ROLE OF INTRACELLULAR CALCIUM IN *PASTEURELLA HAEMOLYTICA* LEUKOTOXIN - INDUCED NEUTROPHIL PLASMA MEMBRANE DAMAGE AND SYNTHESIS OF LEUKOTRIENE B_4

By

LAURA ANNETTE KELLUM

Bachelor of Science

University of Oklahoma Health Sciences Center

Oklahoma City, Oklahoma

1991

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 1995

ROLE OF INTRACELLULAR CALCIUM IN *PASTEURELLA HAEMOLYTICA* LEUKOTOXIN -INDUCED NEUTROPHIL PLASMA MEMBRANE DAMAGE AND SYNTHESIS OF LEUKOTRIENE B_4

Thesis Approved:

Cynthia R. Clarke
Thesis Adviser

Kenneth D Chinkenbeard

James M Matthews

Thomas C. Collins
Dean of the Graduate College

ACKNOWLEDGMENTS

I have been blessed with a grand opportunity to do research and further my education with financial support. Many people have made the pursuit of my Master's Degree a positive experience. First and foremost, I would sincerely like to thank my major advisor, Dr. Cyril Clarke, without whom I am sure I would not have finished my degree in the timely manner. He has unselfishly offered his intelligent insight, keen guidance, honesty, and personal concern for my academic as well as personal growth. I also wish to express gratitude to my other committee members; Dr. Kenneth Clinkenbeard and Dr. Jerry Malayer, for their assistance, encouragement, and friendship, which made the process of earning my degree enjoyable as well as educational. I feel very fortunate to have had such intelligent and kind individuals on my committee. Also, many members of the Department of Physiological Sciences have been fantastic during the past 18 months and I am sure that these instrumental relationships will continue throughout my doctoral degree.

I would like to express special appreciation to my family and friends, without whose support and encouragement I would not have been able to complete the requirements for my degree in the manner that I have. In particular, I would like to thank my parents and my sister Jenny, who have provided invaluable love and understanding.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Pneumonic pasteurellosis is a disease of feedlot and stocker cattle that is responsible for considerable economic loss to beef producers and consumers (Allan *et al*, 1985; Yates, 1982). Caused by *Pasteurella haemolytica*, the disease is characterized by acute fibrinopurulent pneumonia involving massive accumulation of neutrophils (Yates, 1982), which are responsible for much of the lung pathology (Slocombe *et al*, 1985; Breider *et al*, 1988; Frank, 1989). Infiltrating neutrophils do not provide an effective host defense response, but undergo degranulation and lysis, releasing lysosomal enzymes and reactive oxygen products that aggravate tissue damage.

Eicosanoids, including leukotriene B_4 (LTB_4), are considered to be important chemotactic agents responsible for influx of neutrophils into infected lungs (Clarke *et al*, 1994). Leukotoxin (LKT), a protein exotoxin produced by log-phase *P. haemolytica*, stimulates the release of LTB_4 and other 5-lipoxygenase products from bovine neutrophils (Clinkenbeard *et al*, 1994; Henricks *et al*, 1992). This toxin is a member of the family of RTX pore-forming cytolysins, which are characterized by tandemly arranged repeats of a nine amino acid sequence (Welch, 1991). At low concentrations, LKT is a potent neutrophil activating agent (Czuprynski & Noel, 1990), but at high concentrations it causes severe membrane defects and neutrophil lysis (Clinkenbeard *et al*, 1989b). Therefore, LKT contributes to the chemoattraction of neutrophils into sites of *P. haemolytica* infection as well as the impairment of neutrophil-mediated host defenses.

Exposure of bovine neutrophils to LKT causes a concentration-dependent increase in intracellular calcium concentration ($[Ca^{2+}]_i$), which can be inhibited by the calcium channel blocker, verapamil (Ortiz-Carranza &Czuprynski, 1992). Considering the effect of $[Ca^{2+}]_i$ on activities of enzymes responsible for release of arachidonic acid (AA) from

phospholipid membranes (Mayer & Marshall, 1993) and subsequent oxidation of AA to leukotrienes (Musser & Kreft, 1992), Ca^{2+} may serve as an important second messenger in LKT-mediated inflammatory responses. Elucidation of the molecular pathogenesis of LKT-induced eicosanoid synthesis will facilitate targeting of relevant signal transduction mechanisms and use of specific anti-inflammatory agents to attenuate the severity of the neutrophil-mediated inflammatory response and enhance the efficacy of concurrent antibacterial therapy. Therefore, the objectives of this study were to determine whether LKT-induced synthesis of LTB_4 by bovine neutrophils is dependent on increased $[Ca^{2+}]_i$ and to study the mechanism of this response.

CHAPTER II

LITERATURE REVIEW

Pathogenesis of Bovine Respiratory Disease

Mortality and high morbidity resulting from bovine respiratory disease (BRD) are responsible for greater economic losses to the feedlot industry than any other disease in North America (Wikse, 1990; Yates, 1982). This disease is usually characterized by severe pneumonic signs, including; fever, dyspnea, respiratory rales, nasal discharge, and depression. The most common pathological findings are fibrinopurulent pneumonia and varying degrees of pluritis and pulmonary abscesses (Wikse, 1990; Hungerford, 1990; Radostitis *et al*, 1994).

Several infectious agents have been implicated in BRD but *Pasteurella haemolytica* serotype A1 is considered to be the primary etiologic organism, with other bacteria, such as *Pasteurella multocida* and *Haemophilus somnus*, being isolated less frequently (Wikse, 1990; Confer *et al*, 1988; Yates, 1982; Radostitis *et al*, 1994). *P. haemolytica* is a normal inhabitant of the nasopharyngeal mucosa of many healthy cattle (Yates, 1982) and is considered to be an opportunistic pulmonary pathogen of the normally sterile environment of the lung (Wikse, 1990). When pulmonary defenses are depressed by stress, nutritional deficiencies, and/or viral infections, *P. haemolytica* is able to colonize and proliferate, first in the upper respiratory tract and then in the lung, producing severe acute fibrinopurulent bronchopneumonia. This disease syndrome is also referred to as shipping fever, in recognition of the role of transportation stress in its etiology (Frank *et al*, 1987; Radostitis *et al*, 1994; Wikse, 1990).

Neutrophils are recognized to be the primary effector cells of pulmonary injury in BRD. Although alveolar macrophages constitute the first line of defense against *P. haemolytica* in the lung, once these cells have been overwhelmed the predominant

phagocyte is the neutrophil (Clinkenbeard *et al*, 1992). After experimental intratracheal or aerosol inoculation, neutrophils infiltrate the lung within 2 to 4 hours, causing a marked increase in the neutrophil/macrophage ratio (Gosset *et al*, 1984; Walker *et al*, 1985; Lopez *et al*, 1986). Instead of eliminating invading organisms, infiltrating neutrophils contribute to severe pathological changes. The destructive effect of neutrophil infiltration has been demonstrated by Slocombe *et al* (1985), who reported that neutrophil depletion of calves prior to intratracheal inoculation of *P. haemolytica* protects against development of severe lung pathology. Breider *et al* (1988) subsequently reported that while neutrophil-deficient calves inoculated with *P. haemolytica* still had extensive intra- and inter-lobular edema, intra-alveolar hemorrhage, atelectasis, and focal areas of alveolar septal necrosis, they did not exhibit the severe fibrinopurulent alveolitis and bronchiolitis observed in calves with normal numbers of circulating neutrophils. Therefore, it is apparent that neutrophil chemotaxis into sites of *P. haemolytica* infection does not result in a competent host defense response but that reactive products released by degranulation and lysis of neutrophils aggravate tissue damage (Whiteley *et al*, 1992).

Role of Eicosanoids in Bovine Respiratory Disease

Although all of the chemotactic factors involved in neutrophil attraction into sites of *P. haemolytica* infection have not yet been identified, eicosanoids are known to play a major role. Synthesis of eicosanoids involves release of arachidonic acid (AA) from membrane phospholipids by phospholipases and then oxidation of AA by cyclo-oxygenase to produce thromboxanes and prostaglandins or by lipoxygenases to produce hydroxyeicosatetraenoic acids and leukotrienes (Holzman, 1991; Higgins & Lees, 1984; Moncada & Vane, 1979). Products of cyclo-oxygenase and lipoxygenases, collectively referred to as eicosanoids, have many different functions that contribute to generation of an inflammatory reaction (Holzman, 1991). Prostaglandins and thromboxanes, through their

action on patency and permeability of peripheral vasculature, as well as sensitization of pain receptors, are responsible for inducing most of the cardinal signs of inflammation; including erythema, swelling, increased temperature, and pain (Higgins and Lees, 1984). Prostaglandins and thromboxanes also modulate platelet aggregation and blood clotting. Although lipoxygenase products have been implicated in every phase of the inflammatory response (Ford-Hutchison, 1985; Higgins, 1985), they are principally involved in leukocyte-mediated mechanisms. In particular, leukotriene B_4 (LTB_4) is a potent chemotactic agent (Clarke *et al*, 1994; Heidle *et al*, 1989) that binds with high affinity, stereospecificity, and saturability to receptors on neutrophil cell membranes (Goldman *et al*, 1982) and acts as both a chemokinetic and aggregating agent (Ford-Hutchison, 1980). Indeed, LTB_4 appears to be an important chemoattractant of neutrophils in BRD. In studies conducted by Heidel *et al* (1989), intradermal injection of LTB_4 was followed by rapid accumulation of bovine neutrophils. In another study (Clarke *et al*, 1994), conducted using a soft-tissue infection model established by inoculation of *P. haemolytica* into chambers implanted subcutaneously in cattle, an inhibitory effect of dexamethasone on both neutrophil influx into inoculated chambers and concentration of LTB_4 in infected chamber fluids, together with the temporal relationship between these two events, strongly suggested a chemotactic role for LTB_4.

Calcium-Mediated Control of Leukotriene B_4 Synthesis

Regulation of LTB_4 synthesis occurs by modulation of signal transduction and product feedback control and activation of 5-lipoxygenase (5-LO). Mammalian lipoxygenases exhibit regional specificity during interaction with a substrate and are designated as 5-LO, 12-lipoxygenase, or 15-lipoxygenase. Each enzyme inserts an oxygen molecule at carbon-5, -12, or -15, respectively. In resting cells, 5-LO exists in cytosol in a dormant ferrous state. When activated by hydroperoxidases, ATP, and Ca^{2+}, 5-LO

translocates to the cell membrane and associates with calcium activating proteins to oxidize arachidonic acid to many products, including LTB_4 (McMillin & Walker, 1992; Musser & Kreft, 1992; Lewis *et al*, 1990; Rouzer *et al*, 1988). Further control is exerted via oxygen radical products causing initial auto acceleration of enzyme activity and eventual self inactivation (Holzman, 1991; Higgins & Lees; 1984; Moncada & Vane, 1979). In addition to promoting translocation of 5-LO, Ca^{2+} also stimulates LTB_4 synthesis by simulating release of AA substrate from phospholipid membranes (Mayer & Marshall, 1993).

