ROLES OF PHOSPHOLIPASE A_2 AND PHOSPHOLIPASE D IN *PASTEURELLA HAEMOLYTICA* LEUKOTOXIN-INDUCED SYNTHESIS OF LEUKOTRIENE B_4 BY BOVINE NEUTROPHILS

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CHAPTER I

INTRODUCTION

Shipping fever is an acute and often fatal pneumonic disease of cattle in which there is widespread destruction of pulmonary tissue. Pulmonary lesions are characterized histologically by massive exudation of neutrophils and fibrin-rich plasma into airways and alveoli. Paradoxically, it appears that this excessive host inflammatory response is principally responsible for the severe lung pathology, rather than the direct effects of the causative bacterium, *Pasteurella haemolytica.*

Chemotactic agents responsible for the rapid influx of neutrophils into sites of infection have not all been conclusively identified, but both *in vivo* and *in vitro* studies have shown that eicosanoids are known to play a major role. Eicosanoids are derived from arachidonic acid (AA), which is released from membrane phospholipids by the action of phospholipases. The free arachidonic acid is then metabolized via one of two major pathways: by cyclo-oxygenase to prostaglandins and thromboxanes or by lipoxygenase to hydroxyeicosatetraenoic acids and leukotrienes. Leukotriene B_4 (LTB_4), a product of 5-lipoxygenase (5-LO), is recognized to be a potent and reliable chemotactic agent for bovine neutrophils.

There is reliable evidence (Clinkenbread *et al*, 1994) that synthesis of LTB_4 by bovine neutrophils is stimulated by the direct effects of *Pasteurella haemolytica* leukotoxin (LKT) and that this effect is dependent on increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) resulting from influx of extracellular Ca^{2+}. LKT-induced production of LTB_4 is increased substantially by provision of exogenous AA, suggesting that release of AA from membrane phospholipids is the rate-limiting step in eicosanoid

synthesis. Such release of AA from phospholipid membranes is accomplished by phospholipases, particularly phospholipase A_2 (PLA_2), which is regulated by signal transduction pathways involving $[Ca^{2+}]_i$ and phosphorylation as well as by other phospholipases.

The overall goal of this dissertation research was to examine the role of PLA_2 in LKT-induced synthesis of LTB_4 and to identify important mechanisms of PLA_2 regulation. A clear understanding of the involvement of phospholipases in the molecular pathogenesis of pneumonic pasteurellosis would facilitate identification of mechanisms that can be targeted using specific anti-inflammatory agents. The use of such agents to suppress the uncontrolled pulmonary exudation is likely to have the benefit of restoring effective neutrophil phagocytic function as well as enhancing the efficacy of antibacterial therapy.

CHAPTER II

LITERATURE REVIEW

Bovine Respiratory Disease

Bovine respiratory disease (BRD), commonly referred to as shipping fever, is an acute fibrinopurulent, necrotizing, bronchopneumonia that is caused by a combination of various stressful factors and infectious agents including bacteria, viruses, and mycoplasma (Rehmtulla and Thomson, 1981; Frank, 1989; Whitely *et al*, 1992). An accurate accounting of annual losses due to shipping fever is not available, but it has been estimated that these approximate $800 million in the United States (Weekly *et al*, 1998), making it the most economically important disease to the beef feedlot industry in North America.

Outbreaks of bovine pneumonic pasteurellosis usually occur within two weeks after calves arrive in the feedlot and commonly last for about 2 - 3 weeks thereafter (Yates, 1982). Morbidity ranges from 3 - 45% while mortality is usually 1 - 2% (Yates, 1982; Conlon *et al*, 1995; Ribble *et al*, 1995a). The clinical signs of bovine pneumonic pasteurellosis have been well described by Wikse and Baker (1996). Cardinal signs include depression, anorexia, dyspnea, nasal discharge, abnormal lung sounds, coughing, and high fever. The severity of clinical signs may vary from inapparent to rapidly fatal disease. Generally, feedlot calves exhibiting depression and body temperature > 40.5°C without any obvious etiology are initially diagnosed as suffering from shipping fever (Ribble *et al*, 1995b).

Although the etiology of BRD appears to be multifactorial, usually involving a combination of transportation and/or cold weather, viruses, and bacteria (Frank, 1989), the primary etiological agent responsible for inducing pulmonary lesions is *Pasteurella haemolytica* biotype A serovar 1 (A1) (Rehmtulla and Thomson, 1981; Wilkie and Shewen, 1988). *P. haemolytica* A1 is the most frequently isolated bacterium in cases of shipping fever: according to a diagnostic study of 304 cattle that died of BRD in Texas and Oklahoma, *P. haemolytica* was isolated from 71.1%, *P. multocida* was isolated from 21.1%, and *Haemophilus somnus* was isolated from 7.8% of cases (Welsh, 1993). Similar results were generated by a survey of Colorado feedlots (Wilkie and Shewen, 1988). Although the involvement of predisposing factors in the etiology of BRD complicates establishment of experimental infections, several researchers have been able to produce lung pathology similar to that observed in natural cases by transthoracic or intratracheal inoculation of *P. haemolytica* A1 alone (Panciera and Corstvet, 1984; Ames *et al*, 1985). Furthermore, vaccination of calves with various *P. haemolytica* antigens affords a degree of protection against natural infection, although much work still needs to be accomplished before a reliable vaccine is developed. Stress factors and other infectious agents, particularly viruses such as parainfluenza 3, infectious bovine rhinotracheitis (IBR), bovine virus diarrhea (BVD), and bovine syncytial virus, are believed to predispose cattle to BRD by enhancing colonization and proliferation of *P. haemolytica* A1 in the upper respiratory tract (URT) and lung (Yates, 1982; Whitely *et al*, 1992).

The pathological characteristics of pneumonic pasteurellosis have been studied in natural and experimentally infected cases of shipping fever (Panciera and Corstvet, 1984; Ames *et al*, 1985). Classical features include massive fibrin exudation and extensive neutrophil infiltration into airways and alveoli, venous, arterial, and capillary thrombosis, foci of coagulation necrosis at the terminal bronchioles, and alveoli surrounded by bacteria and degenerating inflammatory cells. When shipping fever was experimentally

produced by intratracheal inoculation, atelectasis and neutrophil infiltration of alveoli and bronchioles occurred prior to development of fibrinous pneumonia, which involved exudation of fibrin into alveoli, capillary thrombosis, and areas of necrosis bordered by fusiform macrophages (Friend *et al*, 1977). The role of neutrophil infiltration in the pathogenesis of BRD has received much attention, and there is evidence that mobilization of neutrophils fails to combat bacterial infection and that degranulation and lysis of these phagocytes release damaging products that directly aggravate lung injury (Breider *et al*, 1988; Slocombe *et al*, 1985).

Pathogenic mechanisms proposed to explain the development of severe lung pathology in pneumonic pasteurellosis recognize three major stages: first, environmental stressors combined with viral infection depress URT defenses allowing rapid multiplication of *P. haemolytica* A1. Initial proliferation and colonization of *P. haemolytica* A1 in the nasopharynx and tonsils has not been extensively investigated, but several probable mechanisms have been proposed (Confer *et al*, 1995; Whitely *et al*, 1992). Based on studies of other gram-negative bacteria, two major factors are believed to contribute to bacterial colonization: alterations in mucociliary function and adhesion of bacteria to epithelial cell surfaces. The latter may be affected by host and bacterial factors. For example, viral infection may cause degradation of the fibronectin layer that covers normal epithelial surfaces, thus preventing binding of bacteria to epithelial cell adhesins (Proctor, 1987; Woods, 1987; Briggs and Frank, 1992). Bacterial virulence factors that may promote colonization and adherence include fimbriae or pili, capsule polysaccharide, outer membrane proteins, lipopolysaccharide, neuraminidase, and neutral glycoprotease (Confer *et al*, 1995).

The second phase in the pathogenesis of pneumonic pasteurellosis is believed to involve extension of the infection from the URT into the lung. Lung colonization apparently occurs by inhalation of aerosols containing bacteria derived from rapid multiplication in the nasopharynx (Frank *et al*, 1989). Considering that intranasal

inoculation in the absence of stress or viral infection usually fails to produce pneumonia, it is clear that lung colonization also depends on depression of host defenses by agents other than *P. haemolytica*. In particular, the protective function of alveolar macrophages must be compromised before extension of the infection into lung parenchyma can occur.

The third pathogenic phase is characterized by severe, acute inflammation and involves cells and mechanisms that are not unique to the lung. The central role of peripheral neutrophils in the development of the acute inflammatory response is well supported. After intrabronchial or aerosol inoculation in calves, neutrophils were found to be the predominant cell type infiltrating the lung at 2 to 4 hours (Walker *et al*, 1985). Experimental aerosol exposure to *P. haemolytica* induces a marked increase in the neutrophil/macrophage ratio (Lopez *et al*, 1986). These changes correlate well with reported histologic changes in which small airways become plugged with purulent exudate (Lopez *et al*, 1986). There is reliable evidence indicating that mobilization of neutrophils does not effectively combat infection, but contributes to development of lung lesions. In experiments conducted by Slocombe *et al* (1985) and Breider *et al* (1988), neutrophil depletion prior to inoculation with *P. haemolytica* protected calves from the development of gross fibrinopurulent pneumonic lesions, although less severe inflammatory changes still occurred. Thus, the neutrophil-mediated inflammatory response itself appears to be a major determinant of pathogenicity. Excessive inflammation decreases the efficacy of antibacterial therapy because it changes the composition of interstitial fluid and compromises host defenses (Clarke *et al*, 1994). Virulence factors of *P. haemolytica* that appear to be primarily responsible for the acute inflammatory response are lipopolysaccharide and leukotoxin (LKT).

Roles of Lipopolysaccharide and Leukotoxin in Generation of Inflammatory Responses to *Pasteurella haemolytica* Infection

Lipopolysaccharide

Lipopolysaccharide produced by *P. haemolytica* (LPS) is similar to lipopolysaccharides from other gram-negative bacteria in that it consists of an oligosaccharide core, an O antigenic polysaccharide chain, and biologically active lipid A, which is embedded in the outer leaflet of the outer membrane. LPS of *P. haemolytica* serovar A1 shares identical O-chain polysaccharides with serovars 6 and 9 and has core oligosaccharides that are similar to those of serovars 6, 8, 9, and 12. Using immunohistochemical techniques, Whitely *et al* (1990) demonstrated that when calves were inoculated intratracheally with live *P. haemolytica* A1, LPS was released into the inflammatory exudates and could be identified in neutrophils, alveolar macrophages, endothelial cells, pulmonary intravascular macrophages, and on epithelial cell surfaces. The wide distribution of LPS in so many cell types suggests that LPS may mediate many different inflammatory responses.

A number of studies have provided evidence in support of LPS playing an important role in the pathogenesis of vascular necrosis and thrombosis (reviewed in Whitely *et al*, 1992). Exposure of bovine pulmonary artery endothelial cells to LPS *in vitro* caused dose-dependent degeneration of cells characterized by changes in cell shape, cell retraction, cell membrane damage, and pyknosis (Paulsen *et al*, 1989; Breider *et al*, 1990). LPS may also induce endothelial cell damage indirectly: alveolar macrophages stimulated by LPS release cytokines, such as tumor necrosis factor-α (TNF_α) and interleukin-1 (IL-1) (Bienhoff *et al*, 1992), both of which enhance LPS-mediated endothelial cell damage (Sharma *et al*, 1992a,b). TNF_α and IL-1 also stimulate neutrophil degranulation and release of free radicals (Strieter *et al*, 1989; Yoshimura *et*

al, 1987), which aggravate endothelial cell injury. Endotoxin-mediated endothelial cell damage probably plays a crucial role in activation of the coagulation cascade and stimulation of platelet aggregation, thus inducing microvascular thrombosis that occurs extensively in pneumonic pasteurellosis.

LPS is able to cause vascular thrombosis independent of its effect on endothelial cells. Breider and Yang (1994) demonstrated that *P. haemolytica* endotoxin caused expression of thromboplastin (coagulation Factor III) in endothelial cells. The lipid A component of LPS is capable of activating Hageman factor leading to activation of the coagulation cascade (Morrison and Ulevitch, 1978). Furthermore, LPS-activated platelets release thromboxane A_2 (Heffner *et al*, 1987), a potent vasoconstrictor and platelet aggregator identified as the major eicosanoid responsible for pulmonary hypertension resulting from endotoxemia (Ball *et al*, 1983). Indeed, intravenous infusion of *P. haemolytica* endotoxin caused increased circulating levels of AA, eicosanoids (thromboxane B_2 (TXB_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)), serotonin, and histamine in calves (Emau *et al*, 1986; Emau *et al*, 1987) and in sheep (Emau *et al*, 1984). Incubation of isolated bovine lung parenchyma with LPS resulted in release of LTB_4 and prostaglandin E_2 (PGE_2).

Aside from direct and indirect damage to the vascular system, LPS potentiates the inflammatory response to *P. haemolytica* infection by inducing release of a wide range of proinflammatory mediators, free oxygen radicals, and proteases. Purified LPS activates bovine alveolar macrophages to express and secrete TNF_α, IL-1, and interleukin-8 (IL-8) (Yoo *et al*, 1995a; Lafleur *et al*, 1998), and to produce reactive oxygen intermediates and nitric oxide (Yoo *et al*, 1996). TNF_α is not chemotactic for neutrophils, but it may facilitate neutrophil influx by up-regulating expression of leukocyte adhesion proteins (intracellular adhesion molecule-1) on endothelial cells and neutrophils (Billingham, 1987). Interleukin-8 is not only a potent chemotactic factor for neutrophils but also

activates neutrophils to produce oxidative radicals, release proteases, and undergo degranulation (Peveri *et al*, 1988; Thelen *et al*, 1988).

Leukotoxin

Initially identified as a cytotoxic component in *P. haemolytica* culture supernatant (Benson *et al*, 1978), LKT was later named for its selective toxicity for ruminant leukocytes (Renshaw, 1984; Chang *et al*, 1986; Chang and Renshaw, 1986; Kaehler *et al*, 1980; Berggren *et al*, 1981; Shewen and Wilkie, 1982). More recently, sensitivity of bovine platelets to LKT has been demonstrated (Clinkenbeard and Upton, 1991). All 16 serovars and several untypable strains of *P. haemolytica* produce LKT, which is believed to be one of the most important virulence factors contributing to lung injury in bovine pneumonic pasteurellosis (Whitely *et al*, 1992; Confer *et al*, 1995). Whitely *et al* (1990) demonstrated in experimentally challenged calves that LKT was associated with cell membranes of degenerating inflammatory cells in the lung. The resistance of calves to experimental challenge of *P. haemolytica* was closely correlated with their high titers of antibody against LKT (Gentry *et al*, 1985; Shewen and Wilkie, 1988; Sreevatsan *et al*, 1996). Although some protection against experimental challenge has been achieved by a vaccine that did not contain LKT (Confer, 1993), the addition of recombinant LKT to a supernatant vaccine enhanced protection against experimental challenge (Conlon and Shewen, 1991). Thus, protection against the effects of LKT appears to attenuate the severity of the disease, thus suggesting that LKT is an important virulence factor in the pathogenesis of pneumonic pasteurellosis. A recent experiment conducted by Tatum *et al* (1998) provided further evidence in support of an important role for LKT: experimental infection with a LKT-deficient *P. haemolytica* mutant strain did not elicit significant pulmonary neutrophilic infiltration or lesions.

Leukotoxin is a heat–labile, oxygen-stable, pH-stable, non-dialyzable, water-soluble glycoprotein that is produced by *P. haemolytica* during log-phase growth (Shewen and Wilkie, 1985; Chang *et al*, 1986a; Chang *et al*, 1987; Lo *et al*, 1987; Gentry and Srikumaran, 1991). The molecular mass of LKT, predicted from analysis of the structural gene, is 102 kDa (Highlander *et al*, 1989), but native LKT tends to aggregate to multimers with molecular mass of about 400 kDa or greater, as judged by gel exclusion chromatography (Baluyut *et al*, 1981; Himmel *et al*, 1982; Mosier *et al*, 1986; Chang *et al*, 1987). Clinkenbeard *et al* (1995) reported that the molecular mass of highly aggregated LKT in phosphate buffer was about 8000 kDa. The low leukotoxic activity of these very large aggregates could be increased up to 20-fold by treating LKT preparations with bovine serum albumin or chaotropic agents such as guanidine, which desegregate LKT to multimers with molecular mass of 400-800 kDa (Waurezyniak *et al*, 1994; Clinkenbeard *et al*, 1995).

Leukotoxin belongs to the RTX (Repeats in ToXin) toxin family, which is composed of two distinct groups of toxins produced by gram-negative bacteria; hemolysins and leukotoxins. Hemolysins have been isolated and characterized from *Escherichia coli* (Schmidt *et al*, 1996), *Actinobacillus spp.* (Chang *et al*, 1989, Frey *et al*, 1993), *Bordetella pertussis* (Glaser *et al*, 1988), *Proteus vulgaris* (Koronakis *et al*, 1987; Welch, 1987), *Morganella morganii* (Koronakis *et al*, 1987), and *Moraxella bovis* (Gray *et al*, 1995). *Actinobacillus spp* and *P. haemolytica* produce leukotoxins (Kraig *et al*, 1990; Strathdee and Lo, 1987) that are distinguished from hemolysins by their narrower range of target cell selectivity. *P. haemolytica* LKT is specifically cytolytic for ruminant leukocytes and platelets (Shewen and Wilkie, 1982; Clinkenbeard and Upton, 1991) whereas *Actinobacillus spp* LKT is specifically cytolytic for human and primate leukocytes (Simpson *et al*, 1988; Mangan *et al*, 1991).

