MECHANISMS OF *ESCHERICHIA COLI* ALPHA-HEMOLYSIN-INDUCED INCREASE IN INTRACELLULAR CALCIUM CONCENTRATION IN HL60 CELLS

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BL3	Bovine lymphoma cell line
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Intracellular calcium ion concentration
cADPR	Cyclic adenosine triphosphate
CD	Cell designate
CICR	Calcium-induced calcium release
Ehx	Enterohemorrhagic *E. coli* toxin
ER	Endoplasmic reticulum
G_i	G-inhibitory protein
HL60	Human leukemic cell line
IP_3	Inositol 1,4,5-triphosphate
IP_3-R	Inositol 1,4,5-triphosphate receptor
K^+	Potassium ion
KDa	Kilo dalton
HlyA	Alpha (α)-hemolysin
$HlyA_{c-}$	C-mutant alpha (α)-hemolysin
hlyA	One of four genes in the HlyA operon
hlyB	One of four genes in the HlyA operon
hlyC	One of four genes in the HlyA operon
hlyD	One of four genes in the HlyA operon
5-LO	5-Lipoxygenase pathway
La^{3+}	Lanthanum ion
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte functional antibody
LKT	leukotoxin
Mg^{2+}	Magnesium ion
Na^+	Sodium ion
NAADP	Nicotinic acid adenine dinucleotide phosphate
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMN	Polymorphornuclear
sRBC	Sheep red blood cells
ROCC	Receptor-operated calcium channels
RTX	Repeats in toxin
Ry-R	Ryanodine receptor
SDS-PAGE	Sodium dodecyl polyacrylamide gel electrophoresis
Tg	Thapsigargin

VOCC	Voltage-operated calcium channels
Wm	Wortmannin
XeC	Xestospongin C

CHAPTER I

INTRODUCTION

Alpha-hemolysin (HlyA) is a Repeat-in-ToXin (RTX) protein exotoxin produced by uropathogenic strains of *Escherichia coli*. The RTX family of bacterial exotoxins constitute a group of related cytolytic proteins that are produced by a wide variety of gram-negative human and animal pathogens. While diverse in their associated diseases and in their target cell specificities, they share common structural and functional features, genetic organization, and many cellular effects. Two distinct groups of RTX toxins have been identified, based on their target cell specificity: leukotoxins and hemolysins. Leukotoxins display the highest target cell specificity, affecting only ruminant leukocytes and platelets, whereas hemolysins attack a wide variety of animal species and cell types. HlyA affects a variety of target cells, ranging from ruminant erythrocytes (RBCs) to human leukocytes, which constitute the first line of defense against bacterial infection. Irrespective of their target cell specificity, RTX toxins injure target cells by interacting with plasma membranes, causing flux of ions and solutes across phospholipid bilayers (Welch *et al*, 1981). Studies conducted by Czuprynski, *et al* (1991), Ortiz-Carranza & Czuprynski (1992), and Cudd *et al* (1999) indicated that exposure of neutrophils to the RTX toxin, *Mannheimia haemolytica* leukotoxin (LktA), resulted in increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as well as Ca^{2+}-dependent stimulation of oxidative activity. Increased $[Ca^{2+}]_i$ also stimulates synthesis and release of inflammatory eicosanoids, such as LTB_4, which is a potent chemotactic agent for bovine

neutrophils. Stimulation of LTB_4 release follows Ca^{2+}-dependent activation of phospholipase A_2 and phospholipase D, which provide the arachidonic acid substrate used in the production of eicosanoids (Wang *et al*, 1998; Wang *et al*, 1999). Calcium-dependent release of inflammatory mediators from human leukocytes and platelets exposed to HlyA has also been observed (Konig *et al*, 1994), thus demonstrating that many of the effects of RTX toxins on target cells, particularly at sublytic concentrations, appear to be mediated by increase in $[Ca^{2+}]_i$. Therefore, the mechanisms involved in HlyA-induced increase in $[Ca^{2+}]_i$ must be clearly understood before rational strategies to combat the effects of this important virulence factor can be developed.

Increase in $[Ca^{2+}]_i$ of HlyA-exposed leukocytes could be caused by influx of Ca^{2+} across the plasma membrane (down a concentration gradient from 1.0 - 1.5 mM to 0.1 μM in the cytosol) and/or by release of Ca^{2+} from intracellular stores. The former mechanism has been the subject of several investigations that have suggested the involvement of voltage-operated Ca^{2+} channels (VOCC), based on inhibitory effects of the L-type VOCC inhibitor, verapamil (Ortiz-Carranza and Czuprynski, 1992; Hsuan *et al*, 1998). However, these inhibitory effects have been observed at drug concentrations considerably higher than those required to inhibit Ca^{2+} flux in excitable cells, such as neurons and muscle cells, thus suggesting that verapamil may inhibit Ca^{2+} flux nonspecifically, by inhibiting receptor-operated Ca^{2+} channels (ROCC) or perhaps even by direct interaction with toxin-induced membrane pores.

Therefore, the overall goal of this research project was to study the mechanisms involved in HlyA-induced increase in $[Ca^{2+}]_i$, including the involvement of VOCC and

ROCC in influx of extracellular Ca^{2+} and the contribution of Ca^{2+} released from intracellular Ca^{2+} stores.

CHAPTER II

LITERATURE REVIEW

Escherichia coli Alpha-Hemolysin – An RTX Toxin

Certain strains of *Escherichia coli* secrete a cytolytic toxin known as HlyA. These *E. coli* strains were first identified by the presence of clear zones of beta hemolysis around colonies cultured on blood agar plates and production of a heat labile, filterable hemolytic substance by bacteria growing in liquid media (Lovell and Rees, 1969; Cavalieri and Snyder, 1982). Synthesized during exponential growth periods (Williams, 1979), it is now believed that HlyA is a prominent virulence factor in human extraintestinal *E. coli* infections (Hacker *et al*, 1983; Knapp *et al*, 1986), especially pyelonephritis cases caused by uropathogenic *E. coli* (Laestadius *et al*, 2002).

HlyA belongs to a family of RTX toxins (Repeats-In-Toxin) that increase the permeability of target cell plasma membranes to a variety of ions, causing target cell swelling and lysis (Jorgensen *et al*, 1983; Bhakdi *et al*, 1986; Welch *et al*, 1995). HlyA is considered the structural model for the RTX family of toxins. It has three functional domains that are conserved in sequence among other RTX members: a large hydrophobic, N-terminal domain; a region predicted to be β-turn rich; and a carboxy-terminal domain consisting of glycine-rich tandem repeats (Welch *et al*, 1995), the consensus sequence of which has repeating units represented by L-X-G-G-X-G-N-D-X (Boehm *et al*, 1990a). This 110 KDa protein exotoxin is encoded by the *hlyCABD*

genetic operon. The product of the *hlyA* structural gene is activated by HlyC and secreted extracellularly via the sec-independent pathway involving both HlyB and HlyD in conjunction with the downstream TolC gene product (Welch *et al,* 1995).

Target Cell Specificity

HlyA is capable of causing lysis of a broad range of target cell types from a variety of animal hosts (Cavalieri and Snyder, 1982) including sheep erythrocytes (sRBC), human granulocytes (Boehm *et al,* 1990b), and renal epithelial cells (Laestadius *et al,* 2002; Uhlen *et al,* 2000). This non-discriminating target cell specificity is in sharp contrast to the leukotoxin members of the RTX family (Welch *et al,* 1995). For example, *Mannheimia haemolytica* (previously, *Pasteurella haemolytica*) leukotoxin (LktA), demonstrates a very narrow target cell range, affecting only ruminant leukocytes and platelets (Kaehler *et al,* 1980). A recently discovered member of the RTX family, enterohemorrhagic *E. coli* toxin (Ehx), also has a relatively narrow target cell specificity, affecting only human, sheep, and bovine erythrocytes, but not human leukocyte-derived cell lines (Bauer and Welch, 1996).

Effects on Target Cells

Based on varying effects of HlyA on the integrity of target cells and their membranes, three types of actions have been recognized: subpermeabilizing, permeabilizing, and lytic actions (Welch *et al,* 1995). While these actions have been

considered to be toxin concentration-dependent, it is probable that the degree of cell damage caused by exposure to RTX toxins is also incubation time-dependent (Clarke CR, personal communication). Therefore, depending on when HlyA-induced effects are measured, the same cells may first exhibit prelytic/sublytic responses and then undergo lysis as incubation time progresses.

Sublytic/prelytic effects of HlyA have been studied in many different cell types, but especially in neutrophils. At low toxin doses, neutrophils exhibit a marked chemiluminescent response, indicative of an oxidative burst, along with decreased chemotaxis and phagocytosis (Cavalieri *et al*, 1984). Low-dose exposure causes the loss of ATP from neutrophils along with other intracellular constituents necessary for phagocytic function (Welch *et al*, 1995). These permeabilizing lesions formed prior to lysis apparently are not repaired by the cell, and intracellular concentrations of ATP never return to normal following HlyA challenge. Therefore, at low levels of exposure, it appears that HlyA impedes the primary host defense function of neutrophils without immediately lysing them, while at higher doses rapid cell lysis occurs (Bhakdi *et al*, 1989).

Other target cell types react similarly to HlyA exposure. Rat mast cells released histamine at toxin concentrations that were similar to those causing neutrophil chemiluminescence (Scheffer *et al*, 1985). When exposed to subpermeabilizing concentrations of HlyA, monocytes released measurable amounts of interleukin-1β (Bhakdi *et al*, 1990). Sublytic effects of HlyA on renal proximal tubular cells included depletion of ATP stores and increased generation of superoxide radicals (Keane *et al*, 1987). Endothelial cells were permeabilized at low concentrations of HlyA, allowing

passive influx of $^{45}Ca^{2+}$ and production of prostacyclins from the arachidonic acid metabolic pathway in a Ca^{2+}-dependent manner (Suttrop *et al,* 1990).

Research conducted previously by Clarke and coworkers demonstrated that exposure of bovine neutrophils to *M. haemolytica* LktA stimulated the release of eicosanoids, such as leukotriene B_4 (LTB_4): These studies implicated LTB_4 as an important chemotactic agent for bovine neutrophils as well as a mediator of inflammation during *M. haemolytica* infection (Wang *et al,* 1998; Cudd *et al,* 1999). Synthesis of LTB_4 involves two key enzymes, phospholipase A_2 (PLA_2) and 5-lipoxygenase (5-LO). PLA_2 catalyzes the hydrolysis of cell membrane phospholipids to liberate arachidonate, which then serves as a substrate for 5-LO – catalyzed synthesis of LTB_4. In addition to generation of eicosanoid mediators, phospholipase action may contribute to LKT-induced loss of plasma membrane integrity via the formation of lysophospholipids. Wang *et al,* (1998) demonstrated conclusively, through studies using radiolabel release assays in conjunction with lactate dehydrogenase (LDH) leakage from exposed bovine neutrophils, that PLA_2 was involved in LKT-induced synthesis of LTB_4. This was shown to be a Ca^{2+}-dependent action, as removal of Ca^{2+} from the incubation medium resulted in a decrease in radiolabel and LDH release (Wang *et al,* 1999). Ca^{2+}-dependence was also demonstrated by using the Ca^{2+}-specific chelator EGTA. LKT-induced effects were restored when the Ca^{2+} concentration exceeded the chelating capacity of the EGTA present in the media.

Depending on the proximity of phagocytes to infecting organisms and the duration of infection, a range of RTX toxin-induced responses can be expected to occur *in vivo*. For example, neutrophils initially responding to an infection involving a RTX-

producing bacterium would be primed for respiratory burst activity. As neutrophils migrated closer to the site of infection, they would be exposed to higher permeabilizing concentrations of HlyA, causing influx of extracellular Ca^{2+}, stimulation of signal transduction pathways, and premature degranulation. Neutrophils located at the site of infection would be exposed to very high concentrations of toxin, which would cause serious impairment of their host defense capability and lysis (Welch *et al*, 1995).

Interaction of HlyA with Target Cell Membranes

HlyA has been demonstrated through immunological studies to be associated with the membranes of its target cells (Bhakdi *et al*, 1986; Bhakdi *et al*, 1989; Eberspacher *et al*, 1989), where it acts to increase the cation permeabilities of whole cells, such as erythrocytes, and artificial lipid bilayers (Boehm *et al*, 1990b; Jorgensen *et al*, 1983). Earlier studies, particularly those employing erythrocytes and artificial lipid bilayers, suggested that HlyA integrates into the plasma membrane to form transmembrane pores.

Evidence for Formation of Transmembrane Pores

Insertion of the N-terminal hydrophobic region of HlyA into a target cell plasma membrane has been postulated by a number of researchers to create a transmembrane pore capable of conducting ions (Figure 1). This toxin-induced ion channel is believed to consist of eight membrane-spanning alpha-helical runs per toxin molecule, based on computer models capable of predicting secondary protein structure based on amino acid

sequence. In support of this theory, Ludwig *et al* (1991) reported that amino acid substitutions in the N-terminal hydrophobic region resulted in a loss of hemolytic activity and that non-hemolytic isolates had mutations in this region. Interaction of HlyA with target membranes apparently is facilitated also by acylation of the protein, an activation step accomplished by LktC (Wagner *et al*, 1983; Welch *et al*, 1995; Rowe *et al*, 1994), and by the nine amino acid repeat region located immediately proximal to the export signal at the carboxyl terminus of the protein (Rowe *et al*, 1994; Welch 1995). Irrespective of the length of this region, which varies in the number of repeats among RTX family members (Lo *et al*, 1987), it has been shown to be essential for hemolytic activity (Felmlee *et al*, 1985). The principal function of the repeat region is to bind Ca^{2+}, presumably prior to export from the bacterial cell (Boehm *et al*, 1990a; Boehm *et al*, 1990b). Deletion of gene sequences encoding for this repeat region results in the production and export of a toxin that is non-hemolytic and incapable of associating with erythrocyte membranes (Ludwig *et al*, 1991).

Based on the observation that HlyA aggregates in solution, Ostolaza *et al* (1991) proposed that creation of a membrane pore involved integration and organization of the toxin into the membrane in the form of an oligomer, such as occurs with many gram-positive cytolysins (Bhakdi and Tranum-Jensen, 1986; Bhakdi and Tranum-Jensen, 1988). However, attempts to physically isolate an assembled HlyA oligomer by solubilizing toxin-treated membranes with deoxycholate, followed by sucrose-density gradient centrifugation and SDS-PAGE analysis, were unsuccessful (Bhakdi *et al*, 1986). Similar approaches had been successful in identifying stable oligomers of *Staphylococcus aureus* α-toxin (Bhakdi *et al*, 1981). Thus, it was proposed that HlyA integrates into the

plasma membrane in the form of a monomer (Boehm *et al*, 1990b), forming a functional pore with an estimated diameter of 1-3 nm (Benz *et al*, 1989; Menestrina *et al*, 1987). Evidence in support of HlyA monomers forming transmembrane pores is based principally on experiments conducted using planar lipid bilayers and erythrocytes. When added to lipid bilayers, HlyA monomers formed water-filled transmembrane pores in a voltage-dependent process (Menestrina *et al*, 1987). The properties of these pores were shown quantitatively to account for the observed effects of HlyA on erythrocytes.

Theories involving toxin-induced pore formation are consistent with the results of osmotic protection studies (Bhakdi *et al*, 1986; Clinkenbeard *et al*, 1989; Iwase *et al*, 1990; Ehrmann *et al*, 1991), the theoretical premise of which is that extracellular solutes with molecular diameters larger than the size of a pore formed in the target cell membrane will prevent oncotic lysis of target cells. Utilizing moderate to high molecular weight sugars as osmotic protectants, Bhakdi *et al* (1986) postulated the existence of pores in the form of protein lined, static-sized channels with diameters of approximately 3 nm, based on the observation that dextran-4 protected erythrocytes from hemolysis after 45 minutes of RBC exposure.

However, studies conducted by Moayeri and Welch (1994) indicated that osmotic protection, as a means of lesion size determination, was sensitive to environmental factors such as temperature, HlyA concentration, and incubation time, thus suggesting that membrane permeability may be related to a dynamic phenomenon more consistent with loss of membrane integrity due to the action of enzymes or a detergent effect of HlyA. Indeed, Ostolaza *et al* (1993) proposed that leakage of ions and diffusion of water across toxin-exposed membranes did not occur via toxin-formed pores but as a result of

detergent effects of HlyA on the membrane. These and other studies employing lipid bilayers concluded that the interactions between RTX toxins and target cell membranes were fairly nonspecific in nature and did not involve specific binding to membrane receptors, an interpretation that largely has been refuted by subsequent and more recent studies described below.

Involvement of Cell Membrane Receptors and Other Proteins in Binding of RTX Toxins

Interaction of RTX toxins with target cell membranes appears to be mediated by binding to specific cell surface receptors (Bhakdi *et al*, 1989), although, as described above, there is evidence that these toxins may disrupt the integrity of protein-free liposomes and planar lipid bilayers (Bhakdi *et al*, 1988), thus suggesting the possibility of non specific toxin-membrane interactions. The probable involvement of membrane surface receptors provides a rational explanation for the target cell specificities of RTX toxins. HlyA is active against an exceptionally broad range of target cells, including both human and animal leukocytes and erythrocytes. This non-discriminating target cell specificity is in sharp contrast to the leukotoxin members of the RTX family, which are capable only of causing lysis of relatively few target cell types, including leukocytes and platelets (Welch *et al*, 1995). However, despite the wide target cell specificity of HlyA, thus suggesting the absence of specific toxin-membrane interaction, Lally *et al* (1997) reported compelling evidence indicating that interaction with HL60 cells is mediated by specific binding to β_2 integrins, which serve as cell membrane surface receptors. HlyA-

induced lysis could be inhibited by monoclonal antibodies to αCD11a and αCD18, which constitute the LFA-1 receptor, a member of the β_2 integrin family.

There is evidence even that exposure to HlyA may cause subpermeabilizing effects on neutrophils that may not be related directly to integration of α-helical regions of the molecule into the cell membrane. Apparently, these effects are mediated by binding to cell surface receptors and activation of intracellular signal transduction pathways (Bhakdi *et al*, 1988). For example, HlyA has been shown to induce phosphoinositol turnover. Furthermore, the effects of HlyA can be inhibited by *Bordatella pertusis* toxin, which causes long-lasting activation of the G-inhibitory protein (G_i) for adenylate cyclase (Welch *et al*, 1995; Sperelakis, 1998). G_i involvement suggests G-protein linked receptor activation and stimulation of signal transduction pathways associated with leukotriene formation and oxidative burst (Grimminger *et al*, 1991a).

Effects of HlyA on ion permeability of Target Membranes

Bhakdi *et al* (1986) originally hypothesized that the membrane lesions produced by HlyA served as cation-selective channels. Based on this study, as well as the research conducted by Jorgensen *et al* (1983), which demonstrated a rapid efflux of cellular K^+ and influx of $^{45}Ca^{2+}$ preceding HlyA-induced hemolysis, cell damage and hemolysis was hypothesized to result from selective Ca^{2+} uptake (Bhakdi *et al*, 1986). Reports indicating that extracellular Ca^{2+} was required for induction of hemolysis by HlyA (Cavalieri *et al*, 1984) appeared to be compatible with this proposed hypothesis, which

was also supported by experiments conducted by Boehm *et al* (1990b), suggesting that lysis of target cells was the result of cation influx/efflux followed by influx of water due to the disruption of membrane integrity.

Experiments employing artificial lipid bilayers indicated that HlyA caused single channel conductance changes characterized by nonlinear dependence of membrane conductance on HlyA concentration over time (Benz *et al*; 1989). Other planar-lipid bilayer experiments described a linear dependence of membrane conductance on toxin concentration. These HlyA-mediated ion channels in planar lipid bilayers had limited longevity and displayed rapid switching between the open/closed states (Benz *et al* 1989).

