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Proteolytic modification of the Ca²-release mechanism of sarcoplasmic reticulum in skeletal muscle

Ute Goerke

Portland State University

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Title: Proteolytic Modification of the Ca\textsuperscript{2+}-Release Mechanism of Sarcoplasmic Reticulum in Skeletal Muscle

APPROVED BY THE MEMBERS OF THE THESIS COMMITTEE:

Jonathan J. Abramson, Chairperson

Pavel K. Smejtek

David H. Peyton

Calcium ions are important mediators in the mechanism of contraction and relaxation of muscle fibers. Depolarization of sarcolemma and transverse tubule causes an increase of myoplasmic Ca\textsuperscript{2+} concentration which induces contraction of the myofibrils. In skeletal muscle fibers, the intracellular Ca\textsuperscript{2+} concentration is regulated by an extensive membrane system, the sarcoplasmic reticulum (SR). Ca\textsuperscript{2+}-release from SR is initiated by depolarization of the transverse tubule via a process referred to as excitation-contraction coupling. The Ca\textsuperscript{2+}-
release channel located in the junctional SR plays an important role in this mechanism.

Two different proteins of molecular weight of 400 kDa and 106 kDa have been suggested to constitute the Ca\textsuperscript{2+}-release channel. Focusing on these two proteins, the effect of proteolytic modification of the Ca\textsuperscript{2+}-release mechanism was studied. The proteolytic enzymes used were type III trypsin (Sigma), sequence-grade trypsin (Boehringer), thrombin, kallikrein, and chymotrypsin.

Passive Ca\textsuperscript{2+}-release from 'native' and proteolytically modified SR was measured spectrophotometrically using the Ca\textsuperscript{2+}-sensitive dye arsenazo III. Tryptic cleavage (type III trypsin from Sigma) of the Ca\textsuperscript{2+}-release channel stimulated release from SR (trypsin:SR protein=1:200 (w/w) for varying times of proteolysis) with maximal enhancement of Ca\textsuperscript{2+} efflux occurring after 18 min of proteolysis. Since trypsin specifically cleaves peptide bonds at arginine and lysine bonds, the first approach was to determine the cleavage site responsible for enhancement of Ca\textsuperscript{2+}-release from SR. The arginine specific protease, thrombin, did not affect Ca\textsuperscript{2+}-release from SR at thrombin to SR protein ratios varying from 1:20 to 1:2000 following 15 min of proteolysis. The observation that sequence-grade trypsin (Boehringer) in contrast to the type III trypsin (Sigma) does not stimulate Ca\textsuperscript{2+}-release from SR suggested that proteolytic contaminants
in the type III trypsin (Sigma) caused this effect. Possible impurities, kallikrein and chymotrypsin did not enhance release at concentrations likely to be found in the Sigma-trypsin. Also, chymotrypsin did not stimulate Ca\textsuperscript{2+}-release from SR at higher concentrations.

The degradation of specific SR membrane bound proteins was also examined by SDS-PAGE with 5-15% gradient gels. The type III trypsin (Sigma) appeared to cleave proteins faster and into slightly different patterns than the sequence-grade trypsin (Boehringer). Multiple cleavaged of the 400 kDa protein was observed inspite of no stimulation of Ca\textsuperscript{2+}-release when ultrapure trypsin was used. Both types of trypsin cleaved the 400 kDa protein several times within 30 sec. The initial cleavage of this protein did not correlate with the stimulation of Ca\textsuperscript{2+}-release from SR.
PROTEOLYTIC MODIFICATION OF THE Ca$^{2+}$-RELEASE MECHANISM
OF SARCOPLASMIC RETICULUM IN SKELETAL MUSCLE

by

UTE GOERKE

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TO THE OFFICE OF GRADUATE STUDIES:

The members of the committee approve the thesis of Ute Goerke presented August 10, 1992.

Jonathan J. Abramson, Chairperson

Pavel K. Smejtek

David H. Peyton

APPROVED:

Erik Bodegom, Chair, Department of Physics

Roy W. Koch, Vice Provost for Graduate Studies and Research
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CHAPTER I

THE $\text{Ca}^{2+}$ - RELEASE CHANNEL

INTRODUCTION

Calcium ions play an important role in many different metabolic processes occurring in cells. $\text{Ca}^{2+}$ is involved in the control of enzyme activity, neurotransmitter release, secretion, calcium activated potassium channel opening, and the control of muscle contraction.

Muscle contraction and force development depends on the intracellular $\text{Ca}^{2+}$ concentration. Calcium ions function as mediators between the cell membrane and the contractile proteins, the myofibrils. Depolarization of the surface membrane of the muscle cell, caused by nervous or spontaneous stimulation, results in a rise of the intracellular $\text{Ca}^{2+}$ concentration, inducing contraction of the myofibrils.

This mechanism is common for most muscle types. The mechanism in detail, however, differs in essential points (Fleischer and Inui, 1989). This thesis focuses on calcium ion regulation of the sarcoplasmic reticulum from white rabbit skeletal fast-twitch muscle.
MORPHOLOGY OF A MUSCLE FIBER

Skeletal muscle consists of bundles of fibers which are attached to the bone via tendons. The cell surface membrane is referred to as the sarcolemma. The fiber contains a fluid called the sarcoplasm.

When bundles of skeletal muscle fibers are examined under the light microscope, a striated pattern is observed. Hence skeletal muscle is also known as striated muscle. The myofibrils that run the length of the fiber parallel to the axis of contraction produce the appearance of striats caused by periodical changes in density.

Besides the myofibrils, muscle fibers contain mitochondria, lipid droplets, and glycogen granules. An extensive membrane system (transverse tubules and sarcoplasmic reticulum) surrounds the myofibrils by forming a substructure referred to as the sarcomere.

Sarcolemma

In skeletal muscle contraction is initiated by a neural impulse arriving at the neuromuscular junction. The synapse when excited releases the messenger acetylcholine which then diffuses across the gap between the synapse and sarcolemma. The acetylcholine receptor in the sarcolemma opens an ion channel that allows sodium ions to cross the membrane, which results in depolarization of the sarcolemma.
**T-Tubules**

At each end of a sarcomere are invaginations of the cell surface membrane called transverse tubules (t-tubules). They conduct the action potential from the sarcolemma into the muscle fiber.

**Sarcoplasmic Reticulum**

The sarcoplasmic reticulum (SR) is a membrane system surrounding the myofibrils. It regulates the \( \text{Ca}^{2+} \) concentration inside the cell responding to depolarization of the t-tubules. It is specialized for cyclical uptake, storage, and release of \( \text{Ca}^{2+} \) ions. The SR is roughly divided into two parts: longitudinal and junctional SR. The terminal cisternae (junctional SR) form a saclike region adjacent to the t-tubules. Longitudinal SR is a tubular system connecting the terminal cisternae of each end of the sarcomere. The association of the t-tubule and terminal cisternae membrane is referred to as a triad. The gap between those two membranes is approximately 12 to 17 nm (Martonosi, 1984).

**Myofibrils**

The myofibrils consists of parallel aggregated proteins that form two distinct types of filaments, the thin and thick filaments (Ebashi, 1974). The thin filaments consist of two strands of fibrous actin with myosin binding sites at regular intervals and two smaller proteins, troponin and tropomyosin. The thick filament possesses the myosin head. In the sliding
Figure 1. The internal membrane system of a skeletal muscle fiber. Adapted from Peachey, 1965.
filament theory, the myosin head attaches to and disconnects from actin in a cyclic process. During attachment the myosin head undergoes mechanical movement which pulls the thin filament along the thick filament and causes contraction of the muscle fiber.

THE MECHANISM OF CONTRACTION: SYSTEMS FOR STUDY

Introduction

How muscle fibers contract has been studied at different levels, from assigning function to cell organelles down to understanding the precise molecular mechanism by which proteins interact with each other. An overview of experimental methods used to study these interactions is described by Fleischer and Inui (1989) and is summarized here.

Intact and Cut Fibers

Intact fibers have been used to study tension development and relaxation. In a typical experiment, the fiber is mounted in a strain gauge that measures isometric force. Basic information about the influence of hormones and drugs at the cellular level have been obtained using this technique.

Microinjections of photoluminescent proteins or calcium-sensitive dyes have provided important information on the role of calcium in the contractile process. Ashley and Ridgeway (1968, 1970) were among the first to use this method. They demonstrated an increase of the intrafibrous $\text{Ca}^{2+}$ concentration after electrical stimulation of the muscle
fiber. Solutions of aequorin, a protein which emits light in the presence of calcium ions, were injected into the large muscle fibers of the barnacle *Balanus nubilus*. The electrically stimulated fiber produced a faint glow.

Since then, this technique has been much improved. Cut fiber segments under voltage clamp conditions in a double Vaseline gap chamber have been used to study the coupling between electrical events at the sarcolemma and the increase in intracellular Ca\(^{2+}\) concentration (Melzer et al., 1987). More sensitive dyes such as quin-2 and fura-2 have also facilitated the measurement of intracellular Ca\(^{2+}\)-fluxes.

The observation made by several laboratories (Blinks et al., 1982; Martonosi, 1984) is that a few milliseconds after the rising phase of the action potential the Ca\(^{2+}\) concentration increases from about 0.1 µM at rest to ~10 µM. When the maximal Ca\(^{2+}\) concentration is reached tension starts to develop.

**Skinned Fibers**

Skinned fibers have been prepared by either mechanical peeling, or by chemical treatment. Such fibers retain a functional SR while direct access to the sarcoplasm facilitates more direct measurements of the influence of drugs and other ligands on the Ca\(^{2+}\) release mechanism of SR. In this type of experiment, tension can be measured and changes in myoplasmic Ca\(^{2+}\) concentrations can be assayed using isotopic...
and spectrophotometric methods.

