A STUDY ON SOME STRUCTURAL AND FUNCTIONAL PROPERTIES OF THE SARCOPLASMIC RETICULUM FROM SKELETAL MUSCLE

by

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CHAPTER 1

INTRODUCTION AND LITERATURE DISCUSSION

It is a well-established fact that the sarcoplasmic reticulum (SR) plays a very important role in the exitation-contraction coupling of skeletal muscle by regulating the concentration of free Ca^{2+} in the sarcoplasm (Hasselbach, 1964; Sandow, 1965; Weber et al, 1966; Ebashi & Endo, 1968 and Fuchs, 1974). Depolarization of the sarcolemma by nervous stimul= ation spreads over the entire surface of the muscle cell by means of a regenerating process and also to the interior of the cell via the T-tubules. This wave of depolarization is followed by the release of Ca^{2+} from the SR by which process muscle contraction is induced. To understand the exact role of the SR in the contraction-relaxation cycle it is necessary to clarify the structural organization of the SR mem= brane and to establish the role of its different components. Furthermore, it is important to know by what physiological mechanism depolarization of the T-tubule can induce the release of adequate amounts of Ca^{2+} to activate the contractile apparatus.

Therefore, the aim of this study in the first place is to further elucidate the structure and function of the protein components of the SR membrane. Secondly, we tried to establish a figure for the Ca^{2+}-permeability of the membrane and to test the hypothesis that Ca^{2+} release is the result of a direct electrical coupling

between the T-tubule and the SR membrane.

In the rest of Chapter 1, a historical background of the development of SR research is given, as well as a literature discussion on the functional role of different protein components of the SR membrane. The literature concerning the Ca^{2+}-permeability of SR membranes as well as different experimental procedures for the release of Ca^{2+} from the SR, is reviewed. In Chapter 2 the different experimental procedures used in the course of this study are described. Chapter 3 is concerned with experimental results in a study of the functional and structural roles of the protein components of the SR, as well as Ca^{2+}-flux studies in the presence and absence of ATP. A three-compart= mental passive flux curve is presented with a discuss= ion on the possible nature of these compartments. Results obtained in experiments to produce uneven ion distributions, and thus a potential difference across the membrane, in an effort to produce Ca^{2+} release from the vesicles, are also included. In Chapter 4 a relationship between the number of calcul= ated ATPase molecules per unit membrane and the number of 90 Å particles observed in freeze-fractured mem= brane surfaces, is deduced. Furthermore, a value for the Ca^{2+}-permeability of the SR membrane is calc= ulated and the possible physiological significance of Ca^{2+} release as result of ion substitutions, is discussed.

1.1 Historical background

About twenty years ago three very important public=
ations laid the ground-work for the present tremend=
ous interest in muscle physiology. In 1951 Marsh
published a short communication describing the disc=
overy of a "relaxing factor" in the supernatant of a
muscle homogenate. He reported that the muscle hom=
ogenate which is usually in a shrunken form in the
presence of ATP, undergoes swelling when the super=
natant containing the relaxing factor, plus ATP, are
added. The second paper appeared in 1953 by Hanson
& Huxley in which they suggested that the two major
muscle proteins, actin and myosin, are organized into
two sets of parallel, interdigitating filaments.
Thirdly, reporting the results of electron microscopic
studies, Bennett & Porter (1953), described the pres=
ence of a tubular network, the sarcoplasmic reticulum
within the interfibrillar space of skeletal muscle.

Knowledge about the sarcoplasmic reticulum and its
role in the excitation-contraction coupling was
extremely limited up to that time. In 1882 Retzius
and in 1902 Veratti described the existance of a
reticular structure within the myofibril (cf. Hassel=
bach, 1964). This structure was named the reticular
apparatus by Veratti, who regarded it as an intra=
cellular extension of the muscular nerve-supply (cf.
Langer, 1968). The present term, sarcoplasmic reticulum
(SR), was given to this structure by Bennett & Porter
(1953).

The first indication that calcium plays an important role in muscle contraction was presented by Ringer in 1883 (cf. Langer, 1968) when he noted that the frog heart does not develop tension unless Ca^{2+} is present in the perfusion medium. The role of Ca^{2+} was further defined by Niedergerke (1955) who injected various cations directly into single, isolated frog skeletal muscle fibres by means of a micro-pipette. Ca^{2+} was the only ion of those physiologically present that produced shortening of the fibre. This raised the possibility that local release of Ca^{2+} into the myo= filamentous region is responsible for the initiation of contraction.

A further step in establishing the important role of Ca^{2+} in the excitation process started with the ex= periments of Huxley & Taylor (1958). With local electrical stimulation of frog skeletal muscle in the region of the Z-line of a sarcomere, they produced a contraction limited to a single I-band or to adjacent I-bands of separate sarcomeres. Stimulated by this observation, Podolsky & Constantin (1964) applied Ca^{2+} to the Z-line and I-band region and reported single I-band contractions. These studies firmly established the role of Ca^{2+} and gave credence to the concept that electrical depolarization brings about a release of Ca^{2+} which then initiates contraction.

In living muscle, contraction is followed by relax= ation. The question then remains by what physio= logical mechanism this contraction-relaxation cycle

can be brought about. It was pointed out that ATP has a double function inducing both contraction and relaxation of actomyosin threads. It was further demonstrated that a reversible cycle of contraction-relaxation of glycerinated muscle fibres could be obtained by simply raising and lowering the temper= ature of the medium. All these factors, however, are maintained constant in living muscle and cannot therefore be the mechanism of the contraction-relax= ation cycle (Ebashi & Endo, 1968).

The big break-through started with the discovery of the "relaxing factor" by Marsh in 1951. In a study on the effect of water retention of a muscle homogen= ate, he found that the homogenized muscle, which was normally shrunken by ATP, could be induced to swell by the addition of ATP plus a muscle extract obtained by a simple procedure. The swelling was considered to be analogous to the relaxation process. Bendall (1953) reported that this same muscle extract could induce the relaxation of glycerinated fibres.

These results suggested that the muscle extract con= tained the physiological factor responsible for in= ducing relaxation of muscle. This physiological factor was termed the "relaxing factor" and efforts were subsequently concentrated in attempting to ident= ify the active component from such extracts. Work by Kumagai and by Ebashi showed that this factor is identical with a known fraction of muscle i.e. Kielley-Meyerhof's granular ATPase (Ebashi & Endo, 1968).

Kielley & Meyerhof reported an ATPase fraction in skeletal muscle which was apparently soluble but could be sedimented by centrifugation at 18 000 g for 1 to 2 hours. The particulate nature of the relaxing factor was confirmed by Portzehl (1957).

Bozler in 1954 reported for the first time that EDTA in the presence of ATP could induce relaxation of glycerinated muscle fibres. However, at that time it was not interpreted as the effect of deprivation of the contractile system of Ca^{2+} (Ebashi & Endo, 1968). According to these authors it was Weber in 1959 who first pointed out that a minute amount of Ca^{2+} is necessary for the functioning of the acto=myosin-type ATPase of myofibrils. Ebashi (1960) showed that the relaxing activities of various Ca^{2+}-chelating agents on glycerinated fibres is a strict function of their Ca^{2+}-binding capacity.

Another break-through in this field also came in 1960 with the work of Ebashi who showed that this relaxing factor could accumulate Ca^{2+} in the presence of ATP. This initial observation was subsequently repeated and extended by numerous authors. As result of the particulate nature of the relaxing factor it was con=cluded that this factor was derived from some cellular organella. Electron microscopic studies on these particles revealed their vesicular structure (Nagai et al, 1960). These observations indicated that the relaxing factor described by Marsh nine years earlier was, in fact, a suspension of vesicles derived from the

sarcoplasmic reticulum (Hasselbach & Makinose, 1961 and Ebashi & Lipmann, 1962). This provided a good explanation for the results obtained by Huxley & Taylor (1958) with their local stimulation experi= ments. They showed that the T-tubule is the path= way through which electrical events are mediated to the interior of the cell.

These results led also to the conclusion that the sarcoplasmic reticulum exerts its relaxing effect by removing Ca^{2+} from the contractile system. It was postulated that the sarcoplasmic reticulum is the site of linkage between excitation and contraction. That would mean that the Ca^{2+} associated with a certain part of the sarcoplasmic reticulum is released under the influence of the electrical current induced by depolarization of the surface membrane. This re= leased Ca^{2+} subsequently activates the contractile system (Ebashi & Endo, 1968; Fuchs, 1974).

Since these discoveries much work has been done on sarcoplasmic reticulum function. Most of this work has been directed toward the Ca^{2+}-uptake mechanism by isolated SR, structure and function of the ATPase enzyme of the SR and also on elucidating the structure of the membrane itself as well as the T-tubule - terminal cisternae association.

1.2 General organization and function of the sarcotubular system *in vivo*

1.2.1 Skeletal muscle: The sarcotubular system

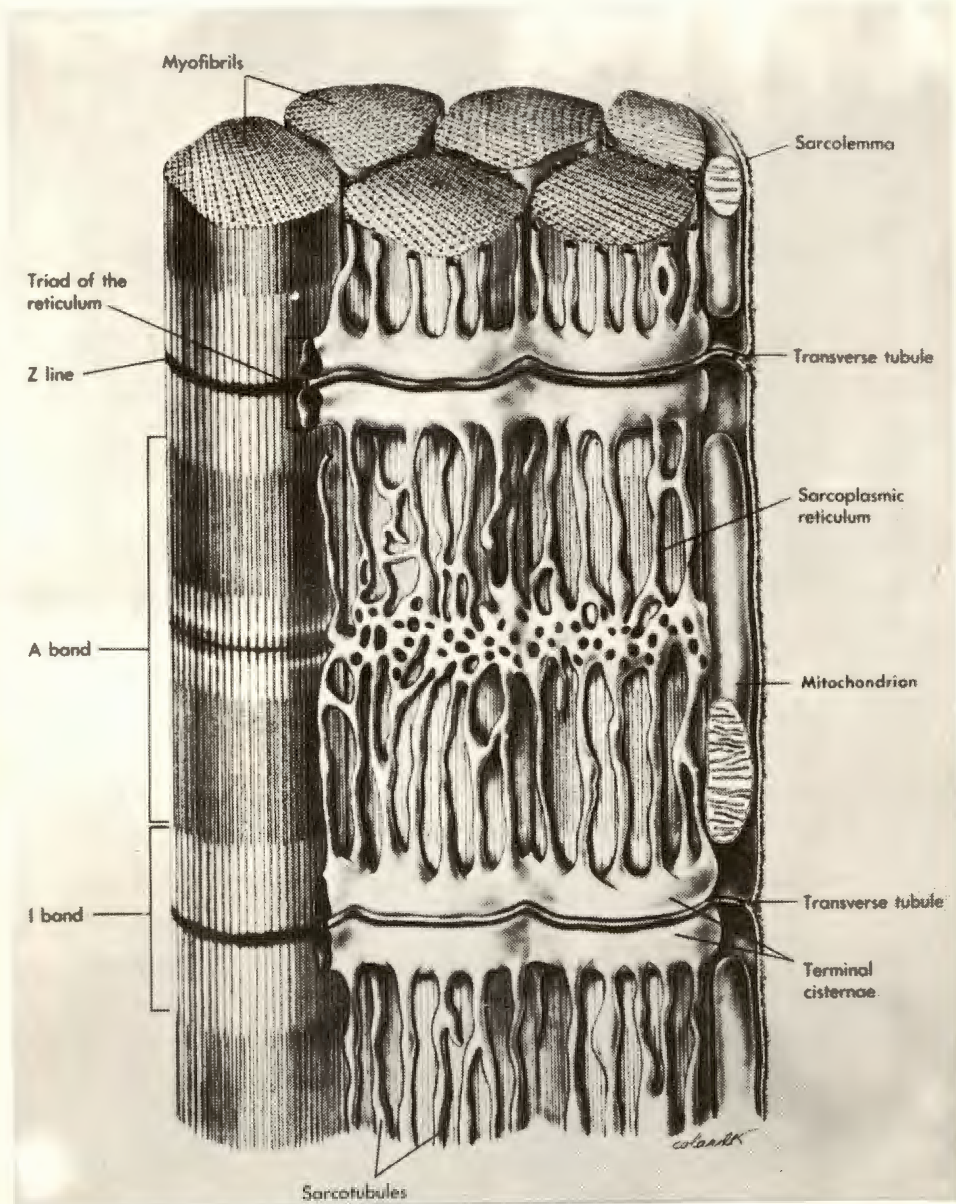

Myofibrils
Sarcolemma
Triad of the reticulum
Z line
Transverse tubule
Sarcoplasmic reticulum
A band
Mitochondrion
I band
Transverse tubule
Terminal cisternae
Sarcotubules

Plate 1: Schematic representation of the distribution of the sarcoplasmic reticulum around the myofibrils of skeletal muscle. The longitudinal sarcotubules are confluent with transverse elements called the terminal cisternae. A slender transverse tubule (T-tubule) extending inward from the sarcolemma is flanked by two terminal cisternae to form the so-called triads of the reticulum. In frog muscle there are two to each sarcomere, located at the A-I junctions. (Modified after L. Peachey: J. Cell. Biol., 25:209, 1965, from D.W. Fawcett and S. McNutt. Drawn by Sylvia Colard Keene. Reproduction of plate by the kind permission of W.B. Saunders Co., publishers and by the authors of Bloom, W. & Fawcett, D.W. 1968. A textbook of histology, 9th Ed.).

closely surrounds the myofibrils and consists of two main parts i.e. the transverse tubules (T-tubules) and the sarcoplasmic reticulum. A close association exists between these two parts in skeletal muscle, called the triad consisting of the T-tubule as central element with two adjacent sac-like structures of the SR, the terminal cisternae (plate 1, page 8).

1.2.1.1 T-system

The T-system is essentially invaginations of the sarco= lemma and is recognizably different from the SR for three reasons:

(a) Its dimensions are smaller.
(b) Its limiting membrane is more prominent.
(c) Its content is never continuous with the content of the SR (Franzini-Armstrong & Porter, 1964 and Peachey, 1965).

Although the openings of the T-tubules have rarely been seen electron microscopically in mammal skeletal muscle, these openings have been seen in preparations from fish skeletal muscle (Franzini-Armstrong & Porter 1964). Huxley (1964), too, has used electron micros= copy to demonstrate the entrance of ferritin part= icles from the external medium into the transverse tubules of frog muscle. The opening of the T-tubules are at the level of the Z-line or the A-I junction. It was observed that the T-tubules are continuous across the width of the fibre.

1.2.1.2 Sarcoplasmic reticulum (plate 1)

The SR consists mainly of two interconnected parts i.e. sac-like structures, the terminal cisternae and longitudinal tubules, the L-tubules. On both sides of each T-tubule in vertebrate skeletal muscle, two terminal cisternae are situated in close association with the T-tubules, forming a triad. There is no direct connection between the different parts of the triad and, in fact, the T-tubule and terminal cist=ernae are separated with a gap of 120 - 140 Å (Franzini-Armstrong, 1970), and, at periodic intervals of about 300 Å the SR membrane forms small projections, the tips of which are joined to the T-tubule membrane by some amorphous material. These projections and amorphous material have become known as junctional or SR feet. The feet are disposed in two parallel rows, two such rows being present on either side of the T-tubule. The feet cover at the most 30% of the T-tubule surface area and 3% of the total SR area (Franzini-Armstrong, 1970).

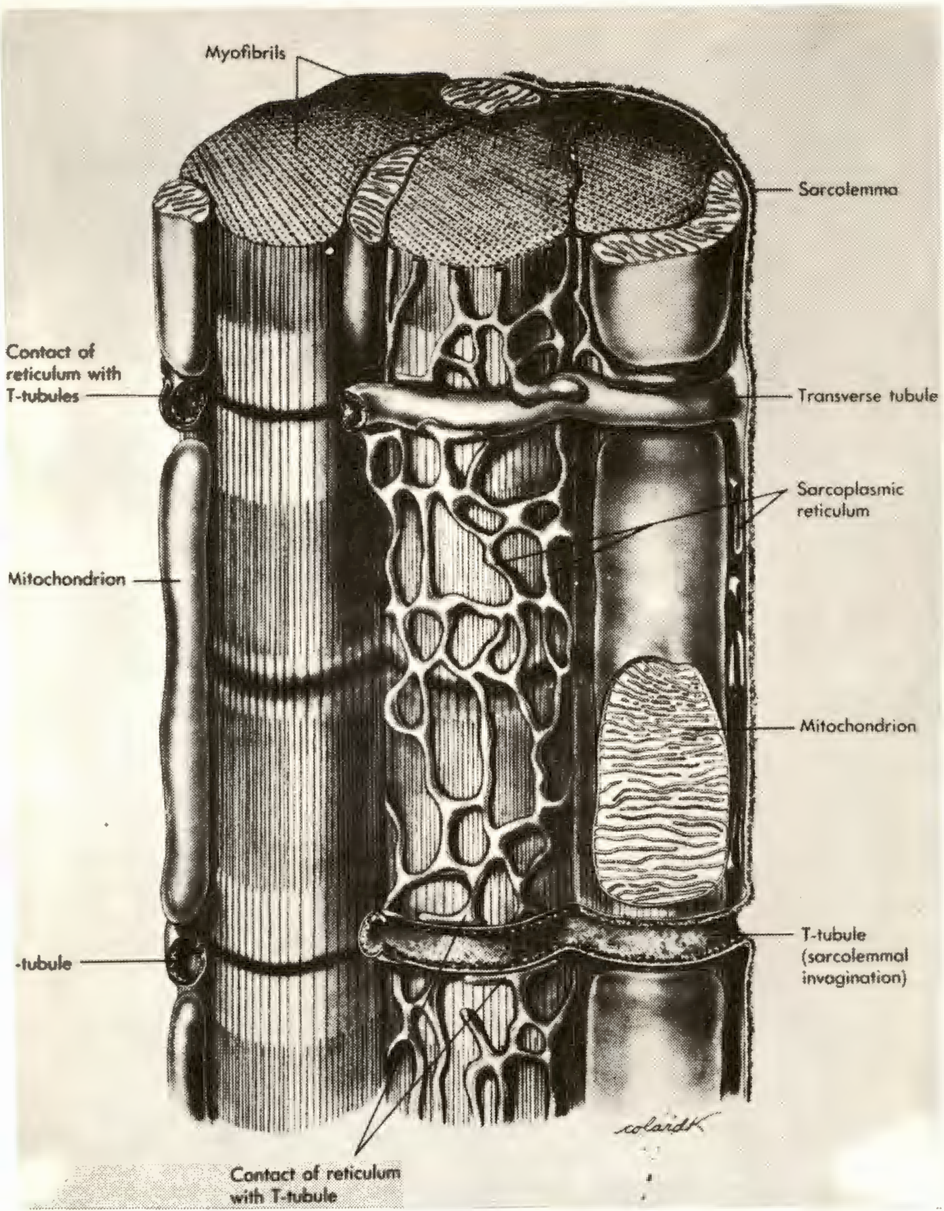
From the terminal cisternae the L-tubules extend lengthwise along the myofibrillar surface and connect with the terminal cisternae of the adjacent triad (plate 1). Near the centre of the A-band the L-tubules fuse to form continuous collars around the myofibrils, called fenestrated collars. Initially it was thought that these fenestrations represent perforations in one of the two membranes of the SR collar and thus are places where the internal content of the reticulum is in continuity with the sarcoplasm.

However, this proved to be wrong as was shown by Peachey (1965) who found that these fenestrations represent openings of a meshlike structure formed by the L-tubules in this area.

The morphological organization of the sarcoplasmic membrane meets the requirements which its function demands. In fast-contracting muscles each myofibril is surrounded by the tubules and cisternae of the sarcoplasmic membrane. Consequently, the Ca^{2+} ions do not have to diffuse farther than 0,5 μ (Hasselbach & Suko, 1974). Furthermore, the surface of the sarco=plasmic membranes is enlarged by this structural arrangement up to more than 10 000 cm^2/cm^2 of surface area, for a muscle fibre 2 cm in diameter which is an area approximately 40 - 50 times larger than the plasm membranes around the fibres. The volume of the SR is about 15% of the fibre volume (Peachey, 1965).

1.2.2 Cardiac muscle

The tubules of the T-system in cardiac muscle are larger than the corresponding structures in skeletal muscle. The openings of the T-tubules are on the level of the Z-lines and penetrate to the centre of the muscle fibre. According to Bloom & Fawcett (1968) no point in the cardiac muscle fibre is more than 2 - 3 μ from the extracellular space, either at the outer surface of the fibre or in one of the T-tubule invaginations. These channels apparently, also provide additional surface for the exchange of metabolites (plate 2, page 13).


Myofibrils
Sarcolemma
Contact of
reticulum with
T-tubules
Transverse tubule
Sarcoplasmic
reticulum
Mitochondrion
Mitochondrion
-tubule
T-tubule
(sarcolemmal
invagination)
Contact of reticulum
with T-tubule
colardK

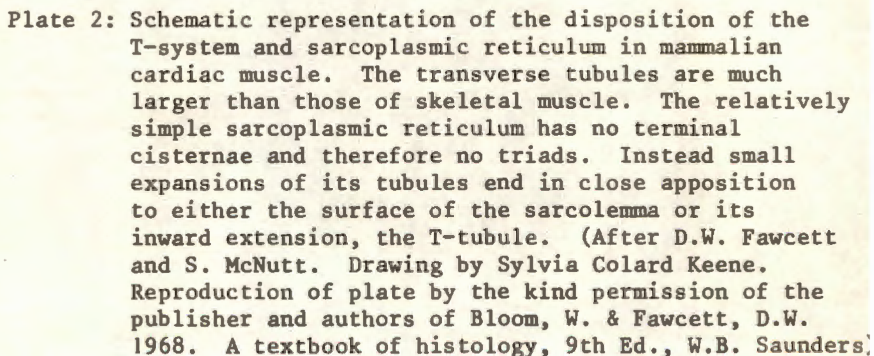


Plate 2: Schematic representation of the disposition of the T-system and sarcoplasmic reticulum in mammalian cardiac muscle. The transverse tubules are much larger than those of skeletal muscle. The relatively simple sarcoplasmic reticulum has no terminal cisternae and therefore no triads. Instead small expansions of its tubules end in close apposition to either the surface of the sarcolemma or its inward extension, the T-tubule. (After D.W. Fawcett and S. McNutt. Drawing by Sylvia Colard Keene. Reproduction of plate by the kind permission of the publisher and authors of Bloom, W. & Fawcett, D.W. 1968. A textbook of histology, 9th Ed., W.B. Saunders)

The cardiac sarcoplasmic reticulum is rather poorly developed. It consists of a rather simple reticul= ated tubular system arranged around the myofibril. No structures comparable to the terminal cisternae are present. Instead small branches of the SR are closely associated with the membranes of the T-tubules. This same type of contact also exists between the SR and the sarcolemma. Though the sum of all these points of contact may be quite large, it is still much smaller than the contact area in the triad of skeletal muscle (Bloom & Fawcett, 1968).