Observations relating to single-channel conductance of artificial lipid bilayers are compatible with the osmotic lysis noted in erythrocytes following rapid K^+ efflux (Menestrina *et al,* 1987). Changes in conductance were voltage-dependent and proportional to the conductivity of the media. These cation-selective channels opened at a rate that was exponentially proportional to the applied voltage (Menestrina *et al,* 1987). Clinkenbeard *et al* (1989) concluded that *M. haemolytica* LktA creates pores in the plasma membranes of bovine BL3 cells that allow leakage of intracellular K^+ within minutes of exposure, but that larger cytosolic proteins were retained by the cell. This phenomenon was referred to as toxin-induced molecular sieving, which causes a relative increase in the intracellular osmotic pressure, resulting in cell swelling (Clinkenbeard *et al,* 1989). Following cell swelling, further membrane damage resulted in leakage of larger cytosolic components.

Calcium as a Second Messenger

Calcium has long been recognized as an essential mediator of many cellular functions, including fertilization, development, differentiation, adhesion, growth, division, movement, contraction, and secretion. Clearly, Ca^{2+} plays a very versatile role in many biological systems, including mechanisms of action of bacterial toxins (Cudd *et al*, 1999). Investigating the involvement of Ca^{2+} in RTX toxin-induced effects on target cells has required use of a variety of technical approaches, including alteration of extracellular Ca^{2+} concentration using chelators and use of pharmacological agents that specifically stimulate or inhibit Ca^{2+} homeostatic mechanisms. Pharmacological agents used include those that interact with specific types of Ca^{2+} channels to inhibit conductance (e.g., $LaCl_3$, verapamil), those that chelate Ca^{2+} (e.g., EGTA), or those that serve as ionophores to promote flux of Ca^{2+} across cell membranes (e.g., A23187). Other agents used include those that specifically inhibit or promote uptake or release of Ca^{2+} from intracellular stores (e.g., Mg^{2+}, xestospongin C, thapsigargin).

Changes in $[Ca^{2+}]_i$, which constitute an important signal transduction pathway for many different cell responses, typically result from opening of Ca^{2+} channels or activity of Ca^{2+} transporters (Berridge *et al*, 1998). These are localized either in the plasma membrane, or inside the cell in membranes of various organelles, especially the sarcoplasmic/endoplasmic reticulum (ER). Plasma membrane Ca^{2+} channels are differentiated according to their activation mechanism and include voltage-operated,

receptor-operated, and mechanically activated Ca^{2+} channels, as well as store-operated Ca^{2+} channels, which open in response to depletion of internal Ca^{2+} stores (Berridge *et al,* 1998). Ca^{2+} release from ER stores occurs via inositol 1,4,5-triphosphate (IP_3) receptors and ryanodine (Ry) receptors. Another less characterized channel known as the sphingolipid Ca^{2+}-release-mediating protein of ER (SCaMPER) is reported to release Ca^{2+} in response to an increase in sphingolipid concentrations. The differential expression of these channels provides cells with alternative strategies for responding to a diverse range of stimuli, thus producing Ca^{2+} signals that are tissue-specific (Berridge *et al,* 1998). When activated, both Ca^{2+}-entry and Ca^{2+}-release channels respond for a short time only to produce brief and well-controlled pulses of Ca^{2+} that create localized increases in $[Ca^{2+}]$. These events have been visualized by confocal microscopy techniques in living cells (Lipp and Niggli, 1997) and have a limited spatial range estimated to be 1-6 μm, with the $[Ca^{2+}]$ declining rapidly as the distance from the site of the channel increases. Regulation of cellular activities relies on close proximity of the Ca^{2+} channels to their effector mechanisms, allowing changes in $[Ca^{2+}]_i$ to have highly specific effects (Bootman and Berridge, 1995).

Release of Ca^{2+} from Intracellular Stores

In a normal resting state, cells utilize ATP-driven Ca^{2+} pumps to maintain low $[Ca^{2+}]_i$ of 100 nM or less. These pumps establish a steep $[Ca^{2+}]$ gradient between the cytosol and the extracellular environment as well as the intracellular stores (da Silva 2000). Three intracellular receptor-mediated pathways are responsible primarily for

mobilizing Ca^{2+} stores: those activated by IP_3 (Streb *et al,* 1983), cyclic adenosine diphosphoribose (cADPR), or nicotinic acid adenine dinucleotide phosphate ($NAADP^+$), initially described by Lee and coworkers (Lee, 1997, Lee *et al,* 1989). IP_3 and cADPR act as second messengers and endogenous ligands of the IP_3 and ryanodine (Ry) receptors, respectively. It has been demonstrated that the intracellular concentrations of IP_3 and cADPR increase upon stimulation of receptors on the plasma membrane, but similar results have not yet been reported for $NAADP^+$ (da Silva *et al,* 2000). The IP_3 and cADPR systems are highly conserved (Lee, 1997), perhaps because it is irrelevant to the cell which of the three Ca^{2+}-mobilizing system is activated when a global Ca^{2+} response is required. However, it is possible also that these apparently redundant mechanisms may act independently to produce localized changes in $[Ca^{2+}]_i$ that differ in amplitude and duration, thus producing finely tuned physiological responses.

Under resting conditions, the intracellular $[IP_3]$ is kept relatively low, but levels increase rapidly following stimulation of cell surface receptors and subsequent activation of the enzyme, phospholipase C (PLC), by G-proteins or by receptor- and non receptor-associated tyrosine kinases (Berridge, 1993). Activated PLC catalyzes the hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate to produce two secondary messengers, IP_3 and 1,2-diacylglycerol (da Silva *et al,* 2000). Pharmacological tools available to study IP_3-mediated Ca^{2+} release include compounds such as heparin and xestospongin C, which are potent and specific antagonists of the IP_3 receptor (IP_3-R) (Gafni *et al,* 1997).

cADPR is a cyclic derivative of β-nicotinamide adenine dinucleotide, which is synthesized by ADP-ribosyl cyclase (Lee *et al,* 1989). Occurring in both membrane-bound and soluble forms, cADPR mediates Ca^{2+} release by binding to Ry receptors (Ry-

R), historically identified with mechanisms of Ca^{2+}-induced Ca^{2+} release (CICR) (Fabiato, 1983). Three isoforms of Ry-R have been identified and specific stimulatory effects of cADPR on Ry-R subisotypes 2 and 3 have been demonstrated (Meszaros *et al,* 1993). Pharmacological studies have confirmed that cADPR is a physiological modulator of CICR (Galione, 1992) and that cADPR stimulation of the Ry-R/Ca^{2+} channel is promoted by Ca^{2+}, calmodulin, inorganic phosphate, and palmitoyl-CoA, and inhibited by high concentrations of Mg^{2+} (Lee, 1997; and Guse *et al,* 1996). Pharmacological tools used to study cADPR-mediated Ca^{2+} release include Ry and caffeine, which activate Ry-R, and ruthenium red, procaine, and various synthetic derivatives of cADPR, such as 7-deaza-8-Br-cADPR, which inhibit Ry-R (Guse, 2000). The ionic conductance of Ry-R generally is similar to that reported for the IP_3-R (Bezprozvanny and Erlich, 1994).

In contrast to the IP_3- and cADPR-mediated signaling pathways, many aspects of $NAADP^+$-mediated Ca^{2+} signaling are still unknown. For example, neither the identity of the enzyme involved in intracellular synthesis of $NAADP^+$ nor the intracellular receptor of $NAADP^+$ has been identified (da Silva *et al,* 2000).

Influx of Ca^{2+} Across the Plasma Membrane

A variety of channels in the plasma membrane have been identified that have specific conductances to Ca^{2+}. Depending on whether the conductances change in response to transmembrane voltage or to binding of specific ligands, they are broadly classified as voltage-operated Ca^{2+} channels (VOCC) or receptor-operated Ca^{2+} channels

(ROCC) (Tsein and Tsein, 1990; Spedding and Paoletti, 1992; Berridge, 1997). VOCC usually require steep membrane depolarization 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). Subclassifications of VOCC include L-type channels that are activated by high voltage and inhibited by dihydropyridine antagonists, T-type channels that are low-voltage operated and inhibited by low extracellular Ni^{2+} concentration, N-type channels that are high voltage activated and inhibited by ω-conotoxin, and P-type channels that are moderately high-voltage activated but not inhibited by dihydropyridine antagonists or ω-conotoxin (Tsein and Tsein, 1990).

ROCC open in direct response to binding of a ligand (Tsein and Tsein, 1990), although the function of these may also be dependent on changes in membrane potential. Nonspecific antagonism of ROCC can be accomplished using large trivalent cations, such as La^{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). $LaCl_3$ has been shown to completely block Ca^{2+} entry in human neutrophils exposed to fMLP, an activator of ROCC, while VOCC blockers had no effects on Ca^{2+} entry into the cell (Rosales and Brown, 1992).

In a variety of cell types, release of Ca^{2+} from intracellular stores and depletion of these stores triggers influx of Ca^{2+} into the cell via Ca^{2+} channels in the plasma membrane, a process termed capacitative Ca^{2+} entry (CCE) (Putney, 1986; Putney, 2001). Hoth and Penner (1992) measured the Ca^{2+} current associated with store depletion and termed it Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}). This I_{CRAC} pathway has been characterized as one of relatively low conductance (Zweifach *et al,* 1993) and susceptible

to bivalent and trivalent cation blockers, such as Ni^{2+} and La^{3+}. However, I_{CRAC} pathways were shown to be insensitive to L-type and N-type Ca^{2+}-channel blockers (Yao *et al*, 1994). Studies employing a *Drosophila* model suggested that the transient receptor potential (*trp*) gene product may function as a CCE channel (Friel, 1996; Putney *et al*, 2001). Over-expression of Trp has been shown to augment CCE (Kiselyov *et al*, 1998; Liu *et al*, 2000). Furthermore, there is general agreement from several studies that Trp3 and other closely related membrane proteins can be activated in an IP_3- and IP_3-R-dependent manner (Putney *et al*, 2001).

CCE has subsequently been confirmed to occur in numerous cell types, but mainly in non-excitable cell types such as endothelial, epithelial, and blood cells (Holda and Blatter, 1997; Putney, 2001). Recent evidence indicates that CCE plays an important role in many aspects of cellular Ca^{2+} signaling, such as volume regulation, mitogenesis, regulation of adenylate cyclase, and sustained Ca^{2+} oscillations (Berridge, 1995). Although the universal importance of CCE is generally recognized, until recently no consensus had been reached concerning the specific cellular mechanisms involved in this pathway. Several hypotheses have evolved to explain the mechanism of CCE (Putney and Bird, 1993; Clapham, 1995; Berridge, 1995) and these can be classified according to two proposed mechanisms for the retrograde Ca^{2+} entry signal: (1) Release of a diffusible second messenger, termed calcium influx factor (CIF), that is produced when Ca^{2+} stores become depleted and that stimulates Ca^{2+} influx across the plasma membrane. (2) Depletion of stored Ca^{2+} causes conformational changes that bring ER receptors into close proximity with plasma membrane receptors. To date, specific and direct pharmacological activators of CCE have not been identified. However, there are

pharmacological agents that will activate this pathway by virtue of their ability to deplete intracellular Ca^{2+} stores (Putney *et al*, 2001), such as thapsigargin, which inhibits uptake of Ca^{2+} by the smooth endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump (Putney, 2001).

Mechanisms of HlyA-Induced Increase $[Ca^{2+}]_i$ in

Most studies investigating the effects of HlyA on target cells have indicated that extracellular Ca^{2+} is necessary for induction of toxicity and that many of these effects can be mimicked using calcium ionophores, such as A23187. In experiments involving *in vitro* exposure of renal epithelial cells to HlyA, the toxin induced constant, low-frequency oscillations in $[Ca^{2+}]_i$ that could be inhibited by nifedipine, an L-type VOCC inhibitor (Uhlen *et al*, 2000). Furthermore, nifedipine and verapamil, another VOCC inhibitor, have been reported to inhibit increase in $[Ca^{2+}]_i$ in LktA-exposed bovine neutrophils (Ortiz-Carranza and Czuprynski, 1992; Hsuan et al, 1998). These experiments suggest that the increase in $[Ca^{2+}]_i$ induced by RTX toxins occurs by influx of Ca^{2+} via VOCC in the plasma membrane, presumably in response to a change in transmembrane potential. However, conclusions based on these studies suggesting the involvement of VOCC may not be correct because of the high concentrations of VOCC inhibitors required to produce inhibitory effects, relative to the concentrations required to inhibit excitable cells, such as neurons. Indeed, it is possible that these blockers may be acting on VOCC located on intracellular vesicular membranes or that they may be exerting nonspecific inhibitory effects on RTX toxin-induced transmembrane pores.

There is evidence that not all HlyA-induced effects are entirely dependent on influx of extracellular Ca^{2+}, but that release of intracellular, stored Ca^{2+} may also be involved. For example, Grimminger *et al* (1991b) suggested that that the marked capacity of low doses of HlyA to induce degranulation, respiratory burst, and lipid mediator generation in human neutrophils probably involved signal transduction mediated by products of phophatidyl inositol hydrolysis, which stimulate release of stored Ca^{2+} via the IP_3-R. Furthermore, Uhlen *et al* (2000) reported that HlyA-induced oscillations in $[Ca^{2+}]_i$ of renal epithelial cells could be inhibited by 2-aminoethoxydiphenyl borate, an IP_3-R antagonist and that inhibition of PLC, the enzyme that produces IP_3, caused a similar effect. These studies suggest that HlyA-induced increase in $[Ca^{2+}]_i$ may result from a combination of mechanisms involving both influx of extracellular Ca^{2+} across the plasma membrane and release of Ca^{2+} from intracellular stores. Considering the relevance of $[Ca^{2+}]_i$ to effects of HlyA on target cell structure and function, these mechanisms need to be specifically elucidated before pharmacological inhibitors can be developed to protect cells against the pathogenic effects of RTX toxins.

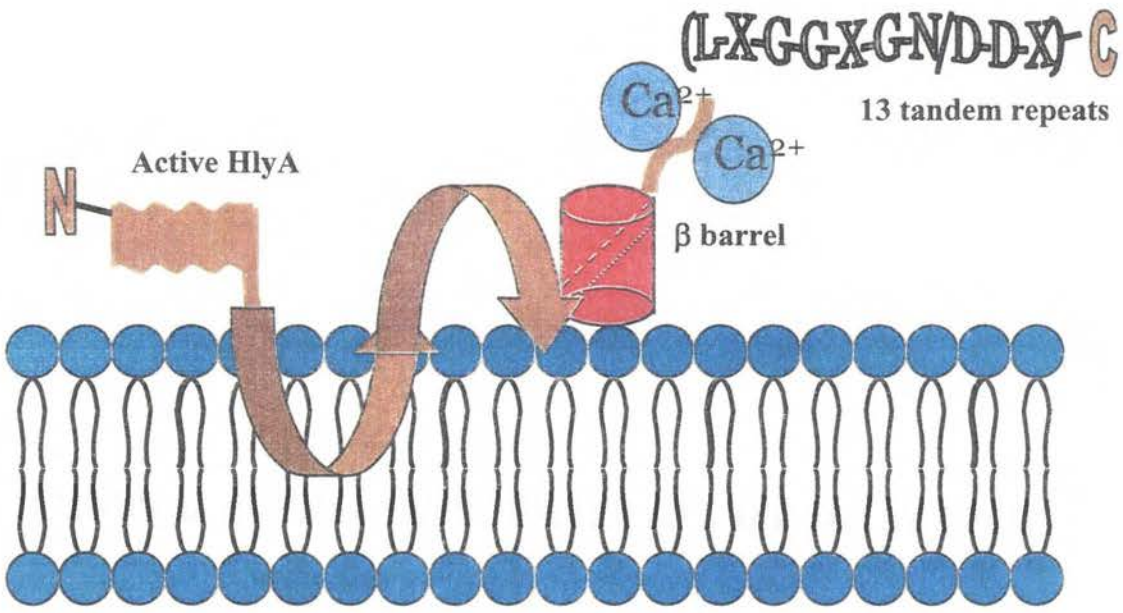

Figure 1. HlyA is postulated to associate with HL60 and other target cell membranes in a calcium-dependent manner. Amino acid analyses (Felmlee, 1985) suggests that N-terminal phobic runs integrate into the outer leaflet of the plasma membrane. Other HlyA structures include β-barrel and Ca^{2+}-binding domains. Binding to Ca^{2+} depends on the carboxy-terminal tandem repeats that are characteristic of the RTX toxin family. Figure adapted from Forestier and Welch, 1991.

CHAPTER III

HYPOTHESIS AND EXPERIMENTAL OBJECTIVES

Phase I

Previous studies conducted by Cudd *et al* (1999) and others (Ortiz-Carranza *et al*, 1992) reported inhibitory effects of VOCC blockers on both RTX toxin-induced increase in $[Ca^{2+}]_i$ and subsequent release of LTB_4, thus suggesting that influx of Ca^{2+} via VOCC may be an important event in the molecular pathogenesis of HlyA. Based on these observations, the initial hypothesis upon which this research was based was that increase in $[Ca^{2+}]_i$ induced by HlyA was caused by influx of extracellular Ca^{2+} through VOCC in the plasma membrane and that opening of these VOCC occurred in response to changes in membrane potential following HlyA-induced influx of monovalent cations (Figure 2).

This hypothesis was tested by measuring the effect of HlyA on $[Ca^{2+}]_i$, $[Na^{+}]_i$, and membrane potential of individual and suspended populations of HL60 cells, using fluorescent indicators, and the ability of verapamil, a VOCC blocker, to inhibit any HlyA-induced responses. To eliminate the potential contribution of other virulence factors produced *by E. coli*, such as LPS, effects of HlyA were compared to those produced by an HlyA-deficient mutant strain of *E. coli*.

Specific Phase I experimental objectives (addressed in Chapter IV) were to:

(1) prepare HlyA and negative toxin controls from strains WAM 582, WAM 783 and WAM 971, develop methods for the assay of toxin activity, and assess HlyA storage stability;

(2) confirm that exposure of HL60 cells to HlyA caused increased $[Ca^{2+}]_i$;

(3) investigate the effects of HlyA exposure on $[Na^+]_i$;

(4) determine whether exposure to HlyA caused changes in plasma membrane voltage potential; and

(5) determine whether any HlyA-induced increase in $[Ca^{2+}]_i$ and/or $[Na^+]_i$ could be inhibited by the VOCC blocker, verapamil.

Phase II

While the results of the studies conducted in Phase I confirmed that exposure of HL60 cells to HlyA caused changes in $[Ca^{2+}]_i$ and $[Na^+]_i$, it was clear that these effects were not associated with changes in membrane potential or exclusive involvement of VOCC. Based on further studies that implicated receptor-operated Ca^{2+} entry, the initial Phase I hypothesis was rejected in favor of an alternate hypothesis that HlyA-induced increase in $[Ca^{2+}]_i$ is not caused by voltage-dependent mechanisms but is caused instead by receptor-operated influx of extracellular Ca^{2+} through the plasma membrane and by release of Ca^{2+} from intracellular stores.

This alternate hypothesis was tested by investigating the affects of pharmacological inhibitors of Ca^{2+} transport across the plasma membrane and the membranes of intracellular storage vesicles, and by comparing the effects of HlyA on HL60 cells with similar cells (K562 cells) that do not express relevant cell surface receptors.

Specific Phase I experimental objectives (addressed in Chapter V) were to:

(1) explore the involvement of ROCC in HlyA challenge;

(2) investigate whether release of intracellular Ca^{2+} stores and capacitative Ca^{2+} entry contribute to HlyA-induced changes in $[Ca^{2+}]_i$; and

(3) determine whether HlyA binding to surface-expressed β_2 integrin receptors is involved in receptor-operated Ca^{2+} entry.