Release of AA from phospholipid membranes is accomplished by phospholipases, particularly phospholipase A_2 (PLA_2). Several different types of mammalian PLA_2 enzymes have been differentiated, based on structure and amino acid sequence (Mayer & Marshall, 1993). Two of these, designated Type II and Type IV, are nonpancreatic enzymes that metabolize phospholipids to lysophospholipids and fatty acids, including AA. Type II PLA_2 has a molecular mass of 14 kDa, may be extracellular or cell membrane associated, and is activated by increased intracellular calcium concentration $[Ca^{2+}]_i$. Type IV PLA_2 has a molecular mass of 85 kDa, is arachidonoyl-selective, and is localized intracellularly, but translocates to the cell membrane under the influence of increased $[Ca^{2+}]_i$ (Musser & Kreft, 1992). Thus, the activities of both types of PLA_2 are either directly or indirectly dependent on Ca^{2+}, which appears to be the principle second messenger responsible for stimulation of LTB_4 synthesis.

Pasteurella haemolytica leukotoxin

P. haemolytica produces several virulence factors that are believed to contribute to the development of severe pneumonic pasteurellosis (Confer *et al*, 1990). These include fimbriae, a polysaccharide capsule, lipopolysaccharide, and leukotoxin (LKT), which has particular relevance to the participation of neutrophils in the pathogenesis of disease. Leukotoxin is a protein exotoxin that is produced during the logarithmic growth phase of *P.*

haemolytica and is cytocidal only to ruminant leukocytes (Kaehler *et al*, 1980) and platelets (Clinkenbeard & Upton, 1991). At low concentrations, LKT causes neutrophil activation and degranulation in similar fashion to that caused by standard activating agents such as opsonized zymosan, as evidenced by the generation of oxygen radicals and the release of lysosomal enzymes (Maheswaren *et al*, 1992; Czuprynski *et al*, 1991). At higher concentrations, LKT is inhibitory or lethal to neutrophils, causing cell swelling, loss of membrane ruffling, development of a finely porous surface, and formation of large membrane defects prior to lysis (Clinkenbeard *et al*, 1989a).

The importance of LKT in the pathogenesis of BRD is illustrated by comparison of pneumonias caused by *P. haemolytica* with those caused by *P. multocida,* which produces endotoxin but no LKT. The pathology of the latter is characterized by suppurative bronchopnemonia without necrosis and fibrin exudation (Ames *et al*, 1985). This suggests that LKT has a specific role in producing the characteristic lesions of *P. haemolytica* pneumonia, which are principally neutrophil-mediated (Whiteley *et al*, 1991).

Several studies have confirmed that generation of neutrophil chemotactic factors, especially LTB_4, is promoted by the action of LKT. Exposure of bovine neutrophil suspensions to dilutions of crude LKT-containing culture supernatant caused increased release of 5-, 12-, and 15-eicosatetraenoic acids and LTB_4 in a dose-dependent manner (Henricks *et al*, 1992). Release of these eicosanoids was not due to decreased cellular retention and occurred in the absence of exogenous AA, suggesting that LKT stimulates synthesis of eicosanoids by promoting release of AA from phospholipid membranes. A more recent experiment confirmed the observations of Henricks *et al* (1992) and provided further support for the primary role of phospholipases (Clinkenbeard *et al*, 1994). The results of this experiment indicated that LTB_4 synthesis by bovine neutrophils was closely correlated with LKT-induced membrane damage and lysis and that both of these effects could be inhibited by neutralizing monoclonal anti-LKT antibody. Furthermore, when neutrophils were incubated with exogenous AA, LKT-induced synthesis of LTB_4 occurred

more rapidly and to a greater degree than when exogenous AA was not provided. Thus, the release of AA from phospholipid membranes by phospholipases appears to be the rate limiting step in LKT-induced eicosanoid synthesis. Considering the importance of Ca^{2+} in activation of PLA_2 and 5-LO, LKT-induced synthesis of LTB_4 probably involves calcium-mediated signal transduction.

Effect of Leukotoxin on Cell Membrane Permeability and Intracellular Calcium Concentration

Pasteurella haemolytica leukotoxin is a member of a family of widely deseminated cytolytic toxins known as "repeats-in-toxin" or RTX toxins. These exotoxins share extensive sequence homology, particularly in a region of tandem 9-amino acid repeats (Strathdee *et al*, 1987; Lo *et al*, 1985). Studies conducted using *Escherichia coli* α-hemolysin, a RTX toxin that has been more extensively studied than LKT, have indicated that the toxic mechanism of action involves the formation of functional pores that allow electrolyte flux across the plasma membrane of the target cell. Exposure of erythrocytes to this toxin leads to rapid influx of Ca^{2+} and low molecular weight sugars such as mannitol and sucrose, as well as a rapid efflux of K^+ (Bhakdi *et al*, 1986). The effect of α-hemolysin on membrane permeability characteristics has led to the hypothesis that α-hemolysin damages membranes by partial insertion into the lipid bilayer and formation of a discrete hydrophylic transmembrane pore with an approximate diameter of 3 nm. Clinkenbeard *et al* (1989a, 1989b) have reported similar effects of LKT on bovine lymphoma cells and bovine neutrophils. Exposure to LKT caused leakage of K^+, cell swelling, and then formation of large membrane defects. Incubation in hypertonic media containing carbohydrates with molecular weights ≥ 505 kD prevented LKT-induced cell swelling but not K^+ leakage, suggesting existence of stable pores with a functional diameter of approximately 0.9-1.2 nm. However, more recent studies of the moleculer

pathogenesis of α-hemolysin have reported that membrane pore size may be dependent on temperature, time, and toxin concentration, consistent with production of more heterogenous membrane lesions than previously postulated (Moayeri & Welch, 1994).

Regardless of whether LKT forms discrete or heterogenous transmembrane pores, it is clear that the permeability characteristics of membranes are changed, allowing transmembrane flux of electrolytes, particularly cations. In an experiment involving isolated bovine neutrophils, Ortiz-Carranza and Czuprynski (1992) determined that sublethal concentrations of LKT caused dose-dependent increases in $[Ca^{2+}]_i$. Treatment with verapamil, a calcium channel blocker, inhibited LKT-induced increase in $[Ca^{2+}]_i$ as well as luminol-dependent chemiluminescence response, thus suggesting the involvement of voltage-operated calcium channels.

Several different types of calcium channels exist in different cell types, with those in neurons and muscles being the best studied. Calcium-specific channels are classified according to whether they are voltage- or receptor-operated (Spedding & Paoletti, 1992). The former may be inhibited by calcium channel blockers such as verapamil, nifedipine, and diltiazem. Verapamil is a phenylalkylamine that inhibits voltage-dependent L-type channels by binding to 1a and 1b sites on calcium channel proteins. Nifedipine is a dihydropyridine while diltiazem is a benzothiazepine, both of which have selectivity for separate binding sites on the alpha-1 subunit of voltage-dependent L channels. Although the alpha-1 binding sites of nifedipine and diltiazem are different, the sites are allosterically linked (Spedding & Paoletti, 1992). Voltage-dependent calcium channels have been reported not to occur in neutrophil plasma membranes (Spedding & Paoletti, 1992), raising the possibility that the inhibitory effect of verapamil on LKT-mediated increase in $[Ca^{2+}]_i$ may result from inhibition of other types of verapamil-sensitive channels or from interaction with voltage-dependent calcium channels located in intracellular organelle membranes.

Another calcium antagonist, lanthanum chloride ($LaCl_3$), also inhibits transmembrane flux of Ca^{2+}, but in a manner that is distinct from the voltage-dependent calcium channel blockers. Lanthanum chloride has a very high affinity for calcium binding sites and, therefore, competes with Ca^{2+} for several Ca^{2+} channels without being transported across the plasma membrane (Thomson & Dryden, 1981; Gould *et al*, 1982). Lanthanum chloride was completely able to prevent the entry of extracellular Ca^{2+} into Ca^{2+}-depleted human neutrophils in contrast to verapamil, nifedipine and diltiazem (Montero *et al*, 1991; Rosales & Brown, 1992).

Potential Role of Anti-inflammatory Agents in Treatment of Bovine Respiratory Disease

More research is necessary to definitively determine the role of neutrophil $[Ca^{2+}]_i$ and calcium channels in the molecular pathogenesis of leukotoxin-induced inflammation. Once identified, calcium channels and calcium-mediated signal transduction pathways would be logical targets for pharmacological agents used in the therapy of BRD. Pharmacological inhibition of these calcium-mediated processes would inhibit the excessive inflammatory reactions associated with BRD and improve the efficacy of concurrently administered antibacterial agents.

Inflammation associated with pneumonic pasteurellosis has several implications relevant to antibacterial efficacy. *In vivo* efficacy of antibacterial agents may be compromised by binding to cell debris and other constituents of purulent exudates (Vandaux & Waldovogel, 1980; Barza, 1981). Also, inflammation results in changes in vascular permeability caused by inflammatory mediators and release of neutrophil-derived proteolytic enzymes (Clarke *et al*, 1989a; Clarke *et al*, 1989b). Accumulation of fibrin and exudate results in increased drug diffusional distances and lower drug concentrations in infected tissue (Clarke *et al*, 1992). Therefore, the rationale for using anti-inflammatory

agents concurrently with antibacterial agents is to attenuate the detrimental effects of inflammation on efficacy of antibacterial agents.

Several reports have described the use of non steroidal and steroidal anti-inflammatory agents in the treatment of BRD (Clarke *et al*, 1992; Christie *et al*, 1977; Radostitis *et al*, 1994). Unfortunately, the use of non steroidal anti-inflammatory drugs (NSAIDs) has not resulted in favorable therapeutic outcomes, possibly because of their incomplete inhibition of AA metabolism. The NSAIDs exert their anti-inflammatory effect by inhibiting cyclo-oxygenase; they generally have negligible effect on production of lipoxygenase products (Higgins & Lees, 1984). Indeed, increased availability of AA substrate arising from inhibition of cyclo-oxygenase is believed to cause an increase in leukotriene synthesis because of substrate diversion. Corticosteroid anti-inflammatory agents indirectly inhibit the release of AA from membrane phopholipids by phospholipases and, therefore, ultimately inhibit the synthesis of eicosanoids by both cyclo-oxygenase and lipoxygenase (Higgins & Lees, 1984). However, corticosteroids also inhibit many other important humoral and cell-mediated host defense reponses that contribute to the animal's ability to erradicate *P. haemolytica.* In contrast, specific inhibition of calcium channels and/or calcium-mediated signal transduction could block AA release and metabolism as well as preserve the functional integrity of neutrophils by preventing damage to their membranes.

Several studies have explored the possible use of calcium channel blockers as anti-inflammatory agents. Non infectious inflammation, induced by injection of formalin into rat's paws, was inhibited by pretreatment with nitrendipine and nicardipine, both inhibitors of L-type calcium channels (Gurdal *et al*, 1992). However, in experiments involving pretreatment of rabbits with calcium channel blockers prior to eye irritation with capsaisin, results were mixed with diltiazem exhibiting the most anti-inflammatory activity and verapamil the least (Gonzalez *et al*, 1993).

