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EFFECTS OF STAPHYLOCOCCAL ALPHA TOXIN ON RAT PAPILLARY MUSCLES

by

Gang Xu

A Thesis
Submitted to the Faculty
of Graduate Studies and Research
through the Department of Biological Sciences
in Partial Fulfillment for the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada
1991
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ABSTRACT

The effect of staphylococcal alpha toxin on myocardial contractile function was examined in rat papillary muscles of the right ventricle. When muscles were stretched at L_{max}, 26°C and in 1.0 mM extracellular Ca^{2+}, a low concentration (10 HU/mL) of purified toxin significantly altered the developed force which was stabilized within 30 minutes while the passive tension remained unchanged. The peak tension, the maximal rate of force development, and the maximal rate of relaxation were all reduced, while the timing parameters of the twitch were unaltered.

Heat inactivated toxoid is neither hemolytic nor lethal, but has similar cardiac dysfunctional effects as the toxin. The developed force of the muscles was decreased within 30 minutes exposure to the toxoid, while the passive force was unchanged. The twitch characteristics after toxoid treatment were similar to these after toxin treatment.

A transient increase of the developed force was linearly related to toxin concentration.

The rest potentiation experiments performed after treatment of 30 minutes of higher toxin concentration (100 HU/mL) suggest that the toxin inhibited Ca^{2+} movement from sarcolemma to sarcoplasmic reticulum. A slight increase in Ca^{2+} transportation from the uptake compartment of sarcoplasmic reticulum to the release compartment of sarcoplasmic reticulum was also observed. The recirculation fraction of Ca^{2+} from sarcoplasmic reticulum to the myofilaments remained unchanged.
DEDICATION

This thesis is dedicated to my parents.
ACKNOWLEDGEMENTS

I would like to thank all those who helped me complete this project. I am deeply indebted to my supervisor, Dr. H.B. Feckrell, for his invaluable guidance, enthusiasm, and support. I would like to express my particular appreciation to Dr. Paul Taylor, my co-supervisor, for his tremendous academic assistance and direction. I am very grateful to Dr. B. Zielinski and Dr. R. Thibert for serving on my committee. I am very appreciative to Dr. C.J. Mao and Dr. H. Tang for their numerous technical assistance and contributions. Many thanks to my fellow graduate student Lana Kantor for all the friendly talks, and especially for her assistance with the English when I just arrived at the New World. The technical assistance of Paul Fazekas and James Gamble is also much appreciated. Special thanks to my parents for their great moral support from the other side of the Earth.
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LIST OF ABBREVIATIONS

°C  degree centigrade
+ dF/dT  maximal rate of force development
- dF/dT  maximal rate of force relaxation
FPLC  fast pressure liquid chromatography
HU  hemolytic unit
KD  kilodalton
L₀  the muscle’s length at which the developed force was just detectable
L_max  the muscle’s length at which the developed force was maximal
Min  minute(s)
mM  millimolar
msec  millisecond(s)
PBS  phosphate buffered saline
pI  isoelectric point
RBC  red blood cells
S  Svedberg
S.E.  standard error
Sec  second(s)
INTRODUCTION

The objective of this study was to establish a model of rat papillary muscles to study the effects of the staphylococcal alpha toxin on cardiac muscle.

*Staphylococcus aureus* is an important human pathogen. This Gram positive organism produces a large number of extracellular proteins of which the alpha toxin is the most potent (Arbuthnott, 1970; Mollby, 1983; Kinsman et al., 1981; O’Reilly et al., 1986; Patel et al., 1987). The alpha toxin is hemolytic, demembranocytic, cytotoxic for many cell types, and lethal for a number of laboratory animals as well as humans (for review, see Freer and Arbuthnott, 1983; Wiseman, 1975). However, although it is closely related to the pathogenicity of *Staphylococcus aureus*, the role of alpha toxin in disease processes is not clear. Therefore, my study has focused on physiological function of the staphylococcal alpha toxin.

It is generally agreed that the primary cellular target of the toxin is the membrane. Until now, most of the studies on alpha toxin have used erythrocytes. The erythrocyte is not only very sensitive to alpha toxin, but is also a very simple and efficient model for the study of events that occur on the cellular membrane since erythrocytes contain few organelles. However, hemolysis does not seem to be the lethal factor. When animals were injected with lethal doses of alpha toxin, they showed respiratory distress and spastic paralysis, but the degree of hemolysis was insufficient to explain rapid death even in the rabbit, which is one of the most sensitive species (Arbuthnott, 1970). Recently, rapidly occurring circulatory disturbances have been suggested as a primary lethal factor (Thelestam and Blomqvist, 1988).

Using low concentration (3 HU/mL) of pure alpha toxin, Adamo et al. (1989) proved that the alpha toxin has a potent cardiovascular effect that may contribute to its lethality. Their studies suggested that constriction of the
blood vessels deprived the hearts of oxygen, and the alpha toxin may have a direct effect on cardiac muscles since isolated atrial (vascular-poor tissue) preparations were also affected by the toxin.

The rat papillary muscles were selected in the present study since the influence of the blood vessels was minimized as diffusion was not a limiting factor. The rat papillary muscles provided an ideal model to study the response of the excitable cells to the toxin with biophysical methods that were noninvasive to the function of the muscle. Also, it is a well studied model. More precise control over muscle loading can be achieved.
LITERATURE REVIEW

2.1 Staphylococcal Alpha Toxin

*Staphylococcus aureus* has long been recognized as an important human pathogen. It produces many extracellular proteins, most of which are harmful to the cells and tissues of animals and humans. These proteins can be divided into two groups: the first group includes those that help the bacteria to colonize the host; and the second group includes those that affect the normal tissues and cells as well as their functions (Blair, 1962). The first group contains mainly enzymes such as coagulase, leukocidin, hyaluronidase, phospholipases, lipases, proteases and a variety of esterases (Blair, 1962; Freer and Arbuthnott, 1983). The second group contains many exotoxins, including alpha, beta, gamma, and delta toxins; Panton-Valentine leukocidin; exfoliatins A and B; and a number of enterotoxins (Freer and Arbuthnott, 1986; Woodin, 1970; Bergdolli, 1983; Wiseman, 1975). The alpha toxin is the most potent protein produced by *S. aureus*. It is of primary importance in the pathogenesis of infections caused by *S. aureus* (Arbuthnott, 1970; Mollby, 1983). Alpha toxin deficient mutants of *S. aureus* do not inhibit weight gain to the same extent as the parent strain in the newborn mouse model (Kinsman et al., 1981). Using genetic approaches to inactivate the alpha toxin gene, Foster's group has established that the alpha toxin is an important virulence factor in *S. aureus* infections (O'Reilly et al., 1986). They showed that the alpha toxin is a major factor in forming subcutaneous lesions and in causing the death of mice injected intraperitoneally with *S. aureus* (Patel et al., 1987).

The alpha toxin is membrane damaging to a variety of natural and artificial membranes, dermonecrotic, cytotoxic and lethal (Fisher and Goshi, 1963; Jackson, 1963; Wiseman, 1975; Blair, 1962; Cooper et al., 1966; Berngeimer and Schwartz, 1963; Goshi et al., 1963; Jeljaszewicz et al., 1968). Rabbit erythrocytes are more sensitive to alpha toxin than
erythrocytes of any other species. Maharaj and Fackrell (1980) found that band 3 is the receptor for alpha toxin on the rabbit erythrocyte membrane. It seems that the primary target of alpha toxin is the cell membrane. Alpha toxin is sometimes referred to as alpha hemolysin. However, aside from being hemolytic, alpha toxin is also cytolytic, dermonecrotic, neurotoxic and lethal. Therefore, the term "hemolysin" is not appropriate. Some other terms were also proposed by some authors such as, "cytolytic toxin" by Bernheimer (1974), "membrane-damaging toxin" by McCartney and Arbuthnott (1978). However, the term alpha toxin is used by most researchers at present time.

Beta toxin is most hemolytic to sheep and ox erythrocytes (Bigger, 1933). It demonstrated a "hot-cold" phenomenon, i.e., the hemolysis becomes much more evident when temperature is changed from 37°C to 4°C (Bigger et al., 1927; Walbum, 1921). The beta toxin is serologically distinct from alpha toxin (Glenny and Stephens, 1935). It is Mg$^{2+}$ dependent phospholipase C, specific for sphingomyelin (Doery et al., 1963; Wiseman and Caird, 1967; Maheswaran et al., 1967).

Gamma toxin is hemolytic towards rabbit, human, and sheep erythrocytes (Guyonnet and Plommet, 1970; Fackrell, 1974). This hemolytic activity is inhibited by several lipids and sterols in which the 3-beta-hydroxy group is esterified (Bernheimer, 1974). The toxin is also lethal for guinea pigs (Fackrell and Wiseman, 1976). Fackrell and Wiseman (1976) showed a release of nitrogen and acid soluble phosphorous from gamma toxin treated human erythrocytes. This suggests that gamma hemolysin may have phospholipase activity.

Delta toxin affects most cell types which have been tested (Marks and Vaughn, 1950; Kayser and Renaud, 1965; Fackrell, 1974). It is electrophoretically heterogeneous and dissociates into subunits in nonionic detergents,
which explains the wide range of reported molecular weights of 68-200 KD (Waldvogel, 1985). It damages cell membranes via its high surface activity and hydrophobicity (Freer and Arbuthnott, 1983; Bernheimer, 1974). Delta toxin is lethal and dermonecrotic (Kapral et al., 1976; Freer and Arbuthnott, 1983). It may aggregate on the membrane and form transmembrane channels which result in the loss of ions (Bhakoo et al., 1982). The morphological changes in cells treated with delta toxin were described by Thelestam and Mollby (1975 a,b). The toxin produces small lesions initially, and then the size of the lesions increases gradually. This toxin may play a role in the pathogenesis of acute diarrhea in some staphylococcal infections since it inhibits water absorption and simultaneous stimulation of cyclic AMP production in the rabbit and guinea pig ileum (Rogolsky, 1979).

2.1.1 Physiochemical characteristics and basic biochemistry of alpha toxin

Alpha toxin is a single polypeptide chain protein (Bernheimer and Schwartz, 1963; Lominski et al., 1963; Arbuthnott, 1970; Six and Harshman, 1973; Freer and Arbuthnott, 1986). An early report that the alpha toxin contains a carbohydrate (Goshi et al., 1963) has never been confirmed. The toxin is positively charged (Coulter, 1966). Molecular weight estimations range from 10 to 45 KD, as reported by several laboratories and summarized by Wiseman (1975) and Freer and Arbuthnott (1986). The variation is probably due to the different methods and reagents used. For example, Six and Harshman (1973) used four different methods to determine the molecular weight of a single alpha toxin product and reported molecular weight to be in the range of 26-31 KD. However, when the nucleotide sequence of the alpha toxin gene was determined (Gray and Kehoe, 1984), it predicted an amino acid sequence giving a molecular size of 33 KD. This resolved the conflicting data reported previously.

There are three forms of alpha toxin which have been reported frequently by sedimentation coefficient studies: a 3S soluble and active form; a 12S insoluble inactive form; and a 12S aggregate form (Arbuthnott et al., 1967).
The major component has a sedimentation coefficient of approximately 3S (Bernheimer and Schwartz, 1963; Lominski et al., 1963; Arbuthnott et al., 1967; Six and Harshman, 1973; Coulter, 1966). A minor component has been found to have sedimentation coefficient between 10.9-16S (Bernheimer and Schwartz, 1963; Lominski et al., 1963). It is generally agreed that both aggregated and soluble forms of 12S alpha toxin are biologically inactive (Arbuthnott et al., 1967; Lominski et al., 1963; Bhakdi et al., 1983), although Bernheimer (1974) reported that the 12S toxin is sometimes hemolytic. Arbuthnott et al. (1967) and Hugo et al. (1987) reported that the active 3S component and the inactive 12S aggregate can be transformed to the other form under certain conditions.

The values for the isoelectric point (pI) of the major alpha toxin component were reported within the range of 8.4-8.7 (Wadstrom, 1968; Goode and Baldwin, 1974; Fackrell and Wiseman, 1976; Dalen, 1975; 1976). In these studies, it was found that alpha toxin could be separated into several components of different isoelectric points. Wadstrom (1968), and McNiven et al. (1972) found that the main component of pI 8.5 accounted for 80-90 percent of the hemolytic activity.

Results of amino acid analyses from different laboratories are quite similar (Bernheimer and Schwartz, 1963; Coulter, 1966; Fackrell and Wiseman, 1976; Watanabe and Kato, 1974; Kato and Watanabe, 1980), which show that half-cystine is absent and the amount of lysine, aspartic and glutamic acids are relatively large. Gray and Kehoe (1984) determined the complete DNA sequence of a cloned alpha toxin gene from S. aureus and predicted amino acid sequence of alpha toxin. They also found the primary product of a signal sequence involved in secretion, and three short regions of high hydrophobicity, which may interact with the hydrophobic region of target cell membranes.
The secondary structure of alpha toxin was determined by circular dichroism spectroscopy. It contains 61% beta sheet, 28% random coil and 11% alpha helix in the monomers and 58, 30 and 12% respectively in the oligomers (Ikigai and Nakae, 1985).

2.1.2 Production and purification

Strain Wood 46 of S. aureus is most commonly used for the production of alpha toxin. Goode and Baldwin (1974) reported that the alpha toxin produced by different stains of S. aureus is very similar in its various properties.

