STUDIES ON THE REGULATION OF CALCIUM TRANSPORT

IN CARDIAC AND SKELETAL MUSCLE

SARCOPLASMIC RETICULUM

by

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B.Sc., The University of British Columbia, 1979

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

in

THE FACULTY OF PHARMACEUTICAL SCIENCES

DIVISION OF PHARMACOLOGY AND TOXICOLOGY

We accept this thesis as conforming to the

required standards

THE UNIVERSITY OF BRITISH COLUMBIA

SEPTEMBER 1983

ABSTRACT

The sarcoplasmic reticulum (SR) membrane plays a critical role in excitation-contraction coupling in both skeletal and cardiac muscle. In an attempt to examine the function and regulation of SR, we examined the unique role that it plays in both normal and diseased tissue. In our elucidation of the role of the SR in normal dog heart muscle, we attempted to "purify" the crude microsomes enriched in SR according to the method of Jones et al (1979), in order to minimize contamination by sarcolemmal and mitochondrial membranes; both of these membranes have been shown to contain ATPase activity, and may contribute to spurious results. Both sarcolemmal and mitochondrial membrane contamination were decreased in the Ca-oxalate loaded, purified SR preparation. In addition, a 3-5-fold enhancement of both Ca^{++}-uptake and $(Ca^{++}-Mg^{++})$-ATPase activity was observed in the purified preparation compared to crude. When analyzed on SDS-PAGE, the absence of a 95,000 MW protein in the purified preparation was evident. Regulation of the purified SR preparation by cAMP-dependent protein kinase (cAMP-PK) and calmodulin (CAM) appeared identical to the stimulation typically observed in crude preparations with the exception of lower stimulation of Ca^{++}-uptake at higher (1.0 μM) free Ca^{++} concentrations. A comparison of the two preparations with respect to membrane phosphorylation revealed that incubation of either purified or crude SR with cAMP-PK or the catalytic (C) subunit of cAMP-PK resulted in similar time-course profiles. In the presence of CAM, the total level of phosphoprotein

incorporation was decreased in the crude preparation compared
to the purified, and both the intensity and time to complete
phosphorylation of phospholamban were markedly diminished in
purified SR. Nevertheless, both crude and purified SR were
similar in that CAM-dependent phosphorylation was both slower
and decreased compared to that observed with either cAMP-PK or
C subunit incubation.

An examination of the role of CAM in normal rabbit
skeletal SR was undertaken as a result of the disputed claims
of the mode of CAM binding to the skeletal SR membrane. Our
studies revealed that, unlike the claims of Campbell and
MacLennan (1982), a considerable amount of CAM remained bound
to the SR membrane following extensive washing with EGTA.
SDS-PAGE of all supernatants revealed a number of bands which
migrated in the region of CAM (15.5-19.5 Kdaltons); following
high-speed centrifugation of all the supernatants, the
molecular weight bands assumed to be CAM did not appear in the
EGTA-washed fSR or sSR supernatants. An RIA for CAM revealed
that measureable levels of CAM were present in all
supernatants. Higher levels of CAM were released into the
supernatant from sSR than from fSR. In addition, CAM was
liberated from supernatants boiled in the presence or absence
of EDTA regardless of prior EGTA-washing, although the levels
of CAM derived from vesicles that been pre-treated with EGTA
were approximately 33% less than the levels obtained from
vesicles which were not treated with EGTA. CAM RIA revealed
that this EGTA-extractable CAM was not present in the

supernatant of EGTA-washed SR. It was concluded that the stimulator present in boiled supernatants of fast and slow SR was, indeed, CAM, whereas the stimulator reported by Campbell and MacLennan (1982) was most likely CAM that originated from the boiling of contaminant SR membrane fragments present in EGTA-washed SR supernatants.

The final aspect of SR regulation investigated was Ca^{++}-uptake in the skeletal muscle of streptozotocin-diabetic rats. It was found that, similar to the reports in rat cardiac SR, skeletal SR from diabetic rats had significantly depresssed levels of Ca^{++}-uptake, and elevated levels of free carnitine and long-chain acylcarnitines, compared to controls. It was concluded that the diabetic state may result in generalized pathophysiology, a result of which may be the non-specific decrease in Ca^{++}-uptake in SR.

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ACKNOWLEDGEMENTS

TABLE OF CONTENTS

In both heart and skeletal muscle, contraction and relaxation are controlled by the intracellular distribution of Ca^{++}, regulated at least in part by a specialized membranous network surrounding the myofibrils, the sarcoplasmic reticulum (SR). Muscle relaxation is believed to occur when cytosolic Ca^{++} is taken up by the SR, thus lowering the free Ca^{++} concentration below that needed to activate the contractile proteins (10^{-7} M). Muscle contraction, on the other hand, at least in skeletal muscle, is thought to require SR as its source of "activator" Ca^{++} for binding to troponin, the Ca^{++} receptor of the contractile proteins. In cardiac muscle, the internal Ca^{++} store has not been unequivocally localized to the SR and may be associated with SR, sarcolemma, or both. Because of the unique role of the SR in mediating both contraction and relaxation, the kinetics of calcium sequestration and release by this organelle have been intensively investigated.

I Morphology of the SR

The SR is frequently divided into two categories (Sommer and Waugh, 1976) which are distinct morphologically and, possibly, functionally: (1) junctional SR and (2) longitudinal SR. Junctional SR is that portion of SR which closely abuts the plasmalemma at the level of the transverse (T) tubules to form a triad: two elements of junctional SR form couplings (terminal cisternae) on opposite sides of a T tubule. The T tubules, running perpendicular to the SR, are not part of the SR but

are, rather, continuous with the surface membrane. The triad is considered to be a key control point for at least two steps in excitation-contraction coupling: (1) the site of storage and release of activator calcium and (2) the site at which the action potential is translated into a "trigger" for calcium release (Endo, 1977; Fabiato and Fabiato, 1977). Longitudinal or "free" SR represents that SR not associated with the T tubules: it is electronlucent and forms the meshwork surrounding the A and I bands.

The SR membrane itself is a lipid bilayer consisting of several kinds of phospholipids: 60-75% phosphatidylcholine, 10-20% phosphatidylethanolamine, 5-10% phosphatidylserine, 10% phosphatidylinositol, and smaller amounts of other lipids (Meissner and Fleischer, 1971; MacLennan et al, 1971). There are four major types of protein embedded in the membrane: (1) the 100,000 MW ATPase, which participates directly in active Ca^{++} transport and accounts for 60-90% of the total protein in the skeletal SR membrane (Inesi, 1972; Meissner et al, 1973) and 35-40% of protein in cardiac SR (Suko and Hasselbach, 1976), (2) calsequestrin, a 66,000 MW (55,000 MW in cardiac SR; Campbell et al, 1983) highly acidic protein able to bind 900 nmole Ca^{++}/mg protein and thought to be responsible for calcium storage in the SR lumen (MacLennan and Wong, 1971), (3) two high-affinity calcium binding proteins (MW = 53,000 & 160,000), and (4) a low molecular weight proteolipid (12,000 MW). The relative content of these four types of protein varies among the different populations of vesicles isolated by isopycnic

centifugation and may explain the discrepency in estimates of amount of a particular protein. For example, Meissner (1975) distinguished three different fractions of rabbit skeletal SR by sucrose density gradient centrifugation: "heavy", "intermediate", and "light". Subsequent work has revealed that calsequestrin in vitro is found almost entirely in the "heavy" fraction (Caswell et al, 1976; Campbell et al, 1980); in situ, it is found in the terminal cisternae (Campbell et al, 1980). This has led to the conclusion that "heavy" vesicles are derived from the terminal cisternae. Vesicles derived from this SR fraction, then, may cause investigators to overestimate the amount of calsequestrin as a proportion of total protein. It is of interest that immunofluorescence localization studies in skeletal muscle in situ have identified the $(Ca^{++}-Mg^{++})$-ATPase throughout the longitudinal SR but not in terminal cisternae (Jorgensen et al, 1979), suggesting that the former may be largely concerned with calcium uptake, whereas junctional SR, with its large Ca^{++}-calsequestrin store, may function as the site of Ca^{++} release.

II The $(Ca^{++}-Mg^{++})$-ATPase of the SR membrane

Much of the current understanding of the role of SR in mediating muscle relaxation stems from the discovery of an ATP-dependent sequestration of calcium by vesicles derived from skeletal muscle cell homogenates (Hasselbach and Makinose, 1961; 1962; 1963). Vesicles derived from skeletal SR demonstrated a Ca^{++}-stimulated, Mg^{++}-dependent ATPase activity

which was able to transport 2 moles of Ca^{++} inward for each mole of ATP hydrolyzed against a 1000-5000 fold Ca^{++} concentration gradient (Hasselbach and Makinose, 1962). Two different ATPase activities were soon distinguished: a Mg^{++}-dependent or "basal" ATPase which required only Mg^{++} for its activation (Hasselbach, 1964), and a Ca^{++}-stimulated Mg^{++}-dependent ATPase whose activity was correlated with calcium transport. Only the latter will be discussed here.

(A) Structure

The $(Ca^{++}-Mg^{++})$-ATPase was first successfully purified by MacLennan (1970) as a lipoprotein complex. The enzyme has since been shown to be a single polypeptide chain of 100,000 MW which can be cleaved by trypsin into fragments of 50,000 and 45,000 MW (Migala et al, 1973). The latter fragment is cleaved by prolonged digestion into two fragments of approximately 30,000 and 20,000 MW (Stewart et al, 1976). Each of the three fragments has been shown to contain different functional sites; of particular note are the sites of phosphorylation (the 30,000 MW fragment; Thorley-Lawson and Green, 1973) and Ca^{++} binding (the 20,000 dalton fragment; Pick and Racker, 1979).

The enzyme appears to be asymmetrically embedded in the SR membrane, with one part protruding from the cytoplasmic surface while the remainder resides in the lipid bilayer (deMeis, 1981). In addition, it is thought that the ATPase is self-associated in the membrane as oligomers, with each ATPase peptide able to perform the entire catalytic cycle of ATP

hydrolysis and Ca^{++} transport (Moller et al, 1982). Aggregation of the ATPase monomers into the oligomer appears to be promoted by the prevailing lipid environment. This was demonstrated indirectly by the finding that almost complete replacement of the enzyme's endogenous lipids by synthetic lipid (Warren et al, 1974) or nonionic detergents (Dean and Tanford, 1978) resulted in a monomeric ATPase which retained full activity. When detergent was removed (LeMaire et al, 1978) and/or endogenous phospholipids replaced, the ATPase protein self-associated into larger aggregates.

(B) Kinetic Properties of the $(Ca^{++}-Mg^{++})$-ATPase

Hasselbach and Makinose (1962) found that skeletal SR vesicles catalyzed an exchange of inorganic phosphate (Pi) between ADP and ATP in the presence of Ca^{++} and Mg^{++}. This prompted speculation of a high-energy phosphoprotein intermediate (EP) formed during the active transport of Ca^{++}. Kinetic analysis by Yamamoto and Tonomura (1967; 1968) showed that a protein of SR vesicle preparations was phosphorylated when incubated with ^{32}P-ATP and quenched with trichloroacetic acid. Phosphoprotein levels, at steady state, depended on the concentrations of Ca^{++} and ATP and paralleled the Ca^{++}-dependent ATPase activity. When the pre-steady state of the ATPase was followed in skeletal SR by a rapid-mixing device, the reaction intermediate EP was quickly formed in the presence of suboptimal ATP and calcium concentrations, and reached a maximum within 50 msec at $20^{\circ}C$ (Froelich and Taylor,

1976). Inorganic phosphate, assumed to be liberated from EP, was monitored and shown to lag initially (coinciding with the rapid increase in EP formation) and then increase (coinciding with the completion of EP formation). Thus, it is now well established that EP is the true intermediate of the ATPase reaction.

(c) Reaction Mechanism of the $(Ca^{++}-Mg^{++})$-ATPase

Based on the kinetic analyses of EP formation and the release of the products ADP and Pi, a simple reaction mechanism which represented the coupling between the ATPase and calcium transport was suggested by Tada et al (1978):

$$E + ATP + 2Ca(out) \rightleftharpoons E;ATP;Ca_2(out)$$

$$E + ADP + Pi + 2Ca(in) \longleftarrow E\sim P;Ca_2(in) + ADP$$
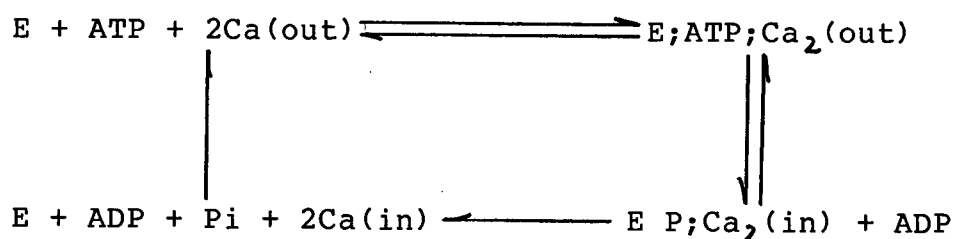
where "in" and "out" represent the inside and outside of the SR membrane vesicles, respectively. Two moles of Ca^{++} and one mole of ATP were thought to bind to one mole of the ATPase enzyme (E) at the outer surface of the membrane, forming the Michaelis complex $E;ATP;Ca_2^{++}$. Calcium is then translocated inside the membrane when EP is formed. Dephosphorylation of EP is accompanied by the release of Ca^{++} from the enzyme into the vesicle interior. This scheme has since been broadened by the findings of (1) at least two forms of EP, an ADP-sensitive (E_1P) and ADP-insensitive (E_2P) (Shikegawa et al, 1978), which exhibit different affinities for Ca^{++}, and (2) high and low

affinity binding sites for Ca^{++} on the SR membrane. A revised mechanism is presented below (Tada and Katz, 1982):

$$E_1;Ca(out) \underset{}{\overset{ATP}{\rightleftharpoons}} E_1;ATP;Ca(out) \underset{}{\overset{ADP}{\rightleftharpoons}} E_1 \sim P;Ca(in)$$

with vertical reactions: left side $E_1;Ca(out) \rightleftharpoons E_2;Mg$ (step (i)) involving Ca^{++}, Mg^{++}; right side $E_1 \sim P;Ca(in) \rightleftharpoons E_2 \sim P;Mg$ (step (iv)) involving Ca^{++}, Mg^{++}

$$E_2;Mg \underset{P_i}{\overset{}{\rightleftharpoons}} E_2;Mg;P_i \underset{H_2O}{\overset{}{\rightleftharpoons}} E_2 \sim P;Mg$$
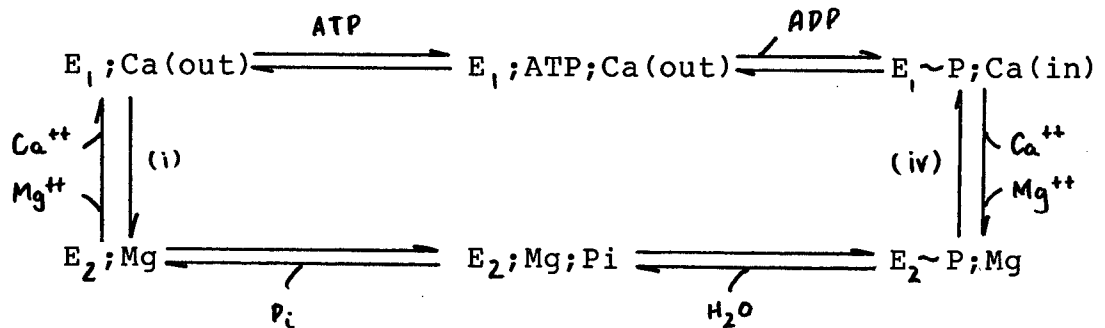
In this scheme, EP is now the sum of E_1P and E_2P and calcium is translocated from outside to inside the membrane when $E_1 \sim$ P;Ca(in) is formed and is released within the vesicle interior when $E_1 \sim P;Ca(in)$ is converted to $E_2 \sim P;Mg$. The two major rate-limiting steps are the conversion of E_2 to E_1 (step i) and the decomposition of $E_1 \sim P$ (step iv), under saturating concentrations of Ca^{++}, ATP, and alkali metals (Na* and K*). Both alkali metals and divalent cations (Ca^{++}, Mg^{++}) accelerate EP decomposition; in the absence of alkali metals, the rate-limiting step becomes E_2P decomposition (step v), and not step iv).

Activation of the skeletal SR pump displays a sigmoidal dependence on the Ca^{++} concentration in the medium, with half-maximal saturation at 0.5 μM Ca^{++} (deMeis, 1971). The Km for calcium in cardiac SR is somewhat higher ~1-2 μM (Shikegawa et al, 1976). Two high-affinity Ca^{++} binding sites have been shown to be exposed on the outer surface of skeletal SR vesicles (Meissner and Fleischer, 1973) and appear to operate

through a cooperative mechanism such that binding of the first Ca^{++} ion results in a protein conformational change which then increases the affinity for binding of a second Ca^{++} ion (Inesi et al, 1980). Once the enzyme becomes phosphorylated, the high-affinity sites are converted to a low-affinity state (Ikemoto, 1974), thereby allowing Ca^{++} to be released inside the vesicles.

Mg^{++} has also been shown to play at least two important roles (Yamamoto et al, 1979): (1) to accelerate the decomposition of EP formed during the reaction (Inesi et al, 1974), and (2) to form an equimolar complex with ATP and serve as the true substrate for the Ca^{++}-dependent ATPase. This latter role of Mg^{++} was inferred by a number of observations: Weber et al (1966) demonstrated that Mg^{++} concentrations that exceeded those of ATP resulted in optimal activity whereas the Ca^{++}-dependency profile of the enzyme was not altered over a wide range of Mg^{++} concentrations. Froelich and Taylor (1975) found that the initial velocity of EP formation was proportional to the amount of Mg-ATP complex. In addition to the requirement of the enzyme for divalent cations, two physical parameters, pH and temperature, also play a critical role. The transition temperature of $20°C$ (Johnson and Inesi, 1969) appears to be related to the phase transition of the SR phospholipid bilayer, while enzymatic activity exhibits a bell-shaped curve with an optimal pH between 6.5-7.5.
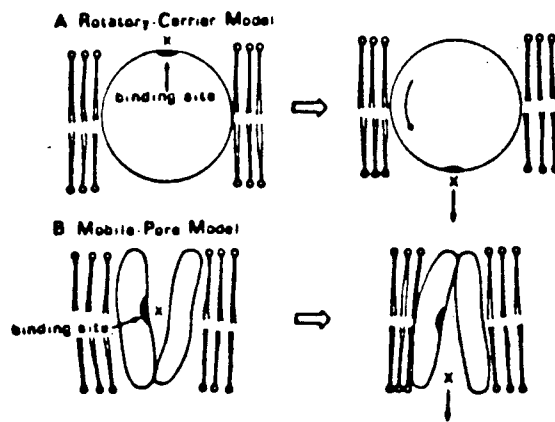
FIGURE 1. Two molecular models for active transport of Ca^{2+}, taken from Yamamoto et al (1979). In each model, X represents the Ca^{2+} to be translocated. (A) Rotary-carrier model. (B) Mobile-pore model. For explanation, see text.

(D) Molecular Models of Calcium Transport

In the process of hydrolytic cleavage of ATP by the SR ATPase, Ca^{++} is translocated from outside to inside the membrane. A number of models of Ca^{++} transport have thus been suggested on the basis of the known structure of the enzyme, and can be divided into two categories (Yamamoto et al, 1979): "rotary" and "mobile pore" models (Figure 1). "Rotary" models propose a rotation within the lipid bilayer able to translocate bound calcium, while "mobile pore" models postulate that the enzyme, through a conformational change, forms a pore through which calcium is transported. Thus far, these models have had little experimental support and have been deemed unsatisfactory primarily due to thermodynamic objections (Tada et al, 1978; Yamamoto et al, 1979).