A possible impediment to using calcium channel blockers as anti-inflammatory agents is the detrimental effect that they may have on host defense responses in general. Calcium channel blocker therapy has been associated with iatrogenic gingival hyperplasia, a disorder that is associated with immunosuppression (Lawrence *et al*, 1994). Furthermore, nitrendipine has been reported to significantly inhibit fMLP-induced adhesion of neutrophils to endothelial cells as well as other critical functions of neutrophils (Perry *et al*, 1993) and lymphocytes. *In vivo* studies involving viral infections in human subjects (Teitz & Thompson, 1995) and protozoal infections in mice (Kalra *et al*, 1993) have provided further information concerning the potentially immunosuppressive effects of calcium channel blockers. Although pneumonic pasteurellosis presents a suitable potential indication for calcium channel blockers because the inflammatory response itself is a major contributor to tissue damage, attenuation of the inflammatory reaction must be accomplished without excessive suppression of beneficial host defense responses.

CHAPTER III

EXPERIMENTAL OBJECTIVES

The hypothesis upon which this research was based is that LTB_4 production and cell membrane damage caused by exposure of bovine neutrophils to *P. haemolytica* LKT is mediated by increased $[Ca^{2+}]_i$ resulting from influx of extracellular calcium through voltage-dependent calcium channels.

This hypothesis was tested by characterizing the effect of LKT on $[Ca^{2+}]_i$ of isolated bovine neutrophils and by examining the effect of different types of calcium antagonists on LKT-induced responses. To eliminate the potential contribution of other virulence factors of *P. haemolytica*, responses to partially purified LKT were compared with those produced by similarly purified culture supernatant of a LKT-deficient mutant of *P. haemolytica*. The specific objectives were:

(1) to determine whether neutrophil LTB_4 synthesis and membrane damage following exposure to LKT were dependent on increased $[Ca^{2+}]_i$; and

(2) to investigate the role of voltage-dependent L-type calcium channels and exchange of extracellular and intracellular calcium in LKT-induced increase in $[Ca^{2+}]_i$, membrane damage, and LTB_4 synthesis.

CHAPTER IV

MATERIALS AND METHODS

Preparation of *P. haemolytica* Wildtype Leukotoxin

,Leukotoxin (LKT) preparations were made by P. Clinkenbeard, Department of Pathology, Oklahoma State University from a *P. haemolytica* biotype A, serotype 1 wildtype strain (LKT) and an isotonic leukotoxin-deficient mutant strain A [LKT(-)], produced by allelic replacement of lktA with β-lactamase bla gene by Dr. George Murphy and L. Whitworth, Department of Pathology, Oklahoma State University (Murphy *et al*, 1995) These strains were grown in 150 ml BHI broth to an OD_{600nm} of 0.8-1.0. Bacteria collected from the BHI cultures were inoculated into 250 ml RPMI 1640 medium (pH 7.0, 2.2 g/l $NaHCO_3$) containing 0.5% bovine serum albumin (A-6003 fraction V, essentially fatty acid free, Sigma Chemical Co., St. Louis, MO) to an OD_{600nm} of 0.25. The RPMI cultures were grown at 37°C, and 70 oscillations/min to an OD_{600nm} of 0.8-1.0, and the culture supernatants were harvested following centrifugation at 8,000 x g for 30 minutes (Sorvall GS3 rotor, DuPont Co., Wilmington, DE). This and all subsequent steps were conducted at 4°C. Culture supernatants were concentrated by addition of solid ammonium sulfate (361 g/L) to yield 60% saturation and the precipitated material was collected by centrifugation at 8,000 x g for 45 minutes (Sorvall GS3 rotor). Precipitates were resuspended in 3 ml of 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0 buffer, and then dialyzed against 500 ml of the same buffer overnight. Dialyzed concentrated culture supernatants were stored frozen at -135°C. Control uninoculated concentrated culture supernatants were similarly prepared.

Assay of *P.haemolytica* Wildtype Leukotoxin

P.haemolytica LKT activity was assayed by measuring cytoplasmic lactate dehydrogenase (LDH) released from target cells. In each particular experiment, isolated bovine neutrophils or tissue culture bovine lymphoma cells (BL3 cells, CRL 8037, American Type Culture Collection, Rockville, MD) were exposed to LKT in 1.5 ml microfuge tubes or 96-well round bottom microtiter plates at 37°C. Exposure was terminated by centrifugation (2 minutes at 5,650 x g for microfuge tubes and 5 minutes at 700 x g for microtiter plates) and the concentration of extracellular LDH was assayed by transfer of 100 µl of incubation supernatant to wells of a flat bottom 96-well microtiter plate. The plate was warmed to 37°C, 100 µl LDH assay reagent [LD-L 228 - 50 ml, (Sigma Chemical Co., St Louis, MO), rehydrated by addition of 25 ml H_2O] at 37°C was added, and the LDH activity was measured in a thermally-controlled kinetic microtiter plate reader (Thermomax, Molecular Devices Palo Alto, CA) at 340 nm for 2 minutes at 37°C. Data were reported as mOD/minute. Maximal LDH leakage was determined by replacing LKT with Triton x100 (final concentration was 0.1% v/v), and background LDH leakage was determined by replacing LKT with appropriate buffer control. Percent specific leakage of LDH was calculated using the formula:

$$\%\ \text{specific leakage LDH} = \frac{(\text{LKT-induced LDH leakage} - \text{background LDH leakage}) \times 100}{\text{maximal LDH leakage} - \text{background LDH leakage}}$$

Quantitation of Leukotoxic Activity

The amount of leukotoxic activity used in experiments was quantified as toxic units (TU) using BL3 cells. These cells were cultured at 37°C and 5% CO_2 in 50% Leibovitz L-5 and 50% Eagle minimal essential medium containing 10% fetal bovine

serum, 2 mM L-glutamine, 50 mg/l gentamicin, and 2.2 g/l $NaHCO_3$ as described elsewhere (Clinkenbeard *et al*, 1989b). LKT was serially diluted, leukotoxic activity measured by LDH leakage as described above, and the reciprocal of the dilution causing 50% maximal leakage of LDH determined graphically. One TU caused leakage of 50% LDH from 4 x 10^5 BL3 cells in 200 µl at 37°C in 1 hour. Each assay was conducted in triplicate and the TU were determined for each experiment.

Preparation of Neutrophil Suspensions

Whole bovine blood was collected in 1% sodium citrate by venipuncture and then centrifuged in 50 ml polypropylene conical tubes (Corning Incorporated, Corning, NY) at 600 x g for 30 min at 4°C. The plasma, buffy coat, and the top portion of the red cell column were aspirated to leave 10 ml of cell suspension, which was then subjected to two cycles of hypotonic lysis (Weiss *et al*, 1989). In the first cycle, 20 ml of sterile, distilled water was added to the red cell suspension, which was mixed for 60 seconds and tonicity was then restored by adding an equal volume of double-strength phosphate buffered saline (PBS). Suspensions were then centrifuged (Sorvall RC5C, HS-4 rotor, DuPont Co., Wilmington, DE) for 10 min at 200 x g and the supernatant discarded. Neutrophils were resuspended with 5 ml of PBS and subjected to the second cycle of hypotonic lysis in which 10 ml of sterile distilled water was added, the cell suspension was mixed for 60 seconds, and 10 ml of double strength PBS added to restore isotonicity. Thereafter, cells were resuspended in 5 ml of $CaCl_2$-free Hank's balanced salt solution (HBSS) (Sigma Chemical Co., St Louis, MO) containing 0.5 mM $MgCl_2$ (Sigma Chemical Co., St Louis, MO) and 50 µM EGTA (Sigma Chemical Co., St Louis, MO). Cells were enumerated by hemocytometer and viability was assessed by trypan blue exclusion. Differential counts were conducted on stained smears (Diff-Quik, Baxter Healthcare Corp., Miami, FL).

Preparations were then diluted to a final concentration of 6 x 10^6 viable neutrophils/ml HBSS.

Neutrophil Loading with Fluorescent Calcium Indicator

Intracellular calcium concentration was measured using the membrane-permeable acetoxymethyl (AM) ester of the fluorescent calcium indicator, Fluo-3 (Molecular Probes Inc., Eugene, OR). Membrane permeable Fluo-3 AM diffuses across the neutrophil membrane, but is retained within the cytosol after it is hydrolyzed intracellularly to the membrane impermeable free acid (Kao *et al*, 1989). Neutrophil suspensions contained in 50 ml polypropylene conical centrifuge tubes (Corning Incorporated, Corning, NY) and protected from light were incubated with Fluo-3 AM for 30 minutes at 22°C while constantly mixing on a cell rotator (Angenics, Cambridge, MA). Sufficient Fluo-3 AM (in DMSO containing 0.14% pluronic acid) was added to the cell suspensions to achieve a final concentration of 5 μM. Loaded cells were then centrifuged at 200 x g and 4°C for 10 minutes, the supernatant was discarded, and the cells were resuspended in 10 ml PBS before centrifuging again at 200 x g and 4°C for 10 minutes. After discarding the supernatant, cells were resuspended in 3 ml HBSS, enumerated by hemocytometer and then resuspended in HBSS to 1 x 10^7 cells/ml.

Exposure of Isolated Neutrophils to Leukotoxin

The effects of LKT and other stimulators on $[Ca^{2+}]_i$, neutrophil membrane damage, and LTB_4 synthesis were tested in 96-well flat bottom microtiter plates (Corning Glass Works, Corning, NY). Ten microliters of 25 mM $CaCl_2$ (Sigma Chemical Co., St. Louis, MO) and 2 μl antifluoroscein antibody (Molecular Probes, Eugene, OR), diluted 1:5 in PBS, were added in sequence to 250 μl of cell suspension. The antifluoroscein

antibody quenched fluorescence of extracellular indicator. Thereafter, 25 µl of diluted LKT, LKT-deficient control, 40 µM 4-bromo A23187 (Sigma Chemical Co., St. Louis, MO) in DMSO, 0.5% Triton x100, or PBS were added to wells, lids were placed on the plates, and the plates were incubated at 37°C. Fluorescence (490 nm excitation, 523 nm emission) was measured at times indicated for each specific experiment, using a spectrofluorometer (Cytofluor 2300 Fluorescence Measurement System, Millipore Corp., Bedford, MA). Experiments were terminated by centrifugation at 200 x g at 4°C for 5 minutes and aliquots of supernatants were removed for assay of LDH and LTB_4. All experiments included triplicate wells for each of the treatments.

Intracellular Calcium Dependence of Leukotoxin-Induced Neutrophil Membrane Damage and Synthesis of Leukotriene B_4

The effects of LKT on $[Ca^{2+}]_i$, neutrophil membrane integrity and LTB_4 synthesis were investigated by exposing neutrophil suspensions to dilutions (1:10, 1:50, 1:100, and 1:1000) of LKT or LKT-deficient control, measuring fluorescence and then incubating for 120 minutes before harvesting samples for assay of LDH and LTB_4.

