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CHARACTERIZATION OF CALMODULIN EFFECTS ON CALCIUM
TRANSPORT IN CARDIAC MICROSOMES ENRICHED IN
SARCOPLASMIC RETICULUM

by

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ABSTRACT

Calmodulin prepared from red cell hemolysate stimulates Ca^{2+} -transport in cardiac microsomal preparations enriched in sarcoplasmic reticulum (S.R.) in a concentration-dependent manner (Katz and Remtulla, 1978). The present study was performed to characterize this calmodulin regulation of Ca^{2+} -transport. K^+ and Na^+ have also been found to enhance Ca^{2+} -transport in microsomal preparations enriched in S.R. (Jones et al., 1978). It was observed that in the presence of K^+ (110 mM) and Na^+ (110 mM) stimulation of Ca^{2+} -transport activity by calmodulin was greatly reduced. This result was obtained at all free Ca^{2+} concentrations studied. That this was not an ionic effect was indicated by the decreased antagonism of calmodulin stimulation by Li^+ (110 mM).

Kinetic characterization determined that calmodulin significantly enhanced the maximal Ca^{2+} activation without affecting the apparent Ca^{2+} affinity of the Ca^{2+} -transport process in cardiac S.R.. K^+ was found to enhance the $V_{\text{Ca}^{2+}}$, as well as lowering the apparent Ca^{2+} affinity. Examination of the initial rate of Ca^{2+} -transport in cardiac S.R. confirmed that calmodulin stimulation is due mainly to an increase in the $V_{\text{Ca}^{2+}}$. CyclicAMP-dependent protein kinase, on the other hand, has been shown to increase the $V_{\text{Ca}^{2+}}$, as well as decrease the apparent K_m for Ca^{2+} (Hicks et al., 1979), suggesting a different mechanism of action.

Experiments were performed to investigate whether calmodulin was indigenous to the preparations used. Attempts were therefore made to isolate calmodulin from dog cardiac microsomal preparations enriched in S.R. by methodology used to isolate calmodulin from other sources (Jung, 1978; Depaoli-Roach et al., 1979). These extracts were then tested to determine their ability to stimulate Ca^{2+} -transport into S.R.. It was observed that neither boiling nor treatment with 0.6mM EGTA could extract calmodulin from these preparations. This result indicates that the microsomal preparations utilized do not contain indigenous calmodulin.

Since calmodulin does not appear to be a component of the S.R., it was postulated that binding to sites on the membrane must occur in order for calmodulin to augment Ca^{2+} -transport. It was also suggested

that K^+ and Na^+ may decrease calmodulin stimulation of Ca^{2+} -transport by altering this binding. Studies were therefore performed using ^{125}I -labelled calmodulin to determine the degree of binding to microsomal preparations in the presence and absence of K^+ (110mM), Na^+ (110mM), and Li^+ (110mM). It was found that ^{125}I -calmodulin binds to microsomal cardiac S.R. in a Ca^{2+} concentration-dependent manner. At Ca^{2+} concentrations above $10^{-7}M$, this binding was significantly decreased ($p < 0.05$, students "t" test) in the presence of K^+ or Na^+ . Li^+ , previously shown not to alter Ca^{2+} -transport augmentation by calmodulin, did not alter calmodulin binding to a significant extent. K^+ and Na^+ therefore may inhibit calmodulin stimulation of Ca^{2+} -transport in these preparations by decreasing calmodulin binding to the S.R.. The lack of inhibition by Li^+ (110mM) indicates that this result is not due to a non-specific ionic effect.

Our studies therefore have shown that calmodulin is not an indigenous protein of the sarcoplasmic reticulum preparations used. Calmodulin, though, has been found to bind, in a Ca^{2+} -dependent manner, to these preparations. This binding is altered by monovalent cations previously shown to inhibit calmodulin stimulation of Ca^{2+} -transport.

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LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
$^{\circ}\text{C}$	degree centigrade
$(\text{Ca}^{2+}-\text{Mg}^{2+})\text{-ATPase}$	Mg^{2+} -dependent calcium stimulated ATPase
cAMP	cyclic adenosine 5'-monophosphate
Ci	Curie
cpm	counts per minute
E	enzyme
EDTA	ethylene diamine tetraacetate, disodium salt
EGTA	ethyleneglycol-bis-(β -aminoethyl ether) N,N'-tetra-acetic acid
EP	phosphorylated enzyme intermediate
<u>et al</u>	and others
g	gram
$\times g$	acceleration of gravity
IO	inside-out
K_{diss}	dissociation constant
K_{m}	Michaelis-Menten constant
m	milli
μ	micro
M	molar
mg	milligram
min	minute
ml	milliliter
μl	microliter
mM	millimolar concentration
μM	micromolar concentration
MW	molecular weight
NAD^{+}	nicotinamide adenine dinucleotide
nmoles	nanomoles
P_i	inorganic phosphate
pmoles	picomoles
RBC	red blood cell
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

S.E.M.	standard error of the mean
S.R.	sarcoplasmic reticulum
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl aminomethane)
$V_{Ca^{2+}}$	maximum velocity of Ca^{2+} -transport
V_{max}	maximum velocity of enzyme reaction
%	percent
/	per

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INTRODUCTION

Cardiac sarcoplasmic reticulum plays an integral part in heart function. Calcium released from this intracellular organelle is involved in muscle fibril contraction, while reuptake of calcium by the sarcoplasmic reticulum is believed to mediate the subsequent relaxation of the muscle (Ebashi et al, 1969). Reaccumulation of calcium within the sarcoplasmic reticulum is an active process involving a Mg^{2+} - Ca^{2+} -adenosine triphosphatase (ATPase) enzyme, which is embedded in the sarcoplasmic reticulum membrane (Hasselbach and Makinose, 1961). In recent years various regulatory mechanisms controlling the uptake process have been identified. A cAMP-dependent protein kinase appears to modulate calcium transport by phosphorylation of a low MW sarcoplasmic reticulum membrane protein, phospholamban (Kirchberger et al, 1978). Monovalent cations such as K^{+} and Na^{+} also effect the transport mechanism, probably by increasing the rate of dephosphorylation of the Mg^{2+} - Ca^{2+} -ATPase enzyme (Shigekawa and Pearl, 1976; Jones et al, 1978).

Recently a calcium-dependent regulatory protein, termed calmodulin, has been shown to modulate the activity of a number of enzymes including erythrocyte membrane Mg^{2+} - Ca^{2+} -ATPase activity (Gopinath and Vincenzi, 1977). Subsequently it was shown that calmodulin enhanced Ca^{2+} -uptake into inside out vesicles prepared from red cell membranes (Sarkadi et al, 1979). In view of this it was decided to investigate the role of calmodulin in the regulation of calcium transport in cardiac sarcoplasmic reticulum (S.R.). The interaction of calmodulin with various mediators of calcium uptake was also investigated. Calcium uptake was measured in a microsomal preparation enriched in sarcoplasmic reticulum obtained from canine cardiac ventricle muscle.

BACKGROUND

I. Calmodulin

It is now becoming apparent that a calcium binding protein, termed calmodulin (Cheung et al, 1978), acts as a mediator in many cellular Ca^{2+} -regulated events. The presence of this activator protein in animal tissue was first demonstrated by Cheung in 1967. It was shown that a heat stable, nondialyzable factor from bovine brain homogenate was necessary to maintain cyclic nucleotide phosphodiesterase activity during

purification of the enzyme from crude extract. Using a cyclic nucleotide phosphodiesterase preparation free of activator Kakiuchi et al (1970) showed that the enzymes catalytic activity had an absolute requirement for both Ca^{2+} and the activator protein fraction. Further purification of the activator fraction showed that an acidic protein (MW approximately 18,000) which forms a complex with Ca^{2+} was the true activator of phosphodiesterase (Liu et al, 1974; Teo and Wang, 1973).

Independently, Bond and Clough (1973) demonstrated that hemolysate from human erythrocytes, if added to erythrocyte membrane preparations could stimulate Mg^{2+} - Ca^{2+} -ATPase activity. The stimulatory activity was found to be due to an activator protein found predominantly in the cytosol of erythrocytes. The activator was purified to homogeneity, and shown to be a calcium binding protein of MW approximately 18,000 (Jarrett and Penniston, 1977; Jung, 1978; Luthra et al, 1976). A comparison was made of the ability of the erythrocyte activator and the phosphodiesterase activator to stimulate erythrocyte Mg^{2+} - Ca^{2+} -ATPase (Gopinath and Vincenzi, 1977; Jarrett and Penniston, 1977). It was found that the properties of the two activators were similar, suggesting that the two were the same protein. It has subsequently been shown that calmodulin is ubiquitous in the animal kingdom, and recently has been isolated from plants (Jarrett et al, 1980).

a) Properties of Calmodulin:

Estimates of the molecular weight of calmodulin have varied between 15,000 and 19,000 depending on the methodology and conditions employed (Watterson et al, 1976; Lui et al, 1974; Vanaman et al, 1977; Dedman et al, 1977). One reason for the variation is that SDS-polyacrylamide gel electrophoresis and sedimentation equilibrium MW determinations can be altered by changing EGTA or Ca^{2+} concentrations. As the Ca^{2+} concentration increases the density of the calmodulin increases due to conformational changes in the molecule due to Ca^{2+} -binding (Lui and Cheung, 1976; Wolff et al, 1977). Recently, Vanaman et al (1977) have worked out the amino acid sequence of bovine brain calmodulin, arriving at a MW of 16,723. The sequence as shown in figure 1, contains 148 residues with 27 glutamate and 23 aspartate residues accounting for

Figure 1:

Amino Acid Sequence of Calmodulin from Bovine Brain, as Determined by Vanaman et al (1977).

Ac(ala, asx) GLN LEU THR GLU GLU GLN ILE ALA GLU PHE LYS glu ALA PHE
 SER LEU PHE ASP lys ASP GLY THR ILE THR THR LYS GLU LEU GLY THR VAL MET
 ARG ser LEU GLY GLN ASN PRO THR glu ala GLU LEU GLU ASX MET ILE ASN GLU
 VAL ASP ala ASP GLY ASX GLY THR ILE ASP PHE pro GLU PHE LEU thr MET MET
 ALA ARG lys MET LYS ASP thr asp SER GLU GLU GLU ILE arg GLU ala PHE ARG
 VAL PHW ARG VAL PHE ASP LYS ASP GLY ASN GLY TYR ILE SER ALA ala GLU LEU
 ARG his VAL MET thr asx leu GLY GLU tml LEU THR ASP GLU GLU VAL ASP GLU
 MET ILE ARG GLU ALA ASN ile ASP gly ASP GLY glx VAL ASX TYR GLX GLX PHE
 VAL GLN MET MET thr ALA lys COOH

the acidic nature of calmodulin. The molecule can be divided into four symmetrical homologous domains, each containing a Ca^{2+} -binding site (Vanaman et al, 1977; Kretsinger, 1979). Each site contains a hydrophobic core, with six oxygen containing amino acid side chains acting as the calcium binding ligands. The binding sites appear identical to the Ca^{2+} -binding sites of the calcium binding proteins troponin C and parvalbumin (Vanaman, 1980) and a common evolutionary origin has been suggested (Kretsinger, 1980; Barker et al, 1977; Wang et al, 1975).

The regulation of various enzymatic functions by calmodulin requires the presence of Ca^{2+} and has been described by the following non-stoichiometric sequence.

When Ca^{2+} binds to calmodulin the helical content of the molecule increases, thereby producing a conformational change (Wolff and Brostrom, 1979). It is thought that this change allows calmodulin to bind to the enzyme, resulting in activation. The Ca^{2+} -dependent binding of calmodulin

to the enzyme is a reversible process dependent of the concentration of Ca^{2+} in the medium. In some enzyme systems, such as phosphorylase kinase (Cohen, 1978) and cyclic nucleotide phosphodiesterase (Cheung, 1970; Wang, 1980) calmodulin is actually a subunit of the enzyme. In this instance, Ca^{2+} binding to calmodulin present in the enzyme complex would result in activation.

Recently, Klee and Haiech (1980) have suggested that binding of Ca^{2+} to calmodulin is dependent on previously bound Ca^{2+} , and have suggested the following stoichiometric equation:

Since Cheung first demonstrated that calmodulin mediates Ca^{2+} -regulated phosphodiesterase activation many researchers have looked at its involvement in other Ca^{2+} -mediated events. It is now known that calmodulin has widespread actions which usually involve activation of an enzyme system controlling some cellular function. Listed in table 1 are most of the cellular functions and enzymes which have been shown to date to involve calmodulin regulation.

II. Calcium Transport in Sarcoplasmic Reticulum

The central role of Ca^{2+} as an inducer of the contractile response in skeletal muscle was first suggested by Heilbrunn and Wiercinski (1947). This was later confirmed by other researchers, who demonstrated the ability of Ca^{2+} to control myofibril contractility (Weber and Herz, 1961; Bozler, 1954) and identified a calcium binding protein, troponin, in the thin filaments of the myofibrils (Ebashi and Endo, 1968).