Mechanically skinned fibers are permeable to substrates but retain an functionally intact sarcotubular membrane system. In these fibers, Ca\(^{2+}\) release can be stimulated by addition of Ca\(^{2+}\) (Ca\(^{2+}\)-induced release), caffeine, or potassium. Ca\(^{2+}\) and caffeine trigger Ca\(^{2+}\) release by a direct action with the SR, while potassium-induced contractions occur via depolarization of the t-tubule (Donaldson, 1985). Skinned fiber experiments have been helpful in understanding the coupling of t-tubules to terminal cisternae.

**Isolation of the Myofibrils**

Myofibrils are readily isolated by adding high concentration of salts to muscle homogenates and by removing membrane fractions by centrifugation. Early studies focused on the molecular mechanism and reactions of contractile proteins (Ebashi, 1974). When the myoplasmic free Ca\(^{2+}\) concentration is low, the interaction between actin and myosin is blocked by the troponin-tropomyosin complex. At elevated intracellular free Ca\(^{2+}\) concentrations, the troponin-tropomyosin complex dissociates, and actin and myosin interact with each other.

**Isolated Subcellular Membrane Fractions**

Several different methods have been developed for the isolation of subcellular muscle fractions. These fractions have been used to characterize their biochemical and functional properties.
Triads have been isolated by several groups. Mitchell et al. (1983) were able to purify morphologically intact triads from rabbit fast-twitch skeletal muscle. A heavy microsomal fraction was prepared which was then enriched in triads by performing two sequential 90-min sucrose-gradient centrifugations. A microsomal fraction that contained primarily triads was first isolated by Caswell et al. (1976). This group disrupted the triads by using a French press technique, and obtained a preparation enriched in t-tubules (Lau et al., 1977). Rosemblatt et al. (1981) used t-tubules purified by density-gradient centrifugation to raise a polyclonal antibody against this fraction. Cross sections of rabbit skeletal muscle were stained with these antibodies. Using a fluorescent conjugated secondary antibody, the t-tubule in the muscle could be localized. They appeared as striated pattern.

Although it is difficult to isolate sarcolemma, which is only a small fraction of the cellular mass and is associated with extracellular basal lamina, preparations of sarcolemma have been developed (Seiler and Fleischer, 1982).

Since fast-twitch skeletal muscle has an extensive sarcotubular system, SR and t-tubules are readily isolated from this muscle type. Furthermore, SR subfractions enriched in terminal cisternae and longitudinal tubules were obtained by density-gradient centrifugation (Meissner, 1975).

Preparation of membrane fractions have been used to
investigate the properties of these different membrane fractions. The results obtained from such experiments are summarized in the following sections.

Sarcolemma and t-tubules. Supporting results from earlier experiments with skinned fibers, Ikemoto et al. (1984, 1985) demonstrated that an intact triad (t-tubule SR junction) is needed to trigger rapid depolarization-induced Ca^{2+} release.

In cardiac and smooth muscle, a voltage-sensitive Ca^{2+} channel was found to regulate the inward movement of Ca^{2+} into the cell during the action potential (Schwartz et al., 1985; Rios and Brum, 1987). This high-affinity dihydropyridine (DHP) receptor is mainly localized in the t-tubule membrane. Its specific function in skeletal muscle is still unknown.

Sarcoplasmic reticulum. The regulation of the intracellular Ca^{2+} concentration is controlled primarily by the SR in both skeletal and cardiac muscle. Subfractionating of SR by density-gradient centrifugation allowed identification of two distinct fractions: light SR (LSR) and heavy SR (HSR). It has been shown that LSR is enriched in longitudinal SR while HSR primarily contains membranes from the terminal cisternae (Meissner, 1975).

In LSR, 90% of the total protein consists of the calcium pump protein. The lumen of this membrane fraction is devoid of proteins. The Ca^{2+} pump, Ca^{2+},Mg^{2+}-ATPase, with a molecular mass of 105 kDa, establishes a Ca^{2+} concentration gradient across the membrane via hydrolysis of ATP (active Ca^{2+}
transport). The principal function of this membrane is to keep the myoplasmic Ca\textsuperscript{2+} concentration low during muscular relaxation.

The HSR contains \textasciitilde80\% calcium pump membrane and \textasciitilde20\% junctional face membrane. The second most common protein component found in the SR is calsequestrin. It is localized within the isolated terminal cisternae and it appears to be associated with the junctional face membranes (Brunschwig et al., 1982; Franzini-Armstrong et al., 1987; Saito et al., 1984). Calsequestrin binds calcium ions with high capacity and low affinity. This relates to its function in Ca\textsuperscript{2+} storage. At rest much of the Ca\textsuperscript{2+} content of the SR is bound to calsequestrin and localized in the terminal cisternae.

An interesting characteristic of the terminal cisternae is that it is relatively leaky to Ca\textsuperscript{2+}. This 'leakiness' can be blocked by known inhibitors of the Ca\textsuperscript{2+} release mechanism of SR, such as ruthenium red. This and other observations support the hypothesis that fast Ca\textsuperscript{2+} release from SR is localized in the terminal cisternae and is caused by a specific Ca\textsuperscript{2+} release channel. Responding to an appropriate message this Ca\textsuperscript{2+} channel protein can either close or open in order to maintain or reduce the myoplasmic Ca\textsuperscript{2+} concentration gradient. Ca\textsuperscript{2+} flux measurements across isolated SR vesicles membranes showed that Ca\textsuperscript{2+} release is faster in vesicles derived from the HSR than from the LSR fractions. Ca\textsuperscript{2+} release
channels are mainly localized in the terminal cisternae (Meissner, 1984).

Although much progress has been made by isolation of subcellular membrane fragments, a number of questions remain unanswered.

It is difficult to prepare highly purified membrane fractions. Thus, issues concerning the exact protein components constituting particular membranes remain controversial. Furthermore, relatively harsh methods have to be used to isolate membrane bound proteins. This may introduce artifacts due to proteins which are no longer in their native state.

It also appears that even under non physiological conditions favoring Ca\(^{2+}\) release, the release rate in isolated SR preparations is not comparable to the expected rate of Ca\(^{2+}\) release observed in more intact preparations (i.e. intact muscle fibers). The difference between the rate of activating Ca\(^{2+}\) release in vivo and the 'passive' Ca\(^{2+}\) release in isolated vesicular preparations in vitro is 1,000 to 10,000 fold (Martonosi, 1984).

**Purified membrane channels**

On the level of identification and characterization of the proteins involved in the Ca\(^{2+}\) release process, patch-clamp methodology has yielded many significant observations. Vesicles are incorporated into an artificial membrane
following a well controlled fusion event. Purified channel proteins also can be reconstituted, and their gating properties can then be compared with SR vesicles following fusion to the bilayer (Tsien, 1983).

EXCITATION-CONTRACTION COUPLING

Introduction

In spite of the avalanche of recent studies, the molecular mechanism that couples excitation of the t-tubule to contraction induced by Ca\(^{2+}\) released from SR remains unknown. This interaction is referred to as excitation-contraction coupling (E-C coupling).

Three functional steps have to be realized to make E-C coupling possible, first, a voltage change at the t-tubule membrane has to initiate the process. The last step is the release of Ca\(^{2+}\) from SR. At least one intermediate stage is necessary to transmit the signal from the t-tubules to SR.

Transmission of the signal could be principally realized in three ways: mechanical, electrical or/and chemical.

Mechanical Coupling

The discovery of physical connections referred to as "feet" between the SR membrane and t-tubules on electron micrographs led the suggestion that E-C coupling is mechanically mediated (Franzini-Armstrong, 1986). Considerable effort has been directed toward identifying these "feet"
proteins.

The next step supporting this hypothesis was the finding that a protein identified as the high affinity ryanodine receptor appears to form a four-leaf clover structure similar to the feet (Inui et al., 1987a, Campbell et al., 1980). Incorporation of the purified ryanodine receptor into an artificial membrane revealed a Ca\(^{2+}\) channel with properties similar to that expected of the physiological Ca\(^{2+}\) release protein (Lai et al., 1988, 1989). In addition, junctional t-tubules show diamond-shaped clusters of particles that correspond in position to the subunits of the feet protein and may be the DHP receptor. In the proposed mechanical mechanism for E-C coupling, the foot serves as a plunger to control the Ca\(^{2+}\) release channel and the DHP receptor constitutes the voltage sensor. (Block et al., 1988).

Such a large junctional complex spanning the gap between the t-tubules and the SR membrane is possibly the mediator of a mechanical interaction between these two membranes.

Electrical Coupling

This theory suggests that a signal is transmitted through an ionic pathway between the t-tubule and SR membranes. Theoretical considerations by Eisenberg (1987) suggest that the capacitive coupling between t-tubule and SR is very unlikely. However, since the foot protein apparently touches the t-tubule membrane an electric signal may propagate along
it towards the SR membrane.

An (exclusively) electrical coupling hypothesis claims that depolarization of the t-tubule causes a potential change at the SR membrane which opens Ca\(^{2+}\) release channels. This is unlikely because the SR membrane is leaky and no gradient other than Ca\(^{2+}\) exists across the SR membrane. It is therefore highly unlikely that a membrane electrical potential difference could be maintained across the SR (Martonosi, 1984). Furthermore, the Ca\(^{2+}\) release channel is only slightly voltage dependent when examined in bilayer reconstitution experiments (Smith et al., 1986).

**Chemical Coupling**

The third possibility of coupling is a chemical mediated interaction. Below I summarize possible mechanism.