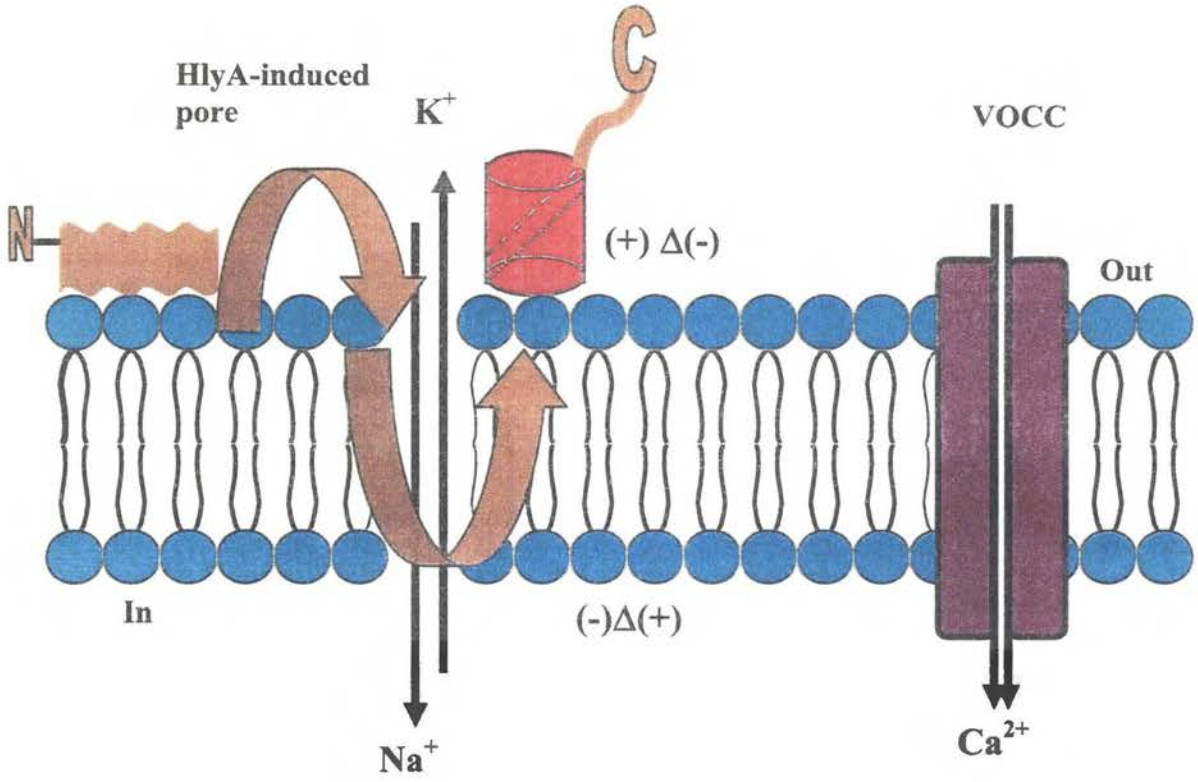

Figure 2. Initial hypothesis describing formation of a transmembrane pore by insertion of hydrophobic N-terminal amino acids into the plasma membrane. Flux of monovalent cations through this toxin-induced pore was postulated to cause changes in transmembrane potential that stimulated opening of VOCC and influx of extracellular Ca^{2+}.

CHAPTER IV

ROLE OF VOLTAGE-OPERATED CALCIUM CHANNELS IN *ESCHERICHIA COLI* ALPHA HEMOLYSIN-INDUCED INCREASE IN INTRACELLULAR CALCIUM CONCENTRATION OF HL60 CELLS

Introduction

Escherichia coli normally represents a major component of the intestinal flora in humans, but some strains may colonize and infect extra-intestinal sites, such as the urinary tract. In particular, strains producing HlyA can cause severe damage to the kidneys, resulting in permanent scarring and loss of function (Laestadius *et al*, 2002). Other HlyA-related illnesses include peritonitis and meningitis (Menestrina *et al*, 1994).

HlyA is a member of the family of RTX toxins, characterized by amino acid glycine-rich tandem repeats proximal to the carboxyl terminus of these proteins (Bohach *et al*, 1988). Hemolytic and/or cytotoxic effects of RTX toxins on mammalian target cells are Ca^{2+}-dependent (Boehm *et al*, 1990), as evidenced by the requirement that Ca^{2+} be bound to the toxin molecule for full expression of activity and the role of increased $[Ca^{2+}]_i$ in mediating effects on target cells, including renal epithelial cells and leukocytes (Uhlen *et al*, 2000; Grimminger *et al*, 1991b).

Previous studies reported that inhibitors of VOCC inhibited increase in $[Ca^{2+}]_i$ in renal epithelial cells exposed to HlyA and in neutrophils exposed to another RTX toxin, *Mannheimia haemolytica* LktA. These observations suggest that increase in $[Ca^{2+}]_i$ is

caused by influx of extracellular Ca^{2+} through VOCC in the plasma membranes of target cells, presumably in conjunction with a change in membrane potential caused by transmembrane flux of monovalent ions, such as Na^{+}. However, this proposed involvement of VOCC in RTX-toxin induced increase in $[Ca^{2+}]_i$ may not be correct because high concentrations of VOCC inhibitors generally are required to produce inhibitory effects (compared with the concentrations required to inhibit excitable cells, such as neurons) and the lack of credible evidence indicating that VOCC even exist in non-excitable neutrophils (von Tscharner *et al*, 1986). It is possible that VOCC inhibitors may be exerting nonspecific inhibitory effects on RTX toxin-induced transmembrane pores, thus limiting the potential use of these agents in the therapy of RTX toxin-mediated diseases.

Therefore, the objectives of this phase of study were to: (1) develop methods for preparation, assay, and storage of HlyA and negative toxin controls; (2) confirm that exposure of HL60 cells to HlyA caused increased $[Ca^{2+}]_i$; (3) investigate the effects of HlyA exposure on the monovalent ion, $[Na^{+}]_i$; (4) determine whether exposure to HlyA caused changes in plasma membrane voltage potential; and (5) determine whether any HlyA-induced increase in $[Ca^{2+}]_i$ and/or $[Na^{+}]_i$ could be inhibited by VOCC inhibitors.

Materials and Methods

Preparation of *E. coli* HlyA

HlyA and $HlyA_c$- negative control were prepared by harvesting culture supernatants from *Escherichia coli* host DH1 (Forrestier *et al*, 1991) expressing

*hly*CABD (WAM 582) and *hly*ABD (WAM 783) genes, respectively. In contrast to WAM 582, which produced the active 110 kDa toxin protein (Rowe *et al*, 1994), WAM 783 produced a nonacylated inactive form of the toxin. Another control strain, WAM 971, contained only the plasmid used as a vector during transfection.

Bacterial strains were cultured on Luria agar (Sigma Chemical Co.) and selected on the basis of resistance to 20 μg/ml chloramphenicol (C^m). Isolated colonies were then cultured in 10 ml Luria Broth (LB) base and 20 μg/ml C^m overnight at 37°C at 160 oscillations/minute. These cultures were expanded into 1 L LB containing 20 μg/ml C^m with shaking (160 osc/min.) and grown to an optical density (OD_{600}) of 0.85. Preliminary studies revealed that the bacterial strains grew exponentially after an initial 2-hour lag period (Figure 3) and that production of HlyA (as measured by lytic activity) occurred in parallel with bacterial replication. Maximal production of active HlyA was achieved after 7 hours of incubation.

Further preparation of toxin was conducted at 4°C. Culture supernatants were collected following centrifugation at 8,000 rpm for 30 minutes (Sorvall RC 5B Plus using Superlite GS-3 rotor by DuPont) and concentrated by addition of ammonium sulfate (361 g/l) with gentle stirring for one hour to yield 60% saturation. The precipitated material was collected by centrifugation at 8,500 rpm for 45 minutes (Sorvall RC 5B Plus, using Superlite GS-3 rotor by DuPont), resuspended in 5 ml phosphate buffered saline (PBS), and dialyzed for 4 hours against 1 liter of PBS for x3 buffer changes. Dialyzed, concentrated culture supernatants were aliquoted and stored frozen at -135°C until used.

The purity of toxin preparations was assessed by SDS-PAGE. Briefly, toxin preparations were mixed 1:2 in SDS-PAGE sample buffer (Sigma Chemical Co.) and

boiled for 5 min. Samples were subjected to SDS-PAGE on 10% gels, which were then silver stained (Daiichi Silver Stain II, Integrated Separation Systems). Preparations produced multi-banded lanes (Figure 4), consistent with the multimeric protein aggregates demonstrated in previous studies (Ostolaza *et al*, 1991).

Assay of HlyA activity

Prior to freezing, baseline lytic activity of each HlyA preparation was assessed using sheep erythrocytes (sRBC) and HL60 cells, by measuring the release of hemoglobin (Hb) and lactate dehydrogenase (LDH), respectively, after exposure to HlyA or toxin controls (Figure 5). Lysis of sRBC represents a common method of estimating HlyA activity and served as a basis for comparison with other published reports. Lysis of HL60 cells provided a measure of activity that was directly relevant to the present study and was necessary to calculate appropriate dilutions of toxin in each of the experiments. HL60 lytic activity of thawed samples was assessed on a weekly basis to ensure that HlyA preparations used in experiments were at least 70% of initial activity.

Target cells were exposed to HlyA and control preparations in 96-well round bottom microtiter plates by serial dilution in assay buffer (pH 7.2, 1 mM $CaCl_2$ in modified Hank's Buffered Saline Solution [HBSS], Sigma Chemical Co.) to a total volume of 150 µl. Target cells (100 µl of either 1% (v/v) sRBC or $4x10^4$/ml HL60) were added to all wells and the plates were incubated at 37°C for 1 hour. Toxic exposure was terminated by centrifugation (7 min at 700 x g) and 100 µl of assay supernatant was

transferred to a flat-bottom 96-well plate in order to determine the amount of Hb or LDH release.

Hb concentration in supernatants was determined at room temperature using a plate reader (ThermoMax, Molecular Devices) set at OD_{550}. Maximal Hb leakage was achieved by replacing HlyA with 50 µL of 0.01% saponin solution (Sigma Chemical Co.). Background absorbance was determined by replacing HlyA with 100 µl assay buffer. Likewise, the amount of LDH released was determined at 37°C using a thermally controlled microtiter plate reader at OD_{340} (ThermoMax, Molecular Devices), as previously described (Clinkenbeard *et al*, 1994). Percent specific release of either Hb or LDH was calculated using the following equation:

$$\% \text{ Specific release} = 100 \times (A-B)/(C-B)$$

where A, B, and C are the toxin-induced release, background absorbance, and maximum release values, respectively. HlyA activity was expressed as toxic units (TU), where 1 TU was defined as the dilution of HlyA required to cause 50% maximal release. Comparison between % specific release of Hb and LDH from sRBC and HL60 cells, respectively, revealed that sRBC were more sensitive to the effects of HlyA, suggesting that the mechanisms of intoxication may differ between these two cell types (Figure 5).

Alpha hemolysin storage and optimization studies

Preliminary experiments were conducted to estimate the time course of HL60 cell intoxication and to determine the stability of toxin preparations under storage conditions.

Measurement of % specific release of LDH from HL60 cells exposed to 5 TU/ml HlyA before and after 15, 30, 60, 90 and 120 minutes of incubation at 37°C indicated that maximum release was achieved between 60 – 90 minutes (Figure 6).

Stability of frozen HlyA was investigated by measuring the % specific release of LDH after 4, 6, 8, 14, 21 and 25 days of storage at -135°C. Although activity declined as storage time increased, a predictable relationship between these variables could not be established, thus necessitating performance of LH60 activity assays immediately prior to conducting each set of experiments.

Preparation of HL60 cells

HL60 cells were obtained from American Type Cell Culture (ATCC) and cultured in Dulbecco's Modified Eagles Medium (DMEM) with 10% FBS (pH 7.1) in an incubator at 37°C and 5% CO_2. Harvested cells were resuspended in HBSS containing 1 mM Ca^{2+} (Ca-HBSS, pH 7.1), washed x3 in 50 ml of Ca-HBSS by centrifugation at 1,082 x g, and then resuspended in 10 ml Ca-HBSS at a concentration of 4.8 x 10^6/ml, as determined by hemocytometer.

Measurement of intracellular cation concentrations in isolated single cells

Intracellular concentrations of Ca^{2+} and Na^+ in single isolated cells were measured using the ratiometric fluorescent indicators, Fura-2 and Sodium Benzofuran Isophthalate (SBFI), respectively. For $[Ca^{2+}]_i$ determination, HL60 cells suspended in Ca-HBSS in a

light-tight 50 ml culture tube were incubated at room temperature for 30 minutes with 5 μM Fura-2 (Molecular Probes). Loading of fluorescent indicator was terminated by centrifugation (10 minutes at 200 x g) and loaded cells were washed twice in 50 ml Ca-HBSS and then resuspended to a final volume of 10 ml.

For $[Na^+]_i$ determination (Harootunian *et al,* 1989), HL60 cells were suspended in 10 ml of 10 mM HEPES buffered DMEM (pH 7.1, 4.8 x 10^6 cells/ml) in a light-tight 50 ml culture tube, which was incubated for 45 minutes with 5 μM SBFI (Molecular Probes) at 37°C (5% CO_2). Loading of fluorescent indicator was terminated by centrifugation (10 minutes at 250 x g) and loaded cells were washed twice in 50 ml of 10 mM HEPES buffered DMEM and then resuspended in 10 ml of 150 mM Na^+-HEPES (130 mM Na-Gluconate, 30 mM NaCl, 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgSO_4$, pH adjusted to 7.1 with n-methyl-glucamine). Depending on the experimental requirements, cells were suspended in 150 mM K^+-HEPES for experiments requiring absence of Na^+ or mixed in different ratios with 150 mM Na^+-HEPES to obtain required Na^+ concentrations.

Fluorescence intensities of HL60 cells exposed to HlyA or controls were measured using a fluorescence microscopy system for ratiometric determination of intracellular ion concentrations. Cells were immobilized on poly-L-lysine (Sigma Chemical Co., St. Louis) coated cover slips (12 mm-diameter) installed in open cell chambers: Cell suspension (500 μl) were pipetted onto each cover slip and allowed to settle by gravity for 1-5 minutes before replacing the suspension buffer with the appropriate experimental buffer.

Microscopic fields including at least 6 cells were imaged in real time using an inverted microscope fitted with an intensified charge-coupled device camera (Photon

Technology International, New Jersey). Digitized images were collected before and after exposure to HlyA and controls by monitoring the 510 nm fluorescence emissions generated by 360/380 nm and 365/380 nm excitation of fura-2 or SBFI loaded cells, respectively. These images were stored and then analyzed using software (Image Master 2.0, Photon Technology International, New Jersey) for measurement of fluorescence intensity in selected regions of interest in the microscopic field.

The following equation was used to calculate $[Ca^{2+}]_i$ (Grynkiewicz *et al*,1985):

$$[Ca^{2+}]_i \text{ or } [Na^+]_i = K_d\,(R - R_{min}/R_{max} - R)\,\beta$$

where R is the ratio in emission intensities observed at the 2 excitation wavelengths. The maximal ratio (R_{max}) was determined by exposure to the Ca^{2+} ionophore, 4-Br-A23187 (10 μM), or to gramicidin (10 μM) in the presence of 1 mM Ca^{2+} (Ca-HBSS) or 150 mM Na^+-HEPES, respectively. The minimal ratio (R_{min}) was determined in a separate cell chamber by incubating HL60 cells in the absence of extracellular Ca^{2+} for 20 minutes prior to image acquisition. No autofluorescence was detected at any of the excitation wavelengths and, therefore, no background subtractions were required. The dissociation constants (K_d) used for Fura-2- or SBFI-loaded HL60 cells were 150.5 nM and 3.8 mM, respectively. Beta (β) is a ratio-generated constant calculated using the 380 nm excitation wavelength at zero [cation] and saturating fluorescent indicator conditions. Preliminary wavelength scans of loaded cells were conducted to confirm appropriate selection of excitation wavelengths.

Measurement of intracellular Ca^{2+} concentrations in cell suspensions

For cell suspension (population) studies, Fluo-3 was used for measurement of $[Ca^{2+}]_i$. HL60 cells were loaded using the acetoxymethyl ester of Fluo-3 (Fluo-3/AM, Molecular Probes Inc., Eugene), as described for Fura-2. Briefly, Fluo-3 suspensions 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 Ca-HBSS, enumerated by hemocytometer and then resuspended in Ca-HBSS to 1 x 10^7 cells/ml.

The effects of HlyA and controls on $[Ca^{2+}]_i$ in Fluo-3-loaded HL60 cells 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 toxin or control preparations 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 (Victor^2, Perkin Elmer).

Experiments were terminated by centrifugation at 200 x g at 4°C for 5 minutes. All experiments included quadruplicate wells for each of the primary treatments.

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

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

using a K_d value of 358 nM, as determined in previous studies (Cudd *et al*, 1999). 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:

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

Estimation of changes in voltage potential of isolated HL60 cells

Changes in voltage membrane potential were studied using the fluorescent indicator, bis-1,3-dibutylbarbituric acid trimethine oxonol ($DiBAC_4$, Molecular Probes,

Eugene). Changes in membrane potential cause a shift in the oxonol chromophore between a membrane binding site and the aqueous region on the surface of the membrane, as described by George *et al* (1988). HL60 cells harvested in 10mM HEPES buffered DMEM (pH 7.1) were prepared as described above. Cells were loaded with fluorescent indicator by adding 200 µl of 250µM $DiBAC_4$ in DMSO to 10 ml suspension to yield 50 µM $DiBAC_4$. The suspension then was mixed gently for 1 minute before incubating in the dark at 37°C with 5% CO_2 for 29 minutes. Loading was terminated by washing 3 times in 10mM HEPES buffered DMEM (centrifugation for 10 minutes at 200 x g). The final pellet was resuspended in 10 ml 1 mM Ca-HBSS.

Change in voltage potential was assessed by measuring the intensity of fluorescence emission at 512 nm generated by excitation at 488 nm. Based on the linear correlation between emission intensity and voltage potential and the absence of any specific interest in quantifying voltage potential, non-calibrated raw intensity data were used to assess the effects of HlyA on this response variable. The positive control employed in these studies was 60 mM KCl, which causes membrane depolarization.

Effects of HlyA on $[Na^+]_i$, $[Ca^{2+}]_i$, and plasma membrane potential and the role of VOCC.

The involvement of VOCC in HlyA-induced increase in $[Ca^{2+}]_i$ was studied by investigating whether HlyA was capable of causing transmembrane flux of the monovalent cation, Na^+, and changes in membrane voltage, the principal triggers most likely to cause opening of VOCC, and whether any HlyA-induced effects on

transmembrane cation flux could be inhibited by using the VOCC inhibitors, verapamil and nifedipine. The ability of HlyA to induce an increase in $[Ca^{2+}]_i$ was first confirmed by exposing HL60 cells loaded with Fura-2 to HlyA (0.25, 0.5, and 1 TU/ml), $HlyA_c$, and 4Br-A23187 (40 μM), and measuring fluorescence emission of individual cells at 2 second intervals, before and for 15 minutes after toxin addition. Effects of HlyA on $[Na^+]_i$ were studied by exposing SBFI-loaded HL60 cells to HlyA (1 and 5 TU/ml), $HlyA_c$, or gramicidin, and measuring $[Na^+]_i$ of individual cells before and at 5 second intervals thereafter for 20 minutes. In an attempt to correlate effects of HlyA on monovalent cation flux with changes in membrane voltage potential, HL60 cells loaded with $DiBAC_4$ were then exposed to HlyA (1 and 5 TU/ml), $HlyA_c$, and KCl, and the fluorescence emissions of individual cells were measured at 5-second intervals before and for a period of 20 minutes after toxin exposure. KCl served as a positive control to confirm the functionality of the fluorescent indicator. Finally, effects of VOCC inhibitors on HlyA-induced increase on $[Ca^{2+}]_i$ were tested, both in individual cells as well as in cell suspensions, by exposing HL60 cells to HlyA in absence or presence of verapamil or nifedipine. HL60 cells were pretreated with drug solvent (ethanol) or 0.5, 5, or 50 μM verapamil for 20 minutes prior to HlyA exposure.

In anticipation of the possibility that the hypothesized mechanism of HlyA intoxication involving influx of Ca^{2+} via VOCC would not be supported by the experiments described above, an additional study was designed to explore other mechanisms of HlyA-induced increase in $[Ca^{2+}]_i$, such as influx of Ca^{2+} across the plasma membrane via ROCC and release of Ca^{2+} from intracellular stores: The possible involvement of these other mechanisms was explored in a preliminary experiment by

pretreating HL60 cells with 50 μM verapamil, 500 μM $LaCl_3$, or 500 μM $MgCl_2$ before exposing Fluo-3 – loaded cells to 1 TU/ml HlyA or $HlyA_{c-}$. Lanthanum chloride and $MgCl_2$ are inhibitors of plasma membrane Ca^{2+} channels (including both VOCC and ROCC) and release of Ca^{2+} via Ry-R on vescicular Ca^{2+} stores, respectively.