In 1951 it was shown by Marsh that an aqueous extract of skeletal muscle could also alter contractility of myofibrillar bundles. This extract was subsequently shown to consist of microsomal vesicles,

Table 1:

Regulatory Functions of Calmodulini) Specific Enzymes

Phosphodiesterase

Adenylate Cyclase

Ca²⁺-Mg²⁺-ATPase

Phosphorylase Kinase

Phospholipase A₂NAD⁺ Kinase

Myosin Light Chain Kinase

a) Skeletal Muscle

b) Smooth Muscle

c) Platelet

Tryptophan 5'-monooxygenase

References

Cheung (1970)

Brostrom et al (1975)

Gopinath and Vincenzi (1977)

Jarrett and Penniston (1977)

Cohen (1978)

Wong and Cheung (1979)

Anderson and Cormier (1978)

Yagi et al (1978)Dabrowski et al (1975)Adelstein et al (1975)

Yamauchi and Fujisawa (1979)

ii) Cellular Functions

Microtubule assembly and disassembly

Welsh et al (1978)Ca²⁺-transport

a) Sarcoplasmic Reticulum

b) Erythrocyte Membrane

c) Synaptic Membrane

Katz and Remtulla (1978)

Hinds et al (1978)Kuo et al (1979)

Neurotransmitter Release

Grab et al (1979)

Insulin Release

Sudgen et al (1979)

Protein Phosphorylation

Schulman and Greengard (1978)

Prostaglandin Metabolism

Wong and Cheung (1980)

Axoplasmic Transport

Iqbal and Ochs (1980)

Brady et al (1980)

presumably composed of sarcoplasmic reticulum membranes (Ebashi and Lipmann, 1962). The microsomal vesicles contain a Mg^{2+} -dependent ATPase activity and are capable of removing Ca^{2+} from medium, in the presence of ATP and Mg^{2+} (Hasselbach and Makinose, 1962; Ebashi and Lipmann, 1962). It was thus suggested that the sarcoplasmic reticulum alters myofibril contractility by changing cytoplasmic Ca^{2+} levels, either by actively accumulating cytoplasmic Ca^{2+} , or by releasing accumulated Ca^{2+} into the cytoplasm. Comparative studies performed by Fawcett (1961) indicated that cardiac muscle also contained sarcoplasmic reticulum, which is qualitatively similar to that of skeletal muscle. Harigaya and Schwartz (1969), as well as Repke and Katz (1972) have since confirmed that, although Ca^{2+} -transport in cardiac S.R. is slower than skeletal muscle, both transport systems are basically similar. Mg^{2+} - Ca^{2+} -ATPase activity in cardiac S.R. has a V_{max} for Ca^{2+} 3-6 times lower than skeletal muscle S.R. (Shigekawa et al., 1976). As well phosphoprotein levels that can be obtained in cardiac preparations are 4 times lower than fast skeletal S.R.. This indicates that cardiac S.R. has 4 times fewer active sites than skeletal muscle. Since the turnover rate of Mg^{2+} - Ca^{2+} -ATPase does not differ at saturating Ca^{2+} concentrations in skeletal or cardiac S.R. the slower Ca^{2+} -transport rate in cardiac S.R. must be due to a lower density of Ca^{2+} -pumping sites. Therefore it can be concluded that cardiac and skeletal muscle S.R. is qualitatively, but not quantitatively, similar.

a) Properties of Sarcoplasmic Reticulum:

Sarcoplasmic reticulum in skeletal and cardiac muscle is an intracellular membranous compartment in close association with the myofibrils (Porter and Palade, 1957). This membrane system performs two important functions; 1) transport of Ca^{2+} from the myofibrillar space to the interior of the organelle, thereby allowing muscle relaxation, and 2) release of accumulated Ca^{2+} , which results in muscle activation. A portion of the S.R. membrane, termed the junctional S.R., is located in close proximity to the transverse tubules (T-tubules) of the sarcolemma membrane. It has been postulated that this segment of the S.R. acts as a sensor, which detects T-tubule membrane depolarization (Franzini -

Armstrong, 1980). The mechanism by which Ca^{2+} is released from the S.R. in response to membrane depolarization has yet to be elucidated. Schneider and Chandler (1973) suggest that the charge movements occurring within the T-tubule membrane have a direct effect of producing an increase in the S.R. permeability to Ca^{2+} . Fabiato and Fabiato (1975) hypothesized the "trigger" Ca^{2+} mechanism, whereby low levels of Ca^{2+} which enter the cell during depolarization trigger a massive release of Ca^{2+} from the S.R.

The S.R. membrane itself is a lipid bilayer consisting primarily of phospholipid. It contains four main proteins, the 100,000 MW ATPase protein, a high affinity Ca^{2+} binding protein, calsequestrin, and low molecular weight proteolipids (MacLennan et al, 1974). The ATPase protein, comprising 90% of S.R. protein, is uniformly distributed along the surface of the membrane which is in closest contact with the myofibrils (Bray et al, 1978). It is embedded within the membrane (Deamer and Baskin, 1969) and appears aggregated into tetramers with other ATPase proteins (Hidalgo and Ikemoto, 1977). The low molecular weight proteolipids are also intrinsic proteins and are randomly distributed throughout the S.R. membrane. Calsequestrin and the high affinity Ca^{2+} -binding protein are extrinsic proteins, which are loosely associated with the inner surface of the membrane (Ikemoto et al, 1971). As of yet no discernable function has been identified for the proteolipids, calsequestrin, or the high affinity Ca^{2+} binding protein. It has been suggested that calsequestrin may act as a calcium storage site, but Repke et al (1976) present data which does not support this. In cardiac S.R. a 22,000 MW protein, recently termed phospholamban, which bands on SDS-PAGE in the region MacLennan identified as proteolipids, plays a role in the regulation of Ca^{2+} -uptake (Tada et al, 1975). This will be discussed in a later section.

b) Mg^{2+} - Ca^{2+} -ATPase:

Sarcoplasmic reticulum has been shown to contain a Ca^{2+} -dependent ATPase, as well as a Ca^{2+} -independent ATPase (Inesi et al, 1976). This discussion will concern only the Ca^{2+} -dependent ATPase activity.

It has been fairly well established that the Ca^{2+} -ATPase protein plays a central role in Ca^{2+} -transport in S.R.. Meissner and Fleisher

(1973), as well as Repke et al (1976) have shown that purified S.R. ATPase which has been reconstituted into membrane vesicles will actively transport calcium. The transport process is dependent on ATP and Mg^{2+} (Hasselbach, 1962) and it appears that a Mg .ATP complex acts as the substrate for the Ca^{2+} -ATPase activity (Yamamoto and Tonomura, 1967). Another absolute requirement for activity is calcium binding to specific sites on the S.R. ATPase. Ikemoto (1974) demonstrated the presence of both high and low affinity Ca^{2+} -binding sites, although only the high affinity sites appear to be specifically involved in ATPase activation (Meissner et al, 1973). Recently, Inesi et al (1979) have demonstrated a co-operative mechanism of Ca^{2+} -binding. They suggest that binding of Ca^{2+} to a first site causes a conformational change in the ATPase, resulting in the development of higher affinity at a second binding site. This co-operative mechanism of Ca^{2+} -binding and transport, shown in Table 2, is a modification of the reaction sequence first proposed by Kanazawa et al (1971).

Table 2

Mg^{2+} - Ca^{2+} -ATPase reaction sequence as proposed by Inesi et al (1979)

Inesi et al suggest that a first cytoplasmic Ca^{2+} ion rapidly binds to the enzyme, followed by high affinity ATP binding (1,2). This results in a slower protein transition, which forms a second high affinity Ca^{2+} -binding site (3). Upon binding of a second Ca^{2+} ion (4) the bound ATP hydrolyzes forming a high energy phosphorylated intermediate (5) (Hasselbach, 1964). Kanazawa et al (1970) provide evidence suggesting

that the formation of the phosphorylated intermediate is coupled to the translocation of Ca^{2+} across the membrane. Once this occurs a reduction in the binding affinity results, and Ca^{2+} dissociates from the enzyme (7,8) (Yamada and Tonomura, 1972). Yamada and Ikemoto (1979) suggest that dissociation of the second Ca^{2+} ion is the rate limiting step of the entire reaction sequence. The final step involving decomposition of the phosphorylated intermediate (12) is greatly accelerated in the presence of Mg^{2+} . Therefore Mg^{2+} has two roles in ATPase activation, i) to accelerate the decomposition rate of the phosphorylated enzyme, and ii) to act as the true substrate for the enzyme.

From the reaction scheme and earlier studies by Hasselbach (1964) and other workers it has been suggested that 2 moles of Ca^{2+} are transported per mole of ATP hydrolyzed. Studies by Suko (1973) suggest a ratio of 1:1. A fixed ratio has been questioned by Tate et al (1980) who provide evidence suggesting a flexible stoichiometry. Depending on factors, such as pH and temperature, the stoichiometric relationship between Ca^{2+} -transport and energy transduction may be a dynamic entity.

c) Regulation of Cardiac Sarcoplasmic Reticulum Calcium Transport:

Calcium transport by cardiac S.R. is regulated by a number of systems. These appear to act mainly on the Mg^{2+} - Ca^{2+} -ATPase enzyme, not only through its reaction mechanism, but also through a regulatory system associated with the enzyme.

One obvious regulator of Ca^{2+} -transport is the true substrate of the Mg^{2+} - Ca^{2+} -ATPase enzyme, $\text{Mg} \cdot \text{ATP}$. Kanazawa et al (1971) showed there is an increased formation of the phosphorylated intermediate with increasing ATP concentration. They suggest that this is not due to an increased rate of ATP binding, but rather to an accelerated transition of the enzyme-ATP complex to a second complex, which can rapidly convert to the phosphorylated intermediate. The presence of Mg^{2+} is required for this phosphorylation to occur. As well, Mg^{2+} plays a regulatory role in Ca^{2+} -transport by accelerating the rate of decomposition of the phosphorylated intermediate (Inesi et al, 1974).

This increases the turnover rate of the enzyme, thereby increasing the rate of calcium uptake.

As would be expected, Ca^{2+} itself can alter S.R. Ca^{2+} -transport. Increases in cytoplasmic Ca^{2+} will immediately increase the rate of ATP hydrolysis and Ca^{2+} -transport (Hasselbach, 1964). It is also possible that Ca^{2+} at the inner surface of the membrane may inhibit Ca^{2+} -transport. Yamada and Tonomura (1972) have found that Ca^{2+} can competitively inhibit Mg^{2+} -dependent decomposition of the phosphorylated intermediate. This decomposition of the enzyme phosphate complex is thought to be associated with the interior of the membrane (Sumida and Tonomura, 1974).

Two physiological parameters, pH and temperature, may also control the Ca^{2+} -transport mechanism. Alteration of either parameter results in an alteration of the coupling ratio between Ca^{2+} -uptake and Ca^{2+} -ATPase activity (Tate *et al.*, 1980). The sensitivity of the transport mechanism to pH and temperature may be due to an alteration of the lipid-protein interaction in the S.R. membrane.

d) Regulation by Monovalent Cations:

It is now becoming apparent that monovalent cations, such as K^+ and Na^+ , play an important role in the regulation of S.R. Ca^{2+} -transport. Earlier studies (DeMeis, 1969; Katz and Repke, 1967) suggested that the alkali metals inhibited Ca^{2+} -uptake into S.R. vesicles. They were unable, however, to show a similar decrease in Mg^{2+} - Ca^{2+} -ATPase activity. With the development of better experimental techniques it is now well established that these cations have a marked stimulatory effect on the Ca^{2+} -transport process (Shigekawa and Pearl, 1976; Jones *et al.*, 1977; Duggan, 1977; Lopaschuk *et al.*, 1980). Mg^{2+} - Ca^{2+} -ATPase activity and ATP hydrolysis in cardiac S.R. vesicles is enhanced to such a degree by K^+ that Jones *et al.* (1977) refer to the enzyme as a K^+ - Ca^{2+} -ATPase. The alkali metals appear to exert their effect by increasing the turnover rate of the Mg^{2+} - Ca^{2+} -ATPase pump (Shigekawa and Pearl, 1976). Kinetic studies suggest that they can regulate Ca^{2+} -transport by two mechanisms. The phosphorylated ATPase can be in two forms, an ADP-sensitive (E''' -P in table 2) and ADP-insensitive form (E-P in table 2) (Shigekawa *et al.*, 1978). In the absence of K^+ an appreciable portion of the phosphorylated

enzyme is insensitive to ADP (E-P). If K^+ is added the E-P can convert to the the ADP-sensitive form, which can react with ADP to give the enzyme plus ATP. As well, the presence of alkali metals result in an increased decomposition rate of the ADP-insensitive complex (Shigekawa and Akowitz, 1979). These steps result in an increase turnover of the phosphorylated intermediate, which increases the reaction rate of the enzyme. This work was recently confirmed by Yamada and Ikemoto (1980) who suggest that multiple forms of the E-P complex exists depending on K^+ and Mg^{2+} concentrations. They also demonstrate that K^+ will shift the equilibrium of the sequential ADP-sensitive to ADP-insensitive E-P reaction back toward the ADP-sensitive form.