RTX toxins are composed of tandem repeats of highly conserved nine amino acid sequences (L-X-G-G-X-G-(N/D)-X), which serve as Ca^{2+}-binding motifs (one calcium

ion per repeat) (Strathdee & Lo, 1987; Boehm *et al*, 1990; Baumann *et al*, 1993). RTX toxins are similar with respect to DNA homology and arrangement of genes responsible for toxin structure, mechanisms of activation, and secretion (Welch, 1991). In the case of LKT, there is a four-gene cluster (LktCABD) that encodes for LKT structure (LktA) and the proteins required for activation (LktC) and secretion (LktB and LktD). The arrangement of this gene cluster is analogous to that of the *E. coli* hemolysin locus (HlyCABD) (Chang *et al*, 1987; Lo *et al*, 1987; Strathdee and Lo, 1987, Highlander *et al*, 1989; Highlander *et al*, 1990).

Aside from variation in LKT selectivity between different cell types and different species, there is also variation in the sensitivity of cell types within the population of target cells. For example, bovine neutrophils and monocytes are more sensitive to LKT than macrophages (O'Brien and Duffus, 1987; Stevens and Czuprynski, 1995). The selectivity of LKT for certain target cells appears to be related to the presence of specific receptors on target cell surfaces. Lally *et al* (1997) have identified a cell surface receptor for *Actinobacillus actinomycetemcomitans* leukotoxin (AA-LKT) and α-hemolysin that is a member of the β_2-integrin family, lymphocyte function-associated antigen 1 (LFA-1). They have proposed that the reason why α-hemolysin has a wider target cell selectivity than AA-LKT is that α-hemolysin also binds to other molecules that are present on many different cells. It is also possible that α-hemolysin may be less stringent with regard to the requirement for a protein receptor on target cell surfaces because it apparently binds to lipid vesicles that do not contain any proteins (Ostolaza and Goni, 1995). However, binding of RTX toxins to cells is not necessarily correlated with cytolytic activity. Clinkenbeard *et al* (unpublished data) have observed that LKT failed to bind to human HL 60 promyelocyte cells, but did bind to human Raji lymphoma cells, which are resistant to the cytolytic effect of LKT. Similarly, AA-LKT binds to human erythroleukemic cells and mouse SP2 myeloma cells, but neither cell types are susceptible to this toxin (Taichman *et al*, 1991; Sato *et al*, 1993).

Although the precise mechanism whereby LKT interacts with target cells has not yet been elucidated, most LKT-induced effects are probably related to the ability of RTX toxins to form transmembrane pores. Formation of pores in neutrophil plasma membranes is hypothesized to occur by transmembrane integration of hydrophobic runs of amino acids located near the N-terminus (Bhakdi and Tranum-Jensen, 1988; Forestier and Welch 1991) (Figure 1). Transmembrane pores allow rapid diffusion of monovalent ions down their concentration gradients, resulting in osmotic imbalances that cause severe cell swelling and cell membrane damage (Clinkenbeard *et al*, 1989a). LKT-induced cell swelling and leakage of large cytoplasmic proteins can be prevented by suspending cells in hypertonic solutions of sucrose (75 mM), but leakage of intracellular K^+ continues unaffected (Clinkenbeard *et al*, 1989b). Although the model of pore formation involving insertion of the toxin into the membrane is consistent with the results of osmotic protection experiments, it cannot by itself explain the observation, based on the effects of the related RTX *E. coli* α-hemolysin, that pore size increases with higher toxin concentration and longer exposure times (Moayeri and Welch, 1994; Styrt *et al*, 1990a). Furthermore, the initial LKT-induced events, leakage of intracellular K^+ and cell swelling, occur independently of extracellular Ca^{2+} whereas leakage of large cytoplasmic proteins and cell lysis requires Ca^{2+}. Thus, loss of membrane integrity appears to be a dynamic process involving initial formation of discrete pores and subsequent formation of larger membrane defects consistent with Ca^{2+}-mediated enzymatic membrane degradation.

Effects of Leukotoxin on Neutrophil Function

The effects of LKT on bovine neutrophils are concentration-dependent. At high concentrations, LKT causes neutrophils to lose their chemiluminescence response (Czuprynski and Noel, 1990), swell, lose their membrane ruffling, develop a finely

porous surface, and form large membrane defects prior to lysis (Clinkenbeard *et al*, 1989c). At lower sublytic concentrations, LKT causes a range of effects consistent with neutrophil activation, including degranulation, generation of reactive oxygen species, and release of inflammatory mediators. Most of these sublytic effects appear to be mediated by influx of extracellular Ca^{2+}, which diffuses down its concentration gradient from extracellular fluid (1500 μM) into the cytosol (0.1 μM in resting cells) causing a rapid rise in $[Ca^{2+}]_i$ (Ortiz-Carranza and Czuprynsky, 1992; Hsuan *et al*, 1998). Inhibitors of voltage-operated channels, such as verapamil, are reported to inhibit LKT-induced increase in $[Ca^{2+}]_i$ (Ortiz-Carranza and Czuprynsky, 1992; Hsuan *et al*, 1998), but these inhibitory effects occur only at high inhibitor concentration, suggesting that Ca^{2+} influx may occur via toxin pores and that the effects of the inhibitors may be nonspecific. Regardless of the mechanism of Ca^{2+} influx, it is likely that LKT-induced increase in $[Ca^{2+}]_i$ serves as a second messenger affecting an array of neutrophil functions.

There are two major mechanisms by which neutrophils can cause tissue damage: degranulation and release of toxic free radicals. Bovine neutrophils, like other phagocytes, are armed with granules that are toxic to tissues when released. Exposure of bovine neutrophils to *P. haemolytica* causes secretion and/or release of primary granules, specific granules, and cytosolic enzymes (Watson *et al*, 1995). The involvement of LKT in the release of these granules has been confirmed (Styrt *et al*, 1990b; Czuprynski *et al*, 1991; Maheswaran *et al*, 1992). Although it is possible that LKT causes granule release simply by causing cell lysis, degranulation may also be induced by LKT at sublytic concentrations, possibly via Ca^{2+}-dependent activation.

Generation of free oxygen radicals is an important mechanism by which phagocytes control bacterial pathogens (Nathan, 1983). In response to various stimuli, leukocytes undergo a process called a respiratory burst, which is characterized by rapidly increased oxygen consumption and production of reactive oxygen radicals, including

superoxide anion, hydroxyl radical, singlet oxygen, and hydrogen peroxide (Babior, 1973; Aida and Onoue, 1984). These reactive oxygen intermediates, which may be released into the extracellular space, are toxic to both eukaryotic and prokaryotic cells. Maheswaran *et al* (1992) demonstrated that LKT caused generation of reactive oxygen intermediates in bovine neutrophils, as measured by the superoxide dismutase-inhibitable reduction of ferricytochrome C. Using a luminol-dependent chemiluminescence assay, Czuprynski *et al* (1991) reported that dilute LKT (up to 1:8192) stimulated the respiratory burst in bovine neutrophils. However, other studies have reported that chemiluminescence may be decreased in neutrophils incubated with LKT or *P. haemolytica* whole cell (Chang *et al*, 1985; Czuprynski and Noel, 1990). Furthermore, LKT may suppress the respiratory burst of neutrophils in response to known agonists (Czuprynski and Noel 1990; Styrt *et al*, 1990b; Maheswaran *et al*, 1992). These discrepancies in results probably arise from a failure to distinguish between lytic and sublytic effects of LKT; cytolysis induced by LKT is known to suppress the respiratory burst activity of neutrophils (Maheswaran *et al*, 1992).

LKT has recently been reported to induce apoptosis in bovine neutrophils (Stevens and Czuprynski, 1996). Exposure of neutrophils to sublytic concentrations of LKT caused marked cytoplasmic membrane blebbing and chromatin condensation and margination, both of which are hallmarks of apoptosis. These morphological changes were LKT concentration-dependent and could be inhibited by anti-leukotoxin monoclonal antibody. Apoptosis is a process of cell death characterized by various morphological and biochemical alterations, including blebbing of the cytoplasmic membranes (zeosis), chromatin condensation, and DNA fragmentation (Cohen and Duke, 1992). It occurs under both physiological and pathologic conditions and does not cause inflammation because apoptotic cells are phagocytosed before they lyse. The significance of LKT-induced leukocytic apoptosis in the pathogenesis of bovine pneumonic pasteurellosis is not known, but it is reasonable to assume that impairment of this important line of host

defense would facilitate colonization of bacteria in the lung. The mechanism whereby LKT causes leukocytes to undergo apoptosis has yet to be determined.

The effects of LKT on expression of inflammatory cytokines have been reported recently (Yoo *et al*, 1995b). Exposure of bovine alveolar macrophages to sublytic concentrations of purified LKT caused increased expression and secretion of IL-1 and TNF-α *in vitro*. Both IL-1 and TNF stimulate neutrophil degranulation and the generation of reactive oxygen intermediates (Billingham, 1987) and make endothelial cells more susceptible to neutrophil-mediated cell damage (Varani *et al*, 1988). Caswell *et al* (1998) reported that the expression of IL-8 increased in the lungs of animals suffering from pneumonic pasteurellosis but not in those with viral pneumonia. IL-8 has been implicated as an important neutrophil chemoattractant in human pneumonia (Strandiford *et al*, 1996) and is believed to serve as a chemoattractant in bovine pneumonic pasteurellosis.

The Role of Eicosanoids in the Pathogenesis of Pneumonic Pasteurellosis

Considering that the salient pathological features of pneumonic pasteurellosis are neutrophil accumulation, edema, and deposition of fibrin, the pathogenesis of the disease could be well explained by the effects of eicosanoids on pulmonary tissue. Eicosanoids are derived from arachidonic acid (5,8,11,14-eicosatetraenoic acid, Figure 2), which serves as the common substrate for generation of a range of eicosanoids. Eicosanoids are a group of 20 carbon lipids including prostanoids (thromboxanes, prostacyclins and prostaglandins) and leukotrienes, both of which are important inflammatory mediators. Prostaglandins and prostacyclins cause increased vascular permeability leading to edema in sites of inflammation. Thromboxane A_2 is a potent vasoconstrictor and promotes platelet aggregation and fibrin deposition. Leukotrienes include LTA_4, LTB_4, LTC_4, LTD_4 and LTE_4. LTB_4 is a potent chemotactic factor for neutrophils, monocytes, and

macrophages. LTC_4, LTD_4 and LTE_4 are collectively termed slow-reacting substances of anaphylaxis, which stimulates contraction of smooth muscle and enhances vascular permeability.

Metabolism of AA to prostaglandins, prostacyclins, and thromboxanes occurs via the "cyclic pathway" whereas production of leukotrienes occurs via the "linear pathway." The pathway by which arachidonic acid is metabolized is dependent on the type of cells and the nature of stimulation. The linear pathway appears predominant in neutrophils while the products of both linear and cyclic pathways are detected in monocytes (Hsueh *et al*, 1981). In the cyclic pathway, arachidonic acid is first catalyzed by prostaglandin H_2 synthase (PGH_2 synthase) to produce PGH_2. PGH_2, the immediate precursor of all series-2 prostaglandins, prostacyclins, and thromboxanes, is oxidized by cyclo-oxygenases in a cell type-specific manner to produce prostacyclins (PGI_2), prostaglandins (PGE_2, $PGF_{2\alpha}$, PGD_2), or thromboxanes (TXA_2, TXB_2). For example, in blood platelets, PGH_2 is exclusively catalyzed by thromboxane synthase to produce TXA_2, but in vascular endothelial cells, PGH_2 is predominantly oxidized by prostacyclin synthase pathway to prostacyclins.

In the linear pathway, AA is converted to hydroperoxyeicosatetraenoic acids (HPETEs) by 5-, 12-, and 15-lipoxygenases. HPETEs are catalyzed by 12- and 15-lipoxygenase to generate hepoxins and lipoxins in various systems, but there is little information about their functions and biological significance. 5-Lipoxygenase catalyzes the oxygenation of AA to produce 5-HPETE and the dehydration of hydroperoxide intermediate to produce the epoxide, leukotriene A_4. Leukotriene A_4 is hydrolyzed to LTB_4 by leukotriene A_4 hydrolase or may be conjugated with glutathione by leukotriene C_4 synthase to produce LTC_4. Leukotriene C_4 is further metabolized to produce LTD_4 and LTE_4.

Exposure of bovine neutrophils to LKT *in vitro* induces increased production of LTB_4 (Clinkenbeard *et al*, 1994). Synthesis of LTB_4 was closely correlated with LKT-induced neutrophil membrane damage and lysis (as detected by leakage of the 160 kDa cytoplasmic protein, lactate dehydrogenase [LDH]), suggesting a common mechanism, and both events could be inhibited by the neutralizing monoclonal anti-LKT antibody, MM601. These experiments confirmed the earlier observations of Henricks *et al* (1992), who reported that bovine neutrophils exposed to *P. haemolytica* culture supernatants synthesized LTB_4 and 5-hydroxyeicosatetraenoic acid. Considering that studies conducted *in vivo* have confirmed that LTB_4 is a potent chemotactic agent for bovine neutrophils (Heidel *et al*, 1989), the potential importance of LTB_4 in the pathogenesis of neutrophil mediated lung damage in pneumonic pasteurellosis is indisputable. Indeed, Clarke *et al* (1994) demonstrated that inoculation of *P. haemolytica* A1 into subcutaneous tissue chambers markedly increased synthesis of LTB_4 and that treatment with corticosteroids inhibited both LTB_4 synthesis and neutrophil accumulation.

In addition to stimulating production of LTB_4, *P. haemolytica* infection also stimulates production of prostaglandins and thromboxanes (Clarke *et al*, 1994). However, the cellular sources of these eicosanoids have not been definitively identified; neutrophils do not produce thromboxanes in response to LKT or calcium ionophores, such as A23187 (Clinkenbeard *et al*, 1994). Prostaglandins appear to be derived principally from endothelial cells, not as a result of the action of LKT (LKT is not toxic to endothelial cells), but due to the effects of LPS (Paulsen *et al*, 1989). Thromboxanes are probably synthesized by platelets, which are susceptible to LKT (Clinkenbeard and Upton, 1991; Steven and Czuprynski, 1995).

The Role of Phospholipases in LKT-Induced Eicosanoid Synthesis

Considering that the level of free AA in resting inflammatory cells, such as neutrophils, is very low (< 3 pmole per million cells), hydrolysis of membrane phospholipids to liberate AA is believed to be the rate-limiting step in the synthesis of eicosanoids and, therefore, serves as a reasonable target for development of strategies intended to attenuate the acute inflammatory response in pneumonic pasteurellosis. Membrane phospholipids (Figure 3) consist of a glycerophosphate in which the hydroxyl groups are esterified with long-chain fatty acids and the phosphoryl moiety forms a phosphodiester bond with a polar head group, commonly choline, ethanolamine, inositol, or serine. Phospholipid structures are designated by a stereospecific nomenclature (*sn*) based on L- glycerol- 3 - phosphate. Thus, the fatty acid esterified to carbon-1 of the glycerol backbone is termed the *sn*-1 fatty acid, which is commonly saturated, e.g., palmitate. The fatty acid esterified to carbon-2 of the glycerol backbone is the *sn*-2 fatty acid, which is often polyunsaturated, e.g., arachidonate (Burch, 1995).

Hydrolysis of membrane phospholipids is achieved by phospholipases, which were first identified in pancreatic juice and cobra venom in the early 1900s (Wittcoff, 1951). Based on their sites of phospholipid hydrolysis, they are classified as phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), and phospholipases B (PLB), C (PLC), and D (PLD) (Waite, 1990) (Figure 3). Phospholipase A_1 hydrolyzes the *sn*-1 fatty acid, PLA_2 hydrolyzes the *sn*-2 fatty acid, whereas PLB hydrolyzes fatty acid residues in both sites. These three types of phospholipases are hydrolases that cleave the fatty acid chains of membrane phospholipids to liberate free fatty acid and lysophospholipids. These products may serve as intracellular second messengers themselves and/or as precursors for the generation of pro-inflammatory lipid mediators, including prostaglandins, leukotrienes, platelet-activating factors, and other bioactive lipids (Glaser *et al*, 1993). PLC hydrolyzes phospholipids to release the head group

phosphates and diacylglycerols (DAG). Diacylglycerols may be further hydrolyzed to release free fatty acid by the action of diglyceride lipase (Irvine, 1987; Axelrod *et al*, 1988). PLD cleaves phospholipids to generate the head group and phosphatidic acid (PA). Phosphatidic acid is a potential second messenger that is involved in many intracellular signaling pathways (reviewed in English *et al*, 1996). Both PLC and PLD are phosphodiesterases.

Phospholipase A_2

The PLA_2 enzyme complex constitutes a group of ubiquitous enzymes that hydrolyze phospholipids in the *sn*-2 position to generate free fatty acids and lysophopholipids. However, they differ in distribution, regulation, function, structure, mechanism, and role of divalent metal ions in their actions. PLA_2 enzymes are generally divided into two major groups: extracellular or secretory PLA_2 ($sPLA_2$) and intracellular or cytosolic PLA_2 ($cPLA_2$).

Secretory PLA_2 enzymes occur in mammalian pancreatic juice and snake and bee venoms and are secreted from cells as a result of various physiological and/or pathophysiological conditions. They generally have low molecular masses (14-18 kDa), contain a high number of disulfide bridges (up to 7), have no arachidonate preference, and require millimolar levels of calcium for catalysis (Dennis, 1994; Waite, 1990). Based on their amino acid sequences, $sPLA_2$ enzymes are classified into three groups, termed Group I, Group II, and Group III (Davidson and Dennis, 1990; Ward and Pattabiraman, 1990). Group I enzymes, represented by enzymes in mammalian pancreatic juice and the venoms of cobras and kraitts, differ from Group II, represented by enzymes in rattlesnake and viper venom, in the arrangement of the cysteines in the primary structure and the cross-linking disulfide bonds. Group I enzymes have a disulfide bond between Cys-11

and Cys-77 whereas the Group II enzymes have a disulfide bridge between Cys-50 and the Cys at the C-terminal end (Heinrikson *et al*, 1977). Both Group I and Group II have a high content of disulfide bonds, a calcium "binding loop" formed at positions 28 (Tyr), 30 (Gly), 32 (Glu), and 49 (Asp), and a helical region at the N-terminal region (Slotboom *et al*, 1982). Group III includes PLA_2 enzymes isolated from lizard and bee venoms. The enzyme activity of all three groups is calcium-dependent, but Group I is optimally active in the acidic pH range while Group II and Group III prefer alkaline pH. PLA_2 in pancreatic juice is secreted as a proenzyme. While the characterization of $sPLA_2$ groups has been largely based on studies of non-human enzymes, a large number of Group I and Group II enzymes have also been found in human tissues. Human $sPLA_2$ enzymes isolated from synovial fluid, platelets, and placenta have been classified as Group II enzymes (Hara *et al*, 1988; Kramer *et al*, 1989; Lai and Wada, 1988).