**Ca\(^{2+}\) induced Ca\(^{2+}\) release.** In heart muscle, Ca\(^{2+}\)-induced Ca\(^{2+}\) release represents the E-C coupling mechanism. In the t-tubule membrane, the DHP receptor functioning as an inward directed Ca\(^{2+}\) channel causes an increase in the myoplasmic Ca\(^{2+}\) concentration which then triggers Ca\(^{2+}\)-release from the SR. This mechanism does not apply in skeletal muscle. The DHP receptor in skeletal muscle appears not to function as a Ca\(^{2+}\) channel (Schwartz et al., 1985). Further, Armstrong et al. (1972) demonstrated that skeletal muscle fibers twitch for long periods of time in the absence of extracellular Ca\(^{2+}\). This observation seems to negate the possibility that
physiological Ca\(^{2+}\) release is dependent on extracellular Ca\(^{2+}\) in skeletal muscle. It is therefore unlikely that Ca\(^{2+}\) induced Ca\(^{2+}\) release is physiologically relevant. Despite all these criticisms of a physiological role for Ca\(^{2+}\) in E-C coupling, the Ca\(^{2+}\) release channel is sensitive to the Ca\(^{2+}\) concentration. The channel is closed at submicromolar Ca\(^{2+}\) concentration and is opened at -10 µM Ca\(^{2+}\) (Endo et al., 1970, 1985). It is possible that Ca\(^{2+}\) modulates Ca\(^{2+}\) release stimulated by some other primary messenger.

**Phosphorylation by inositol 1,4,5-triphosphate.** An alternative chemical mediator for E-C coupling model is inositol 1,4,5-triphosphate (IP\(_3\)). This model proposes that depolarization of the t-tubule stimulates phospholipase C activity that hydrolyzes phosphatidylinositol(4,5)-bisphosphate (PI\((4,5)P_2\)) to IP\(_3\) (Vergara et al., 1985; Volpe et al., 1985). The IP\(_3\) quickly diffuses through the triadic junctional gap. When it reaches the SR membrane it opens IP\(_3\)-sensitive calcium channels and induces Ca\(^{2+}\) release from SR.

Experiments have shown that IP\(_3\) concentrations increase during excitation of intact muscle fibers. In addition, IP\(_3\) causes calcium release and contraction in skinned fibers, and opens the calcium release channels of SR in reconstitution experiments. However, it was shown that the rate and amount of IP\(_3\) produced is too little to be physiologically relevant (Walker et al., 1987).
**Sulfhydryl oxidation.** The sensitivity of the Ca\(^{2+}\) release channel to oxidation of sulfhydryls suggests this as a physiologically relevant Ca\(^{2+}\) release mechanism from SR (Trimm et al., 1986). Oxidation of two SH to a S-S opens the channel, while a reduction of the disulfide formed closes the channel. Stimulators and inhibitors of Ca\(^{2+}\) induced Ca\(^{2+}\) release affect SH induced Ca\(^{2+}\) release of SR vesicles in a similar way. In favor of this model, binding of heavy metals to endogenous SH groups causes Ca\(^{2+}\) release specifically from terminal cisternae SR vesicles (Salama and Abramson, 1988).

The physiological relevance of SH oxidation depends on whether any endogenous SH oxidants can cause SR Ca\(^{2+}\) release (Trimm et al., 1986). The observation that reducing agents (glutathione, dithiothreitol) do not appreciably affect muscle E-C coupling (Brunder et al., 1988) suggests that SH oxidation may not be physiologically relevant in inducing Ca\(^{2+}\) release.

**Perspectives**

It is difficult to directly demonstrate the exact molecular mechanism of E-C coupling under physiological conditions. Alternative, indirect methods for unravelling this puzzle need to be employed to clarify the mechanism of Ca\(^{2+}\) release from SR.
THE IDENTIFICATION OF THE \textit{Ca}^{2+}-RELEASE CHANNEL

\textbf{Introduction}

It is still unknown which particular protein(s) is (are) involved in the \textit{Ca}^{2+}-release mechanism of SR. However, several potential \textit{Ca}^{2+}-release channels have been proposed.

In general, ligand-binding (e.g. binding of biotinylated SH-reagents or ryanodine binding) specifically to the channel has been used to identify the \textit{Ca}^{2+} release protein. These ligands can modify channel properties, or they can be employed as probes to detect modifications of the channel (e.g. reduction in binding of radiolabeled ryanodine). Ligands can be also used as markers to localize the labelled protein in membrane fractions or in purification processes such as density-gradient centrifugation. Ligands immobilized in columns have also been used to purify the \textit{Ca}^{2+} release channel. Finally, the purified proteins can be used for developing immunoaffinity probes.

In this section, I discuss the results of studies that deal with two putative \textit{Ca}^{2+}-release channel proteins, the 400 kDa and the 106 kDa proteins.

\textbf{Characterization of the 'Native' \textit{Ca}^{2+}-Release Channel}

The transport properties of the \textit{Ca}^{2+} release channel from HSR has been characterized by rapid mixing methods (Ikemoto et al., 1985; Meissner et al., 1986, Meissner, 1985) and by lipid
bilayer reconstitution experiments (Smith et al., 1986, 1988, Rousseau et al., 1986). Ca\(^{2+}\) release is inhibited by micromolar calmodulin and ruthenium red and millimolar Mg\(^{2+}\), and is stimulated by micromolar Ca\(^{2+}\) and millimolar adenine nucleotides. The reconstituted protein shows high single channel conductance for both mono- and divalent cations.

**400 kDa Protein**

An important step in studies of the Ca\(^{2+}\)-release channel in SR was the discovery that the Ca\(^{2+}\)-release channel possesses a high-affinity binding site for the plant alkaloid ryanodine. Ryanodine affects the Ca\(^{2+}\) permeability of the receptor. Low concentrations open the channel (<10 µM) while higher concentrations close it (Meissner, 1985).

Tritiated ryanodine has been used to mark the channel during purification procedures. Isolation of the receptor by density gradient centrifugation (Smith et al., 1988), sequential column chromatography (Inui et al., 1987b), and immunoaffinity chromatography (Campbell et al., 1987) revealed a ryanosine-labeled protein of about 400,000 Da. Incorporation of this protein into a lipid bilayer, yielded single channel characteristics similar to the 'native' Ca\(^{2+}\) release channel from SR (Smith et al., 1988).

The 400 kDa protein appears to be arranged in an oligomeric complex of four proteins to form the putative channel (Inui et al., 1987a). Furthermore, electron
micrographs of the purified reconstituted receptor show a structure similar to the junctional SR membrane feet.

106 kDa Protein

Ligand-binding to another site has been demonstrated to affect Ca$^{2+}$ release from SR (Zaidi et al., 1989a). Reactive disulfides induce Ca$^{2+}$ release from SR by oxidizing free sulfhydryls on the channel. This effect is reversed by addition of reducing agents such as glutathione or dithiothreitol. Interestingly, ryanodine binding is inhibited by binding of reactive disulfide reagents, while other sites responsible for inhibition (Mg$^{2+}$, ruthenium red, adenine nucleotides) maintain their functionality. Purification of the Ca$^{2+}$ release channel by biotin-avidin chromatography and by polyclonal-affinity columns revealed a protein with molecular weight of about 106 kDa. Addition of proteolytic inhibitors during SR preparation, purification processes and crossreaction tests with antibodies showed that the 106 kDa protein is neither a part of the ATPase nor a fragment of the 400 kDa protein. Incorporation in planar bilayers revealed cationic channels with properties similar to the 'native' Ca$^{2+}$ release channels (Zaidi et al., 1989a; Hilkert et al., 1992). Assuming that oxidation of free sulfhydryls is the physiological mechanism for Ca$^{2+}$ release during E-C coupling, the 106 kDa protein is most likely the Ca$^{2+}$-release channel.
106 kDa versus 400 kDa Protein

It is unlikely that two totally different proteins have the same functional properties in the Ca\textsuperscript{2+} release process. It is possible that the 106 kDa protein is a proteolytic fragment of the 400 kDa protein. Alternatively, it is possible that in purifying one of these two proteins, the other protein is copurified. Hence, the properties attributed to one of these two proteins may be due to the presence of the other protein (Zaidi et al., 1989b). The question of which of these two proteins is the Ca\textsuperscript{2+} release channel, still remains open.

PROTEOLYTIC MODIFICATION OF SR

Introduction

Proteolytic modification of the SR can be used as a tool to understand function better and to identify the Ca\textsuperscript{2+} release protein from SR. Of particular interest are those proteolytic enzymes which alter the transport properties of the SR in a well-defined manner. Visualization of the proteolytic fractions by gel-electrophoresis can then be used to help identify the Ca\textsuperscript{2+}-release channel.

Trypsin

Originally, trypsin was used to study the ATPase of SR. It was found that tryptic cleavage uncouples active Ca\textsuperscript{2+} transport across the membrane from the hydrolysis of ATP. It was hoped that this would provide some important clues about
the molecular mechanism underlying the Ca\textsuperscript{2+} pumps function. In contrast, Shoshan-Barmatz et al. (1987) demonstrated that the inhibition of Ca\textsuperscript{2+} accumulation in SR vesicles is caused by an activation of the Ca\textsuperscript{2+} release channel (CRC), not by an uncoupling of the ATPase.

Tryptic modification now became an important tool to investigate changes in the properties of the CRC. The rate of Ca\textsuperscript{2+} release from SR vesicle was greatly enhanced by tryptic proteolysis (Trimm et al., 1988). The functional properties of the sites involved in the activation (cAMP, doxorubicin, micromolar Hg\textsuperscript{2+}, and Ca\textsuperscript{2+}) and inhibition (millimolar Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and micromolar ruthenium red) of the Ca\textsuperscript{2+} release system are, however, not affected by proteolysis (Trimm et al., 1988; Chu et al., 1988).

In many papers, the high-molecular weight (400,000 Da) protein is discussed as the putative CRC. SDS-polyacrylamide gels demonstrate a very high sensitivity of the high molecular weight protein to tryptic digestion. While the ATPase is cleaved at a later stage, the 400 kDa is cleaved rapidly, and protein bands of lower molecular weight appear following immunoblot analysis. The stimulation of Ca\textsuperscript{2+} release appears to be caused by a later cleavage of the 400 kDa protein (Trimm et al., 1988), or it may be correlated with cleavage of the 106 kDa protein.