Although it has been well established that the alkali metals can alter Ca^{2+} -transport in S.R. its physiological relevance, if any, is not known. Ca^{2+} -uptake and enzyme phosphorylation experiments to date have been performed on fragmented S.R. preparations, where the external ion concentration can be easily regulated. In these experiments the activation occurs in the range of 0-50mM K^+ or Na^+ . In vivo the S.R. is bathed in high K^+ , in the range of 100-175mM (Sreter, 1963). It is not known whether K^+ (or Na^+) levels in vivo can change to such a degree as to be physiologically significant as a regulator of Ca^{2+} -transport.

e) Regulation by a Cyclic AMP-dependent Protein Kinase:

Probably the most unique feature concerning the regulation of cardiac S.R. is the ability of the cAMP-dependent protein kinase to stimulate Ca^{2+} -transport (Kirchberger et al, 1972). Cyclic AMP-dependent protein kinase, which is present in cardiac cells (Walsh et al, 1968; Wray et al, 1973), will phosphorylate a protein contained in the cardiac S.R. membrane (LaRaia and Morkin, 1974; Wray et al, 1973). The phosphorylation of this 22,000 MW protein, termed phospholamban (Katz et al, 1975), results in stimulation of the calcium pump (Kirchberger et al, 1974). Figure 2 (Hicks et al, 1979) is a schematic representation of the proposed mechanism of action of cAMP-dependent protein kinase. According to this scheme phosphorylation of phospholamban, which is associated with the Mg^{2+} - Ca^{2+} -ATPase enzyme, results in a conformational change in the protein. This increases the Ca^{2+} -sensitivity of the ATPase enzyme by decreasing

Figure 2:

Possible mechanism by which phospholamban modulates the activity of the calcium pump of the cardiac sarcoplasmic reticulum. Upper: Dephosphorylation of phospholamban interacts with the calcium pump ATPase in the membrane (shaded), conferring positive co-operativity on the two Ca^{2+} binding sites and lowering the Ca^{2+} -sensitivity of the latter. Lower: Phosphorylation of phospholamban reduces its interaction with the calcium pump ATPase, increasing the Ca^{2+} sensitivity of calcium uptake and allowing the Ca^{2+} binding sites of the calcium pump to interact independently with Ca^{2+} . These effects increase calcium transport rate at low Ca^{2+} concentrations. Reproduced from Hicks et al (1979).

the positive co-operativity between the two Ca^{2+} binding sites. The increase in the Ca^{2+} -sensitivity of the ATPase enzyme results in stimulation of Ca^{2+} -transport by the pump. In other words, cAMP-dependent protein kinase acts on the ATPase reaction sequence itself. This is supported by the work of Tada et al (1979) who demonstrated that phosphorylation of phospholamban increases the V_{max} of the calcium pump, but not the level of the phosphorylated intermediate of Mg^{2+} - Ca^{2+} -ATPase.

Cyclic AMP-dependent protein kinase regulation of cardiac S.R. Ca^{2+} -transport has generated considerable interest due to the possibility that it may mediate catecholamine action on the heart. Current biochemical evidence, as summarized by Katz (1979) (figure 3), suggests that catecholamines act through cAMP-dependent protein kinase to 1) phosphorylate troponin I, which decreases the Ca^{2+} -sensitivity of troponin C, and 2) to phosphorylate an S.R. protein, phospholamban, which results in stimulation of Ca^{2+} -uptake. Together the two processes can result in augmentation of the rate and degree of tension development in cardiac muscle. Although cAMP-dependent protein kinase had been shown to be involved in the two processes mentioned above, as of yet no experimental evidence clearly demonstrates the involvement of catecholamines. In vitro experiments using fragmented S.R. preparations have failed to show an augmentation of Ca^{2+} -transport using adrenergic agonists (Yu and Triester, 1969). Messineo and Katz (1979) have shown that β -adrenergic blocking agents, such as propranolol, will inhibit Ca^{2+} -uptake into S.R., but in concentrations which far exceed clinically observed serum levels.

The possible role of phospholamban in cardiac S.R. calcium transport is now becoming widely accepted. Whether cAMP-dependent protein kinase regulation of the S.R. Ca^{2+} pump is unique to cardiac S.R. is still controversial. Kirchberger et al (1972) failed to show any significant stimulation of Ca^{2+} -transport by fast skeletal muscle S.R. in the presence of the kinase enzyme. Schwartz et al (1976) were able to show slight stimulation of Ca^{2+} -transport, but not a concomittant phosphorylation of any S.R. vesicle protein. Efforts to isolate phospholamban from fast

Figure 3:

Proposed integration of the effects of catecholamines on the cardiac contractile proteins and sarcoplasmic reticulum in terms of their physiological response. Reproduced from Katz (1979).

skeletal muscle S.R. (Kirchberger and Tada, 1976) were unsuccessful, and only small amounts could be obtained from slow skeletal muscle.

The more pronounced role of cAMP-dependent protein kinase in cardiac S.R. conforms with the physiological role of catecholamines in muscle. In fast skeletal muscle catecholamines will only slightly increase the tension developed, unlike the marked changes seen in cardiac muscle (Goffert and Ritchie, 1952). If indeed cAMP-dependent protein kinase does mediate catecholamine action, one would expect this more pronounced action in cardiac S.R. preparations.

f) Regulation of Ca^{2+} -Transport by Calmodulin:

One of the many apparent functions of calmodulin is the regulation of various Ca^{2+} -transport events. Calmodulin will stimulate Ca^{2+} -uptake into synaptosomes (Ochs and Igbal, 1978) as well as inside-out vesicles derived from erythrocyte membranes (Larsen and Vincenzi, 1978). Previous work in this laboratory demonstrated that calmodulin obtained from bovine brain will stimulate Ca^{2+} -uptake into cardiac S.R. vesicles (Katz and Remtulla, 1978). The maximum activation seen by calmodulin was additive to the maximum activation of Ca^{2+} -uptake seen with cAMP-dependent protein kinase. This suggests that the calmodulin stimulation of Ca^{2+} -transport is not a result of increased cAMP levels due to activation of adenylate cyclase.

LePeuch et al (1979) found that calmodulin will phosphorylate phospholamban through a protein kinase. This kinase enzyme differs from cAMP-dependent protein kinase in that it is Ca^{2+} -dependent, and the catalytic subunit appears to be membrane bound. The protein inhibitor of cAMP-dependent protein kinase will not reverse the Ca^{2+} -calmodulin phosphorylation of phospholamban. This led LePeuch et al to believe that regulation of cardiac S.R. calcium transport occurred through two separate protein kinases, each phosphorylating a distinct site on phospholamban. Phosphorylation experiments performed by Kranias et al (1980) support this hypothesis. They found that calmodulin- Ca^{2+} -dependent phosphorylation and cAMP-dependent phosphorylation both occurred on the same protein. As well, phosphorylation of cardiac S.R. by Ca^{2+} and calmodulin, in the presence of cAMP-dependent protein kinase, was enhanced above the level obtained when cAMP-dependent protein kinase was present alone.

III) Objectives of the present study

Although we know that calmodulin regulates cardiac S.R. Ca^{2+} -transport, little is known concerning its mechanism of action. It has been shown that calmodulin is involved in phosphorylation of phospholamban, but what effect this has on the Ca^{2+} -pump mechanism remains to be elucidated. As well, the monovalent cations play an important role in the translocation of Ca^{2+} . The interaction between calmodulin regulation and monovalent cation regulation of Ca^{2+} -transport has not been previously studied.

In this study we used cardiac microsomes, enriched in S.R., to characterize calmodulin stimulation of Ca^{2+} -transport. These vesicles are believed to have an intact Ca^{2+} -transport mechanism, which is characteristic of Ca^{2+} -transport in vivo. Using this system we studied the interaction of calmodulin and monovalent cations on the transport system. Kinetic analysis and initial rate studies were performed to determine what effect calmodulin and K^+ had on the Ca^{2+} -sensitivity of the pump. These results can be compared to results previously obtained by Hicks *et al* (1979) studying cAMP-dependent protein kinase stimulation of cardiac S.R. Ca^{2+} -transport. From this it can be determined whether calmodulin-dependent phosphorylation of S.R. has an effect on Ca^{2+} -transport similar to cAMP-dependent protein kinase phosphorylation.

Since calmodulin appears to regulate Ca^{2+} -transport it is important to determine whether it is a cytosolic protein, or an integral protein of the S.R.. Therefore experiments were performed to determine if calmodulin was endogenous to the S.R. membrane. This was done using a number of techniques which have been used to isolate calmodulin from other sources. If calmodulin is a cytosolic protein then it must either interact directly with the S.R., or through an intermediate which interacts with the S.R.. Therefore ^{125}I -labelled calmodulin was prepared in order to determine if calmodulin will bind to S.R. in a Ca^{2+} -dependent manner. The effect of monovalent cations on calmodulin binding to S.R. was also determined.

Although much work must be done to reveal the mechanism of calmodulin regulation of Ca^{2+} -transport in cardiac S.R. the above objectives should partially clarify this problem.

MATERIALS AND METHODS

A) Materials:

1) Animals:

Dogs of either sex between six months and four years of age were used throughout the study. Hearts were removed from pentobarbital anaesthetized dogs and placed in cold normal saline solution (4°C). The ventricles were cut into 2-4g pieces, quick frozen in methyl butane on dry ice, and stored at -80°C until use.

2) Chemicals:

i) Radioisotopes

- ^{45}Ca in the form of $(^{45}\text{Ca})\text{Cl}_2$ (10 Ci/mmol), γ - ^{32}P -ATP (16.4 Ci/mmol), and ^{125}I in form of $\text{Na}(^{125}\text{I})$ in NaOH pH 7-11 (15mCi/ μg) were purchased from Amersham corporation (Toronto, Ontario).

ii) Chromatography columns

-Sephadex G-15, g-25, g-75, g-200, DEAE Sephadex A50, and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals.

-AG 50 WX8 (200-400 mesh) analytical grade cation exchange resin was purchased from Bio-Rad laboratories.

iii) SDS-polyacrylamide gel electrophoresis

-sodium dodecyl sulphate and mercaptoethanol were purchased from Sigma chemicals.

-Sodium phosphate (monobasic and dibasic) was purchased from Fisher Scientific Co.

-all other reagents were obtained from Bio-Rad.

iv) Filtration equipment

-Immersible-CX separator filters and circular vacuum filters (0.45 μ , 0.8 μ , 2.0 μ , and 5.0 μ) were obtained from Millipore Co.

-PM-10 filters (10,000 MW cutoff) were obtained from Amicon.

v) Assay Chemicals

- CaCl_2 dihydrate, L-ascorbate, Trizma oxalate, sodium carbonate, and sodium bicarbonate were purchased from Analar BDH Chemicals.

-Lithium chloride, lanthanum chloride, and activated charcoal were purchased from Fisher Scientific Co.

-2-methyl butane was purchased from MCB manufacturing chemists.

-Aquasol scintillation fluid was purchased from New England Nuclear.

-Sodium hydroxide (10N) was obtained from Baker Chemicals.

-The followin chemicals were purchased from Sigma[®] chemical:

Trizma Adenosine Triphosphatase (equine muscle)

Phosphodiesterase 3',5'-cyclic nucleotide activator from bovine heart

Adenosine 3',5'-cyclic nucleotide monophosphate (Tris salt)

cAMP-dependent protein kinase from beef heart (Type II)

L-Histidine free base	Bovine serum albumin
EGTA	Sucrose
EDTA	Trizma base
Chloramine T	Trizma HCl
Imidazole	Trizma phosphate
Magnesium chloride	Triton X-100
Potassium chloride	Dithiothreitol
Sodium chloride	Potassium iodide
Sodium azide	Urea
Sodium potassium tartrate	Copper sulfate

B) Methods

1) Preparation of Calmodulin from Outdated Human Erythrocytes:

Calmodulin was prepared by a modification of the method of Vincenzi and co-workers (Jung, 1978). One hundred ml of outdated human blood, obtained from the Canadian Red Cross, was divided into four 50ml plastic centrifuge tubes. The tubes were centrifuged at 3,000Xg for 5 minutes at 4°C in a Beckman[®] Model J2-21 centrifuge. The supernatant was discarded and the packed cells washed with normal saline (0.9% NaCl). The tubes were recentrifuged (3,000Xg for 5 minutes), the supernatant discarded, and the normal saline wash subsequently repeated three times. Washed packed cells were then lysed with 500ml 15.7mM Imidazole pH 7.4 and the resultant hemolysate centrifuged at 30,000Xg for 30 minutes. The supernatant was filtered through 2µm filters using a Millipore[®] pump, and NaCl added to bring the hemolysate to 0.3M NaCl. The hemolysate was then passed through a DEAE-Sephadex[®] A50 column (2.6X 35 cm), pre-equilibrated with 0.3 M NaCl and 20mM Imidazole (pH 6.8). The hemolysate was eluted from the column using the equilibrating buffer. Calmodulin was eluted from the column with a linear NaCl

gradient (0.3-0.8 M NaCl) and 8ml fractions collected using a Bromma[®] LKB 7000 Ultrarac fraction collector. These fractions were assayed for their ability to stimulate $(\text{Mg}^{2+}-\text{Ca}^{2+})$ -ATPase activity of human EDTA-washed red cell membranes. Active fractions were pooled and concentrated 4 times using Millipore[®] Immersible CX membranes (10,000 MW CUTOFF). A Sephadex[®] G-15 column (2X20cm) was used to desalt the calmodulin solution. Purity of the solution was determined by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and a Gelman ACD 16 densitometer used to determine the percent of calmodulin protein present in the polyacrylamide gels. Absence of salt in the calmodulin solution was verified using atomic absorption spectrophotometry. The calmodulin solution was stored in aliquots at -80°C .

