MECHANISM OF CALCIUM UPTAKE AND BINDING OF SARCOPLASMIC RETICULUM AND MITOCHONDRIA

by

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Introduction

It is now clearly established that calcium plays a pivotal role in controlling a number of cellular processes. In muscle, calcium is the trigger which causes the myofibrils to contract and develop force. The sarcoplasmic reticulum, an intricate and interconnected intracellular membrane system, is believed to maintain the cytoplasmic calcium concentration at 2 x 10^{-7}M during rest and release calcium (up to 10^{-5}M) in response to a signal from the T-tubules (Endo, 1977). The calcium diffuses to the myofibrils, binds to troponin on the thin filament and thereby allows myosin and actin to interact. The sarcoplasmic reticulum then rebinds the calcium until a new contraction cycle is initiated.

The mechanism by which the sarcoplasmic reticulum binds and releases calcium has been the subject of intense study in recent years. The relaxing factor (Marsh, 1952) found in muscle homogenates was later identified as fragments of the sarcoplasmic reticulum (Hasselbach and Makinose, 1961; Ebashi and Lipmann, 1962). These fragments were shown to be capable of binding calcium in an ATP dependent transport process. A model describing the steps of this process has gradually evolved and some of the key features of this model will be described below.

Although the sarcoplasmic reticulum is believed to be the primary modulator of the intracellular calcium level in white skeletal muscle, the mitochondria may also play some role, particularly in red skeletal and cardiac muscle. It has been known for some time that mitochondria from a variety of tissues have the capacity to accumulate a large amount of calcium in their matrices (Vasington and Murphy, 1962; DeLuca and Engstrom, 1961). The importance of this process in muscle remains unanswered. However, the decreasing content and specific activity of the sarcoplasmic reticulum of white vs. red vs. cardiac muscle and the parallel increase in mitochondrial content of these tissues suggests that the mitochondria may be involved in controlling calcium movement in red and cardiac muscle.

The purpose of this report is to describe the mechanisms of calcium binding and release by sarcoplasmic reticulum and mitochondria. The literature on this subject is extensive, and no attempt will be made to cover all aspects. Further details may be obtained from several recent reviews (Carafoli, 1973; Carafoli, 1974; MacLennan, 1975; MacLennan and Holland, 1975; Carafoli and Crompton, 1976; Inesi and Malan, 1976; Hasselbach, 1977; Bygrave, 1977; Endo, 1977; Korenbrot, 1977).

Mechanism of Calcium Uptake by the Sarcoplasmic Reticulum

Fragments of the sarcoplasmic reticulum can be obtained from muscle homogenates by differential centrifugation. These membranes, composed of 60% protein and 40% phospholipid, spontaneously form closed vesicles after the homogenization. The protein composition of the sarcoplasmic reticulum is well defined (Figure 1). The ATPase, comprising 60 or 70% of the total protein, contains two high affinity calcium binding sites and is embedded in the membrane. Calsequestrin contains a large number of calcium binding sites, but they are of low affinity. The high affinity calcium binding protein has one calcium binding site. Both of these latter proteins are believed to be either bound to the inner surface of the membrane or in the vesicles' internal space. In addition, a low molecular weight proteolipid is found embedded in the sarcoplasmic reticulum membrane, but it does not appear to contain any calcium binding sites. These four proteins account for more than 90% of the total sarcoplasmic reticulum protein.

PROTEIN	SIZE	CALCIUM BINDING SITES	LOCATION
ATPASE	102,000	2/MOLE, K_D = <1 μM	MEMBRANE
CALSEQUESTRIN	44,000	43/MOLE, K_D = 800 μM	INTERNAL?
HIGH AFFINITY CA^{++} BINDING PROTEIN	56,000	1/MOLE, K_D = 3 μM	INTERNAL?
PROTEOLIPID	6,000–12,000	NONE	MEMBRANE

FIGURE 1

Proteins of the sarcoplasmic reticulum.

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Although binding of calcium by these proteins is important, the critical process occurring in the sarcoplasmic reticulum is active transport or uptake. Calcium is moved from outside the vesicles across the membrane and deposited on the inside (Figure 2). This movement requires ATP and magnesium and is catalyzed by the ATPase protein. The movement of calcium is tightly coupled to ATP hydrolysis, with 2 calcium ions being transported for each ATP split (Hasselbach, 1964). The critical importance of the ATPase protein in this process has been demonstrated by the reconstitution of calcium transport from a mixture of the purified ATPase with phospholipids (Racker, 1972; Warren *et al.*, 1974).

