Modification of the Ca²⁺ Release System of Skeletal Muscle Sarcoplasmic Reticulum Vesicles via Sulfhydryl Oxidation and Tryptic Proteolysis

Jonathan Lee Trimm
Portland State University

Let us know how access to this document benefits you.
Follow this and additional works at: https://pdxscholar.library.pdx.edu/open_access_etds

Recommended Citation

10.15760/etd.1347

This Dissertation is brought to you for free and open access. It has been accepted for inclusion in Dissertations and Theses by an authorized administrator of PDXScholar. For more information, please contact pdxscholar@pdx.edu.
MODIFICATION OF THE Ca^{2+} RELEASE SYSTEM OF SKELETAL MUSCLE SARCOPLASMIC RETICULUM VESICLES VIA SULPHHYDRYL OXIDATION AND TRYPIC PROTEOLYSIS.

by

JONATHAN L. TRIMM

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
in
ENVIRONMENTAL SCIENCES and RESOURCES / PHYSICS

Portland State University

©1988
TO THE OFFICE OF GRADUATE STUDIES:

The members of the Committee approve the dissertation of Jonathan L. Trimm presented March 11, 1988.

Jonathan J. Abramson, Chairman

Pavel K. Smejtek

Arnold D. Pickar

Mary L. Taylor

John H. Golbeck

Gary R. Brodowicz, Graduate Council Representative

APPROVED:

Pavel K. Smejtek, Director, Environmental Sciences and Resources Program

Bernard Ross, Vice Provost for Graduate Studies
AN ABSTRACT OF THE DISSERTATION OF Jonathan L. Trimm for the degree of Doctor of Philosophy in Environmental Sciences and Resources / Physics, presented March 11, 1988.

Title: Modification of the Ca²⁺ Release System of Skeletal Muscle Sarcoplasmic Reticulum Vesicles via Sulfhydryl Oxidation and Tryptic Proteolysis.

APPROVED BY MEMBERS OF THE DISSERTATION COMMITTEE:

Jonathan J. Abramson, Chairman

Pavel K. Smejtek

Arnold D. Pickar

Mary L. Taylor

John H. Golbeck

Gary R. Brodowicz
The sarcoplasmic reticulum (SR) is an intracellular membrane system found in muscle cells which regulates the concentration of Ca\(^{2+}\) ions in muscle cytoplasm. ATP-dependent Ca\(^{2+}\) pumps actively transport Ca\(^{2+}\) from the cytoplasm across the membrane, where it is stored within the lumen of the SR. When the cell is in the relaxed state, the cytoplasmic Ca\(^{2+}\) concentration is \(10^{-7}\) M. In response to excitation of the cell at the neuromuscular junction, the normally polarized membrane of the cell depolarizes. Depolarization of the surface membrane and the associated transverse tubular system signals the SR, via a poorly understood mechanism, to release its stored Ca\(^{2+}\). The released Ca\(^{2+}\) activates the contractile elements of the cell.

Isotopic and spectrophotometric assays show that micromolar concentrations of heavy metal ions (particularly Ag\(^{+}\) and Hg\(^{2+}\)) stimulate ATPase activity but inhibit Ca\(^{2+}\) uptake in isolated SR vesicles. Both effects are caused by increased Ca\(^{2+}\) permeability of the membrane, apparently the result of activation of the Ca\(^{2+}\) release system of the vesicles due to heavy metal binding to a critical sulfhydryl group associated with the Ca\(^{2+}\) release channels. Cu\(^{2+}\)-catalyzed co-oxidation of this sulfhydryl with exogenous cysteine to form a mixed disulfide also results in activation of the Ca\(^{2+}\) release system. The rate and extent of Cu\(^{2+}\)/cysteine-induced release is maximal at physiological
pH and is inhibited by local anaesthetics and Mg\textsuperscript{2+}, suggesting that the redox state of this sulfhydryl may play a role in activation of the Ca\textsuperscript{2+} release system of intact muscle.

Modification of the SR with the proteolytic enzyme trypsin also increases the Ca\textsuperscript{2+} permeability of the SR, and enhances the rate of Ca\textsuperscript{2+} release activated by cAMP, doxorubicin, Hg\textsuperscript{2+}, and Cu\textsuperscript{2+}/cysteine. The rates of release activated by all reagents are stimulated by a factor of five after five minutes exposure to trypsin. Hg\textsuperscript{2+} and Cu\textsuperscript{2+}/cysteine-activated release are not stimulated further, while cAMP- and doxorubicin-activated release continue to increase up to maximum of 20-fold stimulation after 15 minutes exposure to trypsin. Inhibitors of the Ca\textsuperscript{2+} release system such as Mg\textsuperscript{2+} and ruthenium red still inhibit release from proteolytically modified SR, and the binding affinities of activators and inhibitors to their sites are not significantly altered by proteolysis; only the rates of Ca\textsuperscript{2+} transport are affected. The most probable mechanisms of tryptic stimulation of Ca\textsuperscript{2+} release are (1) removal of a regulatory protein or subunit of the Ca\textsuperscript{2+} release system, making more channels available for transport; (2) increasing the single channel unitary conductance; (3) increasing the open time of activated channels. The biphasic character of proteolytic stimulation of cAMP- and doxorubicin-activated release (as opposed to monophasic stimulation of Hg\textsuperscript{2+} and
Cu²⁺/cysteine-activated release) suggests that more than one of the above parameters are involved in tryptic stimulation of the Ca²⁺ release system.
ACKNOWLEDGEMENTS

To the Faculty, Staff, and Students of Portland State University, especially the Physics Department and Environmental Sciences Program: thank you for several fine and enjoyable years. Individuals who deserve extra mention are my advisor, Dr. Jon Abramson, who is a good scientist, a good teacher, and a good friend, and Mrs. Dawn Dressler, who gave me a job when I needed one. The members of my thesis committee offered often challenging and usually useful comments on my progress: Drs. Pavel Smejtek and Arnold Pickar from Physics; Dr. Mary Taylor from Biology; Dr. John Golbeck from Chemistry; and Dr. Gary Brodowicz from the Department of Health and Physical Education. Dr. Joann Loehr of the Oregon Graduate Center helped me a great deal during my first few years in research, while Dr. Guy Salama of the University of Pittsburgh has contributed much in recent years. Fellow students who helped both materially and by their presence and friendship were Lyle Weden, Rich Findlay, Jan Stuart, Desmond Crooks, Hui Xiong, Ed Buck (whose computer did the final printing of this thesis), Dave English, Sanjay Khandekar, and Benito U. Marsan. Thanks also to Joe Cronin, (more than) a talented research technician, and Garo Arakelian, Rudi Zupan, Lee Thannum, and Brian McLoughlin of the Science Support Shop. The Oregon and Alaska affiliates of the American Heart Association, the Medical Research Foundation of Oregon, and the Dant Foundation awarded grants to Dr. Abramson which funded my research. Finally, I'd like to thank my friends and family both at PSU and beyond for providing the other half of the life experience as well as occasional assistance with my work. Garry Whyte helped with, among other things, the long term loan of a computer, while Glenn Lebrasseur helped with timely repairs of said computer. To my good friends Jim, Heather, Cheryl, Stuart, Peter, Molly, Bill, Denny, and Kenny, and all other companions in adventure, large and small: thanks for the memories and helping to keep the spark alive. This work is as much about perseverance as it is about muscle, and for teaching me that lesson, I must thank my family, especially my parents, to whom this is dedicated.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Approval Page</th>
<th>ii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
</tbody>
</table>

## CHAPTER

### I OVERVIEW OF SARCOPLASMIC RETICULUM

- Introduction ........................................ 1
- Brief History and Summary .......................... 3
- Roles of Electricity and the Sarcolemma ......... 11
- The Contraction and Relaxation Factors ......... 13
- The Role of the T-Tubules ......................... 17
- Contemporary Excitation-Contraction Coupling .... 20
- Modulators of Ca²⁺ Permeability of the SR ........ 27
- Overview of Dissertation .......................... 32
- Relevance of Dissertation ........................ 38

### II Heavy Metals Cause Rapid Ca²⁺ Release From Isolated Sarcoplasmic Reticulum Vesicles

- Summary ........................................... 42
- Introduction ...................................... 42
- Materials and Methods ............................ 43
<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>II (CONTINUED)</td>
<td></td>
</tr>
<tr>
<td>RESULTS</td>
<td>49</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>60</td>
</tr>
<tr>
<td>III SULFHYDRYL OXIDATION INDUCES RAPID Ca(^{2+}) RELEASE FROM ISOLATED SARCOPLASMIC RETICULUM VESICLES</td>
<td>65</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>65</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>66</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>70</td>
</tr>
<tr>
<td>RESULTS</td>
<td>80</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>100</td>
</tr>
<tr>
<td>IV TRYPDIC PROTEOLYSIS STIMULATES ACTIVATION OF Ca(^{2+}) RELEASE FROM ISOLATED SARCOPLASMIC RETICULUM VESICLES</td>
<td>104</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>104</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>106</td>
</tr>
<tr>
<td>RESULTS</td>
<td>112</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>138</td>
</tr>
<tr>
<td>V ANALYSIS OF PROTEINS MODIFIED BY TRYPsin AND SULFHYDRYL OXIDATION</td>
<td>144</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>144</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>144</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>146</td>
</tr>
<tr>
<td>RESULTS</td>
<td>155</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>162</td>
</tr>
<tr>
<td>VI CONCLUDING REMARKS</td>
<td>166</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>178</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Concentration of reagent which causes 50% release in 30 seconds</td>
<td>56</td>
</tr>
<tr>
<td>II. Oxidation of mercaptans in the presence of Cu^{2+}</td>
<td>87</td>
</tr>
<tr>
<td>III. Ca^{2+} release activity of mercaptans and disulfide compounds</td>
<td>88</td>
</tr>
<tr>
<td>IV. Sulfhydryl oxidants induce Ca^{2+} release</td>
<td>91</td>
</tr>
<tr>
<td>V. Summary of Ca^{2+} release response of native and TMSR vesicles to cAMP</td>
<td>114</td>
</tr>
<tr>
<td>VI. Summary of kinetic analysis of cAMP- and doxorubicin-activated Ca^{2+} release from native and TMSR</td>
<td>133</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

FIGURE PAGE
1. Morphology of a muscle cell . . . . 5
2. Schematic diagram of a muscle cell . . . . 6
3. Calcium uptake and ATPase activity as a function of Hg2+ concentration . . . . 50
4. Ca2+ release induced by Hg2+ . . . . 53
5. Calcium release induced by air oxidation catalyzed with cupric phenanthroline . . . . 59
6. ATP-driven Ca2+ uptake and Cu2+/cysteine-induced Ca2+ release from SR vesicles . . . . 81
7. Dependence of Ca2+ release rate on concentrations of cysteine, cysteamine, and homocysteine . . . . 83
8. Dependence of Ca2+ release rate on Cu2+ concentration . . . . 86
9. Reversal of Cu2+/cysteine-induced Ca2+ release with dithiothreitol . . . . 92
10. Dependence of Cu2+/cysteine-induced Ca2+ release rate on total Mg2+ concentration . . . . 95
11. Rate of Cu2+/cysteine-induced Ca2+ release as a function of pH . . . . 97
12. Rate of Cu2+/cysteine-induced Ca2+ release as a function of ionic strength . . . . 98
13. Ca2+ release from passively loaded native and tryptically modified SR vesicles . . . . 113
14. Ca2+ release rate in the absence and presence of cAMP as a function of time of proteolysis . 116
FIGURE

15. Effective rate constant of Ca\(^{2+}\) release from HSR and LSR in the presence and absence of ruthenium red, as a function of time of proteolysis . . . . . . 118

16. Rate constant of Ca\(^{2+}\) release from native and TMSR vesicles as a function of external Ca\(^{2+}\) concentration. . . . . . . . . . 122

17. Rate of Ca\(^{2+}\) release from native and TMSR vesicles as a function of Mg\(^{2+}\) . . . . 125

18. Rate of Ca\(^{2+}\) release from native and TMSR as a function of ruthenium red concentration . 127

19. Rate of Ca\(^{2+}\) release from native and TMSR as a function of cAMP concentration . 129

20. Rate of Ca\(^{2+}\) release from native and TMSR as a function of doxorubicin concentration . 131

21. Rate of Ca\(^{2+}\) release from native and TMSR as a function of Hg\(^{2+}\) concentration . 134

22. Parallel time course of tryptic stimulation of cAMP- and Hg\(^{2+}\)-induced Ca\(^{2+}\) release . . 136

23. Autoradiograph of \(^{35}\)S-cysteine-labeled SR in a 7% SDS polyacrylamide gel . . . . 156

24. Log (molecular weight) vs mobility in gel for proteins in Figure 23 . . . . . 157

25. Tryptically modified SR proteins in an 8% SDS polyacrylamide gel . . . . . 158

26. Log (molecular weight) vs mobility for proteins in Figure 25 . . . . . 159
CHAPTER I

OVERVIEW OF SARCOPLASMIC RETICULUM

INTRODUCTION

The defining characteristic of the members of the animal kingdom is their ability to move in and manipulate their environment. The anatomical structures which give animals these powers are muscles. Muscle tissue comes in three basic types: smooth muscle, which is found in stomach, arteries, etc.; heart or cardiac muscle; and skeletal muscle, which is characterized as fast or slow depending on its mitochondrial content. Skeletal and cardiac muscle are also called striated muscle because, under a light microscope, the muscle fibers appear transversely striped with light and dark bands. The great variety of animal species, from insects to reptiles to birds to fishes and humans, has led to the development of a large variety of each muscle type. Some highly developed muscle varieties are found in only a few species; the human vocal chord muscles are an example of such specialization. The most obvious muscle variety is skeletal muscle, and from a human perspective the most interesting class of skeletal muscle is vertebrate skeletal muscle. Vertebrate skeletal muscle is
muscle but important differences often exist, for example in
the exact structure of the connections between the
intracellular membrane systems.

All cells are defined physically by cell membranes;
within a cell there are intracellular membrane systems which
carry out specific functions for that cell. Nerve cells
carry signals from the brain to the muscle cell. Neural
signals pass from the nerve cell to the muscle cell's
surface membrane, the sarcolemma. The sarcolemma
communicates with an intracellular membrane system, the
sarcoplasmic reticulum (SR), which in turn controls the
contractile state of the cell by regulating the $\text{Ca}^{2+}$
concentration in the cell's interior fluid, the sarcoplasm.
The communication link between the sarcolemma and the
sarcoplasmic reticulum is the general area of focus of this
thesis.

The purpose of this chapter is to summarize the current
views of neural control of skeletal muscle activity and to
provide a brief historical overview of the development of
this field. Three main themes will be developed: 1) the
role of $\text{Ca}^{2+}$ in the contraction of muscle; 2) the role of
the SR in regulating the intracellular (sarcoplasmic) $\text{Ca}^{2+}$
concentration; and 3) the mode of communication between the
sarcolemma and the SR. The reader is referred to general
texts and review articles for discussion of tangentially
related topics. For work which is closely or directly
related to this thesis, original sources are cited. I have tried to select only work which clearly illustrates the most important points, and do not claim to present a complete survey of the field.

CLASSICAL EXCITATION-CONTRACTION COUPLING I: BRIEF HISTORY AND SUMMARY

In 1791, the Italian anatomist Luigi Galvani of the University of Bologna performed an experiment which has since been refined and repeated many times, and still yields new information (for discussion of the original experiment see ref. 1). Galvani placed the severed leg of a frog on brass hooks outside his laboratory window during a thunderstorm. The frog leg twitched as he had expected, but continued to twitch after the storm abated. The muscle was lying in such a way as to make contact with the brass hooks and an iron lattice, and it was soon found that muscle would twitch anytime it was in contact with two dissimilar metals. Galvani was convinced that the muscle created "animal electricity" which flowed between the metals, but in 1794 Volta showed that electric charge flows between two dissimilar metals without the aid of muscle tissue. Volta argued just the opposite: electric current flowed through the frog leg from one metal to the other, and in so doing caused the muscle to twitch. From a contemporary perspective, Volta's explanation appears to be closer to the
truth, but Galvani was right in his belief that muscles generate electric phenomena in their operation. A more detailed, modern summary of Galvani's experiment is given below, following a current description of muscle morphology (see ref. 2 for more details).

Muscle Morphology

As mentioned above, striated muscle is so called because under a light microscope the muscle cells or fibers appear to be striped with light and dark bands. The structure of a striated muscle fiber, summarized by Peachey in 1965 (3), is shown in Figures 1 and 2. The contractile unit of a striated muscle fiber is a cylindrical bundle of contractile filaments known as the sarcomere, which is typically 1 μm in diameter and 2 μm in length. A collection of sarcomeres joined end-to-end forms a myofibril, which may or may not run the length of the fiber, and a collection of myofibrils in parallel forms a muscle fiber. Under a light microscope, the most distinct dark bands are formed where two sarcomeres join together end-to-end; these are called the Z-bands. The fiber is encased in a membrane called the sarcolemma. The myofibrils tend to be grouped in such a way that the Z-bands of parallel myofibrils are in alignment; at points on the sarcolemma corresponding to the Z-bands there are tubular invaginations of the sarcolemma which penetrate into the interior of the fiber. These are called transverse tubules or T-tubules. The T-tubules form a network of
Figure 1. Morphology of a muscle cell (adapted from Peachey, 3). Several sarcomeres are shown in their relation to each other and to the SR and T-tubules. Region 1 is a triadic junction, consisting of terminal cisterna, T-tubule, and terminal cisterna of two contiguous sarcomeres. Region 2 is longitudinal reticulum, region 3 is intermediate reticulum, region 4 is a terminal cisterna, and region 5 is a T-tubule.
Figure 2. Schematic diagram of a muscle cell.
tubular membranes, the branches of which completely encircle the myofibrils at the Z-bands. Each sarcomere is thus bounded by T-tubules at each end. Surrounding each sarcomere, also bounded by T-tubules at each end, is the closed membrane system known as sarcoplasmic reticulum (sarcoplasm is the intracellular fluid of a muscle cell; a reticulum denotes a network forming a closed system). Each sarcoplasmic reticulum is divided into morphologically distinct regions: at each end, in contact with the T-tubules, are the terminal cisternae; in between are the longitudinal reticulum and the intermediate reticulum. Peachey (3) calculated that each of these domains of the SR has a volume of 4 to 5% of the total fiber volume and surface areas of 40 to 50 times that of the sarcolemma. The transverse tubular system has a volume of ~0.3% of the volume of the cell and surface area ~7 times that of the sarcolemma of a fiber 100 μm in diameter. The pattern of SR-transverse tubule-SR found where two different sarcoplasmic reticulum systems from adjacent sarcomeres make contact with a T-tubule at a Z-band is called a triad, and will be discussed in more detail below.

The contractile filaments of the sarcomere are of two complementary types known as actin and myosin, packed in a double hexagonal array (for review see refs 2, 3, 4, and 5). The Z-bands are formed by knitting together one end each of two groups of actin filaments; the free ends extend into the
middles of the two adjoining sarcomeres. Myosin filaments are interdigitated among the free ends of the actin filaments which extend into the middle of the sarcomere from each end. The myosin filaments are suspended by their "heads" which form cross bridges with actin filaments. The heads of myosin filaments can hydrolyze the high energy bond of the terminal phosphate group of ATP and use the liberated energy to slide along the actin filaments with a ratchet-like mechanism (see figure 2). In resting muscle, contraction is prevented by troponin, which is found at intervals along the actin filaments. When the muscle is activated by any process which causes the sarcoplasmic reticulum to release its Ca$^{2+}$, the released Ca$^{2+}$ binds to troponin. In contrast to free troponin, the Ca$^{2+}$-troponin complex does not inhibit muscle contraction. When the Ca$^{2+}$ concentration of the sarcoplasm is ~5 μM, the troponin molecules form a complex with the Ca$^{2+}$ and the myosin heads use the chemical energy of ATP to move along the actin filaments. The ATP-driven movement of the myosin filaments along the actin filaments pulls the ends of the sarcomere together, producing a shortening of the sarcomere. The microscopic shortening of many sarcomeres in series produces a macroscopic shortening of the myofibrils, and the combined shortening of many myofibrils in parallel gives rise to the macroscopic forces generated by muscle fibers.
**Excitation-contraction coupling**

The sarcolemma of a muscle fiber at rest is electrically polarized (~88 mV, inside negative) by the action of ionic pumps and channels in the membrane. In Galvani's experiment, the electric (Galvanic) current passing through the frog leg produced local depolarizations of the sarcolemmata of some of the muscle fibers. Voltage-gated Na⁺ channels in the sarcolemmata opened in response to the local depolarizations and waves of depolarization swept over the sarcolemmata and down the transverse tubules of the affected fibers. Depolarization of the transverse tubules (T-tubules) of the muscle cells signaled the sarcoplasmic reticulatae of the fibers to release their stored Ca²⁺ into the sarcoplasm. Ca²⁺ in the sarcoplasm bound to troponin and relieved troponin's inhibitory effect on the actin and myosin filaments, and the stored chemical energy of ATP was converted to mechanical work as the affected muscle fibers contracted. Of course, in an intact frog, depolarization of the sarcolemmata would arise from signals which originate in the brain and reach the muscles via nerve cells. Activated nerve cells release the neurotransmitter acetyl choline at the neuromuscular junction. Acetyl choline-activated Na⁺ channels in the sarcolemma open in response to the acetyl choline and the sarcolemma undergoes a local depolarization at the neuromuscular junction. Voltage-activated Na⁺ channels propagate the wave of depolarization, the
The sarcoplasmic reticulum releases its Ca$^{2+}$, and the muscle contracts (see refs. 2, 3, 4, 5, and 6).

The Sarcoplasmic Reticulum

The term "sarcoplasmic reticulum" dates back at least to the time of Emilio Veratti, another Italian anatomist. In 1902 Veratti published a paper, the English translation of which reads, "Investigations on the Fine Structure of Striated Muscle Fiber" (7). In that paper, Veratti described various methods of staining muscle fibers and showed hand drawn details of muscle fiber structure visualized under the light microscope. One of the features he described was a "reticulum in the sarcoplasm" which was similar in structure to the endoplasmic reticulum. Unfortunately, Veratti's elegant structural descriptions were forgotten even as the revolutions in physics and chemistry, which began in the early 1900s, led to new discoveries of biochemical and biophysical aspects of muscle function. By the 1940s, according to Porter (8), reviews of muscle structure and function neglected to mention the "reticulum in the sarcoplasm" altogether. A history of its rediscovery in the context of the development of modern views of muscle physiology follows.
CLASSICAL EXCITATION-CONTRACTION COUPLING II:
THE ROLES OF ELECTRICITY AND THE SARCOLEMMA

In contrast to the lack of attention which Veratti's structural work received, Galvani's experiment, perhaps due to the controversy it generated and the fact that it sparked development of whole new fields of study (especially electrodynamics), created much sustained interest in the function of electricity in muscle physiology. The small dimensions of muscle fibers made electrical measurements difficult to obtain, but throughout the 1800s and early 1900s, in parallel with new developments in physics and chemistry, experimenters continued to refine techniques both of electrically stimulating muscle contraction and of isolating and handling muscle fibers.

In 1946, using glass microelectrodes to impale isolated muscle fibers, Graham and Gerard measured the normal resting potential across the sarcolemma and found it to be \(-60 \pm 20\) mV, inside negative (9). Using a similar technique, Kuffler (10) measured potential changes upon application of acetylcholine. Nastuk and Hodgkin, in 1950, used a refined version of Graham and Gerard's technique and found that the resting potential across the sarcolemma is \(88 \pm mV\), inside negative (11). All groups of investigators found that electrical stimulation of these fibers produced transient depolarizations of the fibers which went as far as 40 mV, inside positive, for a total change in potential of about
130 mV. This abrupt transient change in membrane potential across the sarcolemma after electrical stimulation is called an action potential, after a similar phenomenon found earlier in the giant nerve axon of the squid (12, 13). The action potentials measured by all three groups were always accompanied by contraction of the fibers. Later work showed that the action potential is also associated with movement of Na⁺ and K⁺ ions across the sarcolemma (14). It had been known for a long time that a sudden increase in the K⁺ concentration of the bathing solution of muscle tissue would cause contraction of the muscle; Hodgkin and Horowicz showed that sudden increase in external K⁺ causes an action potential across the sarcolemmæ of isolated fibers (15, 16). The resting potential across the sarcolemma is the result of unequal concentrations of Na⁺ and K⁺ across the membrane, generated by a Na⁺/K⁺ exchange protein. The action potential is primarily the result of Na⁺ channels opening in the sarcolemma in response to membrane depolarization, which is initiated by acetyl-choline gated Na⁺ channels. (More extensive reviews can be found in references 2, 4, 5, and 17.)

Following the work of Gerard and Graham and that of Kuffler, the coupling of depolarization of the sarcolemma to muscular contraction was undoubtedly a popular topic of speculation. One popular theory appears to have been that of a "contraction factor", which was assumed to be released
into the sarcoplasm by the sarcolemma as a result of depolarization. Upon release into the sarcoplasm by the sarcolemma, the contraction factor was assumed to diffuse into the sarcoplasm and activate the contractile machinery. In 1948 however, Hill (18) pointed out a serious weakness in this theory. Hill's calculations showed that a small molecule released from the sarcolemma, for example acetyl choline or Ca$^{2+}$ (Hill's guess!) could not diffuse into the center of a muscle fiber in the time it takes for depolarization of the sarcolemma to induce the transient contraction of a single twitch. In 1949, Hill showed that the tension generated by a single twitch of a stretched fiber is equal to that of a sustained tetanus, indicating that all of the contractile machinery may operate in a single twitch (19). Therefore, the contractile signal must propagate into the interior of the fiber via some process faster than simple diffusion of a contraction factor from the sarcolemma into the fiber interior. This conclusion was confirmed some years later by the rediscovery of the SR and T-tubules and elucidation of their physiological roles.

CLASSICAL EXCITATION-CONTRACTION COUPLING III: 
THE "CONTRACTION FACTOR" AND THE "RELAXATION FACTOR"

In 1951, Marsh (20) described the preparation of a "water soluble and non-dialysable [hence large on a molecular scale] substance", present in a suspension of
muscle cell homogenate, which would cause relaxation of isolated muscle fibers in the presence of ATP. In 1953, Bendall showed that Mg\(^{2+}\) as well as ATP is required to achieve the relaxing effect (21). In 1954, Bozler (22) reported that addition of 1 mM of the Ca\(^{2+}\) chelator EDTA to the bathing solution of an isolated muscle fiber mimics the effect of the relaxing factor while addition of 2 mM Ca\(^{2+}\) reverses the relaxing effect of EDTA; he concluded that the muscle fibers relax when the relaxing factor "inactivates bound Ca\(^{2+}\)" on the contraction factor. It was not then suspected that Ca\(^{2+}\) is the contraction factor, though this was soon shown to be the case.

In 1959, Weber reported some correlation between the free Ca\(^{2+}\) concentration in the bathing solution of isolated muscle fibers and contraction of the fibers (23). In a more detailed study, in 1960, Ebashi reported a strong correlation between the amount of Ca\(^{2+}\) bound to various analogues of EDTA in suspensions of muscle fibers and relaxation of the fibers (24). In that paper, Ebashi also claimed that the "particulate fraction of skeletal muscle [i.e. the nondialysable, water soluble fraction], which has relaxing activity, also strongly concentrates Ca\(^{2+}\) in an ATP-coupled reaction and thereby removes it from the medium". Ebashi proposed that relaxation of muscle in vivo is due to Ca\(^{2+}\) uptake by the particulate relaxing factor. Later, using an electron microscope, Ebashi and Lipmann
showed that the particulate fraction is vesicular in nature, probably derived from the endoplasmic reticulum (25). Weber (26) showed that the endoplasmic reticulum fragments of muscle homogenate are capable of accumulating sufficient Ca\(^{2+}\) stores, at sufficient rates, to account for regulation of the contractile state of the actomyosin complexes of the sarcomeres. In summary, the endoplasmic reticulum of muscle, now known as the sarcoplasmic reticulum, forms closed vesicles which are far too large to pass through a dialysis membrane when muscle is homogenized in aqueous solution. The SR sequesters Ca\(^{2+}\) within its lumen via an active (i.e. energy-requiring) process which, in intact muscle, leads to muscle relaxation. The process by which the SR takes up Ca\(^{2+}\) from the sarcoplasm became the next topic of inquiry.

**Mechanism of Ca\(^{2+}\) Uptake by SR**

In 1961, Ebashi proposed that the mechanism of Ca\(^{2+}\) uptake by the SR vesicles involves Ca\(^{2+}\) binding to groups which are exposed by a combined action of Mg\(^{2+}\) and ATP (27). According to Martonosi and Feretos (28), Hasselbach proposed, also in 1961, that the SR lowers the free Ca\(^{2+}\) concentration in the sarcoplasm by actively transporting Ca\(^{2+}\) into the lumen of the SR, where it is unable to interact with myosin. It was clear that the SR lowers the free Ca\(^{2+}\) concentration in the sarcoplasm and thereby causes muscle relaxation, but the mechanism by which the SR does
so was controversial: is Ca$^{2+}$ uptake due to ATP and Mg$^{2+}$-induced exposure of Ca$^{2+}$ binding sites, as Ebashi had proposed, or is Ca$^{2+}$ uptake due to Mg$^{2+}$ and ATP-catalyzed active Ca$^{2+}$ transport into the lumen of the SR, as proposed by Hasselbach? The issue was resolved in favor of Hasselbach's active transport mechanism by Martonosi and Feretos (28) in 1964. They showed that the Ca$^{2+}$ uptake capacity of the SR is dramatically increased in the presence of oxalate, a Ca$^{2+}$ precipitating anion. The reasoning is thus: one would not expect that oxalate would increase the number of available binding sites, but if Ca$^{2+}$ is concentrated in a volume containing oxalate, Ca$^{2+}$-oxalate crystals will form and precipitate, and the free Ca$^{2+}$ concentration will remain low. The Ca$^{2+}$ uptake capacity of an active transport system will be stimulated under such conditions, but since oxalate would not be expected to increase the number of Ca$^{2+}$ binding sites under Ebashi's proposal, oxalate would not increase the Ca$^{2+}$ uptake capacity of a Ca$^{2+}$ binding system. The protein component of the SR which, in the presence of ATP and Mg$^{2+}$, is responsible for transporting Ca$^{2+}$ from the sarcoplasm into the lumen of the SR is called the Ca$^{2+}$,Mg$^{2+}$-ATPase (commonly called the Ca$^{2+}$ pump), which was isolated and characterized in 1970 by MacLennan (29). The nature of the SR, and the mechanism by which the SR sequesters Ca$^{2+}$ had been
established. The next logical questions were: what are the stimulus for, and mechanism of, Ca\textsuperscript{2+} release from the SR?

**CLASSICAL EXCITATION-CONTRACTION COUPLING IV:**

**THE T-TUBULES CONDUCT THE CONTRACTION SIGNAL FROM THE SARCOLEMMA TO THE SARCOPLASMIC RETICULUM**

In the discussion above it was shown that the SR regulates the sarcoplasmic Ca\textsuperscript{2+} concentration, and Ca\textsuperscript{2+} is the final messenger which results in muscle contraction. However, the link between depolarization of the sarcolemma and Ca\textsuperscript{2+} release from the SR has not been established.

Hill's work (18, 19) had shown that the signal to release Ca\textsuperscript{2+} from the SR must be conducted into the interior of the fiber via some process other than diffusion of a messenger from the sarcolemma into the fiber interior. Rediscovery of the transverse tubular system by the electron microscopists prompted speculation that the T-tubules conduct the contraction signal from the sarcolemma into the interior of fast twitch fibers. Early investigators reported electron micrographs which appeared to show that the T-tubules are invaginations of the sarcolemma (30; see Porter, 8 or Peachey, 3 for review). This was confirmed in the mid 1960s by showing that fluorescent dyes (31) and electron-dense molecules such as ferritin and horseradish peroxidase (32), added to the external bathing solutions of isolated muscle fibers, penetrate into the lumina of the T-tubules but not
into the sarcoplasm. Hence, the lumina of the T-tubules are topologically external to the muscle fibers, and it follows that the sarcolemmal and T-tubular membranes are continuous. With proof that the T-tubules are continuous with the sarcolemma, the central issue of excitation-contraction coupling became whether muscular contraction is preceded by depolarization of the T-tubules.