Alpha toxin is produced in a biphasic manner in liquid batch cultures. It is secreted at low levels in the late logarithmic stage; most of alpha toxin appears during the subsequent period of slower cell growth (Duncan and Cho, 1971; Coleman and Abbas-Ali, 1977). The maximum toxin concentration represents 1.6 to 2.0% of the dry weight of the cells (Duncan and Cho, 1971). The yield of alpha toxin is related to factors such as CO₂ tension, and glucose and amino acids composition of the medium. Parker et al. (1926) obtained maximum toxin yield in the presence of 8 to 10 percent CO₂. Burnet (1930) increased the yield of toxin by incubation in CO₂ and air, and addition of 0.3% agar to the medium (Gladstone, 1938; Dalen, 1973). But Goode and Baldwin (1973) found that CO₂ was not essential. Duncan and Cho (1972) have shown that production of alpha toxin is maximal at a glucose concentration of 0.2%.

There are numerous methods of purification of alpha toxin. These methods often involve electrophoresis, ion exchange chromatography, gel filtration and precipitation. Lind et al. (1987) have described a FPLC method resulting in a 3S alpha toxin of high purity. Harshman et al. (1988) have presented a very simple purification procedure using column chromatography on controlled pore glass beads followed by gel filtration. This method produces highly purified alpha toxin.
2.1.3 Biological activities

The sensitivity of erythrocytes to lysis by alpha toxin varies greatly. The rabbit erythrocytes are approximately 100 times more sensitive than erythrocytes of chicken, guinea pig, human, horse, or monkey (Bernheimer, 1974; Jeljaszewicz, 1972). The minimum dermonecrotic dose in rabbit skin is 0.03 micrograms (Watanabe and Kato, 1974). Subcutaneous administration of small doses results in what appears to be hemolysis under the skin, which progresses to severe necrosis, sloughing of the tissue, and heavy scab formation after several days (Wiseman, 1975).

Alpha toxin damages leucocytes (Gladstone, 1966; Maheswaran et al., 1967; McGee et al., 1983; Suttrop et al., 1987), platelets (Siegel and Cohen, 1964; Bernheimer et al., 1972; Machangu and Bobel, 1983; Bhakdi et al., 1988), macrophages (Gladstone, 1966), hepatocytes (Bernheimer et al., 1972; McEwen and Arion, 1985), human diploid fibroblasts, amnion cells, Hela cells, rabbit kidney cells (Artenstein et al., 1963), mouse adrenocortical (Y1) tumor cells (Thelestam and Blomqvist, 1983). It also stimulates pulmonary arachidonic acid cascade (Seeger et al., 1985). Thelestam (1983) found that those cells of epithelial origin were more susceptible to alpha toxin than cells of other origins.

Alpha toxin is lethal to many animals, including mice, rats, cats, rabbits, dogs, guinea pigs, frogs and fish (Kellaway et al., 1930; Thal and Egner, 1956; Wiegershausen, 1962; Lominski et al., 1965; Masek et al., 1967). The severity of effects of alpha toxin is dose dependent. The LD$_{50}$ of intravenous infection is approximately 1 $\mu$g for mouse and 4 $\mu$g for rabbit (Fackrell and Wiseman, 1974; Freer and Arbuthnott, 1986). The lethal effect of alpha toxin is the least understood. It has been attributed to both circulatory disturbances and neurotoxicity.
Alpha toxin affects the central nervous system where it induces abnormal EEG readings just prior to death in rabbits and rats (Edelwejn and Jeljaszewicz, 1973; Edelwejn et al., 1976; Lipman and Harshman, 1985). It damages the myelin sheaths in mouse brain and isolated rabbit vagus nerves (Szmigielski et al., 1979; Harshman et al., 1981; Harshman et al., 1985). However, the distribution of radiolabeled alpha toxin after intravenous administration to mice was studied with whole body and microautoradiography, and no radioactivity was found in the central nervous system (Blomqvist et al., 1987). Since the transfer of alpha toxin molecules across the blood-brain barrier seems unlikely, the myelin damage of alpha toxin may not be relevant to the lethal effect. It appears more likely that rapidly occurring circulatory disturbances are primary to the neurotoxic effect (Thelestam and Blomqvist, 1988). In 1963, Wiegershausen found alpha toxin caused constriction of blood vessels through its action on the smooth muscle of the vasculature. Thal and Ehner (1961) also suggested alpha toxin caused vasospasm of the smooth muscle of the blood vessels, and constriction of the coronary vessels.

Alpha toxin causes contraction of smooth muscle from rabbit jejunum and guinea pig ileum (Brown et al., 1959), taenia coli of guinea pigs (Gulda and Kadlec, 1974), heart, auricle strips, and blood vessels of various species (Wiegershausen, 1962), and isolated rabbit and rat aorta (Svihovec et al., 1967). Wurzel et al. (1966) described the contraction as "spastic paralysis". The action of alpha toxin on smooth muscle was irreversible when muscle preparations were exposed to alpha toxin for sufficient time (Brown et al., 1959; Thal and Egner, 1961). Svihovec et al. (1967) reported that 20 minutes of perfusion were required for contraction of the arteries. The action of alpha toxin on smooth muscle was unresponsive to K⁺ or Ca²⁺ ions or isoproteranol (Cassidy et al., 1974; Wurzel et al., 1966). In contrast, however, Rahal et al. (1968), Kadlec and Capek (1969), and Szmigielski et al. (1971) suggested that alpha toxin inhibited the (Na⁺-K⁺)-dependent ATPase in smooth muscle. Gulda et al. (1978) proposed that alpha toxin inhibited the
active $\text{Ca}^{2+}$ uptake by smooth muscle microsomes and fragments of plasma membrane and liberated bound calcium, which caused spasmogenic action of smooth muscles.

The effects of alpha toxin on skeletal muscle and cardiac muscle have been rarely studied. Lominski et al. (1962) reported that the alpha toxin caused flaccid paralysis of skeletal muscle of mice in vivo and in vitro. In 1979, Cassidy and Harshman showed that radiolabeled alpha toxin can bind to skeletal muscle. Gulda et al. (1973) observed that alpha toxin decreased the action potential, the resting potential, and the threshold of excitability, and caused fibrillation in isolated frog myocardial fibres. They concluded that the alpha toxin resulted in an increased permeability of the membrane to calcium. In 1985, Brill also suggested that the alpha toxin increased the permeability of sarcolemma to calcium. However, the information about action of alpha toxin on skeletal and cardiac muscle is far from complete.

2.1.4 Mode of action

It is generally accepted that the membrane damaging properties of alpha toxin are essential to all its biological activities. The erythrocyte is the most used cell model for study of alpha toxin. The process of hemolysis could be divided into three stages (Cassidy and Harshman, 1976; Freer and Arbuthnott, 1986). The first stage is pre-lytic phase, in which the binding of toxin is relatively rapid and time dependent. Binding of $^{125}$I-labelled toxin to both rabbit and human erythrocytes reached a maximum level after 5-10 minutes at 1°C (Phimister and Freer, 1984). The binding could be prevented by preincubation with anti-binding antibodies, which can not inhibit hemolysis if treated after addition of toxin (Lo and Fackrell, 1979). A lag period precedes initiation of the membrane damage. The lag was about 10 minutes in rabbit RBC (Harshman and Sugg, 1985). The hemolysis could be prevented by another population of antibodies even after addition of toxin, named indirect hemagglutinating antibodies (Lo and Fackrell, 1979). These antibodies probably prevent the rearrangement of the toxin monomers on the surface of the cells’ membrane, which leads to the formation of trans-
membrane channels. High concentrations of extracellular Ca\(^{2+}\) could also prevent membrane damage by alpha toxin (Harshman and Sugg, 1985; Blomqvist and Thelestam, 1986). But the binding itself was insensitive to Ca\(^{2+}\). The second stage is lytic phase. In this stage, there is a flux of small molecules across the toxin-treated cell membrane, such as K\(^+\), \(^{86}\)Rb\(^+\), glucose-6-phosphate, nucleotides, alpha-amino- isobutyric acid, sucrose, and inulin etc. In rabbit RBC the leakage results in an influx of water across the membrane. The last step is lysis of the cells with hemoglobin release. The whole sequence takes place in about 20-30 minutes, depending on the concentration of the toxin (Thelestam and Blomqvist, 1988).

Alpha toxin binds to membranes as monomeric form. The toxin oligomers formed either by heat aggregation in solution, or on phosphotidylcholine-cholesterol liposomes do not damage membranes (Hugo et al., 1987; Bhakdi et al., 1983; Arbuthnott et al., 1967). After dissociation of the oligomers, the activity of 3S toxin can be fully restored (Hugo et al., 1987). The oligomers are thought to be 12S hexamers and have a ring like structure (Arbuthnott et al., 1967; Bhakdi et al., 1983).

Alpha toxin monomers bind to the target first, then undergo oligomerization (Harshman, 1979; Ikigai and Nakae, 1987). The 3S alpha toxin polymerizes to the 12S ring formation on rabbit, human, horse and rat hepatocyte plasma membranes as revealed by electron microscopy (Freer et al., 1968; Bernheimer et al., 1972; Bernheimer et al., 1974). The ringlike structures were isolated from toxin treated membranes and appeared in electron microscope as hollow cylinders with outer diameters of 8-10 nm and inner diameters of 3-5 nm (Fussle et al., 1981). The oligomers were shown to have a molecular weight of approximately 200,000, implying they could contain six toxin monomers (Cassidy and Harshman, 1976a,b). Ikigai and Nakae (1985) demonstrated conformational changes occurred concomitantly with the transformation of the monomer to the hexamer. Tobkes et al. (1985) proposed a hypothetical model for the assembly of alpha toxin.
into cell membranes. They suggested that the tertiary structure of the alpha toxin changed. Monomeric alpha toxin polypeptide can assemble into an oligomeric membrane channel. The monomer contains two main domains connected by a hinge. Monomers turn partly inside out before forming hexamers, in which the hydrophilic residues that were formerly on the surface of the monomer line the channel or take part in subunit-subunit and domain-domain interactions. Hydrophobic residues that were occluded between the two domains in the monomer are revealed during assembly and form the outer surface of the channel. When alpha toxin interacts with biological membranes, the conformational change could be induced by the toxin receptors. This model is supported by data presented by Gray and Kahoe (1984), whose alpha toxin gene sequence analysis shows a centrally located region in the toxin that consisting 32 percent glycine, which may constitute the hinge region (Tobkes et al., 1985).

Cassidy and Harshman (1976a,b), Harshman (1979), and Barei and Fackrell (1979) found that ringlike structures on membranes could only be seen at high concentrations of alpha toxin. It should be noted that the earlier electron microscope studies by Freer et al. (1973) employed erythrocyte ghosts rather than intact erythrocytes. The work of Arbuthnot et al. (1973) and Cassidy et al. (1974) showed that only 5 percent of added toxin bound to intact erythrocytes, while 60-70 percent bound to ghosts. Alpha toxin may bind to ghosts non-specifically, forming the ringlike structure. Barei and Fackrell (1979) proposed two binding mechanisms, low affinity of non-specific binding which could occur at high toxin concentrations and high affinity of specific binding to receptors which could occur even at low toxin concentrations. This could also explain variable susceptibilities among various cell types of different species.

In recent years, some researchers have postulated the existence of specific receptors for alpha toxin on the cell membranes. Cassidy and Harshman (1976a,b) showed that ^125I-labelled alpha toxin which bound
irreversibly to rabbit red blood cells could be inhibited if cells were preincubated with native toxin. The binding was time- and temperature-dependent. They found that there were approximately 5,000 high affinity binding sites per rabbit erythrocyte. These receptors may be proteinaceous since pronase treatment of erythrocytes decreased the number of binding sites (Cassidy and Harshman, 1976a). This corresponded with the finding of Kato et al. (1975) that pretreatment of rabbit erythrocytes with pronase decreased hemolysis by 97 percent. Such high affinity binding sites were not found on human erythrocytes (Cassidy and Harshman, 1976a,b). In 1979, Barei and Fackrell demonstrated that there were 125,000 receptors per rabbit erythrocyte. The discrepancy in the receptor number may be because Cassidy and Harshman used \(^{125}\text{I}\)-labelled toxin which retained 10 percent of hemolytic activity, while Barei and Fackrell used heat-inactivated toxoid. The toxoid is presumably a hexamer and therefore the number of receptor could be in the order of 20,000. Cassidy and Harshman (1979) isolated high molecular weight complexes which were stable in SDS or urea solutions. Barei and Fackrell (1979) found the numbers of receptors on erythrocytes from different species were correlated to their sensitivity to alpha toxin. Kato et al. (1975) postulated that a flavine mononucleotide binding glycoprotein, which is pronase sensitive, on the erythrocyte membrane, was the receptor for alpha toxin. However, they later suggested that a ganglioside extracted from human erythrocyte was the receptor (Kato and Naiki, 1976).

In 1980, Maharaj and Fackrell suggested that the alpha toxin receptor is band 3, the integral erythrocyte membrane glycoprotein. Band 3 exists as a dimer, spans the erythrocyte membrane, and is involved in anion transport (Steck, 1978; Cabanchik et al., 1978). In previous studies, Cassidy and Harshman (1976a,b), and Kato et al. (1975) found alpha toxin receptor was pronase sensitive. The pronase degrades only glycoporphin and band 3 (Brethescher, 1973; Bender et al., 1971). Trypsin, which cleaves glycoporphin, but not band 3, does not alter the hemolytic sensitivity of the erythrocytes (Brethescher, 1973). Alpha-chymotrypsin to which band 3 is susceptible was
found to increase erythrocyte resistance to alpha toxin. Also, erythrocytes coated with Concanavalin A (Con A), *Lens culinaris* (LCA) and *Ricinus communis* (RCA) all exhibited marked resistance to lysis by alpha toxin (Maharaj and Fackrell, 1980). All these lectins bind specifically to band 3 (Findlay, 1974; Marchesi et al., 1976; Adair and Kornfield, 1974). Ketsis and Grand (1982) showed band 3 was the only substantial receptor for Concanavalin A in the erythrocytes. Preincubation of alpha toxin with purified band 3 reduced hemolytic activity. Antibodies to band 3 protected rabbit erythrocytes from lysis. All the evidence suggests that band 3 is the receptor for alpha toxin.