The amount of calcium accumulated depends on the conditions used for measurement. When sufficient calcium is present in the incubation medium, approximately 0.1 to 0.15 μmoles are bound per mg of sarcoplasmic reticulum protein. The inclusion of oxalate stimulates uptake to the 2 to 5 μmoles/mg level due to formation of insoluble calcium oxalate precipitates inside the vesicles. Uptake rates as high as 10-12 μmoles per mg have been obtained with some purified fractions of sarcoplasmic reticulum vesicles (Meissner, 1975).

The ATP hydrolysis accompanying transport was shown to occur in at least two steps with the demonstration that a phosphorylated intermediate could be formed (Yamamoto and Tonomura, 1967). In the first step the terminal phosphate from ATP is attached to the ATPase protein (Figure 3). This bond is stable in acid. The phosphate is attached to the β carboxyl group of an aspartic acid residue at the active site (Degani and Boyer, 1973), and a 31 residue tryptic peptide containing this phosphorylated aspartate has been isolated and partially sequenced (Allen and Green, 1976). In the second step the phosphate is released from the ATPase. It has been shown that the rate of phosphorylation of the ATPase is very rapid while the overall rate of ATP hydrolysis is slow (Coffey *et al.*, 1975). This has been interpreted to mean that the splitting of the phosphorylated intermediate is slow and rate limiting. Froehlich and Taylor (1975) and Kurzmack and Inesi (1977) used rapid quenching techniques and found evidence for two phosphate containing intermediate steps (Figure 4). Their work showed that there was a rapid burst of phosphate produced soon after mixing ATP with sarcoplasmic reticulum fragments. They proposed a conversion of the phosphorylated ATPase from an acid stable to an acid labile form. Mixing acid with the latter form would liberate the phosphate and give rise to the rapid burst. The physiological rate limiting step of the ATPase reaction would be the conversion of the acid labile phosphorylated intermediate to the free enzyme plus inorganic phosphate.

The overall coupling of the ATP hydrolysis to calcium transport is shown in Figure 5. The two lines represent the boundary of the membrane and outside refers to the side facing the cytoplasm in the cell. Two calcium ions and one Mg ATP combine with the ATPase enzyme (E) near the outer surface (Steps 1 and 2). The order of addition is not obligatory, with Mg ATP capable of binding to the calcium free enzyme. The binding of either calcium or Mg ATP does not affect the affinity for the other (Yates and Duance, 1976). The magnesium is apparently bound at the same time as the ATP since the rate of formation of the phosphorylated intermediate is magnesium dependent. The magnesium and ADP are then released at the outer surface (Step 3). The ATPase changes in shape to allow the calcium binding site to move near the inner surface of the membrane (Step 4). Calcium is released on the inner side and the ATPase remains phosphorylated. The enzyme then goes through a conformational change, allowing it to increase its affinity for magnesium (Step 6). A magnesium ion is bound at the inner surface (Step 7) and the enzyme–Mg–phosphate complex moves its active site to the outside (Step 8). The phosphate and magnesium ion are released (Step 9), and this release requires the participation of phospholipid (Martonosi *et al.*, 1971, 1974). The enzyme finally

$$2\ Ca^{2+}_{OUT} + 1ATP \xrightarrow[Mg^{2+}]{MG\text{-}CA\text{-}ATPASE} 2\ Ca^{2+}_{IN} + ADP + P_I$$

FIGURE 2

Calcium transport by sarcoplasmic reticulum.

$$MG\text{-}CA\text{-}ATPASE + ATP \rightleftharpoons MG\text{-}CA\text{-}ATPASE\sim P + ADP \quad (RAPID)$$

$$MG\text{-}CA\text{-}ATPASE\sim P + H_2O \rightleftharpoons MG\text{-}CA\text{-}ATPASE + P_I \quad (SLOW)$$

PHOSPHORYLATION RATE	300 S^{-1}
OVERALL HYDROLYSIS RATE	4 S^{-1}

FIGURE 3

ATP hydrolysis by sarcoplasmic reticulum.

$$E + ATP \rightleftharpoons E \cdot ATP \overset{ADP}{\rightleftharpoons} \underset{(ACID\ STABLE)}{E\sim P} \rightleftharpoons \underset{(ACID\ LABILE)}{E \cdot P} \overset{RATE\ LIMITING}{\rightleftharpoons} E + P_I$$

FIGURE 4

ATP hydrolysis by sarcoplasmic reticulum.

undergoes a change whereby it has a high affinity for calcium and the cycle can be repeated (Step 10). The whole cycle results in a net movement of 2 Ca^{++} inside and 1 Mg^{++} outside. It has been proposed that there may also be a movement of 2 K^{+} outside to achieve a charge balance.

