PASTEURELLA HAEMOLYTICA LEUKOTOXIN - INDUCED CHANGES IN BOVINE NEUTROPHILS MEDIATED BY INTRACELLULAR CALCIUM

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AA	Arachidonic acid
$AACOCF_3$	Arachidonyl trifluoromethyl ketone
A23187	4Br-A23187
B	Fragmented DNA fraction of DPA assay
BRD	Bovine respiratory disease
$[Ca^{2+}]_i$	Intracellular calcium concentration
CICR	Calcium induced calcium release
DAG	Diacylglycerol
DPA	Diphenylamine
ER	Endoplasmic reticulum
F_{bkg}	Background fluorescence
F_{max}	Maximum fluorescence
F_{min}	Minimum fluorescence
F_{mn}	Manganese bound fluorescence
IP_3	Inositol 1, 4, 5-trisphosphate
IP_3R	Inositol 1, 4, 5-trisphosphate receptors
Kd	Dissociation constant
LDH	Lactate Dehydrogenase
5LO	5-Lipoxygenase
LKT	Leukotoxin
LKT (-)	Leukotoxin deficient mutant

LTB_4	Leukotriene B_4
NMDAR	N-methyl-D-aspartate receptors
PAF	Platelet activating factor
PIP_2	Phosphotidylinositol 4,5-bisphosphate
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol myristate acetate
S	Supernatant from pelleted whole cells in DPA assay
T	Intact chromatin fraction of DPA assay
Tg	Thapsigargin
$rhC5_a$	Recombinant human C5a
ROC	Receptor operated calcium channels
RTX	Repeats in ToXin
VOC	Voltage operated calcium channels
XeC	Xestospongin C

CHAPTER I

INTRODUCTION

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

The virulence factor of *P. haemolytica* that appears to be responsible for the acute neutrophil-mediated inflammatory reaction as well as the failure of neutrophils to clear the infection is leukotoxin (LKT). Produced by log-phase *P. haemolytica*, LKT is a member of the family of RTX pore-forming cytolysins, which are characterized by tandemly arranged repeats of a nine amino acid sequence (Welch, 1991). Although LKT may affect many neutrophil functions, three actions in particular are probably responsible for causing the excessive and ineffective neutrophil response: stimulation of leukotriene B_4 (LTB_4) synthesis, plasma membrane damage, and apoptosis.

Eicosanoids, particularly leukotriene B_4 (LTB_4), are considered to be important chemotactic agents responsible for influx of neutrophils into infected lungs (Clarke *et al*, 1994). LTB_4 is a potent chemotactic agent for bovine neutrophils (Heidel *et al*, 1989) and is produced by neutrophils in response to LKT (Clinkenbeard *et al*, 1994; Henricks *et al*, 1992). Failure of these infiltrating phagocytes to combat the infection can be explained by the apoptotic (Stevens and Czuprynski, 1996) and necrotic effects (Clinkenbeard *et al*,

1989) of LKT on bovine neutrophils, which have been demonstrated *in vitro*. Considering that LKT-induced synthesis of LTB_4 by bovine neutrophils and plasma membrane damage are dependent on extracellular Ca^{2+} and that Ca^{2+} is required by enzymes involved in apoptosis, it is likely that intracellular Ca^{2+}-mediated signal transduction serves as a common pathway whereby LKT accomplishes its primary pathological effects on neutrophils. Therefore, a clear understanding of the nature and mechanism of LKT-induced changes in intracellular calcium concentration ($[Ca^{2+}]_i$) would facilitate identification of potential targets for pharmacological inhibition of the undesirable effects of LKT while leaving intact the Ca^{2+} pathways that are involved in essential neutrophil functions. Therefore, selective attenuation of neutrophil-mediated inflammation in pneumonic pasteurellosis could possibly limit the development of severe lung pathology and improve the efficacy of antibacterial agents, the action of which may be compromised by constituents in inflammatory exudate (Clarke *et al*, 1991).

CHAPTER II

LITERATURE REVIEW

Pathogenesis of Bovine Respiratory Disease

Mortality and high morbidity resulting from bovine respiratory disease (BRD) are responsible for greater economic losses to the North American feedlot industry than any other disease (Yates, 1982; Weekly *et al*, 1998). This disease is usually characterized by severe pneumonic signs, including; fever, dyspnea, respiratory rales, nasal discharge, and depression. The most common pathological findings are fibrinopurulent pneumonia and varying degrees of pluritis and pulmonary abscesses (Wikse, 1990; Hungerford, 1990; Radostitis *et al*, 1994).

Several infectious agents have been implicated in BRD, but *P. haemolytica* serotype A1 is considered to be the primary etiologic organism, with other bacteria, such as *Pasteurella multocida* and *Haemophilus somnus*, being isolated less frequently (Yates, 1982; Confer *et al*, 1988; Wikse, 1990; Radostitis *et al*, 1994). *P. haemolytica* is a normal inhabitant of the nasopharyngeal mucosa of many healthy cattle (Yates, 1982), but not of the lung, and is considered to be an opportunistic pulmonary pathogen (Wikse, 1990). When pulmonary defenses are depressed by stress, nutritional deficiencies, and/or viral infections, *P. haemolytica* is able to colonize and proliferate, first in the upper respiratory tract and then in the lung, producing severe acute fibrinopurulent bronchopneumonia. This disease syndrome is also referred to as shipping fever, in recognition of the role of transportation stress in its etiology (Frank *et al*, 1987; Wikse, 1990; Radostitis *et al*, 1994).

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

The Importance of *Pasteurella haemolytica* Leukotoxin in Bovine Respiratory Disease

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

heat-labile, pH-stable, non-dialyzable, water-soluble glycoprotein produced by *P. haemolytica* during the logarithmic growth phase (Shewen and Wilkie, 1985; Chang *et al*, 1986; Chang *et al*, 1987; Lo *et al*, 1987; Gentry and Srikumaran, 1991). This exotoxin is cytocidal only to ruminant leukocytes, platelets and erythrocytes (Kaehler *et al*, 1980; Clinkenbeard and Upton, 1991). Based on analysis of the structural gene of LKT, the predicted molecular mass of the protein is 102 kDa, but native LKT aggregates in multimers with molecular mass of 400 kDa or greater (Highlander *et al*, 1989; Baluyut *et al*, 1981; Himmel *et al*, 1982; Mosier *et al*, 1986; Chang *et al*, 1986). The tendency of LKT to aggregate was investigated by Clinkenbeard and Waurzyniak, who determined that treatment of LKT with chaotropic agents such as guanidine and urea results in disaggregation of multimers of LKT to proteins with fewer LKT units and a higher toxic activity (Clinkenbeard *et al*, 1995; Waurezyniak *et al*, 1994).

The importance of LKT in the pathogenesis of BRD is illustrated by comparison of pneumonias caused by *P. haemolytica* with those caused by *P. multocida*, which produces endotoxin, but no LKT. The pathology of the latter is characterized by suppurative bronchopnemonia without necrosis and fibrin exudation (Ames *et al*, 1985). In a more recent experiment, Tatum and coworkers (1998) compared the pneumonias caused by LKT and a LKT-deficient strain of *P. haemolytica* and found that the LKT-deficient strain did not elicit significant pulmonary neutrophilic infiltration or lesions. This suggests that LKT has a specific role in producing the characteristic lesions of *P. haemolytica* pneumonia, which are principally neutrophil-mediated (Whiteley *et al*, 1991).

Role of Leukotriene B_4 in Bovine Respiratory Disease

Although all of the chemotactic factors involved in neutrophil attraction into sites of *P. haemolytica* infection have not yet been identified, eicosanoids are known to play a

major role. Synthesis of eicosanoids involves release of arachidonic acid (AA) from membrane phospholipids by phospholipases and then oxidation of AA by cyclo-oxygenase to produce thromboxanes and prostaglandins or by lipoxygenases to produce hydroxyeicosatetraenoic acids and leukotrienes (Moncada and Vane, 1979; Higgins and Lees, 1984; Holzman, 1991). Products of cyclo-oxygenase and lipoxygenases, collectively referred to as eicosanoids, have many different functions that contribute to generation of an inflammatory reaction (Holzman, 1991). Prostaglandins and thromboxanes, through their action on patency and permeability of peripheral vasculature as well as sensitization of pain receptors, are responsible for inducing most of the cardinal signs of inflammation; including erythema, swelling, increased temperature, and pain (Higgins and Lees, 1984). Prostaglandins and thromboxanes also modulate platelet aggregation and blood clotting. Although lipoxygenase products have been implicated in every phase of the inflammatory response (Ford-Hutchison, 1985; Higgins and Lees 1984), they are principally involved in leukocyte-mediated mechanisms. In particular, LTB_4 is a potent chemotactic agent (Heidle *et al*, 1989; Clarke *et al*, 1991) that binds with high affinity, stereospecificity, and saturability to receptors on neutrophil cell membranes (Goldman *et al*, 1982) and acts as both a chemokinetic and aggregating agent (Ford-Hutchison, 1985). Indeed, LTB_4 appears to be an important chemoattractant of neutrophils in BRD. In studies conducted by Heidel *et al*, intradermal injection of LTB_4 was followed by rapid accumulation of bovine neutrophils (Heidle *et al*, 1989). In another study, Clarke *et al* (1991) used a soft-tissue infection model established by inoculation of *P. haemolytica* into chambers implanted subcutaneously in cattle. In this experiment, an inhibitory effect of dexamethasone on both neutrophil influx into inoculated chambers and concentration of LTB_4 in infected chamber fluids, together with the temporal relationship between these two events, strongly suggested a chemotactic role for LTB_4.

Leukotoxin-induced Leukotriene B_4 production

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

Role of Phospholipase A_2 in Leukotoxin-induced Leukotiene B_4 production

Release of AA from phospholipid membranes is accomplished by phospholipases, particularly phospholipase A_2 (PLA_2). Several different types of mammalian PLA_2 enzymes have been differentiated, based on structure and amino acid sequence (Mayer and Marshall, 1993). Two of these, designated Type II and Type IV, are nonpancreatic enzymes that metabolize phospholipids to lysophospholipids and fatty acids, including AA.

Type II PLA_2 has a molecular mass of 14 kDa, may be extracellular or cell membrane associated, and is activated by increased $[Ca^{2+}]_i$ (Musser and Kreft, 1992). Type IV PLA_2 has a molecular mass of 85 kDa, is arachidonoyl-selective, and is localized intracellularly, but translocates to the cell membrane under the influence of increased $[Ca^{2+}]_i$ (Musser and Kreft, 1992). Indeed, type IV PLA_2 has been shown to be involved in LKT-induced LTB_4 production in bovine neutrophils (Wang *et al* , 1998). Wang *et al* (1998) determined that when bovine neutrophils labeled with $[^3H]$AA were exposed to LKT, radioactivity associated with AA and AA metabolites was released from whole cells and a redistribution of radioactivity within neutrophil membranes occurred that was consistent with hydrolysis of phospholipid substrates by phsopholipases. Furthermore, removal of Ca^{2+} from the extracellular medium resulted in a decrease in both ^3H AA release and plasma membrane damage, indicating that phospholipase activation is Ca^{2+}-dependent. The specific involvement of phospholipase A_2 (PLA_2) in these LKT-induced effects, including synthesis of LTB_4, was confirmed using the specific PLA_2 inhibitor, arachidonyl triflouromethyl ketone ($AACOCF_3$). Regulatory pathways involved in activation of PLA_2 by LKT appear to be complex: activation is mediated via phosphatidic acid (PA), a product of phospholipase D (PLD), the activity of which is also increased by LKT in a Ca^{2+}-dependent manner (Wang *et al*, unpublished data). Thus, the activity of type IV PLA_2 is either directly or indirectly dependent on Ca^{2+}.

Role of 5-Lipoxygenase in LKT-induced LTB_4 Production

Regulation of LTB_4 synthesis could also involve activation of 5-lipoxygenase (5-LO). Mammalian lipoxygenases exhibit regional specificity during interaction with a substrate and are designated as 5-LO, 12-lipoxygenase, or 15-lipoxygenase. Each enzyme

inserts an oxygen molecule at carbon-5, -12, or -15, respectively. In resting cells, 5-LO exists in cytosol in a dormant ferrous state. When activated by hydroperoxidases, ATP, and Ca^{2+}, 5-LO translocates to the cell membrane and associates with calcium activating proteins to initiate the oxidation of arachidonic acid to many products, including LTB_4 (Rouzer *et al*, 1988; Lewis *et al*, 1990; McMillin and Walker, 1992; Musser and Kreft, 1992). Further control is exerted via oxygen radical products causing initial auto-acceleration of enzyme activity and eventual self inactivation (Moncada and Vane, 1979; Higgs and Lees; 1984; Holzman, 1991). Therefore, in addition to promoting translocation and activation of PLA_2 to release the AA substrate from phospholipid membranes, Ca^{2+} could also stimulate LTB_4 synthesis by simulating the translocation and activation of 5-LO to metabolize AA to LTB_4 (Mayer and Marshall, 1993).

Effect of Leukotoxin on Cell Membrane Permeability

P. haemolytica leukotoxin is a member of a family of cytolytic toxins known as "Repeats in ToXin" or RTX toxins. These exotoxins share extensive sequence homology, particularly in a region of tandem 9-amino acid repeats (Lo *et al*, 1987; Strathdee *et al*, 1987). Studies conducted using *Esherichia coli* α-hemolysin, a RTX toxin that has been more extensively studied than LKT, have indicated that the toxic mechanism of action involves the formation of functional pores that allow electrolyte flux across the plasma membrane of the target cell. Exposure of erythrocytes to this toxin leads to rapid efflux of K^+ and influx of Ca^{2+} as well as higher molecular weight compounds such as mannitol and sucrose (Bhakdi *et al*, 1986). The effect of α–hemolysin on membrane permeability characteristics has led to the hypothesis that α-hemolysin damages membranes by partially inserting into the lipid bilayer and forming discrete transmembrane pores with an approximate diameter of 3 nm. Clinkenbeard *et al* (1989) have reported similar effects of

LKT in bovine neutrophils. Exposure to LKT caused a leakage of K^+, cell swelling, and then formation of large membrane defects. Incubation of bovine neutrophils in hypertonic media containing carbohydrates with molecular mass of about 505 kDa prevented LKT-induced cell swelling, but not K^+-leakage, suggesting existance of stable pores with a functional diameter of approximately 0.9-1.2 nm. However, more recent studies of the molecular pathogenesis of α–hemolysin have reported that membane pore size may be dependent on temperature, time, and toxin concentration, consistent with production of more heterogenous membrane lesions than previously postulated (Moayeri and Welch, 1994).

Leukotoxin-Induced Apoptosis in Bovine Neutrophils

Aside from the effects of LKT on neutrophil LTB_4 synthesis and membrane integrity, LKT has also been reported to cause apoptosis (Stevens and Czuprynski, 1996). Apoptosis is a process of cell death that is morphologically and functionally distinct from cell lysis, which is characteristic of oncosis. While oncosis is primarily a degenerative process ocurring in cells that lie in close proximity to the source of tissue injury, apoptosis is a controlled process of cell death that involves self destruction of isolated cells (Wyllie *et al*, 1980; Majno and Joris, 1995). Necrosis usually occurs rapidly and is characterized by electron-lucent cytoplasm, mitrochondrial swelling, and loss of plasma membrane integrity without severe damage to the nucleus. In contrast, the distinguishing characteristics of apoptotic cells are fragmented nuclei with condensed chromatin, fragmented and condensed cytoplasm, organelle relocation and compaction, plasma and nuclear membrane blebbing, and the formation of apoptotic bodies (Wyllie *et al*, 1980; Majno and Joris, 1995).

Biochemically, apoptosis involves DNA fragmentation that results in characteristic elution "ladders" on gel electrophoresis (Wyllie *et al*, 1980; Eastman, 1996). The Ca^{2+}-

dependent/Mg^{2+}-dependent endonuclease (DNase I) has been implicated in the fragmentation of DNA, indicating that Ca^{2+} may be involved in apoptotic signaling pathways (Eastman, 1996; McConkey and Orrenius, 1996). In addition, calcium chelators, calcium channel blockers, and calmodulin antagonists have been reported to inhibit induction of apoptosis (Trump and Berezesky, 1996; McConkey and Orrenius, 1996). Furthermore, increased $[Ca^{2+}]_i$ has been demonstrated to promote the transcription of genes encoding the surface cell death receptors Fas and FasL (Vignaux *et al*, 1995). However, in aged neutrophils, Ca^{2+} ionophores have been reported to inhibit apoptosis, suggesting that increased $[Ca^{2+}]_i$ may not be a universal signal for apoptosis (Whyte *et al*, 1993). The involvement of Ca^{2+} in apoptosis may be further complicated by the sequential interactions of enzyme systems. For example, there is evidence that inhibition of a Ca^{2+}-dependent nuclear serine protease also blocks endonuclease activation, indicating that the protease either indirectly or directly activates endonuclease (Dowd, 1995). Aside from Ca^{2+}, other regulatory mechanisms, such as protein kinase C, cAMP, protein tyrosine kinases, cyclin-dependent kinases, intracellular acidification, and oxygen radicals may be involved (Trump and Berezesky, 1995; McConkey and Orrrenius, 1996). The lack of consensus as to the signal transduction pathways involved in apoptosis suggests that there is redundancy in activation pathways. Indeed, it has been demonstrated that Fas/APO-1 and tumor necrosis factor receptor 1 (TNFRF1) receptors both result in the eventual stimulation of sphingomyelinase through different pathways. Sphingomyelinase then catalyzes the formation of ceramide to lead to apoptosis via a common pathway of endonuclease activation (Hannun, 1994; Cliftone *et al*, 1995; Cuvillier *et al*, 1996; Nagata, 1997).

Using light microscopy, Stevens and Czuprynski (1996) have reported that bovine neutrophils exposed to very low doses of LKT *in vitro* displayed morphological signs of

apoptosis, such as zeiosis and membrane budding, but they were unable to demonstrate DNA fragmentation upon gel electrophoresis, which is considered a more reliable diagnostic sign of apoptosis. More recently, Sun *et al* (in press) have confirmed by gel electrophoresis that bovine lymphocytes exposed to low concentrations of LKT *in vitro* undergo apoptosis. However, Clarke *et al* (1998) failed to identify morphological signs of apoptosis in neutrophils harvested from subcutaneous tissue chambers infected with *P. haemolytica* or in lung sections from cattle with experimental pneumonic pasteurellosis. Electron microscopic examination of these *in vivo* samples revealed changes in neutrophil morphology consistent with necrosis rather than apoptosis. Clearly, further research into the influences of incubation time and LKT concentration on apoptosis of bovine neutrophils and the role of Ca^{2+} in this effect must be conducted before a more definitive understanding of the importance of apoptosis in the pathogenesis of pneumonic pasteurellosis emerges.

Role of Intracellular Calcium Concentration in Leukotoxin-Induced Effects on Bovine Neutrophils

Considering the importance of Ca^{2+} in activation of enzyme systems responsible for synthesis of LTB_4, the extracellular Ca^{2+}-dependency of LKT-induced LTB_4 synthesis and neutrophil plasma membrane damage, and the involvement of Ca^{2+} in apoptosis, it is likley that these processes are mediated by increased $[Ca^{2+}]_i$. Indeed, several investigators have reported that exposure of bovine leukocytes to LKT results in increased $[Ca^{2+}]_i$ (Clinkenbeard *et al*, 1989, Ortiz-Carranza and Czuprynski, 1992; Hsuan *et al*, 1998), although LKT-induced increase in $[Ca^{2+}]_i$ has yet to be correlated with the important neutrophil responses described above.

Mechanisms whereby $[Ca^{2+}]_i$ can be increased can be broadly divided into those involving influx of extracellular Ca^{2+} and those involving release of vesicular Ca^{2+} stored in intracellular organelles (Berridge, 1997). Influx of extracellular Ca^{2+} may occur via pores formed by integration of LKT into the plasma membrane, regardless of whether such pores have a discrete and constant size or whether they are more heterogenous and dynamic in morphology, or influx may occur through Ca^{2+}-specific channels (Fig. 1).

The Role of Voltage Operated Channels in Leukotoxin-Induced Increases in Intracellular Calcium Concentration

Types of channels through which Ca^{2+} may diffuse down its concentration gradient from extracellular fluid into the cytosol include voltage-operated calcium (VOC) channels and receptor-operated calcium (ROC) channels (Tsien and Tsien, 1990; Spedding and Paoletti, 1992; Berridge, 1997). VOC channels are the most extensively studied class of calcium channels, with those in neurons and muscles being the best studied (Table 1). Common features of most VOC channels are that they require steep membrane depolorization for activation, exhibit high-affinity conductance of Ca^{2+}, and can be selectively modulated by neurotransmitters, G-proteins, and diffusible messengers (Tsein and Tsein, 1990). VOC channels can be further divided into several subclasses: L-type VOC channels are high-voltage activated and inhibited by dihydropyridine antagonists, T-type VOC channels are low-voltage operated and inhibited by low extracellular Ni^{2+} concentration, N-type calcium channels are high voltage activated and inhibited by ω-conotoxin, and P-type are moderately high-voltage activated, but not inhibited by dihypropyridine antagonists or ω-conotoxin (Tsein and Tsein, 1990).

Verapamil and nifedipine, two L-type VOC channel blockers, have been reported to inhibit LKT-induced $[Ca^{2+}]_i$ increase in bovine neutrophils (Ortiz-Carranza and Czuprynski, 1992; Hsuan *et al*, 1998). Verapamil is a phenylalkylamine that inhibits voltage-dependent L channels by binding to 1a and 1b sites on the calcium channel proteins. Nifedipine is a dihydropyridine that has selectivity for separate binding sites on the alpha-1 subunit of voltage-dependent L channels. However, conclusions based on these studies suggesting that VOC are involved in LKT-induced $[Ca^{2+}]_i$ influx may not be correct, because such channels apparently do not occur in neutrophil plasma membranes and very large concentrations of VOC channel blockers were required to produce an inhibitory effect. Indeed, it is possible that these blockers may be acting on VOC channels located on intracellular vesicular membranes or that they may be exerting nonspecific inhibitory effects on LKT transmembrane pores.

In contrast to VOC channels, ROC channels open in direct response to binding of an external ligand (Tsein and Tsein, 1990) (Table 2). Two classes of ROC channels are distinguished by their dependence on membrane potential. All ROC channels are activated by ligand binding, but depolarization of the membrane enhances activation of N-methyl-D-aspartate receptors (NMDAR), while it has no effect on the activation of ATP receptors (ATPR). Antagonists of ROC channels include $LaCL_3$, which competes with Ca^{2+} for binding to channel proteins without being transported through the channel (Thomson and Dryden, 1981; Gould *et al*, 1982; Rosales and Brown, 1992). ROC channels are much less selective for Ca^{2+} than VOC channels, which are not affected by $LaCl_3$. $LaCl_3$ has been shown to completely block Ca^{2+} entry in human neutrophils exposed to fMLP, an activator of ROC channels, while VOC blockers had no effects on Ca^{2+} entry into the cell (Rosales and Brown, 1992).

The Role of G-proteins in Leukotoxin-Induced Increase in Intracellular Calcium Concentration

Trimeric G-proteins are classified into three major families based on the amino acid sequences of their α subunits (Table 2) (Birnbaumer, 1990). Specific G-proteins affect Ca^{2+} channels differently; Type I, G_s-proteins activate Ca^{+2} channels, whereas Type II, G_i- and G_o-proteins inactivate Ca^{+2} channels. These effects can be distinguished by the stimulatory action of cholera toxin and inhibitory action of pertussis toxin on Type I and Type II G-proteins, respectively (Birnbaumer, 1990). Hsuan *et al* (1998) recently reported that exposure of bovine neutrophils to pertussis toxin resulted in inhibition of LKT-induced $[Ca^{2+}]_i$ increase, thus suggesting that Type II G-proteins may be involved in the signal transduction process. Considering that Type II G_o-proteins inhibit Ca^{2+} channels but activate phospholipase C, it is probable that LKT activated G-proteins are involved in a receptor-mediated increase in $[Ca^{2+}]_i$. Receptor-mediated activation of phospholipase C (PLC) constitutes an important initial step in a sequence of signal transduction events resulting in release of vesicular Ca^{2+}.

The release of calcium from vesicular stores is mediated by inositol 1,4,5-trisphosphate receptors (IP_3R) or ryanodine receptors (RyR) in muscle cells (Majerus, 1992; Berridge, 1993; Berridge, 1997). The formation of IP_3 is the focal point of two major signaling pathways. IP_3 can be formed by either the tyrosine kinase receptor or G-protein linked receptor pathways (Majerus, 1992; Berridge, 1993). Both of these receptors transduce signals that activate different types of PLC, all of which hydrolyze the membrane phospholipid precurser, phosphotidylinositol 4,5-bisphosphate (PIP_2), to IP_3 and diacylglycerol (DAG) (Rhee and Choi, 1992; Cockcroft and Thomas, 1992). IP_3 binds to the IP_3 receptor (IP_3R) on the endoplasmic reticulum (ER) to release calcium, whereas

DAG activates protein kinase C to phosphorylate and activate selected serine and threonine proteins in the cell (Majerus, 1992; Berridge, 1993). The IP_3R can be blocked by membrane impermeable heparin and by a novel class of membrane permeable IP_3R blockers, xestospongins (Gafni *et al*, 1997).

Calcium Induced Calcium Release from Vesicular Stores

Aside from IP_3, Ca^{2+} itself controls IP_3R-mediated release of vescicular Ca^{+2} (Finch *et al*, 1991). Evidence of dual agonist control includes an experiment using flash photolysis in which IP_3 had little effect on the release of vesicular Ca^{2+} in the absence of cytosolic Ca^{2+}. However, as the $[Ca^{2+}]_i$ was increased, the effect of IP_3 on the release of Ca^{2+} was enhanced until a maximum was reached, at which time Ca^{2+} became inhibitory (Iino and Endo, 1992). In another experiment, Yao and Parker (1992) divided a *Xenopus* oocyte into excitable and unexcitable regions by releasing IP_3 into half of the cell only and then demonstrated that a regenerative Ca^{2+} wave could be propagated only through the excitable, IP_3 containing portion of the cell.

Not only is calcium required for dual agonist control of IP_3 receptor activation, but Ca^{2+} can activate the receptor itself in the process of Ca^{2+}-induced calcium release (CICR). This process can be identified in *Xenopus* oocytes as the second of a two-phase Ca^{2+} release. In the first phase, IP_3 stimulates localized release of Ca^{2+} from the ER. When the Ca^{2+} concentration reaches a certain level, the second phase is initiated and involves an all-or-none response in which Ca^{2+} stimulates its own release until an inhibitory Ca^{2+} concentration is achieved (Parker and Yao, 1991; Lechleiter *et al*, 1991; Lechleiter and

Clapham, 1992). CICR can be distinguished from IP_3-induced Ca^{+2} release using IP_3R inhibitors, such as heparin (Finch *et al*, 1991).

Increased $[Ca^{2+}]_i$ has also been shown to activate non-selective voltage operated ion channels in human neutrophils exposed to N-formyl-methionyl-leucyl-phenylalanine (fMLP) or platelet-activating factor (PAF) (von Tscharner *et al*, 1986). In this study, the researchers observed an initial increase in $[Ca^{2+}]_i$ due to calcium release from the ER, which was followed by membrane depolarization. If extracellular Ca^{2+} was not available, the $[Ca^{2+}]_i$ increase induced by fMLP was significantly lower. These non-selective channels were not activated by either fMLP or IP_3 directly. However, although these data suggest the contribution of non-selective ion channels to increased $[Ca^{2+}]_i$ caused by a receptor-mediated agonist, other researchers have concluded that there is no evidence of VOC channels occurring in the plasma membrane of human neutrophils and that VOC channel blockers do not inhibit fMLP induced $[Ca^{2+}]_i$ increase (Fukushima and Hagiwara, 1985; Rosales and Brown, 1992).