Action potentials of the sarcolemma only propagate when the membrane is depolarized beyond a "threshold" value (33). In 1958, Huxley and Taylor found that subthreshold electrical stimulation of the sarcolemma at a Z-band will cause a local contraction of the fiber, confined to the region in the immediate vicinity of the Z-band which was stimulated (33). Peachey reported electron micrographs in 1965 (3) indicating that the T-tubules are distributed along the Z-bands. In 1967, Gage and Eisenberg (34) showed that osmotically shocking a muscle fiber by incubating it in a salt solution containing 400 mM glycerol for one hour and then rapidly immersing the fiber in a similar solution but without glycerol uncouples depolarization of the sarcolemma from contraction of the fiber. The T-tubules of these osmotically shocked fibers were found to be completely destroyed, yet the SR and sarcolemma were only minimally affected (35). Addition of caffeine, which was known to increase the Ca²⁺ permeability of the SR, would still induce
contraction of "detubulated" fibers (caffeine contraction; 36).

Clearly, since caffeine activates contractures of detubulated fibers, the contractile machinery of muscle fibers is not impaired by osmotic shock, nor is the function of the sarcoplasmic reticulum per se. Since osmotic destruction of the T-tubules uncouples depolarization of the sarcolemma from muscle contraction, and since direct electrical stimulation of the T-tubules of intact fibers at the triadic junction (33) leads to local contraction, it became apparent that the T-tubules conduct the contraction signal (depolarization of the sarcolemma) into the interior of the fiber, and that the mechanism of conduction is via depolarization of the T-tubules. This has been confirmed in a number of ways. For example, with a preparation of isolated triads, using ionic substitution to generate and dissipate membrane potentials, it was recently reported that depolarization of the T-tubules of isolated triads leads to Ca$^{2+}$ release from the associated SR vesicles (37).

The Terminal Cisternae are the Loci of the Ca$^{2+}$ Release Function

Given that depolarization of the T-tubules leads to Ca$^{2+}$ release from the SR, the question arose: is the Ca$^{2+}$ release function limited to the region of the SR in contact with the T-tubules, or is the whole SR involved? This question was resolved by Fairhurst and Hasselbach, who
fractionated isolated SR vesicles on a discontinuous sucrose
gradient and found that caffeine induces significant Ca\textsuperscript{2+}
release from the heaviest fraction only (38). Later work
showed that this heavy fraction is derived from the terminal
cisternae of the SR. The intermediate reticulum is lighter,
and the longitudinal reticulum is the lightest of the three
fractions (39, 40). The heavy and light SR (HSR and LSR)
fractions, as they are often called, constitute an important
diagnostic indicator of the Ca\textsuperscript{2+} release system. That is,
any method of activating Ca\textsuperscript{2+} release from HSR but not LSR
is probably activating the Ca\textsuperscript{2+} release system. Given the
complexity of the SR, this diagnostic indicator helps
greatly in distinguishing between perturbations of the Ca\textsuperscript{2+}
release system and perturbation of other functions of the
SR, particularly the Ca\textsuperscript{2+} pumping function (see Chapters II,
III, and IV).

CONTEMPORARY EXCITATION-CONTRACTION COUPLING

It has been established that 1). depolarization of the
sarcolemma initiates muscle fiber contraction; 2). the T-
tubules conduct the contractile signal (depolarization of
the sarcolemma) into the interior of the muscle fiber by
themselves depolarizing; 3). Ca\textsuperscript{2+} is the final messenger in
the chain of signals leading to muscle contraction; 4). the
sarcoplasmic reticulum regulates the sarcoplasmic Ca\textsuperscript{2+}
concentration. The link between depolarization of the T-
tubular network and activation of the Ca$^{2+}$ release system of the SR has, however, proven to be a stubborn mystery which remains to be solved.

**Approaches to Studying Activation of Ca$^{2+}$ Release from SR**

There are two basic model systems for investigating activation of the Ca$^{2+}$ release system of the SR. Muscle fibers can be isolated and their morphological, chemical, and physical properties can be probed (see refs 2-6, 16, and 17 for review), or the internal membrane systems (T-tubules, ref. 41, and SR, refs. 20-29) can be isolated and studied separately. There are advantages and disadvantages to both approaches. Using isolated muscle fibers as a model system, the investigator is sure that all of the essential molecular machinery is present. Also, the sarcolemma can be stripped away if desired (6), yielding direct access to the SR and contractile machinery, or the T-tubules can be disrupted as described above (34, 35, 36). A brief review of the previous sections reveals the importance of work with fibers in the development of the present understanding of excitation-contraction coupling. Yet, with regard to measuring ion fluxes across the SR or any of the other membrane systems, the presence of non-essential contractile machinery can only complicate collection and interpretation of data. A simpler approach is to directly measure properties of isolated SR and T-tubule vesicles. This simplifies data analysis and thereby reduces the chance of
introducing experimental artifacts. In addition, isolated SR vesicles can be further fractionated, and vesicles derived from the various domains of the SR can be separated. In this way, it was found that the terminal cisterna appears to be the locus of the Ca\(^{2+}\) release system, while longitudinal reticulum appears to be the primary locus of the Ca\(^{2+}\) uptake function (see previous section and refs. 38, 39, 40). Isolated vesicles can also be fused to artificial membranes, allowing for direct measurement of the electrical properties of individual Ca\(^{2+}\) channels (42, 43). The dominant risk associated with studying the Ca\(^{2+}\) release system of isolated SR vesicles is that the basic properties of the entire system may be altered during the isolation procedure. The two model systems, i.e. isolated membranes and muscle fibers, must be considered complementary approaches. A summary of the present state of knowledge of T-tubule/SR coupling, gathered from both approaches, follows.

**The T-Tubules and SR do not Appear to be Electrically Coupled**

Electron micrographic studies of the triadic junction indicate that the T-tubules are separated from the terminal cisternae of the SR in the triadic junction by an aqueous gap of about 120 to 140 angstroms (44, 45). The two membrane systems are held at this distance by "feet" proteins (also called bridging proteins (46) and spanning
proteins (47)). The separation between the two membrane systems appears to be too large to provide efficient capacitive coupling between them (45), given that the sarcolemmal capacitance of "detubulated" muscle fibers is not substantially different from that of intact fibers (5). Other facts weigh against a purely electrical coupling between SR and T-tubule. First, several investigators have tried to create ionic conditions which would electrically mimic "depolarization" of isolated SR vesicles; in experiments designed to minimize osmotic shock, ionic "depolarization" of isolated SR vesicles does not induce Ca$^{2+}$ release (37, 48), though ionic depolarization of triads does cause Ca$^{2+}$ release from the SR vesicles which are attached to T-tubule fragments (37). Fabiato used a variety of electrical techniques to stimulate the SR of skinned fibers and to measure potential changes in the interior of these fibers. Fabiato did not detect changes in potential in response to Ca$^{2+}$ release from SR, nor did direct electrical stimulation activate Ca$^{2+}$ release (49). The permeability of the SR to K$^+$ (48, 50) and Cl$^-$ (51) is apparently too large to allow any ionic transport systems to develop a significant membrane potential. Taken together, the facts indicate that the coupling between SR and T-tubule is not electrical.

The T-tubules and SR may be Electromechanically Linked
In 1973, Schneider and Chandler reported detection of charge movement on the T-tubule in response to changes in the T-tubule's membrane potential (52). Assuming that the charges moved between fixed positions, the density of the charged groups was calculated to be roughly equal to the density of the feet proteins described by Franzini-Armstrong (44), and Schneider and Chandler proposed that the charged groups may be mechanically linked, via the feet proteins, to the activation mechanisms of the \( \text{Ca}^{2+} \) channels of the SR. This is a very popular theory, but due to experimental difficulties, data confirming or disproving the assumptions inherent in the theory is lacking.

A "Triggering Substance" may be Released by the T-tubules

An alternate interpretation of the data presented by Schneider and Chandler is that the voltage-dependent charge movement they observed is evidence of voltage gated mechanisms in the T-tubules which mediate release of some "triggering" substance upon depolarization. The trigger would diffuse across the gap between the two membrane systems and bind to activating sites on the SR, leading to \( \text{Ca}^{2+} \) release. Diffusion of a small molecule or ion across a gap of this size would take a few tens of microseconds at most (45), only a fraction of the total time involved in excitation-contraction coupling. Two such triggers have been proposed: \( \text{Ca}^{2+} \) itself (39, 53-58) and inositol 1,4,5-
trisphosphate (59, 60). The evidence in support of these proposals is presented below.

Ca\(^{2+}\)-Induced Ca\(^{2+}\) Release. A number of investigators have reported that addition of a small amount of Ca\(^{2+}\) to isolated skinned fibers whose SR had been previously loaded with Ca\(^{2+}\) induces contraction of the fiber. Addition of Ca\(^{2+}\) to a fiber which had not been previously loaded would not lead to contraction, as the SR would take up the added Ca\(^{2+}\). Addition of Ca\(^{2+}\) to preloaded fibers led to contractions which were stronger than the amount of added Ca\(^{2+}\) could have produced alone, indicating that the added Ca\(^{2+}\) induced Ca\(^{2+}\) release from the preloaded SR (53-56).

The original investigators regarded Ca\(^{2+}\)-induced Ca\(^{2+}\) release as an unimportant anomaly from the point of view of the function of SR in vivo because, under the original conditions, the rate of Ca\(^{2+}\) release was too slow to be of significance physiologically (53, 54). Subsequent work with isolated vesicles defined conditions which led to faster release rates (39, 57, 58), but in all cases, in the presence of physiological Mg\(^{2+}\) concentrations (61, 62), the rate of Ca\(^{2+}\) release was still too slow to be of physiological importance. However, later reports showed that the Ca\(^{2+}\) release rates in the presence of physiological Mg\(^{2+}\) concentration could be dramatically increased by increasing the rate of mixing of the added Ca\(^{2+}\) (55). Ca\(^{2+}\)-induced Ca\(^{2+}\) release has since become a well-documented
mechanism of Ca\textsuperscript{2+} release from the SR of cardiac muscle (for review, see ref. 56), but corresponding data for skeletal muscle is less abundant (see refs 4, 5, and 6 for reviews of skeletal literature).

Briefly, the theory of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release is that extracellular Ca\textsuperscript{2+}, which is present in the lumina of the T-tubules in resting muscle, is released upon depolarization of the T-tubule. The released Ca\textsuperscript{2+} would diffuse across the gap of the triadic junction and bind to Ca\textsuperscript{2+} receptors, leading to opening of the Ca\textsuperscript{2+} channels of the SR. In addition to the evidence listed above, some additional data has been collected in support of this theory. First, the T-tubule membranes have Ca\textsuperscript{2+} pumps (41, 63) and voltage-gated Ca\textsuperscript{2+} channels (64, 65) embedded in them, which could conceivably mediate Ca\textsuperscript{2+} uptake into the T-tubules and release of triggering Ca\textsuperscript{2+} upon depolarization. Second, the permeability of Ca\textsuperscript{2+}-loaded isolated HSR vesicles is highly Ca\textsuperscript{2+} dependent (57, 58, and Chapter IV), and Ca\textsuperscript{2+} channels of the terminal cisternae which have been incorporated into artificial membranes are strongly Ca\textsuperscript{2+} dependent (42, 43). The Ca\textsuperscript{2+} flux rates through these channels and the density of these channels is sufficiently large that these channels could mediate Ca\textsuperscript{2+} release from the SR in excitation-contraction coupling (66).

**Inositol 1,4,5-Trisphosphate-Induced Ca\textsuperscript{2+} Release.**

Inositol 1,4,5 trisphosphate (IP\textsubscript{3}) triggers Ca\textsuperscript{2+} release
from the endoplasmic reticulum of several tissue types (67, 68), and several reports exist which claim that IP₃ induces Ca²⁺ release from isolated SR vesicles (59, 60). By analogy with the mechanism of transmission of nerve signals across synapses and Ca²⁺ release from endoplasmic reticulum, a model of IP₃-activated Ca²⁺ release from SR (in vivo) was proposed: depolarization would trigger production of IP₃ at the T-tubule. The IP₃ would then diffuse across the T-tubule/SR gap, bind to a receptor, and activate the Ca²⁺ release mechanism of the SR, leading to muscle contraction. The IP₃ would be broken down by an enzyme, IP₃-5 phosphatase, and Ca²⁺ would be taken back into the SR, leading to muscle relaxation (59). This theory is controversial, considering its sketchy supporting evidence and reports which claim that IP₃ does not induce Ca²⁺ release from SR vesicles (69). Despite its drawbacks, this theory will no doubt receive more attention in the future.

MODULATORS OF THE Ca²⁺ PERMEABILITY OF THE SR

As seen above, Ca²⁺ and IP₃ are potential triggers of Ca²⁺ release because of their abilities to increase the permeability of the SR to Ca²⁺, presumably by interacting with the Ca²⁺ release system. In order to be considered a potential trigger, a substance would have to be capable of undergoing a rapid change in concentration in the time interval between depolarization of the T-tubules and
activation of Ca\textsuperscript{2+} release from the SR. Such a change in concentration is possible for both Ca\textsuperscript{2+} and IP\textsubscript{3}, but has never been directly observed in either case. Nonetheless, both are modulators of the Ca\textsuperscript{2+} release system regardless of their status as triggers. Other substances are known to modulate the Ca\textsuperscript{2+} permeability of the SR as well, though most are not potential triggers, either because their concentrations do not change significantly during contraction or because they are not normally found in muscle tissue. Some of these modulators inhibit release while others stimulate or activate release. Below is a brief discussion of the known modulators of the Ca\textsuperscript{2+} release system of the SR, classified by whether they are normally found in muscle or not ("physiological" or "non-physiological") and whether they are inhibitors or activators of the Ca\textsuperscript{2+} release system.

**Physiological Modulators of Ca\textsuperscript{2+} Permeability of the SR.**

**Inhibitors of Ca\textsuperscript{2+} Release.** Mg\textsuperscript{2+} is a well-known inhibitor of Ca\textsuperscript{2+} release from isolated SR vesicles (38, 57, 58) and a well-documented inhibitor of muscle fiber contraction (53-56). Its free concentration in muscle fibers in vivo is not known with certainty; estimates range from 0.6 (61) to 6 mM (62), with the former figure the more reliable of the two. Calmodulin is a Ca\textsuperscript{2+}-modulated protein which has recently been reported to inhibit Ca\textsuperscript{2+} release from isolated SR vesicles (70). Its effect on Ca\textsuperscript{2+} release
from SR in situ has not been reported, and its role in muscle has not yet been established, if indeed it has one. High Ca\(^{2+}\) concentrations, in the range of 100 \(\mu\)M or more, inhibits Ca\(^{2+}\) release from isolated vesicles (58, 71, Chapter IV). In the Ca\(^{2+}\)-induced release theory, this fact is cited as being a possible negative feedback mechanism which would prevent uncontrolled Ca\(^{2+}\) release from the SR (71).

Activators of Ca\(^{2+}\) Release. As noted above, Ca\(^{2+}\) in micromolar concentrations activates Ca\(^{2+}\) release from SR. Adenine nucleotides, such as ATP, cAMP, and non-hydrolyzable analogues of ATP stimulate Ca\(^{2+}\)-induced Ca\(^{2+}\) release (39, 57, 58, 72) and stimulate Ca\(^{2+}\) release induced by heavy metals (73, 74), sulfhydryl oxidation (75, Chapter III), and ryanodine (76). The effect of IP\(_3\) on the Ca\(^{2+}\) permeability of the SR has been described above.

Non-Physiological Modulators of Ca\(^{2+}\) Release.

Inhibitors of Ca\(^{2+}\) Release. Ruthenium red (Ru red), a carbohydrate stain which inhibits Ca\(^{2+}\) uptake by mitochondria (77), has been shown to inhibit the Ca\(^{2+}\) release mechanism of isolated SR vesicles (57, 58, 74, 75, Chapters III and IV). The local anaesthetic tetracaine inhibits Ca\(^{2+}\) release from both isolated vesicles (57, 74, 75, Chapter III) and muscle fibers (78). Inhibitors of Ca\(^{2+}\) release, in particular Ru red and Mg\(^{2+}\), play important diagnostic roles in studying the Ca\(^{2+}\) release system of SR.
That is, any means of inducing Ca\(^{2+}\) release from SR which is inhibitable by Ru red and Mg\(^{2+}\) presumably activates the Ca\(^{2+}\) release system.

**Activators of Ca\(^{2+}\) Release.** Caffeine activates muscle fiber contraction (36, 78, 79) and Ca\(^{2+}\) release from isolated SR vesicles (38, 57, 80); it is often used for standardizing results of muscle fiber studies because of its ability to directly cause Ca\(^{2+}\) release from the SR without affecting the other components of the fibers (36, 79).

**Heavy metals,** in particular Hg\(^{2+}\) and Ag\(^{+}\) (73, 74), cause rapid Ca\(^{2+}\) release from isolated SR vesicles and cause contraction of muscle fibers (81). Their site of action appears to be a sulfhydryl group. Heavy metal-activated Ca\(^{2+}\) release is inhibited by all known inhibitors of Ca\(^{2+}\) release and hence appears to involve a direct interaction with the Ca\(^{2+}\) release system (74). In Chapter III of this dissertation, I show that *sulfhydryl oxidation* with Cu\(^{2+}\) and cysteine activates the Ca\(^{2+}\) release system by the Cu\(^{2+}\)-catalyzed covalent attachment of cysteine to the Ca\(^{2+}\) release system (75). Cu\(^{2+}\)/cysteine-activated Ca\(^{2+}\) release is the only "rapid" covalent method of Ca\(^{2+}\) release which does not inhibit the activity of the Ca\(^{2+}\) pump. The high sensitivity of this sulfhydryl may indicate that it is intimately involved in gating the Ca\(^{2+}\) release protein(s). In Chapter V it is shown that Cu\(^{2+}\) catalyzes the covalent attachment of \(^{35}\)S-cysteine to several SR proteins, some of
which are probably involved in Ca\(^{2+}\) release. **Doxorubicin** is a potent antineoplastic agent (82) which also causes Ca\(^{2+}\) release from isolated SR vesicles and contraction of isolated fibers (83). \(^{14}\)C-labeled doxorubicin in the presence of ultraviolet illumination can be covalently attached to several proteins in the SR, some of which appear to be involved in Ca\(^{2+}\) release (84). **Ryanodine** is a neutral plant alkaloid which produces irreversible contraction of skeletal muscle (85). It causes Ca\(^{2+}\) release from the terminal cisternae of isolated SR vesicles (38, 86, 87) and binds very specifically to a protein component of the terminal cisternae of the SR (88–90). The apparent specificity of ryanodine binding to protein(s) involved in Ca\(^{2+}\) release may provide the information necessary to isolate the Ca\(^{2+}\) release system and hence to study the properties of the Ca\(^{2+}\) release system without having to correct for complicating artifacts introduced by the other (often uncharacterized and unknown) proteins present in the SR.

**Covalent Modification of the SR**

Covalent modification of the proteins involved in Ca\(^{2+}\) release via chemical and enzymatic methods may provide important clues regarding the functions of these proteins. As shown in Chapter III and noted above, sulfhydryl oxidation rapidly activates the Ca\(^{2+}\) release system. The rapidity of Cu\(^{2+}\)/cysteine-induced release suggests that the
sulfhydryl involved may be part of the gating mechanism of the channel. On a somewhat longer time scale (minutes instead of seconds), it has recently been reported that chemical modification of lysine and arginine groups (91, 92) results in increased Ca$^{2+}$ permeability of the SR. Finally, as reported in Chapter IV, limited modification of the SR with the proteolytic enzyme trypsin stimulates the activation of the Ca$^{2+}$ release system. Apparently, the modulatory sites which bind Ca$^{2+}$, Mg$^{2+}$, Ru red, and doxorubicin are unaffected by tryptic proteolysis. Differences between the degree of tryptic stimulation of Ca$^{2+}$ release induced by these reagents and Ca$^{2+}$ release induced by heavy metals and Cu$^{2+}$/cysteine may provide a means of identifying the functional units of the Ca$^{2+}$ release system.

OVERVIEW OF THIS DISSERTATION

The focus of this dissertation is the Ca$^{2+}$ release system of the SR, as it exists in isolated SR vesicles. In particular, this research is a study of the effects of chemical and enzymatic modification of the SR on the Ca$^{2+}$ release system. This work, begun in the summer of 1981, was originally aimed at delineating the functions of the Ca$^{2+}$ pump. At that time, Lyle Weden (a former graduate student also working under Dr. Abramson's supervision) and I re-examined reports that SR vesicles do not actively take up
Ca\textsuperscript{2+} in the presence of Hg\textsuperscript{2+}, even though ATP is hydrolyzed (93). The original interpretation of this result was that Hg\textsuperscript{2+} uncouples the energy transducing mechanism of the ATP hydrolytic site on the Ca\textsuperscript{2+} pump from Ca\textsuperscript{2+} translocation across the membrane. In a paper published in the Proceedings of the National Academy of Sciences (73) we showed that the original interpretation of the inhibition of Ca\textsuperscript{2+} accumulation by the SR in the presence of Hg\textsuperscript{2+} was incorrect; the reason Ca\textsuperscript{2+} is not actively taken up by SR vesicles in the presence of Hg\textsuperscript{2+} is because the permeability of SR vesicles is greatly increased by Hg\textsuperscript{2+}. In the presence of Hg\textsuperscript{2+}, Ca\textsuperscript{2+} is still actively transported into the SR, but it immediately leaks out of the vesicles, leading to no net Ca\textsuperscript{2+} uptake. Hg\textsuperscript{2+} binds strongly to sulfhydryl groups (94), and other heavy metals which bind strongly to sulfhydryls, particularly Ag\textsuperscript{+} and Cu\textsuperscript{2+} (94), were also shown to increase the Ca\textsuperscript{2+} permeability of the SR, hence it was concluded that the heavy metals cause increases in the Ca\textsuperscript{2+} permeability of the SR by perturbing a critical sulfhydryl on a transmembrane protein, and it was conjectured that the protein(s) affected were perhaps the Ca\textsuperscript{2+} release protein(s). This work is presented in Chapter II. Later work, not performed by me, confirmed that heavy metals activate the Ca\textsuperscript{2+} release system of isolated SR vesicles (74).
I had hoped to exploit the binding of Ag⁺ and Hg²⁺ to the Ca²⁺ release system as a means of identifying the protein(s) responsible for Ca²⁺ release. At that time, the identity of the protein(s) responsible for Ca²⁺ release was an almost completely open question. An issue which needed to be settled before heavy metals could be used to identify this protein(s) was whether a heavy metal ion, once bound to the sulfhydryl group which activates Ca²⁺ release, would transfer to another, unoccupied, sulfhydryl group. The following experiment was devised in order to assay for this: Ca²⁺ was actively taken up by SR vesicles in the presence of ATP and Mg²⁺. A heavy metal ion was added (i.e. Ag⁺, Hg²⁺, or Cu²⁺) and Ca²⁺ release was observed. Then cysteine, a sulfhydryl-containing amino acid, was added. One of two results were expected: 1) cysteine would have no effect, which would indicate that the sulfhydryl(s) involved in Ca²⁺ release do not exchange bound metal ions or 2) addition of the cysteine would lead to Ca²⁺ re-uptake by the SR in the presence of the residual ATP, which would indicate that the bound metal ion can be displaced from the critical sulfhydryl group. Ag⁺- and Hg²⁺-induced release were slightly affected by the exogenous cysteine, but Cu²⁺-induced release was dramatically enhanced (to my great surprise). In fact, cysteine and a concentration of Cu²⁺ which is too low to cause release by itself will cause rapid Ca²⁺ release. Subsequent work and supporting evidence
gathered by Jan Stuart, another graduate student under Dr. Abramson's supervision, showed that Cu²⁺/cysteine activates the Ca²⁺ release system of the SR. The mechanism of activation of the Ca²⁺ release system with Cu²⁺/cysteine also appears to be sulfhydryl perturbation, and based on observations shown in Chapter IV, appears to be the same sulfhydryl involved in heavy metal-induced Ca²⁺ release. Cu²⁺ is known to catalyze the co-oxidation of sulfhydryl pairs to form disulfide bonds (95); the nature of the perturbation of the SR with Cu²⁺/cysteine appears to be Cu²⁺-catalyzed co-oxidation of the sulfhydryl associated with the Ca²⁺ release system and the sulfhydryl of the added cysteine to form a mixed disulfide. My part of this work was published in the Journal of Biological Chemistry (75) and is presented in Chapter III; Ms. Stuart's work has been submitted to Archives of Biochemistry and Biophysics for publication (96).

At the time of completion of the Cu²⁺/cysteine work the identity of the Ca²⁺ release protein(s) was still an open question. Since the mechanism of Cu²⁺/cysteine-induced Ca²⁺ release appears to result in covalent attachment of cysteine to the Ca²⁺ release protein (75, 95, Chapter III), it was hoped that the Ca²⁺ release protein could be labeled with ³⁵S-cysteine and then the other sulfhydryl groups of the SR could be blocked with N-ethyl maleimide. In this way the problem of label exchange between SR sulfhydryls is
overcome, possibly allowing identification of the Ca\textsuperscript{2+} release protein. Preliminary experiments showed that many proteins covalently bind \textsuperscript{35}S-cysteine when in the presence of Cu\textsuperscript{2+}, and no single protein could be unequivocally identified as the Ca\textsuperscript{2+} release protein. In an attempt to explore this issue further, I began experiments designed to proteolytically modify the Ca\textsuperscript{2+} release protein with trypsin. Earlier investigators had reported that trypsin does not alter the Ca\textsuperscript{2+} permeability of the SR (97), and it was my hope that after a well-defined period of exposure to trypsin, Cu\textsuperscript{2+}/cysteine would no longer activate Ca\textsuperscript{2+} release. Tryptic inhibition of Cu\textsuperscript{2+}/cysteine-induced release was (hopefully) to be correlated with disappearance of \textsuperscript{35}S-label from some protein component of the SR. A correlation between disappearance of label on a particular protein and inactivation of release would have strongly implicated such a protein in Ca\textsuperscript{2+} release. Instead, the opposite happened: Cu\textsuperscript{2+}/cysteine-activated release was stimulated by tryptic modification of the SR, as were several other methods of activating Ca\textsuperscript{2+} release.

The earlier study of tryptic modification of the SR (97) had focused on the effects of trypsin on Ca\textsuperscript{2+} uptake and the Ca\textsuperscript{2+} pump. It was observed that tryptic proteolysis inhibits Ca\textsuperscript{2+} uptake, and the investigators concluded that trypsin uncouples the Ca\textsuperscript{2+} transport site on the Ca\textsuperscript{2+} pump from the site of ATP hydrolysis, in a manner similar to the
(erroneous) mechanism proposed for heavy metal inhibition of Ca\textsuperscript{2+} uptake. A control experiment was run to rule out the possibility that the reason for the reduced Ca\textsuperscript{2+} uptake wasn't in fact an increase in Ca\textsuperscript{2+} permeability of the membrane. However, as explained in Chapter IV, the experimental conditions for that control masked the increased permeability of the membrane caused by trypsin, leading to the erroneous conclusion that trypsin does not increase the Ca\textsuperscript{2+} permeability of the SR. That conclusion, and the conclusion that tryptic modification uncouples ATP hydrolysis from active Ca\textsuperscript{2+} transport, have been proven invalid in several ways. In a very recent study (published while my work was in progress), it has been shown that, although the Ca\textsuperscript{2+} pump is modified by trypsin, it still supports active Ca\textsuperscript{2+} uptake when isolated and reconstituted into artificial vesicles (98). Also, as demonstrated in Chapter IV, the Ca\textsuperscript{2+} uptake capacity of tryptically modified SR vesicles is restored upon addition of the Ca\textsuperscript{2+} release inhibitor ruthenium red. These data, coupled with the direct measurement of increased Ca\textsuperscript{2+} permeability due to tryptic proteolysis, clearly indicate that the reason tryptically modified SR's Ca\textsuperscript{2+} uptake capacity is reduced is that trypsin stimulates the Ca\textsuperscript{2+} release system, and that Ca\textsuperscript{2+} transported into the lumen of the SR by the Ca\textsuperscript{2+} pump simply leaks back out via the Ca\textsuperscript{2+} release system. The
research presented in Chapter IV has been submitted to the Journal of Biological Chemistry for publication (99).

In Chapter V I give a brief description of some of the proteins which are covalently modified by Cu²⁺/cysteine and by tryptic proteolysis. These experiments can only be considered preliminary, with their ultimate value being applications which will require much more extensive groundwork. The motivation behind both sets of experiments was originally to identify the Ca²⁺ release protein. However, as discussed previously, ryanodine appears to be a specific label of the Ca²⁺ release protein, and in the past year several papers have been published which detail methods of isolating a ryanodine binding protein (86-90) and of reconstituting this protein into an artificial membrane, where it exhibits channel-like characteristics (100). The data presented in Chapter V do not conflict with the results of the ryanodine binding studies but do not, on their own, constitute an independent identification of the Ca²⁺ release channel. Moreover, it seems unlikely, after a critical review of the data, that the Cu²⁺/cysteine/trypsin approach could constitute an independent scheme for identification of the Ca²⁺ release protein. However, if adapted for the purpose of probing the purified Ca²⁺ release channel, studies similar to those reported in Chapter V could aid greatly in identifying the functional domains of this protein, and ultimately may help to elucidate the molecular
mechanisms involved in regulation of the Ca\(^{2+}\) permeability of the SR. Such studies would be well beyond the scope of this dissertation but are discussed for the sake of completeness and to indicate possible further applications of the findings of this research.

**RELEVANCE OF THIS DISSERTATION**

The data presented in Chapters II and III show that the Ca\(^{2+}\) release system is activated by perturbation of a critical sulfhydryl. Previous research on Ca\(^{2+}\) release from the SR had been limited to phenomenological descriptions of conditions which result in Ca\(^{2+}\) release; few attempts had been made to identify functional groups involved in activation of the release system. Prior to publication of our finding that heavy metals induce Ca\(^{2+}\) release from SR (73), Schneider and Chandler's electromechanical model of Ca\(^{2+}\) release activation was the only attempt to describe, on a molecular level, the coupling of depolarization of the T-tubules to Ca\(^{2+}\) release from the SR (52). The critical sulfhydryl implicated in heavy metal- and oxidation-activated Ca\(^{2+}\) release is the first functional group to be implicated in the activating mechanism of the Ca\(^{2+}\) release system, and remains the only one. Corroborating evidence supporting the hypothesis that the critical sulfhydryl is involved in gating the Ca\(^{2+}\) channel is provided by Pessah et al (76). They have shown that Ag\(^{+}\) displaces ryanodine bound
to the SR, and have suggested that the critical sulfhydryl which is perturbed by heavy metals and Cu$^{2+}$/cysteine is very near, or is part of, the ryanodine binding site. An attractive possibility is that the redox state of this sulfhydryl group is the determinant, \textit{in vivo}, of whether the Ca$^{2+}$ release system is activated. This hypothesis is discussed in greater detail in the Discussion of Chapter III.

Strong oxidants and heavy metals represent two of industrial society's biggest challenges to environmental quality. Some of the research presented in this dissertation examines specific effects of oxidants and heavy metals on a simple biological membrane system. As outlined in Chapter II, even in such a simple system as isolated SR, the effects of heavy metals are sufficiently complicated that highly regarded and competent researchers drew erroneous conclusions from initial studies. As a result of the research presented in this dissertation, a more accurate picture of the nature of these challenges to organic integrity has been generated.