Neutralization of LKT activity by murine antileukotoxin monoclonal antibody MM601 (gift from S.Srikumaran, University of Nebraska) was tested by first preincubating 25 µl undiluted and 1:100, 1:10,000; and 1:1,000,000 dilutions of LKT with 25 µl of 1:500 diluted MM601 ascitic fluid or irrelevant murine IgG1 monoclonal antibody (Sigma Chemical Co., St. Louis, MO) at 4°C for 30 min, as previously described (Gentry & Srikumaran, 1991). Thereafter, 25 µl aliquots of preincubated LKT were added to neutrophil suspensions to achieve final LKT dilutions of 1:20, 1:2,000, 1:200,000, and 1:20,000,000, fluorescence was measured and plates were incubated for 120 minutes before assay of LDH and LTB_4.

Time dependent effects of LKT were examined by incubating neutrophil suspensions exposed to 1:100 or 1:100,000 dilutions of LKT for 5, 30, 60, 90, and 120 minutes. Separate plates containing all duplicates of LKT dilutions and relevant controls were used for each incubation period. At the end of each incubation period, fluorescence was measured, plates were centrifuged and supernatants were harvested for assay of LDH and LTB_4. This experiment was repeated using more closely spaced incubation times; 5, 15, 30, 45, and 60 minutes.

Exchange of Extracellular and Intracellular Calcium in LKT-Induced Increase in Intracellular Calcium Concentration and the Role of Voltage-Dependent L-Type Calcium Channels

The dependence of LKT-induced responses on extracellular calcium was investigated by altering the concentration of calcium in the neutrophil suspension media. Neutrophils were suspended in calcium-free HBSS, HBSS with 1 mM $CaCl_2$, HBSS with 1 mM EGTA, or HBSS with 3 mM $CaCl_2$ and 1 mM EGTA. Additional $CaCl_2$ was not added as in previous experiments. Fluorescence was measured immediately after addition of 25 µl 1:100 LKT and LDH and LTB_4 assays were performed after 120 minutes of incubation.

The involvement of voltage-dependent calcium channels was explored using the specific calcium channel blockers, verapamil and nifedipine. Furthermore, the role of extracellular calcium was studied using $LaCl_3$, which inhibits exchange of extracellular and intracellular calcium. Twenty five microliters of either 5 mM, 0.5 mM, 50 µM, 5 µM or 0.5 µM verapamil in DMSO, nifedipine in DMSO, or $LaCl_3$ in water were added to neutrophil suspensions, which were then incubated for 20 minutes before adding 25 µl 1:100 LKT. Fluorescence was measured and suspensions were then incubated further for 120 minutes before concentrations of LDH and LTB_4 were measured.

The effect of verapamil on neutrophils subjected to plasma membrane perturbation by an agent other than LKT was studied. Neutrophil suspensions received 25µl of 0 mM, 5 µM, 10µM, and 5mM verapamil dilutions in DMSO, were incubated for 20 minutes, and were than stimulated by addition of 25µl of 100 mM digitonin solution in DMSO. Fluorescence was measured and suspensions were then incubated further for 120 minutes before concentrations of LTB_4 were measured.

Determination of Intracellular Calcium Concentration

Cytosolic calcium was determined using the formula (Kao *et al*, 1989):

$$[Ca^{2+}]_i = \frac{K_d (F-F_{min})}{F_{max} - F}$$

Maximum fluorescence (F_{max}) was determined by exposing control cells to 4-bromo A23187, measuring fluorescence after quenching by addition of 20 µl 20 mM $MnCl_2$ (F_{Mn}), and then using the formula:

$$F_{max} = \frac{(F_{Mn}-F_{bkg})}{0.2} + F_{bkg}$$

where F_{bkg} is the fluorescence emitted by Fluo-3 - loaded cells in the absence of an excitation beam. Minimum fluorescence (F_{min}) was determined using the formula:

$$F_{min} = \frac{(F_{max} -F_{bkg})}{40} + F_{bkg}$$

The Ca^{2+}-Fluo-3 dissociation constant (K_d) was determined by M. Shelton at Oklahoma State University by using a commercially prepared kit (Molecular Probes, Eugene, OR) which measured the fluorescence of 4-bromo-A23187 exposed bovine neutrophils

suspended in a range of EGTA-containing Ca^{2+} buffers at pH 7.2 and 22°C. The calibration curve for Fluo-3 was linear and indicated a Kd of 358 nM (Figure 1).

Leukotriene B_4 Immunoassay

Concentrations of LTB_4 were determined using a commercially prepared radioimmunoassay kit (NEN Research Products, Du Pont, Medical Products Department, Boston, MA) that has been previously validated (Clinkenbeard *et al*, 1994).

Statistical Analyses

Data were analyzed using SYSTAT intelligent software for the Macintosh, Version 5.2 (SYSTAT Inc, Evanston, IL) (Wilkinson, 1992). Unpaired t-tests were used to compare each response variable at each LKT dilution in studies comparing LKT to LKT (-) mutant as well as in studies comparing LKT incubated with irrelevant IgG1 to LKT incubated with MM601. General linear analyses of variance followed by separation of means using Scheffe's test for each variable were used to evaluate the effects of extracellular calcium concentration. Time-dependent trends in response variables were identified using the method of orthogonal polynomials. The effects of verapamil, nifedipine and $LaCl_3$ on LKT-induced effects as well as the effect of verapamil on digitonin-induced effects were analyzed using the general linear model. Thereafter, each drug treatment was compared with its relevant control using Dunnet's test. Differences between means and order of polynomials were considered significant at the $P < 0.05$ level.

CHAPTER V

RESULTS

Exposure of isolated bovine neutrophils to *P. haemolytica* wildtype LKT resulted in significant increases in $[Ca^{2+}]_i$, LTB_4 production, and LDH release at all LKT dilutions tested compared to the LKT deficient controls (Figure 2). All LKT dilutions were highly lytic and produced similar increases in $[Ca^{2+}]_i$ and LTB_4 synthesis. Leukotoxin was effectively inactivated by pre-incubation with the anti-LKT monoclonal antibody, MM601, except for the 1:20 dilution of LKT, which overwhelmed the neutralizing effect of the antibody (Figure 3). Leukotoxin preparations pretreated with irrelevant murine IgG control produced concentration-dependent effects on $[Ca^{2+}]_i$, LTB_4 synthesis, and LDH release.

Exposure of neutrophils to the 1:1,000 dilution of LKT caused an immediate increase in $[Ca^{2+}]_i$, followed within 15 minutes by rapid and parallel increases in LTB_4 synthesis and LDH leakage, which continued to increase over the 60 minute incubation period (Figure 4). Neutrophils exposed to a concentration of LKT that was too low to cause significant LDH release (1:100,000) still increased synthesis of LTB_4, although at lower levels than observed when higher concentrations of LKT were used (Figure 5). After the initial increase in LTB_4 production, synthesis gradually declined over the 120 minute incubation period.

Exposing neutrophils to LKT in Ca^{2+}-free buffer resulted in significantly smaller increases in $[Ca^{2+}]_i$ and LTB_4 synthesis in comparison with responses observed when neutrophils were exposed to LKT in buffer containing 1 mM $CaCl_2$ (Figure 6). When 1 mM EGTA and no $CaCl_2$ was added to the suspension buffer, further reductions in increases in $[Ca^{2+}]_i$ and LTB_4 synthesis were measured.

Inhibitory effects of verapamil on increase in $[Ca^{2+}]_i$ and LTB_4 synthesis were only observed at the highest drug concentration tested (0.5 mM) (Table I). Lower drug concentrations also inhibited LDH release but this effect was not consistent at the 50 μM drug concentration. Nifedipine inhibited LTB_4 synthesis at the highest concentration tested but did not significantly affect the other responses. In contrast to the calcium channel blockers, $LaCl_3$ caused relatively greater inhibition of $[Ca^{2+}]_i$ and LDH release.. Although significant, the inhibitory effect of $LaCl_3$ on LTB_4 synthesis was relatively small. The effects of verapamil on digitonin-induced increase in $[Ca^{2+}]_i$ and LTB_4 synthesis were similar to those observed when neutrophils were exposed to LKT: high verapamil concentration (0.5 mM) significantly decreased both of these responses in digitonin-exposed neutrophils (Figure 7).

CHAPTER VI

DISCUSSION & CONCLUSIONS

A previous study demonstrated that LKT-induced synthesis of LTB_4 by isolated bovine neutrophils was dependent on extracellular Ca^{2+} and that this response was closely correlated with neutrophil lysis (Clinkenbeard *et al*, 1994). Another study reported that exposure of bovine neutrophils to sublytic concentrations of LKT caused an increase in $[Ca^{2+}]_i$ (Ortiz-Carranza & Czuprynski, 1992). The present study explored the possible involvement of intracellular Ca^{2+} as a second messenger in LKT-induced neutrophil plasma membrane damage and synthesis of LTB_4.

Comparison between responses elicited by LKT and LKT-deficient control preparations confirmed that increased $[Ca^{2+}]_i$, LTB_4 synthesis, and cell lysis were all caused by LKT and not by other constituents of the partially purified culture supernatants, such as lipopolysaccharide. The LKT-deficient *P. haemolytica* mutant used in the preparation of the negative control was produced by allelic replacement of the *lkt A* gene, a mutation that had no effect on growth rate, lipopolysaccharide production, or capsule formation (Murphy *et al*, 1995). Therefore, except for the absence of the 102-kDa LKT protein, the LKT-deficient preparations were identical to those prepared from wild-type culture supernatants. The neutralizing effect of the anti-LKT monoclonal antibody, MM601, further confirmed that LKT in purified *P. haemolytica* supernatant was responsible for increased $[Ca^{2+}]_i$, LTB_4 synthesis, and cell membrane damage.

Studies utilizing MM601-treated controls demonstrated a positive correlation between LKT concentration, $[Ca^{2+}]_i$, and neutrophil responses (LTB_4 synthesis and LDH release), thus suggesting a signal transduction role for intracellular Ca^{2+}. This relationship was further investigated by examining the effects of LKT on bovine neutrophils as a function of time. In studies involving exposure of neutrophils to lytic

concentrations of LKT over a period of 60 minutes, the temporal relationship between the initial increase in $[Ca^{2+}]_i$ and subsequent synthesis of LTB_4 and release of LDH are consistent with the contention that both of these responses were dependent on increased $[Ca^{2+}]_i$. This conclusion is consistent with the knowledge that intracellular calcium is involved in the release of AA from membrane phospholipids by phospholipases as well as the oxidation of AA by lipoxygenase to LTB_4 (Holzman, 1991; Higgins & Lees, 1984; Moncada & Vane, 1979). Simultaneous release of LDH and synthesis of LTB_4 can be explained by Ca^{2+}-dependent translocation and activation of phospholipase A_2, resulting in generation of membrane-damaging lysophospholipids and release of the eicosanoid precursor, AA. Further investigations involving the use of sublytic dilutions of LKT indicated that LTB_4 synthesis could be stimulated in the absence of significant LDH release. This suggests either that intracellular Ca^{2+}-dependent leukotriene synthesis and plasma membrane damage may not necessarily result from activation of a mechanism or enzyme common to both responses, or that sufficient arachidonic acid may be released from mild membrane damage for a significant LTB_4 production without significant LDH leakage. Nevertheless, although not conclusive, all time-dependent studies supported the hypothesis that LKT-induced synthesis of LTB_4 and plasma membrane perturbation are mediated via signal transduction involving intracellular Ca^{2+}. The rapid decrease in $[Ca^{2+}]_i$ after initial exposure to lytic dilutions of LKT was surprising, considering the abundance of Ca^{2+} in the extracellular medium and the loss of plasma membrane integrity, as evidenced by leakage of LDH. It is possible that this decrease in $[Ca^{2+}]_i$ may have been artifactual or that plasma membrane damage could have resulted in leakage of the fluorescent Ca^{2+} indicator prior to the detection of plasma membrane damage by LDH leakage as the molecular weight of Fluo-3 is 1.1 kD and the molecular weight of LDH is 160 kD.