Ca^{2+} Signaling and the Treatment of Bovine Respiratory Disease

More research is necessary to definitively determine the role and mechanism of $[Ca^{2+}]_i$ increase in the molecular pathogenesis of LKT-induced inflammation. Considering the important enzymatic and cellular events regulated by increased $[Ca^{2+}]_i$, it is reasonable to assume that increased $[Ca^{2+}]_i$ constitutes a control mechanism that is common to many LKT-induced events that contribute to lung pathology. Once identified, Ca^{2+}-mediated control mechanisms would be logical targets for pharmacological agents used in the therapy of BRD. Selective pharmacological inhibition of these calcium-mediated processes

responsible for the excessive and damaging inflammatory responses would improve the efficacy of concurrently administered antibacterial agents, without compromising beneficial host defense reactions.

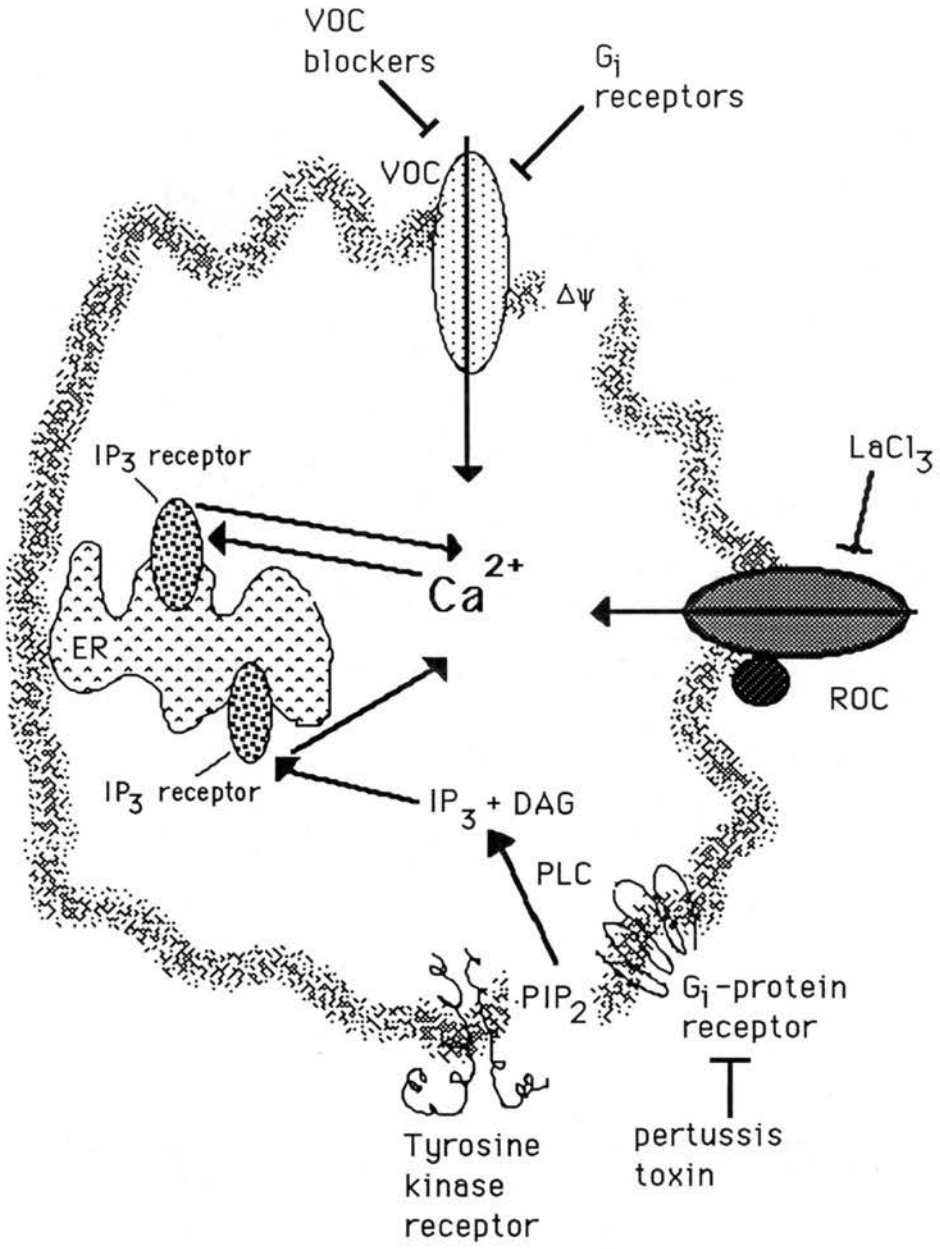


Fig. 1- A simplified overview of mechanisms of calcium entry into the cytosol of a cell from extracellular and intracellular sources.

TABLE 1 - Ca^{2+} entry pathways in vertebrate cells: A simplified summary

Type	Properties	Function/location
Voltage-operated Ca^{2+} channels		
L-type	High-voltage activated, blocked by dihydrophyridine (DHP) antagonists	Excitation-contraction coupling in some endocrine cells and some neurons
T-type	Low-voltage activated, slowly deactivating, blocked by low $[Ni^{2+}]_o$	Contributes to pacemaker activity and repetative firing in heart and neurons
N-type	High-voltage activated, blocked by ω-conotoxin	Identified in most neurons, triggers transmitter release
P-type	Moderately-high voltage activated, blocked by funnel web spider toxin, not blocked by DHP or ω-conotoxin	Mediated transmitter release and high-threshold spiking in some neutrons
Receptor operated Ca^{2+} channels		
NMDAR	Ligand-gated, indirectly voltage-activated (by relief of Mg^{2+} block)	mediated neuronal Ca^{2+} entry in response to conjunction of transmitter and membrane depolarization
ATPR	Ligand-gated without indirect voltage-gating	Allows cation influx and Ca^{2+} entry for smooth muscle activation

TABLE 2 - The major families of trimeric G proteins

Type	Sub-type	α Subunits	Functions	Modified by bacterial toxin
I	G_s	α_s	Activates adenylyl cyclase, activates Ca^{2+} channels	Cholera activates
II	G_i	α_i	Inhibits adenylyl cyclase, activates K^+ channels	Pertussis inhibits
	G_o	α_o	Activates K^+ channels, inactivates Ca^{2+} channels, activates PLCβ	
III	G_q	α_q	Activates PLCβ	No effects

Families are determined by amino acid sequence relatedness of the α subunits.

CHAPTER III

HYPOTHESIS AND EXPERIMENTAL OBJECTIVES

The hypothesis upon which this research is based is that the pathogenic effects of LKT on bovine neutrophils, including excessive LTB_4 production, cell membrane damage, and apoptosis, are mediated via unregulated increase in $[Ca^{2+}]_i$, resulting from influx of extracellular Ca^{2+} and release of vesicular Ca^{2+} (Fig. 2). This hypothesis was tested by determining whether these LKT-induced pathogenic effects on bovine neutrophils are dependent on increased $[Ca^{2+}]_i$ and by studying the characteristics and mechanisms of $[Ca^{2+}]_i$ alteration in relation to LKT concentration. To eliminate the potential contributions of other virulence factors of *P. haemolytica*, effects of LKT were compared with those of a negative control prepared using a LKT-deficient mutant strain of *P. haemolytica*..

Specific objectives of the experiment were:

(1) to determine whether LKT-induced production of LTB_4 by bovine neutrophils and neutrophil plasma membrane damage are dependent on increased $[Ca^{2+}]_i$;

(2) to study the involvement of Ca^{2+} in LKT-induced neutrophil apoptosis and the relationship between LKT concentration and apoptosis versus oncosis;

(3) to characterize the nature of LKT-induced increase in $[Ca^{2+}]_i$ by comparison with other activators of neutrophils and by manipulation of $[Ca^{2+}]_i$; and

(4) to study the mechanisms of LKT-induced $[Ca^{2+}]_i$ increase by investigating the involvement of voltage operated calcium channels and the contribution of vesicular calcium.

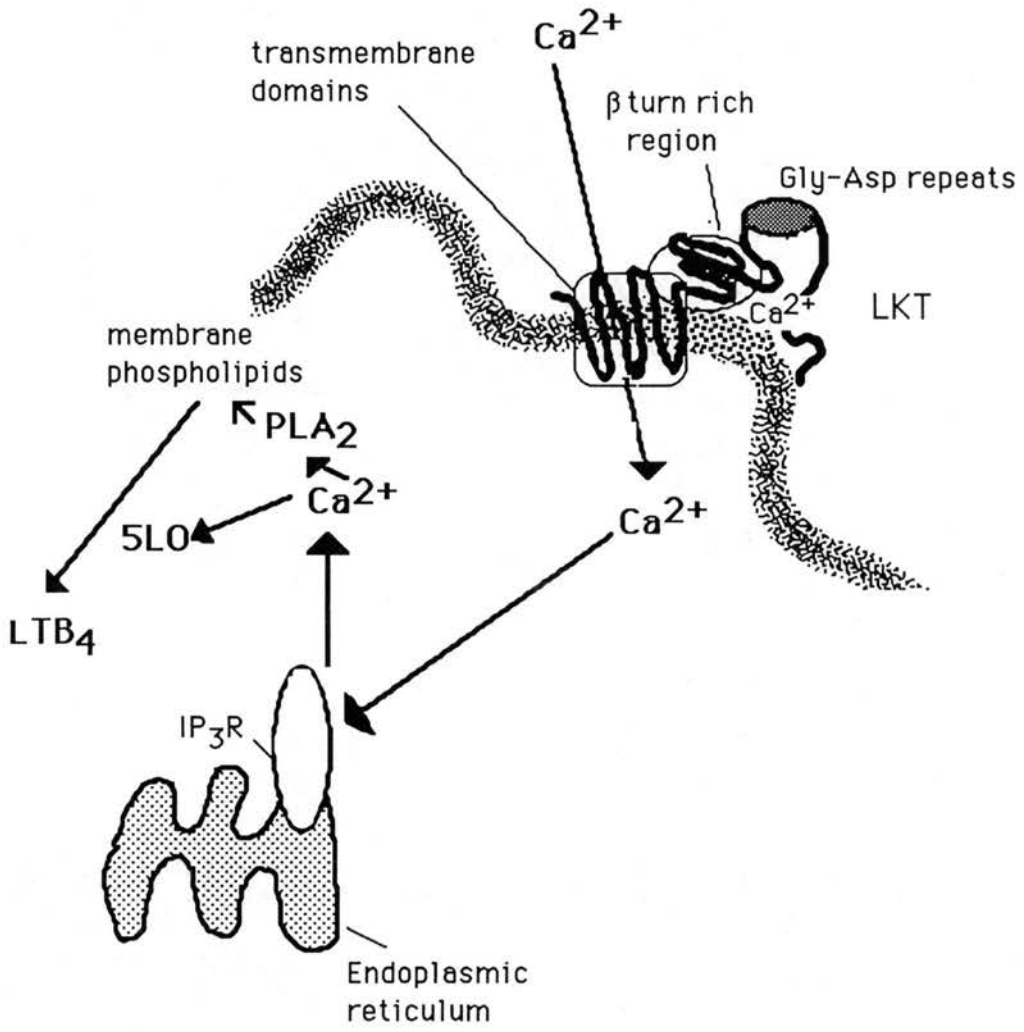


Fig.2- Hypothesized mechanism of LKT induced $[Ca^{2+}]_i$ increase, plasma membrane damage and LTB_4 production.

CHAPTER IV

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

Introduction

Pasteurella haemolytica is the causative agent of bovine shipping fever, which is characterized by acute fibrinous pneumonia involving massive accumulation of neutrophils (Yates, 1982). Infiltrating neutrophils do not provide an effective host defense response, but undergo degranulation and lysis, releasing lysosomal enzymes and reactive oxygen products that aggravate tissue damage (Slocombe *et al*, 1985; Breider *et al*, 1988; Frank 1989). Eicosanoids, including LTB_4, are considered to be important chemotactic agents responsible for influx of neutrophils into infected lungs (Clarke *et al*, 1994). LKT, a protein exotoxin produced by log-phase *P. haemolytica*, stimulates the release of LTB_4 and other 5-lipoxygenase products from bovine neutrophils (Henricks *et al*, 1992; Clinkenbeard *et al*, 1994). This toxin, which is a member of the family of RTX pore-forming cytolysins (Welch, 1991), also causes membrane perturbation and neutrophil lysis (Clinkenbeard *et al*, 1989). Leukotoxin-induced plasma membrane damage may contribute to the availability of AA, which serves as the substrate for eicosanoid production. The importance of LKT and neutrophil infiltration in disease production is supported by observations that experimental infection of neutrophil-sufficient calves with an isogenic leukotoxin-deficient mutant (Tatum *et al*, 1998) or inoculation of neutrophil-deficient

calves with LKT-producing *P. haemolytica* (Breider *et al*, 1988) does not result in development of pneumonic lesions typical of shipping fever.

Exposure of bovine neutrophils to LKT causes a concentration-dependent increase in $[Ca^{2+}]_i$ (Ortiz-Carranza and Czuprynski, 1992). Considering the effect of $[Ca^{2+}]_i$ on activities of enzymes responsible for release of AA from phospholipid membranes (Mayer and Marshall, 1993) and subsequent oxidation of AA to leukotrienes (Musser and Kreft, 1992), Ca^{2+} may serve as an important second messenger in LKT-mediated inflammatory responses. Therefore, the primary objective of this study was to determine whether LKT-induced production of LTB_4 by bovine neutrophils is dependent on increased $[Ca^{2+}]_i$. The association of membrane damage with LKT-induced LTB_4 production was also explored.

Materials and Methods

Preparation of *P. haemolytica* Wildtype Leukotoxin and Leukotoxin-Deficient Control

P. haemolytica biotype A, serotype 1 wildtype strain and an isogenic leukotoxin-deficient (LKT(-)) mutant strain A, produced by allelic replacement of lktA with β–lactamase bla gene (Murphy *et al*, 1995), were grown in 150 ml BHI broth to a density of 600 nm (OD_{600}) of 0.8-1.0. Bacteria collected from the bacterial cultures were inoculated into 250 ml RPMI 1640 medium (pH 7.0, 2.2 g/l $NaHCO_3$) containing 0.5% bovine serum albumin (A-6003 fraction V, essentially fatty acid free, Sigma Chemical Co., St. Louis) to an OD_{600} of 0.25. The RPMI cultures were grown at 37°C, and 70 oscillations/min to an OD_{600} of 0.8-1.0, and the culture supernatants were harvested following centrifugation at 8,000 x *g* for 30 minutes (Sorvall GS3 rotor, DuPont Co., Wilmington). This and all subsequent steps were conducted at 4°C. Culture supernatants were concentrated by

addition of solid ammonium sulfate (361 g/L) to yield 60% saturation and the precipitated material was collected by centrifugation at 8,000 x *g* for 45 minutes (Sorvall GS3 rotor). Precipitates were resuspended in 3 ml of 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0 buffer, and then dialyzed against 500 ml of the same buffer overnight. Dialyzed concentrated culture supernatants were stored frozen at -135°C.

LKT 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 2 hours of incubation. The mean (± SD) activity of undiluted LKT preparations used in this study was 2.34 x 10^5 ± 1.75 x 10^5 TU/ml.

Preparation of Neutrophil Suspensions

Whole bovine blood was collected in 1% sodium citrate by venipuncture and then centrifuged in 50 ml polypropylene conical tubes (Corning Incorporated, Corning) at 600 x *g* for 30 min at 4°C. The plasma, buffy coat, and the top portion of the red cell column were aspirated to leave 10 ml of cell suspension, which was then subjected to two cycles of hypotonic lysis (Holden-Stauffer *et al*, 1989, Clinkenbeard *et al*, 1994). In the first cycle, 20 ml of sterile, distilled water was added to the suspension of red cells and neutrophils remaining in the tube, the diluted suspension was mixed for 60 seconds, and tonicity was then restored by adding an equal volume of double-strength phosphate buffered saline (PBS). Suspensions were then centrifuged (Sorvall RC5C, HS-4 rotor, DuPont Co., Wilmington) for 10 min at 200 x *g* and the supernatant discarded. Neutrophils were resuspended with 5 ml of PBS and subjected to the second cycle of hypotonic lysis in which 10 ml of sterile distilled water was added, the cell suspension was mixed for 60 seconds, and 10 ml of double strength PBS added to restore isotonicity. After centrifuging

the cells again, the supernatant was discarded, and cells were resuspended in 20 ml PBS. The resuspended cells were then centrifuged for 10 minutes at 200 x *g*. Thereafter, the supernatant was discarded and cells were resuspended in 5 ml of $CaCl_2$-free Hank's balanced salt solution (HBSS) (Sigma Chemical Co., St. Louis) containing 0.5 mM $MgCl_2$ (Sigma Chemical Co., St. Louis) and 50 μM EGTA (Sigma Chemical Co., St. Louis). Cells were enumerated by hemocytometer, and viability was assessed by trypan blue exclusion. Differential counts were conducted on stained smears (Diff-Quik, Baxter Healthcare Corp., Miami). The lowest viability and highest mononuclear cell contamination of suspensions used in the experiments were 98% and 6%, respectively. Preparations were then diluted to a final concentration of 6 x 10^6 viable neutrophils/ml HBSS.

Neutrophil Loading with Fluorescent Calcium Indicator

$[Ca^{2+}]_i$ was measured using the membrane-permeable acetoxymethyl (AM) ester of the fluorescent calcium indicator, Fluo-3 (Molecular Probes Inc., Eugene). Membrane permeable Fluo-3 AM diffuses across the neutrophil membrane, but is retained within the cytosol after it is hydrolyzed intracellularly to the membrane impermeable free acid (Kao *et al*, 1989). Neutrophil suspensions contained in 50 ml polypropylene conical centrifuge tubes (Corning Incorporated, Corning) and protected from light were incubated with Fluo-3 AM for 30 minutes at 22°C, while constantly mixing on a cell rotator (Angenics, Cambridge). Sufficient Fluo-3 AM (in DMSO containing 0.14% pluronic acid) was added to the cell suspensions to achieve a final concentration of 5 μM. Loaded cells were then centrifuged at 200 x *g* and 4°C for 10 minutes, the supernatant was discarded, and the cells were resuspended in 10 ml PBS before centrifuging again at 200 x *g* and 4°C for 10

minutes. After discarding the supernatant, cells were resuspended in 3 ml HBSS, enumerated by hemocytometer and then resuspended in HBSS to 1 x 10^7 cells/ml.

Exposure of Isolated Neutrophils to Leukotoxin

The effects of LKT and LKT(-) control on $[Ca^{2+}]_i$, neutrophil membrane damage, and LTB_4 production were tested in 96-well flat bottom microtiter plates (Corning Glass Works, Corning). Ten microliters of 25 mM $CaCl_2$ (Sigma Chemical Co., St. Louis) and 2 µl antifluoroscein antibody (Molecular Probes, Eugene), diluted 1:5 in PBS, were added in sequence to 250 µl of cell suspension. The antifluoroscein antibody quenched fluorescence of extracellular indicator. Thereafter, 25 µl of diluted LKT preparation, 25 µl of diluted LKT(-) control, 10 µl of 40 µM 4-bromo A23187 (Sigma Chemical Co., St. Louis) in dimethyl sulfoxide (DMSO), 25 µl of 1% Triton X 100, or 25 µl of PBS were added to wells, lids were placed on the plates, and the plates were incubated at 37°C. Fluorescence (490 nm excitation, 523 nm emission) was measured at times indicated for each specific experiment, using a spectrofluorometer (Cytofluor 2300 Fluorescence Measurement System, Millipore Corp., Bedford). Experiments were terminated by centrifugation at 200 x *g* at 4°C for 5 minutes and aliquots of supernatants were removed for assay of LDH and LTB_4. All experiments included quadruplicate wells for each of the primary treatments.

The specific objective of the initial experiment was to study the effects of the LKT preparation on $[Ca^{2+}]_i$, neutrophil membrane integrity, and LTB_4 production and to confirm that these effects resulted from exposure to LKT and not to other bacterial products or constituents in the partially purified culture supernatant. LKT-induced responses were distinguished by comparison with LKT(-) control preparations, which contained all constituents of the partially purified culture supernatant except LKT. Neutrophil

suspensions were exposed to dilutions (1:10, 1:50, 1:250, 1:500, 1:1000, 1:2500, 1:5000, and 1:10000) of LKT or LKT(-) control preparations, $[Ca^{2+}]_i$ was measured 2 minutes after exposure, and then suspensions were incubated for 120 minutes before samples were harvested for assay of LDH and LTB_4.

After establishing that effects of LKT preparation on neutrophil $[Ca^{2+}]_i$, LTB_4 production, and LDH release were caused by LKT, subsequent experiments investigated the association between these responses and the contribution of extracellular calcium to increased $[Ca^{2+}]_i$. The temporal association between LKT-induced changes in $[Ca^{2+}]_i$, LTB_4 production, and LDH release and the relationship between plasma membrane damage and LTB_4 production were examined by measuring responses before exposure and after 5, 15, 30, 45, 60, 90, and 120 minutes of incubation with LKT or LKT(-) (1:50 dilutions) at 37°C. Separate plates containing all duplicates of LKT dilutions and relevant controls were used for each incubation period. At the end of each incubation period, $[Ca^{2+}]_i$ was measured, plates were centrifuged, and supernatants were harvested for assay of LDH and LTB_4.

The dependence of LKT-induced responses on influx of extracellular Ca^{2+} was investigated by altering the concentration of calcium in the neutrophil suspension media, by replacing Ca^{2+} with other divalent cations to confirm that the requirement for Ca^{2+} was specific. Neutrophils were suspended in Ca^{2+}-free HBSS, HBSS with 1 mM $CaCl_2$, HBSS with 1 mM EGTA, or HBSS with 3 mM $CaCl_2$ and 1 mM EGTA. Additional $CaCl_2$ was not added as in the other experiments. The $[Ca^{2+}]_i$ was measured 2 minutes after addition of 25 µl 1:50 LKT or LKT(-), and LDH and LTB_4 assays were performed after 120 minutes of incubation.

The specificity of the dependence of LKT-induced responses on influx of extracellular calcium was investigated by replacing calcium in the neutrophil suspension

media with other divalent cations. Neutrophils were suspended in calcium-free HBSS or HBSS with 1 mM of either $CaCl_2$, $BaCl_2$, $MgCl_2$, or $ZnCl_2$. Additional $CaCl_2$ was not added as in the other experiments. The $[Ca^{2+}]_i$ was measured 2 minutes after addition of 25 µl 1:50 LKT or LKT(-) and LDH and LTB_4 assays were performed after 60 minutes of incubation.

Calculation of Intracellular Calcium Concentration.

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

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

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

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

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

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

The Ca^{2+}-Fluo-3 dissociation constant (K_d) was determined using a commercially prepared kit (Molecular Probes, Eugene) which measured the fluorescence of 4-bromo-A23187 - exposed bovine neutrophils suspended in a range of EGTA-containing Ca^{2+} buffers at pH

7.2 and 22°C. The calibration curve for Fluo-3 was linear (r^2 = 0.990) and indicated a K_d of 358 nM.

Estimation of Plasma Membrane Damage.

The effect of *P. haemolytica* LKT on neutrophil plasma membrane integrity was assayed by measuring extracellular release of cytoplasmic 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 X 100 (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:

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

Leukotriene B_4 Immunoassay

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

Statistical Analyses

Data were analyzed using SYSTAT intelligent software for the Macintosh, Version 5.2 (SYSTAT Inc., Evanston, IL). Unpaired t-tests were used to compare LKT- and LKT(-)-induced responses at each toxin dilution or incubation time in studies assessing concentration and time-dependent effects of LKT. The effects of extracellular calcium concentration on LKT-induced responses were evaluated using the general linear model followed by comparison of mean pairs using Scheffe's test. Effects of other divalent cations were tested using the general linear model followed by *a priori* comparison of selected mean pairs using Fisher's LSD Test: LKT-induced responses were compared with cation-free controls and LKT-induced responses were compared with corresponding LKT (-) controls. Differences between means were considered significant at the $P < 0.05$ level.

Results

Exposure of isolated bovine neutrophils to *P. haemolytica* wildtype LKT preparation resulted in significant increases in $[Ca^{2+}]_i$, LTB_4 production, and LDH release whereas exposure to LKT(-) control had no effect on the observed responses (Fig. 3). The LKT-induced responses were concentration-dependent and decreased in parallel as LKT concentration was decreased. When exposed to a lytic concentration of LKT, $[Ca^{2+}]_i$ increased rapidly, reaching a maximum at 5 minutes, before declining thereafter (Fig. 4). The initial increase in $[Ca^{2+}]_i$ was followed by slower increases in LTB_4 production and LDH leakage. No significant effects were observed when neutrophils were exposed to LKT(-) during the 120 minute incubation period.

Exposing neutrophils to LKT in Ca^{2+}-free buffers was used to examine the extracellular Ca^{2+}-dependence of changes in $[Ca^{2+}]_i$, LTB_4 production, and LDH release (Figs. 5a, 5b, 5c). Compared with responses observed when neutrophils were exposed to LKT in buffer containing 1 mM $CaCl_2$, all responses were significantly lower in Ca^{2+}-free buffer and Ca^{2+}-free buffer containing EGTA. Intracellular calcium concentration and LTB_4 production of neutrophils exposed to LKT in Ca^{2+}-free buffers were no different from the corresponding responses of cells exposed to LKT(-). Although the absence of Ca^{2+} in the suspension buffer caused significant decreases in LDH release by LKT-exposed neutrophils, the values obtained in Ca^{2+}-free buffers were still significantly higher than those of neutrophils exposed to LKT(-) control. The LKT-induced increases in $[Ca^{2+}]_i$, LTB_4 production, and LDH release were restored when the calcium chelating effect of EGTA was saturated by addition of 3 mM $CaCl_2$, although LTB_4 production was significantly lower than that obtained in the presence of Ca^{2+} and the absence of EGTA. Replacement of Ca^{2+} with other divalent cations indicated that LKT-induced production of LTB_4 and LDH release were mediated specifically by Ca^{2+} (Table 3): with the exception of addition of $BaCl_2$, only when $CaCl_2$ was added were production of LTB_4 and release of LDH significantly increased compared with responses obtained in the absence of extracellular cation. The small but significant increase in LTB_4 production by neutrophils suspended in buffer containing $BaCl_2$ coincided with increased $[Ca^{2+}]_i$ (as measured by Fluo-3 fluorescence intensity), indicating that LTB_4 synthesis still may have been mediated via Ca^{2+}, possibly following displacement of intracellular stores of Ca^{2+} by Ba^{2+}.

Discussion

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

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

Studies utilizing a range of LKT and LKT(-) dilutions demonstrated a positive correlation between LKT concentration, $[Ca^{2+}]_i$, LTB_4 production, and LDH release. This relationship was further investigated by examining the effects of LKT on bovine neutrophils as a function of time. The temporal relationship observed between the initial, rapid increase in $[Ca^{2+}]_i$ and subsequent production of LTB_4 and LDH release supports the hypothesis that these responses are dependent on increased $[Ca^{2+}]_i$. Simultaneous release of LDH and production of LTB_4 could be explained by Ca^{2+}-dependent translocation of phospholipase A_2 to cell membranes, generation of membrane-damaging lysophospholipids and AA, oxidation of AA by 5-lipoxygenase, and carrier-mediated

release of LTB_4 down a concentration gradient (Holtzman, 1991; Higgins and Lees, 1984; Moncada and Vane, 1979; Lam *et al*, 1990). Indeed, a recent study has confirmed that exposure of isolated bovine neutrophils to LKT causes an increase in cytosolic phospholipase A_2 activity (Wang *et al*, 1998).

