- Weller, P.F., Lee, C.W., Foster, D.W., Corey, E.J., Austen, K.F., & Lewis, R.A. (1983). Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: predominant production of leukotriene C₄. Proceedings of the National Academy of Sciences, U.S.A., 80, 7626-7630.
- Welsch, D.J., Creely, D.P., Mathis, K.J., Hauser, S.D., & Isakson, P.C. (1995). Molecular cloning and expression of human leukotriene C₄ synthase. In B. Samuelsson, P.W. Ramwell, R. Paoletti, G. Folco, E. Granström, & S. Nicosia (Eds.), Advances in Prostaglandin, Thromboxane, and Leukotriene Research, Volume 23, (pp. 167-169). New York: Raven Press.
- Wenzel, S.E., Trudeau, J.B., Riches, D.W.H., Westcott, J.Y., & Henson, P.M. (1993). Peritoneal lavage fluid alters patterns of eicosanoid production in murine bone marrow-derived and peritoneal macrophages: dependency on inflammatory state of the peritoneum. Inflammation, 17, 743-756.
- Wong, A., & Crooke, S.T. (1991). Regulation of 5-lipoxygenase activity in rat basophilic leukemia cells. In S.T. Crooke & A. Wong (Eds.), Lipoxygenases and Their Products, (pp. 67-87). San Diego: Academic Press, Inc.

- Woods, J.W., Evans, J.F., Ethier, D., Scott, S., Vickers, P.J., Hearn, L., Heibin, J.A., Charleson, S., & Singer, I.I. (1993). 5-lipoxygenase and 5-lipoxygenase-activating protein are localized in the nuclear envelope of activated human leukocytes. Journal of Experimental Medicine, 178, 1935-1946.
- Yokota, K. (1991). Cellular mechanism of synergistic stimulation of PGE₂ production by phorbol diester and Ca²⁺ ionophore A23187 in cultured madin-darby canine kidney cells. Archives of Biochemistry and Biophysics, 288, 192-201.