All the steps in this scheme (Figure 5) are shown to be reversible. The reversibility has been demonstrated by the catalysis of an ATP-ADP exchange (Steps 2 and 3), a phosphate-$H^{18}OH$ exchange (Step 9), and ATP formation when calcium loaded vesicles were incubated with phosphate and ADP (Steps 9 through 2) (Panet and Selinger, 1972).

Mechanisms of Calcium Release from the Sarcoplasmic Reticulum

The means by which calcium is released from the sarcoplasmic reticulum to activate contraction is not understood at present. A number of possibilities have been proposed (Figure 6) and their merits discussed (Inesi and Malan, 1976). An obvious suggestion is that calcium might be released by a reversal of the transport process. The movement of calcium outside from loaded vesicles in the presence of ADP and phosphate occurs at a rate similar to that of calcium uptake. Unfortunately, this rate is about one tenth that observed for physiological calcium release so it is much too slow (Katz *et al.*, 1977). Also the ADP level does not vary that much in a living cell to allow a significant release.

A second mechanism which has been proposed is an ionophore might be involved. Ionophores are compounds which can embed themselves in membranes to facilitate the passage of ions from one side of the membrane to the other. Ionophores isolated from bacterial sources do indeed cause loaded sarcoplasmic

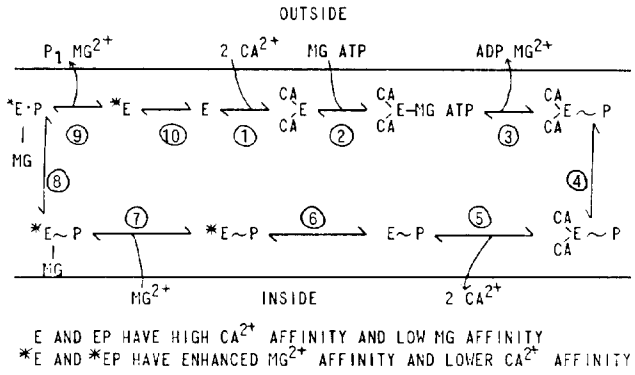

FIGURE 5

Mechanism of Ca++ transport by sarcoplasmic reticulum. Redrawn from MacLennan and Holland (1975).

1. PUMP REVERSAL
2. IONOPHORE RELEASE
3. CA^{2+} TRIGGERED RELEASE
4. DEPOLARIZATION OF SARCOPLASMIC RETICULUM
5. ATPASE MONOMER–OLIGOMER TRANSFORMATION

FIGURE 6

Possible mechanisms of calcium release from the sarcoplasmic reticulum.

reticulum vesicles to release calcium but no such ionophore has been shown to occur in muscle.

A third idea proposed involves a calcium triggered release of calcium. Experiments using skinned muscle fibers have demonstrated that the application of a calcium containing solution on the fiber causes a large release of calcium from the sarcoplasmic reticulum. The idea has been developed that calicum in the T tubules might move across these membranes and trigger the release of calcium from the sarcoplasmic reticulum. The problem with this model is that rather high concentrations of calcium are necessary to trigger the release, and, it appears unlikely that the T tubules or other extracellular sites contain sufficient calcium for this to occur.

A fourth suggestion is the sarcoplasmic reticulum undergoes a depolarization much like the sarcolemma does during the action potential. To date there has been no evidence that there is any membrane potential across the sarcoplasmic reticulum, so this idea only remains a hypothesis at present.

A final mechanism has recently been proposed to be involved in calcium release. Structural studies on the sarcoplasmic reticulum have shown 35 Å surface particles on the membrane and 90 Å particles visible when the membrane is fractured between the two phospholipid layers. The number of 35 Å particles at the surface agrees reasonably well with the number of ATPase molecules in the membrane (Scales and Inesi, 1976). However, the number of 90 Å particles is only one-third or one-fourth the number of ATPases and it has been suggested that these particles may be ATPase trimers or tetramers. Further evidence for this idea was obtained by Vanderkooi and coworkers (1977). They labeled two different preparations of the ATPase with fluorescent labels, mixed the proteins in an artificial membrane, and observed the energy transfer between the different molecules. Their results indicated the adjacent ATPase molecules were very close and diluting the membrane with additional lipid had no effect. This would be consistent

with the idea the ATPase molecules existed as oligomers. Dilution of the labeled membranes with unlabeled ATPase abolished the energy transfer. Vanderkooi and coworkers proposed the ATPase molecules were in a dynamic equilibrium between monomer and tetramer states and one of these states would be conducive to calcium uptake while the other might facilitate calcium release. While this is an attractive model, further work will be necessary to demonstrate whether this type of process actually occurs.