Simpson and Fackrell (1985) further demonstrated that receptor site was located in the 35 KD C-terminal chymotryptic fragment of band 3. They suggested the carbohydrate moiety of rabbit erythrocyte band 3 is a receptor. The human erythrocytes, which also contain band 3, are much more resistant to alpha toxin than rabbit erythrocytes. This was explained by Fackrell et al. (1985), who showed band 3 receptor site was somehow masked on human erythrocytes. Treatment with trypsin and pronase may unmask the carbohydrate moiety of human erythrocyte band 3 (Simpson and Fackrell, 1985).

However, some researchers still doubt the existence of the receptor for alpha toxin. Using fully hemolytic $^{125}\text{I}$-alpha toxin, Phimister and Freer (1984) suggested there was no specific alpha toxin receptor on rabbit erythrocytes. Reichwein et al. (1987) could not demonstrate any saturation of binding sites.
2.2 Cardiac Physiology

The heart pumps blood around the cardiovascular system by rhythmical contraction. There is a pacemaker in the sinus node in the right atrium. This produces regular electrical pulses (action potentials) which are conducted quickly through the whole heart. The action potential causes muscle contraction.

The blood flow forced by the heart is influenced by the heart rate and stroke volume. The heart rate is regulated by the nervous system and is sensitive to stretch. The stroke volume is based on the force of contraction, which is length- and load-dependent (Jewell, 1977; Taylor and Rudel 1970; Sugiura et al., 1989). The end result of excitation-contraction coupling is the formation of tension-generating crossbridges between the overlapping parts of the thick (myosin) and thin (actin) filaments that make up the contractile system. The muscle stiffness varies directly with the number of attached crossbridges such that shortening reduces this number, and thus muscle stiffness, during contraction (Julian and Sikkubsm, 1975). Other important inotropic length-dependent factors include the intracellular Ca$^{2+}$ concentration is length-dependent (Sugiura et al., 1989), calcium-triggered calcium release from the sarcoplasmic reticulum (Fabriato and Fabiato, 1975), the myofibrillar calcium sensitivity (Hibberd and Jewell, 1982), and the affinity of troponin for calcium (Honig and Reddy, 1975).

2.2.1 The cellular calcium distribution

The cellular distribution of Ca$^{2+}$ is determined by the maximum binding capability per milligram of protein for Ca$^{2+}$ of the different cellular structures. Actomyosin can maximally bind up to 10$^{-1}$ mM Ca$^{2+}$ per kilogram of cardiac cells (Lullmann et al., 1983). Under physiological conditions, the contribution of the actomyosin-bound Ca$^{2+}$ to the total Ca$^{2+}$ content will remain far below this value. The sarcoplasmic reticulum, if maximally saturated, would only yield 2x10$^{-2}$ mM per kilogram of cells and thus 1% of the total Ca$^{2+}$. The mitochondria contain little Ca$^{2+}$ due to the concentration range of the
cytosolic Ca$^{2+}$ concentration. At an extracellular Ca$^{2+}$ concentration of 1 mM, the amount bound to the outer leaflet of the sarcolemma is about 1 mM per kilogram of cells, and thus it contains more than half of total cellular Ca$^{2+}$. The amount of Ca$^{2+}$ bound to the inner leaflet of the sarcolemma is far lower since it faces the low intracellular Ca$^{2+}$ concentration. The high affinity, potential-dependent binding site which is located in the sarcolemma can bind up to about 10$^{-1}$ mM Ca$^{2+}$ per kilogram of cells. Conclusively, most of the cellular Ca$^{2+}$ is in the outer leaflet on high affinity, potential-dependent binding site of sarcolemma. There are only minor amounts of Ca$^{2+}$ in intracellular organelles under physiological conditions (Lullmann et al., 1983).

2.2.2 Excitation-contraction coupling

There are several important aspects in regulation of excitation-contraction coupling.

1. Ca$^{2+}$ influx during action potentiation. There are different ion channels in the muscle membrane. These channels are composed of protein and each one is specific to one ion, i.e. Na$^+$, K$^+$, or Ca$^{2+}$. During action potentiation, the membrane potential is changed from about -85 mv to positive and the calcium flows inward through these Ca$^{2+}$ channel.

2. Na$^+$/Ca$^{2+}$ exchange. The extracellular concentrations of Ca$^{2+}$ and Na$^+$ have strong influence on the contractility of the muscle. It has been suggested there is a Na$^+$/Ca$^{2+}$ exchange mechanism in the sarcolemma, and with every Ca$^{2+}$ transported outward, there are 2 to 3 Na$^+$ flowing inward (Reuter, 1974; Mullins, 1979).

3. Ca$^{2+}$ release from sarcoplasmic reticulum. The contractile filaments are activated by Ca$^{2+}$ release from sarcoplasmic reticulum during action potentiation. It is believed that this Ca$^{2+}$ release is regulated by calcium current (Nabauer et al., 1989). However, some researchers argue that it is
regulated by a charge-coupled release mechanism during action potentiation (Cannell et al., 1987).

4. Ca\textsuperscript{2+} uptake by the sarcoplasmic reticulum. During the relaxation phase, the Ca\textsuperscript{2+} is taken back by an ATPase which has a high affinity to Ca\textsuperscript{2+} and is on the membrane of the sarcoplasmic reticulum (Chapman, 1979).

The Ca\textsuperscript{2+} handling is also regulated by a negative feedback mechanism. The Ca\textsuperscript{2+} channels are inactivated by intracellular Ca\textsuperscript{2+}. The high intracellular Ca\textsuperscript{2+} concentrations reduce the Ca\textsuperscript{2+} influx (Braveny and Sumbera, 1970; Bassingthwaigte et al., 1976).

2.2.3 Calcium compartment model for cardiac muscle

In 1987, Schouten et al. proposed a calcium compartment model in heart muscle based under studies on rat cardiac muscles (Schouten et al., 1987). This model postulates Ca\textsuperscript{2+} transport among the three hypothetical Ca\textsuperscript{2+} compartments, i.e. an uptake and a release compartment both presumably located within the sarcoplasmic reticulum and an exchange compartment located at the sarcolemma.

There are two phases in this model as shown in Figure 1B: alpha phase and beta phase. Both phases are involved with the force generation. The transport of Ca\textsuperscript{2+} from uptake compartment to release compartment is termed alpha phase. The calcium needed for force recovery at normal pacing frequency (0.2 Hz) is mainly from the alpha phase.

The beta phase represents the Ca\textsuperscript{2+} transport between the release compartment and exchange compartment. In beta phase, Ca\textsuperscript{2+} flows inward within 100 seconds after the beating, and outward after 100 seconds. The force increases to the maximum at the rest interval of about 100 seconds. The beta phase contributes to the force recovery at steady frequency of
Figure 1

A: Typical rest interval-force curve. The alpha and beta phases can be identified from rest potentiation analysis.

B: Calcium compartment model of rat cardiac muscles. Three calcium compartments are depicted.

\[ r = \text{recirculation fraction between beats}. \]

\[ 1-r = \text{fraction of Ca}^{2+} \text{ extruded with each beat}. \]

\[
\text{alpha phase} = \text{Ca}^{2+} \text{ movement from uptake compartment to release compartment.}
\]

\[
\text{beta phase} = \text{Ca}^{2+} \text{ movement between exchange compartment and release compartment.}
\]

(reprint from Schouten et al., 1987).
0.2 Hz less than the alpha phase, but it contributes significantly to the maximal potentiation at long rest intervals (100-120 seconds).

There is a Ca\(^{2+}\) efflux during the contraction (1-r in Figure 1B). The fraction of Ca\(^{2+}\) available to the myofilaments is called recirculating fraction (r in Figure 1B).

The Ca\(^{2+}\) handling among the compartments can be reflected by interval-force relationship. A typical curve is shown in Figure 1A. The alpha and beta phases on this curve represent the alpha and beta phases in the model (the experiment is explained at sections 3.13 and 3.14).

When cardiac tissues are not beating for a while, all intracellular Ca\(^{2+}\) compartments are in equilibrium with an extremely low cytosolic Ca\(^{2+}\) concentration (10\(^{-7}\) M); only the outer layer of the sarcolemma is in equilibrium with the extracellular Ca\(^{2+}\) concentration which is about 10\(^{-3}\) M Ca\(^{2+}\) (Lullmann et al., 1983).

2.2.4 The peak force, resting tension, and extracellular and intracellular Ca\(^{2+}\) concentration.

The magnitude of peak force is directly related with the extracellular Ca\(^{2+}\) concentration. Schouten (1985) demonstrated that at 0.2 Hz stimulation frequency, the peak force was just detectable in 0.2 mM Ca\(^{2+}\), and in about 2.5 mM Ca\(^{2+}\) maximal force was reached. Further increase of extracellular Ca\(^{2+}\) concentration up to 10 mM resulted in a small decrease of peak force, often accompanied by spontaneous contractions. The positive relation between the magnitude of peak force and the extracellular Ca\(^{2+}\) concentration is due to the influence of the extracellular Ca\(^{2+}\) concentration on the intracellular free Ca\(^{2+}\) concentration. It is well known that there is a positive association between the intracellular free calcium concentration and the magnitude of peak force (Yue, 1987). However, when the cardiac muscle is overloaded with calcium, it displays spontaneous subcellular mechanical
oscillations which is due to spontaneous cyclic release of calcium from the sarcoplasmic reticulum (Stern et al., 1989). In rat ventricular muscles, the resting tension is also related to extracellular Ca\(^{2+}\) concentration. A stepwise increase of extracellular Ca\(^{2+}\) concentration results in an increase in resting tension (Lullmann et al. 1983).
MATERIALS AND METHODS

3.1 **Reagents and Buffers**

Heparin was purchased from Sigma Chemical Co., St. Louis, MO. Ether for anesthesia was obtained from Mallinckrodt Canada Inc., Pointe-Claire, Quebec. All other chemicals were analytical reagent grade.

Phosphate buffered saline (PBS) consisted of 145 mM NaCl and 10 mM sodium phosphate buffer pH 7.4. For the perfusion of hearts and papillary muscles, a modified Krebs-Henseleit bicarbonate buffer was used, which was composed of 1.0 mM CaCl$_2$.2H$_2$O, 117.1 mM NaCl, 5 mM KCl, 1.2 mM MgCl$_2$.H$_2$O, 2.4 mM Na$_2$SO$_4$, 2 mM NaH$_2$PO$_4$.2H$_2$O, 27 mM NaHCO$_3$, and 10 mM glucose. The buffer was oxygenated with a gas mixture of 95% oxygen and 5% carbon dioxide from 1 hour prior to use. The buffer was adjusted to pH 7.4 with 1 N NaOH.

3.2 **Cultures**

Cultures of *Staphylococcus aureus* strain wood 46 were used to produce alpha toxin. The cultures met the requirement established by Baird-Parker in 1972. The Canadian Communicable Disease Centre in Ottawa Phage-typed the strain. It was stored at -90°C. The cultures were incubated at 37°C for 36 hours on rabbit blood agar plates in an atmosphere of 10% CO$_2$ and 90% air. Those colonies which showed the largest areas of hemolysis were subcultured on rabbit blood agar plates and the used for production of alpha toxin.

3.3 **Production of Alpha Toxin**

Alpha toxin was produced according to the method of Harshman et al. (1988). The colony showing the largest zone of lysis on blood agar plates was inoculated into one litre flask containing 500 mL yeast extract dialysate
medium which had pH 7.5 and was composed of yeast extract, 25 g/L; acid-hydrolyzed casein, 20 g/L; glucose, 2.5 g/L; thiamin, 0.13 mg/L; and nicotinic acid, 1.2 mg/L. Then the flask was incubated with shaking at 37°C for 18 hours. The cells were removed by centrifugation for 10 minutes at 5,000 x g. The culture supernatant was adjusted to pH 6.8 with NaOH and was ready to add to the controlled glass-pore beads for column chromatography (CPG-10, 120/200 mesh, mean pore diameter 340 Å, Electronucleonics Inc., Fairfield, NJ).

Before adding the supernatant to the column, the glass-pore beads were washed with a 1-5% bleach solution which was followed by copious rinses with distilled water and equilibrated with 0.01 M KHPO₄ buffer at pH 6.8. The glass-pore beads were then poured into a column and the culture supernatant was applied. Flow rate was controlled at 10-20 mL/min. Effluent fractions of 100 mL each were collected and tested for hemolytic activity using 50% endpoint method (section 3.6).

After the saturation of the column with toxin, the column was washed with 0.01 M KHPO₄ buffer of pH 6.8. Then the bound alpha toxin was eluted with 1.0 M KHPO₄ buffer of pH 7.5. Fractions that showed positive in the hemolytic assay were combined and brought to 90% saturation by the addition of solid (NH₄)₂SO₄ (66.2 g/100 mL of solution). The solution was stored overnight at 4°C.