1.2.3 Functional role of the sarcotubular system

The classical experiments on local stimulation of skeletal muscle fibres by Huxley & Taylor (1958), led to recognition of the fact that the T-tubule is the likely pathway for inward spread of excitation. It was found by Howell (1969) and Krolenko (1969) that treatment of the muscle fibres with hypertonic sol= utions of glycerol destroys the T-system while the excitation mechanims of the sarcolemma stay intact. In such fibres the mechanical response to excitation is eliminated.

Experiments by Constantin (1970) and Conzâlez-Serratos (1971), showed that the inward transmission of the action potentials of the sarcolemma is through a Na^{+}-dependent regenerative response, probably similar to the surface action potential. Since then, various laboratories have produced evidence in support of this idea (Fuchs, 1974).

Presumably the same mechanism is true for cardiac muscle, but studies on this muscle are complicated by a high resistance of the T-tubules to glycerol treat= ment. A concentration as high as 1 M had no notice= able effects on the T-tubules of cardiac muscles (Nie= meyer & Forssman, 1971). Although the reason for this is not clear, they mentioned two morphological factors that may be relevant:

(a) The basement membrane of cardiac muscle extends into the T-tubule, and
(b) the cardiac T-tubule is much shorter and wider than skeletal T-tubules.

As was indicated by the experiments of Podolsky & Constantin (1964), the release of intracellular Ca^{2+} and especially the release of Ca^{2+} at the level of the triad (i.e. Z-line or A-I junction), is essential for muscle contraction. It follows then that inward spread of the action potential in the T-tubule must be accompanied by Ca^{2+} release from the terminal cisternae of the SR. Indeed, such inward spread of the action potential must initiate the release of Ca^{2+} from the SR. The mechanism of this process remains obscure. However, two general mechanisms are consid= ered in this connection:

(a) A chemical coupling between the T-tubule and SR membrane where a chemical substance to the nature of the carriers in the neuro-muscular junction or perhaps Ca^{2+} itself, might play a role in triggering the re= lease of SR Ca^{2+}.

(b) A direct electrical coupling between the two membranes with the SR Ca^{2+} permeability, voltage dependent.

Whatever the mechanism, it must be situated in the triadic junction. From extensive electron micros= copic studies Franzini-Armstrong (1970-1971) con= cluded that on morphological grounds alone, one cannot decide whether coupling is through ionic current flow via the junctional feet or chemical transmission across the junctional gap. Although she concluded that the triad is "badly designed" for direct electrical current she proposed the existence of aqueous channels in the SR feet through which an electrical current may be transmitted from the T-tubule to the terminal cisternae. The present status in the literature of the various possibilities will be discussed more extensively in 1.3.2 (page 32).

From the SR the Ca^{2+} diffuses to the contractile pro= teins, actin and myosin, of the myofibril. Via the troponin - tropomyosin control mechanism, interaction between actin and myosin is produced and shortening of the muscle is effected. An excellent review article on possible mechanisms involved, was published by Fuchs (1974) but, as it falls outside the scope of this study it will not be discussed further.

By taking up Ca^{2+} by means of an ATP-dependent Ca^{2+} pump, the SR is also capable of lowering the intra= cellular Ca^{2+} concentration to about 10^{-7} M and thus

produces muscle relaxation. As a result of autoradiographic studies Winegrad (1970) concluded that intracellular Ca^{2+} movement follows a rather complex cycle instead of a simple exchange between SR and sarcoplasm. He proposed the following pathway for Ca^{2+} movement:

(a) Ca^{2+} released by the terminal cisternae of the SR diffuses to the thin filaments (actin),

(b) accumulation by the L-tubules, and

(c) transport of Ca^{2+} through the reticulum back to the terminal cisternae.

These results indicate that there is a separation of function into "release" sites (terminal cisternae) and "relaxation" sites (L-tubules) (Fuchs, 1974). The idea of a separation of function in the SR is supported by results obtained by Meissner (1975) who separated the isolated SR vesicles by means of a continuous sucrose gradient into two fractions, light and heavy microsomes. He concluded that the heavy SR originates from the terminal cisternae and has a low Ca^{2+}-uptake activity while the light SR originates from the L-tubules and has a high Ca^{2+}-uptake activity. (For a more detailed discussion see 1.3.1, page 24).

Although all of these theories have not been proven beyond any doubt, a relatively clear picture is emerging of some of the key-aspects of the contraction-relaxation cycle in skeletal muscle. In other types

of muscle like cardiac muscle, however, the picture is much more obscure. From the discussion in 1.2.2 it is clear that the cardiac SR is not as well-developed by far as its skeletal muscle counterpart. Further= more, a number of functional differences between cardiac and skeletal muscle exist, with the result that the exact role of cardiac SR is still in doubt.

The effect of extracellular Ca^{2+} on the contraction of muscle cells is one of the most striking functional differences between cardiac and skeletal muscle. As early as 1883 Ringer demonstrated the necessity of extracellular Ca^{2+} for normal contraction of cardiac muscle cells (Langer, 1968). Since then, this effect has been clearly shown by various authors (Lüttgau & Niedergerke, 1958; Schwartz et al, 1973). On the other hand, extracellular Ca^{2+} is not so important in skeletal muscle (Schwartz et al, 1973), an indication that the intracellular Ca^{2+} sinks in skeletal muscle are much more effective than in cardiac muscle. This difference led some investigators to believe that a specific pool of calcium associated with the cell membrane in cardiac muscle may control the process of contraction whereas relaxation is carried on mainly by the SR. In skeletal muscle both contraction and relaxation seem to be under control of the SR.

From the difference in shape of the action potential (Fig. 1) it can be seen that in the case of the cardiac muscle, an additional phase (phase 2) exists which is believed to be associated with a slow inward

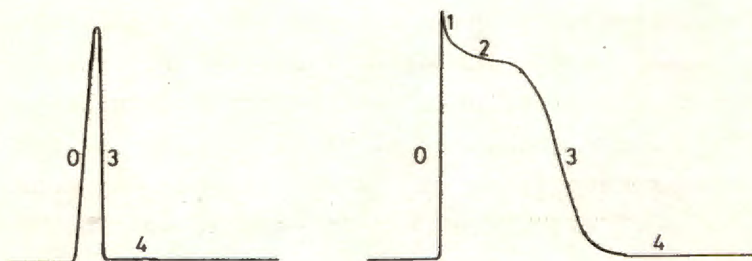


Figure 1: Schematic representation of action potentials of skeletal (left) and cardiac muscle (right). The duration of the action potential in skeletal muscle is about 2 msec. while it lasts for 500 msec. in cardiac muscle. The additional phase i.e. phase 2 in the cardiac muscle action potential is associated with a slow inward Ca^{2+} current as well as a decrease in potassium conductance.

calcium current, as well as a decrease in potassium conductance. This is another indication of the importance of extracellular calcium. Despite these findings, Solaro & Briggs (1974) estimated the funct= ional capabilities of cardiac SR and determined that this SR is able to accumulate enough Ca^{2+} to provide for muscle contraction from the results of *in vitro* experiments. Some doubt, however, remains whether the rate of accumulation and release of Ca^{2+} by cardiac SR is fast enough to explain the contraction-relaxation cycle.

Based on experimental evidence and in an effort to tie all these phenomena together, the theory of Ca^{2+}-triggered Ca^{2+} release has been proposed (Ebashi et al, 1969; Ford & Podolsky, 1970 and Endo et al, 1970). According to this theory the Ca^{2+} entering the muscle cell with each action potential could serve as a trig= ger for the release of more Ca^{2+} at a rapid rate from the internal Ca^{2+} stores, especially the SR. However, quite a controversy exists and Endo (1975) reported that the required concentrations of triggering Ca^{2+} are too high to be consistent with the physiological mechanism of excitation-contraction coupling in skel= etal muscle. Nevertheless, it is possible that this mechanism may play a role in other muscle types such as smooth muscle and cardiac muscle (Fabiato & Fabiato, 1975).

Also in isolated SR, certain possible functional dif= ferences between skeletal and cardiac muscle SR have

been observed. The lability of isolated cardiac SR in comparison to skeletal SR shows a striking differ= ence. In our experience cardiac SR maintains its activity well for 24 hours but loses it rapidly after that - up to 50% loss of activity during the next 24 hours while skeletal SR maintains its Ca^{2+}-uptake activity for at least seven days. In order to reduce this lability and produce a more active and stable preparation of cardiac SR we tried various substances to reduce oxidation, proteolysis and the possible effect of free fatty acids. For antioxidants we used various concentrations of butylated hydroxytoluene (BHT) as well as saturation of the media with N_2. To reduce the effect of proteolytic enzymes, 5×10^{-6} M phenylmethylsylfonylfluoride (PMSF) was used and for the possibility of harmful effects of free fatty acids 5 mg/ml albumin was added. None or very little improvement was observed (results not shown).

From the literature (Pretorius et al, 1969; Katz et al, 1970) it is clear that cardiac SR preparations produce another serious problem, namely, severe cont= amination by other cell organelles, especially mito= chondria. This contamination was estimated by Pre= torius (1972) to be as high as 50% in certain prep= arations. To reduce this contamination we implemented sucrose gradients which resulted in a 25% increase in activity (results not shown). Much work has still to be done before a stable and reliable preparation of cardiac SR will be available. To evaluate the funct= ional capability of cardiac SR, it is essential that

such a preparation should be obtained. To avoid ambiguities resulting from the problems in connection with cardiac SR, the experimental work for the present study has been done exclusively on isolated skeletal SR.

1.3 Literature discussion and motivation for the present study

During the past 25 years, since the report on the "relaxing factor" by Marsh (1951), numerous reports have appeared concerning various aspects of the struct=ure and function of the sarcoplasmic reticulum both *in vivo* and in the isolated vesicular form. A number of questions concerning the role of the sarcotubular system in the excitation-relaxation cycle have been elucidated mainly in skeletal muscle. However, a number of new and important questions were raised and some of them have not yet been answered. The reason for the slow progress in certain fields of sarcoplasmic reticulum research must be attributed to severe tech=nical difficulties in studying a system as delicate as the sarcotubular system.

During the first few years most of the research on isolated SR was directed towards Ca^{2+} uptake, ATPase activity and electron microscopy. By taking up calcium the SR effects relaxation in the muscle. Studies on Ca^{2+} uptake in isolated SR represent the relaxation process. As Ebashi & Endo (1968) remarked: "It is rather ironic that recognition of the essential

role of Ca ions in contraction has resulted mainly from the investigation into the mechanism of relax= ation".

It is generally accepted that the energy for the process of Ca^{2+} uptake is derived from the hydrolysis of ATP by the ATPase enzyme of the SR membrane. This enzyme is the main protein component of the membrane but certain minor protein components are also present. So, for the first part of this project, we undertook a study of the functional and structural roles of the sarcoplasmic reticulum protein components.

A study of the literature concerning sarcoplasmic reticulum function revealed a strikingly small number of publications concerning Ca^{2+}-release mechanisms, the actual process responsible for the initiation of muscle contraction. Again, a lack of suitable tech= nical methods for the effective study of this very important physiological process must be held respons= ible for the lack in knowledge. For the second and main part of this project a study of the permeability and Ca^{2+}-release mechanisms in sarcoplasmic reticulum vesicles was made.

1.3.1 Functional and structural roles of sarcoplasmic reticulum protein components

Sarcoplasmic reticulum constitutes a membrane system highly specialized for Ca^{2+} transport. Although Ostwald & MacLennan (1974) claimed to have isolated

seven proteins from sarcoplasmic reticulum, it is clear from studies on highly purified SR preparations that only three protein components are present in any sig= nificant amounts (Meissner et al, 1973; Inesi & Scales, 1974 and Meissner, 1974). Just the fact that Mac= Lennan (1970) and Ikemoto et al (1971) reported a several-fold purification of the ATPase protein which makes up 70-90% of the total SR protein, raises doubts about the purity of their SR preparation. These three components are the ATPase protein, calcium-binding protein (M_{55}-protein) and calsequestrin (M_{45}-protein). The morphological arrangement of these proteins in the membrane as well as possible functional relationships between them, have not yet been estab= lished.

It was suggested that these proteins should be class= ified into two groups: a group of membrane-associated proteins which are loosely attached to the membrane and are easily removed under relatively mild conditions e.g. M_{45}- and M_{55}-proteins. These proteins can be released from the vesicles by washing the SR with solutions containing EGTA or EDTA at pH 8,0 (Duggan & Martonosi, 1970; Meissner et al, 1973). The other group consists of intrinsic membrane proteins which make up the primary structure of the membrane together with the phospholipid, most notably the ATPase protein. These proteins require extreme conditions for solubil= ization (Meissner et al, 1973).

1.3.1.1 ATPase protein

A most important component of the SR membrane is the Ca^{2+}-dependent ATPase which is tightly-coupled to active transport of the divalent cation. The first successful separation of ATPase and the other proteins was performed by Martonosi (1969) and Martonosi & Halpin (1971) on SDS-polyacrylamide gel electrophoresis. Different bands were obtained but only three of these bands were of major content i.e. ATPase, M_{55}- and M_{45}-proteins. The biggest fraction was a protein with a molecular mass of 106 000 and was deduced to be the ATPase since ^{32}P from ATP-γ-^{32}P was incorporated only in this component (MacLennan, 1974).

MacLennan (1970) was the first to succeed in isolating the ATPase enzyme with a twofold increase in its act= ivity. Several other laboratories succeeded in purifying the ATPase by different procedures (Ikemoto et al, 1971; Meissner et al, 1973). Unfortunately, much inconstancy is found in the literature regarding the percentage of total SR protein accounted for by ATPase, as figures varying from 16% to 90% have been given (MacLennan, 1970; McFarland & Inesi, 1970 & 1971; Martonosi & Halpin, 1971; MacLennan & Wong, 1971; Meissner & Fleischer, 1971). By using a differ= ent separation procedure we are in this study able to show with much more certainty that the Ca^{2+} ATPase accounts for 70-90% of the total SR protein.

Two structural features of the SR vesicles have been

attributed to the ATPase protein:

(i) The 35-40 Å particles seen on the outer surfaces of negatively stained vesicles. These particles were first described by Ikemoto et al (1966). Later Marto=nosi (1968); Inesi & Asai (1969) and Ikemoto et al (1971) also described these particles. Studies with trypsin digestion showed that the particles are definitely of a protein nature (Ikemoto et al, 1971). From studies on the changes in membrane proteins as a function of time during the first 8-10 days in newly-born rabbits, Sarzala et al (1975) reported that the vesicles of skeletal muscle SR of newly-born animals are smooth. The 40 Å particles appear for the first time from 5-8 days after birth and are strictly correl=ated with an increase in the activity of the Ca^{2+} ATPase and Ca^{2+}-uptake activity of the vesicles. By dializing SR vesicles against EDTA the minor protein components can be removed, leaving only the 106 000 dalton ATPase protein associated with the membrane. In such membrane preparations the 40 Å particles are still present, suggesting that these structures are related to the 106 000 dalton protein (Inesi & Scales, 1974).

(ii) The second structural feature is 90 Å particles seen on the concave faces of the outer segments of freeze-fracture replicas (Deamer & Baskin, 1969). By freezing the vesicles very rapidly in liquid freon and cleaving them with a blade in a special freeze-fracture apparatus the "two-layered" membrane structure will split

down the middle revealing two faces, one convex and one concave, of the interior membrane structure (plate 5, page 98).

The outer concave fracture faces in rabbit skeletal muscle SR normally reveal numerous 90 Å particles while the convex surfaces are smooth. Sarzala et al (1975) showed that, like the 40 Å particles, the appearence of these 90 Å particles is strictly correlated with increasing ATPase activity during the early postnatal development of rabbits. Further= more, Jilka et al (1975) showed that introduction of ATPase protein to the structure of phospholipid vesicles not only increased the membrane permeability to Ca^{2+} but also led to the appearance of 90 Å part= icles on the freeze-fracture faces of the phospho= lipid vesicles. Earlier, Inesi & Scales (1974) showed that removal of the minor protein components of SR vesicles by dializing them against EDTA, did not result in the removal of these 90 Å particles.

If both the 40 Å and 90 Å particles are related to the ATPase protein, some correlation should exist between the number of 40 Å particles and the number of 90 Å particles. Although it is relatively easy to count the 90 Å particles on the freeze-fracture faces, counting of the 40 Å particles on the outer surface produces much difficulty. Despite these problems Deamer & Baskin (1969) already noticed a discrepancy between the number of 90 Å particles and the more numerous 40 Å particles.

In 1974 Inesi & Scales made a comparison between the number of 90 Å particles counted on electron micro= graphs and a calculated value for the number of ATPase molecules per μ^2 of membrane surface. According to this comparison the number of ATPase molecules per μ^2 is very close to the 5 700 of the 90 Å particles per μ^2.

As a result of a better estimation of the fraction of total protein comprised by ATPase in the SR membrane, we feel that in this study, we are able to make a better estimation of this relationship. A discussion concerning this subject is presented in Chapter 4, page 92.

1.3.1.2 Calsequestrin or M_{45}-protein

The molecular mass of calsequestrin is estimated between 44 000 and 55 000 (MacLennan & Wong, 1971; Meissner et al, 1973; Inesi & Scales, 1974; Margreth et al, 1974; Sarzala et al, 1974). This protein was first isolated and characterized by MacLennan & Wong (1971). It is an acidic protein with a high capacity for binding Ca^{2+} and it makes up 5-10% of total SR protein (MacLennan & Wong, 1971; Meissner et al, 1973). In the presence of 100 mM KCl calsequestrin has a high capacity for binding Ca^{2+} (850 nmoles Ca^{2+}/mg) but not a high affinity (dissociation constant about 800 μM) according to Ostwald & MacLennan (1974). It was suggested that calsequestrin may play an important role in binding calcium ions actively translocated

into the lumen of the vesicle (MacLennan & Wong, 1971; Jilka et al, 1975; Garcia et al, 1975). Meissner (1975) reported that a purified preparation of sarco= plasmic reticulum from rabbit skeletal muscle consists of a heterogeneous population of vesicles. By using a 25-45% linear sucrose gradient he collected "light" and "heavy" vesicles from the upper and lower ends of the gradient. Light vesicles contained about 90% ATPase protein and no M_{45}- and M_{55}-fractions while heavy vesicles contained 55-65% ATPase protein, 20-25% Ca^{2+}-binding protein and 5-7% M_{55}-protein. From the paper it is not clear which one of these proteins is calsequestrin and which one is Ca^{2+}-binding protein. However, the light vesicles without the minor protein components were able to accumulate more Ca^{2+} in the presence of ATP than heavy vesicles. In the absence of ATP the heavy vesicles bound about three times as much Ca^{2+} as the light vesicles.

Interestingly enough, Meissner (1975) showed with electron microscopy that only vesicles from the heavy fraction contained electron-dense material while the light vesicles appeared to be empty. Washing the heavy vesicles with EDTA or EGTA, a procedure known to remove the minor protein components, resulted in empty vesicles consisting mainly of ATPase protein and phospholipid. He interpreted his findings as consist= ent with the view that:

(a) the electron-dense material inside heavy vesicles may be identical with the two minor protein components, and

(b) light and heavy vesicles may be derived from longitudinal sections and terminal cisternae of SR, respectively.

In the present study we simplified this centrifugation technique to a discontinuous multiple-zonal gradient and we were able to separate the vesicles of a normal preparation of rabbit skeletal SR even more success=fully into four different types with different protein compositions. Making use of the functional properties of these different fractions and also of the functional properties of SR vesicles from rabbit, lobster and chicken skeletal muscle, which differ markedly in protein composition, we were able to confirm some of Meissner's results. However, contrary to Meissner, we found differences in localization of Ca^{2+}-binding protein and calsequestrin with the Ca^{2+}-binding protein associated with the light vesicles and calsequestrin with the heavy vesicles. No explanation can be given for this discrepancy. These results should make it possible to separate possible functions of the two minor components. In Chapter 4 a more detailed discussion on these possible functions is presented.

1.3.1.3 Ca^{2+}-binding or M_{55}-protein

A second acidic protein that binds Ca^{2+} was first isolated by MacLennan et al in 1972 (MacLennan & Hol=land, 1975). This protein has a molecular mass of 55 000 dalton and is the only protein that binds Ca^{2+} with a high affinity but low capacity - 16-22 nmoles/mg

with a dissociation constant between 2,5 and 4,0 μM in the presence of 0,1 M KCl (Ostwald & MacLennan, 1974). The function of the M_{55}-protein is as yet, unknown. However, Jilka et al (1975) speculated that a possible function for both the minor proteins could be in regulating membrane permeability.

1.3.2 Permeability and Ca^{2+}-release mechanisms in sarcoplasmic reticulum vesicles

Since the discovery that vesicles from the SR induce relaxation in isolated muscle proteins, the involve= ment of SR in the relaxation process has been studied extensively (Hasselbach & Makinose, 1961; Ebashi & Lipmann, 1962). It was shown that the isolated SR vesicles exhibit ATP-dependent Ca^{2+} transport *in vitro*, reducing the Ca^{2+} concentration in the medium to levels (Weber et al, 1966) and at rates (Scarpa & Inesi, 1972) compatible with relaxation of myofibrils in physiological conditions (Weber, 1966).

The question of Ca^{2+}release, however, has been elusive for a long time. In this regard it is known that skeletal muscle SR is able to store an amount of calcium which is sufficient for activation of myo= fibrils (Ohnishi & Ebashi, 1964). Therefore, it is simply assumed that upon membrane excitation, Ca^{2+} is released from SR, thereby raising the Ca^{2+} concen= tration in the myoplasm and initiating contraction.

In recent years various experiments have been performed

demonstrating that Ca^{2+} release from SR can occur by means of different mechanisms. Although these mech= anisms are of great interest and possibly of physio= logical relevance, few of them can explain the real problem of Ca^{2+} release by the SR as result of T-tubule depolarization.

1.3.2.1 Reversal of the Ca^{2+} pump

The release of Ca^{2+} from SR vesicles loaded by active transport is very slow if the activity of the pump is stopped by procedures reducing Ca^{2+} or ATP concentra= tions in the outside medium (Weber et al, 1966; Bar= logie et al, 1971). This very slow Ca^{2+} efflux must be attributed to low Ca^{2+}-permeability of the membranes since the hydrolysis of membranal lipids or the binding of surface-active agents induces a very fast calcium release. However, the effect of these membrane modify= ing treatments cannot be reversed. On the other hand, in the presence of ADP and inorganic phosphate, a very specific and reversible Ca^{2+} release from loaded vesicles, occurs at rates comparable to those of inward active transport (Barlogie et al, 1971 and Masuda & De Meis, 1974).