2) Preparation of Cardiac Microsomes Enriched with Sarcoplasmic Reticulum Vesicles:

Sarcoplasmic reticulum enriched vesicles were prepared using the methodology of Harigaya and Schwartz (1969) with slight modifications. The entire procedure was performed at 4°C . Frozen dog ventricle (4g) was minced and put into 30ml of buffer 1 (10mM NaHCO_3 , 5mM NaN_3 , and 0.2 mM ascorbate pH 6.8) contained in a glass homogenizer tube. This was homogenized using a teflon pestle at 1500 r.p.m. for 15 seconds. The homogenized solution was transferred to a 50ml Beckman[®] plastic centrifuge tube and, along with three other similar homogenate solutions, was centrifuged at $10,000\times g$ for 20 minutes in a Beckman[®] Model J2-21 centrifuge. The supernatant from the four tubes was then divided into 8 Corex[®] tubes and centrifuged at $40,000\times g$ for 60 minutes. The resultant supernatant was discarded and the pellet resuspended by gentle homogenization in 2ml of Buffer 2 (0.6 M KCl, 2.0 mM tris Cl pH 7.3, 1.0 mM MgCl_2). The suspensions were pooled, divided into 2 corex[®] tubes and buffer 2 added to bring the volumes up to 10ml. These tubes were recentrifuged at $40,000\times g$ for 60 minutes, the supernatant was discarded, and the two pellets pooled and suspended in 10 ml Buffer 3 (10mM tris Cl pH 7.3). After recentrifugation at $40,000\times g$ for 60 minutes, the supernatant was discarded, and the two pellets pooled and suspended in 3 ml of Buffer 4 (40% sucrose 10 mM tris Cl pH 7.4, 5 mM dithiothreitol). The sucrose solution had previously

been purified by passage through a Bio-Rad[®] AG 50w-X8 cation exchange resin column in the Na⁺ form. The concentrated microsomal suspension (1.2-1.5 mg/ml) was then divided into 4 test tubes. These were either used immediately, or quick frozen, by immersing the aliquot in methyl butane on dry ice, and stored at -80°C. When used the concentrated aliquots were diluted with buffer 4 such that the final protein content ranged from 0.4-0.6mg/ml. All microsomal suspensions were used within two weeks of preparation. The activity of the suspension, as measured by the ability to transport Ca²⁺ into the S.R. vesicles, was similar in fresh and previously frozen aliquots.

3) Measurement of Calcium Uptake by Cardiac Microsomes Enriched in Sarcoplasmic Reticulum Vesicles:

Oxalate facilitated ATP-dependent calcium uptake by S.R. was measured by the method of Tada et al (1974) with a few modifications. An incubation medium was prepared such that when 0.3 ml was diluted to the final incubation volume of 0.5 ml a solution of 40 mM histidine-HCl pH 6.8, 5mM MgCl₂, 5 mM tris -ATP and 2.5 mM tris-oxalate was obtained. Depending on the experiment, calmodulin or monovalent cations such as 110 mM K⁺ were then added to the incubation medium. When monovalent cation strength was altered the osmolarity of the incubation medium was maintained by sucrose addition. The microsomes enriched in S.R. vesicles (30-50µg) were then pre-incubated in the incubation medium for 11 minutes at 30°C. Calcium uptake by the vesicles was initiated by the addition of CaCl₂ containing (⁴⁵Ca)Cl₂ (10 Ci/mmmole). The desired free calcium concentration was maintained by the addition of ethylene-bis-(β-aminoethyl ether) -N,N'-tetraacetate (EGTA) and the free Ca²⁺ concentration present determined by the equations of Katz et al (1970) taking into consideration temperature, pH, Mg²⁺ concentration, and ATP concentrations. Calcium uptake activity was terminated, usually after 5 minutes, by filtering a 0.41 ml aliquot of the incubation medium through Millipore[®] filters (type HA 0.45µ pore size) with the aid of a Millipore[®] pump. The filters were then washed twice with 10 ml of 40mM tris-Cl pH 6.8, dried for 5 minutes at 60°C, and placed in 10 ml of Aquasol[®] scintillation fluid. Samples were counted for 10 minutes in an Isocap[®]

scintillation counter.

4) Calculation of Calcium Uptake Activity by Cardiac Microsomes Enriched in Sarcoplasmic Reticulum:

The rate of Ca^{2+} -uptake by the microsomal preparation is expressed as nmoles Ca^{2+} taken up per mg protein per minute. This is determined by the following formula:

$$\frac{\text{Sample Counts} - \text{Blank Counts}}{\text{Total Counts} - \text{Blank Counts}} \times \text{dilution factor} \times \text{Total } (\text{Ca}^{2+}) \times \frac{\text{mg protein}}{\text{incubation time}}$$

where

Sample Counts = $^{45}\text{Ca}^{2+}$ counts obtained per individual samples.

Total Counts = total $^{45}\text{Ca}^{2+}$ counts present in the incubation medium

Blank Counts = $^{45}\text{Ca}^{2+}$ counts obtained in the absence of microsomal protein

Total (Ca^{2+}) = total calcium concentration present in the incubation medium

Dilution factor = $\frac{\text{correction for incubation volume}}{\text{aliquot volume}} = \frac{0.5\text{ml}}{0.41\text{ ml}} = 1.21$

mg protein = weight of microsomal protein present in the incubation medium.

Incubation time = length of time the microsomal protein was incubated in the presence of CaCl_2 .

5) Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

i) Preparation of 10% polyacrylamide gels:

SDS-PAGE was performed according to the protocol of Weber and Osborne (1968). A 10% acrylamide stock solution was prepared by dissolving 22.2g acrylamide and 0.6g bis-acrylamide in 100 ml distilled H_2O , and filtered through a Whatman #1 filter. The acrylamide stock solution (13.5 ml) was gently mixed with 15 ml Gel buffer (0.2 M Na phosphate buffer, pH 7.0, 0.2% SDS). To this, 1.5ml of 1.5% ammonium persulfate and 0.045 ml TEMED (N,N,N',N'-tetramethylene-diamine) Neat reagent were added and gently mixed. The mixture was then pipetted into glass gel tubes (2.5ml/tube) sealed at one end with Parafilm[®]. Before the gel solution set, one drop of distilled

H₂O was added to the top of each gel.

ii) Preparation of Calmodulin Protein Samples:

Previously frozen calmodulin samples (40-80 μ g/ml) were lyophilized using a Virtis[®] lyophilizer, such that approximately 20 μ g of solid protein was obtained per sample. This was resuspended in 70 μ l of distilled H₂O. To this was added 30 μ l of a previously prepared solution containing 5X buffer (0.5 M Na phosphate buffer, pH 7.0, 5% SDS, 40% glycerol): 0.03% bromophenol blue: and 2-mercaptoethanol Neat reagent in a ratio of 20:5:5. The samples were then capped, gently boiled for 5 minutes, cooled to 20°C, and pipetted onto the top of each gel. Electrophoresis buffer (Gel buffer diluted 1:1) was then carefully added to fill each gel tube.

iii) Electrophoresis, Staining, and Destaining of Samples:

The gel tubes were then placed in a Pharmacia[®] EPS 500/400 Electrophoresis Gel Column Apparatus. A current of 8 mA/tube was applied to the apparatus for 8 hours, or until the tracking dye had migrated to the bottom of the gel tubes. The polyacrylamide gels were then removed from the tubes by forcing gel buffer between the gel and the tube with a syringe. The gels were placed in individual test tubes containing stain 1 (0.0125% Coomassie brilliant blue, 10% glacial acetic acid, and 25% isopropyl alcohol). After 16 hours stain 1 was replaced with stain 2 (0.0125% Coomassie brilliant blue, 10% glacial acetic acid, and 10% isopropyl alcohol), and the gels left for an additional 24 hours. The gels were destained and stored in a 10% acetic acid solution. Molecular weight of the protein bands was determined by comparing the R_f value (distance travelled by the band/ migration distance of the dye) to a standard curve of the R_f vs. log molecular weight of the standards.

6) Iodination of Calmodulin:

Radioiodination of calmodulin was achieved by the method of Hunter and Greenwood (1962). The entire procedure, including elution of the ¹²⁵I-calmodulin through a Sephadex[®] G-25 column, was performed at 4°C. The column was prepared by soaking 5 g of Sephadex[®] G-25 in 0.05 M NaH₂PO₄·2H₂O pH 7.4 + 2 g/l B.S.A., and packing it into a 1 × 30 cm jacketed column maintained at 4°C. The iodination reaction was performed

in a vial containing 2 mCi Iodine-125 (Amersham NaI(^{125}I) in NaOH pH 7-11, 13-17 mCi/ μg). To this was added 10 μl (15 μg) pure calmodulin, and 10 μl of 5 mg/ml chloramine T in 0.05 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ pH 7.4. Within 15 seconds of the chloramine T addition, 10 μl of 24 mg/ml Na metabisulfite 0.05 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ pH 7.4 was added, followed by 0.2 ml of 10 mg/ml KI in 0.05 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ pH 7.4. The contents of the vial were then pipetted onto the surface of the Sephadex[®] G-25 column. The iodination vial was rinsed with a further 0.2 ml KI solution, which was also added to the column. The ^{125}I -calmodulin was eluted with 0.05 M $\text{Na}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ pH 7.4 + 2 g/l B.S.A. and 6 drop fractions collected in polystyrene tubes (Amersham/Searle[®]). These tubes were counted in a Nuclear-Chicago[®] 1185 Gamma counter for 1 second, and the fraction from the first peak with the highest activity were pooled and stored at -70°C . A 15 μl sample of the iodinated calmodulin was suspended in 1 ml of 10% trichloroacetic acid (TCA) and counted in the Gamma counter. This was then centrifuged and 0.5 ml of the supernatant counted separately. It was determined that approximately 50% of the ^{125}I in the sample was bound to calmodulin.

7) Measurement of ^{125}I -calmodulin Binding to Sarcoplasmic Reticulum Enriched Microsomes:

Binding studies were performed using incubation procedures similar to those utilized in the Ca^{2+} -uptake experiments. Microsomal S.R. (approximately 150 μg /0.5 ml incubation volume) was pre-incubated for 7 minutes at 30°C in Corex tubes containing 40 mM histidine-Cl pH 6.8, 5 mM MgCl_2 , 110 mM KCl, 5 mM ATP, and 2.5 mM tris oxalate. The iodinated calmodulin (approximately 80,000 cpm) was added to the incubation medium with the buffered calcium solution. The amount of Ca^{2+} necessary to maintain a desired free- Ca^{2+} concentration in the presence of 0.4 mM EGTA was determined by the equations of Katz et al (1970). After 5 minutes of incubation the medium was centrifuged at 40,000 $\times g$ for 40 minutes and the supernatant discarded. The pellet was carefully washed with 40 mM tris-Cl pH 7.2, containing 0.4 mM EGTA and the Ca^{2+} concentration that was present in the incubation medium (0.01 μM -10 mM). The pellet was then suspended in 0.75 ml of tris-Cl solution by homogenization with a Teflon pestle (200 RPM $\times$ 20 seconds). The content of

the Corex tube was pipeted into polystyrene tubes (Amersham/Searle). The Corex tube was then rinsed with a further 0.75 ml of tris-Cl solution, which was also pipeted into the polystyrene tube. These tubes were counted for 10 minutes in a Gamma spectrophotometer (Nuclear-Chicago[®] 1185) which measured the photopeak for ^{125}I .

Binding of ^{125}I -calmodulin to the microsomal S.R. was expressed as counts per minute/ mg sarcoplasmic reticulum protein.

8) Miscellaneous Methods:

EDTA-washed human erythrocyte membranes were prepared by the method of Blostein (1968).

Measurement of Mg^{2+} - Ca^{2+} -ATPase activity was performed by the technique of Katz and Blostein (1975).

Determination of Na^{+} concentrations in calmodulin samples was carried out by Atomic Absorption Spectrophotometry, according to the operation manual procedure of the Varian Techtron A.A.-5 Spectrophotometer. The process was performed on samples containing calmodulin in solution, or calmodulin precipitated with 6% TCA.

Procedures used for isolating calmodulin from cardiac S.R. membranes are described in detail in the results section.

Protein assays were performed by the method of Lowry et al (1951) using Bovine Serum Albumin as a standard.

Statistical analysis was by the student's "t" test for ungrouped data.

In a number of figures appearing in the results (figure 5,8,9, 10,11,14) experimental data was presented as typical experiments, rather than the average of a number of experiments. This is due to the variability from experiment to experiment in the control rate of Ca^{2+} -uptake in cardiac microsomes enriched in sarcoplasmic reticulum. The rate of tris-oxalate facilitated Ca^{2+} -uptake in the presence of $1\text{ }\mu\text{M}$ free Ca^{2+} and absence of KCl ranged from 5.0 to 30 nmoles/mg/min depending on the microsomal preparation used. In the presence of 110 mM KCl the rate of tris-oxalate facilitated Ca^{2+} -uptake in the presence of $1\text{ }\mu\text{M}$ free Ca^{2+} ranged from 10 to 50 nmoles/mg/min. When the

effect of calmodulin on Ca^{2+} -uptake by the cardiac microsomal S.R. was determined, maximal stimulatory concentrations of calmodulin were used. Depending on the calmodulin preparation used, this ranged from 2.25 to 6.5 μ g/0.5ml final incubation volume.

RESULTS

1) Isolation of Calmodulin:

In the majority of experiments performed in this study calmodulin was isolated from hemolysates of human erythrocytes using a modification of the method of Jung (1978). Fractions collected from the DEAE Sephadex[®] column were tested for their ability to stimulate Mg^{2+} - Ca^{2+} -ATPase activity in EDTA-washed red cell membranes. Figure 4 shows a typical preparation in which Mg^{2+} - Ca^{2+} -ATPase activity is enhanced by a narrow range of fractions, while Mg^{2+} -ATPase activity remains unaltered. This suggests that the fractions are specifically stimulating Mg^{2+} - Ca^{2+} -ATPase.