The involvement of calcium in LKT-induced LTB_4 production and LDH release was further investigated by altering the concentration of extracellular calcium in the suspension buffer and by replacing extracellular calcium with other divalent cations. In comparison with neutrophils suspended in Ca^{2+}-containing buffer, neutrophils in Ca^{2+}-free buffers released significantly less LTB_4 and LDH. Furthermore, LKT-exposed neutrophils suspended in buffers containing divalent cations other than Ca^{2+} did not produce LTB_4 or release LDH, with the exception of those exposed to $BaCl_2$; these neutrophils still appeared to respond via a mechanism involving displacement of stored intracellular Ca^{2+} by Ba^{2+}. Evidence that LKT-induced LTB_4 production and LDH release require extracellular Ca^{2+} and that this requirement always is coupled with increase in $[Ca^{2+}]_i$ further supports the contention that LKT-induced effects involve influx of extracellular Ca^{2+}. This role of free extracellular Ca^{2+} should not be confused with the necessity that RTX toxins be bound to Ca^{2+} to possess an active conformation (Boehm *et al*, 1990). This binding of Ca^{2+} to the protein occurs with very high affinity and, considering that LKT was prepared in the presence of Ca^{2+}, subsequent alterations in extracellular Ca^{2+} were unlikely to affect activity of the toxin molecule.

The significant decrease in LDH release that was observed when Ca^{2+} was removed from the suspension buffer of neutrophils and the absence of any significant LDH release in the presence of other divalent cations confirms the earlier observation that LKT-induced neutrophil lysis is calcium-dependent (Clinkenbeard *et al*, 1994). However, in the current study, removal of Ca^{2+} from the suspension buffer of neutrophils exposed to a highly lytic concentration of LKT (> 90% specific LDH release) had less effect on LDH release than on LTB_4 production, which was no different from that of neutrophils exposed

to LKT(-). The difference between the effect of extracellular Ca^{2+} on these responses suggests that LKT-induced plasma membrane damage and leukotriene production may not be as closely coupled as was previously hypothesized (Clinkenbeard *et al*, 1994) and suggests that, at high concentrations of LKT, disruption of membrane integrity may result from a combination of calcium-dependent and calcium-independent pathways: probably, calcium-mediated degradation of the membrane by phospholipases contributes to Ca^{2+}-independent osmotic imbalances, cell swelling, and lysis resulting from flux of monovalent cations through pores formed by interpolation of hydrophobic runs of the RTX toxin in the plasma membrane (Clinkenbeard *et al*, 1989; Forester and Welch, 1991). Recent research involving exposure of [^{3}H]AA-labeled neutrophils to LKT (Wang *et al*, 1998) has revealed that release of radioactivity resulting from the action of phospholipases does not fully explain LDH release, thus suggesting that LKT-induced loss of plasma membrane integrity is a complex process involving a number of molecular pathogenic pathways.

In conclusion, this study demonstrated that exposure of isolated bovine neutrophils to *P. haemolytica* LKT induces an increase in $[Ca^{2+}]_i$, which constitutes an essential step in the transduction of a signal resulting in synthesis and release of LTB_4. Furthermore, LKT-induced increase in $[Ca^{2+}]_i$ contributes to plasma membrane damage and release of LDH, although other mechanisms apparently also participate in this response.

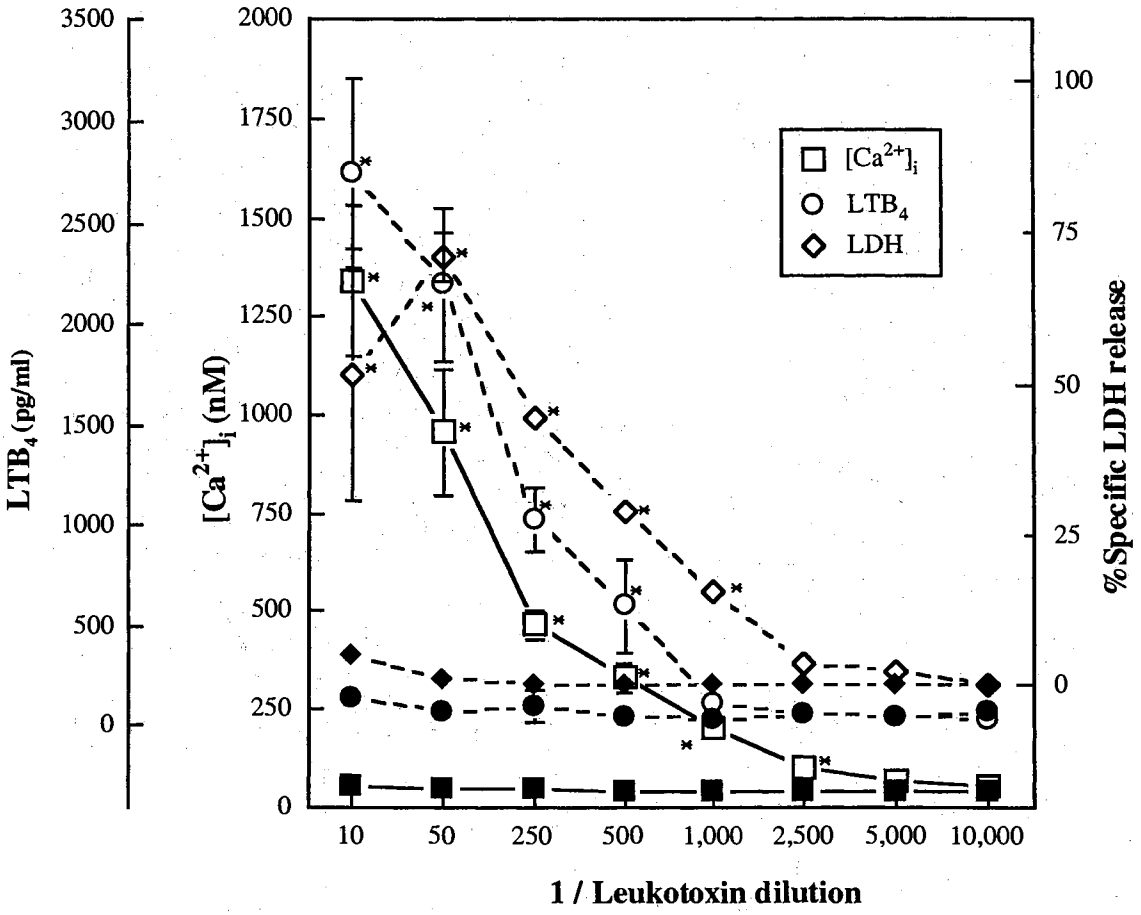


Figure 3. Effect of LKT (open symbols) and LKT(-) control preparations (solid symbols) on neutrophil $[Ca^{2+}]_i$, LTB_4 production, and LDH release. Isolated bovine neutrophils were exposed to dilutions of LKT or LKT(-) and responses were measured at 2 minutes ($[Ca^{2+}]_i$) and at 120 minutes (LTB_4, LDH) after exposure. *LKT-induced effects were significantly higher than corresponding LKT(-) control values among quadruplicate samples within the same plate ($p<0.05$).

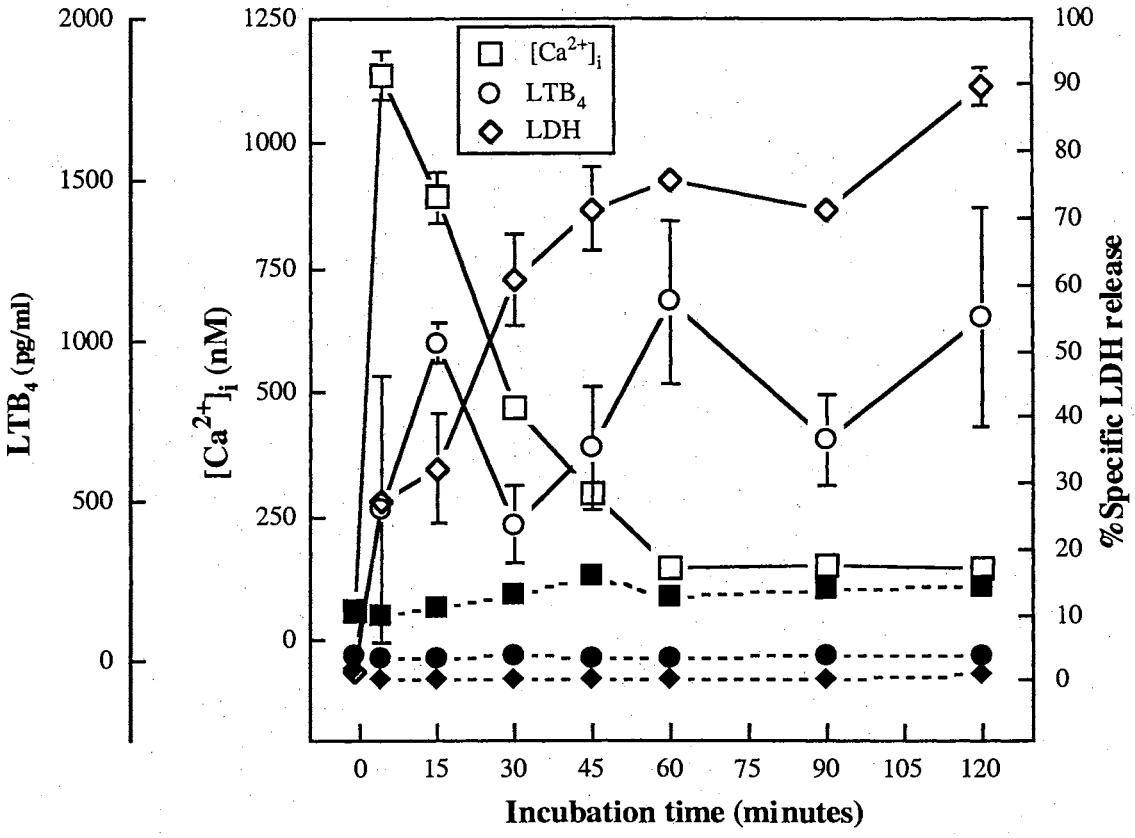


Figure 4. Time-dependent increase in $[Ca^{2+}]_i$, LTB_4 production, and LDH release after exposure of isolated bovine neutrophils to LKT or LKT(-) (lytic, 1:50 dilution). With the exception of values measured before addition of LKT, all LKT-induced responses were significantly different from corresponding LKT(-) control values as measured by quadruplicate samples in a single plate ($p<0.05$).

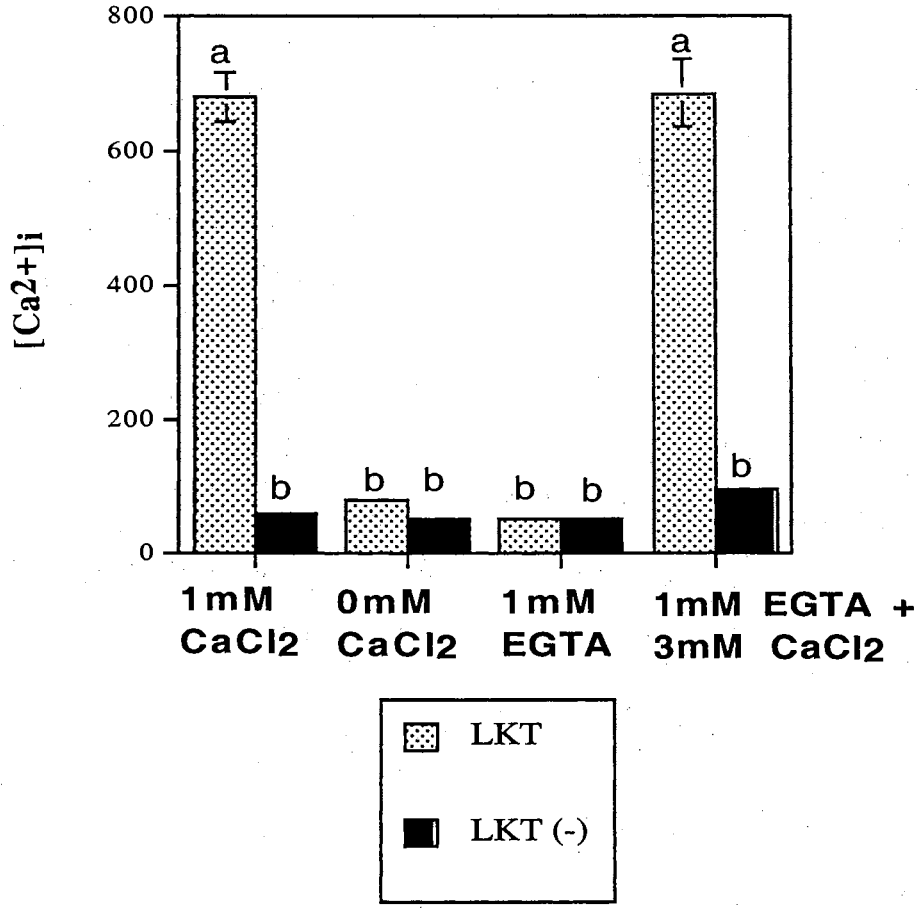


Figure 5a. Extracellular calcium dependence of LKT-induced effects on $[Ca^{2+}]_i$. Isolated neutrophils were exposed to a 1:500 dilution of LKT in buffer suspensions containing 1 mM $CaCl_2$, no Ca^{2+}, 1 mM EGTA and no Ca^{2+}, or 1 mM EGTA and 3 mM $CaCl_2$. ^{a,b} Values with the same individual letter annotations were not significantly different among quadruplicate replicates within a single plate ($p<0.05$)

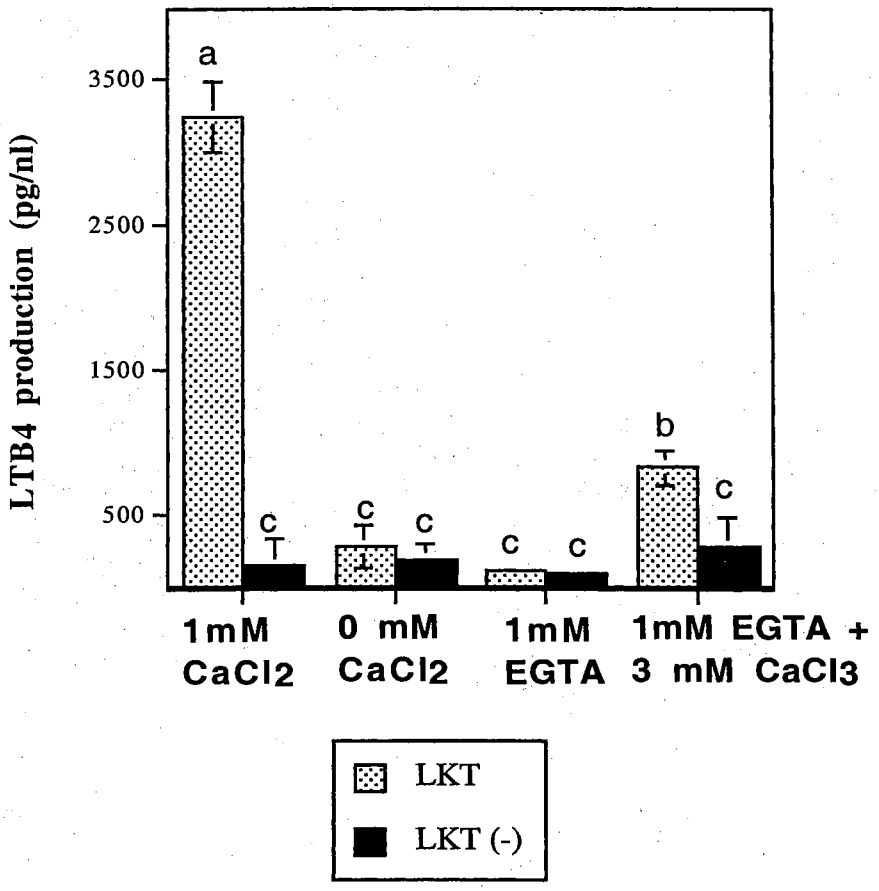


Figure 5b. Extracellular calcium dependence of LKT-induced effects on LTB_4 production. Isolated neutrophils were exposed to a 1:500 dilution of LKT in buffer suspensions containing 1 mM $CaCl_2$, no Ca^{2+}, 1 mM EGTA and no Ca^{2+}, or 1 mM EGTA and 3 mM $CaCl_2$. ^{a,b,c} Values with the same individual letter annotations were not significantly different among quadruplicate replicates within a single plate ($p<0.05$)

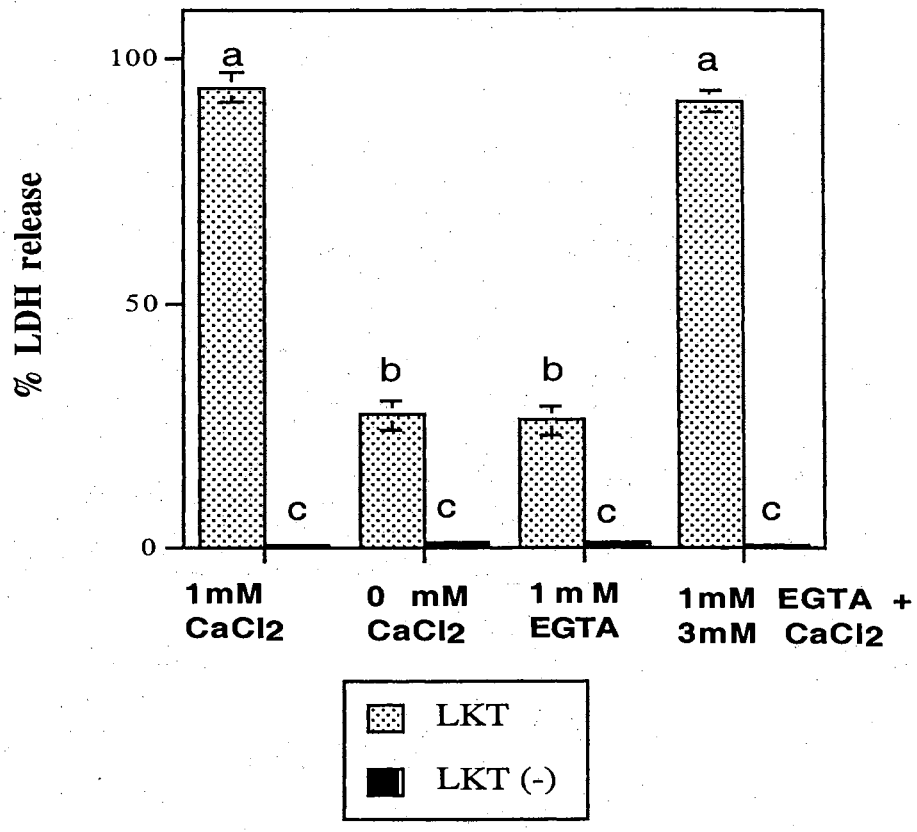


Figure 5c. Extracellular calcium dependence of LKT-induced effects on LDH release. Isolated neutrophils were exposed to a 1:500 dilution of LKT in buffer suspensions containing 1 mM $CaCl_2$, no Ca^{2+}, 1 mM EGTA and no Ca^{2+}, or 1 mM EGTA and 3 mM $CaCl_2$. [a,b,c] Values with the same individual letter annotations were not significantly different among quadruplicate replicates within a single plate ($p<0.05$)

Table 3. Effect of replacing Ca^{2+} with other divalent cations on LKT-induced responses. Neutrophils were suspended in cation-free HBSS (No cations) or HBSS with 1 mM of either $CaCl_2$, $BaCl_2$, $MgCl_2$, or $ZnCl_2$. Data are presented as means ± SD.

		No cation	**$CaCl_2$**	**$BaCl_2$**	**$MgCl_2$**	**$ZnCl_2$**
$[Ca^{2+}]_i$ (nM)	LKT	24.2 ± 0.7	175.9 ± 7.6†*	61.3 ± 1.9†*	27.5 ± 1.0	32.2 ± 0.7†
	LKT(-)	22.4 ± 0.0	24.1 ± 0.2	48.1 ± 0.7	27.5 ± 0.6	34.8 ± 1.3
LTB_4(pg/ml)	LKT	112 ± 34	1317 ± 136†*	219 ± 77†*	96 ± 11	104 ± 38
	LKT(-)	118 ± 23	37 ± 10	90 ± 12	97 ± 13	85 ± 23
% Specific LDH Release	LKT	1.7 ± 1.7	49.3 ± 4.3†*	0.0 ± 0.0	5.1 ± 1.2*	0.0 ± 0.0
	LKT(-)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

† Value significantly higher from corresonding No cation control value (n=4).

* Value significantly higher than corresponding LKT(-) control value (n=4).

CHAPTER V

INVOLVEMENT OF CALCIUM IN LEUKOTOXIN-INDUCED NEUTROPHIL APOPTOSIS AND THE EFFECT OF LKT CONCENTRATION ON APOPTOSIS VERSUS ONCOSIS.

Introduction

P. haemolytica is the causative organism of BRD, which presents as a severe fibrinoplurulent pneumonia in feedlot or stocker cattle (Allen *et al*, 1985; Wilkie and Shewen, 1988; Frank *et al*, 1989). Although neutrophils usually constitute a component of the host defense system, in BRD neutrophils are not only ineffective in clearing the organism, but contribute to the development of severe lung pathology (Yates *et al*, 1982; Slocombe *et al*, 1985; Breider *et al*, 1988). While there are many virulence factors produced by *P. haemolytica* that contribute to persistance of the organisms in the host, one of the most important of these is LKT, an exotoxin produced by *P. haemolytica* during log phase growth (Shewen and Wilkie, 1985; Confer *et al*, 1995).

LKT is an RTX exotoxin that shares many characteristics with other RTX toxins, such as the ability to form pores in the plasma membranes of susceptible target cells (Kaehler *et al*, 1980; Shewen and Wilkie, 1982; Clinkenbeard *et al*, 1989; Welch, 1991). Bovine neutrophils have been shown to undergo degranulation and lysis and to release chemotactic factors when exposed to LKT (Clinkenbeard *et al*, 1989; Czuprynski *et al*, 1990; Clinkenbeard *et al*, 1994). The effects of LKT on susceptible cells may be catagorized as high-concentration, lytic effects and low-concentration, sublytic effects. Lytic effects on bovine neutrophils include K^+ leakage down it's concentration gradient, loss of membrane ruffling, cell swelling, increase in $[Ca^{2+}]_i$, LTB_4 production, and

membrane lysis (Clinkenbeard *et al*, 1989; Ortiz-Carranza and Czuprynski, 1992; Clinkenbeard *et al*, 1994). At sublytic concentrations, LKT is a potent leukocyte stimulator (Czyprynski *et al*, 1990). Recent studies utilizing light microscopy have suggested that sublytic concentrations of LKT can also induce apoptosis in bovine neutrophils *in vitro* (Stevens and Czuprynski, 1996). However, the reliability of these findings was compromised by the absence of confirmatory gel electrophoresis and morphological exam .

Apoptosis and oncosis are two distinct cell death processes. While oncosis involves limited nuclear changes, the salient characteristics of oncosis are organelle and cell swelling which result in cell lysis, followed by the leakage of cytoplasmic components into surrounding tissue and inflammation (Majno and Joris, 1995; Wyllie *et al*, 1980). In contrast, apoptosis is a process which appears to be regulated by the nucleus, resulting in eventual cell shrinkage and phagocytosis of the cell without the release of cellular contents into surrounding tissues (Trump and Berezesky, 1996; Majno and Joris, 1995; Wyllie *et al*, 1980). The universal signal responsible for apoptosis induction in all cells has not been determined, but increased $[Ca^{2+}]_i$ has been shown to induce apoptosis in many systems (Trump and Berezesky, 1995; Trump and Berezesky, 1996), thus suggesting that increased $[Ca^{2+}]_i$ may serve as an essential signal for LKT-induced apoptosis (Ortiz-Carranza and Czuprynski, 1992; Hsuan *et al*, 1998). Therefore, the objectives of this study were to confirm that LKT induces apoptosis in bovine neutrophils by use of definitive mophological, electrophoretic, and chemical methods and to determine whether LKT-induced apoptosis is dependent on Ca^{2+}.

Materials and Methods

Preparation of *P. haemolytica* Leukotoxin

LKT and LKT(-) were prepared from culture supernatants of *P. haemolytica* biotype A, serotype 1 wildtype strain and a leukotoxin-deficient mutant strain A, as described in Chapter IV. Lytic concentrations of LKT caused > 50% lysis of 2.5 x 10^6 neutrophils in 250 µl buffer after 2 hours incubation while sublytic doses of LKT caused <1% lysis from similarly diluted and incubated neutrophils.

Preparation of Neutrophil Suspensions

Two healthy beef calves (200 ± 50 kg) served as blood donors for isolation of neutrophils. Neutrophils were isolated by hypertonic lysis as described previously in Chapter IV, and suspended in 5 ml of Ca^{2+}- and Mg^{2+}-free HBSS. Cells were enumerated by hemocytometer and viability was assessed by trypan blue exclusion. Differential counts were conducted on stained smears. Preparations were then diluted to a final concentration of 1 x 10^7 viable neutrophils/ml HBSS.

Exposure of Neutrophils to LKT and Control Stimulators

The ability of LKT to induce apoptosis or necrosis in bovine neutrophils was investigated by exposing cells to lytic (1:50) and sublytic (1:2,500) concentrations of LKT and to positive (staurosporin 1mM) and negative (PBS; LKT(-) 1:50; LKT(-) 1:2,500) controls. Bovine neutrophil suspensions (1 x 10^7 cells/ml) were incubated with each

stimulator and 1mM $CaCl_2$ for a predetermined duration (5, 15, 30, 120, 240 minutes) and then centrifuged at 200 x g for 10 minutes. Cell pellets were subjected to examination by transmission electron microscopy (TEM; all sampling times), gel electrophoresis (240 minute sample only), or diphenylamine assay (DPA; 240 minute sample only). DPA assays included triplicate cuvettes for each of the treatments, whereas TEM and gel electrophoresis were conducted on single samples.

The contribution of extracellular Ca^{2+} to LKT-induced apoptosis was investigated by incubating bovine neutrophils with LKT (1:50 or 1:2,500 dilutions), LKT (-) (1:50 or 1:2,500 dilutions), PBS, staurosporin (1mM), or A23187 (40 μM) in buffer containing 1mM $CaCl_2$ or in Ca^{2+}-free buffer containing 1mM EGTA for 240 minutes prior to DPA analysis. DPA analysis included triplicate cuvettes for each of the treatments.

Transmission Electron Microscopy

Fixative solution containing 1.6% glutaraldehyde in 0.2M sodium cocodylate buffer was added to pelleted samples, and after washing in 0.2M cocodylate buffer containing sucrose, the samples were then post-fixed with 1% osmium tetroxide in 0.2M sodium cacodylate buffer. After washing the samples with 0.1M sodium cacodylate buffer 3 times, samples were dehydrated in ethanol and propylene oxide, and embedded in epoxy resin. Thin sections were stained with 2.5% aqueous uranyl acetate and Reynold's saturated lead citrate solution (Reynold, 1963).

Ninety-six individual cells within each treatment were scored by TEM as either positive or negative for 9 morphological criteria indicative of apoptosis (chromatin aggregation, convoluted nuclear outline, perinuclear dilatation, apoptotic bodies, cytoplasmic condensation, cytoplasmic protrusion, cytoplasmic budding, lucent cytoplasmic vacuoles, and lack of organelles) and 7 morphological criteria indicative of oncosis (loosely marginated chromatin, pyknosis, karyolysis, nuclear membrane rupture,

swollen mitochondria/rupture of mitochondrial membranes, dilated endoplasmic reticulum, and plasma membrane lysis) (Wyllie *et al*, 1980). A cell scored as positive for 3 or more morphological criteria for either apoptosis or oncosis was recorded as apoptotic or oncotic, respectively.

DNA Extraction and Fragment Analysis by Gel Electrophoresis or DPA

DNA was extracted from treated neutrophils using a DNA Extraction Kit (Stratagene) which utilizes a modified salt precipitation method (5 x 10^7 cells/sample, diluted to 45 ml after incubation). After initial precipitation of DNA in ethanol, the sample was centrifuged at 10,000 x g for 20 minutes at 4°C, washed with 100% ethanol, centrifuged again, washed with 70 % ethanol, centrifuged, and decanted. The pellet was then resuspended in TBE, subjected to gel electrophoresis on a 1% agarose gel at 50V for 7 hours, and stained with 0.1% ethidium bromide for 15 minutes (Sun *et al*, In Press).