Tryptic stimulation of Ca$^{2+}$ release may yield much information about which domains of the Ca$^{2+}$ release proteins are actually involved in activation of Ca$^{2+}$ release. The binding characteristics of the various activators and inhibitors of Ca$^{2+}$ release tested do not appear to change as a result of tryptic modification of the Ca$^{2+}$ release system;
a possible explanation of the tryptic stimulation of Ca\textsuperscript{2+} release may be that trypsin modifies some regulatory protein or its binding site on the Ca\textsuperscript{2+} release system. The possible existence of regulatory proteins which modulate the Ca\textsuperscript{2+} release mechanism has only been explored to a limited extent (70). From this perspective, it is interesting that the ryanodine binding proteins implicated in Ca\textsuperscript{2+} release (88-90) also bind calmodulin (101), which inhibits Ca\textsuperscript{2+} release (70). It is my hope that trypsin will prove to be a valuable tool in the dissection of the Ca\textsuperscript{2+} release system of the SR; certainly, it would seem to be promising.

At the very least, my work with trypsin and heavy metals has cleared up misunderstandings of earlier work and has perhaps steered others from pursuing fruitless lines of research. More optimistically, my work along these lines and with sulfhydryl oxidation sheds some light, on a molecular level, on how the Ca\textsuperscript{2+} release system of the sarcoplasmic reticulum is regulated. As mentioned above, the recent schemes for isolating the Ca\textsuperscript{2+} release proteins (100) may offer new applications of my discoveries.
CHAPTER II

HEAVY METALS INDUCE RAPID Ca\(^{2+}\) RELEASE FROM
ISOLATED SARCOPLASMIC RETICULUM VESICLES

SUMMARY

In this chapter, it is shown that various heavy metals cause rapid release of Ca\(^{2+}\) from SR vesicles. This increase in Ca\(^{2+}\) permeability of the SR is correlated with stimulation of Ca\(^{2+}\),Mg\(^{2+}\)-ATPase activity, and appears to be due to heavy metal binding to a sulfhydryl on a protein in the SR. It is also shown that air oxidation of sulfhydryl pairs to form disulfides causes a very large increase in the Ca\(^{2+}\) permeability of SR vesicles.

INTRODUCTION

Shamoo and MacLennan (93, 102) have reported that HgCl\(_2\) and CH\(_3\)HgCl inhibit ATPase activity and active Ca\(^{2+}\) uptake in SR vesicles in the presence of the Ca\(^{2+}\) precipitable anion oxalate. Ca\(^{2+}\) uptake, however, decreased more quickly with increasing Hg\(^{2+}\) concentration than ATPase activity did. Normally, ATP hydrolysis is tightly coupled to Ca\(^{2+}\) uptake; Shamoo and MacLennan concluded that Hg\(^{2+}\) and CH\(_3\)Hg\(^{+}\) inhibit Ca\(^{2+}\) uptake before ATPase activity by uncoupling the normally tightly coupled hydrolysis of ATP from transport of
Ca\textsuperscript{2+} into the lumina of the isolated SR vesicles. In this chapter, similar experiments are carried out in the absence of oxalate. Under these conditions, the concentration of Hg\textsuperscript{2+} which inhibits Ca\textsuperscript{2+} uptake actually leads to a stimulation of ATPase activity. Passive Ca\textsuperscript{2+} efflux studies show that the stimulation of ATPase activity at this concentration of Hg\textsuperscript{2+} is due to Hg\textsuperscript{2+} activation of a Ca\textsuperscript{2+} permeability pathway. Thus, Hg\textsuperscript{2+}-induced increase of Ca\textsuperscript{2+} permeability is probably the mechanism of decreased Ca\textsuperscript{2+} uptake reported by Shamoo and MacLennan, and not uncoupling of Ca\textsuperscript{2+} translocation from ATP hydrolysis, as they had originally reported (93, 102). This work was published in the Proceedings of the National Academy of Sciences in 1983 (73).

MATERIALS AND METHODS

Preparation of SR vesicles

SR vesicles were isolated from rabbit white skeletal muscle following a protocol similar to the method of MacLennan (29). A 2 to 3 kg male New Zealand White rabbit was stunned, bled, and the back and hind leg muscles were removed. The muscles were trimmed of fat and connective tissue, red muscle was discarded, and the remaining white muscle was put into ice-cold Buffer A (120 mM NaCl, 10 mM imidazole, adjusted to pH 7.4 with NaOH; generally, 8 liters of this buffer were required per rabbit and was made up the
night before the prep was to be done, and stored on ice until used). The muscle was ground in a meat grinder and homogenized in a Waring blender in three volumes of Buffer A for 15 seconds on low speed, 30 seconds on high speed, and then again for 30 seconds on high speed after a wait of 30 seconds. The soupy suspension was centrifuged for 10 minutes at 1600xg (large GSA rotor in Sorvall centrifuge at 3100 rpm). The supernatant (~1500 ml) was strained through four layers of cheesecloth and the pelleted cell debris was discarded. The pH was adjusted with dry imidazole to 7.4 and the supernatant was centrifuged at 10,000xg for 15 minutes (8,000 rpm in large GSA rotor). The supernatant was again strained through four layers of cheesecloth and the brown mitochondrial pellet was discarded. The supernatant was centrifuged at 44,000xg for 50 minutes (70 minutes after the Ti19 rotor came up to full speed of 19,000 rpm in the Beckman ultracentrifuge) in completely filled tubes, balanced in opposing pairs. The clear supernatant was carefully pipetted off and discarded, and the pellet (except for the brown mitochondrial ring) was scraped off, homogenized in a Wheaton tissue homogenizer, and suspended at ~10 mg/ml. The pH was again adjusted to 7.4 with dry imidazole and the suspension was centrifuged at 7500xg for 10 minutes (11,000 rpm in Ti60 rotor). The large flocculent myosin pellet was discarded; the supernatant was removed with a Pasteur pipette (being careful to avoid disturbing
the pellet) and spun down at 78,000xg for 30 minutes (35,000 rpm in the Ti60 rotor). This pellet, which contained SR vesicles, was suspended at ~25 mg/ml in 100 mM KCl, 50 mM HEPES, and 5 mM MgCl$_2$ (adjusted to pH 7.0 with KOH) and stored in small aliquots in liquid nitrogen.

In all phases of isolation, the following precautions were observed: 1). the muscle was excised as quickly as possible and cooled as quickly as possible and 2). all glassware was rinsed with Buffer A (or the KCl buffer) prior to contact with the muscle, in order to reduce osmotic shock and avoid contamination from tap water. All buffers were prepared with distilled, de-ionized water. Generally, an SR preparation requires a full day; in order to facilitate the process, all buffers were prepared the day before and stored on ice. All rotors and the Sorvall centrifuge were cooled the day before also. The Beckman centrifuge was turned on and cooled the day of the preparation, first thing in the morning. Since the cold room is usually ~8°C, the rotors need some time in the appropriate centrifuge, with the temperature set at 0°C, in order to cool completely.

**Protein concentration determination**

The concentration of SR protein was determined by the method of Lowry et al. (103).
**Assays**

Isotopic measurements of Ca\(^{2+}\) uptake and Ca\(^{2+}\),Mg\(^{2+}\) ATPase activity were made at 37°C in a buffer consisting of 100 mM KCl, 50 mM HEPES, 5 mM MgCl\(_2\), and 30 μM CaCl\(_2\), adjusted to pH 7.0 with KOH. Uptake and ATPase activity were initiated by addition of 1 mM Na\(_2\)ATP (previously adjusted to pH 7.0 with NaOH) to a solution containing 0.2 mg/ml SR vesicles.

Ca\(^{2+}\) uptake as a function of [Hg\(^{2+}\)] (or [Ag\(^{+}\)]) was measured with \(^{45}\)CaCl\(_2\) (\(10^5\) cpm/nmol of Ca\(^{2+}\)) in the following way: ATP was added to a \(^{45}\)Ca\(^{2+}\)/SR (0.2 mg protein/ml) suspension containing the indicated concentration of Hg\(^{2+}\). The mixture was placed in a water bath held at 37°C. Uptake was stopped after two minutes by filtering 200 μl of the suspension through a Millipore 0.45 μm type HA filter (28). The vesicles, and hence the intravesicular \(^{45}\)Ca\(^{2+}\), are trapped on the filters but the extravesicular solution passes through. The filters were washed with 5 ml of wash solution (100 mM KCl, 50 mM HEPES, 5 mM MgCl\(_2\), adjusted to pH 7.0 with KOH) in order to remove extravesicular \(^{45}\)Ca\(^{2+}\). They were then dried and radioactivity was determined in Aquasol-2 universal liquid scintillation cocktail (New England Nuclear) using a Beckman LS-9000 liquid scintillation counter. Each measurement of radioactivity was performed in triplicate and the mean and standard deviation of the count rate was calculated.
calibrate the count rate with the Ca\(^{2+}\) content, an aliquot of the same vesicle/\(^{45}\text{Ca}\)\(^{2+}\) mixture was placed on a filter which was not washed prior to \(^{45}\text{Ca}\)\(^{2+}\) counting. The count rate per nmol Ca\(^{2+}\) could thus be determined and used as a standard for computing the Ca\(^{2+}\) uptake capacity of the vesicles in terms of nmol Ca\(^{2+}\) per mg SR protein.

ATPase activity was measured in the presence of unlabeled CaCl\(_2\) with isotopically labeled \(^{32}\text{P}\)-ATP. To start the reaction, 1 mM of \(^{32}\text{P}\)-ATP, with the labeled phosphate occupying the terminal position, was added to a small aliquot of SR (0.2 mg/ml) suspended in the uptake buffer, which contained varying concentrations of HgCl\(_2\). ATP hydrolysis was terminated after two minutes by addition of a large volume of ice-cold trichloracetic acid. The precipitated protein was pelleted by centrifugation, and the supernatant, which contained \(^{32}\text{P}\)-ATP, ADP, and \(^{32}\text{P}\)-PO\(_4\), was drawn off and diluted into a butyl acetate/ammonium molybdate mixture (104). The radioactively labeled inorganic phosphate, which was liberated by ATP hydrolysis, forms a complex with molybdate ion, while the non-hydrolyzed \(^{32}\text{P}\)-ATP and ADP do not. The \(^{32}\text{P}\)-labeled phosphomolybdate complex was extracted into the butyl acetate phase by extended vortex mixing. The phases were separated by centrifugation and samples from the butyl acetate phase containing \(^{32}\text{P}\)-phosphomolybdate were assayed for radioactivity by liquid scintillation counting. The rate of
production of labeled phosphate (extracted into the butyl acetate phase) is directly proportional to the rate at which ATP is hydrolyzed by the Ca\textsuperscript{2+} pump. Each measurement was performed in triplicate and the mean and standard deviation were calculated.

**Isotopic Measurements of Ca\textsuperscript{2+} Release.** SR vesicles were suspended at a protein concentration of 5 mg/ml in a "passive loading buffer" consisting of 100 mM KCl, 50 mM HEPES, 5 mM MgCl\textsubscript{2}, and 5 mM \textsuperscript{45}CaCl\textsubscript{2} (adjusted to pH 7.0 with KOH) and incubated on ice for 16-24 hr in order to allow transmembrane Ca\textsuperscript{2+} equilibration. Ca\textsuperscript{2+} efflux from the passively loaded vesicles was initiated by diluting an aliquot into 25 volumes of a similar buffer which contained the indicated concentration of HgCl\textsubscript{2} (or other heavy metal salt or reagent) but no added Ca\textsuperscript{2+}, held at 37°C in a water bath. Samples were removed at the indicated times and filtered through Millipore 0.45 µm type HA filters. Filters were washed in order to remove residual extravesicular \textsuperscript{45}Ca\textsuperscript{2+}, dried, and radioactivity was determined by scintillation counting. Each measurement was performed in triplicate.

**Spectrophotometric Assay of Ca\textsuperscript{2+} Release.** Ca\textsuperscript{2+} release from SR vesicles was assayed by dilution of passively loaded vesicles into 40 volumes of a medium containing 100 mM KCl, 20 mM HEPES, 0.1 mM arsenazo III (ARS III), 5 mM MgCl\textsubscript{2}, and varying concentrations of CuCl\textsubscript{2}, HgCl\textsubscript{2}, or AgNO\textsubscript{3}. 
Extravesicular Ca\textsuperscript{2+} concentrations were monitored continuously by following the differential absorption changes of ARS III at 675 nm - 685 nm with a time-sharing dual wavelength spectrophotometer (105). The use of this dye and the controls demonstrating its specificity for Ca\textsuperscript{2+} in similar SR experiments have been previously reported (106). The reagents used to modulate the Ca\textsuperscript{2+} permeability of the SR vesicles produced negligible changes in the absorption of the Ca\textsuperscript{2+} indicator and did not appear to interfere with measurements of free Ca\textsuperscript{2+} concentration. Upon completion of Ca\textsuperscript{2+} efflux, the Ca\textsuperscript{2+} ionophore A23187 was added to the vesicles in order to determine the total Ca\textsuperscript{2+} content of the vesicles, then a known concentration of Ca\textsuperscript{2+} was added in order to calibrate the response of the dye to Ca\textsuperscript{2+}. The rate of Ca\textsuperscript{2+} efflux from SR vesicles is proportional to the time rate of change of the differential absorption signal of the dye. Calibration of the dye permits calculation of the rate of Ca\textsuperscript{2+} efflux expressed in units of nmol Ca\textsuperscript{2+} released per second per mg SR protein (see Chapters III and IV for representative examples of efflux traces).

RESULTS

Hg\textsuperscript{2+} stimulates ATP hydrolysis but inhibits Ca\textsuperscript{2+} uptake

As shown in Figure 3, micromolar concentrations of HgCl\textsubscript{2} cause a stimulation of Ca\textsuperscript{2+},Mg\textsuperscript{2+}-ATPase activity but a decrease in the total amount of Ca\textsuperscript{2+} accumulated. Optimal
Figure 3. Calcium uptake (---) and ATPase activity (----) as a function of \( \text{Hg}^{2+} \) concentration. Uptake and ATPase activity were measured isotopically in the presence of various concentrations of \( \text{Hg}^{2+} \). Values shown are mean ± SD of three trials at each \( \text{Hg}^{2+} \) concentration.
ATPase activity occurs at a Hg$^{2+}$/SR ratio of 5 μmol Hg$^{2+}$/10$^8$ μg SR protein; at this Hg$^{2+}$/SR ratio, the rate of ATP hydrolysis is four times faster than the rate of ATP hydrolysis in the absence of Hg$^{2+}$. The ATPase comprises about 70% of the protein in the SR and has a molecular weight of about 10$^8$. On a molar basis, optimal ATPase activity occurs at a ratio of about seven Hg$^{2+}$ ions to one ATPase molecule. Further increase in the mercury concentration inhibits both ATPase activity and active Ca$^{2+}$ uptake. AgNO$_3$ also stimulates ATPase activity and inhibits Ca$^{2+}$ uptake into SR vesicles (data not shown), though Ag$^+$ is not quite as potent at stimulating ATPase activity as Hg$^{2+}$. The molar ratio of either heavy metal to SR protein is critical: lower protein concentrations require less metal, while higher protein concentrations require higher metal concentrations, suggesting that some reactive group(s) is being titrated by the metal ions.

**Hg$^{2+}$ Activates Ca$^{2+}$ Release from SR Vesicles**

It has been reported that ionic detergents exhibit ionophoric activity (107) and also stimulate ATPase activity (29). The mechanism by which these detergents stimulate ATP hydrolysis appears to be due to their ionophoric activity. In the presence of any Ca$^{2+}$ ionophore, or any pathway of Ca$^{2+}$ permeability, Ca$^{2+}$ pumped into the lumen of an SR vesicle will rapidly leak out, and will not accumulate.
within the vesicles. Since Ca\(^{2+}\) translocation via the Ca\(^{2+}\) pump is tightly coupled to ATP hydrolysis, any means of dissipating the Ca\(^{2+}\) gradient across the membrane (thus lowering the electrochemical potential against which the pump must work) will stimulate ATP hydrolysis. The stimulation of ATP hydrolysis and coincident inhibition of Ca\(^{2+}\) uptake in the presence of Hg\(^{2+}\) thus suggest that Hg\(^{2+}\) may activate a Ca\(^{2+}\) permeability pathway. This hypothesis was directly tested by measuring the rate of Ca\(^{2+}\) release from SR vesicles in the presence of Hg\(^{2+}\) (Figure 4). SR vesicles were passively loaded with \(^{45}\)Ca\(^{2+}\) and then diluted into a similar, but Ca\(^{2+}\)-free medium (control), or alternately into a similar, Ca\(^{2+}\)-free medium containing 10 \(\mu\)M Hg\(^{2+}\). The \(^{45}\)Ca\(^{2+}\) content of the vesicles was assayed at intervals using the Millipore filtration technique described in the Methods. The final ratio of Hg\(^{2+}\) to SR was identical to that which causes optimal ATPase stimulation (refer to Figure 3). The data summarized in Figure 4 clearly indicates that the SR is more permeable to Ca\(^{2+}\) in the presence of Hg\(^{2+}\). If all the Ca\(^{2+}\) were to equilibrate, only 4% of the original Ca\(^{2+}\) within the vesicles would remain. In the presence of Hg\(^{2+}\), the transmembrane Ca\(^{2+}\) concentrations nearly reach equilibrium within 30 seconds after initiation of Ca\(^{2+}\) efflux. In contrast, Ca\(^{2+}\) equilibrium takes on the order of half an hour for control vesicles, not treated with Hg\(^{2+}\).
Figure 4. Calcium release induced by Hg$^{2+}$. SR vesicles were passively loaded with $^{45}$Ca$^{2+}$ at 5 mg/ml by incubation and efflux was assayed by the Millipore filtration technique. Values given are mean ± SD of three trials at each time point.
**Hg²⁺-Induced Ca²⁺ Release is Reversed by Mercaptoethanol**

β-mercaptoethanol binds Hg²⁺ with high affinity (94). This fact was exploited in order to determine whether heavy metal-induced Ca²⁺ release is a reversible effect. SR vesicles were suspended in the ⁴⁶Ca²⁺ "passive loading buffer" at a protein concentration of 5 mg/ml. 250 μM HgCl₂ was added and the suspension was brought to room temperature for 15 minutes. After this time interval, 2.5 mM β-mercaptoethanol was added, and this preparation was incubated for an additional 30 minutes at room temperature, followed by incubation at O°C for 16-24 hours to allow transmembrane Ca²⁺ equilibration. Control vesicles were incubated in Hg²⁺ but not treated with β-mercaptoethanol. Ca²⁺ efflux from the passively loaded SR vesicles was measured by diluting them 1:25 into a similar but Ca²⁺- and Hg²⁺-free medium. When the SR was incubated in Hg²⁺ but without β-mercaptoethanol, Ca²⁺ efflux followed a time course almost identical to the 10 μM Hg²⁺ curve shown in Figure 4 (note that final concentrations of Hg²⁺ and SR are identical to those of the earlier experiment). In the presence of the 10-fold excess of β-mercaptoethanol, the Ca²⁺ remaining in the SR at the first time point (t = 30 sec) was 40-50% of that at t = 0, and the values found at all later time points paralleled the "no Hg²⁺" curve in Fig. 4; i.e., they were all lower by 40-50%. It appears that some, but not all, of the Hg²⁺ can be displaced by a large
excess of reducing agent. Those vesicles in which the Hg$^{2+}$ was removed by 2-mercaptoethanol appear to be as impermeable to Ca$^{2+}$ as normal untreated SR vesicles.

Heavy Metals Induce Ca$^{2+}$ Release by Sulfhydryl Binding

To determine the nature of the binding site which initiates Hg$^{2+}$-induced Ca$^{2+}$ release from SR vesicles, the relative potency of several heavy metals causing Ca$^{2+}$ release was examined. Table I shows the concentrations of various agents which cause release of 50% of passively loaded Ca$^{2+}$ in 30 sec. Cd$^{2+}$, Zn$^{2+}$, and CH$_3$Hg$^+$ are much less potent than Cu$^{2+}$, Hg$^{2+}$, and Ag$^+$. Ca$^{2+}$ efflux induced by Cd$^{2+}$, Zn$^{2+}$, and CH$_3$Hg$^+$ and by N-ethyl maleimide and Ba$^{2+}$ were measured isotopically. The reagents were incubated in SR passively loaded with $^{45}$Ca$^{2+}$ at 25 times the concentration shown in this table prior to Ca$^{2+}$ efflux assay, then diluted 25-fold into efflux medium. In order to increase the time resolution of the measurement of Ca$^{2+}$ release due to the more potent reagents and to avoid problems of Cu$^{2+}$-catalyzed sulfhydryl oxidation (see next Chapter III), Ca$^{2+}$ release in the presence of Cu$^{2+}$, Ag$^+$, and Hg$^{2+}$ was measured spectrophotometrically. Passively loaded (with non-labeled Ca$^{2+}$) vesicles were diluted 40-fold into a solution containing the indicated concentrations of metal ion. The sequence of Ca$^{2+}$ release potency—Hg$^{2+}$ > Ag$^+$ > Cu$^{2+}$ > Cd$^{2+}$ > Zn$^{2+}$ > CH$_3$Hg$^+$ > N-ethylmaleimide > Ba$^{2+}$—is very similar to the binding sequence of heavy metals to such sulfhydryl-
TABLE I

CONCENTRATION OF REAGENT WHICH CAUSES 50% RELEASE IN 30 SECONDS

For study of Ca\textsuperscript{2+} release induced by Cu\textsuperscript{2+}, Ag\textsuperscript{+}, or Hg\textsuperscript{2+}, SR vesicles were suspended at 8 mg/ml in 100 mM KCl, 20 mM HEPES, 1 mM MgCl\textsubscript{2} and 1 mM non-radioactive CaCl\textsubscript{2} and passively loaded by incubation. Ca\textsuperscript{2+} release was initiated by 1:40 dilution into a similar but Ca\textsuperscript{2+}-free medium which contained the indicated concentration of heavy metal and Ca\textsuperscript{2+} release was assayed spectrophotometrically. In the case of the other reagents listed, SR vesicles were suspended at 5 mg/ml in 100 mM KCl, 50 mM HEPES, 5 mM MgCl\textsubscript{2}, 5 mM \textsuperscript{45}CaCl\textsubscript{2}, pH 7.0, and incubated overnight on ice. 30 minutes prior to efflux assay, a concentration of reagent equal to 25 times that indicated was added to the vesicles. Ca\textsuperscript{2+} efflux was initiated by diluting the passively loaded vesicles 1:25 into a similar but Ca\textsuperscript{2+}-free buffer.

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>CONCENTRATION (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hg\textsuperscript{2+}</td>
<td>4</td>
</tr>
<tr>
<td>Ag\textsuperscript{+}</td>
<td>10</td>
</tr>
<tr>
<td>Cu\textsuperscript{2+}</td>
<td>10</td>
</tr>
<tr>
<td>Cd\textsuperscript{2+}</td>
<td>15</td>
</tr>
<tr>
<td>Zn\textsuperscript{2+}</td>
<td>20</td>
</tr>
<tr>
<td>CH\textsubscript{3}Hg\textsuperscript{+}</td>
<td>30</td>
</tr>
<tr>
<td>NEM</td>
<td>40</td>
</tr>
<tr>
<td>Ba\textsuperscript{2+}</td>
<td>no release</td>
</tr>
</tbody>
</table>
containing reagents as S-methylcysteine and penicillamine (94). Methylmercuric chloride is far less potent than the more hydrophilic mercuric chloride, while N-ethylmaleimide, a relatively specific sulfhydryl reagent, causes release of Ca\(^{2+}\) at a somewhat higher concentration. Ariki and Shamoo (108) report that there are at least 5 reactive sulfhydryl groups per ATPase molecule, hence the concentration of reactive sulfhydryl groups on ATPase molecules at a protein concentration of 0.2 mg/ml is about 7 \(\mu\)M. The more effective reagents, such as Hg\(^{2+}\), Ag\(^{+}\), and Cu\(^{2+}\), activate the Ca\(^{2+}\) permeability pathway at a concentration which is comparable to the concentration of reactive sulfhydryl groups on the ATPase.

Air Oxidation of Sulfhydryl Pairs Increases the Ca\(^{2+}\) Permeability of the SR

Hg\(^{2+}\), Ag\(^{+}\), and Cu\(^{2+}\) are often bifunctional; i.e., they can react with two sulfhydryl-containing ligands to form a linear complex. Given this, it seems possible that the extreme reactivity of Hg\(^{2+}\), Ag\(^{+}\), and Cu\(^{2+}\) relative to the other reagents which cause Ca\(^{2+}\) release may be due to binding to two neighboring sulfhydryls. If so, a reagent which can react with two sulfhydryls simultaneously may increase the Ca\(^{2+}\) permeability of the SR. Cupric phenanthroline (in a ratio of two phenanthrolines to one cupric ion) is known to catalyze the air oxidation of two neighboring sulfhydryl groups to form a disulfide (109).
Figure 5 shows the effect of cupric phenanthroline on the permeability of SR vesicles passively loaded with "Ca²⁺. In this experiment, SR vesicles at 5 mg/ml were incubated overnight in 100 mM KCl, 50 mM HEPES, 5 mM MgCl₂, and 5 mM "CaCl₂, with additional 5 μM CuCl₂ and 10 μM o-phenanthroline (Sigma), or 5 μM CuCl₂ alone or 10 o-phenanthroline alone or with no additional reagents, the latter three conditions serving as controls. Ca²⁺ release was initiated by 25-fold dilution into 100 mM KCl, 50 mM HEPES, and 5 mM MgCl₂ and assayed by the Millipore filtration technique. The results of all three control experiments were identical. Attempts to reverse the Cu²⁺/phenanthroline effect by adding a large excess of β-mercaptoethanol in a manner similar to that described in the Hg²⁺ and Ag⁺ experiments was unsuccessful, in contrast to the heavy metal experiments. The inability of this disulfide reducing agent to reverse the oxidation caused by cupric phenanthroline may indicate that Cu²⁺-phenanthroline catalyzes oxidation of sulfhydryls beyond disulfide formation, to higher oxidation states of sulfur which are not reducible with β-mercaptoethanol. Alternately, the irreversibility of Cu²⁺-phenanthroline induced Ca²⁺ release may indicate that formation of disulfides from neighboring sulfhydryls induces a conformational change of the protein which buries the disulfide formed, inhibiting its reduction.
Figure 5. Calcium release induced by air oxidation catalyzed with cupric phenanthroline. SR vesicles at 5 mg/ml were incubated in $^{45}$Ca$^{2+}$ overnight as described previously with (——) and without (—\(-\)) 5 \(\mu\)M CuCl$_2$ and 10 \(\mu\)M o-phenanthroline. Control experiments, in which CuCl$_2$ alone and phenanthroline alone were incubated with the vesicles, were also performed in identical fashion. Ca$^{2+}$ release was measured by the Millipore filtration technique. Values given are mean \(\pm\) SD. All populations of control vesicles showed release comparable to the vesicles which contained neither Cu$^{2+}$ nor phenanthroline.
DISCUSSION

In this chapter it has been shown that, at the proper ratio of metal ion to protein, heavy metals stimulate the ability of the Ca\(^{2+}\) pump to hydrolyze ATP, yet inhibit the ability of isolated SR vesicles to actively accumulate Ca\(^{2+}\). In several previous studies, it was reported that higher concentrations of Hg\(^{2+}\) and sulfhydryl reagents inhibit the pump. At such high Hg\(^{2+}\) to SR protein ratios it was noted that actively accumulated Ca\(^{2+}\) was released from the vesicles (28, 38, 110, 111, 112), yet no systematic study of changes of the Ca\(^{2+}\) permeability of isolated SR vesicles due to sulfhydryl reagents was undertaken. Shamoo and MacLennan (93) showed that the isolated, succinylated Ca\(^{2+}\) pump protein can be inserted into an artificial membrane and thereby increasing the membrane's Ca\(^{2+}\) permeability. They also observed that the Ca\(^{2+}\) ionophoric activity of the reconstituted pump is inhibited by Hg\(^{2+}\) (93). Noting that Hg\(^{2+}\) inhibits Ca\(^{2+}\) uptake, they concluded that Hg\(^{2+}\) uncouples the Ca\(^{2+}\) transport function of the Ca\(^{2+}\) pump from its ATP hydrolysis function. They did not directly measure the permeability of the SR membrane in the presence of Hg\(^{2+}\).

Two interesting facts emerge from a review of the data reported in previous studies: 1) Hg\(^{2+}\) and other sulfhydryl reagents cause Ca\(^{2+}\) release from actively loaded vesicles and 2) Hg\(^{2+}\) inhibits passive Ca\(^{2+}\) transport through a modified reconstituted Ca\(^{2+}\) pump. These facts suggest that
the Hg$^{2+}$-induced release of actively accumulated Ca$^{2+}$ is mediated by some protein other than the Ca$^{2+}$ pump, and thus represents more than simple diffusion back through the inhibited pump, as had been tentatively suggested (28). The data shown in Figures 4 and 5 and in Table I are the first systematic examination of the effect of Hg$^{2+}$ and other sulfhydryl reagents on the Ca$^{2+}$ permeability of the SR. Clearly, the SR is more permeable in the presence of Hg$^{2+}$, etc., perhaps due to activation of the mechanism by which Ca$^{2+}$ exits the SR *in vivo*, in response to depolarization of the T-tubules. Later work (74) supports the hypothesis that Hg$^{2+}$ activates the Ca$^{2+}$ release channel.

In the absence of oxalate, hydrolysis of ATP by the Ca$^{2+}$ pump is stimulated, while Ca$^{2+}$ uptake is inhibited by Hg$^{2+}$ (Figure 3). Shamoo and MacLennan reported that, at the same Hg$^{2+}$:SR ratio, in the presence of oxalate, Ca$^{2+}$ uptake was inhibited but ATP hydrolysis was not affected (93). It appears that, in both cases, Ca$^{2+}$ uptake is inhibited by Hg$^{2+}$ because Ca$^{2+}$ which is actively transported into the lumen of the SR simply leaks back out via this other pathway. In the absence of oxalate, the Ca$^{2+}$ gradient which is normally generated by active uptake into the vesicles is dissipated or greatly reduced by the existence of this Ca$^{2+}$ efflux path. Under normal conditions (i.e. no Hg$^{2+}$, relatively Ca$^{2+}$-impermeable vesicles, no oxalate) the vesicles may accumulate internal free Ca$^{2+}$ concentrations of
up to a few millimolar, while reducing the external free Ca^{2+} concentration to less than a micromolar. This represents a considerable electrochemical potential difference against which the pump must work. Dissipation of this potential by dissipating the Ca^{2+} gradient allows the pump to work at near-optimal activity indefinitely. This is the mechanism by which ionic detergents stimulate ATPase activity (29, 107), and, given the activation of a Ca^{2+} permeability pathway by Hg^{2+} (Figure 4), is probably the mechanism by which Hg^{2+} stimulates ATPase activity (Figure 3). Oxalate is often used to dissipate the transmembrane Ca^{2+} gradient: the SR is freely permeable to oxalate and the solubility product of Ca^{2+}-oxalate is $\approx 3 \times 10^{-9}$ M; with a few mM oxalate present, the internal Ca^{2+} concentration never exceeds a few micromolar, in contrast to the millimolar concentration found in vesicles loaded actively in the absence of oxalate or Ca^{2+} permeability pathways. In the presence of oxalate, activation of a Ca^{2+} permeability pathway does not significantly reduce the already negligible difference in Ca^{2+} electrochemical potential across the membrane, hence stimulation of the Ca^{2+} pump above its already near-optimal activity would not be expected. This explains why Shamoo and MacLennan did not observe stimulation of ATPase activity due to Hg^{2+} (93, 102).

Hg^{2+} reacts strongly with sulfhydryl groups (94), and in order to confirm the nature of the binding site which
triggers heavy metal-induced $\text{Ca}^{2+}$ release, $\text{Ca}^{2+}$ release induced by a variety of heavy metals, sulfhydryl reagents, and other metals was studied (Table I). Those heavy metals that were most effective in causing $\text{Ca}^{2+}$ release are known to bind most strongly to sulfhydryl groups, thus it appears that the binding site is a sulfhydryl group on a protein in the SR. This is also suggested by the fact that $\text{Ca}^{2+}$ release can also be triggered by incubation of the SR with low concentrations of $\text{Cu}^{2+}$-phenanthroline, which catalyzes oxidation of neighboring sulfhydryl groups to form a disulfide group (109). If $\text{Cu}^{2+}$-phenanthroline is catalyzing disulfide formation to cause release, this effect should in principle be reversed by addition of a large excess of the reducing agent $\beta$-mercaptoethanol. This is not, however, the case, though the reason for this discrepancy has not been delineated. Several possible reasons, such as occlusion of the disulfide by conformational change of the protein or oxidation of the sulfhydrlys to oxidation states not reducible by mercaptoethanol, have been suggested.