The source of the calcium involved in the intracellular calcium signal was investigated by altering the concentration of extracellular calcium available to the

neutrophils. In comparison with neutrophils suspended in Ca^{2+}-containing buffer, neutrophils in Ca^{2+}-free buffer produced significantly less LTB_4, thus demonstrating dependence of this effect on extracellular Ca^{2+}. Addition of EGTA to the buffer caused further reduction in LKT-induced effects, suggesting that release of Ca^{2+} from intracellular stores may also contribute to increased cytosolic $[Ca^{2+}]_i$. Incubation of cells in buffer containing EGTA not only chelates extracellular Ca^{2+}, but also causes rapid depletion of intracellular calcium stores as Ca^{2+} rapidly diffuses down a concentration gradient from intracellular organelles to extracellular buffer (Rosales & Brown, 1992). Therefore, both extra- and intra-cellular sources of calcium apparently contribute to increased $[Ca^{2+}]_i$ and the signal transduction event resulting from exposure to LKT.

Verapamil has been previously reported to inhibit the LKT-induced luminol-dependent chemiluminescence response of bovine neutrophils (Ortiz-Carranza & Czuprynski, 1992), suggesting that influx of extracellular Ca^{2+} into the cytosol may occur via L-type voltage-independent calcium channels. The present study also demonstrated inhibitory effects of verapamil (and another inhibitor of calcium channels, nifedipine), but only at drug concentrations that were much higher than those required to inhibit Ca^{2+} flux across nerve and cardiac muscle plasma membranes. Either verapamil inhibits calcium influx in a non-specific manner or the target L-type calcium specific channels are inaccessible and only reached at high concentrations. The latter is most likely considering the recent identification of L-type voltage-dependent channels on neutrophil organelle membranes (Rosales & Brown, 1992) and the evidence indicating that these do not occur in neutrophil plasma membranes (Tscharner *et al*, 1986). The inhibitory effect of verapamil on digitonin-induced increase in $[Ca^{2+}]_i$ and LTB_4 synthesis could be used to further support to the suspicion that L-type calcium channels located on intracellular membranes are involved as low concentrations of digitonin perturb the plasma membrane in a non-specific manner allowing influx of extracellular calcium. Apparently, the rise in $[Ca^{2+}]_i$ is supplemented by additional Ca^{2+} flux through

verapamil-inhibitable channels. Indeed, influx of extracellular Ca^{2+} can serve as a trigger for release of Ca^{2+} from intracellular stores (Berridge, 1993), although this mechanism involves ryanodine receptor-linked channels: the association between extracellular Ca^{2+} flux and L-type voltage-dependent channels on organelle membranes has yet to be investigated.

Exposure to $LaCl_3$ produced inhibitory effects that were different from those caused by the calcium channel blockers. In contrast to the calcium channel blockers, which primarily inhibited LTB_4 synthesis, $LaCl_3$ had relatively more effect on LDH release. Furthermore, 0.5 mM $LaCl_3$ caused a 53% reduction in $[Ca^{2+}]_i$ response versus the 23% reduction caused by the same concentration of verapamil. Considering that $LaCl_3$ binds to calcium binding sites with higher affinity than Ca^{2+} and, therefore, is recognized to inhibit Ca^{2+} flux accross the plasma membrane, (Weiss, 1974, Rosales & Brown, 1992) influx of extracellular Ca^{2+} makes the major contribution to LKT-induced increase in $[Ca^{2+}]_i$ and is primarily responsible for plasma membrane damage and LDH leakage. Assuming that the calcium channel blockers interact with calcium channels on organelle membranes, leukotriene B_4 synthesis, therefore, apparently relies upon the contribution of intracellular Ca^{2+} stores for transduction of the activating signal. This evidence in support of different Ca^{2+}-dependent mechanisms for LKT-induced LTB_4 synthesis and plasma membrane damage is consistent with the results of the time-dependent studies discussed above.

In conclusion, this study demonstrated that exposure of isolated bovine neutrophils to *P. haemolytica* LKT induces an increase in $[Ca^{2+}]_i$ followed by LTB_4 synthesis and plasma membrane damage. Thus, changes in $[Ca^{2+}]_i$ may constitute an important stage in the signal transduction process responsible for LKT-induced neutrophil lysis and inflammatory response. Furthermore, LKT-induced increase in $[Ca^{2+}]_i$ is dependent on the contributions of influx of extracellular Ca^{2+} and mobilization of intracellular Ca^{2+} stores. Although LTB_4 synthesis and plasma membrane damage are

both Ca^{2+}-dependent, the precise mechanisms responsible for these effects appear to differ: membrane damage is primarily dependent on influx of extracellular Ca^{2+} whereas LTB_4 synthesis depends on supplementary Ca^{2+} released from intracellular organells via L-type voltage-dependent channels.

Further studies are needed to better define the specific mechanisms whereby LKT causes increase in $[Ca^{2+}]_i$ before calcium antagonists can be suggested as possible therapeutic agents for treatment of pneumonic pasteurellosis. Nevertheless, based on these investigations, L-type calcium channel blockers are unlikely to be efficacious because only high and potentially toxic concentrations are anti-inflammatory. At these concentrations other host defense functions, such as those involving natural killer cells and other leukocytes would be depressed (Teitz & Thompson, 1995; Gurdal *et al*, 1992), thus compromising the animal's ability to fight the infection. Enzymes responsible for membrane damage and generation of lipid inflammatory mediators are likely to provide better targets for anti-inflammatory therapies designed to depress neutrophil chemotaxis and preserve phagocytic function.

REFERENCES

Allan EM, Gibbs HA, Wiseman A, Selman IE. Seqential lesions of experimental bovine pneumonic pasteurellosis. Vet Rec 117:438, 1985.

Ames TR, Markham RJF, Asibo-Opuda J, Leininger JR, Maheswaren SK. Pulmonary response to intratracheal challenge with *Pasteurella haemolytica* and *Pasteurella multocida.* Can J Comp Med 49:395,1985.

Barza M. Principles of tissue penetration of antibiotics. J Antimicrob Chemoth. 8(suppl.C);7, 1981.

Berridge, MJ. Inisitol phosphate and calcium silgnalling. Nature 361:315, 1993.

Bhakdi S, Mackman N, Nicaud J, Holland IB. Escherichia coli alpha-hemolysin may damage target cell membranes by generating transmembrane pores. Infect Imun 52:63, 1986.

Breider MA, Walker RD, Hopkins FM, et al. Pulmonary lesions induced by *Pasteurella haemolytica* in neutrophil sufficient and neutrophil deficient calves. Can J Vet Res 52:205, 1988.

Christie BM, Pierson RE, Braddy PM, et al. Efficacy of corticosteroids as supportive therapy for bronchial pneumonia in yearling cattle. Bovine Pract 12:115, 1977.

Clarke CR, Lauer AK, Barron SJ, et al. The role of eicosanoids in the chemotaxic response to *Pasteurella haemolytica* infection. J Vet Med B 41:483, 1994.

Clarke CR, Barron SJ, Ayalew S, et al. Response of *Pasteurella haemolytica* to erythromycin and dexamethazome in calves with established infection. Am J Vet Res 53:684, 1992.

Clarke CR, Short CR, Corstvet RE, et al. Interaction between *Pasteurella haemolytica*, sulfadiazine/trimethoprim and bovine viral diarrhea virus. Am J Vet Res 50:1557, 1989a.

Clarke CR, Short CR, Corstvet RE, et al. Effect of *Pasteurella haemolytica* infection on the distribution of sulfadiazine and trimethoprim into tissue chambers implanted subcutaneuosly in cattle. Am J Vet Res 50:1551, 1989b.

Clinkenbeard, KD, Mosier, DA, and Confer, AW Transmembrane Pore Size and Role of Cell Swelling in Cytotoxicity Caused by *Pasteurella haemolytica* Leukotoxin. Infect Immun 57(2):420, 1989a.

Clinkenbeard KD, Mosier DA, Confer AW. Effects of *Pasteurella haemolytica* leukotoxin on isolated bovine neutrophils. Toxicon 27(7):797,1989b.

Clinkenbeard KD, Clarke CR, Hague CM, et al. *Pasteurella haemolytica* leukotoxin-induced synthesis of eicosanoids by bovine neutrophils in vitro. J Leuko Biol 56:644, 1994.

Clinkenbeard KD, Clarke CR, Morton, RJ, et al. Role of *Pasteurella haemolytica* leukotoxin in virulence and immunity in shipping fever pneumonia. Compend Cont Ed Pract Vet 14:1249, 1992.

Clinkenbeard KD, Upton ML. Lysis of bovine platelets by *Pasteurella haemolytica* leukotoxin. Am J Vet Res 52(3):453, 1991.

Confer AW, Panciera RJ, Clinkenbeard KD, Mosier DA. Molecular aspects of virulence of *Pasteurella haemolytica.* Can J Vet Res 54:S48, 1990.

Czuprynski CJ, Noel EJ, Ortiz-Carranza, et al. Activation of bovine neutrophils by partially purified *Pasteurella haemolytica* leukotoxin. Infect Immun 59(9):3126, 1991.

Czuprynski CJ, Noel EJ. Influence of *Pasteulla haemolytica* A1 crude leukotoxin on bovine neutrophil chemoluminescence. Infect Immun 58(5):1485, 1990.

Ford-Hutchison AW. Leukotrienes: their formation and role as inflammatory mediators. FESEB 44:25, 1985.

Frank GH, Briggs RE, GilletteKG. *Pasteurella haemolytica* serotype 1 colonization of the nasal passages of virus-infected calves. Am J Vet Res 48(12):1674, 1987.

Frank GH. Pasteurellosis in cattle. In *Pasteurela and Pasteurellosis* (C. Adlam and JM Rutter, eds) Academic Press, London, 197-222, 1989.

Gentry MJ, Srikumaran S. Neutralizing monoclonal antibodies to Pasteurella haemolytica leukotoxin affinity-purified from crude culture supernatants. Microb Pathog 10:411, 1991.

Goldman DW, Goetzl EJ. Specific binding of leukotriene B_4 to receptors on human polymorphonuclear leukocytes. J Immunol 129:1600, 1982.

Gonzalez GG, Garcia-de-la-Rubia P, Gallar J, Belmonte C. Reduction of capsaisin-induced ocular pain and neurogenic inflammation by calcium antagonists. Invest Opthalmol Vis Sci 34(12):3329, 1993.

Gosset KA, Potter ML, Enright FM, et al. Assessment of the inflammatory response in bovine pneumonic pasteurellosis by bronchoalveolar lavage cytology. Proc Am Assoc Vet Lab Diagn 257, 1984.