Once the active fractions were concentrated 4-fold it was necessary to desalt the samples. Initial samples were desalted by dialysis against low concentrations of EDTA. In most instances this resulted in an unexplained loss in the ability of calmodulin to stimulate Mg^{2+} - Ca^{2+} -ATPase in EDTA-washed red cell membranes. It was then decided to desalt the calmodulin samples by passage through a G-15 Sephadex[®] column. In order to insure that the sample was relatively free of salt it was subjected to atomic absorption spectrophotometry. This was performed either with the calmodulin free in solution, or precipitated out with 6% TCA. In all calmodulin samples it was determined that there was 0.6 mM or less Na^+ present. If the samples were desalted through a G-25 Sephadex[®] column the calmodulin was retained close to the void volume of the column. Once the void volume eluted, Na^+ will begin to be eluted from the column. In the only sample desalted with the G-25 Sephadex[®] column it was determined that 9 mM Na^+ was still present in solution. To ensure that calmodulin activity was maintained during the concentrating and desalting procedure the sample was again tested for its ability to stimulate Mg^{2+} - Ca^{2+} -ATPase in EDTA-washed red cell membranes. Table 3 indicates that these calmodulin preparations markedly stimulate red cell Mg^{2+} - Ca^{2+} -ATPase activity without altering Mg^{2+} -ATPase activity. Depending on the degree of stimulation, the sample was either concentrated further or used as is. During the course of the study, calmodulin obtained from beef heart became commercially available from the Sigma[®] Chemical Co.. Table 3 shows that

Figure 4:

Determination of Fractions Containing Calmodulin Using the ATPase Assay of EDTA-washed Red Cell Membranes.

ATPase assay, calmodulin preparation, and preparation of EDTA-washed red cell membranes were as described in methods. The top line (●—●) represents Mg^{2+} - Ca^{2+} -ATPase activity in each fraction in the presence of $50 \mu\text{M}$ Ca^{2+} , while the bottom line (●---●) represents Mg^{2+} -ATPase activity. ATPase activity is calculated as counts/minute of $^{32}\text{P}_i$.

Table 3

Effect of Various Calmodulin Preparations on Mg^{2+} -ATPase and Mg^{2+} - Ca^{2+} -ATPase activity of EDTA-washed Human Erythrocyte Membranes

Sample	Mg^{2+} -ATPase Activity (pmoles/mg/min)	Mg^{2+} - Ca^{2+} -ATPase Activity (pmoles/mg/min)	% increase
Control	2566	3562	39
Calmodulin C (2.5 μ g/ml)	-	5432	-
Calmodulin D (3.26 μ g/ml)	1949	5620	188
Calmodulin E (6.2 μ g/ml)	2578	8458	228
Sigma [®] calmodulin (20 μ g/ml)	2225	5003	125
Sigma [®] calmodulin (40 μ g/ml)	2497	6302	152

-calmodulin samples C,D, and E are three typical calmodulin preparations from hemolysate of human erythrocytes. The calmodulin purchased from Sigma^r Chemical Co., is derived from beef heart.

-ATPase activity was measured in the presence and absence of 50 μ M Ca^{2+} as described in methods.

- data was normalized due to differences in membrane activity between experiments.

this calmodulin will also stimulate Mg^{2+} - Ca^{2+} -ATPase, but that much higher concentrations are necessary to produce a similar effect. The reason for this decreased potency will be discussed in a later section.

Early on in the study it was found that calmodulin in the absence of K^+ in the incubation medium could substantially stimulate Ca^{2+} -transport in cardiac microsomal S.R.. It was decided that this assay could be used to determine relative activity of the calmodulin sample prepared. Figure 5 demonstrates the calmodulin concentration-dependent increase in Ca^{2+} -uptake observed with a typical sample preparation under these conditions.

In addition to the determination of the activity of the calmodulin-preparation it was necessary to ensure that the sample was relatively pure. In order to do this, samples were subjected to SDS-polyacrylamide gel electrophoresis. Shown in figure 6 are 3 densitometer tracings obtained from SDS-PAGE gels. The first tracing consists of compounds of known MW used as a reference standard. The tracing of calmodulin preparation A and calmodulin preparation B are indicative of what was obtained for most of the calmodulin samples. The sample protein migrated either to a single band of 18,000 MW, or to a major band at 18,000 MW with minor bands at 50-65,000 MW. As determined by densitometry, the 18,000 MW peak in calmodulin preparation A comprised 85% of the total protein present. The 18,000 MW band is thought to be calmodulin, and will co-migrate with a red cell calmodulin preparation isolated in the laboratory of DR. B. Roufogalis.

2) Characterization of Cardiac Microsomes Enriched in S.R.:

Ca^{2+} -uptake was measured at varying concentrations of S.R. prepared as described in the methods. Figure 7 illustrates that Ca^{2+} -uptake increased linearly as the microsomal S.R. concentration increased. In other words, the rate of Ca^{2+} -uptake was not affected by variation in the S.R. concentration used. Most of the experiments described were performed at microsomal S.R. concentrations between 0.05 and 0.1 mg/ml. The majority of Ca^{2+} -uptake experiments performed during the course of the study involved incubation of the S.R. for 5 minutes in the presence of Ca^{2+} . To obtain an accurate measurement of the amount of Ca^{2+} -trans-

Figure 5

Effect of Red Cell Calmodulin on Ca^{2+} -uptake in Microsomal Preparations
Enriched in Sarcoplasmic Reticulum

Ca^{2+} -uptake was determined as described in methods in the absence of KCl, and in the presence of $1\text{ }\mu\text{M}$ free Ca^{2+} . The calmodulin used ($2.25\text{ }\mu\text{g}/50\text{ }\mu\text{l}$) was a typical preparation.

This result is typical of 9 experiments performed. Ca^{2+} -uptake activity in these experiments in the absence of added calmodulin ranged from 5.0 to 30.0 nmoles/mg/min.

Figure 6

Densitometer Tracings of Calmodulin Preparations Subjected to SDS-Polyacrylamide Gel Electrophoresis.

SDS-PAGE was performed as described in the methods. Calmodulin preparations A and B represent two calmodulin samples obtained by the method of Jung (1978). The number by each peak represents the apparent molecular weight of the protein which comprises that peak. Molecular weight standard proteins include phosphorylase B (94,000 MW), Bovine Serum Albumin (67,000 MW), Ovalbumin (43,000 MW), Carbonic Anhydrase (30,000 MW), Soybean Trypsin Inhibitor (20,100 MW), and α -lactalbumin (14,000 MW).

standards **calmodulin
preparation A** **calmodulin
preparation B**

Figure 7

Effect of Varying Concentrations of the Microsomal Preparation Enriched in Sarcoplasmic Reticulum on Ca^{2+} -uptake by the S.R. Preparation.

Ca^{2+} -uptake was determined as described in methods, in the presence of $1 \mu\text{M}$ free Ca^{2+} and 110 mM KCl.

ported/minute it was necessary that Ca^{2+} -uptake be linear over the period of incubation. Initial rate studies shown in figure 15 (discussed later) confirm that Ca^{2+} -uptake was indeed linear over the incubation periods utilized. At the time this study was being performed other researchers in the laboratory were attempting to purify the crude microsomal S.R. preparation using sucrose density gradient centrifugation techniques. Cardiac S.R. preparations at various stages of purification were tested to determine the ability of cAMP-dependent protein kinase to stimulate Ca^{2+} -uptake. As indicated in table 4 the crude microsomal S.R. was qualitatively similar to the purified S.R. fractions in the ability of cAMP-dependent protein kinase to stimulate Ca^{2+} -uptake. As well, the crude microsomal S.R. was found to be higher in Ca^{2+} -transport activity than the other S.R. preparations tested. These results are further evidence that the Ca^{2+} -uptake activity observed in crude microsomal preparations is due to the presence of S.R. and not due to contamination from other organelles.

3) Monovalent Cation Stimulation of Ca^{2+} -transport in Cardiac S.R.:

The effect of K^+ and Li^+ on Ca^{2+} -transport in microsomal S.R., in the presence and absence of calmodulin, is shown in figure 8. In the presence of 110 mM KCl, Ca^{2+} -uptake activity is enhanced compared to that activity noted in the presence of 110 mM LiCl, or in the presence of no monovalent cations (200 mM sucrose was used to maintain osmolarity) at every free calcium concentration studied. In the presence of 110 mM KCl, calmodulin increased Ca^{2+} -uptake activity by 20-40% as noted previously (Katz and Remtulla, 1978). However, in the presence of 110 mM LiCl or in the absence of monovalent cations, calmodulin stimulated Ca^{2+} -uptake to a much greater degree. In fact, in the absence of KCl, calmodulin restored Ca^{2+} -uptake to the maximum activity noted in the presence of 110 mM KCl alone.

Similar experiments were performed in the presence and absence of KCl using commercially obtained Sigma[®] calmodulin in place of red cell calmodulin. As shown in figure 9, the Sigma[®] calmodulin, at concentrations comparable to the amount of red cell calmodulin needed to produce maximal activation, only slightly enhanced Ca^{2+} -uptake at all

Table 4

Ca²⁺-uptake by Various Cardiac Muscle Preparations in the Presence and Absence of cAMP-dependent Protein Kinase

<u>Sample</u>	<u>Ca²⁺-uptake Activity (nmoles/mg/min)</u>		<u>% increase</u>
	<u>-cAMP-dependent protein kinase</u>	<u>+cAMP-dependent protein kinase</u>	
Washed particles ¹	4.26	6.44	51
Crude microsomal S.R. ²	8.96	14.23	59
Purified S.R. level 1 ³	2.20	3.88	76
Purified S.R. level 2	2.29	4.12	79

1-washed particles, prepared by Betty Richter, consist of dog heart homogenized in Na phosphate buffer, followed by a KCl buffer and tirs buffer wash.

2-crude microsomal S.R. was prepared as described in methods.

3-purified S.R., prepared by Betty Richter, involves placing the crude microsomal S.R. onto a sucrose density gradient and assaying the resultant layers.

-Ca²⁺-uptake was measured as described in the methods, in the presence of 1 μ M free Ca²⁺ and absence of KCl. The experiment was performed either in the absence or presence of cAMP (1 μ M) and cAMP-dependent protein kinase (type 1, 25 μ g/0.5 ml final incubation volume).

Figure 8

Effect of Monovalent Cations on Ca^{2+} -uptake in the Presence, and Absence of Calmodulin

Ca^{2+} -uptake was determined as described in methods in the presence and absence of 110 mM KCl, 110 mM LiCl, or 200 mM sucrose (0 KCl, 0 LiCl) with (●—●) and without (●---●) calmodulin (3.0 μ g/0.5 ml final incubation volume).

This result is typical of the average of 4 experiments performed. Ca^{2+} -uptake activity in these experiments, in the absence of added KCl and calmodulin at 1 μ M free Ca^{2+} , ranged from 4.25 to 13.38 nmoles/mg/min.

Figure 9

Effect of K^+ on Ca^{2+} -uptake in the presence and absence of Sigma[®] Calmodulin

Ca^{2+} -uptake was determined as described in methods in the presence and absence of 110 mM KCl with (●—●) and without (●---●) Sigma[®] calmodulin (5.0 μ g/0.5 ml final incubation volume).

This result is typical of 3 experiments performed.

Ca^{2+} -uptake activity in these experiments, in the absence of added KCl and calmodulin at 1 μ M free Ca^{2+} , ranged from 6.6 to 8.8 nmoles/mg/min.

Ca^{2+} concentrations studied. When the amount of Sigma[®] calmodulin was increased to 15 $\mu\text{g}/0.5$ ml incubation tube it was possible to obtain a 34.1% increase in Ca^{2+} -uptake by the microsomal S.R. in the absence of K^+ , at 1 mM free Ca^{2+} . The cost and availability of the Sigma[®] calmodulin made it unfeasible to try higher concentrations over a range of Ca^{2+} concentrations.

The effect of increasing K^+ concentrations on calmodulin stimulation of Ca^{2+} -transport in cardiac microsomal S.R. was investigated (figure 10). Increasing the K^+ concentration increased the degree of Ca^{2+} -uptake to a maximum at 30 mM KCl, following which Ca^{2+} -uptake decreased slightly. Calmodulin, though, stimulated Ca^{2+} -uptake to a greater degree in the absence of added KCl. As the KCl concentration was increased the degree of calmodulin stimulation of Ca^{2+} -uptake decreased, from over 100% stimulation at 0 K^+ to 15-25% at 110mM KCl. A qualitatively similar result was obtained with increasing NaCl concentrations (figure 11). Maximal Ca^{2+} -uptake occurred at 100 mM NaCl. Calmodulin had the highest stimulatory effect at 0 Na^+ , and no stimulation was noted at NaCl concentrations above 80 mM.

4) Effect of Calmodulin on the Kinetic Parameters of Ca^{2+} -transport in Cardiac Microsomal Preparations Enriched in S.R.:

Calmodulin (2 $\mu\text{g}/0.5$ ml) had no significant effect on the apparent K_d for Ca^{2+} (0.516 mM in the absence of calmodulin and 0.676 mM in the presence of calmodulin), but significantly increased the $V_{\text{Ca}^{2+}}$ of this preparation (20.2 nmoles/mg/min in the absence of calmodulin and 48.2 nmoles/mg/min in the presence of calmodulin). (figure 12). By contrast, KCl (110 mM) significantly altered the apparent K_d for Ca^{2+} (0.516 mM in the absence of KCl and 2.32 mM in the presence of 110 mM KCl) (figure 13).