DPA analysis of DNA fragmentation involves initial centrifugation to separate DNA that has extensively fragmented from large, chromosome-length DNA followed by colorimetric assay of deoxyribose fragments in a method previously described (Sun *et al*, In Press). Briefly, after exposure to stimulators, neutrophils were diluted to 5 x 10^6 cells/ml and 0.5 ml was pipetted into a 1.5-ml microcentrifuge tube. Cells were then centrifuged for 10 minutes at 200 x g and the supernatant was removed and labeled S. The pellet was then resuspended in 0.5 ml of TTE and centrifuged at 13,000 x g for 10 minutes to separate intact chromatin (supernatant) from fragmented chromatin (pellet). The supernatant (labeled T) was removed and 0.5 ml of TTE was added to the pellet (labeled B). All fractions were then incubated with 0.5 ml of TCA overnight at 4°C. Thereafter, precipitated DNA was pelleted by microcentrifugation at 13,000 x g for 10 minutes and supernatants were discarded. Pellets were then hydrolysed by adding 80 µl of 5% TCA to

each sample and heating in a 90°C heating block for 15 minutes. One hundred-sixty µl of DPA was then added to each microcentrifuge tube and allowed to develop for 4 hours at 37°C. Colored solution from each tube was then transfered to a 96-well flat-bottomed ELISA plate and optical density was read at 600 nm in a thermally-controlled kinetic microtiter plate reader (Thermomax, Molecular Devices, Palo Alto, CA). Percent fragmented DNA was then calculated using the formula:

$$\% \text{ Fragmented DNA} = \frac{(S + B) \times 100}{S + T + B}$$

Statistical Analyses

Data were analyzed using SYSTAT intelligent software for the Macintosh, Version 5.2 (SYSTAT Inc, Evanston, IL) (Wilkinson, 1992). The effects of various stimulators on DNA fragmentation, as measured by DPA analysis, were compared using the general linear model followed by comparison of mean pairs using Scheffe's test. Differences between means were considered significant at the $P < 0.05$ level among quadruplicate samples within a single plate.

Results

Examination by TEM showed that bovine neutrophils exposed to lytic concentrations of LKT (1:50) for 5 minutes contained changes consistent with oncosis, such as swollen mitochondria, lysis of plasma membrane, loose margination of chromatin, and dilated endoplasmic reticulum (Fig. 6). In contrast, neutrophils exposed to sublytic dilutions of LKT (1:2,500) for 5 minutes contained changes consistent with early

apoptosis, such as margination of chromatin, lucent vacuoles, convoluted nuclear membrane, and perinuclear dilation (Fig. 7) (Wyllie *et al*, 1980). The apoptotic changes seen in neutrophils exposed to sublytic doses of LKT were similar to those observed in neutrophils exposed to the apoptosis inducer, staurosporin (Fig. 8). Neutrophils exposed to either PBS or LKT (-) (1:50 or 1:2,500) did not contain changes associated with either necrosis or apoptosis. Normal morphological features of these controls included normal membrane ruffling, cytoplasm of normal density, dense and unactivated vacuoles, undilated mitochondria, and normal endoplasmic reticulum (Fig. 9).

Neutrophils exposed to lytic concentrations of LKT incubated for 120 minutes had changes consistent with late oncosis, including nuclei undergoing karyolysis, nuclear membrane rupture, swollen mitochondia, dilated endoplasmic reticulum, and lysis of plasma membranes (Fig. 10). In contrast, neutrophils exposed to sublytic concentrations of LKT or staurosporin for 120 minutes had changes consistent with late apoptosis, such as margination of chromatin, convoluted nuclear membrane, perinuclear dilitation, apoptotic bodies, convoluted plasma membranes, and lucent vacuoles (Figs. 11 & 12). Neutrophils exposed to either PBS or LKT (-) (1:50 or 1:2,500) for 120 minutes were normal in structure and had none of the changes charateristic of apoptosis or oncosis (Fig. 13).

Examination of the temporal relationship between apoptotic or oncotic changes and LKT concentration revealed that oncosis was evident in >51% of cells exposed to a lytic concentration of LKT after only 5 minutes of incubation. However, changes associated with apoptosis were visible in only 9.4% and 3.1% of cells incubated for 5 minutes with a sublytic concentration of LKT and staurosporin, respectively, while no oncotic changes were observed. After 15 minutes of incubation, 37.5% and 44.8% of cells exposed to a sublytic concentration of LKT and staurosporin, respectively, were apoptotic (Fig. 14).

The classic confirmatory test for the presence of apoptosis is evidence of DNA fragmentation in the form of a distinctive 200 base-pair ladder on agarose gel

electophoresis. This technique showed fragmentation of DNA in bovine neutrophils exposed to a sublytic concentration of LKT (1:2,500), A23187, or the apoptosis inducer, staurosporin, but not in neutrophils exposed to lytic concentrations of LKT (1:50), LKT (-) (1:50 and 1:2,500), or PBS (Fig. 15).

An additional test for DNA fragmentation is the DPA staining of deoxyribose in samples of neutrophils in which intact, chromosomal length DNA has been separated from fragmented DNA by centrifugation. Results from DPA analysis of neutrophils exposed to LKT (1:50 and 1:2,500), LKT (-) (1:50 and 1:2,500), PBS, staurosporin, and A23187 revealed that sublytic concentrations of LKT (1:2,500), staurosporin, and A23187 induced DNA fragmentation after 240 minutes of incubation.

The calcium ionophore, A23187, induced apoptosis in neutrophils, as demonstrated by DNA fragment analysis, suggesting that increased $[Ca^{2+}]_i$ was involved in the induction of apoptosis. The role of calcium in apoptosis was further investigated by exposing neutrophils to stimulators in the presence or absence of extracellular Ca^{2+}. Although removal of extracellular Ca^{2+} caused a significant decrease in A23187-induced apoptosis, apoptosis was still evident in these Ca^{2+}-free samples. Furthermore, replacement of Ca^{2+} in the buffer with the Ca^{2+} chelator, EGTA, did not affect apoptosis induced by sublytic concentrations of LKT or staurosporin (Fig. 16).

Discussion

It has been well established that neutrophil-mediated inflammation in BRD contributes to the development of severe disease and not to the effective clearing of invading *P. haemolytica* (Yates *et al*, 1982; Slocombe *et al*, 1985, Wilkie and Shewen, 1988; Breider *et al*, 1988). One of the virulence factors of *P. haemolytica* responsible for the impaired function of neutrophils is LKT (Shewen and Wilkie, 1985; Confer *et al*,

1995). Many studies have investigated the mechanisms whereby LKT impairs the function of neutrophils and it has been determined that LKT can induce many effects in bovine neutrophils in a concentration-dependent manner. At high concentrations, LKT has been shown to be cytolytic for bovine neutrophils while at low concentrations LKT induces neutrophil activation and oxidative burst (Clinkenbeard *et al*, 1989; Czuprynski *et al*, 1990). While it is easy to understand how neutrophil lysis leads to impairment of neutrophil function, it is more difficult to understand how activation by low concentration LKT can compromise the host defense capacity of neutrophils. Recently, evidence of apoptosis in bovine neutrophils exposed to low concentrations of LKT has been reported (Stevens and Czuprynski, 1996). This induction of cell death by apoptosis would account for impairment of neutrophil functions at low concentrations, explaining why neutrophils are not effective at clearing *P. haemolytica,* regardless of LKT concentration. Using definitive analytical methods, the present study confirmed that low concentrations of LKT induce apoptosis in bovine neutrophils and that apoptosis occurs more slowly than oncosis, which is a consequence of exposure to high concentrations of LKT.

Apoptosis is known to be mediated by Ca^{2+} in various systems (Trump and Berezesky, 1995; Dowd, 1995). The most common biochemical feature of apoptosis is endonuclease activation, which degrades DNA into the distinctive domain-sized DNA fragments to yield DNA ladders upon gel electrophoresis (McConkey and Orrenius, 1996; Eastman, 1996). Considering that there are many Ca^{2+} dependent endonucleases that have been implicated in apoptosis and that LKT has been shown to increase $[Ca^{2+}]_i$ in neutrophils exposed to both high and low concentrations of LKT (Clinkenbeard *et al*, 1994; Ortiz-Carranza and Czuprynski, 1992; Hsuan *et al*, 1998), it was considered possible that increased $[Ca^{2+}]_i$ may be involved in apoptotic death of bovine neutrophils exposed to LKT. The results of the present study indicated that LKT and the calcium ionophore A23187 induce DNA fragmentation in the absence of extracellular Ca^{2+}, which

implies that Ca^{2+} is not an essential requirement for LKT-induced apoptosis. However, the results also illustrate that a higher percentage of apoptosis is induced by A23187 or LKT in the presence of Ca^{2+}, indicating that DNA fragmentation is promoted by Ca^{2+}. This suggests that LKT-induced apoptosis is controlled by signal transduction pathways that do not require, but are influenced by, increased $[Ca^{2+}]_i$.

The signal transduction pathways involved in apoptosis regulation appear to vary depending upon the stimulus and cell system. Regulation by Ca^{2+}, protein kinase C, cAMP, ceramide, protein tyrosine kinases, cyclin-dependent kinases, intracellular acidification, and oxygen radicals have all been indicated (McConkey and Orrenius, 1996). The difference in the Ca^{2+} dependency observed in the present experiment between LKT-, staurosporin- and A23187-induced apoptosis suggests that LKT may induce apoptosis via differing cell surface receptors that activate different signaling cascades, which later diverge upon a common caspase (Thornberry *et al*, 1992; Enari *et al*, 1995). The onset of apoptosis associated with low concentrations of LKT was relatively rapid, suggesting the possible activation of the Fas/APO-1 (apoptosis activating protein 1) receptor mediated process of apoptosis. The Fas/APO-1 receptor activated process of apoptosis bypasses the long sequence of enzymes associated with tumor necrosis factor receptor 1 (TNFR1) to immediately activate preexisting caspases (Thornberry *et al*, 1992; Enari *et al*, 1995).

In conclusion, our results indicate that low concentrations of LKT induce apoptosis while high concentrations of LKT induce oncosis in bovine neutrophils, both contributing to an ineffective host defense response. Furthermore, the results indicate that LKT-induced apotosis in bovine neutrophils may be influenced by increased $[Ca^{2+}]_i$ although Ca^{2+} does not act as an essential signal transducer for LKT-induced apoptosis as has been previously demonstrated with LKT-induced oncosis.

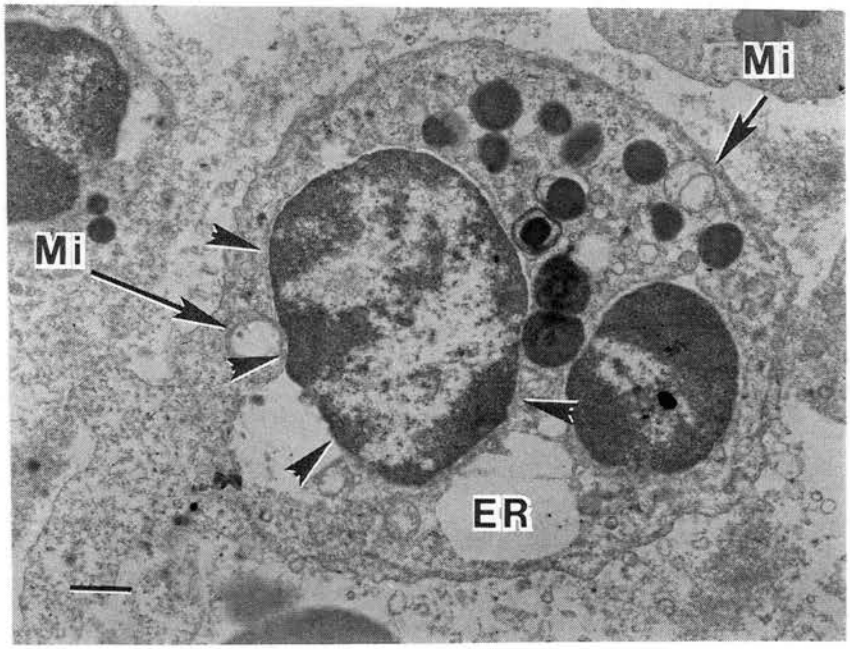


Figure 6. A transmission electron micrograph of a bovine neutrophil exposed to a lytic dose of LKT (1:50) for 5 minutes. Note classic changes associated with oncosis such as swollen mitochondria (Mi), loose margination of chromatin (arrowheads), and dilated endoplasmic reticulum (ER). Bar = 10 μm.

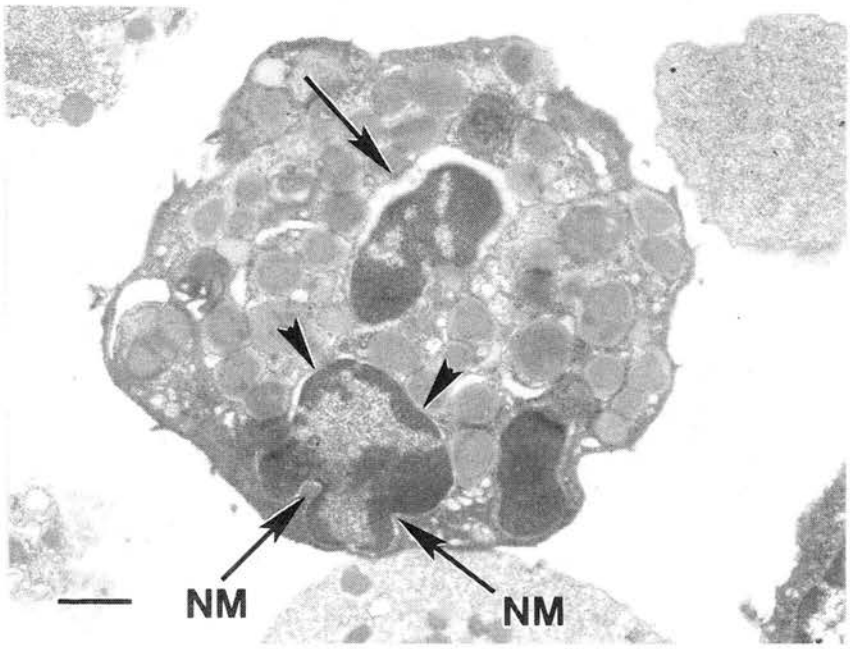


Figure 7. A transmission electron micrograph of neutrophils exposed to sublytic dilution of LKT (1:2,500) for 5 minutes. Note the early apoptotic changes such as margination of chromatin (arrowheads), convoluted nuclear membrane (NM), and perinuclear dilation (arrow). The apoptotic changes seen in neutrophils exposed to sublytic doses of LKT correspond to similar changes seen neutrophils exposed to the apoptosis inducer staurosporin. Bar = 10 μm.

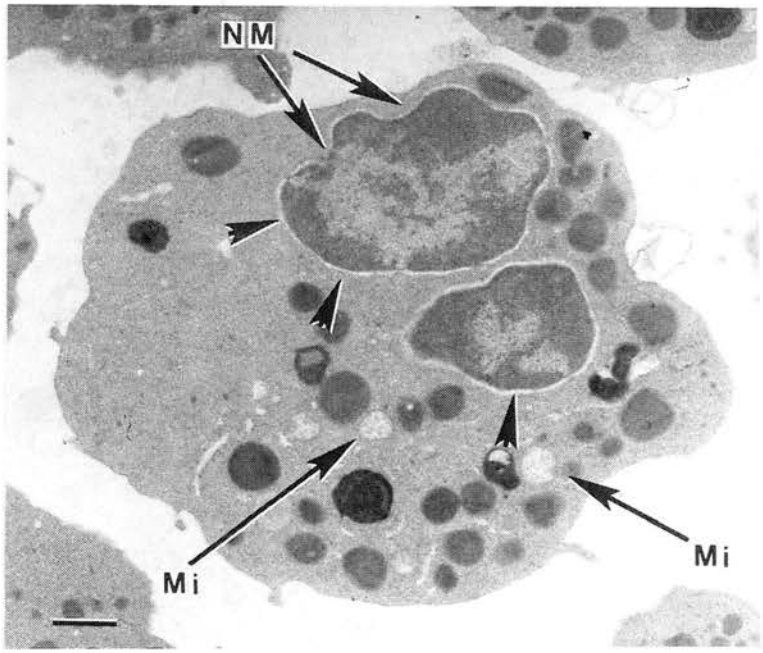


Figure 8. A transmission electron micrograph of a neutrophil exposed to stauroporin (1mM) for 5 minutes. Note the early apoptotic changes such as margination of chromatin (arrowheads), convoluted nuclear membrane (NM), and slightly swollen mitochondria (Mi). Bar = 10 μm.

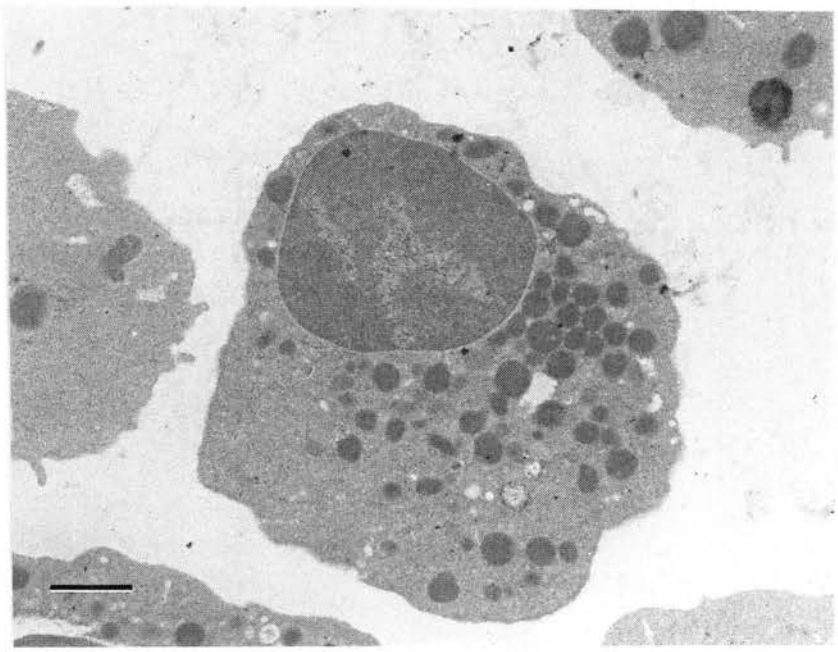


Figure 9. A transmission electron micrograph of a bovine neutrophil exposed to PBS for 5 minutes. Neutrophils exposed to either PBS, LKT (-) (1:50 or 1:2,500) all had normal structure. Bar = 10 μm.

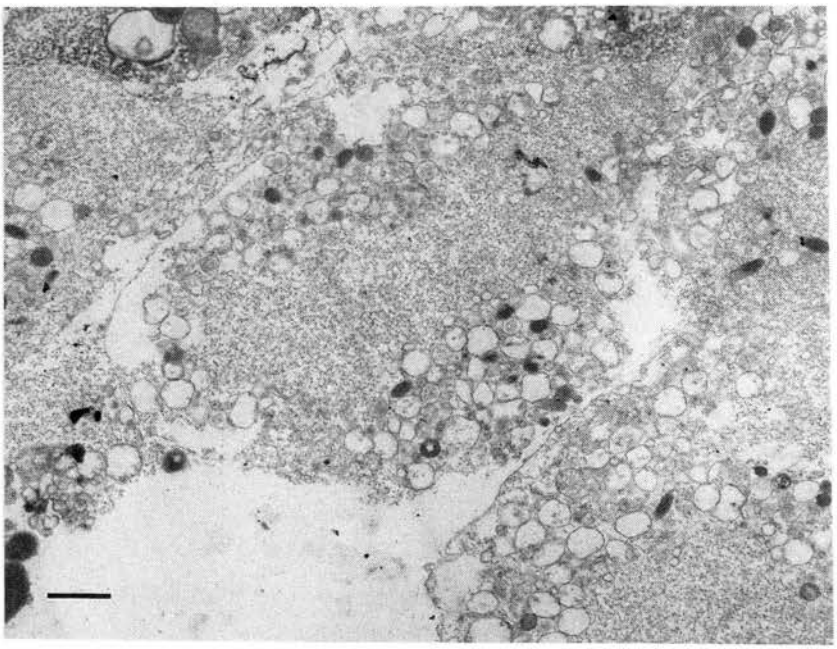


Figure 10. A transmission electron micrograph of a bovine neutophil exposed to LKT (1:50) for 120 minutes. These preparations contained mostly ruptured membranes and dilated/ruptured organelles. Bar = 10 μm.

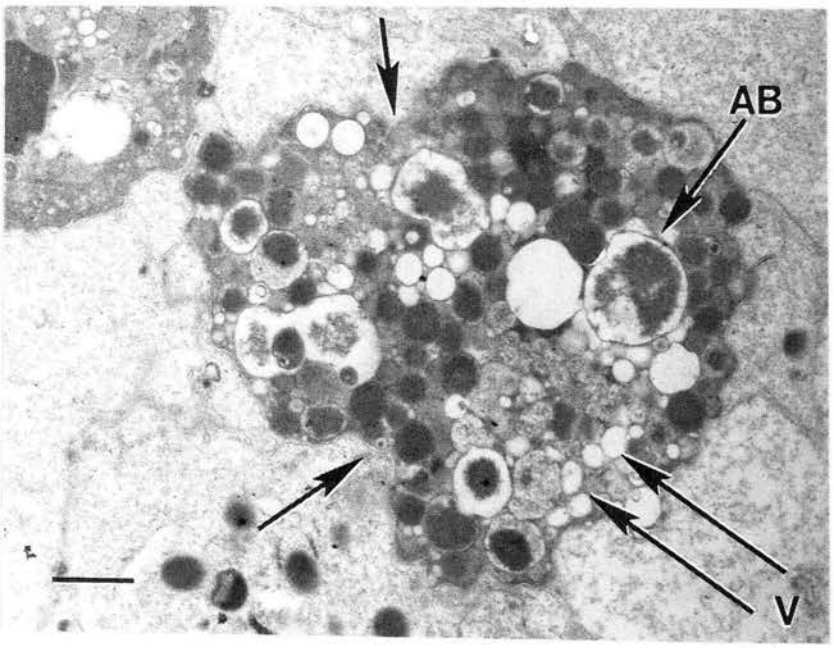


Figure 11. Transmission electron micrograph of a bovine neutrophil exposed to LKT (1:2,500) for 120 minutes. Note the late apoptotic features of apoptotic bodies (AB), convoluted plasma membranes (arrows), and lucent vacuoles (V). The changes seen in neutrophils exposed to sublytic doses of LKT for 120 minutes were consistant with changes seen in neutrophils exposed to staurosporin for 120 minutes. Bar = 10 μm.

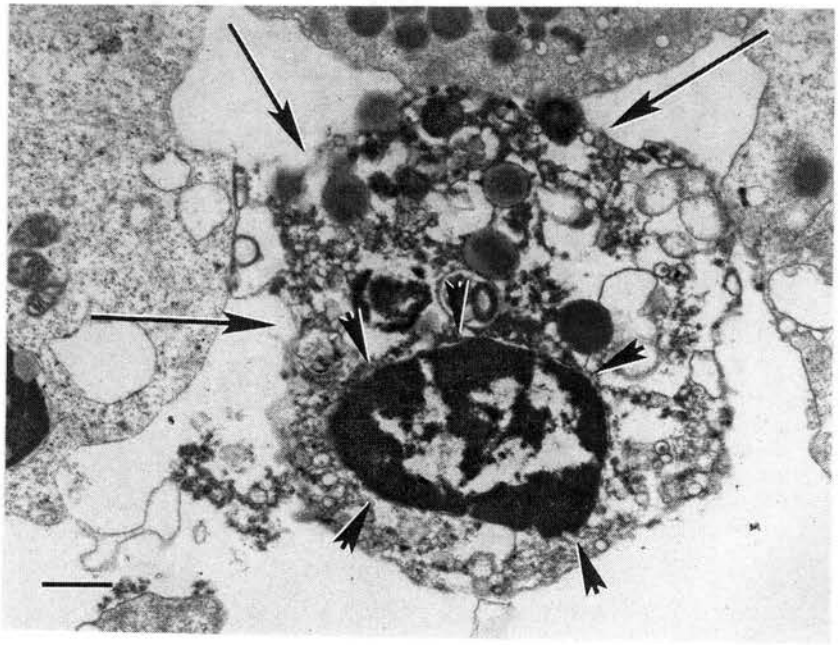


Figure 12. A transmission electron micrograph of a bovine neutrophil exposed to staurosporin (1mM) for 120 minutes. Note the presence of changes associated with late apoptosis such as margination of chromatin (arrowheads) and convoluted plasma membranes (arrows). Bar = 10 μm.

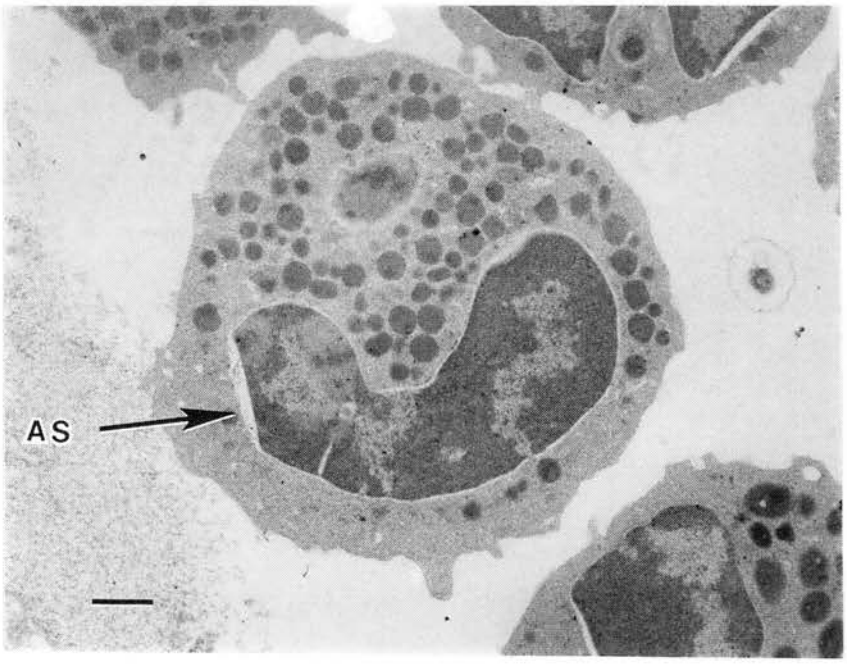
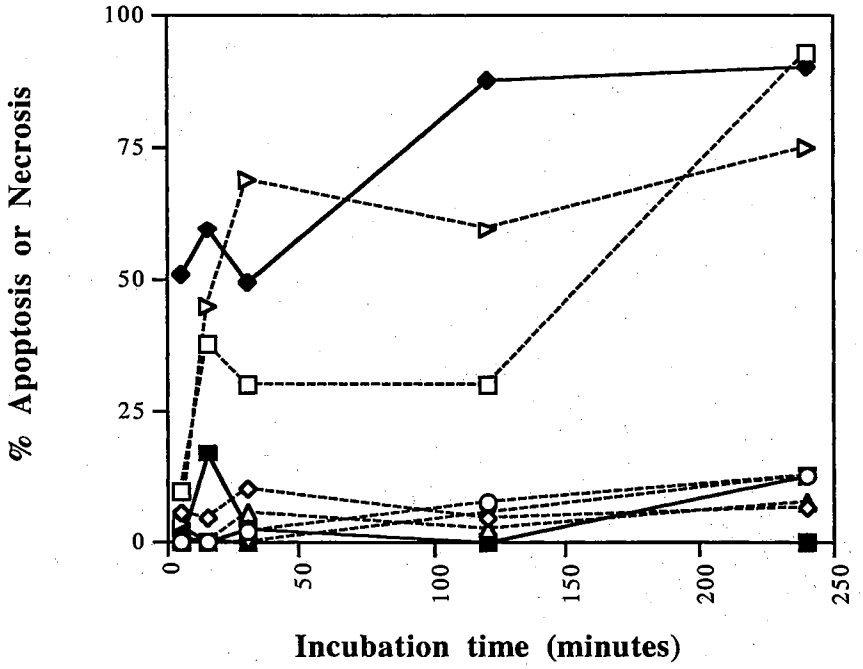


Figure 13. A transmission electron micrograph of a bovine neutrophil exposed to PBS for 120 minutes. Note the normal structure and absence of changes consistent with apoptosis or necrosis. The presence of artifactual swelling in the perinuclear space (AS) is visible in this figure. Neutrophils exposed to either PBS or LKT (-) (1:50 and 1:2,500) for 120 minutes were similar in morphological features. Bar = 10 μm.