Intracellular $cPLA_2$s are present in the cytosol of various types of cells, have high molecular mass (31-110 kDa), contain no disulfide bonds (resistant to dithiothreitol), require either micromolar or no calcium for optimum activity, and appear to be selective for hydrolysis of phospholipids containing AA (Clark *et al*, 1991; Glaser *et al*, 1993). These $cPLA_2$ enzymes share no homology with the $sPLA_2$ identified so far and are, therefore, classified into a separate Group IV (Dennis, 1994). The most notable $cPLA_2$ was isolated from the cytosol of the human monocytic U937 cell line (Kramer *et al*, 1989). It has a molecular mass of 110 kDa, as determined by SDS-PAGE analysis (85 kDa predicted from cDNA), and requires submicromolar Ca^{2+} for optimum activity. This $cPLA_2$ is composed of 749 amino acid residues that include 12 possible sites of Ser/Thr phosphorylation and four possible sites for disulfide bonds (Clark *et al*, 1991).

The current classification of PLA_2 enzymes into four groups does not include all enzymes that have been identified to date. Hazen *et al* (1990) have isolated a $cPLA_2$ from canine myocardium that is Ca^{2+}-independent but shows a preference for

arachidonyl-containing phospholipids. A similar enzyme has been identified in P388 D_1 murine macrophage-like cells (Ackermann *et al*, 1994). There are numerous other Ca^{2+}-dependent and Ca^{2+}-independent intracellular PLA_2 activities reported in the literature. Obviously these enzymes constitute a much more diverse group than was originally anticipated and a more appropriate classification system will have to be developed.

Although the combined actions of phospholipase C and diglyceride lipase yield free arachidonic acid in certain cell systems (Irvine, 1982), the release of arachidonic acid in neutrophils is PLA_2-mediated (Walsh *et al*, 1981). Neutrophils contain at least two types of PLA_2, a 14 kDa $sPLA_2$ and a 85 kDa $cPLA_2$; both have been implicated in generation of free arachidonic acid leading to eicosanoid synthesis. Using permeabilized human neutrophils, Bauldry and Wooten (1996) reported that stimulation by *N*-formyl-met-leu-phe (FMLP) caused $cPLA_2$-mediated release of AA and subsequent synthesis of LTB_4. Cytosolic PLA_2 has also been implicated in the release of AA and generation of eicosanoids in platelets (Kramer *et al*, 1993; Mounier *et al*, 1993). Thromboxane B_2 production was closely correlated with increased $cPLA_2$ activity in cell lysates (Kramer *et al*, 1993) whereas TXB_2 synthesis was not affected by deletion of platelet $sPLA_2$ (Mounier *et al*, 1993). Furthermore, thrombin-induced release of AA and eicosanoid generation in platelets could be inhibited by a $cPLA_2$ inhibitor but not by a $sPLA_2$ inhibitor (Bartoli *et al*, 1994). However, in human monocytes, deletion of $cPLA_2$ had no effect on leukotriene formation (Marshall *et al*, 1997), indicating that the involvement of $sPLA_2$ versus $cPLA_2$ varies between cell type, even within the leukocyte series.

Consistent with their diversity of structural characteristics, PLA_2 enzymes accomplish a variety of functions. Mammalian pancreatic $sPLA_2$ digest phospholipids in the gut. PLA_2 enzymes in snake and bee venoms promote tissue damage in bite or sting victims. Group II and Group IV enzymes in mammalian cells play an important role in

membrane repair and remodeling, which involves deacylation-reacylation cycles. Aside from their important role in basic lipid metabolism, PLA_2 enzymes participate in many physiological and pathological events involving signal transduction pathways.

Group II $sPLA_2$ is inducible and secreted in response to cytokine stimulation. Exposure to IL-1 stimulates gene transcription and protein synthesis resulting in time-dependent increase in $sPLA_2$ activity (Kerr *et al*, 1989; Gilman and Chang, 1990). The stimulatory effects of IL-1 can be suppressed by glucocorticoids (Schalkwijk *et al*, 1991), which exert their inhibitory effect by promoting expression of lipocortins (Ambrose and Hunninghake, 1990; Solito *et al*, 1991). Lipocortins are Ca^{2+}/lipid-binding proteins that inhibit the association of PLA_2 and its phospholipid substrates.

The regulation of Group IV $cPLA_2$ occurs via a variety of signal transduction pathways involving intracellular Ca^{2+} and/or enzyme phosphorylation as well as "cross-talk" between different phospholipases. $cPLA_2$ has a Ca^{2+}/lipid-binding (CaLB) domain that shares homology with that of protein kinase C (PKC), GTP-activating protein, and PLC (Clark *et al*, 1991; Sharp *et al*, 1991). However, it appears that Ca^{2+} is not involved directly in regulation of the catalytic activity of the enzyme, but promotes translocation of $cPLA_2$ from the cytosol to cell membranes, where the substrates are located (Dennis, 1994; Channon and Leslie, 1990). Recent studies have demonstrated that Ca^{2+}-dependent translocation results in association of $cPLA_2$ with the nuclear envelope and endoplasmic reticulum rather than the plasma membrane (Schievella *et al*, 1995; Peters-Golden *et al*, 1996; Pouliot *et al*, 1996). Considering the role of Ca^{2+} in increasing the functional capacity of $cPLA_2$, it is probable that LKT-induced increase in $[Ca^{2+}]_i$ results in $cPLA_2$-mediated hydrolysis of membrane phospholipids and synthesis of inflammatory eicosanoids.

Both *in vivo* and *in vitro* studies have indicated that phosphorylation of $cPLA_2$ is an important mechanism whereby enzymatic activity is controlled (Lin *et al*, 1993). Although there are many possible phosphorylation sites on $cPLA_2$, the one located at Ser-505 has been demonstrated to be of importance in regulation of catalytic activity. Mitogen-activated protein (MAP) kinase, which can be activated by tyrosine kinase, G-protein coupled receptors, and phorbol ester, phosphorylates $cPLA_2$ at Ser-505. Mutation at Ser-505 deletes MAP-mediated phosphorylation as well as subsequent agonist-stimulated release of AA from intact cells. Furthermore, deletion of the phosphate group by alkaline phosphatase also causes a loss of activity. MAP is not the only kinase implicated in this regulation; protein kinase C can also phosphorylate $cPLA_2$ (Nemenoff *et al*, 1993; Xing *et al*, 1994).

In addition to Ca^{2+}-mediated translocation and phosphorylation of $cPLA_2$ and possibly in association with these regulatory mechanisms, cross-talk among phospholipases has been proposed to play an important role in the control of AA release from phospholipid substrate. In particular, regulation of $cPLA_2$ by PLD has been demonstrated (Kennedy *et al*, 1996; Fujita *et al*, 1996; Bauldry and Wooten, 1997).

Phospholipase D

Phospholipase D is a ubiquitous enzyme that catalyzes the hydrolysis of phospholipids to generate PA and the corresponding head group. Phosphatidylcholine (PC), the major phospholipid component in mammalian cells, is the preferred substrate (Lambeth, 1994), although catalysis by PLD of phosphatidylethanolamine (PE) and phosphatidylinositol (PI) has been reported (Kiss and Anderson, 1989). The PA can be rapidly metabolized to 1, 2–diacylglycerol (DAG) by the action of phosphatidate phosphohydrolase (PAP) and to lysophosphatidic acid by the action of specific PLA_2.

In the presence of primary alcohols, PLD catalyzes the transphosphatidylation reaction in which the phosphatidyl group is transferred to the primary alcohol (ethanol, propanol, or butanol), generating relatively stable phosphatidylalcohols. Phosphatidylethanol (PET) is generated in the presence of ethanol. Since PA is unstable and can be produced by diglyceride kinase-mediated phosphorylation of DAG, production of phosphatidylalcohol, such as PET, is considered conclusive evidence of PLD activity.

Phospholipase D activity has been detected in all mammalian cells examined, including neutrophils (Balsinde *et al*, 1989; Siddiqi *et al*, 1995; Wang *et al*, 1991). Although purification of PLD is difficult, much progress has been made in this regard. PLD purified from porcine lung microsomes has a molecular mass of 190 kDa and is specific for PC (Okamura and Yamashita, 1994). This enzyme did not have an essential requirement for Ca^{2+} or Mg^{2+}, but could be stimulated by these ions as well as by fatty acids. A PLD extracted from porcine brain membranes had a molecular mass of 95 kDa and was markedly stimulated by phosphatidylinositol 4,5-bisphophate (PIP_2) and by the small G protein ADP-ribosylation factor (ARF) (Brown *et al*, 1995), as was a PLD solubilized from HL-60 cell membranes (Brown *et al*, 1993). A 120 kDa PLD has recently been cloned by screening a HeLa cell cDNA library (Hammond *et al*, 1995).

As with PLA_2 enzymes, the classification of PLD enzymes is complicated by the diversity of isozymes isolated not only from different cells and species but also from different locations in the cell. For example, most PLD located in the cytosol can hydrolyze PC, PE, and PI and requires Ca^{2+} for optimum activity while membrane-associated PLD prefers PC and has Ca^{2+}-independent activity (Wang *et al*, 1991). PLD has also been found in the endoplasmic reticulum, golgi body, and nuclei of certain cells and tissues (Provost *et al*, 1996). Nevertheless, a classification including two major classes of PLD has been proposed, based on the stimulatory effects of phosphoinositide (Jenco *et al*, 1998). One class of PLD isoforms (including PLD_1 and PLD_2) is

phosphoinositide-dependent and can be stimulated by small GTP-binding proteins (ARF, Rho family) and by protein kinase C. The other class is stimulated by free fatty acids instead of phosphoinositide.

Numerous agonists are capable of increasing PLD activity. Mechanisms of activation involve G-protein-coupled receptors, tyrosine kinase receptors, and pathways that do not involve receptors. The receptor-mediated mechanisms usually involve the Rho family of small GTP-binding proteins (RhoA, Ras, and cdc42), ARF, and protein kinase C, which directly activates PLD. For example, one probable activation pathway is that binding of an agonist to its receptor results in activation of phospholipase C, particularly PI-specific PLC, leading to generation of diacylglycerol and inositol triphosphate (IP_3). IP_3-mediated release of intracellular Ca^{2+} along with DAG then activates PKC, which activates PLD directly or activates small GTP-binding proteins (Rho family and ARF). However, in some instances, receptor-mediated activation of PLD may be PKC independent. Indeed, the recent discovery of a 50 kDa cytosolic factor that collaborates with ARF in activation of neutrophil and HL-60 PLD (Lambeth *et al*, 1995; Bourgoin *et al*, 1995) serves as further evidence of the complexity of PLD activation.

The role of Ca^{2+} in regulation of PLD has not been clearly elucidated. PLD isolated from porcine lung was Ca^{2+}-independent (Okamura and Yamashita, 1994), but the cytosolic PLD isolated from human granulocytes requires Ca^{2+} for activity (Balsinde *et al*, 1989; Siddiqi *et al*, 1995). Calcium was unable to activate PLD in permeabilized neutrophils and in extracts from neutrophils. However, the Ca^{2+} ionophore, A23187, stimulated PLD activity in granulocytes as well as in a number of other cell types (Lambeth, 1994). Furthermore, there is evidence that chelation of extracellular and intracellular Ca^{2+} inhibits receptor-mediated activation of PLD in neutrophils (Kessels *et al*, 1991) and other types of cells (Lin and Gilfillan, 1992; Huang *et al*, 1991; Balboa *et*

al, 1995). Phorbol ester-induced PLD activation was not blocked in intact neutrophils by removal of Ca^{2+}, but A23187 was synergistic with phorbol 12-myristate 13-acetate (PMA) in stimulating PLD activity in HL 60 cells. Therefore, these rather confusing and sometimes contradictory results suggest that Ca^{2+} may directly activate PLD in certain types of cells exposed to agonists while in other circumstances it may be required only for optimal activation of PLD. Probably, the requirement of Ca^{2+} by PLD is isoform dependent.

Despite the many questions that remain concerning regulation of PLD, it is clear that PLD itself serves as an important regulator of other phospholipases, particularly PLA_2. PA, the product of PLD catalysis, serves as an important intracellular messenger (English *et al*, 1996) and is known to stimulate a broad spectrum of cellular responses, including mobilization of Ca^{2+}, stimulation of the respiratory burst in neutrophils, activation of specific protein kinases and phospholipases, and promotion of mitogenesis in fibroblasts. PA may exert its effects by contributing to production of diacylglycerol and lysophosphatidic acid, both of which are involved in signal transduction pathways. Recently, the involvement of PLD in $cPLA_2$-dependent release of arachidonic acid in human neutrophils has been reported (Bauldry and Wooten, 1997). The release of AA was closely correlated with PA production. When PA production was inhibited by addition of ethanol, AA release decreased. Cross talk between PLA_2 and PLD has also been demonstrated in rat neutrophils and MDCK cells (Fujita *et al*, 1996; Kennedy *et al*, 1996).

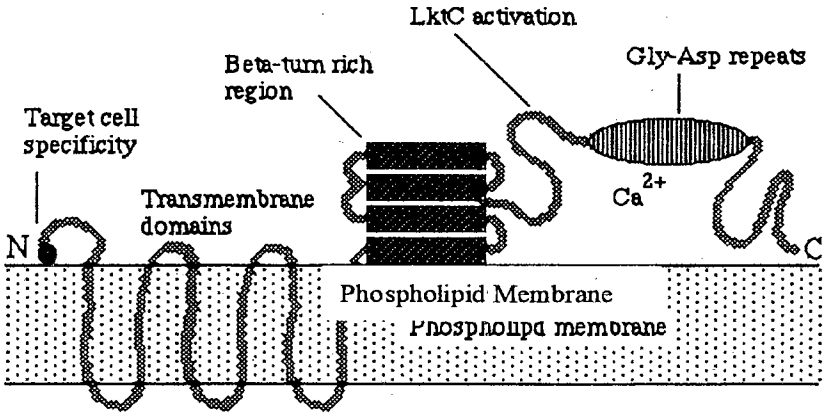

Figure 1. Molecular model for interaction of LKT with phospholipid membranes, adapted from Forestier and Welch (1991). The model proposes that the transmembrane domains, either singly or in cooperation with other LKT molecules, assume the structure of a pore that allows passage of monovalent and possibly divalent cations.

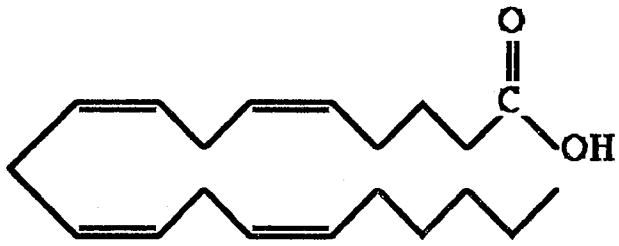

Figure 2. Structure of 5,8,11,14-eicosatetraenoic acid (arachidonic acid, AA))

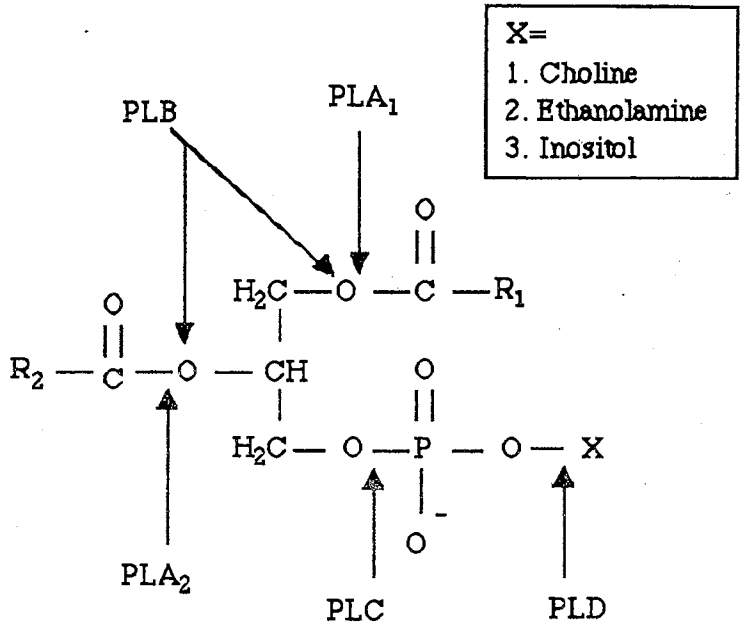

Figure 3. Phospholipid structure and catalytic sites of action of PLA_2, PLA_1, PLD, PLB and PLC

CHAPTER III

HYPOTHESIS AND EXPERIMENTAL GOALS

The hypothesis upon which this research was based is that *P. haemolytica* LKT causes excessive activation of PLA_2, which results in synthesis of chemotactic eicosanoids, thereby amplifying the inflammatory reaction and aggravating pulmonary tissue damage and that LKT-induced activation of PLA_2 is achieved via Ca^{2+}-mediated activation of PLD (Figure 4).