In spite of drastic cleavage of the high molecular weight
protein its ultrastructure as a foot visualized by electron microscopy is not altered (Chu et al., 1988). Further, mild proteolysis does not affect the sedimentation characteristics (30S) of ryanodine-receptor complex (Meissner, 1989). Extensive exposure to trypsin leads to a reduction of the protein size (10-15S). These and other results suggest that the fragments are stabilized by multiple noncovalent interactions which can only be dissociated with strong detergents.

The alteration of ryanodine-binding due to proteolysis was demonstrated as another characteristic of the Ca^{2+} release channel (Shoshan-Barmatz and Zarka, 1988). Mild proteolysis does not affect ryanodine binding, while further degradation prior to the assay leads to a complete loss of high-affinity binding capacity. This loss of high-affinity ryanodine binding is not correlated with an enhancement of Ca^{2+} release. Moreover, low-affinity binding is not altered by extensive proteolysis.

The effect of trypsin on a single channel incorporated in a lipid bilayer was demonstrated by Meissner et al. (1989). Addition of trypsin to the cytoplasmic side of the bilayer initially increased the channel open time and was followed by a complete and irreversible loss of channel activity.

The large degree of variability from one laboratory to the next is likely to be caused by different experimental conditions and protocols. Shoshan-Barmatz and Zarka (1988)
reported that high concentration of NaCl in the buffer prevents loss of ryanodine binding. This also results in an altered pattern as seen on a SDS-polyacrylamide gel. So far, no conclusion can be drawn about the change in molecular structure and functionality of CRC caused by tryptic proteolysis. Nevertheless, tryptic proteolysis remains a sensitive and important tool to study the mechanism of Ca\textsuperscript{2+}-release.

**Calpain II**

Calpain II is an endogenous cytosolic protease. It was used to study the high molecular weight protein (Rardon et al., 1990). In contrast to trypsin, the 400 kDa protein was cleaved into only two fragments of 315 and 150 kDa by calpain II. This caused a change in the ultrastructure of the feet. Ryanodine-binding was not affected. But the channel open time increased while the unitary conductance was maintained. Rardon et al. suggest that proteolysis with calpain II only destroys the ability of the channel to inactivate.

**Summary**

Proteolytic modification of the Ca\textsuperscript{2+} release mechanism of SR is a potentially sensitive tool to analyze the molecular mechanism of Ca\textsuperscript{2+}-release and better understand E-C coupling.
OVERVIEW

The identification of the Ca\(^{2+}\) release protein is an important step in understanding E-C coupling. The sensitivity for ryanodine and for active sulfhydryl reagents has been utilized to purify possible Ca\(^{2+}\) release proteins, the 400 kDa and 106 kDa protein. I had hoped to identify one of these two proteins as the Ca\(^{2+}\) release protein by proteolytic modification of the Ca\(^{2+}\) release mechanism.

It has been demonstrated that trypsin stimulates Ca\(^{2+}\) release from SR vesicles (Trimm et al., 1988). I tried to characterize this effect better. Using a dual-wavelength spectrophotometer, Ca\(^{2+}\) efflux from native and proteolytic modified SR was assayed. Since trypsin cleaves peptide bonds at arginine and lysine residues, the first approach was to determine which of these two cleavage sites is responsible for enhancement of Ca\(^{2+}\) release. Thus, the arginine specific protease thrombin was tested. It appeared not to stimulate Ca\(^{2+}\) release. Further investigations revealed that only type III trypsin from Sigma, but not sequence-grade trypsin from Boehringer, affected the Ca\(^{2+}\) release mechanism. This suggests contaminants in the Sigma-trypsin are probably responsible for stimulation of Ca\(^{2+}\) efflux. The possible impurities, kallikrein and chymotrypsin, were tested at appropriate concentration. No stimulation could be observed. The results of these experiments are described in Chapter III.
The next attempt was to correlate the stimulation of Ca\textsuperscript{2+} release caused by tryptic proteolysis with the degradation of one of the two possible Ca\textsuperscript{2+} release proteins, the 400 kDa and 106 kDa protein. For comparison purposes thrombin, kallikrein, chymotrypsin and the two types of trypsin were tested. Proteolytic fragments of SR proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands either were visualized by staining with coomassie-blue or were transferred onto sheets of nitrocellulose and were detected with antibodies to the 106 kDa and 400 kDa protein. Results of these experiments are presented in Chapter IV. It appeared to be difficult to inhibit these proteases when SDS-sample buffer was added. Since staining with the polyclonal antibody to 106 kDa protein was faint, no information could obtained about cleavage of the 106 kDa protein. However, the disappearance of the 400 kDa protein band does not correlate well with the stimulation of Ca\textsuperscript{2+} release from SR.

Although I could not draw any further conclusion about the identity of the Ca\textsuperscript{2+} release protein, these experiments demonstrate that tryptic modification of SR is a promising tool to identify and characterize the Ca\textsuperscript{2+} release channel.
CHAPTER II

METHODS AND MATERIALS

SR PREPARATION

Skeletal muscle SR vesicles were isolated following the method of MacLennan (1970). White muscle was taken from the back and the hind legs of a 5-7 lb male rabbit. The fat and the connective tissue were trimmed off. The muscle was put into ice-cold buffer A (120 mM NaCl, 10 mM imidazole, pH 7.4). It was then ground in a meat grinder. The suspension was diluted into three volumes of buffer A and homogenized in a Waring blender for 15 seconds on low speed, followed by 30 seconds on high speed. It was then centrifuged at 1,600 g (3,100 rpm in the large Sorvall GSA rotor) for ten minutes. The supernatant was strained through four layers of cheesecloth and its pH was adjusted to 7.4 with dry imidazole. The pelleted cell debris was discarded. The supernatant was again centrifuged at 10,000 g (8,000 rpm in the GSA rotor) for 14 minutes. The supernatant was then filtered through four layers of cheesecloth and the brown mitochondrial pellet was discarded. The suspension was then ultracentrifuged at 44,000 g (19,000 rpm in the Beckman type 19 rotor) for 70 minutes. The pellet (without the brown mitochondrial ring) was scrapped off, resuspended at about 10 mg/ml in buffer A, and
homogenized with a glass homogenizer. This was again centrifuged at 7,500 g (11,000 rpm in the Beckman Ti60 rotor, total volume 150 ml) for 10 min. The myosin pellet was discarded and the supernatant was then centrifuged at 78,000 g (35,000 rpm in the Ti60) for 30 minutes. The pellet, which is the SR fraction, was suspended into a buffer containing 20 mM HEPES, 100 mM KCl at pH 7.0 at a final protein concentration of about 15 to 25 mg/ml. The final concentration was determined by the method of Lowry et al. (1953). The SR suspension was stored in small aliquots in liquid nitrogen.

PASSIVE CA\(^{2+}\) EFFLUX

**Passive Ca\(^{2+}\) Loading of SR Vesicles**

SR was diluted in 1 mM CaCl\(_2\), 20 mM HEPES, 100 mM KCl at pH 7.0 to a final concentration of 10 mg/ml. For passively loading of the vesicles with CaCl\(_2\), the suspension was frozen in liquid nitrogen and then thawed at room temperature (freeze-thaw technique).

**Proteolytic Modification of SR Vesicles**

With Ca\(^{2+}\) loaded SR vesicles at room temperature were subjected to treatment with different proteolytic enzymes. In passive Ca\(^{2+}\) efflux measurements, the enzymes type III trypsin from Sigma, sequence-grade trypsin from Boehringer, kallikrein and chymotrypsin were used. Time dependent modification was carried out at an enzyme to SR protein ratio of 1:200 (w:w).
In these experiments in which the concentration of protease was varied, SR vesicles were exposed to the proteolytic enzyme for either 15 or 18 min. At specified time intervals, an aliquot of proteolytically (trypsin, thrombin) modified SR was added to a ten-fold excess of soybean trypsin inhibitor and the sample was kept on ice until Ca\(^{2+}\) efflux was assayed. Kallikrein and chymotrypsin were not inhibited. Ca\(^{2+}\) release was monitored immediately after the indicated time of incubation.

**Ca\(^{2+}\) Efflux measurements**

Passive Ca\(^{2+}\) release from SR vesicles was assayed as followed:

Native or proteolytic modified SR was diluted into 40 volumes of Ca\(^{2+}\) free 'efflux medium' (20 mM HEPES, 100 mM KCl, 100 mM arsenazo III, pH 7.0) containing various concentrations of Ca\(^{2+}\) permeability modulators like MgCl\(_2\) and doxorubicin. The change in the absorbance of the Ca\(^{2+}\) sensitive dye arsenazo III (Ars III) was monitored as a function of time with a time-sharing dual wavelength spectrophotometer. The absorbance difference at 675 nm and at 685 nm wavelength is proportional to the change of the free external Ca\(^{2+}\) concentration. At the end of each recording, the Ca\(^{2+}\) ionophore A23187 (2 µg/ml) was added to release the intravesicular residual Ca\(^{2+}\). A known amount of CaCl\(_2\) (2 µM) was then added three times to the efflux medium. The average of these three measurements was
used to calibrate the response of the dye to Ca$^{2+}$.

Ca$^{2+}$ efflux rates were calculated from the initial slopes of the time courses of passive Ca$^{2+}$ release from SR vesicles.

**SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS**

**Preparation of 5-15% Gradient Slab Gels**

5-15% gradient slab gels (16.0 cm x 18.0 cm x 1.5 mm) were poured using the following instruments: SG Series Gradient Makers (Hoefer Scientific Instruments), polystaltic pump (Buchler Instruments) and a vertical slab gel system (Hoefer Scientific Instruments). Their arrangement is shown in Figure 2. The gradient maker is mounted slightly above the magnetic stirrer without contact to avoid heating up of the acrylamide solutions. Reservoir A and B are connected by a tubing. Chamber B contains a stir bar. The solution flowing from reservoir A is diluted and mixed in chamber B. This mixture then flows through tubing into the space between the glass plates of the slab gel system. The polystaltic pump forces the solution through the tubing.