The research presented in this chapter indicates that micromolar concentrations of heavy metals do not uncouple translocation of $\text{Ca}^{2+}$ across the membrane from hydrolysis of ATP by the pump, as was previously thought (93, 102). Instead, heavy metals induce a large increase in the permeability of SR vesicles. At the concentration of $\text{Mg}^{2+}$ used in the transport assays reported here, the rate and
extent of Ca\textsuperscript{2+} release are dramatic compared with Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (57, 113, 114). As demonstrated elsewhere (74) heavy metals appear to cause this change in permeability by binding to the "physiological release protein".
CHAPTER III

SULPHYDRYL OXIDATION INDUCES RAPID Ca\(^{2+}\) RELEASE FROM ISOLATED SARCOPLASMIC RETICULUM VESICLES

SUMMARY

In this chapter it is demonstrated that micromolar concentrations of cupric ion (Cu\(^{2+}\)) and mercaptans such as cysteine, cysteamine, and homocysteine trigger large and rapid Ca\(^{2+}\) release from skeletal muscle sarcoplasmic reticulum (SR) vesicles. At the concentrations used, Cu\(^{2+}\) alone does not induce Ca\(^{2+}\) release, nor does cysteine alone; both are required to induce Ca\(^{2+}\) release from SR. Cu\(^{2+}\) is known to catalyze the autoxidation of cysteine to its disulfide form cystine, and Cu\(^{2+}\)/mercaptan-induced Ca\(^{2+}\) release appears to be caused by Cu\(^{2+}\) catalyzed formation of a mixed disulfide between the exogenous mercaptan and a critical sulfhydryl on a trans-membrane protein. Supporting evidence for this interpretation are: 1) the order of Ca\(^{2+}\)-releasing reactivity of the mercaptans is the same as the order in which these compounds undergo oxidation to disulfide forms in the presence of Cu\(^{2+}\); 2) Ca\(^{2+}\) efflux induced by cysteine and Cu\(^{2+}\) can be reversed by the addition of the disulfide reducing agent dithiothreitol; 3) hypochlorous acid and plumbagin, both potential sulfhydryl
oxidants, induce rapid Ca\textsuperscript{2+} efflux from SR vesicles; 4) Cu\textsuperscript{2+}, which catalyzes H\textsubscript{2}O\textsubscript{2} oxidation of cysteine, enhances H\textsubscript{2}O\textsubscript{2}-induced release.

Oxidation-induced Ca\textsuperscript{2+} release from SR can be partially reversed or blocked by ruthenium red or the local anaesthetics procaine and tetracaine. The Ca\textsuperscript{2+} efflux rates are strongly Mg\textsuperscript{2+} dependent and are significantly higher in heavy SR than in light SR. These data indicate that oxidation-induced Ca\textsuperscript{2+} efflux occurs via the "Ca\textsuperscript{2+} release channel" and it is suggested that the oxidation state of a critical sulfhydryl group on this protein may be the principal means by which the Ca\textsuperscript{2+} permeability of the SR is regulated in vivo.

INTRODUCTION

Most theories of physiological Ca\textsuperscript{2+} release from SR have one thing in common. Efflux of Ca\textsuperscript{2+} from the SR is presumed to be mediated by a Ca\textsuperscript{2+} channel distinct from the Ca\textsuperscript{2+} pump (Ca\textsuperscript{2+},Mg\textsuperscript{2+}-ATPase). In most studies of Ca\textsuperscript{2+} release from SR, the conditions which lead to Ca\textsuperscript{2+} release have been described phenomenologically in terms of the agents which induce the changes in Ca\textsuperscript{2+} permeability, with little or no discussion on a molecular level as to the mechanisms by which these agents regulate the opening and closing of the "Ca\textsuperscript{2+}-release channel".
In Chapter II it was reported that micromolar concentrations of heavy metal ions (i.e. Cu\(^{2+}\), Hg\(^{2+}\), Ag\(^{+}\), Cd\(^{2+}\), or Zn\(^{2+}\)) trigger the rapid release of Ca\(^{2+}\) by apparently binding to a sulfhydryl group on an integral membrane protein of SR vesicles (also see ref. 73); the potency of these heavy metals to induce Ca\(^{2+}\) release from SR was found to be similar to their relative binding affinities to sulfhydryl groups. In an extension of that study, Ag\(^{+}\) induced release was examined as a function of pH, Mg\(^{2+}\), and ionic strength; in light, heavy, and intermediate SR; and in the presence of known blockers of Ca\(^{2+}\) efflux (74). The data strongly suggest that Ag\(^{+}\) acts at the physiological site of Ca\(^{2+}\) release (74). Other investigators have reported that less reactive sulfhydryl reagents like organic mercurials (50-100 \(\mu\)M) also induce Ca\(^{2+}\) release from SR (28, 115).

It has been shown in both Chapter II and in subsequent work reported by other investigators (116) that cupric phenanthroline, which is known to catalyze the air oxidation of sulfhydryls to disulfides (109), can induce Ca\(^{2+}\) release from the SR, though it has been suggested (116) that the mode of action of cupric phenanthroline may involve cross-linking of the Ca\(^{2+}\),Mg\(^{2+}\)-ATPase and not the oxidation of sulfhydryls on a separate Ca\(^{2+}\) channel, as suggested in Chapter II.
Clearly, heavy metals do not play a role in physiological Ca$^{2+}$ release from SR. However, given that they seem to strongly interact with the Ca$^{2+}$ release channel, they are useful as probes of the functions of this channel and especially the role, if any, of this critical sulfhydryl in the regulation of the Ca$^{2+}$ permeability of the SR in vivo. In the present chapter it is shown that cysteine and other biologically common mercaptans, in the presence of Cu$^{2+}$, induce large and rapid increases in the Ca$^{2+}$ permeability of the SR. Cu$^{2+}$ is known to catalyze the oxidation of cysteine to its disulfide form, cystine (95, 117). The relative potency of the mercaptans to cause release (in the presence of Cu$^{2+}$) is shown to parallel the rate at which these compounds undergo Cu$^{2+}$-catalyzed oxidation to disulfides. Hypochlorous acid (118) and plumbagin (119), both of which can act as sulfhydryl oxidants in the absence of Cu$^{2+}$, are shown to induce Ca$^{2+}$ release from SR vesicles, though at somewhat lower rates. Also Cu$^{2+}$, which is known to stimulate sulfhydryl oxidation by H$_{2}$O$_{2}$ (123), is shown to enhance Ca$^{2+}$ release from SR induced by millimolar H$_{2}$O$_{2}$. The rate of Ca$^{2+}$ efflux induced by Cu$^{2+}$ and cysteine is strongly dependent on free [Mg$^{2+}$] and is optimal at physiological pH and ionic strength. Ca$^{2+}$ efflux induced by Cu$^{2+}$/cysteine is also much faster in heavy SR vesicles than in light, and can be partially blocked or reversed by known inhibitors of Ca$^{2+}$ release, namely
ruthenium red (114, 57), procaine, and tetracaine (57). These properties of Cu²⁺/cysteine-induced Ca²⁺ release from SR are very similar to Ag⁺ and Ca²⁺-induced Ca²⁺ release (74), suggesting a common site of action. The rapid increase in Ca²⁺ permeability of the SR induced by sulfhydryl oxidation may be 1) coincidental with no physiological significance or 2) an indication that sulfhydryl oxidation is the chemical reaction which links the depolarization of the T-tubule membrane with Ca²⁺ release from the SR or 3) an indication that this critical sulfhydryl is involved in regulation of the Ca²⁺ channel though its normal function does not involve undergoing oxidation. A preliminary report on the work presented here has appeared in abstract form (120) and a complete study was published in the Journal of Biological Chemistry in 1986 (75).

MATERIALS AND METHODS

Preparation of SR Vesicles

Rabbit skeletal sarcoplasmic reticulum vesicles were prepared in a manner similar to the method of MacLennan (29), described in detail in Chapter II. One additional change was made to this procedure: 25 µM dithiothreitol (DTT) was added to 4 of the 8 liters of NaCl/Imidazole to protect against oxidation and heavy metals in the isolation procedure, especially the initial steps where the SR is
exposed to metal scalpel blades, blender blades, and meat grinder blades. The buffer used in later steps was free of DTT in order to avoid artifacts associated with DTT. Light and heavy SR (LSR and HSR) vesicles were prepared by further fractionation of the crude SR preparation on a sucrose step gradient as described below. SR, LSR, and HSR vesicles were suspended at approximately 30 mg protein/ml in 100 mM KCl plus 20 mM HEPES at pH 7.0 and frozen in liquid nitrogen until used. The final concentration of DTT in each fraction was approximately 2 μM. Protein concentrations were determined by the method of Lowry et al. (103).

**Preparation of Light and Heavy SR Vesicles.**

Light and Heavy SR vesicles were isolated by fractionating the (crude) SR vesicle preparation of Chapter II on a discontinuous sucrose gradient. Since even "ultrapure" sucrose contains slight heavy metal contaminants, and since the Ca²⁺ release system is sensitive to low concentrations of heavy metals, the sucrose to be used for the gradient fractionation was first subjected to an ion-exchange protocol in order to remove as much of the heavy metals as possible before exposing the SR to the sucrose, then DTT was added to the sucrose solutions in order to prevent oxidation and to protect further against heavy metals.
Preparation of an Ion-Exchange Column for Removing Heavy Metals from Sucrose

A coarse grade (50-100 mesh) Chelex-100 column was prepared in the followed way:

1) Several tablespoons of Chelex 100 (Bio-Rad) were suspended in ~500 ml of a solution of 20 mM HEPES, titrated to pH 7.0 with KOH. The suspension was gently mixed by manual agitation, the Chelex beads were allowed to settle, the excess HEPES solution was poured off, and more HEPES solution was added. This was repeated several times, until the pH of the Chelex suspension was 7.0.

2) A 2.5 X 75 cm glass chromatography column (Bio-Rad) was rinsed several times with dilute HCl and distilled water. The clean column was supported upright with a stand and partially filled with a solution of 20 mM HEPES, pH 7.0.

3) The Chelex/HEPES suspension (pH 7.0) was slowly poured into the column and the beads allowed to settle until a bed of Chelex beads of thickness approximately 3-4 cm formed on the bottom of the column. Excess HEPES solution was allowed to run through the column until only a few mm covered the beads. Care was taken to keep the beads wet at all times, both during the pouring of the column and while running sucrose through.
Preparation of Ion-Free Sucrose

Ion-free (excepting pH buffer) sucrose solution was prepared as follows:

1) 400 ml of a 52 % sucrose (w/v), 20 mM HEPES, pH 7.0 solution was prepared by weighing out 208 grams of sucrose and adding a solution of 20 mM HEPES, pH 7.0 (previously adjusted with KOH) until the volume was 400 ml.

2) The sucrose/HEPES solution was slowly poured into the column and the beads were allowed to settle. The column was opened and sucrose was allowed to run through. The first 25 to 50 ml were discarded, and the rest were collected in an acid-washed glass beaker rinsed with 1 mM EGTA. The flow rate of the sucrose through the column was approximately 10 ml per minute.

3) The solution collected after this treatment is approximately 45 % (w/w) sucrose. Concentrated dithiothreitol (DTT) was added to a final concentration of 100 μM, the pH was adjusted, if necessary, to 7.0, and the sucrose concentration was checked with an Abbe refractometer (note: a 52 % w/v sucrose solution is about 46 % w/w; after running through the column, adding DTT, and adjusting pH, the concentration of sucrose is nearly 45 %, w/w). Sucrose/HEPES was added to bring the sucrose concentration to 45 %, w/w.

4) Using the known densities of sucrose solutions as a guide (CRC Handbook of Chemistry and Physics, "Density of
Sucrose Solutions"), the 20 mM HEPES and 45 % sucrose solutions were mixed in order to make solutions with the following concentrations of sucrose (note: all solutions will have the indicated sucrose concentration and 20 mM HEPES, pH 7.0):

25 % Sucrose (50 ml)
   25.5 ml 45 % Sucrose
   24.5 ml HEPES

29 % Sucrose (90 ml)
   54 ml 45 % Sucrose
   36 ml HEPES

35 % Sucrose (140 ml)
   104.1 ml 45 % Sucrose
   35.9 ml HEPES

41 % Sucrose (90 ml)
   80.4 ml 45 % Sucrose
   9.6 ml HEPES

45 % Sucrose (50 ml)
   50 ml 45 % Sucrose
   no HEPES

250 mM Sucrose (50 ml; 8.25 % sucrose)
   7.85 ml 45 % Sucrose
   42.2 ml HEPES

The sucrose concentrations were checked with an Abbe refractometer. These volumes of solutions are enough for two sucrose gradients, in case a mistake was made in pouring
the first one. The solutions were made fresh no more than two days prior to the preparation of the light and heavy SR vesicles.

**Fractionation of Crude SR into Light and Heavy SR on a Discontinuous Sucrose Gradient**

The sucrose solutions prepared above and the buckets of a Beckman SW28 swinging-bucket rotor (lined with fresh disposable polyallomer tubes) were were put on ice and allowed to cool for an hour or more. A discontinuous sucrose gradient was formed by adding 4, 7, 11, 7, and 4 ml of the 45 %, 41 %, 35 %, 29 %, and 25 % sucrose solutions, in that order, to each bucket. The solutions were added with the aid of a 25 ml Pasteur pipette whose tip had been filed off at an angle of ~30° relative to the axis of the pipette (in contrast to the 90° angle found on standard models). Extreme care was taken to add the sucrose solutions slowly in order to minimize mixing between the layers.

A normal crude SR preparation yields about 500-600 mg of SR. After the last centrifugation step of a crude SR preparation (see previous chapter), prior to resuspension in KCl, about 200-250 mg (i.e. about 40 % of the final SR pellet) of SR to be fractionated into light and heavy SR was suspended in 250 mM sucrose, 20 mM HEPES, pH 7.0 (instead of KCl) to obtain a final volume of no more than 9 ml. The SR/250 mM sucrose suspension was layered onto the tops of
the previously poured sucrose gradients (no more than 1.5 ml per bucket), the buckets were balanced with 250 mM sucrose in opposing pairs and mounted on the rotor head. The SR/sucrose density gradient was centrifuged at 22,000 rpm for 18-20 hours.

The swinging bucket rotor containing the sucrose gradients was allowed to come to a stop without braking. The polyallomer tubes were withdrawn from the buckets and distinct bands of SR were observed at the 29 / 35 % interface (light SR) and at the 35 / 41 % interface (heavy SR). 10 ml syringes fitted with 18 gauge needles were used to pierce the tubes and draw off the protein without mixing. The fractionated vesicles in sucrose were diluted into ~0.5 volumes (or more) of 100 mM KCl, 20 mM HEPES, pH 7.0 (no DTT), and centrifuged at 35,000 rpm in a Ti60 rotor for 60 minutes. The pellets were suspended in the same buffer and centrifuged again at 35,000 rpm for 30 minutes, resuspended in the same buffer, and stored in small aliquots in liquid nitrogen.

**Ca²⁺ Release Experiments**

Ca²⁺ release experiments were performed with SR vesicles which were loaded with Ca²⁺ either passively, or actively in the presence of ATP. Extravesicular Ca²⁺ concentrations were measured through the differential absorption changes of Arsenazo III (ARS III) at 675nm - 685nm (106) with a time-sharing dual wavelength
spectrophotometer (105). This technique was described in detail in Chapter II. Measurements of Ca\textsuperscript{2+} efflux from actively loaded SR vesicles were performed as follows: SR vesicles were suspended at a protein concentration of 0.25 mg/ml in 100 mM KCl, 20 mM HEPES, pH 7.0, .1 mM ARS III, and various concentrations of MgCl\textsubscript{2} at room temperature. Two additions of Ca\textsuperscript{2+} were made to verify the linearity of the dye's response to Ca\textsuperscript{2+} (final [Ca\textsuperscript{2+}] = 15 or 20 μM as indicated) and the differential absorption changes of the dye was continuously monitored. ATP (.5 mM) was added to initiate active Ca\textsuperscript{2+} uptake and the time course of Ca\textsuperscript{2+} uptake was recorded until Ca\textsuperscript{2+} loading was completed (as judged by a leveling-off of the dye signal). Ca\textsuperscript{2+} release was induced by sequentially adding various concentrations of cysteine (or other mercaptans) then Cu\textsuperscript{2+}, or by adding a sulfhydryl oxidizing agent. The maximum efflux rates were calculated by determining the slope of the efflux curve at its steepest phase. The Ca\textsuperscript{2+} efflux rate was calculated as in Chapter II and in some cases the rate constant of efflux was calculated by dividing the rate of efflux (expressed in units of nmol Ca\textsuperscript{2+} effluxed per second per mg SR protein) by the total amount of Ca\textsuperscript{2+} released (in nmol Ca\textsuperscript{2+} per mg protein) to give units of sec\textsuperscript{-1}.

**Effects of pH and Ionic Strength**

Cu\textsuperscript{2+}/cysteine-induced Ca\textsuperscript{2+} release was measured as a function of pH and ionic strength using passively loaded SR
vesicles. For pH-dependent Ca\textsuperscript{2+} efflux, SR vesicles were Ca\textsuperscript{2+} loaded by incubating SR vesicles (10 mg protein/ml) in a medium containing (in mM): 100 KCl, 0.8 MgCl\textsubscript{2}, 0.8 CaCl\textsubscript{2}, 10 HEPES and 10 PIPES at pH values ranging from 6.75 to 8.0. For measurements of Ca\textsuperscript{2+} efflux as a function of ionic strength the vesicles were suspended at 10 mg protein/ml in solutions containing (in mM) 20 HEPES, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, pH 7.25, and various concentrations of KCl and sucrose to obtain a final osmolarity of approximately 225 mOsm. In both studies, the vesicles were incubated on ice for 12 to 18 hours to allow complete equilibration of free Ca\textsuperscript{2+} concentration across the SR membrane. The passively loaded SR vesicles were rapidly diluted by a factor of 40 in similar but Ca\textsuperscript{2+}-free media containing 0.1 mM ARS III at room temperature. The differential absorption of the Ca\textsuperscript{2+} indicator was continuously monitored to follow the passive efflux of Ca\textsuperscript{2+} from the lumen of the SR (at 1 mM Ca\textsuperscript{2+}) to the extravesicular medium (at 25-35 \textmu M Ca\textsuperscript{2+}). Sequential additions of cysteine then CuCl\textsubscript{2} initiated a rapid rise (under optimal conditions) of the Ca\textsuperscript{2+}-dye signal (Ca\textsuperscript{2+} efflux) which gradually leveled off after a period of time. Upon completion of the experiment, the Ca\textsuperscript{2+} ionophore A23187 was added (0.5 ug/ml) to abolish any residual Ca\textsuperscript{2+} gradient across the SR membrane and thus to determine the total internal Ca\textsuperscript{2+} content of the vesicles. At this point, a known amount of Ca\textsuperscript{2+} was added in order to provide an
internal calibration of the Ca\(^{2+}\) dye response for that particular experiment. The rate constant of efflux was calculated from the rate of Ca\(^{2+}\) efflux (in nmol Ca\(^{2+}\)/mg protein per second) divided by the total internal Ca\(^{2+}\) loading of the vesicles (in nmol Ca\(^{2+}\)/mg protein). The percentage of Ca\(^{2+}\) effluxed was calculated by dividing the amount of Ca\(^{2+}\) effluxed due to Cu\(^{2+}\)/cysteine by the total internal Ca\(^{2+}\) loading of the vesicles. Each measurement of efflux under a given set of conditions was repeated four times and the mean efflux rate and standard deviation were calculated.

**Measurement of Sulfhydryl Oxidation Rates with DTNB**

The reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's Reagent) has been used extensively to quantitatively determine sulfhydryl concentration at pH above 8.0 (121). DTNB provides a convenient optical assay for studying sulfhydryl group oxidation since it does not interact with disulfides and because it exhibits a large change in absorption at 410 nm upon reacting with sulfhydryls. Rates of sulfhydryl oxidation of cysteine, cysteamine, homocysteine, and glutathione (at room temperature) were measured in the presence of Cu\(^{2+}\) using DTNB with the following protocol. An aliquot of a solution (0.9 ml) containing 100 mM KCl, 5 mM HEPES, and either 1 or 10 mM MgCl\(_2\), pH 7.0 (Solution A) was mixed with 0.1 ml of a solution B containing 1 mM DTNB dissolved in 120 mM Tris-
HCl, pH 8.5. The final pH of the mixture (A+B) was 8.1. The absorbance at 410 nm of this mixture provided the "reference" base line absorption against which other mixtures were compared. The sulfhydryl compound (30 μM) to be tested was added to 0.9 ml of solution A plus 2, 4, or 6 μM Cu²⁺. At chosen time intervals, 0.1 ml of solution B was added and the absorption of the new mixture was recorded. The concentration of non-oxidized sulfhydryl groups left in the medium was computed by comparing the absorption of the reaction mixture with that of a control reaction mixture which did not contain Cu²⁺. The resulting plot of remaining free sulfhydryl groups vs time was linear, as expected (this reaction is zero order with respect to [cysteine] (117)); the rate of sulfhydryl oxidation was computed from the slope of this line.

Materials

The buffers HEPES and PIPES, and the sulfhydryl reducing agent dithiothreitol (DTT) were purchased from Research Organics (Cincinnati, OH). Imidazole was purchased from Aldrich Chemical Co. Hydrogen peroxide (H₂O₂), MgCl₂ and CaCl₂ were purchased from J.T. Baker Chemicals. ATP and the Ca²⁺ ionophore A23187 were purchased from Calbiochem-Behring. Vacuum-distilled hypochlorous acid (HOCl) was a gift of Dr. J. Michael Albrich of the Oregon Graduate Center. Chelex-100 (50-100 mesh; sodium form) was obtained from Bio-Rad. Ultrapure sucrose was purchased from Schwarz-
Mann. Superoxide dismutase, catalase, and all other reagents were purchased from Sigma Chemical.

RESULTS

Sulfhydryl Oxidation with Mercaptans Induces Ca\textsuperscript{2+} Release from SR Vesicles

In Figure 6, the differential absorption changes (\(\Delta A = A_{675} - A_{685}\)) of ARS III were used to monitor ATP-dependent Ca\textsuperscript{2+} uptake followed by Cu\textsuperscript{2+}/cysteine-induced Ca\textsuperscript{2+} release from SR vesicles. Two equal additions of Ca\textsuperscript{2+} to the SR reaction mixture produced equal changes in \(\Delta A\) verifying the linearity of the dye response in the medium. An addition of ATP initiated active Ca\textsuperscript{2+} uptake by SR vesicles as inferred by the decrease in \(\Delta A\). Sequential additions of Cu\textsuperscript{2+} (2 \(\mu\)M) then cysteine (10 \(\mu\)M) induced a partial but rapid release of the accumulated Ca\textsuperscript{2+}, recorded by a rapid rise in \(\Delta A\). Reversing the order of cysteine and Cu\textsuperscript{2+} additions did not alter the rate or extent of Ca\textsuperscript{2+} release. However, the addition of either reagent alone or of a premixed solution of Cu\textsuperscript{2+} and cysteine to Ca\textsuperscript{2+}-loaded SR vesicles did not induce Ca\textsuperscript{2+} release. Indeed, after a few minutes a mixture of Cu\textsuperscript{2+} and cysteine in these proportions contains little or no reduced cysteine because of the Cu\textsuperscript{2+}-catalyzed oxidation of cysteine to form cystine (95, 117). Addition of oxidized cysteine (cystine), did not induce Ca\textsuperscript{2+} release from SR.
Figure 6. ATP-driven Ca²⁺ uptake and Cu²⁺/cysteine-induced Ca²⁺ release from sarcoplasmic reticulum vesicles. SR vesicles were suspended at a protein concentration of 0.25 mg/ml in 100 mM KCl, 20 mM HEPES, 1mM MgCl₂, and 0.1 mM ARS III. Two aliquots of 10 μM CaCl₂ were then added and the difference in absorbance between 675 nm and 685 nm was monitored as a measure of external [Ca²⁺]. Uptake was initiated by addition of 0.5 mM ATP (free [Mg²⁺] ~0.6 mM). When uptake was judged to be complete, small amounts of Cu²⁺ and cysteine were added sequentially, triggering Ca²⁺ release. Ca²⁺ release rates were calculated from the steepest slope of the release curve. Addition of 2 μM ruthenium red (RR) caused immediate re-uptake of 90% of the released Ca²⁺. Addition of the Ca²⁺ ionophore A23187 caused complete release of the remaining internal Ca²⁺.
Upon completion of Ca\textsuperscript{2+} efflux (indicated by a leveling-off of the absorption changes of the Ca\textsuperscript{2+} indicator), subsequent addition of 2 \mu M ruthenium red resulted in the active re-uptake of the released Ca\textsuperscript{2+}. Alternatively, ruthenium red added at the beginning of the experiment or just after ATP-dependent Ca\textsuperscript{2+} uptake blocked Cu\textsuperscript{2+}/cysteine-induced Ca\textsuperscript{2+} release (not shown). Similarly, other inhibitors of Ca\textsuperscript{2+} release like procaine (10 mM) or tetracaine (1 mM) blocked or reversed Cu\textsuperscript{2+}/cysteine-induced Ca\textsuperscript{2+} release (not shown). At the end of each experimental run, the Ca\textsuperscript{2+} ionophore A23187 was added to release all the Ca\textsuperscript{2+} stored in the lumen of the SR and to verify that the total Ca\textsuperscript{2+} released (after A23187) was equal to the total Ca\textsuperscript{2+} taken up by the vesicles. Under the given experimental conditions, the total Ca\textsuperscript{2+} actively sequestered by the SR vesicles was consistently in the range of 100 ± 10 nmoles Ca\textsuperscript{2+}/mg protein.

The experiment shown in Figure 6 was repeated with cysteine and the structurally related mercaptans cysteamine and homocysteine. In Figure 7, the Cu\textsuperscript{2+} concentration was kept constant at 2 \mu M while the concentrations of cysteine, cysteamine and homocysteine were varied over a wide range of values. For each experimental run, the rate of Ca\textsuperscript{2+} efflux induced by the addition of Cu\textsuperscript{2+} and the mercaptan was calculated from the maximum slope of the efflux trace and the rates of Ca\textsuperscript{2+} efflux were plotted as a function of
Figures 7a. and 7b. Dependence of Ca$^{2+}$ release rate on concentrations of cysteine, cysteamine, and homocysteine. SR vesicles were suspended at 0.25 mg protein/ml in 100 mM KCl, 20 mM HEPES, 1 mM MgCl$_2$ and 0.1 mM ARS III, pH = 7.0. Ca$^{2+}$ uptake was initiated by addition of 0.5 mM ATP (free [Mg$^{2+}$] ~0.6 mM) and Ca$^{2+}$ release was initiated by adding the indicated amounts of mercaptan followed by addition of 2 μM CuCl$_2$. Ca$^{2+}$ efflux rates were calculated from the steepest slope of the efflux curve. (Note the difference in scale between the two figures.)
mercaptan concentration. The relationship between rate of efflux and mercaptan concentration exhibits several interesting features:

(1) At its optimal concentration, cysteine is more reactive than cysteamine and both are much more effective in triggering release than homocysteine. (Note the change in scale between Figures 7A and 7B).

(2) Ca\(^{2+}\) efflux rates first increase with increasing mercaptan concentration, reaching a maximum rate, then decreasing with further increase in mercaptan concentration.

(3) Cu\(^{2+}\)/mercaptan-induced Ca\(^{2+}\) release is inhibited by excess mercaptan (~10-fold or more over [Cu\(^{2+}\)]). Thus, the mercaptans act as both agonists (at low concentrations) and as inhibitors (at high concentrations) of Ca\(^{2+}\) release.

These features are consistent with Cu\(^{2+}\)-catalyzed formation of mixed disulfide bonds between the thiol group on the added cysteine and a critical thiol group on the Ca\(^{2+}\) channel. For fixed protein and Cu\(^{2+}\) concentrations, at low cysteine concentrations the ratio of cysteine: Cu\(^{2+}\): channel appears to favor formation of mixed disulfides, while at higher cysteine concentrations the relatively small amount of Cu\(^{2+}\) spends more of its time catalyzing the formation of cystine and less time catalyzing mixed disulfide formation.
On the other hand, the Cu$^{2+}$-catalyzed oxidation of cysteine is known to be approximately first order with respect to Cu$^{2+}$ (95, 117). Assuming that the mechanism of mixed disulfide formation is the same as that of cystine formation, a plot of Ca$^{2+}$ efflux rate vs [Cu$^{2+}$] should be roughly linear. In Figure 8 this prediction is borne out: Ca$^{2+}$ efflux rates vs [Cu$^{2+}$] are plotted at two fixed concentrations of cysteine, 5 and 20 μM; the protein concentration was 0.25 mg/ml in each case. The curve is linear over a range of Cu$^{2+}$ concentration from zero up to equimolarity with the added cysteine.

The rate at which mercaptans autoxidize in the presence of Cu$^{2+}$ parallels their ability to induce Ca$^{2+}$ release from SR, as shown in Tables II and III. This is a noteworthy observation, but it is emphasized that in the first case (autoxidation of mercaptans, Table II), the reaction is between two identical molecules, while in the second (Ca$^{2+}$ efflux induced by Cu$^{2+}$ and mercaptan) Ca$^{2+}$ efflux rates are used to assay for the relative rates of reaction between dissimilar molecules (i.e. the mercaptan and the Ca$^{2+}$ release protein). For Table II, the rate of sulphydryl oxidation of cysteine, cysteamine, homocysteine, and glutathione was measured with DTNB as described above. Cysteine and cysteamine self-oxidized rapidly compared to homocysteine, while glutathione oxidation was too slow to detect over the
Figure 8. Dependence of Ca²⁺ release rate on Cu²⁺ concentration. SR vesicles were suspended at 0.25 mg protein/ml in 100 mM KCl, 20 mM HEPES, 3 mM MgCl₂, 0.1 mM ARS III, pH 7.0. 15 μM Ca²⁺ was added, followed by 0.5 mM ATP (free [Mg²⁺] ~2.5 mM). When uptake was complete, 5 or 20 μM cysteine was added, followed by various amounts of CuCl₂. Release rates were calculated from the steepest portion of the efflux curve.
The mercaptans listed were suspended at 30 μM in 100 mM KCl, 5 mM HEPES, and either 1 or 10 mM MgCl₂, pH 7.0 (.9 ml total volume). 2 μM CuCl₂ was added and then at times indicated, 0.1 ml of 120 mM Tris-HCl, 1 mM DTNB, pH 8.5 was added (final pH = 8.1). The concentration of unoxidized -SH groups in solution is proportional to the absorbance of light by DTNB at 410 nm; absorbance at 410 nm was monitored in a Beckman model DU-7 spectrophotometer. The rate of oxidation of -SH groups was calculated from the slope of the resulting absorbance vs time plot.

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>[Cu²⁺]</th>
<th>[Mg²⁺]</th>
<th>OXIDATION RATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYSTEINE</td>
<td>2 μM</td>
<td>1 mM</td>
<td>.060 μM/sec</td>
</tr>
<tr>
<td></td>
<td>4 μM</td>
<td>1 mM</td>
<td>.108 μM/sec</td>
</tr>
<tr>
<td></td>
<td>6 μM</td>
<td>1 mM</td>
<td>.162 μM/sec</td>
</tr>
<tr>
<td></td>
<td>4 μM</td>
<td>10 mM</td>
<td>.133 μM/sec</td>
</tr>
<tr>
<td>CYSTEAMINE</td>
<td>4 μM</td>
<td>1 mM</td>
<td>.070 μM/sec</td>
</tr>
<tr>
<td>HOMOCYSTEINE</td>
<td>4 μM</td>
<td>1 mM</td>
<td>.004 μM/sec</td>
</tr>
</tbody>
</table>
TABLE III

**Ca²⁺ RELEASE ACTIVITY OF MERCAPTANS AND DISULFIDE COMPOUNDS**

SR vesicles were suspended at 0.25 mg protein/ml in 100 mM KCl, 20 mM HEPES, and 1 mM MgCl₂. 20 μM CaCl₂ was added followed by .5 mM ATP (free [Mg²⁺] = 0.6 mM). Upon completion of Ca²⁺ uptake, various concentrations of mercaptan or disulfide compound and 2 μM CuCl₂ were added sequentially. Mercaptans are listed in their order of effectiveness at inducing Ca²⁺ release.