The inorganic salt was removed by low-speed centrifugation at 400 x g for 5 minutes and the alpha toxin was collected by high-speed centrifugation at 15,000 x g for 15 minutes. The pellet containing the alpha toxin was dissolved in about 5-10 mL of 100 mM NaHPO₄ buffer, pH 7.2, and was applied to a Sephadex G-75 column (18 x 1.7 cm). The column was developed with 100 mM phosphate buffer, pH 7.2, with flow rate of 1.0 mL/min, and fractions of 5.0 mL were collected. The alpha toxin from
fractions showing hemolytic activity was precipitated by the addition of solid
(NH₄)₂SO₄ (66.2 g/100 mL of solution) and stored overnight in cold room.
The alpha toxin was then recovered from the ammonium sulfate solution by
centrifugation at 15,000 x g for 10 minutes and the precipitate was dissolved
in 2-5 mL of 100 mM NaHPO₄ buffer, pH 7.2. The final solution contained
2-10 mg of alpha toxin per milliliter of buffer and could be stored at -70°C.

3.4 Production of Alpha Toxoid

Alpha toxoid was produced by heating alpha toxin in a water bath at
60°C for 30 minutes. The term "toxoid" was introduced by Burnet (1931) to
represent the non-hemolytic form of alpha toxin.

3.5 Preparation of Erythrocytes

Blood from rabbits was collected into flasks containing an equal volume
of Alsever’s solution (Colligan et al., 1991). The flasks were shaken to prevent
clot formation and stored at 4°C. Upon use, the erythrocytes were packed
by centrifugation of blood samples at 600 x g for 3-5 minutes and the
supernatant was removed. Then the erythrocytes were washed three times
in 6 volumes of PBS by centrifugation and the top layer of white cells (buffy
coat) was removed. The washed erythrocytes were resuspended to 2% (v/v)
solution in PBS.

3.6 Hemolytic Titration of Alpha Toxin and Determination of
Amount of Alpha Toxoid

To determine the hemolytic titration of alpha toxin, the 50% end point
method was used. One hemolytic unit (HU) of toxin was defined as the
amount required to lyse 50% of 1% suspension of rabbit erythrocytes in
1 mL of PBS after 1 hour at 37°C. Fifty microlitres of alpha toxin were serially
diluted in 50 microlitres of PBS in microplates. Fifty microlitres of PBS and
100 microlitres of 2% suspension of rabbit erythrocytes were added to each
well. The mixture was incubated at 37°C for 1 hour and centrifuged at
200 x g for 5 minutes. One hundred microlitres supernatant from each well
were taken out and the absorbance was read at 540 nm. The percentage of hemolysis of each well could be obtained by comparison of its turbidity to standards. The standards were prepared from a lysate of 2% erythrocytes in distilled water. The 50% end points were determined from the dilution-percentage curves. One hemolytic unit (HU) is the reciprocal of that dilution of the toxin which causes 50% hemolysis.

Although alpha toxoid is not hemolytic, the amount of toxoid is also expressed as hemolytic unit(s). One hemolytic unit of alpha toxoid represents the amount of toxoid made by one hemolytic unit alpha toxin.

3.7 Kinetic Hemolytic Assay

The lysis of erythrocytes by alpha toxin was measured by decrease in absorbance at 650 nm. A series of different amounts of alpha toxin and alpha toxoid were added to wells of microplate. Each well had two hundred microlitres of a 2% erythrocytes and final amount of each well after addition of alpha toxin/alpha toxoid and PBS was 250 microlitres. The microplate was put into a microplate autoreader (EL 309, BIO-TEK INSTRUMENTS) immediately after the mixture of erythrocytes with toxin or toxoid and the absorbance was read at 650 nm. The microplate autoreader was controlled by an IBM XT microcomputer with a "COLLECT" program. Up to 12 wells of the microplate can be read simultaneously. The absorbance was read for 20 minutes with 400 points per well. Another computer program called "ANALYSE" calculated a variety of parameters such as maximal instantaneous rate of lysis; time to maximal instantaneous rate of lysis; maximal absorbance; absorbance of 50% of maximal absorbance; time to maximal absorbance; and slope and Y intercept of regression.

3.8 Animal Handling

Adult male and female Wistar rats weighing 200 to 400 g were housed at 23°C with 12 hour light and dark cycles and maintained on Purina
laboratory rat chow and water *ad libitum*. The animals were maintained in accordance with the guidelines set by the Canadian Council on Animal Care.

3.9 **Preparation of Papillary Muscles**

The rats were anaesthetized with ether. The abdominal incisions were made and rats were injected with 0.2 mL of heparin (0.6% w/v heparin sodium in saline) via the inferior vena cava to prevent blood clotting. Thirty seconds after injection, the thoracic cavities were opened and the hearts were rapidly removed and placed into ice cold normal saline (0.8% w/v) to inhibit contractions and then put into a dissection chamber. The aortae were cannulated and the hearts were perfused with modified Krebs-Henseleit bicarbonate buffer at room temperature. After residual blood was removed which took about 1 minute, the K⁺ concentration in the perfusate was increased to 15 mM to arrest the beating of the hearts. Then the right ventricle was opened under a dissecting microscope. The free wall was pulled back so that the papillary muscles were exposed. Only thin and long muscles with uniform sides and no branches were selected. During dissection, contact of the papillary muscles with the operational implements and stretch of the papillary muscles were very carefully minimized because the muscles are very vulnerable. The papillary muscles were dissected in such a way that a portion of the right ventricular wall which surrounded the base of the papillary muscle and a portion of the tricuspid valve which connected the tip of the papillary muscle were excised from the hearts and remained connected with the papillary muscles. The block and valve would be used later when muscles were suspended in muscle bath.

3.10 **Length Clamp and Experimental Setup for Papillary Muscles**

Figure 2 depicts the length clamp and experimental setup for the studies of rat papillary muscles. Immediately after dissection from right ventricles, the papillary muscles were mounted in a muscle bath which was on the stage of a dissecting microscope. The block of tissue at the base of the muscles was suspended in a cradle and a hole on the tricuspid valve was inserted
Figure 2

A schematic diagram of the system setup.
with a tungsten wire hook which was connected to the arm of a force-displacement transducer (Grass FT03. Grass Instrument Co., Quincy, MA). The length of the muscles could be adjusted by moving the position of the cradle with micromanipulators.

Before treated with toxin or toxoid, the muscles were perfused first with the modified Krebs-Henseleit buffer with calcium concentration of 1.0 mM. Through a pump system, the oxygenated buffer was recirculated between muscle bath and the glass reservoir. The buffer was oxygenated with a gas mixture of 95% oxygen and 5% carbon dioxide in the reservoir. The muscles were perfused at about 10 mL/minute.

The temperature of the buffer in the muscle bath was monitored by a thermocouple embedded in the muscle bath, and was controlled by a heater and a cooler that pumped warm or cold water into the space in the water jacked reservoir.

It was found that the alpha toxin lost hemolytic activity when incubated with buffer that was oxygenated by bubbling. Therefore, when muscles were treated with alpha toxin, two reservoirs were used. First one contained only normal buffer that was oxygenated and second one contained preoxygenated buffer with alpha toxin, i.e. no bubbling in second reservoir. The alpha toxin concentration in this second reservoir was 10 times higher than that desired for treatment of the muscles. By adjusting the filling speeds of the buffer from two reservoirs into the muscle bath, the muscle was perfused with the buffer in which 90% from the first reservoir and 10% from the second one. Then the desired concentration was obtained and the buffer still contained oxygen. This method eliminated the possible damage of alpha toxin by bubbled buffer. Moreover, this manipulation gave rapid and accurate addition of alpha toxin to muscles.
The muscles were stimulated by an electric stimulator via two platinum wire electrodes which were parallel to the longitudinal section of the muscles. At beginning, the stimulation voltage was increased from 0 V to the threshold at which the contraction was just stimulated. Then the voltage was increased 50% from this threshold. The range of the usually used voltage was 20-80 V. The duration of each stimulation was 4 ms throughout the whole experiment.

During equilibration, the muscles were given a moderate resting tension and equilibrated for an hour at 1.0 Hz stimulation frequency (1 beat every 1 second). This frequency effectively allowed the muscles to recover from surgery.

After equilibration, the stimulation frequency was changed to 0.2 Hz (1 beat every 5 seconds). Preparations of rat ventricular myocardium show a negative force-frequency relationship. The lower the frequency, the higher the developed force. However, too low frequency causes muscle instability. 0.2 Hz was selected for our experiments because the muscles developed greater force and still remained stable.

3.11 Data Recording

The force signals generated by the muscles were transmitted by the force displacement transducer, amplified by a Grass amplifier (model 7P122D) and converted to digital form by an IBM personal computer data acquisition and control adapter distribution panel with 12 bit analog-digital (A/D) converter. The amplified force signals were also sent to an oscilloscope so that the signals could be observed in real time. The signals were digitized by the A/D converter. The data were processed through a program called "Unkelscope" (Unkel Software Inc., Lexington, MA) which presented the force signals as voltage. The change of the voltage reflected the change of the force. Figure 3 illustrates the relationship between force (g) and change of the voltage (v) at different sensitivities of the amplifier. The data were
Figure 3

Calibration of transducer. The changes of voltages were measured when the weight attached to the transducer produced a steady DC voltage output. The sensitivity was changed by allowing the gain on the pre-amplifier adhered to the transducer was changed at different sensitivity of the transducer. The relation of the changes in the force and the change of the voltages was plotted, and the slopes of the fitted lines were calculated.
sampled at 200 Hz and shown on the screen. There were at least 120 points for each twitch function. The selected data were saved on floppy discs. With the "Unkelscope" program, the peak force, the maximal rate of force development (+dF/dT), the maximal rate of force deactivation (-dF/dT), and various timing parameters could be calculated.

### 3.12 Twitch Characterization

The twitch curve has two phases, a contractile phase which consists the ascending limb and a relaxation phase which consists the descending limb. The force parameters included: the peak tension, the maximal rate on contractile phase (+dF/dT), and maximal rate on relaxation phase (-dF/dT). The timing parameters analysed were: time to maximal rate of force development (T0-T1), time from maximal rate of force development to peak tension (T1-T2), time to peak tension (T0-T2), time from peak tension to maximal rate of relaxation (T2-T3), time from maximal rate of relaxation to complete relaxation (T3-T4), and time from peak tension to complete relaxation (T2-T4). The T0, T1, T2, T3, and T4 represent the time at which muscle starts to contract, the time at which the positive maximal rate is arrived, the time at which the peak tension is arrived, the time the negative maximal rate is arrived, and the time at which the contraction is completed respectively. A typical twitch curve recorded from a normal rat papillary muscle is shown on Figure 4, at which these parameters are pointed.

### 3.13 Interval-Force Relationship

The interval-force relationship was examined at 22 rest intervals (0.80, 1.00, 1.50, 2.00, 2.50, 3.50, 4.50, 6.00, 8.00, 11.00, 15.00, 20.00, 27.00, 36.00, 49.00, 66.00, 90.00, 121.00, 130.00, 150.00, and 163.00 seconds). The ratio of the developed force of first beat after rest interval and the developed force of last beat before rest interval (i.e. force/baseline) was calculated at each rest interval as shown in Figure 5. The rest intervals versus force/baseline was plotted (Figure 1A). The alpha phase and beta phase were isolated from the interval-force curve by a computer program called
Definition of parameters in the analysis of the twitch characteristics. The maximal rates (±dF/dT) and timing parameters are described on a typical twitch function curve. T₀: Time at which the muscle starts to contract. T₁: Time at which the positive maximal rate is arrived. T₂: Time at which the peak tension is arrived. T₃: Time at which the negative maximal rate is arrived. T₄: Time at which the muscle completes contraction. + dF/dT: Maximal rate of force development. -dF/dT: Maximal rate of force relaxation.
Figure 5

**Protocol for generating rest interval-force data.** The interval-force data were collected by plotting force of first beat after a rest interval with force of last beat before interval (force/baseline) at various rest intervals. In the experiment, the muscle was stimulated with 0.2 Hz. Then a different rest interval was given. The potentiation = The force of beat 1/The force of last beat before rest interval. After measuring the potentiations at various rest intervals (0.8-163 seconds), the interval-force relationship was obtained as the curve in Figure 1A.

The potentiation decay experiment was measured by similar protocol. After a rest interval of 121 seconds, a series of beats was collected. The force at each beat was divided by force of baseline. Then the force/baseline at beat n versus force/baseline at beat n + 1 was plotted and the potentiation decay was calculated. An example of the calculation of potentiation decay is given at Figure 6.
"Eureka" (Borland Software Inc., California). The mathematical model used in separating two phases (Taylor et al., 1989) was:

\[ f(t) = F(1-M\exp(-at)-(1-M)\exp(-bt)) \]

F, M, t, a and b represent maximum ratio of force/baseline, mixing constant, length of rest interval, constant for alpha phase, and constant for beta phase respectively.

3.14 Potentiation Decay

The potentiation decay was studied after 121 seconds rest interval. The force/baseline at beat n versus force/baseline at beat n + 1 was plotted, and 11 beats were analyzed for each muscle. The slope of its linear relation was considered as recirculating fraction (r in figure 1B). The recirculating fraction is the percentage of the Ca\(^{2+}\) available to the myofilaments in the total amount of Ca\(^{2+}\) released by the release compartment. One example is given on Figure 6.