Results

Growth patterns of the WAM 582 and WAM 783 bacterial strains were virtually identical, but only culture supernatant from WAM 582 caused any lysis of target sRBC or HL60 cells, thus confirming that the $HlyA_{c-}$ produced by WAM 783 served as a suitable negative control for the partially purified HlyA produced by WAM 582. Comparison between lytic effects of HlyA on sRBC versus HL60 cells revealed that the former were much more sensitive: Percent specific lysis values of 50% were achieved at 1:64 and 1:4,200 dilutions of 1 TU/ml HlyA incubated with HL60 and sRBC, respectively. The relative sensitivities of these cells confirmed the necessity of basing selection of HlyA dilutions used in each experiment on activity against HL60 cells rather than using the more widely employed sRBC assay.

Single HL60 cell fluorescence studies confirmed that HlyA exposure causes an oscillatory increase in $[Ca^{2+}]_i$, which was more pronounced at the lower toxin concentrations (Figure 7). $HlyA_{c-}$ failed to produce any change in $[Ca^{2+}]_i$. Exposure of HL60 cells to HlyA also caused significant oscillatory increases in $[Na^{+}]_i$ that were similar in magnitude and frequency to those caused by the positive control, gramicidin (Figure 8). Again, the lower 1 TU/ml HlyA concentration paradoxically produced a

greater response than that produced by the higher 5 TU/ml concentration, which was no different from that of the $HlyA_{c-}$ negative control. However, these effects of HlyA on the distribution of monovalent and divalent cations across the plasma membrane failed to affect membrane voltage potential, as measured by fluorescence intensity of $DiBAC_4$ (Figure 9). Exposure of these HL60 cells to KCl, the positive control, confirmed that the assay methodology would have identified any changes in membrane potential in response to HlyA had they occurred.

Studies involving imaging of individual cells revealed that the VOCC inhibitor, verapamil, significantly attenuated the increase in $[Ca^{2+}]_i$ induced by HlyA, but only for a brief period after initial exposure to the toxin (Figure 10). Similarly, verapamil significantly inhibited HlyA-induced increase in $[Na^+]_i$, but only when used at the high 50 μM concentration (Figure 11). Studies involving HL60 cell suspensions were less definitive regarding inhibitory effects of verapamil: The 0.5 μM and 5 μM concentrations of verapamil failed to cause significant inhibition of HlyA-induced increase in $[Ca^{2+}]_I$ (Figure 12). However, when the much larger 50 μM concentration of verapamil was employed, HlyA-induced increase in $[Ca^{2+}]_i$ was enhanced, at both the 1 TU and 4 TU toxin concentrations. Clearly, the lack of a concentration-dependent inhibitory effect of verapamil on HlyA-induced increase in $[Ca^{2+}]_i$ and the inhibitory effects of verapamil on HlyA-induced increase in $[Na^+]_i$ were not representative of a specific action on VOCC, thus bringing into question previous assumptions that inhibitory effects of verapamil on RTX toxin-induced increase in $[Ca^{2+}]_i$ involved influx of extracellular Ca^{2+} through VOCC. Indeed, when the effects of verapamil were compared with those produced by lanthanum and $MgCl_2$, it was clear from the significant

inhibitory effects of the latter two agents that other mechanisms of $[Ca^{2+}]_i$ must be investigated (Figure 13).

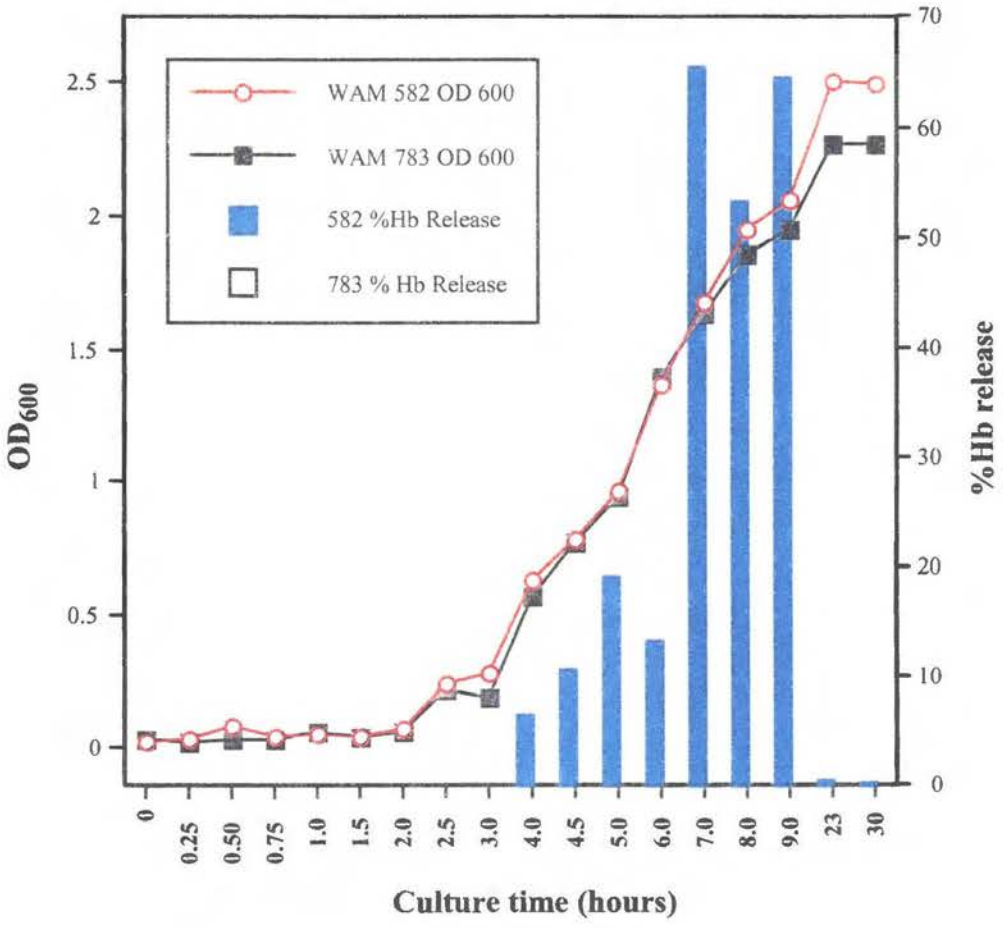

Figure 3. Growth rate (OD_{600}) of *Escherichia coli* WAM 582 and WAM 783 strains and ability of culture supernatants to cause lysis of sRBC (%Hb release). After a 2-hour lag period, strains grew exponentially. Maximum HlyA production, estimated by sRBC lytic activity of culture supernatants, was achieved by 7 hours of WAM 582 culture at 37°C, 5% CO_2.

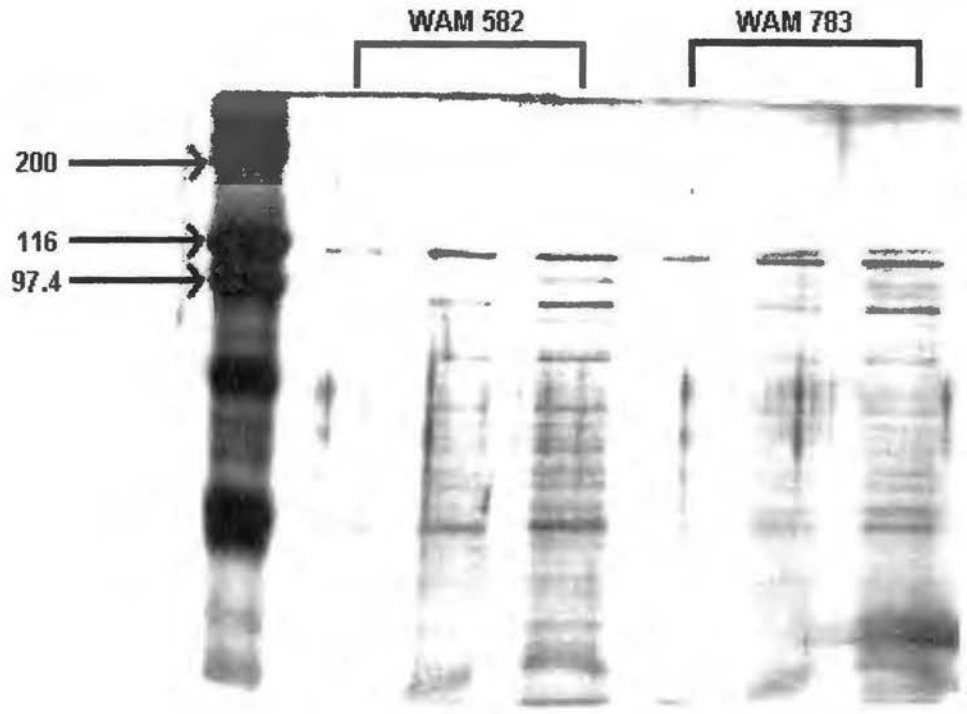

Figure 4. SDS-PAGE of *E. coli* WAM 582 and WAM 783 strains (n = 3), indicating multimeric banding pattern characteristic of partially purified preparations of RTX toxins. Previous studies have determined that HlyA is a 110 kDa exoprotein.

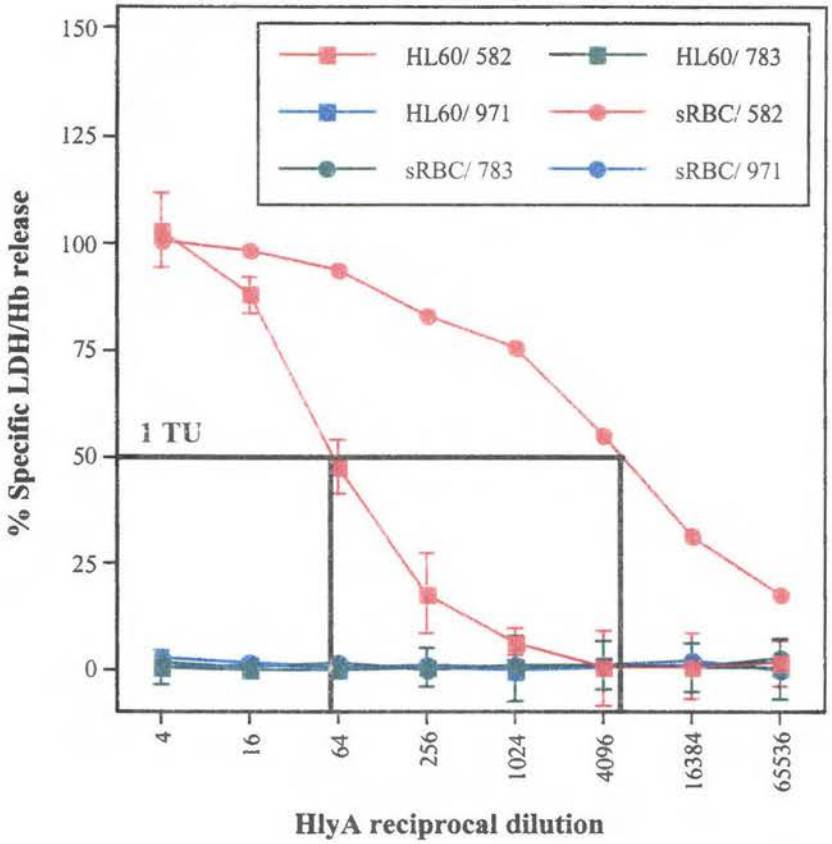

Figure 5. Effects of WAM 582 (HlyA), WAM 783 ($HlyA_{c-}$), and WAM 971 toxin preparations on the release of LDH and Hb from exposed HL60 cells and sRBC, respectively. Isolated HL60 cells were exposed to dilutions of toxin preparations for 60 minutes (n = 3). HlyA caused lysis of both sRBC and HL60 cells, while preparations from the control strains, WAM 783 and WAM 971, had no effect on cell integrity. HlyA activities of 1 TU were achieved by dilutions of approximately 1:64 and 1:4,096 for HL60 cells and sRBC, respectively, indicating that sRBC are relatively more sensitive to the lytic effects of HlyA.

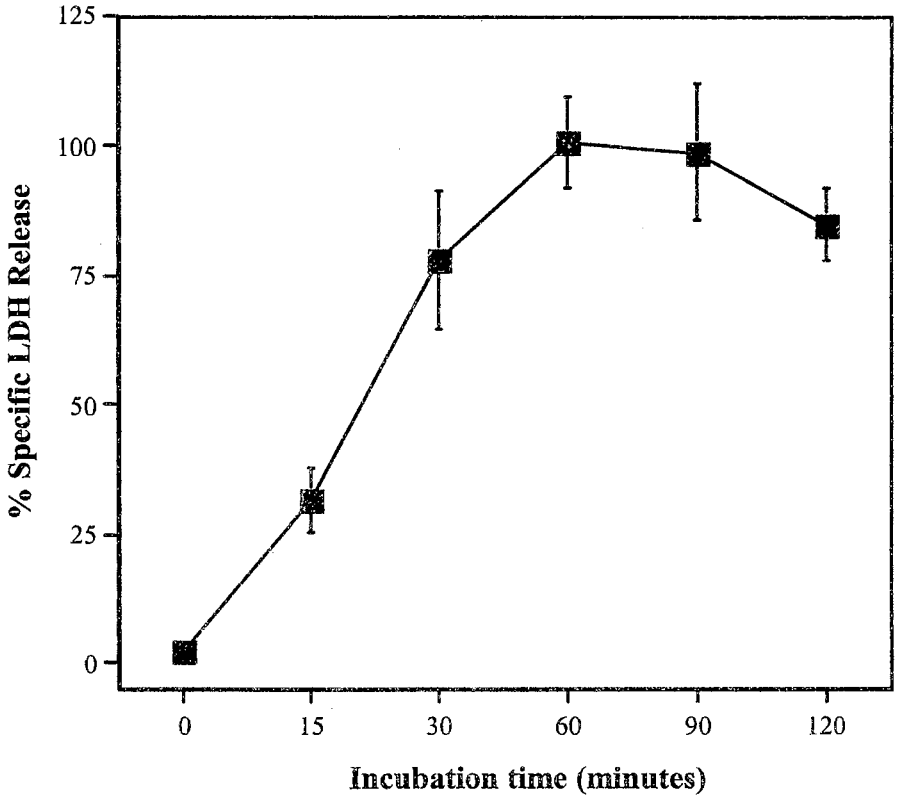

Figure 6. Mean (± SD) % specific LDH release from HL60 cells exposed to 5 TU/ml HlyA (n = 3). Maximal effects were achieved by 60 minutes of incubation.

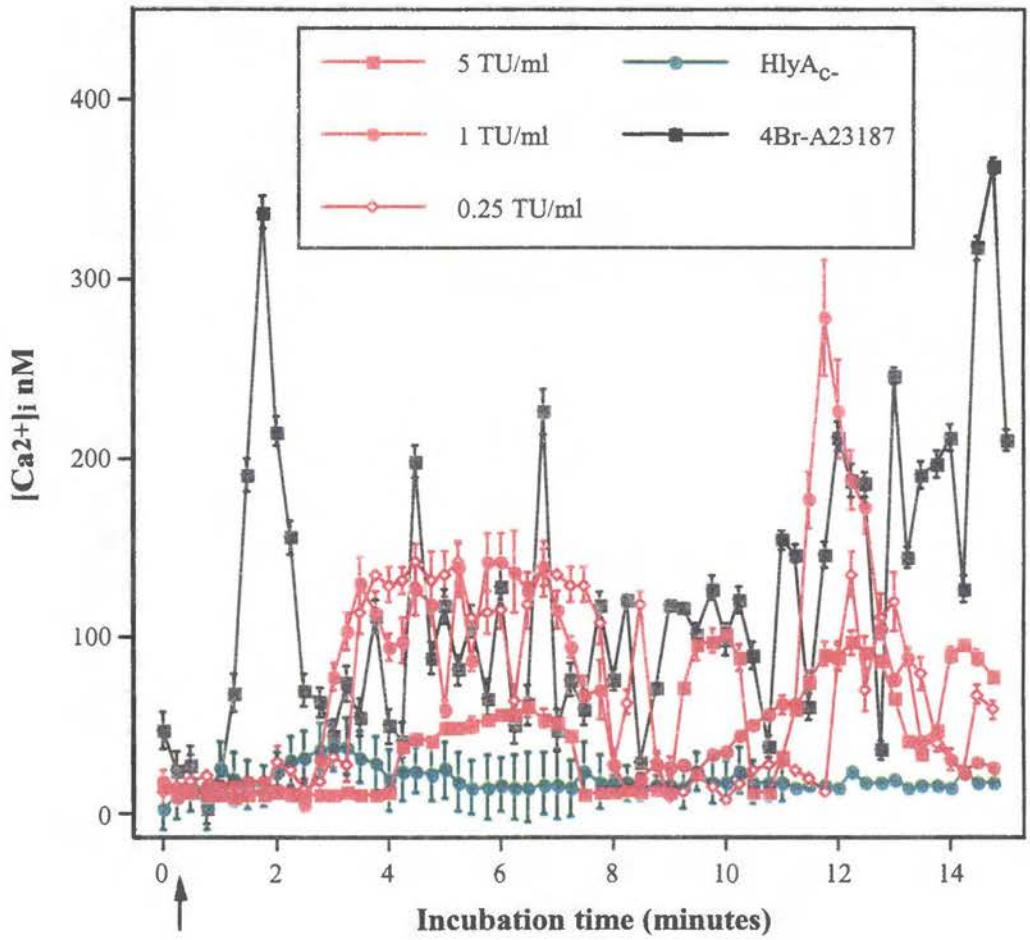

Figure 7. Mean (± SD) $[Ca^{2+}]_i$ of individual HL60 cells (n = 6) before and after addition (arrow) of different concentrations (TU/ml) of HlyA, 4Br-A23187, or $HlyA_{C-}$. Lower concentrations of HlyA and 4Br-A23187 caused higher oscillatory increases in $[Ca^{2+}]_i$ compared with 5 TU/ml HlyA.

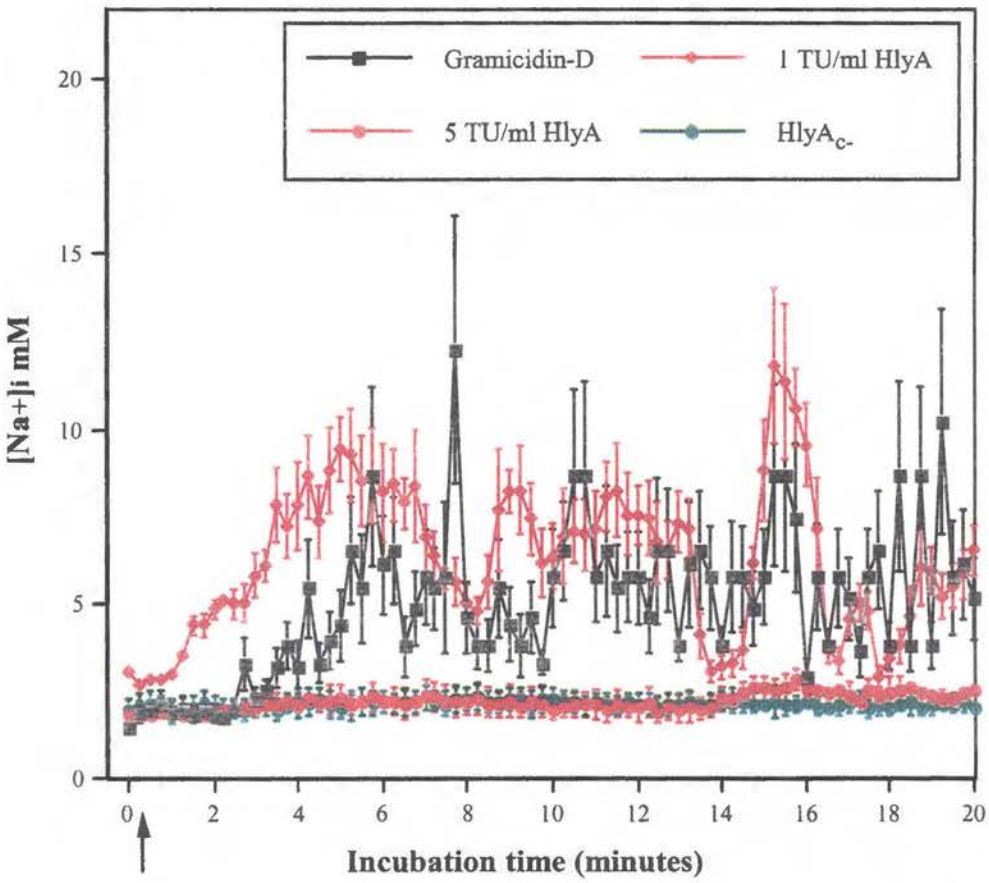

Figure 8. Mean (± SD) $[Na^+]_i$ of individual HL60 cells (n = 6) before and after addition (arrow) of different concentrations (TU/ml) of HlyA, gramicidin-D, or $HlyA_{c-}$. The lower 1 TU/ml concentration of HlyA and Gramicidin-D caused higher oscillatory increases in $[Na^+]_i$ compared with the 5 TU/ml HlyA.