The Ca^{2+} release is accompanied by the formation of ATP, indicating that potential energy derived from the transmembrane calcium gradient can be utilized for the phosphorylation of ADP and that for every two Ca^{2+} ions that leave the vesicle, one phosphate group is transferred to ADP to form ATP (Makinose & Hasselbach, 1971). It was also shown that a phosphorylated form

of the SR ATPase is an intermediate step during both outward translocation of Ca^{2+} under these conditions and inward translocation during active transport (Yamamoto & Tonomura, 1967 & 1968; Makinose, 1972; Yamada & Tonomura, 1972).

The occurrence of Ca^{2+} release in the presence of ADP and inorganic phosphate clearly demonstrates that the Ca^{2+} pump of sarcoplasmic reticulum can be reversed. However, such a mechanism of release is unlikely to play a physiological role since:

(a) the rates of efflux are too slow to provide sufficient calcium during the short time of mechanical coupling, and

(b) it is not apparent how the sarcoplasmic concen= tration of ADP would significantly increase as an immediate consequence of membrane depolarization.

1.3.2.2 Ionophore-induced release

Several antibiotics have been found to increase the permeability of natural and artificial membranes to cations, due to the selective formation of cation-antibiotic complexes which are lipid soluble (Hender= son et al, 1969). Some of these antibiotics facil= itate diffusion of ions across membranes by function= ing as carriers of ions, and have been descriptively named ionophores.

Two ionophores of particular relevance to the question

of Ca^{2+} release from SR are the antibiotics X537A and A23187 described by Berger et al and by Reed respect= ively (cf. Scarpa et al, 1972). These two antibiotics, isolated from different strains of streptomyces, possess the ability to function as divalent cation ionophores. Addition of either of the two to loaded SR vesicles produces immediate Ca^{2+} release (Entman et al, 1972; Scarpa & Inesi, 1972; Scarpa et al, 1972), while neither ATPase inhibition nor structural damage to the vesicles is apparent (Scarpa et al, 1972). In isol= ated muscle preparations of skeletal, cardiac and smooth muscle, addition of the ionophore X537A in μM concentrations causes marked contractile effects. In the case of skeletal muscle this effect is attributed to Ca^{2+} release from the SR while in cardiac and smooth muscle a more complicated mechanism involving β-receptors is involved. Other antibiotics which are known to facilitate transmembrane diffusion of mono= valent, but not divalent cations, have no effect on Ca^{2+} release by isolated SR (Scarpa et al, 1972).

The ionophore-induced release definitely demonstrates that a transmembrane gradient of Ca^{2+} is formed in SR vesicles as a consequence of ATP-dependent Ca^{2+} uptake. It also shows that the accumulated Ca^{2+} can be released at a very rapid rate, if the SR permeability to Ca^{2+} is suitably increased. However, the involvement of ionophores in the physiological mechanism of excitation-contraction coupling, is an open question since no low molecular weight muscle component, meeting the

definition of a calcium ionophore, has as yet been isolated from muscle.

The possibility that the proteins of the SR membrane might act as Ca^{2+} ionophores in artificial lipid membranes was also investigated (MacLennan & Holland, 1975). Studies on the M_{55}- and M_{45}-proteins were negative. According to them the ATPase protein in a succinylated form, to induce solubility, acted as a Ca^{2+}-dependent and selective ionophore in the lipid membrane. The increase in conductance was 200-fold for the Ba^{2+}, Ca^{2+}, Sr^{2+} series, and nothing for monovalent ions. However, nothing is known about the ionophoric activity of ATPase in the intact SR mem= brane.

1.3.2.3 Ca^{2+} release as a function of pH

Another Ca^{2+}-release mechanism in isolated skeletal SR with possible physiological implications, was reported by Nakamaru & Schwartz (1972). By raising the pH of a medium containing SR vesicles, preloaded with Ca^{2+} in the presence of ATP, a Ca^{2+}-release process as a function of pH was observed: 57 nmoles Ca^{2+} were released per 1 pH unit change per mg protein. This process was reversible to a certain extent and was not the result of influences on the ATP-membrane inter= action. According to Nakamaru & Schwartz (1972), Carter et al suggested that intracellular pH may be a function of membrane potential. If this can be proven *in situ*, Ca^{2+} release from the SR may also be

a function of intracellular pH changes. However, as yet this could not be shown.

1.3.2.4 Ca^{2+}-triggered Ca^{2+} release

One of the most useful preparations to study the mechanism of Ca^{2+} release is the mechanically skinned muscle fibre preparation originally devised by Natori in 1954 (cf. Endo & Blinks, 1973). In these prepar= ations the sarcolemma is peeled off the fibre, while the SR remains with the myofibrils. The remaining structure then exhibits phenomena which are dependent on the relation between SR and myofibrils. In the skinned fibres release of Ca^{2+} from SR can be directly demonstrated by use of a radioactive calcium isotope (Ford & Podolsky, 1972(a) & 1972(b)) or by measuring the bioluminescence of the Ca^{2+}-sensitive protein Aequorin (Endo & Blinks, 1973).

Studying the Ca^{2+}-releasing effect of caffeine, Ebashi et al (1969) noticed that addition of a small amount of Ca^{2+} greatly enhances the effect of caffeine. This result was interpreted as showing that Ca^{2+} ions them= selves release more Ca^{2+} from the SR. This effect was subsequently confirmed by various authors (Endo et al, 1970; Ford & Podolsky, 1970, 1972(a) & 1972(b); Endo & Blinks, 1973). Sr^{2+} showed a similar Ca^{2+}-releasing action, although the effective concentration was about fifty times greater than for Ca^{2+} (Endo et al, 1970). On the other hand, free Mg^{2+} inhibits the Ca^{2+}-triggered release (Endo et al, 1970; Ford & Podolsky, 1970; Endo, 1975(a)).

These observations offer a very attractive hypothesis viewing excitation-contraction coupling as a Ca^{2+}-triggered regenerative process, involving Ca^{2+} release from SR. This phenomenon may play a physiological role by means of either of two possible mechanims:

(a) depolarization of the T-system might cause Ca^{2+} to enter from outside or Ca^{2+} bound to the membrane of the T-system to be released, the amount which may be too small to activate the contractile system by itself but still it acts on the SR to induce the release of more Ca^{2+} from the latter system; or

(b) depolarization of the T-system may cause depolar=ization of the SR with release of some Ca^{2+} (Endo & Nakajima, 1973) which in turn acts on the SR to release more Ca^{2+} (Thorens & Endo, 1975).

Either of these mechanisms would mean that these two processes, depolarization and Ca^{2+}-triggered release, should be dependent upon each other. However, Thorens & Endo (1975), showed that these mechanisms are pract=ically independent for the following reasons:

(a) Ca^{2+}-triggered release is strongly affected by free Mg^{2+} concentration while "depolarization"-induced release is not influenced by a change in free Mg^{2+} concentration.

(b) Ca^{2+}-triggered release requires a certain level of preloading of the SR. No such requirement exists for "depolarization"-induced release.

(c) They found that different substances separately inhibit either Ca^{2+}-triggered release or "depolar= ization"-induced release of Ca^{2+} without appreciably affecting the other. Ford & Podolsky (1972a) and Thorens & Endo (1975) found that procaine effectively inhibit Ca^{2+}-triggered release without affecting "depolarization"-induced Ca^{2+} release. On the other hand Thorens & Endo (1975) found that concentrations of 40 mM sucrose and higher, as well as other sugars like glucose, fructose and xylose, completely in= hibit "depolarization"-induced release while little or no effect is observed in Ca^{2+}-triggered release.

Endo (1975) found that the required concentrations of triggering Ca^{2+} are too high to be consistent with the physiological mechanism of excitation-contraction coupling in skeletal muscle. According to Fabiato & Fabiato (1975), the concentration of free Ca^{2+} necessary to induce transient contraction in skeletal muscle fibres, is more than enough to saturate the troponin system and induce contraction without any contribution from the SR system. Furthermore, Rich & Langer (1975) conducted studies on physiologically-perfused skeletal and heart muscle and concluded that there is no support for the hypothesis that Ca^{2+} may act as a trigger for the release of more Ca^{2+} in skeletal or cardiac preparations where the sarcolemma is still intact. It is therefore unlikely that Ca^{2+} is the primary mediator for transmitting information from the T-tubule to the SR to induce the release of

Ca^{2+} in skeletal muscle (Thorens & Endo, 1975). How= ever, in cardiac muscle fibres with disrupted sarco= lemmal membranes, a transient contractile response could be obtained with free Ca^{2+} concentrations much lower than required to activate myofibrils directly (Fabiato & Fabiato, 1975). Thus, a physiological role for the Ca^{2+}-triggered release in special circ= umstances such as in the presence of caffeine, and in heart and smooth muscle, can not be excluded. In the present study the objective was to reproduce Ca^{2+}-triggered release in isolated SR as well as investigate the effect of elevated free Mg^{2+} concentration. The results obtained in this investigation are presented in Chapter 3 (page 58).

1.3.2.5 The effect of caffeine

The contractile effects of caffeine on skeletal muscle are mediated through Ca^{2+} release from SR (Weber, 1968; Endo et al, 1970). The following results seem to confirm this mechanism of caffeine:

(a) Purified phospholipase C, which is known to destroy the ability of isolated SR to accumulate Ca^{2+} and thus also its ability to induce relaxation, abolishes caf= feine-induced contraction of the skinned fibres whereas the contractile ability of the fibre is largely retained.

(b) The concentration of free Ca^{2+} in and around the skinned fibres was measured with murexide, a Ca^{2+}-

binding dye, and shown to increase during caffeine-induced contraction (Endo et al, 1970).

In fact, such a release of Ca^{2+} can be induced by caffeine in isolated SR vesicles (Fairhurst & Has=selbach, 1970; Ogawa, 1970). Certain analogies between Ca^{2+}-triggered release and caffeine-induced Ca^{2+} release were clearly pointed out by Endo (1975b).

(a) In both cases Ca^{2+} release is facilitated by lowering the concentration of free Mg^{2+} or raising the concentration of free Ca^{2+} in the medium.

(b) The two phenomena potentiate each other.

(c) Both mechanisms require that the SR be preloaded with Ca^{2+} at near maximal levels and are inhibited by procaine.

It is apparent that, under physiological conditions, caffeine renders the Ca^{2+}-triggered release operative even at low sarcoplasmic concentrations of Ca^{2+}. Under normal circumstances the caffeine mechanism does not seem to have any physiological function.

1.3.2.6 "Depolarization" of sarcoplasmic reticulum

There is now fairly solid information concerning the signal conveyed along the T-tubule (Fuchs, 1974), but the link between this signal and Ca^{2+} release from the SR remains obscure. One attractive possibility is a direct electrical coupling between these two

components of the sarcotubular system. However, Franzini-Armstrong (1970 & 1971) concluded on morphol= ogical grounds that the triadic junction is "badly designed" for a direct electrical link between the T-tuble and terminal cisternae. She proposed the existence of aqueous channels in the SR feet for the conductance of electrical current to the SR with every action potential in the T-tubule. Another possibility was raised by Schneider & Chandler (1973) who suggested that a link in excitation-contraction coupling involves an ionic current or movement of charge across the T-system membrane which could play a role in triggering the Ca^{2+}-release mechanism. Whatever the mechanism it must cause the release of considerable amounts of Ca^{2+} from the terminal cisternae of the SR, which would mean that the permeability of the SR has to be voltage-dependent.

Again the Natori-fibre, a skinned muscle preparation, deprived of all surface membranes developed by Natori, seems to be a useful tool to study directly the effects of electrical stimulation on the SR (Endo & Blinks, 1973). In such a preparation Constantin & Podolsky (1966 & 1967) obtained local contractions by applying current pulses to the interior of the fibre. Furthermore, by adding droplets of 140 mM KCl directly to the skinned fibre, transient local contractions could be elicited. According to these authors this reaction can be explained in the following way: Since the surface membrane of the fibre is removed

under paraffin oil, the composition of the internal medium of the cell should remain unchanged with a high content of K^+ and a low concentration of permeant anions. Increasing the concentration of permeant anions by adding KCl, for instance, would result in diffusion of Cl^- across the internal membrane system with the result that an uneven distribution of charges would occur. This will tend to drive the potential of the myofibrillar space positive, with respect to the internal membrane space and thus creating a depolarizing effect. This explanation would mean that the SR membrane is more permeable to Cl^- than to K^+. In Chapter 4 results obtained in the present study with isolated SR vesicles are discussed which could be explained by assuming a similar permeability difference of the SR membrane for K^+ and Cl^-. Adding droplets of potassium priopionate or methylsulphate on the skinned fibres which should mimic a repolarizing effect, Constantin & Podolsky (1967) could not produce any contractile response. These experiments are based on the assumption that the SR membrane is less permeable to propionate than to chloride.

Ford & Podolsky (1970), also using the Natori-fibre induced quick contractions when fibres that had been loaded in a buffered Ca^{2+} solution, containing propionate as the major anion, were exposed to a high concentration (120 mM) of chloride. By replacing propionate in the bathing solution with chloride, Glenn et al (1974) also found transient contractile

responses in mechanically-disrupted cardiac cells. However, some doubt remains whether contributions of membranes other than the SR, are completely elimin= ated. One possibility is that remaining T-tubules could reseal to form closed compartments retaining Na^+-pump activity and electrical properties similar to those of the sarcolemma (Constantin & Podolsky, (1967). Electrical stimulation or changes in ionic composition could cause depolarization of the resealed tubules which could generate a stimulus for the SR.

Attempts to eliminate this possibility were made by Endo & Nakajima (1973) by using partially skinned muscle fibres, on which remnants of interrupted sarcolemma prevent the T-tubules from resealing. By treating these preparations with caffeine the SR could be unloaded and then reloaded with known amounts of Ca^{2+} in a buffered medium to a desired level. Preincubating these fibres in a medium containing methanesulfonate (MS^-) and by assuming that the SR membrane permeability for MS^- is less than for Cl^- and replacing the preincubation medium with one containing Cl^- would produce an uneven distribution of anions rendering the inside of the SR more negative than the outside, thus producing a "depolarizing" effect. This procedure resulted in a transient contractile response of the fibre. That this was the result of the release of Ca^{2+} by the SR was proven by the lack of a further contractile response on addition of caffeine. Replacing anions in the

reverse direction, did not produce any contractile response.

These results were very promising, however, the primary involvement of the SR membrane in releasing Ca^{2+} upon depolarization would be best illustrated if Ca^{2+} release could be obtained from isolated SR vesicles. The first effort to obtain such a release of Ca^{2+} was made by Lee et al (1966) who applied direct electrical stimulation to SR suspensions and obtained the following results:

In the presence of ATP the SR took up Ca^{2+} actively. When monophasic square waves were applied with platinum electrodes to the suspension, Ca^{2+} uptake was decreased. When the vesicles were stimulated electrically after they had been loaded with Ca^{2+}, release of Ca^{2+} was observed. Cessation of the electrical current was followed by re-uptake of Ca^{2+}. Similar results were obtained by Scales & McIntosh (1968).

On re-examining these results Miyamoto & Kasai (1973) found that by using platinum electrodes as stimulating electrodes, the Ca^{2+}-release effect was dependent on the total current passed through the suspension. On the other hand, when silver-silverchloride electrodes were used, no effect was observed even if the voltage and current were the same as in the case of the platinum electrodes. In addition, apparent re-uptake of Ca^{2+} after cessation of electrical stimulation

using platinum electrodes was shown to be due to a binding of Ca^{2+} to denatured SR vesicles which did not need an energy supply such as ATP. They concluded that the effect of electrical stimulation on SR vesicles was attributable to the irreversible denaturation of SR, due to the oxidation caused by chlorine generated at the platinum electrode. However, in the light of Ca^{2+} released by the SR when a depolarizing effect was created by changing the ionic environment (Constantin & Podolsky, 1967; Ford & Podolsky, 1970; Endo & Nakajima, 1973) these results with electrical stimulation by using silver-silverchloride electrodes, are very difficult to evaluate.

In a series of very interesting experiments, Kasai & Miyamoto (1973) tested the effect of preincubating isolated SR in a medium containing KMS and subsequent replacement of the KMS by KCl. This was done by collecting the SR vesicles on a filter by millipore filtration after loading them with Ca^{2+}. The vesicles on the filter were washed with washing solutions containing various anions and subsequently tested for ^{45}Ca content. They found that preincubation with KMS and washing with KCl was the only procedure accompanied by the release of a considerable amount of Ca^{2+}. Assuming a lesser membrane permeability for MS^- this procedure would produce a "depolarizing" effect. Reversing anion substitutions by incubating the vesicles in a medium containing Cl^- and washing them with KMS, did not produce any significant release of Ca^{2+}.

Any change in permeability accompanied by the release of significant amounts of Ca^{2+} of physiological import= ance, should occur in the presence of ATP which is normally present in high concentrations in the cell. In the present study a series of experiments are described, designed to obtain first, a sufficiently precise value for the permeability of SR membranes to calcium, and then to investigate the effect of changes in the electrolyte composition of the reaction media during Ca^{2+} fluxes in the absence and the presence of ATP.

CHAPTER 2

METHODS AND MATERIALS

Most of the methods described in this chapter are standard techniques widely used in many laboratories. Some of these methods have been adapted in some minor ways. The basic method for studying ATP-independent inward and outward Ca^{2+} fluxes was reported by Inesi et al (1973). In this case, however, the method has been redesigned totally and can be regarded as a new technique (see 2.6, page 54). The method of Meiss= ner (1975) to separate SR vesicles into different populations differing in structure and function, was simplified considerably to give an even more success= ful separation of SR vesicles into four instead of two different populations. This method is described in paragraph 3.1 (page 59). The method for the release of Ca^{2+} sequestered by ATP-dependent uptake described in paragraph 2.7 (page 56), is a newly devised technique.

2.1 Preparation of SR vesicles from skeletal muscle

SR vesicles were prepared from white skeletal muscle of rabbit hindlegs as previously described by Eletr & Inesi (1972), with minor alterations. According to this method the rabbit was stunned with a sharp blow behind the head and killed by cutting the carotid arteries. Soon after excision the muscle was washed and cooled in 0,1 mM EDTA solution, pH 7, on ice. 170 g. of trimmed muscle was then homogenized in a

Waring blendor in 500 ml of medium 1 (10 mM MOPS; 10% sucrose; 0,1 mM EDTA, pH 7) for 15 seconds every 5 minutes for 1 hour. During the homogenization the pH was kept between 6,5 and 7,0 by adding a few drops of 5% NaOH when necessary.
Then the homogenate was centrifuged at 15 000 g for 20 minutes. The supernatant was collected and filtered through a one-inch thick layer of washed gauze to eliminate remaining low-density lipid aggreg= ates. The filtered supernatant was centrifuged at 40 000 g for 90 minutes and the resulting sediment was resuspended in 48 ml of medium 2 (10 mM MOPS; 0,6 M KCl, pH 6,8) with a glass homogenizer. The suspension was incubated at 2-4°C for 40 minutes and then centrifuged for 10 minutes at 15 000 g. After this centrifugation the top layer, approximately 10% of the total volume, was carefully discarded to eliminate small amounts of low-density lipids that may still be associated with the preparation. The bottom part of the supernatant was also discarded to prevent contamination of the preparation with parts of the pellet. The remaining supernatant was col= lected and centrifuged for 90 minutes at 40 000 g. This final sediment was then resuspended in 16 ml of medium 3 (10 mM MOPS; 30% sucrose, pH 6,8). The entire procedure was carried out in a cold room at 2 - 4°C. The SR vesicles maintained a high level of Ca^{2+}-uptake activity for about one week when stored under refrigeration.

The SR vesicles prepared as described above, were

separated into two different populations by a method of continuous-flow centrifugation by Meissner (1975). By simplifying this technique to a multiple zonal sucrose gradient the vesicles could be divided into four different SR fractions with different structural and functional characteristics. This technique is described in more detail in chapter 3.1 (page 59).

2.2 Protein determination was done in two ways:

2.2.1 Biuret protein determination (Gornall et al, 1949)

The biuret reagent containing 6 mM cupric sulphate ($CuSO_4 \cdot 5H_2O$), 21 mM potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) and 0,75 M NaOH was mixed in a 4:1 ratio with the SR suspension. After allowing 30 minutes for the colour development the sample was read against a blank at 555 mμ. The protein concen= tration was read from a curve standardized with the Kjeldahl nitrogen-determination method (Inesi & Scales, 1974).

2.2.2 Lowry protein determination (Lowry et al, 1951)

For the Lowry protein determination the following solutions were made:

Solution 1 : 0,19 M Na_2CO_3
0,1 M NaOH

Solution 2 : 0,43 M sodium tartrate

Solution 3 : 20 mM cupric sulphate

These three solutions were mixed in 50:1:1 ratio to form solution 4. To 5 ml of solution 4, 1 ml sample or blank was added and equilibrated in a waterbath at $37^{o}C$ for 3 minutes. 5 ml 1N Phenol reagent was added and the incubation was continued for 20 minutes. The optical density was read at 700 mμ and compared to a bovine serum albumin standard.

2.3 ATP-dependent Ca^{2+} transport

ATP-dependent Ca^{2+} uptake was performed both in the absence ("Ca^{2+} uptake") and in the presence ("Ca^{2+} loading") of potassiumoxalate (see addendum for explanation of these terms).

2.3.1 Ca^{2+} uptake was performed in a medium contain= ing 125 mM KCl; 20 mM MOPS, pH 6,8; 0,1 mM ^{45}Ca-$CaCl_2$; 0,1 mM EGTA; 2,5 mM $MgCl_2$ and 200-300 μg SR protein/ml in a final volume of 2 ml. Uptake was started by the addition of 2,5 mM ATP. Serial samples were taken by Millipore filtration and the concentration of residual calcium in the medium was estimated by determining the radio-activity in the filtrate by means of liquid scintillation counting.

2.3.2 Ca^{2+} loading was performed in a medium contain= ing 125 mM KCl; 20 mM MOPS, pH 6,8; 5 mM $K_2C_2O_4$ (potassium oxalate); 2 mM ^{45}Ca-$CaCl_2$; 2 mM EGTA and 200-300 μg SR protein/ml in a final volume of 2 ml. The reaction was started by the addition of 2,5 mM ATP·Mg. Serial samples were taken by Millipore

filtration and the filtrate was treated as in the case of Ca^{2+} uptake

2.4 ATPase activity was determined in reaction mixtures similar to those used for Ca^{2+}-uptake studies. To determine maximum activity 0,1% Triton X-100, a nonionic detergent, per 0,1 mg SR protein was used to increase the membrane permeability.

2.4.1 ATPase activity in presence of Triton X-100

The following reaction mixture was used : 0,1 mM $MgCl_2$; 0,4 mM $CaCl_2$; 0,1 - 0,2 mg SR protein/ml. Initially, no $CaCl_2$ was added and the Mg^{2+}-dependent ATPase activity was started by adding 5 mM ATP. A few samples were taken according to a time schedule to determine the "basic" or Mg^{2+}-dependent ATP splitting. After about 6 minutes, 0,4 mM $CaCl_2$ was added to activate the Ca^{2+}-dependent ATPase and serial samples were taken. The reaction was stopped by addition of ice-cold TCA to a final concentration of 5%.