A 5-15% sodium dodecyl sulfate-polyacrylamide gradient gel is made as follows. Two solutions of 5% and 15% acrylamide were prepared according to Table I. Ammonium persulfate and the polymerization catalyst TEMED were added just before adding the solutions to the reservoirs of the gradient mixer. The sucrose in the 15% acrylamide solution helps prevent mixing due to convection which is caused by heating during
Figure 2. Apparatus for formation of gradient polyacrylamide slab gels (adapted from Hames and Rickwood, "Gel electrophoresis of proteins - a practical approach", IRL press 1984). A and B refer to the two chambers of the gradient maker. Mixing chamber B contains the stir bar and is positioned above the magnetic stirrer. Reservoirs A and B are connected by a tubing which is controlled by a two-way tab. During formation of the gel, both tabs are open and the peristaltic pump forces the mixture through the tubing. The end of the tubing is inserted between the two glass plates.
<table>
<thead>
<tr>
<th>SDS-ACRYLAMIDE MIXTURES FOR ONE LAEMMLI GEL</th>
</tr>
</thead>
</table>

**5-15% gradient gel (running gel)**

<table>
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<tr>
<th>Component</th>
<th>15%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide, .8% N,N'-methylene-bis-acrylamide</td>
<td>7.25 ml</td>
<td>2.4 ml</td>
</tr>
<tr>
<td>1 M tris[hydroxymethyl]-aminomethane (tris) pH 8.8</td>
<td>5.43 ml</td>
<td>5.43 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>1.52 ml</td>
<td>6.35 ml</td>
</tr>
<tr>
<td>10% sodium dodecyl sulfate (SDS)</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>10% ammonium persulfate (APS)</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>N,N,N',N'-tetramethyl-ethylenediamine (TEMED)</td>
<td>5 ul</td>
<td>5 ul</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2.25 g</td>
<td>-</td>
</tr>
</tbody>
</table>

**4% stacking gel**

<table>
<thead>
<tr>
<th>Component</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide, .8% N,N'-methylene-bis-acrylamide</td>
<td>1.33 ml</td>
</tr>
<tr>
<td>1 M tris[hydroxymethyl]-aminomethane (tris) pH 6.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>10% sodium dodecyl sulfate (SDS)</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>10% ammonium persulfate (APS)</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>N,N,N',N'-tetramethyl-ethylenediamine (TEMED)</td>
<td>35 ul</td>
</tr>
</tbody>
</table>

Polymerization.

The 5% acrylamide solution is added to chamber A and the 15% acrylamide solution is added to chamber B up to the same level of the solution in chamber A. The magnetic stirrer and polystaltic pump are turned on and the taps are opened. The mixture is pumped into the region between the glass plates at a flow-rate of 2 to 5 ml/min. The resulting gel is a 5 to 15% acrylamide gel with 15% acrylamide at the bottom and 5% acrylamide at the top. A thin layer of deionized water is
carefully poured onto the top of the sample gel to obtain an even interface. After polymerization the water is removed. According to the method of Laemmli, a 4% stacking gel (see table I) with 10 sample wells was polymerized in place. The pH of the stacking gel (6.8) is chosen so that the in the electric field migrating polypeptides stack up at the interface between the stacking and running gel (pH 8.8). Such gels with different pH for stacking and running gels is referred to as discontinuous buffer system.

Sample Preparation

SR vesicles (10 mg/ml) were treated with proteolytic enzymes (type III trypsin from Sigma, sequence-grade trypsin from Boehringer, kallikrein, chymotrypsin, or thrombin) at an enzyme to SR protein ratio of 1:200 in absence and presence of 1 mM CaCl₂. Several protease inhibitors were tested in order to achieve complete inhibition. For both types of trypsin, a 10-fold excess of soybean trypsin inhibitor and/or 1 mM diisopropanyl fluorophosphate (DIFP) was added. To inhibit chymotrypsin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and/or aprotinin equimolar to chymotrypsin was tested. Kallikrein was inhibited by addition of 100 µM leupeptin or a ten-fold excess of soybean trypsin inhibitor. At the indicated time intervals, samples were pipetted from the suspension of proteolytically modified SR and added to the specified inhibitors.

An equal volume of sample buffer (4% SDS, 62.5 mM Tris pH
6.8, 10% sucrose, 0.02% bromophenol blue in deionized H₂O) was added to each sample. In some experiments the sample buffer contained the reducing agent mercaptoethanol (5%) which was added shortly before the sample was added to the buffer. Before the samples were loaded on the gel, the samples were kept on ice or were incubated at room temperature, 30°C, or 95°C for various periods of time.

**Electrophoresis**

Electrophoresis was carried out in a Hoefer vertical slab gel apparatus. The glass plate sandwich with a 1.5 mm thick gradient gel is mounted in between two reservoir buffers (25 mM Tris, pH 8.8, 1.918 M Glycine, 0.1% SDS). Negatively charged SDS solubilized proteins loaded on the gel migrate through the gel by an electric field which is applied between the lower and upper reservoir.

Excess of the anionic detergent SDS is used to denature the proteins of the SR membrane. SDS solubilized proteins migrate under a constant electric field such that the mobility of the proteins is proportional to the logarithm of the apparent molecular weight of the proteins (Laemmli, 1970).

The gels were run with a current between 5 and 35 mA for about 4 to 14 hours. The gels were stained with Coomassie blue or the proteins were transferred from gels by Western blotting. For calibration, molecular weight standards migrated in one lane of each gel. For gels which were stained with
Coomassie blue, the following standards from Boehringer were used: \( \alpha_2 \) macroglobulin: non-reduced 340 kDa, reduced 170 kDa; \( \beta \)-galactosidase: 116.4 kDa; fructose-6-phosphate kinase: 85.2 kDa; glutamate dehydrogenase: 55.6 kDa; aldolase: 39.2 kDa; triosephosphate isomerase: 26.6 kDa; trypsin inhibitor: 20.1 kDa; and lysozyme: 14.3 kDa. The following prestained standards were used when proteins were transferred onto nitrocellulose: \( \alpha_2 \) macroglobulin: 180 kDa; \( \beta \)-galactosidase: 116 kDa; fructose-6-phosphate kinase: 84 kDa; pyruvate kinase: 58 kDa; fumerase: 48.5 kDa; triosephosphate isomerase: 26.6 kDa.

**Staining with Coomassie Blue**

Some of the gels were stained in a solution containing 0.1% coomassie brilliant blue R dissolved in glacial acetic acid:methanol:d\( \text{H}_2\text{O} \) = 1:5:4 overnight.

They were then destained in a mixture of glacial acetic acid:methanol:d\( \text{H}_2\text{O} \) = 1:5:5. The destaining solution was changed two or three times over a period of 4-6 hours. As a last step the destaining solution was diluted 1:1 with \( \text{H}_2\text{O} \), and gel was incubated for ~2 hours.

**Western blot**

Following separation of proteins on SDS gel electrophoresis, they were electrophoretically transferred onto nitrocellulose (12 x 13 cm, pore size: 0.45 um from Schleicher & Schuell). These nitrocellulose sheets were later
used for staining with antibodies to particular proteins.

The procedure for transferring proteins was as follows. The gel was first soaked in transfer buffer (20% methanol in reservoir buffer containing 25 mM Tris, pH 8.8, 1.918 M Glycine, 0.1% SDS) for about 20 minutes. The sheet of nitrocellulose was then floated in deionized water and then rinsed in transfer buffer. The nitrocellulose and the gel were then stacked between sheets of filter paper, filter backing paper and a foam sponge to hold the stack in the gel cassette with equal pressure over the whole area. Transferring the proteins was carried out in the transfer buffer described above at about 75 mA for 20 hours.

Immunoblotting was used to visualize the 106 kDa and the 400 kDa proteins on the nitrocellulose. Primary antibodies which bound to the these proteins were then detected with the appropriate anti-IgG (anti rabbit or anti mouse) secondary antibodies conjugated with the enzyme alkaline phosphatase (AP) or horseradish peroxidase (HRP). Incubation with a color developing solution allows visualization of the particular protein of interest. The detailed procedure was as follows:

The nitrocellulose was incubated in blocking buffer (2% blot-qualified BSA in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for AP-linked secondary antibodies or 5% dry non-fat milk in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) for HRP-linked secondary antibodies ) for at least 1 hour to saturate nonspecific protein binding sites. In all following
procedures Tween-20 was included in all AP linking incubations, and was omitted from all HRP incubations. The blocking buffer was then replaced with TBS(T) containing the appropriate dilution of primary antibody (1:1000 polyclonal anti 400 kDa, 1:50 monoclonal anti 400 kDa, 1:750 polyclonal anti 106 kDa) and was incubated overnight. The membrane was then washed five to six times for five to ten minutes to remove unbound antibodies. The nitrocellulose was then submerged in TBS(T) containing the appropriate secondary antibody (anti mouse AP-linked for monoclonal anti 400 kDa, anti rabbit AP-linked or HRP-linked for the polyclonal anti 106 kDa and for polyclonal anti 400 kDa). After incubation of 30 min, the nitrocellulose was again washed six times for five to ten minutes. The membrane was then exposed to the color development solution to visualize bound antibodies. For AP-linked secondary antibodies: 66 ul of 50 mg/ml nitro blue tetrazolium (NBT) in 70% dimethylformamide and 33ul of 50 mg/ml 5-bromo-4-chloro-3-indoly1 phosphate (BCIP) in dimethylformamide was added to 10 ml alkaline phosphatase (AP) buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2) and for HRP-linked secondary antibodies: 6 mg of 4 chloro-1-naphtol dissolved in 2 ml methanol and 4 ul of 30% H2O2 added to 10 ml of TBS. The reaction was stopped by rinsing with deionized water.
MATERIALS

The materials used were purchased from the following vendors.

SR Preparation

Sigma Chemical: NaCl, KCl.
Aldrich Chemical Company: Imidazole.
Research Organics: HEPES.
Western Oregon Rabbit Co.: Rabbit.