Apoptosis	Necrosis
LKT 1:2500	LKT 1:2500
LKT 1:50	LKT 1:50
LKT (-) 1:2500	LKT (-) 1:2500
LKT (-) 1:50	LKT (-) 1:50
PBS	PBS
Staurosporin 1mM	Staurosporin

Figure 14- Bovine neutrophils were exposed to LKT (1:50 or 1:2500), LKT (-) (1:50 or 1:2500), PBS, or staurosporin 1mM for the indicated times before fixation and TEM was performed. Ninety-six individual cells within each time and treatment were then morphologically analyzed for apoptotic (open symbols) or oncotic changes (closed symbols) and reported as a percentage.

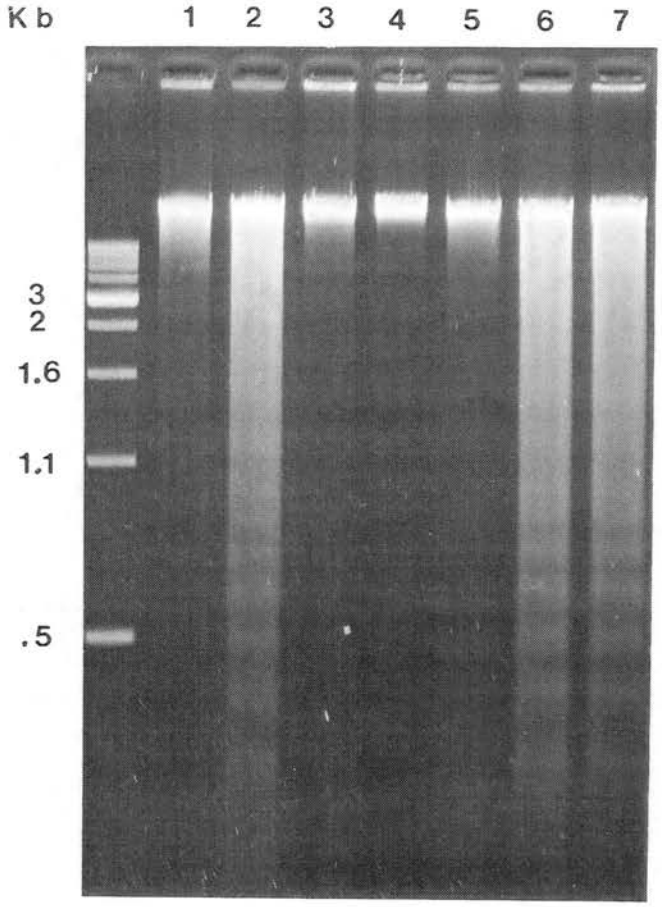


Figure 15. Bovine neutrophils treated with either LKT (lytic 1:50) (lane 1), LKT (sublytic 1:2,500 dose) (lane 2), LKT (-) (1:50) (lane 3), LKT (-) (1:2,500 dose) (lane 4), PBS (lane 5), A23187 40 μm (lane 6), or staurosporin 1mM (lane 7) prior to DNA extraction. 2μg of isolated DNA was then analysed by 1% agarose gel electrophoresis to reveal DNA fragment ladders in LKT (1:2,500), A23187, and staurosporin treatments.

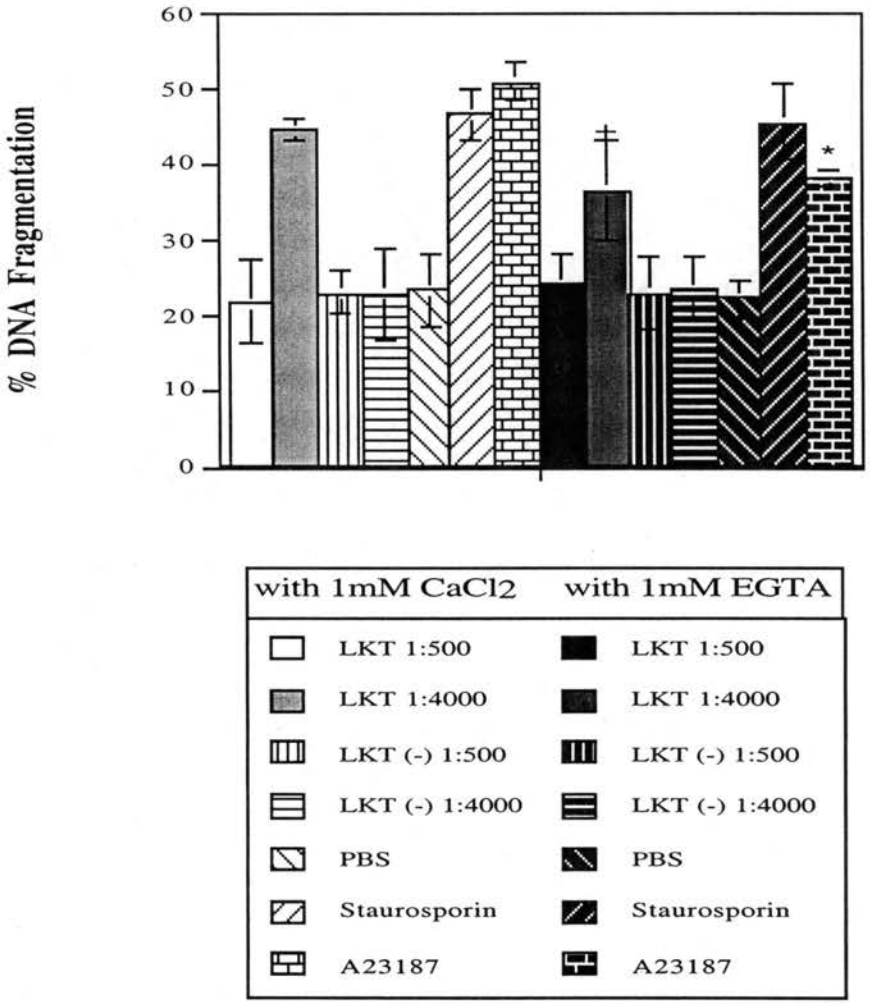


Figure 16- Bovine neutrophils exposed to LKT (1:50 or 1:2,500 dilutions), LKT(-)(1:50 or 1:2,500 dilutions), PBS, Staurosporin 1mM, or A23187 49 μM either in the presence of 1mM $CaCl_2$ (white background) or 1mM EGTA (black background) for 240 minutes prior to DNA fragment analysis by DPA assay. * indicates a significant difference among quadruplicate sample tubes ($p<.05$) and + indicates marginal significance ($P<.10$) between 1mM $CaCl_2$ and 1mM EGTA groups.

CHAPTER VI

PASTEURELLA HAEMOLYTICA LEUKOTOXIN-INDUCED INCREASE IN LEUKOTRIENE B_4 PRODUCTION BY BOVINE NEUTROPHILS IS MEDIATED BY SUSTAINED AND UNREGULATED INCREASE IN INTRACELLULAR CALCIUM CONCENTRATION

Introduction

Pneumonic pasteurellosis is a disease of feedlot and stocker cattle caused by *P. haemolytica* and is characterized by acute fibrinopurulent pneumonia and massive accumulation of neutrophils (Allen *et al*, 1985). Infiltrating neutrophils do not provide an effective host defense response, but undergo degranulation and lysis, releasing lysosomal enzymes and reactive oxygen products that aggravate tissue damage (Yates, 1982; Slocombe *et al*, 1985; Breider *et al*, 1988). Eicosanoids, including LTB_4, are considered to be important chemotactic agents responsible for influx of neutrophils into infected lungs (Clarke *et al*, 1994). LKT, a protein exotoxin produced by log-phase *P. haemolytica*, stimulates the release of LTB_4 and other 5-lipoxygenase products from bovine neutrophils (Welch, 1991; Henricks *et al*, 1992; Clinkenbeard *et al*, 1994). This toxin is a member of the family of RTX pore-forming cytolysins, which are characterized by tandemly arranged repeats of a nine amino acid sequence (Welch, 1991). At sublytic concentrations, LKT is a potent neutrophil activating agent that stimulates LTB_4 production (Henricks *et al*, 1992; Czuprynski *et al*, 1991; Clinkenbeard *et al*, 1994). However, at lytic concentrations, LKT causes severe membrane defects and neutrophil lysis, impairing neutrophil phagocytosis (Clinkenbeard *et al*, 1989; Clinkenbeard *et al*, 1994). Therefore, LKT

contributes to the chemoattraction of neutrophils into sites of *P. haemolytica* infection as well as the impairment of neutrophil-mediated host defenses.

Previous experiments in chapter 4 have demonstrated that increased $[Ca^{2+}]_i$ precedes membrane damage and release of LTB_4 in bovine neutrophils exposed to LKT. Considering the role of Ca^{2+} in the activation of enzymes responsible for the release of AA and subsequent oxidation of AA to leukotrienes, intracellular Ca^{2+} apparently serves as an second messenger in LKT-mediated inflammatory responses (Mayer and Marshall, 1993; Musser and Kreft, 1992). Ca^{2+} is known to be a important second messenger for many normal physiological functions of neutrophils; the mechanisms of Ca^{2+} increase, the sources of Ca^{2+}, and the enzymes regulated by increased $[Ca^{2+}]_i$ vary according to the specific stimulus (Dore *et al*, 1990; Dore *et al*, 1992; Rosles and Brown, 1992), but usually these physiological responses are narrowly regulated with regard to the magnitude and duration of $[Ca^{2+}]_i$ increase. The hypothesis developed to explain the role of $[Ca^{2+}]_i$ in the pathogenic responses of neutrophils to LKT is that LKT activates neutrophils by causing sustained and unregulated increase in $[Ca^{2+}]_i$, which results in uncontrolled synthesis of inflammatory mediators.

Therefore, the objectives of this study were: 1) to compare the effects of LKT on $[Ca^{2+}]_i$, LTB_4 production and cell membrane damage with those caused by other pharmacological and physiological stimulators of neutrophil function; and 2) to confirm that excessive increases in $[Ca^{2+}]_i$ are necessary for uncontrolled synthesis of inflammatory mediators.

Materials and Methods

Preparation of *P. haemolytica* Leukotoxin

LKT and LKT(-) were prepared from culture supernatants of *P. haemolytica* biotype A, serotype 1 wildtype strain and a leukotoxin-deficient mutant strain A, as described previously in Chapter IV. Leukotoxin activity was quantified as toxic units (TU) using BL3 cells, as described previously (Clinkenbeard *et al*, 1989). One TU caused 50% LDH leakage from 4 x 10^5 BL3 cells in 200 µl at 37°C in 1 hour. Each assay was conducted in triplicate and the TU were determined for each experiment. In this experiment, lytic doses of LKT caused >50% lysis of 2.5 x 10^6 neutrophils in 250 µl buffer after 2 hours incubation while sublytic doses of LKT caused <10% lysis from similarly diluted and incubated neutrophils.

Preparation of Neutrophil Suspensions

Two healthy beef calves (200 ± 50 kg) served as blood donors for isolation of neutrophils. Neutrophils were isolated by hypertonic lysis as described previously in Chapter IV, and suspended in 5 ml of Ca^{2+}- and Mg^{2+}-free HBSS. Cells were enumerated by hemocytometer and viability was assessed by trypan blue exclusion. Differential counts were conducted on stained smears. Preparations were then diluted to a final concentration of 6 x 10^6 viable neutrophils/ml HBSS.

Loading of Neutrophils with Fluorescent Calcium Indicator and Intracellular Calcium Concentration Determination

Cells were loaded with Fluo-3 AM and $[Ca^{2+}]_i$ was measured as previously described in Chapter IV. Thereafter, loaded cells were resuspended in 3 ml HBSS, enumerated by hemocytometer, and then resuspended in HBSS to 1 x 10^7 cells/ml.

Exposure of Isolated Neutrophils to Stimulators

The effects of LKT on $[Ca^{2+}]_i$, neutrophil membrane integrity, and LTB_4 synthesis were compared with those of other physiological and pharmacological stimulators by exposing neutrophil suspensions to dilutions (1:10, 1:1,000) of LKT, LKT(-) (1:10), 4-bromo A23187 (4Br-A23187) (0.1 mM), epinephrine (1 μM), phorbol myristate acetate (PMA) (10 μg/ml), recombinant human complement C_{5a} (rhC_{5a}; 0.1 μM), recombinant bovine interferon gamma ($rBoINF_\gamma$; 55 u/ml), and PBS in 96-well flat bottom microtiter plates. Measurements of $[Ca^{2+}]_i$, LDH release, and LTB_4 synthesis were conducted in parallel using one plate of Fluo-3 - loaded neutrophils and five plates of neutrophils not loaded with Fluo-3. Ten microliters of 25 mM $CaCl_2$ and 2 μl antifluoroscein antibody diluted 1:5 in PBS were added in sequence to 250 μl of cell suspension. Thereafter, the individual stimulators were added to respective wells, lids were placed on the plates, and the plates were incubated at 37°C. Fluorescence of Fluo-3 - loaded cells (490 nm excitation and 523 emission) was measured before addition of each stimulator and at 1, 5, 10, 15, 30, 45, 60, 75, 90, and 120 minutes thereafter, using a spectrofluorometer. Plates with neutrophils not loaded with Fluo-3 were centrifuged and supernatant collected for LDH or LTB_4 measurements before addition of each stimulator or at 15, 45, 75, or 120

minutes thereafter. All experiments included triplicate wells for each of the treatments within a single plate.

The relevance of duration of $[Ca^{2+}]_i$ increase to production of LTB_4 was tested by artificially terminating A23187 or LKT-induced $[Ca^{2+}]_i$ increase using extracellular EGTA. Ten microliters of 25 mM $CaCl_2$ and 2 µl antifluorescein antibody were added in sequence to Fluo-3 - loaded bovine neutrophils (250 µl) in 96-well plates. Intracellular Ca^{2+} concentration was then manipulated by adding 100 µl 2.5 mM EGTA at 5 minutes before or 2, 10, 30, or 60 minutes after stimulation of neutrophils with either 10 µl 1 mM 4Br-A23187 or 25 µl LKT (1:100). Fluorescence was measured at regular intervals during the course of the experiment. Release of LTB_4 was measured at the conclusion of the experiment.

The relationship between magnitude the of $[Ca^{2+}]_i$ increase and synthesis of LTB_4 was tested by exposing neutrophils to stimulators in a range of extracellular Ca^{2+} concentration, thus allowing different maxima of $[Ca^{2+}]_i$ to be achieved. Fluo-3 - loaded bovine neutrophils were suspended in 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mM Ca-EGTA with 0, .017, .038, .065, .100, .150, .225, .351, .602, 1.35, or 39.8 µM of free calcium concentration respectively (Molecular Probes Inc., Eugene, OR). Antifluorescein antibody (2 µl) was added to 250 µl of cell suspension, followed by 10 µl 1 mM 4Br-A23187 or 25 µl of LKT (1:10). Fluorescence was measured before stimulation and at 2, 5, 10, 20, 30, 40, 50 and 60 minutes thereafter. Production of LTB_4 was measured at 60 minutes.

The possibility that leakage of Fluo-3 and extracellular quenching of the indicator by antifluorescein antibody affected the estimation of time-dependent changes in $[Ca^{2+}]_i$ was tested by exposing 250 µl aliquots of Fluo-3 - loaded neutrophil suspensions (containing 1 mM $CaCl_2$) to A23187 or LKT (1:10 or 1:1,000 dilutions) in the absence of antifluorescein antibody, after addition of antibody, or prior to addition of antibody 50

minutes after exposure to the stimulator. Fluorescence intensity was recorded before initial exposure to stimulators and at frequent intervals thereafter.

The ability of LKT-exposed neutrophils to respond to repeated exposure of stimulators was investigated by exposing 250 µl aliquots of Fluo-3 - loaded neutrophil suspensions, containing 1 mM $CaCl_2$ and 2 µl antifluorescein antibody, to LKT (1:10 or 1:1000 dilutions) for 30 minutes and then stimulating them again by further addition of LKT (1:10 or 1:1,000 dilutions), LKT(-) (1:10), 4Br-A23187, or DMSO (vehicle control for 4Br-A23187).

Estimation of Plasma Membrane Damage

The effect of *P. haemolytica* LKT on neutrophil plasma membrane integrity was assayed by measuring extracellular release of cytoplasmic LDH, as previously described in Chapter IV.

Leukotriene B_4 Immunoassay

Concentrations of LTB_4 were determined using a commercially prepared radioimmunoassay kit that has been previously validated (Clinkenbeard *et al*, 1994).

Statistical Analyses

Data were analyzed using SYSTAT intelligent software for the Macintosh, Version 5.2 (Wilkinson, 1992). Time-dependent effects of LKT and other stimulators on $[Ca^{2+}]_i$ were analyzed by the general linear model followed by comparison between pre-stimulation values and each of the post-stimulation values using Dunnet's test. The effects of different

stimulators at selected sampling times, the effects of artificial manipulation of $[Ca^{2+}]_i$ on LTB_4 production, and the effects of antifluorescein antibody on stimulator-induced change in $[Ca^{2+}]_i$ were analyzed using the general linear model followed by mean separation using Scheffe's test. Differences between means were considered significant at the $P < 0.05$ level.

Results

Exposure of bovine neutrophils to lytic dilutions of LKT caused an initial dramatic increase in $[Ca^{2+}]_i$, followed by a slower decline, although a higher than normal $[Ca^{2+}]_i$ was maintained for the duration of the experiment (Fig. 17) A23187 or PMA caused similar sustained increases in $[Ca^{2+}]_i$, but maximum concentrations were achieved more slowly and did not decline thereafter (Fig. 17). Marginally lytic dilutions of LKT (< 20% specific LDH release), A23187, and PMA all stimulated LTB_4 synthesis (Fig. 18), which had increased substantially by 15 minutes. In contrast, rhC_{5a} caused a small brief increase in $[Ca^{2+}]_i$ that failed to stimulate synthesis of LTB_4. No changes in neutrophil responses were recorded after exposure to PBS, LKT (-), epinephrine, or $rBoINF_\gamma$ (data not shown).

Addition of EGTA to neutrophil suspensions at various times before or after exposure to LKT or A23187 caused dramatic decreases in $[Ca^{2+}]_i$ (Figs. 19 and 20). The magnitude and duration of stimulator-induced increase in $[Ca^{2+}]_i$ were increased when longer incubation periods were allowed to elapse before addition of EGTA. The magnitude and duration of $[Ca^{2+}]_i$ increase were directly proportional to the amount of LTB_4 produced by neutrophils (Figs. 19 and 20).

The Ca^{2+} concentration of buffers in which neutrophils were suspended was directly proportional to LKT- and A23187-induced increase in $[Ca^{2+}]_i$ and subsequent production of LTB_4 (Figs. 21, 22, 23, and 24). Close examination of the magnitudes of $[Ca^{2+}]_i$ increase and LTB_4 production indicated the existence of two relative degrees of response; high and low, thus suggesting that a threshold of increased $[Ca^{2+}]_i$ must be exceeded before significant production of LTB_4 occurred (Figs. 21, 22, 23, and 24).

Experiments conducted in the presence or absence of antifluorescein antibody (Fig.25) indicated that the decline in $[Ca^{2+}]_i$ after initial exposure to lytic dilutions of LKT may not be completely explained by a loss of plasma membrane integrity, leakage of the 1.1 kD Fluo-3, and quenching of fluorescence by antifluorescein antibody. Furthermore, once $[Ca^{2+}]_i$ had declined after initial exposure to LKT, neutrophils were still capable of mounting a $[Ca^{2+}]_i$ response to A23187. These results suggest that the decline in $[Ca^{2+}]_i$ was not completely due to cell lysis, although lowering of relative $[Ca^{2+}]_i$ through cell swelling and lysis is possible . The increase in $[Ca^{2+}]_i$ in cells previously stimulated with LKT further indicated that $[Ca^{2+}]_i$ is not at equilibrium with external cell Ca^{2+} as one would expect with pore formation or calcium ionophores. However, addition of more LKT did not result in an additional response from the cell as did the addition of more calcium ionophore (Figs. 26 and 27).

Discussion

Previous experiments have demonstrated that exposure of bovine neutrophils to LKT caused an increase in $[Ca^{2+}]_i$, which was directly correlated with LTB_4 synthesis and plasma membrane damage, thus indicating that changes in $[Ca^{2+}]_i$ constitute an important

stage in the signal transduction process responsible for LKT-induced neutrophil responses. The present study investigated whether the pattern of changes in $[Ca^{2+}]_i$ resulting from exposure to LKT could be distinguished from those caused by other physiological or pharmacological stimulators of bovine neutrophils and whether the pathological effects of LKT on neutrophils are mediated by unregulated and excessive increase in $[Ca^{2+}]_i$.

Comparison of the effects of different stimulators on bovine neutrophils as a function of time demonstrated differences in $[Ca^{2+}]_i$ increase patterns that were dependent on the specific stimulator. Exposure to rhC_{5a} resulted in a significant increase in $[Ca^{2+}]_i$, in a manner consistent with the earlier observations of Dore *et al* (1990). However, in contrast to LKT, A23187, and PMA, the rhC_{5a}-induced $[Ca^{2+}]_i$ increase was more rapid in onset, had a shorter duration, and achieved a lower maximum concentration. This relatively brief and low peak in $[Ca^{2+}]_i$ was not associated with any significant loss of membrane integrity or production of LTB_4, in contrast to the other stimulators, which all caused significant LTB_4 synthesis. Assuming that rhC_{5a}-induced $[Ca^{2+}]_i$ increase represents an activation stimulus that promotes normal defensive functions of neutrophils, it is clear that the ability of LKT, A23187, and PMA to induce LTB_4 synthesis is related to the magnitude and duration of $[Ca^{2+}]_i$ increase caused by these stimulators and that these effects on $[Ca^{2+}]_i$ repesent excessive and pathological responses. The signal transduction pathways that mediate stimulation of human neutrophils by rhC_{5a} apparently involve G_i-proteins. Although recent studies by other researchers have implicated G_i-proteins in LKT-induced $[Ca^{2+}]_i$ increase, the marked difference between rhC_{5a}- and LKT-induced $[Ca^{2+}]_i$ increases observed in the present study indicate that mechanisms other than or in addition to those involving G_i-proteins must be sought to fully explain the action of LKT.

When bovine neutrophils were exposed to the protein kinase C inducer, PMA, or the calcium ionophore, A23187, the resulting increases in $[Ca^{2+}]_i$ were slower in onset and of longer duration than that caused by LKT. The differences in changes in $[Ca^{2+}]_i$ caused by LKT, A23187, and PMA again suggest that the mechanism of LKT-induced $[Ca^{2+}]_i$ increase cannot fully be explained by activation of protein kinase or diffusion of Ca^{2+} across the plasma membrane, as is caused by A23187.

Although neutrophils are reported to have β-adrenoreceptors (Galant and Allred, 1980), the present study failed to demonstrate any effect of epinephrine on $[Ca^{2+}]_i$ or LTB_4 production. These results contradict those of another study in which inhibitory effects of propranalol, a β-adrenoreceptor antagonist, on LKT-induced effects on bovine neutrophils were observed (Ortiz-Carranza and Czuprynski, 1992). Likewise, despite evidence that rBoIFN-γ is capable of activating bovine neutrophils (Roth and Frank, 1989), exposure of bovine neutrophils to rBoIFN-γ in the present study had no significant effect on $[Ca^{2+}]_i$ or LTB_4 production.

Comparison of the effects of the various stimulators on change in $[Ca^{2+}]_i$ and LTB_4 production reveal a direct relationship between duration and magnitude of $[Ca^{2+}]_i$ increase and LTB_4 production. This relationship was confirmed by the studies in which duration and magnitude of $[Ca^{2+}]_i$ increase in response to A23187 or LKT were artificially manipulated, either by incubating cells in media containing different Ca^{2+} concentrations or addition of extracellular EGTA: LTB_4 was synthesized only when $[Ca^{2+}]_i$ was allowed to increase substantially be be maintained for a prolonged period of time, as occurred after exposure to A23187, PMA, and LKT.

The ability to experimentally manipulate LKT-induced $[Ca^{2+}]_i$ increase by addition of extracellular EGTA or changing extracellular Ca^{2+} concentration confirms that influx of

extracellular Ca^{2+} across the plasma membrane constitutes an important mechanism involved in the increased $[Ca^{2+}]_i$ resulting from LKT exposure. This conclusion is consistent with the hypothesis that LKT forms pores in neutrophil plasma membranes through which Ca^{2+} is able to diffuse down its concentration gradient. However, comparison between A23187- and LKT-induced $[Ca^{2+}]_i$ increases indicated that diffusion cannot fully explain LKT-induced rise in Ca^{2+}: exposure to A23187 results in an increase in $[Ca^{2+}]_i$ that is characteristic of diffusion down a concentration gradient until equilibrium is reached whereas exposure to LKT causes a more rapid increase in $[Ca^{2+}]_i$, followed by a progressive decrease in $[Ca^{2+}]_i$. Additional experiments involving exposure of neutrophils to LKT in the presence or absence of antifluorescein antibody and repeated simulation of neutrophils confirmed that this decline in $[Ca^{2+}]_i$ may not be completely explained by cell swelling and lysis. Rather, the rapid initial increase in $[Ca^{2+}]_i$ and the subsequent decrease suggest that in addition to transmembrane diffusion of Ca^{2+}, LKT-induced changes in $[Ca^{2+}]_i$ may be modulated by active transport processes. These may contribute to the initial rise in $[Ca^{2+}]_i$ as well as an attempt to restore normal $[Ca^{2+}]_i$ subsequently. Modulating effects such as these could be accomplished by release and re-uptake of Ca^{2+} from intracellular vesicular stores.

In conclusion, the similarities between LKT-, A23187-, and PMA-induced changes in $[Ca^{2+}]_i$ and LTB_4 synthesis suggest that a large sustained increase in $[Ca^{2+}]_i$ is necessary to cause significant production of LTB_4 by bovine neutrophils. Furthermore, the character of $[Ca^{2+}]_i$ increase induced by the pore forming cytolysin, LKT, is different from those induced by the protein kinase C agonist, PMA, the Ca^{2+} ionophore, A23187, and the physiological stimulator, rhC_{5a}. This evidence supports the hypothesis that LKT

stimulates uncontrolled synthesis of LTB_4 by causing a sustained and unregulated increase in bovine neutrophil $[Ca^{2+}]_i$. Although LKT-induced increase in $[Ca^{2+}]_i$ results, in part, from influx of extracellular Ca^{2+}, the specific pattern of the response suggests the contributions of the release of vesicular Ca^{2+}.