Mechanisms of Calcium Uptake By Mitochondria

The understanding of the means by which mitochondria accumulate calcium is complicated by the fact these organelles perform many biological functions (ATP synthesis, fatty acid oxidation, etc.) in addition to calcium binding and they contain a double membrane. The mitochondrion is surrounded by an outer membrane and contains an inner membrane which is extensively folded into what are known as crista. The space between the inner and outer membrane is referred to as the peripheral space while the space which the inner membrane surrounds is referred to as the matrix.

Calcium is accumulated by mitochondria using either ATP or electron transport for energization (Figure 7). Inhibitors of either ATP formation or electron transport alone will not block calcium uptake if the other system is operative. Mitochondria are capable of lowering the calcium concentration of the medium to the micromolar range and can successfully compete with troponin for calcium (Carafoli *et al.*, 1975).

The level of mitochondrial calcium loading depends on the conditions of incubation (Figure 8). In the absence of permeant anions such as phosphate or acetate, approximately 80 μmoles of calcium are taken up per mg of protein (Lehninger, 1970). Permeant anions stimulate uptake and move across the mitochondrial membranes, resulting in a several fold higher binding capacity. If ATP or ADP is included along with a permeant anion, the mitochondria become "massively loaded" with levels approaching 3 μmoles/mg (Figure 8). This calcium accumulating ability is similar to that of the sarcoplasmic reticulum as mentioned above.

Studies have been conducted to determine the location of the calcium binding sites in the mitochondria (Figure 9). The outer membrane contains 50-60 sites per mg of protein with a dissociation constant of 100-150 μ Molar (Carafoli and Gazzotti, 1973). The inner membrane has a similar number of sites and with a dissociation constant of 50 to 80 μ Molar. The inner membrane also contains 4-7 sites with a high calcium affinity. These latter sites are believed to somehow be related to the transport of calcium. It has also been shown the inner membrane alone is capable of accumulating calcium (Pedersen and Coty, 1972) and thus the transport protein must be associated with this membrane. In addition, some soluble proteins may be in the matrix or peripheral space which have calcium binding properties, and one such protein has been identified (Sottocasa *et al.*, 1972).

Evidence that a calcium carrier exists in the inner mitochondrial membrane is based on several observations (Bygraves, 1977). First, the rate of calcium uptake is saturable, i.e., the rate of uptake reaches a maximum level as calcium concentration in the medium is increased. Second, the transport mechanism shows ion specificity with only calcium, strontium, and manganese ions being translocated at significant rates. Third, the transport process can be specifically inhib-

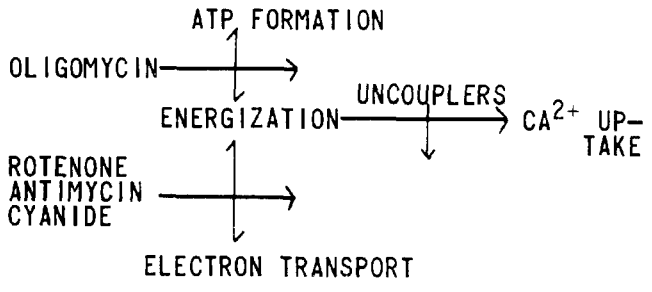

FIGURE 7

Calcium transport and energy transformations in mitochondria. Redrawn from Carafoli (1974).

PERMEANT ANION	ION MOVEMENT	CAPACITY (N MOLES/MG PROTEIN)
NONE	H^+ EJECTION	80
PHOSPHATE OR ACETATE	ANION ENTRY	200–300
PHOSPHATE + ATP(ADP)	ANION ENTRY	3000

FIGURE 8

Mitochondrial calcium loading under different conditions. Redrawn from Lehninger (1970).