The amount of Pi liberated from the ATP in the super= natant was determined by the method of Fiske & Subbarow (1925) as a function of ATPase activity.

In some instances the basic and extra ATPase determin= ation were separated into two different experiments.

2.4.2 ATPase activity in the absence of Triton X-100

In cases where the coupling between Ca^{2+} uptake and ATP hydrolysis was studied, the ATPase activity was determined in the absence of Triton X-100 but in the presence of 5 mM $K_2C_2O_4$. In these instances the basic and extra ATPase activities were mostly done separately. Also, $MgCl_2$ was not added as such to the reaction mixture but the reaction was started by the addition of 5 mM ATP·Mg. In all other aspects the experiments were performed as described in 2.4.1.

2.5 Gel electrophoresis was performed according to the method of Inesi & Scales (1974). The SR vesicles were solubilized by boiling a mixture of 0,5 mg protein/ml; Tris-sodiumacetate buffer, pH 7,4; 0,2% SDS for 5 minutes. To prevent oxidation 2 mM DTT was added after the samples cooled down.

Electrophoresis was performed in separating gels containing 7% acrylamide; 1,85% bisacrylamide; 4,5% Tris; 0,03% Temed; 0,11 M HCl; 0,1% SDS and 0,7% $(NH_4)_2S_2O_8$ (ammoniumpersulphate) with a final pH 7,4. Stacking gels contained 4% acrylamide; 1,2% Tris; 0,11% Temed; 0,11 M HCl. 0,001% ribo=flavine and 8% sucrose.

The running buffer contained 0,5% Tris; 0,164% CH_3COONa and 0,1% SDS; final pH 7,4.

Pyronine Y was used as a tracking dye and a current

of 7-8 mA/gel was applied. After 7-8 hours of elec=
trophoresis, the gels were fixed overnight in 50% TCA and subsequently stained for 5 - 6 hours with 0,25% Coomassie brilliant blue R dissolved in 20% methanol and 20% TCA. The gels were destained in a solution of 20% methanol and 5,6% acetic acid.

Each gel was loaded with 50 - 100 μg protein.

2.6 ATP-independent ("passive") Ca^{2+} fluxes

Both inward and outward passive fluxes were studied. The methods used are modifications of those reported by Inesi et al (1973). In addition, passive Ca^{2+}-exchange experiments have also been performed.

2.6.1 Passive Ca^{2+} influx

Skeletal SR vesicles in medium 3 (see 2.1) were washed and resuspended in a medium containing 60 mM KCl; 10 mM $MgCl_2$; 20 mM MOPS, pH 6,8 to a final concentra=
tion of 6-8 mg SR protein/ml. This suspension was preincubated for 1 hour at 37°C and then diluted 20-fold with a medium of identical composition but containing 1 mM ^{45}Ca-$CaCl_2$. Serial samples were filtered through millipore filters, HA 0,45 μ and the vesicles (0,4 - 0,8 mg) collected on the filters, were washed three times with 3 ml of an ice-cold medium containing 300 mM sucrose and 0,1 mM EGTA, pH 6,8.

The filters were solubilized in a dioxane-based

scintillation fluid and the calcium in the vesicles was then estimated by determining the radioacitivity in a liquid scintillation counter.

2.6.2 Passive Ca^{2+} efflux

The SR vesicles were equilibrated for 1 hour at 37°C with the same media as described in 2.6.1 containing 1 mM ^{45}Ca-$CaCl_2$, and then diluted with media of identical composition but containing no added calcium. Serial samples were taken and filtered as described.

2.6.3 Passive outward and inward Ca^{2+} exchange

SR vesicles were equilibrated passively with media as described above containing 1 mM ^{45}Ca-$CaCl_2$. After a preincubation period of one hour the vesicles were diluted 20-fold with a medium of identical composition to the preincubation medium except that 1 mM "cold" calcium i.e. no ^{45}Ca, was added. By taking serial samples, filtering them as described and determining the residual ^{45}Ca in the vesicles an estimate of the passive outward exchange could be made.

For inward exchange, preincubation was performed in media containing cold $CaCl_2$ and then diluting the samples 20-fold with identical media containing 1 mM ^{45}Ca-$CaCl_2$. Serial samples were taken again and filtered as described.

All changes in the basic composition of the preincub= ation and diluting media are described in the legends to the various figures.

2.7 Methods for the release of calcium sequestered by ATP-dependent Ca^{2+} uptake

Ca^{2+} uptake was performed at 37°C as described in paragraph 2.3.1. When the steady state level was reached after 60 seconds the ionic composition of the reaction mixture was drastically changed by the addition of small volumes (200 - 400 μl) of different cations in high concentrations. Serial samples were taken by Millipore filtration and the residual calcium was estimated by determining the radioactivity in the filtrate.

2.8 Liquid scintillation cocktails

In cases where the radioactivity in the vesicles on the filter was determined, the filters were dissolved in a dioxane-based cocktail of the following compos= ition:

p-Xylene	450 ml
Dioxane	1 350 ml
Ethoxyethanol	1 350 ml
Naphtalene	250 g
P.P.O.	15 g
P.O.P.O.P.	0,3 g

All chemicals used, were scintillation grade.

For determining radioactivity in the filtrate either the cocktail described above or Instagel (Packard Instruments Co.) was used.

2.9 Chemicals

Sucrose was of ultrapure enzyme grade purchased from Schwartz-Mann, poly-glutamic acid and poly-lysine were purchased from Sigma, washed several times by alternative precipitations and solubilizations, and finally converted to the appropriate salt.

All other chemicals were of analytical reagent grade and purchased from various distributors. Only double glass-distilled water was used during the experiments.

2.10 Statistical analysis

Statistical analysis, where indicated in the legends to the various figures, was performed according to the method of Rao (1958). According to this method the particular curves which are in the form of growth curves, were transformed to straight lines going through the zero point, i.e. the intercept of X- and Y-axes. By comparing the slopes of these straight lines by means of covariance analysis, the significance of differences between the straight lines and thus the curves themselves, could be estimated.

CHAPTER 3

RESULTS

In order to understand the mechanism by which the SR exerts its physiological function, it is important to clarify the relationship between structure and function of the different membrane components. The enzymatic activity in the membrane is coupled to one of the protein components, the ATPase enzyme. Through the action of this enzyme the ATP-dependent Ca^{2+} pump is enabled to function. From the literature discuss= ion in chapter 1.3.1 it is clear that much confusion exists about the structural and functional roles of the other protein components. In the present study a series of experiments was performed in order to elucidate some of the properties of all these proteins. The results of these experiments are described in 3.1.

Another functional aspect of the SR and, in fact, of the whole sarcotubular system, is the mechanism by which depolarization of the T-tubule can induce Ca^{2+} release by the terminal cisternae of the sarcoplasmic reticulum. (For the structural arrangement of these structures in skeletal muscle see plate 1, page 9). By manipulating the ion composition of the reaction media in both ATP-dependent and ATP-independent Ca^{2+} flux experiments, an effort was made in the present study to evaluate the hypothesis that Ca^{2+} release by the SR can be effected by a direct electrical coupling between the T-tubule and the terminal cisternae.

The results of these experiments are presented in 3.2.

3.1 Functional and structural roles of SR protein components

By means of continuous-flow centrifugation Meissner (1975) separated SR vesicles on a continuous gradient between 25% and 45% into three fractions. Vesicles recovered from the upper end of the gradient (10-15% of the total protein), were termed "light" vesicles, and those from the lower end were termed "heavy" vesicles (also 10-15% of the total). The remainder of the vesicles were of intermediate density and were banded between the light and heavy fraction. To simplify this procedure a multiple zonal sucrose gradient was prepared instead of the continuous-flow technique.

This multiple zonal gradient consisted of 4 layers of sucrose of different densities, layered in the following fashion (Fig. 2):

4 ml 28% sucrose
4 ml 32% sucrose
3 ml 39% sucrose
1 ml 43% sucrose

SR was prepared from rabbit skeletal muscle in the normal way (see 2.1, page 48).

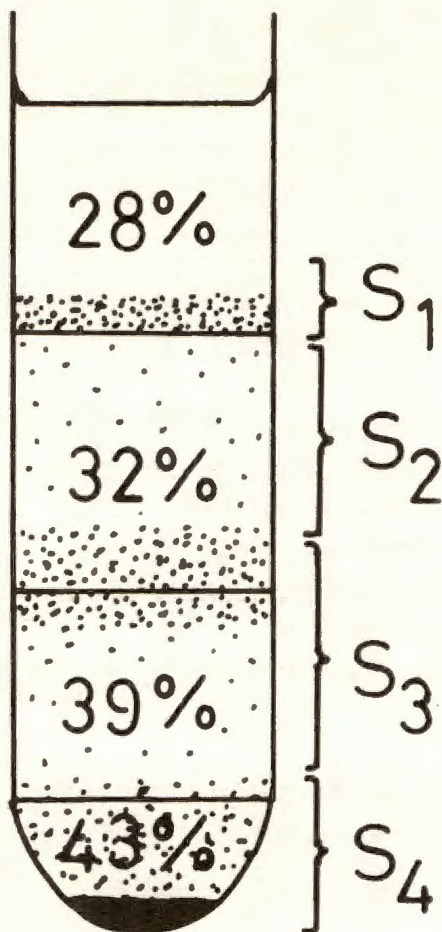


Figure 2: Schematic representation of multiple zonal sucrose gradient and different fractions of SR. Four concentrations of sucrose 43%, 39%, 32% and 28% were used. Centrifugation in Beckman SW 41 rotor at 68 000 g for 20 hours. Fraction 1 (S_1) accounts for less than 1% of the total SR protein, while S_2, S_3 and S_4 account for 72%, 16% and 11%, respectively.

Instead of resuspending the final pellet in 30% sucrose it was resuspended in 28% sucrose and used as such as the top layer of the discontinuous gradient. The concentration of SR in this top layer was 4-5 mg SR protein/ml. The gradient was spun at 68 000 g for 20 hours in a Beckman SW 41 rotor.

After the centrifugation was completed, 4 fractions were recovered.

Electrophoresis, according to the technique described in 2.5, was carried out on SR preparations from rabbit hindleg muscle, lobster muscle and the pectoralis muscle from chicken, as well as on the different fractions from the multiple zonal gradient described above. SR was prepared from lobster and chicken breast muscle according to the same procedure described for rabbit hindleg muscle (see 2.1).

The electrophoretic resolutions of the protein components of rabbit, lobster and chicken SR are shown in plate 3. In all three preparations the main band corresponds to a protein of 106 000 daltons, the ATPase enzyme (MacLennan, 1970; McFarland & Inesi, 1970, 1971; Martonosi & Halpin, 1971; MacLennan & Wong, 1971; Meissner & Fleischer, 1971). In addition, the rabbit SR contains the two minor components named 'calcium-binding protein' and 'calsequestrin' (MacLennan & Wong, 1971; Meissner et al, 1973; Ostwald

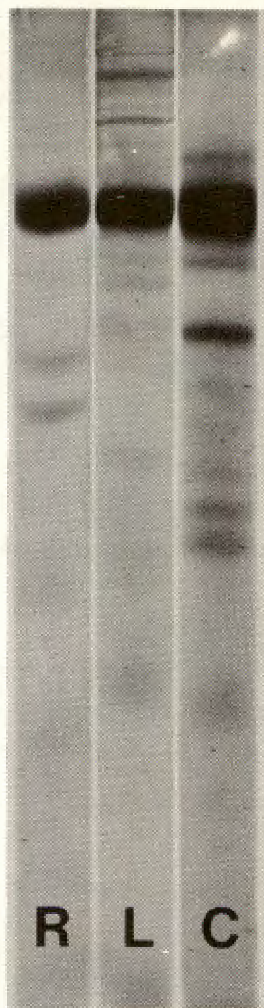


Plate 3: Electrophoretic separation of SR proteins solubilized in SDS. SR from rabbit (R), lobster (L) and chicken (C) skeletal muscle. Electrophoresis performed according to technique described in 2.5. The top heavy band represents the ATPase protein. In the rabbit preparation this band is followed by the lighter bands, the M55- and M45-proteins.

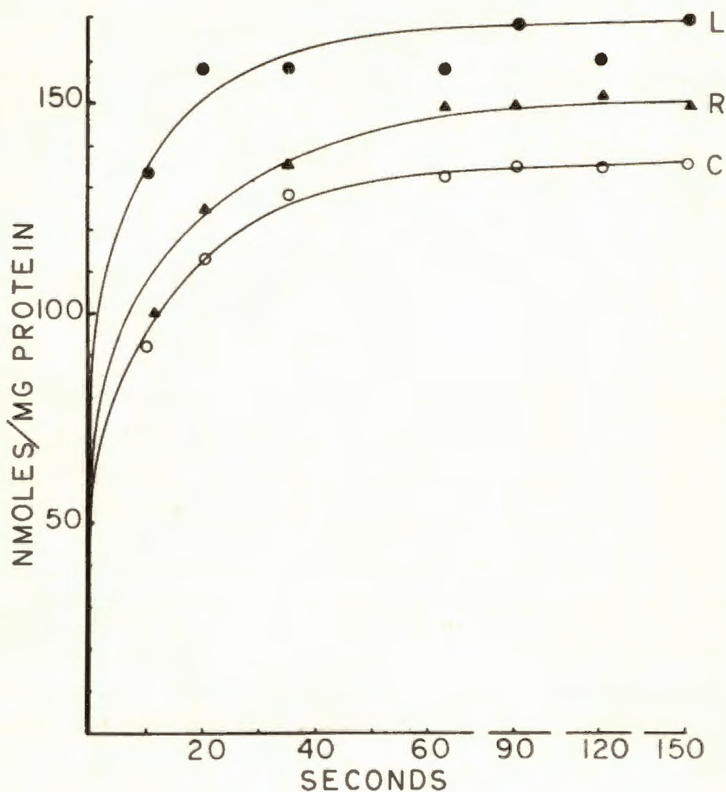


Figure 3: Calcium uptake by SR in the presence of ATP. Reaction mixture: 0,1 - 0,3 mg SR protein/ml; 20 mM MOPS, pH 6,8; 120 mM KCl; 2,5 mM $MgCl_2$; 0,1 mM EGTA; 0,1 mM ^{45}Ca-$CaCl_2$ and 2,5 mM ATP. Controls contained SR but no ATP. L = lobster, R = rabbit and C = chicken SR. Temp. = 25°C.

& MacLennan, 1974; Sarzala et al, 1974). These two proteins are absent in the lobster and chicken prepar= ations, which contain other minor bands which migrate with different velocities. With regard to functional activity, Fig. 3 shows that all three preparations take up calcium in the presence of ATP, with no relation to the presence of any of the minor protein components. In fact, the lobster SR, which contains the lowest amount of minor protein components is the most active.

It was suggested that one of the minor protein compo= nents of rabbit SR, calsequestrin, plays a role in binding calcium ions, actively translocated into the lumen of the vesicles (MacLennan & Wong, 1971). Therefore, one would expect that the maximal levels of active calcium uptake are related to the amount calsequestrin present.

In this regard we found that the vesicles in the four fractions separated on the multiple zonal gradient contain various amounts of calsequestrin (plate 4). Fractions 1, 2, 3 and 4 account for 1%, 72%, 16% and 11% of the SR preparation and contain different amounts of calsequestrin in each fraction. The relative ATPase contents are 69%, 90%, 81% and 69% respectively, as determined by densitometer scanning (see table 1, page 66).

Calculated from densitometer scan plots of the gels

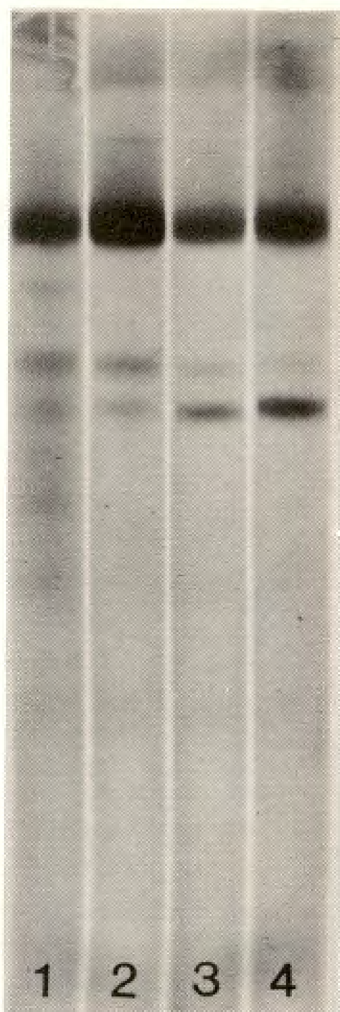


Plate 4: Electrophoretic separation of fractions of rabbit SR obtained by 20 hour centrifugation on a multiple zonal sucrose gradient: 29% (1); 32% (2); 39% (3) and 43% (4). SR protein solubilized in SDS. Electrophor= esis performed according to technique described in 2.5.

in plate 4, the relative amounts of protein components as a percentage of the total protein is given in Table 1.

TABLE 1

Relative Amounts of Different Protein Components in the Four SR fractions Separated by Discontinuous Sucrose gradient

	%ATPase	$\%M_{45}$-protein	$\%M_{45}$-protein (calsequestrin)
Normal SR	80	5	15
S_1	69	19	12
S_2	90	5,3	4,7
S_3	81	3	16
S_4	69	1	30

The ATPase activities of fractions 2, 3 and 4 are more or less proportional to the relative content of ATPase as indicated by the electrophoretic gels (Fig. 4).

In this regard, the S_1 fraction proved to be an exception. Although its minimal yield severely limited experimentation, some ATPase activity determinations were performed. These results indicate a surprisingly high Mg^{2+}-activated ATPase activity relative to the total activity - the Mg^{2+}-activated ATPase activity,

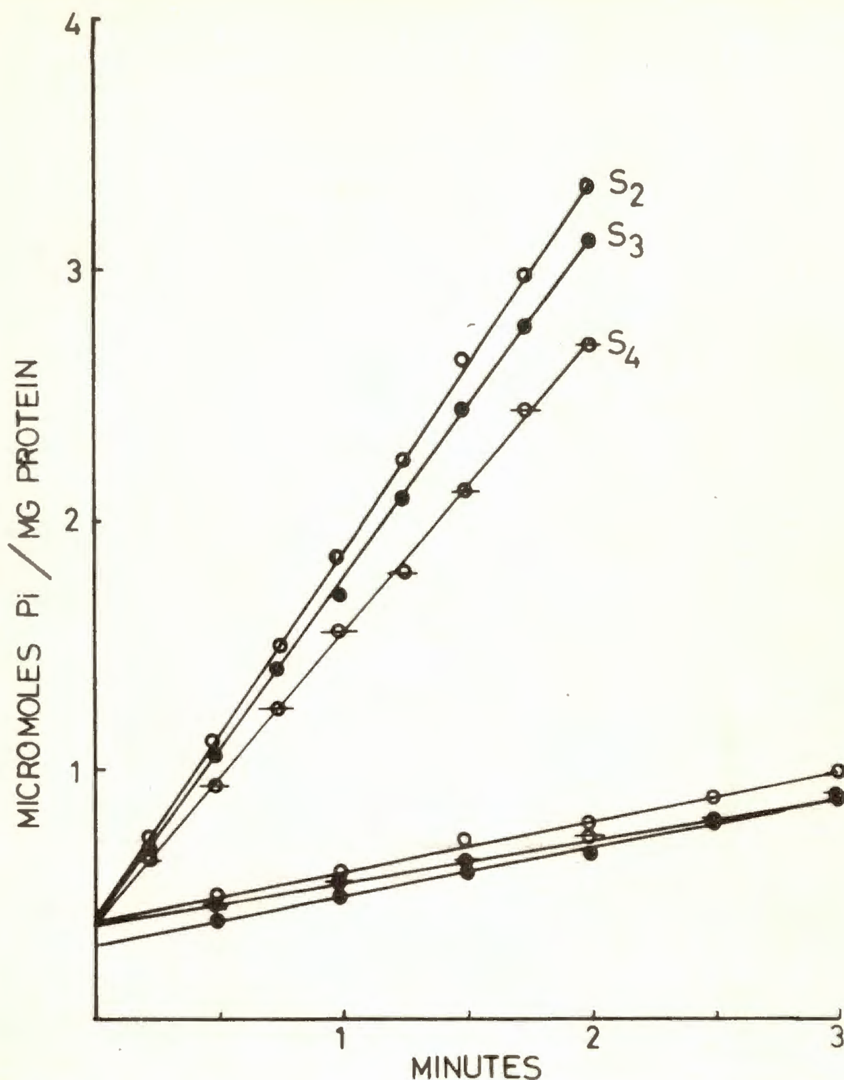


Figure 4: ATPase activity of the different fractions of a multiple zonal sucrose gradient. Reaction mixture: 20 mM MOPS, pH 6,8; 2 mM EGTA; 5 mM $K_2C_2O_4$; 120 mM KCl; 0,3 mg SR protein/ml; 2,5 mM ATP·Mg. Ca^{2+}-ATPase determinations contain 2 mM $CaCl_2$. Solid lines: Ca^{2+}-Mg^{2+}-ATPase activity; broken lines: Mg^{2+}-activated ATPases. Temp. = 25°C. This figure represents the average values of four separate experiments.