III Comparative Features of Cardiac and Skeletal Muscle Contractility

(A) Excitation-contraction coupling in cardiac and skeletal muscle

Excitation-contraction (E-C) coupling is essentially the process by which electrical activity at the sarcolemma (the action potential) is translated into calcium release required for contraction. Relaxation, as discussed briefly earlier, results when the free calcium concentration drops below the 10^{-7} M concentration required to activate the contractile

proteins. <u>A priori</u>, there is no reason to expect much
similarity in the E-C coupling in skeletal and cardiac muscle.
Skeletal muscle, typically, is under neural control: force
generation is accomplished by the "voluntary" activation of
motor units. A single neuron may contract as few as half a
dozen fibres or as many as a few thousand. Neural "fine-tuning"
is achieved via a complex interplay of afferent and efferent
polysynaptic feedback loops. The heart, in contrast, does not
have a "voluntary" innervation system but contracts
rhythmically with its entire muscle mass activated in an
"all-or-none" fashion. Action potentials are initiated in
spontaneously depolarizing pacemaker cells in the sino-atrial
node which are then propagated to adjacent cells by low
resistance gap junctions in the intercalated discs (Noble,
1975). Free Ca^{++} concentration does not rise as rapidly as it
does in fast skeletal cells nor does it saturate the
contractile proteins to produce a 100% state of activation in a
single twitch. Incomplete activation can be viewed as
advantageous since the heart's force of contraction is
regulated by a number of factors (which do not affect skeletal
muscle contractility) which allow for a reserve contractile
capacity. These include action potential duration, stretch
(Starling's law), external Ca^{++} and Na^{+} concentration, hormones
and neurotransmitters (Chapman, 1979). Nevertheless, upon
closer inspection, particularly at the level of the contractile
proteins and SR, the E-C coupling mechanism in the two muscle
types appears fundamentally similar. In both cardiac and

skeletal muscle, excitation is initiated by activation of a sodium channel resulting in rapid depolarization. In skeletal muscle, the depolarization is quickly followed by repolarization as sodium current is inactivated and potassium current is activated. The transient action potential "spike" is then transmitted along the sarcolemma, down the T tubular network to the SR which, in turn, releases Ca^{++}. At the cessation of neural stimulation, Ca^{++} begins to be pumped back into the SR. In heart, the sodium channel is similarly inactivated within a few milliseconds, but repolarization does not ensue immediately because of the activation of a slow inward calcium current (i_{si}) which results in the characteristic plateau in the action potential of cardiac muscle.

In cardiac muscle, unlike skeletal muscle, the entry of Ca^{++} from the extracellular fluid is essential for the maintenance of contractility (Ringer, 1883). The estimated intracellular calcium concentration present in the heart with each beat (2 μM) is subthreshold for direct activation of the myofilaments but has led to speculation that an internal pool of activator Ca^{++} is present. Due to the relative sparsity of SR in cardiac muscle (see below) and an increased percentage of surface membrane components (e.g. SL and T tubules) compared to skeletal muscle, both SR and SL and its components have been proposed as the source of activator Ca^{++}.

In skeletal muscle, the role of the SR as the source of activator Ca^{++} for contraction remains largely undisputed.

Studies by Huxley and Taylor (1958) demonstrated that a depolarizing solution administered into the T tubular system of skeletal muscle resulted in a localized contraction at the hemisarcomeres, suggesting that depolarization at the triad led to Ca^{++} release by SR. In cardiac muscle, however, the predominant role of the SR in Ca^{++} release remains controversial for the reasons discussed above. Lullman and Peters (1977), for example, have proposed the the sarcolemma may serve as the site of bound Ca^{++} which could be released upon depolarization. Work by Langer and his associates (Langer and Frank, 1972; Rich and Langer, 1975; Frank et al, 1977) has suggested that the glycocalyx, a layer of glycoprotein on the cell surface, functions as an intermediary Ca^{++} binding store which is in rapid equilibrium with both the extracellular space and the integral proteins which possibly serve as transmembrane Ca^{++} channels. Any change in bound Ca^{++} alters the amount of Ca^{++} which subsequently enters the cell and affects contractility. Langer's work, however, fails to consider the importance of the slow inward Ca^{++} current (Langer et al, 1982), unlike the work of Fabiato and Fabiato (1977) which has incorporated the i_{si} as the trigger Ca^{++} required to induce a more massive release of Ca^{++} from cardiac SR. Although such criticisms as variations in E-C coupling between species and the use of inappropriate in vitro models (e.g. "skinning" of cardiac muscle cells) have been levelled at their work, the "calcium-induced calcium release" hypothesis is now thought to most closely model in vivo E-C coupling in the heart (Wohlfart

and Noble, 1982). The calcium-induced calcium release mechanism does not appear to be operative in skeletal muscle, as demonstrated by Thorens and Endo (1975). The phenomenon could be demonstrated only in the presence of an abnormally low Mg concentration, a high SR Ca^{++} pre-load, and a large (10 μM) Ca^{++} "trigger".

(b) Morphological and biochemical characteristics of skeletal and cardiac SR

Morphological differences in SR between cardiac and skeletal muscle are apparent: ultrastructural studies in mammals have revealed that the SR is less abundant in cardiac than in skeletal muscle, occupying approximately 7% of the myofibrillar volume; this comprises 9-30 times less volume than that found in skeletal muscle (Chapman, 1979). In addition, the T tubular diameter is much larger (5-20 nm) in cardiac muscle (Sommer and Waugh, 1976). Apart from these two major dissimilarities, the overall "geometry" of the SR from both muscle types appears similar. Biochemical studies, however, have tended to dispute this. Ca^{++}-uptake, Ca^{++}-dependent ATPase activity and levels of maximal phosphorylation are consistently 2-4 times lower in cardiac SR than in the comparable activities of fast skeletal muscle (Martonosi, 1972; Shikegawa et al, 1976; Levitzki et al, 1982). A comparison of skeletal and cardiac SR separated on SDS polyacrylamide gels reveals two striking differences: a much higher percentage of total protein represented by the ATPase in skeletal SR (up to 80% vs. 35-40%

in cardiac SR) and a number of additional protein bands in cardiac SR not observed in skeletal SR. The former result has been linked to the finding of a decreased number of ATPase particles, as revealed by freeze-fracture electron microscopy, on the cytoplasmic "P" faces of cardiac muscle SR preparations. The latter finding has prompted speculation that the unaccounted-for additional proteins may play a role in reducing cardiac SR enzymatic activity (Jones and Besch, 1979). Considerable differences have also been observed in the phospholipid and fatty acid components of the the two SR preparations (Hidalgo et al, 1979). It is generally accepted that the major activities of the SR, Ca^{++}-ATPase, and calcium uptake, are markedly influenced by the surrounding phospholipid bilayer. Differences noted include a greater percentage of phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyelin in cardiac SR, while phosphatidylcholine levels are decreased (Chamberlain et al, 1983). Cardiac SR also has increased levels of fatty acids with two or more double bonds (Levitzki et al, 1982).

Other factors have been suggested to account for the reduced enzymatic activities of cardiac SR. These include (i) the lability of the heart muscle ATPase, possibly due to the increased number of membranous and proteinaceous impurities (Levitzki et al, 1976), and (ii) the simple teleological explanation that the cardiac muscle contraction-relaxation cycle of several hundred milliseconds demands an ATPase which subserves somewhat different functions than the enzyme found in

skeletal muscle (Jones and Besch, 1979). Although the amino acid composition of the Ca^{++}-ATPase in dog heart is very similar to that of rabbit skeletal SR (Tada et al, 1977), the calcium-pump protein of the two muscle types was found to be distinct antigenically (DeFoor et al, 1980). Although all of the above may contribute to the discrepency in ATPase function between cardiac and skeletal SR, it is now widely accepted that the principal reason is the lower density of pump sites in cardiac SR as opposed to fast skeletal muscle SR (Jones and Besch, 1979; Chamberlain et al, 1983).

IV. Comparative Aspects of Fast-Twitch and Slow-Twitch Skeletal Muscle

Throughout the preceding discussion, comparison of the properties of cardiac and skeletal muscle (and their respective SR) rested exclusively on a comparison between cardiac and fast-twitch skeletal muscle. This is somewhat misleading since mammalian skeletal muscle consists of both fast and slow fibres, in various proportions, and the differences between slow skeletal and cardiac muscle are less marked than those between the two types of skeletal muscle (Ebashi et al, 1974; Wang et al, 1979). As will be described below, many of the differences between fast and slow skeletal SR are similar to the documented differences between fast-twitch and cardiac SR.

The two general categories of skeletal muscle in mammals, fast-twitch and slow-twitch, have been established to describe first, the time to relaxation (short for fast-twitch and long

for slow-twitch fibres; Close, 1972), and second, resistance to fatigue during repetitive stimulation (low for fast-twitch fibres and high for slow). Both of these physiological variables have biochemical and histochemical correlates (Eisenberg and Kuda, 1976): time to relaxation is associated with myosin ATPase activity and the volume of SR, for example, whereas fatiguability correlates with oxidative and glycolytic capacity. As summarized by Bell et al (1980), fast-twitch fibres have an increased myosin ATPase activity, greater SR volume, a briefer and faster propagating action potential, and larger amounts of glycogen and glycolytic enzymes. Such muscle is capable of short periods of intense activity but fatigues very rapidly with depletion of glycogen stores. This latter point has also distinguished between two types of fast-twitch fibres: those with high glycolytic and oxidative enzyme concentrations ("red" fast muscle, the red associated with a high myoglobin content) or those with predominantly glycolytic enzymes ("white" fast muscle). As a consequence of the absence or presence of oxidative enzymes, the fast muscle will fatigue sooner or later, respectively. In contrast, slow-twitch muscles are more efficient for a given work load: they possess a higher density of capillaries and therefore maintain a high resting blood flow. Typically, this type of muscle is red (again due to it's high myoglobin content) and possesses a high concentration of oxidative enzymes, such as succinic dehydrogenase.

(A) Characteristics of Fast-Twitch and Slow-Twitch SR

As briefly mentioned in the preceding section, the density of the SR membrane is higher in fast-twitch than in slow-twitch skeletal muscle (Luff and Atwood, 1971). Predictably, the density of (Ca^{++}-Mg^{++})-ATPase sites, as determined ultrastructurally, is diminished in slow, as compared to fast skeletal muscle SR (Bray and Rayns, 1976; Jorgensen et al, 1982) which, as in the case of cardiac SR, may explain the decreased Ca^{++} uptake and ATPase activity as well as lower phosphoprotein levels compared to fast SR (Pette and Heilmann, 1979; Zubrzycka-Gaarn et al, 1982). In addition, Borchman et al (1982) have suggested that the decreased Ca^{++}-ATPase activity in slow SR may be due to a decreased bilayer fluidity: levels of cholesterol and sphingomyelin are substantially increased whereas phosphatidylcholine levels are significantly decreased in the slow SR membrane.

Unlike the comparison between cardiac and fast skeletal SR, there are a few intriguing features of the fast versus slow system. First, although the density of the calcium pump protein is decreased in slow SR, the basal (Mg^{++}-ATPase) activity is dramatically elevated (Sreter, 1969). The significance of this is unknown but may mirror the finding of a multitude of protein bands in slow SR not observed in fast SR (Pette and Heilmann, 1977). Second, it has been observed that fast-twitch muscle can be transformed into a slow-twitch-like fibre when subjected to chronic stimulation resembling the firing frequency of a slow motoneuron (Salmons and Vrbova, 1969). The resulting SR preparation obtained from such a fibre

resembles slow SR ultrastructurally and biochemically (Pette and Heilmann, 1977). Van Winkle and Entman (1979) suggest that the transformation may promote altered gene expression such that a different ATPase isozyme, with its associated membranous framework, is manifested.

V. Regulation of the $(Ca^{++}-Mg^{++})$-ATPase in SR

The calcium-pump protein and its associated property of calcium transport is regulated by a variety of factors which appear to be similar in both skeletal and cardiac SR. One prominent regulator is MgATP. As described earlier, Mg^{++} plays at least two important roles in ATP hydrolysis: the acceleration of phosphorylated intermediate decomposition and its association with ATP to serve as true substrate for the enzyme. ATP is able to serve not only as substrate but also as a regulator controlling enzyme activity. Kanazawa et al (1971) demonstrated that steady-state ATP hydrolysis was accelerated by ATP, in that Vmax and Km values increased with ATP concentration. Dupont (1977) has found that the ATPase of skeletal SR has high and low affinity binding sites for ATP, the high affinity site can be phosphorylated while the low affinity site can serve as a regulatory site. Stimulation of ATPase activity by high ATP concentrations appears to be due to an accelerated conversion of E2-El (deMeis and Vianna, 1979).

Calcium ions are also able to alter their own transport by SR. Increases in cytoplasmic Ca^{++} stimulate ATP hydrolysis and concomitant Ca^{++} transport (Hasselbach, 1964). Once transported

into the lumen of the SR vesicle, Ca^{++} also is able to decrease ATP hydrolysis and its own further uptake (Weber, 1971). This effect of Ca^{++} appears to be related to a Ca^{++}-dependent accumulation of E_1P inhibiting the formation of E_2P (Yamada and Tonomura, 1972).

Monovalent cations have a marked stimulatory effect on Ca^{++} transport in both skeletal (Shikegawa and Pearl, 1976) and cardiac (Jones et al, 1977) muscle SR. The alkali metals appear to exert their effect by increasing turnover of the pump, possibly by stimulating the decomposition rate of the ADP-sensitive phosphoenzyme (E_1P) (Shikegawa and Akowitz, 1979). Subtle differences between cardiac and skeletal muscle SR with repect to the influence of monovalent cations have been reported (Wang et al, 1981). These authors report that in Ca^{++}-deficient skeletal SR preparations, pre-incubation with K^+ did not affect initial phosphorylation levels when Ca^{++} and ATP were subsequently added; in cardiac SR, the phosphoenzyme level rose. In addition, K^+ was found to inhibit EP formation in skeletal SR preparations containing bound Ca^{++}, whereas inhibition was only observed in cardiac SR preparations at low ($<1 \mu M$) Ca^{++} concentrations. In vivo, the SR is bathed in a high K^+ concentration (100-175 mM; Sreter, 1963); thus, the physiological relevance of regulation by monovalent cations is not known.

Thus far, I have discussed regulatory features which appear to act in parallel in both cardiac and skeletal muscle. Further treatment of the regulation of SR must now diverge into

more idiosyncratic regulatory mechanisms underlying the two muscle types.

A. Regulation of Cardiac SR

The sarcoplasmic reticulum of cardiac muscle has been shown to possess at least two mechanisms which are not seen in the SR of fast-twitch (and possibly slow twitch; see later) muscle: stimulation of calcium transport, Ca^{++}-dependent ATPase activity, and phosphoprotein formation by (1) cAMP-dependent protein kinase and (2) calmodulin.

(i) Cyclic AMP-dependent protein kinase regulation of cardiac SR

Ever since the discovery that catecholamines (CAs) activated particulate adenylate cyclase preparations from heart in accord with their in vivo potency (Sutherland et al, 1968), it has been postulated that the inotropic action of CAs was mediated by cyclic AMP (cAMP). Cyclic AMP serves as the intracellular second messenger for these regulatory processes in that beta adrenergic activation of a sarcolemmal adenylate cyclase results in an elevation of intracellular cAMP levels (Sutherland and Rall, 1960). This elevation leads to stimulation of cAMP-dependent protein kinases, which consist of a holoenzyme made up of two regulatory and two catalytic subunits (Rosen and Erlichman, 1975). When the regulatory subunits bind cAMP, the catalytic subunits dissociate into free, active forms (Erlichman et al, 1971). Cyclic

AMP-dependent protein kinases, in turn, catalyze the phosphorylation of a number of proteins resulting in functional modifications.

The mechanical response of cardiac muscle to agents which increase intracellular cAMP levels includes (1) an enhanced level and rate of tension development and (2) an increased rate of relaxation (Katz et al, 1975). Suspicions of the involvement of SR in the cAMP-mediated inotropism were confirmed in 1969 by Entman et al who found a stimulation of SR Ca^{++} transport by the cyclic nucleotide. Subsequent work by Kirchberger et al (1972; 1974) and Tada et al (1975) confirmed that both calcium uptake and the Ca^{++}-dependent ATPase of cardiac SR were stimulated three-fold in the presence of cAMP and exogenously added cAMP-dependent protein kinase and were closely correlated with the phosphorylation of a 22,000 MW protein termed phospholamban (Figure 2). The molecular weight of the phosphoprotein has recently been disputed with the suggestion that it is either an 11,000 MW dimer (LePeuch et al, 1979), a trimer of 11,000, 8000, and 4000 daltons (Louis et al, 1982) or a 5500 MW tetramer (Kirchberger and Antonetz, 1982a). Phospholamban has properties characteristic of a phosphoester (i.e. the Pi is incorporated primarily into serine) and therefore easily distinguished from the acyl phosphate (EP) intermediate of the ATPase (Tada et al, 1975).

Stimulation of calcium transport in cardiac SR as a consequence of phospholamban phosphorylation appears to be due to three interdependent factors: (1) effects on the
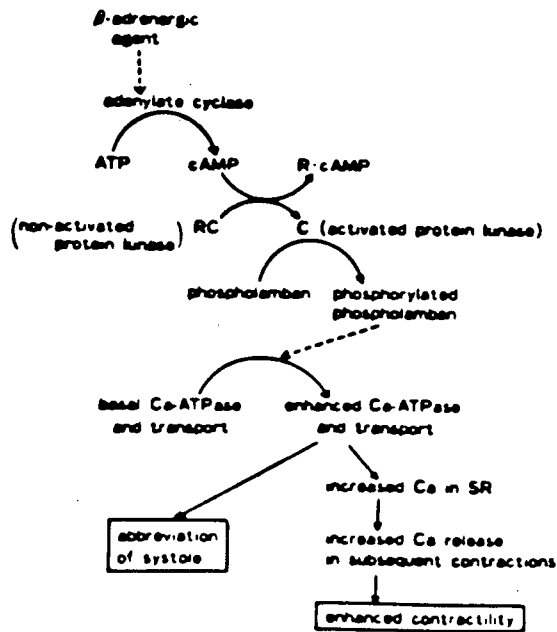
FIGURE 2. A possible mechanism for β-adrenergic effects on contractility, mediated by enhanced Ca^{++} uptake by the sarcoplasmic reticulum (SR). RC represents protein kinase in soluble form or in association with SR membranes. R represents the regulatory subunit, C the catalytic subunit of the protein kinase. Taken from Tada et al (1982).

interaction between the two Ca^{++} binding sites on the ATPase (Hicks et al, 1979), (2) direct effects on the acyl phosphate intermediate (Tada and Katz, 1982), and (3) a possible interaction with phosphorylated Troponin I (Kranias and Solaro, 1982).

Hicks et al (1979) proposed that phospholamban phosphorylation results in a conformational change in the ATPase enzyme such that the positive cooperativity between the two Ca^{++} binding sites is decreased. The resulting increase in Ca^{++} sensitivity of the enzyme (Tada et al, 1974) stimulates Ca^{++} transport. More recent work by Tada et al (1979; 1982) suggests that the alteration in Ca^{++} kinetics may be more the result of phosphorylated phospholamban interacting with key elementary steps of the ATPase reaction mechanism. These workers found that steady-state EP levels were not altered by phospholamban phosphorylation at saturating concentrations of Ca^{++} and ATP. However, below 10 μM Ca^{++}, EP levels were reduced while the rate of Pi liberation was increased, suggesting the the rate-limiting step of EP decomposition was enhanced. Among the steps at which EP is decomposed (steps iv, v, and vi in equation 1), step iv appears to be the one accelerated since the rate of decay of E_2P was found to be enhanced (Tada et al, 1982).