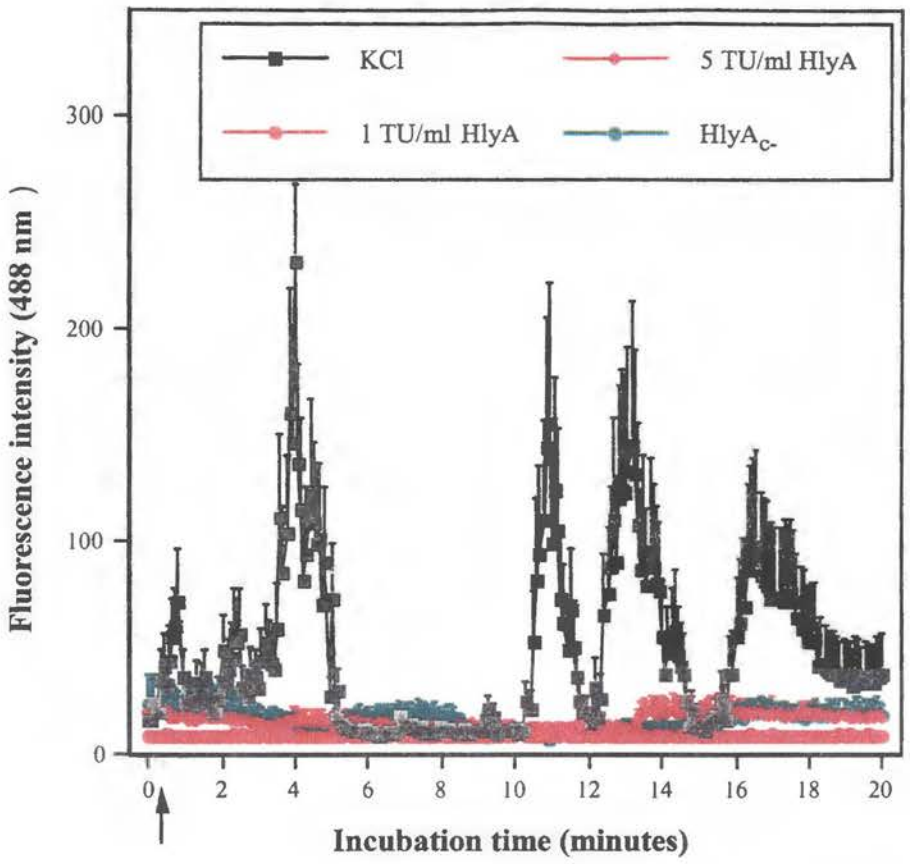

Figure 9. Mean (± SD) fluorescence intensity emitted by $DiBAC_4$ indicating changes in plasma membrane voltage potential of individual HL60 cells (n = 6) before and after addition (arrow) of different concentrations (TU/ml) of HlyA, KCl, or $HlyA_{c-}$. No effects on membrane potential were observed after exposure to HlyA.

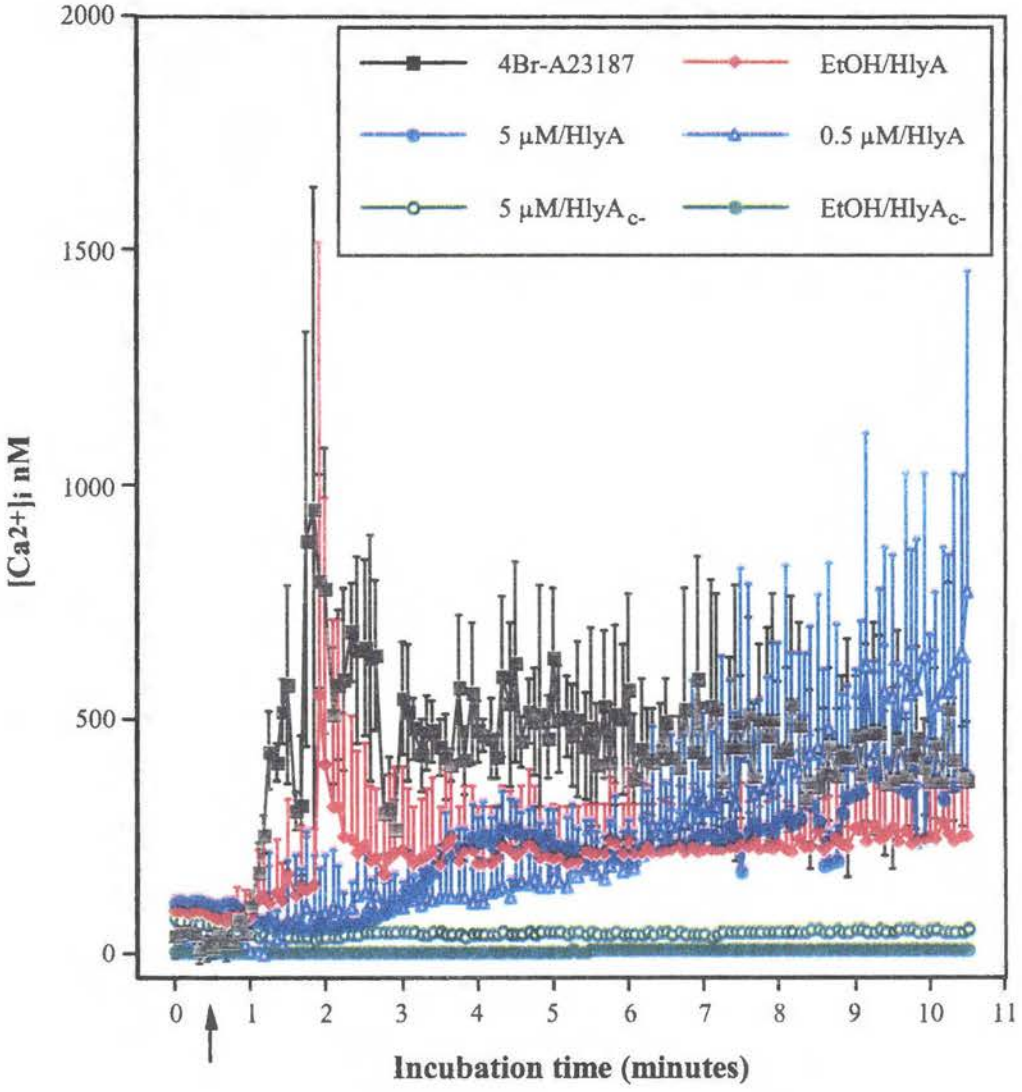

Figure 10. Mean (± SD) effects of the VOCC inhibitor, verapamil, on HlyA-induced increase in $[Ca^{2+}]_i$. Individual cells (n = 6) were incubated for 20 minutes with different concentrations of verapamil (5 μM or 0.5 μM) or the drug solvent, ethanol (EtOH), before exposure to HlyA, $HlyA_{c-}$, or 4Br-A23187. Both concentrations of verapamil significantly inhibited HlyA-induced increase in $[Ca^{2+}]_i$ at 2 minutes after addition of toxin, however, there were no significant effects of verapamil at 4, 6, 8, and 10 minutes thereafter (as determined by unpaired t tests, $P < 0.05$).

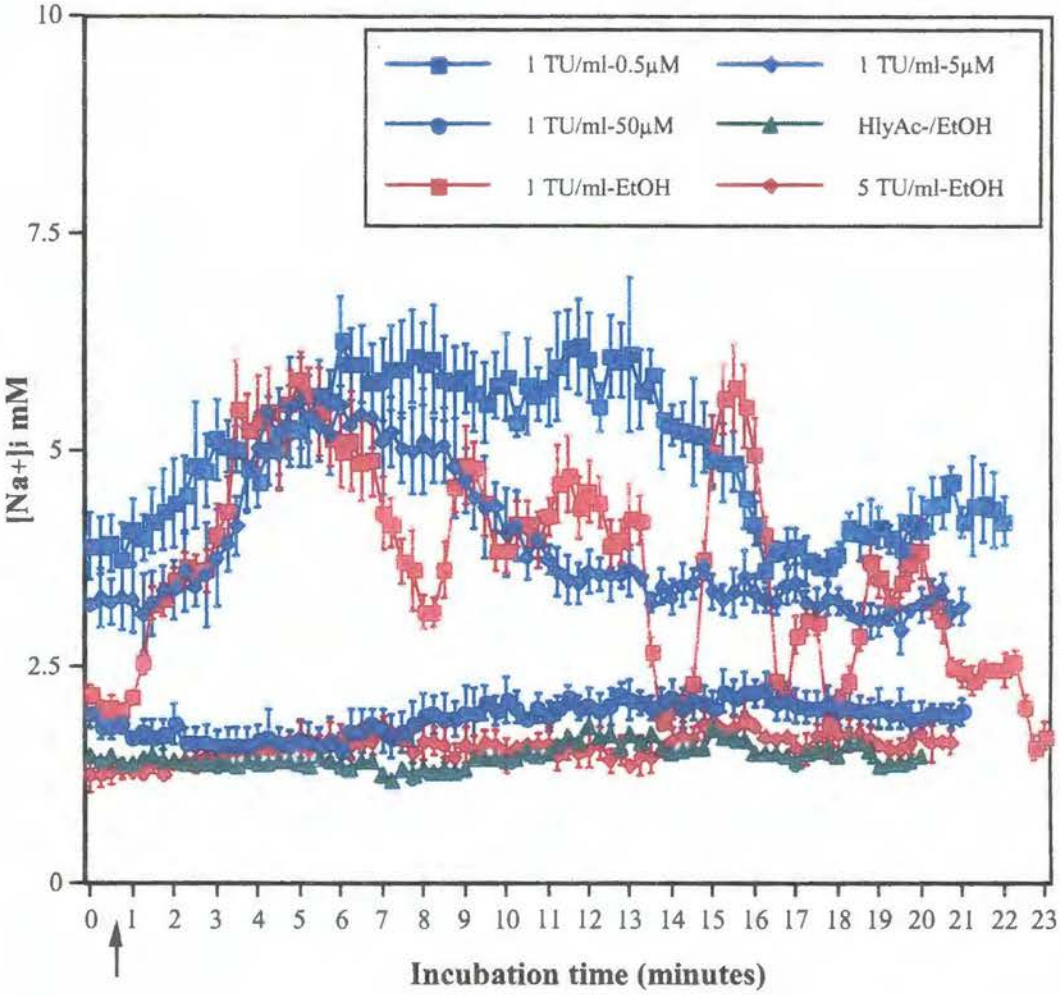

Figure 11. Mean (± SD) effects of the VOCC inhibitor, verapamil, on HlyA-induced increase in $[Na^+]_i$. Individual cells (n = 6) were incubated for 20 minutes with different concentrations of verapamil (0.5 µM, 5 µM, or 50 µM) or drug solvent (EtOH), before exposure to HlyA (1 TU/ml) or $HlyA_c$.. The highest concentration of verapamil (50 µM) significantly inhibited HlyA-induced increase in $[Na^+]_i$ at 2 minutes after addition of toxin, however, there were no significant effects of verapamil at 4, 6, 8, and 10 minutes thereafter (as determined by unpaired t tests, $P < 0.05$).

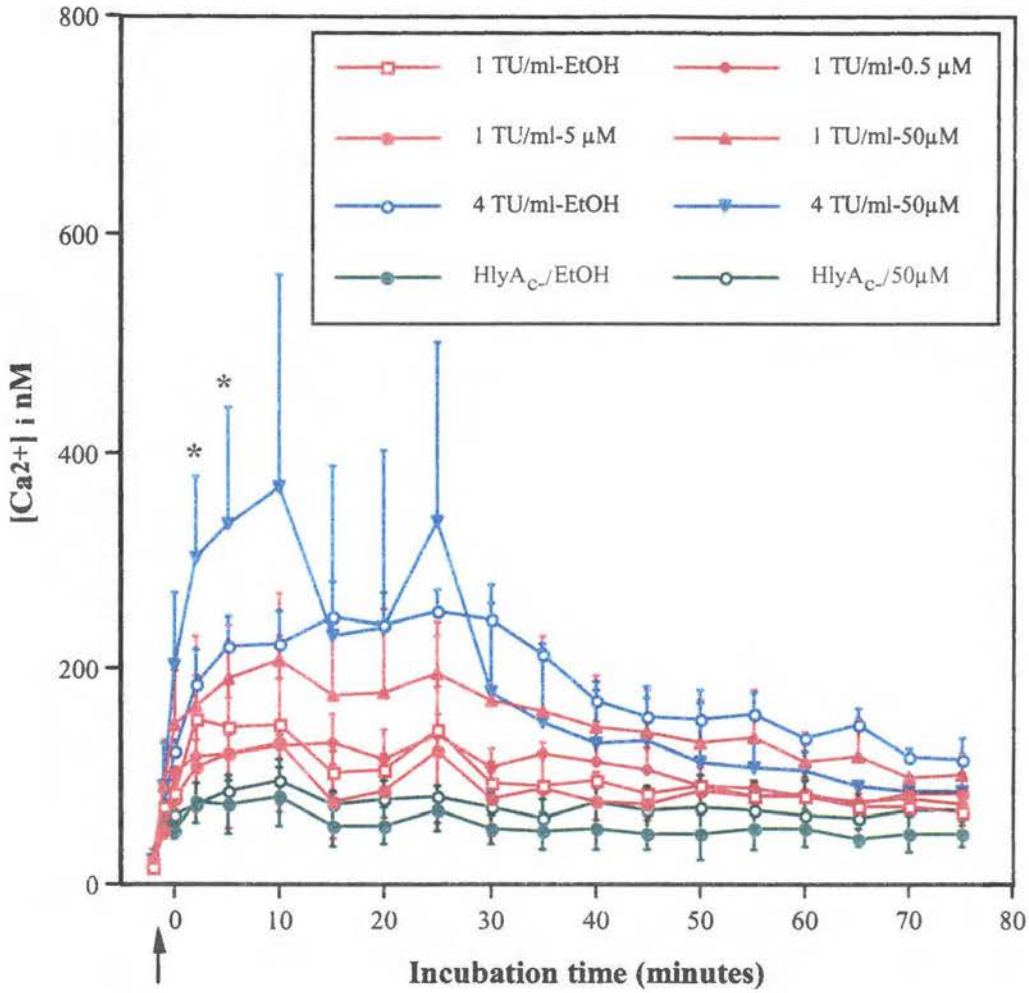

Figure 12. Mean (± SD) effects of the VOCC inhibitor, verapamil, on HlyA-induced increase in $[Ca^{2+}]_i$. Cell suspensions (n = 3) were incubated for 20 minutes with different concentrations of verapamil (0.5 µM, 5 µM, or 50 µM) or drug solvent (EtOH), before exposure to HlyA (1 TU/ml or 4 TU/ml) or HlyA$_{c-}$. At lower concentrations, verapamil had no significant effects on HlyA-induced increase in $[Ca^{2+}]_i$. However, at 50 µM verapamil enhanced the increase in $[Ca^{2+}]_i$ induced by both the 1 TU/ml and 4 TU/ml concentrations of HlyA, as determined by unpaired t tests, $P < 0.05$*.

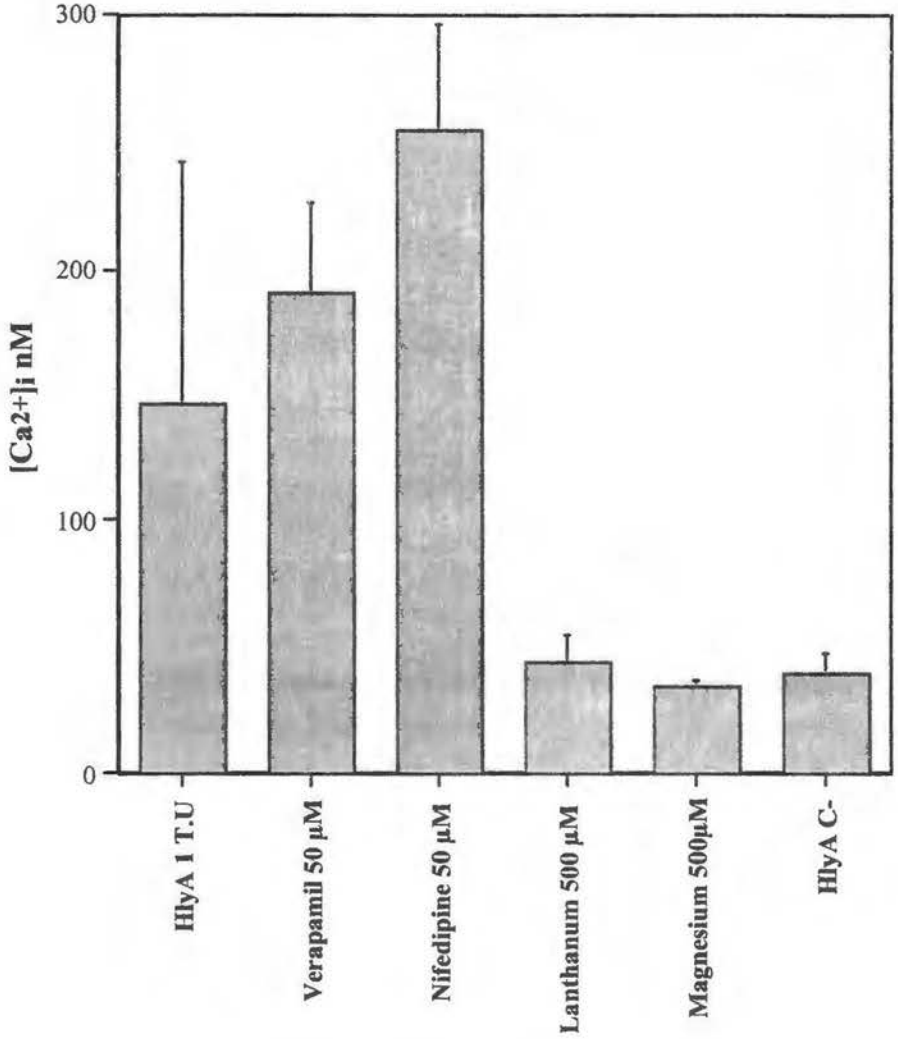

Figure 13. Mean (± SD) effects of verapamil, nifedipine, lanthanum, and $MgCl_2$ (Magnesium) on HlyA-induced increase in $[Ca^{2+}]_i$ of suspended populations of HL60 cells (n = 3). In contrast to the VOCC inhibitors, verapamil and nifedipine, pretreatment with La^{3+} and Mg^{2+} significantly attenuated the HlyA-induced increase in $[Ca^{2+}]_I$, as determined by Scheffe's test ($P < 0.05$).

Discussion

Previous studies reported that pharmacological inhibitors of VOCC were capable of inhibiting the increased $[Ca^{2+}]_i$ induced by RTX toxins (Cudd *et al*, 1999; Ortiz-Carranza and Czuprynski, 1992; Hsuan *et al*, 1998), including HlyA (Uhlen *et al*, 2000). These observations served as the basis for the hypothesis underlying the present phase of study; that increase in $[Ca^{2+}]_i$ induced by HlyA is caused by influx of extracellular Ca^{2+} through VOCC in the plasma membrane and that opening of these VOCC occurs in response to changes in membrane potential following HlyA-induced transmembrane flux of monovalent cations, including Na^+.