Ca$^{2+}$ Efflux Assays

Sigma Chemical: KCl, doxorubicin.
Research Organics: HEPES.
Mallinckrodt: CaCl$_2$.
Aldrich Chemical Company: Arsenazo III.
Calbiochem: A23187.

SDS-Polyacrylamide Gel Electrophoresis

BioRad: >99.9% acrylamide, N,N'-methylene-bis-acrylamide.
Sigma Chemical: Tris[hydroxymethyl]aminomethane, sodium dodecyl sulfate, ammonium persulfate, mercaptoethanol, bromophenol blue, glycine, prestained molecular weight standards, Tween 20 (polyoxyethylenesorbitan monolaureate), 30% H$_2$O$_2$, Coomasie brilliant blue R.
Eastman: N,N,N',N'-tetramethylethlenediamine (TEMED).
ICN: Ultrapure sucrose.
Boehringer: Molecular weight standards.
EM Science or Mallinckrodt: Glacial acetic acid.
VWR: Methanol.
Schleicher & Schuell: 0.45 um pore size nitrocellulose.
Cappel: Goat anti rabbit secondary antibody HRP-linked.
Promega: Goat anti rabbit secondary antibody AP-linked,
goat anti mouse secondary antibody AP-linked, NBT, BCIP, blot
qualified BSA, 4-chloro-1-naphtol.

Proteases and Protease Inhibitors
Sigma Chemical: β-chymotrypsin from bovine pancreas, type
III trypsin (bovine pancreas), kallikrein, thrombin (bovine
pancreas), soybean trypsin inhibitor, DIFP, PMSF, aprotinin.
Boehringer: Sequence-grade trypsin, leupeptin.

Primary Antibodies
Primary antibodies to the 106 kDa and 400 kDa protein
were provided by:
Dr. Guy Salama: rabbit anti-106 kDa polyclonal
antibody
Dr. Gerhard Meissner: rabbit anti-400 kDa polyclonal
antibody
Dr. John Sutko: mouse anti-400 kDa monoclonal antibody
CHAPTER III

SPECTROPHOTOMETRIC ASSAY OF Ca$^{2+}$ PERMEABILITY

INTRODUCTION

In this chapter, stimulation of Ca$^{2+}$ release from SR vesicles by several different proteolytic enzymes is compared. The effect of two different types of trypsin (obtained from Sigma and Boehringer), chymotrypsin, kallikrein, and thrombin are described. Stimulation of Ca$^{2+}$ release by limited tryptic proteolysis of the Ca$^{2+}$ release channel has previously been reported (Trimm et al., 1988).

In the first part of this chapter, I demonstrate that Ca$^{2+}$ release is only stimulated following proteolysis with the type III trypsin from Sigma. No stimulation was observed with sequence grade trypsin from Boehringer.

The second section of this chapter describes the results of two different approaches to examine the cause of the stimulation of Ca$^{2+}$ release of Sigma-TMSR. Before it was known that sequence-grade trypsin does not stimulate Ca$^{2+}$ release, I tried to determine which cleavage site of the Ca$^{2+}$ release protein is responsible for enhancement of Ca$^{2+}$ release. Trypsin cleaves at lysine and arginine residues. Thus, the arginine specific protease thrombin was chosen to test the
influence of proteolysis on enhancement of Ca\(^{2+}\) release. At a later stage in these studies, I observed that sequence-grade trypsin does not stimulate Ca\(^{2+}\) release. This suggests that impurities in the type III trypsin cause the observed enhancement. The vendor (Sigma) suggested that either chymotrypsin or kallikrein might be the proteolytic contaminant in the type III trypsin. Kallikrein preferably cleaves peptide bonds at lysine and arginine residues. Chymotrypsin is specific for tyrosine and tryptophan. Proteolysis by thrombin, kallikrein, and chymotrypsin did not stimulate Ca\(^{2+}\) release from SR.

**TRYPTIC MODIFICATION OF THE CA\(^{2+}\) RELEASE SYSTEM**

Passively loaded SR vesicles were exposed to trypsin for 18 min at room temperature (21°C). A ten-fold excess of soybean trypsin inhibitor was then added, and Ca\(^{2+}\) efflux was assayed. Figure 3 shows spectrophotometric traces of Ca\(^{2+}\) efflux of untreated (traces 1 and 4) and of Boehringer (traces 2 and 5) or Sigma (traces 3 and 6) trypsin modified SR (TMSR: tryptically modified SR). The efflux medium (100 µM Ars III, 20 mM HEPES, 100 mM KCl, pH 7.0, 2 mM MgCl\(_2\)) either contained 0 (traces 1 to 3) or 20 µM doxorubicin (traces 4 to 6). At the end of each run, the ionophore A23187 (2 µg/ml) was added to release internal residual Ca\(^{2+}\), and 2 µM CaCl\(_2\) was added to calibrate the response of the dye. The total amount of
Figure 3: Spectrophotometric recordings of Ca$^{2+}$ efflux from untreated and Sigma- and Boehringer-trypsin exposed SR in the absence and presence of doxorubicin. SR vesicles (10 mg/ml) were passively loaded with CaCl$_2$ by a freeze-thaw procedure. A fraction was then exposed either to Type III trypsin from Sigma (Sigma-TMSR) or sequence-grade trypsin from Boehringer (Boehringer-TMSR) at a trypsin to SR ration of 0.005 (w/w) for 18 min. Addition of a 10-fold excess of soybean trypsin inhibitor stopped proteolysis. To induce release, native and TMSR was diluted 40-fold in efflux medium (100 µM Ars III, 20 mM HEPES, 100 mM KCl, pH 7.0, 2 mM MgCl$_2$) which contained either 0 or 20 µM doxorubicin. Extravesicular Ca$^{2+}$ concentration was spectrophotometrically measured by monitoring the differential absorbance of arsenazo III at 675 nm and 685 nm. At the end of each recording, the ionophore, A23187 (2 µg/ml), was added to release intravesicular free Ca$^{2+}$, and a known amount of CaCl$_2$ was added to calibrate the response of the dye. Traces 1 to 3: no doxorubicin; traces 4 to 6: 20 µM doxorubicin; traces 1 and 4: untreated SR; traces 2 and 5: Boehringer-TMSR; traces 3 and 6: Sigma-TMSR. Ca$^{2+}$ efflux rates are calculated from initial slopes (see Table II).
released Ca\(^{2+}\) was approximately 75 nmol / mg SR. Calcium efflux rates were calculated from the initial slope of the traces and are displayed in Table II.

Doxorubicin is known to stimulate release by a direct interaction with the Ca\(^{2+}\) release channel from SR. If trypsin causes stimulation by a mechanism other than affecting the Ca\(^{2+}\) release channel, one would expect that the stimulation by doxorubicin of untreated SR (slope of trace 4 - slope of trace 1 = -0.09 nmol/(mg*s)) and by tryptic modification (slope of trace 3 - slope of trace 1 = -0.19 nmol/(mg*s)) would add up to total enhancement of Ca\(^{2+}\) efflux induced by TMSR (Sigma) in the presence of doxorubicin (slope of trace 6 - slope of trace 1 = .71 nmol/(mg*s)). This is clearly not the case. There is a synergistic interaction between the Ca\(^{2+}\) release activator, doxorubicin, and tryptic modification. Enhanced Ca\(^{2+}\) release

**TABLE II**

\(\text{Ca}^{2+}\) RELEASE RATES FROM NATIVE AND TRYPTICALLY MODIFIED SR IN ABSENCE AND PRESENCE OF DOXORUBICIN

Indicated errors are standard deviations of two to three traces.

<table>
<thead>
<tr>
<th>Efflux rates [nmol/(mg*s)]</th>
<th>no doxorubicin</th>
<th>20 µM doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native SR</td>
<td>0.14 ± 0.03</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>Boehringer-TMSR</td>
<td>0.13 ± 0.05</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>Sigma-TMSR</td>
<td>0.33 ± 0.01</td>
<td>0.85 ± 0.06</td>
</tr>
</tbody>
</table>
is due to an interaction with the Ca\textsuperscript{2+} release mechanism of SR.

Ca\textsuperscript{2+} release from SR is enhanced by proteolysis with Sigma type III trypsin, in both the absence and presence of doxorubicin. SR modified by Boehringer trypsin does not result in enhanced Ca\textsuperscript{2+} release rates. Interestingly, treatment with Boehringer trypsin seems to desensitize the Ca\textsuperscript{2+} release mechanism to activation by doxorubicin.

The time-dependence of efflux rates of induced by tryptic modification is shown in Figure 4. Passively loaded vesicles were exposed to trypsin (from Sigma and Boehringer at a ratio of trypsin:SR=0.005 (mg/mg) at room temperature for the indicated time intervals. Proteolysis was stopped by a ten-fold excess of soybean trypsin inhibitor, and then Ca\textsuperscript{2+} efflux was assayed.

Ca\textsuperscript{2+} release from Sigma-TMSR reaches a maximum (2.24 nmol/(mg*s)) at 18 min compared to unmodified SR (0 sec: 0.41 nmol/(mg*s)). In contrast, modification of SR by Boehringer-trypsin does not enhance Ca\textsuperscript{2+} efflux.