Figure 10

Effect of K^+ on Ca^{2+} -uptake in the Presence of Red Cell Calmodulin

Ca^{2+} -uptake was determined as described in methods at $1 \mu M$ free Ca^{2+} in the presence (●—●) and absence (○---○) of red cell calmodulin ($3.0 \mu g/0.5$ ml final incubation volume).

This result is typical of the average of 5 experiments. Calmodulin significantly stimulated Ca^{2+} -uptake at all KCl concentrations tested ($p < 0.05$ students "t" test, paired data.)

Ca^{2+} -uptake activity in these experiments, in the absence of added KCl and calmodulin, ranged from 5.3 to 18.7 nmoles/mg/min.

Figure 11

Effect of Na^+ on Ca^{2+} -uptake in the Presence and Absence of Red Cell Calmodulin

Ca^{2+} -uptake was determined as described in methods at $1 \mu\text{M}$ free Ca^{2+} in the presence (●—●) and absence (○---○) of red cell calmodulin ($3.0 \mu\text{g}/0.5 \text{ ml}$ final incubation volume).

This result is typical of the average of 4 experiments. Calmodulin significantly stimulated Ca^{2+} -uptake at all NaCl concentrations tested below 55 mM ($p < 0.05$, students "t" test, paired data).

Ca^{2+} -uptake activity in these experiments, in the absence of added NaCl and calmodulin, ranged from 7.7 to $16.7 \text{ nmoles/mg/min}$.

Figure 12

Effect of Calmodulin on the K_d for Ca^{2+} and the $V_{\text{Ca}^{2+}}$ for Ca^{2+} -
Uptake in Microsomal Preparations Enriched in Sarcoplasmic Reticulum

Ca^{2+} -uptake was determined as described in the methods at various free Ca^{2+} concentrations ($0.2 \mu\text{M}$ – $30.0 \mu\text{M}$ free Ca^{2+}) and the data plotted as reciprocals of results obtained in the absence ($\bullet\text{---}\bullet$) and presence ($\circ\text{---}\circ$) of calmodulin. Data obtained in assays performed on separate days was normalized to that activity found in the absence of calmodulin at $1 \mu\text{M}$ free Ca^{2+} .

This result is typical of the average of 3 experiments performed.

Ca^{2+} -uptake activity in these experiments, in the absence of added KCl and calmodulin at $1 \mu\text{M}$ free Ca^{2+} , ranged from 7.4 to 19.3 nmoles/mg/min.

Figure 13

Effect of K^+ on the K_d for Ca^{2+} and the $V_{Ca^{2+}}$ for Ca^{2+} -uptake in Microsomal Preparations Enriched in Sarcoplasmic Reticulum

Ca^{2+} -uptake was determined as described in methods at various free Ca^{2+} concentrations ($0.2 \mu M$ – $30.0 \mu M$ free Ca^{2+}) and the data plotted as reciprocals of results obtained in the absence ($\bullet\text{---}\bullet$) and presence ($\circ\text{--}\text{--}\circ$) of K^+ . Data obtained in assays performed on separate days was normalized to that activity found in the absence of K^+ at $1 \mu M$ free Ca^{2+} .

This result is typical of the average of 3 experiments performed. Ca^{2+} -uptake activity in these experiments, in the absence of added KCl and calmodulin at $1 \mu M$ free Ca^{2+} , ranged from 27.2 to 54.6 nmoles/mg/min.

4) Effect of Calmodulin on the Initial Ca^{2+} -uptake Velocity in Cardiac Microsomal Preparations Enriched in Sarcoplasmic Reticulum:

The time course of calcium uptake at various free Ca^{2+} concentrations in the presence and absence of red cell calmodulin ($2\mu\text{g}/0.5\text{ ml}$ final incubation volume) is shown in figure 15. These experiments were conducted using tris-phosphate instead of tris-oxalate to facilitate kinetic analysis of the data. Increasing the free Ca^{2+} concentration produced an increase in the initial rate of Ca^{2+} -uptake in the microsomal S.R. to a maximum at $10\mu\text{M}$ Ca^{2+} free. Calmodulin stimulated the initial rate of Ca^{2+} -uptake at all free Ca^{2+} -concentrations tested; maximal stimulation of Ca^{2+} -uptake by calmodulin occurred at $5.0\mu\text{M}$ free Ca^{2+} . Figure 16 indicates the calcium uptake velocity obtained in the presence and absence of calmodulin at the various free Ca^{2+} -concentrations studied. Reciprocal plots of this data indicate that calmodulin significantly increased the $V_{\text{Ca}^{2+}}$ of the Ca^{2+} -transport system ($3.07\text{ nmoles/mg/min}$ in the absence of calmodulin and $4.65\text{ nmoles/mg/min}$ in the presence of calmodulin) without changing the apparent K_d for Ca^{2+} ($0.791\mu\text{M}$ in the absence of calmodulin and $0.619\mu\text{M}$ in the presence of calmodulin).

5) Effect of Microsomal S.R. Extracts On Calcium Uptake Activity in Cardiac Microsomal Preparations Enriched in Sarcoplasmic Reticulum:

In order to determine whether calmodulin was indigenous to the S.R. membrane, attempts were made to extract calmodulin from microsomal S.R.. Using methodology developed for isolating calmodulin from other sources (described in table 5) extracts of microsomes enriched in S.R. were prepared. As shown in table 5 when these extracts were added back to intact microsomal S.R., both in the presence and absence of KCl, no stimulation of Ca^{2+} -uptake occurred. This would suggest that calmodulin was not present in the microsomal preparations routinely used. As a control, a calmodulin sample was subjected to the same boiling and isolation procedures. The ability of this sample to stimulate Ca^{2+} -uptake in microsomal S.R. was unaltered by this procedure.

Figure 14

Effect of Calmodulin on the Initial Rate of Calcium Uptake in Microsomal Preparations Enriched in Sarcoplasmic Reticulum.

Ca^{2+} -uptake was measured as described in methods at various times such that no greater than 5% of the total Ca^{2+} present was accumulated by the microsomes during the incubation period. Ca^{2+} -uptake rates are indicated by their respective plots in the presence (●—●) and absence (○--○) of calmodulin (2 μg /0.5 ml final incubation volume) for each free Ca^{2+} concentration used.

This result is typical of 3 experiments performed.

Ca^{2+} -uptake activity in these experiments, in the absence of calmodulin at 1 μM free Ca^{2+} , ranged from 3.75 to 4.47 nmoles/mg/min.

Figure 15

Velocity of Calcium Uptake in Cardiac Microsomal Preparations
Enriched in Sarcoplasmic Reticulum in the Presence and Absence
of Calmodulin

Ca^{2+} -uptake was measured as described in methods at various times in the presence of varying free calcium concentrations and the initial rates of Ca^{2+} -uptake determined as described in methods in the presence (●—●) and absence (○---○) of calmodulin (2 μg /0.5 ml final incubation volume). The insert shows a reciprocal plot of this data.

This result is typical of 3 experiments performed.

Ca^{2+} -uptake activity in these experiments, in the absence of added KCl and calmodulin at 1 μM free Ca^{2+} , ranged from 3.07 to 5.93 nmoles/mg/min.

Table 5

Effect of Microsomal Extracts on Calcium Uptake Activity in
Cardiac Microsomal Preparations Enriched in Sarcoplasmic Reticulum.

<u>Extract Volume (1)</u>	<u>Calcium Uptake as Percent of Control</u>		
	<u>Method A</u> ⁽¹⁾	<u>Method B</u> ⁽²⁾	<u>Method C</u> ⁽³⁾
a) Absence of KCl			
0	100	100	100
10	95.5	88.1	95.9
20	95.6	88.4	93.1
40	107.7	80.6	92.0
50	88.9	88.7	92.0
b) Presence of 110 mM KCl			
0	100	100	100
10	93.3	97.9	104.7
20	95.1	95.8	96.5
30	91.9	92.5	95.5
50	97.0	91.5	94.2

(1) concentrated microsomal S.R. (1.4 mg protein/ml) was boiled gently for 5 min in the presence of 0.6 mM EGTA. The preparation was centrifuged at 40,000g for 30 min and the supernatant desalted by serial dilution through an Amicon PM-10 filter.

((2) same procedure as (1), except the supernatant was desalted by passage through a Sephadex^r G-15 column (2 30 cm).

(3) same procedure as (1), except the microsomal sarcoplasmic reticulum was boiled in the presence of 1 mM EGTA.

Experiments were also performed in an attempt to remove any indigenous calmodulin during the preparation of the microsomal S.R.. Treatment with either 0.02% Triton X-100, 0.02% EGTA, or 9 M Urea in 75 mM tris-Cl before the last tris-Cl wash resulted in a total loss of the ability of the microsomal S.R. to transport Ca^{2+} .

6) Binding of ^{125}I -labelled Calmodulin to Cardiac Microsomes Enriched in Sarcoplasmic Reticulum:

^{125}I -labelled calmodulin was synthesized by the method of Hunter and Greenwood (1962) as described in the methods. Using a Sephadex[®] G-25 column the synthesized ^{125}I -calmodulin and free ^{125}I could be separated due to differential elution. Figure 17 shows the elution profile obtained, the first peak representing the fractions containing ^{125}I -labelled calmodulin. The four fractions that comprised this peak were then pooled and diluted 4 times. As determined by 6% TCA protein precipitability, 49.9% of the total ^{125}I present in these fractions was bound to calmodulin. It was decided that further removal of free ^{125}I from the fractions was unnecessary since in control studies it was found that there was negligible binding of free ^{125}I to cardiac microsomes enriched in S.R.

Initially, measurement of ^{125}I -calmodulin binding to cardiac microsomal S.R. was performed in a similar manner to the Ca^{2+} -uptake assay, except that ^{125}I -calmodulin was substituted for ^{45}Ca in the incubation medium. However, a high degree of non-specific binding of ^{125}I -calmodulin to the filter (Millipore[®]) occurred; the binding of ^{125}I -calmodulin to the filter could be altered by varying the concentration of EGTA present in the incubation medium. Therefore, as an alternate method, the binding technique described in the methods was developed. Using this technique a Ca^{2+} -concentration dependent increase in ^{125}I -calmodulin binding occurred (figure 18). This binding was decreased in the presence of monovalent cations, with 110 mM KCl and 110 mM NaCl having the greatest effect. Table 6 shows the effect of monovalent cations on ^{125}I -labelled binding at 10^{-6} M free Ca^{2+} , the Ca^{2+} concentration used in the majority of the previous studies. Binding of ^{125}I -calmodulin to microsomes enriched in S.R.

was significantly decreased in the presence of 110 mM KCL and 110 mM NaCl ($p < 0.05$, students "t" test) at all free Ca^{2+} concentrations above 10^{-7} M. In the presence of 110 mM LiCl binding was decreased, but was not significantly different from that degree of binding observed in the controls, except at 10^{-7} M free Ca^{2+} concentrations.

In these experiments there was a substantial amount of non-specific binding of ^{125}I -calmodulin to the microsomal S.R.. The amount of ^{125}I -calmodulin which would bind to the microsomal S.R. inactivated by boiling was found to be 64% of that which would bind to intact microsomal S.R. in the absence of Ca^{2+} . However, the binding of ^{125}I -calmodulin to inactive S.R. was not Ca^{2+} -concentration-dependent. Therefore this non-specific binding was subtracted from the values obtained in the results shown (figure 18, table 6).

Figure 16

Separation of Fractions Containing ^{125}I -labelled Calmodulin From
Free ^{125}I Using G-25 Sephadex[®] Column Chromatography

Iodination and isolation of calmodulin are as described in methods. Fractions (8 drops) were counted for 1 second on a Gamma spectrophotometer. The first peak represents ^{125}I -calmodulin eluted from the column, while subsequent peaks consist of free ^{125}I eluted from the column.

Figure 17

Effect of Monovalent Cations on ^{125}I -calmodulin Binding to Cardiac Microsomes Enriched in Sarcoplasmic Reticulum.

^{125}I -calmodulin binding, expressed as CPM/mg sarcoplasmic reticulum protein, was determined in the absence of monovalent cations ($\bullet\text{---}\bullet$), and in the presence of 110 mM KCl ($\circ\text{--}\text{--}\circ$), 110 mM NaCl ($\Delta\text{---}\Delta$), or 110 mM LiCl ($\blacktriangle\text{.....}\blacktriangle$). Result shown is the mean of 3 experiments (maximum variability $\pm$ 1800 counts/mg protein). Non-specific binding of ^{125}I -calmodulin to denatured microsomal S.R., which is equivalent to 64% of the counts obtained in the absence of added Ca^{2+} , was subtracted from the total counts obtained for each incubation sample.

Table 6

Effect of Monovalent Cations on ^{125}I -calmodulin Binding to
Cardiac Microsomes Enriched in Sarcoplasmic Reticulum

<u>Incubation Conditions</u>	<u>Counts ^{125}I-calmodulin/mg S.R. Protein</u>	
	<u>10^{-6} M Ca^{2+}</u>	
Control	7,402 $\pm$ 1795	(5)
110 mM LiCl	6,598 $\pm$ 1595	(3)
110 mM NaCl	4,768 $\pm$ 1582	(3) ^a
110 mM KCl	4,423 $\pm$ 1611	(3) ^a

a-significantly different ($p < 0.05$, student 't' test) than control
- ^{125}I -calmodulin binding as described in the methods was performed
at 1 μM free Ca^{2+} .

-bracketed numbers indicate the "n" value.