3.15 Statistical Analysis

The group t-test was used to determine the overall statistical significance between controlled and experimental groups. The paired t-test was used to study the statistical significance between before and after alpha toxin or toxoid treatment. \( p < 0.05 \) was considered significant (Ehrenfeld and Littauer, 1964).
Figure 6

An example of the calculation of potentiation decay. After a rest interval of 121 seconds, the developed force/baseline was calculated for each of the 11 subsequent beats (Figure 5 shows 8 beats). The force at each beat was divided by force of baseline, i.e., (force of beat 1)/baseline, (force of beat 2)/baseline, and so on. Then the force/baseline at beat n versus force/baseline at beat n + 1 is plotted. The slope of the fitted line represents the recirculation fraction r in the calcium compartment model.
RESULTS

4.1 Hemolytic Activity of Alpha Toxin and Inhibition by Toxoid.

The purposes of the present research were to measure the effect of alpha toxin on cardiac muscles and to compare it to the effect on erythrocytes. Thus the hemolytic effects of alpha toxin and toxoid on rabbit erythrocytes were examined as baselines. However, tremendous effort on hemolytic effects of alpha toxin has been made by many labs and some of the information will be discussed.

The lysis of the erythrocytes by alpha toxin was observed with a turbidometric procedure. The absorbance of the suspension of the erythrocytes at 650 nm decreases with time as erythrocytes are lysed by the toxin. The process can be continually measured by a spectrophotometer that is connected with a microcomputer. Figure 7 shows the hemolytic sensitivity of 1.6% rabbit erythrocytes to various concentrations (130, 65, 32, 16, 8, 4, and 2 HU/mL) of alpha toxin and 3,000 HU/mL alpha toxoid. The response is concentration dependent. Slow hemolysis was seen at a toxin concentration as low as 2 HU/mL, while at higher concentration of 130 HU/mL, the lysis of the erythrocytes occurred within the first minute, and was almost completed within 10 minutes. In contrast, alpha toxoid did not lyse rabbit erythrocytes even at the high concentration of 3,000 HU/mL. Moreover, the hemolytic effect of the alpha toxin was inhibited by the addition of the toxoid at the same time when toxin was added (Figure 8).

The computer also calculates the instantaneous rates of lysis. The maximal rates of hemolysis by alpha toxin at each concentration were then recorded. The maximal rate versus toxin concentration is plotted in Figure 9 and shows that the maximal rate of lysis was toxin concentration dependent. The maximal rate increased rapidly at lower range of toxin concentrations.
Figure 7

Hemolytic effect of alpha toxin. Different concentrations of alpha toxin or toxoid were added to 1.7% rabbit erythrocytes. The mixtures were monitored for hemolytic activity by the Kinetic Hemolytic Assay as described in Material and Methods. (○) 130 HU/mL alpha toxin; (▲) 65 HU/mL alpha toxin; (▲) 32 HU/mL alpha toxin; (●) 16 HU/mL alpha toxin; (●) 8 HU/mL alpha toxin; (●) 4 HU/mL alpha toxin; (●) 2 HU/mL alpha toxin; and (●) 3,000 HU/mL alpha toxoid. The results presented here are typical. Normally 400 data points were collected for each curve. Fewer data points are plotted for graphical clarity.
Inhibition of hemolysis of alpha toxin by toxoid. The alpha toxin and toxoid were added to 0.7% rabbit erythrocytes at same time and the mixtures were monitored for hemolytic activity by the Kinetic Hemolysis Assay as described in Material and Methods. The concentration of alpha toxin was 100 HU/mL. (○) Controlled without toxin and toxoid; (●) 100 HU/mL alpha toxin and no toxoid; (△) 100 HU/mL alpha toxin and 300 HU/mL toxoid; (△) 100 HU/mL alpha toxin and 240 HU/mL toxoid; (+) 100 HU/mL alpha toxin and 160 HU/mL toxoid. The results presented here are typical. Normally 400 data points were collected for each curve. Fewer data points are plotted for graphical clarity.
Figure 9

The maximal rates of hemolysis at different alpha toxin concentrations. The maximal rates of hemolysis were calculated at several different toxin concentrations from 2 to 130 HU/mL. The x-axis is logarithmic. Maximal rate of hemolysis is plotted as units where 1 unit = 0.001 OD/min.
and relatively slowly at higher range of concentrations. The maximal rate at toxin concentration of 130 HU/mL was approximately 11 times higher than the maximal rate of lysis at 2 HU/mL.

The erythrocytes provide a relatively simple model to study how alpha toxin affects membranes. Previous studies (Arbuthnott et al., 1967; Freer et al., 1968) have proposed that the alpha toxin binds to the cellular membrane as a monomer form and then forms a hexamer. These hexamers form transmembrane channels (Thelestance and Blomqvist, 1988) which lead to $K^+$ ($^{86}$Rb) release and eventual osmotic lysis of the cells with hemoglobin release (Freer and Arbuthnott, 1966). It has also been suggested that there are receptors on red blood cells for alpha toxin (Barei and Fackrell, 1979; Cassidy et al., 1974; Simpson and Fackrell, 1985; Cassidy and Harshman, 1976a,b; Kato et al., 1975; Cassidy and Harshman, 1979; Kato et al., 1975; Kato and Naiki, 1976; Maharaj and Fackrell, 1980). The inhibition of lysis by toxoid suggests that toxoid still can bind to the membranes of erythrocytes, and toxin and toxoid have same receptors on erythrocytes’ membranes (Barei and Fackrell, 1979). Therefore, the alpha toxoid still has biological activity although it lost hemolytic activity.

4.2 The Length-tension Relation on Rat Papillary Muscles.

To test the reliability of the new length-clamp equipment, two traditional experiments have been repeated. The first one was to examine the developed force and passive force at different muscles’ lengths.

Figure 10 shows the results from a typical experiment. In this experiment, the muscle's length was initially adjusted to $L_0$, at which the developed force was just detectable. Then the length was increased by 0.05 mm and the developed force and passive tension were recorded when stabilized. The length was again increased and the process was repeated. After measuring the developed force and passive tension at various lengths, these forces
Figure 10

Length-tension relationship of rat papillary muscle at a wide range of length. The developed force and passive tension were measured at a wide range of length (from 0% to 134% of $L_{\text{max}}$). The developed force and passive tension were normalized by cross sectional area of the muscle. The lengths were normalized by $L_{\text{max}}$ (placed at 100%). The data represent a typical experiment.
were plotted with lengths. The length at which the maximum developed force was obtained was defined as L_{max}. The length at L_{max} minus the length at L_0 was called 100% of L_{max}, and each length was calculated as a percentage of L_{max}. The figure shows the developed force was minimal when lengths were less than 50% of L_{max}, developed sharply when lengths were between 50 - 100% of L_{max}, and then decreased when lengths were greater than L_{max}. On the other hand, the passive tension increases minimally before L_{max} and steeply after L_{max}. These results confirmed the literature (see review by Jewell, 1977). The length-dependence of the force is due to several mechanisms which have been summarized in the literature review.

To study the variability of the system, seven muscles were examined in such a way that L_{max} was determined and then muscles were equilibrated sequentially to 95%, 90%, 85%, 80%, 75%, and 70% of L_{max}. Muscles were not examined with lengths longer than L_{max} because the muscles could be easily damaged if they were ever stretched. Damaged muscles show spontaneous and irregular contractions. Since the muscles tested were going to be used for other experiments after this length-tension experiment, these muscles were only examined at lengths less than L_{max}. Both the developed force and passive tension varied from muscle to muscle at each length due to the individuality of the muscle. However, when these forces were normalized to the L_{max} of each muscle, they showed same pattern and consistency as seen in Figure 11.

The experiments above also provided basis for choosing muscle length for later experiments. Since L_{max} optimizes both developed force and passive tension which are critical parameters for the studies of cardiac muscles, the L_{max} was selected as the muscle length for later experiments.
Length-tension relationship of rat papillary muscles with statistical analysis. The developed force and the passive tension were measured at different lengths. The length at which the developed force was just detectable is defined as $L_0$ and the length at which the developed force was maximal is defined as $L_{max}$. An hundred percent of $L_{max}$ represents the length of $L_{max}$ minus $L_0$. An hundred percent of relative force represents the maximal developed force. The values are means ± S.E. of 7 experiments.
Since the system produced results which confirmed the literature (see reviews by Jewell, 1977) and showed good consistency, it is concluded that the system is solid and reliable.

4.3 The Developed Force of Rat papillary Muscle at Different Temperatures.

The second experiment to test the reliability of the system was to examine the developed force at different temperatures. Another purpose of this experiment was to select a temperature that would favor optimal twitch force development. In this experiment, the developed force of papillary muscles was examined at 25°C, 30°C, and 35°C, respectively.

During experiments, the muscles were set at Lmax and equilibrated at 25°C for about 30 minutes, and then the developed force was measured. Afterward, the temperature was changed to 30°C, and developed force was measured after 30 minutes equilibration. Finally the same process was repeated at 35°C. The muscles' length was not changed during the experiments. The results presented in Figure 12 show that the developed force was highest at 25°C, and was significantly reduced at 30°C and 35°C. The changes were linear. The results are consistent with the data presented by Shattock and Bers (1987). The ratio of standard error and mean volume increased at higher temperatures. It was 6.9%, 8.7%, and 12.8% at 25°C, 30°C, and 35°C respectively. Therefore the force at higher temperatures is lower and more changeable. The increase of the force at lower temperatures involves several different mechanisms, including an increase in calcium influx during the action potential, an increase in the subsequent release of calcium triggered by the action potential, and a change in the sensitivity of the myofilaments to activator calcium (Fabiato, 1985).

Since the developed force is maximum and the variability is minimum at lower temperature, the 26°C has been selected for later experiments. The actual range of the temperature during the experiments was 26.0 ± 0.3°C.
Temperature-developed force relationship. The developed force of rat papillary muscles was measured at 25°C, 30°C, and 35°C. The length of muscles was adjusted to $L_{\text{max}}$ at 25°C before measuring the developed force and changing the temperature. The developed force was normalized by cross section areas of the muscles. The values are means ± S.E. of 11 experiments.
The mean and standard deviation of developed force obtained from these eleven muscles at 25°C, 0.2 Hz, and 1.0 mM Ca\(^{2+}\) was 2,425 ± 556 mg/mm\(^2\). The result fits remarkably to the literature. Schouten et al. (1985) reported that the maximum developed force from rat papillary muscles at same condition except at 2.5 mM Ca\(^{2+}\) was 5,200 ± 1,900 mg/mm\(^2\), and it was 50% at 1.0 mM Ca\(^{2+}\). Moreover, the standard deviation of 22.9% in present research is much less than that of 36.5% in Shouten's research. This is probably because more uniform size of muscles was selected in this experiment than in Schouten's experiment in which both papillary and trabecular muscles were used.

Therefore, the system was once again proved to be solid because it confirmed the literature, and the results from several muscles showed minimum variability.

4.4 The Effects of Low Concentration Alpha Toxin (10 HU/mL) on Developed Force and Passive Tension of Rat Papillary Muscles.

Alpha toxin has direct cardiovascular effect (Adamo, 1989; Brill, 1985; Guida, 1973; Wiegershausen, 1962) through constricting the blood vessels and depriving the heart of oxygen (Adamo et al., 1989). Adamo et al. also suggested that there might also be a direct effect of alpha toxin on cardiac muscle. To study this effect, the papillary muscles from rat right ventriculum were isolated and exposed to alpha toxin. The papillary muscles were used because there was almost no effect of vasculature on these muscles as diffusion was not limiting. The muscles were mounted on the muscle bath, stimulated with 0.2 Hz for an hour at 25°C, and then adjusted to L\(_{\text{max}}\). When the developed force was stabilized at L\(_{\text{max}}\), the alpha toxin was added to the buffer at a final concentration of 10 HU/mL. Both developed force and passive tension were measured over a period of 30 minutes. The results are illustrated in Figure 13 and Figure 14. The developed force showed a transient increase within 1-2 minutes, which was not significantly different
Figure 13

Percentage change in developed force in rat papillary muscles due to the administration of 10 HU/mL alpha toxin. Muscles were first equilibrated with modified Krebs-Henseleit buffer and electrically stimulated at 0.2 Hz. The Lmax was determined and muscles were allowed to equilibrate at Lmax until the developed force was stabilized. Then the rat papillary muscles were perfused with a low concentration of alpha toxin (10 HU/mL). The changes in the developed force were measured over time. Data were normalized by baseline values (placed at 100%). The values are means ± S.E. of 8 experiments.
Figure 14

Percentage change in passive tension in rat papillary muscles due to the administration of 10 HU/mL alpha toxin. Muscles were first equilibrated with modified Krebs-Henseleit buffer and electrically stimulated at 0.2 Hz. The $L_{\text{max}}$ was determined and muscles were allowed to equilibrate at $L_{\text{max}}$ until the developed force was stabilized. Then the rat papillary muscles were perfused with a low concentration of alpha toxin (10 HU/mL). The changes in the passive tension were measured over time. Data were normalized by baseline values (placed at 100%). The values are means $\pm$ S.E. of 8 experiments.
from the force at time 0. After this transient increase of first few minutes, there was significant \( p < 0.05 \) decrease in the developed force after 30 minutes alpha toxin treatment. The changes were very similar to the results presented by Adamo et al. (1989), who used isolated rat left atria and 12 HU/mL alpha toxin. In contrast, the passive tension remained relatively stable during the experiments as no significant change in the passive tension during 30 minutes treatment. This experiment indicates that alpha toxin indeed has effect on cardiac muscles. The changes of developed force could be due to the changes of length and/or \( \text{Ca}^{2+} \) handling mechanism. The stability of the passive tension during the experiments suggests that it is the \( \text{Ca}^{2+} \) handling but not length that was affected by alpha toxin. To look inside the \( \text{Ca}^{2+} \) handling mechanism, the twitch characteristics were next analyzed.