PROTEOLYSIS BY THROMBIN, CHYMOTRYPSIN, AND KALLIKREIN

The results of the studies with thrombin are shown in Figure 5. SR vesicles passively loaded with Ca\textsuperscript{2+} were exposed to thrombin at varying thrombin concentrations for 15 min. A ten-fold excess of soybean trypsin inhibitor was then added to
Figure 4. Ca\textsuperscript{2+} efflux rate versus time of proteolysis of Sigma- and Boehringer-TMSR. Passively loaded SR vesicles were exposed either to type III trypsin from Sigma (-o-) or sequence-grade trypsin from Boehringer (-\textbullet-) at a ratio of trypsin:SR=0.005. At the indicated times, a 10-fold excess of soybean trypsin inhibitor was added. For spectrophotometrically measurements. Aliquots of SR were then diluted 40-fold into efflux medium containing 1 mM MgCl\textsubscript{2}. Error bars represent standard deviation of the slopes of two to three different traces.
Figure 5. Ca$^{2+}$ efflux rate versus the concentration of thrombin. Passively loaded SR vesicles were treated with thrombin at the indicated thrombin to SR protein ratios for 15 min. Proteolysis was stopped by adding a ten-fold excess of soybean trypsin inhibitor. Modified SR was diluted 40-fold into the standard efflux medium (100 μM arsenazo III, 100 mM KCl, 20 mM HEPES, pH 7.0) containing 1 mM MgCl$_2$, and Ca$^{2+}$ efflux was measured spectrophotometrically. Error bars indicate standard deviation of the slopes of two to three traces.
stop proteolysis and Ca$^{2+}$ release was spectrophotometrically measured. Under these conditions no enhancement of Ca$^{2+}$ release from SR was observed.

Studies were then carried out to determine whether kallikrein or chymotrypsin, two possible contaminants found in Sigma-trypsin, were responsible for the observed stimulation of Ca$^{2+}$ release. SR vesicles loaded with Ca$^{2+}$ were treated with either chymotrypsin or kallikrein for 15 min. This is close to the time required for maximal stimulation of Ca$^{2+}$ release induced by Sigma type III trypsin. Immediately following the proteolysis, SR vesicles were assayed for Ca$^{2+}$ release. No proteolytic inhibitor was added to stop proteolysis. Sigma suggested that possible proteolytic contaminants in type III trypsin were between 0.5 and 5%. In Figure 6, I show possible modification of Ca$^{2+}$ release at these levels of chymotrypsin and kallikrein.

At higher protease concentrations, possible stimulation for short time intervals of proteolysis by chymotrypsin was studied. SR vesicles passively loaded with Ca$^{2+}$ were exposed to chymotrypsin at a ratio of enzyme:SR=0.005 for the indicated time intervals. Immediately following proteolysis, Ca$^{2+}$ release was assayed. The data are shown in Figure 7. Ca$^{2+}$ release was not stimulated by chymotrypsin. Instead, proteolysis seems to inhibit efflux slightly. The degree of inhibition does not appear to be significant.
Figure 6. Ca$^{2+}$ efflux rate versus the concentration of chymotrypsin and kallikrein. SR vesicles loaded with CaCl$_2$ was exposed to either chymotrypsin (---) or kallikrein (---) at the indicated protease to SR protein ratios. After 15 min of proteolysis, SR was diluted 40-fold into the standard efflux medium containing 1 mM MgCl$_2$. Ca$^{2+}$ efflux was assayed spectrophotometrically. Error bars indicate standard deviation of the slopes of two traces.
Figure 7. Ca$^{2+}$ efflux rates versus time of proteolysis by chymotrypsin. Passively loaded SR vesicles were modified by chymotrypsin at a protease to SR protein ratio of 0.005. At the indicated time intervals, Ca$^{2+}$ release was assayed spectrophotometrically by dilution 40-fold into the standard efflux medium containing 1 mM MgCl$_2$. Error bars represent the standard deviation of two measurements.
DISCUSSION

Type III trypsin from Sigma causes enhancement of Ca\(^{2+}\) release from SR. This effect does not occur when SR is modified by the sequence-grade trypsin from Boehringer. Neither the arginine specific protease thrombin nor possible contaminants, chymotrypsin and kallikrein, stimulates Ca\(^{2+}\) efflux. The impurity found in the type III trypsin which caused stimulation of Ca\(^{2+}\) release was not determined.
CHAPTER IV

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

INTRODUCTION

In this chapter, I attempt to correlate the stimulation of Ca\(^{2+}\) release by Sigma type III trypsin with the degree of degradation of either the 400 kDa or 106 kDa protein. On Coomassie-blue stained SDS-polyacrylamide gels the high-molecular protein can be easily observed. However, the 106 kDa protein migrates either right below or above the Ca\(^{2+}\)-ATPase band, which is a major component in the SR. It is impossible to observe the 106 kDa protein without some type of marker or label. Immunoblot techniques provide reliable information about the identity and degree of degradation of the proteins involved.

EXPERIMENTAL CONDITIONS

SR vesicles either passively loaded with Ca\(^{2+}\) or diluted with deionized water to a protein concentration of 10 mg/ml were subjected to proteolysis for the indicated time periods. An appropriate proteolytic inhibitor was then added, and the samples were added to SDS sample buffer (4% SDS, 62.5 mM Tris pH 6.8, 10% sucrose, 0.02% bromophenol blue in dH\(_2\)O). These
samples were loaded onto a gel and electrophoresed at 5 to 35 mA for 4 to 14 hrs.

In order to compare results from SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with those from Ca\(^{2+}\) efflux assays, proteolysis was done under the same condition. The absence or presence of CaCl\(_2\) did not change the pattern on SDS-PAGE.

SDS is a strongly denaturing detergent. I discovered that it was difficult to inhibit proteolysis in the presence of SDS. This created problems in interpreting some of the data generated and analyzed with PAGE.

**TRYPTIC MODIFICATION**

Sigma type III trypsin appeared to be very difficult to inhibit. Diisopropyl fluorophosphate (DIFP) and phenylmethylsulfonyl fluoride (PMSF) were ineffective in stopping proteolysis. Soybean trypsin inhibitor (10-fold excess) slowed degradation down. However, in the absence of SDS, it completely inhibited the effects of proteolytic degradation. Dilution of TMSR into sample buffer containing 5% mercaptoethanol, followed by heating of the samples to 80°C or 95°C seemed to prevent degradation in the presence of SDS. This unfortunately introduced another artifact, the ATPase coagulated and additional bands above the high molecular weight protein appeared. SDS-polyacrylamide gels of Sigma- and Boehringer-TMSR treated this way are shown in Figure 8 and 9.

SR was tryptically modified at a trypsin to SR ratio of
Figure 8. Time dependent degradation of SR modified by type III trypsin from Sigma. Passively loaded SR vesicles were exposed to type III trypsin from Sigma at a trypsin to SR protein ratio of 0.005. At various time intervals, proteolysis was stopped by a 10-fold excess of soybean trypsin inhibitor. The samples were diluted into Laemmli sample buffer containing 5% mercaptoethanol and were then heated to 80°C for 10 min. Aliquots of 150 µg SR protein per well were loaded onto a 5-15% gradient gel with 4% stacking gel. Lane 0: native SR; lane 1: soybean trypsin inhibitor added before trypsin; lane 4: molecular weight standards - a2 macroglobulin: reduced 170 kDa, β-galactosidase: 116.4 kDa, fructose-6-phosphate kinase: 85.2 kDa, glutamate dehydrogenase: 55.6 kDa, aldolase: 39.2 kDa, triosephosphate isomerase: 26.6 kDa, trypsin inhibitor: 20.1 kDa, lysozyme: 14.3 kDa; time intervals of proteolysis of lane 2: 30 sec; lane 3: 1 min; lane 5: 6 min; lane 6: 12 min; lane 7: 18 min; lane 8: 24 min; lane 9: 30 min.
Figure 9. Time dependent degradation of SR modified by sequence-grade trypsin from Boehringer. The experimental procedure was identical to the one described in the caption of the preceding figure 8. Lane 0: native SR; lane 1: soybean trypsin inhibitor added before trypsin; lane 4: molecular weight standards - α2 macroglobulin: reduced 170 kDa, β-galactosidase: 116.4 kDa, fructose-6-phosphate kinase: 85.2 kDa, glutamate dehydrogenase: 55.6 kDa, aldolase: 39.2 kDa, triosephosphate isomerase: 26.6 kDa, trypsin inhibitor: 20.1 kDa, lysozyme: 14.3 kDa; time intervals of proteolysis of lane 2: 30 sec; lane 3: 1 min; lane 5: 6 min; lane 6: 12 min; lane 7: 18 min; lane 8: 24 min; lane 9: 30 min.
0.005 (mg/mg) for the indicated time intervals. The ability of trypsin inhibitor to inhibit proteolysis was determined by comparing a sample without trypsin and trypsin inhibitor (lane 0) with a control to which trypsin inhibitor was added before trypsin (lane 1). Lane 4 shows molecular weight standards. Other lanes demonstrate degradation of the SR proteins for 30 sec (lane 2), 1 min (lane 3), 6 min (lane 5), 12 min (lane 6), 18 min (lane 7), 24 min (lane 8), and 30 min (lane 9).

Calsequestrin is not affected by trypsin. The ATPase is cleaved into fragments A and B of molecular weight of 57 kDa and 52 kDa, respectively. At longer time intervals, fragment A is further cleaved into 34 kDa and 23 kDa fragments referred to as A1 and A2. Complete degradation of the ATPase into A and B already appeared after 30 sec of proteolysis by Sigma-trypsin, while Boehringer-trypsin requires 6 min of exposure to cause comparable degradation of the Ca$^{2+}$-ATPase. Cleavage of the A-fragment into A1 and A2 occurs on a similar time scale for both types of trypsin.

A high molecular weight protein which is presumably the 400 kDa junctional foot protein could be visualized on the Coomassie-blue stained gels. It is very sensitive to proteolysis. Immunoblot with a 400 kDa monoclonal antibody clearly showed that even with the less active Boehringer-trypsin, the 400 kDa protein disappeared following 30 sec of proteolysis (data not shown).

The difference between the two types of trypsin is more
than just a kinetic difference. The distinct sensitivity of the two kinds of trypsin is not simply caused by the Sigma trypsin having a higher activity for cleavage of lysine and arginine residues. Careful examination of the gels (Figure 8 and 9) show the appearance of bands on one gel that are not evident on the other gel (i.e. treatment with the two types of trypsin yield slightly different digestion products).