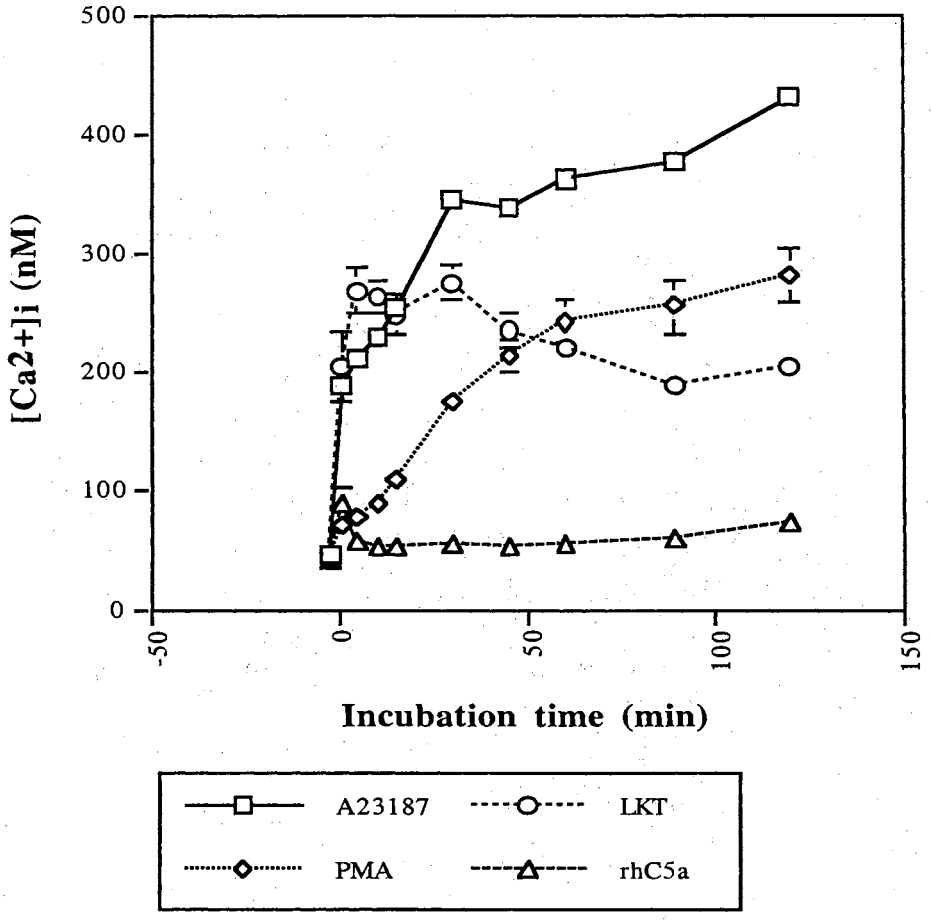


Figure 17- Effect of exposure of bovine neutrophils to rhC5a, A23187, PMA, and LKT (1:10) on $[Ca^{2+}]_i$ measured at various times. Error bars are standard deviations for triplicate samples within a single plate for each time point.

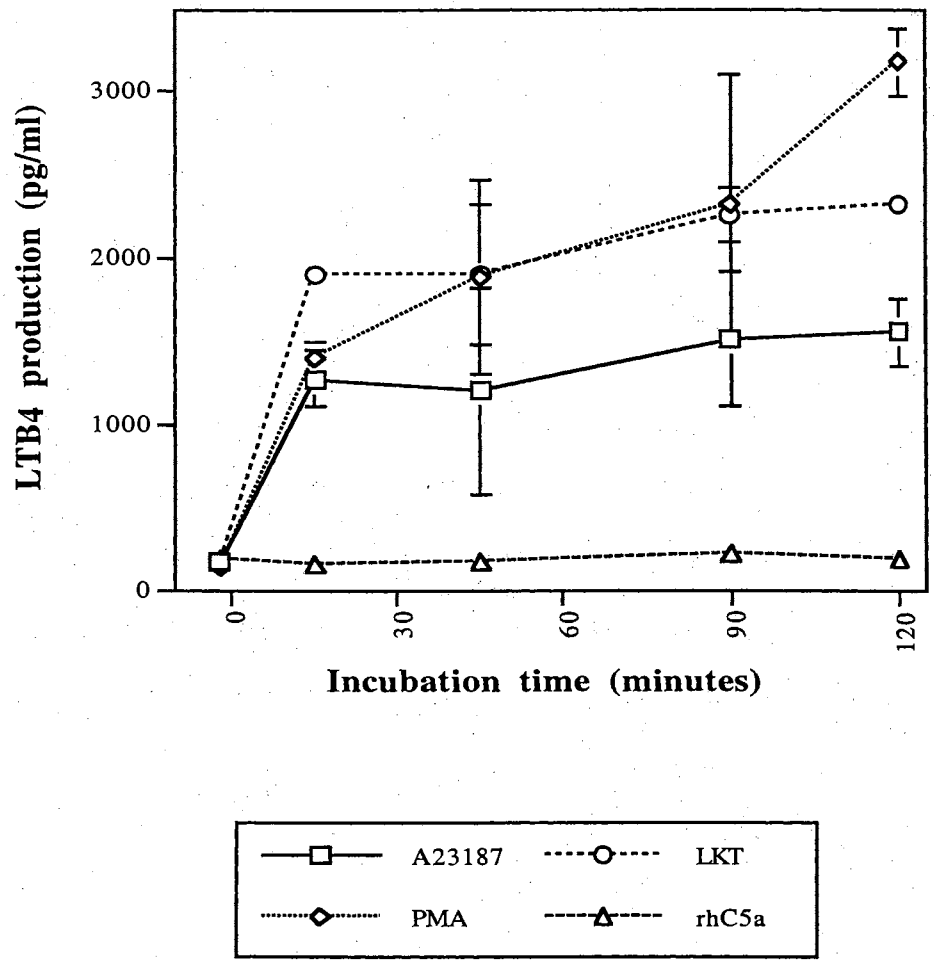


Figure 18- Effect of exposure of bovine neutrophils to rhC5a, A23187, PMA, and LKT (1:10) on LTB_4 production measured at various times. Error bars represent standard deviations for triplicate samples in a single plate for each times point. All treatments were significantly different ($p<0.05$) from each other at 120 minutes.

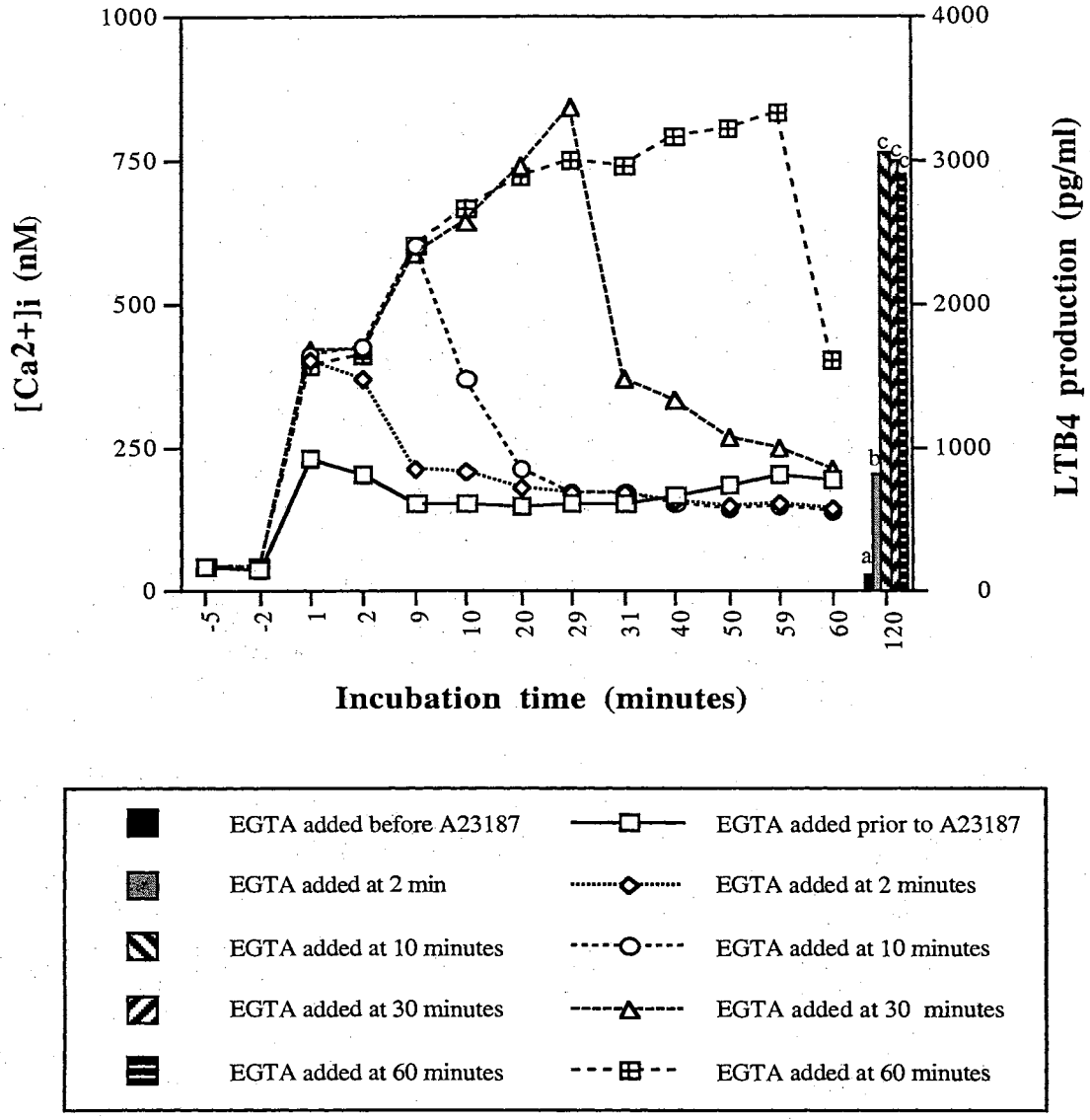


Figure 19- The effect of EGTA addition at various times on A23187-induced effects in bovine neutrophils. EGTA was added prior to and 2, 10, 30, and 60 minutes after A23187 stimulation with $[Ca^{2+}]_i$ measured at various times and LTB_4 production measured at 120 minutes. Error bars represent standard deviations for triplicate samples within a single plate. a, b, c Indicates statisically ($p<0.05$) similar groups of LTB_4 production.

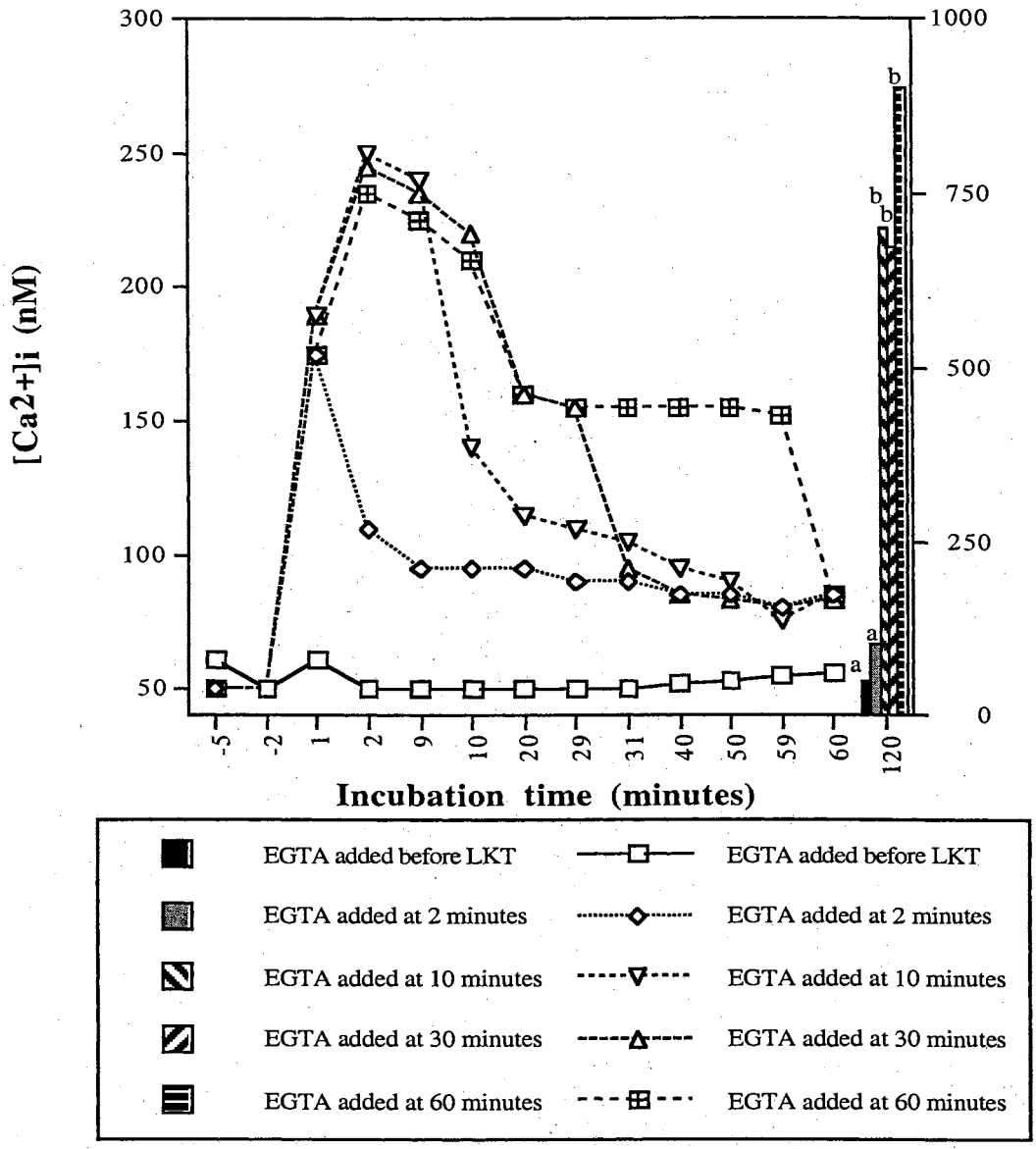


Figure 20- The effect of EGTA addition at various times on LKT-induced effects in bovine neutrophils. EGTA was added prior to and 2, 10, 30, and 60 minutes after LKT stimulation with $[Ca^{2+}]_i$ measured at various times and LTB_4 production measured at 120 minutes. Error bars represent standard deviations for triplicate samples within a single plate. a, b, c Indicates statisically ($p<0.05$) similar groups of LTB_4 production.

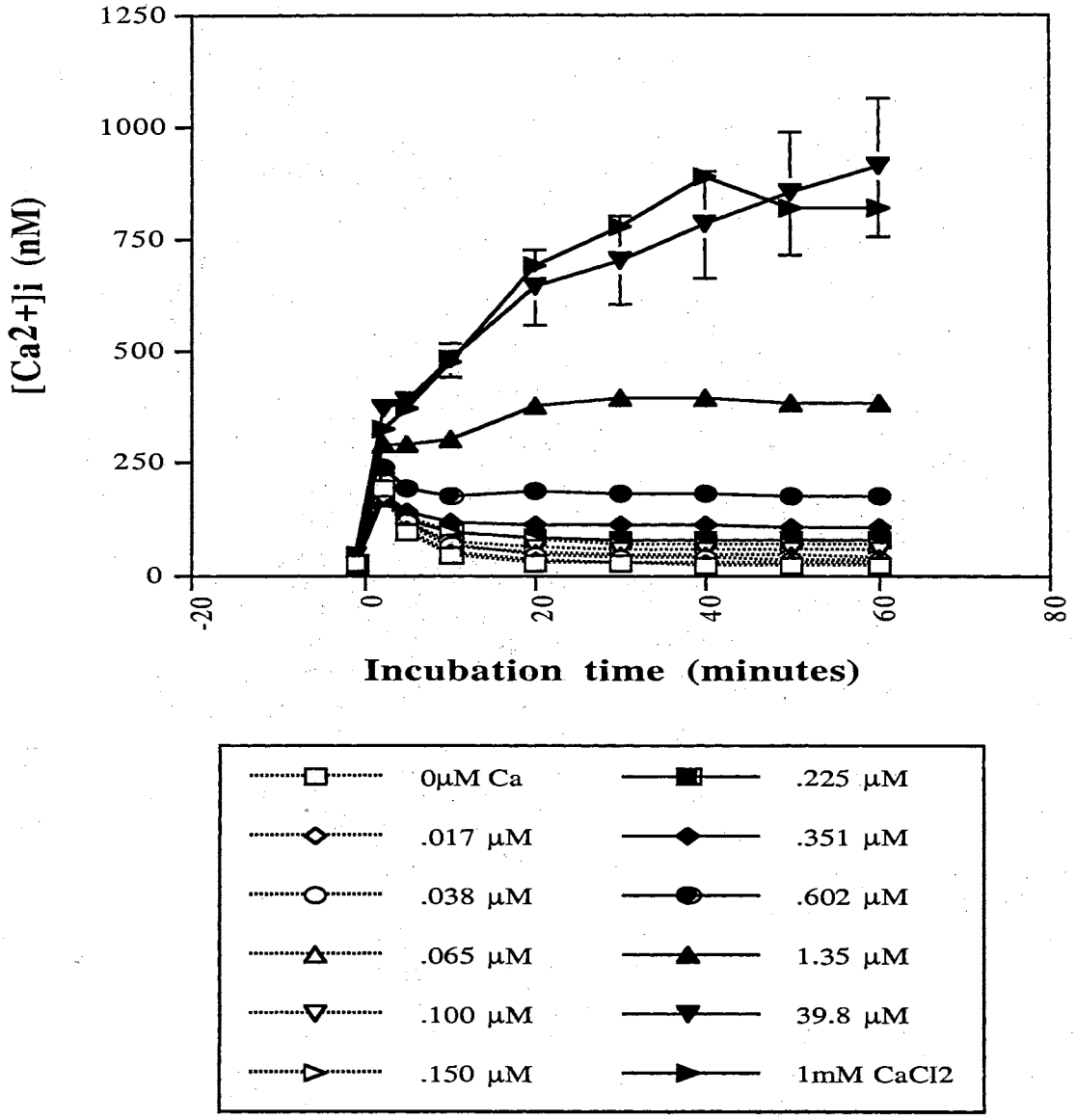


Figure 21- $[Ca^{2+}]_i$ measured at various times in bovine neutrophils suspended in a range of Ca^{2+} buffers prior to exposure to A23187. Error bars represent standard deviation for triplicate samples within a single plate. Cells suspended in 1.35 μM and 39.8 μM free Ca^{2+}-samples yeilded significantly different ($p<0.05$) $[Ca^{2+}]_i$ from all other treatments at 5-60 minutes but not from each other.

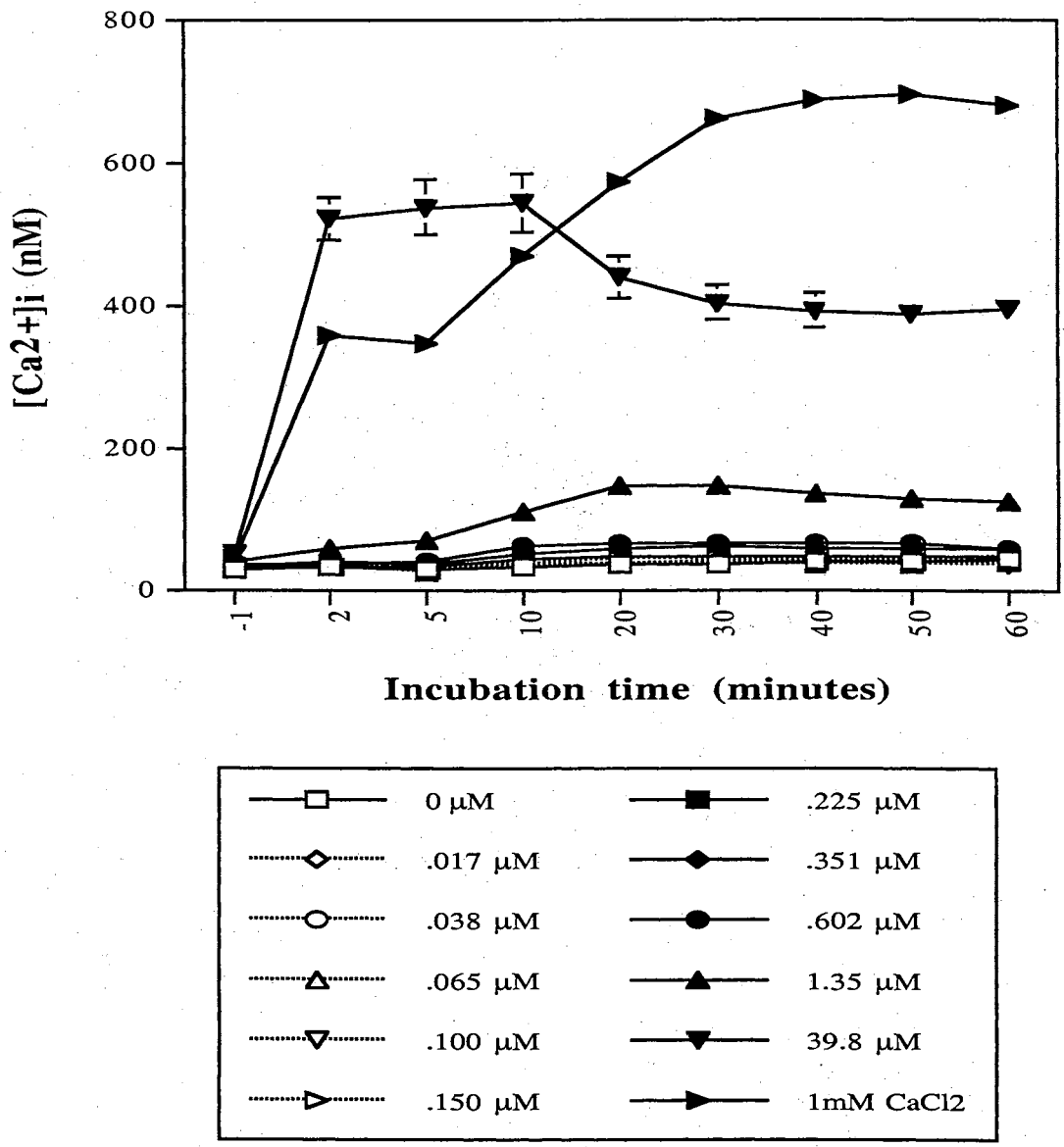


Figure 22- $[Ca^{2+}]_i$ measured at various times in bovine neutrophils suspended in a range of Ca^{2+} buffers prior to exposure to LKT . Error bars represent standard deviation for triplicate samples within a single plate. Cells suspended in 1.35 μM and 39.8 μM free Ca^{2+}-samples yeilded significantly different ($p<0.05$) $[Ca^{2+}]_i$ from all other treatments at 5-60 minutes but not from each other.

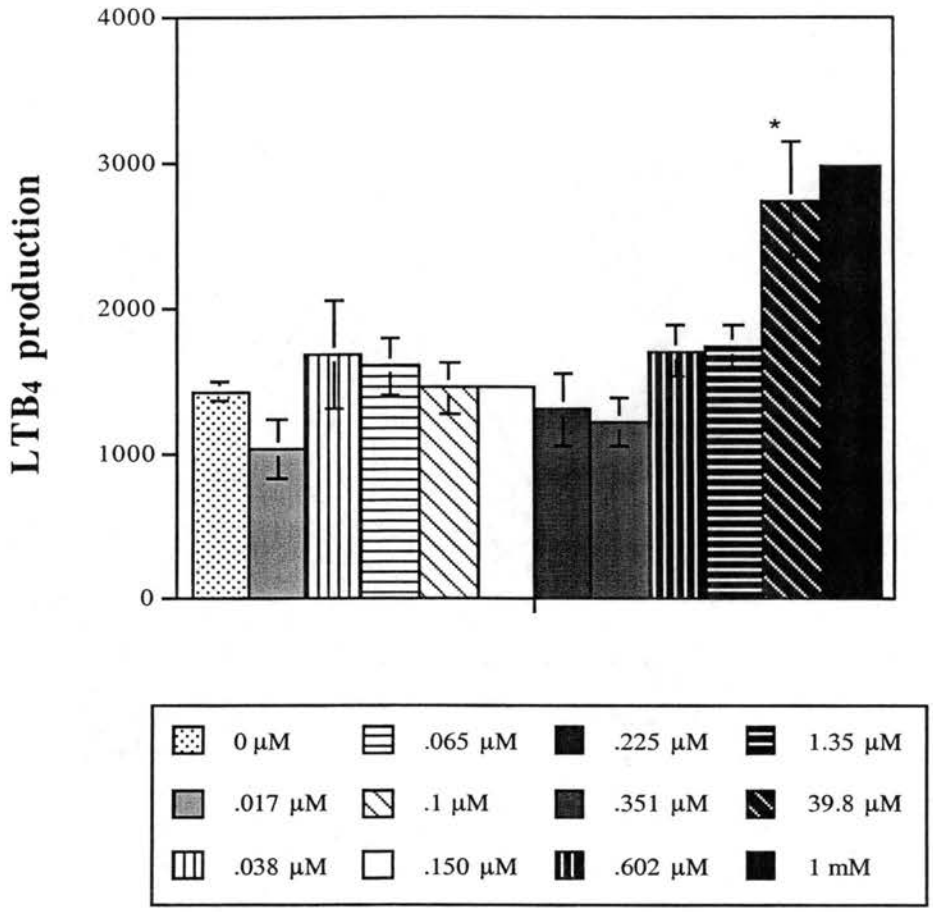


Figure 23- Bovine neutrophils suspended in various Ca^{2+} buffers prior to exposure to A23187. LTB_4 samples were taken at 60 minutes. Error bars represent standard deviation from triplicate samples within a single plate. *Indicates values significantly ($p<0.05$) different from all other values.

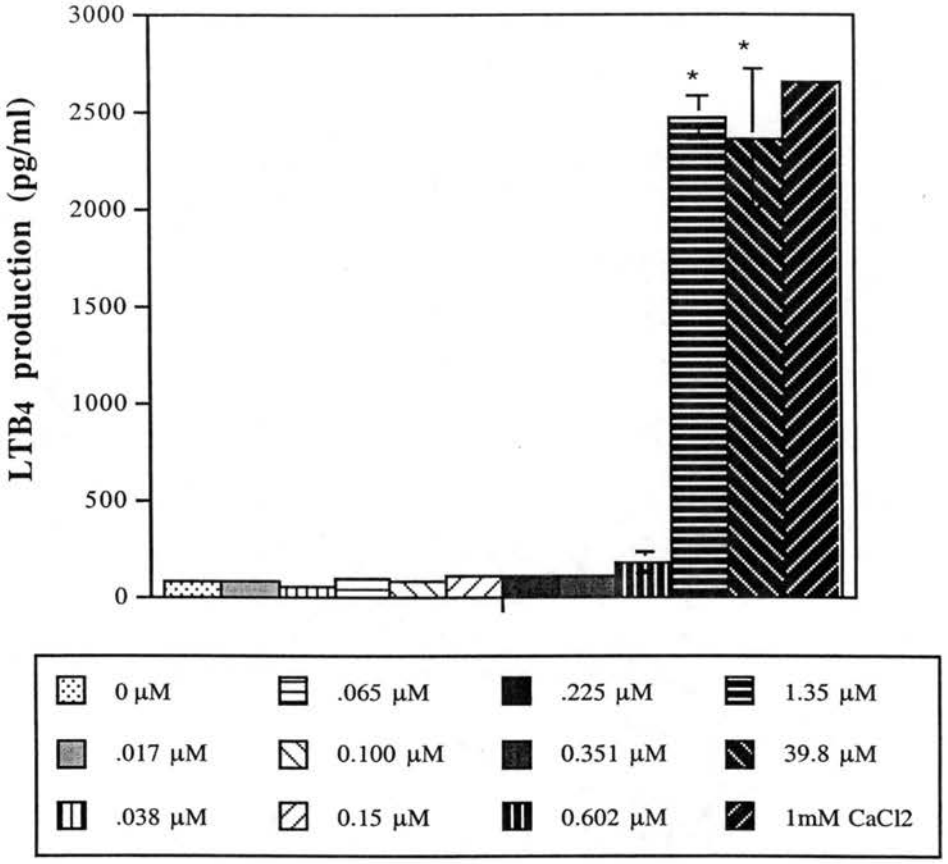


Figure 24- Bovine neutrophils suspended in various Ca^{2+} buffers prior to exposure to LKT. LTB_4 samples were taken at 60 minutes. Error bars represent standard deviation from triplicate samples within a single plate. *Indicates values significantly ($p<0.05$) different from all other values.