4.5 The Changes in Twitch Force Characteristics after Alpha Toxin (10 HU/mL) Treatment.

The study in twitch characteristics could provide information regarding the calcium handling mechanism and a better understanding of effects of alpha toxin on cardiac muscles.

This study determined the magnitude and shape of the twitch function curve, which are represented by the tension and the positive and negative maximal rates of force development, and timing parameters. The characteristics of the twitch curve were analyzed before and after 30 minutes of alpha toxin (10 HU/mL) treatment. The reason for selecting 30 minutes is that the developed force was stable at this time.

Figure 15 shows the changes on the shape of the twitch function by superimposing two twitch function curves which were before and after toxin treatment from a single muscle. The curves are raw data recorded during the experiment. Peak developed force was reduced after toxin treatment as were the maximal rates of force development \((+dF/dT)\) and relaxation \((-dF/dT)\).
Figure 15

A superimposed twitch contraction from the same muscle before and after 30 minutes of alpha toxin (10 HU/mL) treatment. The curves were center matched for better comparison. The magnitude and the shape of the twitch function curve has been changed after toxin treatment. The positive and negative maximal rates were reduced and the timing parameters were not altered. For statistical analysis, see Table 1 and Table 2.
Before toxin treatment
30 min after toxin treatment
To quantitatively study these parameters, the results from before and after toxin treatment were analyzed with paired t-test. As seen in Table 1, not only was peak developed force significantly reduced (p < 0.05), there were also significant parallel decreases in both positive and negative maximal rates of force development (p < 0.05). The change of the shape of the twitch response indicates that the reduction of peak force is related to the calcium handling mechanism which may be affected by alpha toxin. The decreases of the maximal rates reflect the changes of processes of activation and deactivation, which may be due to the decrease of Ca$^{2+}$ amount released from sarcoplasmic reticulum to the myofilaments and/or the decrease of sensitivity of myofilaments to calcium.

As shown in Table 2, there were no significant changes on all timing parameters (p > 0.05). The unchanged timing indicates that the decreased developed force was caused by the decrease of the amount of calcium released from the sarcoplasmic reticulum. It also implies that the sarcoplasmic reticulum is not the target of alpha toxin, because the recycling of calcium by the sarcoplasmic reticulum is time-dependent and the impairment of sarcoplasmic reticulum should be reflected by the changes of the timing on relaxation phase (Poggesi et al., 1987). Therefore, the decrease of the amount of the calcium was probably induced by toxin impeding the calcium pathways rather than by affecting the sarcoplasmic reticulum itself.

4.6 The Effects of Low Concentration Alpha Toxoid (10 HU/mL) on Developed Force and Passive Tension of Rat Papillary Muscles.

When alpha toxin is heated at 60°C for 30 minutes, it loses its hemolytic activities (Figure 7). This inactivated alpha toxin, called toxoid, is a hexameric form of the alpha toxin (Harshman, 1979). Although toxoid has no hemolytic effect, it can bind to the receptor on erythrocytes. The preincubation of erythrocytes with toxoid prevents the hemolysis by alpha toxin (Barei and Fackrell, 1979). It is proposed that there are two domains on alpha toxin, a
Table 1

Twitch force characteristic data before and after 30 minutes of alpha toxin (10 HU/mL) treatment.

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>After</th>
<th>p &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tension (mg/mm²)</td>
<td>2252.8 ± 618.0</td>
<td>1872.9 ± 497.8</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>+Maximal Rate (mg/mm²/ms)</td>
<td>18.1 ± 4.4</td>
<td>16.1 ± 3.8</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>-Maximal Rate (mg/mm²/ms)</td>
<td>10.3 ± 2.6</td>
<td>9.2 ± 2.3</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

The tension and positive and negative maximal rates were collected from twitch function curves of same muscles before and after 30 minutes of toxin (10 HU/mL) treatment. The values are means ± S.E. of 8 experiments. All parameters show significant differences by paired t-test (not significant by group t-test since the difference among individuals is much bigger than the difference between before and after toxin treatment).
Table 2

Twitch force characteristic data of timing parameters before and after 30 minutes of alpha toxin (10 HU/mL) treatment.

<table>
<thead>
<tr>
<th>Timing Parameters (msec)</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Contraction Phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0 - T1</td>
<td>61 ± 4</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>T1 - T2</td>
<td>102 ± 8</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>T0 - T2</td>
<td>163 ±10</td>
<td>155 ±10</td>
</tr>
<tr>
<td><strong>Relaxation Phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2 - T3</td>
<td>92 ± 7</td>
<td>92 ± 7</td>
</tr>
<tr>
<td>T3 - T4</td>
<td>300 ±23</td>
<td>276 ±23</td>
</tr>
<tr>
<td>T2 - T4</td>
<td>392 ±30</td>
<td>368 ±29</td>
</tr>
</tbody>
</table>

The timing parameters were collected from twitch function curves of same muscles before and 30 minutes after toxin (10 HU/mL) treatment. The values are means ± S.E. of 8 experiments. There are no significant differences of all parameters (p>0.05) by paired t-test.
binding domain located in C-terminal and a hemolytic and lethal domain located in N-terminal (Blomqvist et al., 1987). After heat treatment, the activity of hemolytic domain is lost. To examine how alpha toxin affects cardiac muscle, the heated, hemolytically inactive toxin was applied on rat papillary muscles. At the same time, this experiment would also provide information concerning the comparison of effects and mechanisms of alpha toxin on erythrocytes and cardiac muscles.

The experiments were performed at a stimulation frequency of 0.2 Hz, a temperature of 26°C, and a muscle length of L_max. One HU toxoid implies this amount toxoid is obtained from 1 HU alpha toxin. Ten HU/mL of toxin was heated and used for the experiment, and the developed force and passive tension were examined.

Although toxoid is not hemolytic, it has direct effect on rat papillary muscles. As seen in Figure 16, the developed force showed a progressive decrease after a few minutes and arrived at a plateau in about 30 minutes. Figure 17 showed that there was no significant change of passive tension within 30 minutes of toxoid treatment. The responses are quite similar in comparison with changes after 10 HU/mL toxin treatment (Figure 13 and Figure 14). Like alpha toxin, toxoid probably affects Ca^{2+} handling system. The finding that the toxoid showed a similar cardiac dysfunctional effect is fundamentally different from the studies on erythrocytes. This suggests that the cardiac dysfunctional effect of alpha toxin is heat stable and its mechanism is maybe different from that of hemolysis. To further compare the cardiac dysfunctional effect of alpha toxoid with that of alpha toxin, the twitch characteristics after toxoid treatment were analyzed.
Figure 16

Percentage change in developed force in rat papillary muscles due to the administration of alpha toxoid. Muscles were first equilibrated with modified Krebs-Henseleit buffer and electrically stimulated at 0.2 Hz. The L_max was determined and muscles were allowed to equilibrate at L_max until the developed force was stabilized. Then the rat papillary muscles were perfused with a low concentration of alpha toxoid (10 HU/mL). The changes in the developed force were measured over time. Data were normalized by baseline values (placed at 100%). The values are means ± S.E. of 6 experiments.
Figure 17

Percentage change in passive tension in rat papillary muscles due to the administration of alpha toxoid. Muscles were first equilibrated with modified Krebs-Henseleit buffer and electrically stimulated at 0.2 Hz. The \( L_{\text{max}} \) was determined and muscles were allowed to equilibrate at \( L_{\text{max}} \) until the developed force was stabilized. Then the rat papillary muscles were perfused with a low concentration of alpha toxoid (10 HU/mL). The changes in the passive tension were measured over time. Data were normalized by baseline values (placed at 100%). The values are means ± S.E. of 6 experiments.
4.7 The Changes in Twitch Force Characteristics after Alpha Toxoid (10 HU/mL) Treatment.

The changes in twitch characteristics after 10 HU/mL toxoid treatment have also been analyzed to help understanding how alpha toxin affects the calcium handling mechanism.

First, the changes of the shape of the twitch curve were analyzed. Figure 18 shows the peak tension and slopes of contraction and relaxation phases are reduced after 30 minutes toxoid treatment. The figure is superimposition of two raw twitch function curves recorded during experiment before toxoid treatment and after 30 minutes of toxoid treatment from a same muscle. The quantitative study of these parameters with the paired t-test is summarized in Table 3. It shows significant decreases in all three parameters including peak tension (p < 0.001), positive maximal rate (p < 0.001) and negative maximal rate (p < 0.01) after 30 minutes of toxoid treatment. On the other hand, Table 4 shows there was no significant changes on timing parameters by paired t-test. The result reveals that, like treated with toxin, the treatment of toxoid interferes with the intracellular calcium handling mechanism.

The reduced maximal rates of contraction and relaxation phases indicate that there is either a reduction of amount of Ca$^{2+}$ released from the sarcoplasmic reticulum to myofilaments and/or reduction of sensitivity of myofilaments to calcium.

The changes in twitch characteristics after toxoid treatment are very similar to the changes after toxin treatment. It once again indicates that the alpha toxin and toxoid have similar cardiac dysfunctional effect.

4.8 Effects of Alpha Toxin at Higher Concentrations.

The previous studies of hemolysis by alpha toxin showed that the hemolysis caused by alpha toxin is concentration dependent (Figure 7).
Figure 18

A superimposed twitch contraction from the same muscle before and after 30 minutes of alpha toxoid (10 HU/mL) treatment. The curves were center matched for better comparison. The magnitude and the shape of the twitch function curve has been changed after toxoid treatment. The positive and negative maximal rates were reduced and the timing parameters were not altered. For statistical analysis, see Table 3 and Table 4.
Table 3

Twitch force characteristic data before and after 30 minutes of alpha toxoid (10 HU/mL) treatment.

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>After</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Force (mg/mm²)</td>
<td>1624.6 ± 85.6</td>
<td>1265.3 ± 95.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>+Maximal Rate (mg/mm²/ms)</td>
<td>14.1 ± 1.4</td>
<td>11.0 ± 1.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>-Maximal Rate (mg/mm²/ms)</td>
<td>7.7 ± 1.5</td>
<td>6.8 ± 1.1</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The tension and positive and negative maximal rates were collected from twitch function curves of same muscles before and after 30 minutes of toxoid (10 HU/mL) treatment. The values are means ± S.E. of 6 experiments. All parameters show significant differences by paired t-test (not significant by group t-test since the difference among individuals is much bigger than the difference between before and after toxin treatment).
Table 4

Twitch force characteristic data of timing parameters before and after 30 minutes of alpha toxoid (10 HU/mL) treatment.

<table>
<thead>
<tr>
<th>Timing Parameters (msec)</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction Phase</td>
<td></td>
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<tr>
<td>T0 - T1</td>
<td>64 ± 4</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>T1 - T2</td>
<td>127 ± 13</td>
<td>112 ± 8</td>
</tr>
<tr>
<td>T0 - T2</td>
<td>158 ± 15</td>
<td>143 ± 15</td>
</tr>
<tr>
<td>Relaxation Phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2 - T3</td>
<td>104 ± 8</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>T3 - T4</td>
<td>340 ± 40</td>
<td>306 ± 34</td>
</tr>
<tr>
<td>T2 - T4</td>
<td>444 ± 45</td>
<td>403 ± 37</td>
</tr>
</tbody>
</table>

The timing parameters were collected from twitch function curves of same muscles before and after 30 minutes of alpha toxoid (10 HU/mL) treatment. The values are means ± S.E. of 6 experiments. There are no significant differences of all parameters (p>0.05) by paired t-test.
Therefore, the cardiac dysfunctional effects by various different concentrations alpha toxin were examined.

Figure 19 shows changes in developed force from four typical examples which used 10, 25, 50, and 100 HU/mL alpha toxin respectively. The transient increase of the developed force was present at all these experiments. It took about 2 to 4 minutes for the developed force to reach peak value. Immediately after this peak value, the developed force progressively decreased and then stabilized in about 10 to 20 minutes. The relation between toxin concentration and peak of transient increase of developed force is linear as depicted in Figure 20. Therefore, the cardiac dysfunctional effect of alpha toxin is also concentration-dependent. There was a transient increase on the developed force of the muscles. However, the muscles did not lose the ability to contract after 30 minutes of high concentration toxin (100 HU/mL) treatment, and developed force was even back to the pretreated level. As the statistical analysis in Table 5 and 6, the developed force was significantly increased at 2 minutes (p < 0.01), but not significantly altered at 30 minutes (p > 0.1) after 100 HU/mL toxin treatment. The twitch characteristics at 30 minutes were also unchanged. The transient increase phase is short and thus difficult to track with experiments such as the interval-force relationship experiment. However, the twitch characteristics at transient phase after 100 HU/mL toxin treatment was analyzed. As shown in Table 5, the peak force and positive and negative maximum rates of force development were all significantly increased (p < 0.01). This implies that the transient increase of the developed force also involved with Ca²⁺ handling such as Ca²⁺ release from sarcoplasmic reticulum and/or sensitivity of myofilaments to Ca²⁺. The twitch curves from before and after 2 and 30 minutes of toxin (100 HU/mL) treatment were superimposed in Figure 21, which summarized changes of the shape of the twitch function.