Initial studies were supportive of this hypothesis and confirmed that HlyA does cause marked increases in both $[Ca^{2+}]_i$ and $[Na^+]_i$. In individual HL60 cells, these increases in monovalent cation concentration were characterized by oscillatory patterns with frequencies in the range of 1 – 4 minutes. These patterns of response were very similar to those observed in renal epithelial cells exposed to lower sublytic concentrations of HlyA (Uhlen *et al*, 2000; Laestadius *et al*, 2002). Low frequency Ca^{2+} oscillations in renal epithelial cells are believed to activate host defense mechanisms, particularly synthesis of cytokines, such as interleukin-6 and interleukin-8. In contrast to low concentrations of HlyA, exposure of renal epithelial cells to high concentrations caused sustained increases in $[Ca^{2+}]_i$ and cell lysis. In the present study, exposure to high concentrations of HlyA in the range of 4 – 5 TU/ml generally caused relatively little increase in $[Ca^{2+}]_i$, possibly because these cells were intoxicated so rapidly that they were incapable of responding in a more controlled manner. Indeed, the resistance of HL60 cells to HlyA-induced lysis, relative to sRBC, and the Ca^{2+} oscillations caused by

exposure to low concentrations of HlyA suggest that HL60 cells and other related leukocytes may be capable of mounting a coordinated defense against HlyA involving controlled flux of Ca^{2+} across the plasma and organelle membranes.

Although HlyA was observed to increase both $[Ca^{2+}]_i$ and $[Na^+]_i$, in accordance with the study hypothesis, subsequent experiments involving investigation of HlyA effects on membrane voltage potential and effects of verapamil failed to support a significant role for VOCC: HlyA did not cause any change in membrane voltage potential, the trigger generally responsible for opening of VOCC. Furthermore, verapamil, the VOCC inhibitor, was unable to inhibit HlyA-induced increase in $[Ca^{2+}]_i$ in a selective and dose-dependent manner. While it is possible that VOCC may still be involved in the mechanism of intoxication employed by HlyA, it is probable that inhibitory effects of verapamil and other VOCC inhibitors are due to nonspecific effects on a variety of other membrane transport mechanisms, including those responsible for HlyA-induced increase in $[Na^+]_i$.

Other Ca^{2+} transport mechanisms that may be involved include ROCC in the plasma membrane and release of Ca^{2+} from intracellular stores. Transport of Ca^{2+} across the plasma and organelle membranes is closely coordinated (Putney *et al*, 2001): Entry of Ca^{2+} across the plasma membrane usually triggers mobilization of intracellular Ca^{2+} stores through activation of Ry-R channels, in a process called Ca^{2+}-induced Ca^{2+} release. Furthermore, release of Ca^{2+} from intracellular stores of non-excitable cells, such as leukocytes, may stimulate influx of Ca^{2+} through ROCC in the plasma membrane. In addition to Ry-R, release of Ca^{2+} from intracellular stores may also involve IP_3 (Berridge, 1993). Considering that eukaryotic cell types employ $[Ca^{2+}]_i$ as an ubiquitous signaling

mechanism for a wide range of cell functions, it is reasonable to expect that multiple mechanisms of Ca^{2+} transport are involved in HlyA intoxication of HL60 cells. Indeed, the preliminary experiments testing the relative effects of verapamil, $LaCl_3$, and $MgCl_2$ provide persuasive circumstantial evidence that influx of Ca^{2+} via ROCC and release of Ca^{2+} from the ER via Ry-R activation are important contributors to HlyA-induced increase in $[Ca^{2+}]_i$. Clearly, the present hypothesis describing an exclusive role for VOCC in HlyA-induced increase in $[Ca^{2+}]_i$ must be abandoned in favor of a more comprehensive alternative hypothesis addressing contributory roles of ROCC and release of Ca^{2+} from intracellular stores.

CHAPTER V

ROLES OF RECEPTOR-OPERATED CALCIUM CHANNELS AND INTRACELLULAR CALCIUM STORES IN INCREASED INTRACELLULAR CALCIUM CONCENTRATION INDUCED BY *ESCHERICHIA COLI* ALPHA HEMOLYSIN

Introduction

Investigation into the mechanisms involved in HlyA-induced $[Ca^{2+}]_i$ increase requires consideration of the contributions of ROCC and the role of intracellular Ca^{2+} store release. A wide range of cell types possess the ability to generate intracellular Ca^{2+} signals upon binding of ligands (such as neurotransmitters, hormones, and growth factors) to receptors on the cell surface (Braun *et al,* 2001). Release of Ca^{2+} from intracellular stores, particularly the ER, also involves receptors, including IP_3-R and Ry-R. Inositol triphosphate receptors are sensitive to IP_3, which is a catalytic product of PLC, a membrane-associated enzyme (Ackerman *et al,* 1999). Ryanodine receptors are also responsible for Ca^{2+} release from ER stores and are modulated by ryanodine itself, caffeine, Ca^{2+}, Mg^{2+}, and calmodulin (Berridge, 1993). Depletion of ER Ca^{2+} stimulates replenishment of intracellular stores, which is accomplished through Ca^{2+} channels in the plasma membrane, a process termed capacitative calcium entry (Putney, 1986). The resultant increase in $[Ca^{2+}]_i$ governs a wide range of important cellular processes, including cell growth and differentiation as well as apoptosis and cell death, and it is

probable that these mechanisms are involved in cellular responses to HlyA, both those that mediate activation of host defenses as well as those that ultimately result in cell lysis.

Interaction of HlyA with the target cell surface has been demonstrated to involve the LFA-1 heterodimer surface protein, collectively termed β_2-integrin (Lally *et al,* 1997). Based on research performed by Lally and coworkers, as well as the results of the first phase of the present research (see Chapter IV), it is postulated that binding of HlyA to LFA-1 promotes intimate contact with the cell and activation of a signal transduction process that culminates in the release of Ca^{2+} from intracellular stores. Considering the possible involvement of IP_3-R – mediated release of Ca^{2+} from the ER, it is likely that transduction of this signal relies on activation of PLC, the γ-isoform (PLCγ_2) of which is expressed by HL60 cells (Bianchini *et al,* 1993). Binding to β_2-integrin is believed to be linked to activation of tyrosine kinase, which in turn phosphorylates PLCγ_2, thus promoting generation of IP_3. However, control of other types of Ca^{2+} influx channels may also be linked to activation of PLC-linked receptors (Zhu *et al* 1998).

Therefore, the objectives of this phase of study were: (1) to investigate the mechanisms of HlyA-induced increase in $[Ca^{2+}]_i$ by employing specific pharmacological inhibitors of several alternative cellular pathways involved in release of intracellular stored Ca^{2+} and receptor-mediated transport of Ca^{2+} across the plasma membrane; and (2) to compare the effects of HlyA on HL60 cells and a similar cell line (K562) that does not express LFA-1 surface receptors.

Preparation of *E. coli* HlyA

Culture supernatants were harvested from *E. coli* strains WAM 582, WAM 783, and WAM 971, and HlyA and toxin controls were prepared as described previously in Chapter IV. Similarly, methods for toxin storage and assay of activity were identical to those used previously.

Preparation of HL60 and K562 cells

In vitro cell lines were obtained from American Type Cell Culture (ATCC). Methods for culture, harvesting, and preparation of HL60 and K562 cells were identical to those employed previously for performance of HL60 cell suspension (population) studies. Cells were loaded with Fluo-3 for determination of $[Ca^{2+}]_i$, the assays were calibrated, and $[Ca^{2+}]_i$ values were calculated using the methods and formulae described previously in Chapter IV.

Pretreatment of HL60 cells with pharmacological inhibitors

Assays were conducted in 96-well plates. Wells containing 4×10^4 HL60 or K562 cells and anti-fluoroscein Ab (Calbiochem, La Jolla, CA) in 100 µl of assay buffer were exposed to 5, 1, 0.5, or 0.1 TU/ml of HlyA or corresponding concentrations of

$HlyA_{c-}$. Changes in $[Ca^{2+}]_i$ were monitored before and after exposure (-2, -1 ,0, 2, 5 and every 5 minutes thereafter for a minimum of 60 minutes) to toxin preparations at room temperature.

Initial experiments compared the effects of HlyA on HL60 cells and K562 cells. Thereafter, pharmacological inhibitors were used in a systematic fashion to explore the involvement of relevant ROCC and intracellular Ca^{2+} release mechanisms. Prior to addition of HlyA or $HlyA_{c-}$, cells were incubated with the pharmacological inhibitors (genistein, wortmannin, xestospongin, $MgCl_2$, thapsigargin, $LaCl_3$, or SK&F-96365) or solvent controls for 20 minutes (or as otherwise indicated below) in the dark.

Genistein (Calbiochem, La Jolla, CA) is a naturally occurring isoflavanoid phytoestrogen that acts as a strong inhibitor of protein tyrosine kinases (PTK) responsible for activation of PLCγ (Husain and Jafri, 2002). An 8 mg/ml stock solution prepared in EtOH:DMSO (4:1) and stored at -20°C was diluted to 300 µg/ml in EtOH and added to HL60 cell suspensions to achieve final concentrations of 30, 10, 1, and 0 µM. Wortmannin (Wm, Sigma Chemical, St. Louis, MO), derived from *Penicillium fumiculosum*, is an inhibitor of phosphatidylinositol kinases, which are responsible for synthesizing the substrate of PLC-catalyzed production of IP_3 (Broad *et al*, 2001). Wortmannin was prepared as a 300 µM stock solution in EtOH:DMSO (4.6:0.4) and added to HL60 cell suspensions to achieve final concentrations of 30, 10, 1, and 0 µM. Release of Ca^{2+} from ER by activation of IP_3-R was inhibited using xestospongin C (XeC, Gafni *et al*, 1997), which was prepared as a 200 µM stock solution in EtOH. Final concentrations of XeC in HL60 cell suspensions were 20, 2, 0.2 or 0 µM. The divalent cation, Mg^{2+}, was used to inhibit release of Ca^{2+} from ER via Ry-R. HL60 cell

suspensions were exposed to 5 mM Mg^{2+} by addition of appropriate volumes of a 50 mM stock solution of $MgCl_2$ in 1 mM Ca-HBSS immediately prior to HlyA or $HlyA_c$ exposure. Thapsigargin (Tg), a sesquiterpene lactone that inhibits active uptake of Ca^{2+} into the ER by sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (Thastrup *et al*, 1990), was used to deplete ER Ca^{2+} stores prior to exposure to HlyA. Thapsigargin was prepared by addition of a 5 mM stock solution in EtOH to cell suspensions to achieve final concentrations of 0.5, 0.1, 0.05, and 0 mM. The period of pretreatment was 30 minutes. The non-selective ROCC inhibitor, lanthanum (La^{3+}) (Sigma Chemical, St. Louis, MO) competes with Ca^{2+} for binding to extracellular sites, including Ca^{2+} channels. Due to the large valence shell of La^{3+}, it precludes entry of Ca^{2+} through these channels. $LaCl_3$ was used at 1, 0.5, 0.05 or 0 mM and was incubated with HL60 cell suspensions for 15 minutes prior to addition of toxin in the presence of 0.5 mM Ca-HBSS. The final assay volume was 250 µl. Finally, SKF-96365 was used as a specific inhibitor of ROCC involved in capacitative calcium entry (Krautwurst *et al.* 1993; Leung *et al*, 1993). A 200 µM stock solution of SKF-96365 in ddH_2O was added to HL60 cell suspensions to yield final concentrations of 20, 15, 5 or 0 µM. Cells were pretreated for 10 minutes prior to addition of toxin in the presence of 1 mM Ca-HBSS.

Results

Exposure of K562 cells, which do not express β_2-integrin (Lally *et al*, 1997), to a wide range of HlyA concentrations did not induce cell lysis, as determined by release of the endogenous enzyme, LDH (Figure 14). However, exposure of sRBC and HL60 cells

to the same HlyA concentrations caused rapid and serious damage to these cells, as determined by Hg and LDH release, respectively. Exposure of K562 cells to HlyA did cause a small increase in $[Ca^{2+}]_i$ compared with $HlyA_{c-}$ - stimulated cells (Figure 15). This increase in $[Ca^{2+}]_i$ was observed during K562 cell exposure to 5 TU/ml HlyA and was absent in the presence of either 1 or 0.5 TU/ml HlyA. However, despite the ability of a high dose of HlyA to cause increased $[Ca^{2+}]_i$, this effect was not correlated with cell lysis (Figure 15) and was much less marked than that observed in HL60 cells (Figure 16).

Pretreatment of HL60 cells with genistein, the PLCγ inhibitor, significantly attenuated the increase in $[Ca^{2+}]_i$ caused by all concentrations of HlyA tested (Figure 17). Similarly, PLCγ substrate depletion using the phosphatidylinositol kinase inhibitor, wortmannin, depressed HlyA-induced increase in $[Ca^{2+}]_i$, but the Ca^{2+} response was not completely ablated and significant increases in $[Ca^{2+}]_i$ still occurred in the presence of HlyA (Figure 18). Xestospongin C, the inhibitor of IP_3-R – mediated Ca^{2+} release from the ER, significantly inhibited HlyA-induced $[Ca^{2+}]_i$ increase, although $[Ca^{2+}]_i$ still was significantly higher than the corresponding $HlyA_{c-}$ treatment at the 5 TU/ml dose of HlyA (Figure 19). Incomplete inhibition by XeC and wortmannin, both of which suppress IP_3-mediated Ca^{2+} release from ER, suggested that there may be redundancy in mechanisms employed by HlyA to increase $[Ca^{2+}]_i$. This suspicion was confirmed by experiments investigating the involvement of the parallel Ry-R – mediated Ca^{2+} release mechanism: A high concentration of Mg^{2+} (5 mM in the presence of 1 mM Ca^{2+}) caused a 33% reduction in the Ca^{2+} response induced by 5 TU/ml HlyA and a 50% reduction when HL60 cells were exposed to 1 TU/ml HlyA (Figure 20). The contribution of intracellular stored Ca^{2+} to HlyA-induced increase in $[Ca^{2+}]_i$ was further confirmed by the effect of

Tg, the SERCA inhibitor (Figure 21). In the absence of HlyA, Tg induced capacitative Ca^{2+} entry. In the presence of HlyA, the effect of Tg depended on the relative balance established between capacitative Ca^{2+} entry, which promoted increased $[Ca^{2+}]_i$, and depletion of ER Ca^{2+} stores, which limited the availability of stored Ca^{2+} that could contribute to an increase in $[Ca^{2+}]_i$ via IP_3-R and Ry-R mechanisms. The balance between these effects shifted towards the latter as the concentration of Tg was increased.

Involvement of ROCC in HlyA-induced $[Ca^{2+}]_i$ increase was investigated using $LaCl_3$ and SKF-96365. Exposure of HL60 cells to 5, 1, and 0.5 TU/ml HlyA in the presence of 1 mM La^{3+}, the nonspecific inhibitor of Ca^{2+} channels in the plasma membrane, caused a reduction in the Ca^{2+} influx and a decrease in $[Ca^{2+}]_i$ over a 60 minute time period (Figure 22). Treatment with less than 1 mM La^{3+} had no effect, indicating the requirement for a minimum threshold concentration. In contrast to $LaCl_3$, 5 μM SKF-96365 had no effect on HlyA-induced $[Ca^{2+}]_i$ increase, even at the higher concentrations of 15 μM and 20 μM (Figure 23).

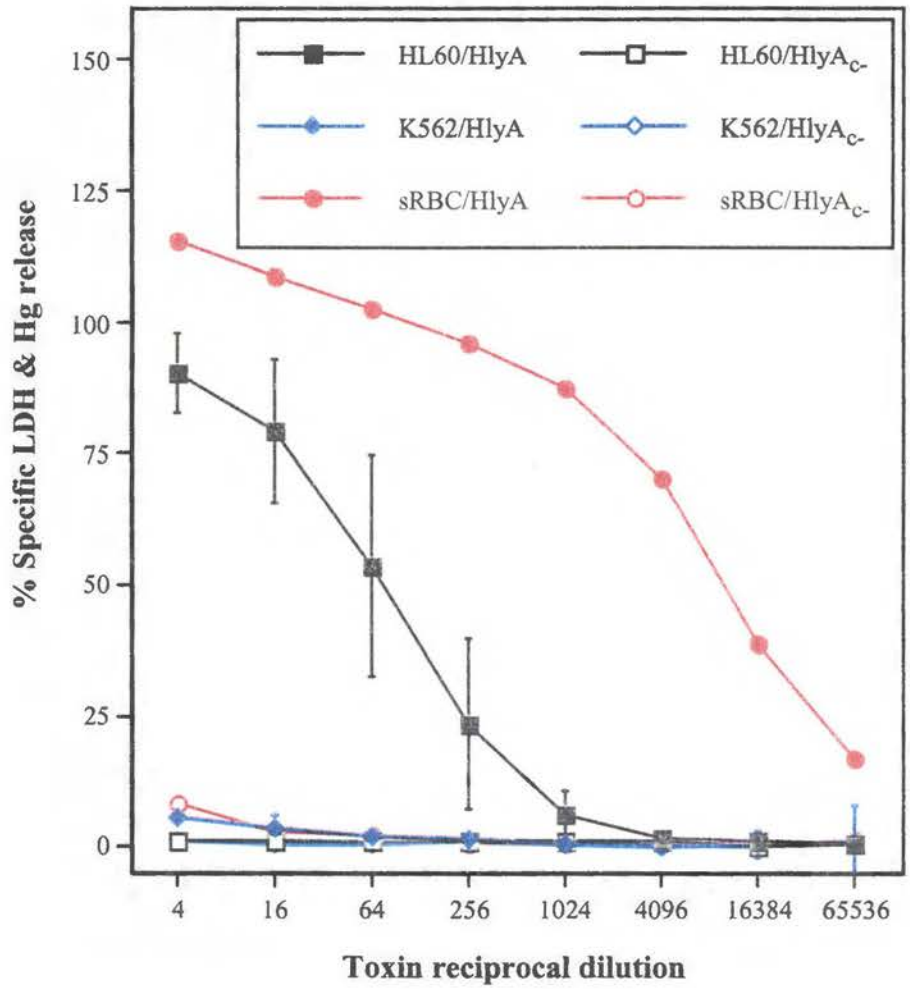

Figure 14. Mean (± SD) % specific LDH and Hb release resulting from exposure of sRBC, HL60 and K562 cells to HlyA or HlyA$_{c-}$ (n = 3). K562 cells, which do not express LFA-1 receptors, were resistant to the membrane damaging effects of HlyA.