	NUMBER OF CA^{++} SITES	K_D
OUTER MEMBRANE	50–60/MG PROTEIN	100–150 μM
INNER MEMBRANE	50–60/MG PROTEIN 4–7/MG PROTEIN	50–80 μM 1 μM

FIGURE 9

Calcium binding sites in mitochondria. Data from Carafoli and Gazzotti (1973).

ited by La^{+3} or ruthenium red, the former competitively and the latter noncompetitively. Studies with these inhibitors (Reed and Bygraves, 1974), have demonstrated the extremely low concentration of calcium transport protein in mitochondria, of the order of picomoles per mg protein (Figure 10). This concentration is about a thousand fold lower than the concentration of the ATPase protein in the sarcoplasmic reticulum. Because of this extremely low mitochondrial carrier concentration, it is not surprising difficulties have been encountered in its isolation. The specific inhibition of calcium transport by antibodies directed against a calcium binding glycoprotein suggests it may be the carrier or directly associated with it (Panfili *et al.*, 1976).

The actual mechanism of transport remains controversial. The proton gradient which develops as a result of electron transport (Mitchell, 1968) has been proposed to be involved in calcium transport. Moyle and Mitchell (1977) have shown one H^+ is ejected for each one Ca^{++} that is moved inside. They believe the movement of 2 Ca^{++} inward is accompanied by one phosphate in the same direction. Bygrave (1977) has proposed the driving force for calcium transport is due to the electrical component resulting from the proton gradient. The membrane potential between the interior and exterior of the mitochondrian is 100-200 mv, with the interior being negative (Rottenberg, 1975). Thus the positively charged calcium ions would be electrophoretically attracted to the negatively charged interior. Lehninger (1974) has suggested the lack of protons inside the mitochondria draws permeable anions (such as phosphate) inward which can donate protons to the matrix. This negative inside anion gradient then draws calcium inwardly. His mechanism suggests there are carriers for both the anions and the calcium. Puskin and coworkers (1976) have presented evidence to suggest there are two types of calcium carriers, one for moving calcium ions inside and another for extruding calcium to the outside. These studies demonstrate no consensus has been reached concerning the model for mitochondrial calcium ion accumulation.

LOCATION	INNER MEMBRANE
CARRIER CONCENTRATION	SEVERAL P MOLES/MG PROTEIN
AFFINITY	2–4μM
SPECIFICITY	$CA^{2+} > SR^{2+} > MN^{2+}$
ENERGY SOURCE	MEMBRANE POTENTIAL OR ATP
INHIBITORS	LA^{3+} (COMPETITIVE) RUTHENIUM RED (NONCOMPETITIVE)

FIGURE 10

Properties of the mitochondrial calcium transport system.

Mechanisms of Calcium Release from Mitochondria

The signals for calcium release from mitochondria are not known. Two different ideas have emerged. Some recent work by Crompton and coworkers (1976) showed small concentrations of sodium ions (less than 20 mM) stimulated calcium efflux from mitochondria. They proposed two calcium carriers exist with one that moves calcium inward and the other which couples sodium influx with calcium efflux. The sodium would move into the cell cytoplasm during the depolarization of the plasmalemma from extracellular locations.

A second mechanism for calcium release is caused by anaerobic conditions. Thomas and coworkers (1969) demonstrated calcium loaded mitochondria discharged calcium when held in the absence of oxygen. In their system ATP was also absent, so that the mitochondria would not be maintained in an energized state (Figure 7). Since ATP levels do not oscillate widely in the cell, it appears unlikely this release mechanism would be operative under physiological conditions.

A serious drawback in the understanding of the relationship between calcium movements in skeletal muscle and the role of mitochondria in these movements is the fact most of the experimental work has been conducted with liver, kidney or heart mitochondria. Jacobus and coworkers (1975) have demonstrated major differences exist between liver and heart mitochondria. They found calcium uptake was much more rapid in liver than heart mitochondria and liver mitochondria transported calcium in preference to synthesizing ATP while the reverse was true with heart mitochondria. Thus care must be exercised in extrapolating the mechanisms and properties of mitochondria from other tissues to those in skeletal muscle.

Conclusions

Both the sarcoplasmic reticulum and mitochondria have active transport systems capable of removing calcium from the cytoplasm of the muscle cell. The mechanism for calcium uptake is well understood for the sarcoplasmic reticulum but few details are known concerning mitochondrial calcium accumulation. ATP provides the energy for transport in the sarcoplasmic reticulum while either respiration or ATP furnish the driving force for mitochondrial calcium uptake. The concentration of the calcium carrier protein in mitochondria is extremely low and in contrast to the sarcoplasmic reticulum where the transport ATPase constitutes the majority of the total protein. In both the sarcoplasmic reticulum and mitochondria the physi-

ological release mechanisms are only conjectures at present.

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