Based on a number of observations, Tada et al (1982) have proposed a model of the action of phospholamban phosphorylation on the ATPase. During Ca^{++} transport, the ATPase subunit containing the active site (the 30,000 MW fragment) undergoes a

conformational change which promotes the translocation of the Ca^{++} binding subunit (the 20,000 MW fragment) from outside to in. Phospholamban phosphorylation is proposed to accelerate the rate at which the 30,000 MW fragment undergoes its conformational change, resulting in an increased rate of translocation of the Ca^{++} binding subunit.

The third possibility is that phospholamban phosphorylation interacts with phosphorylated Troponin I (TnI), a component of the regulatory complex of the thin filaments. This suggestion has arisen from the finding of a cAMP-dependent phosphorylation of TnI (England, 1975) mediating a decrease in the Ca^{++} sensitivity of Troponin C and of cardiac actomyosin ATPase activity (Solaro et al, 1976). The physiological effect of a reduced affinity of the troponin complex to Ca^{++} would be an enhanced rate of dissociation of Ca^{++} from the contractile proteins, resulting in an accelerated rate of relaxation. Kranias and Solaro (1982) therefore propose that both phospholamban and TnI phosphorylation act in concert to enhance the rate of relaxation by SR (Figure 3). Nevertheless, the physiological relevance of TnI phosphorylation remains in question following the finding of phosphorylation extending beyond the time required for a catecholamine-mediated inotropic effect (England, 1976).

(ii) Calmodulin mediated regulation of cardiac SR

Calmodulin, a heat-stable acidic Ca^{++} binding protein with a molecular weight of approximately 16,800 (Klee and Vanaman,
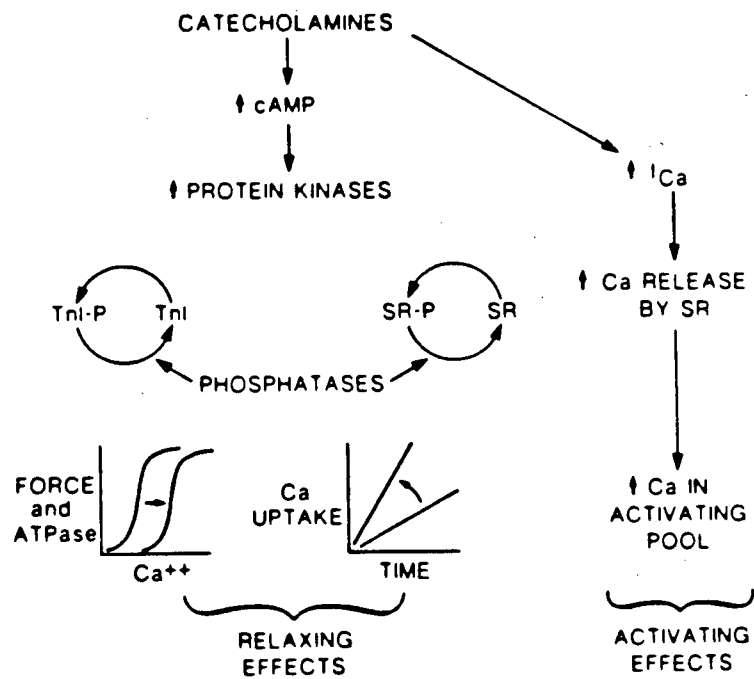
FIGURE 3. A scheme describing possible mechanisms
for the activating and relaxing effects of catechol-
amines on the heart. Taken from Kranias & Solaro (1983).

1982) has been isolated from a number of tissues, including brain, heart, and testis. Discovered independently by Cheung and Kakiuchi and Yamazaki in 1970 as an activator of phosphodiesterase, calmodulin (CAM) has since been shown to serve as a multifunctional intracellular modulator of a number of Ca^{++}-dependent enzymes as shown in Table 1. It appears that each CAM molecule binds 4 Ca^{++} ions, with dissociation constants in the range of 1-100 μM (Wolff et al, 1977). The binding of Ca^{++} alters the conformation of CAM, increasing its helical content (Dedman et al, 1977) and exposing hydrophobic regions (LaPorte et al, 1980). In this conformation, the Ca^{++}-CAM complex can bind to target enzymes (Niggli et al, 1977) and, through an unknown mechanism, increase their activities.

Within the last ten years, a number of seemingly diverse pharmacological agents, among them: the phenothiazine trifluoperazine (TFP) and the histamine releaser, compound 48/80, have been shown to inhibit the CAM-induced activation of a variety of enzymes. All of these "anti-CAM" (Vincenzi 1981) agents show several common structural characteristics, namely a large hydrophobic region and a charged amino group at physiological pH (Figure 4). The charged amino group is presumed to interact with negative charges on the highly acidic CAM, while the hydrophobic regions uncovered when Ca^{++} binds to CAM are thought to interact with the lipophilic regions on the anti-CAM agent (Prozialeck and Weiss, 1982). Although claims of specificity have been made (Levin and Weiss, 1977), it is now

TABLE 1. Calmodulin-dependent enzymes and processes that are inhibited by phenothiazine antipsychotics. Taken from Weiss _et al_ (1982)

Enzymes
  Adenylate cyclase
  (Ca^{2+} + Mg^{2+})-ATPase
  15-Hydroxyprostaglandin dehydrogenase*
  Myosin light chain kinase
  NAD kinase
  Phosphodiesterase
  Phospholipase A$_2$
  Phosphorylase b kinase
  Protein kinase
  Tryptophan hydroxylase

Processes
  ADH-mediated water transport
  α-Adrenergic responses
  Calcium uptake
  Catecholaminergic function
  Chloride secretion in intestine
  DNA synthesis
  Endocytosis
  Exocytosis
  Insulin release
  Leukocyte function
  Neuromuscular transmission
  Neurotransmitter release
  Phospholipid methylation
  Platelet function
  Release of trichocyst in paramecium
  Smooth muscle contraction

  * Calmodulin inhibits 15-hydroxyprostaglandin dehydrogenase.
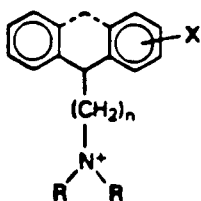
FIGURE 4. Generalized structural characteristics of calmodulin inhibitors. This generalized structure depicts a positively-charged group (N$^+$) attached to two hydrophobic groups. The hydrophobic groups need not necessarily be attached to each other at the ring. X represents a moiety that increases the hydrophobicity of the ring. For high potency n should be at least three carbons long. Taken from Prozialek & Weiss (1982).

well accepted that the highly lipophilic anti-CAM agents also act nonspecifically (Roufogalis, 1981) in that they inhibit basal, CAM-independent enzyme activity (Luthra, 1982) possibly by membrane perturbing effects (Raess and Vincenzi, 1980; Ho et al, 1983). Thus, caution must be exerted when inferring the presence of a CAM-dependent process solely by the use of these agents.

The first observation that CAM plays a role in regulating Ca^{++} fluxes across membranes was the observation by Bond and Clough (1973) that a protein in human red cells activated the erythrocyte (Ca^{++}-Mg^{++})-ATPase, such that Ca^{++} was actively transported from the cell. Our laboratory was the first to demonstrate a stimulation by CAM of both ATP-dependent Ca^{++} transport (Katz and Remtulla, 1978) and (Ca^{++}-Mg^{++})-ATPase activity (Lopaschuk et al, 1980) in cardiac SR. This work has subsequently been reproduced by a number of workers (LePeuch et al, 1979; Kranias et al, 1980; Kirchberger and Antonetz, 1982b). The calmodulin-dependent regulatory system requires Ca^{++} and also depends on a specific kinase which, as first shown by LePeuch et al (1979), also phosphorylates phospholamban but at a site distinct from that phosphorylated by the cAMP-dependent system; an additive increase in the amount of phosphorylation was observed when cardiac SR was incubated in the presence of both CAM and cAMP-dependent protein kinase. The mechanism of stimulation of transport and ATPase activity appears somewhat similar to that seen with cAMP-dependent protein kinase: an increase in ATPase turnover

FIGURE 5

Scheme depicting the known and postulated regulatory mechanisms of cardiac SR Ca^{++} transport. Cyclic AMP-dependent protein kinase phosphorylates phospholamban which then, perhaps through a series of intermediate steps, stimulates both calcium transport and (Ca^{++}-Mg^{++})-ATPase activity of the SR calcium pump protein. Calmodulin, in the presence of Ca^{++}, also phosphorylates phopholamban by presumably interacting with a membrane-bound kinase. The possibility remains that Ca^{++}-calmodulin circumvents phospholamban to act directly on the ATPase or on an, as yet, unidentified intermediate step.
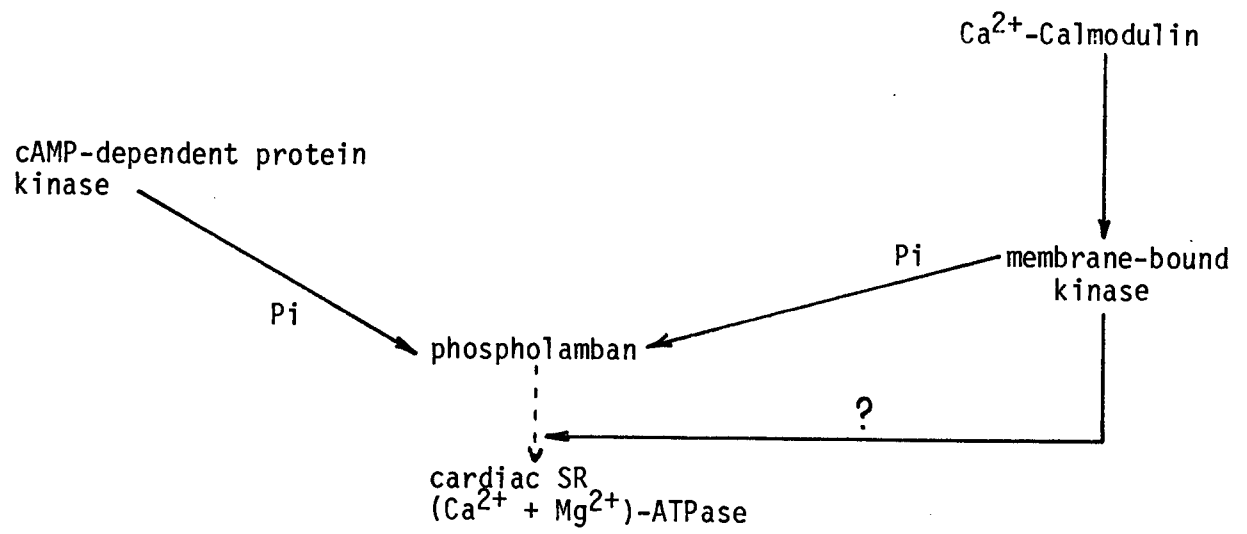
rate via enhancement of the rate of dephosphorylation of the acylphosphate (EP) intermediate (Lopaschuk et al, 1980). The possibility still exists, according to Katz (1980), that CAM acts directly on EP, bypassing the kinase phospholamban system (Figure 5).

The physiological relevance of CAM-mediated stimulation of the $(Ca^{++} -Mg^{++})$-ATPase in the cardiac SR membrane is unknown. Recently, a sarcolemmal $(Ca^{++} -Mg^{++})$-ATPase has been identified in myocardium and postulated to handle a part of the extrusion of Ca^{++} from the cardiac cell along with the Na/Ca exchanger (Caroni and Carafoli, 1981). The enzyme is stimulated by CAM (Caroni and Carafoli, 1981) and may serve the in vivo function of regulating the rise of intracellular Ca^{++} that occurs with each heart beat. Regulation of the SR ATPase by CAM, however, can not be as easily explained. Clearly, an enhancement of the SR calcium pump will mediate an enhanced rate of relaxation, similar to that seen in the cAMP-dependent protein kinase mediated system. Yet, speculations range from CAM having an important role as a modulator of the beat-to-beat rise and fall in Ca^{++} with each contractile cycle (Kirchberger and Antonetz, 1982b) to a role as a back-up mechanism in the event of pathological alterations (Lopaschuk et al, 1980; Louis and Maffitt, 1982), to its being a ubiquitous contaminant of cardiac membrane preparations (Hartweg and Bader, 1983). Clearly, more information will have to be acquired before a definitive role for CAM in cardiac SR is determined.

B. Regulation of Skeletal SR

Unlike the regulatory mechanisms documented in cardiac SR, skeletal muscle SR regulation is not nearly as well characterized and has been the subject of ongoing controversy. Schwartz et al (1976) and Bornet et al (1977) reported that cAMP-dependent protein kinase stimulated Ca^{++} transport in fast or slow-twitch skeletal muscle SR. Fabiato and Fabiato (1978), using skinned fast skeletal fibres of the cat, also reported a cAMP-mediated relaxation of tension. On the other hand, a number of other laboratories, including our own, have been unable to replicate the findings of a cAMP-mediated increase in fast-twitch skeletal SR Ca^{++} transport (Kirchberger and Tada, 1976; Caswell et al, 1978; Katz and Wong, 1982), although Kirchberger and Tada (1976) reported a stimulation of slow skeletal SR by cAMP-dependent protein kinase. The regulation of skeletal SR is further complicated by reports of phosphorylation of a 100,000 MW membrane protein by cAMP-dependent protein kinase (Kranias et al, 1980; 1983) or phosphorylase b kinase (Schwartz et al, 1976). The cAMP-mediated phosphorylation of the 100,000 MW protein has been found by Kranias et al (1983) to stimulate both the initial rates of EP formation and decomposition. Nevertheless, the finding of phosphorylase b kinase phosphorylation suggests, perhaps, that both kinases may be glycogenolytic contaminants of skeletal SR preparations. As yet, the physiological relevance of a cAMP-dependent stimulation of skeletal muscle remains unclear since the effects of catecholamines on

contractility in fast skeletal muscle, in particular, are much lower than those observed in heart and have been postulated to be mediated purely by local vasoconstriction (Bowman and Zaimis, 1958).

Recently, a calmodulin-dependent phosphorylation has been noted in fast skeletal SR (Chiesi and Carafoli, 1982; Campbell and MacLennan, 1982; Kirchberger and Antonetz, 1982c). The proteins phosphorylated include a 20 and 57-60 kDalton protein, the former, an acidic proteolipid that is not identical with phospholamban (Chiesi and Carafoli, 1983). Work in our laboratory (Katz and Wong, 1982) and by others (Carafoli, 1980) has confirmed a lack of stimulation of Ca^{++} transport by CAM. The role of CAM-mediated phosphorylation, therefore, remains even less understood than in cardiac muscle SR and has prompted a number of speculations of the role of CAM in Ca^{++} release from the SR (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1982).

VI Disease States and the Sarcoplasmic Reticulum

Pathological changes in sarcoplasmic reticulum Ca^{++} transport ability are suspected to play important roles in the pathogenesis of a number of disorders in both heart and skeletal muscle. Whether these changes represent primary or secondary events in the development of the disease process is, however, not known (Heffron, 1979).

A. Skeletal Muscle

One of the strongest cases for the primary involvement of skeletal SR in the pathogenesis of a disease state is in Duchenne Muscular Dystrophy (DMD), the most common of the human muscular dystrophies. DMD is a progressive, crippling disease of young males characterized by recessive sex-linked inheritance and an early onset of symptoms which include proximal muscle weakness and atrophy, a myocardial involvement, hypertrophy of the calves, muscular contractions, mental retardation and death due to respiratory or cardiac failure usually before thirty years of age (Walton and Gardner-Medwin, 1974). One of the earliest biochemical signs of DMD is an increase in serum enzymes derived from muscle, especially creatine kinase, which are thought to enter the circulation either from necrotic muscle or due to altered permeability of muscle SL (Furukawa and Peter, 1978). Initially, the SL was suspected as being important in the early pathogenesis of the disease due to the early appearance of creatine kinase, but a number of observations have since contested the primacy of SL

defects and placed the burden, instead, on the SR network. These observations include: (1) ultrastructural evidence of morphological changes in the SR preceding those of the SL (Cullen and Fulthorpe, 1975), (2) although creatine kinase may be leaked through the SL, carnitine, a much smaller molecule, appears to be retarded (DiMauro and Rowland, 1976), and (3) a reported elevation (a suspected early event) of intracellular Ca^{++} levels in DMD skeletal muscle (Oberc and Engeel, 1977; Maunder et al, 1977). This latter observation, in particular, has directed attention towards the SR as a result of the proposition of a "calcium hypothesis" of DMD pathogenesis (Ebashi and Sugita, 1979; Cullen et al, 1979). Briefly, the calcium hypothesis suggests that a defect in calcium regulation by skeletal muscle cells, resulting in a substantial, sustained elevation of intracellular Ca^{++}, may elicit direct or indirect pathological alterations in the skeletal muscle cell. Direct damage by the increased intracellular Ca^{++} may be due to hypercontraction of sarcomeres in the immediate vicinity of the elevated [Ca]i in addition to stretching and rupturing of sarcomeres in neighbouring myofibrillar regions of the cell; all of these changes predispose to necrosis (Cullen and Fulthorpe, 1975). Indirect damage by the elevated [Ca]i may be the activation of intracellular Ca^{++}-dependent proteases, resulting in hydrolysis of myofibrillar elements and other organelles (Ebashi and Sugita, 1979).

Perhaps the clearest phenomenon of DMD implicating a role of SR is the finding of a number of independent laboratories of

a defect (a decrease compared to control) in SR Ca^{++} transport in both human (Takagi et al, 1973) and animal (Sreter et al, 1967; Martonosi, 1968; Dhalla and Sulakhe, 1973) muscular dystrophy. To exclude the possibility that these apparent functional changes reflect SR vesicle contamination by membranes of infiltrating fat and connective tissue cells, caffeine-induced transient isometric tension response studies were performed in single "skinned" DMD skeletal muscle fibres with the finding of a defect in SR Ca^{++} regulation (Wood et al, 1978).

A defect in the ability of dystrophic skeletal muscle SR to rapidly sequester and/or store intracellular Ca^{++} may explain the delayed relaxation time and partial loss of normal tension development observed in dystrophic muscle (Roc et al, 1967) and the possible calcium-mediated cytotoxicity brought about by the sustained increase in [Ca]i. The precise nature of the putative SR defect of skeletal muscle remains to be demonstrated. Possible sites of the SR defect include the SR ATPase protein and/or proteins associated with the pump (Verjovski-Almeida and Inesi, 1979), the documented regulators of the ATPase activity (calmodulin, cAMP-dependent protein kinase, etc.), SR proteins associated with Ca^{++} release or facilitating Ca^{++} storage (e.g. calsequestrin) (Etienne et al, 1980), and alterations in the lipid environment of the ATPase protein in the SR membrane (Hanna et al, 1981).

Malignant hyperthermia (MH) is another disease entity in which an SR Ca^{++} handling disorder may play a prominent role.

As summarized by Gronert (1980), in MH, skeletal muscle in response to certain anesthetics (halothane, succinylcholine) and drugs suddenly and unexpectedly increases its aerobic and anaerobic metabolism, resulting in an intense production of heat, CO_2, and lactate. Muscular rigidity, increased muscle permeability (i.e. elevated serum creatine kinase) and sympathetic stimulation then ensue with mortality occurring in almost 30% of reported cases. It is currently believed that control of intracellular [Ca] is lost in MH (Gallant et al, 1979), the resultant increase in metabolism is symptomatic of the body's attempt to reverse the abnormally high Ca^{++} concentrations. The resultant damage to muscle and other organelles may be presumed, as in DMD, to result from the sustained Ca^{++} levels and consequent toxic accumulation in organelles.