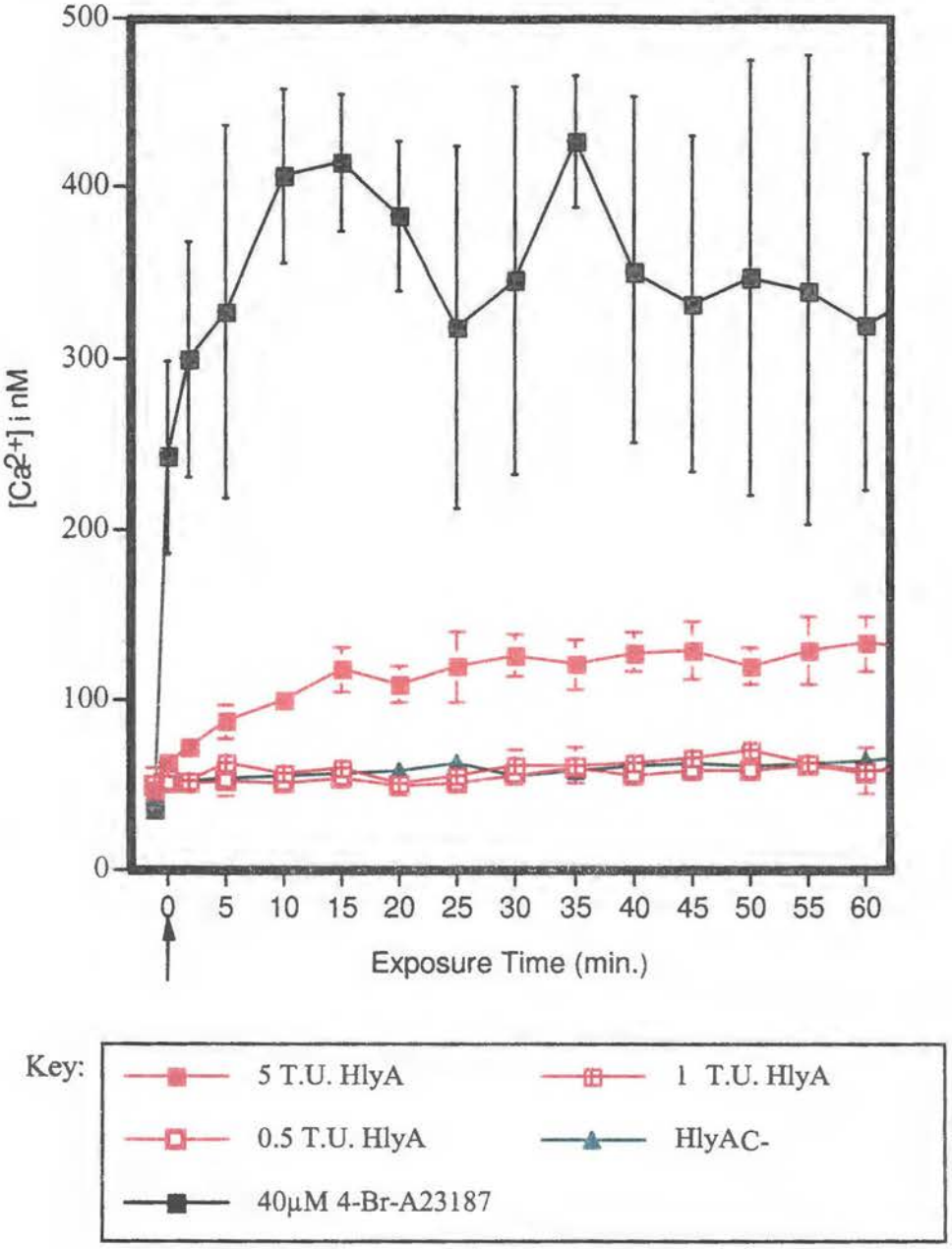

Figure 15. Mean (± SD) effect of HlyA on $[Ca^{2+}]_i$ of K562 cell suspensions (n = 3). The response induced by HlyA at the 5 TU/ml was significantly higher than that induced by $HlyA_c$- but significant lower than that induced by 4Br-A23187, using a repeated measures analysis of variance ($P < 0.05$). Arrow indicates addition of HlyA or $HlyA_c$-.

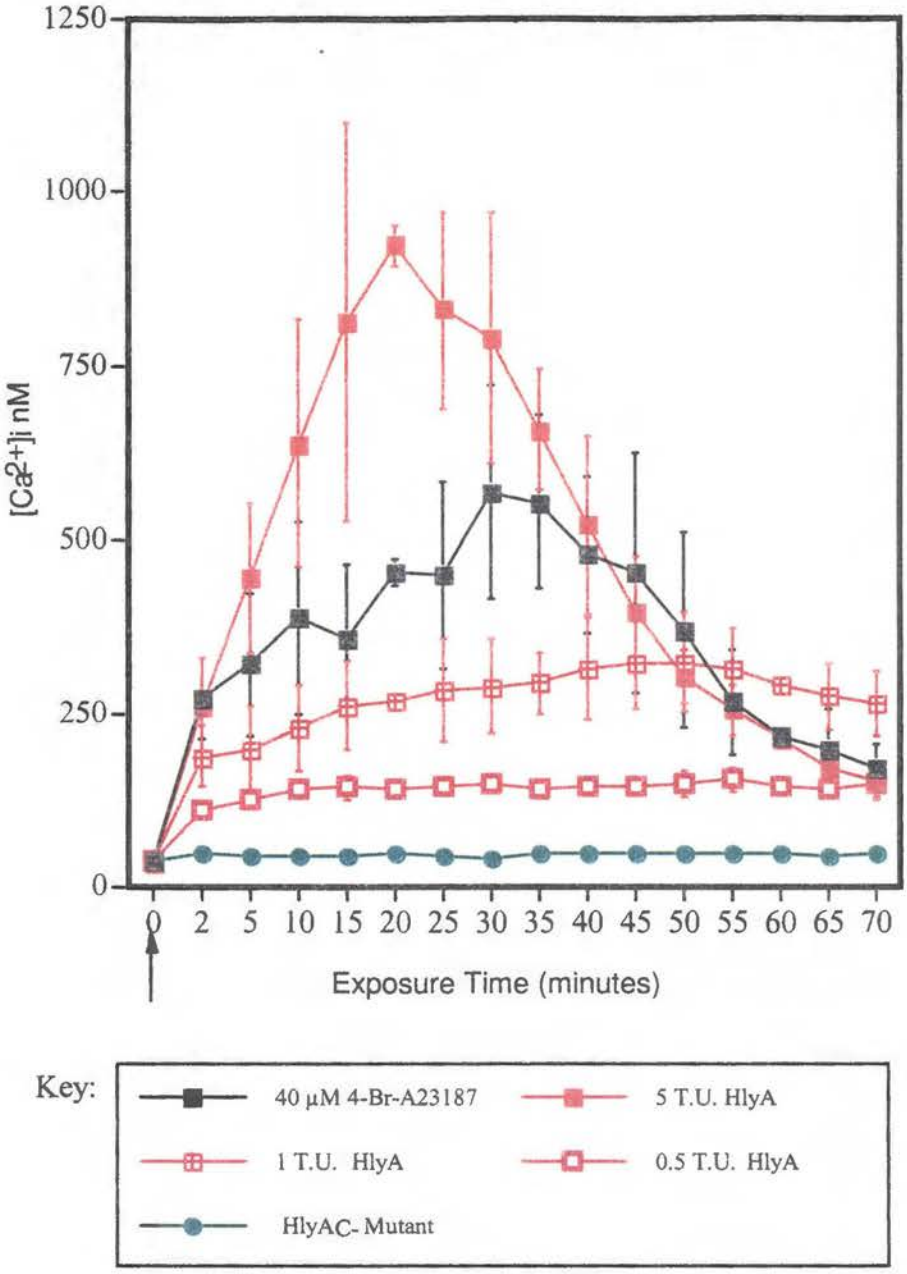

Figure 16. Mean (± SD) effects of HlyA on $[Ca^{2+}]_i$ of HL60 cell suspensions (n = 3). Responses induced by HlyA at the 0.5, 1, and 5 TU/ml were all significantly higher than that induced by $HlyA_{c-}$, using a repeated measures analysis of variance ($P < 0.05$). When compared with the responses of K562 cells plotted in Figure 15, HL60 cells demonstrated a large concentration-dependent response when exposed to HlyA, relative to the effect of 4Br-A23187. Arrow indicates addition of HlyA or $HlyA_{c-}$.

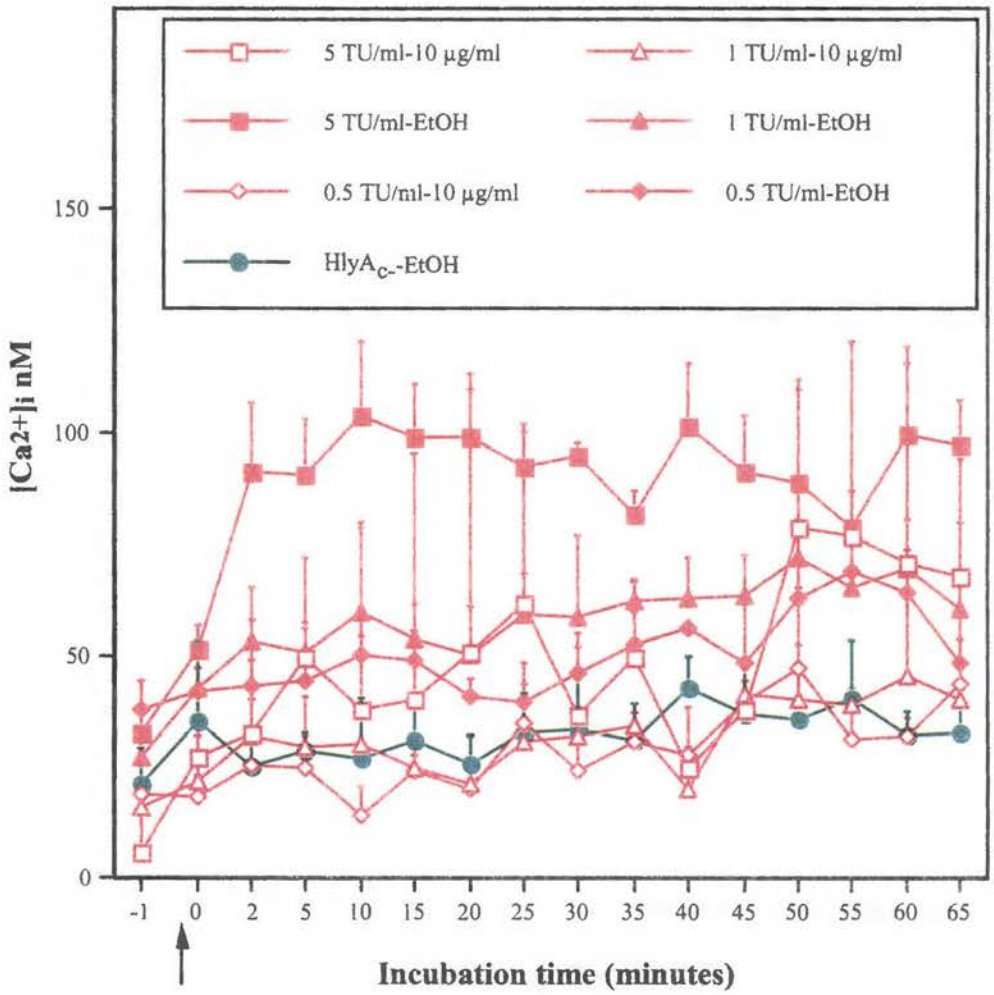

Figure 17. Mean (± SD) effects of genistein (10 µg/ml) on HlyA-induced $[Ca^{2+}]_i$ increase. HL60 cell suspensions (n = 3+ were exposed to 0.5, 1, and 5 TU/ml HlyA. Mean $[Ca^{2+}]_i$ values in genistein-treated cells were significantly lower than corresponding untreated cells (EtOH), using repeated measures analysis of variance ($P < 0.05$). Arrow indicates addition of HlyA or $HlyA_{c-}$.

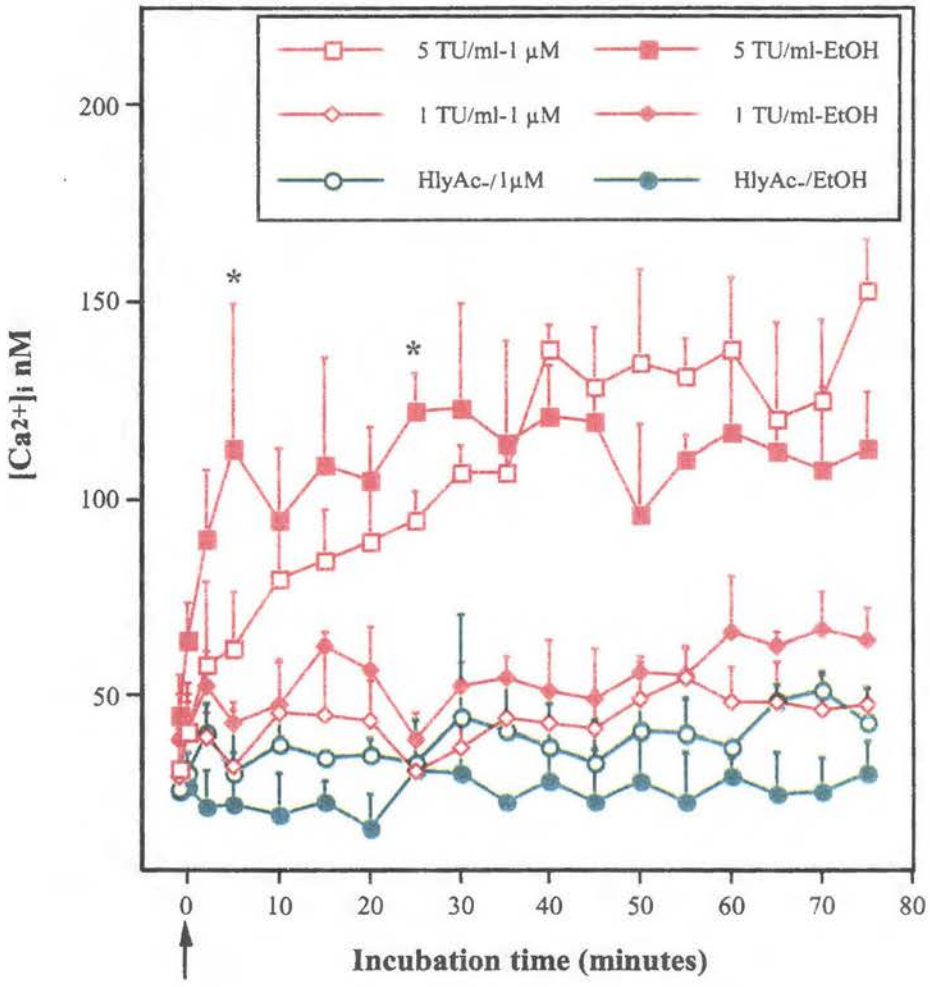

Figure 18. Mean (± SD) effects of wortmannin (1 μM) on HlyA-induced $[Ca^{2+}]_i$ increase. HL60 cell suspensions (n = 3) were exposed to 1 and 5 TU/ml HlyA. *Significant ($P < 0.05$) effects of wortmannin versus corresponding untreated control values (EtOH) were identified at discrete time points, using unpaired t tests. Arrow indicates addition of HlyA or $HlyA_{c-}$.

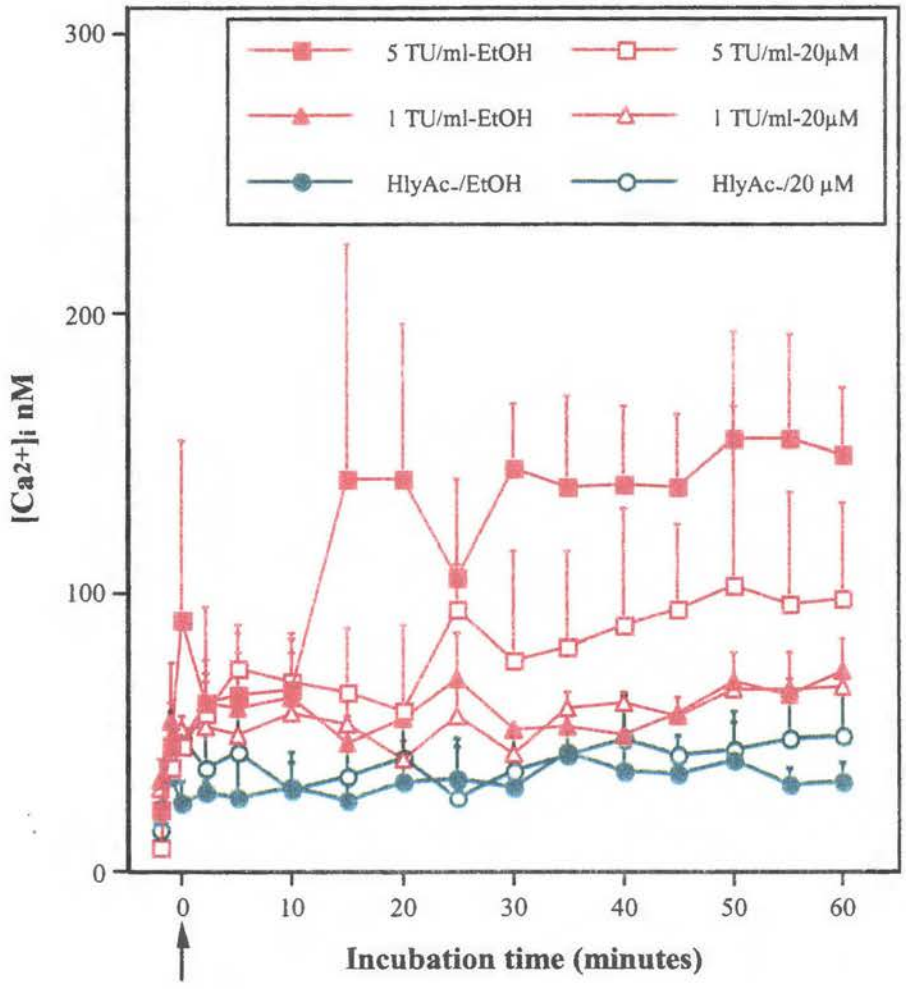

Figure 19. Mean (± SD) effects of XeC (20 μM) on HlyA-induced $[Ca^{2+}]_i$ increase. HL60 cell suspensions (n = 3) were exposed to 1 and 5 TU/ml HlyA. Mean $[Ca^{2+}]_i$ values of cells treated with XeC and exposed to 5 TU/ml HlyA were significantly lower than corresponding untreated cells (EtOH), using repeated measures analysis of variance ($P < 0.05$). Arrow indicates addition of HlyA or $HlyA_c$..

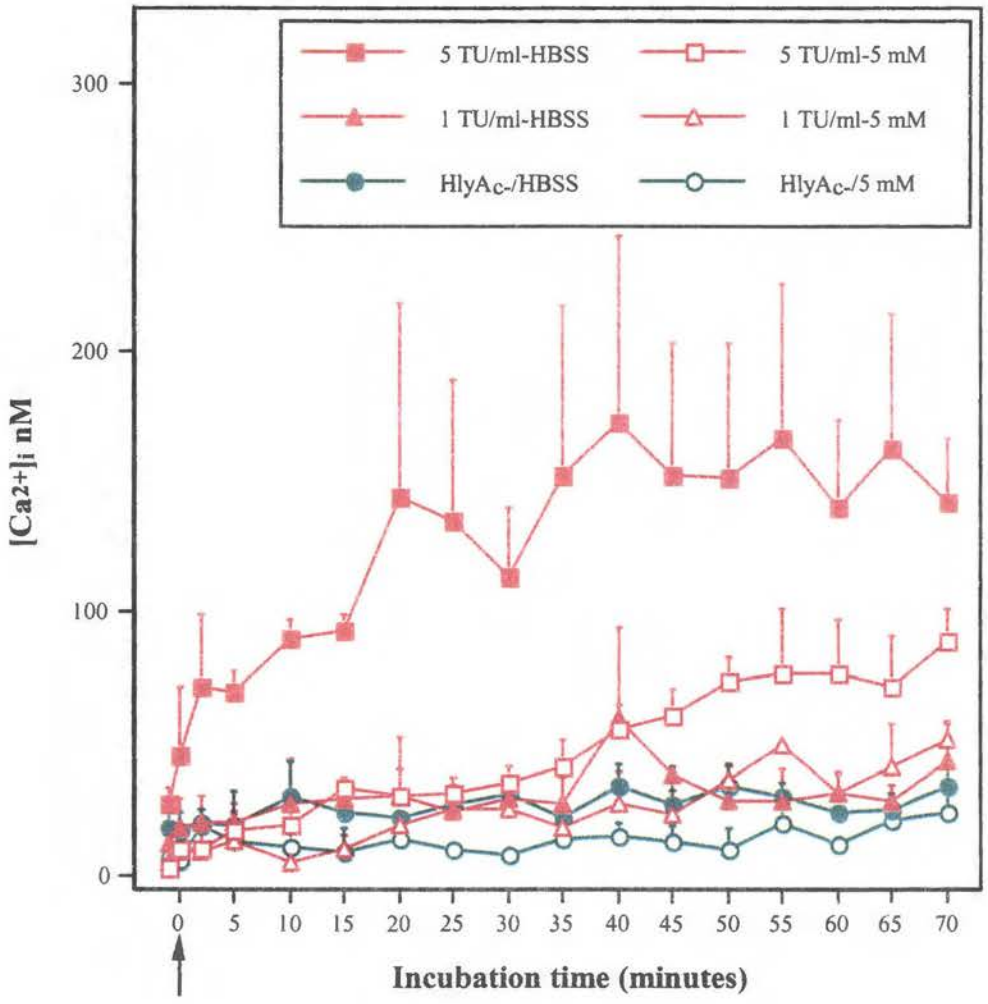

Figure 20. Mean (± SD) effects of Mg^{2+} (5 mM) on HlyA-induced $[Ca^{2+}]_i$ increase. HL60 cell suspensions (n = 3) were exposed to 1 and 5 TU/ml HlyA. Mean $[Ca^{2+}]_i$ values of cells treated with Mg^{2+} and exposed to 5 TU/ml HlyA were significantly lower than corresponding untreated cells (HBSS), using repeated measures analysis of variance ($P < 0.05$). Arrow indicates addition of HlyA or $HlyA_{c-}$.