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>STRUCTURE</th>
<th>CONCENTRATION (μM)</th>
<th>RELEASE RATE (nmol/sec)/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYSTEINE</td>
<td>COOH-CH-CH₂-SH</td>
<td>5</td>
<td>11.0</td>
</tr>
<tr>
<td>CYSTEAMINE</td>
<td>NH₂-CH₂-CH₂-SH</td>
<td>5</td>
<td>3.3</td>
</tr>
<tr>
<td>NH₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOMOCYSTEINE</td>
<td>COOH-CH-CH₂-CH₂-SH</td>
<td>20</td>
<td>0.8</td>
</tr>
<tr>
<td>MERCAPTO-PROPIonic ACID</td>
<td>COOH-CH₂-CH₂-SH</td>
<td>100*</td>
<td>3.3</td>
</tr>
<tr>
<td>MERCAPTO-ETHANOL</td>
<td>HO-CH₂-CH₂-SH</td>
<td>100</td>
<td>0.6</td>
</tr>
<tr>
<td>NH₂ CH₃</td>
<td>PENICILLAMINE</td>
<td>100**</td>
<td>0.4</td>
</tr>
<tr>
<td>CH₃</td>
<td>COOH-CH-C-SH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COENZYME A</td>
<td>300</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>GLUTATHIONE</td>
<td>300</td>
<td>trace</td>
<td></td>
</tr>
</tbody>
</table>

Notes: * Not effective at 20 μM.
** Requires 3 μM CuCl₂. (continued overleaf)
TABLE III (continuation)

RELATED BUT INEFFECTIVE COMPOUNDS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>METHIONINE</td>
<td>( \text{NH}_2 - \text{COOH-CH-CH}_2 - \text{CH}_2 - \text{S-CH}_3 )</td>
</tr>
<tr>
<td>CYSTEIC ACID</td>
<td>( \text{NH}_2 - \text{COOH-CH-CH}_2 - \text{SO}_2 \text{H}_2 )</td>
</tr>
<tr>
<td>CYSTINE</td>
<td>( \text{NH}_2 - \text{COOH-CH-CH}_2 - \text{S-S-CH}_2 - \text{CH-COOH} )</td>
</tr>
<tr>
<td>CYSTAMINE</td>
<td>( \text{NH}_2 - \text{CH}_2 - \text{CH}_2 - \text{S-S-CH}_2 - \text{CH}_2 - \text{NH}_2 )</td>
</tr>
</tbody>
</table>

time scale of these experiments. As shown in Table III, glutathione is also ineffective at inducing Ca\(^{2+}\) release when in the presence of Cu\(^{2+}\), while cysteine, cysteamine, and homocysteine are effective, and to degrees comparable to their Cu\(^{2+}\)-catalyzed autoxidation reactivity. The efflux experiments summarized in Table III were performed essentially in the same manner as those of Figures 6 and 7, with the exception that mercaptans other than cysteine were added, at the concentrations indicated.

The chemical reactivity of sulfhydryl groups is known to be enhanced by the close proximity of an amine, thus it is not surprising that cysteine and cysteamine are more reactive than homocysteine (homocysteine's hydrocarbon chain is one carbon longer than those of cysteine and cysteamine). Penicillamine's sulfhydryl is attached to a tertiary carbon,
which is also known to greatly reduce reactivity (122). Consistent with sulfhydryl oxidation-induced Ca\(^{2+}\) release, the oxidized forms of cysteine and cysteamine were not effective at inducing Ca\(^{2+}\) release either in the presence or absence of Cu\(^{2+}\), nor was methionine, the S-methyl derivative of homocysteine. The sulfur atom of methionine can form strong complexes with heavy metal ions but this configuration does not generally lead to oxidation (122).

In addition to Cu\(^{2+}\)/mercaptan-induced Ca\(^{2+}\) release from SR, we have observed Ca\(^{2+}\) release induced by sulfhydryl oxidants, summarized in Table IV. Plumbagin most likely acts by oxidizing neighboring sulfhydryls to disulfides (119) and although less certain, it is probable that hypochlorous acid (HOCl) acts in the same way. Hydrogen peroxide (\(\text{H}_2\text{O}_2\)) is a well-known thiol oxidant whose activity as a thiol oxidant is greatly stimulated by the presence of Cu\(^{2+}\) ion (123), thus the stimulation of \(\text{H}_2\text{O}_2\)-induced release with Cu\(^{2+}\) again implies sulfhydryl oxidation.

If Ca\(^{2+}\) release is caused by the oxidation of a critical sulfhydryl to form a mixed disulfide, then if the disulfide is still accessible, a disulfide reducing agent should in principle reverse the effect of Cu\(^{2+}\) and cysteine. In Figure 9, SR vesicles were suspended in a standard KCl buffer with 1 mM MgCl\(_2\), loaded with 20 \(\mu\text{M}\) Ca\(^{2+}\) using 1 mM Mg\(^{2+}\)-ATP, then Ca\(^{2+}\) efflux was induced by addition of 10 \(\mu\text{M}\) cysteine and 2\(\mu\text{M}\) Cu\(^{2+}\) (free [Mg\(^{2+}\)] at time of DTT addition
TABLE IV

SULPHHYDRYL OXIDANTS INDUCE Ca²⁺ RELEASE

SR vesicles were suspended at .25 mg protein/ml in 100 mM KCl, 20 mM HEPES, and 3 mM MgCl₂. 20 µM CaCl₂ was added followed by .5 mM ATP (free [Mg²⁺] ~2.5 mM). Upon completion of Ca²⁺ uptake, addition of either 50 µM plumbagin or 100 µM HOCl induced Ca²⁺ release; addition of 2 mM H₂O₂ induced release which was stimulated by prior addition of 2 µM Cu²⁺.

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>CONCENTRATION</th>
<th>RELEASE RATE (nmol/sec)/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLUMBAGIN</td>
<td>50 µM</td>
<td>3.5</td>
</tr>
<tr>
<td>HOCl</td>
<td>100 µM</td>
<td>0.1</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>2 mM</td>
<td>0.2</td>
</tr>
<tr>
<td>H₂O₂ (+ 2 µM Cu²⁺)</td>
<td>2 mM</td>
<td>0.8</td>
</tr>
</tbody>
</table>

1.2 mM). 2 mM dithiothreitol (DTT) (124) was added after the extravesicular Ca²⁺ concentration had reached a new steady-state (indicating completion of the channel-activating reaction, the rate of Ca²⁺ efflux from the vesicles being equal to the rate of Ca²⁺ pumping). Addition of the DTT led to re-uptake of most (~80%) of the released Ca²⁺ within three minutes.

By-Products of Cu²⁺-Catalyzed Cysteine Oxidation do not Induce Ca²⁺ Release

Clearly, the Cu²⁺-catalyzed oxidation of cysteine leads to Ca²⁺ release from SR. The two end products of this
Figure 9. Reversal of Cu²⁺/cysteine-induced Ca²⁺ release with dithiothreitol (DTT). SR vesicles were suspended at 0.25 mg protein/ml in 100 mM KCl, 20 mM HEPES, 0.1 mM ARS III, 1 mM MgCl₂ and 20 μM Ca²⁺. Ca²⁺ uptake was initiated by addition of 1 mM Mg-ATP (free [Mg²⁺] ~1.2 mM at time of DTT addition). Efflux was initiated by sequential addition of 10 μM cysteine and 2 μM CuCl₂, and after completion of efflux, 2 mM DTT was added, resulting in slow re-uptake of ~80% of the released Ca²⁺.
process are cystine and \( \text{H}_2\text{O}_2 \), with the latter presumably being formed via superoxide radical as an intermediate \((95, 117)\). As noted in Table III, cystine does not induce \( \text{Ca}^{2+} \) release from SR. The concentration of \( \text{H}_2\text{O}_2 \) produced by cysteine oxidation is equal to the original cysteine concentration \((117)\). Experiments similar to that shown in Figure 6 indicate that even 50 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (10 times the optimal concentration of cysteine, at 2 \( \mu \text{M} \) \( \text{Cu}^{2+} \)) does not induce \( \text{Ca}^{2+} \) release, whether in the presence of 2 \( \mu \text{M} \) \( \text{Cu}^{2+} \) or not (not shown). Superoxide dismutase catalyzes the aqueous disproportionation of superoxide radical to \( \text{H}_2\text{O}_2 \) and molecular oxygen, while catalase catalyzes the aqueous reduction of \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \). Neither of these enzymes had any discernible effect on \( \text{Cu}^{2+}/\text{cysteine-induced Ca}^{2+} \) efflux rates, even at molar enzyme concentrations equal to the concentration of \( \text{Cu}^{2+} \) (data not shown). Thus, the side products (i.e. \( \text{H}_2\text{O}_2 \) and superoxide radical) and end product (i.e. free cystine in solution) of \( \text{Cu}^{2+} \)-catalyzed autoxidation of cysteine to cystine do not appear to participate in \( \text{Cu}^{2+}/\text{cysteine-induced Ca}^{2+} \) release from SR.

Properties of \( \text{Cu}^{2+}/\text{Cysteine-Induced Ca}^{2+} \) Release

The rate of \( \text{Ag}^{+} \)-induced \( \text{Ca}^{2+} \) release is strongly affected by \( \text{Mg}^{2+} \), pH, ionic strength, and upon where the SR vesicles were derived from, (i.e. from longitudinal SR (LSR) or from terminal cisternae (HSR)) \((75)\). Similarly, \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release is strongly affected by \( \text{Mg}^{2+} \) and the
morphological lineage of the vesicles (39, 70). The rate of Ca\textsuperscript{2+} release induced by Cu\textsuperscript{2+}/cysteine was examined as a function of these parameters and strong similarities between Cu\textsuperscript{2+}/cysteine-induced Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} release induced by these other two methods was found. The rate of Ca\textsuperscript{2+} efflux induced by Cu\textsuperscript{2+}/cysteine is plotted as a function of total Mg\textsuperscript{2+} in the medium in Figure 10. In this study, SR vesicles (.25 mg protein/ml) were suspended in 100 mM KCl, 20 mM HEPES, 0.1mM ARS III, 15 \mu M CaCl\textsubscript{2}, and 0.5, 1, 3, 5, 7.5, or 10 mM MgCl\textsubscript{2}. Ca\textsuperscript{2+} uptake was initiated by addition of 0.5 mM ATP, then when the uptake was complete, 10 \mu M cysteine and 2 \mu M CuCl\textsubscript{2} were added to induce release. The ratio of ATP to ADP at the time of addition of Cu\textsuperscript{2+} and cysteine was about 4 to 1 so that the free Mg\textsuperscript{2+} concentration was about 0.2, 0.6, 2.6, 4.6, 7.1, and 9.6 mM, respectively. As shown in the figure, the rate and extent of Ca\textsuperscript{2+} release was inhibited with increasing free Mg\textsuperscript{2+}, a characteristic similar to Ag\textsuperscript{+}-induced Ca\textsuperscript{2+} release from SR vesicles and Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from both isolated SR vesicles (57) and from SR in skeletal muscle fibers (78). This is in contrast to the rate of Cu\textsuperscript{2+}-catalyzed cysteine oxidation, which is slightly stimulated by high Mg\textsuperscript{2+}, as shown in Table II. The lower rates of efflux at higher [Mg\textsuperscript{2+}] thus seem to be due to inhibition of Ca\textsuperscript{2+} transport across the membrane and not inhibition of sulfhydryl oxidation.
Figure 10. Dependence of Cu\(^{2+}\)/cysteine-induced Ca\(^{2+}\) release rate on total Mg\(^{2+}\) concentration. SR vesicles were suspended at 0.25 mg/ml protein in 100 mM KCl, 20 mM HEPES, 0.1 mM ARS III, 15 μM CaCl\(_2\), and various concentrations of MgCl\(_2\). External Ca\(^{2+}\) concentration was followed by monitoring differential absorbance changes at 675 - 685 nm. Ca\(^{2+}\) uptake was initiated by addition of 0.5 mM ATP; upon completion of uptake, Ca\(^{2+}\) was initiated by addition of 10 μM cysteine and 2 μM CuCl\(_2\). The free [Mg\(^{2+}\)] is lower for all points above 1 mM by approximately 0.5 mM; for the 0.5 mM MgCl\(_2\) point the free [Mg\(^{2+}\)] is ~0.2 mM, while for the 1 mM point the free [Mg\(^{2+}\)] is ~0.6 mM.
To examine Ca$^{2+}$ release from SR as a function of pH and ionic strength, SR vesicles (10 mg protein/ml) were passively loaded with Ca$^{2+}$ by incubation overnight in .8 mM Ca$^{2+}$ and various concentrations of sucrose, KCl, buffers, and pH (see figure captions). The vesicles were diluted by a factor of 40 (final [protein] = 0.25 mg/ml) into Ca$^{2+}$-free but otherwise identical media containing 0.1 mM ARS III. Ca$^{2+}$ efflux was initiated by addition of 10 μM cysteine and 2 μM Cu$^{2+}$ and monitored spectrophotometrically as described previously. The rate constants (efflux rate in nmol Ca$^{2+}$/second per mg protein divided by Ca$^{2+}$ loading in nmol Ca$^{2+}$/mg protein) and extent (%) of Cu$^{2+}$/cysteine-induced Ca$^{2+}$ release as functions of pH and ionic strength are plotted in Figures 11 and 12. The rate of Ca$^{2+}$ release has a broad maximum at around pH 7.0 to 7.25 (Figure 11), and decreases with decreasing ionic strength (Figure 12); both characteristics are similar to Ca$^{2+}$ release triggered by Ag$^+$ (74), and are consonant with conditions prevailing in vivo. The rate constant reported at pH 7.25, 100 mM KCl, 0.8 mM MgCl$_2$, corresponds to an efflux rate of ~1.6 nmol Ca$^{2+}$ per second per mg protein.

The rate of Cu$^{2+}$/cysteine-induced Ca$^{2+}$ release from actively-loaded heavy SR and light SR vesicles was measured spectrophotometrically. Light or heavy SR vesicles were suspended at 0.25 mg protein/ml in 100 mM KCl, 20 mM HEPES, 1 mM MgCl$_2$, pH 7.0, with 0.1 mM ARS III and loaded with 20
Figure 11. Rate of Cu^{2+}/cysteine-induced Ca^{2+} release as a function of pH. SR vesicles were suspended at 10 mg/ml in 100 mM KCl, .8 mM MgCl_2, .8 mM CaCl_2, 10 mM HEPES and 10 mM PIPES, at the indicated pH. After incubation on ice for 12 to 18 hours the vesicles were diluted fortyfold into similar solutions containing 0.1mM ARS III but no added Ca^{2+} (final [protein] = 0.25 mg/ml). Ca^{2+} fluxes were monitored by measuring differential absorbance changes at 675 - 685 nm. Ca^{2+} efflux was triggered by addition of 10 μM cysteine and 2 μM CuCl_2. Upon completion of Ca^{2+} release, 0.5 μg/ml A23187 was added in order to determine the total Ca^{2+} content of the vesicles and then an internal calibration was made by adding a known amount of Ca^{2+} at the end of the experiment. The percentage of Ca^{2+} efflux (—△—) was calculated by dividing the amount of Ca^{2+} effluxed after Cu^{2+}/cys by the total internal Ca^{2+} content of the vesicles. Efflux rate constants (—○—) were calculated by dividing the Ca^{2+} efflux rate (in nmol Ca^{2+}/second per mg protein) by the total Ca^{2+} loading of the vesicles (in nmol Ca^{2+}/mg protein).
Figure 12. Rate of Cu²⁺/cysteine-induced Ca²⁺ release as a function of ionic strength. SR vesicles were suspended at 10 mg/ml in 20 mM HEPES, 1 mM MgCl₂, 1 mM CaCl₂, pH 7.25 with the indicated concentrations of KCl and sucrose. After incubation on ice for 12 to 18 hours the vesicles were diluted fortyfold into similar solutions which contained 0.1 mM ARS III but no added Ca²⁺. Ca²⁺ fluxes were measured by monitoring ΔA675-685. Ca²⁺ efflux was triggered by adding 10 µM cysteine and 2 µM CuCl₂. Ca²⁺ efflux rate constants and percent effluxed (— △—) were calculated as in figure 11.
μM CaCl₂ by addition of 0.5 mM Mg²⁺-ATP (free [Mg²⁺] ~ 1 mM) as described in Figure 6; Ca²⁺ release was initiated by addition of 10 μM cysteine and 2 μM CuCl₂. Under these conditions, the Ca²⁺ efflux rate from heavy SR was 9.8 nmol Ca²⁺/mg protein per second while the rate of efflux from light SR was about 2.3 nmol Ca²⁺/mg protein per second.

**DISCUSSION**

In this chapter it has been demonstrated that various mercaptans in the presence of Cu²⁺ induce rapid Ca²⁺ release from SR. These results have been confirmed with Millipore filtration techniques using ⁴⁰Ca²⁺ as a tracer, with another metallochromic indicator, antipyrylazo III, and with a Ca²⁺ sensitive electrode (data not shown). Ca²⁺ release from SR induced by Cu²⁺/cysteine shares many similarities with Ag⁺ and Ca²⁺-induced Ca²⁺ release: all three methods of inducing Ca²⁺ release are inhibited by procaine, tetracaine, and ruthenium red. Also all three show similar responses to free Mg²⁺. In addition, Cu²⁺/cysteine-induced Ca²⁺ release is significantly faster in HSR than LSR, another common characteristic of Ag⁺ and Ca²⁺-induced Ca²⁺ release.

Ag⁺ and the other heavy metals appear to induce Ca²⁺ release from SR by binding to a critical sulfhydryl group which is closely associated with Ca²⁺ release system of the SR (Chapter II, 74, 75), but the Cu²⁺/mercaptan effect seems to be due to Cu²⁺ catalyzed cross-linking of the
exogenous mercaptan's sulfhydryl group with this critical sulfhydryl associated with the Ca\textsuperscript{2+} release system. Arguments supporting this oxidative mechanism of Ca\textsuperscript{2+} release are:

1) Ca\textsuperscript{2+} release induced by Cu\textsuperscript{2+} and cysteine is reversed by addition of an excess of the disulfide reducing agent dithiothreitol (DTT; Figure 9).

2) The rates of Cu\textsuperscript{2+}-catalyzed oxidation of mercaptans (Table II) parallels the rates of Cu\textsuperscript{2+}-assisted Ca\textsuperscript{2+} release induced by these mercaptans (Table III).

3) The Ca\textsuperscript{2+} release rate induced by Cu\textsuperscript{2+} and cysteine appears to be first order with respect to Cu\textsuperscript{2+} (Figure 8), as expected for Cu\textsuperscript{2+}-catalyzed oxidation (95, 117).

4) Hypochlorous acid (118) and plumbagin (119), both potential sulfhydryl oxidants, induce Ca\textsuperscript{2+} release from SR in the absence of Cu\textsuperscript{2+}, while Cu\textsuperscript{2+}, which enhances the rate of H\textsubscript{2}O\textsubscript{2} oxidation of cysteine, stimulates Ca\textsuperscript{2+} release induced by 2 mM H\textsubscript{2}O\textsubscript{2}. (Table IV).

An issue of interest is the possible role of redox reactions in excitation-contraction coupling in skeletal muscle. The data presented here indicate that formation of a mixed disulfide between exogenous mercaptan and a sulfhydryl associated with the Ca\textsuperscript{2+} release channel induces Ca\textsuperscript{2+} release from SR. The pH profile of Cu\textsuperscript{2+}/cysteine-
induced $\text{Ca}^{2+}$ release (Figure 11) suggests that an analogous process may occur in $\text{Ca}^{2+}$ release from SR in vivo. Yet $\text{Cu}^{2+}$/cysteine-induced $\text{Ca}^{2+}$ release from isolated vesicles the reverse process (i.e. closing the channel with DTT) are too slow to be of physiological significance. If sulfhydryl redox reactions control the opening and closing of the $\text{Ca}^{2+}$ channel, these reactions would almost certainly be enzymatically catalyzed. The activities of several non-transport proteins are known to be regulated by the oxidation states of sulfhydryl pairs which can switch from the dithiol to the disulfide state and back on a time scale of a few milliseconds. For example, lipoamide dehydrogenase is regulated by intracellular $\text{NAD(H)}$ (125) and glutathione reductase is regulated by intracellular $\text{NADP(H)}$ (125). Also, Robillard and Konings (126) describe a dithiol/disulfide exchange reaction which may be the means of regulating the activities of a number of microbial membrane transport systems. In view of the parallels between $\text{Ca}^{2+}$- and oxidation-induced $\text{Ca}^{2+}$ release, and the fact that the molecular mechanism underlying $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release is unknown, it is suggested that the underlying mechanism of $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release may involve oxidation or some other perturbation of this critical sulfhydryl. For example, $\text{Ca}^{2+}$ may regulate an enzyme responsible for catalyzing sulfhydryl redox reactions linked to $\text{Ca}^{2+}$ release or, if $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release involves direct binding of
Ca\(^{2+}\) to the Ca\(^{2+}\) channel, this critical sulfhydryl may be one of the functional groups involved in gating the channel, whether it undergoes oxidation or not.

In analyzing these results, the interpretation emphasized has been one in which Cu\(^{2+}\) acts as a catalyst in the formation of a mixed disulfide between the exogenous mercaptan and an essential sulfhydryl on the Ca\(^{2+}\) channel. It should be noted, however, that the Cu\(^{2+}\) catalyzed oxidation of cysteine does transiently produce Cu\(^{+}\). Under aerobic conditions, however, Cavallini et al (95) place an upper limit on the number of copper atoms in the +1 state at 20% while Zwart et al (117) estimate this number to be more like 2%. The +1 oxidation state of copper is reportedly more stable in an aerobic environment when the Cu\(^{+}\) ion is at the center of a coordination complex between two or more high affinity ligands (such as sulfhydryl groups) (127). However, given the low initial concentration of Cu\(^{2+}\) and the necessarily even lower Cu\(^{+}\) concentration, Cu\(^{+}\) binding or complex formation would seem an unlikely mechanism even without the data supporting the oxidation hypothesis (i.e. the cysteine dependence in Figure 7, the reversal with DTT, and plumbagin-, HOCl-, and Cu\(^{2+}/\text{H}_2\text{O}_2\)- induced release).

Another possible explanation for the effects of Cu\(^{2+}\) and cysteine would be that Cu\(^{2+}\) catalyzes a thiol/disulfide exchange reaction between the cysteine thiol and a protein disulfide (128), though the oxidative mechanism again seems
more likely. Finally, in Chapter V it is shown that several proteins are covalently labeled by $^{35}$S-cysteine when in the presence of Cu$^{2+}$, indicating positively that covalent attachment of cysteine to SR proteins does occur when Cu$^{2+}$ is present.

Whether or not sulfhydryl oxidation is engaged in the in vivo gating of the Ca$^{2+}$ channel of the SR, it is interesting to note that the mercaptan penicillamine (which is either a sulfhydryl oxidant or reductant, depending on conditions) has been used to slow down the onset of muscular dystrophy in chickens (129). Also, elevated levels of Ca$^{2+}$ have been implicated in the onset of this disease (130). I suggest that, in this clinical context in the absence of Cu$^{2+}$, penicillamine may protect the same critical sulfhydryls involved in Cu$^{2+}$/cysteine-induced release from pathological oxidation; the result of this protection would be maintenance of normal resting-state Ca$^{2+}$ permeability levels for the membrane, thus lower cytosolic Ca$^{2+}$ concentrations. Seen in this light, oxidation-induced Ca$^{2+}$ release from SR may hold important clues for understanding muscular dystrophy in addition to understanding the functions of normal muscle.
CHAPTER IV

TRYPTIC PROTEOLYSIS STIMULATES ACTIVATION OF Ca^{2+} RELEASE FROM ISOLATED SARCOPLASMIC RETICULUM VESICLES

INTRODUCTION

A number of investigators have studied the effects of tryptic proteolysis on the SR. Most of the effort has centered on structure-function relationships between the tryptic fragments of the Ca^{2+},Mg^{2+}-ATPase in the hope of elucidating the mechanisms of active Ca^{2+} transport (131, 132, 97). The effect of proteolysis on passive Ca^{2+} transport has received less attention. Shoshan-Barmatz et al (98) have shown that tryptic proteolysis of the SR inhibits ATP-driven Ca^{2+} uptake of isolated SR vesicles by greatly increasing the Ca^{2+} permeability of the SR. The paths of Ca^{2+} permeability created by proteolysis do not appear to be fragments of the ATPase, and it was suggested, though not demonstrated, that the increase in Ca^{2+} permeability is due to cleavage of the physiological Ca^{2+} release channel of the SR. In another study, Toogood et al (133) showed that proteolysis of the SR "exposes an ionophore" which can passively transport Ca^{2+} across the membrane of preloaded vesicles, also suggesting that this ionophore may be a fragment of the Ca^{2+} channel of the SR.
Neither of these reports are conclusive with regard to the identity of the ionophore or mechanism of increased Ca\textsuperscript{2+} permeability. Toogood et al (133) established that the ionophore is a channel or pore, while Shoshan-Barmatz et al (98) showed that the main Ca\textsuperscript{2+} permeability pathway(s) created by limited proteolysis are not fragments of the Ca\textsuperscript{2+},Mg\textsuperscript{2+}-ATPase.

In the present chapter it is shown that tryptic proteolysis, under somewhat milder conditions than the previous studies cited above (in terms of time exposed to trypsin and concentration of trypsin), stimulates the activation of passive Ca\textsuperscript{2+} transport across the membrane of isolated SR vesicles. Ca\textsuperscript{2+} efflux from tryptically modified SR (TMSR) vesicles in the presence of micromolar Ca\textsuperscript{2+}, micromolar heavy metals, micromolar anthraquinones, and millimolar adenine nucleotides is faster than from native SR by a factor of as much as 25, yet Ca\textsuperscript{2+} release from TMSR is inhibited by micromolar ruthenium red, millimolar Ca\textsuperscript{2+}, and Mg\textsuperscript{2+}. Kinetic analysis shows that the binding characteristics of these modulators of Ca\textsuperscript{2+} release do not change despite the drastic change in transport rate. The data indicate that limited tryptic proteolysis stimulates the activation mechanism of the Ca\textsuperscript{2+} release channel of the SR without changing the binding affinities for these modulators of Ca\textsuperscript{2+} release.
MATERIALS AND METHODS

Preparation of SR Vesicles

Rabbit skeletal sarcoplasmic reticulum vesicles were prepared according to the method of MacLennan (29). Light and heavy density SR (LSR and HSR) vesicles were prepared by the method of Meissner (39), with an additional final centrifugation step in order to lower the sucrose concentration to ~10 mM (for full description, see below). All SR preparations were suspended at a protein concentration of 25 to 30 mg/ml in 100 mM KCl, 20 mM HEPES at pH 7.0, and frozen in liquid nitrogen until used. Protein concentrations were determined by the method of Lowry et al (103).

Ca²⁺ Permeability Studies

Passive Ca²⁺ Loading of SR Vesicles. The passive efflux of Ca²⁺ from SR vesicles (both native and tryptically modified) was measured isotopically with radiolabeled §Ca²⁺ and spectrophotometrically with the Ca²⁺ indicating dye arsenazo III (ARS III). In both cases, the SR vesicles were passively loaded with Ca²⁺ by incubation. For spectrophotometric studies, SR vesicles were suspended at a protein concentration of 10 mg/ml in 100 mM KCl, 20 mM HEPES, 1mM CaCl₂, pH 7.0. The vesicles were incubated for 18 to 24 hours on ice in order to allow equilibration of free Ca²⁺ concentration across the membranes. For isotopic
studies the vesicles were suspended at a protein concentration of 5 mg/ml in 100 mM KCl, 20 mM HEPES, and 1 mM CaCl₂ with a small addition of the radioisotope ⁴⁵Ca²⁺. The specific activity of the ⁴⁵Ca²⁺ was ~1 μCi/μmole Ca²⁺ (1000 cpm/nmole).

**Tryptic Modification of SR Vesicles.** Tryptic modification of the SR was carried out on passively loaded vesicles after first warming them to room temperature (21 to 25 °C, as indicated in the captions) then adding trypsin to obtain a final trypsin:SR protein ratio of 0.005 to 0.0075 (w/w); control vesicles were warmed and treated exactly as the modified vesicles, but were not subjected to trypsin. For measurement of permeability vs time of proteolysis, Ca²⁺ efflux was initiated by diluting the vesicles into an efflux-inducing medium at predetermined times after addition of the trypsin (see below). In other studies it was desirable to create a population of vesicles which had all undergone the same extent of modification. In these cases, proteolysis was stopped at the indicated time by addition of an excess of soybean trypsin inhibitor (STI). The permeability of the tryptically modified SR (TMSR) vesicles were then assayed in the presence of various concentrations of Ca²⁺ release activators as described below.

**Spectrophotometric Assay of Ca²⁺ Permeability.** Ca²⁺ efflux from the native or TMSR vesicles was assayed by dilution of the passively loaded vesicles into 40 volumes of
a medium containing 100 mM KCl, 20 mM HEPES, 0.1 mM arsenazo
III (ARS III), and varying concentrations of Ca$^{2+}$
permeability modulators such as adenine nucleotides (39, 57,
58, 72, 134), HgCl$_2$ (Chapter II, 73), doxorubicin (83), Cu$^{2+}$
and cysteine (Chapter III, 75), MgCl$_2$ (38, 57, 58) or
ruthenium red (Chapter III, 57). Extravesicular Ca$^{2+}$
concentrations were monitored spectrophotometrically and
Ca$^{2+}$ release rates were calculated as described in Chapter
II. The Ca$^{2+}$ content of passively loaded longitudinal SR
(LSR) and terminal cisternae SR (HSR) vesicles differed
significantly. In order to compare the relative
permeabilities of LSR and HSR, the efflux rates were
normalized to the Ca$^{2+}$ content of the vesicles and an
"effective rate constant" (sec$^{-1}$) was calculated by dividing
the initial rate of Ca$^{2+}$ efflux from the vesicles (nmol Ca$^{2+}$
per mg SR protein per sec) by the total Ca$^{2+}$ content of the
vesicles (nmol Ca$^{2+}$ per mg SR protein).

**Isotopic Assay of Ca$^{2+}$ Permeability.** The effect of
external Ca$^{2+}$ on Ca$^{2+}$ efflux from native and TMSR vesicles
was studied isotopically. SR vesicles were passively loaded
with $^{45}$Ca$^{2+}$ by incubation. The loaded vesicles were
tryptically modified as described above. Samples were
prepared for assay of Ca$^{2+}$ permeability in the following
way. Small aliquots of native SR or TMSR were diluted 50-
fold into 100 mM KCl, 20 mM HEPES, and (to retard premature
efflux) 10 mM MgCl$_2$. The diluted Ca$^{2+}$-loaded SR vesicle
suspension was then immediately applied to a 0.65 μm Millipore filter. Excess liquid was drawn through the filter under vacuum, resulting in an even distribution of loaded SR vesicles over the surface of the filter. Residual extravesicular "Ca²⁺ was washed from the filter by passing 2 ml of the KCl/HEPES/10 mM Mg²⁺ buffer through the filter. Ca²⁺ efflux from the vesicles was initiated by drawing a Ca²⁺ efflux-activating medium through the SR vesicle/filter matrix for pre-set time intervals ranging from 20 msec to 1 sec with the aid of a Bio-Logic RFS-4 rapid filtration apparatus (BioLogic, Grenoble, France; 135). The efflux buffer contained 100 mM KCl, 20 mM HEPES, 1 mM EGTA, 1 mM cAMP, and various concentrations of CaCl₂ to give buffered free Ca²⁺ concentrations over a range of 10 nM to 1 mM. Free Ca²⁺ concentrations were calculated with the aid of binding constants and an algorithm reported by Fabiato and Fabiato (136). The first order rate constants of Ca²⁺ efflux at each free external Ca²⁺ concentration were calculated by linear regression of \( \text{Ln(CPM}(t) - \text{CPM}(\infty)) \) vs time.