This hypothesis was tested by characterizing the effects of LKT on activities of PLA_2 and PLD of isolated bovine neutrophils and by examining the effects of specific inhibitors of phospholipase functions. To eliminate the potential contribution of other virulence factors of *P. haemolytica*, particularly LPS, effects of LKT were compared with those produced by a LKT-deficient mutant strain of *P. haemolytica*.

Specific objectives of the experiment were:

(1) to determine whether LKT caused increased activity of PLA_2, by measuring the effect of LKT on the release of arachidonate from isolated bovine neutrophils, the distribution of phospholipid substrates in neutrophil membranes, and the effects of an inhibitor of $cPLA_2$; and

(2) to determine whether LKT causes an increase in PLD activity in bovine neutrophils and to study the regulatory role of PLD in LKT-induced activation of PLA_2.

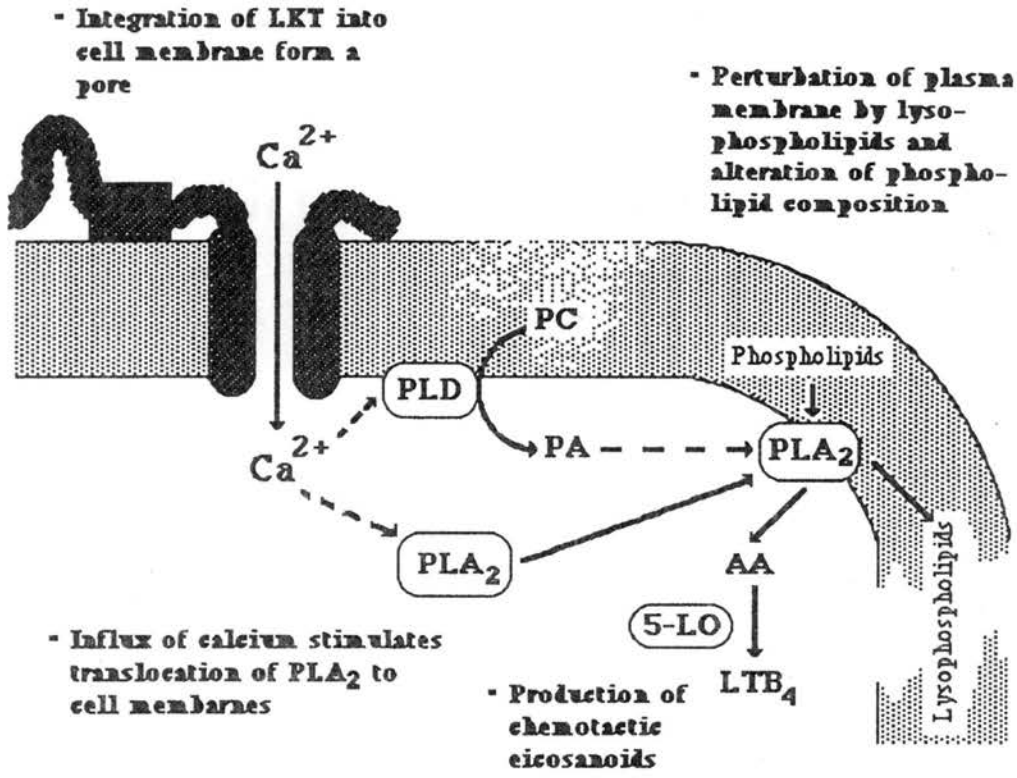

Figure 4. Hypothesized model describing involvement of PLA_2 and PLD in LKT-induced synthesis of LTB_4.

CHAPTER IV

PASTEURELLA HAEMOLYTICA LEUKOTOXIN-INDUCED INCREASE IN PHOSPHOLIPASE A_2 ACTIVITY IN BOVINE NEUTROPHILS

Introduction

Pasteurella haemolytica is the primary etiologic agent of pneumonic pasteurellosis (Collier *et al*, 1962), a disease that causes substantial economic losses to the cattle feedlot and stocker industry (Hird *et al*, 1991). Pulmonary lesions caused by *P. haemolytica* infection are characterized by extensive infiltration of neutrophils and exudation of fibrin into airways and alveoli (Yates, 1982). Mobilization of neutrophils fails to effectively combat infection, and degranulation and lysis of these phagocytes releases damaging products that aggravate pulmonary damage (Slocombe *et al*, 1985; Breider *et al*, 1988).

Chemotaxis of neutrophils and their inability to clear the infection may both be due to the action of *P. haemolytica* leukotoxin (LKT). This pore-forming RTX cytotoxin is produced by log-phase bacteria and causes lysis of ruminant leukocytes and platelets (Clinkenbeard *et al*, 1989; Clinkenbeard and Upton, 1991). Exposure of bovine neutrophils to low concentrations of LKT stimulates release of chemotactic eicosanoids, such as leukotriene B_4 (LTB_4) (Henricks *et al*, 1992). Previous studies reported that LKT-induced synthesis of LTB_4 by isolated bovine neutrophils was closely correlated with membrane damage and lysis (Clinkenbeard *et al*, 1994), suggesting a common mechanism for these two important effects of LKT.

Eicosanoids are derived from the oxidation of arachidonic acid (AA), which is released from membrane phospholipids via the action of phospholipases. Hydrolysis of the ester linkage at the *sn*-2 position of plasma membrane phospholipids by phospholipase A_2 (PLA_2) is believed to be the rate-limiting step in eicosanoid synthesis (Glaser *et al*, 1993). The action of phospholipases may also contribute to LKT-induced loss of plasma membrane integrity: hydrolysis of phospholipids by PLA_2 leads to elaboration of lysophospholipids, which are known to cause detergent-like effects on membranes (Weltzien, 1979).

Mammalian leukocytes contain several types of PLA_2 enzymes. The type most commonly involved in eicosanoid production is high molecular weight (85 kDa) cytosolic PLA_2 ($cPLA_2$) (Bauldry and Wooten, 1996). If $cPLA_2$ is involved in LKT-induced effects on bovine neutrophils, this enzyme would constitute a rational target for therapy to suppress the uncontrolled pulmonary exudation that contributes to lung damage. Therefore, the objectives of this study were to determine whether LKT caused increased activity of PLA_2, by measuring the effect of LKT on the release of arachidonate from isolated bovine neutrophils, the distribution of phospholipid substrates in neutrophil membranes, and the effects of an inhibitor of $cPLA_2$.

Materials and Methods

Preparation of *P. haemolytica* leukotoxin

P. haemolytica biotype A, serotype 1 wildtype strain and an isogenic leukotoxin-deficient mutant strain A, produced by allelic replacement of lktA with β-lactamase bla gene (Murphy, 1995), were grown in 150 ml BHI broth to an optical density at 600 nm (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 $NaHCO_3$) 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 × g for 30 minutes (Sorvall GS3 rotor, DuPont Co., Wilmington). 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 × 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.

Leukotoxin activity was quantified as toxic units (TU) using BL3 cells, as described previously (Clinkenbeard *et al*, 1994). One TU was defined as the amount of LKT that caused 50% maximal leakage of lactate dehydrogenase (LDH) from 4×10^5 BL3 cells in 200 µl at 37°C after 1 hour of incubation. The mean activity of undiluted LKT preparations used in this study was $6.6 \pm 1.9 \times 10^5$ TU/ml.

Preparation of bovine neutrophils

Two healthy beef calves (200 ± 50 kg) served as blood donors for isolation of neutrophils. Neutrophils were isolated by hypotonic lysis as previously described (Weiss *et al*, 1989). Briefly, the whole venous blood was collected in 60 ml syringes containing 5 ml 10% sodium citrate and then centrifuged in 50 ml polypropylene conical tubes (Corning Incorporated, Corning) at 600 × g and 4°C for 30 minutes (Centra-GP8R, IEC, Boston). The plasma, buffy coat and top layer of red blood cells were aspirated, leaving approximately 10 ml of the cell pellet in each tube. In the first cycle of hypotonic lysis, 20 ml cold (4°C) sterile distilled water was added to each tube, the cell suspension was

mixed for 50 - 60 seconds, 20 ml double-strength phosphate-buffered saline (PBS) was added to restore the tonicity, and the suspension was then centrifuged at 200 × g and 4°C for 10 minutes. The cell pellet was resuspended in 5 ml PBS after the supernatant was discarded. Thereafter, 10 ml water was again added to each tube, suspensions were mixed for 50-60 seconds, tonicity was balanced with 10 ml double-strength PBS, and then centrifuged at 200 × g and 4°C for 10 minutes. The cell pellet was washed once with PBS and twice with modified Ca^{2+}-free Hank's balanced salt solution (HBSS, Sigma Chemical Co., St. Louis), containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 50 μM ethylene glycol-bis (-aminoethylether) N, N, N', N'- tetraacetic acid (EGTA). The cell pellet in each tube was resuspended in 3 ml modified HBSS. The concentration of neutrophils was estimated by hemocytometer and adjusted to 2×10^7 cells/ml. The viability and purity of neutrophil suspensions were assessed by trypan blue exclusion. Proportions of viable neutrophils were greater than 95%.

Incorporation of [^{3}H] AA into neutrophils

Incorporation of [^{3}H] AA into bovine neutrophils was accomplished using a modification of the method described by Ramesha and Taylor (1991). Briefly, [^{3}H] AA (100 μCi/ml or 0.0010 mmol/ml ethanol, Dupont NEN research products, Boston) was added to neutrophils in suspension (2.0 ± 10^7 cells/ml) at 0.5 μCi/ml, and the suspension was then incubated at 37°C for 30 minutes. Thereafter, the suspension was centrifuged (200 × g, 10 min), the cell pellet was washed twice with cold HBSS, and the neutrophils were resuspended in HBSS containing 0.5 mM $MgCl_2$, 50 μM EGTA, and 1mM $CaCl_2$ at $1.0\text{-}1.5 \times 10^7$ cells/ml.

Effect of LKT on neutrophil phospholipase activity and membrane integrity

Concentration- and time-dependent effects of LKT and controls were tested in 1.5 ml polypropylene microcentrifuge tubes. LKT-induced responses were distinguished by comparison with leukotoxin-deficient control preparation (LKT(-)). Concentration-dependent effects of LKT on PLA_2 activity and membrane integrity were studied by incubating [^{3}H] AA loaded neutrophils with dilutions (1:10, 1:100, 1:200, 1:500, 1:1,000; 1:2,000, 1:5,000, 1:10,000) of LKT or LKT(-) for 60 minutes. The relationships between period of incubation and LKT-induced stimulation of phospholipases and loss of membrane integrity were measured by incubating neutrophils with LKT (1:100), LKT(-) (1:100), or the calcium ionophore, A23187 (2.5 μM) for 0, 5, 15, 30, 60, or 90 minutes. Experiments included 4 replicates for each of the primary treatments.

Release of radioactivity served as a measure of phospholipase activity in intact neutrophils. At the completion of each incubation period, the experiment was terminated by centrifugation at 10,000 × g for 5 minutes, 100 μl supernatant was suspended in 5 ml liquid scintillation cocktail (Atomlight, Dupont NEN Research Products, Boston), and radioactivity was measured for 3 minutes by liquid scintillation counting (Model LC5000TD, Beckman Instruments). Percent specific release of radioactivity related to tritium-labeled AA and metabolites was calculated using the formula:

$$\%\ \text{specific}\ ^3\text{H release} = \frac{(\text{LKT-induced radioactivity in supernatant} - \text{Bkg}) \times 100}{\text{Tot} - \text{Bkg}}$$

where Bkg is the radioactivity released by suspensions exposed to Hanks buffered saline solution (HBSS) and Tot is the total radioactivity added to the sample.

The effect of *P. haemolytica* LKT on neutrophil plasma membrane integrity was assayed by measuring extracellular release of LDH. 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), 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) 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:

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

Effect of LKT on distribution of ^{3}H-labeled membrane phospholipids

The ability of LKT to activate phospholipases was further explored by comparing the effect of LKT on the distribution of ^{3}H-labeled substrate and products in neutrophil membranes with those of positive (A23187) and negative (LKT(-)) controls. Bovine neutrophil suspensions were incubated at 37°C for 90 minutes with LKT (1:100), LKT(-) (1:100), A23187 (5 µM), and dimethylsulfoxide (DMSO, 2% final concentration), which served as a solvent control for A23187. Stimulation was terminated by addition of 3 ml chloroform-methanol (1:2, v/v) and 0.1 ml 9% formic acid, and lipids were extracted using a modification of the method of Bligh and Dyer (1959). Briefly, the mixture was

vortexed for 2 minutes, 2 ml chloroform was added and the mixture was vortexed for 30 seconds followed by further addition of 1 ml water and mixing for 30 seconds. After centrifugation at 600 × g for 10 minutes, the chloroform phase was removed and evaporated under a stream of nitrogen. Lipid precipitates were resuspended in 50 µl chloroform-methanol (9:1, v/v) and separated by thin-layer chromatography (TLC) on Silica Gel G plates (250 mm thickness, 20 × 20 cm plates, Alltech Associates Inc, Deerfield) by development for 2 hours in a solvent system consisting of chloroform-ethanol-water-triethylamine (30:34:8:35) (Korte and Casey, 1982). Silica gel bands (5 mm in width) were scraped into scintillation vials and radioactivity was measured by liquid scintillation counting. Identification of lipids in radioactive bands was accomplished by comparison with parallel tracks containing standards (phosphatidyl choline, PC; phosphatidyl inositol, PI; phosphatidyl ethanolamine, PE; fatty acids, FFA; and neutral lipids, NL) (Sigma Chemical Co., St. Louis). Radioactivities in eluted bands corresponding to lipid standards were reported as counts per minute (cpm) and as percentages of total radioactivity spotted onto the plates. Preliminary experiments confirmed that the distribution of ^{3}H-labeled membrane constituents in untreated neutrophils did not change substantially between 30 and 120 minutes of incubation.

Effect of $cPLA_2$ inhibition on LKT-induced effects

The $cPLA_2$ inhibitor, arachidonyl trifluoromethyl ketone ($AACOCF_3$) was used to confirm the involvement of $cPLA_2$ in LKT-induced eicosanoid synthesis (Street *et al*, 1993; Riendeau *et al*, 1994). The effect of $AACOCF_3$ on the release of radioactivity from [^{3}H] AA - loaded neutrophils was tested by adding appropriate volumes of $AACOCF_3$ (2 mM in 20% ethanol) to neutrophil suspensions to achieve concentrations of 0, 20, 40, 80, and 160 µM, preincubating for 15 minutes, and then exposing neutrophil suspensions (n = 4) to LKT (1:100) or A23187 (5 µM) for 30 minutes at 37°C.

Thereafter, neutrophil suspensions were treated as described above for estimation of [^{3}H] AA release. Data were reported as percent decrease in supernatant activity expressed as a proportion of [^{3}H] AA release measured at 0 μM $AACOCF_3$.

The effect of $AACOCF_3$ on the distribution of radioactivity in [^{3}H] AA-loaded neutrophils was investigated by preincubating neutrophil suspensions with 120 μM $AACOCF_3$ or DMSO solvent control (2% final concentration, v/v) for 15 minutes, and then exposing suspensions (n = 4) for 90 minutes at 37°C to LKT (1:100) or LKT(-) (1:100). Thereafter, lipids were extracted and separated by TLC, as described above.

Finally, the effect of $AACOCF_3$ on LKT-induced production of LTB_4 was examined by preincubating unlabeled neutrophil suspensions with 120 μM $AACOCF_3$ for 15 minutes, and then exposing suspensions (n = 4) for 120 minutes at 37°C to LKT (1:100) or A23187 (5 μM). Experiments were terminated by centrifugation at 600 × g for 10 minutes at 4°C and LTB_4 in 50 μl supernatant was assayed by radioimmunoassay (Dupont NEN Research Products, Boston), as described previously (Clinkenbeard *et al*, 1994).

Involvement of calcium in LKT-induced activation of PLA_2

The extracellular Ca^{2+} dependence of LKT-induced release of [^{3}H] AA and AA metabolites and LDH from radiolabeled neutrophils was tested 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 $CaCl_2$ and $MgCl_2$ were not added as in previous experiments. The % specific ^{3}H and % specific LDH release were estimated after 30 minutes of incubation at 37°C, as described above.

Statistical analyses

Statistical analyses were conducted using a commercially available microcomputer program (Systat, Evanston). Concentration- and time-dependent effects of LKT and/or A23187 on [^{3}H] AA and LDH release, and the effects of these stimulators on the distribution of radioactivity in neutrophil membranes and LTB_4 synthesis were compared to corresponding negative controls using t tests. The effect of $AACOCF_3$ on release of [^{3}H] AA from intact neutrophils was investigated by comparing the response at each dosage level with that of the inhibitor-free control, using Dunnett's test. The effects of $AACOCF_3$ on the distribution of radioactivity in LKT-exposed neutrophils and extracellular Ca^{2+}-dependency of LKT induced responses were investigated using the general linear model followed by comparison of means using Turkey's test. Differences between means were declared significant at the $P < 0.05$ level.

Results

LKT caused [^{3}H] AA and AA metabolites release (6.19 ± 0.50% at a dilution of 1:10) and LDH leakage (77.09 ± 21.92% at a dilution of 1:10) from bovine neutrophils in a dose-dependent manner whereas LKT(-) failed to stimulate either [^{3}H] AA and AA metabolites release or LDH leakage across the range of dilutions tested (Figure 5). When compared with the response to A23187, exposure of [^{3}H] AA-loaded neutrophils to LKT resulted in lower maximum % specific [^{3}H] AA release (8.69 ± 2.14 at 30 minutes of incubation versus 37.11 ± 9.66 at 60 minutes for A23187) but higher maximum % specific LDH release (79.38 ± 1.77 versus 47.92 ± 2.79 for A23187) (Figure 6). Maximal responses for both [^{3}H] AA release and LDH release were achieved more rapidly by LKT-exposed neutrophils than by those exposed to A23187.