If muscles are exposed to a very high concentration of alpha toxin, they will stop functioning in a few minutes. The concentration of toxin required to
Figure 19

Percentage change in developed force of rat papillary muscles due to the administration of different concentrations of alpha toxin. Muscles were first equilibrated with modified Krebs-Henseleit buffer and electrically stimulated at 0.2 Hz. The $L_{\text{max}}$ was determined and muscles were allowed to equilibrate at $L_{\text{max}}$ until the developed force was stabilized. Then the rat papillary muscles were perfused with various concentrations of alpha toxin and changes in the developed force were measured over time. Data were normalized by baseline values (placed at 100%). The data represent four typical experiments.
Figure 20

The maximal increases of the developed force of rat papillary muscles after different alpha toxin concentrations treatment. The maximal increases of the developed force of rat papillary muscles at toxin concentrations from 10 to 100 HU/mL. The data of the developed force were normalized by baseline values. The data are from 4 typical experiments.
Table 5

Twitch isoe characteristic data before and after 2 minutes of alpha toxin (100 HU/mL) treatment.

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Force (mg/mm²)</td>
<td>1429.1 ± 204.4</td>
<td>1675.6 ± 253.1 (p&lt;0.01)</td>
</tr>
<tr>
<td>+Maximal Rate (mg/mm²/ms)</td>
<td>12.6 ± 1.3</td>
<td>14.8 ± 1.7 (p&lt;0.01)</td>
</tr>
<tr>
<td>-Maximal Rate (mg/mm²/ms)</td>
<td>6.7 ± 0.7</td>
<td>7.7 ± 0.8 (p&lt;0.01)</td>
</tr>
</tbody>
</table>

The tension and positive and negative maximal rates were collected from twitch function curves of same muscles before and after 2 minutes of toxin (100 HU/mL) treatment. The values are means ± S.E. of 5 experiments. All parameters show significant differences by paired t-test (not significant by group t-test since the difference among individuals is much bigger than the difference between before and after toxin treatment).
Table 6
Twitch force characteristic data before and after 30 minutes of alpha toxin (100 HU/mL) treatment.

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>After</th>
<th>(p&gt;0.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Force (mg/mm²)</td>
<td>1429.1 ± 204.4</td>
<td>1551.7 ± 216.1</td>
<td></td>
</tr>
<tr>
<td>+Maximal Rate (mg/mm²/ms)</td>
<td>12.6 ± 1.3</td>
<td>13.9 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>-Maximal Rate (mg/mm²/ms)</td>
<td>6.7 ± 0.7</td>
<td>6.9 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

The tension and positive and negative maximal rates were collected from twitch function curves of same muscles before and 30 minutes after toxin (100 HU/mL) treatment. The values are means ± S.E. of 5 experiments. All parameters show no significant differences by paired t-test.
Figure 21

A superimposed twitch contraction from the same muscle before and after 2 and 30 minutes of alpha toxin (100 HU/mL) treatment. The curves were center matched for better comparison. The magnitude and the shape of the twitch function curve has been changed after 2 minutes but not after 30 minutes of toxin treatment. After 2 minutes, the positive and negative maximal rates were increased and the timing parameters were not altered. All parameters were not altered after 30 minutes. For statistical analysis, see Table 5 and Table 6 (timing parameters were not shown).
destroy each individual muscle varies, the results from six experiments give a mean ± standard error of 207 ± 64 HU/mL. The changes of developed force and passive tension from one experiment is shown in Figure 22. In this experiment, the concentration of alpha toxin was 200 HU/mL. There was also a transient increase at first few minutes, the increase was about 65% above the developed force before toxin treatment. Suddenly at the 7th minute, the contraction of the muscle was progressively decreased and diminished within one minute. It is interesting to notice that the passive tension, which should be increased at higher intracellular Ca\(^{2+}\) concentrations (Lurmann et al., 1983), did not show significant change and could be maintained at least a few minutes after muscle stopped contracting. Also, the spontaneous contraction, which is characteristic of the calcium overloading (Stern et al., 1989), was not observed. Therefore it is suggested that the alpha toxin does not overload calcium to the muscles.

4.9 The Interval-force Relationship after Alpha Toxin (100 HU/mL) Treatment.

One question arose from experiment 4.8 was if the muscles were the same after the transient increase of developed force, since the developed force was not significantly changed after 30 minutes of 100 HU/mL toxin treatment. Although the transient increase of developed force was too short to be measured with the interval-force relationship experiment, the muscles can be examined with this method after developed force was stabilized, i.e. 30 minutes after toxin treatment. This experiment could also provide valuable information regarding how and where alpha toxin affects cardiac muscle since this experiment elucidates the mechanism of calcium handling within intracellular calcium compartments.

The experiment was performed as described in Materials and Methods. Five muscles were untreated and used as controls and another five muscles were treated with 100 HU/mL alpha toxin. The developed force was tested at 22 different rest intervals (from 0.8 to 163 seconds). The ratio of the
Effect of a very high concentration alpha toxin (200 HU/mL) on developed force and passive tension of rat papillary muscle. Muscle was first equilibrated with modified Krebs-Henseleit buffer and electrically stimulated at 0.2 Hz. The $L_{\text{max}}$ was determined and muscle was allowed to equilibrate at $L_{\text{max}}$ until the developed force was stabilized. Then the rat papillary muscle was perfused with a very high concentration of alpha toxin (200 HU/mL). The changes in the developed force and the passive tension were measured over time. Data were normalized by baseline value (placed at 100%). The data represent a typical experiment.
developed force after a test rest interval and the developed force on the immediately prior beat versus rest intervals from controlled and toxin treated muscles was plotted in Figure 23. The force/baseline of controlled muscles and toxin treated muscles arrived plateau of the force/baseline at almost same time, but peak of the force/baseline was reduced after toxin treatment. The averages from the raw data were analyzed by a computer program called "Eureka" (Borland Inc., California) with a mathematical equation which was used to separate the alpha and beta phases of the slow Ca\(^{2+}\) transport in the muscles cells. The raw data from both controlled and toxin treated muscles fit the fitted data obtained from computer very well and therefore it is adequate to use fitted parameters to isolate the alpha and beta phases.

In Figure 24, the data are plotted on log scaled x-axis and a biphasic function can be seen. Both phases, i.e. alpha and beta phases, contribute to the force recovery between beats (Schouten et al., 1987). The figure shows that the alpha toxin affects both phases. There were significant increases of force/baseline at rest intervals less than 5 seconds and significant decreases at longer rest intervals. Further mathematical isolation of alpha and beta phases which is shown in Figure 25 and Figure 26 reveals that there was a slight increase at alpha phase and a major decrease at beta phase. It suggests that alpha toxin mainly affects sarcolemma because beta phase is closely related with the sarcolemma (Figure 1). The Ca\(^{2+}\) transport from sarcolemma to release compartment was reduced, which caused the reduction of maximal potentiation from 2.38 to 1.76.

The fitted data in Figure 27 and Figure 28 show the isolation of each phase in controlled and treated muscles plotted on a logarithmic x-axis. The maximal potentiation, the contributions of the alpha and beta phases to the maximal potentiation and to the potentiation at 5 seconds rest interval (normal pacing frequency) were all changed after toxin treatment.
**Figure 23**

**Interval-force relationship of controlled and toxin treated muscles.**

Raw interval-force data (symbols) and fitted data (solid and dashed lines) are plotted on a linear x-axis. The values of raw data from both controlled and toxin treated (100 HU/mL) muscles are means ± S.E. of 5 experiments. The fitted data were calculated as described in "Rest interval-force relationship" of Materials and Methods. The fitted equation is

\[ f(t) = F(1-M\exp(-at)-(1-M)\exp(-bt)) \]

The equation fitted the raw data well and the maximal error is less than 1%.
Figure 24

Rest interval-force relationship plotted on a logarithmic x-axis. Raw (symbols) and fitted (curves) rest interval-force are data plotted on a linear x-axis. The values of raw data from both controlled and toxin treated (100 HU/mL) muscles are means ± S.E. of 5 experiments. The ★ represents significant difference (p < 0.05) of mean of toxin treated muscles from mean of control muscles. The fitted data were calculated as described in "Rest interval-force relationship" of Materials and Methods. The fitted equation is

\[ f(t) = F(1 - M \exp(-at) - (1-M) \exp(-bt)) \]

The equation fitted the raw data well and the maximal error is less than 1%.
Isolated alpha phases from controlled and toxin treated muscles. Mathematically isolated alpha phases overlaid from the controlled and toxin (100 HU/mL) treated muscles plotted on a logarithmic x-axis. The alpha phase was isolated according to the equation of $f(t) = F(1 - M \exp(-at) - (1-M)\exp(-bt))$ in which $b = 0$. 
**Figure 26**

**Isolated beta phases from controlled and toxin treated muscles.** Mathematically isolated beta phases overlaid from the controlled and toxin (100 HU/mL) treated muscles plotted on a logarithmic x-axis. The alpha phase was isolated according to the equation of $f(t) = F(1-M\exp(-at)-(1-M)\exp(-bt))$ in which $a = 0$. 

Fitted interval-force data from controlled muscles. The fitted interval-force data from controlled muscles show the total curve and also the mathematically isolated alpha and beta phases.
Figure 28

Fitted interval-force data from toxin treated muscles. The fitted interval-force data from toxin (100 HU/mL) treated muscles show the total curve and also the mathematically isolated alpha and beta phases.
The percent contribution of the alpha process and beta process to the maximal potentiation was also altered after alpha toxin treatment (Table 7). The contribution of the alpha process was increased from 29% to 42%. The contribution of the beta process was decreased from 71% to 58%. Therefore, the alpha phase became more dominant and beta phase became less dominant after toxin treatment. Since the change on beta phase was bigger, the maximal potentiation was reduced.

When the interval-force relation is analysed at a rest interval of 5 seconds which is the interval of normal pacing frequency (0.2 Hz), the contribution of alpha phase was increased from 69% to 75% while that of beta phase was reduced from 31% to 25% (Table 8). These changes may explain the changes in the developed force. The transient increase of the developed force in first few minutes toxin treatment was probably caused by the increase in alpha phase. The change in the beta phase did not affect developed force during the first few minutes since the beta phase influences the force recovery at longer time. However, this change did show an effect on the developed force after 10-20 minutes. The combined results of the changes on alpha and beta phases may cause the stability of the developed force after the transient increase.

4.10 Potentiation Decay

The recirculating fraction of calcium during excitation-contraction coupling before and at after least 30 minutes of 100 HU/mL alpha toxin treatment was analyzed by the studies of potentiation decay after 121 seconds rest interval. This rest interval was chosen because the maximal potentiation was produced at this interval and the maximal potentiation is important for accurate determination of the recirculating fraction (Schouten, 1985). For each muscle, the developed force of 11 beats after rest interval was collected and force/baseline at beat n versus force/baseline at beat n + 1 was plotted. The relation is linear since the percentage of Ca$^{2+}$ used for subsequent contraction is constant. Therefore, the slope of this linear relation represents
Table 7

The maximal potentiation and ratios of contributions by alpha and beta processes.

<table>
<thead>
<tr>
<th></th>
<th>Maximal Potentiation</th>
<th>Contribution (Alpha:Beta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>2.38</td>
<td>29:71</td>
</tr>
<tr>
<td>After</td>
<td>1.76</td>
<td>42:58</td>
</tr>
</tbody>
</table>

The maximal potentiation and contributions of alpha and beta processes to the maximal potentiation before and after 30 minutes of alpha toxin (100 HU/mL) treatment were calculated from the fitted data.
Table 8

The contributions of alpha and beta processes to the potentiation at test interval of 5 seconds.

<table>
<thead>
<tr>
<th></th>
<th>Contribution (Alpha:Beta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>69:31</td>
</tr>
<tr>
<td>After</td>
<td>75:25</td>
</tr>
</tbody>
</table>

The contributions of alpha and beta phases to the potentiation at test interval of 5 seconds before and after 30 minutes of alpha toxin (100 HU/mL) treatment were calculated from the fitted data.
the recirculating fraction "r" in Figure 1B (Shouten et al., 1987, Schouten, 1985). The group t-test was applied to the constant r obtained from controlled and toxin treated muscles. Each group had 5 muscles. The result in Table 9 illustrated that there was no significant change (p > 0.5) after toxin treatment. It implies that the amount of Ca$^{2+}$ recirculated between beats remained unchanged since the r constant represents the amount of calcium recirculating to the myofilaments. It seems that the developed force was not altered after 30 minutes toxin (100 HU/mL) treatment because the amount of Ca$^{2+}$ released to myofilaments was not changed. This may be the combined results of (1) the increase of the alpha phase, and (2) the decrease of the beta phase.
Table 9

Calculation of recirculating fraction from controlled and toxin treated muscles.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction &quot;r&quot;</td>
<td>0.7352 ±0.0155</td>
<td>0.7436 ±0.0367 (p&gt;0.5)</td>
</tr>
</tbody>
</table>

The recirculating fraction was calculated as described in "Potentiation decay" of Materials and Methods. The values from both controlled and toxin (100 HU/mL) treated muscles are means ± S.E. of 5 experiments. There is no significant difference (p>0.05) by group t-test.
DISCUSSION

The main objective of this study was to study the effect of alpha toxin on cardiac muscles.

The rat papillary muscles provide a good system to study the effect of alpha toxin on excitable cells. The cardiac muscles did not lose the ability to contract at toxin concentrations between 10-100 HU/mL. Instead, a new equilibrium was established, and the mechanism of the toxin was further studied with these live cells.