Claims for the primary abnormality of SR in MH have arisen on the basis of the prominent clinical observation of muscle rigidity and the increased sensitivity of MH muscle to develop caffeine-induced contractures (Kalow et al, 1972); caffeine is thought to specifically induce Ca^{++} release from the SR (Weber, 1968).

Ca^{++} transport by SR appears to also be decreased in both human and porcine models of MH (Gronert et al, 1979; Hidalgo et al, 1982) but the differences noted are not dramatic (Gronert, 1980). Gronert (1980) suspects that the noted decrease in transport ability is more of a secondary effect since (1) the decrease in transport is not severe enough to account for the

entire MH syndrome, (2) with clinical concentrations of halothane, Ca^{++} binding (i.e. oxalate-independent) was increased in both MH and normal SR, whereas at higher concentrations of halothane, Ca^{++} binding and Ca^{++}-oxalate uptake in both types of SR were depressed (Gronert et al, 1979), and (3) the specific action of dantrolene sodium in preventing and reversing MH along with the finding of an increased sensitivity of MH muscle to caffeine implicates Ca^{++} release (and the terminal cisternae of the SR) as opposed to the Ca^{++} uptake process (Nelson, 1978).

Somewhat unrelated to the finding of impaired SR function due to presumed pathological increases in intracellular Ca is the noted decrease in skeletal SR Ca^{++} transport observed in a variety of experimentally-induced vitamin D deficient states. Muscle weakness and atrophy are prominent features of nutritional vitamin D deficiency or diseases in which the metabolism of vitamin D is altered (e.g. uremia) (Smith and Stern, 1969). Speculation has therefore arisen that the abnormal muscle function may be the result of impaired muscle cell Ca^{++} turnover (Stanbury, 1965), perhaps at the level of E-C coupling (Curry et al, 1974). The noted decrease in SR Ca^{++} transport in states of both nutritional vitamin D deficiency (Curry et al, 1974; Pointon et al, 1979) and experimental uremia (Heimberg et al, 1976; Boland et al, 1983), and reversal of such impaired SR function by in vivo administration of calcitriol (1,25-dihydroxycholecalciferol) (Matthews et al,1977; Boland et al, 1983), the active metabolite of vitamin

D, have implicated the SR as an important target organ for vitamin D. Three observations, however, suggest that both the inhibition of Ca^{++} transport and reversal of such impaired function by calcitriol may be more the result of systemic metabolic changes than a primary SR defect. First, Ca^{++}-ATPase activity in SR derived from both controls and vitamin D deficient animals remains unchanged (Curry et al, 1974; Boland et al, 1983b). Second, despite clear evidence of defective mineralization in rats treated with ethane 1-hydroxy 1,1 diphosphonate (EHDP), an inhibitor of calcitriol formation in the kidney (Hill et al, 1973), no change in Ca^{++}-uptake activity was observed in skeletal muscle SR of these animals (Pointon et al, 1979). Third, muscle tissue has been shown not to contain specific calcitriol receptors (Boland et al, 1983b) thus discounting such tissue as a specific target organ for vitamin D.

B. Cardiac Muscle

Similar to the findings in skeletal muscle SR cited above, certain pathological states of the myocardium are correlated with an inhibition of the heart's SR Ca^{++} transport system; whether the change represents a primary or secondary event in the development of the disease process is, again, unclear, but most investigators theorize that the alteration of transport is a secondary manifestation of the disease (Dhalla, 1976; Katz, 1975).

The alterations in SR calcium uptake and related changes

in E-C coupling have been most thoroughly investigated in ischemic heart disease (Dhalla et al, 1982). Ischemic heart disease refers to a spectrum of clinical disorders of the heart resulting from imbalance between the myocardial need for oxygen and the adequacy of its supply (Robbins and Cotran, 1979). At least 95% of all cases examined present atherosclerotic narrowing of the coronary arteries (Hillis and Braunwald, 1977). Involvement of the SR is thought to begin as a correlate of the rapid loss of contractility (Scwartz et al, 1973) accompanying interruption of myocardial blood flow. The mechanism reponsible for the depression of contractile performance is not known, although this has not stopped investigators from proposing a plethora of possibilities, of which I will briefly discuss four: cellular depletion of high energy phosphate compounds, acidosis, Ca^{++} overload, and the accumulation of long-chain fatty acids.

Cellular depletion of high energy phosphate compounds (e.g. ATP, creatine phosphate) is believed to occur in the ischemic myocardium either as a result of the inhibitory effect of elevated Ca^{++} concentrations (see below) on mitochondrial oxidative phosphorylation (Dhalla et al, 1982) or through the selective loss of the adenine ring as ATP degrades to ADP, AMP, and adenosine, the latter rapidly diffusing through the cardiac cell membrane (Oliver, 1978). Although some workers insist that such loss of high energy phosphates is the major cause of cardiac cell death and the resulting depression of contractility (Guyton, 1981), others (Katz, 1975; Dhalla et al,

1978) hold that ATP depletion is not a critical factor, at least initially. Insofar as a depression of the SR Ca^{++}-ATPase is concerned, the average cellular ATP levels in the failing myocardium still fall well above the Km of 0.1 mM, thus excluding an impairment of the Ca^{++} pump following ATP "depletion" (Katz, 1975).

The fall in intracellular pH that occurs when the myocardium becomes ischemic is also thought to be a likely explanation for the reduced cardiac contractility (Steenbergen et al, 1977). Under resting conditions, cardiac muscle utilizes fats mainly for its energy, with approximately 70% of the normal metabolism being derived from fatty acids (Oliver, 1978). Under anaerobic or ischemic conditions, however, the metabolic demands of the heart are dramatically altered such that anaerobic glycolysis becomes the primary energy source. Under these conditions, H^+ will be produced during the metabolism of glucose to lactate. The fall in pH influences the cardiac cell negatively in a number of ways (Mandel et al, 1982), affecting mitochondria, myosin ATPase activity, and the binding of Ca^{++} to troponin C and sarcolemma. Of particular relevance, however, is the noted decrease in SR Ca^{++}-uptake activity (Nakamura and Schwartz, 1972; Sorenson and deMeis, 1977; Fabiato and Fabiato, 1978) under acidic conditions, suggesting a possible explanation of the decrease in contractility as a state of acidosis develops in the ischemic myocardium. The mechanism of the effect of pH on SR Ca^{++} transport has recently been investigated by Mandel et al (1982)

who found that formation and decomposition of E~P became depressed under acidic conditions. The retardation of these two rate-limiting steps in the reaction sequence of the $(Ca^{++}-Mg^{++})$-ATPase may, then, be one of the contributing factors to the inhibition of myocardial function in ischemia.

Another factor thought to be important in the ischemic myocardium is a pathological rise in intracellular Ca^{++} ($[Ca]i$), termed calcium overload (Dhalla et al, 1978). As discussed in the section on pathological alterations in skeletal SR, a rise in $[Ca]i$ appears to be closely correlated with muscle tissue necrosis. The concept of a Ca^{++}-induced myocardial necrosis (Fleckenstein et al, 1974) has also been introduced to account for the decline in contractility of the ischemic myocardium. In the original formulation by Fleckenstein et al (1974), it was suggested that mitochondrial damage resulting from Ca^{++} overload, coupled with the activation of Ca^{++}-dependent ATPases, would result in sharp decreases in high energy phosphates, impairing cellular function. Dhalla (1976) has suggested that pathological states such as ischemia alter cardiac membrane structure and function. This, then, leads either to a Ca^{++} overload or deficiency (Figure 6) both of which are drastic circumstances in the cardiac cell and result in contractile failure and inhibition of Ca^{++} transport via any of the Ca^{++}-associated pathological consequences such as Ca^{++}-induced inhibition of mitochondrial oxidative phosphorylation, tissue necrosis, or the release of Ca^{++}-activated proteases, phospholipases, and lysosomal enzymes
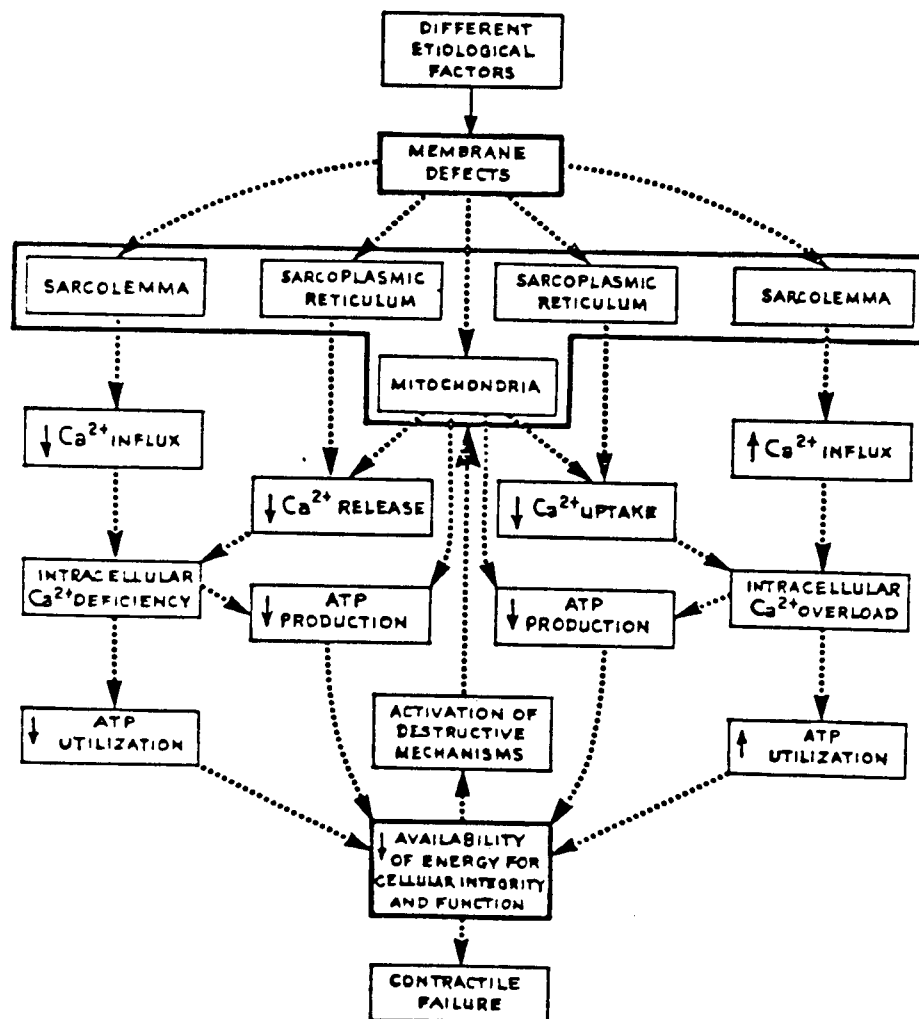
FIGURE 6. Postulated sequence of steps in a scheme involving membrane defects in heart failure due to intracellular calcium deficiency or intracellular calcium overload. Taken from Dhalla (1976).

(Dhalla et al, 1982).

The newest postulate invoked to explain contractile failure in ischemic myocardium is the suggestion of long-chain fatty acid (and their derivatives) accumulation in the heart (Katz and Messineo, 1981). It is proposed that mitochondrial fatty acid oxidation becomes inhibited in the ischemic myocardium, possibly due to a build-up of intracellular [Ca], with the resultant accumulation of long-chain fatty acids and their acyl carnitine derivatives (Idell-Wenger and Neely, 1978). Of particular interest is the rapid accumulation of palmitylcarnitine, a potent inhibitor of both sarcolemmal Na^+, K^+ ATPase and sarcoplasmic reticulum (Ca^{++}-Mg^{++})-ATPase (Pitts et al, 1978; Adams et al, 1979). The strongly charged carnitine moiety in association with long-chain (hydrophobic) fatty acids imparts a highly ampiphilic character to the molecule, allowing for its intercalation into the membrane and ability to disrupt enzymatic activity, possibly due to its detergent-like action (Adams et al, 1979). It has therefore been suggested that the elevated levels of palmitylcarnitine and consequent inhibition of SR Ca^{++} transport may be a major contributing factor in the fall of contractility in ischemic myocardium.

Lopaschuk et al (1983) have recently invoked the notion of an accumulation of long-chain acylcarnitines to explain the mechanism whereby both contractile function (Fein et al, 1980; Vadlamudi et al, 1982) and cardiac SR Ca^{++} transport (Penpargkul et al, 1981; Lopaschuk et al, 1983) are depressed in the chronic experimentally-induced diabetic rat. In

diabetes, the heart extracts its energy almost exclusively from the oxidation of fatty acids (Randle, 1978), a by-product of which are elevated levels of acylcarnitines (Feuvray et al, 1979) which serve as intermediates in the transport of acyl CoA into the inner mitochondrial matrix (Fritz, 1959). Lopaschuk et al (1983) found that as the course of the diabetic state progressed, the levels of long-chain acylcarnitines rose. This correlated with the corresponding time-dependent inhibition of heart function and SR Ca^{++} transport. It is of interest that levels of long chain acylcarnitines are also elevated in the skeletal muscles of diabetic rats (Stearns, 1980). A corresponding decrease in SR Ca^{++} transport and skeletal muscle contractility, if observed, may suggest a role for the involvement of acylcarnitine derivatives in the muscle pathophysiology of diabetes.

Objectives of the Study

The main objectives of this current study were (i) to further explore the nature of the calmodulin and cAMP-dependent protein kinase modulation of cardiac SR calcium transport, (ii), to determine the role, if any, of calmodulin in skeletal muscle SR systems and (iii), study Ca^{++} transport in skeletal muscle SR obtained from a disease model where alterations in calcium translocation have been noted.

The following is the rationale for these studies:

1) Much of the work on cardiac microsomes enriched in SR has been plagued by doubts about purity. In contrast to skeletal muscle, where the SR is the more extensively developed membrane system, in cardiac SR, the sarcolemma contributes a much greater percentage of the total membrane mass. In preparing the SR vesicles, contamination by SL and mitochondrial membranes, both of which contain $(Ca^{++}-Mg^{++})$-ATPase activity, is of real concern and may lead to spurious results. We therefore undertook a purification of cardiac SR for the sake of investigating of the effects of the indigenous regulators (e.g., cAMP-dependent protein kinase, calmodulin) on Ca^{++} transport, $(Ca^{++}-Mg^{++})$-ATPase activity and membrane phosphorylation in a system that is less contaminated with other organelles.

2) The groups of Carafoli (Chiesi and Carafoli, 1982) and

MacLennan (Campbell and MacLennan, 1982) have recently investigated the role of calmodulin in skeletal muscle SR. Both groups have determined the presence of CAM-dependent protein phosphorylation which appears not to result in the stimulation of Ca^{++} transport. Our laboratory had assumed that the inability of CAM to stimulate Ca^{++} transport was related to its high endogenous level in SR membranes. Chiesi and Carafoli (1982) demonstrated the presence of CAM in fast skeletal SR by first boiling the membranes and then passing the denatured suspension down a CAPP-Sepharose calmodulin-affinity column. They concluded that CAM was tightly bound to the membrane and only removable by harsh treatment such as boiling. It was of great interest, therefore, that Campbell and MacLennan (1982) stated that CAM was indeed bound to the membrane but was easily dislodged by treatment with EGTA. Their supposition was partly based on the original observations of MacLennan (1972) that centrifugation and re-suspension of fast SR vesicles three times in the presence of 1 mM EGTA resulted in a decreased ability of the SR pellet to transport Ca^{++}. When the resultant supernatant was added back to the SR transport system, Ca^{++} uptake could be partially restored. The more recent work by MacLennan (Campbell and MacLennan, 1982) confirmed that the supernatant of 1 mM EGTA-washed fSR contained CAM on the basis of two observations. First, EGTA-washed fSR demonstrated an enhanced level of phosphorylation in the presence of CAM compared to "unwashed" SR vesicles (presumably due to the depletion of CAM by EGTA), and second, supernatant derived from

1 mM EGTA-washed SR stimulated phosphorylation of fSR. Our work, then, was an attempt to resolve the controversy of the nature of the binding of CAM to the skeletal SR membrane.

3) It has been proposed that the depression of Ca^{++}-uptake activity noted in cardiac SR obtained from chronically diabetic rats may be due to the accumulation of long-chain acylcarnitines which act as disruptive ampiphiles in the lipid environment of the Ca^{++} pump protein (Lopaschuk et al, 1983). As total tissue levels of long-chain acylcarnitines have also been reported to be elevated in chronically diabetic rats (Stearns, 1980), it was of interest to investigate whether the Ca^{++} transport ability of skeletal muscle SR was also affected and, if so, what role the acylcarnitine derivatives might play in the pathophysiology of diabetes and regulation of both skeletal and cardiac SR function.

MATERIALS AND METHODS

A) MATERIALS

(i) Animals:

a) Studies utilizing cardiac microsomes enriched in sarcoplasmic reticulum.

Dogs (10-30 kg) of either sex were used for preparation of cardiac microsomes enriched in sarcoplasmic reticulum. Hearts were removed from pentobarbital-anesthetized dogs and placed in ice-cold normal saline. The ventricles were cut into 2-4 g pieces, and either used immediately for preparation of sarcoplasmic reticulum, or quick-frozen in 2-methylbutane on dry ice, and stored at -80°C until use.

b) Studies utilizing skeletal microsomes enriched in sarcoplasmic reticulum.

New Zealand White rabbits of either sex were used throughout the study. Animals, some of which were injected with 500 U of heparin 10 minutes prior to sacrifice, were killed by a sharp blow to the base of the skull. Vastus lateralis (fast) and soleus (slow) muscle were excised from the hind limbs and placed in ice-cold 10 mM tris maleate, pH 6.8 for microsomal SR preparation.

c) Studies utilizing skeletal microsomes enriched in

sarcoplasmic reticulum derived from diabetic animals.

Female Wistar rats (Charles River Canada^R) between 150-200 g were used. Diabetes was induced by a single tail-vein injection of streptozotocin (50 mg/kg) dissolved in citrate buffer, pH 4.0. Control animals were injected with citrate buffer alone. Animals were then housed for 90 days during which time food and water were available ad libitum. Rats were sacrificed at the end of the 90 day period and the tibialis anterior (fast) muscle was isolated from both hindlimbs, and placed in ice-cold 10 mM tris maleate buffer, pH 6.8 for preparation of microsomes enriched in sarcoplasmic reticulum. At the time of sacrifice, serum samples were withdrawn and assayed for insulin [Becton-Dickinson Insulin ^{125}I-Radioimmunoassay Kit^R] and glucose [Ames Reagent Kit for Blood Glucose^R].

(ii) Chemicals

a. Radioisotopes: (^{45}Ca)Cl$_2$ (10 Ci/mmole) was purchased from Amersham^R radiochemicals. ^{125}I-RIA kit for calmodulin was obtained from New England Nuclear^R..

b. Reagents: the following chemicals were purchased from Sigma Chemical Company^R: ammonium bicarbonate, L-ascorbic acid, bromphenol blue, citric acid, compound 48/80, deoxycholic acid, EDTA (disodium salt), EGTA, Folin and Ciocalteu's phenol reagent, glycerol, glycine, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES),

magnesium chloride, potassium chloride, silver nitrate, sodium azide, sodium carbonate, sucrose, N,N,N',N'-tetramethylethylenediamine (TEMED), trichloroacetic acid, Trizma adenosine triphosphate, Trizma base, Trizma hydrochloride, Trizma maleate, and Trizma oxalate.

- Sodium dodecyl sulfate was purchased from Biorad^R.

- Sodium chloride was purchased from Amachem^R.

- The following reagents were purchased from Fisher^R: glacial acetic acid, glutaraldehyde, & cello-seal.