Gould, RJ, Murphy, KKM, and Snyder SH. [3H] Nitrendipine-labeled calcium channels discriminate inorganic calcium agonists and antagonists. Proc. Natl. Acad. Sci. U.S.A. 79, 3656, 1982.

Gurdal H, Sara Y, Tulunay FC. Effects of calcium channel blockers on formalin-induced nociception and inflammation in rats. Pharmacology 44(5):290, 1992.

Heidle JR, Taylor SM, Laegreid WW, et al. In vivo chemotaxis of bovine neutrophils induced by 5-lipoxygenase metabolites of arachidonic and eicosapentaenoic acid. Am J Physiol 34:671, 1989.

Henricks PAJ, Binkhorst GJ, Drijver AA, et al. *Pasteurella haemolytica* leukotoxin enhances production of leukotriene B_4 and 5-hydroxyeicosatetraenoic acid by bovine polymorphonuclear leukocytes. Infect Immun 60(8):3238, 1992.

Higgins AJ. The biology, pathophysiology and control of eicosanoids in inflammation. J Vet Pharmacol Therap 8:1, 1985.

Higgins AJ, Lees P. The acute inflammatory process, arachidonic acid metabolism and the mode of action of antiinflammatory drugs. Equine Vet J 16(3):163, 1984.

Holzman MJ. Arachidonic acid metabolism: implications of biological chemistry for lung and disease. Am Rev Respir Dis 143:188, 1991.

Hungerford TG. Diseases of Livestock, 9th ed. McGaw-Hill, Sydney. 432,1990.

Kaehler KL, Markham RJF, Muscoplat CC, Johnson DW. Evidence of species specificity in cytocidal effects of *Pasteurella haemolytica*. Infect Immun 30:615, 1980.

Kalra A, Dubey ML, Ganguly NK, Mohan K, Mahajan RC. Altered course of Plasmodium berghei infection by nifedipine treatment. APMIS 101(9):667, 1993.

Kao JPY, Harootunian AT, Tsein RY. Photochemically generated cytosolic calcium pulses and their detection by fluo-3. J Bioc Chem 264(14):8179, 1989.

Lawrence DB, Weart CW, Laro JJ, Neville BW. Calcium channel blocker-induced gingival hyperplasia: case report and review of this iatrogenic disease. J Fam Pract. 39(5):483, 1994.

Lewis RA, Austen F, Soberman RJ. Leukotrienes and other products of the 5-lipoxygenase pathway. New Eng J Med 323(10):645, 1990.

Lo RCY, Shewen PE, Strathdee CA, Greer CN. Cloning and expression of the leukotoxin gene of *Pastreurella haemolytica* A1 in *Escherichia coli* K-12. Infect Immun 50:667, 1985.

Lopez A, Maxie MG, Ruhnke L, et al. Cellular inflammatory response in the lungs of calves exposed to bovine viral diarrhea virus, *Mycoplasma bovis* and *Pasteurella haemolytica*. Am J Vet Res 47:1283, 1986.

Maheswaren SK, Weiss DJ, Kannan MS, et al. Effects of *Pasteurella haemolytica* A1 leukotoxin on bovine neutrophils: degranulation and generation of oxygen-derived free radicals. Vet Immunol Immunopath 33:51, 1992.

Mayer RJ, Marshall LA. New insights on mammalian phospholipase A_2(s); comparison of arachidonyl-selective and -nonselective enzymes. FASEB J 7:339, 1993.

McMillin RM, Walker ERH. Designing therapeutically effective 5-lipoxygenase inhibitors. TiPS 13(8):323, 1992.

Mocada S, Vane JR. Pharmacology and endogeneous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin. Pharm Rev 30:293, 1979.

Montero, M., Alvarez, J., and Garcia-Sancho, J. Agonist-induced Ca2+ influx in human neutrophils is secondary to the emptying of intracellular calcium stores. Biochem. J. 277:73, 1991.

Moayeri M, Welch RA. Effects of temperature, time, and toxin concentration on lesion formation by the *Escherichia coli* hemolysin. Infect Immun 62(10):4124, 1994.

Murphy GL, Whitworth LC, Clinkenbeard KD et al. Hemolytic activity of the Pasteurella haemolytica leukotoxin. Infect Immun 63(8):3209, 1995.

Musser JH, Kreft AF. 5-lipoxygenase: properties, pharmacology, and the quinolinyl (briged) aryl class of inhibitors. J Med Chem 35(14):2501, 1992.

Ortiz-Carranza O, Czuprynski CJ. Activation of bovine neutrophils by *Pasteurella haemolytica* leukotoxin is calcium dependent. J Leuk Biol 52:558, 1992.

Perry I, Buttrum SM, Nash GB. Effect of activation on adhesion of flowing neutrophils to cultured endothelium: time course and inhibition by a calcium channel blocker (nitrendipine). Br J Pharmacol 110(4):1630, 1993.

Radostitis OM, Leslie KE, Fetrow J. Health and production management in beef feedlots. In Herd health: Food animal production medicine, 2nd ed. WB Saunders, Philadelphia. 413, 1994.

Rosales C, Brown EJ Calcium channel blockers nifedipine and diltiazem inhibit Ca2+ release from intracellular stores in neutrophils. J Biol Chem 267:1443, 1992.

Rouzer CA, Rands E, Kargman S *et al.*. Characterization of cloned humen leukocyte 5-lipoxygenase expressed in mammalian cells. J Biol Chem 263:10135, 1988.

Slocombe RF, Malark J, Ingersoll R, et al. Importance of neutrophils in the pathogenesis of acute pneumonic pastreurellosis in calves. Am J Vet Res 46:2253, 1985.

Spedding M, Paoletti R. III. Classification of calcium channels and the site of action modifying channel function. Pharmacol Rev 44(3):363, 1992.

Strathdee CA, Lo RYC. Extensive homology between the leukotoxin of *Pasteurella haemolytica* A1 and the alpha-hemolysin of *Escherichia coli.* Infect Immun 55:3233, 1987.

Tscharner, VV, Prod'hom B, Baggiolini M et al. Ion channels in human neutrophils activated by a rise in free cytosolic calcium concentration. Nature. 324:369, 1986.

Teitz NW, Thompson J. Possible concentration-dependent suppression of immune response by verapamil. Arch Fam Med 4(4):368, 1995.

Thomson CM, Dryden WF. Different actions of calcium channel blocking agents on resting membrane conductance in developing skeletal muscle. Can. J. Physiol. Pharmacol. 59:335, 1981.

Vandaux P, Waldovogel FA. Gentamicin inactivation in purulent exudates: Role in cell lysis. J Infect Dis 142:586, 1980.

Walker RD, Hopkins FM, Schultz TW, et al. Changes in leukocyte populations in pulmonary lavage fluids of calves after inhalation of *Pasteurella heamolytica*. Am J Vet Res 46:2429, 1985.

Weiss JW, Kraemer R, Schmit K. Isolation of granulocytes and mononuclear cells from the blood of dogs, cats, horses, and cattle. Vet Clin Pathol 18:33, 1989.

Welch, RA. Pore-forming cytolysins of Gram-negative bacteria. Mol Microbol 5:521, 1991.

Whiteley LO, Maheswaren SK, Weiss DJ, Ames TR, Kannan MS. *Pasteurella haemolytica* A1 and Bovine respiratory disease: Pathogenesis. J Vet Int Med 6(1):11, 1992.

Whiteley LO, Maheswaren SK, Weiss DJ, Ames TR. Morphological and morphometrical analysis of the acute response of the bovine alveolar wall to *Pasteurella haemolytica* A1-derived endotoxin and leukotoxin. J Comp Path 104:23, 1991.

Wilkinson L. Statistics, Version 5.2 Edition, Systat Inc., Evanston, IL, 116, 1992.

Wikse SE. The Bronchopneumonias. In Large animal internal medicine (Smith BP ed.) CV Mosby Co.; St. Louis. 582, 1990.

Yates WDG. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can J Comp Med 46:225,1982.

APPENDICES

APPENDIX A

TABLES

TABLE 1

EFFECTS OF VERAPAMIL, NIFEDIPINE, AND LANTHANUM CHLORIDE ON LEUKOTOXIN-INDUCED INCREASE IN INTRACELULAR CALCIUM CONCENTRATION ($[Ca^{2+}]_i$), LEUKOTRIENE B_4 SYNTHESIS (LTB_4), AND PERCENT SPECIFIC RELEASE OF LDH (% LDH Release). ALL DATA ARE PRESENTED AS MEAN ± SD.

	$[Ca^{2+}]_i$ (nM)	LTB_4 (pg/ml)	% LDH Release
Verapamil			
0.5 mM	110.1 ± 26.5*	289 ± 69*	14.9 ± 7.9*
50 μM	129.2 ± 17.0	2077 ± 503	17.9 ± 3.4
5 μM	130.2 ± 12.5	2815 ± 270	9.5 ± 1.4*
0.5 μM	118.7 ± 7.1	2624 ± 1269	13.7 ± 7.8*
50 nM	137.5 ± 4.3	2854 ± 881	27.7 ± 5.3
DMSO solvent	143.1 ± 5.0	2188 ± 781	27.1 ± 5.1
Nifedipine			
0.5 mM	114.1 ± 11.8	487 ± 45*	14.6 ± 8.7
50 μM	139.0 ± 0.7	1426 ± 110	13.7 ± 4.8
5 μM	127.4 ± 8.4	2140 ± 563	21.1 ± 3.0
0.5 μM	116.3 ± 9.6	2918 ± 415	21.0 ± 7.8
50 nM	133.9 ± 4.0	2723 ± 677	17.1 ± 0.3
DMSO solvent	120.2 ± 17.8	2188 ± 431	15.5 ± 0.8
$LaCl_3$			
0.5 mM	64.9 ± 12.0*	1378 ± 154*	2.8 ± 1.0*
50 μM	125.7 ± 4.9	1694 ± 342	48.9 ± 2.4*
5 μM	125.5 ± 8.4	1992 ± 665	51.7 ± 8.6*
0.5 μM	120.9 ± 5.7	2183 ± 528	56.0 ± 5.5
50 nM	139.1 ± 12.0	1870 ± 74	45.6 ± 7.2*
Water solvent	139.1 ± 10.4	2292 ± 284	62.7 ± 3.6
Buffer control	53.2	318	5.5

* Significantly different from corresponding solvent control value.