Thin-layer chromatography revealed that incubation of bovine neutrophils with [^{3}H] AA resulted in labeling of the phospholipid, FFA, and NL components of lipid membranes (Table 1). In neutrophils exposed to LKT(-), the highest proportion of radioactivity was associated with PC (38.07 ± 0.44%) and lower proportions were associated with the FFA (7.02 ± 0.41%) and NL (14.63 ± 1.46%) constituents. Exposure to LKT caused a significant decrease in labeled PC and increases in labeled FFA and NL, consistent with metabolism of phospholipid substrate by phospholipases and production of free arachidonate and LTB_4, which elute together with the FFA and NL standards (Bauldry *et al*, 1988), respectively. Exposure to A23187 caused an even greater transfer in radioactivity from PC to NL components, consistent with this ionophore's ability to induce Ca^{2+}-mediated production of eicosanoids.

Pretreatment of neutrophils with $AACOCF_3$ confirmed the involvement of $cPLA_2$ in LKT-induced synthesis of eicosanoids. When [^{3}H] AA-loaded neutrophils were pretreated with the inhibitor and subsequently exposed to LKT, the amount of radioactivity released decreased as the concentration of $AACOCF_3$ was increased (Figure 7). At a concentration of 160 μM, the amount of radioactivity released was 76.19 ± 2.66% of the value measured without the inhibitor. The calcium ionophore, A23187, was even more susceptible to the inhibitory effects of $AACOCF_3$; at 160 μM, release of radioactivity was decreased by approximately 50%. The effects of $AACOCF_3$ on the distribution of radioactivity in neutrophil membranes further supported the involvement of $cPLA_2$, by demonstrating inhibitory effects on LKT-induced decrease in PC substrate and increase in NL product (Table 2). However, $AACOCF_3$ also caused significant increases in labeled FFA, suggesting that this inhibitor may affect enzymes and processes in addition to those involved in release of AA by $cPLA_2$. Nevertheless, treatment of unlabeled neutrophils with $AACOCF_3$ demonstrated that preservation of PC substrate is

correlated with LTB_4 synthesis, as the LKT- and A23187-induced releases of LTB_4 from intact neutrophils were both inhibited (Figure 8).

Release of radioactivity and LDH from LKT-exposed neutrophils was Ca^{2+}-dependent (Figure 9). Removal of Ca^{2+} from the incubation medium caused decreases in both [^{3}H] AA and LDH release. Further decreases in both responses were produced when EGTA was added to the Ca^{2+}-free medium; LKT-induced effects were restored when a high concentration of Ca^{2+}, exceeding the chelating capacity of EGTA, was added. These results were consistent with Ca^{2+}-dependent catalysis of membrane phospholipids by $cPLA_2$.

Table 1

Mean (± SD) counts per minute (cpm) and percentages of total radioactivity in each elution profile (n = 3) corresponding to phosphatidyl choline (PC), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE), free fatty acids (FFA), and neutral lipid (NL) standards. Bovine neutrophil suspensions were incubated at 37°C for 90 minutes with LKT (1:10), LKT(-) (1:10), A23187 (5 μM), and dimethylsulfoxide (DMSO), and lipid extracts were subjected to thin-layer chromatography.

	LKT(-)		LKT		DMSO		A23187	
	cpm	%	cpm	%	cpm	%	cpm	%
PC	103052 ± 8979	38.07 ± 0.44	82032 ± 10299	29.62* ± 0.26	109896 ± 2618	40.50 ± 0.60	47168 ± 1754	20.50* ± 0.63
PI	51956 ± 3421	19.22 ± 0.53	65867 ± 9046	23.76* ± 0.14	47165 ± 1629	17.39 ± 0.89	38001 ± 1762	16.53 ± 1.01
PE	51011 ± 488	18.93 ± 1.57	46701 ± 5022	16.89 ± 0.58	33471 ± 26570	12.23 ± 9.69	24943 ± 8584	10.90 ± 3.96
FFA	19056 ± 2722	7.02 ± 0.41	27139 ± 5794	9.73* ± 0.80	18755 ± 26344	7.02 ± 9.91	30373 ± 7755	13.24 ± 3.50
NL	39825 ± 7241	14.63 ± 1.46	49186 ± 5791	17.77* ± 0.33	33818 ± 3854	12.45 ± 1.26	40533 ± 7391	17.54* ± 2.55

* Values significantly different from corresponding negative control values

Table 2

Mean (± SD) counts per minute (cpm) and percentages of total radioactivity in each elution profile (n = 4) corresponding to phosphatidyl choline (PC), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE), free fatty acids (FFA), and neutral lipid (NL) standards. Bovine neutrophils, loaded with [^{3}H]-AA, were pre-incubated with 120 μm $AACOCF_3$ or DMSO solvent control (2% final concentration, v/v) for 15 minutes, and then exposed (n = 4) for 90 minutes at 37°C to LKT (1:10) or LKT(-) (1:10)

	LKT(-)		LKT		LKT + $AACOCF_3$	
	cpm	%	cpm	%	cpm	%
PC	63706 ± 18981	37.53[a] ± 0.88	52620 ± 6935	31.82[b] ± 1.22	64093 ± 3762	34.85[c] ± 1.41
PI	35564 ± 10708	20.92[a] ± 0.35	32501 ± 3593	19.70[a] ± 0.97	35283 ± 10919	19.08[a] ± 5.37
PE	35314 ± 10382	20.83[a] ± 0.45	27884 ± 4742	16.81[b] ± 1.02	23865 ± 2639	12.94[c] ± 0.73
FFA	6476 ± 1785	3.83[a] ± 0.08	8775 ± 494	5.34[b] ± 0.34	12415 ± 941	6.74[c] ± 0.17
NL	17877 ± 5256	10.53[a] ± 0.57	24042 ± 2598	14.56[b] ± 0.09	24557 ± 3072	13.30[c] ± 0.54

Values within the same row that have different letter superscripts are significantly different.

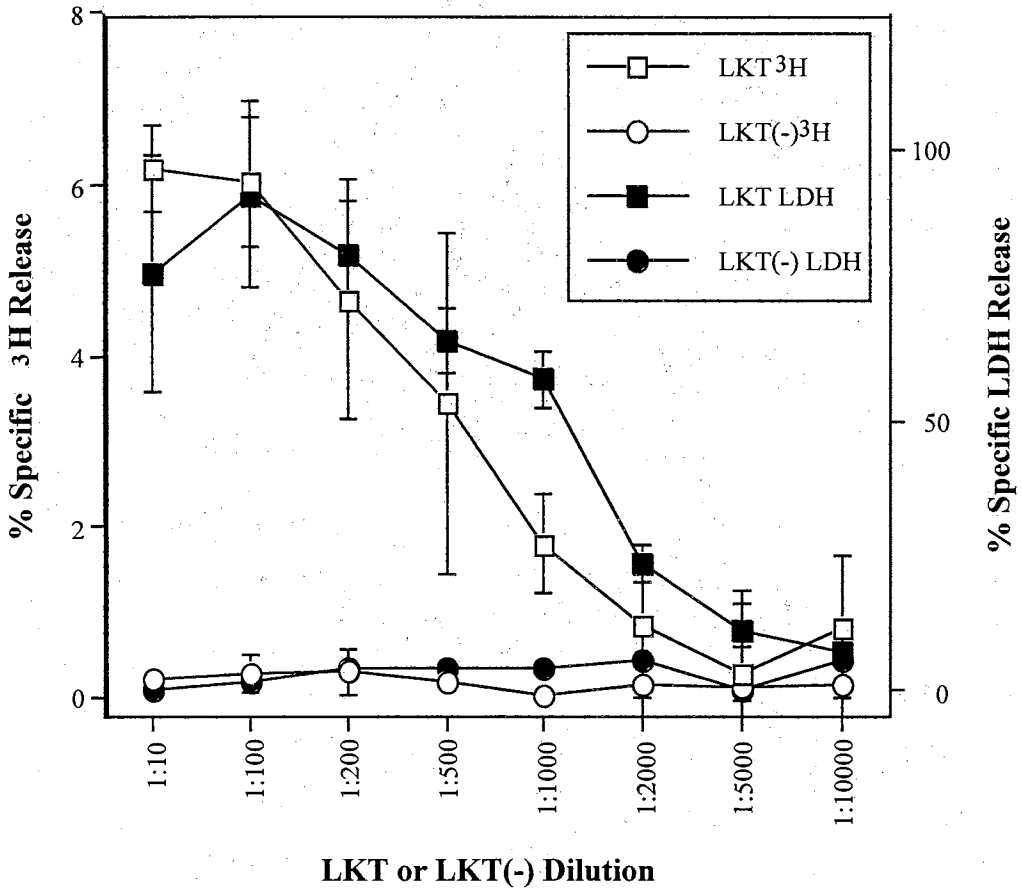

Figure 5. Effect of LKT and LKT(-) control preparations on release of [^{3}H] AA and LDH. Isolated neutrophils, loaded with [^{3}H] AA, were exposed to dilutions of LKT or LKT(-) for 60 minutes (n = 4). Mean values describing release of [^{3}H] AA caused by LKT dilutions = 1:1,000 were significantly different from corresponding LKT(-) values. Except for the 1:10,000 dilution, all % specific LDH release values were significantly different from corresponding negative control values.

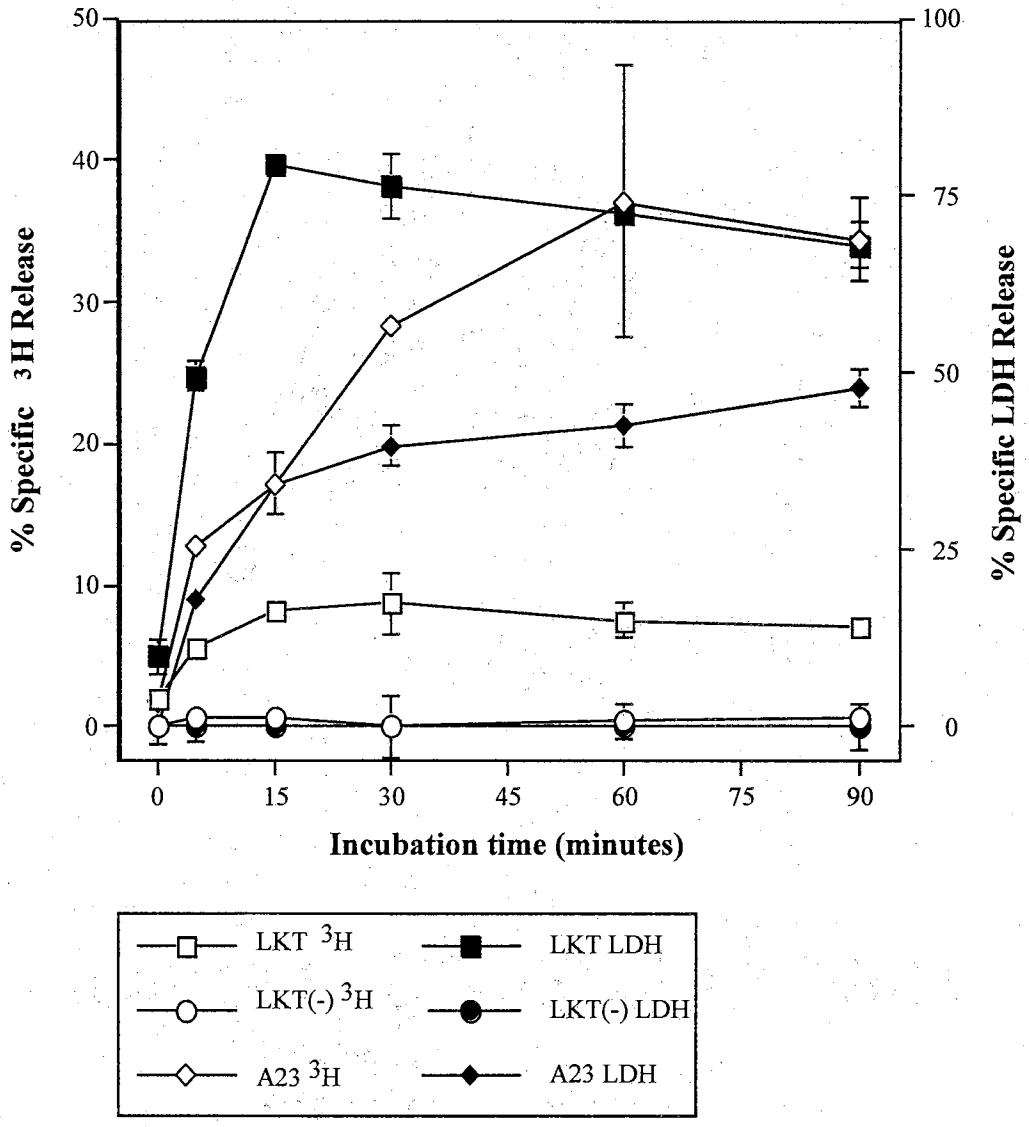

Figure 6. Time-dependent effects on release of [^{3}H] AA and LDH after exposure of isolated bovine neutrophils to LKT (1:10 dilution), LKT(-) (1:10 dilution), or A23187 (2.5 μM). All LKT and A23187 values were derived from samples incubated for ≈ 5 minutes were significantly different from corresponding LKT(-) values.

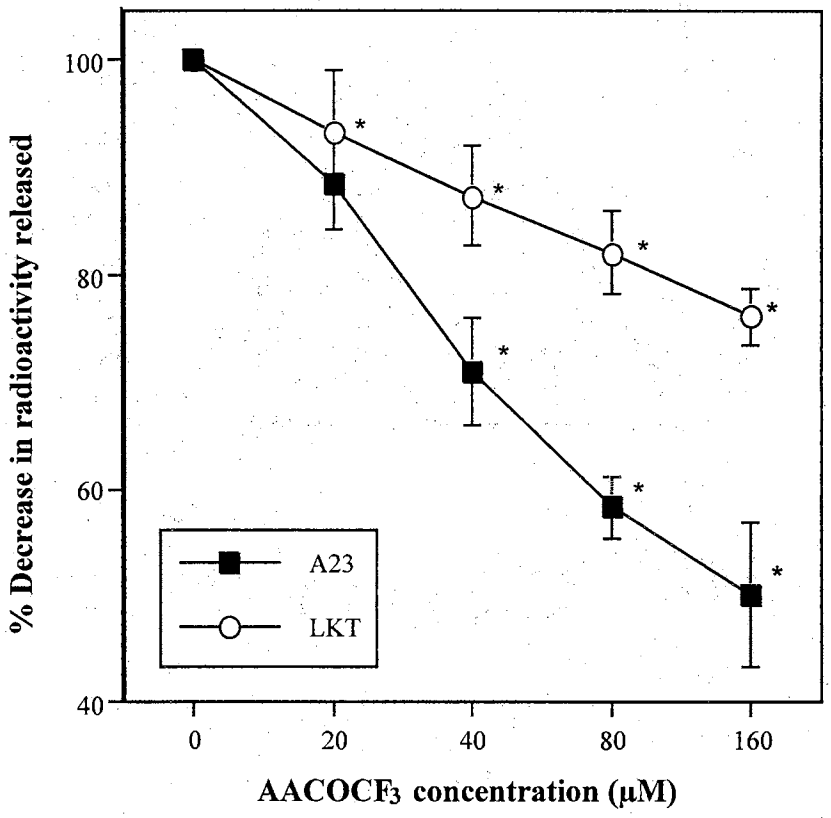

Figure 7. Mean (± SD) percent decrease in radioactivity released from isolated bovine neutrophils exposed to LKT or A23187 (A23) in the presence of different concentrations of $AACOCF_3$ (n = 4). *Radioactivity (dpm) values are significantly different from corresponding inhibitor-free (0 μM $AACOCF_3$) value.

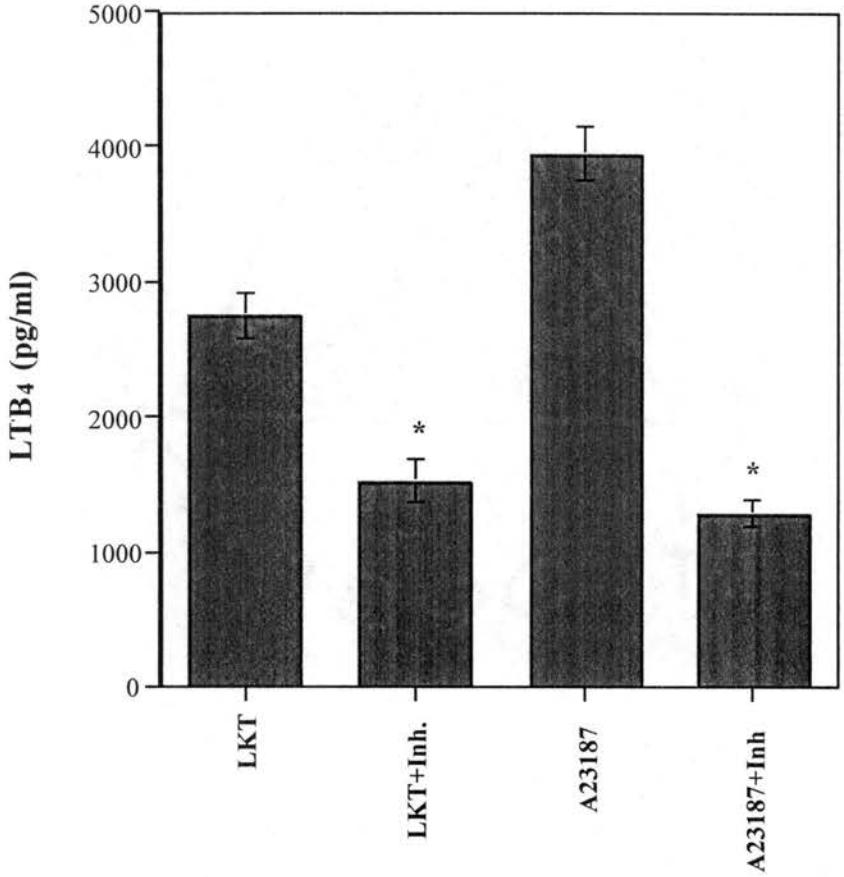

Figure 8. Effect of $AACOCF_3$ (Inh.) on synthesis of LTB_4 induced by exposure of isolated bovine neutrophils to LKT (1:10) or A23187 (5μM) for 120 minutes (n = 4).