- Biorad^R electrophoretic reagents included acrylamide, ammonium persulfate, bisacrylamide, and High and Low Molecular Weight Standards.

- Calmodulin used was purchased from either Calbiochem^R or Sigma Chemical Co.^{R}

- Calcium chloride and sodium fluoride were obtained from Analar^R.

- Trifluoperazine dihydrochloride was a generous gift of Smith, Kline, & French.^{R}

- Aquasol scintillation fluid was purchased from New England Nuclear^R.

- 2-Methylbutane was obtained from MCB Manufacturing Chemists.^{R}

(iii) Apparatus

- The assay of inorganic phosphate was performed spectrophotometrically at 660 nm using a Technicon^R Autoanalyzer model I and recorded on a Technicon^R chart recorder.

- Immersible-Cx separator filters were obtained from Millipore Co.®

- Electrophoresis was performed with a slab-gel apparatus on slabs of 1.5-mm thickness and 15-cm length. Constant current was supplied by a Pharmacia® power supply (Model EPS 500/400). Gels were dried under vacuum using a Bio-Rad® gel dryer, model 224.

B. METHODS

(1) Preparative Methods

(a) Preparation of Skeletal Muscle and Cardiac Muscle Microsomes Enriched in Sarcoplasmic Reticulum

Rabbit fast and slow skeletal muscle and dog cardiac muscle microsomes enriched in sarcoplasmic reticulum were prepared by a modification of the method of Sumida et al (1978). All preparation was performed at 4°C. Muscle was homogenized with a teflon pestle for 15 seconds at 1500 rpm in 10 mM tris maleate, pH 6.8. The homogenate was then centrifuged at 4000g for 10 min, and the supernatant passed through cheesecloth. Following re-centrifugation at 15,000g for 20 min, the supernatant was again passed through cheesecloth. This supernatant was then centrifuged at 40,000g for 80 min. The resulting pellet was then re-suspended in 10 mM tris maleate, pH 6.8, containing 0.6M KCl and centrifuged at 40,000g for 100 min. The final pellet was suspended in 10 mM tris maleate containing 40% sucrose, quick-frozen in 2-methylbutane on dry ice, and stored at -70°C until use.

(b) Preparation of a Purified Cardiac Microsomal Preparation Enriched in Sarcoplasmic Reticulum

Cardiac microsomes enriched in sarcoplasmic reticulum prepared by the method of Sumida et al (1978) were subject to further purification using the method of Jones et al (1979)

with modifications. Freshly prepared or frozen aliquots of crude cardiac sarcoplasmic reticulum (10-25 mg) were incubated at 37°C for 5 minutes in a medium containing 50 mM histidine-Cl pH 6.8, 15 mM $CaCl_2$, 5 mM tris-oxalate, 16 mM tris-EGTA pH 6.8, 5 mM $MgCl_2$, 100 mM KCl, and 5 mM NaN_3. Loading of the vesicles with calcium oxalate was initiated by the addition of 5 mM ATP. After 5 minutes, a second aliquot of 5 mM tris-oxalate was added and the reaction terminated after 5 minutes by placing the reaction tubes on ice. The sample was then centrifuged at 4°C at 40,000xg for 90 minutes, and the pellet suspended in a medium containing 0.25 M sucrose, 0.30 M KCl, and 0.1 M tris base, pH 7.2, and layered onto a discontinuous sucrose density gradient consisting of consecutive layers of 0.6 M, 1.0 M, and 1.5 M sucrose (all containing 0.3 M KCl and 0.1 M tris base, pH 7.2). The preparation was then centrifuged for 2 hours at 27,000 rpm in an SW 27 rotor (Beckman^R Instruments). The pellet sedimenting through the 1.5 M sucrose layer was then re-suspended in 10 mM tris maleate, pH 6.8 and either used freshly prepared or quick-frozen in 2-methylbutane on dry ice and stored at -80°C until use.

(c) Preparation of Boiled Skeletal Sarcoplasmic Reticulum Extracts

Approximately 3-8 ml of fast or slow sarcoplasmic reticulum vesicles (1-3 mg/ml) were placed in a 95° water bath and incubated for five minutes in either the presence or absence of 0.2 mM EDTA. Samples were then centrifuged at

40,000xg for 30 minutes and the clear supernatant was isolated. When EDTA was present, 0.2 mM $CaCl_2$ was added in order to chelate it. Samples were placed in dialysis membrane tubing (Spectrapor[R] 1) and dialyzed against 10 mM NH_4HCO_3 pH 7, for 48 hours at 4°C. Extracts were then lyophilized, re-suspended in a small volume of 10 mM tris maleate, pH 6.8, and frozen until use.

(d)   Preparation of EGTA-Washed Skeletal Sarcoplasmic Reticulum Extracts

Isolation of supernatant from fast and slow sarcoplasmic reticulum washed in 1 mM EGTA was performed according to the method of MacLennan (1972). Aliquots of fast and slow SR (3-8 ml of 1-3 mg/ml) were incubated at 4°C with 1 mM EGTA for 15 minutes and then centrifuged at 40,000xg for 50 min. The pellet was suspended and gently homogenized with a Teflon pestle and re-centrifuged at 40,000xg for 50 min. This process was repeated a third time. Following the final centrifugation, the supernatant was withdrawn and the pellet homogenized and suspended in approx. 2 ml of 10 mM tris maleate pH 6.8, containing 40% sucrose. The pellet (EGTA-washed SR) was then used for preparation of boiled extracts (see above). $CaCl_2$ (1 mM) was added to the supernatant for chelation following which this extract was dialyzed against 10 mM NH_4HCO_3 pH 7, for 48 hours at 4°C, lyophilized, and the pellet re-suspended in a small volume of 10 mM tris maleate buffer. A summary of the preparation of the various extracts is presented in Figure 7.
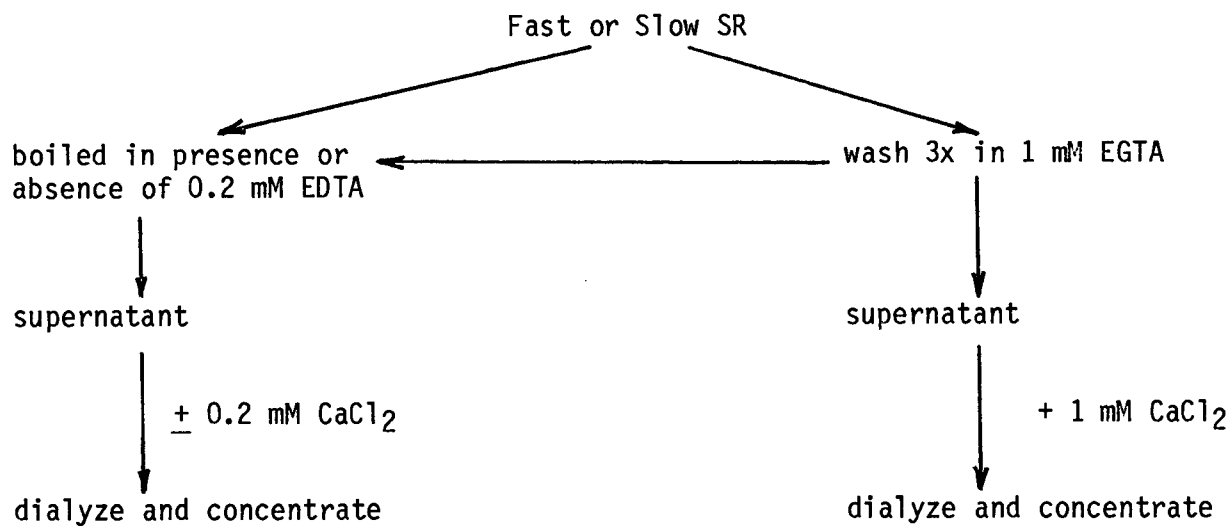
Fig. 7: Methodology for preparation of supernatants used for skeletal SR studies.

(e) Preparation of Red Cell Membranes

Human blood, not more than four days old, was obtained from the Canadian Red Cross. Erythrocytes, collected by centrifugation at 2500xg for 5 min., were then prepared by one of two methods (all preparation performed at 4°C):

(i) Method 1

Following removal of the plasma and buffy coat, the red blood cells were washed twice in two volumes of normal saline. Osmotic lysis was initiated by the addition of 10 volumes of ice-cold distilled, de-ionized water. After 30 minutes, the cells were centrifuged at 38,000xg for 20 minutes. The supernatant was aspirated and the following washes were initiated (each wash consisting of a 38,000xg x 20 min. centrifugation followed by aspiration of supernatant): twice in 1 mM tris-EDTA, pH 7.4, once in 10 mM tris-EDTA, pH 7.4, three times in 2 mM tris-Cl, pH 7.4, and the final white pellet suspended in 2 mM tris-Cl, pH 7.4 containing 4 μM EDTA. Membranes were quick-frozen in 2-methylbutane on dry ice and stored for one week at -80°C.

(ii) Method 2

Red cell membranes were prepared by the method of Niggli et al (1981) with some modification. Erythrocytes were washed twice in five volumes of 130 mM KCl and 20 mM tris-Cl, pH 7.4. The cells were then hemolyzed in 5 volumes of 1 mM K-EDTA, 10 mM tris-Cl, pH 7.4, and centrifuged at 18,000xg for 10 minutes. This step was repeated five times, following which the

membranes were washed in 10 mM HEPES, pH 7.4, and centrifuged at 18,000xg for 10 min. This latter step was repeated four times. The ghosts were retained in 10 mM HEPES, pH 7.4, quick-frozen in 2-methylbutane on dry ice, and stored for 1 week at -80°C.

(2) Analytical Methods

(a) Measurement of Calcium Uptake by Skeletal and Cardiac Muscle Microsomes Enriched in Sarcoplasmic Reticulum.

ATP-dependent Ca^{++}-uptake into skeletal and cardiac SR was measured by the method of Tada et al (1974) with a few modifications. Oxalate-facilitated Ca^{++}-uptake was determined in an incubation medium containing 10-30 ug SR protein, 40 mM histidine-HCl, pH 6.8, 5 mM $MgCl_2$, 5 mM tris ATP, 2.5 mM tris oxalate, 110 mM KCl and a variety of free Ca^{++} concentrations. The desired free Ca^{++} concentrations(containing 10 Ci/mmole $^{45}CaCl_2$) were maintained by the addition of ethylene glycol bis (β-aminoethyl ether)-N,N' tetraacetate (EGTA), and determined by the Fortran program, Mult.S (see below). Samples were preincubated for 7 minutes at 30°C and the reaction initiated by the addition of $^{45}CaCl_2$. The reaction was terminated after 5 min, in the case of control skeletal and cardiac muscle SR, and 1 min., in the case of purified cardiac and diabetic skeletal muscle SR, by filtering an aliquot of the reaction mixture through a 0.45 μm Millipore filter (HA 45, Millipore^R Co.). The filter was then washed twice with Aquasol (New England

Nuclear), and counted for radioactivity in a liquid
scintillation counter.

- Calculation of Calcium Uptake Activity by Skeletal Muscle
Microsomes Enriched in Sarcoplasmic Reticulum

The rate of calcium uptake by the microsomal preparation
is expressed in nmoles of Ca^{++} taken up per mg protein per
minute. This is determined by the following calculation:

$$\frac{(sample\ counts\ -\ blank)}{(total\ counts\ -\ blank)} \times \frac{dilution\ factor}{incubation\ time} \times \frac{total\ calcium}{mg\ protein}$$

where:

sample counts= ^{45}Ca counts (dpm) obtained per sample

total counts= total ^{45}Ca counts (dpm) present in the incubation
  media

blank= ^{45}Ca counts (dpm) obtained in the absence of microsomal
  protein

dilution factor= correction for incubation volume sampled (=
  1.21)

incubation time= length of time (5 min) microsomal protein is
  incubated in the presence of $CaCl_2$

total calcium= total amount of calcium present in the
  incubation medium (= 62.5 nmoles)

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

(b) Assay of $(Ca^{++}-Mg^{++})$-ATPase Activity in Cardiac Microsomes
Enriched in Sarcoplasmic Reticulum

(Ca^{++}-Mg^{++})-ATPase activity was measured by a modification of the method of Katz and Blostein (1975) using the incubation conditions of Tada et al (1979). Purified cardiac sarcoplasmic reticulum (0.6-3.6 μg) was pre-incubated in 40 mM histidine-Cl buffer pH 6.8, 5 mM $MgCl_2$, 110 mM KCl, 10 mM NaF, and 5.7 μM A23187 at $30°C$ for 5 minutes. The free calcium concentration was maintained by the addition of Ca-EGTA. The reaction was started by the addition of 200 μM ATP containing ^{32}P-ATP (10 Ci/mmole; 100,000 dpm/sample) and terminated in one minute by the addition of ice-cold 5% TCA containing 5 mM Na_2ATP and 2 mM KH_2PO_4. A suspension of activated charcoal (1.5 g Fisher Norit[R] A/10 ml) was added to each sample, vortexed, and incubated at $4°C$ for 15 minutes with occasional vortexing. Samples were then centrifuged at 1500xg for 5 minutes, and an aliquot of the clear supernatant counted for radiactivity. Results are expressed as nmoles ^{32}Pi released/ mg protein/ min.

(c)   Phosphorylation of Cardiac Membrane Vesicles

Crude and purified (10-40 μg protein) microsomes enriched in sarcoplasmic reticulum were preincubated at $30°C$ for 10 minutes in a medium containing 40 mM histidine-Cl pH 6.8, 110 mM KCl, 4 mM $MgCl_2$, 10 mM NaF, and 5.7 μM A23187. Calmodulin (1 μM) or cyclic AMP (1 μM) and cAMP-dependent protein kinase (20 μg), or the catalytic subunit of cAMP-dependent protein kinase (0.5 μM) were present in some reaction tubes. The reaction was initiated by the addition of 0.2 mM ATP containing ^{32}P-ATP (10 Ci/mmole; 1-2 x 10^6 dpm/sample) and terminated after various

time intervals with a quenching solution of 5% TCA, 5 mM Na$_2$ATP, and 2 mM KH$_2$PO$_4$. Bovine Serum Albumin (0.125% final concentration) was then added to each sample, the tubes mixed, and centrifuged at 1500xg for 5 minutes. The supernatant was decanted and the pellet applied onto glass microfibre filters (Whatman^R GF/A), washed with 30 ml of the TCA quench solution, dried, and counted for radioactivity.

(d) Measurement of "patent" and "latent" (Na$^+$-K$^+$)-ATPase activity

(Na$^+$-K$^+$)-ATPase activity measured either in the absence ("patent") or presence ("latent") of detergent was determined in accordance with the methods of Besch et al (1976) and Jones et al (1979) with modification. Latent Na$^+$-K$^+$ATPase activity was "unmasked" by preincubating cardiac SR membranes (0.15-0.20 mg/ml) in the presence of Triton X-100 (0.05%) for twenty minutes at room temperature. Activity was then assessed at 37°C by adding membrane protein (15-20 μg/ml) to a medium containing 50 mM histidine, pH 7.4, 3 mM MgCl$_2$, 1 mM tris-EGTA, 100 mM NaCl, 10 mM KCl, and 3 mM tris ATP. The release of inorganic phosphate was then measured using the semi-automated method of Fiske and Subbarow (1925) as modified by Raess and Vincenzi (1980) (see below). (Na$^+$-K$^+$)-ATPase activity was taken as that activity inhibited by 1 mM ouabain.

(e) Measurement of Cytochrome C Oxidase Activity

Cytochrome C Oxidase activity was measured

spectrophotometrically using the method of Wharton and Tzagaloff (1967). To each of 2 cuvettes, 100 μl of 10 μM potassium phosphate buffer, pH 7.0, 70 μl 1% ferrocytochrome C, and 0.83 ml of water was added. The ferrocytochrome C was reduced with ascorbic acid and dialyzed overnight prior to its addition to the reaction medium. Ten μl of 0.1 M potassium ferrocyanate was added to the "blank" cuvette in order to oxidize the ferrocytochrome present. Both cuvettes were incubated for 10 minutes at 37°C at which time the reaction was initiated by the addition of 10 μl cardiac SR (approximately 5 μg protein) to the "reaction" cuvette. The decrease in absorbance at 550 nm was used as an index of the rate of oxidation of ferrocytochrome C and was monitored for 3 minutes. Cytochrome C Oxidase activity of the SR preparation was determined using the following first order rate equations:

$$k = \frac{\ln \text{ (absorbance at time 0) min}}{\text{(absorbance at 1 minute)}}$$

$$\text{Specific Activity} = k \frac{\text{(concentration of cytochrome c)}}{\text{(concentration of SR)}}$$

The specific activity was expressed as nmole of cytochrome c oxidized per mg SR protein per minute.

(f) Measurement of Free and Long-Chain Acylcarnitines in Skeletal Muscle Sarcoplasmic Reticulum Isolated From Diabetic Rats

Aliquots of microsomal SR (containing 1-4 mg protein) were pooled and centrifuged at 40,000xg for 45 min. at 4°C. The pellet was then suspended in cold 6% perchloric acid. A 100 μl

aliquot was neutralized with 2M tris base and used to determine the levels of total carnitine present. The remainder of the sample was then centrifuged at 12,000g for 10 min. A 200 μl aliquot of the subsequent supernatant was aso neutralized with tris base and was used to measure the levels of acid-soluble free carnitine. The pellet obtained was washed with 6% perchloric acid and was used to determine the levels of (acid insoluble) long-chain acylcarnitines. Total carnitine and long-chain acylcarnitines were assayed as free carnitine following alkaline hydrolysis of the samples by incubation at 70°C for 1 hour in the presence of 1 M tris base and 0.4 N KOH (pH approx. 13). Following incubation, samples were neutralized with 0.6 N HCl and were assayed for carnitines.

Free carnitine was measured using the radioisotopic procedure developed by McGarry and Foster (1976), which uses ^{14}C-acetyl-CoA, carnitine acetyltransferase, and sodium tetrathionate. The samples were incubated for thirty minutes following which a 0.3 ml aliquot of Dowex 1X8-400 anion exchange resin was added and the samples vortexed and placed on ice. The samples were vortexed twice at 10 min intervals, and centrifuged at 3000g for 5 min. A 0.7 ml aliquot of the supernatant was then placed in Aquasol^R and measured for radioactivity in a liquid scintillation counter. The amount of ^{14}C-acetyl-CoA in the supernatant fraction is stoichiometrically related to the amount of carnitine present in the sample. Levels of free carnitine and long-chain

acylcarnitines are expressed as nmoles/mg sarcoplasmic
reticulum.

(g)   Assay of (Ca^{++}-Mg^{++})-ATPase Activity in Red Blood Cell
Membranes

EDTA-washed red cell membranes (50-250 μg protein) were
freeze-thawed twice and then incubated at 37°C for 30 minutes
in the presence of 44 mM tris-Cl, pH 7.4, 4.0 mM $MgCl_2$, 0.1 mM
ouabain, 0.17 mM $CaCl_2$, and 0.15 mM EGTA resulting in a free
Ca^{++} concentration of 10 μM as determined by MULT-S. The
reaction was initiated by the addition of 2 mM ATP, allowed to
proceed for 15 minutes, and stopped by the addition of 0.5 ml
2% SDS to yield a final reaction volume of 0.71 ml. The
anti-calmodulin agents, trifluoperazine dihydrochloride (60 μM)
and Compound 48/80 (1 μg/ml), when used, were freshly prepared
and assayed in the dark (Roufogalis, 1981). When calmodulin (60
nm) or the extracts derived from skeletal muscle SR were to be
assayed, they were added to membranes 10 minutes prior to the
start of the reaction.