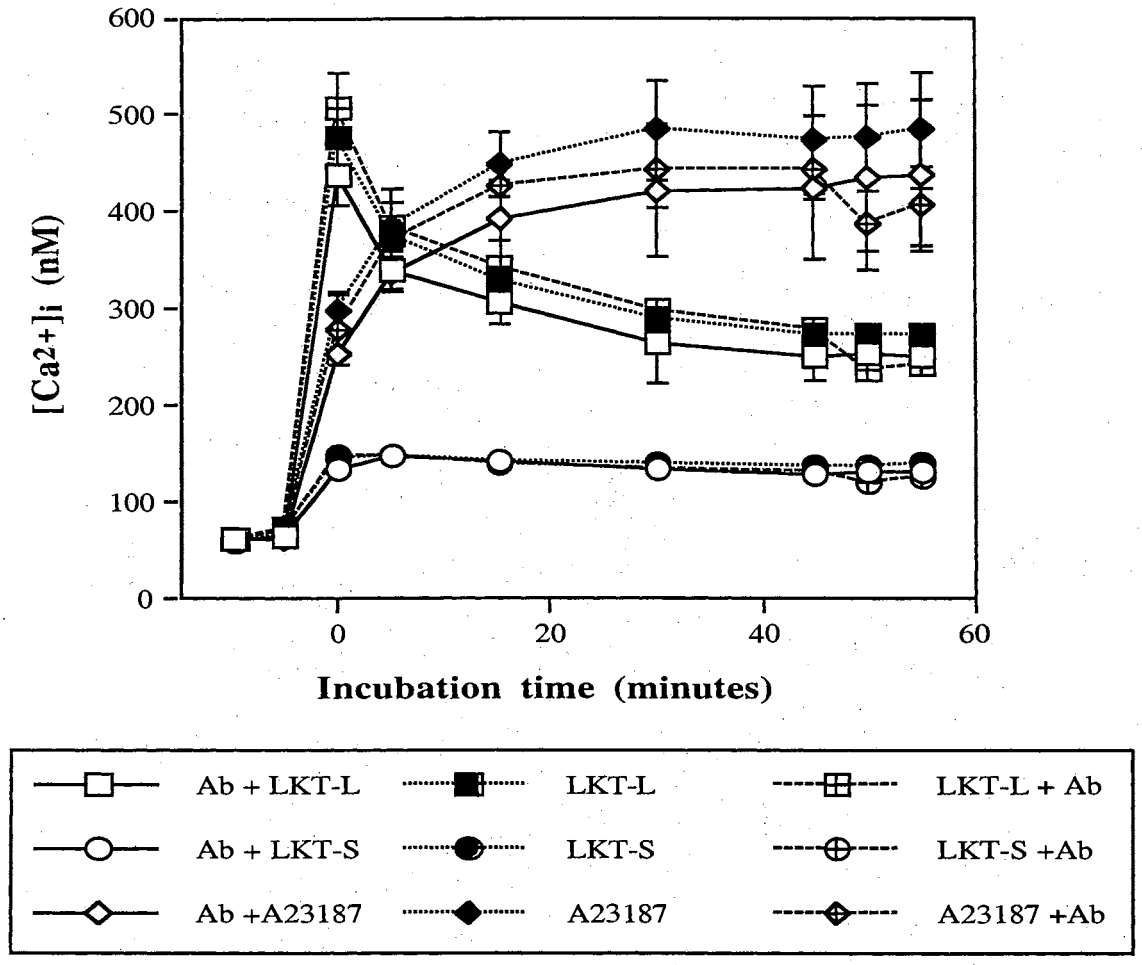


Figure 25- The effects of 1:100 (LKT-L, specific LDH release >40% at 60 minutes after exposure) and 1:10,000 (LKT-S, specific LDH release <10% at 60 minutes after exposure) dilutions of LKT and of A23187 on $[Ca^{2+}]_i$ with antifluorescein antibody added wither prior to stimuli exposure (Ab+LKT-L, Ab+LKT-S, Ab+A23187), 30 minutes after stimuli exposure (LKT-L+Ab, LKT-L+Ab, A23187+Ab), or not at all (LKT-L, LKT-S, A23187). Error bars are standard deviations for triplicate sampleswithin a single plate. There were no significant differences ($p<0.05$) within stimuli treatments for antibody treatments.

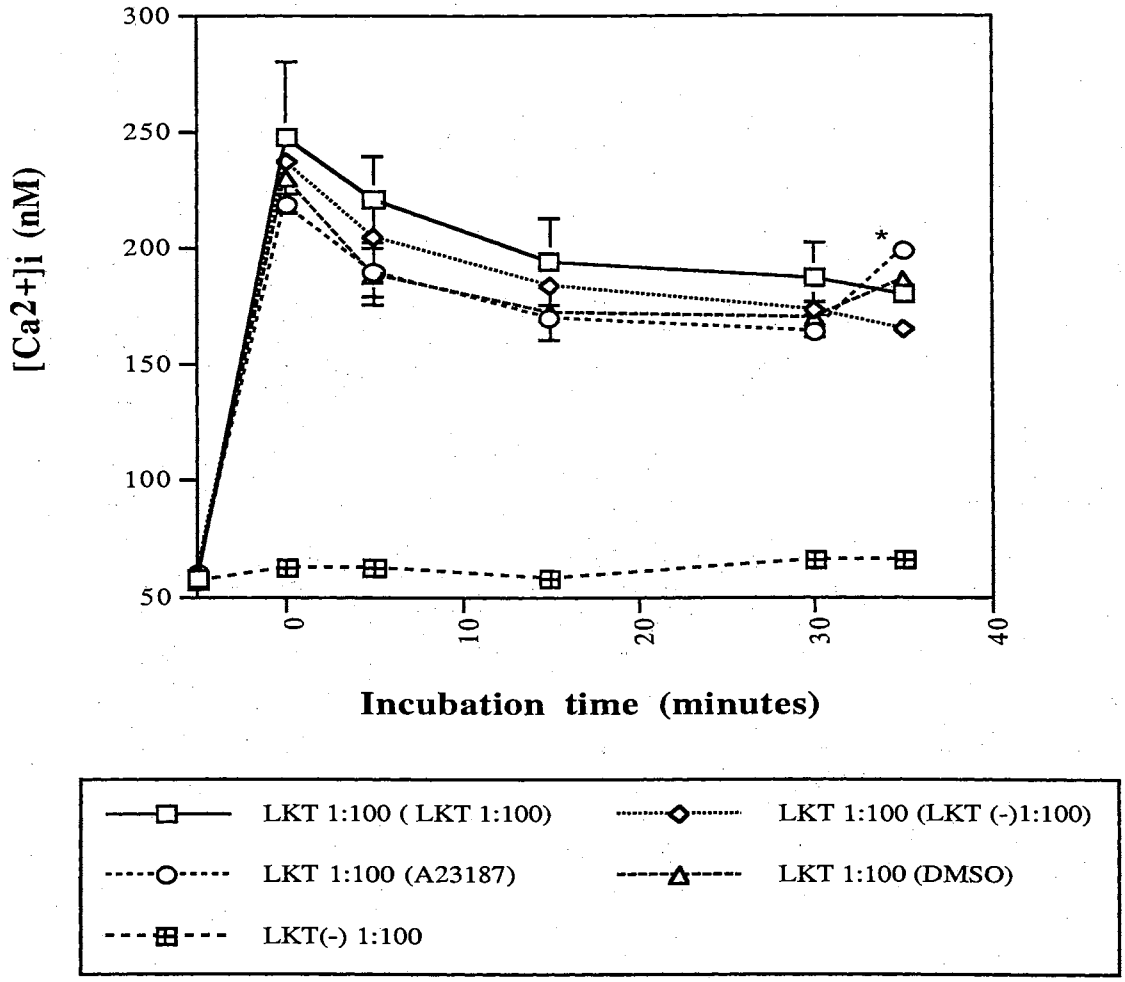


Figure 26- The effect of LKT 1:100, LKT(-) 1:100, A23187, and DMSO on $[Ca^{2+}]_i$ in neutrophils previously exposed to LKT 1:100. Isolated bovine neutrophils were exposed to LKT 1:100 and fluorescence was measured at preincubation, 0, 5, 15, 30, and 35 minutes respectively. LKT 1:100, LKT(-) 1:100, A23187 in DMSO or DMSO was added respectively at 30 minutes as indicated. Error bars represent standard deviations for quadruplicate samples within a single plate. *Indicates significant differences ($p<0.05$) between $[Ca^{2+}]_i$ values just prior to and after second stimuli.

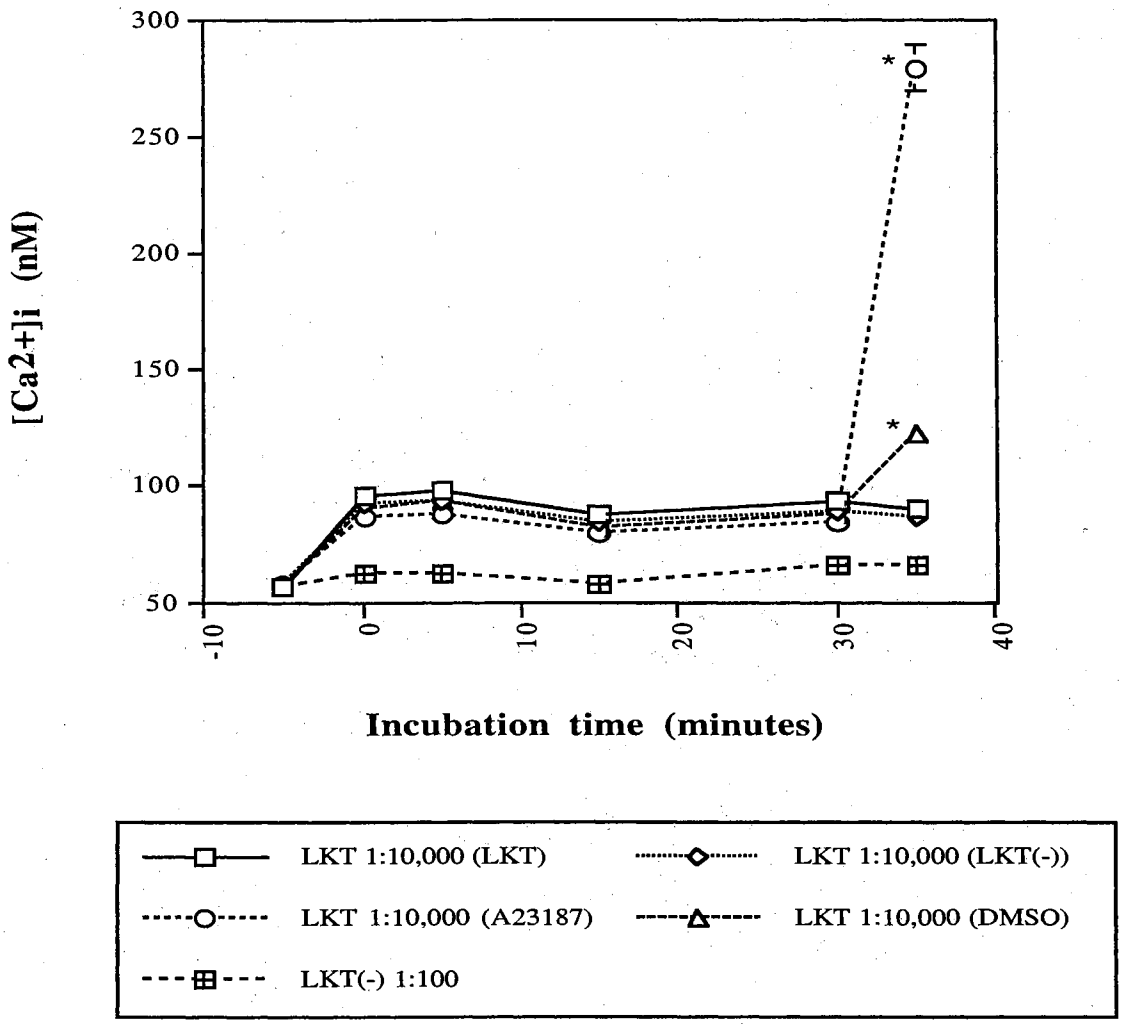


Figure 27- The effect of LKT 1:10,000, LKT(-) 1:10,000, A23187, and DMSO on $[Ca^{2+}]_i$ in neutrophils previously exposed to LKT 1:10,000. Isolated bovine neutrophils were exposed to LKT 1:10,000 and fluorescence was measured at preincubation, 0, 5, 15, 30, and 35 minutes respectively. LKT 1:10,000, LKT(-) 1:10,000, A23187 in DMSO or DMSO was added respectively at 30 minutes as indicated. Error bars represent standard deviations for quadruplicate samples within a single plate. *Indicates significant differences ($p<0.05$) between $[Ca^{2+}]_i$ values just prior to and after second stimuli.

CHAPTER VII

CONTRIBUTION OF VESICULAR CALCIUM TO INCREASE IN INTRACELLULAR CALCIUM CONCENTRATION CAUSED BY *PASTEURELLA HAEMOLYTICA* LEUKOTOXIN.

Introduction

The massive influx of neutrophils into lungs of cattle with pneumonic pasteurellosis (Allan *et al.*, 1985) fails to clear the offending bacterium, *Pasteurella haemolytica*, but instead contributes to the development of severe lung pathology (Yates 1982, Slocombe *et al*, 1985; Breider *et al*, 1988). A virulence factor of *P. haemolytica* that contributes substantially to neutrophil-mediated lung injury is leukotoxin, a protein exotoxin produced during log phase growth. LKT is a member of the RTX family of pore forming toxins, which are characterized by tandemly arranged repeats of a nine amino acid sequence (Welch, 1991). Lytic doses of LKT cause severe membrane defects and neutrophil lysis, impairing neutrophil phagocytosis (Clinkenbeard *et al*, 1989), while sublytic doses of LKT are potent neutrophil activating agents, stimulating LTB_4 production and neutrophil chemotaxis into the infected tissue (Ortiz-Carranza and Czuprynski, 1992; Clinkenbeard *et al*, 1994; Clarke *et al*, 1994).

Previous studies have concluded that increased $[Ca^{2+}]_i$ serves as an essential signal transduction event in LKT-induced LTB_4 production, membrane damage, and neutrophil activation (Ortiz-Carranza and Czuprynski, 1992; Clinkenbeard *et a*l, 1994; Cudd *et al*, unpublished data). Furthermore, experiments in cchapter 4 involving the use of Ca^{2+} chelators have demonstrated that influx of extracellular Ca^{2+} across the plasma membrane

contributes to LKT-induced increase in $[Ca^{2+}]_i$. Whether transmembrane flux of Ca^{2+} occurs via LKT-indued pores or via calcium channels has not been conclusively determined. Moreover, comparison of LKT-induced effects with other stimulators, such as A23187, have suggested that influx of extracellular Ca^{2+} does not fully explain the specific pattern of LKT-induced $[Ca^{2+}]_i$ increase and that release and re-uptake of vesicular Ca^{2+} may also be involved. Therefore, the specific objectives of this study were: (1) to investigate the involvement of calcium channels in LKT-induced transmembrane flux; and (2) to assess the importance of vesicular Ca^{2+} stores in LKT-induced $[Ca^{2+}]_i$ increase.

Materials and Methods

Preparation of *P. haemolytica* Leukotoxin

LKT and LKT(-) were prepared from culture supernatants of *P. haemolytica* biotype A, serotype 1 wildtype strain and a leukotoxin-deficient mutant strain A, as described previously in Chapter IV. Leukotoxin activity was quantified as toxic units (TU) using BL3 cells, as described previously (Clinkenbeard *et al*, 1989). Each assay was conducted in triplicate and the TU were determined for each experiment. In this experiment, lytic doses of LKT caused >50% lysis of 2.5 x 10^6 neutrophils in 250 µl buffer after 2 hours incubation while sublytic doses of LKT caused <10% lysis from similarly diluted and incubated neutrophils.

Preparation of Neutrophil Suspensions

Two healthy beef calves (200 ± 50 kg) served as blood donors for isolation of neutrophils. Neutrophils were isolated by hypertonic lysis as previously described in

Chapter IV and suspended in 5 ml of Ca^{2+} and Mg^{2+} -free HBSS. Cells were enumerated by hemocytometer and viability was assessed by trypan blue exclusion. Differential counts were conducted on stained smears. Preparations were then diluted to a final concentration of 6 x 10^6 viable neutrophils/ml HBSS.

Neutrophil Loading with Fluorescent Calcium Indicator and Intracellular Calcium Concentration Determination

Cells were loaded with Fluo-3 AM and $[Ca^{2+}]_i$ was measured as previously described in Chapter IV. Thereafter, the cells were resuspended in 3 ml HBSS, enumerated by hemocytometer and then resuspended in HBSS to 1 x 10^7 cells/ml.

Exposure of Isolated Neutrophils to Stimulators

The relative contributions of vesicular Ca^{2+}, influx of extracellular Ca^{2+} via ROC and VOC channels, and Ca^{2+}–induced Ca^{2+} release to LKT-, A23187, and rhC_{5a}-induced increase in $[Ca^{2+}]_i$ were explored using specific inhibitors of these pathways. With the exception of experiments involving xestospongin C, which were conducted in glass tubes to avoid chelation of xestospongin C to plastic (Gafni *et al*, 1997; Pessah, personal communication), neutrophil suspensions were aliquoted (final volume after addition of treatments = 250 µl) into 96-well microtiter plates and incubated with the various inhibitors for 45 minutes prior to sequential addition of 10 µl 25 mM $CaCl_2$, 2 µl antifluoroscein antibody diluted 1:5 in PBS, and the individual stimulators. Neutrophils were incubated at 37° C and fluorescence was measured prior to stimulator exposure and at various times thereafter using a spectrofluorometer. After 120 minutes of incubation, plates were

centrifuged and supernatant collected for LDH measurement. All experiments included quadruplicate wells for each of the treatments.

The roles of ROC channels, intracellular Ca^{2+} stores, and CICR in stimulator-induced increase in $[Ca^{2+}]_i$ and LDH release were investigated by incubating neutrophils with either $LaCl_3$ (1 mM), thapsigargin, or $MgCl_2$ (5 mM), respectively, prior to exposure to LKT (1:500), LKT (-) (1:500) control, 4Br-A23187 (0.1 mM), rHC_{5a} (0.1 μM), or PBS, as described above. Fluorescence was measured prior to addition of the stimulator and at specific times thereafter. Thapsigargin-treated neutrophils were incubated with 1 mM EGTA to deplete intracellular Ca^{2+} stores and 1 μM thapsigargin to prevent active replenishment of vesicular Ca^{2+}. Prior to addition of $CaCl_2$, antifluoroscein antibody, and stimulators, neutrophils were washed with PBS to remove EGTA. Removal of EGTA did not affect the inhibitory function of thapsigargin, which remained tightly bound to the Ca^{2+}-ATPase pump responsible for replenishment of vesicular Ca^{2+} (Lytton *et al*, 1991). After stimulation, the cells were incubated at 37°C for 120 minutes prior to measurement of LDH release.

The relative contributions of release of vesicular Ca^{2+} via IP_3 receptors and CICR were studied by incubating neutrophils with xestospongin C (7 mM), an IP_3 receptor blocker, $MgCl_2$ (5 mM), an inhibitor of Ca^{2+}-induced Ca^{2+} release, or both xestospongin C and $MgCl_3$ prior to addition of LKT. Results were compared to cells incubated with thapsigargin or no inhibitors. Fluorescence was measured prior to exposure of neutrophils to the stimulator and at specific times thereafter. Release of LDH was measured at 120 minutes of incubation.

The involvement of VOC channels in LKT-induced increase in $[Ca^{2+}]_i$ was explored using the calcium channel blocker, verapamil. Twenty five microliters of either 0.5 mM, 5 μM, 50 nM verapamil, or DMSO control solvent were added to neutrophil

suspensions, which were then preincubated for 20 minutes before adding 25 µl 1:50 LKT or LKT(-). Fluorescence was recorded after 2 minutes of incubation and suspensions were then incubated further for 120 minutes before concentrations of LTB_4 were measured. Release of LDH was not investigated because preliminary experiments indicated that verapamil interfered with the LDH release assay.

Estimation of Plasma Membrane Damage.

The effect of *P. haemolytica* LKT on neutrophil plasma membrane integrity was assayed by measuring extracellular release of cytoplasmic LDH, as previously descibed in Chapter IV.

Leukotriene B_4 Immunoassay

Concentrations of LTB_4 were determined using a commercially prepared radioimmunoassay kit that has been previously validated (Clinkenbeard *et al*, 1994).

Statistical Analyses

Data were analyzed using SYSTAT intelligent software for the Macintosh, Version 5.2 (Wilkinson, 1992). The effects of inhibitors on stimulator-induced increases in $[Ca^{2+}]_i$ and LDH release were compared with the responses of corresponding controls using unpaired t-tests. Effects of verapamil were tested using the general linear model followed by *a priori* comparison of selected mean pairs using Fisher's LSD Test: LKT-induced responses were compared with verapamil-free controls and LKT-induced responses were

compared with corresponding LKT(-) controls. Differences between means were considered significant at the $P < 0.05$ level.

Results

Depletion of intracellular Ca^{2+} stores using thapsigargin and EGTA, inhibition of CICR using $MgCl_2$, or inhibition of ROC channels using $LaCl_3$ all attenuated the increases in $[Ca^{2+}]_i$ caused by LKT, A23187, or rhC_{5a} (Figs. 28, 29, and 30), thus suggesting that a variety of mechanisms involving release of vesicular Ca^{2+} and influx of extracellular Ca^{2+} across the plasma membrane may contribute to the rate and magnitude of $[Ca^{2+}]_i$ increase induced by these stimulators.

The involvement of vescicular Ca^{2+} release was confirmed further by demonstrating that preincubation with inhibitors of IP_3 receptor-mediated Ca^{2+} release (xestospongin) or CICR ($MgCl_2$) inhibited LKT-induced $[Ca^{2+}]_i$ increase and that when these two inhibitors were combined they produced an inhibitory effect that was similar in degree to that caused by inhibition of vesicular Ca^{2+} uptake using thapsigargin (Fig.31). The relevance of vesicular Ca^{2+} release to effects of LKT on neutrophil integrity was demonstrated by the inhibitory effect of thapsigargin on LDH release of LKT-exposed cells (Fig. 32).

Verapamil, the voltage-operated calcium channel blocker, inhibited LKT-induced increase in $[Ca^{2+}]_i$ as well as subsequent production of LTB_4. However, these effects were observed only at high verapamil concentration (Table 4).

Discussion

Several investigators have demonstrated that a variety of effects of LKT on bovine neutrophils, such as LTB_4 production, membrane damage, and neutrophil activation, are dependent on extracellular Ca^{2+} (Clinkenbeard *et al*, 1994; Ortiz-Carranza and Cyuprynski, 1996). Furthermore, this reliance on extracellular Ca^{2+} has been correlated with increased $[Ca^{2+}]_i$, suggesting that LKT induces an influx of Ca^{2+} across the plasma membrane (Ortiz-Carranza and Cyuprynski, 1996; Clinkenbeard *et al*, 1994). Based on evidence that verapamil inhibits LKT-induced increase in $[Ca^{2+}]_i$, influx of extracellular Ca^{2+} has been postulated to occur via voltage-operated calcium channels (Gerbig *et al*, 1989; Ortiz-Carranza and Cyuprynski, 1992).

Comparison between the effects of various stimulators, including LKT, A23187, PMA, and rhC_{5a}, on the rate and degree of increase in $[Ca^{2+}]_i$ (see Chapter VI) has indicated that the response to LKT cannot be explained entirely by influx of extracellular Ca^{2+} across the plasma membrane down a concentration gradient from extracellular to intracellular locations; the rate of increase is too rapid to fit the characteristic $[Ca^{2+}]_i$ increase pattern of diffusional processes. Although exposure of bovine neutrophils to rhC_{5a} resulted in a peak in $[Ca^{2+}]_i$ that was much lower than that produced by LKT, the responses were similar with regard to the initial rate of increase in $[Ca^{2+}]_i$, thus suggesting that the responses to the two stimulators may share common mechanisms. Considering that C_{5a} is known to act via a $G_{i/o}$ protein-mediated pathway that results in release of vesicular Ca^{2+} (Shirato *et al*, 1988), it is possible that similar pathways may act in cooperation with influx of extracellular Ca^{2+} to cause a rapid and excessive increase in $[Ca^{2+}]_i$ when neutrophils are exposed to LKT. Indeed, a recent investigation of the

$[Ca^{2+}]_i$ when neutrophils are exposed to LKT. Indeed, a recent investigation of the mechanism of Ca^{2+} entry into LKT-stimulated cells indicated that LKT-induced increase in $[Ca^{2+}]_i$ could be inhibited by pertussis toxin, an inhibitor of $G_{i/o}$ proteins, thus implicating a signal transduction pathway involving release of Ca^{2+} from intracellular stores (Hsuan *et al*, 1998). Generally, $G_{i/o}$ proteins operate via inhibition of adenylate cyclase, activation of K^+ channels, inactivation of VOC, and activation of phospholipase C to release Ca^{2+} from internal calcium stores (Hescheler *et al*, 1988; Rosenthal *et al*, 1988; Birnbaumer, 1990).

Many transduction pathways rely on vesicular Ca^{2+} stores for transmission of intracellular signals. $G_{i/o}$-proteins promote Ca^{2+} release from intracellular stores via receptor-mediated activation of PLC, which metabolizes phosphatidylinositol 4,5-bisphosphate (PIP) to yield DAG and IP_3 (Berridge, 1993). Calcium is stored in several intracellular locations, including the endoplasmic reticulum (ER), mitochondria, and intracellular Ca^{2+} binding proteins. Although mitochondria have a large capacity for Ca^{2+} uptake and storage, release and uptake of Ca^{2+} by mitochondria are too slow to suit the requirements of intracellular signalling. Intracellular Ca^{2+} binding proteins are important in processing of the Ca^{2+} signal but are not important sources of Ca^{2+} buffering, as Ca^{2+} binds too tightly to Ca^{2+} binding proteins to respond rapidly to activating stimuli. Stores of Ca^{2+} in the ER have a great capacity for Ca^{2+} and are able to release Ca^{2+} rapidly through an inositol trisphosphate (IP_3)-mediated channel or slowly through a Ca^{2+} leak channel. After Ca^{2+} release, the ER is replenished with Ca^{2+} by a Ca^{2+}-ATPase pump in the ER membrane (Carafoli, 1987). Upon thapsigargin binding, the refilling of the ER is inhibited but the slow leak of Ca^{2+} out of the ER continues until the ER is depleted of Ca^{2+}. If ER is depleted of Ca^{2+}, IP_3 binding to the IP_3 receptor fails to cause release of

inhibitor, xestospongin, which were observed in the present study confirmed that LKT causes a release of vesicular Ca^{2+} that involves IP_3 receptor-mediated channels on vesicular membranes.

Although Ca^{2+} release from the ER is an IP_3 receptor-mediated event, it has been shown that cytosolic Ca^{2+} is required for IP_3 receptor activation by IP_3 (Yao and Parker, 1992). Furthermore, Ca^{2+} is not only required for IP_3 receptor activation, but Ca^{2+} can itself induce vesicular Ca^{2+} release through IP_3 receptor mediated channels. While initially stimulatory to IP_3 receptors, sustained and elevated $[Ca^{2+}]_i$ becomes inhibitory to IP_3 receptors (Finch *et al*, 1991). The inhibitory effects of xestospongin and $MgCl_2$ observed in the present experiment indicate that LKT-induced increase in $[Ca^{2+}]_i$ involves both IP_3-mediated release and CICR. When these agents were combined, the inhibitory effect on LKT-induced $[Ca^{2+}]_i$ increase was additive and similar to that produced by depleting intracellular Ca^{2+} stores using thapsigargin, thus indicating that IP_3 receptor-mediated events and CICR events together account for all the release of Ca^{2+} from the ER. Considering the dual requirement of IP_3 receptor-mediated release of Ca^{2+} (i.e., IP_3 + Ca^{2+}) and the contribution of CICR, it is reasonable to hypothesize that release of vesicular Ca^{2+} occurs in close association with influx of extracellular Ca^{2+}, which acts as the trigger for the rapid and excessive LKT-induced increase in $[Ca^{2+}]_i$.