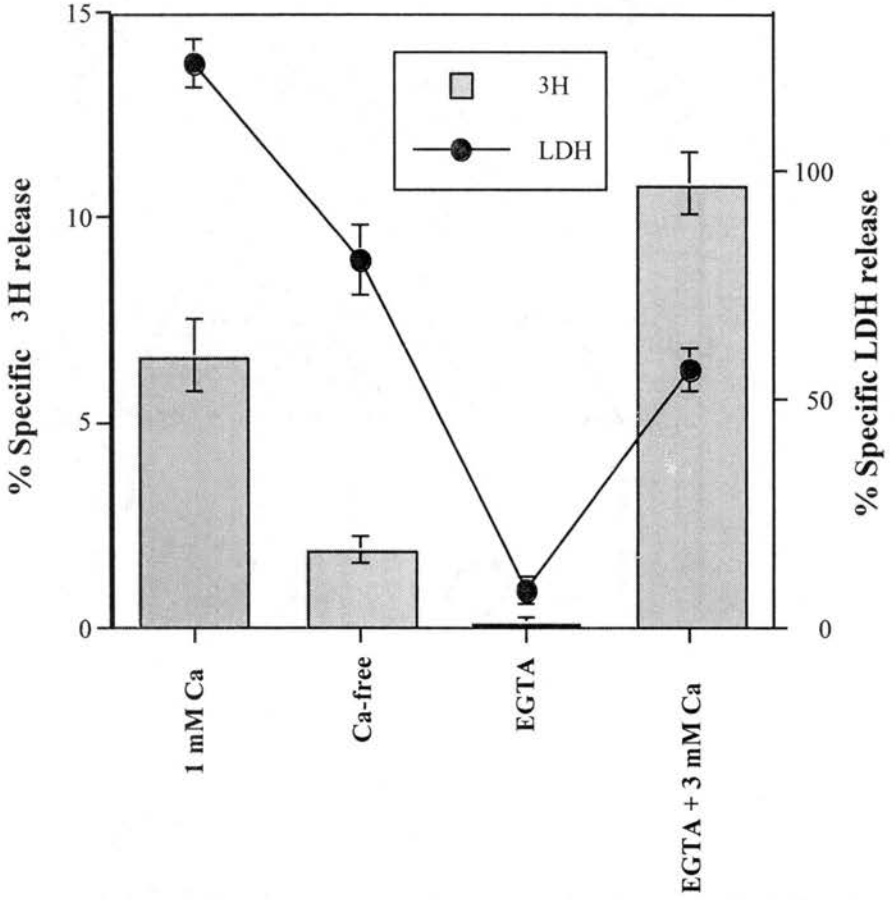

Figure 9. Extracellular calcium dependence of LKT-induced effects on % specific ^{3}H AA release and % specific LDH release. Isolated neutrophils were exposed to a 1:10 dilution of LKT in buffer suspensions containing 1 mM $CaCl_2$ (1 mM Ca), no Ca^{2+} (Ca-free), 1 mM EGTA and no Ca^{2+} (EGTA), or 1 mM EGTA and 3 mM $CaCl_2$ (EGTA + 3 mM Ca). All treatments within each of the response variables were significantly different from one another.

Discussion

Pasteurella haemolytica LKT is a member of the repeats-in-toxin (RTX) group of exotoxins that are produced by a number of gram-negative bacteria. Previous studies have indicated that LKT causes cytolysis of ruminant leukocytes and platelets (Clinkenbeard *et al*, 1989; Clinkenbeard and Upton, 1991) and, at sublytic concentrations, induces degranulation of neutrophils and generation of reactive oxygen derivatives (Maheswaran *et al*, 1992; Czuprynski *et al*, 1991). Furthermore, exposure of bovine neutrophils to LKT stimulates the release of eicosanoids such as LTB_4 (Henricks *et al*, 1992; Clinkenbeard *et al*, 1994), which has been implicated as an important chemotactic agent for bovine neutrophils (Heidel *et al*, 1989) and a mediator of inflammation in *P. haemolytica* infection (Clarke *et al*, 1994).

Synthesis of LTB_4 involves two important enzyme systems; PLA_2 and 5-lipoxygenase (5-LO). The former catalyzes the hydrolysis of cell membrane phospholipids to liberate AA, which is then further oxidized by the latter to LTB_4 via the intermediate, 5(S)-hydroperoxy-6,8,11,14-(E,E,Z,Z)-eicosatetraenoic acid (5-HPETE) (Ford-Hutchinson *et al*, 1994). Hydrolysis of membrane phospholipids by PLA_2 is believed to be the rate limiting step in eicosanoids synthesis (Glaser *et al*, 1993) and, therefore, serves as a relevant focus for investigation of the mechanism of LKT-induced synthesis of LTB_4 by bovine neutrophils.

Release of radioactivity from [^{3}H] AA-loaded cells provides a convenient and sensitive method of studying phospholipase activity in a wide variety of cell types, including neutrophils. Incorporation of [^{3}H] AA into the PC, PI, and PE fractions of the lipid membrane in the present study was consistent with the results of previous studies investigating remodeling of phosphoglycerides in neutrophils (Chilton and Murphy, 1986; Ramesha and Taylor, 1991). This incorporation profile is not related to pool size,

but reflects rates of phosphoglyceride turnover. In human neutrophils, relatively short incubation periods, such as that used in the present study, result in preferential incorporation of activity into PI and PC, whereas longer incubation periods result in more radioactivity being incorporated into PE. All three of these phosphoglycerides serve as important substrates for the PLA_2 enzymes found in neutrophils (Mayer and Marshall, 1993). Thus, the results of the present study demonstrating LKT-induced release of incorporated [^{3}H] AA and redistribution of radioactivity from PC substrate to FFA and NL products provide strong evidence of activation of PLA_2 by LKT. Although statistically significant, the extent to which LKT activated PLA_2 was lower than that predicted from previous studies (Clinkenbeard *et al*, 1994) that indicated that exposure of bovine neutrophils to LKT caused the production of large quantities of LTB_4. However, this discrepancy can be explained by the limitations of measuring only the release and redistribution of labeled AA and metabolites. Comparison between gas chromatographic and radiometric assays led to the conclusion that radiometric assay substantially underestimates PLA_2 activity because it does not take into account the AA released from endogenous, unlabeled phosphoglyceride pools (Ramesha and Taylor, 1991).

Release of AA from neutrophil membrane phosphoglycerides is believed to occur primarily via the actions of two types of PLA_2; type II secretory PLA_2 ($sPLA_2$) and type IV cytosolic PLA_2 ($cPLA_2$) (Mayer and Marshall, 1993; Bauldry *et al*, 1988). Low MW $sPLA_2$ (~14 kDa) requires mM concentrations of Ca^{2+} for optimal catalytic activity (Glaser *et al*, 1993; Dennis, 1994), and has no fatty acid specificity in the *sn*-2 position of phosphoglyceride substrates, but prefers PE and, to a lesser degree, PI and PC (Diez *et al*, 1994). In contrast to $sPLA_2$, $cPLA_2$ has a higher MW (~85 kDa), requires nM concentrations of Ca^{2+} for translocation from the cytosol to the nuclear envelope, and has no phospholipid substrate preference but prefers AA in the *sn*-2 position.

Inhibitory effects of $AACOCF_3$ on LKT-induced release of [^{3}H] AA and LTB_4 from intact neutrophils and decrease in PC and increase in NL fractions of neutrophil membranes provides further evidence that exposure of bovine neutrophils to LKT activates PLA_2. $AACOCF_3$, an analogue of AA in which the COOH group is replaced with $COCF_3$, is a slow, tight-binding inhibitor of $cPLA_2$ (Street *et al*, 1993); it has no inhibitory effect on $sPLA_2$ (Street *et al*, 1993: Bartoli *et al*, 1994). However, the decreases in PE and PI and the increase in FFA membrane fractions of cells exposed to LKT in the presence of $AACOCF_3$ suggest that while $AACOCF_3$ inhibits $cPLA_2$, it may promote hydrolysis of phospholipid substrates other than PC. A more definitive understanding of the specificity of $AACOCF_3$ for enzymes involved in release of membrane phospholipids and membrane remodeling would require identification of catalysis products using an analytical method that has greater resolution than TLC.

Experiments involving human neutrophils permeabilized with another pore-forming bacterial toxin, *Staphylococcus aureus* α-toxin, have provided strong evidence that $cPLA_2$ is primarily responsible for providing AA precursor for LTB_4 synthesis (Bauldry and Wooten, 1996). However, the involvement of $cPLA_2$ in providing substrate for leukotriene synthesis is less certain in other leukocyte types. In human monocytes, $cPLA_2$ appears to play a more important role in synthesis of prostaglandins than leukotrienes; $sPLA_2$ apparently is primarily responsible for providing the substrate for leukotriene formation (Marshall *et al*, 1997). Nevertheless, it is clear from the inhibitory effect of $AACOCF_3$ on LTB_4 synthesis observed in the present study that, in bovine neutrophils, $cPLA_2$ plays an important role in leukotriene biosynthesis.

A previous study (Clinkenbeard *et al*, 1994) demonstrated that LKT-induced synthesis of LTB_4 is dependent on extracellular Ca^{2+}. The present study further extends our understanding of the involvement of Ca^{2+} in LKT-induced LTB_4 synthesis by confirming that Ca^{2+} is necessary for activation of PLA_2. Influx of Ca^{2+} into LKT-

exposed neutrophils causes increased intracellular Ca^{2+} concentration, which serves as the stimulus for the oxidative burst (Ortiz-Carranza and Czuprynski, 1992). Calcium-dependency of PLA_2 function is consistent with the role of Ca^{2+} in stimulating translocation of $cPLA_2$ or modulating the catalytic activity of $sPLA_2$. The further reduction in responses observed in the present study when EGTA was added to the Ca^{2+}-free suspension medium suggests that intracellular stores of Ca^{2+} may also contribute to the LKT-induced increase in intracellular Ca^{2+}; extracellular EGTA causes rapid depletion of intracellular Ca^{2+} stores as Ca^{2+} rapidly diffuses down a concentration gradient from intracellular organelles to extracellular buffer (Rosales and Brown, 1992).

The involvement of PLA_2 in LDH release appears to be more complex than that of LTB_4 synthesis. Extracellular leakage of large MW LDH serves as a measure of plasma membrane integrity. The parallel LKT concentration-dependencies of LDH release and [^{3}H] AA and AA metabolites release suggested that a single mechanism, such as the elaboration of both AA and membrane-damaging lysophospholipids, may explain both responses. However, close examination of the relationship between incubation time and LDH release from LKT- or A23187-exposed neutrophils indicated that LKT caused more LDH release yet less [^{3}H] AA and AA metabolites release than did A23187. The moderate degree of membrane damage caused by A23187 suggested that Ca^{2+}-mediated activation of phospholipases and production of lysophospholipids probably contributed to membrane damage, but other mechanisms must be investigated to fully explain the lytic effect of LKT.

The central role of neutrophils in the development of fulminating pneumonic pasteurellosis is well supported. Experimental aerosol exposure to *P. haemolytica* induces rapid infiltration of neutrophils into the lung (Walker *et al*, 1985) and a marked increase in the neutrophil/macrophage ratio (Lopez *et al*, 1986). These changes correlate well with reported histologic changes in which small airways become plugged with

purulent exudate (Lopez *et al*, 1986). There is reliable evidence indicating that mobilization of neutrophils does not effectively combat infection, but contributes to development of lung lesions. Neutrophil depletion prior to inoculation with *P. haemolytica* protected calves from the development of gross fibrinopurulent pneumonic lesions (Slocombe *et al*, 1985), although less severe inflammatory changes still occurred (Breider *et al*, 1988). Thus, the neutrophil-mediated inflammatory response itself appears to be a major determinant of *P. haemolytica* pathogenicity and identification of the mechanisms whereby LKT induces the synthesis of leukotrienes that cause chemoattraction of neutrophils into infected tissue is crucial to understanding the pathogenesis of pneumonic pasteurellosis. The results of the present study support the hypothesis that LKT-induced LTB_4 synthesis involves Ca^{2+}-dependent activation of $cPLA_2$.

This study, together with further elucidation of the role of other PLA_2 enzymes and 5-LO in LKT-induced synthesis of LTB_4, is expected to identify pathways and mechanisms that can be targeted to develop strategies for the control of infections caused by *P. haemolytica* and other similar bacteria. Antibacterial therapy of these infections may fail because inflammatory responses caused by bacteria change the composition of interstitial fluid and compromise host defenses (Clarke *et al*, 1994), thus decreasing antibiotic activity (Vandaux and Waldvogel, 1980). The use of agents to suppress uncontrolled pulmonary exudation mediated by inflammatory pathways such as activation of $cPLA_2$ is likely to have the benefit of restoring effective neutrophil phagocytic function as well as enhancing the efficacy of antibacterial therapy.

CHAPTER V

ROLE OF PHOSPHOLIPASE D IN *PASTEURELLA HAEMOLYTICA* LEUKOTOXIN-INDUCED INCREASE IN PHOSPHOLIPASE A_2 ACTIVITY IN BOVINE NEUTROPHILS

Introduction

Pasteurella haemolytica biotype A serotype 1 is the primary bacterial agent of bovine pneumonic pasteurellosis or shipping fever (Collier *et al.*, 1962), a disease that is characterized by extensive infiltration of neutrophils and exudation of fibrin into airways and alveoli (Yates, 1982). Instead of clearing the bacterial infection, mobilized neutrophils aggravate lung injury (Breider *et al*, 1988; Slocombe *et al*, 1985), probably by undergoing degranulation and lysis resulting in the release of inflammatory mediators, superoxides, and proteolytic enzymes.

A bacterial virulence factor that appears to contribute substantially to infiltration of neutrophils into sites of *Pasteurella haemolytica* infection is *P. haemolytica* leukotoxin (LKT). Leukotoxin is a pore-forming RTX cytotoxin produced by log-phase bacteria that is specifically cytolytic to ruminant leukocytes and platelets (Clinkenbeard *et al*, 1989; Clinkenbeard *et al*, 1991). Exposure of bovine neutrophils to LKT stimulates not only degranulation and production of superoxides (Maheswaran *et al*, 1992) but also synthesis of chemotactic eicosanoids, such as leukotriene B_4 (LTB_4) (Clinkenbeard *et al*, 1994). Leukotriene B_4 is a potent chemotactic agent for bovine neutrophils (Heidel *et al*, 1989)

and has been implicated as an important mediator of *P. haemolytica*-induced inflammation (Clarke *et al*, 1994).

Leukotriene B_4 is derived from oxidation of arachidonic acid (AA), which is released from membrane phospholipids by the action of phospholipases (Needleman *et al*, 1986). A previous study has demonstrated that exposure of bovine neutrophils to LKT results in increased activity of cytosolic phospholipase A_2 ($cPLA_2$) and subsequent synthesis of LTB_4; a specific inhibitor of $cPLA_2$ inhibited both the release of membrane AA as well as production of LTB_4 (Wang *et al*, 1998). Leukotoxin-induced effects on $cPLA_2$ were dependent on extracellular Ca^{2+}, consistent with the role of calcium in promoting translocation of $cPLA_2$ from the cytosol to cell membranes. However, it is unlikely that regulation of $cPLA_2$ occurs entirely via the direct effects of intracellular Ca^{2+}. In human neutrophils, PLA_2 acts in concert with phospholipase D (PLD), which occupies a central position in the signaling cascade leading to neutrophil activation and synthesis of eicosanoid mediators in response to physiological stimulators (Bauldry and Wooten, 1997). Future research exploring the use of anti-inflammatory agents to attenuate LKT-induced inflammation depends on elucidation of the principal regulatory mechanisms controlling phospholipid metabolism. Therefore, the objectives of this study were to determine whether LKT causes an increase in PLD activity in bovine neutrophils and to study the regulatory role of PLD in LKT-induced activation of PLA_2.

Materials and Methods

Preparation of *P. haemolytica* Leukotoxin

P. haemolytica LKT and LKT-negative control (LKT(-)) preparations were prepared as described previously (Wang *et al*, 1998), using a *P. haemolytica* biotype A,

serotype 1 wildtype strain and its isogenic LKT-deficient mutant (produced by allelic replacement of LktA with a β-lactamase bla gene) (Murphy *et al*, 1995). Leukotoxin activity was quantified as toxic units (TU) using BL3 cells, as described previously (Clinkenbeard *et al*, 1994). One TU was defined as the amount of LKT that caused 50% maximal leakage of lactate dehydrogenase (LDH) from 4 x 10^5 BL3 cells in 200 µl at 37°C after 1 hour of incubation. The mean activity of undiluted LKT preparations used in this study was $6.6 \pm 1.9 \times 10^5$ TU/ml. LKT and LKT(-) preparations were divided into aliquots and stored frozen at -135°C until use.

Preparation of bovine neutrophils

Two healthy beef calves (200 ± 50 kg) served as blood donors for isolation of neutrophils. Neutrophils were isolated by hypotonic lysis as described previously (Wang *et al*, 1998) and suspended in Ca^{2+}- and Mg^{2+}-free HBSS. Cell concentration and viability were estimated by hemocytometer and trypan blue exclusion. Proportions of viable neutrophils were greater than 95%.