**Kinetic Analysis of Ca²⁺ Transport Activation.** Several investigators (58, 137) have analyzed the kinetics of activation of the Ca²⁺ channel with adenine nucleotides. Similar analyses of cAMP- and doxorubicin- activated Ca²⁺ release, from both native SR and TMSR, were performed for the purpose of determining the specific effects of
proteolysis on the binding of these compounds to their activation sites. The assumption underlying these analyses is that activation of Ca$^{2+}$ release with cAMP and doxorubicin follows a rate equation similar in form to that proposed by Hill in his study of oxygen binding to hemoglobin (138):

$$R([\text{activator}]) = \frac{R_{\text{max}} \times [\text{activator}]^h}{K_{\text{act}} + [\text{activator}]^h}$$

where $R$ is the "response" of the channels to a given concentration of activator, found by subtracting the rate of Ca$^{2+}$ efflux in the absence of activator from the rate of efflux in the presence of that concentration of activator, $R_{\text{max}}$ is the Ca$^{2+}$ efflux response to saturating concentration of activator, $K_{\text{act}}$ is the binding constant of the activator to its site, and $h$ is the Hill coefficient, indicating the degree of cooperativity between activator binding sites. When $h$ is equal to 1, this equation reduces to a form similar to the Michaelis-Menten equation. The rate equation shown above (the Hill equation) can be transformed to several linear forms. One such transformation, proposed by Hill, yields a straight line when $\ln(R/(R_{\text{max}}-R))$ is plotted as a function of $\ln([\text{activator}])$ (Hill plot). Another linear form of the Hill equation is similar to that of Hanes' transformation of the Michaelis-Menten equation, and a modified Hanes plot ($[\text{activator}]^h/R$ vs $[\text{activator}]^h$) should also be linear, to the degree that the Hill equation accurately represents activation of Ca$^{2+}$ transport. Using
these two linear forms of the Hill equation, I have developed an iterative technique for determining the kinetic parameters of activation of Ca$^{2+}$ release. The rate of Ca$^{2+}$ efflux from SR vesicles is measured as a function of the concentration of activator. Using an initial estimate of $R_{\text{max}}$, the efflux rate vs [activator] data are arranged in a form suitable for a Hill plot (i.e. $\log(R/(R_{\text{max}}-R))$ vs $\log([\text{activator}])$). Linear regression analysis yields an estimate of the Hill coefficient $h$ (slope) and $K_{\text{act}}$ (the X-intercept is $-\log(K_{\text{act}})$). The estimated Hill coefficient is then used to arrange the raw data in a form suitable for a modified Hanes plot: $[\text{activator}]^{b}/R$ vs $[\text{activator}]^{b}$. Linear regression analysis yields a new estimate of $R_{\text{max}}$ (1/slope) and $K_{\text{act}}$ (from the X-intercept). The new estimate of $R_{\text{max}}$ is used to perform a new Hill analysis and the process is reiterated until the two independent estimates of $K_{\text{act}}$ converge. Typically, 20 iterations were required for convergence. The adaptation of the Hill equation to Ca$^{2+}$ transport activation and the Pascal program performing the iterative calculations are summarized in Appendices A and B.

Materials

The buffer HEPES was purchased from Research Organics (Cincinnati, OH). Imidazole was purchased from Aldrich Chemical Co. MgCl$_2$ and CaCl$_2$ were purchased from J.T. Baker Chemicals. Ultra pure sucrose was purchased from Schwartz-Mann. The Ca$^{2+}$ ionophore A23187 was purchased from
Calbiochem-Behring. Trypsin (bovine pancreas), soybean trypsin inhibitor, and all other reagents were purchased from Sigma Chemical.

RESULTS

Tryptic Modification Enhances Activation of Ca\textsuperscript{2+} Release.

Adenine nucleotides (134, 58, 137) are known to activate Ca\textsuperscript{2+} efflux from SR vesicles while ruthenium red is a known inhibitor of Ca\textsuperscript{2+} release (57). Figure 13 shows spectrophotometric traces of Ca\textsuperscript{2+} efflux from native SR and TMSR vesicles in the absence and presence of cAMP and ruthenium red. The efflux rates calculated from these traces are displayed in Table V. In trace 1, passively loaded SR vesicles were diluted into 40 volumes of a Ca\textsuperscript{2+}-free medium containing 1 mM MgCl\textsubscript{2} and Ca\textsuperscript{2+} efflux from the vesicles was monitored spectrophotometrically. In trace 2 the dilution (efflux) medium contained 0.5 mM cAMP and 1 mM MgCl\textsubscript{2}. Traces 3, 4, and 5 are recordings of Ca\textsuperscript{2+} efflux from TMSR. In trace 3 the efflux medium was the same as in trace 1, while in trace 4 the efflux medium was the same as in trace 2; in trace 5 the efflux medium contained 0.5 mM cAMP, 1 mM MgCl\textsubscript{2} and 1 µM ruthenium red. Comparison of the initial slopes of these traces shows that tryptic proteolysis increases the permeability of the vesicles in the absence of any Ca\textsuperscript{2+} releasing activator by a factor of \textsuperscript{4} (slope of trace 3/slope of trace 1) while the
Figure 13. Ca\(^{2+}\) release from passively loaded native and trypically modified SR (TMSR) vesicles. SR vesicles (10 mg/ml) were passively loaded with 1 mM Ca\(^{2+}\) by incubation. A portion were then exposed to trypsin (trypsin:SR = 0.005 w/w) for 12 minutes at 22 °C (TMSR). The Ca\(^{2+}\) permeabilities of native and TMSR vesicles were assayed by 40-fold dilution into 100 mM KCl, 20 mM HEPES, 1 mM MgCl\(_2\), 0.1 mM of ARSIII, pH 7.0, 22°C, with optional additions of 0.5 mM cAMP and 1 μM Ru red. Extravesicular [Ca\(^{2+}\)] was monitored by following the differential absorption of the dye at 675 and 685 nm. Traces 1 and 2: Ca\(^{2+}\) efflux from native SR vesicles in the absence and presence of 0.5 mM cAMP. Traces 3 and 4: Ca\(^{2+}\) efflux from TMSR vesicles in the absence and presence of 0.5 mM cAMP. Trace 5: efflux from TMSR in the presence of both 0.5 mM cAMP and 1 μM Ru red. The Ca\(^{2+}\) ionophore A23187 was added at the end of each run to release residual internal Ca\(^{2+}\), then a known amount of Ca\(^{2+}\) was added in order to calibrate the response of the dye to Ca\(^{2+}\). Ca\(^{2+}\) efflux rates (see Table V) were calculated from the initial slopes of the efflux traces.
TABLE V

SUMMARY OF Ca²⁺ RELEASE RESPONSE OF NATIVE AND TMSR VESICLES TO CAMP.

This table summarizes the results of Figure 13. Rates shown are given in nmol Ca²⁺ released/mg protein/sec. The "response" of the SR to cAMP is the rate of Ca²⁺ efflux in the presence of cAMP minus the efflux rate in the absence of cAMP (for native SR this is the slope of trace 2 minus the slope of trace 1, while for TMSR this is the slope of trace 4 minus the slope of trace 3). The stimulation factor is the ratio of the response of the TMSR vesicles to that of the native vesicles.

<table>
<thead>
<tr>
<th>Trace#</th>
<th>Rate</th>
<th>Response</th>
<th>Stim. factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.27</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.0</td>
<td>2.36</td>
<td>23.6</td>
</tr>
<tr>
<td>5</td>
<td>0.09</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

permeability of native vesicles is increased by 0.5 mM cAMP by a factor of ~1.6 (slope of trace 2/slope of trace 1). If the enhancement of efflux via proteolysis is due to nonspecific degradation of the membrane or of some component other than the Ca²⁺ channels, we would expect the effects of proteolysis and cAMP to be additive, and therefore the Ca²⁺ efflux rate from TMSR vesicles in the presence of 0.5 mM cAMP would be ~6 times greater than that of native vesicles in the absence of cAMP (the rate due to nonspecific "leakiness" + the rate due to transport through the Ca²⁺ channel). Instead, as summarized in Table V, the combined
effects of proteolysis and cAMP are more than
multiplicative: the rate of efflux from TMSR vesicles in the
presence of cAMP is ~20 times greater than the rate of
efflux from native vesicles in the absence of cAMP (slope of
trace 4/slope of trace 1). The response of the channels to
0.5 mM cAMP (as defined above in the method of kinetic
analysis: slope of trace 4 minus slope of trace 3 and slope
of trace 2 minus slope of trace 1) is greater in TMSR by a
factor of ~24. Trace 5 shows that ruthenium red clearly
inhibits Ca\(^{2+}\) release from the TMSR vesicles even in the
presence of cAMP. Proteolysis apparently enhances cAMP
activation of Ca\(^{2+}\) transport through the Ca\(^{2+}\) channels while
the site of ruthenium red inhibition remains functional.

The combination of trypsin to SR ratio, temperature,
and time of proteolysis shown in Figure 13 was chosen so as
to maximize the effect of proteolysis on cAMP activation of
Ca\(^{2+}\) efflux. Figure 14 shows the time course of proteolytic
stimulation of Ca\(^{2+}\) efflux in the presence and absence of
cAMP. In both cases, the Ca\(^{2+}\) efflux rate rises to a
maximum at about 18 minutes of proteolysis, followed by a
somewhat slower decline in efflux rate with further
proteolysis. Proteolysis for much longer times leads to
more rapid efflux again, though this enhancement of efflux
with more extensive proteolysis is not inhibited by
ruthenium red (not shown).
Figure 14. Ca\textsuperscript{2+} efflux rate in the absence and presence of cAMP as a function of time of proteolysis. SR vesicles were incubated overnight in 1 mM CaCl\textsubscript{2}, KCl, and HEPES as in Figure 13. The vesicles were then exposed to trypsin at a trypsin:SR ratio of 0.005 in a water bath held at 22 °C. At the indicated times, an aliquot of SR was diluted 40-fold into 100 mM KCl, 20 mM HEPES, 1 mM MgCl\textsubscript{2}, 0.1 mM ARSIII, and either no cAMP (---) or 0.2 mM cAMP (----); the external [Ca\textsuperscript{2+}] was monitored spectrophotometrically. Ca\textsuperscript{2+} efflux rates were calculated from the initial slopes of the efflux traces.
The Ca\textsuperscript{2+} channels of the SR are believed to be localized primarily in the terminal cisternae (39). If proteolytic enhancement of Ca\textsuperscript{2+} release is due to modification of the Ca\textsuperscript{2+} channel then cAMP activation of Ca\textsuperscript{2+} release from vesicles derived from the terminal cisternae of the SR (HSR vesicles) should be stimulated by proteolysis, while cAMP-activated Ca\textsuperscript{2+} release from longitudinal SR (LSR) vesicles should not be stimulated by proteolysis. As shown in Figure 15a, the time course of tryptic stimulation of Ca\textsuperscript{2+} efflux from HSR vesicles is similar to tryptic stimulation of the native SR preparation (refer to Figure 14). There is a gradual, and then accelerated, increase in rate, followed by gradual decline. In contrast, though efflux from LSR vesicles is also stimulated by proteolysis (Figure 15b), the degree of stimulation is less, and the rate of efflux increases without ever reaching a maximum. Another important difference is that Ca\textsuperscript{2+} efflux from HSR vesicles is strongly inhibited by ruthenium red at all stages of proteolysis, while efflux from LSR is only weakly inhibited. Given that proteolysis does not appear to affect the mechanism of ruthenium red inhibition of efflux (trace 5, Figure 13), the difference between the efflux rate in the absence of ruthenium red and the rate in the presence of ruthenium red is presumably proportional to Ca\textsuperscript{2+} release channel activity. Some stimulation of Ca\textsuperscript{2+} channel activity appears in LSR,
Figure 15. Effective rate constant of Ca\textsuperscript{2+} efflux from HSR and LSR in absence and presence of ruthenium red, as a function of time of proteolysis. HSR (15a; this page) and LSR (15b; overleaf) vesicles were passively loaded with 1 mM Ca\textsuperscript{2+} by incubation as described above. The vesicles were warmed to room temperature in a water bath (21 °C) and trypsin was added (trypsin:SR protein = 0.0075 w/w). At the indicated times following addition of trypsin, an aliquot of vesicles was diluted 40-fold into a solution containing 100 mM KCl, 20 mM HEPES, 1 mM MgCl\textsubscript{2}, 1 mM cAMP, 0.1 mM ARSIII, pH 7.0, (○) and optional addition of 10 μM Ru red (△). Extravesicular [Ca\textsuperscript{2+}] was monitored spectrophotometrically and the rates of Ca\textsuperscript{2+} efflux were calculated as described above. "Effective" rate constants were obtained by dividing the efflux rates by the total Ca\textsuperscript{2+} content of the vesicles.
Fig 15b. Effective rate constant of Ca$^{2+}$ release from LSR vesicles in presence and absence of ruthenium red as a function of time of proteolysis.
but it is small compared to that of HSR, and perhaps represents some HSR contamination in the LSR preparation. Since proteolytic stimulation of Ca$^{2+}$ release activity occurs predominantly in HSR vesicles and is inhibited by ruthenium red, the stimulation appears to be due to modification of the Ca$^{2+}$ release channel or some related protein. Furthermore, since the Ca$^{2+}$ pump is more heavily concentrated in LSR than in HSR, yet efflux from proteolytically modified LSR vesicles is much slower than from modified HSR, it appears that tryptic fragments of the Ca$^{2+}$ pump created by limited proteolysis do not provide a significant pathway for Ca$^{2+}$ efflux. It is possible that the slight stimulation of efflux from LSR is due to extensive proteolysis of a small percentage of Ca$^{2+}$ pumps. This would explain the modest enhancement of non-ruthenium red-inhibitable Ca$^{2+}$ efflux from LSR at relatively short times and the non-ruthenium red-inhibitable stimulation of Ca$^{2+}$ efflux from both HSR and LSR at much longer times of proteolysis (not shown).

**Characterization of Tryptically Modified SR**

It is apparent that limited proteolysis stimulates activation of Ca$^{2+}$ transport through the Ca$^{2+}$ channels of the SR, but the mechanism of this stimulation is unclear. Several possibilities exist. For example, the increase in transport rate may be due to the removal of inhibitory mechanisms, such as the Mg$^{2+}$ inhibition site. Alternately,
stimulation may be the result of modification of regulatory sites which gate the Ca\textsuperscript{2+} channel, and there may be an increase in cooperativity between activator binding sites or more Ca\textsuperscript{2+} channels may be exposed to activating agents as a result of proteolysis. In an effort to determine the mechanism by which proteolysis stimulates Ca\textsuperscript{2+} release activation, the Ca\textsuperscript{2+} release response of both native and tryptically modified SR vesicles to known modulators of the Ca\textsuperscript{2+} release channels is characterized below.

Adenine nucleotide-activated Ca\textsuperscript{2+} release from SR vesicles is strongly dependent on the external Ca\textsuperscript{2+} concentration (39). Figure 16 indicates that, in the presence of 1 mM cAMP, both native SR and TMSR vesicles show peak Ca\textsuperscript{2+} transport activation between 1 and 10 μM extravesicular free Ca\textsuperscript{2+}, though again release from TMSR is much faster than from native SR. Both the activation (at micromolar Ca\textsuperscript{2+}) and inhibition (at millimolar Ca\textsuperscript{2+}) sites appear to be intact after proteolysis, indicating that tryptic stimulation of Ca\textsuperscript{2+} release is not the result of modification of either of these sites.

Mg\textsuperscript{2+} is a well-documented inhibitor of Ca\textsuperscript{2+} efflux from isolated SR vesicles (39, 57, 58). A possible explanation for the stimulation of Ca\textsuperscript{2+} release in the presence of 1 mM MgCl\textsubscript{2}, as seen in Figures 13, 14, and 15 above, is that the inhibitory Mg\textsuperscript{2+} binding site is altered by proteolysis. This hypothesis was tested by measuring the
Figure 16. Rate constant of Ca$^{2+}$ release from native and TMSR vesicles as a function of external Ca$^{2+}$ concentration. SR vesicles (5 mg protein/ml) were passively loaded with $^{45}$Ca$^{2+}$ by incubation in 100mM KCl, 20 mM HEPES, and 1 mM $^{45}$CaCl$_2$ overnight on ice. A Bio-Logic RFS-4 rapid filtration apparatus was used to determine the rate constants of Ca$^{2+}$ efflux from native (16a; this page) and TMSR (trypsin:SR = 0.005 for 10 minutes at 22 °C. 16b; overleaf) vesicles in the presence of the indicated concentrations of external free Ca$^{2+}$, 100 mM KCl, 20 mM HEPES, and 1 mM cAMP as described in the Methods section of this paper.
Figure 16b. Rate constant of Ca\(^{2+}\) efflux from tryptically modified SR vesicles as a function of external Ca\(^{2+}\) concentration.
rate of Ca\(^{2+}\) efflux from tryptically modified SR vesicles in the presence of varying concentrations of Mg\(^{2+}\), in the absence (Figure 17a) and presence (Figure 17b) of 0.1 mM cAMP. In both 17a and 17b the shapes of the Mg\(^{2+}\) inhibition curves for native and TMSR vesicles are nearly identical, though the rate (vertical) scales differ in both cases by a factor of 20. The binding characteristics of the Mg\(^{2+}\)/inhibitory site complex thus appear to be unchanged, indicating that the mechanism of Mg\(^{2+}\) inhibition of Ca\(^{2+}\) release from isolated SR vesicles is also unaffected by this limited degree of proteolysis.

Ruthenium red is a potent inhibitor of Ca\(^{2+}\) release from SR (57) and, as shown in Figure 13, trace 5, is also a potent inhibitor of Ca\(^{2+}\) release from tryptically modified SR. Figure 18 shows the rate of Ca\(^{2+}\) release from both native and TMSR vesicles in a medium containing 0.6 mM Mg\(^{2+}\), 0.3 mM cAMP, and various concentrations of ruthenium red. Ruthenium red clearly inhibits Ca\(^{2+}\) release from TMSR as well as from native SR. Also note that even at relatively high [ruthenium red], Ca\(^{2+}\) efflux is still evident in both populations of vesicles, indicating a path of Ca\(^{2+}\) efflux independent of the Ca\(^{2+}\) channels. Ca\(^{2+}\) transport through this independent path is also increased by proteolysis, though only by a factor of 2. The behavior of this independent path is similar to the Ca\(^{2+}\) efflux pathways of LSR, as shown in Figure 15b. It contributes a very minor
Figure 17. Rate of Ca\textsuperscript{2+} release from native and TMSR as a function of [Mg\textsuperscript{2+}]. SR vesicles were passively loaded by overnight incubation in 100 mM KCl, 20 mM HEPES, and 1 mM CaCl\textsubscript{2}, pH 7.0. Half of the incubated vesicles were exposed to trypsin (trypsin:SR = 0.005) for 10 minutes in a water bath held at 23 °C; proteolysis was terminated by addition of a 5-fold excess of soybean trypsin inhibitor. Passive Ca\textsuperscript{2+} efflux from the native SR vesicles (---) and TMSR vesicles (——) was measured spectrophotometrically as in previous figures. In 17a (this page), the efflux medium contained 100 mM KCl, 20 mM HEPES, 0.1 mM ARSIII and the indicated concentrations of MgCl\textsubscript{2}; in 17b (overleaf) the efflux medium was similar, but it contained an addition of 0.1 mM cAMP.
Figure 17b. Rate of $\mathrm{Ca}^{2+}$ release from native and TMSR as a function of $\mathrm{Mg}^{2+}$ in presence of 0.1 mM cAMP.
Figure 18. Rate of Ca$^{2+}$ release from native and TMSR vesicles as a function of ruthenium red. SR vesicles (10 mg/ml) were passively loaded with 1 mM Ca$^{2+}$ by incubation as described above. A portion of the loaded vesicles were exposed to trypsin (trypsin:SR = 0.005) at 23 °C for 12 minutes. Ca$^{2+}$ efflux from native SR (—x—) and TMSR (—o—) vesicles was initiated and monitored spectrophotometrically following a 40-fold dilution into 100 mM KCl, 20 mM HEPES, 0.6 mM MgCl$_2$, 0.3 mM cAMP, 0.1 mM ARSIII, pH 7.0, and the following concentrations of Ru red (in μM): 0, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, and 5. Bars indicate the range of 2 repetitions at each [Ru red].
component of the total Ca\textsuperscript{2+} flux and probably represents, as suggested above, a small population of extensively modified Ca\textsuperscript{2+} pumps.

Figures 19a and 19b show the rate of Ca\textsuperscript{2+} efflux from native and tryptically modified SR vesicles as a function of cAMP concentration. The data points with error bars indicate the mean and standard deviation of three measurements of efflux rate at each cAMP concentration. The solid curve was generated from the kinetic parameters found by the iterative algorithm described in the method of kinetic analysis. These parameters are reported in Table VI. As shown in this table, the binding constant for cAMP and the Hill coefficient for cAMP activation of Ca\textsuperscript{2+} efflux are essentially unchanged after limited tryptic proteolysis, but the maximum response of the channel to cAMP is greater by a factor of 20.

Doxorubicin is a potent antineoplastic agent (82) which has also been shown to activate rapid Ca\textsuperscript{2+} release from SR vesicles (83). The Ca\textsuperscript{2+} permeability of the SR as a function of [doxorubicin] was examined in both native SR (Figure 20a) and proteolytically modified SR (Figure 20b). The data points and error bars represent the mean and standard deviation of three measurements of Ca\textsuperscript{2+} efflux rate at each doxorubicin concentration. The solid curve was generated with the parameters listed in Table VI, found by the iterative algorithm described previously. Apparently,
Figure 19. Rate of Ca\(^{2+}\) release from native and TMSR as a function of cAMP concentration. SR vesicles were incubated at 10 mg protein/ml in 100 mM KCl, 20 mM HEPES, 1 mM CaCl\(_2\), pH 7.0 on ice overnight. 19a (this page), the vesicles were diluted 40-fold into a medium containing the indicated concentrations of cAMP, 100 mM KCl, 20 mM HEPES, 1 mM MgCl\(_2\) and 0.1 mM ARSIII, pH 7.0, at 22 °C. 19b (overleaf), the vesicles were exposed to trypsin for 10 minutes (trypsin:SR = 0.005, w/w) at 25 °C prior to dilution. Points plotted are the mean ± the standard deviation of three determinations of efflux rate. The solid curves were generated from the parameters listed in Table VI, found by the iterative technique described in the text.
Figure 19b. Rate of Ca\textsuperscript{2+} release from TMSR as a function of cAMP concentration.
Figure 20. Rate of Ca\textsuperscript{2+} release from native and TMSR as a function of doxorubicin concentration. SR vesicles were passively loaded with 1 mM CaCl\textsubscript{2} in 100 mM KCl, 20 mM HEPES by overnight incubation. Native SR vesicles (20a, this page) and trypsinized SR vesicles (trypsin:SR = 0.0075, temp. = 22°C, time = 10 minutes; 20b, overleaf) were diluted 40-fold into 100 mM KCl, 20 mM HEPES, 1 mM MgCl\textsubscript{2}, and 0.1 mM ARSIII. Ca\textsuperscript{2+} efflux was monitored spectrophotometrically and efflux rates were calculated by the initial slopes of the efflux traces. Points plotted are the mean ± standard deviation of three determinations of efflux at each [doxorubicin]. The solid curve is the best-fit curve to the Hill equation as determined by the iterative technique described in the text. The kinetic parameters thus determined are listed in Table VI.
Figure 20b. Rate of Ca²⁺ release from TMSR as a function of doxorubicin concentration.
TABLE VI
SUMMARY OF KINETIC ANALYSIS OF CAMP AND DOXORUBICIN ACTIVATION OF Ca^{2+} EFFLUX.

The data of Figures 19 and 20 were subjected to the iterative kinetic analysis described in the Methods section of this chapter. The kinetic parameters ± standard deviations reported here (defined in the description of the kinetic analysis) were used to generate the solid curves of Figures 19 and 20.

<table>
<thead>
<tr>
<th>Act.</th>
<th>SR</th>
<th>( R_{\text{max}} )</th>
<th>( K_{\text{act}} )</th>
<th>Hill Coef</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAMP</td>
<td>Native</td>
<td>0.21 ± 0.01</td>
<td>0.45 ± 0.08 mM</td>
<td>1.04 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>TMSR</td>
<td>4.14 ± 0.23</td>
<td>0.54 ± 0.06 mM</td>
<td>0.79 ± 0.08</td>
</tr>
<tr>
<td>Doxo</td>
<td>Native</td>
<td>0.30 ± 0.02</td>
<td>21.4 ± 8.8 \mu M</td>
<td>1.20 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>TMSR</td>
<td>4.66 ± 0.18</td>
<td>20.4 ± 5.7 \mu M</td>
<td>1.25 ± 0.14</td>
</tr>
</tbody>
</table>

The binding characteristics of doxorubicin are unaffected by proteolysis, though the maximum response increases dramatically.

In Chapters II and III it was shown that heavy metals (73, 74) and sulfhydryl oxidation with Cu^{2+} and cysteine (75) induce rapid Ca^{2+} release from isolated SR vesicles, apparently by activating the Ca^{2+} release channels. Figure 21 summarizes the Ca^{2+} release response of native and TMSR vesicles to HgCl_{2}. As indicated by the different scales on the graph, the response of TMSR to Hg^{2+} is about 5 times greater than that of native SR. Apparently, the degree of
Figure 21. Rate of Ca$^{2+}$ release from native and TMSR as a function of Hg$^{2+}$ concentration. SR vesicles were passively loaded with 1 mM CaCl$_2$ in 100 mM KCl and 20 mM HEPES, pH 7.0 by incubation on ice. After transmembrane [Ca$^{2+}$] equilibration, a portion of the vesicles were exposed to trypsin (trypsin:SR = 0.005, w/w) for 15 minutes at 23 °C. Ca$^{2+}$ efflux was initiated by diluting an aliquot of native SR (●) or TMSR (△) 40-fold into 100 mM KCl, 20 mM HEPES, 1 mM MgCl$_2$, 0.1 mM ARSTII, and the indicated concentration of HgCl$_2$. Efflux rates were calculated as described previously. Note difference in scales.
tryptic stimulation of Hg^{2+}-induced release is much less than the 20-fold stimulation seen in cAMP- or doxorubicin-activated release. This observation is confirmed in a related experiment (Figure 22), where the time courses of proteolytic stimulation of Hg^{2+}-activated and cAMP-activated Ca^{2+} efflux were measured in parallel using the same batch of vesicles. As seen in the figure, though the rate of efflux induced by Hg^{2+} in native SR was much faster than that of cAMP-activated efflux, the ratio of efflux rate after proteolysis to the rate before proteolysis is about the same for both activators for the first 5 minutes, at which time both means of inducing efflux are stimulated by a factor of 5. At this time, however, stimulation of Hg^{2+}-induced release levels off, while the time rate of stimulation of cAMP-activated efflux actually accelerates, rising to a stimulation factor of 23 over the next 5 minutes. Similar experiments show that proteolytic stimulation of doxorubicin-activated efflux follows a time course similar to that of cAMP, while stimulation of Cu^{2+}/cysteine-induced efflux (75) follows a time course similar to of Hg^{2+} (not shown).
Figure 22. Parallel time course of tryptic stimulation of cAMP- and Hg^{2+}-induced Ca^{2+} release. SR vesicles were passively loaded with 1 mM CaCl_2 by incubation as described previously. The vesicles were warmed to room temperature (23 °C) and the rate of Ca^{2+} release from native vesicles in the presence of 3 μM Hg^{2+} (- - -) or 0.5 mM cAMP (- - --) and 1 mM Mg^{2+} was measured in triplicate spectrophotometrically, as previously described. At t = 0, trypsin was added to the vesicles for a final trypsin:SR protein ratio of 0.005 and efflux was measured spectrophotometrically alternately in the cAMP medium or in the HgCl_2 medium at intervals of 1.5 minutes. Figure 22a (this page) shows the initial efflux rate as a function of time of proteolysis, while Figure 22b (overleaf) shows the stimulation of efflux due to proteolysis, i.e. the rate of efflux from the TMSR vesicles at the time of proteolysis indicated divided by the mean rate of efflux from the native vesicles.
Figure 22b. Parallel time course of tryptic stimulation of cAMP- and Hg²⁺-induced Ca²⁺ release. Ca²⁺ release rate as a function of time of proteolysis divided by rate of release from native vesicles.
DISCUSSION

The results of this study show that limited tryptic proteolysis stimulates activation of passive Ca\(^{2+}\) transport across the SR membrane by a factor of 20 or more. Aside from the rate of Ca\(^{2+}\) transport, the kinetic parameters (binding affinity and Hill coefficient) of Ca\(^{2+}\) transport activation with cAMP and doxorubicin are unaffected by proteolysis. Activation with micromolar Hg\(^{2+}\) and Ca\(^{2+}\) and inhibition with millimolar Ca\(^{2+}\), Mg\(^{2+}\), and micromolar ruthenium red also seem to be unaffected by proteolysis. Apparently, the sites responsible for activation and inhibition of the Ca\(^{2+}\) release system remain intact, yet the rate of Ca\(^{2+}\) transport is enhanced by tryptic proteolysis dramatically.

In earlier work, Scott and Shamoo (97) reported that tryptic proteolysis does not stimulate Ca\(^{2+}\) efflux from isolated vesicles. This appears to be at odds with the findings of both the present report and those of Shoshan-Barmatz et al (98) and Toogood et al (133). A fundamental difference between the studies performed by Scott and Shamoo and those in which tryptic enhancement of Ca\(^{2+}\) efflux is observed is that, in Scott and Shamoo's work, 1 M sucrose was present during proteolytic modification and was not removed prior to Ca\(^{2+}\) transport assay. Shoshan-Barmatz et al exposed the SR to trypsin in a manner very similar to
that of Scott and Shamoo, but removed the sucrose by centrifugation prior to assaying Ca\textsuperscript{2+} permeability. Toogood et al. monitored changes in Ca\textsuperscript{2+} permeability with SR vesicles which had been actively loaded using ATP in the presence of the Ca\textsuperscript{2+}-precipitating anion oxalate; their medium contained 160 mM sucrose. The SR used in the present report was not exposed to sucrose at all, except for the HSR and LSR preparations (39), which were washed with KCl to bring the sucrose concentration down to \textasciitilde 10 mM. It has been observed that substitution of sucrose for KCl inhibits Ca\textsuperscript{2+} release induced by Ag\textsuperscript{+} (74) and by oxidation via Cu\textsuperscript{2+} and cysteine (Chapter III, 75). Perhaps the high concentration of sucrose present in Scott and Shamoo's Ca\textsuperscript{2+} efflux assays were sufficient to inhibit efflux from even tryptically modified SR.

The present chapter does not reveal the molecular mechanism by which trypsin increases the response of the Ca\textsuperscript{2+} release system to activators of Ca\textsuperscript{2+} release. This stimulation may be due to direct modification of some component or regulatory (inhibitory) subunit of the Ca\textsuperscript{2+} system or it may be the result of stimulation of some related process. It is not likely that the stimulation of Ca\textsuperscript{2+} release is caused by modification of an obligatory co- or counter- ion transport system, since the permeability of the native SR to K\textsuperscript{+} and Cl\textsuperscript{−} is sufficiently large that it would not appear to limit the rate of Ca\textsuperscript{2+} release (139).
Yet, the lack of an effect on the binding characteristics of Ca\(^{2+}\) transport activators suggests that the activating mechanism of the Ca\(^{2+}\) channel is unaffected by trypsin and that some other indirect mechanism may be involved.