DISCUSSION

1) Activity and Purity of our Red Cell Calmodulin:

The present study was carried out to characterize the regulatory role of calmodulin on Ca^{2+} -transport in cardiac S.R.. In order to do this it is necessary to use an active and relatively pure calmodulin preparation. In this study, calmodulin activity was determined by its ability to stimulate Mg^{2+} - Ca^{2+} -ATPase activity in EDTA-washed red cell membranes. At 50 μM free Ca^{2+} , maximal concentrations of calmodulin resulted in a 236% increase in Mg^{2+} - Ca^{2+} -ATPase activity (table 4). This is comparable to results obtained by a number of other researchers. Katz et al (1979) showed that maximal calmodulin concentrations would stimulate Mg^{2+} - Ca^{2+} -ATPase 111% in identical membranes, at 0.1 μM free Ca^{2+} . Niggli et al (1979) using EDTA treated membranes, which they refer to as hypotonic ghosts, obtained a 301% stimulation of Mg^{2+} - Ca^{2+} -ATPase activity in the presence of 2 μg calmodulin/mg ghost protein and 50 μM Ca^{2+} . Earlier studies by Gopinath and Vincenzi (1977) using red cell membranes prepared in low osmolarity imidazole buffer showed that calmodulin derived either from red cells or brain tissue would stimulate Mg^{2+} - Ca^{2+} -ATPase activity 370%. In all of the above studies the red cell ghosts were prepared such that during isolation some, if not all, of the calmodulin normally associated with the membrane was freed.

When determining the purity of the sample preparation using SDS-polyacrilamide gel electrophoresis, calmodulin migrated to the apparent MW of 18,000. Vanaman et al (1977) determined the amino acid dequence of calmodulin and arrived at a MW of 16,723. The discrepancies in these two values is probably due to the ability of EGTA or Ca^{2+} to alter the mobility of calmodulin on SDS gels (as discussed in the introduction). Molecular weight determination of calmodulin by a number of other researchers using SDS-PAGE have produced values that vary between 15,000 and 19,200 MW (Wolff and Brostrom, 1979). The fact that our samples co-migrate with calmodulin prepared by other workers (see results) suggests that the 18,000 MW band is indeed calmodulin. The small amount of a 50,000 MW to 65,000 MW contaminant protein often

seen in the sample has not been identified. Earlier preparations, which contained larger quantities of this protein were found to inhibit Ca^{2+} -transport in cardiac microsomal S.R. once the maximal stimulatory concentration of calmodulin was exceeded. This result led to the conclusion that the 51,000 MW or 65,000 MW peak in calmodulin preparation A (figure 6) may have been an inhibitor of calmodulin or Mg^{2+} - Ca^{2+} -ATPase. Au (1978) reported that a 56,000 MW and 35,000 MW inhibitor of Mg^{2+} - Ca^{2+} -ATPase could be isolated from pig red cell hemolysate along with the Mg^{2+} - Ca^{2+} -ATPase activator; similar to our results, if the preparations were diluted the effect of the inhibitor was masked by the activator which was present in higher concentrations. As our study progressed and experimental techniques improved the amount of contaminant protein in the calmodulin preparation was decreased or totally eliminated (figure 6, calmodulin preparation B).

2) Effect of Storage on Calmodulin Activity

During the course of this study a commercial calmodulin preparation became available from the Sigma[®] Chemical Company. When compared to our calmodulin preparation it displayed a decreased potency in the stimulation of Mg^{2+} - Ca^{2+} -ATPase activity in EDTA-washed erythrocyte membranes (table 3) and Ca^{2+} -transport in microsomal S.R. (figure 9). The decreased activity of Sigma[®] calmodulin was probably due to the lyophilization and storage of the preparation. We have found that lyophilization of a solution containing calmodulin and storage at room temperature, or -20°C , results in a substantial loss in activity. A pure lyophilized calmodulin sample obtained from Dr. Penniston which was stored at room temperature showed a similar decrease in potency. When subjected to SDS-PAGE the presence of smaller MW breakdown products was detected. Kretsinger (1980) reported similar results using calmodulin prepared in his laboratory; in order to maintain calmodulin activity over time it was necessary to store calmodulin at -70°C . The presence and absence of Ca^{2+} in the calmodulin preparation during storage can also alter activity. Since our calmodulin samples were not lyophilized we did not encounter any problems with decreases in

potency over time.

3) Calmodulin Stimulation of Ca^{2+} -transport in Cardiac Microsomes Enriched in Sarcoplasmic Reticulum:

As mentioned earlier, previous work in this laboratory has shown that calmodulin will stimulate Ca^{2+} -transport in cardiac S.R. (Katz and Remtulla, 1978). This has been verified in this study, as well as by the work of LePeuch et al (1979), Kranias et al (1980), and Carafoli et al (1980). LePeuch et al (1979) have shown that calmodulin will catalyze a membrane bound protein kinase phosphorylation of phospholamban, the same protein phosphorylated by cAMP-dependent protein kinase. They also showed that the phosphorylation due to the two kinases occurred at distinct sites on phospholamban. Kranias et al (1980) have obtained similar results. LePeuch et al therefore postulate that calmodulin stimulates cardiac S.R. Ca^{2+} -transport through the stimulation of a Ca^{2+} -dependent, cyclic AMP-independent protein kinase that phosphorylates phospholamban. This explanation differs from the explanation presented by our laboratory (Lopaschuk et al, 1980), although we did not measure phosphorylation under conditions that would enable us to verify this result. One of the reasons LePeuch et al postulated this mechanism of calmodulin action was that they could not show an increase in Mg^{2+} - Ca^{2+} -ATPase activity in the presence of calmodulin. Unlike LePeuch et al we have shown in our laboratory that calmodulin will stimulate Mg^{2+} - Ca^{2+} -ATPase activity in cardiac S.R.. Kranias et al have since confirmed this result. Therefore, calmodulin may be interacting directly with the ATPase enzyme itself, rather than through the phosphorylation of phospholamban.

4) Effect of Monovalent Cations on Ca^{2+} -transport in Cardiac S.R.

Early on in the study it was determined that monovalent cations, especially K^{+} , had a pronounced effect on the ability of calmodulin to stimulate Ca^{2+} -transport in cardiac microsomal S.R.. It was decided to further investigate this finding since there is strong evidence that K^{+} is also a regulator of S.R. Ca^{2+} -transport (Shigekawa and Pearl, 1976; Jones et al, 1978; Shigekawa and Akowitz, 1979; and Yamada and Ikemoto, 1980). In the presence of 110 mM KCl calmodulin will

stimulate (between 10-40%) Ca^{2+} -transport in microsomal S.R. at various free Ca^{2+} concentrations (figure 8). If KCl is replaced with an equimolar sucrose solution, calmodulin stimulates Ca^{2+} -transport 100-240% at the same free Ca^{2+} -concentrations. It therefore appears that K^+ is decreasing calmodulin stimulation of S.R. Ca^{2+} -uptake. This can also be observed if Ca^{2+} -transport into microsomal S.R. is measured at increasing KCl concentrations in the presence and absence of calmodulin (figure 9). The possibility exists that K^+ maximally stimulates Ca^{2+} -transport in S.R. and that addition of calmodulin would therefore not result in a further increase. This seems unlikely, however, since cAMP-dependent protein kinase which also stimulates Ca^{2+} -transport in S.R. will significantly increase Ca^{2+} -uptake in the presence of K^+ . This suggests that the presence of K^+ does not maximally stimulate Ca^{2+} -transport.

When studying the effect of KCl on calmodulin stimulation of Ca^{2+} -uptake by microsomal S.R. the effect of the Cl^- concentration must be considered. Kasai and Miyamoto (1976), as well as Campbell and Shamoo, (1980) have demonstrated that chloride ions will release Ca^{2+} from isolated sarcoplasmic reticulum vesicles. Therefore, increasing the Cl^- concentration by increasing the KCl levels could result in an increased Ca^{2+} release from S.R.. It is possible that calmodulin stimulation of Ca^{2+} -uptake although appearing to be inhibited by KCl could be due to an increased Ca^{2+} -release from S.R. by chloride ions. This seems unlikely since replacement of KCl with NaCl or LiCl results in a smaller stimulation of Ca^{2+} -uptake, as well as a less pronounced decrease in calmodulin stimulation of Ca^{2+} -uptake, while Cl^- concentrations remain the same. One has to be careful in interpreting these results, however, since Inesi and Malan (1976) reported that Ca^{2+} can be released from isolated S.R. when K^+ is exchanged for a less permeable cation. Therefore, the less pronounced effect of Na^+ and Li^+ on Ca^{2+} -accumulation may be a result of an increase in Ca^{2+} release due to decreased permeability of these ions. An increase in Ca^{2+} release by NaCl or LiCl would not, however, explain why in the presence of these ions a less pronounced decrease in the stimulation of Ca^{2+} -uptake by calmodulin was observed. It thus seems likely that a more direct inter-

action exists between calmodulin and monovalent cation regulation of S.R. Ca^{2+} -accumulation.

Studies in this laboratory have shown that calmodulin will increase the turnover rate of the S.R. Ca^{2+} -pump (Lopaschuk et al, 1980). this is due to an increased decomposition of the phosphoprotein intermediate which is the rate limiting step of the Mg^{2+} - Ca^{2+} -ATPase reaction sequence. Work done by Shigekawa and Pearl (1976) and Jones et al (1978) has shown that K^{+} also increases the turnover of the phosphorylated intermediate of the Ca^{2+} -pump. The possibility exists that calmodulin and K^{+} act in a similar manner to stimulate Ca^{2+} -uptake. The decreased stimulation of the Ca^{2+} -pump by calmodulin in the presence of KCl may be due to the similar mechanism of action of these two potential regulators. Since the S.R. under physiological conditions is bathed in high K^{+} , calmodulin normally may not stimulate Ca^{2+} -transport. Therefore the physiological role of calmodulin may be as a 'backup' to K^{+} regulation of S.R. Ca^{2+} -transport. Under certain conditions such as disease states where K^{+} levels are decreased calmodulin may then become important in regulating the Ca^{2+} -pump.

5) Kinetic Properties of Calmodulin and K^{+} Regulation of Ca^{2+} -transport in Cardiac S.R.:

In cardiac microsomes enriched in sarcoplasmic reticulum calmodulin stimulated Ca^{2+} -transport mainly by increasing the $V_{\text{Ca}^{2+}}$ and had no significant effect on the apparent K_d for Ca^{2+} (figure 12). This differs from results obtained in inside-out erythrocyte vesicles where calmodulin not only increased the V_{max} but also caused a shift in the K_{diss} for Ca^{2+} from high to low (Sarkadi et al, 1978; MacIntyre and Green, 1978). The effect of calmodulin on erythrocyte membrane Mg^{2+} - Ca^{2+} -ATPase parallels its effect on erythrocyte Ca^{2+} -transport. Membranes prepared using EDTA, which have a Mg^{2+} - Ca^{2+} -ATPase activity of both low and high affinity (Schatzman, 1975; Quist and Roufogalis, 1975), will shift to the high Ca^{2+} -affinity form in the presence of calmodulin (Jarrett and Penniston, 1978; Katz et al, 1979). In cardiac microsomes enriched in S.R. an effect of calmodulin on the K_{diss} for

Ca^{2+} may depend on the method of preparation of the microsomes. However, due to the sensitivity of the microsomes, our attempts to prepare the S.R. vesicles in the presence of EDTA or EGTA resulted in complete loss of Ca^{2+} -uptake activity. The effect of calmodulin on the $V_{\max}$ and K_d for Ca^{2+} of the Mg^{2+} - Ca^{2+} -ATPase activity in S.R. has not been determined.

When comparing the kinetics of Ca^{2+} -translocation, it cannot be assumed that changes in Ca^{2+} -activation and Ca^{2+} -affinity produced by calmodulin in erythrocyte membranes would also result in S.R.. Indeed, calmodulin has been shown to have varying effects on the kinetic parameters of other enzyme systems. In investigations of cyclic AMP-dependent phosphodiesterase, calmodulin has been reported to either lower the apparent K_m for cAMP (Brostrom and Wolff, 1976), effect solely the $V_{\max}$ (Gnegy et al., 1976), or effect both kinetic parameters (Uzonov et al., 1976). Calmodulin increases the $V_{\max}$ of adenylate cyclase without changing the apparent K_m for ATP (Lynch and Cheung, 1979). It has also recently been reported by Lynch and Cheung (1979) that stimulation of erythrocyte Mg^{2+} - Ca^{2+} -ATPase by calmodulin is due to an increase in $V_{\max}$ with only a small alteration of the apparent K_m for ATP. Therefore, it does not seem improbable that calmodulin could increase the $V_{\max}$ of Ca^{2+} -transport in cardiac S.R. without altering the K_d for Ca^{2+} .

Since K^+ and calmodulin both appear to regulate Ca^{2+} -transport in S.R. the effect of K^+ activation on the kinetic parameters of the transport process was simultaneously investigated. Unlike calmodulin, K^+ not only increased the $V_{\text{Ca}^{2+}}$, but also increased the apparent K_m for Ca^{2+} (figure 13). The decrease in Ca^{2+} -affinity in the presence of K^+ is similar to the results obtained by Yamado and Ikemoto (1979), as well as Shigekawa and Akowitz (1979), in studies on the Mg^{2+} - Ca^{2+} -ATPase activity of skeletal muscle S.R.. They demonstrated that K^+ will shift the ADP-insensitive Mg^{2+} - Ca^{2+} -ATPase phosphorylated intermediate (E-P) towards the ADP-sensitive phosphorylated intermediate. They also demonstrated that of the two forms of the E-P the ADP-insensitive form has the higher affinity for Ca^{2+} . Therefore, the addition of K^+ will lower the affinity of the enzyme system for Ca^{2+} . Since Mg^{2+} - Ca^{2+} -ATPase plays an integral role in cardiac muscle S.R.