Radiolabeling of bovine neutrophils

Activity of PLA_2 was assayed by measuring the release of [^{3}H] AA from radiolabeled cell membranes. Bovine neutrophils were labeled using a modification of the method described by Ramesha and Taylor (1991). Briefly, [^{3}H] AA (100 µCi/ml or 0.0010 mmol/ml ethanol, Dupont NEN Research Products, Boston) was added to neutrophils in suspension (2.0×10^7 cells/ml) at 0.5 µCi/ml, and the suspension was then incubated at 37°C for 30 minutes. Thereafter, the suspension was centrifuged (200 × g, 10 min) and the cell pellet washed twice with cold HBSS before resuspending the

neutrophils in HBSS containing 0.5 mM $MgCl_2$, 50 μM EGTA, and 1mM $CaCl_2$ at a concentration of 1.0-1.5 × 10^7 cells/ml.

Activity of PLD was assayed by measuring the release of radioactivity from cells labeled with 1-O-alkyl-[1'-2'-^{3}H]-2-lyso-glycerol-3-phosphatidylcholine ([^{3}H] lyso-PC, 30Ci/mmol, Dupont NEN Research Products, Boston). [^{3}H] lyso-PC was suspended by sonication in Ca^{2+}-free HBSS (7 μCi of [^{3}H] lyso-PC in 70 μl ethanol was mixed with 10 ml HBSS buffer) and then added to the tube containing the bovine neutrophil pellet. The cell concentration was ≈ 3.0 × 10^7 cells per ml. Cell suspensions were incubated at 37°C for 45 min, washed twice with cold HBSS, and finally suspended in modified HBSS (1mM Ca^{2+}, 0.5 mM Mg^{2+}, 50μM EGTA) at 1.5 × 10^7 cells/ml.

Effect of LKT on neutrophil PLD activity

Concentration- and time-dependent effects of LKT and controls were tested in 10 ml glass tubes. LKT-induced responses were distinguished by comparison with LKT(-). Concentration-dependent effects of LKT on PLD activity were studied by incubating [^{3}H] lyso-PC-loaded neutrophils with dilutions (1:100, 1:1000, 1:50000) of LKT or LKT(-) for 15 minutes. The relationship between period of incubation and LKT-induced stimulation of PLD was measured by incubating neutrophils with LKT (1:1000) or LKT(-) (1:1000) for 0, 2, 5, 10 or 15 minutes. Experiments included at least 3 replicates for each of the primary treatments.

Stimulation was terminated by addition of 3 ml chloroform-methanol (1:2 v/v) and 0.1 ml 9% formic acid, and lipids were extracted using a modification of the method of Bligh and Dyer (1959). Briefly, the mixture was vortexed for 2 minutes, 2 ml chloroform were added and the mixture was vortexed for 30 seconds followed by further addition of 1 ml water and mixing for 30 seconds. After centrifugation at 600 × g for 10 minutes, the

chloroform phase was removed and evaporated under a stream of nitrogen. Lipid precipitates were resuspended in 30 µl chloroform-methanol (9:1, v/v) and separated by thin-layer chromatography (TLC) on channeled Silica Gel G 60 TLC plates (250 mm thickness, 20 × 20 cm plates, Fisher Scientific Co., St. Louis) by development for 70 minutes in the organic phase of a solvent system consisting of ethyl acetate : iso-octane : acetic acid : water (110:50:20:100, v/v) (Bauldry and Wooten, 1997). Silica gel bands corresponding to phosphatidic acid (PA), the primary product of PLD-catalyzed hydrolysis of phosphatidylcholine, were identified by parallel elution of PA standard (Sigma Chemical Co., St. Louis) and scraped into scintillation vials for measurement of radioactivity by liquid scintillation counting (Model LC5000TD, Beckman Instruments). Radioactivities in eluted bands corresponding to lipid standards were reported as counts per minute (cpm) and as percentages of total radioactivity spotted onto the plates.

Effect of ethanol on LKT-induced PA production

Ethanol does not inhibit PLD, but promotes transphosphatidylation activity, resulting in the production of phosphatidylethanol (PET) instead of PA. To confirm that LKT-induced PA production was due to PLD activation, [^{3}H] lyso-PC-labeled bovine neutrophils were exposed to LKT (1:500) in the presence of ethanol (0 - 2.5%, v/v). After incubation at 37^{o}C for 15 min, production of PA and PET were measured by TLC, as described above using PA and PET (Avanti Polar-Lipids, Inc, Alabaster, AL) standards for identification of product bands.

Regulation of PLA_2 activity by PLD

The regulatory influence of PLD on PLA_2 activity was investigated by studying the effect of the PLD product, PA, on release of radioactivity from [^{3}H] AA-labeled neutrophils. Initially, labeled neutrophils were exposed to LKT in the presence or absence of ethanol (0-2.5%, v/v) to inhibit PLD-catalyzed production of PA. Thereafter, effects of PA produced by PLD were confirmed by exposing neutrophils to LKT in the presence of ethanol with or without added PA. At the completion of each incubation period (15 minutes at 37°C), experiments were terminated by centrifugation at 10,000 × g for 5 minutes, 100 µl supernatants were suspended in 5 ml liquid scintillation cocktail (Atomlight, Dupont NEN Research Products, Boston), and radioactivity was measured for 5 minutes by liquid scintillation counting. Percent specific release of radiolabeled phospholipid substrate was calculated using the formula:

$$\%\ \text{specific}\ ^3\text{H release} = \frac{(\text{LKT-induced radioactivity in supernatant} - \text{Bkg}) \times 100}{\text{Tot} - \text{Bkg}}$$

where Bkg is the radioactivity released by suspensions exposed to Hanks buffered saline solution (HBSS) and Tot is the total radioactivity added to sample.

Involvement of calcium in LKT-induced activation of PLD

The extracellular Ca^{2+} dependence of LKT-induced production of PA from radiolabeled neutrophils was tested 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 $CaCl_2$ and $MgCl_2$ were not added as in previous experiments. The production of PA was estimated after 15 minutes of incubation at 37°C, as described above.

Statistical analyses

Statistical analyses were conducted using a commercially available microcomputer program (The SAS system, SAS institute Inc., Cary, NC). Concentration- and time-dependent effects of LKT on PA and PET production were compared to corresponding negative controls using unpaired t tests. The effect of PA on release of [^{3}H] AA from intact neutrophils was investigated by comparing the response at each dosage level with that of the ethanol-free control, using Dunnett's test. The effects of extracellular Ca^{2+}-dependency of LKT induced responses were investigated using the general linear model followed by comparison of means using Turkey's test. Differences between means were declared significant at the $P < 0.05$ level.

Results

LKT caused production of PA in isolated bovine neutrophils in a concentration- and time-dependent manner whereas LKT(-) failed to stimulate PA production (Figures 10, 11). PA production in bovine neutrophils appeared more sensitive to LKT than [^{3}H] AA release. As the concentration of LKT was decreased from 1:100 to 1:1000, release of [^{3}H] AA decreased by approximately 70% (Figure 5 in Chapter IV) while PA production decreased by only 20% (Figure 10). In the presence of ethanol, LKT-induced PA production decreased while production of PET (the product of PLD transphosphatidylation activity) increased in an ethanol concentration-dependent manner

(Figure 12). These results confirmed that exposure of bovine neutrophils to LKT resulted in increased PLD activity.

The effects of ethanol on release of [^{3}H] AA from LKT-exposed neutrophils, with or without addition of PA, provided strong evidence that PLD regulates PLA_2 activity. As ethanol concentration was increased between 0 and 1%, LKT-induced [^{3}H] AA release decreased (Figure 13). Preliminary experiments had determined that ethanol is cytotoxic to neutrophils at high concentration, but at concentrations less than 2%, there were no adverse effects on cell membrane integrity, as measured by LDH release (Figure 14). When exogenous PA was added in the presence of 1% ethanol, release of radioactivity from [^{3}H] AA-labeled neutrophils was restored (Figure 15). The effect of exogenous PA on PLA_2 activity was concentration-dependent.

The effect of LKT on PLD activity in bovine neutrophils was Ca^{2+}-dependent (Table 3). Removal of Ca^{2+} from the incubation medium caused decreased PA production by [^{3}H] Lyso-PC-labeled neutrophils. LKT-induced effects were only partially restored (the significant level was $P < 0.1$ instead of $P < 0.05$) when a high concentration of Ca^{2+} that exceeded the chelating capacity of EGTA was added.

Table 3

Extracellular calcium dependence of LKT-induced production of phosphatidic acid (PA). Isolated neutrophils were exposed to LKT in buffer suspensions containing 1mM $CaCl_2$ and 0 mM EGTA, 0 mM $CaCl_2$ and 1 mM EGTA, 0 $CaCl_2$ and 0 mM EGTA, or 3 mM $CaCl_2$ and 1 mM EGTA.

Incubation condition	% Total cpm corresponding to PA
1 mM $CaCl_2$, 0 mM EGTA	1.06 ± 0.09*
0 $CaCl_2$, 0 mM EGTA	0.427 ± 0.03
0 mM $CaCl_2$, 1 mM EGTA	0.435 ± 0.05
3 mM $CaCl_2$, 1 mM EGTA	0.535 ± 0.03[a]

*Value significantly different from values of the other three treatments.
[a]Value significantly different from the value of treatment with 0 $CaCl_2$, 0 mM EGTA ($P < 0.1$).

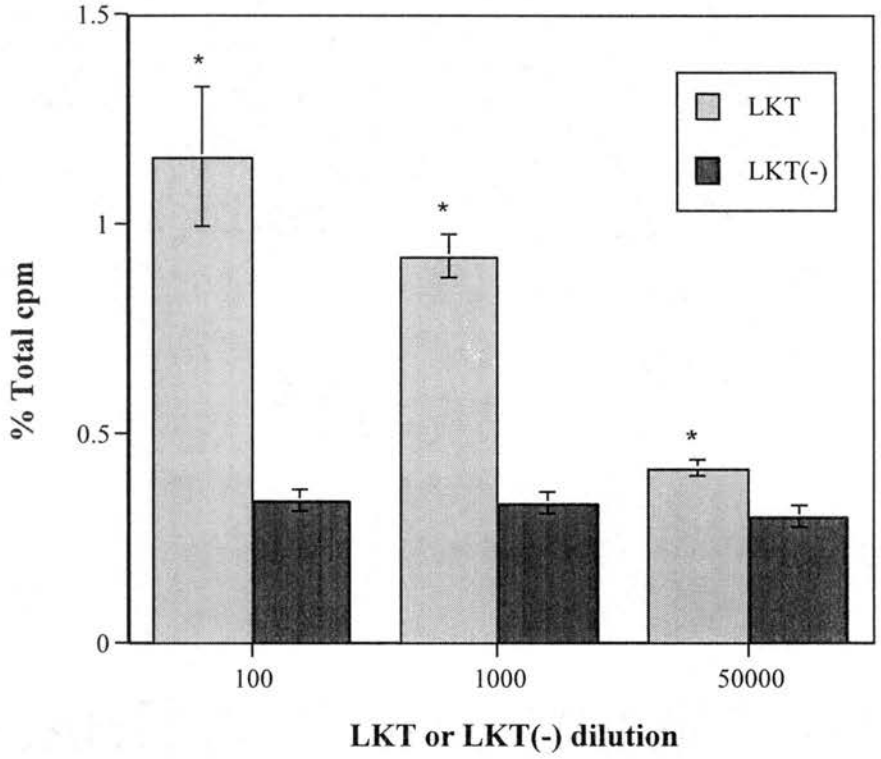

Figure 10. Effect of LKT and LKT(-) control preparation on production of PA in isolated bovine neutrophils. Neutrophils were loaded with [^{3}H] lyso-PC, exposed to dilutions of LKT or LKT(-) for 15 minutes, and subjected to TLC (n = 3). % Total cpm = proportion of total radioactivity corresponding to PA standard. *Mean (± SD) LKT values were significantly higher than corresponding LKT(-) values.

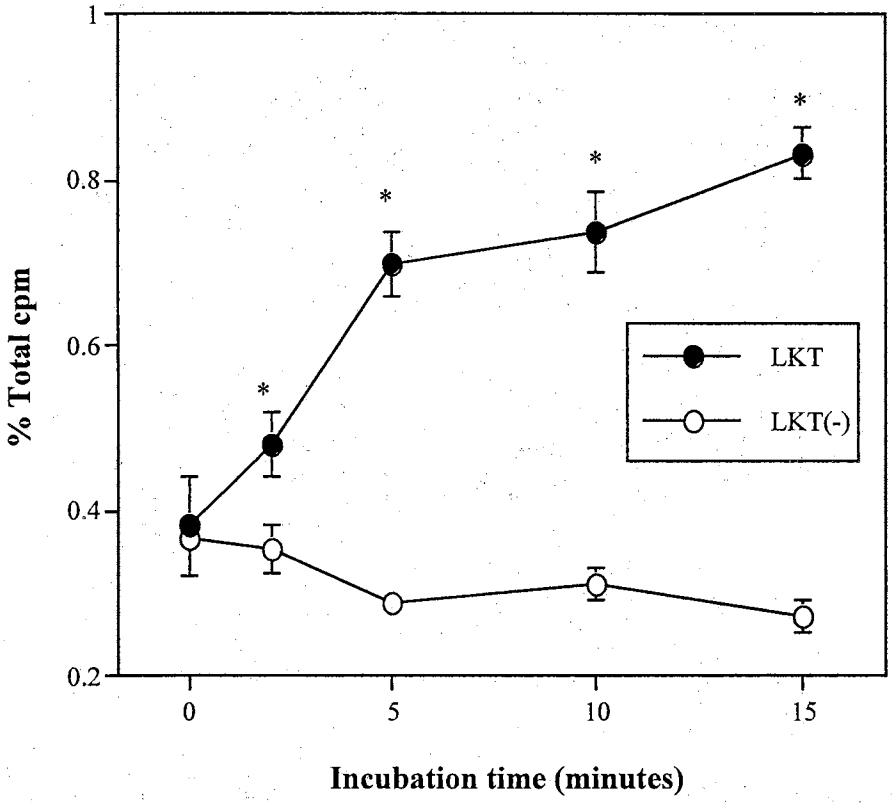

Figure 11. Time-dependant effects of LKT or LKT(-) on production of PA in isolated bovine neutrophils. Neutrophils were loaded with [^{3}H] lyso-PC, exposed to 1:1000 dilutions of LKT or LKT(-) for various periods, and subjected to TLC (n = 3). % Total cpm = proportion of total radioactivity corresponding to PA standard. *All mean (± SD) LKT values derived from samples incubated for ≈ 2 minutes were significantly higher than corresponding LKT(-) values.

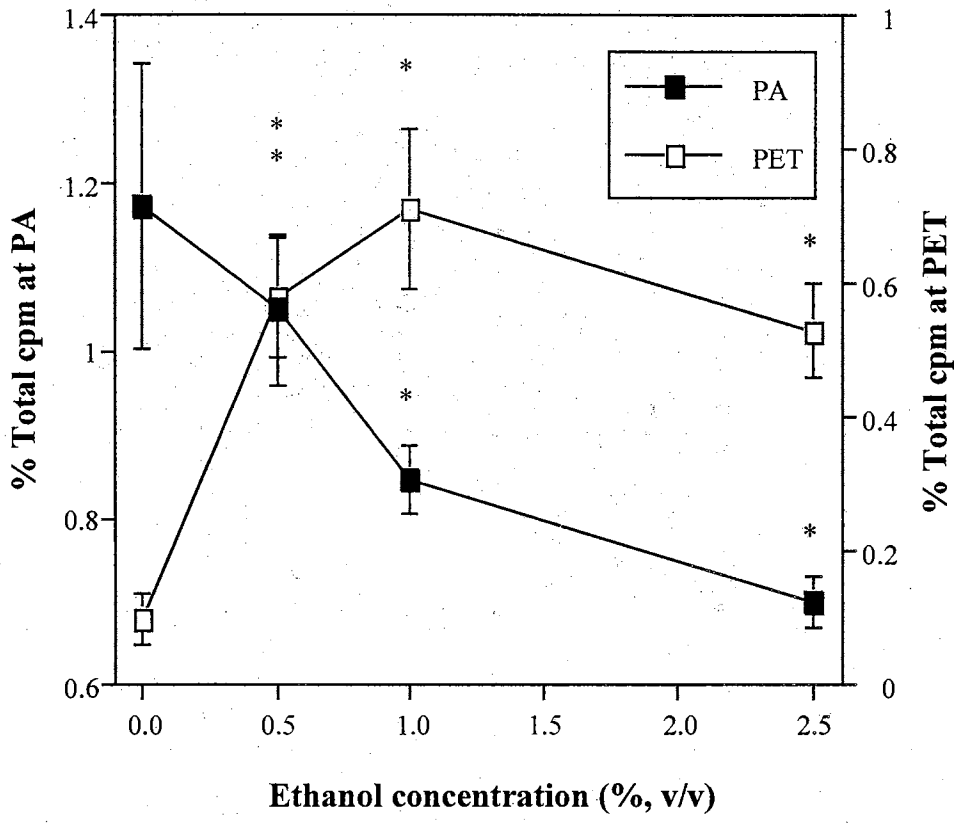

Figure 12. Effects of ethanol on production of PA and PET by isolated bovine neutrophils. Neutrophils were loaded with [^{3}H] lyso-PC, exposed to a 1:500 dilution of LKT for 15 minutes in the presence or absence of ethanol, and subjected to TLC (n = 3). % Total cpm at PA = proportion of total radioactivity corresponding to PA standard. % Total cpm at PET = proportion of total radioactivity corresponding to PET standard. *All mean (± SD) LKT values for each product were significantly different from the corresponding 0% ethanol value.