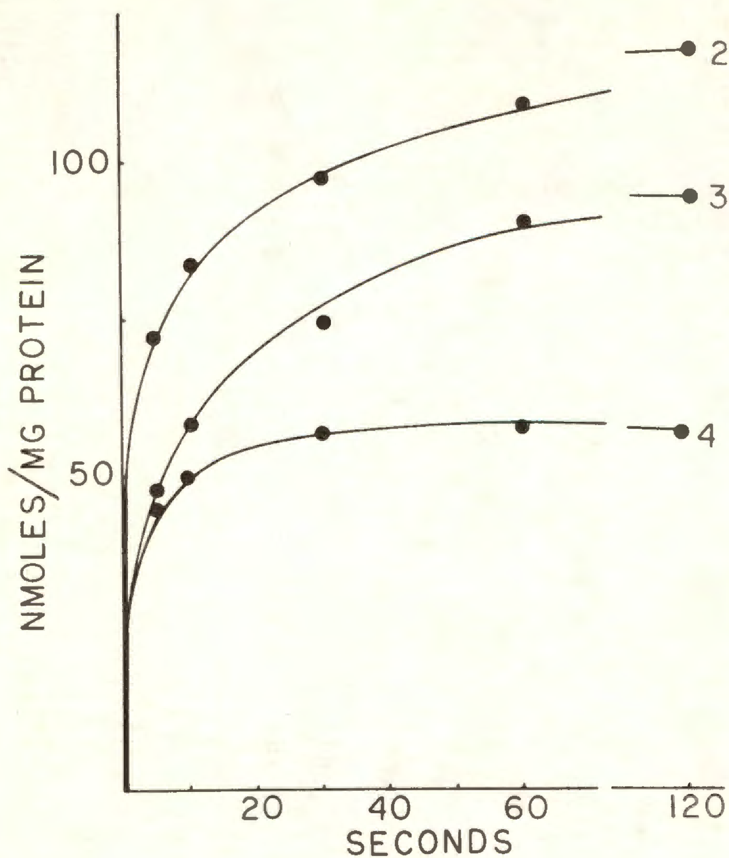


Figure 5: ATP-dependent Ca^{2+} uptake by the SR vesicles from different subfractions of multiple zonal sucrose gradient (see page 60). Reaction mixture: 20 mM MOPS, pH 6,8; 120 mM KCl; 0,1 mM $^{45}Ca-CaCl_2$; 0,1 mM EGTA; 2,5 mM $MgCl_2$; 0,2 - 0,3 mg SR protein/ml and 2,5 mM ATP. Temp. = 25°C. Each curve on this figure represents average values of four individual experiments.

accounts for 74% of the total ATPase activity, compared to 15% in S_2. The Ca^{2+}-activated ATPase on the other hand was much lower than expected from the electro= phoretic pattern. The Ca^{2+}-ATPase activities of the different fractions were as follows: $S_1 = 0,34 \pm 0,04$; $S_2 = 1,17 \pm 0,11$; $S_3 = 1,01 \pm 0,08$ and $S_4 = 0,86 \pm 0,05$ µmoles Pi/mg/min.

The SR vesicles with higher calsequestrin content retain a lower ability to maintain calcium gradients in ATP dependent reactions (Fig. 5). However, in the absence of ATP the vesicles with a higher calse= questrin content, display a higher Ca^{2+}-binding capacity. The S_2 fraction binds 14 $\pm$ 1 nmoles Ca^{2+}/mg protein while the S_4 fraction binds 22 $\pm$ 2 nmoles/mg protein.

3.2 Calcium fluxes through the SR membrane

3.2.1 Passive Ca^{2+} fluxes

3.2.1.1 Basic method

Inward calcium fluxes were initiated by the addition of ^{45}Ca-$CaCl_2$ to suspensions of SR vesicles (see 2.6.1, page 54). Net influx proceeded over a period of several minutes reaching maximal levels in approxim= ately 30 minutes at 37°C (Fig. 6). The amount of calcium taken up passively by SR vesicles in a medium containing 1 mM ^{45}Ca-$CaCl_2$; 20 mM MOPS, pH 6,8; 60 mM KCl and 10 mM $MgCl_2$, was 14,0 $\pm$ 1,0 nmoles/mg protein.

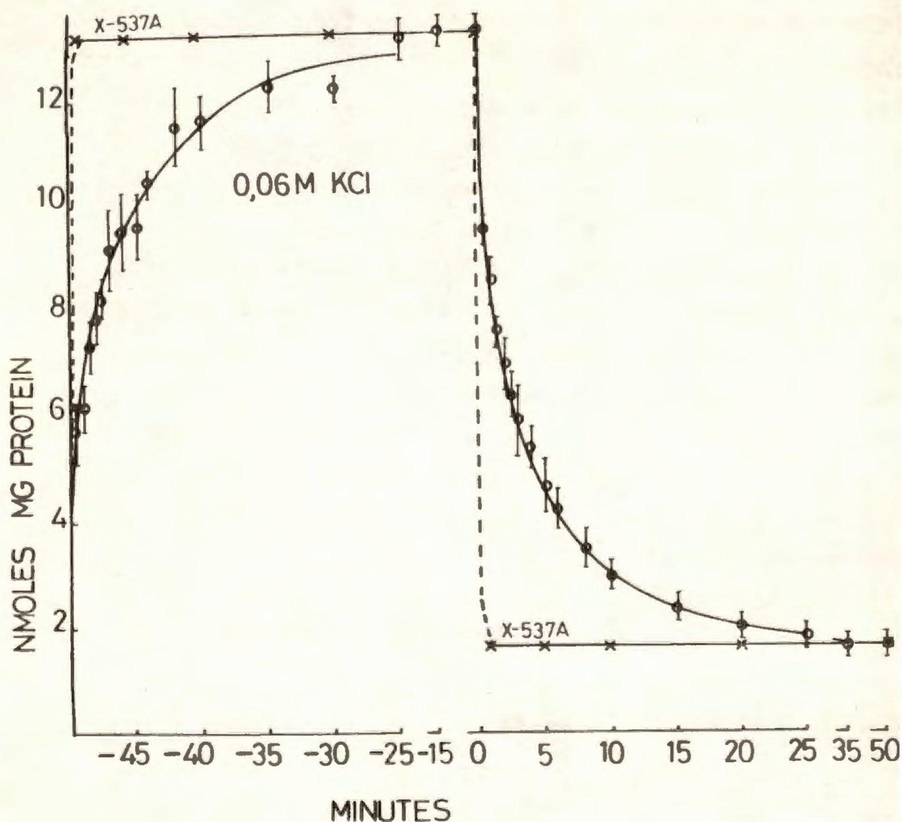


Figure 6: Net calcium influx and efflux in the absence of ATP. Influx was started by adding 1 mM ^{45}Ca-$CaCl_2$ to SR (0,4 - 8,0 mg protein/ml) suspended in 60 mM KCl; 20 mM MOPS, pH 6,8 and 10 mM $MgCl_2$. Equilibrium reached by the passive fluxes was verified by adding 20 µM X537A. Temperature: 37°C. The error bars indicate standard deviation from the mean. The influx curve represents the average value of eight experiments while the efflux curve represents nine experiments.

Outward calcium fluxes were initiated by diluting the preloaded vesicles with the same media described above but containing no added calcium (see 2.6.2, page 55).

Following dilution, net efflux proceeded over several minutes and equilibrium was reached in approximately 30 minutes at 37°C (Fig. 6). To determine whether equilibrium was reached 20 μM of the ionophore X537A (Caswell & Pressman, 1972; Scarpa et al, 1972 and Scarpa & Inesi, 1972), was added. In these cases equilibrium was reached within a few seconds and the same calcium level was maintained throughout the 30 minutes (Fig. 6).

Kinetic analysis of the experimental data yields similar results for influx and efflux data. Owing to lower scatter in the measurements and closer relation to the process of release, analysis of the outward fluxes is presented here.

Semilogarithmic plots of the ratios between the calcium remaining in the vesicles at various time intervals and the total amount of calcium undergoing equilibration ($\frac{Ca_n - Ca_{eq}}{Ca_o - Ca_{eq}}$; where Ca_n = Ca in vesicles after times n; Ca_{eq} = Ca in vesicles at equilibrium and Ca_o = Ca in vesicles after passive influx, i.e. at the start of efflux), display three distinct kinetic components: a first ("very fast") component which is not resolved in a time scale of seconds, a second ("fast") component with a half time of 90 seconds

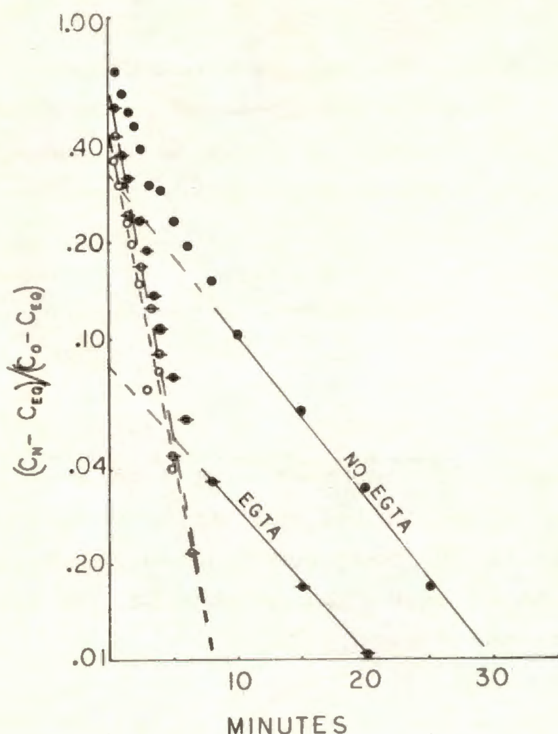


Figure 7: Semilogarithmic plots of net calcium efflux from SR vesicles in the absence of ATP. The efflux was started by dilution of SR vesicles preincubated with media containing 1 mM $^{45}Ca-CaCl_2$, as described in Fig. 5. The dilution media contained 0,5 mM (-●-) or no (●) EGTA. The dotted lines represent extrapolation for the "slow" and "fast" kinetic components of the efflux. The curve of Ca^{2+} efflux without EGTA represents nine experiments while the curve of efflux with EGTA represents six experiments.

and a third ("slow") component with a half time of 7-9 minutes (Fig. 7).

The first component accounts for 20% (2,8 nmoles Ca^{2+}/mg protein) of the net amount of calcium displaced during the flux. This amount can be increased step=wise if the concentration EGTA in the solution with which the filters are washed after separation from the dilution media, is reduced proportionally. There=fore, the "very fast" component may be contributed to calcium binding to the outer surface of the membrane.

The second component (half time = 90 seconds) accounts for 45% (6,3 nmoles Ca^{2+}/mg protein) of the net amount of calcium displaced during the flux. The 90 seconds time constant remains constant when the dilution media contains EGTA (Fig. 7) or high concentrations of other cations (Fig. 8) but is profoundly altered by the presence of a calcium ionophore like X537A (Scarpa et al, 1972) which induces rapid equilibration of the calcium gradient (Fig. 6). Therefore, membrane permeability is the rate-limiting factor for this component, which evidently corresponds to equilibration of the intravesicular calcium concentration with the 1 mM Ca^{2+} concentration of the outside medium in passive influx experiments. In fact, based on an intravesicular volume of 5 µl/mg of SR protein deter=mined in our preparation according to the method of Duggan and Martonosi (1970), 6,3 nmoles calcium/mg protein correspond to an intravesicular concentration of approximately 1 mM.

The third component (half time = 7 - 9 minutes) accounts for 35% (4,9 nmoles Ca^{2+}/mg protein) of the net amount of calcium displaced during the flux. The magnitude of the "slow" component is drastically reduced when the outward fluxes are initiated with dilution media containing EGTA (Fig. 7) or other negatively charged poly-electrolytes. A similar reduction of the slow component is also observed when low affinity calcium-binding sites are screened by high concentrations of monovalent cations e.g. 0,3 M KCl (Fig. 8A and 8B). This effect of high concentrat= ions of KCl must be attributed to competitive binding of K^{+} to Ca^{2+}-binding sites because this reduction of the slow component is absent in passive influx and efflux curves in the presence of 20 mM $CaCl_2$ (Fig. 8C).

The "slow" flux component is observed only in the presence of a high free calcium concentration in the external medium and is a complex phenomenon related to calcium interaction with low affinity binding sites in the SR membrane.

These passive fluxes and the relative sizes of the different components are not sensitive to changes in pH and remain unchanged between pH 6,3 and 7,5.

3.2.1.2 Isotope exchange

During passive influx, Ca^{2+} moves from the large extravesicular volume to the small intravesicular volume. As the intravesicular Ca^{2+} concentration

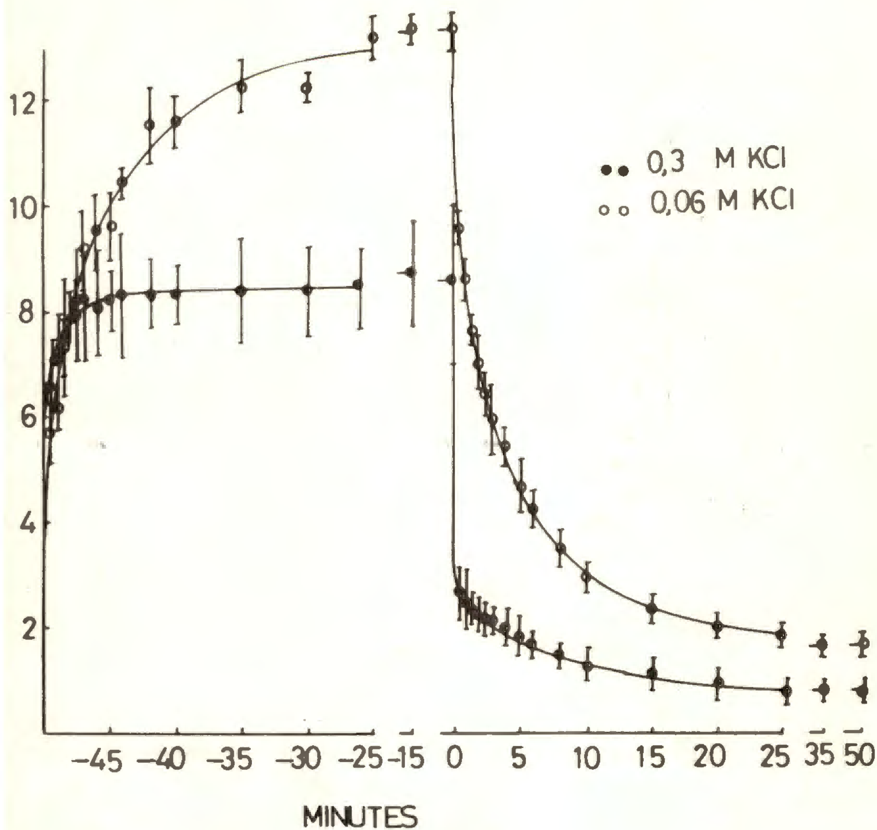


Figure 8A: Net calcium influx and efflux in the absence of ATP. Dilution-media as described in Fig. 6 with Ca^{2+} and KCl concentrations as follows: 1 mM $CaCl_2$ and 60 mM KCl (o); 1 mM $CaCl_2$ and 300 mM KCl (•). Error bars indicate standard deviation from mean. Each curve represents six experiments. Temperature: 37°C.

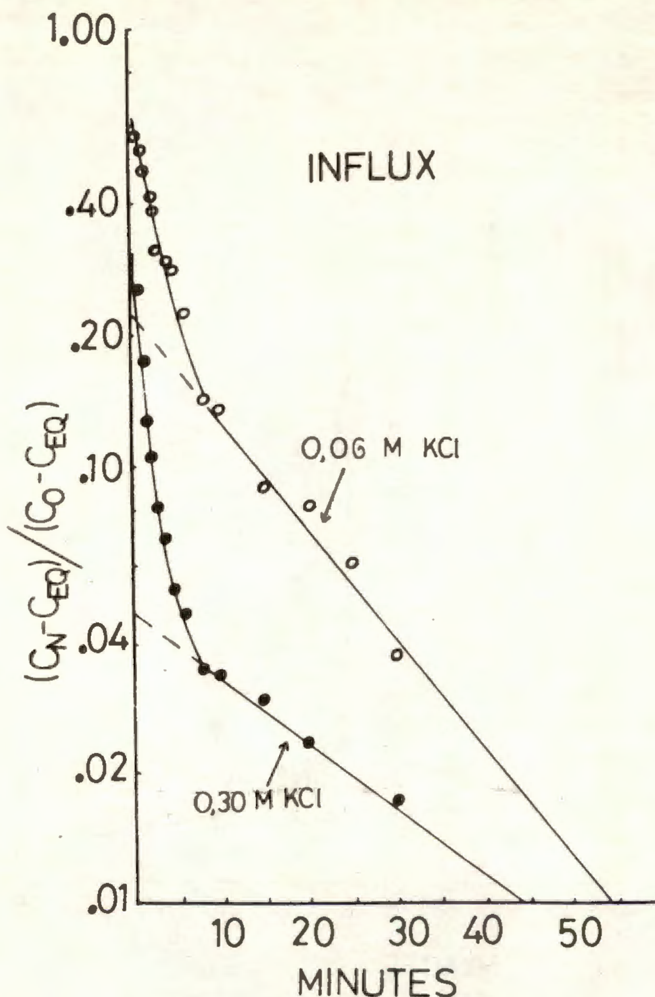


Figure 8B: Semilogarithmic plots of net Ca^{2+}- influx curves presented in Fig. 8A. From this curve it is clear that in presence of 1 mM ^{45}Ca-$CaCl_2$ a sharp reduction in the third component occurs in presence of 300 mM KCl compared to the situation in the presence of 60 mM KCl. This reduction can be explained in terms of competitive binding of Ca^{2+} and K^+ to low affinity binding sites on the surface of the SR membranes.

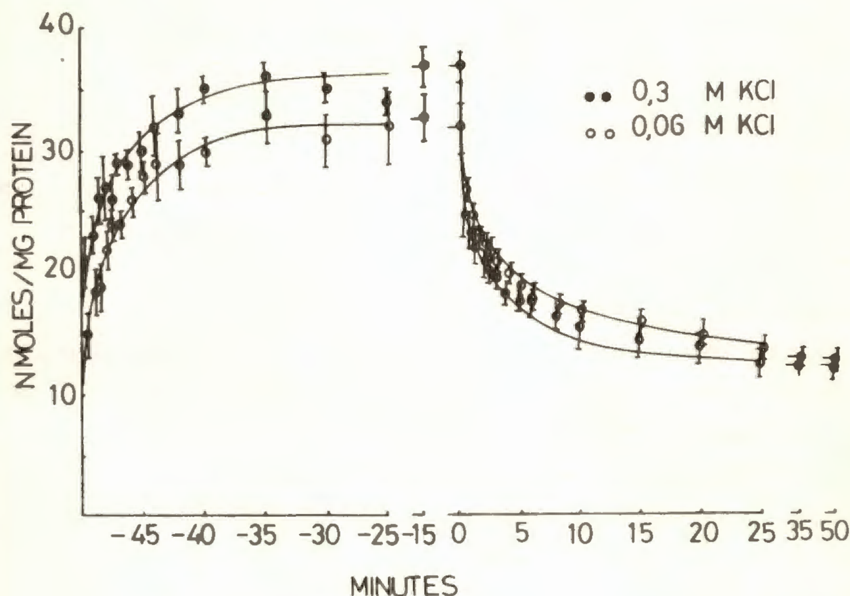


Figure 8C: Net calcium influx and efflux in the absence of ATP. Experimental conditions as described in Fig. 6 with Ca^{2+} and KCl concentrations as follows: 20 mM $^{45}Ca-CaCl_2$ and 60 mM KCl (o); 20 mM $^{45}Ca-CaCl_2$ and 300 mM KCl (•). The error bars indicate standard deviation from the mean. Each curve represents six experiments. Temperature: 37°C. Statistical analysis of these curves indicates that for both influx and efflux, the net Ca^{2+} flux in the presence of 60 mM KCl differs significantly on a 5% basis from the net Ca^{2+} flux in the presence of 60 mM KCl. However, kinetic analysis on a semilogarithmic curve fails to indicate any change in the relative sizes of the different compartments as in the case of Ca^{2+} fluxes in presense of 1 mM ^{45}Ca (see Fig. 8A).

increases, backfluxes may increase to such an extent that the properties of the different components may be influenced. On the other hand, Ca^{2+} moving passively out of the vesicles, move from a small to a large volume and subsequently the dilution factor will be so great that backfluxes will not be a significant factor.

In order to assess the effect of continuously changin calcium concentrations inside the vesicles during net fluxes, a series of experiments was carried out in which the calcium concentration inside and outside th vesicles was kept constant, and fluxes of radioactive calcium tracer were measured. This was accomplished by following the efflux of radioactive calcium tracer from vesicles pre-equilibrated in media containing 1 mM ^{45}Ca-$CaCl_2$ and subsequently diluted with media containing 1 mM (non-radioactive) $CaCl_2$.

Kinetic analysis of calcium exchange in both directio (Fig. 9) again shows the three components observed in net fluxes. It is important to notice that the slopes and therefore the time constants of the various components are not significantly changed as compared to those observed in net fluxes.

3.2.1.3 Changes in electrolyte composition of the reaction media

Stimulated by the observations of Ford & Podolsky (1970) and Endo & Nakajima (1973) a series of passive

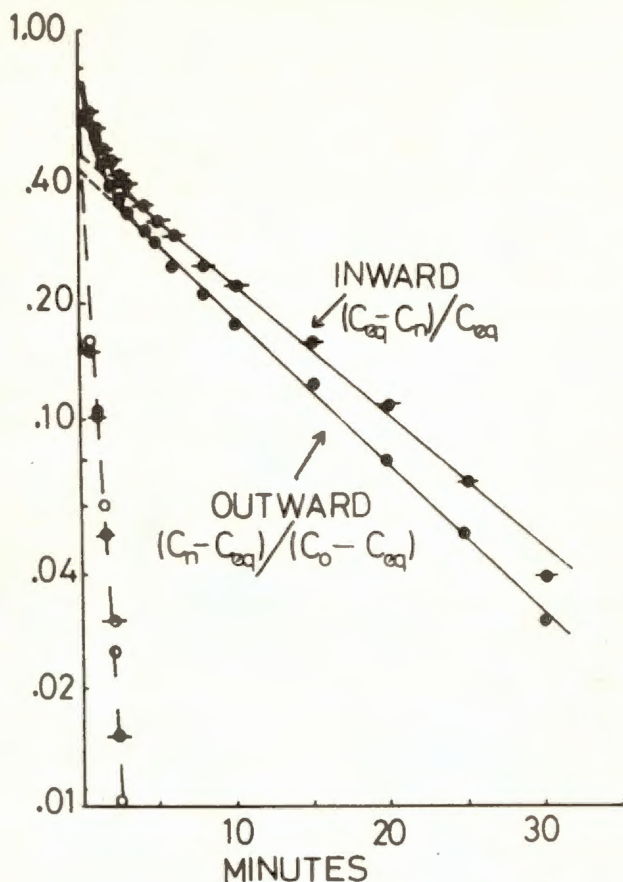


Figure 9: Semilogarithmic plots of calcium exchange in SR vesicles. The SR (7-8 mg/ml) was first equilibrated with a medium containing 60 mM KCl; 20 mM MOPS, pH 6,8; 10 mM $MgCl_2$ and 1 mM ^{45}Ca-$CaCl_2$ for outward exchange and with 0,1 mM $CaCl_2$ (non-radioactive) for inward exchange. Isotope (^{45}Ca) exchange was then started by 20-fold dilution with a medium containing 60 mM KCl; 20 mM MOPS, pH 6,8; 10 mM $MgCl_2$ and 1 mM non-radioactive $CaCl_2$ for outward exchange and 1 mM ^{45}Ca-$CaCl_2$ for inward exchange. Temperature = 37°C. Each curve represents four separate experiments. The broken lines represent the fast component of both inward and outward exchange.

flux measurements was performed to study the effect of chloride substitution with methanesulfonate in the preincubation media. In these experiments SR vesicles were preincubated in the presence of calcium and potassium methanesulfonate. Calcium efflux was started by dilution with media containing potassium chloride assuring that faster diffusion of chloride into the vesicles, as opposed to outward diffusion of the large anion, methanesulfonate, would produce a transient transmembrane potential and alter the kinetics of calcium release. Preliminary experiments on this subject were reported by Kasai & Miyamoto (1973). In their experiments the washing of the vesicles with KCl after preincubation in a medium containing KMS plus ^{45}Ca-$CaCl_2$ and filtration on a millipore filter, produced a sharp increase in Ca^{2+} efflux above the normal efflux observed when washing was done with a medium containing KMS. Preincubation with KCl and washing with KCl did not produce the same amount of Ca^{2+} release.