Influx of extracellular Ca^{2+} across the plasma membrane may occur through pores formed by integration of LKT into the membrane and/or via calcium channels. There are several types of Ca^{2+} channels in the plasma membrane, including ROC and VOC channels. ROC channels are activated directly by certain external ligands to open Ca^{2+} channels and can be inhibited by the nonspecific inhibitor, $LaCl_3$ (Thomson and

Drydon,1981). VOC channels, which are the most extensively studied class of Ca^{2+} channels, are activated by depolarization and selectively modulated by neutrotransmitters, G_s-proteins, and diffusable messengers. VOC channels can be further divided into L, T, N, and P subtypes. L-type, N-type and P-type VOC channels are activated by high to moderately high voltage while T-type VOC channels are activated by low voltage. L-type VOC channels are sensitive to dihydropyridines such as verapamil and nifedipine but not $LaCl_3$ (Tsein and Tsein, 1990).

The results of the present experiment, as well as those of other investigators (Ortiz-Carranza and Czuprynski, 1992), suggest that both VOC and ROC channels are involved in LKT-induced $[Ca^{2+}]_i$ increase, since both verapamil and $LaCl_3$ exerted inhibitory effects. However, these effects were only observed at high concentrations and, in the case of verapamil, much higher than those required to inhibit Ca^{2+} flux across nerve and cardiac muscle plasma membranes. It is possible that verapamil inhibits calcium influx in a non specific manner given the evidence that L-type voltage-dependent channels do not occur in neutrophil plasma membranes (Berridge, 1993).

In conclusion, the results of the present study indicate that LKT-induced increase in $[Ca^{2+}]_i$ involves both influx of extracellular Ca^{2+} and release of vesicular Ca^{2+} that together produce an excessive and unregulated intracellular signal. Release of Ca^{2+} from vesicular stores probably occurs in response to influx of extracellular Ca^{2+} and involves both IP_3-mediated and Ca^{2+}–induced Ca^{2+} release. Although it is possible that ROC and VOC channels may be involved in LKT-induced transmembrane influx of Ca^{2+}, the high concentrations of inhibitors that are required to inhibit this response suggest nonspecific interactions of the channel blockers with pores produced by integration of LKT into the plasma membrane. Formation of such pores would be consistent with the hypothesized mechanism of action of the RTX toxin group, of which LKT is a member.

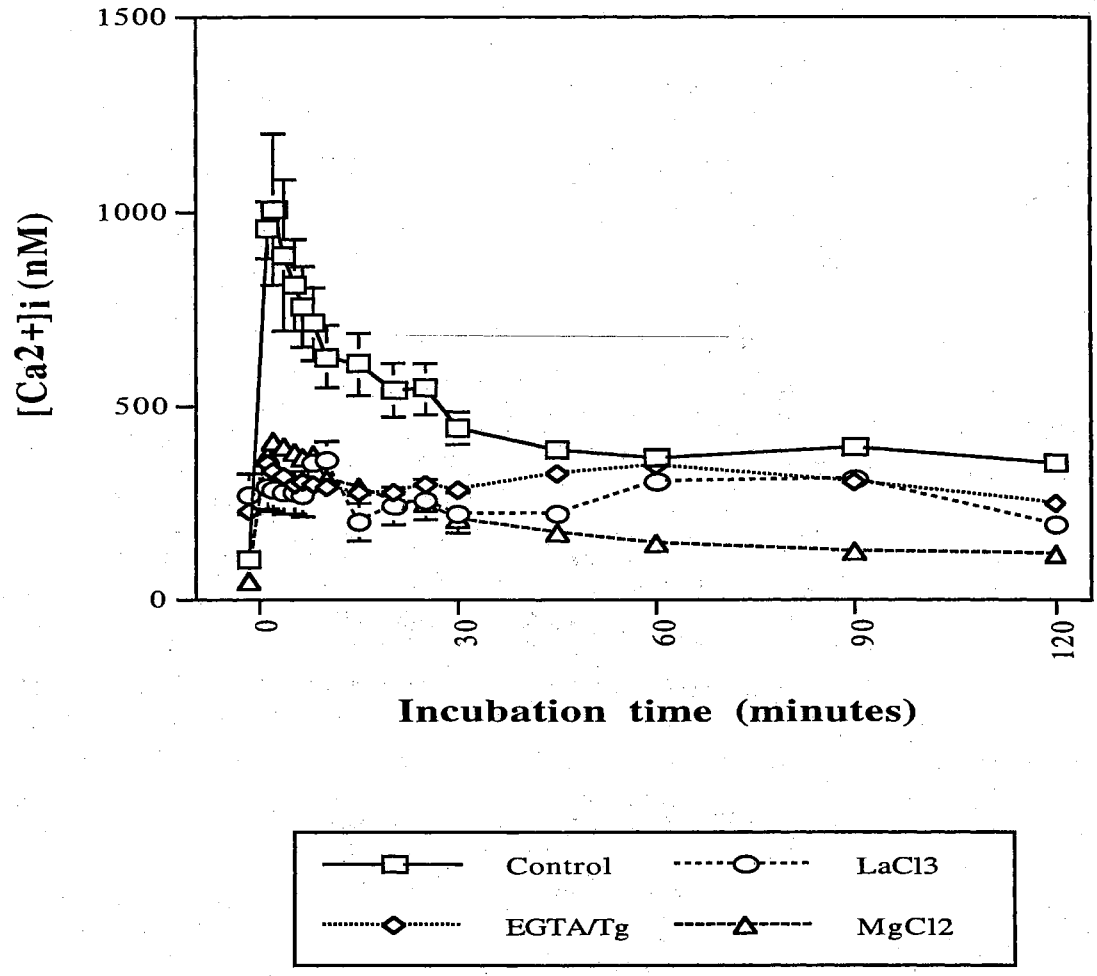


Figure 28- Inhibition of LKT-induced $[Ca^{2+}]_i$ increase in bovine neutrophils with thapsigargin, $LaCl_3$, and $MgCl_2$. Cells were incubated with inhibitors or diluent for a negative control then exposed to LKT and fluorescence was measured at various times. Error bars indicate standard deviation for quadruplicate samples. All inhibitors demonstrated significant differences ($p < 0.05$) from the control at 5 minutes.

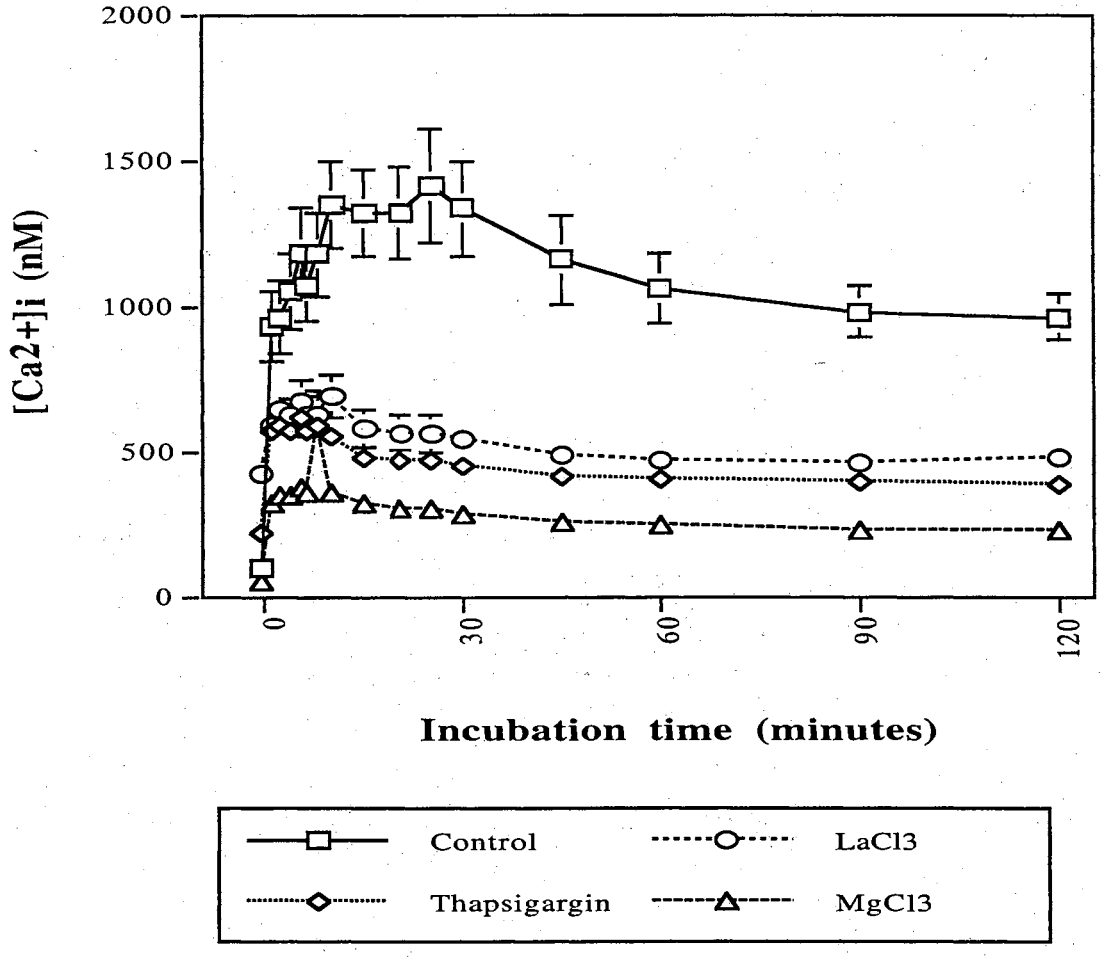


Figure 29- Inhibition of A23187-induced $[Ca^{2+}]_i$ increase in bovine neutrophils with thapsigargin, $LaCl_3$, and $MgCl_2$. Cells were incubated with inhibitors or diluent for a negative control then exposed to A23187 and fluorescence was measured at various times. Error bars indicate standard deviation for quadruplicate samples. All inhibitors demonstrated significant differences ($p < 0.05$) from the control at 5-120 minutes.

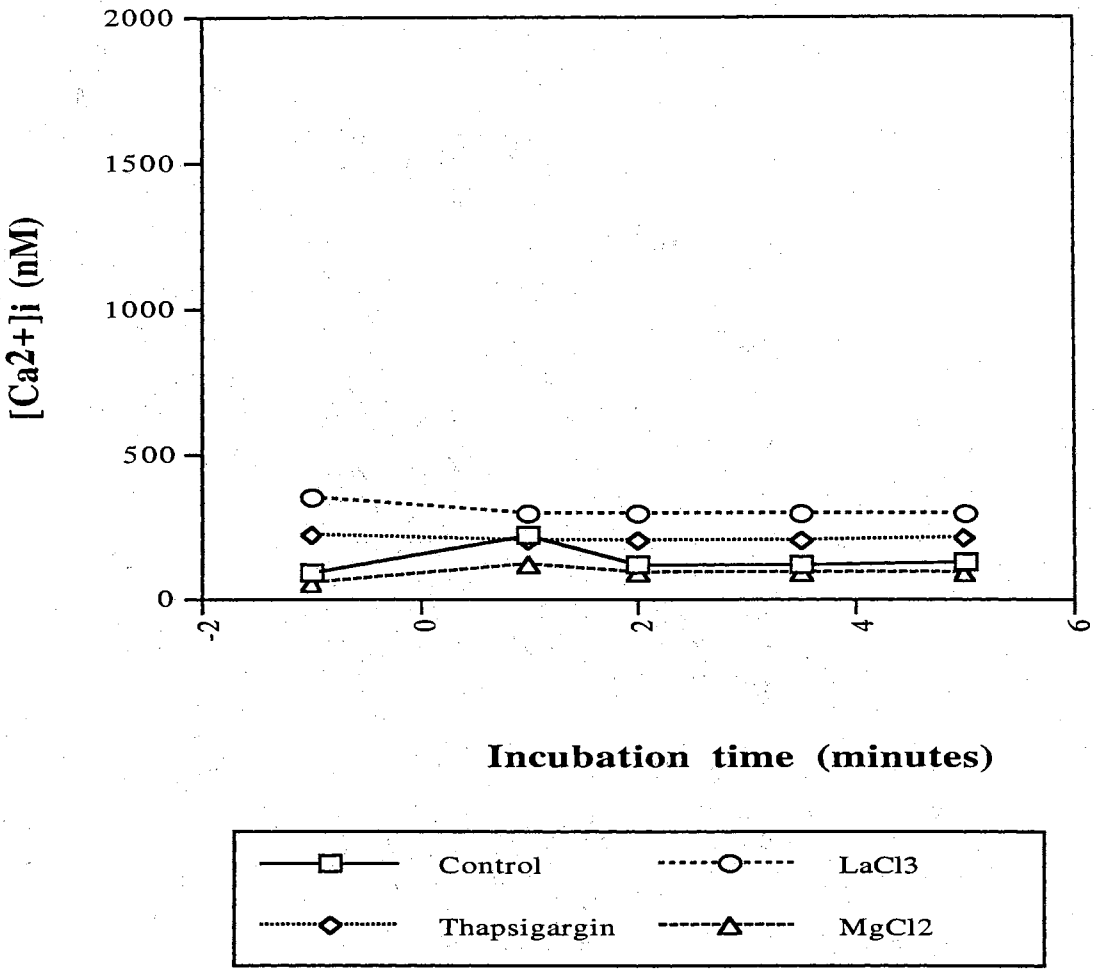


Figure 30- Inhibition of rhC5a-induced $[Ca^{2+}]_i$ increase in bovine neutrophils with thapsigargin, $LaCl_3$, and $MgCl_2$. Cells were incubated with inhibitors or diluent for a negative control then exposed to rhC5a and fluorescence was measured at various times. Error bars indicate standard deviation for quadruplicate samples. $[Ca^{2+}]_i$ levels were compared within treatments at each time point and yielded no significant differences ($p < 0.05$) within any treatment except at 1 minute in control cells.

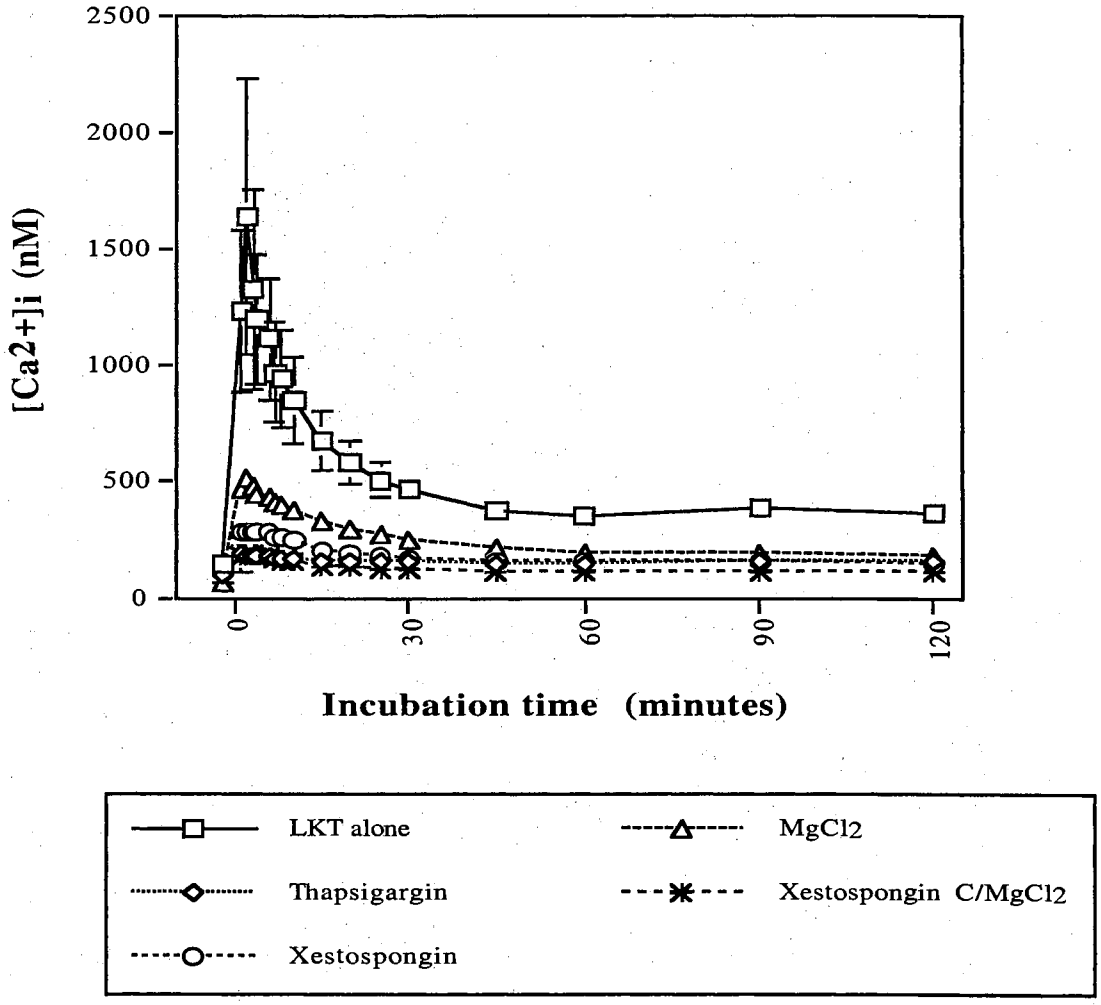


Figure 31- Inhibition of LKT-induced increases in $[Ca^{2+}]_i$ with thapsigargin, xestospongin, $MgCl_2$, or xestospongin/$MgCl_2$. Cells were incubated with inhibitors then exposed to LKT and fluorescence was measured at various times. All inhibitor treatments demonstrated significantly lower ($p < .05$) $[Ca^{2+}]_i$ than the control of LKT only at 6 minutes.

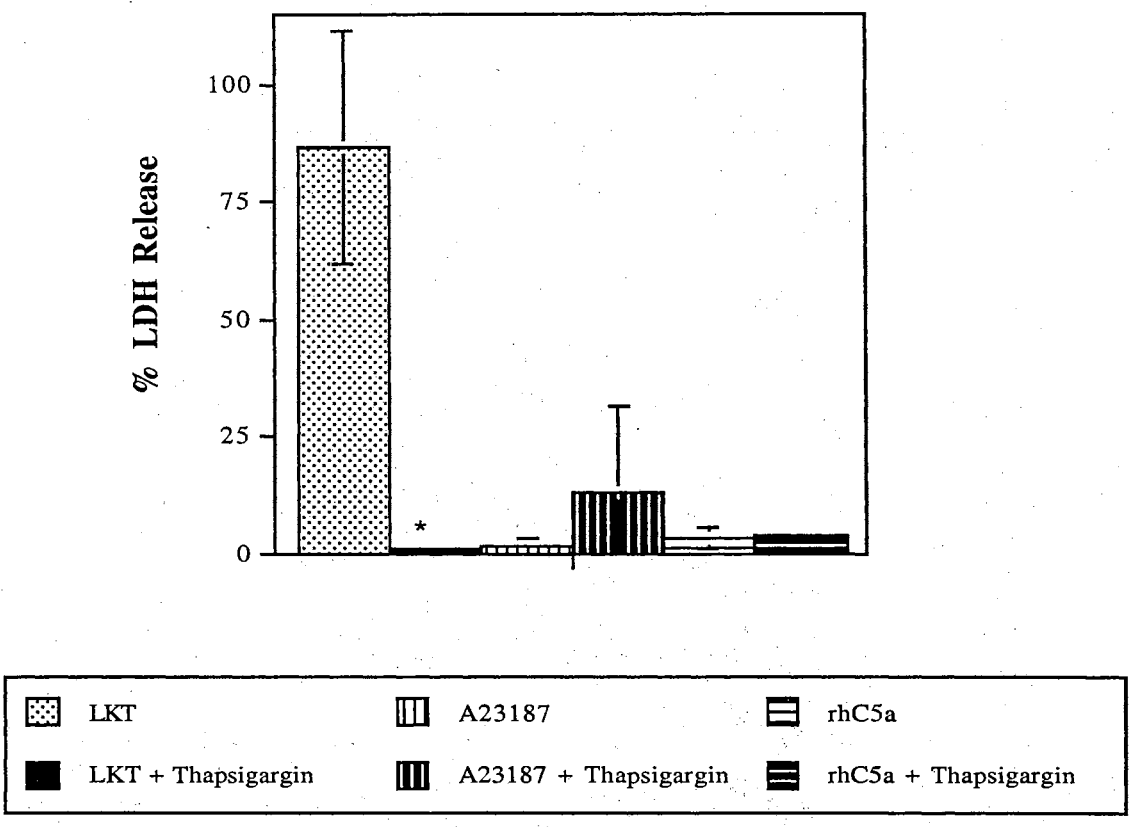


Figure 32- Inhibition of LKT-. A23187-. and rhC5a-induced increases in LDH leakage with thapsigargin. Cells were incubated with thapsigargin or diluent then exposed to LKT, A23187, or rhC5a and LDH leakage was measured at 120 minutes. Error bars indicate standard deviation for quadruplicate samples. * Indicates treatments with significant differences ($p < 0.05$) from treatments with the same stimulator without thapsigargin.

TABLE 4. Effects of verapamil on LKT-induced increase in $[Ca^{2+}]_i$ and LTB_4 production. Neutrophils were preincubated with verapamil, exposed to LKT or LKT(-), and responses were measured at 2 ($[Ca^{2+}]_i$) and 120 (LTB_4) minutes of incubation.

		Verapamil concentration in cell suspension			
		Solvent control	5 nM	0.5 μM	0.05 mM
$[Ca^{2+}]_i$ (nM)	LKT	215.0 ± 20.3*	239.9 ± 15.4*	212.7 ± 20.5*	184.5 ± 4.0†*
	LKT(-)	45.8 ± 4.5	46.8 ± 6.6	47.0 ± 7.3	35.4 ± 2.1
LTB_4 (pg/ml)	LKT	1316 ± 507*	1789 ± 593*	1529 ± 304*	157 ± 61†
	LKT(-)	865 ± 435	793 ± 336	574 ± 121	471 ± 289

* Value significantly different from corresponding LKT(-) control value (n=4).

† Value significantly different from corresonding No cation control value (n=4).

CHAPTER VIII

SUMMARY CONCLUSIONS

The central role of neutrophils in the pathogenesis of BRD has been well established. Influx of neutrophils into lungs infected with *P. haemolytica* not only fails to clear the infection, but contributes to the development of severe lung pathology (Yates *et al*, 1982; Slocombe *et al*, 1985; Breider *et al*, 1988; Wilkie and Shewen, 1988). One of the virulence factors of *P. haemolytica* responsible for the impaired function of neutrophils is LKT (Shewen and Wilkie, 1985; Confer *et al*, 1995). LKT can induce many effects in bovine neutrophils, depending upon the concentration of LKT; high concentrations are cytolytic, while low concentrations induce neutrophil activation and oxidative burst (Clinkenbeard *et al*, 1989; Czuprynski *et al*, 1990). The intracellular signal transduction mechanisms that mediate these effects of LKT on bovine neutrophils are believed to be Ca^{2+}–dependent. It has been reported that exposure of bovine neutrophils to LKT causes an increase in $[Ca^{2+}]_i$ (Ortiz-Carranza and Czuprynski, 1992) and that LKT-induced synthesis of LTB_4 and release of LDH by isolated bovine neutrophils are dependent on extracellular Ca^{2+} (Clinkenbeard *et al*, 1994. The overall goal of this study was to determine whether an increase in $[Ca^{2+}]_i$ served as the essential signal transduction mechanism for LKT-induced production of LTB_4 by bovine neutrophils, plasma membrane damage, and apoptosis, and to confirm that the LKT-induced increase in Ca^{2+} was excessive and unregulated and caused by both influx of extracellular Ca^{2+} across the plasma membrane as well as release of intracellular vesicular Ca^{2+}. This knowledge will aid in the development of strategies to control unregulated production of the chemoattractant, LTB_4, and impairment of neutrophil functions associated with BRD.

Initially, isolated neutrophils were used to study the intracellular Ca^{2+}-dependency of LKT-induced production of LTB_4 and plasma membrane damage. Exposure of neutrophils to LKT caused a rapid and concentration-dependent increase in $[Ca^{2+}]_i$, followed by simultaneous plasma membrane damage and production of LTB_4. Removal of extracellular Ca^{2+} or replacement of Ca^{2+} with other divalent cations, inhibited LKT-induced increases in $[Ca^{2+}]_i$, LTB_4 production, and membrane damage, thus indicating that increase of $[Ca^{2+}]_i$ associated with influx of extracellular Ca^{2+} are necessary to produce these LKT-induced neutrophil responses.

The Ca^{2+}-dependency of LKT-induced apoptosis was studied by first identifying the concentrations of LKT at which apoptotic changes could be observed by EM, gel electrophoresis, and a DNA fragmentation assay. Thereafter, the effect of chelation of extracellular Ca^{2+} on the ability of LKT to produce apoptosis was investigated. Only when exposed to low concentrations of LKT were apoptotic changes observed. Neutrophils exposed to low concentrations of LKT in Ca^{2+}-free buffers containing EGTA did not demonstrate any significant difference in the amount of DNA fragmentation present when compared to neutrophils exposed to LKT in the presence of Ca^{2+}. These results indicated that low concentrations of LKT induce apoptosis in bovine neutrophils but that Ca^{2+} is not an essential requirement for but does influence this process.

Having demonstrated that increased $[Ca^{2+}]_i$ serves as an essential signal transduction mechanism for LKT-induced production of LTB_4 and loss of plasma membrane integrity, the specific characteristics of this signal were compared with those produced by other stimulators to confirm that LKT induces an excessive and unregulated response. In contrast to rhC_{5a}, which caused a transient, two-fold increase in $[Ca^{2+}]_i$ and no effects on LTB_4 synthesis and membrane integrity, exposure to LKT resulted in a rapid,

sustained, five-fold increase in $[Ca^{2+}]_i$, followed by LTB4 synthesis. Although more rapid in onset, these responses to LKT were similar to those produced by A23187 and PMA, suggesting that LKT causes an unregulated stimulation of Ca^{2+}-mediated signal transduction and excessive inflammatory responses. Manipulation of the magnitude and duration of LKT- and A23187-induced $[Ca^{2+}]_i$ increase confirmed that a high and sustained $[Ca^{2+}]_i$ was necessary to stimulate production of LTB4.

The rapid rise in $[Ca^{2+}]_i$ of neutrophils exposed to LKT was not consistent with only passive diffusion of extracellular Ca^{2+} across membrane pores or channels, but was suggestive of an active process involving release of Ca^{2+} from intracellular stores. To test the contribution of vesicular Ca^{2+} release to LKT-induced increase in $[Ca^{2+}]_i$, specific inhibitors were used to isolate the roles of IP_3-mediated Ca^{2+} release and CICR and the involvement of VOC and ROC channels in flux of Ca^{2+} across the plasma membrane. LKT-induced increases in $[Ca^{2+}]_i$ were significantly attenuated by pharmacologically depleting intracellular Ca^{2+} stores using thapsigargin and by selectively inhibiting IP_3-mediated Ca^{2+} release and CICR. Although inhibitors of VOC (verapamil) and ROC ($LaCl_3$) channels attenuated LKT-induced $[Ca^{2+}]_i$ increase and LTB_4 synthesis, these effects were only evident at high drug concentrations, suggesting that the effects may be nonselective and directed at pores formed by the RTX toxin molecule(s). These results support the hypothesis that exposure of bovine neutrophils to LKT causes an influx of extracellular Ca^{2+} that triggers release of intracellular Ca^{2+} from the endoplasmic reticulum and that together these processes contribute to sustained and unregulated increase in $[Ca^{2+}]_i$, which activates the enzymes necessary for excessive LTB_4 production.

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

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