With regard to direct stimulation of the Ca\(^{2+}\) release system, the three major determinants of Ca\(^{2+}\) efflux rate are 1). the number of activatable channels; 2). the single channel unitary conductance; and 3). the probability of finding a channel in the activated state. In studies of rat myotubes it was shown that Na\(^{+}\) transport is stimulated by the proteolytic action of pronase (140). Pronase increases the probability of finding a Na\(^{+}\) channel in the activated state, but does not affect unitary conductance or the number of channels. Examination of Figures 14, 15a, and 22b suggests that tryptic stimulation of Ca\(^{2+}\) release from SR is biphasic, with Hg\(^{2+}\)- and cAMP-activated release being stimulated equally in phase one, while only cAMP-activated release is further stimulated during phase two. Under the conditions of these experiments, Hg\(^{2+}\) is a far more potent activator of Ca\(^{2+}\) release from native SR than cAMP. A plausible explanation of the monophasic stimulation of Hg\(^{2+}\) as opposed to the biphasic stimulation of cAMP is that Hg\(^{2+}\) might increase one of the three factors determining efflux rate to a value near its physical limit without aid of proteolysis, whereas cAMP might not. This would be the
factor which is enhanced during phase two of proteolytic stimulation of cAMP-activated release.

Much attention has been focused on the identity of the Ca\textsuperscript{2+} channels of the SR. Two protein components recently implicated in Ca\textsuperscript{2+} release are the 350 kDa "ryanodine-receptor complex" (89, 90, 100, 141), which is identical with the "spanning protein" found at the triadic junction (44-47, 141) and a 170 kDa doxorubicin-binding protein (84). I have performed SDS polyacrylamide gel analyses (142) of TMSR vesicles as a means of confirming these identifications and have found, in agreement with Caswell's study of the "spanning protein" (141), that the 350 kDa protein associated with SR vesicles is very labile to tryptic proteolysis. In fact, virtually all of this protein is cleaved within one minute of exposure to trypsin under the conditions reported here. Thus, it is possible that cleavage of the 350 kDa protein is responsible for stimulation of Ca\textsuperscript{2+} efflux, though if this is so, most of the stimulation must occur after the second or later cleavage. Another possible explanation for tryptic stimulation of Ca\textsuperscript{2+} release is that trypsin may alter the interaction between some regulatory or activating peptide and the actual Ca\textsuperscript{2+} channel peptide, thus tryptic stimulation of Ca\textsuperscript{2+} release may indicate that more than one protein is involved in regulation of the Ca\textsuperscript{2+} permeability of the SR. Visual inspection of the polyacrylamide gels
indicates that the 170 kDa doxorubicin-binding protein is not cleaved by trypsin under the conditions of this study after even 30 minutes of exposure to trypsin. This fact, taken with the observation that none of the modulatory binding sites of the Ca\textsuperscript{2+} release system are modified by trypsin, may indicate that the 170 kDa protein contains the activating mechanism of the Ca\textsuperscript{2+} release system. If so, its activity is increased by tryptic modification of some other, inhibitory protein, or by altered interaction with the Ca\textsuperscript{2+} channel peptide.

Isolated sarcoplasmic reticulum vesicles present a convenient model for studying the Ca\textsuperscript{2+} regulatory system of skeletal muscle. However, isolated vesicles may not contain the complete Ca\textsuperscript{2+} regulatory system, and it seems likely that some functional characteristics of isolated vesicles may differ from the corresponding characteristics of SR in vivo. For example, isolated vesicles release Ca\textsuperscript{2+} at physiologically significant rates in the absence of Mg\textsuperscript{2+} (137), yet at higher, perhaps more "physiological" free Mg\textsuperscript{2+} concentrations (34), the response of native isolated vesicles to "physiological" Ca\textsuperscript{2+} release activators (eg Ca\textsuperscript{2+}, adenine nucleotides) is too slow to account for the estimated rate of Ca\textsuperscript{2+} release from SR in vivo. Thus, though tryptic modification of SR vesicles results in some loss of functional integrity of the Ca\textsuperscript{2+} regulatory system (evidenced by decreased Ca\textsuperscript{2+} uptake capacity and increased
"basal" Ca$^{2+}$ permeability), the response of tryptically modified SR vesicles to Ca$^{2+}$ release activators is far larger than the response of native vesicles. Given that the pharmacological characteristics of the release system do not change after limited tryptic proteolysis, tryptically modified SR vesicles may offer, in many respects, a more functionally relevant model for study of the Ca$^{2+}$ release system of the sarcoplasmic reticulum than native vesicles.
CHAPTER V

POLYACRYLAMIDE GEL ANALYSIS OF PROTEINS MODIFIED BY 
SULPHHYDRYL OXIDATION AND TRYPIC PROTEOLYSIS

SUMMARY

This chapter shows results of polyacrylamide gel analysis of proteins labeled by $^{35}$S-cysteine in the presence of Cu$^{2+}$ and proteins modified by tryptic proteolysis. These results are discussed in light of recent publications detailing the identification, purification, and reconstitution of the Ca$^{2+}$ release protein using ryanodine as a specific marker of this protein.

INTRODUCTION

In Chapter III it was shown that Cu$^{2+}$ and cysteine activate the Ca$^{2+}$ release system of isolated SR vesicles, apparently via covalent attachment of exogenous cysteine via its sulfhydryl group to the reactive sulfhydryl of the Ca$^{2+}$ release activating mechanism. Cysteine was the first ligand to be covalently attached to the Ca$^{2+}$ release protein which did not inhibit the Ca$^{2+}$ pump or need to be added in a large excess of the number of proteins in the SR, and seemed to offer a means of identifying this protein. However, experiments using SDS polyacrylamide gel electrophoresis
(SDS PAGE) to separate proteins labeled with $^{35}$S-cysteine showed that several proteins bind $^{35}$S-cysteine when in the presence of Cu$^{2+}$, so the attempt to identify the release protein was inconclusive.

Scott and Shamoo (97) had previously reported that the proteolytic enzyme trypsin hydrolyzes the Ca$^{2+}$ pump without changing the permeability of the SR to Ca$^{2+}$. As demonstrated in Chapter IV, this was not correct. Limited tryptic modification of SR vesicles increases the Ca$^{2+}$ permeability of the SR and the response of the Ca$^{2+}$ release system to sulfhydryl reagents by a factor of 5 and increases the response to cAMP and doxorubicin (other Ca$^{2+}$ release activators) by a factor of approximately 20. The use of trypsin in analyzing the Ca$^{2+}$ release system of the SR was initially motivated by Scott and Shamoo's negative result: assuming that trypsin does not modify the Ca$^{2+}$ release system, it was hoped that all but one of the proteins found in the SR would be labile to trypsin, yet labeled by $^{35}$S-cysteine in the presence of Cu$^{2+}$. Such a protein would have been a prime candidate for the Ca$^{2+}$ release protein. Given my observation that trypsin actually stimulates the Ca$^{2+}$ release system (Chapter IV), this was no longer a viable approach for identifying the Ca$^{2+}$ release protein.

The present chapter is a brief summary of the results of the earlier Cu$^{2+}$/35S-cysteine SDS PAGE experiments and of similar SDS PAGE analyses of tryptically modified SR. Both
sets of experiments are in agreement with the recently reported results of the ryanodine method of identifying the release protein (76, 86-90, 100), though not as conclusive, as will be pointed out in the Discussion.

MATERIALS AND METHODS

Preparation of SR vesicles

SR vesicles were isolated from male New Zealand White rabbits as described in Chapter II, with the modifications of procedure indicated in Chapters III and IV. Protein concentrations were determined by the method of Lowry et al (103).

SDS Polyacrylamide Gel Electrophoresis

Polyacrylamide gel analyses of SR proteins were carried out in a manner similar to the method of Laemmli (142). Stock solutions of the following compositions were made up:

4X Lower Tris Buffer:

1.5 M Tris(hydroxymethyl)aminomethane base (Tris)
0.4 % Sodium Dodecyl Sulfate (SDS)
Adjust pH to 8.8 with HCl

4X Upper Tris Buffer:

0.5 M Tris
0.4 % SDS
pH 6.8 (HCl)

50 % Acrylamide, 1.15 % Bis-acrylamide (w/v) (50 % AC)
1.727 % Bis-acrylamide (w/v) (1.727 % Bis)
1.5 % Ammonium Persulfate (w/v)

10X Tris / Glycine Reservoir Buffer:
- 0.25 M Tris
- 1.918 M Glycine
- 1 % SDS (w/v)
  (pH should be ~8.8)

Sample Buffer:
- 25 % 4X Upper Tris Buffer (v/v)
- 2 % SDS (w/v)
- 15 % Sucrose (w/v)

0.4 % Bromphenyl Blue in 4X Upper Tris Buffer

Staining Solution
- 50 % Methanol (v/v)
- 10 % Glacial Acetic Acid (v/v)
- 1 % Coomassie Blue (Brilliant Blue R) (w/v)

Polyacrylamide running gels of varying concentration (7 and 8%, w/v) acrylamide were formed in a Bio-Rad vertical slab gel apparatus with gel dimensions 100 x 160 x 1.5 mm according to the following recipes (recipes are for one gel each):

8 % Gel.
- 7.500 ml 4X Lower Tris Buffer
- 4.800 ml 50 % AC
- 16.905 ml DD H₂O
- 45 µl TEMED
- 750 µl 1.5 % ammonium persulfate
7 % Gel.

7.500 ml 4X Lower Tris Buffer
4.200 ml 50 % AC
17.505 ml DD H₂O
45 μl TEMED
750 μl 1.5 % ammonium persulfate

The order of addition is somewhat critical, in that the AP catalyzes the cross-linking. Therefore, the ammonium persulfate (which was made up fresh from dry reagent the same day) was added last. After pouring the above mixture(s) into the gel apparatus (sealed with Vaseline on the spacers and Parafilm along the bottom) a layer of DD H₂O, about a mm thick, was gently poured on top with as little mixing as possible in order to form a sharp, even interface at the top of the gel. After the mixture gelled (about 10 minutes or less after addition of ammonium persulfate), the H₂O layer was drained off and a 5 % stacking gel (see recipe below) was poured on top of the running gel.

5 % Stacking Gel (recipe for two stacking gels).

6.000 ml 4X Upper Tris Buffer
2.000 ml 50 % AC
310 μl 1.727 % Bis
10.645 ml DD H₂O
45 μl TEMED
1.000 ml ammonium persulfate
Again, the ammonium persulfate was added last. A ten well teflon comb was inserted above the running gel and the 5 % Stacking Gel solution was layered on top of the running gel with the aid of a 30 ml syringe; care was taken to avoid formation of bubbles, especially under the "teeth" of the teflon comb. A layer of DD H₂O was then added as before, and after the stacking gel hardened (~10 minutes), the teflon comb was gently removed, the wells were straightened with the aid of thin spatula, and DD H₂O was poured on top in order to keep the gels hydrated until used.

SR vesicles to be electrophoresed (after tryptic modification or ³⁵S-cysteine labeling) were solubilized in Sample Buffer containing the anionic detergent SDS, then a few microliters of Bromphenyl Blue stain was added per ~150 microliters of SR vesicles in Sample Buffer. The vesicle/Sample Buffer mix (usually in sealed Eppendorf microfuge tubes) was allowed to stand for an hour at room temperature and then ~75 µl of the (now) solubilized SR was added to each well of the stacking gel after draining off the water on top. 10X Tris/Glycine Reservoir Buffer was diluted 10-fold into DD H₂O and the reservoir buffer was added to the upper and lower reservoirs of the apparatus (being careful to not disturb the solubilized SR protein in the wells). A constant-voltage power supply was used to apply a potential difference of 100 to 125 mV between the two reservoirs. The Bromphenyl Blue stain allows tracking
of the most mobile molecules, and when the stain reached the bottom of the gel (usually 2.5 to 3 hours) the electric field was turned off and the gels were placed in Staining Solution for periods ranging from several hours to overnight. The stained gels were destained for several hours in 30 % methanol, 10 % acetic acid under mild agitation and then for a few days in 10 % acetic acid. One lane in each gel was reserved for electrophoresis of proteins of known molecular weight for the purpose of determining the molecular weights of the SR proteins of interest (143, 144). Gels of S-cysteine labeled SR were treated with a fluor as described below, then autoradiographs were taken.

**Tryptic modification of SR vesicles**

SR vesicles were suspended at a protein concentration of 10 mg SR protein per ml of solution containing 100 mM KCl, 20 mM HEPES, and 1 mM CaCl$_2$, titrated to pH 7.0 with KOH. This mixture was allowed to incubate overnight on ice. The mixture was warmed in a water bath to room temperature and at $t = 0$, trypsin was added to obtain a final trypsin:SR protein ratio of 0.005. At pre-selected times, a 150 µg aliquot of the SR/trypsin mixture was drawn off and diluted into 10 ml of an ice-cold solution of soybean trypsin inhibitor dissolved in 100 mM NaCl 20 mM HEPES, titrated to pH 7.0 with NaOH (NaCl was used rather than KCl because the vesicles were to be solubilized ultimately in sodium dodecyl
sulfate (SDS), which precipitates in the presence of K+). The final concentration of soybean trypsin inhibitor in this solution was 0.03 mg per ml, giving a final trypsin:trypsin inhibitor ratio of 1:40. This mixture was centrifuged at 145,000Xg (40,000 rpm in a Beckman Ti50 rotor) for 1.5 hours. The small SR pellet was solubilized in 150 µl Sample Buffer (described above) and subjected to electrophoresis after incubating at room temperature for one hour.

**Reversal of DTT inhibition of Cu²⁺/cysteine-induced Ca²⁺ release with NaAsO₂**

Commercially available ³⁵S-cysteine of sufficient activity to allow use in autoradiography comes as ~10 µM L-cysteine packed in 10 mM DTT in order to retard auto-oxidation of the cysteine. In Chapter III it was shown that DTT reverses Cu²⁺/cysteine-induced Ca²⁺ release. Other experiments (not shown) indicated that as little as 10 µM DTT inhibits Ca²⁺ release activated by 2 µM Cu²⁺ and 5 µM cysteine when the DTT is added to SR prior to addition of Cu²⁺ and cysteine, presumably due to the two thiols of DTT strongly binding the Cu²⁺. Thus, in order to use ³⁵S-cysteine as a covalent label, some method of neutralizing the inhibitory effect of DTT while retaining the Ca²⁺ release activity of the cysteine was sought. Zahler and Cleland (145) had previously reported a method of blocking the two thiols of DTT while retaining activity of monothiols (such as cysteine) using arsenite (AsO₂⁻). Using this
information as a guide, it was ascertained (using the spectrophotometric technique of previous chapters) that 300 \( \mu M \) NaAsO\(_2\) fully restores the Ca\(^{2+}\) efflux activity of 5 \( \mu M \) cysteine and 2 \( \mu M \) Cu\(^{2+}\) in the presence of 15 \( \mu M \) DTT (not shown).

\( ^{35}S \)-Cysteine Labeling of SR

\( ^{35}S \)-cysteine packed in 10 \( mM \) DTT (obtained from New England Nuclear, lot number 21062739) with specific activity 1045 Ci per mmole and radioligand concentration 13.67 mCi per ml ("13 \( \mu M \) cysteine) was prepared for use as a covalent label by first diluting the \( ^{35}S \)-cysteine mixture into unlabeled cysteine and then adding NaAsO\(_2\) to obtain 60 \( \mu l \) of "labeling solution" composed of 0.5 \( mM \) cysteine, 1.5 \( mM \) DTT, and 30 \( mM \) NaAsO\(_2\) (final concentrations).

Isolated SR vesicles were labeled with \( ^{35}S \)-cysteine and prepared for electrophoresis as follows: 0.15 mg SR vesicles were suspended in 0.6 ml of 100 \( mM \) KCl and 20 \( mM \) HEPES at room temperature. 20 \( \mu M \) Ca\(^{2+}\) was then added, followed by 1 mM Mg\(^{2+}\)-ATP and after five minutes of Ca\(^{2+}\) uptake, 6 \( \mu l \) labeling solution was added followed by 6 \( \mu l \) of 0.2 \( mM \) CuCl\(_2\) (final concentrations: 100 \( mM \) KCl, 20 \( mM \) HEPES, 0.25 mg SR per ml, 1 mM MgCl\(_2\), 1 mM ATP, 5 \( \mu M \) \( ^{35}S \)-cysteine, 15 \( \mu M \) DTT, 300 \( \mu M \) NaAsO\(_2\), 2 \( \mu M \) CuCl\(_2\), pH 7.0). The Cu\(^{2+}\), cysteine, and SR were allowed to react for one minute and then the unreacted \( ^{35}S \)-cysteine was scavenged by diluting this mixture into 10 ml of 100 \( mM \) NaCl, 20 \( mM \) HEPES, and 200 \( \mu M \)
N-ethyl maleimide. The SR was pelleted by centrifugation at 100,000 x g (40,000 rpm for 40 minutes in a Beckman Ti50 rotor), solubilized in 150 μl Sample Buffer, and electrophoresed after standing for one hour. A small aliquot of the supernatant was analyzed for radioactivity by liquid scintillation.

**Autoradiography of 35S-cysteine gels**

Polyacrylamide gels of 35S-cysteine-labeled SR were prepared for autoradiography by impregnation of the gel with the fluor En3Hance (New England Nuclear) according to the manufacturer's instructions. The gel impregnated with fluor was suspended in water. A slab of mounting paper (Whatman filter paper, Bio-Rad) was inserted under the gel and the gel on its mounting paper was lifted out of the water. The top surface of the gel was gently sprayed with a jet of water in order to remove any residue of precipitated fluor from the surface. A piece of mylar film (Bio-Rad) was then gently laid over the gel and the whole sheet was sprayed with water and gently smoothed until all wrinkles were removed, taking care to not distort the dimensions of the gel underneath. The mounting paper / gel / mylar sheet sandwich was placed in a Bio-Rad gel dryer and dried under heat and vacuum for three hours.

The dried and mounted fluor-impregnated gel was placed in a Kodak X-ray film holder with a piece of Kodak X-Omat AR X-ray film in contact with the mylar face of the gel. The
gel / X-ray film / film holder sandwich was pressed between two sheets of plexiglass and placed in a dry ice freezer for two days. The exposed film was developed according to the manufacturer's instructions using Kodak GBX developer, Indicator stop bath, and GBX fixer. Developed films were treated with Photoflow 200 (Kodak) prior to drying in order to inhibit formation of spots and surface irregularities.

Estimation of molecular weight

The distance a protein is electrophoresed into an SDS polyacrylamide gel is proportional to the logarithm of its molecular weight (143, 144). The molecular weights of SR proteins were estimated by plotting the log(molecular weight) of known proteins against the distances they electrophorese, then drawing a straight line through the points. The distances SR proteins electrophorese were then plotted on this line and their molecular weights were estimated by finding the molecular weight corresponding to that distance.

Materials

Kodak X-Omat AR film and developing supplies were obtained from Picker International (Seattle Wash.). ³⁵S-cysteine and En³Hance were obtained from New England Nuclear. HEPES was obtained from Research Organics. Protein standards for electrophoresis, gel mounting paper and Mylar film were obtained from Bio-Rad. Bovine
pancreatic trypsin, soybean trypsin inhibitor, ultra-pure acrylamide, N-N'-bisacrylamide, all other reagents were obtained from Sigma.

RESULTS

Figure 23 is a photocopy of an autoradiograph of an 8% polyacrylamide gel into which SR labeled with \[^{35}\text{S}\]-cysteine and solubilized with the anionic detergent SDS has been electrophoresed. In the first lane, \[^{35}\text{S}\]-cysteine, DTT, and NaAsO\(_2\) (in the amounts indicated in the Methods) were added to SR without addition of Cu\(^{2+}\), while in lane 2, 2 \(\mu\)M CuCl\(_2\) was added as described in the Methods. The dark bands signify proteins to which \[^{35}\text{S}\]-cysteine is bound; the darker the band, the greater the degree \[^{35}\text{S}\]-cysteine binding. Three bands are lightly labeled in the absence of Cu\(^{2+}\), while several bands are heavily labeled in the presence of Cu\(^{2+}\). Figure 24 is a plot of Log(molecular weight) of several known proteins (circles) against the distance they traveled into the gel. The distances traveled by several of the labeled bands were also plotted and their molecular weights were estimated according to the protocol outlined in the Methods.

Figure 25 is a photocopy of a Coomassie Blue-stained 7% gel which had had tryptically modified SR electrophoresed into it according to the protocol outlined in the Methods. Lanes 1 and 2 are controls demonstrating feasibility of the
Figure 23. Autoradiograph of $^{35}$S-cysteine-labeled SR in a 7% polyacrylamide gel. SR vesicles were labeled with $^{35}$S-cysteine and electrophoresed into a 7% polyacrylamide gel as described in the text. Molecular weights of several SR proteins were estimated from Figure 24 according to the protocol in the methods and are shown next to the appropriate proteins in this figure.
Figure 24. Log (molecular weight) vs mobility in gel for proteins in Figure 23. Protein standards, molecular weight: A, myosin, 200 kDa; B, β-galactosidase, 116 kDa; C, phosphorylase B, 92.5 kDa; D, bovine serum albumin, 66 kDa; E, carbonic anhydrase, 31 kDa. SR proteins, molecular weight: 1, spanning protein, 290 (350) kDa; 2, doxorubicin binding protein, 160 (170) kDa; 3, doxorubicin binding protein, 150 (160) kDa; 4, Ca$^{2+}$ pump, 100 kDa; 5, unknown, 24 kDa; 6, unknown, 21 kDa; 7, unknown, 18 kDa. Numbers in parentheses are previously-published values.
Figure 25. Tryptically modified SR proteins in an 8% SDS polyacrylamide gel. Lane 1: SR + trypsin inhibitor. Lane 2: SR + trypsin inhibitor + trypsin (added in that order). Lane 3: SR + trypsin + trypsin inhibitor (added one minute after trypsin). Lane 4: protein standards. Molecular weights were estimated using Figure 26 according to the protocol outlined in the Methods.
Figure 26. Log (molecular weight) vs mobility for proteins in Figure 25. Protein standards, molecular weight: A, myosin, 200 kDa; B, β-galactosidase, 116 kDa; C, phosphorylase B, 92.5 kDa; D, bovine serum albumin, 66 kDa; E, carbonic anhydrase, 31 kDa. SR proteins, molecular weight: 1, spanning protein, 290 (350) kDa; 2, doxorubicin binding protein, 165 (170) kDa; 3, doxorubicin binding protein, 145 (160) kDa; 4, Ca²⁺ pump, 100 kDa; 5, calsequestrin, 60 (65) kDa; 6, Ca²⁺ pump fragment, 48 (55) kDa; 7, Ca²⁺ pump fragment 40 (45) kDa; 8, unknown (probably Ca²⁺ pump fragment) 27 kDa. Numbers in parantheses are previously-published molecular weights of SR proteins, where different from those indicated by this gel.
technique. In lane 1, trypsin inhibitor but no trypsin was added prior to the centrifugation step, while in lane 2, trypsin inhibitor and then trypsin were added sequentially prior to centrifugation. The protein patterns of the two lanes are virtually identical, indicating that no proteolysis occurred between addition of trypsin inhibitor and electrophoresis of the proteins, hence trypsin inhibitor prevents proteolysis for the entire duration of the experiment. Lane 3 is of SR subjected to tryptic proteolysis for 1 minute at room temperature at a trypsin:SR protein ratio of 0.005 as described in the legend. Lane 4 shows several proteins of known molecular weight, used for the purpose of calibrating the mobilities of the SR proteins as functions of their molecular weights. Figure 26 is a plot of Log(molecular weight) against mobility of the known proteins, with several bands of both native and tryptically modified SR proteins also plotted in order to determine their molecular weights, as described in the Methods. Referring to Figure 25, lane 3, it appears that nearly all of the Ca²⁺ pump (MW ~100 kDa) is hydrolyzed by trypsin after only one minute of exposure to trypsin. Also, the 290 kDa ("ryanodine binding") protein at the top of the gel is completely hydrolyzed after one minute. The fragments of the pump appear as the heavy bands at ~40 and 48 kDa (Scott and Shamoo (97) report that these fragments are of molecular weight 45 and 55 kDa).
Of particular interest, from the point of view of the Ca\(^{2+}\) release mechanism, is the proteolysis of the 290 kDa protein, which has recently been identified as the ryanodine binding protein (note: the published estimates of the molecular weight of this protein range between 290 and 450 kDa: 76, 86-90). The tryptic fragments of this protein are not identifiable on these gels, probably due to the combined result of a low initial concentration of this protein and of the fragments running down into the region of the gel which is dominated by fragments of the Ca\(^{2+}\) pump and other more abundant proteins. This is an interesting result in itself: given that the molecular weight of this protein is approximately 290 kDa, it should produce tryptic fragments of at least 150 kDa or so upon the first cleavage, yet no new proteins appear above the level of the Ca\(^{2+}\) pump (MW ~100 kDa) even after only one minute of proteolysis. Apparently, this protein is hydrolyzed in several places (so that its fragments have molecular weights of less than 100 kDa) after only one minute of treatment with trypsin. This poses some interesting questions regarding its role in Ca\(^{2+}\) release, as discussed below.

The 65 kDa Calsequestrin band exhibits little proteolysis even after 30 minutes. This protein is generally found inside the SR vesicles (146) and thus would not be expected to undergo proteolysis, as trypsin is too large to easily cross the membrane. Finally, note the
doublet of protein at ~150-165 kDa. This protein shows little proteolysis after 1 minute, and even up to 30 minutes, of proteolysis (not shown).

DISCUSSION

The Cu²⁺/³⁵S-cysteine gel (Figure 23) clearly adds evidence that Cu²⁺ catalyzes the covalent attachment of cysteine to SR proteins. On the other hand, it also indicates that some attachment occurs in the absence of Cu²⁺, though not nearly to the same degree (note: the bands on the photocopies appear much darker than corresponding bands on the autoradiographs, apparently due to the dark background of the autoradiograph). Liquid scintillation analysis of the supernatant from the centrifugation step revealed that most of the ³⁵S-cysteine stays in the supernatant (not shown), yet determination of the protein concentration of the pellet indicated that up to ~95% of the protein was recovered by centrifugation. Hence, most of the ³⁵S-cysteine does not become attached to SR proteins. Given that the Ca²⁺ pump comprises most of the SR protein and that its molecular weight is ~100 kDa, the concentration of Ca²⁺ pump at the time the Cu²⁺ and ³⁵S-cysteine were added was ~2 μM, and the total molar concentration of protein was probably somewhat less than the 5 μM ³⁵S-cysteine. Since the mobility of cysteine in solution is much greater than that of a membrane-bound protein, and given that most of
this cysteine would be oxidized within 30 seconds (see Chapter III), the fact that most of the cysteine does not react with SR is not surprising.

Several proteins are labeled by $^{35}$S-cysteine far more heavily than they are stained by Coomassie Blue (as shown by comparing Figure 23 with Figure 25), suggesting that these proteins contain highly reactive sulfhydryls. Of particular note in this regard are several low molecular weight proteins, a pair of proteins of molecular weight 150 and 165 kDa, the 290 kDa protein, and the high molecular weight proteins at the extreme tops of the gels. In contrast, very little calsequestrin is labeled by $^{35}$S-cysteine, despite its rather heavy Coomassie Blue stain. This simultaneously confirms that calsequestrin is located inside the vesicles (hence not exposed to the radiolabel) and indicates that the labeled proteins are covalently attached to the $^{35}$S-cysteine, since no exchange of label with calsequestrin appears to occur, even after the vesicles have been destroyed and the proteins have been denatured by addition of SDS.

Several groups have recently reported identification, purification, and reconstitution of the Ca$^{2+}$ release protein (76, 86-90, 100). All implicate the 290 kDa protein found near the tops of the gels. Their conclusions are supported by the observation that this protein is strongly labeled by $^{35}$S-cysteine and seems to be very labile to tryptic
proteolysis. However, as shown in Chapter IV, one minute of proteolysis does not produce significant changes in Ca\textsuperscript{2+} permeability, and yet the 290 kDa protein apparently is cleaved several times over this time interval. If this protein is indeed the Ca\textsuperscript{2+} release protein, as indicated by the ryanodine binding studies, it may undergo extensive proteolysis before its transport rate is stimulated. If this protein also contains the regulatory binding sites, it would seem rather remarkable that none of these sites are altered by such extensive proteolysis (see Chapter IV). Perhaps this is an indication that the binding sites reside on another protein or on a domain of the 290 kDa protein which is not sensitive to trypsin. It should be pointed out, however, that in an abstract for the February, 1988 meeting of the Biophysical Society, Chu et al (147) reported that the ultrastructure of this protein as found in the membrane is unchanged by limited tryptic proteolysis. This suggests that even though the protein is very labile to trypsin, the essential structure of the protein (including, perhaps, the modulatory binding sites) is unaltered, despite being cleaved. The conditions under which those experiments were carried out have not appeared in print and so a comparison between that work and the work presented here is difficult to make at this time.

The data presented here support the hypothesis that the 290 kDa protein is involved in Ca\textsuperscript{2+} release but do not rule
out the possibility that other proteins are involved as well. Several proteins are both labile to trypsin and quite heavily labeled by $^{35}$S-cysteine, despite being such minor components of the SR that they do not even show up under Coomassie Blue staining. Actually, being inert to trypsin does not necessarily indicate lack of a role in Ca$^{2+}$ release or regulation of Ca$^{2+}$ permeability. Given that the modulatory binding sites of the Ca$^{2+}$ release system are not altered by tryptic proteolysis, it is not unreasonable to postulate that the 150 and 165 kDa proteins, which bind $^{35}$S-cysteine (Figure 23) and $^{14}$C-doxorubicin (84) and are inert to trypsin, might contain (some of) the regulatory binding sites of the activating mechanism of the Ca$^{2+}$ release system. If so, tryptic stimulation of Ca$^{2+}$ release may be the result of an altered interaction between these peptides and the 290 kDa Ca$^{2+}$ channel peptide. As discussed in the following chapter, subjecting the purified Ca$^{2+}$ channel to an analysis similar to that outlined here may be a fruitful approach to settling such questions.
CHAPTER VI

CONCLUDING REMARKS

When the work presented in this dissertation was first begun in the summer of 1982, the field of Ca\textsuperscript{2+} release from the sarcoplasmic reticulum was much smaller, dominated by physiologists working with isolated muscle fibers. Biophysicists studying isolated SR vesicles were primarily interested in the mechanism of active transport, and found the SR to be an ideal system to study this phenomenon due to its simplicity relative to other biological membranes and the high density of its active transport protein, the Ca\textsuperscript{2+} pump. The questions the biophysicists were addressing mainly concerned the molecular mechanisms by which the pump translocates Ca\textsuperscript{2+} across the membrane, an issue yet to be resolved.

One of the principal tools of a researcher dissecting the inner workings of a biological system is an inhibitor of that system. By finding an inhibitor of an enzyme, the researcher gains information about the sites which catalyze processes carried out by the enzyme. Hg\textsuperscript{2+} had been known as an inhibitor of the Ca\textsuperscript{2+} pump (28, 38, 93, 102, 110, 112), and when I began my work with Dr. Abramson, we (meaning myself, Lyle Weden, and Dr. Abramson) were attempting to use
Hg$^{2+}$ as a probe of the Ca$^{2+}$ pump. Lyle had already carried out some experiments (summarized in Figure 3, Chapter II) which were similar to experiments carried out by other workers previously. These other workers had created conditions which abolished the transmembrane Ca$^{2+}$ gradient, in order to study the pump's activity under optimal conditions, and had used Hg$^{2+}$ to inhibit the pump. In Lyle's study, the SR was allowed to generate the Ca$^{2+}$ gradient, and interesting differences were found between his work and the previous work. Specifically, Lyle found that at low concentrations, Hg$^{2+}$ actually stimulates the activity of the Ca$^{2+}$ pump, and inhibits it only at higher concentrations. A parallel study showed that at Hg$^{2+}$ concentrations which stimulate the pump, active accumulation of Ca$^{2+}$ within the vesicles was inhibited. Dr. Abramson suggested that the explanation for these observations might be that perhaps Hg$^{2+}$ activates some Ca$^{2+}$ permeability pathway: this would abolish the transmembrane gradient, allowing the pump to operate at optimal rate, and would account for the low Ca$^{2+}$ uptake observed in the presence of low concentrations of Hg$^{2+}$. With Dr. Abramson's guidance, I began to study the Ca$^{2+}$ permeability of isolated SR vesicles and found the hypothesis to be correct: Hg$^{2+}$ definitely activates a Ca$^{2+}$ permeability pathway at a much lower concentration than that required to inhibit the pump. The Hg$^{2+}$ binding site appears to be a sulfhydryl group which is
oxidizable by several sulfhydryl oxidants. My contributions to this study are summarized in Figures 4 and 5 and Table I of Chapter II.