Our work with methanesulfonate proved somewhat dis= appointing in that there was no clear indication from the semilogarithmic plots that permeability changes were induced by such manipulation of SR vesicles. However, there was an apparent increased efflux when vesicles, preincubated in a medium containing KMS, were diluted with a medium containing KCl. This apparent increase in efflux of Ca^{2+} was, however, accompanied by an increase in net Ca^{2+} influx in

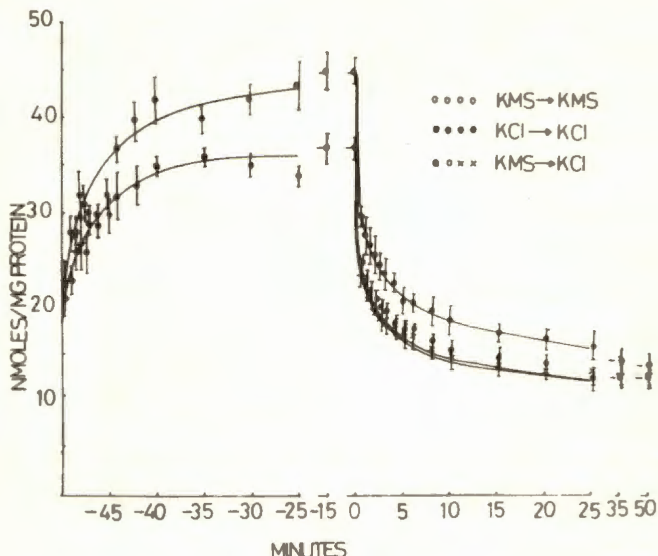


Figure 10: Net calcium influx and efflux in the absence of ATP. Influx was started by adding 20 mM ^{45}Ca-$CaCl_2$ to SR vesicles (0,4-8,0 mg/ml) suspended in 300 mM KCl (•) or KMS (o); 20 mM MOPS, pH 6,8 and 10 mM $MgCl_2$. Efflux was started after equilibration by 20-fold dilution with a medium containing 300 mM KCl (• and ×) or KMS (o); 20 mM MOPS, pH 6,8; 10 mM $MgCl_2$ and no added $CaCl_2$. Temperature : 37°C. Both influx curves represent the average values of six experiments. Statistical analysis of these curves indicates that Ca^{2+} influx in the presence of KMS differs on a 10% basis from Ca^{2+} influx in the presence of KCl. In the case of the efflux curves, statistical analysis reveals the following: Influx in presence of KMS followed by dilution with KCl, thus creating a potential differ= ence across the membrane, differs on a 1% basis from both influx in presence of KMS followed by dilution with a medium containing KMS, as well as influx in presence of KCl followed by dilution with a medium containing KCl. The latter two curves, with a $p > 0,1$ do not differ significantly.

vesicles preincubated in a medium containing KMS (Fig. 10). This increased net influx occurred in the complex third ("slow") component, and dilution of SR vesicles, preincubated in a medium containing KMS with a medium also containing KMS, did not result in release of the extra amount of Ca^{2+} taken up. The mechanism involved, remains obscure. Statistical analysis of the difference between the influx curves failed to show a highly significant difference as result of the scatter occurring in the influx curves. However, a highly significant difference (with a p-value $< 0,01$) exists between the curves representing Ca^{2+} influx in the presence of KMS, followed by dilution with a medium containing KCl and the curves representing Ca^{2+} influx in the presence of KMS, followed by dilution with a medium containing also KMS as well as influx in the presence of KCl, followed by dilution with a medium also containing KCl (see legend to Fig. 10, page 81). According to these analysis there is good agreement between these results and those obtained by Kasai & Miyamoto (1973). Although not statistically highly significant, the higher Ca^{2+} influx in the presence of KMS compared to the influx in the presence of KCl, produces an uncertainty concerning these results. It is interesting to note that Endo & Nakajima (1973), loading partially skinned fibres with Ca^{2+} in the presence of KMS, obtained a faster rate of loading to a higher level than was obtained in the presence of KCl - an effect similar to that observed by us. It is very difficult to compare these results with the

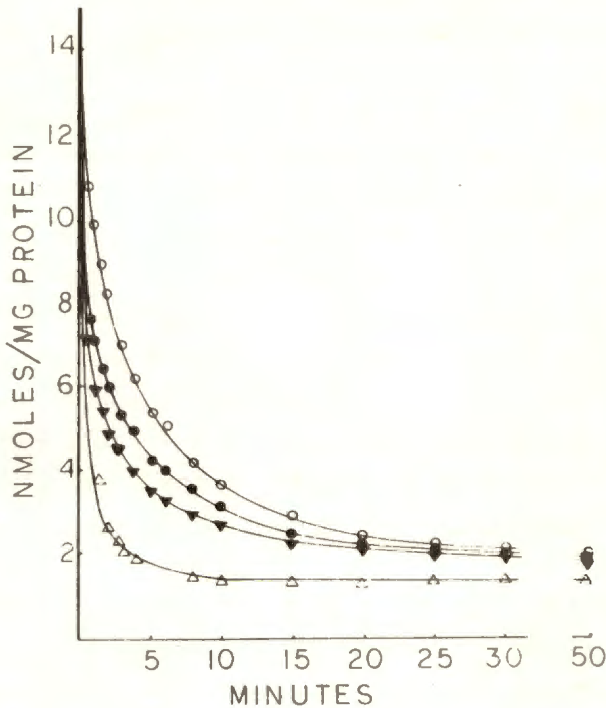


Figure 11: Effects of various cations on calcium efflux from SR vesicles in the absence of ATP. SR (8,0 mg protein/ml) was first equilibrated with a medium containing 60 mM KCl; 20 mM MOPS, pH 6,8; 10 mM $MgCl_2$ and 1 mM ^{45}Ca-$CaCl_2$. Efflux was started by a 20-fold dilution with media containing 20 mM MOPS, pH 6,8; 10 mM $MgCl_2$ and 60 mEq Cl^- with K^+ (o); $(CH_3)_4N^3$ (•), $TRIS^+$ (▼) or poly-lysine (Δ) as corresponding cation. The curve representing efflux affected by K^+ represents nine separate experiments while each of the other curves represents four separate experiments.

findings of Ford & Podolsky (1970) and Endo & Nakajima (1970) on skinned muscle fibres as a result of the big differences in experimental procedures.

These studies were then extended to the use of large cations such as tetramethylammonium (TMAC) or TRIS in the dilution media, which also produced contractions in skinned muscle fibres (Endo & Nakajima, 1973). Assuming that the SR membrane is slowly permeable to large cations, it was expected that an asymmetric charge distribution across the membrane would be prod= uced by pre-equilibration of SR vesicles with $CaCl_2$ (1 mM) and KCl (60 mM) followed by a 20-fold dilution with media containing no added calcium, and either TMAC or TRIS-chloride (100 mM) see Fig. 11.

The possibility that these effects could be the result of osmotic effects produced by the addition of relat= ively impermeable cations, was excluded in a series of experiments where, in addition to the normal constituents of the media, 200 mM sucrose was added. According to Duggan & Martonosi (1970), the SR membrane is permeable to sucrose with the result that the added sucrose should effectively limit osmotic effects produced by the added cations. The added sucrose did not affect the results in any way.

3.2.2 Release of calcium sequestered by active transport

Under normal physiological conditions ATP is present

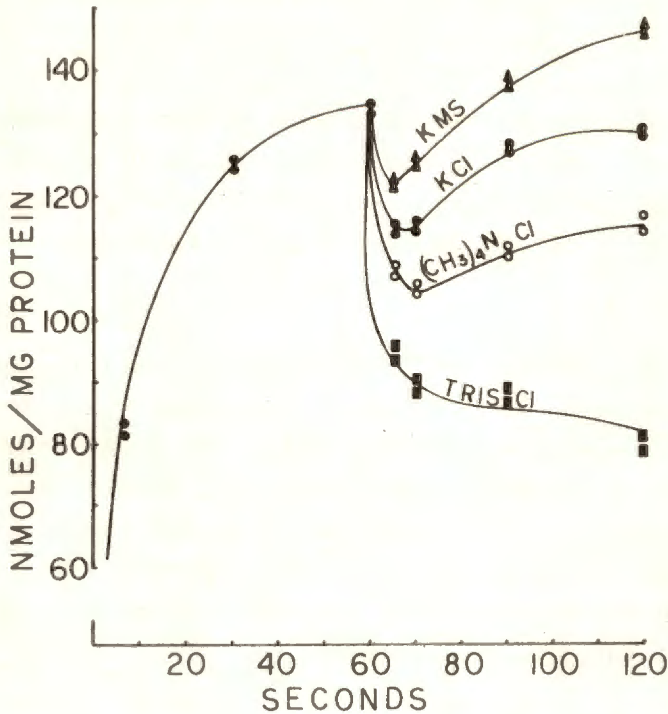


Figure 12: Effects of various cations on calcium release from SR vesicles loaded with calcium in the presence of ATP. Calcium uptake was obtained in the presence of 50 mM KCl; 20 mM MOPS, pH 6,8; 2,5 mM ATP·Mg; 0,1 mM ^{45}Ca-$CaCl_2$·EGTA and 250-350 µg SR protein/ml. After 60 seconds 400 µl of concentrated solutions of KCl, $(CH_3)_4NCl$ of TrisCl were added to reach a final concentration of 250 mM. Temperature : 37°C.

in relatively high concentrations. Any Ca^{2+}-release mechanism of physiological importance should thus be effective in the presence of ATP.

Considering the possible relevance of the observed effects described in 3.2.1.3, page 78 with regard to calcium release in physiological conditions, a series of experiments on ATP-dependent Ca^{2+} fluxes was performed.

Upon addition of ATP, Ca^{2+} uptake by SR vesicles at 37°C occurs with a rapid burst and steady state levels are reached within 60 seconds (Fig. 12). A signif= icant portion of the calcium taken up, contributes to the formation of a transmembrane gradient and is immediately released if the membrane permeability to calcium is increased by the use of a suitable ionophore like X537A (Scarpa et al, 1972).

After the SR vesicles are filled with calcium in the presence of ATP, changing of the electrolyte composition of the reaction mixture by substitution of the entire medium, as was done with skinned fibres, is not experimentally feasible. In the present study the composition of the medium was changed by adding small volumes of concentrated electrolyte solutions (see 2,7, page 56).

It was found that addition of various electrolytes to vesicles filled with calcium in the presence of ATP and KCl, was followed by a release of calcium. This

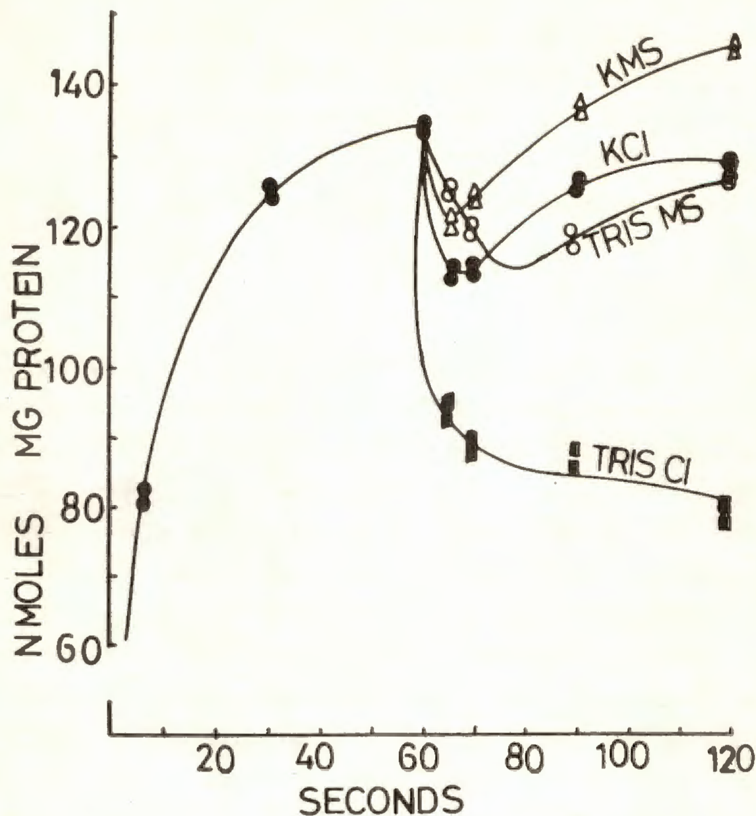


Figure 13: Effects of various cations in combination with anions of different sizes on calcium release from SR vesicles loaded with calcium in the presence of ATP. Experimental conditions were the same as described in the legend to Fig. 11. This figure shows that the relative sizes of the cationic and anionic species are the determining factors for the rate and size of Ca^{2+} release effected.

phenomenon is mostly transient, its extent and duratio depending on the electrolytes added (Fig. 12). In fact the release is more pronounced, the larger the cation (e.g. $Tris^+ > (CH_3)_4N^+ > K^+$) added with chloride. On the other hand, the release is less pronounced the larger the anion ($MS^- < Cl^-$) added with the same cation (Fig. 13).

It appears then that these effects are related to an uneven transmembrane diffusion of cationic and anionic species, producing excess positive charge outside the vesicles. The smaller the differences in permeability between the cationic and anionic species the less and more transient will be the potential difference caused by the uneven distribution of ions. In this latter case, there is a marked reduction in Ca^{2+} release from the SR vesicles. Osmotic mechanisms are unlikely to be involved since, for the same cation, larger anions are less effective. Nevertheless, the possib= ility of osmotic effects was studied in a series of experiments where, in addition to the normal constitu= ents of the reaction media, 200 mM sucrose was added to reduce any possible osmotic effects of the added electrolytes. The added sucrose did not affect the phenomena observed in Fig. 13.

An even greater release (Fig. 14) is produced by positively charged polyvalent cations (e.g. poly-lysin MM 22 000) added at final concentrations as low as 100 mEq, corresponding to a molar concentration of

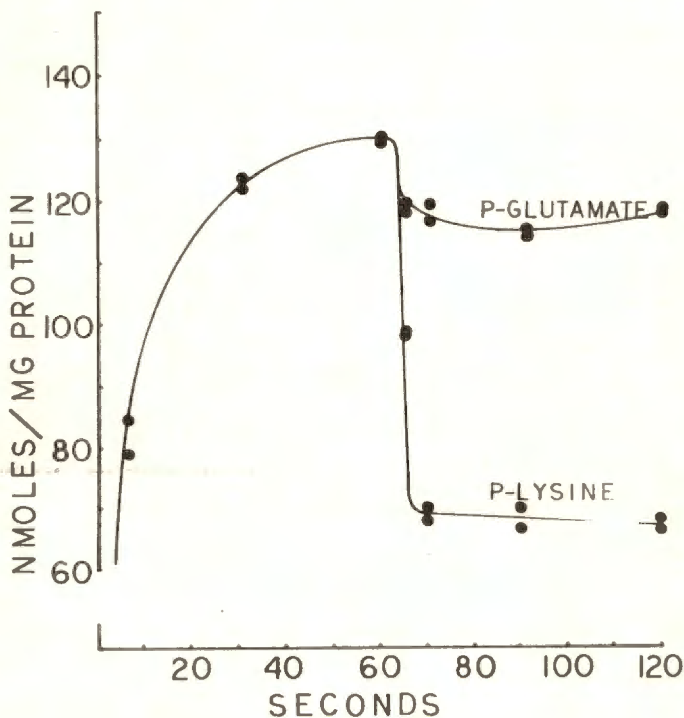


Figure 14: Effects of polyvalent electrolytes on calcium release from SR vesicles loaded in the presence of ATP. Calcium uptake was obtained in the presence of 80 mM KCl; 20 mM MOPS, pH 6,8; 2,5 mM ATP·Mg; 0,1 mM ^{45}Ca-$CaCl_2$·EGTA and 250-350 μg SR protein/ml. After 60 seconds 100 mEq poly-glutamate or poly-lysine were added, with potassium and chloride as respective counterions. Temperature : 37°C. The curves represent the values of two separate experiments.

approximately 0,8 mM according to the average molecular mass of the lysine polymer used in our experiments. However, it is possible that this effect is partially due to asymmetric charge interaction with the outer surface of the vesicles. Negatively charged poly-electrolytes (e.g. poly-glutamate) have little or no effect.

3.2.3 Calcium-induced calcium release

It has been reported (paragraph 1.3.2.4, page 37) in experiments with skinned muscle fibres that the additior of calcium ion itself, triggers the release of calcium previously sequestered by SR (Endo et al, 1970; Ford & Podolsky, 1970). We have found that this mechanism operates also on isolated vesicles preloaded in the presence of ATP (Fig. 15).

As described for skinned muscle fibres, the release does not occur when the concentration of Mg^{2+} in the reaction mixture is of the order of 10^{-3} M (Fig. 15).

The calcium-induced release operates only on vesicles loaded in the presence of ATP, as we observed no compar able effects on passive calcium fluxes.

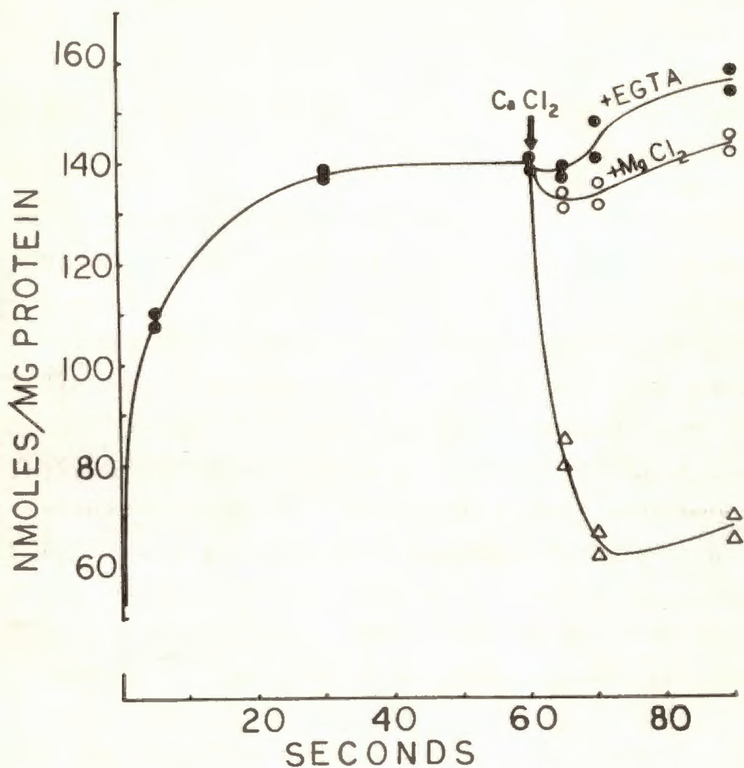


Figure 15: Calcium-induced Ca^{2+} release from SR vesicles loaded in the presence of ATP. Calcium uptake was started as described in the legend to Fig. 13. After 60 seconds, 0,5 mM $^{45}Ca\text{-}CaCl_2$ (Δ), or 0,5 mM $^{45}Ca\text{-}CaCl_2$ + 5 mM $MgCl_2$ (o), or 0,5 mM $^{45}Ca\text{-}CaCl_2$ + 0,5 mM EGTA (•) were added to the reaction mixture. Temperature : 37°C. The values obtained in two separate experiments are presented.

CHAPTER 4

DISCUSSION

4.1 Functional and structural roles of SR protein components

To study the functional and structural roles of the different protein components of the SR membrane in many instances, it is necessary to separate them structurally. The first to separate the protein components into the three major proteins, i.e. ATPase, M_{55}- and M_{45}-protein, was Martonosi (1969) and Martonosi & Halpin (1971) on SDS-polyacrylamide gel electrophoresis (for a discussion of the present status in the literature on this subject see 1.3.1.1, page 26). The biggest fraction was identified as the ATPase protein, an enzyme capable of hydrolizing ATP and thus providing energy to drive the Ca^{2+} pump.

A number of reports appeared during the past few years concerning efforts that have been made to isolate the ATPase (MacLennan, 1970; Martonosi & Halpin, 1971; McFarland & Inesi, 1971 and Sarzala et al, 1974) or the two Ca^{2+}-binding proteins (Ikemoto et al, 1972 and 1974; MacLennan & Wong, 1971). Furthermore, a number of articles appeared on electron microscopy and biochemical manipulations in order to assess the function of some of the protein components. Electron microscopical studies on negatively stained SR vesicles revealed 40 Å particles on the outer surface

of the vesicles (Ikemoto et al, 1966). Although it was reported (Ikemoto et al, 1971) that mild trypsin digestion removed these particles, the treatment did not impair either Ca^{2+} uptake or Ca^{2+} loading activity indicating that these particles do not represent the functional part of the ATPase molecule. By isolating the ATPase enzyme and applying brief sonication treatment, MacLennan et al (1974) reported that the protein formed small vesicles. These vesicles contained the 40 Å particles seen on SR vesicles. Also, when the minor protein components were removed from SR vesicles with EDTA dialysis, the 40 Å particles did not disappear. From these two observ= ations they concluded that these surface particles are in fact part of the ATPase molecule. This conclusion is also supported by Sarzala et al (1975) who studied SR structure during the fetal development of rabbits. The vesicle surfaces of SR isolated from fetuses are smooth and 40 Å particles appear for the first time in the SR vesicles isolated from 5 - 8 day old rabbits, simultaneously with a sharp increase in ATPase and Ca^{2+}- pump activity. Similar results were reported after studies on developing chickens by Boland et al (1973) and Tillack et al (1973).

Recently Meissner (1975) isolated two populations of SR vesicles by a process of zonal centrifugation on a continuous sucrose gradient (see 1.3.1.2, page 29). These vesicles differed in structural and functional features and provided an excellent opportunity to

study the function of various protein components without isolating them first.

By replacing the continuous sucrose gradient with a 4-step discontinuous sucrose gradient between 28% and 43%, we were able to isolate four fractions of SR vesicles differing in their structural and functional characteristics. On gel electrophoresis all the fractions contain one major band corresponding to the ATPase enzyme with a molecular mass of 106 000 dalton (Inesi & Scales, 1974). However, different amounts of the two minor protein components, calsequestrin and Ca^{2+}-binding protein were present in the different fractions (plate 2, page 13 and Fig. 3, page 62).