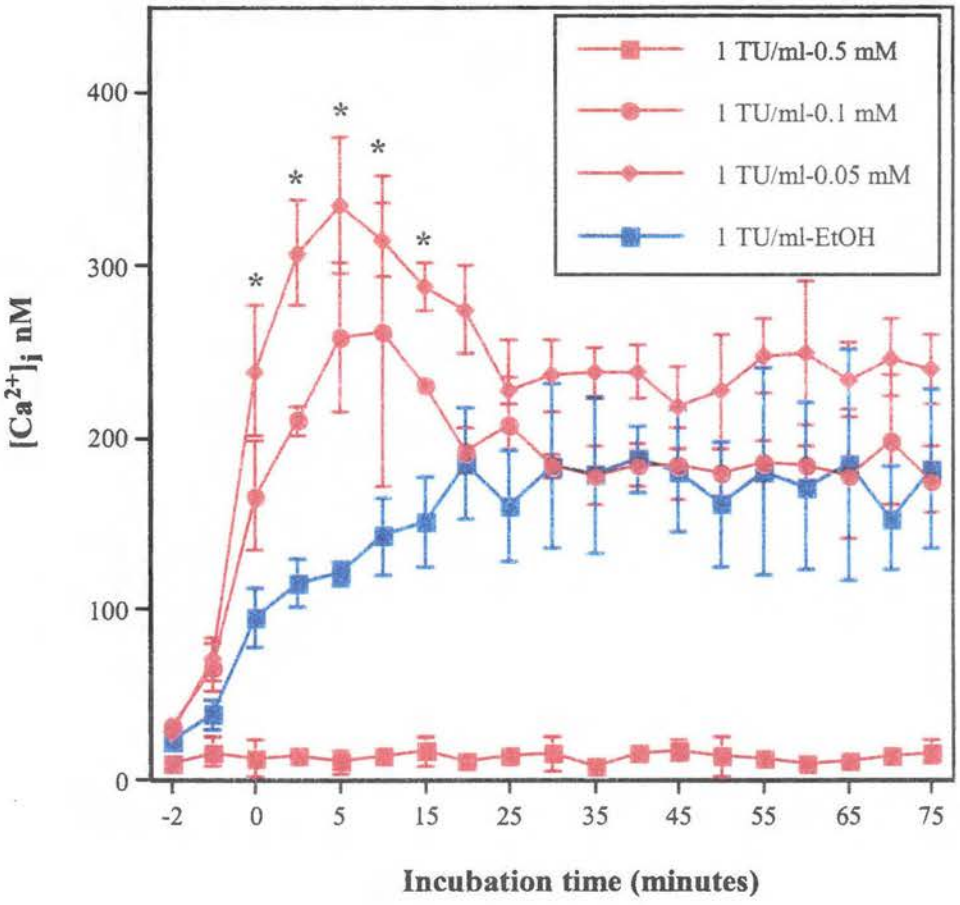

Figure 21. Mean (± SD) effects of Tg (0.05, 0.1, and 0.5 mM) on HlyA-induced $[Ca^{2+}]_i$ increase. HL60 cell suspensions (n = 3) were exposed to 1 HlyA. Mean $[Ca^{2+}]_i$ values of cells treated with 0.5 mM Tg were significantly lower than corresponding untreated cells (EtOH), using repeated measures analysis of variance ($P < 0.05$). *Mean values for cells treated with lower concentrations of Tg (0.05 and 0.1 mM) were determined to be significantly higher than corresponding untreated (EtOH) values, using unpaired t tests ($P < 0.05$). Arrow indicates addition of HlyA.

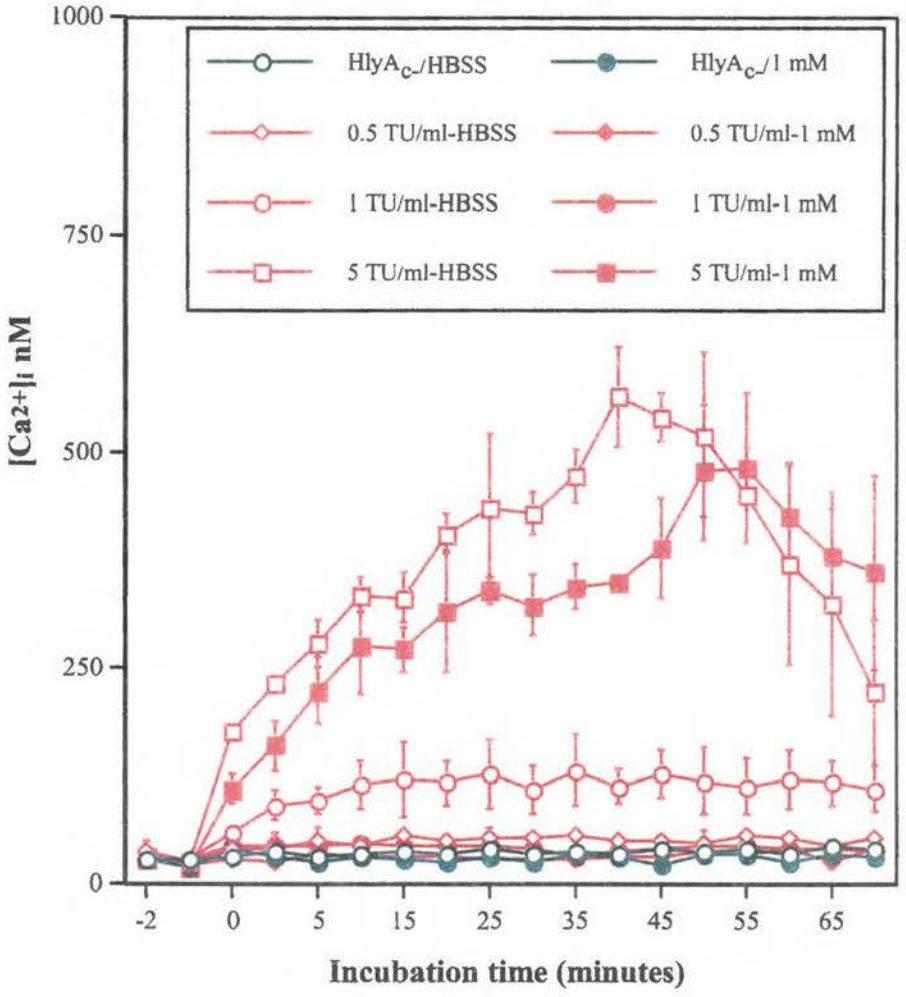

Figure 22. Mean (± SD) effects of La^{3+} (1 mM) on HlyA-induced $[Ca^{2+}]_i$ increase. HL60 cell suspensions (n = 3) were exposed to 0.5, 1, and 5 TU/ml HlyA. Mean $[Ca^{2+}]_i$ values of cells treated with La^{3+} and exposed to 1 and 5 TU/ml HlyA were significantly lower than corresponding untreated cells (HBSS), using repeated measures analysis of variance ($P < 0.05$). Arrow indicates addition of HlyA or $HlyA_{c-}$.

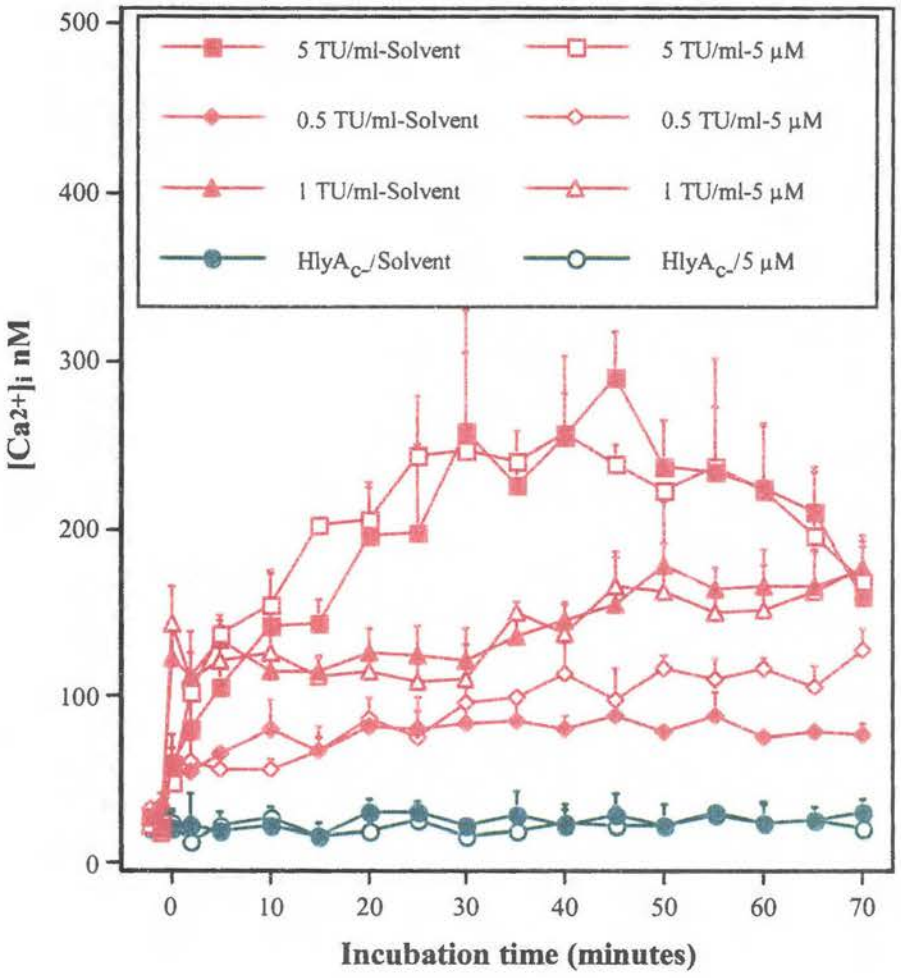

Figure 23. Mean (± SD) effects of SKF 96365 (5 µM) on HlyA-induced $[Ca^{2+}]_i$ increase. HL60 cell suspensions (n = 3) were exposed to 0.5, 1, and 5 TU/ml HlyA. No significant effects of SKF 96365 were identified using repeated measures analysis of variance ($P < 0.05$). Arrow indicates addition of HlyA or $HlyA_{c-}$.

Discussion

Experiments conducted in the initial phase of study (see Chapter IV) concluded that influx of Ca^{2+} through VOCC was unlikely to be the primary means whereby HlyA caused increased $[Ca^{2+}]_i$ in HL60 cells. Instead, preliminary studies suggested that ROCC and release of Ca^{2+} from intracellular stores were more important contributors to this response. Therefore, the overall goal of this phase of research was to explore the specific involvement of mechanisms of intracellular Ca^{2+} control mediated by IP_3-R and Ry-R, and the interaction between these pathways and binding of HlyA to LFA-1 receptors expressed on the surface of HL60 cells. Experiments designed to address this goal employed pharmacological inhibitors of critical steps in these pathways and comparison between HL60 cells and K562 cells, which do not express LFA-1 but are otherwise very similar to HL60 cells.

Previous studies conducted by Lally *et al* (1997) indicated that interaction of HlyA with the plasma membrane of target cells was mediated by specific binding to αCD11a and αCD18, which constitute the LFA-1 receptor. The cell surface β_2-integrin protein, LFA-1, is an important cell adhesion receptor molecule involved in cell signaling (Sirim *et al*, 2001). By interacting with its APC ligand, ICAM-1, the LFA-1 receptor potentiates Ca^{2+} signaling by T-cell receptors (Abraham *et al*, 1999) and promotes T-cell activation. The LFA-1 receptor also mediates activation of macrophage and neutrophilic cell types, leading to degranulation, chemotaxis, and increased phagocytosis of potentially infectious particles. The inability of HlyA to cause significant lysis of K562 cells, which are deficient in expression of LFA-1, provided strong circumstantial

evidence that this β_2-integrin protein serves as the cell surface receptor responsible for mediating many cellular consequences of exposure to HlyA. Although this inability to cause lysis of K562 cells was correlated with lower increases in $[Ca^{2+}]_i$, compared with those observed in HL60 cells, the existence of even a limited increase in $[Ca^{2+}]_i$ suggests that HlyA also employs other parallel pathways for increasing $[Ca^{2+}]_i$ that do not involve binding to LFA-1.

Signal transduction pathways involving cell surface receptors and Ca^{2+} as a second messenger often are mediated by the enzyme PLC, which catalyzes the synthesis of IP_3. The involvement of PLCγ in HlyA-induced $[Ca^{2+}]_i$ was confirmed by the inhibitory action of genistein, possibly by disruption of interactions occurring between the SH_2 domain on PLCγ and the LFA-1 surface protein. However, the specific manner in which PLCγ may interact with LFA-1 has yet to be determined because while wortmannin, the phosphoinositol kinase inhibitor, was able to exert inhibitory effects at discrete times after exposure of HL60 cells to HlyA, these effects were not consistent over the ranges of incubation time and HlyA concentration tested.

The primary pathways for release of Ca^{2+} from intracellular storage sites, particularly the ER, are mediated by IP_3-R and Ry-R. Significant effects of inhibitors on both of these pathways indicated that exposure of HL60 cells to HlyA causes recruitment of parallel and possibly redundant mechanisms to increase $[Ca^{2+}]_i$. Involvement of IP_3 in the HlyA-induced $[Ca^{2+}]_i$ response was directly investigated through the use of the IP_3-R inhibitor, XeC, which significantly inhibited the effect of the high 5 TU/ml concentration of HlyA, although little effect was observed at lower toxin concentrations. The effect of HlyA on $[Ca^{2+}]_i$ was also sensitive to the presence of Mg^{2+}. HL60 cells possess the Ry-

R_3 subtype, which occurs in non-muscle cell types (da Silva and Guse, 2000) and is negatively modulated by high concentrations of Mg^{2+}. It is not yet clear what the relative contributions of IP_3-R and Ry-R may be to HlyA-induced increase in $[Ca^{2+}]_i$. Furthermore, considering that Ca^{2+} itself may modulate the activities of IP_3-R and Ry-R, additional studies are needed to explore whether these mechanisms are activated in response to changes in $[Ca^{2+}]_i$ resulting from influx of extracellular Ca^{2+} across ROCC or whether binding to receptors on the cell surface stimulates release of stored Ca^{2+} via non-Ca^{2+} second messengers, such as IP_3 and cADPR.

Nevertheless, it is clear from the experiments employing Tg that release of Ca^{2+} from intracellular stores is critical to HlyA-induced $[Ca^{2+}]_i$ increase. By inducing intracellular Ca^{2+} release from and uptake into IP_3-sensitive intracellular stores (Lytton *et al,* 1991; Thastrup *et al,* 1990; Bain *et al,* 1991), Tg limited the Ca^{2+} available for intracellular release and, at high concentrations, completely inhibited any increase in $[Ca^{2+}]_i$ after exposure to HlyA. However, the effect of Tg appears to be complex and probably involves the competing influences of capacitative Ca^{2+} entry and limited availability of stored Ca^{2+}, thus explaining the increased $[Ca^{2+}]_i$ observed after pretreatment with 0.05 mM and 0.1 mM, and inhibition of $[Ca^{2+}]_i$ increase after pretreatment with 0.5 mM Tg. Irrespective of the role of capacitative calcium entry in fine-tuning HlyA-induced increase in $[Ca^{2+}]_i$, it is doubtful that it plays a major role in the molecular pathogenesis of HlyA because SKF-96365, the specific inhibitor of ROCC involved in capacitative calcium entry, had no significant effect on changes in $[Ca^{2+}]_i$ induced by HlyA.

While the present experiments demonstrate that release of Ca^{2+} from intracellular stores is a critical contributor to HlyA-induced $[Ca^{2+}]_i$ increase, influx of extracellular Ca^{2+} across channels in the plasma membrane also plays an important role. The involvement of these channels was demonstrated by the inhibitory effects of La^{3+}. Trivalent lanthanides have a large hydrated ionic radius and a cationic charge that promotes high affinity binding to anionic sites on the cell surface, especially those normally occupied by Ca^{2+} (Weiss, 1974). This action results in nonspecific inhibition of Ca^{2+} channels in the plasma membrane, including ROCC. Considering the lack of inhibitory effects of SKF-96365, the present experiments provided little clarification concerning the specific identity of ROCC that may be involved in the transmembrane influx of Ca^{2+} induced by HlyA, thus necessitating future performance of additional more definitive studies.

CHAPTER VI

SUMMARY CONCLUSIONS

Extraintestinal *E. coli* is a major cause of nosocomial infections involving bacteremia, peritonitis, and nephritis (Kreger *et al,* 1980), and HlyA production is recognized to be an important virulence factor in the pathogenesis of these diseases (Cavalieri *et al,* 1984). Exposure of many different cell types to HlyA, particularly leukocytes, leads to increased $[Ca^{2+}]_i$ and subsequent degranulation, oxidative burst, eicosanoid production, and ultimately lysis (Czuprynski *et al,* 1991; Ortiz-Carranza and Czuprynski, 1992). Elucidation of the mechanisms whereby HlyA causes increased $[Ca^{2+}]_i$ is critical to development of effective treatment of these diseases and was the focus of this research.

Numerous researchers have reported (reviewed in Chapter II) that HlyA is capable of directly disrupting the integrity of protein-free liposomes and planar lipid bilayers, suggesting that these effects lead to generation of transmembrane lesions or pore-like structures through which extracellular Ca^{2+} can be transported. Other studies employing inhibitors of VOCC (Cudd *et al,* 1999; Ortiz-Carranza and Czuprynski, 1992; Hsuan et al, 1998) suggested an alternative mechanism for increased $[Ca^{2+}]_i$ involving influx of monovalent cations through membrane pores, changes in membrane voltage potential, and consequent opening of VOCC. While initial experiments confirmed that HlyA causes increases in $[Na^+]_i$ and $[Ca^{2+}]_i$, these two events could not be correlated with any changes in membrane voltage potential, the principal trigger for activation of VOCC.

Furthermore, verapamil inhibited Hly-induced increases in both $[Na^+]_i$ and $[Ca^{2+}]_i$, indicating that VOCC inhibitors lack specificity of action and cannot serve as a credible basis for assuming the primary involvement of VOCC in the pathogenesis of HlyA.

Therefore, further studies were conducted that explored the role of other mechanisms of increased $[Ca^{2+}]_i$, including influx of Ca^{2+} through ROCC and release of Ca^{2+} from intracellular storage sites. Based on evidence that expression of the LFA-1 receptor is necessary for full-blown intoxication by HlyA and the significant effects of pharmacological inhibitors on intracellular Ca^{2+} pathways involving PLC, IP_3-R, and Ry-R, it is clear that both influx of extracellular Ca^{2+} across plasma membrane channels and release of stored Ca^{2+} are important contributors to HlyA-induced increase in $[Ca^{2+}]_i$. The relative roles of intracellular release mechanisms mediated by IP_3-R and Ry-R have yet to be determined, as is the relationship between release of Ca^{2+} from the ER and influx of extracellular Ca^{2+} through plasma membrane channels. While it is possible that these mechanisms of $[Ca^{2+}]_i$ increase represent redundant pathways, it is more likely that the contribution of each varies according to the concentration of HlyA and the duration of exposure, and whether the target cells are subject to sublytic versus lytic attack.

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VITA 2

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