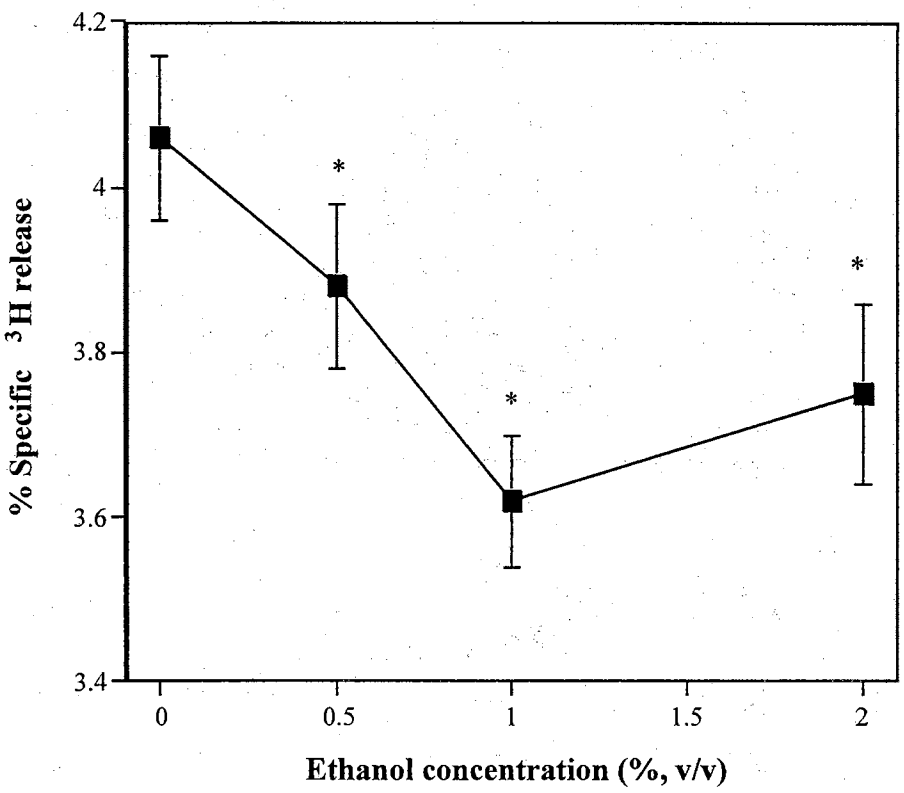

Figure 13. Effect of ethanol on release of [^{3}H] AA and AA metabolites from isolated bovine neutrophils. Neutrophils loaded with [^{3}H] AA were exposed to LKT (1:500) in the presence or absence of ethanol for 15 minutes, and released radioactivity was measured (n = 3). *All mean (± SD) values were significantly different from the corresponding 0% ethanol value.

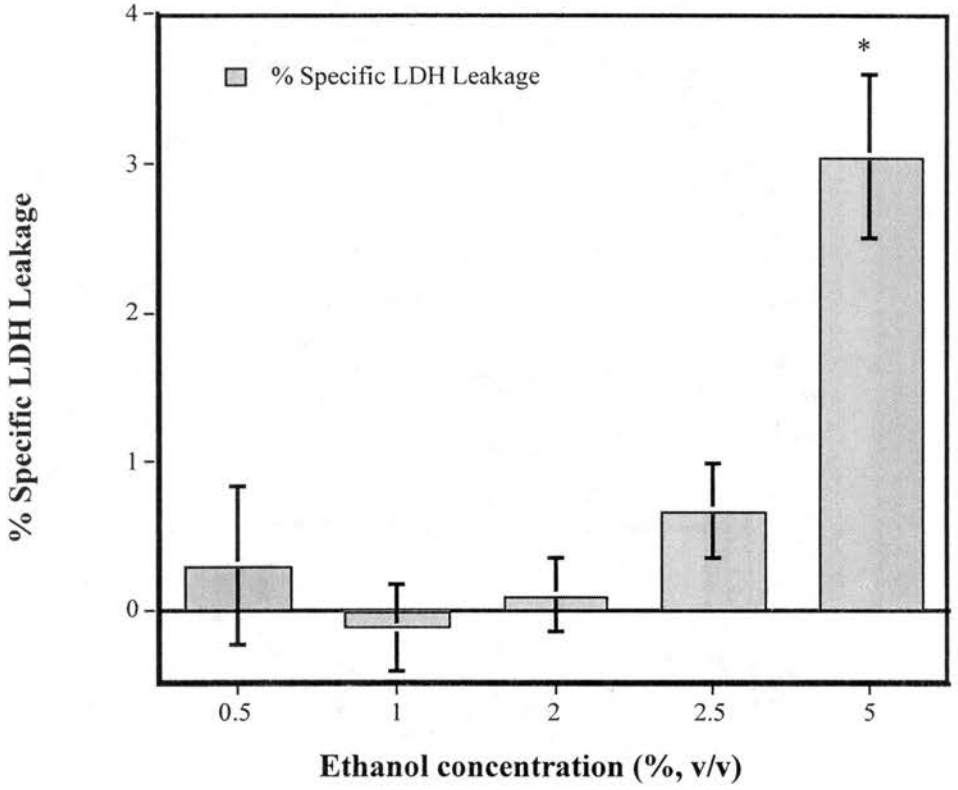

Figure 14. Toxicity of ethanol on isolated bovine neutrophils. Bovine neutrophils (1.5×10^7 cells/ml) were incubated with different concentrations of ethanol as indicated for 60 min. The incubation was stopped by centrifugation and LDH in the supernatant was determined by the method decribed in Chapter IV (n = 4).

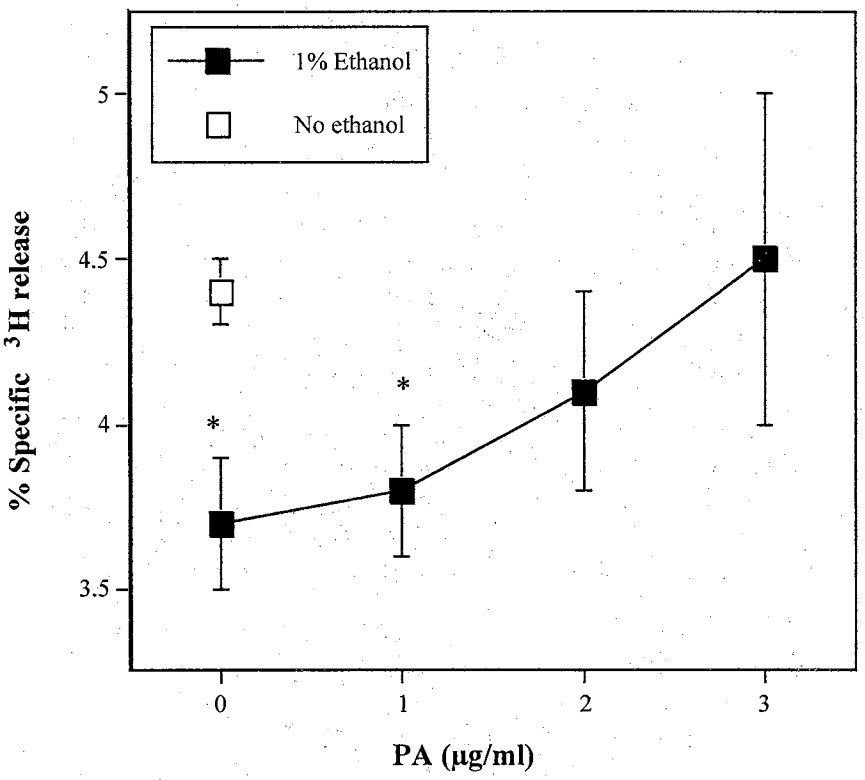

Figure 15. Restoration of [^{3}H] AA release in the presence of ethanol by addition of exogenous PA. Neutrophils loaded with [^{3}H] AA were exposed to LKT (1:500) in the presence or absence of ethanol for 15 minutes and exogenous PA was added before measuring release of radioactivity (n = 3).

Discussion

Previous studies have indicated that bovine neutrophils play a central role in the development of acute pneumonic pasteurellosis. Experimental aerosol exposure of *P. haemolytica* A1 to calves induces rapid infiltration on neutrophils into the lung (Walker *et al*, 1985) and a marked change in the neutrophil/macrophage ratio (Lopez *et al*, 1986). These changes are closely correlated with reported histologic changes in which small airways become plugged with purulent exudate (Lopez *et al*, 1986). There is reliable evidence indicating that mobilization of neutrophils does not effectively combat infection but contributes to development of lung lesions, probably due to release of oxygen-derived free radicals and hydrolytic enzymes. Neutrophil depletion prior to inoculation with *P. haemolytica* protected calves from the development of gross fibrinopurulent pneumonic lesions, although less severe inflammatory changes still occurred (Breider *et al*, 1988; Slocombe *et al*, 1985). Thus, the neutrophil-mediated inflammatory response itself appears to be a major determinant of *P. haemolytica* pathogenicity, and identification of the mechanisms whereby *P. haemolytica* infection induces extensive neutrophil infiltration and degranulation is crucial to understanding the pathogenesis of pneumonic pasteurellosis.

Leukotoxin is believed to be the major virulence factor of *P. haemolytica* responsible for activation of bovine neutrophils in the development of pneumonic pasteurellosis. In contrast to *P. haemolytica* lipopolysaccharide, which causes vascular injury (reviewed in Whitely *et al*, 1992) but is not toxic to bovine neutrophils (Confer and Simons, 1986), LKT is specifically cytolytic to ruminant leukocytes and platelets. Stimulation of bovine neutrophils *in vitro* results in rapid leakage of intracellular K^+ and cell swelling (Clinkenbeard *et al*, 1989), increase of intracellular calcium concentration ($[Ca^{2+}]_i$) (Oritiz-Carranza and Czuprynski, 1992), degranulation (Czuprynski *et al*, 1991), generation of free oxygen radicals (Maheswaran *et al*, 1993), and production of

lipid inflammatory mediators such as LTB_4, which has been implicated as an important chemotactic agent for bovine neutrophils (Heidel *et al*, 1989) in *P. haemolytica* infection (Clarke *et al*, 1994).

Previous studies have indicated that LKT-induced LTB_4 synthesis involves Ca^{2+}-dependent activation of cytosolic phospholipase A_2 ($cPLA_2$) (Wang *et al*, 1998). Although *P. haemolytica* LKT may also induce activation of 5-lipoxygenase, the enzyme complex responsible for oxidizing AA to leukotrienes, $cPLA_2$-mediated AA release appears to be the rate-limiting step in the process of LKT-induced LTB_4 synthesis. In the presence of exogenous AA, LKT induces substantial production of LTB_4, whereas inhibition of $cPLA_2$ in the absence of exogenous AA causes marked inhibition of LTB_4 synthesis (Wang *et al*, 1998).

Calcium-dependent translocation of $cPLA_2$ from the cytosol to cell membranes and protein kinase-mediated phosphorylation are considered to be important mechanisms involved in the regulation of $cPLA_2$ activity (Clark *et al*, 1991; Lin *et al*, 1993). The results of previous experiments investigating LKT-induced effects on $cPLA_2$ activity in the presence or absence of extracellular Ca^{2+} have supported an important signal transduction role for Ca^{2+} (Wang *et al*, 1998). However, it is not clear whether the regulatory effects of $[Ca^{2+}]_i$ are restricted to direct effects on $cPLA_2$ or whether other enzymes may be involved. Indeed, in human neutrophils, PLD is crucial to full expression of $cPLA_2$ hydrolytic activity. PLD is ubiquitous in resting neutrophils, but in stimulated cells it concentrates in the plasma membrane (Morgan *et al*, 1997), where it specifically hydrolyzes PC to yield PA and choline. PA is further metabolized by phosphatidate phosphohydrolase (PAP) to diglycerides (DG) (Exton, 1997). When intact human neutrophils were primed with tumor necrosis factor α and stimulated with N-formyl-Met-Leu-Phe, AA release occurred in parallel with enhanced PA and DG

formation (Bauldry and Wooten, 1997). Therefore, in human neutrophils, PLA_2 activity is induced by the products of PLD catalysis. Similar results have been reported using rat neutrophils (Fujita *et al*, 1996).

The results of the present study provide strong evidence that LKT-induced activation of PLA_2 is mediated by PLD, principally via calcium-dependent production of PA. Although it is possible that PA may promote PLA_2 activity by serving as a substrate, the experiments involving ethanol inhibition of PA production and addition of exogenous PA indicated that production of PA by PLD stimulates hydrolysis of [^{3}H] AA-labeled phospholipid substrate. This effect of PA is consistent with its many regulatory influences on an array of neutrophil functions, such as neutrophil production of free oxygen radicals, degranulation and phagocytosis (reviewed in Olson and Lamberth, 1996). Indeed, PA is considered an important intracellular lipid messenger in many signaling pathways and may facilitate transport of extracellular Ca^{2+} across the plasma membrane as well as mobilization of intracellular Ca^{2+} (reviewed in English *et al*, 1996). Furthermore, studies have indicated that PA can stimulate protein kinase C (PKC) and mitogen-activating protein kinase (MAP) activities, both of which may be involved in phosphorylation of $cPLA_2$. Also, PA is an anionic phospholipid that may alter the physical properties of cell membranes in such a way that $cPLA_2$ activity can be influenced. Thus, there are several mechanisms whereby PA can regulate $cPLA_2$ activity that do not involve serving as a substrate for AA production.

The mechanisms whereby PLD itself is regulated include phospholipase C (PLC)-mediated activation of protein kinase C (PKC), the small G proteins of the ADP-ribosylation factor (ARF) and Rho families, and fluxes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Olsen and Lambeth, 1996; Exton, 1997). Both *in vivo* and *in vitro* studies have indicated that activation of PLC generates diglycerides (DG) and inositol triphosphate (IP_3). The production of DG and resulting increase in $[Ca^{2+}]_i$ caused by IP_3-mediated

mobilization of intracellular Ca^{2+} activate PKC, which in turn activates PLD directly or indirectly via the G proteins. However, the results of the present study suggest a more direct role of Ca^{2+}, possibly via influx of extracellular Ca^{2+} through pores in the plasma membrane caused by LKT. Further support for a more direct role of Ca^{2+} can be found in observations that PLD can be activated by agonists that act via G proteins without the involvement of PKC and that chelation of Ca^{2+} will inhibit activation of PLD by these agonists (Exton, 1997), thus suggesting that Ca^{2+} may exert direct control on PLD activity.

CHAPTER VI

SUMMARY CONCLUSIONS

The pathology of pneumonic pasteurellosis is characterized by extensive infiltration of neutrophils. Several studies (reviewed earlier) have suggested that mobilization of neutrophils does not combat bacterial infection effectively, but contributes to the lung injury. Therefore, identification of pathogenic pathways responsible for neutrophil influx, activation, and lysis has the potential to promote development of strategies for control of *P. haemolytica* infection.

LKT and LPS are believed to be the major virulence factors of *P. haemolytica* contributing to lung injury. Although LPS may play an important role in causing vascular injury and lung edema, relative to LKT it appears to have little effect on neutrophil-mediated inflammation. In contrast, LKT is specifically cytolytic to bovine leukocytes at high concentrations and causes a number of cellular responses in bovine neutrophils at low concentrations, including generation of reactive oxygen radicals, degranulation, apoptosis, and production of eicosanoids. Both *in vitro* and *in vivo* studies have indicated that LTB_4, a potent chemotactic agent for bovine neutrophils, is produced in sites of *P. haemolytica* infection and specifically in response to LKT.

Results of a previous experiment suggested that the rate-limiting step in synthesis of eicosanoids, both prostanoids and leukotrienes, is hydrolytic release of AA from neutrophil membranes (Clinkenbeard *et al*, 1994). The goal of the present experiments was to examine the involvement of various phospholipases in LKT-induced LTB_4

synthesis and to identify important regulatory mechanisms responsible for PLA_2-mediated AA release.

The involvement of PLA_2 in LKT-induced synthesis of LTB_4 was studied using bovine neutrophils labeled with [^{3}H] AA. Incubation of isolated neutrophils with [^{3}H] AA resulted in incorporation of radioactivity in the PLA_2 substrates, PC, PI, and PE. Exposure of radiolabeled neutrophils to LKT caused concentration- and time-dependent release of radioactivity and redistribution of radioactivity in neutrophil membranes consistent with utilization of phosphoglyceride substrate and release of free fatty acid and eicosanoid products. These LKT-induced effects could be inhibited by pretreatment with arachidonyl trifluoromethyl ketone, an inhibitor of type IV, cytoplasmic PLA_2 and were dependent on extracellular calcium. These results support the hypothesis that LKT-induced synthesis of LTB_4 involves calcium-mediated increase in PLA_2 activity.

Mechanisms of regulation of $cPLA_2$ are not well understood, but calcium-mediated translocation of $cPLA_2$ from the cytosol to the cell membrane and interaction between PLD and $cPLA_2$ have been implicated as important mechanisms controlling enzyme activity. Regulation of $cPLA_2$ by products of PLD has been reported in human neutrophils exposed to physiological stimulators (Bauldry and Wooten, 1996), suggesting that similar mechanisms may be involved in bovine neutrophils exposed to the pathological activator, LKT. The effect of LKT on PLD activity was investigated using isolated bovine neutrophils labeled with [^{3}H] lyso-PC. LKT caused concentration- and time-dependent increases in PA production in bovine neutrophils, as measured by TLC. LKT-induced generation of PA and release of AA from cell membranes were inhibited when ethanol was used to promote the alternative PLD-mediated transphosphatidylation reaction, resulting in the production of PET rather than PA. The role of PA in regulation of PLA_2 was then confirmed by means of an add-back experiment, whereby addition of PA in the presence of ethanol restored release of AA from neutrophil membranes.

The regulatory role of Ca^{2+} in LKT-induced release of AA from cell membranes was confirmed by experiments that demonstrated that both PLA_2 and PLD are activated by LKT in a Ca^{2+}-dependent manner. However, further experiments are needed to confirm whether Ca^{2+} may regulate PLA_2 independent of its action on PLD. Similarly, the many possible regulatory pathways involving enzyme activation by phosphorylation must be explored before a comprehensive understanding of the molecular pathogenic pathways involved in LKT-induced eicosanoid synthesis emerges.

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