It is a well-accepted fact that the major protein component, the ATPase enzyme, is responsible for the hydrolysis of ATP to provide energy for the transport of Ca^{2+} across the membrane (Hasselbach & Makinose, 1961).

The relative ATPase contents of fractions 1 to 4 are 69%, 90%, 81% and 69%, respectively (Table 1, page 66) Fractions 2, 3 and 4 display ATPase activities which are proportional to the relative amounts of ATPase enzyme as indicated by the electrophoretic gels. Frac ion 1 is an exception and displays an extremely low Ca^{2+}-activated ATPase activity, 0,34 μmoles Pi/mg/min compared to the 1,17 μmoles Pi/mg/min of fraction 2. On the other hand, fraction 1 has a very high Mg^{2+}-activated ATPase activity, 74% of the total activity,

compared to the 15% (0,2 μmoles Pi/mg/min) of fraction 2. This would indicate that Mg^{2+}-activated ATPase, is associated more with certain parts of the SR system and is therefore unevenly distributed in the SR. A possible functional role for this uneven distribution remains obscure.

The location and functional roles of the two minor protein components, the M_{55}- or calcium-binding protein and the M_{45}-protein (calsequestrin), are much more uncertain. Concerning the functional role it was suggested by MacLennan & Wong (1971) that one of these minor components, calsequestrin, plays a role in binding calcium ions, actively transported into the lumen of the vesicles. Therefore, the maximal levels of active calcium uptake should be proportional to the amount of calsequestrin present. In this regard it is important to note that the SR vesicles with a higher calsequestrin content retained a lower ability to maintain a calcium gradient in the *presence* of ATP (Fig. 5, page 68) but, in experiments on passive Ca^{2+} influx they displayed a greater calcium-binding capacity in the *absence* of ATP (22 nmoles/mg protein compared to 14 $\pm$ 1 nmole Ca^{2+}/mg protein in the normal SR preparation). Regarding the Ca-binding protein associated with the light fractions, we could not detect any role for this protein in either ATP-dependent or ATP-independent Ca fluxes.

These experiments and those performed with lobster and chicken SR suggest that the minor protein components

are not a constant finding in different populations of SR vesicles and in different animal species (see plate 3, page 62). Furthermore, they are not necessary for the active transport of calcium ions in SR vesicles. This conclusion is supported by the finding of Ikemoto et al (1972), that both Ca^{2+} uptake and ATPase activities of fragmented sarcoplasmic reticulum vesicles reach higher levels after the soluble proteins, including calsequestrin, have been removed.

These findings are in direct opposition to the suggestion made by Garcia et al (1975) who concluded that the low Ca^{2+}-uptake activity they found in the SR of barnacle muscle must have been the result of the absence of calsequestrin from these vesicles. However, we can not exclude the possibility that these minor protein components play some physiological role in the excitation-contraction coupling. Jilka et al (1975) suggested a possible function for the M_{55}- and M_{45}-protein could be in regulating membrane permeability. They observed an increase in membrane permeability of SR vesicles to Ca^{2+} after these two proteins had been removed.

With electron microscopy Meissner (1975) observed that the vesicles of the "heavy" fraction are filled with an electron-dense substance while the vesicles of the "light" fraction appear to be empty. In electron micrographs of muscle fibres it appears that the terminal cisternae also contain an electron-dense

material while the L-tubules appear to be empty. On these grounds he concluded that the "light" fraction is derived from the L-tubules while the "heavy" fraction is derived from the terminal cisternae. The higher Ca^{2+}-uptake activity of the "light" fraction is in good agreement with the scheme of Winegrad (1970) who proposed that Ca^{2+} is taken up by the L-tubules and stored and released by the terminal cisternae. Although our results, in many aspects, are in agreement with those of Meissner (1975), we cannot confirm or deny this theory.

Independent of the presence of the minor protein components, electron microscopic inspection of freeze-fracture specimens, reveals the presence of 90 Å particles in all three SR preparations (plate 5, page98).

These 90 Å particles characteristically present on the concave but not on the convex faces, have been identified with the ATPase protein (Deamer & Baskin, 1969). It was pointed out that the number of ATPase molecules and the number of particles per unit membrane area are of the same order of magnitude (Inesi, 1972 and Inesi & Scales, 1974). Having obtained more accurate figures for the fractional value of ATPase per total SR protein, and for the membrane area per protein unit weight, a better estimate of this relation can be given.

The membrane area corresponding to 1 g SR protein

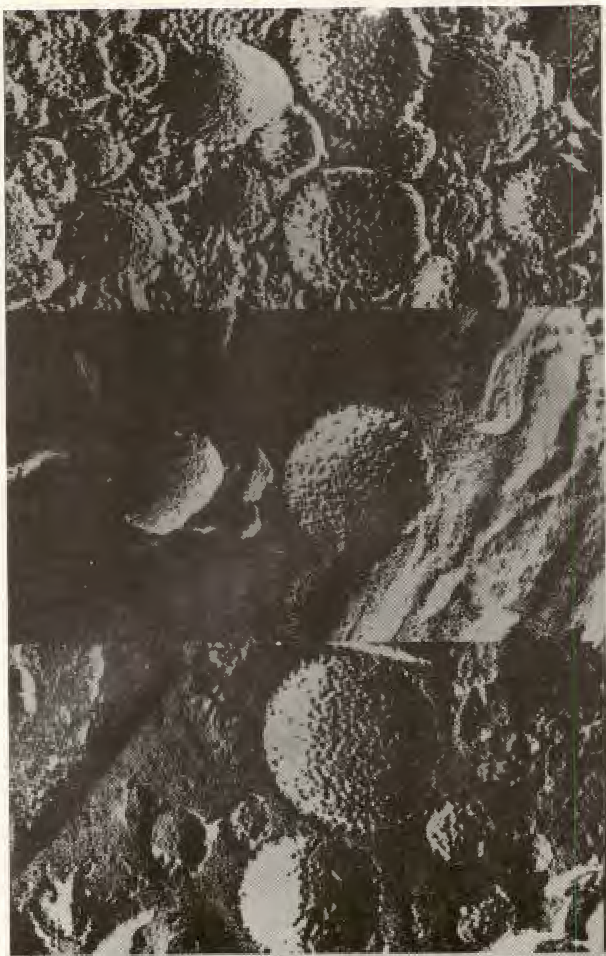


Plate 5: Freeze-fracture replicas of rabbit (top), lobster (middle) and chicken (bottom) SR. The convex and concave faces are clearly visible. Note that many 90 Å particles are present on the concave faces while very few if any particles are visible on the convex faces. Magnification 285 000. (Photograph by courtesy of Dr. D. Scales, U.O.P., San Francisco).

(A_{tot}) is obtained by multiplying the surface area of one vesicle by the number of vesicles in 1 g SR protein.

$$A_{tot} = (4\pi r^2) \cdot \left(\frac{V_{tot}}{\frac{4\pi}{3} r^3}\right)$$

where r is the average radius of one vesicle (750 Å) and V_{tot} is the total volume of the vesicles contained in a unit weight of SR protein, measured as the space excluded to ^{14}C-dextran. We found this volume to be 7,4 ± 0,2 ml/g protein in rabbit SR preparations in which the percentage of total protein accounted for by ATPase was 80 - 82%. In these SR preparations, our estimate for A_{tot} is 2,9 x 10^{14} μ^2/g SR protein. This figure is very close to the calculated value for a model membrane 62 Å thick and composed of 1 g protein and 0,65 g lipid in analogy to SR (2,4 x 10^{14} μ^2, based on a density equal to 1,1). The membrane thick= ness equal to 62 Å was determined by low angle X-ray diffraction by Worthington & Liu (1973).

An average value for the membrane area (A_{ATPase}) occupied by one of the polypeptide chains identified with the ATPase is obtained by dividing A_{tot} by the number of polypeptide chains in 1 g of SR protein.

$$A_{ATPase} = \frac{A_{tot}}{F \cdot \frac{N}{MM}}$$

where F is the fraction of total SR protein accounted for by ATPase, N is the Avogadro number and MM is the molecular mass of the polypeptide chains (106 000 dalton).

Our experimental value for F is 0,80 - 0,82; however, we consider a possible error of 10% (F = 0,72 - 0,92) to account for inaccuracies of gel densitometry, evaluated with known mixtures of standard proteins. Therefore our estimate of A_{ATPase} is 5,7 - 7.1 x 10^{-5} μ^2.

Finally the number of polypeptide chains per unit of SR membrane area (D) may be obtained according to:

$$D = \frac{1}{A_{ATPase}}$$

and found to be 1,41 - 1,75 x $10^4/\mu^2$. This figure exceeds by a factor of 2,5 - 3,1 the density of particles observed in freeze-fracture faces of the same SR preparation, which we found to be 5730 $\pm$ $520/\mu^2$. The calculated density of polypeptide chains and the observed density of particles would be the same only if F was equal to 0,3.

Our observations indicate that either a significant number of polypeptide chains do not appear as particle on the freeze-fracture faces, or the polypeptide chai tend to form olygomers. The alternative is consiste with the different sizes of the particles appearing i the SR freeze-fracture faces. Furthermore, it was previously reported (McFarland & Inesi 1970 and 1971) that the ATPase protein, when solubilized with Triton X-100 as opposed to dodecylsulfate, migrates in the electrophoretic field and sediments in the ultra-centrifuge in the form of monomers, dimers

and prevalently tetramers. Recently, our observations that the ATPase exists and functions in the SR membrane in the form of oligomers, received support in a report by Le Mair, Møller & Tanford (1976). They found that upon solubilization of SR with various detergents, the smallest particle with ATPase activity corresponds to a tri- or tetramer of the 106 000 dalton polypeptide chain.

4.2 Permeability and calcium release in sarcoplasmic reticulum vesicles

In 1951 Marsh published a short communication describ= ing his so-called "relaxing factor" which at that time was thought to be a soluble component of the muscle cell. First the particulate nature of the relaxing factor was established (Portzehl, 1957) and later on, in experiments with EDTA which imitated the physio= logical role of this factor, the essential role of calcium in the contraction-relaxation cycle was established. The fact that the relaxing factor is part of the sarcoplasmic reticulum was not established till the early sixties (Ebashi & Lipmann, 1962). For a more extensive coverage of the history of SR research, see 1.1, page 3.

Much work has since been done, mainly on the mechanism of ATP-dependent Ca^{2+} uptake, hydrolysis of ATP by the ATPase enzyme and the structure of the SR membrane with biochemical and electron microscopical techniques. However, one very important aspect of SR function has

remained very much obscure, the mechanism of Ca^{2+} release from the SR - the actual process that makes muscle contraction possible. The lack of suitable experimental procedures can be blamed for this appare lack in the knowledge of SR function.

It is a well-established fact that action potential developed in the sarcolemma is spread to the interior of the muscle cell by means of the T-tubules (Howell, 1969; Krolenko, 1969). However, the way this impul is transmitted to the terminal cisternae i.e. the mechanism by which T-tubule depolarization can stim= ulate the terminal cisternae to release calcium, is totally obscure. There are at least two possibiliti for this process: (a) a chemical coupling and (b) a direct electrical coupling between the T-tubule and the terminal cisternae, with the SR Ca^{2+} permeability voltage-dependent. Franzini-Armstrong (1970) conclud that on morphological grounds one cannot decide whethe coupling is through ionic current flow via the junc= tional feet, or chemical transmission across the junctional gap. At this stage no information is available on a possible chemical coupling mechanism.

On the other hand, several attempts have been made to assess the possibility of a direct electrical couplin between the T-system and the terminal cisternae. In 1966 Lee et al, made an effort to accomplish Ca^{2+} release from fragmented SR vesicles by direct electri stimulation with the aid of platinum electrodes. The findings, although promising, were proven to be the

result of irreversible denaturation of SR proteins by Miyamoto & Kasai, 1973 (see 1.3.2.6, page 41).

A number of very interesting experiments having a bearing on this problem, was performed with skinned muscle fibres by changing the electrolyte composition to produce asymmetric charge distributions across the internal membranes (Constantin & Podolsky, 1967; Ford & Podolsky, 1970; Endo & Nakajima, 1973). In the case of Endo & Nakajima (1973), partially skinned fibres were incubated in a medium containing Ca^{2+} and KMS. The presumed lesser permeability of MS^{-} should produce an increased negative charge on the inside of the SR when replaced with KCl - this "depolarizing" effect produced Ca^{2+} efflux from the SR (Endo & Blinks, 1973) accompanied by muscle contraction (Endo & Nakajima, 1973).

Similar results were obtained by Kasai & Miyamoto (1973) with isolated SR. Again preincubation was performed in the presence of KMS and, after millipore filtration, the SR vesicles on the filter were washed with a medium containing either KMS or KCl. In the former case no Ca^{2+} efflux was observed but in the latter case Ca^{2+} efflux did occur (see 1.3.2.6, page 41).

Stimulated by these results and by the importance of the question of calcium release, we designed a series of experiments to obtain, first of all, a sufficiently precise value for the permeability of the SR membrane to calcium, and then to investigate the effects of

changes in the electrolyte composition of the reaction media during passive and active calcium fluxes.

4.2.1 Membrane permeability to calcium

Fig. 7, page 72 shows that passive calcium fluxes across the membrane of SR vesicles display three kinetic components. The first or "very fast" component is related to loosely bound calcium to the outside of the SR membrane. This component is very sensitive to EGTA in the washing media. The third or "slow" component is a complex phenomenon which may be related directly or indirectly to calcium binding to the SR membrane. This component is sensitive to competitive binding by high concentrations of KCl (see 3.2.1.1, page 69). As a result of the difference in experimental procedures it is very difficult to compare the sizes of these compartments to those obtained by Carvalho (1966); Carvalho & Leo, (1967) and Cohen & Zelinger (1969).

The second or "fast" component of the passive calcium fluxes corresponds to a net movement of 6,3 nmoles Ca^{2+}/mg SR protein, in vesicles previously equilibrate with media containing 1 mM $CaCl_2$. It is estimated that this value is sufficient to account for intra= vesicular Ca^{2+} concentrations equal to those in the pre-equilibration medium (1 mM), based on an intra= vesicular volume of 5 μl/mg SR protein determined by the method of Duggan & Martonosi (1970). The size of this component remains unchanged in the presence

of EGTA (0,5 mM) or high (300 mM) concentration monovalent cations in the outside medium. On the other hand, its time constant is profoundly influenced by the presence of calcium ionophores (Fig. 6, page 70). It is therefore apparent that this kinetic component is related to equilibration of the calcium concentrat= ions inside and outside the vesicles and that the permeability of the SR membrane is rate limiting in this process.

A possible source of error in this type of experiment is backflux of Ca^{2+} as result of an increase in the Ca^{2+} concentration in the outside medium due to Ca^{2+} efflux from the vesicles. In this case however, the rate constant of the second kinetic component of net outward fluxes approximates that of unidirectional fluxes, due to the very low ratio ($\leq$ 1:500) between intravesicular and extravesicular volumes. This ratio prevents any significant increase of calcium concentration in the external medium as a consequence of net efflux, thereby limiting counterflow. In fact, identical rate constants are obtained in the presence of EGTA which does not penetrate the SR vesicles and effectively prevents counterflow by chelating calcium in the external medium (see Fig. 7, page 72).

The permeability coefficient (P_{Ca}) of the SR membrane to calcium may be derived from the first order rate constant (k) of the second kinetic component of outward fluxes according to:

$$P_{Ca} = k \cdot {}^{V}/_{A}$$

where

V = average volume of SR vesicles = 7,4 $\pm$ 0,2 ml/g SR protein and A = average surface area of SR vesicles = 2,9 x 10^{14} μ^2/protein (see 4.1, page 92). According to the formula

$$\ln {}^{A_o}/_{A} = kt$$

a first order rate constant (k) of 9,2 x 10^{-3} sec^{-1} was calculated for the second ("fast") kinetic compon= ent using values experimentally obtained for A and A_o. Using this value, we estimate P_{Ca} to be 1,5 x 10^{-8} cm sec^{-1}.

Permeability coefficient is related to the diffusion coefficient (D) according to

$$P_{Ca} = {}^{DB}/_{dx}$$

where dx is the thickness of the membrane, assumed to be 60 Å for the SR membrane (Worthington & Liu, 1973) and B is the partition coefficient. Accordingly, the effective diffusion coefficient of calcium within the membrane is estimated to be 9 x 10^{-15} cm^2 sec^{-1}, assuming B = 1.* The limit of this figure is appreci= ated when compared to the diffusion coefficient of calcium in water which is approximately 10^{-5} cm^2 sec^{-1}.

---

*B, the partition coefficient is given by

$$B = \frac{\text{concentration in membrane phase}}{\text{concentration in water phase}},$$

and is thus an expression for the solubility constant for Ca^{2+} in the membrane phase. This value is not known for Ca^{2+} in the SR membrane and is thus assumed to be equal to 1. Therefore, the calculated diffusion coefficient D will be the "effective" rather than "absolute" diffusion coefficient.

The value for P_{Ca} is extremely useful since it can be used to estimate calcium fluxes (ϕ_{Ca}) in conditions of special interest. For the simplest case, neglecting electrical potentials and assuming the volume of the extravesicular medium much larger than the intra= vesicular volume,

$$\phi_{Ca} = P_{Ca} \cdot \Delta C$$

where ΔC is change in Ca^{2+} concentration.

Using this relation calcium efflux from SR vesicles is estimated to be 1,5 x 10^{-14} moles sec^{-1} cm^{-2} in conditions similar to those used by De Boland et al (1975) to obtain values ranging between 10^{-18} and 10^{-15} moles sec^{-1} cm^{-2} for calcium efflux from phospho= lipid vesicles. The difference is attributed to the presence of the SR protein which increases the membrane permeability to calcium, as demonstrated by Jilka et al (1975). This value of 1,5 x 10^{-14} moles sec^{-1} cm^{-2} is nearly one order of magnitude lower than the value obtained by Jilka et al (1975) for passive calcium efflux from SR vesicles *in vitro*. However, big differences in the techniques used, make an objective comparison between the different values very difficult. Jilka et al (1975) loaded the vesicles with the aid of ultrasonic treatment and the effect of such harsh treatment on the structure of the membrane and thus permeability, is very difficult to assess.

Using the same relation, and assuming a 10 mM calcium activity in the space limited by the SR membrane *in vivo*

(instead of the 1 mM in the experimental conditions), a rate of 1,5 x 10^{-13} moles sec^{-1} cm^{-2} is estimated for outward calcium flow from SR in the myoplasm. In this case the possible existence of electrical poten= tials was neglected. However, there is a possibilit that a potential difference exists across the vesicul membrane. In order to assess the forces exerted on the Ca^{2+} ions by such a potential difference the gradient of electrochemical potential is the force that should be considered. According to the definiti of Guggenheim the electrochemical potential, μ, for an ion species like Ca^{2+}, is given by

$$\bar{\mu}_{Ca} = \mu^{o}_{Ca} + RT \ln\gamma_{Ca}c_{Ca} + z_{Ca}F\Psi + P\bar{V}_{Ca} \qquad (1)$$

where μ^{o}_{Ca} = the chemical potential in a standard state; R = gas constant; T = temperature in K; γ_{Ca} = activity coefficient, c_{Ca} = chemical con= centration; z_{Ca} = valency (with sign); F = Faraday constant; Ψ = electrical potential; P = hydrostatic pressure and $\bar{V}_{Ca}$ = partial molar volume. $\gamma_{Ca}c_{Ca}$ as a product forms the chemical activity a_{Ca}, and RT ln a_{Ca},together with μ^{o}_{Ca} represent the chemical potential (Hope, 1971).

The quantities P, c_{Ca}, Ψ and possibly γ_{Ca} may vary from a solution on one side of a membrane, through the membrane to a solution on the other side of the membrane. Assuming there is a thermal equilibrium, T remains constant.