(Ca^{++}-Mg^{++})-ATPase activity was measured by the release of
inorganic phosphate (Pi) using the semi-automated procedure of
Fiske and Subbarow (1925) as modified by Raess and Vincenzi
(1980). Samples were analyzed using a Technicon^R Autoanalyzer
Pump I with a 16 channel manifold and a Technicon^R Sampler II
fitted with a 40-2/1 cam handling 40 samples per hour. Two
large mixing coils provided for adequate mixing between
reagents (see below) and samples. Output of the Autoanalyzer

was connected to a flow-through cell located in a Technicon[R]

Spectrophotometer. Absorbance was monitored at 660 nm and

recorded on a Technicon[R] chart recorder. The reagents used were:

i) acid molybdate solution (130 ml conc. H_2SO_4, 25 g ammonium

molybdate, made up to 1L),

ii) SDS: 6% and 2% (w/v),

iii) 9% ascorbic acid (w/v)

A phosphate standard curve ranging from 0-250 nmoles Pi/ml

KH_2PO_4 was run with each experiment and found to be linear over

the range of concentrations used.

Ca^{++}-dependent ATPase activity was determined by

subtracting the activity obtained in the presence of Ca^{++} and

EGTA from that obtained with EGTA alone.

(h) Determination of calmodulin content of skeletal SR extracts

The levels of calmodulin present in skeletal SR extracts

were determined by radioimmunoassay using a calmodulin

[^{125}I]-RIA kit (New England Nuclear[R]) incorporating the method

of Chafouleas et al (1979). The radioimmunoassay kit is a

competitive inhibition system employing iodine-125 labelled

calmodulin as the tracer and a specific sheep anti-calmodulin

antibody as the binder. Aliquots (100 μl) of skeletal SR

extracts were compared to standard calmodulin concentrations

(0.31 - 20 ng /100 μl) and levels of calmodulin expressed as

ng / starting mg of SR protein.

(3) Electrophoretic Methods ·

(a) Sodium Dodecyl Sulfate Polyacrylamide Slab Gel
Electrophoretic Separation of Proteins

Polyacrylamide slab gels (12.5%) of 1.5 mm thickness were
cast according to the method of Laemmli and Favre (1973), using
a 5% stacking gel. Typically, 75 μl of extract derived from
skeletal SR was boiled for 90 seconds in 25 μl of a medium
consisting of (final concentration): 62.5 mM tris-Cl, pH 6.8,
2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.002%
bromphenol blue. Samples were centrifuged at 10,000xg for 5
minutes and 50-55 μl applied per well. Gels were run at 35 mA
constant current for 6 hours, silver stained according to the
procedure of Morrissey (1981), and dried. The protein standards
used for estimation of molecular weights (in daltons) were
myosin (200,000), β-galactosidase (116,250), phosphorylase b
(92,500), bovine serum albumin (66,200), ovalbumin (45,000),
carbonic anhydrase (31,000), soybean trypsin inhibitor
(21,500), and lysozyme (14,400).

(b) Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
and Autoradiography of Phosphorylated Cardiac SR Preparations

Phosphorylated samples were run concomitantly for analysis
by SDS-gel electrophoresis and autoradiography. The incubation
conditions were identical to those described above except for
the termination of the reaction by an "SDS stop solution"
consisting of (final concentration): 0.083 mM tris-Cl, pH 6.8,

2.5% SDS, 0.12 M sucrose, 0.50 M 2-mercaptoethanol, 0.0067%
bromphenol blue, 1.7 mM Na_2ATP, and 3.3 mM KH_2PO_4. Samples were
heated at 95°C for 90 seconds, centrifuged at 1500xg for 5
minutes and then applied onto the gel. Electrophoresis was
performed as previously described except that some gels were
cast as a 5-20% acrylamide gradient in addition to the usual
12.5% acrylamide concentration. Following electrophoresis, gels
were stained with 0.25% Coomassie Blue in 50% methanol/10%
acetic acid, destained overnight in 7.5% acetic acid/5%
methanol, and then dried under vacuum at 80°C for 2 hours. The
standard proteins were then spotted with ^{32}P-ATP (50 dpm/μl)
and the dried gel placed in contact with X-ray film (Kodak^R
X-OMAT AR) along with intensifying screens (Cronex Lightning
Plus^R) for 7-16 days at -80°C following which the film was
developed.

(4) Miscellaneous Methods

(a) Protein Assay

Microsomal SR or red cell membranes (10-150 μg protein)
were suspended in distilled water to a final volume of 1.5 ml.
To this was added 12.5 μl of a 2% deoxycholate solution.
Following a ten minute incubation at room temperature, 0.5 ml
of cold trichloroacetic acid (24%) was added to precipitate any
protein. The suspension was centrifuged at 3000 x g for thirty
minutes, the supernatant aspirated, and the pellet assayed for
protein using the standard Lowry (1951) protein assay. Bovine

serum albumin was used as the standard protein.

(b) Statistical Analysis

When two samples were compared, statistical analysis was performed using the unpaired Student's t-test. A probability of $p < 0.05$ was used as the level of significance.

(c) Determination of Free Calcium Concentration

Free calcium values were calculated using the Mult.S Fortran program written by Mr. Roland Burton. The program calculates the apparent association constant of calcium and EGTA for the pH selected. Binding of both Mg^{++} and Ca^{++} to ATP and EGTA were calculated by solving a set of simultaneous equations describing the binding. Log association constants used were first to fourth proton association with EGTA: 9.461, 8.851, 2.679, 2.000; CaEGTA: 10.650; HATP: 6.970; CaATP: 4.498; and MgATP: 4.944.

A) Characterization of a purified cardiac SR preparation

Either a freshly prepared or previously frozen (-80°C) preparation of crude cardiac vesicles were subjected to further purification using the method of Jones et al (1979) which employs Ca-oxalate loading followed by sucrose density gradient centrifugation. Typically, 20-25 mg of crude SR yielded 4-5 mg of purified SR.

(i) Assesment of purity using marker enzyme assays

A summary of the marker enzyme assays used to determine the presence of membranes of other than SR origin in the two preparations is shown in Table 2. Purified SR demonstrated at least a three-fold decrease in cytochrome c oxidase activity, a measure of mitochondrial membrane contamination. Initial experiments strictly followed the protocol of Jones et al (1979) which did not include the mitochondrial Ca^{++}-uptake inhibitor, sodium azide. Values of cytochrome c oxidase, therefore, were initially identical in crude and purified SR preparations (data not shown). Only when the azide was included was the decrease in cytochrome c oxidase apparent.

Sarcolemmal membrane contamination was determined by measurement of "patent" and "latent" Na^+,K^+-ATPase activity. As shown in Table 2, both crude and purified SR had approximately equal "patent" ouabain-inhibitable Na^+,K^+-ATPase activity. Following incubation with 0.05% Triton X-100, however, a much greater degree of "latent" activity was

Table 2
Marker enzyme assays performed as described in Methods for determination of
the degree of mitochondrial and sarcolemmal contamination of purified and
crude cardiac sarcoplasmic reticulum preparations. Results shown are typical
of two experiments performed on different SR preparations.

Assay	Crude SR	Pure SR
1. Cytochrome C oxidase activity (nmole of cyt C oxidized/mg SR/min)	12.8	4.0
2. Na^+,K^+-ATPase activity (nmol/mg/min)		
i) "Patent" ouabain (1 mM) inhibited	88	80
ii) "Latent" ouabain (1 mM) inhibited	242	88

recorded in the crude preparation, suggesting the greater
number of sarcolemmal enzyme sites in the crude preparation.

Further comparison of crude and purifed SR utilizing
SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a
marked similarity in protein composition with one notable
exception: the absence of a 95,000 dalton protein in the
purified preparation (Figure 8). The absence of the 95 Kdalton
protein in purified SR was confirmed using either Coomassie
Blue or silver staining techniques (data not shown).

ii) Calcium Uptake and ATPase activity in the Purified Cardiac
SR Preparation

Measurement of Ca^{++}-uptake activity in the purified SR
preparation revealed a 3-5 fold enhancement over the activity
typically obtained with crude SR. Because of this enhanced
activity, only 3-5 µg protein per incubation tube of the
purified preparation was required as opposed to 20-30 µg of
crude SR. The time course of Ca^{++} uptake by purified SR is
shown in Figure 9. In contrast to the crude preparation, which
transported Ca^{++} as a linear function of time for 10 minutes,
purified SR maintained linearity for 1 minute, following which
Ca^{++} uptake deviated from linearity. Further transport studies
utilizing purified SR therefore used 1 minute as the assay
duration in order to insure linearity.

At all free Ca^{++} concentrations tested (0.1 - 2.0 µM),
purified SR demonstrated an enhanced level of Ca^{++}-uptake
activity compared to crude SR (Figure 10). The effect appeared

FIGURE 8

Sodium dodecyl sulfate-polyacrylamide (12.5%) gel
electrophoretic pattern of two different preparations of crude
and purified cardiac sarcoplasmic reticulum, performed as
described in Methods. Approximately 3 μg of protein were
applied of each sample and silver stained according to the
procedure of Morrissey (1981).

MW standards   crude   SR   pure   SR

200,000

116,250

92,500

66,200

45,000

31,000

21,500

14,400
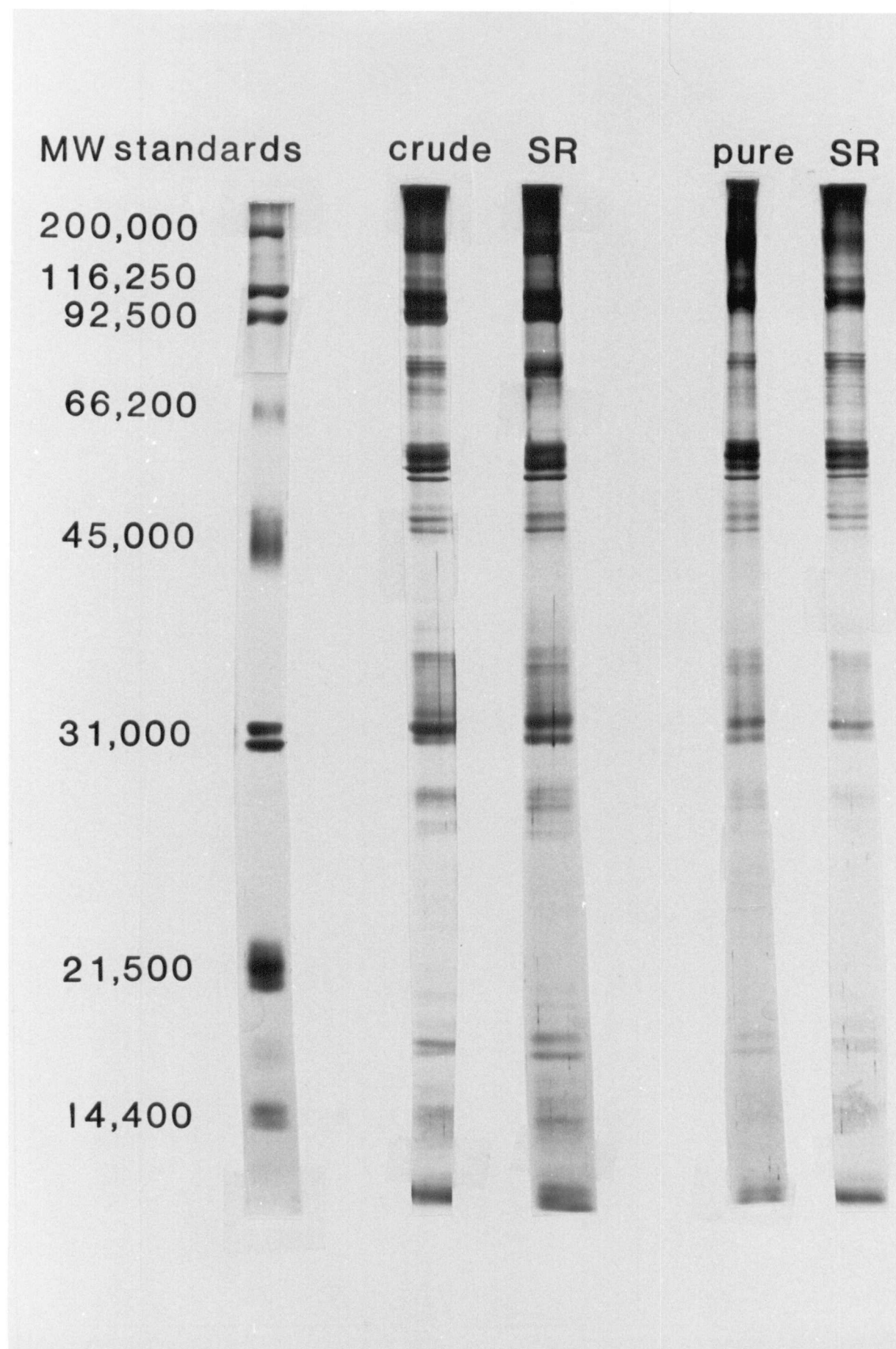
FIGURE 9

Time course of calcium uptake in a crude and purified
preparation of dog cardiac sarcoplasmic reticulum. Calcium
uptake was measured in the presence of 1 µM free Ca^{++} as
described in Methods, in a crude (□————□) and purified
(○————○) SR preparation.
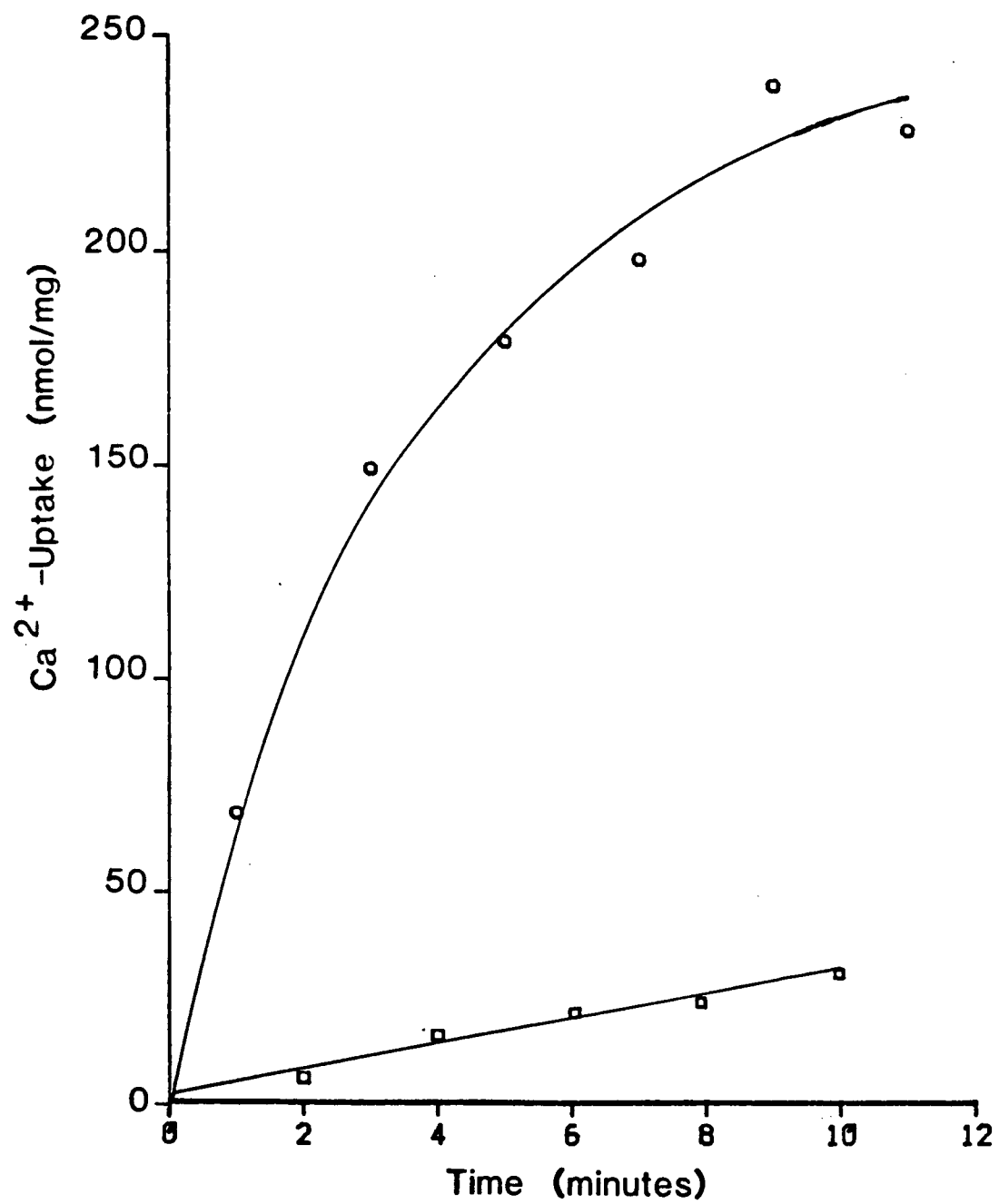
FIGURE 10

Effect of various calcium concentrations on Ca^{++} uptake

activity in purified (○————○) and crude (□————□) cardiac

sarcoplasmic reticulum preparations. Calcium uptake and free

Ca^{++} concentrations were determined as described in Methods.

Result shown is a typical experiment.
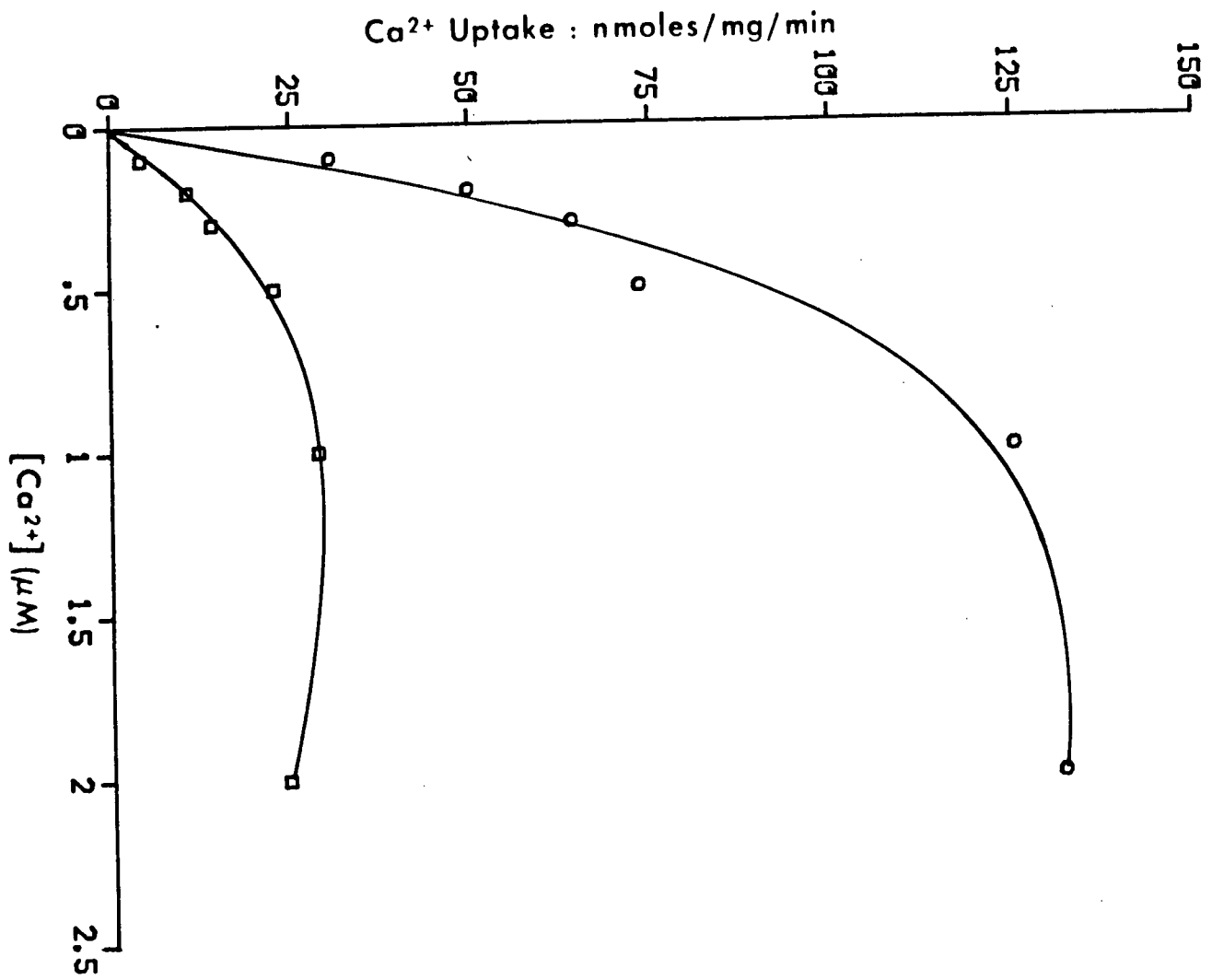
to be one of Vmax, since the Km for Ca^{++} in both preparations was the same (0.3 μM free).