APPENDIX B

FIGURES

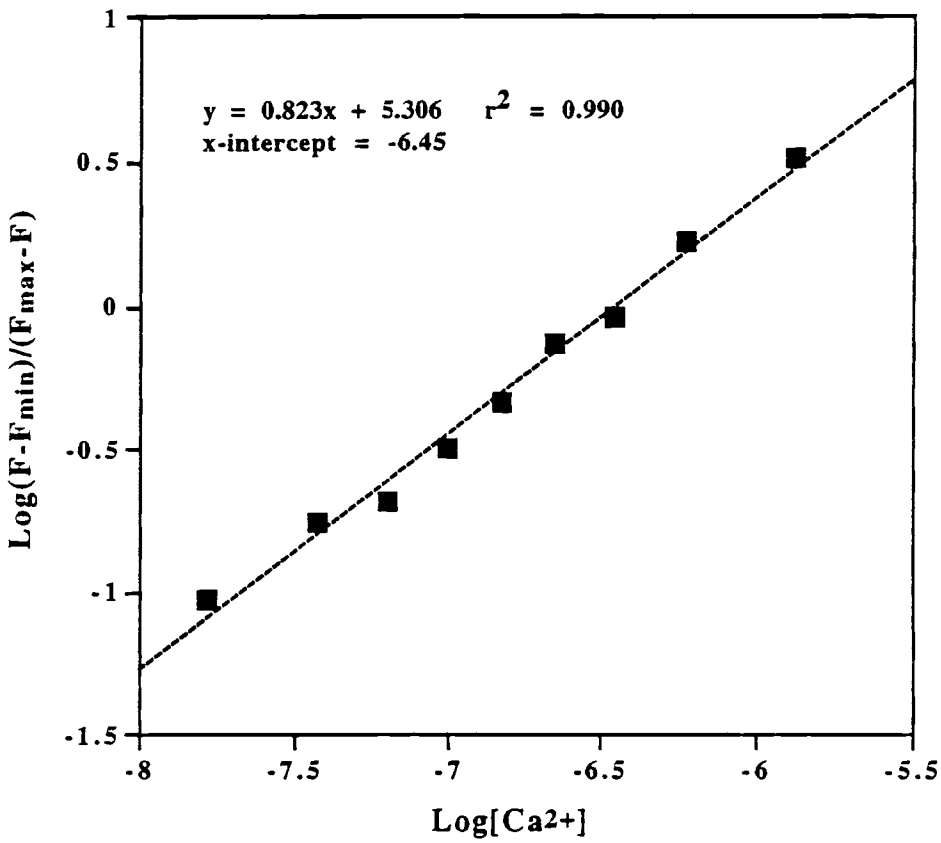

Figure 1. Calibration curve for Fluo-3. As a double log plot, the x-intercept is equal to the apparent log K_d.

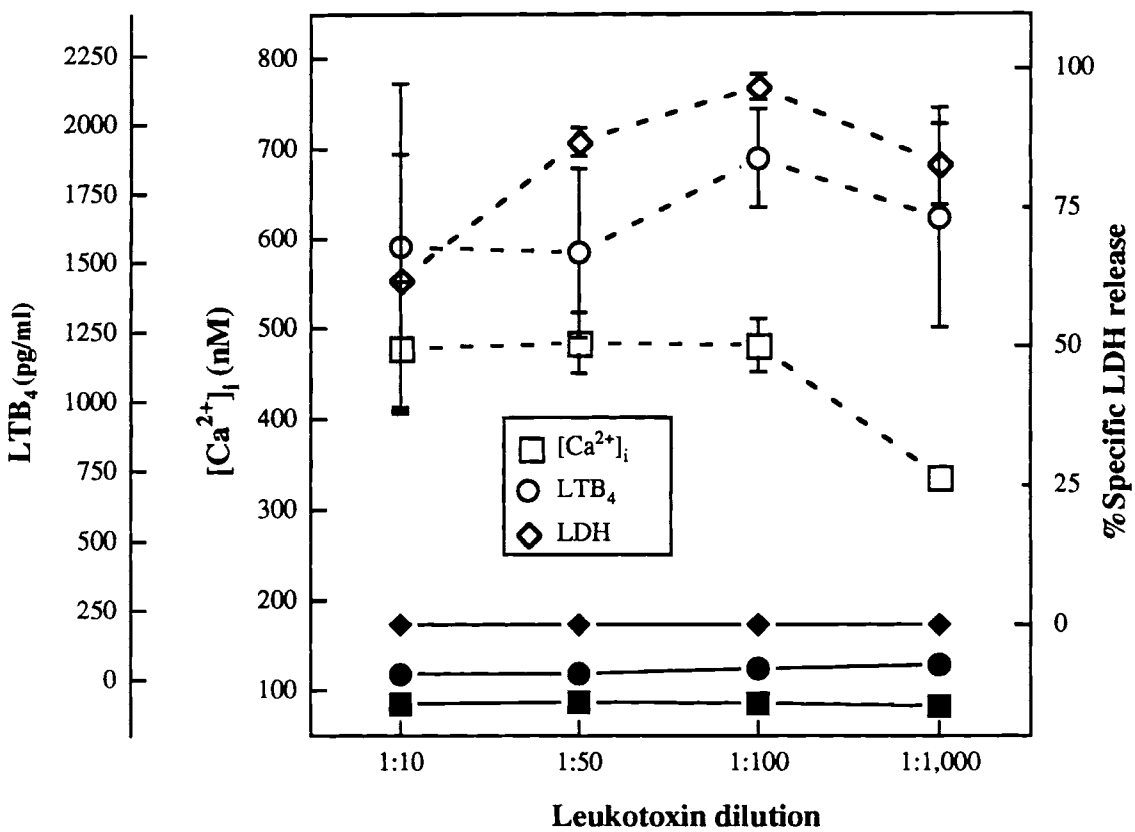

Figure 2. Effect of LKT (open symbols) and LKT-deficient mutant control preparation (solid symbols) on isolated bovine neutrophil responses. LKT-induced effects were significantly higher than LKT-deficient control values at all dilutions.

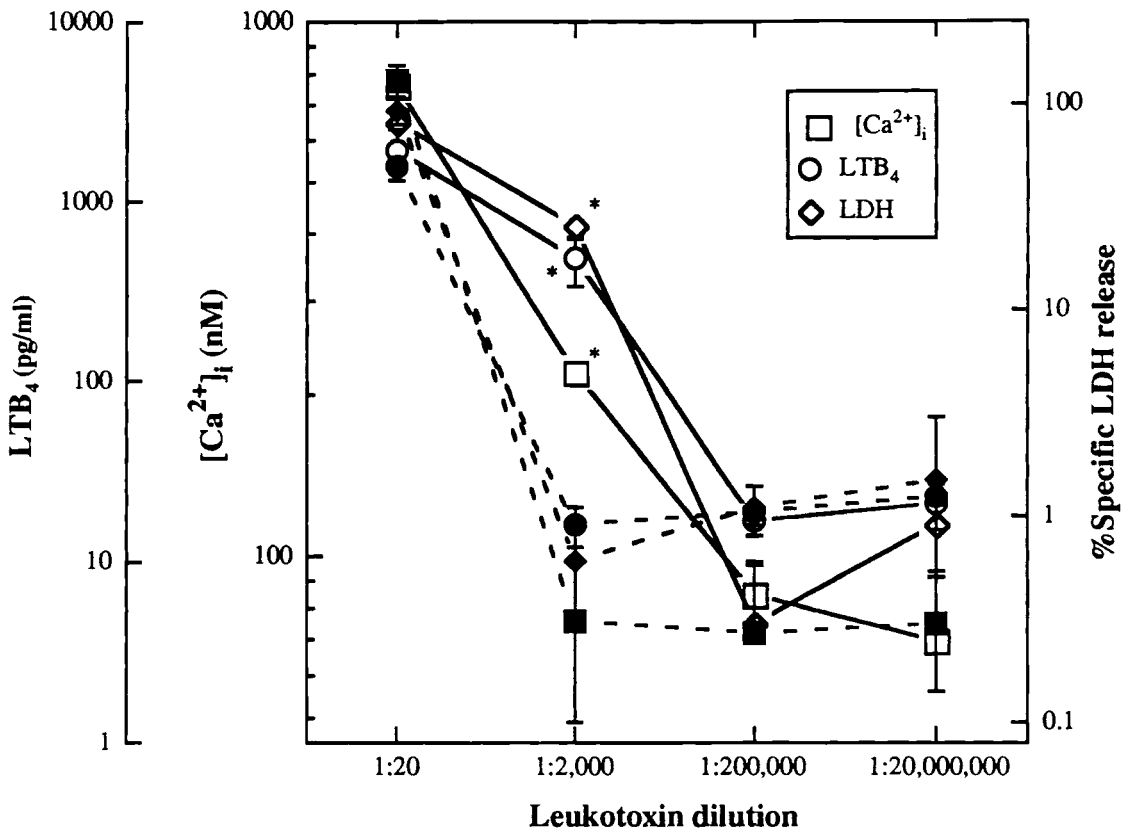

Figure 3. Neutralization of LKT-induced neutrophil responses by antileukotoxin monoclonal antibody, MM601 (solid symbols). The symbols * identify values significantly different from corresponding values measured after exposure to MM601 treated LKT.

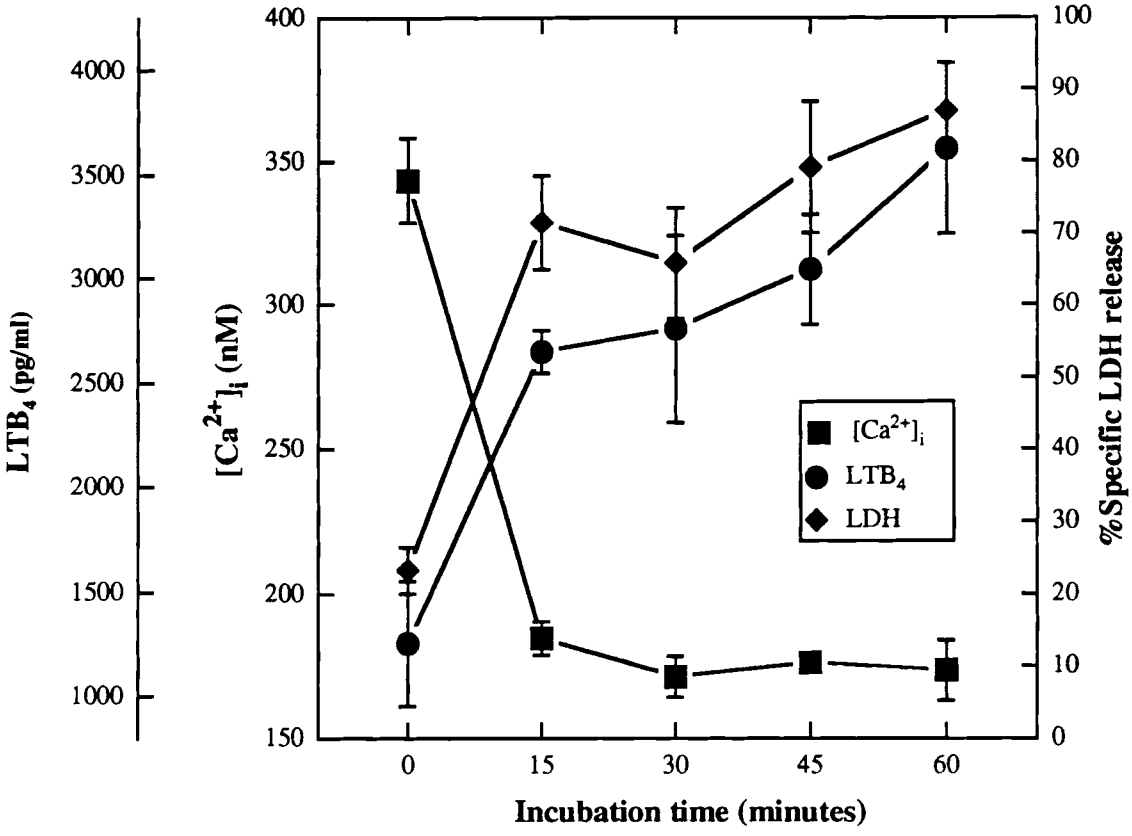

Figure 4. Time-dependent effect of a lytic dilution (1:1,000) of LKT on neutrophil responses.