Although alpha toxin is the most important pathogenicity factor in staphylococcal infections (Arbuthnott, 1970; Mollby, 1983; Kinsman et al., 1981; O'Reilly et al., 1986; Patel et al., 1987), the mechanism of its effects, especially its lethal effect, is not yet clear. However, it is generally agreed that the primary cellular target of the toxin is the membrane. Therefore, tremendous efforts have been made on the toxin’s effects on erythrocytes as they are very sensitive to alpha toxin and provide a very simple model for the study of the events occurring on the cellular membrane. These studies suggest that there are receptors for alpha toxin on erythrocytes (Simpson and Fackrell, 1985; Cassidy and Harshman, 1976a,b; Kato and Naiki, 1976; Barei and Fackrell, 1979; Maharaj and Fackrell, 1980; Adamo and Fackrell, 1989). After binding to the membrane of the erythrocytes, alpha toxin forms transmembrane ion channels (Thelestam and Blomqvist, 1988) which cause the leakage of the ions and then the osmotic lysis of the cells with hemoglobin release (Freer and Arbuthnott, 1986).

However, hemolysis does not seem to be the lethal factor. When animals were injected with a lethal dose of alpha toxin, the degree of hemolysis was insufficient to explain rapid death even in the rabbit, which is the most sensitive species (Arbuthnott, 1970). The symptoms of the intoxication
suggested the cardiovascular system as a lethal target (Wiseman, 1975; Arbuthnott, 1970; Jeljaszewicz et al., 1968).

There are few studies on the effects of alpha toxin on cardiac muscle. In 1973, Gulda et al. suggested that the alpha toxin probably increased the permeability of the membrane of frog myocardial fibres to calcium. Brill (1985) supported the concept that the alpha toxin increased the permeability of Ca$^{2+}$ on the sarcolemma of isolated frog strip myocardium. However, there are two shortcomings in their studies. Firstly, they used impure alpha toxin which could be contaminated with beta, gamma, or delta toxin. Secondly, they used either isolated whole frog hearts or isolated strips of frog myocardium, in which the blood vessels could play an important role since alpha toxin affects blood vessels (Wurzel et al., 1966; Cassidy et al., 1974; Gulda et al., 1978).

Previously, using pure toxin, Adamo et al. (1989) proved that the alpha toxin has a potent cardiovascular effect that may contribute to its lethality. They found that the toxin irreversibly increased coronary pressure, which was followed by increases in systolic and diastolic pressures and a decrease in heart rate. The studies suggested that constriction of the blood vessels deprived the hearts of oxygen. Thus, the hearts needed to contract harder to obtain more oxygen. Their results also suggested that alpha toxin may have a direct effect on cardiac muscles since isolated atrial (vascular-poor tissue) preparations were also affected by the toxin. To study the direct effect of alpha toxin on cardiac muscles, the present study used rat papillary muscles which minimize the influence of the vasculature. The present study shows that a low concentration of the toxin reduced the ability of the cardiac muscles to contract. If these effects are same in vivo and in vitro, the opposite effects of the toxin on smooth muscles and cardiac muscles would create a lethal contribution.
The developed force of the muscles at $L_{\text{max}}$ was significantly reduced within 30 minutes of treatment of low alpha toxin concentration (10 HU/mL) (Figure 13). This implies that the performance of the myofilaments was altered by alpha toxin by changes in muscle length, and/or regulation of calcium. If alpha toxin had changed the length of the muscles, the passive tension would have been changed because the resting tension of the muscle is very sensitive to the change of the length at $L_{\text{max}}$ (Jewell, 1977). Any small change on the sarcomere length should result in a bigger change in passive tension than developed force (Figure 10). Since my experiments were performed at $L_{\text{max}}$ and the passive tension was not changed, it is suggested that the lengths of the muscle were not altered by alpha toxin. Also, the alpha toxin is a membrane damaging protein (Cassidy and Harshman, 1976; Freer and Arbuthnott, 1986; Thelestam and Blomqvist, 1988), that has never been shown to affect intracellular organelles. It is unlikely that alpha toxin has any direct effect on myofilaments. There are several important processes of calcium handling which influence excitation-contraction coupling and thus the developed force. These processes include (1) calcium release from the sarcoplasmic reticulum; (2) calcium entry via the surface membrane; (3) calcium binding by intracellular sites; (4) re-accumulation of calcium into the sarcoplasmic reticulum by active pumping; and (5) active transport of intracellular calcium across the surface membrane to the extracellular space (for review, see Chapman, 1979). Thus, changes in developed force could be due to a change on any aspect of the calcium handling processes.

The first step to investigate the cellular calcium mechanism was to analyse the twitch characteristics after alpha toxin treatment. The twitch characteristics can provide valuable information regarding the $\text{Ca}^{2+}$ movement and handling during a single contraction. Not only the peak developed force, but also the shape of the twitch function is very important.

The maximal rate of force development ($+dF/dT$) is related to the maximal intracellular $\text{Ca}^{2+}$ transient and/or the binding of $\text{Ca}^{2+}$ by myofilaments.
ments. Since it is unlikely that alpha toxin directly affects myofilaments, the decreased positive maximal rate after treatment with a low concentration of alpha toxin (10 HU/mL) (Table 1) indicates that the calcium transient during excitation-contraction coupling was reduced.

The maximal rate of the relaxation phase (-dF/dT) is mainly regulated by the re-accumulation of calcium into the sarcoplasmic reticulum by active pumping after force development. Since there was less calcium available after treatment of toxin as indicated by the decreased positive maximal rate, the uptake of calcium by sarcoplasmic reticulum was also reduced which is shown by the decreased negative maximal rate (Table 1). Therefore, it seems that all the changes of peak tension, positive and negative maximal rates were caused by underloading of the calcium into the intracellular space.

The analysis of timing parameters also supports the role of calcium handling in the cardiac dysfunctional effect of alpha toxin. Firstly, the muscle length was not altered since these timing parameters were not changed (Table 2). Muscle length not only affects the cross bridge numbers, but also the intracellular Ca\(^{2+}\) concentration (Sugiura et al., 1989), calcium-triggered calcium release from the sarcoplasmic reticulum (Fabiat o and Fabiato, 1975), the sensitivity of myofilaments to calcium (Hibberd and Jewell, 1982), and the affinity of troponin for calcium (Honig and Reddy, 1975). These changes could affect the timing parameters (Gamble and Taylor, 1991). Therefore, the unchanged timing parameters do not support any length change of the muscles. Instead, the reduced developed force and the unchanged timing parameters indicate that the amount of calcium released by sarcoplasmic reticulum has been reduced by the treatment of the toxin which is probably due to the interference of the toxin in the calcium pathways to the sarcoplasmic reticulum.
Secondly, if the ability of sarcoplasmic reticulum to release calcium and to recycle calcium had been altered by alpha toxin, the time to reach maximal rate and peak tension (T0-T1 and T0-T2) and the time to complete relaxation (T2-T4) would have been changed (Poggesi et al., 1987). Therefore, the sarcoplasmic reticulum is also unlikely the target of alpha toxin. All the evidence suggests calcium handling as a target for the toxin.

Unlike the hemolytic and lethal effects, the cardiac dysfunctional effect of alpha toxin is heat stable, as alpha toxoid has a similar cardiac dysfunctional effect on the cardiac muscles. The developed force of the rat papillary muscles was reduced within 30 minutes of treatment of alpha toxoid (Figure 16). The response is very similar to the alpha toxin treated muscles (Figure 13). Moreover, the analysis of the passive force and twitch characteristics shows similar results as the toxin (Figures 14,15,17,and 18). Several important considerations have been generated by this unusual finding.

Since the alpha toxoid still has binding ability, immunogenetic ability and cardiac dysfunctional effect, the concept of "biological inactive" of the toxoid is questioned. It seems that the toxoid is just another form of active toxin. Some of the biological activities have been lost on toxoid, which is probably due to the conformational changes upon the heat treatment.

The lethal site and cardiac dysfunctional site are probably two independent regions on the molecule of alpha toxin, since one is heat sensitive and the other is heat stable. Therefore, another conclusion derived from the cardiac dysfunctionality of alpha toxoid is that the cardiac dysfunctional effect of alpha toxin does not account for its lethality.

Since toxoid has no effect on erythrocytes (Barei and Fackrell, 1979; Arbuthnott et al., 1967) and smooth muscles (unpublished data in our lab), the mechanism of the cardiac dysfunctional effect of the toxin is probably
different from its effects on erythrocytes and smooth muscles. It, of course, needs further study.

The transient increase of developed force occurred in the presence of all concentrations of alpha toxin (Figure 19). This transient increase may indicate the calcium overloading immediately after toxin treatment. However, at least two pieces of evidence oppose calcium overloading. Firstly, the passive force was stable throughout the experiment no matter at what concentration (Figures 14, 17, and 22). This result does not favor calcium overloading since the passive force would be increased by the calcium overloading (Lullmann et al., 1983). Secondly, the calcium overloading would induce spontaneous cyclic release of calcium from the sarcoplasmic reticulum (Stern et al., 1989; Chiesi et al., 1981). This would cause spontaneous contraction of the cardiac muscles, which was not observed from the experiments.

It is reasonable to think that higher concentrations of alpha toxin increase muscle damage. But the results show that the developed force was reduced in the presence of low concentration (10 HU/mL) alpha toxin, but was not altered by high toxin concentrations (Figure 19). Although there is a linear relationship between the peak transient increase of the developed force and the toxin concentration (Figure 20), the transient phase only lasted a few minutes and is difficult to analyse. However, the muscles were examined by the interval-force relationship after developed force was stabilized.

On the restitution curve, there is a slight increase on the alpha phase (Figure 25). It reflects that the calcium movement from uptake to release compartment is slightly enhanced. The reason for this slight increase is unknown. However, this may explain the transient increase of the developed force. Since the process of alpha phase is much faster than that of beta phase, the change on alpha phase may be reflected on the developed force
earlier, i.e., a transient increase was seen. After first few minutes, the effect of the changes on slow beta phase showed. The combined results of increased alpha phase and decreased beta phase resulted in the availability of calcium to myofilaments unchanged after 20-30 minutes higher concentration of alpha toxin (100 HU/mL) treatment. Therefore, the developed force did not show change after 20-30 minutes treatment (Figure 19).

The most significant change in the restitution curve after alpha toxin (100 HU/mL) treatment is in the beta phase. Thus it seems that the sarcolemma is the primary target of alpha toxin. The alpha toxin significantly inhibits the calcium movement from sarcolemma into the release compartment. This is consistent with the common idea that the alpha toxin damages cell membranes (Freer et al., 1968; Bernheimer et al., 1974; Wiseman, 1975; Arbuthnott et al., 1973; Cassidy et al., 1974; Harshman, 1979; Barei and Fackrell, 1979). However, the mechanism of the damage may be different in the case of cardiac muscles since (1) alpha toxoid has the same cardiac dysfunctional effect as the toxin; (2) Erythrocytes seem to be destroyed by a sudden influx of small ions, and there is no evidence of calcium overloading in the cardiac system.

The recirculation fraction of Ca$^{2+}$ reveals the beat to beat distribution of Ca$^{2+}$ between the sarcoplasmic reticulum and the myofilaments (Schouten, 1985). It is contributed by calcium from both the exchange compartment and the uptake compartment. The potentiation decay experiment also confirmed that the recirculating fraction of Ca$^{2+}$ was not changed after 30 minutes of treatment with a higher concentration of alpha toxin (100 HU/mL) (Table 9). Rather, after 30 minutes of alpha toxin treatment, there was more calcium transported from the uptake compartment into the release compartment, and less calcium from the exchange compartment transported into the release compartment (Figure 25 and Figure 26). As recirculating fraction was not changed, the availability of the calcium for
myofilaments was not altered and therefore the developed force was not changed.

In evaluation of model of rat papillary muscle, this model is an efficient and sensitive model for studying the mechanism of alpha toxin. The rat papillary muscles provide an ideal model for studying how excitable cells respond to the toxin. The effect of the toxin can be studied with biophysical methods that were noninvasive to the cellular function of the muscle.

The present studies show that the mechanism of the effect of the alpha toxin on rat papillary muscles is different from that on erythrocytes. Therefore, the understanding of the toxin's mechanism would be enhanced if its cardiac dysfunctional mechanism is further elucidated. The next experiments could test the performance of the muscles in the presence of various monoclonal antibodies to alpha toxin and calcium channel blockers. The trabecular muscles could be used to measured the sarcomere length with a laser. However, the experiment on rat papillary muscles is technically demanding since the muscles are so fragile. Great care and good surgical skills are needed. The experiment is also time consuming. It usually takes about six or seven hours to complete an experiment on one muscle.
SUMMARY

Staphylococcal alpha toxin has been considered a cardiovascular toxin, but its effects on the cardiac tissue activity remained poorly understood. Therefore, a model of rat papillary muscles was established to study the mechanism of action of staphylococcal alpha toxin. This model was selected since it contained no blood vessels, which play an important role on the effect of the toxin on whole hearts.

The study shows that the alpha toxin has a cardiac dysfunctional effect through the $\text{Ca}^{2+}$ handling mechanism. Specifically, the availability of the $\text{Ca}^{2+}$ to the contractile myofilaments is reduced after a low concentration toxin (10 HU/mL) treatment.

The alpha toxoid has a similar cardiac dysfunctional effect, which also indicates the difference on the mechanism. It suggests that the cardiac dysfunctional site is probably not altered by the toxin. It also suggests that the lethality of the alpha toxin is not due to its direct effect on cardiac muscles because the alpha toxoid is not lethal.

The study of interval-force relationship reveals that the main target of the toxin on the muscles is sarcolemma. The calcium movement from the exchange compartment on sarcolemma to the release compartment on sarcoplasmic reticulum was significantly inhibited after 100 HU/mL toxin treatment.
LITERATURE CITED


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