$(Ca^{++}-Mg^{++})$-ATPase activity was also enhanced more than three-fold in purified SR compared to crude (Table 3), paralleling the findings of an enhanced Ca^{++}-uptake activity in the former preparation; Ca^{++}-ATPase activity stimulation was approximately two-fold in purified SR.

iii) Regulation of the Purified Cardiac SR Preparation

The effect of two of the documented regulators of cardiac SR, calmodulin (CAM) and cAMP-dependent protein kinase (cAMP-PK) was investigated in the purified SR preparation. As shown in Figure 11, both CAM (1 μM) and cAMP-PK (0.5 mg/ml) stimulated Ca^{++} uptake activity at both 0.2 and 1.0 μM free calcium. As has been previously shown in crude SR (Tada et al, 1975; Kirchberger and Antonetz, 1982b; Plank et al, 1983) the degree of stimulation over basal activity by both CAM and cAMP-PK was more marked at the lower free Ca^{++} concentration; enhancement by CAM of Ca^{++} transport, for example, was 73% at 0.2 μM free Ca^{++} and 40% at 1.0 μM free Ca^{++}. Essentially, the same findings, with respect to stimulation by CAM, were obtained when we examined Ca^{++}-dependent ATPase activity (Figure 12). Stimulation by cAMP-PK, however, was more marked at 1.0 μM free Ca^{++} than at the lower free Ca^{++}. When both CAM and cAMP-PK were present in the incubation, stimulation of ATPase activity was additive at 0.2 μM free Ca^{++}, but there was no further stimulation at the higher free Ca^{++}.

Table 3
Measurement of Ca^{2+}-ATPase activity in crude or purified dog cardiac sarcoplasmic reticulum.

Condition	Mg^{2+}-ATPase Activity (μmole/mg/min)	$(Ca^{2+}-Mg^{2+})$-ATPase Activity (μmole/mg/min)	Ca^{2+}-ATPase Activity (μmole/mg/min)
crude SR	0.160	0.215	0.055
purified SR	0.634	0.740	0.106

$(Ca^{2+} - Mg^{2+})$-ATPase activity was measured at 10 μM free Ca^{2+} as described in Methods. Result shown is a typical experiment.

FIGURE 11

Calcium uptake activity at two different free Ca^{++}

concentrations (0.2 and 1.0 μM) in a purified preparation of

dog cardiac sarcoplasmic reticulum in the presence of 1 μM

calmodulin (CAM) or 1 μM cAMP plus 0.5 mg/ml cAMP-dependent

protein kinase (cAMP). Free calcium concentrations and Ca^{++}

uptake were determined as described in Methods. Result shown

is a typical experiment.
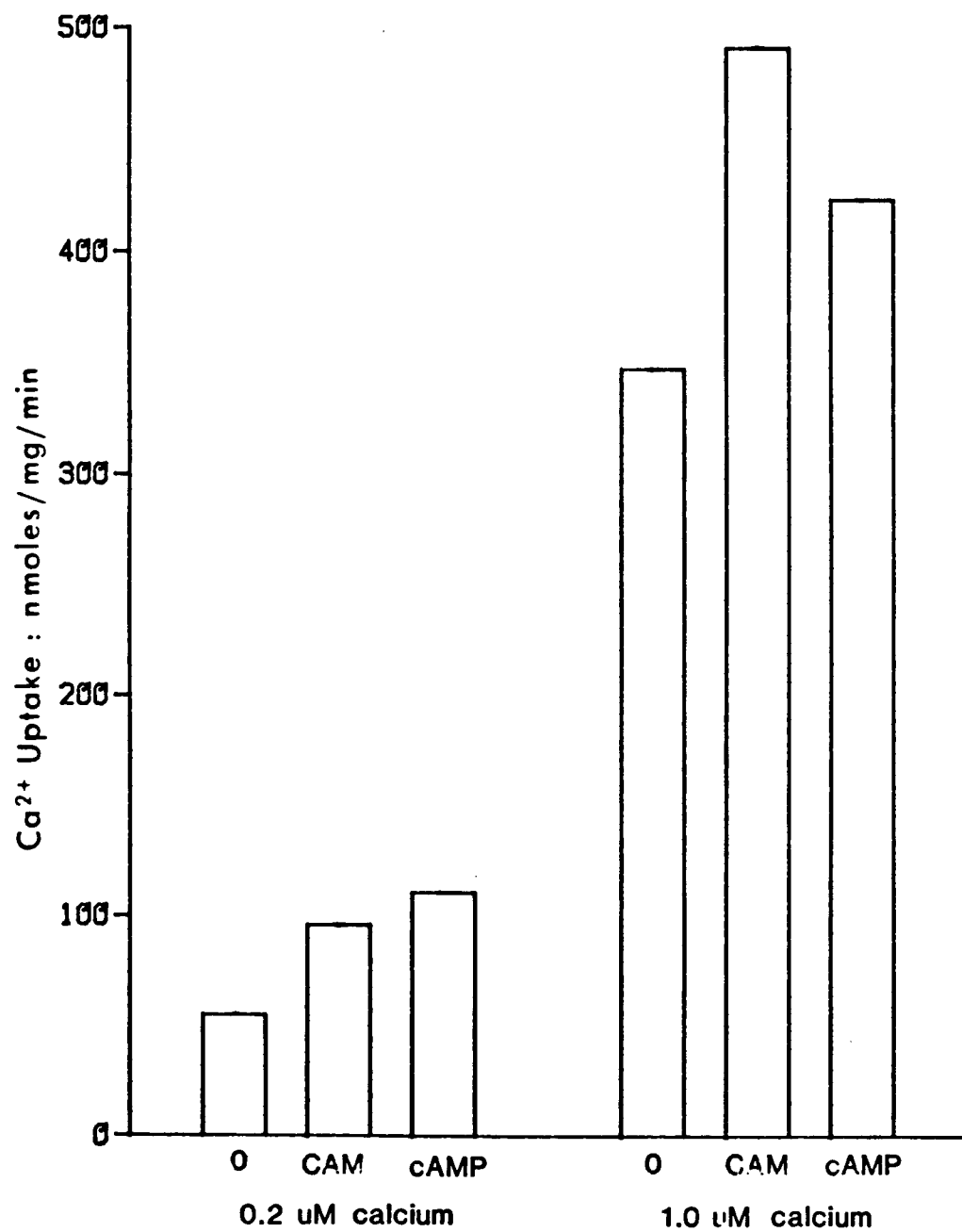
FIGURE 12

Calcium-dependent ATPase activity at two different free Ca
concentrations (0.2 and 1.0 μM) in a purified preparation of
dog cardiac sarcoplasmic reticulum in the presence of 1 μM
calmodulin (CAM), 1 μM cAMP plus 0.5 mg/ml cAMP-dependent
protein kinase (cAMP), or both calmodulin and cAMP plus
cAMP-dependent protein kinase (cAMP/CAM). Calcium ATPase
activity and free Ca^{++} concentrations were determined as
described in Methods. Result shown is a typical experiment.
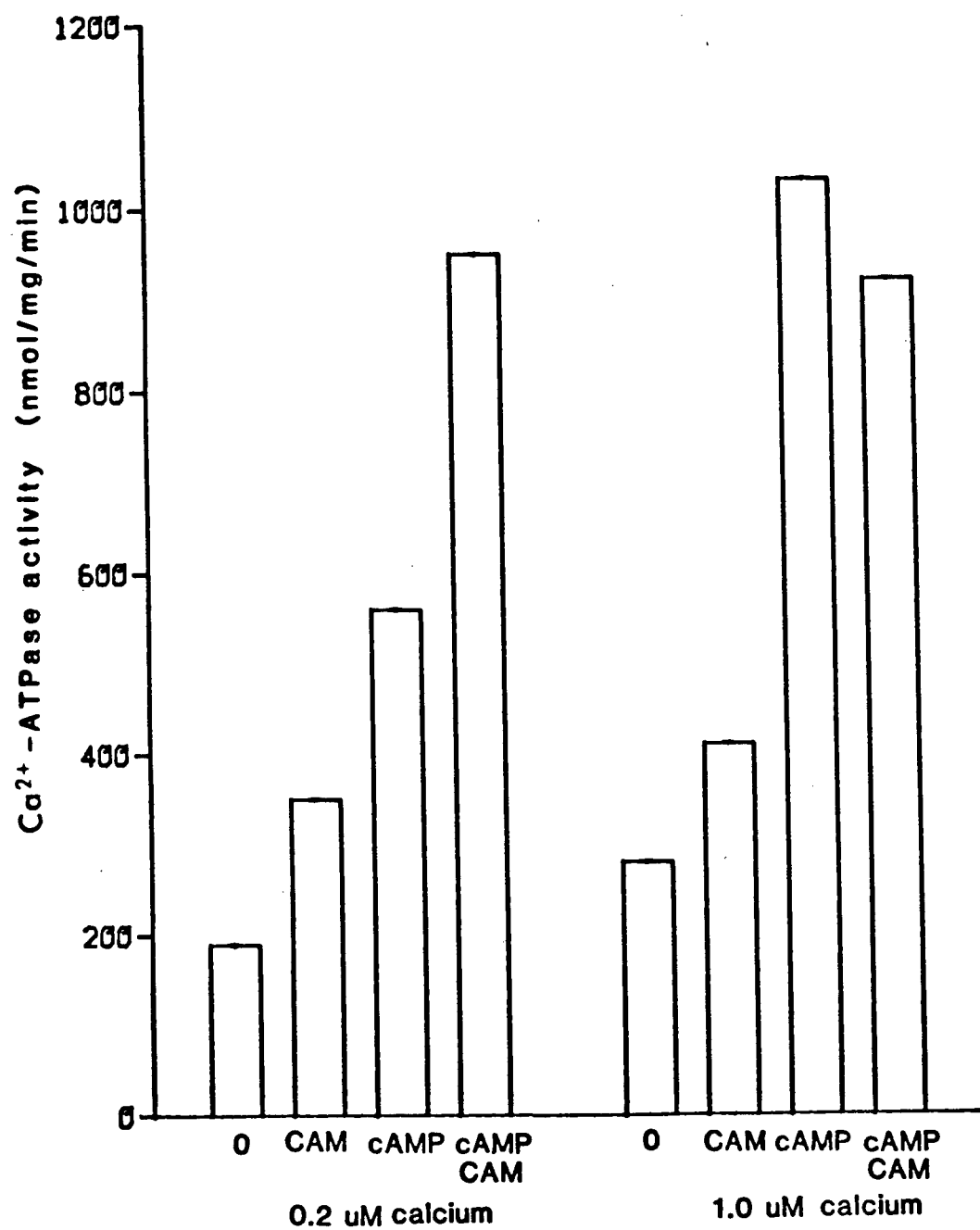
A further study of the regulatory mechanisms of the
purified preparation was carried out by an examination of total
levels of protein phosphorylation. Figures 13 (a) & (b) show
the time-course of phosphorylation of crude and purified
cardiac sarcoplasmic reticulum vesicles performed at $30^{\circ}C$
following preincubation of SR with either cAMP-PK (0.1 mg/ml),
the catalytic ("C") subunit of cAMP-PK (0.5 μM), or CAM (1 μM)
in the presence of 2.0 μM free Ca^{++}. In both crude and purified
preparations, incorporation of phosphate increased with time,
the highest levels of incorporation in both SR preparations
attained in the presence of cAMP-PK. The maximum levels of
cAMP-PK and C subunit phosphorylation, however, in the purified
preparation exceeded that of crude SR by 30% and 55%,
respectively. Although the degree of phosphorylation by C
subunit was not as marked as that seen with cAMP-PK, the
half-time of phosphorylation by both regulators was similar,
estimated at 20-30 seconds. In contrast, CAM-dependent
phosphorylation in both crude and purified SR was slower (t 1/2
= 45-60 sec) and lower compared to cAMP-PK and C subunit
phosphorylation. Unlike the higher levels of cAMP-dependent
phosphorylation noted, CAM-dependent phosphate incorporation
was decreased by 50% in purified SR as compared to crude SR.
The maximum attainable level of Ca^{++}-dependent phosphorylation
was also decreased in purified SR.

Autoradiograms of phosphorylated SR proteins subjected to
SDS-PAGE revealed a marked similarity in the time-course
pattern in both crude and purified SR. As shown in Figure 14,

FIGURE 13

Time-course of phosphorylation in both crude (Figure a) and

purified (Figure b) dog cardiac sarcoplasmic reticulum.

Sarcoplasmic reticulum vesicles (5-15 µg) were preincubated at

30° C for 10 minutes in the presence of 40 mM histidine-Cl, pH

6.8, 110 mM KCl, 4 mM $MgCl_2$, 10 mM NaF, 5.7 µM A23187, and

either EGTA (0.1 mM) (not shown), $CaCl_2$ (2.0 µM free) (■———■),

$CaCl_2$ plus CAM (1 µM) (△———△), cAMP (1 µM) plus

cAMP-dependent protein kinase (0.1 mg/ml) (●———●), or the

catalytic subunit of cAMP-dependent protein kinase (0.5 µM)

(○———○). The reaction was initiated by the addition of 0.2

mM ATP containing ^{32}P-ATP (1-2 x 10^{6} dpm/ sample). Levels of

phosphorylation and free Ca^{++} were determined as described in

Methods. Values are typical of two experiments performed on

different SR preparations.
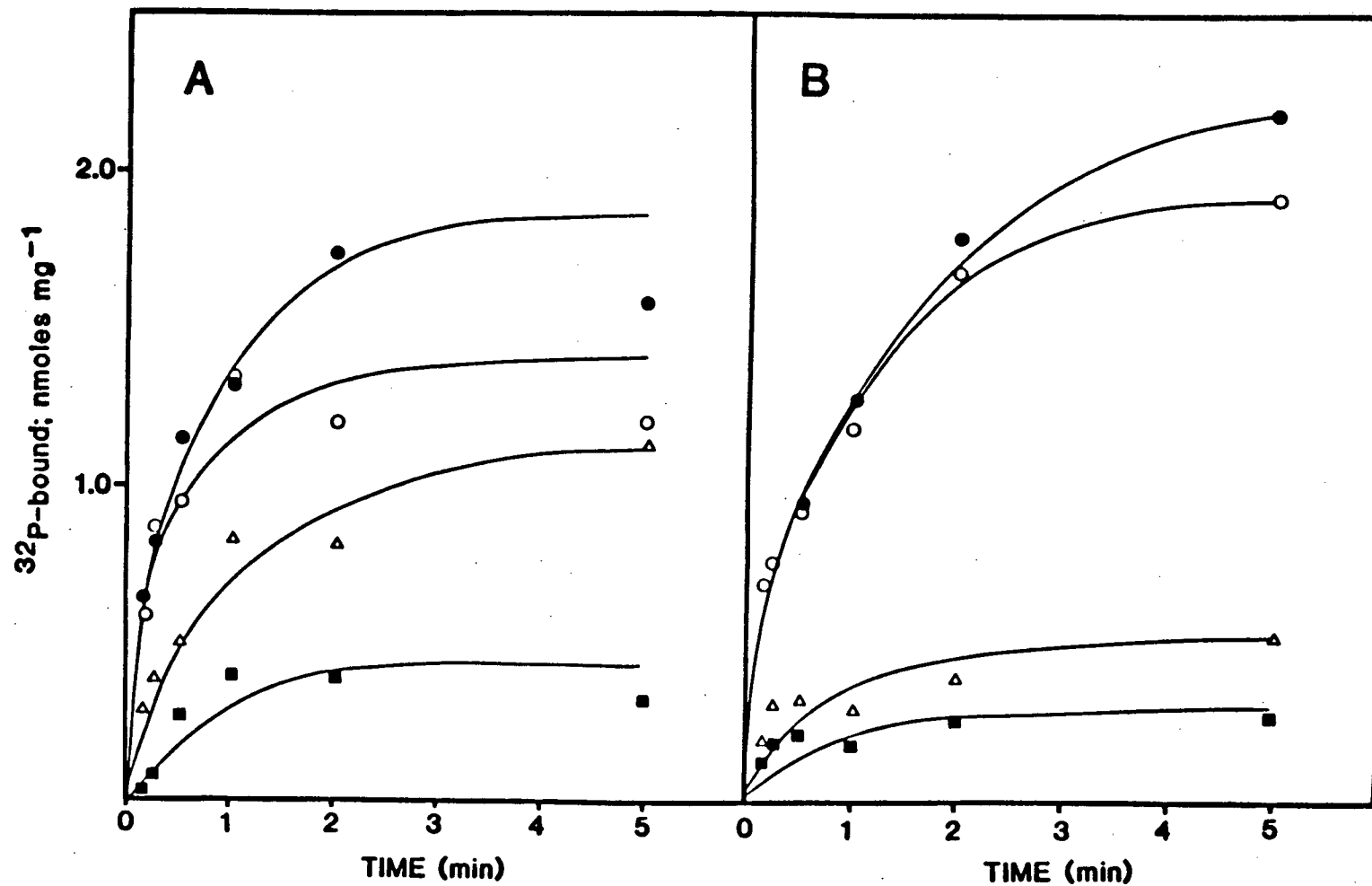
FIGURE 14

^{32}P-Autoradiography of crude dog cardiac SR phosphorylated with ^{32}P-ATP and subjected to autoradiography as described in Methods. Result shown is typical of two different crude or purified SR preparations phosphorylated for various incubation times (10 sec, 30 sec, etc.). The time-course was performed either in the presence of cAMP-dependent protein kinase or the catalytic (C) subunit of cAMP-dependent protein kinase. The molecular weight (in daltons) of phosphorylated bands is shown on the ordinate.