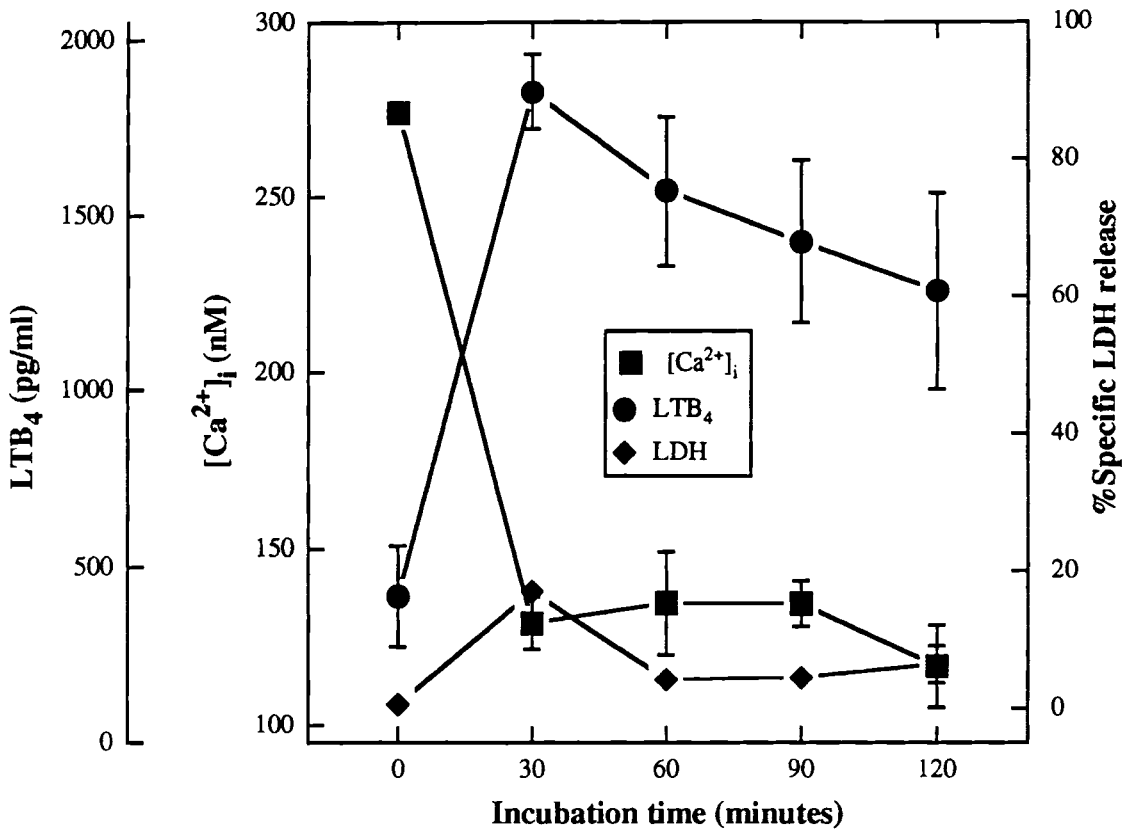

Figure 5. Time-dependent effect of a sublytic dilution (1:100,000) of LKT on neutrophil responses.

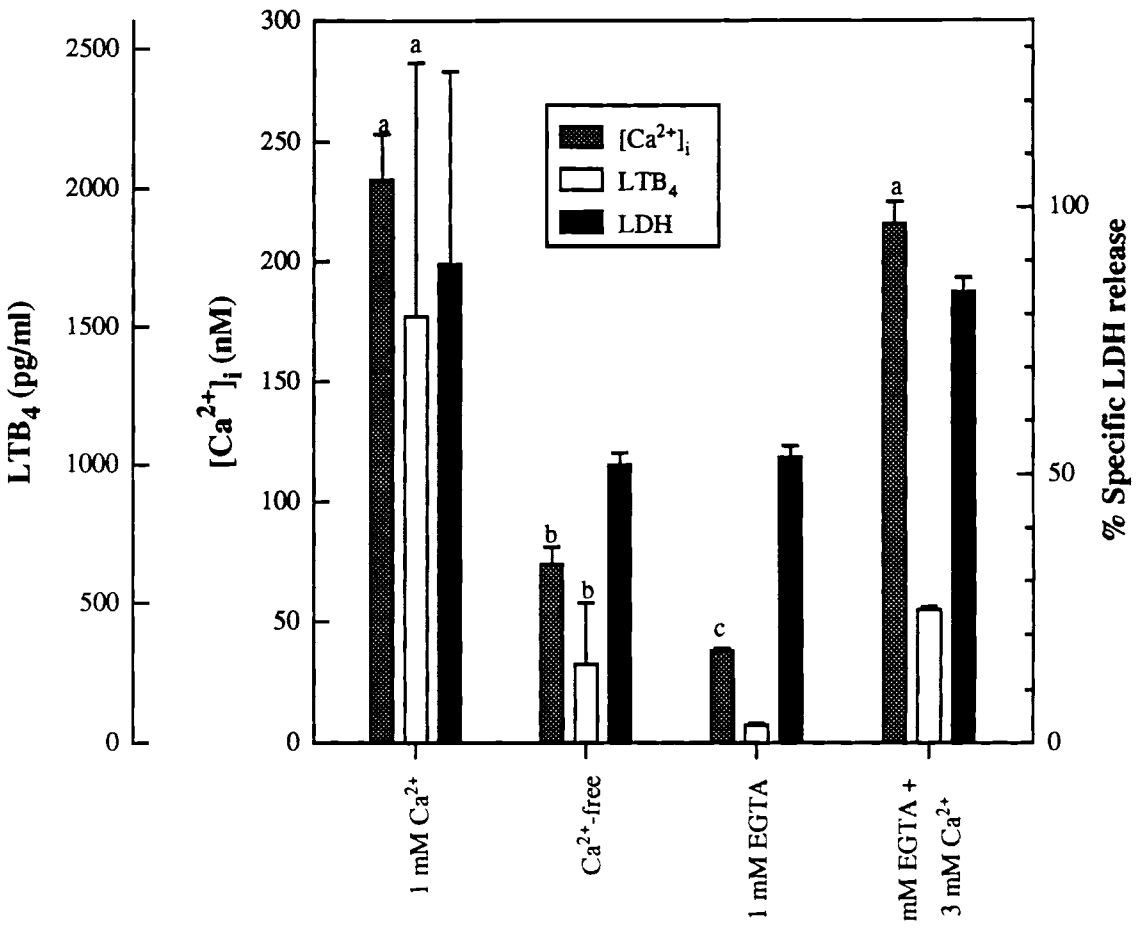

Figure 6. Extracellular calcium-dependence of LKT-induced neutrophil responses. Responses with different letters (a, b, or c) are significantly different from corresponding values.

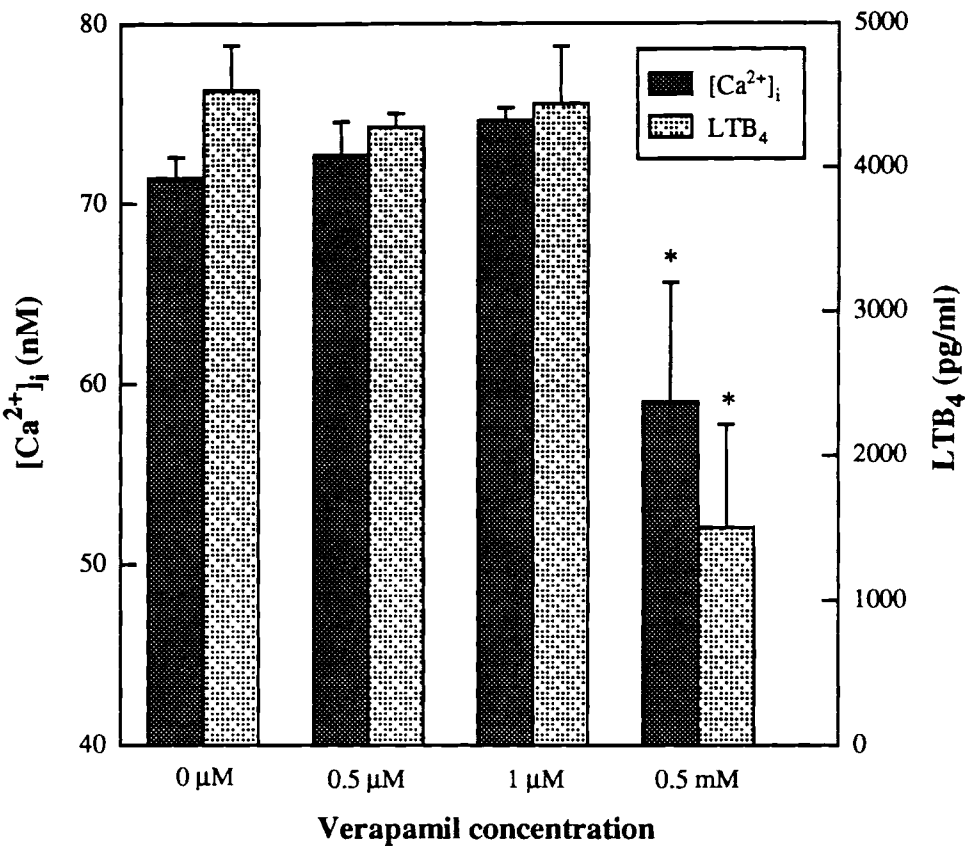

Figure 7. Effect of verapamil on digitonin-induced responses of neutrophils. The symbol * indicates those values that are significantly different from corresponding values.

VITA

Laura Annette Kellum

Candidate for the Degree of

Master of Science

Thesis: ROLE OF INTRACELLULAR CALCIUM IN *PASTEURELLA HAEMOLYTICA* LEUKOTOXIN-INDUCED NEUTROPHIL PLASMA MEMBRANE DAMAGE AND SYNTHESIS OF LEUKOTRIENE B_4

Major Field: Physiological Sciences

Biographical:

Personal Data: Born in Ponca City, Oklahoma, October 28, 1967, daughter of Jerome and Sylvia Kellum.

Education: Graduated from Moore High School, Moore, Oklahoma, in May 1985. Recieved Bachelor of Science Degree in Pharmacy from the Univrsity of Oklahoma Health Sciences Center in Oklahoma City in May, 1991. Completed requirements for the Master of Science degre at Oklahoma State Univrsity in December, 1995.

Professional Experience: Practicing pharmacist from June 1991 - July 1994. Teaching Assistant from August 1994 - December 1995, Department of Physiological Sciences, Oklahoma State University.