Ca^{2+} -transport, it would be expected that K^+ would also lower the Ca^{2+} -affinity of the transport process. Although these researchers did not investigate cardiac S.R. it has been shown by Jones et al (1977) that the overall effects of K^+ on cardiac and skeletal muscle S.R. Ca^{2+} -transport are similar.

6) Effect of Calmodulin and cAMP-dependent Protein Kinase on the Initial Rate of Ca^{2+} -transport in Cardiac S.R.:

In this study the effect of calmodulin on the initial rate of S.R. Ca^{2+} -transport was also determined. Phosphate was used as the precipitating anion to overcome problems associated with the use of oxalate (Hicks et al, 1979). At all Ca^{2+} concentrations tested it was shown that calmodulin increased the initial rate of Ca^{2+} -transport (figure 15). A double reciprocal plot of the initial rates vs Ca^{2+} -concentration (figure 16) confirmed that calmodulin alters the initial rate of Ca^{2+} -transport by increasing the $V_{\text{Ca}^{2+}}$ rather than the apparent K_d for Ca^{2+} .

Since calmodulin and cAMP-dependent protein kinase both increase phosphorylation of phospholamban the kinetic parameters of Ca^{2+} -transport stimulation in cardiac S.R. were compared. Hicks et al (1979) determined the effect of cAMP-dependent protein kinase on the initial rate of Ca^{2+} -transport using the same procedure utilized in our laboratory. Similar to results obtained with calmodulin, cAMP-dependent protein kinase stimulated the initial rate of Ca^{2+} -transport at all Ca^{2+} concentrations tested. However, cAMP-dependent protein kinase, unlike calmodulin, not only increased the $V_{\text{Ca}^{2+}}$ but also decreased the apparent K_m for Ca^{2+} . They suggest that the higher affinity for Ca^{2+} is due to a decrease in the positive co-operativity between the two Mg^{2+} - Ca^{2+} -ATPase Ca^{2+} binding sites. Tada et al (1979) demonstrated that phosphorylation of phospholamban by cAMP-dependent protein kinase did not alter the amount of Mg^{2+} - Ca^{2+} -ATPase phosphoenzyme (E-P), but did accelerate the turnover rate of the calcium pump. Studies in our laboratory (Lopaschuk et al, 1980) have shown that calmodulin also increased the turnover rate of the Ca^{2+} -pump, but this was accompanied

by a slight decrease in E-P levels. Kranias et al (personal communications) have also found that cAMP-dependent protein kinase increased E-P levels in cardiac S.R. preparations. Therefore, even though calmodulin and cAMP-dependent protein kinase can enhance Ca^{2+} -transport the mechanism of this regulation may not be the same.

7) Isolation of Calmodulin from Microsomal Cardiac S.R.:

Calmodulin has previously been isolated from cardiac muscle (Teo et al, 1973) and has been found to be associated with both particulate and supernatant fractions in various tissue homogenate (Kakiuchi et al, 1978). In most systems, calmodulin is loosely associated with membranous or cytoplasmic proteins, and requires the presence of Ca^{2+} for binding (Cheung, 1971). Removal of Ca^{2+} with agents such as EGTA results in dissociation of calmodulin from these proteins. Treatment of membranes in muscle homogenates with high salt concentrations, urea, or TCA will also result in translocation of calmodulin from the particulate to the soluble fraction. In microsomal S.R., the presence of calmodulin associated with the particulate fraction could alter the kinetic parameters of Ca^{2+} -transport as determined by addition of exogenous calmodulin. Therefore it was necessary to determine if the microsomal cardiac S.R. used in this study contained any indigenous calmodulin. Intact microsomal S.R. was subjected to procedures used to isolate calmodulin from other sources (DePaoli-Roach et al, 1979; Au, 1978, Jung, 1978). Boiling in the presence of EGTA would result in solubilization of any calmodulin present. Table 5 indicates that calmodulin could not be isolated from our microsomal preparation. This would indicate that the characterization of calmodulin stimulation of Ca^{2+} -transport in cardiac S.R. preparations is not complicated by the presence of indigenous calmodulin. Since the microsomal S.R. is treated with 0.6 M KCl during preparation it is likely that any calmodulin that may have been associated with the S.R. was solubilized. Carafoli et al (1980) were able to stimulate S.R. Mg^{2+} - Ca^{2+} -ATPase with a heat-treated extract from microsomal cardiac S.R.. However, the microsomal preparation had not previously been treated with high salt concentrations.

8) Calmodulin Binding to Microsomal Cardiac S.R.:

Since calmodulin does not appear to be an integral component of the S.R. it was postulated that binding to a site on the membrane must occur in order for calmodulin to augment Ca^{2+} -transport. It is possible that the monovalent cations decrease calmodulin stimulation of Ca^{2+} -transport by altering this binding. Experiments were therefore performed using ^{125}I -labelled calmodulin to determine the degree of binding to microsomal preparations in the presence and absence of K^+ (110 mM), Na^+ (110mM), and Li^+ (110 mM). Initial experiments, which utilized 0.45 μm Millipore[®] filters to separate bound from free ^{125}I -calmodulin, showed that calmodulin exhibited a high degree of non-specific binding. This non-specific binding was enhanced by increasing EGTA concentrations. A possible explanation is that an alteration in the conformation of calmodulin due to decreased Ca^{2+} levels (Klee, 1977) may either enhance binding to the filter, or obstruct the passage of unbound calmodulin through the filter. When a revised technique was developed to measure calmodulin binding to S.R. without utilization of filters (as described in methods) there was still a substantial amount of calmodulin binding in the absence of Ca^{2+} . This is probably due to a high degree of hydrophobic binding to membrane proteins as suggested by Storm et al (1980). Therefore measurement of specific calmodulin binding to microsomal S.R. was corrected for non-specific binding by subtracting calmodulin bound to microsomal S.R. inactivated by boiling. Binding of ^{125}I -labelled calmodulin to inactive microsomal S.R. was Ca^{2+} -independent, suggesting that it was indeed non-specific in nature.

As indicated in figure 18 ^{125}I -labelled calmodulin binds to microsomal preparations enriched in S.R. in a Ca^{2+} concentration dependent manner. Penniston et al (1980) has demonstrated that ^{125}I -labelled calmodulin will also bind to purified erythrocyte membrane Mg^{2+} - Ca^{2+} -ATPase in a Ca^{2+} -concentration-dependent manner. Since calmodulin stimulates Mg^{2+} - Ca^{2+} -ATPase and Ca^{2+} -transport in both cardiac S.R. and erythrocyte membranes it is possible that calmodulin may be binding to the Mg^{2+} - Ca^{2+} -ATPase enzyme in cardiac S.R. as well.

At Ca^{2+} concentrations (10^{-6} - 10^{-4} M) where monovalent cations were shown to decrease calmodulin stimulation of S.R. Ca^{2+} -transport there is a significant decrease in calmodulin binding in the presence of K^{+} (110 mM) or Na^{+} (110 mM). In the presence of Li^{+} (110 mM), which has a less pronounced inhibitory effect on calmodulin stimulation of S.R. Ca^{2+} -transport, there is less alteration in ^{125}I -calmodulin binding (table 6). This suggests that the monovalent cations decrease calmodulin augmentation of Ca^{2+} -transport in S.R. by decreasing the binding of calmodulin to the S.R. membrane. Since K^{+} and calmodulin both increase the turnover rate of the Ca^{2+} -pump it is conceivable that they both may be competing for similar binding sites on the S.R. membrane. Jones *et al* (1978) have suggested that K^{+} acts by binding directly to the Mg^{2+} - Ca^{2+} -ATPase enzyme in S.R.. Therefore, calmodulin may also stimulate cardiac S.R. Ca^{2+} -transport by binding directly to the Mg^{2+} - Ca^{2+} -ATPase enzyme. On the other hand, the monovalent cations may be inhibiting calmodulin binding less specifically. Wolff and Brostrom (1979) have shown that KCl can cause an ionic strength-dependent conformational change in calmodulin. This non-specific conformational change may alter calmodulin binding to the S.R. membrane. However, this hypothesis does not explain why equal ionic strengths of LiCl would not evoke similar conformational changes.

When analyzing the results of these calmodulin binding studies one must be cautious in interpreting the data. Although the specific activity of the pure calmodulin was determined **prior** to iodination the small yield of ^{125}I -labelled calmodulin made it impossible to insure that calmodulin retained activity subsequent to iodination. As well, high levels of non-specific ^{125}I -labelled calmodulin binding make it difficult to distinguish physiological relevant calmodulin binding to non-specific hydrophobic interaction. Future experiments should concentrate on developing methodology which would enable one to determine the specific activity of ^{125}I -labelled calmodulin, as well as decreasing the levels of non-specific calmodulin binding.

9) Calmodulin and its role in Ca^{2+} -transport in Cardiac S.R.:

Evidence presented in this study, and the work of other researchers (Katz and Remtulla, 1978; LePeuch *et al*, 1979; Kranias *et al*, Carafoli *et al*, 1980), strongly suggest that calmodulin plays a regulatory role in cardiac S.R. Ca^{2+} -transport. Along with K^{+} and cAMP, calmodulin increases the turnover rate of Mg^{2+} - Ca^{2+} -ATPase in S.R. membrane. Figure 19 is a schematic representation of the possible mechanism by which these regulators act. Cyclic AMP activates cAMP-dependent protein kinase, which phosphorylates an S.R. membrane protein, phospholamban, resulting in an increased rate of decomposition of the phosphorylated intermediate of Mg^{2+} - Ca^{2+} -ATPase (E-P). Calmodulin may activate a Ca^{2+} -dependent protein kinase which also phosphorylates phospholamban, resulting in an increased turnover rate of the E-P. As well, calmodulin may act directly on the Mg^{2+} - Ca^{2+} -ATPase enzyme, similar to K^{+} , also enhancing the turnover rate of the E-P. LePeuch *et al* (1979) have suggested that a concerted regulation of cardiac S.R. Ca^{2+} -transport by cAMP and calmodulin exists. Indeed, many other cellular processes exhibit dual regulation by cAMP and calmodulin (Wang and Waisman, 1979). In the complicated regulation of cardiac S.R. Ca^{2+} -transport, K^{+} may be a third regulator which interacts with calmodulin to a greater extent than with cAMP. Regulation of Ca^{2+} -transport by cAMP may become more important during sympathetic stimulation of cardiac muscle, while K^{+} and calmodulin regulation may be more important in the absence of this stimulus. Better models, which represent physiological S.R. Ca^{2+} -transport, must be developed before a hypothesis such as this can be tested.

Figure 18

Possible Mechanisms by Which Calmodulin Regulates the Ca^{2+} -pump of Cardiac Sarcoplasmic Reticulum.

EP, phosphorylated intermediate of Mg^{2+} - Ca^{2+} -ATPase

E, non-phosphorylated intermediate of Mg^{2+} - Ca^{2+} -ATPase

S.R., cardiac sarcoplasmic reticulum

SUMMARY AND CONCLUSIONS

- 1) In the presence of K^+ , calmodulin stimulates Ca^{2+} -transport in microsomal preparations enriched in cardiac S.R. 100 to 250% at physiologically obtainable Ca^{2+} concentrations.
- 2) In the presence of 110mM KCl, calmodulin stimulates Ca^{2+} -transport in microsomal cardiac S.R. 10 to 40% at physiologically obtainable Ca^{2+} concentrations.
- 3) K^+ and Na^+ decrease calmodulin stimulation of Ca^{2+} -transport to a greater degree than Li^+ .
- 4) It therefore appears that monovalent cations, such as K^+ and Na^+ , inhibit calmodulin stimulation of cardiac S.R. Ca^{2+} -transport.
- 5) Calmodulin stimulates Ca^{2+} -transport in microsomal cardiac S.R. by increasing the $V_{Ca^{2+}}$ without altering the apparent K_d for Ca^{2+} , while K^+ increases the $V_{Ca^{2+}}$, as well as increasing the apparent K_d for Ca^{2+} .
- 6) Calmodulin increases the initial rate of Ca^{2+} -transport, by increasing the $V_{Ca^{2+}}$ without altering the apparent K_d for Ca^{2+} . Since cAMP-dependent protein kinase increases the initial rate of Ca^{2+} -transport by increasing the $V_{Ca^{2+}}$ as well as decreasing the apparent K_m for Ca^{2+} it can be concluded that calmodulin and cAMP-dependent protein kinase do not act by similar mechanisms.
- 7) There is no indigenous calmodulin in the microsomal cardiac S.R. used in this study.
- 8) ^{125}I -labelled calmodulin binds to microsomal S.R. in a Ca^{2+} dose dependent manner.
- 9) K^+ (110mM) and Na^+ (110mM) significantly decreased ($p < 0.05$, students "t" test) ^{125}I -labelled calmodulin binding to microsomal cardiac S.R., at calcium concentrations above $10^{-7}M$, while Li^+ (110mM) does not alter calmodulin binding to a significant extent.
- 10) These studies therefore indicate that calmodulin is not an indigenous S.R. protein, but binds in a Ca^{2+} -concentration-dependent manner to sites on the sarcoplasmic reticulum to stimulate Ca^{2+} -transport. This binding is altered by monovalent cations, which also inhibit calmodulin stimulation of Ca^{2+} -transport.

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