The significance of the research presented in Chapter II is twofold. First, it cast a new light on the previous studies of the Ca\(^{2+}\) pump and called into question several conclusions and generally accepted notions about the pump. It showed that Hg\(^{2+}\) does not "uncouple" the ATP hydrolysis function from the Ca\(^{2+}\) translocation function. It also showed that the mechanism of Hg\(^{2+}\)- and other sulfhydryl reagent-activated release of Ca\(^{2+}\) is not due to leakage back through the inhibited pump or to leakage through pre-existing pathways: Hg\(^{2+}\) activates a Ca\(^{2+}\) permeability pathway. Later work showed that this pathway has many characteristics in common with the pathway by which Ca\(^{2+}\) is released in response to added Ca\(^{2+}\). That is, heavy metal-induced Ca\(^{2+}\) release is similar to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (74). Apparently, perturbation of a sulfhydryl on the Ca\(^{2+}\)-induced Ca\(^{2+}\) release protein will activate this protein. Heavy metal activation of this protein is not the result of general denaturation or degradation of the membrane or of this protein, since the uptake capacity of the SR is restored by the Ca\(^{2+}\) release inhibitor ruthenium red.

Prior to publication of our paper showing that heavy metals induce Ca\(^{2+}\) release from isolated SR, only a handful of papers had been published on Ca\(^{2+}\) release from isolated
SR vesicles. In 1973, Kasai and Miyamoto reported that "depolarization" of SR vesicles by ionic substitution led to Ca\(^{2+}\) release (148), but in 1976 Meissner and McKinley (48) showed that the release of Ca\(^{2+}\) under Kasai and Miyamoto's conditions was the result of osmotic rupture of the SR vesicles and not of depolarization-induced activation of a Ca\(^{2+}\) release pathway. Ca\(^{2+}\)-induced Ca\(^{2+}\) release from isolated SR vesicles was first observed by Ohnishi in 1979 (57) and later by Miyamoto and Racker in 1981 (113, 114). Our publication of heavy metal-induced Ca\(^{2+}\) release from isolated vesicles appeared in print in 1983, almost simultaneously with Bindoli and Fleischer's very similar work with organic mercurials (115). Numerous other papers concerning Ca\(^{2+}\) release from isolated vesicles began to appear at about that time, most of which were studies of Ca\(^{2+}\)-induced Ca\(^{2+}\) release.

With repeated demonstrations that isolated SR vesicles could be induced to release Ca\(^{2+}\), it became conceivable that the Ca\(^{2+}\) release system, or at least a major portion of it, could be identified and isolated from SR vesicles. This appeared to be a much simpler problem than attempting to identify and isolate the Ca\(^{2+}\) release system from whole muscle cells. We knew from work described in Chapter II that heavy metals activate the Ca\(^{2+}\) release system at low concentrations, so I designed a series of experiments with the objective of labeling the release system with
radioactive heavy metals, namely Ag⁺, Hg²⁺, and Cu²⁺. The labeled release system would then have been separated from the other SR proteins by electrophoresis. In order to validate the protocol, it was first necessary to determine whether the heavy metals, once bound to the critical sulfhydryl of the release protein, would exchange with other sulfhydryls which become exposed under the denaturing conditions which prevail when running a polyacrylamide gel (see Chapter V). If exchange did occur, the attempt to identify the Ca²⁺ release system with radioactive heavy metals as a label would have had to be abandoned.

The assays which were to determine whether exchange occurs were Ca²⁺ release experiments in which Ca²⁺ was actively taken up by the SR using ATP as energy source, and then the Ca²⁺ was released upon addition of heavy metal. A sulfhydryl-containing compound, namely cysteine, was then added: if heavy metal ion exchange occurred between the added cysteine and the critical sulfhydryl of the Ca²⁺ release system, the Ca²⁺ should have been taken back up into the vesicles. In the course of such an experiment, it was found that cysteine actually enhances, rather than inhibits, Cu²⁺-induced release, shown in Figure 6 of Chapter III. In fact, micromolar cysteine and Cu²⁺ at a concentration too low to cause release by itself will, when added together, induce rapid Ca²⁺ release from isolated SR vesicles.
Following a period of study of the interactions between sulfhydryl groups and Cu\textsuperscript{2+}, including discussions involving Drs. Joann Loehr and Michael Albrich of the Oregon Graduate Center, it became apparent that Cu\textsuperscript{2+}/cysteine-induced Ca\textsuperscript{2+} release might involve the covalent attachment of cysteine to the critical sulfhydryl of the Ca\textsuperscript{2+} release protein which is perturbed by heavy metals. If so, \textsuperscript{35}S-cysteine could be used as a label and the problem of label exchange between the critical sulfhydryl of the Ca\textsuperscript{2+} release protein, and other sulfhydryls which become exposed after solubilization in detergent, could be eliminated. Much of the work presented in Chapter III is devoted to ascertaining whether the reaction which results in Cu\textsuperscript{2+}/cysteine-induced Ca\textsuperscript{2+} release is in fact due to covalent attachment of the cysteine to a protein sulfhydryl via a mixed disulfide bond. This portion of Chapter III, along with the autoradiograph presented in Chapter V (Figure 23), indicates very strongly that Cu\textsuperscript{2+} does indeed catalyze the formation of a mixed disulfide between the exogenous cysteine and the critical sulfhydryl of the Ca\textsuperscript{2+} release protein.

The second half of Chapter III is concerned with establishing that the Ca\textsuperscript{2+} permeability pathway activated by Cu\textsuperscript{2+}/cysteine is the Ca\textsuperscript{2+} release protein, and does not merely represent some general degradation of the membrane or of any transmembrane protein. The effects of pH, Mg\textsuperscript{2+}, ionic strength, and Ca\textsuperscript{2+} release inhibitors such as
tetracaine and ruthenium red indicate that the pathway activated by Cu²⁺/cysteine is indeed the Ca²⁺ release protein; independent studies of the effects of adenine nucleotides, performed by Ms. Jan Stuart, corroborate this conclusion (99). In view of the fact that known modulators of Ca²⁺ release also modulate Ca²⁺ release activated by Cu²⁺/cysteine, it was proposed in Chapter III that sulfhydryl oxidation may be one of the steps linking depolarization of the T-tubule to Ca²⁺ release from the SR in vivo. The work presented in Chapter III, including the proposal that sulfhydryl oxidation may couple depolarization of the T-tubule to Ca²⁺ release from the SR, was published in 1986 (75). Prior to publication of this work, only one other molecular mechanism of physiological Ca²⁺ release had been proposed, that being Schneider and Chandler's electromechanical linkage theory (52). In view of recent information regarding the identity of the Ca²⁺ release protein (76, 86-90, 100, 149), it is entirely possible that the two proposals (as well as the "phenomenological" model of Ca²⁺-induced Ca²⁺ release) reflect different facets of the same mechanism. In another aspect of the work presented in Chapter III, attention was drawn to some previous work which had shown that animals which are genetically predisposed toward muscular dystrophy, when fed a diet which includes sulfhydryl reducing agents, live longer and experience the debilitating effects of the disease later in
their lives (129). Also, elevated Ca\textsuperscript{2+} concentrations in the muscle cells is correlated with onset of the disease (130), and it was suggested that some forms of muscular dystrophy may be the result of pathological oxidation of the critical sulfhydryl of the Ca\textsuperscript{2+} release protein.

The results of the attempt to label the Ca\textsuperscript{2+} release system with \textsuperscript{35}S-cysteine were ambiguous: several proteins covalently bind \textsuperscript{35}S-cysteine. If only one protein had been labeled, the identity of the Ca\textsuperscript{2+} release protein would have been unequivocally established. In an attempt to remove some of the ambiguity of the \textsuperscript{35}S-cysteine labeling studies, the tryptic modification work presented in Chapter IV was begun. It seemed likely that Cu\textsuperscript{2+}/cysteine would not induce Ca\textsuperscript{2+} release from tryptically modified SR (see introduction to Chapter V for rationale). If this were the case, a protein which binds \textsuperscript{35}S-cysteine before proteolysis, but does not bind cysteine after proteolysis, would have been more likely to be the Ca\textsuperscript{2+} release protein than a protein which didn't have these characteristics. The basic premise of this scheme turned out to be unfounded, however: rather than inhibiting Ca\textsuperscript{2+} release, limited tryptic modification of the SR stimulates Ca\textsuperscript{2+} release. This surprising result did not hold as much promise for the purpose of identifying the Ca\textsuperscript{2+} release protein, but was investigated further because it promised to yield some information about the mechanism and regulation of the Ca\textsuperscript{2+} release system.
The significance of the work presented in Chapter IV is, like the heavy metal work, twofold, and in similar ways. First, by establishing the true nature of the effect of trypsic proteolysis on Ca$^{2+}$ uptake by isolated SR vesicles, it reveals previously-held misconceptions (97) about the mechanism by which the Ca$^{2+}$ pump functions. It is not an exaggeration to say that, prior to publication of the work presented in Chapter II, active Ca$^{2+}$ uptake was synonymous with active Ca$^{2+}$ transport. The possibility that inhibition of uptake could be due to activation of Ca$^{2+}$ permeability, rather than inhibition of transport, was never tested correctly, and the result of this neglect was generation of some erroneous theories of active transport (93, 97, 102).

The work presented in Chapters II and IV outline the types of control experiments and the conditions under which they should be carried out in order to distinguish between the two phenomena. This is significant in itself, but the more interesting aspects of Chapters II and IV concern the Ca$^{2+}$ release system, not active Ca$^{2+}$ uptake. Chapter II has been discussed above, and when augmented with Chapter III indicates that Ca$^{2+}$ release from the SR may involve some transient perturbation of a sulfhydryl group. The important fact of Chapter IV is that trypsin stimulates the activation of the Ca$^{2+}$ release system. This suggests the possible existence of a separate protein, associated with the actual channel, whose function is not Ca$^{2+}$ transport per se, but
regulation of the Ca\(^{2+}\) release channel. The channel peptide of the Ca\(^{2+}\) release system has been recently identified as the 300 (350) kDa, ryanodine binding, "spanning protein" found at the triadic junction between the SR and the T-tubules (44-47, 76, 86-90, 100, 149). This protein can be extensively cleaved by trypsin (see Figure 25) and yet, on the whole, the tryptically modified Ca\(^{2+}\) release system of isolated SR vesicles retains most or all of its pharmacological characteristics. One possible extension of my research would be to investigate the Ca\(^{2+}\) transport characteristics of isolated Ca\(^{2+}\) channels incorporated into an artificial membrane after tryptic modification.

The next task in this field is to elucidate the mechanism which couples depolarization of the T-tubules to Ca\(^{2+}\) release from SR. The Ca\(^{2+}\) release channel of SR appears to be in contact with both the T-tubule and SR simultaneously. This unique arrangement lends considerable support to Schneider and Chandler's voltage-dependent charge movement hypothesis (52) but does not prove it nor disqualify the other proposed Ca\(^{2+}\) release mechanisms. The isolated, reconstituted channel is still modulated by Ca\(^{2+}\), supporting the Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism (149), and the work I have presented in Chapter V shows that the channel covalently binds cysteine when in the presence of Cu\(^{2+}\). Probably, the final picture of the mechanism of Ca\(^{2+}\)
release from SR will be a combination of several of the current theories.

All of the research presented in this dissertation has potential utility for dissecting the purified Ca$^{2+}$ channel and hence for determining the mechanism of Ca$^{2+}$ release from the SR in vivo, and perhaps for determining the nature of certain muscular diseases. For example, tryptic proteolysis may have no effect on the transport characteristics of the isolated release protein; this would indicate that some other protein, not present in the purified preparation of Ca$^{2+}$ channel, is intimately connected with the Ca$^{2+}$ release process, perhaps as a regulatory protein. Alternately, if the isolated release protein is stimulated by trypsin, it may be possible to dissect regulatory domains from activating domains. A combination of tryptic modification and $^{35}$S-cysteine labeling experiments may reveal the role of the critical sulfhydryl by showing whether $^{35}$S-cysteine binds to an activating domain or to a regulatory domain. If it should turn out that the $^{35}$S-cysteine binding site is part of an activating domain, this will add weight to the hypothesis (proposed in Chapter III and reference 75) that sulfhydryl oxidation (perturbation) is an aspect of the mechanism which couples depolarization of the T-tubules to Ca$^{2+}$ release from the SR. If the converse is true, then perhaps sulfhydryl oxidation is part of a regulatory mechanism. Regardless of the final outcome, as pointed out
throughout this dissertation, this research represents a significant contribution to the literature of the sarcoplasmic reticulum, in terms of its implications for Ca$^{2+}$ release and for its information regarding the mechanisms of active transport. It has also cast new light on, and generated interest in, the role of sulfhydryl groups and sulfhydryl oxidants in biological systems and their pathologies.
REFERENCES


96. Stuart, J. and Abramson, J.J. submitted.


APPENDIX A

JUSTIFICATION OF THE RATE EQUATION

The Ca\(^{2+}\) channels of the SR catalyze the physical transport of Ca\(^{2+}\) ions from within the lumen of the SR across the SR membrane to the cytosol; the corresponding process for isolated SR vesicles takes Ca\(^{2+}\) from the inside of the vesicles out to the extravesicular space. Under most conditions the channels are at least slightly active, so one usually encounters an appreciable Ca\(^{2+}\) efflux rate from isolated vesicles even in the absence of any activators of the Ca\(^{2+}\) channels; addition of an activator such as cAMP increases the rate of Ca\(^{2+}\) transport. The simplest model of activation of the Ca\(^{2+}\) channels parallels the Michaelis-Menten model of enzyme kinetics, and for this reason the simplest model of activation of the Ca\(^{2+}\) channels of the SR will be called the Michaelis-Menten model (see ref. 43 for more details on this and other kinetic models outlined below). In the Michaelis-Menten model it is assumed that there is one binding site for the activator per functional channel. The rate of Ca\(^{2+}\) transport (in units of moles Ca\(^{2+}\) transported per second per milligram protein) across the membranes of a population of vesicles, in the presence of a concentration of activator [A], is given by:
1) \( V([A]) = V_0 \times [E] + V_a \times [EA] \)

where

\( V([A]) \) is the overall average rate of transport of \( Ca^{2+} \) (through both activated and un-activated channels) for the population at activator concentration \([A] \); \( V_0 \) is the rate of transport through a single, unactivated channel in units of moles \( Ca^{2+} \) transported per second; \( V_a \) is the rate of transport through a single activated channel; \([E] \) is the number of free, unactivated channels per milligram protein; and \([EA] \) is the number of bound, activated channels per milligram protein. The total number of channels per milligram protein, \( E_{total} \), is given by

\[ [E]_{total} = [E] + [EA]. \]

The channels are activated by the binding of activator to its binding site, with a dissociation constant \( K_{act} \), and go back to the unactivated state when the activator/site complex dissociates. The maximum rate of transport, found at saturating concentration of activator, would be:

\( V_{max} = V_a \times [E]_{total}. \)

The minimum, or base rate of transport, found at zero activator concentration, would be:

\( V_{base} = V_0 \times [E]_{total}. \)

Define the response function, \( R([A]) \), by the relationship:

2) \( R([A]) = V([A]) - V_{base} \)
Recalling equation 1 and substituting for $E_{total}$:

$$R([A]) = V([A]) - V_{base}$$

$$= V_o \times [E] + V_a \times [EA] - V_o \times ([E] + [EA])$$

$$= (V_a - V_o)[EA].$$

The maximum response, $R_{max}$, is given by:

$$R_{max} = (V_a - V_o) \times [E]_{total}.$$

Dividing $R([A])$ by $R_{max}$, cancelling common terms, and applying the definition of the dissociation constant, a result similar to the Michaelis-Menten equation is obtained:

$$3) \quad \frac{R}{R_{max}} = \frac{[EA]}{[E]_{total}} = \frac{([E][A])/K_{act}}{[E]x(1 + [A]/K_{act})} = \frac{[A]}{K_{act} + [A]}$$

As noted above, the Michaelis-Menten model is the simplest model of activation of the Ca$^{2+}$ channels of the SR. However, one cannot assume a priori that this equation is an adequate description; it is often found that the functional form of an enzyme has several activator binding sites which may interact with each other.

The functional form of enzymes which have multiple sites for binding the same substrate is often a dimer or trimer or some higher order oligomer of identical or nearly identical subunits; the oxygen carrier hemoglobin, for example, has four nearly identical subunits, each with an oxygen binding site (23). When all sites are unoccupied, the association constants of the activator for the various sites are identical or nearly so. Upon binding of an activator molecule to one of the sites, however, the affinities of the
remaining unoccupied sites for the activator may change; the oxygen binding sites in hemoglobin undergo enormous changes in affinity for oxygen when one or more of the sites is bound. A rate equation for a Ca\textsuperscript{2+} channel with two identical activation sites, which may interact, is developed below as an example of a more complicated activation scheme. For this development it is assumed that each subunit is a channel in itself but that two such subunits are always found closely associated, so that the activation binding sites may communicate. Note that this analysis must reduce to the Michaelis-Menten form in the case where the binding sites are identical and do not interact.

A Ca\textsuperscript{2+} channel dimer with two activation sites has two forms in which one of the sites is bound. It may not be possible to distinguish between these two forms experimentally, but they are in principle different. Let these two forms be known as EA and AE. The equilibrium expression for the binding of one activator molecule to a channel dimer which has both activator sites unoccupied is the sum of the equilibria for binding to each of the empty sites:

\[
[EA] = k_1 [E] [A] \\
[AE] = k_1 [E] [A] \\
[EA]_{\text{total}} = 2 \times (k_1 [E] [A])
\]
Where $[EA]_{\text{total}}$ is the sum of the two singly occupied forms; this quantity is proportional to the average response of the population of vesicles to activator; $k_1$ is the association constant of the activator/site complex for this reaction. As noted above, occupation of one site of the dimer may change the affinity of the other site for the activator. A two-site Ca$^{2+}$ channel dimer with both activator sites occupied is in equilibrium with both forms of the singly-bound channel:

$$[EA_2] = k_2[EA][A]$$
$$[EA_2] = k_2[AE][A]$$

$$2[EA_2] = k_2[A][EA]_{\text{total}} = 2k_2k_1[E][A]^2$$

thus

$$[EA_2] = k_2k_1[E][A]^2$$

where $k_2$ is the association constant for this reaction.

Assume that the response of a channel dimer to an activator is the sum of the responses of its subunits, and that the response of one subunit is independent of the state of the other subunits of the oligomer. Under these conditions the ratio of the response of the population of channels to a given concentration of activator $[A]$ to the maximum response possible would be:

$$\frac{R([A])}{R_{\text{max}}} = \frac{(V_a - V_o)[EA]_{\text{total}} + 2(V_a - V_o)[EA_2]}{2(V_a - V_o)[E]_{\text{total}}}$$

Cancelling common terms and substituting the appropriate expressions yields:
This equation is known as the Adair equation, or rather a version adapted for analyzing channels. Consider the following extreme cases:

1. Binding of activator to each site is independent of the state of the neighboring site in the oligomer, i.e. \( k_1 = k_2 \).

2. Binding of activator to the second site is greatly facilitated by having the first site bound, i.e. \( k_z \sim k_1 \).

3. Binding of the second activator molecule is completely inhibited by the binding of the first, i.e. \( k_z \ll k_1 \).

Case 1: \( k_1 = k_2 \). The Adair equation becomes:

\[
\frac{R([A])}{R_{max}} = \frac{k_1 [A] (1 + k_1 [A])}{(1 + k_1 [A])^2} = \frac{k_1 [A]}{(1 + k_1 [A])}
\]

Switching to dissociation constants and making the substitution \( K_{act} = 1/k_1 \), results in the Michaelis-Menten equation, as expected.

Case 2: \( k_1 \ll k_2 \). Terms involving only \( k_1 \) are neglected and the Adair equation becomes:

\[
\frac{R}{R_{max}} = \frac{k_1 k_2 [A]^2}{1 + k_1 k_2 [A]^2}
\]
Substitution of the averaged dissociation $K_{act}$ for $1/k_1 k_2$ yields the Hill equation, with Hill coefficient 2:

$$\frac{R}{R_{max}} = \frac{[A]^2}{K_{act} + [A]^2}$$

This is a special case of the more general form of the Hill equation, shown in Chapter IV. Note that the form of this equation is the same as that of the Michaelis-Menten equation, with the exception that the latter has a Hill coefficient of 1. In general, for the case where binding of an activator molecule to an oligomer is facilitated by the prior binding of activator to other sites on the oligomer, the Hill coefficient will be between one and the total number of binding sites on the oligomer; such an enzyme is said to bind substrate with positive cooperativity. The Hill coefficient cannot exceed the number of binding sites, though it can be less.

Case 3: $k_2 < k_1$. Terms involving $k_2$ are dropped from the Adair equation:

$$\frac{R([A])}{R_{max}} = \frac{k_1 [A]}{1 + 2k_1 [A]} = \frac{[A]}{K_{act} + 2[A]}$$

This is not of the Hill equation form. Transforming this to a form suitable for a Hill plot, one finds that

$$\log\left(\frac{R}{R_{max}} - R\right) = \log([A]) - \log(K_{act} + 2[A])$$

A Hill plot ( $\log(R/R_{max}-R)$ vs $\log([A])$ ) for this type of interaction between sites would clearly not be linear; were
one to try to fit a line to this plot, the best fit curve would have a slope (Hill coefficient) less than one. Activation with a Hill coefficient of less than one is known as negative cooperativity, indicating that binding is not independent, but rather than being facilitated, the second (or $n^{th}$) activator binding is inhibited by prior activator binding.

The motivation for developing the mathematics of the two-activator site channel dimer given here is to show the limits of information accessible to this kind of analysis. Hill plots are a good place to begin a kinetic analysis, because the results of a Hill plot will indicate whether development of a more complicated theory than simple Michaelis-Menten kinetics is necessary.
APPENDIX B

PASCAL PROGRAM FOR ESTIMATING KINETIC PARAMETERS

The program on the following pages is written in Pascal and is designed to estimate the kinetic parameters $R_{\text{max}}$, $K_{\text{act}}$, and the Hill coefficient $h$ from data representing Ca$^{2+}$ efflux rate vs activator concentration. Using screen prompts, the program first allows the user to set a tolerance for convergence of the equilibrium constants. The procedure Readdata asks for the rate of efflux in the absence of activator ($V_{\text{baae}}$), then an estimate of the maximum efflux rate at saturating activator concentration ($V_{\text{max}}$), and then the raw data. Readdata subtracts the basal efflux rate, $V_{\text{baae}}$, from the raw efflux rates to obtain the response function of the channel, $R([A])$, to that activator. The values of $R([A])$ and $[A]$ are put into separate arrays.

The Hillplot procedure arranges the response and activator data in a form suitable for a regression analysis on a Hill plot, while the Haneplot procedure arranges these data into a form suitable for a regression analysis on a Hanes plot. The program is set up to run the Hill regression first so that an estimate of the Hill coefficient is made and fed into the Haneplot procedure; the Hanes analysis provides a new estimate of $R_{\text{max}}$ which is fed back...
into the Hillplot procedure. Both procedures produce estimates of the binding affinity ($K_{act}$) of the activator/site complex. The process repeats until the estimates of $K_{act}$, which are produced independently by each procedure converge to within the tolerance set at the beginning.
(*Program HaneHill*)

Program HaneHill;
Type Analray = Array[1..50] of Real;
Type Datarray = Array[1..50] of Real;
Var Sary, Vary, Xary, Yary, Vmax, Vbase, Rmax, Diff: Real;
Var LCC, Rmax, Stoich, KS, Xintcept, Yintcept, Slope, SX, SS, SY: Real;
Var Diff1, Diff2, Ratio, KS, Ratio2, Ratio3: Real;
Var A, B, C, I, CTR, Expo, CTR1, CTR2, CTR3, CT, F, G, H: Integer;
Var Flag, Flag1, Flag2, Flag3, Flag4, Flag5, Flag6: Boolean;
Var LCCHP, LCCLB, Nary, Nsray, Ksray, Ksray, Ksray, Ksray, Rmaxi,
    SRmaxi, STC, CTR: Analray;
Var HIX, HILY, HaneX, HaneY: Datarray;

(*Perform Regression Analysis on Data in X and Y Arrays; CTR4 is the number of entries in each array (may vary from run to run); SX, SS, and SY are the standard deviations in X-int, Slope, and Y-int to be output*)

Procedure Regress(Var Xary, Vary: Datarray; Var CT: Integer;
    Var LCC, Xintcept, Yintcept, Slope, SX, SS, SY: Real);
Var K, L: Integer;
Var SumX, SumY, SumXY, SumXX, SumYY, M, Xint, B, Varnc, D, Vinc,
    VarB, VarM, SD, SDM, SDX: Real;
Begin
    SumX := 0;
    SumY := 0;
    SumXY := 0;
    SumXX := 0;
    SumYY := 0;
    Vinc := 0;
    For K := 1 to CT Do Begin
        SumX := SumX + Xary[K];
        SumY := SumY + Yary[K];
        SumXY := SumXY + Xary[K] * Yary[K];
        SumXX := SumXX + SQRT(Xary[K]);
        SumYY := SumYY + SQRT(Yary[K]);
    End;
    LCC := (CT * SumXY - SumX * SumY) / (SQRT((CT * SumXX - SumX) * (CT * SumYY - SumY * SumY)));
    D := CT * SumXX - SQRT(SumX);
    M := (CT * SumXY - SumX * SumY) / D;
    B := (SumXX * SumY - SumX * SumXY) / D;
    Xint := -B / M;
    For L := 1 to CT Do Begin
        Vinc := Vinc + SQRT(Yary[L] * B - M * Xary[L]);
    End;
    Varnc := Vinc / (CT - 2);
    SD := SQRT(Varnc * SumXX / D);
    SDM := SQRT((CT * Varnc) / D);
    SDX := SQRT((SD * Varnc) + SQRT(SDM)) / M;
    Slope := M;
    Xintcept := Xint;
    Yintcept := B;
    SX := SDX;
    SS := SDM;
    SY := SD;
End;
Procedure Readdata(Var Sary, Var Vary, Rary; Datarray; Var I: Integer; Vbase: Real)
Var N: Integer; S, V: Real
Begin
N := 1;
Repeat
Writeln('Enter S');
Readln(S);
Sary[N] := S;
Writeln('Enter V');
Readln(V);
If V > Vmax then Writeln('WRONG!!!!') else
Vary[N] := V;
Rary[N] := Vary[N] - Vbase;
N := N + 1;
Until S = 0;
I := N - 2;
End:

Procedure Hillplot(Var Sary, Rary, Xary, Vary; Datarray; I: Integer; Var Real; Var CTR: Integer)
Var CTR4, P, J: Integer;
Begin
CTR4 := 1;
For J := 1 to CTR4 Do Begin
If Rmax > Rary[J] then begin
Xary[J] := LN(Sary[J]);
Vary[J] := LN(Rary[J] / (Rmax - Rary[J]));
End
Else CTR4 := CTR4 - 1;
End;
Writeln('');
Writeln('****HILL****');
Writeln('Xary    Vary');
For P := 1 to CTR4 do
Writeln(Xary[P]:8, Vary[P]:8);
CTR := CTR4;
End:

Procedure HanePlot(Var Sary, Vary, Rary, Xary, Yary; Datarray; I: Integer; Var Vbase, Rmax, Stoich; Real; Var CTR: Integer)
Var CTR5, Q, J: Integer;
Var Xary, Yary; Datarray;
Begin
CTR5 := 1;
For J := 1 to CTR5 Do Begin
If Rmax > Rary[J] then begin
Xary[J] := EXP(Sstoich*LN(Sary[J]));
Yary[J] := EXP(Sstoich*LN(Sary[J]) / (Rary[J]);
End
Else CTR5 := CTR5 - 1;
End;
Writeln('');
Writeln('**** HanePlot ****');
Writeln('Xary    Yary');
For Q := 1 to CTR5 do
Writeln(Xary[Q]:8, Yary[Q]);
End:
(********** MAIN PROGRAM **********)

Begin
WriteIn('Enter Vbase'); {Vbase is the rate of transport}
ReadIn(Vbase);
WriteIn('Enter Vmax'); {Vmax is the rate with saturating stimulation}
ReadIn(Vmax);
Rmax := Vmax - Vbase;
Rmaxy := Vmax - Vbase;
HillRmax[1] := Rmax;
A := 1;
Readdata(Sary,Vary,Rary,1,Vbase); {Read raw data}
WriteIn('[Substrate]', 'Transport velocity');
For CTR := 1 to 1 Do Begin
  WriteIn(Sary[CTR];10, ', Vary[CTR];10); End;
Reset;
HillPlot(Sary,Vary,Rary,Xary,Vary,1,Rmax,CTR); For F := 1 to CT Do Begin
HillX[F] := Xary[F]; HillY[F] := Yary[F]; End;
Regress(Xary,Yary,CT,CLC,Xintcpt,Yintcpt,Slope,SS,SY);
  Stoich := ABS(Slope);
  Ks := EXP(-Yintcpt);
  Nary[A] := Stoich;
  Naray[A] := SS;
  Ksray[A] := KS;
  Kshray[A] := SYKsH;
  LCCPL[A] := LCC;
  Ratio1 := (Nary[A]-Nary[A-1])/Nary[A];
  Flag5 := Ratio1 < -0.1;
  Flag6 := Ratio1 > 0.1;
  If A >= 2 Then
    If Flag5 then Stoich := 0.9*Nary[A-1] Else
    If Flag6 then Stoich := 1.1*Nary[A-1];
  STC[A] := Stoich;
HillPlot(Sary,Vary,Rary,Xary,Yary,1,Rmax,stoich,CT); For G := 1 to CT Do Begin
Regress(Xary,Yary,CT,CLC,Xintcpt,Yintcpt,Slope,SS,SY);
  KS := -(Xintcpt);
  Karay[A] := KS;
  CCT[A] := CT;
  Kisray[A] := SX;
  Rmax[A] := 1/Slope;
  Rmax0 := SS/SSR(Slope);
  Ratio2 := ABS(Rmax[A]-Rmax0[A-1]/Rmax0[A]);
  Ratio3 := 1/(KS+KS);
  LCCPL[A] := LCC;
  Diff1 := Rmax[A] - Rmax;
  Diff2 := 0.1*Rmax[A];
  Diff3 := -0.1*Rmax[A];
  Flag1 := Diff > Diff3;
  Flag2 := Diff < Diff2;
  If Flag1 then Rmax := 1.1*Rmax else
  If Flag2 then Rmax := 0.9*Rmax else
  Rmax := Rmax[A];
  A := A+1;
HillRmax[A] := Rmax;
  Flag4 := Ratio2 < 0.05;
  Flag := A = 15;
Until Flag4 or Flag;
For B := 1 to 1 Do
Anonymous Program Description: Raw data is analyzed in a Hill Plot to obtain the Hill coefficient of stimulation, then analyzed in a Hanes plot to obtain the maximum response and the binding constant of the stimulant. An initial guess of the maximum velocity is required in order to make things go; the calculated maximum response (Vmax = Vmax (guessed) - Vbase), along with the original data, is used to generate a Hill plot, from which estimates of the Hill Coefficient and the binding constant of the stimulant are obtained. The Hill coefficient is then used as an exponent of the substrate concentration in a modified Hanes plot; a new estimate of Rmax and an independent estimate of Ks are thus obtained. The revised Rmax is used in another iteration of the Hill analysis, etc, etc until the two independent estimates of Ks are nearly equal or until a set number of iterations of the whole routine have been performed.)