The force X_{Ca} on the ions will be the negative of the gradient of $\bar{\mu}_{Ca}$ (Hope, 1971):

$$X_{Ca} = - \text{gradient } \bar{\mu}_{Ca}$$

$$= - RT \frac{\partial(\ln a_{Ca})}{\partial x} - z_{Ca} F \frac{\partial \Psi}{\partial x} - \bar{V}_{Ca} \cdot \frac{\partial P}{\partial x} \qquad (2)$$

where x is the distance inside the membrane from one face of the membrane. The velocity of Ca^{2+} subjected to this force is mobility times the force i.e. u_{Ca}. The force X_{Ca} and finally, the flux ϕ_{Ca} in the x direction is given by velocity times concentration. Thus,

$$\phi_{Ca} = (u_{Ca} X_{Ca}) c_{Ca}$$

$$= - u_{Ca} c_{Ca} \left(\frac{RT}{\gamma_{Ca}} \cdot \frac{\partial \gamma_{Ca}}{\partial x} + z_{Ca} F \frac{\partial \Psi}{\partial x} + \bar{V}_{Ca} \cdot \frac{\partial P}{\partial x}\right) - u_{Ca} RT \frac{\partial c_{Ca}}{\partial x} \qquad (3)$$

In order to integrate equation (3) the assumption was made that the electrical field within the membrane is a constant. This is equivalent to postulating a linear potential gradient (Hope, 1971). Thus,

$$\frac{\partial \Psi}{\partial x} = \frac{(\Psi^{\delta} - \Psi^{o})}{\delta}$$

$$= \frac{\Delta \Psi}{\delta} \qquad (4)$$

where δ = thickness of the membrane. By neglecting the pressure gradient, P and put $\frac{\partial \gamma}{\partial x} = 0$,

$$\phi_{Ca} = -u_{Ca}RT \frac{\partial c_{Ca}}{\partial x} - u_{Ca}c_{Ca}z_{Ca}F \frac{\Delta\Psi}{\delta}$$

$$\phi_{Ca} + u_{Ca}c_{Ca}z_{Ca}F \frac{\Delta\Psi}{\delta} = u_{Ca}RT \frac{\partial c_{Ca}}{\partial x}$$

$$\partial x = \frac{-u_{Ca}RT\partial c_{Ca}}{\phi_{Ca} + u_{Ca}c_{Ca}z_{Ca}F \frac{\Delta\Psi}{\delta}}$$

$$dx = \frac{-u_{Ca}RT\, dc_{Ca}}{\phi_{Ca} + u_{Ca}c_{Ca}z_{Ca}F \frac{\Delta\Psi}{\delta}} \tag{5}$$

Therefore

$$\int_o^\delta dx = -u_{Ca}RT \int_{c^o_{Ca}}^{c^\delta_{Ca}} \frac{dc}{\phi_{Ca} + u_{Ca}z_{Ca}F \frac{\Delta\Psi}{\delta} \cdot c_{Ca}}$$

$$= - \frac{u_{Ca}RT}{u_{Ca}z_{Ca}F \frac{\Delta\Psi}{\delta}} \cdot \ln \left[\phi_{Ca} + u_{Ca}z_{Ca}F \frac{\Delta\Psi}{\delta} \cdot c_{Ca}\right]_{c^o_{Ca}}^{c^\delta_{Ca}}$$

$$= - \frac{\delta RT}{z_{Ca}F\ \Delta\Psi} \cdot [a - b]$$

with $a = \ln\{\phi_{Ca} + u_{Ca}z_{Ca}F \frac{\Delta\Psi}{\delta} \cdot c^o_{Ca}\}$

and $b = \ln\{\phi_{Ca} + u_{Ca}z_{Ca}F \frac{\Delta\Psi}{\delta} \cdot c^\delta_{Ca}\}$,

thus $= - \frac{\delta RT}{z_{Ca}F\ \Delta\Psi} \cdot \ln\left(\frac{\phi_{Ca} + u_{Ca}z_{Ca}F \frac{\Delta\Psi}{\delta} \cdot c^o_{Ca}}{\phi_{Ca} + u_{Ca}z_{Ca}F \frac{\Delta\Psi}{\delta} \cdot c^\delta_{Ca}}\right)$

Thus

$$\delta = \frac{\delta RT}{z_{Ca}\Delta\Psi} \ln\left(\frac{\phi_{Ca} + u_{Ca}c^{o}_{Ca}z_{Ca}F\frac{\Delta\Psi}{\delta}}{\phi_{Ca} + u_{Ca}c^{\delta}_{Ca}z_{Ca}F\frac{\Delta\Psi}{\delta}}\right)$$

$$\frac{z_{Ca}F\,\Delta\Psi}{RT} = \ln\left(\frac{\phi_{Ca} + u_{Ca}c^{o}_{Ca}z_{Ca}F\frac{\Delta\Psi}{\delta}}{\phi_{Ca} + u_{Ca}c^{\delta}_{Ca}z_{Ca}F\frac{\Delta\Psi}{\delta}}\right) \qquad (6)$$

This equation can be rearranged to:

$$\frac{\phi_{Ca} + u_{Ca}c^{o}_{Ca}z_{Ca}F\frac{\Delta\Psi}{\delta}}{\phi_{Ca} + u_{Ca}c^{\delta}_{Ca}z_{Ca}F\frac{\Delta\Psi}{\delta}} = \exp\left(\frac{z_{Ca}F\Delta\Psi}{RT}\right) \text{ since } x = \exp y \text{ if } y = \ln x$$

$$\phi_{Ca} + u_{Ca}c^{o}_{Ca}z_{Ca}F\frac{\Delta\Psi}{\delta} = \{\phi_{Ca} + u_{Ca}c^{\delta}_{Ca}z_{Ca}F\frac{\Delta\Psi}{\delta}\}\cdot\exp\left(\frac{z_{Ca}F\,\Delta\Psi}{RT}\right)$$

$$\phi_{Ca}\,\{1-\exp\left(\frac{z_{Ca}F\,\Delta\Psi}{RT}\right)\} = -u_{Ca}c^{o}_{Ca}z_{Ca}F\frac{\Delta\Psi}{\delta} + u_{Ca}c^{\delta}_{Ca}z_{Ca}F\frac{\Delta\Psi}{\delta}\cdot\exp\left(\frac{z_{Ca}F\Delta\Psi}{RT}\right)$$

$$\phi_{Ca} = -\frac{u_{Ca}z_{Ca}F\frac{\Delta\Psi}{\delta}\cdot c^{o}_{Ca} - c^{\delta}_{Ca}\cdot\exp\left(\frac{z_{Ca}F\,\Delta\Psi}{RT}\right)}{1-\exp\left(\frac{z_{Ca}F\,\Delta\Psi}{RT}\right)} \qquad (7)$$

In this equation c^{o}_{Ca} and c^{δ}_{Ca} are the concentration of Ca^{2+} just within the membrane. By assuming the part= ition coefficient B = 1 as was done previously, c^{o}_{Ca} and c^{δ}_{Ca} become equal to the concentration of Ca^{2+} in the intravesicular and extravesicular media respect= ively. The parameters u_{Ca} and δ can be taken together as the permeability coefficient (Hope, 1971).

Thus equation (7) becomes:

$$\phi_{Ca} = - \frac{P_{Ca} z_{Ca} F \, \Delta\Psi}{RT} \cdot \frac{Ca_{in} - Ca_{out} \cdot \exp\left(\frac{z_{Ca} F \, \Delta\Psi}{RT}\right)}{1 - \exp\left(\frac{z_{Ca} F \, \Delta\Psi}{RT}\right)}$$

Where $P_{Ca} = \frac{u_{Ca} \cdot RT}{\delta}$, with units cm sec^{-1}.

Using this relation it may be estimated that the Ca^{2+} flux, ϕ_{Ca}, which was estimated to be 1,5 x 10^{-13} moles sec^{-1} cm^{-2} for outward Ca^{2+} flow from the SR to the myoplasm would be changed by less than one order of magnitude by transmembrane potentials up to 100 mV in either direction assuming no alteration of permeabilit as a consequence of such potentials.

The value obtained above for calcium efflux from SR *in vivo* (1,5 x 10^{-13} moles sec^{-1} cm^{-2}) is quite similar to that given by Bianchi (1961) for influx of calcium through the sarcolemma in resting muscle (9,4 x 10^{-14} moles sec^{-1} cm^{-2}). On the other hand, these values are four orders of magnitude lower than the efflux of calcium from SR during excitation-contraction coupling. This efflux was estimated to be 10^{-9} to 10^{-8} moles sec^{-1} cm^{-2} in electrically stimulated muscle (Jöbsis & O'Conner, 1966; Ashley & Ridgway, 1970 and Martonosi, 1972). We calculated this value to be approximately 1 x 10^{-9} moles sec^{-1} cm^{-2}, considering that, for each twitch, 0,1 μmole calcium is released from 5 mg of SR protein in 5 msec (Weber, 1966) and that the membrane area corresponding

to 1 mg of SR protein being 2,9 x 10^{11} μ^2/mg (see 4.1) while assuming the partition coefficient B = 1, as was done in the case of *in vitro* Ca^{2+} fluxes. Comparing this value for ϕ_{Ca}, i.e 1 x 10^{-9} moles sec^{-1} cm^{-2}, with the 1,5 x 10^{-13} moles sec^{-1} cm^{-2} from experimental data, it is clear that a mechanism must be operating to increase the SR permeability of calcium upon membrane excitation. This conclusion, although based on different experiments and analysis, is similar to that reached by De Boland et al (1975).

4.2.2 Calcium release from SR vesicles

As described in 4.2 the first experiments that were done to study the possibility of a direct electrical effect on the permeability of SR membranes, were done with skinned muscle fibres (Constantin & Podolsky, 1967; Ford & Podolsky, 1970). In these experiments either droplets of a concentrated KCl solution were added directly to the fibre to produce contraction, or the propionate in the preincubation medium was replaced with high concentrations of KCl. Both these procedures produced transient contractions, due to a "depolarizing" effect creating a negative charge on the inside surface of the membrane. According to Constantin & Podolsky (1967) from theoretical considerations one would expect a resting potential across the internal membrane system as result of a potassium diffusion gradient. This diffusion gradient would result in a potential difference with the internal membrane space positive with respect to the outside.

The interpretation of these experiments is based on the assumption that the SR membrane is more permeable to chloride than to other larger anions (e.g. methane= sulfonate). Following substitution of the bathing medium containing larger anions with a medium contain= ing chloride, the chloride diffuses into SR faster, than the larger anions diffuse outward. This produce net positive charge outside the SR, a condition that may be compared to that occurring during membrane depolarization in intact muscle. On experimental grounds it is not possible to say with certainty whether this effect is a true depolarization i.e. changing an existing potential difference across the membrane, or whether it is a polarization, i.e. creating for the first time, a potential difference across the membrane.

Opposite maneuvers designed to produce net negative charge on the outside surface of the SR membrane system, are not effective (Nakajima & Endo, 1973). Therefore, the calcium release and contraction responses obtained by electrolyte changes in the media appear to be an experimental model reflecting a physiological mechanism and suggest that electrical phenomena affect SR membranes even in the absence of the sarcolemma.

A doubt, however, remains as to whether the intervent= ion of membranes other than SR, is completely elimin= ated in the skinned fibres. For instance, remaining T-tubules could reseal to form closed compartments

retaining Na^+-pump and electrical properties, similar to those of the outer membrane (Constantin & Podolsky, 1967). Depolarization of the resealed tubules would then generate a stimulus for the SR.

Attempts to eliminate this possibility were made by Nakajima & Endo, 1973 (see 1.3.2.6, page 41) by the use of partially skinned fibres on which remnants of interrupted sarcolemma prevent T-tubules from reseal= ing. Nevertheless, the primary involvement of the SR membrane by manipulations of the electrolyte composition of the medium, would be best demonstrated if Ca^{2+} release could be obtained from isolated SR vesicles.

Promising results were obtained by Kasai & Miyamoto (1973) in experiments with isolated SR where the KMS in the incubation medium was replaced with KCl result= ing in a 50% release of Ca^{2+} (see 1.3.2.6, page 41). However, certain ambiguities remained concerning the technique they used which could not differentiate between the different components of the Ca^{2+} associated with the vesicles.

So, having obtained a figure for the Ca^{2+} permeability of the SR membrane we repeated their experiments with our technique to determine whether the Ca^{2+} permeabil= ity of the membrane was changed by ion substitutions. From Fig. 10, page 81, it is clear that an additional amount of Ca^{2+} is released from vesicles preincubated in a medium containing KMS and diluted with a medium

containing KCl, compared to vesicles diluted with a medium containing KMS. Statistical analysis shows that this difference is highly significant ($p < 0,01$). These results then, are in good agreement with those obtained by Kasai & Miyamoto (1973). Kinetic analysis of these results on semilogarithmic plots proved to be somewhat disappointing in that no permeability change of the membrane for Ca^{2+} could be shown.

However, in the *in vivo* situation a high ATP concentration is always present with the result that all changes in Ca^{2+} permeability *in vitro* should be initiated in a medium containing a high ATP concentration in order to have any possible physio= logical significance. With this in mind the SR vesicles were first loaded with Ca^{2+} in the presence of ATP and then various electrolytes were added to the reaction mixtures to change the composition of the medium. Fig. 12, page 85, shows that the addition of chloride and various monovalent cations to vesicles loaded in the presence of potassium chloride, produce sudden calcium release. A more pronounced release is obtained when cations larger than potassium are added, probably reflecting slower transmembrane diffusion of the various cations as compared to that of chloride. An even greater release is produced by the addition of positively (but not negatively) charged polyelectrol= ytes, as shown in Fig. 14, page 89.

In fact, this effect is much reduced when the different cations are combined with anions bigger than chloride,

such as methanesulfonate (Fig. 13, page 87). Con=
sequently, the important factor appears to be the
difference in size and thus the difference in relative
diffusion rates, of the cation and anion. This dif=
ference in diffusion rates creates a depolarizing
effect by asymmetrically distributing charges across
the SR membrane, analogous to the results obtained
with skinned fibres. If such an interpretation is
correct these experiments suggest that the SR membrane
is directly sensitive to electrical effects and that
the physiological release could be induced through
this mechanism.

In another set of experiments we found that such
changes in the electrolyte composition of the medium
effect calcium release from SR vesicles even in the
absence of ATP. Fig. 11, page 83, shows that calcium
efflux following dilution of vesicles previously
equilibrated in media containing Ca^{2+} and potassium
chloride, is more rapid if the dilution media contain
cations larger than potassium. Although the altered
kinetics of release do not allow a precise measurement
of rate constants, these experiments indicate that the
enhanced release is due to an effect on the SR perme=
ability to calcium, rather than to interference with
active transport.

Another type of calcium release observed in skinned
fibres is the "calcium-triggered" Ca^{2+} release (Endo
et al, 1970; Ford & Podolsky, 1970). We were able
to reproduce such a release in isolated SR vesicles

loaded with Ca^{2+}, demonstrating that the calcium-triggered mechanism operates directly on SR. Fig. 15, page 91, shows that if 0,5 mM $CaCl_2$ is added to vesicl loaded with Ca^{2+}, a considerable release of Ca^{2+} is obtained. Similar effects, however, are not observed in the absence of ATP. Therefore, this mechanism of release requires that the calcium pump be activated.

With regard to the physiological relevance of the calcium-triggered release, it was pointed out (Endo, 1975) that the required concentrations of triggering calcium are too high to be consistent with the physiological mechanism of excitation-contraction coupling (see 1.3.2.4, page 37). On the other hand, the calcium-triggered release may play a role in special circumstances such as in the presence of caffeine (Endo, 1975) and in heart muscle preparations (Fabiato & Fabiato, 1975; Glenn et al, 1974).

UMMARY

wo aspects of the structural and functional properties
f the sarcoplasmic reticulum (SR) of skeletal muscle
ere studied.

a) By simplifying a continuous sucrose gradient
echnique described by Meissner (1975), we were able
o separate an SR preparation into 4 subfractions
S_1 - S_4). These fractions differed in their struct=
ral and functional characteristics. Poly-acrylamide
el electrophoresis showed that all four fractions
ontained heavy bands of ATPase protein. However,
hey differed in composition of the minor protein
omponents, i.e. M_{55}- and M_{45}-proteins. This indicates
difference in localization of these minor components
n the *in situ* situation. Structural differences with
egard to the minor protein components were also found
etween rabbit, lobster and chicken SR. On comparing
oth types of preparations with regard to structural
nd functional properties, we could show that only the
TPase protein is essential in ATP-dependent Ca^{2+}
ransport. The ATPase protein accounts for 70-90%
f the total protein content of the membrane.

ith the aid of freeze-fractioning 90 Å particles,
hich were identified as part of the ATPase protein,
ere found on the fracture surfaces. On counting
hese particles, it was revealed that there are
730 $\pm$ 520 μ^{-2}. The number of polypeptide molecules
as estimated to be 1,41 - 1,75 x 10^4 μ^{-2}. There

is thus a factor 3 difference between the calculated value for the polypeptide chains and the number of 90 Å particles on the fracture surfaces. This could mean that (i) a considerable number of polypeptide chains do not appear as particles on the fracture surfaces, or (ii) the ATPase molecules in the membrane exist in an oligomeric condition. Evidence from the literature indicates that this last possibility is the most likely one.

(b) A technique was developed to (i) determine the Ca^{2+} permeability of the SR membrane, and (ii) to determine the effect of ion substitutions on Ca^{2+} release by SR vesicles in the presence and absence of ATP. Plotting passive (ATP-independent) Ca^{2+} fluxes on a semilogarithmic scale, three flux comp= onents could be identified: a "very fast" component representing Ca^{2+} binding to low affinity binding sites; a "fast" component with a $t_{½}$= 90 sec., representing a transmembrane Ca^{2+} flux, and a third "slow" component with a $t_{½}$ = 7 - 9 min., which seems to be a complex phenomenon including extravesicular Ca^{2+} binding.

The Ca^{2+} permeability of the SR membrane was estimated from the second kinetic component of the passive flux curve on $1,5 \times 10^{-8}$ cm sec^{-1}. Ca^{2+} flux *in vivo* was estimated on $1,5 \times 10^{-13}$ moles sec^{-1} cm^{-2}. This is four orders of magnitude slower than necessary for the initiation of muscle contraction. Thus, it can be

oncluded that release of Ca^{2+} from SR *in situ* must
e accompanied by a change in membrane permeability.

a^{2+}-release experiments were performed in the absence
nd presence of ATP. After preincubating the vesicles
n a medium containing KCl and ATP, the ion composition
f the medium was drastically changed by adding small
olumes of concentrated cations. A fast release of
a^{2+} was observed under certain conditions with the
ffect of poly-lysine > Tris > $(CH_3)_4 N^+$ > K^+ with Cl^-
s counterion. The difference in size between the
ation and anion is very important as a difference in
iffusion rate produces an uneven distribution of
harges across the membrane. This is presumably
ollowed by an increase in permeability. The same
ffect is observed in passive Ca^{2+} efflux.

a^{2+}-triggered Ca^{2+} release could be shown in isolated
R vesicles preincubated in a medium containing Ca^{2+}
nd ATP. This Ca^{2+} release is inhibited by a high
oncentration of free Mg^{2+} and is probably not
hysiologically very important in skeletal muscle
nder normal conditions.

OPSOMMING

Twee aspekte van die strukturele en funksionele eien= skappe van die sarkoplasmiese retikulum (SR) van skeletspier is ondersoek.

(a) 'n Kontinue sukrose gradiënttegniek, soos beskryf deur Meissner (1975) is gewysig om 'n SR preparaat in 4 subfraksies (S_1 - S_4) te skei. Die fraksies verskil onderling in strukturele en funksionele eienskappe. Met poli-akrilamied gelelektroforese is gevind dat al vier fraksies swaar bande ATPase proteïen bevat maar verskil in die samestelling van die mindere proteïen= komponente, die M_{55}- en M_{45}-proteïene. Dit dui op 'n verskil in die verspreiding van die proteïenkompo= nente in die SR *in situ*. Strukturele verskille wat proteïensamestelling betref is ook tussen konyn-, kreef- en kuikenspier gevind. 'n Vergelyking van beide tipes preparate se strukturele en funksionele eienskappe toon dat slegs die ATPase proteïen 'n rol speel tydens ATP afhanklike Ca^{2+}-transport. Die ATPase beslaan 70-90% van die totale proteïen in die membraan.

Met behulp van vriesfraksionering is 90 Å partikels, wat met die ATPase proteïen vereenselwig word op die vesikels se fraksioneringsvlakke gevind. Met elek= tronmikroskopiese telling is hulle digtheid op $5730 \pm 520\ \mu^{-2}$ bepaal. Die aantal molekules ATPase is op $1{,}41 - 1{,}75 \times 10^4\ \mu^{-2}$ bereken. Daar is dus

n faktor 3 verskil tussen die berekende aantal mole=
ules en die aantal 90 Å partikels wat daarop dui dat
i) 'n aansienlike aantal polipeptiedkettings nie as
0 Å partikels waargeneem kan word nie, of (ii) dat
ie ATPase molekules in die membraan in 'n oligomeer=
oestand verkeer. Uit die bestaande literatuur blyk
it dat hierdie laaste moontlikheid die mees waarskyn=
ike is.

b) 'n Tegniek is ontwikkel waarvolgens (i) die Ca^{2+}-
eurlaatbaarheid van die SR membraan bepaal kan word
n (ii) die effek van ioonveranderinge op Ca^{2+}-
rystelling in teenwoordigheid en afwesigheid van ATP
agegaan kan word. Deur passiewe (ATP onafhanklike)
a^{2+}-flukse semilogaritmies te plot is drie komponente
an die fluks geïdentifiseer nl. 'n "baie vinnige"
omponent wat binding van Ca^{2+} aan lae affiniteit Ca^{2+}-
indingsplekke verteenwoordig; 'n "vinnige" komponent
et 'n $t_{\frac{1}{2}}$ = 90 sekondes wat 'n transmembraanfluks van
a^{2+} verteenwoordig, en 'n derde, "stadige", komponent
et 'n $t_{\frac{1}{2}}$ = 7 - 9 minute wat 'n komplekse verskynsel
s en waarskynlik ekstravesikulêre Ca^{2+}-bindingsplekke
nsluit.

ie Ca^{2+}-deurlaatbaarheid van die SR membraan is bere=
en uit die tweede kinetiese flukskomponent en word op
,5 x 10^{-8} cm sek^{-1} gestel. Die Ca^{2+}-fluks *in vivo*
s bereken op 1,5 x 10^{-13} mol sek^{-1} cm^{-2}, wat 4 orde-
enhede stadiger is as wat nodig is om spierkontraksie
e inisieer. Hieruit kan dus afgelei word dat vry=
telling van Ca^{2+} deur die SR *in situ* met 'n

verandering in die membraandeurlaatbaarheid gepaard moet gaan.

Ca^{2+}-vrystelling deur die SR is in die aanwesigheid en afwesigheid van ATP nagegaan. Deur Ca^{2+}-opname in aanwesigheid van ATP en KCl te laat verloop en dan die ioonsamestelling van die medium te verander deur die byvoeging van klein hoeveelhede gekonsentreerde katione, is vinnige Ca^{2+}-vrystelling waargeneem. Die effek op Ca^{2+}-vrystelling deur katione was as volg: polilisien > $Tris^{+}$ > $(CH_3)_4N^{+}$ > K^{+} met Cl^{-} as anioon. Die verskil in grootte van die katioon en anioon is baie belangrik deurdat die verskil in diffusiesnelheid 'n oneweredige verspreiding van ladings oor die membraan bewerkstellig. Dit het waarskynlik 'n verandering in deurlaatbaarheid tot gevolg. Dieselfde patroon word met passiewe Ca^{2+}-effluks verkry.

Ca^{2+}- gestimuleerde Ca^{2+}-vrystelling is ook verkry by geïsoleerde SR vesikels gelaai met Ca^{2+} in die teen= woordigheid van ATP. Die Ca^{2+}-vrystelling word opge= hef in teenwoordigheid van 'n hoë konsentrasie vrye Mg^{2+} en speel waarskynlik nie 'n belangrike fisiolo= giese rol onder normale omstandighede in die skelet= spier nie.

:TERATURE

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DDENDUM I

BBREVIATIONS

DP	:	Adenosine diphosphate
TP	:	Adenosine triphosphate
TPase	:	Adenosine triphosphatase
ISACRYLAMIDE	:	N, N'-methylenebisacrylamide
TT	:	1,4-Dithiothreitol
DTA	:	Ethylenediaminetetra-acetic acid
GTA	:	Ethyleneglycol-bis-(β-amino-ethylether) N,N'-tetra-acetic acid
MS	:	Potassiummethanesulfonate
OPS	:	Morpholinopropane sulfonic acid
i	:	Inorganic phosphate
.O.P.O.P.	:	1,4-bis- 2-(4-methyl-5-phenyl=oxazolyl) -benzene
.P.O.	:	2,5-Diphenyloxazole
DS	:	Sodium dodecyl sulfate
R	:	Sarcoplasmic reticulum
CA	:	Trichloroacetic acid
EMED	:	N, N, N', N'-tetramethylenediamine
MAC	:	Tetramethylammoniumchloride
RIS	:	2-amino-2-hydroxymethyl-1,3-propandiol

ADDENDUM II

DEFINITIONS

1. Ca^{2+} uptake: This term is used for the ATP dependent accumulation of Ca^{2+} by isolated sarcoplasmic reticulum vesicles in the absence of oxalate or other Ca^{2+}-precipitating agents.

2. Ca^{2+} loading: ATP-dependent Ca^{2+} accumulation in the presence of oxalate or other Ca^{2+}-precipitating agents.

3. Passive fluxes: The movement of Ca^{2+} ions across the membrane of the sarcoplasmic reticulum in the absence of ATP or other high energy substances

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