PROTEOLYSIS BY CHYMOTRYPSIN, KALLIKREIN, AND THROMBIN

For comparison purposes, the degradation by the proteases thrombin, chymotrypsin, and kallikrein have been studied. Thrombin did not cleave any SR protein (data not shown), while chymotrypsin and kallikrein did cleave SR proteins. Chymotrypsin was inhibited by 1 mM phenylmethylsulfonyl fluoride (PMSF) and/or aprotinin equimolar to chymotrypsin. Also, neither 100 µM leupeptin nor a ten-fold excess of soybean trypsin inhibitor was an effective inhibitor for kallikrein in the presence of SDS sample buffer.

For example, Figure 10 shows SR subjected to kallikrein treatment at a ratio of 0.005 mg kallikrein to 1 mg SR protein for increasing times of digestion. Comparison of the sample not treated with kallikrein (lane 0) and the control in which soybean trypsin inhibitor was added before kallikrein addition revealed that protease activity was not completely inhibited.

Kallikrein did not cleave the ATPase, but the high molecular weight protein was degraded. Bands of slightly
Figure 10. Time dependent degradation of SR by kallikrein. SR vesicles were exposed to kallikrein at a protease to SR protein ratio of 0.006. At various time intervals, a 10-fold excess of soybean trypsin inhibitor was added. Samples were diluted into Laemmli sample buffer without reducing agent. Aliquots of 150 µg of SR protein were loaded onto a 5-15% gradient gel with 4% stacking gel. Lane 0: native SR; lane 1: trypsin inhibitor added prior to kallikrein; lane 3: molecular weight standards – α2 macroglobulin: non-reduced 340 kDa, reduced 170 kDa, β-galactosidase: 116.4 kDa, fructose-6-phosphate kinase: 85.2 kDa, glutamate dehydrogenase: 55.6 kDa, aldolase: 39.2 kDa, triosephosphate isomerase: 26.6 kDa, trypsin inhibitor: 20.1 kDa, lysozyme: 14.3 kDa; time periods of proteolysis of lane 2: 30 sec; lane 4: 1 min, lane 5: 6 min; lane 6: 10 min; lane 7: 18 min; lane 8: 24 min.
lower molecular weight than the 400 kDa appeared as a function of time of proteolysis. These bands are likely to be fragments of the 400 kDa protein.

Chymotrypsin also cleaved the high molecular weight protein (data not shown) and degradated the ATPase. Lack of inhibition, even with PMSF, made it difficult to extract useful information from these experiments.

DISCUSSION

The high molecular weight protein is readily degraded by both types of trypsin, by chymotrypsin, and by kallikrein while only the type III trypsin from Sigma causes an enhancement of Ca\(^{2+}\) release. Thus, the stimulation of Ca\(^{2+}\) release is not likely to be caused by cleavage of the high molecular weight protein unless enhancement of Ca\(^{2+}\) release is due to later cleavage of the high molecular weight protein by the type III trypsin from Sigma.

The 106 kDa protein cannot be visualized on Coomassie-blue stained gels. No information about its degradation could be obtained because immunoblot techniques failed. Artifacts such as appearance of additional bands, too much background, and too little sensitivity of the primary antibody prevented the detection of this protein.

Nevertheless, significant differences in the degradation pattern caused by Sigma- and Boehringer-trypsin was demonstrated. Also, a number of proteases were shown to cleva
the high molecular weight protein without affecting the Ca\textsuperscript{2+} release mechanism. Perhaps the most significant observation is that Boehringer-trypsin causes a similar digestion pattern to the pattern generated by trypsin from Sigma. Yet, these two proteolytic enzymes have a very different effect on Ca\textsuperscript{2+} release. It is, therefore, likely that the difference in proteolysis caused by these two types of trypsin is subtle. The release protein is probably a minor protein component, such as the 106 kDa protein.
CHAPTER V

CONCLUSIONS

The Ca\(^{2+}\) release protein from SR is a key protein involved in E-C coupling. It receives a message to release the Ca\(^{2+}\) stored in the SR when the t-tubule is depolarized. The increase in myoplasmic Ca\(^{2+}\) concentration that results causes muscle contraction.

The identity of the release protein is still uncertain. Two proteins have been proposed to form the Ca\(^{2+}\) release channel from SR. On the basis of \(^{3}\)H-ryanodine binding studies, the high molecular weight 400 kDa junctional foot protein (JFP) has been purified and reconstituted into a lipid bilayer membrane. SH reactivity has been used to isolate the 106 kDa SH activated Ca\(^{2+}\) channel protein. The purpose of this thesis was to utilize proteolytic enzymes in order to help identify which of these two proteins is the Ca\(^{2+}\) release protein from SR.

In Chapter III it is demonstrated that type III trypsin from Sigma stimulates Ca\(^{2+}\) release from SR vesicles by approximately six-fold. Optimal stimulation occurred at a trypsin to SR protein ratio of 1:200 following exposure to trypsin for 18 min (Figure 4, Chapter III). The Ca\(^{2+}\) release
rate increased to a maximum at 18 min of proteolysis and declined when exposed to trypsin for longer time intervals. Since trypsin is known to cleave polypeptides at arginine and lysine residues, the first approach was to determine which of these two cleavage sites was responsible for stimulation of $\text{Ca}^{2+}$ release from SR. Thrombin is specific for arginine residues. However, it had no effect on modifying the $\text{Ca}^{2+}$ release rate (Figure 5, Chapter III).

After completing studies with sequence-grade trypsin from Boehringer it became clear that stimulation of $\text{Ca}^{2+}$ release is apparently caused by a proteolytic contaminant in the type III trypsin from Sigma. Experiments carried out with the sequence-grade Boehringer-trypsin didn't show enhancement of $\text{Ca}^{2+}$ release rate (Figure 4, Chapter III). The vendor (Sigma) suggested kallikrein or chymotrypsin as possible contaminants in this preparation. These two proteases were tested at protease to SR protein ratio varying from 1:100,000 to 1:2,000. This corresponds to a contamination of 0.2-10% in the trypsin used to modify the release characteristics of the SR (Figure 6, Chapter III). Also chymotrypsin (chymotrypsin:SR protein=1:200) was examined for short time periods of proteolysis (Figure 7, Chapter III). These tests demonstrate that at comparable or higher concentrations kallikrein and chymotrypsin there was no effect on the $\text{Ca}^{2+}$ release mechanism of SR. I was not able to determine which proteolytic contaminant in type III trypsin from Sigma was responsible for
stimulation of Ca$^{2+}$ release.

SDS-PAGE (Chapter IV) was used in an attempt to determine whether stimulation of Ca$^{2+}$ release by proteolysis correlated with the cleavage of either the 106 kDa protein or the JFP. The high molecular weight protein is easily visualized by SDS-PAGE with either Coomassie blue staining or Western blot transfers using a monoclonal antibody. Since the 106 kDa protein cannot be resolved in the presence of the Ca$^{2+}$-ATPase on Coomassie blue stained gels, polyclonal antibody techniques were used to monitor the state of the 106 kDa protein.

The high molecular weight protein is very sensitive to proteolysis. All of the proteases tested, except for thrombin (data not shown) cleaved the JFP within 30 sec. Clearly this initial cleavage of the 400 kDa protein does not correlate with the stimulation of Ca$^{2+}$ release from SR vesicles by proteolysis. It is also very difficult using Coomassie blue staining to detect a difference in the digestion pattern of a particular protein component that correlates with the enhancement of Ca$^{2+}$ release from SR (Figure 8 and 9, Chapter IV).

I had hoped to be able to follow cleavage of the high molecular weight protein by using a monoclonal antibody to the 400 kDa protein. However, no protein was visible following the initial cleavage of the 400 kDa protein. It appears as if the antigenic binding site for the monoclonal antibody was destroyed by mild proteolysis.
An alternative approach is to follow cleavage of the high molecular weight protein by detection with a polyclonal antibody to this protein. Unfortunately, the polyclonal antibody provided by Dr. G. Meissner (University of North Carolina) also stains the 106 kDa protein. It is possible that this polyclonal antibody to the JFP can still be used effectively to follow fragments of the JFP and the 106 kDa proteins. Since the polyclonal antibody to the JFP stains the 106 kDa protein more weakly than the JFP, a reduction in the amount of protein used per transfer may help in detecting only fragments of the high molecular weight protein. Comparisons between transfers carried out with high protein concentrations, which should label both the JFP and the 106 kDa protein fragments, and transfers using low amounts of protein may help determine which protein is activated by proteolytic cleavage.

Furthermore, visualization of the 106 kDa protein using a polyclonal antibody to this protein may also reveal useful information. Our polyclonal antibody to the 106 kDa protein is old. Staining and visualizing this faint band is difficult. This problem may be circumvented by using the more sensitive ECL (enhanced chemoluminiscence) technique. This method utilizes the production of light when an agent reacts with a peroxidase linked secondary antibody. After bathing the nitrocellulose in the chemoluminescent reagent, the peroxidase linked protein can be visualized using photographic methods.
Long exposure times should greatly enhance our sensitivity of detecting the 106 kDa protein and its proteolytic fragments.

The following conclusions can be made from the work described in this thesis. The stimulation of Ca^{2+} release is due to a contaminant in the type III trypsin from Sigma. The possible proteolytic contaminants kallikrein and chymotrypsin do not enhance passive Ca^{2+} release from SR. Furthermore, type III Sigma-trypsin appears to be more aggressive than sequence-grade Boehringer-trypsin. Besides the difference in activity, the molecular weight of some proteolytic fragments of SR proteins are distinct for the two different types of trypsin. Both types of trypsin as well as kallikrein and chymotrypsin cleave the high molecular weight protein within 30 sec. The initial cleavage of the JFP does not correlate with enhancement of Ca^{2+} release from SR. The arginine specific protease thrombin neither stimulates release nor cleaves any SR protein. Further studies are required to determine whether cleavage of the 106 kDa protein is responsible for stimulation of the Ca^{2+} release protein.
REFERENCES