| 98,000 |
| 86,000 |
| 68,000 |
| 56,000 |
| 51,000 |
| 44,000 |
| 9000 |

| 10 sec | 30sec | 1min | 2 min | 5 min | | 10 sec | 30sec | 1min | 2 min | 5 min |

cAMP-dependent PK   C Subunit of PK
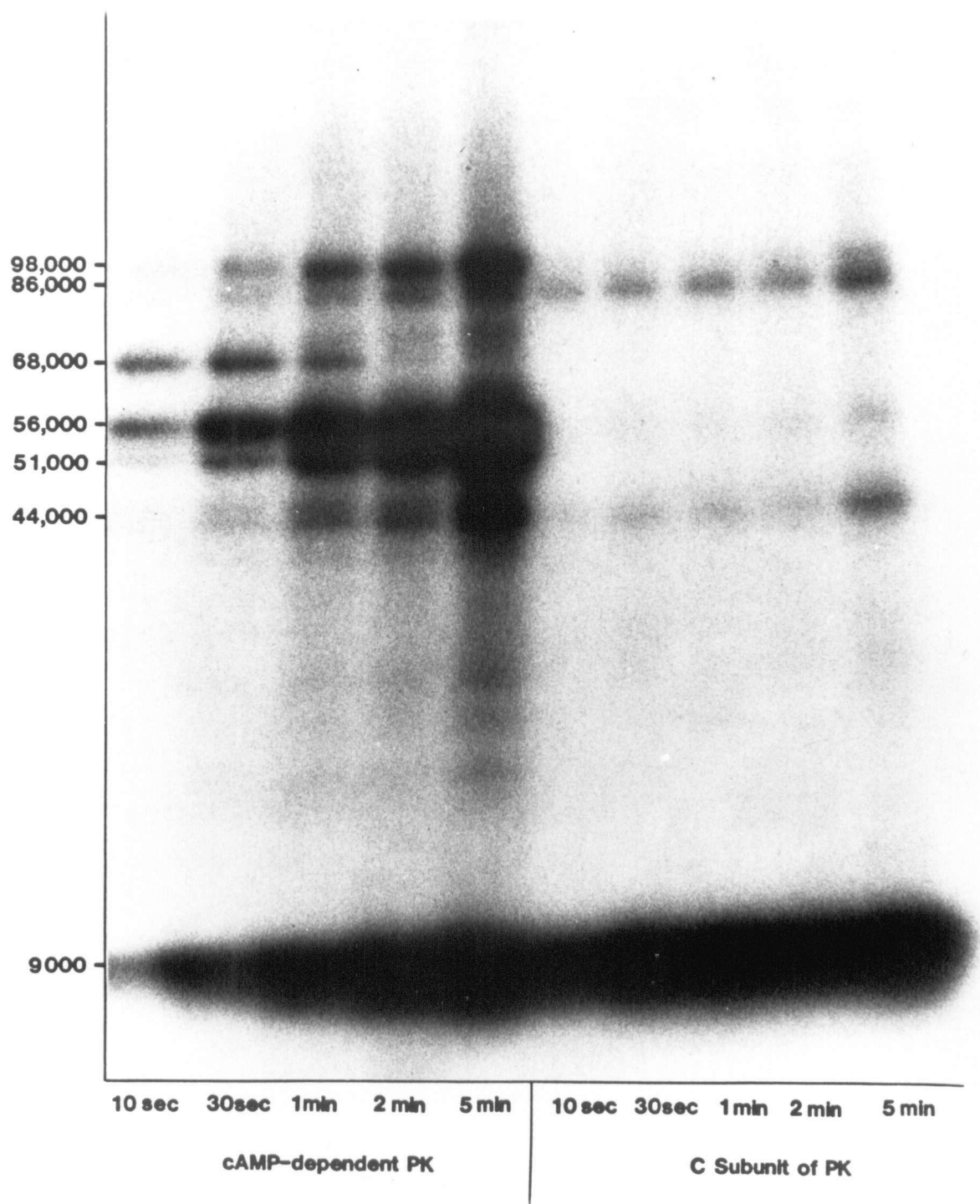
cAMP-PK phosphorylated a number of protein bands, many of which were absent from the pattern observed in the presence of C subunit. These included proteins of 66, 57, and 51 Kdaltons, of which the 66 Kdalton protein decreased in phosphate incorporation with time, while the other two proteins showed a time-dependent increase in phosphorylation. Of particular relevance was the marked phosphorylation of a 9-11 Kdalton protein believed to be phospholamban (LePeuch et al, 1979) in the presence of both cAMP-PK or C subunit. The time-course of phopholamban phosphorylation varied depending on the presence of cAMP-PK or C subunit. The presence of C subunit resulted in almost immediate, complete phosphorylation of phospholamban, achieving maximum intensity by 30 sec. In contrast, the presence of cAMP-PK typically resulted in complete phosphorylation by 2 min.

Unlike the results with cAMP-PK or C subunit, a marked difference in the phosphorylation pattern between crude and purified SR was observed in the presence of 1 μM CAM. As shown in Figure 15, in crude SR, phosphorylation of phospholamban was complete by 30 sec. whereas the level of phosphate incorporation in the purified preparation appeared to steadily increase with time and never plateau. The level of CAM-dependent phosphorylation achieved after 5 minutes in purified SR was not as intense as that seen at steady-state in crude SR, although similar amounts of protein (4 μg) were applied to the electrophoretogram. A number of proteins unique to the crude SR preparation were also phosphorylated by CAM, in

FIGURE 15

^{32}P-Autoradiogram of crude (C) or purified (P) dog cardiac SR phosphorylated with ^{32}P-ATP for the various time intervals shown in the presence of 1 μM calmodulin (CAM) and subjected to autoradiography as described in Methods. Molecular weights (in daltons) of some prominent bands are displayed on the ordinate. Result shown is a typical experiment.

C	P	C	P	C	P	C	P	C	P	C	P
10 sec		15 sec		30 sec		1 min		2 min		5 min	
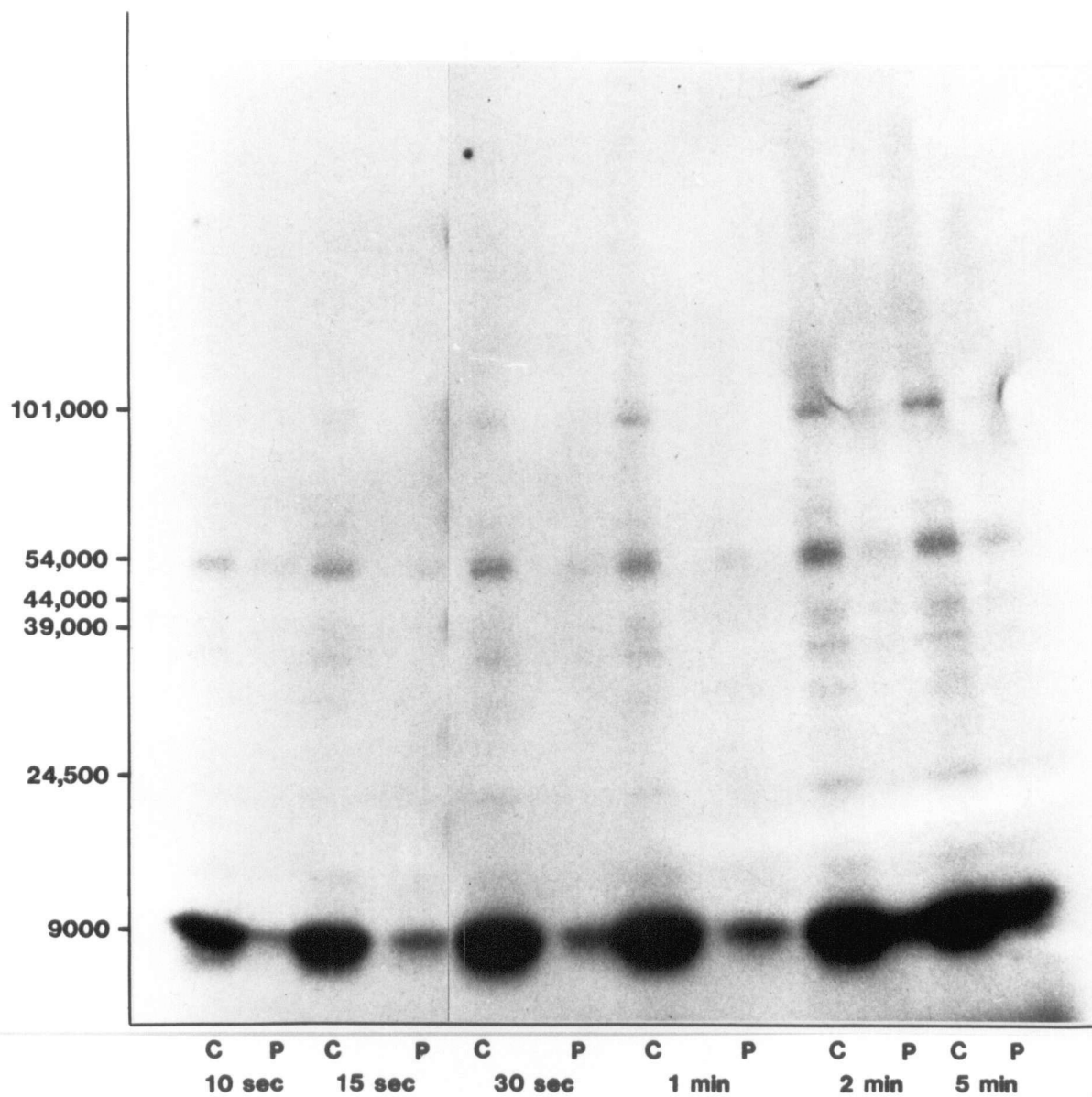
particular, 44, 39, 33, 24.5, and 14.5 Kdalton proteins, whereas only two proteins of 101 and 54 Kdaltons were phosphorylated in both the crude and purified SR. Ca^{++}, in the absence of CAM, produced no phosphorylation of proteins (not shown).

B. Studies on the Role of Calmodulin in Skeletal Muscle Sarcoplasmic Reticulum

Previous studies in our laboratory had indicated that, unlike the case in cardiac SR, exogenous calmodulin did not stimulate Ca^{++} transport in either fast or slow SR preparations. The following series of experiments was therefore conducted in order to determine whether CAM was indiginous to these preparations. The possible presence of CAM was determined in 3 ways: (i) by indirect methods using red cell Ca^{++}-ATPase activation as an indicator, (ii) by direct means using radioimmunoassay techniques and (iii) use of SDS-polyacrylamide gel electrophoresis.

(i) Indirect measurement of calmodulin activity by Ca^{++}-ATPase activation.

The red cell $(Ca^{++}-Mg^{++})$-ATPase is an excellent enzyme for the determination of the presence of CAM in tissue extracts. As shown in Figure 16, pure CAM, in a dose-dependent fashion stimulated Ca^{++}-dependent ATPase activity, half-maximal stimulation attained at 0.5 μg/ml. Subsequent work was performed at a concentration of 1.0 μg/ml (60 nM).

FIGURE 16

Effect of calmodulin on red cell calcium-dependent ATPase activity. Ca^{++}-dependent ATPase activity and free Ca^{++} concentration was determined as described in Methods. Result shown is a typical experiment.
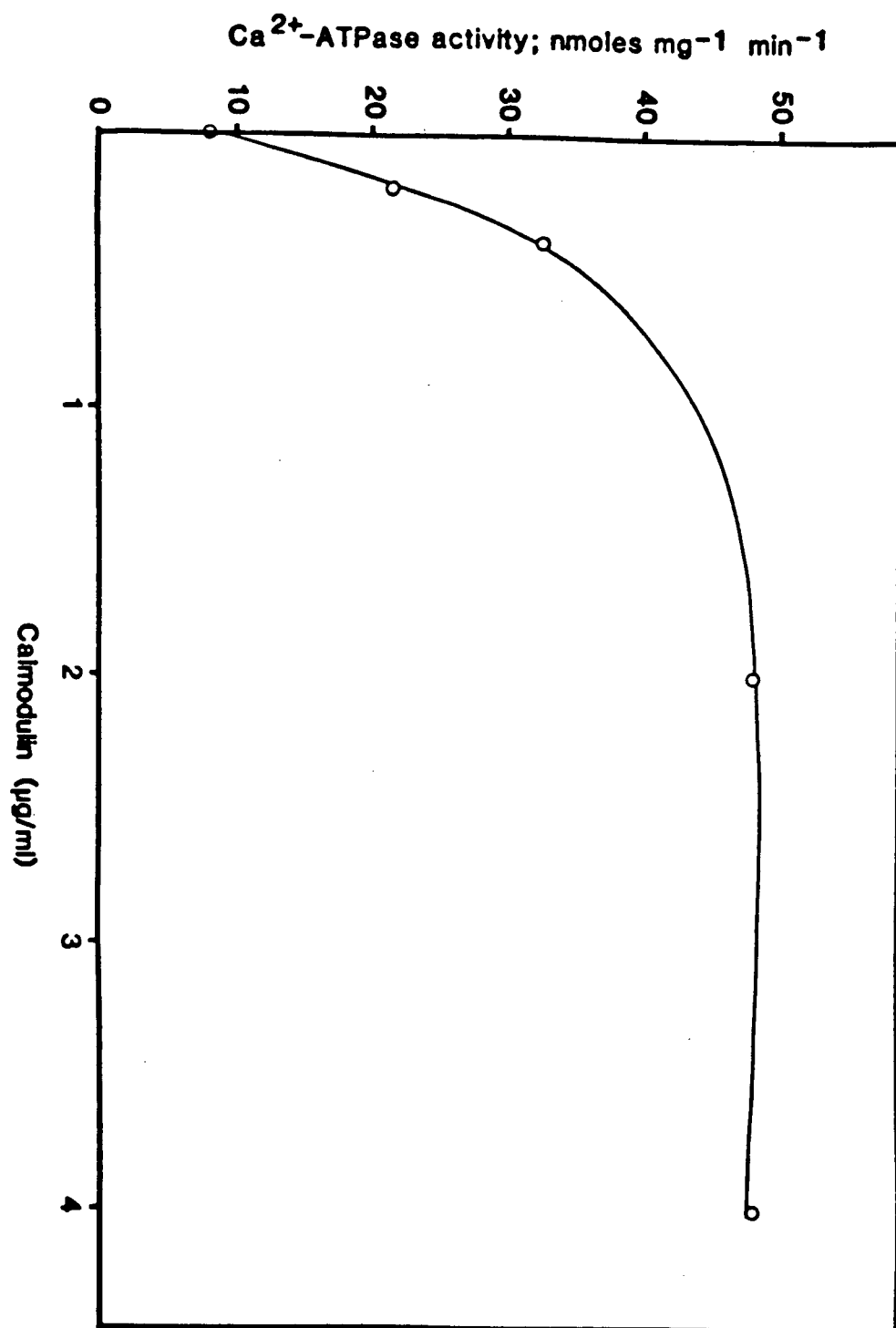
Following the suggestion of Chiesi and Carafoli (1982) that boiling was an effective way of dislodging CAM from SR vesicles, we proceeded to boil both fast (fSR) and slow (sSR) skeletal SR. In addition, we attempted to remove more CAM by both boiling and treatment with 0.2 mM EDTA to chelate any Ca^{++} away from Ca^{++}-CAM complexes. The supernatants thus obtained were then incubated with CAM-depleted red cell ghosts and the effects on the $(Ca^{++}-Mg^{++})$-ATPase activity noted. As shown in Figure 17, both boiled and boiled + EDTA-treated supernatants obtained from rabbit fSR and sSR stimulated $(Ca^{++}-Mg^{++})$-ATPase activity, the EDTA treatment appearing to enhance the stimulation observed over that obtained with boiling alone. The stimulation, nevertheless, did not approach the marked (5.5-fold) enhancement that pure CAM (60 nM) was able to effect on the system.

To determine whether the noted stimulation of $(Ca^{++}-Mg^{++})$-ATPase activity by the supernatants of boiled skeletal muscle SR preparations was attributable to CAM, we investigated whether the anti-CAM agent, trifluoperazine (TFP) could inhibit the ATPase stimulation. As shown in Table 4, stimulation of red cell $(Ca^{++}-Mg^{++})$-ATPase activity by CAM was inhibited approximately 25% by 60 μM TFP but this inhibition was not CAM-specific since marked TFP inhibition of basal (i.e. no added CAM) ATPase activity was also observed. Table 4 further shows that the boiled + EDTA-treated supernatant obtained from fSR stimulated $(Ca^{++}-Mg^{++})$-ATPase activity in a dose-dependent manner; TFP (60 μM) was able to partially

FIGURE 17

Effect of boiled or boiled + 0.2 mM EDTA-treated supernatants (75 μl) derived from fast (fSR) or slow (sSR) rabbit skeletal SR. (Ca^{++}-Mg^{++})-ATPase activity and free Ca^{++} concentration (10 μM) was determined as described in Methods. Result shown is the mean ± S.D. of three experiments.
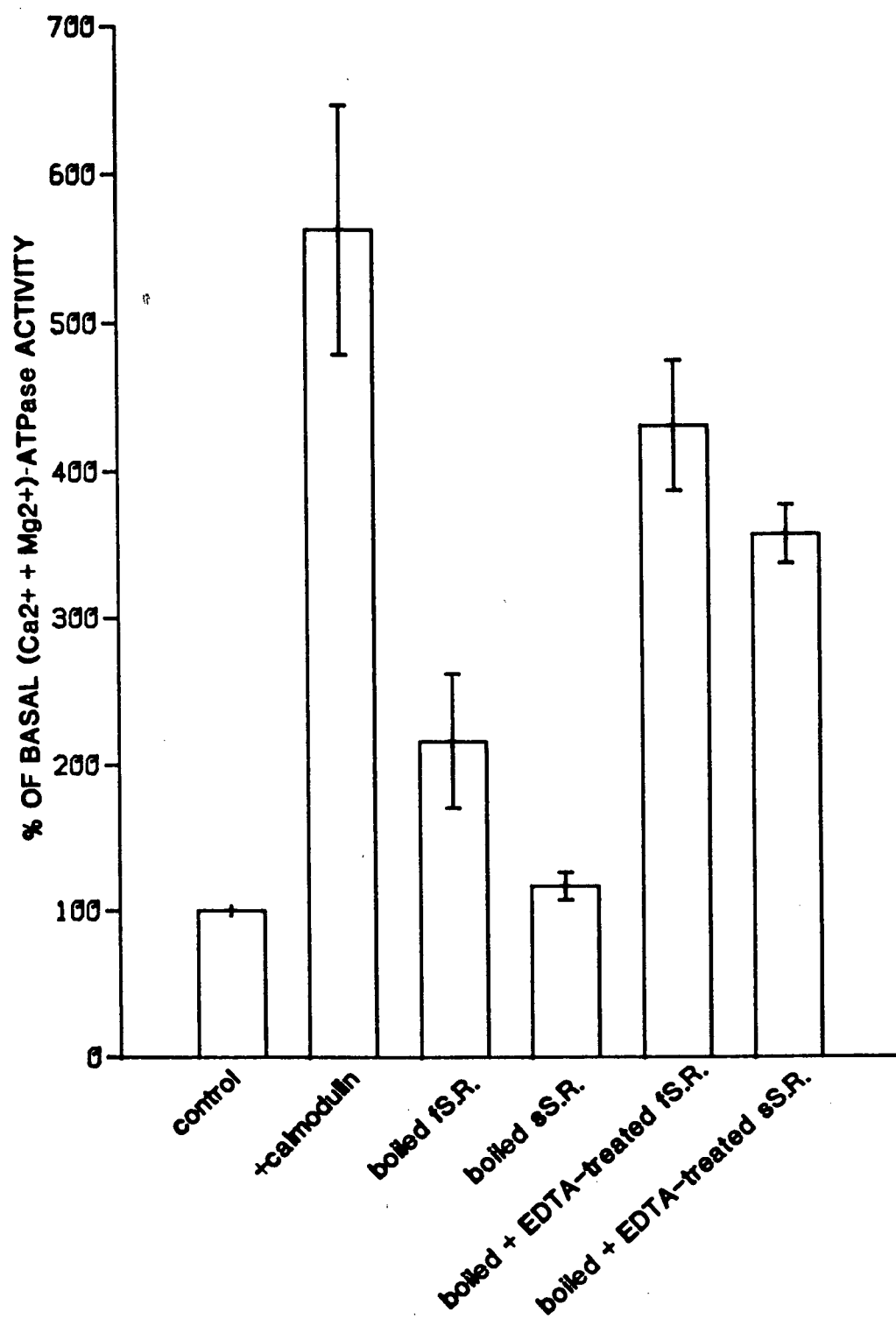
Table 4
Levels of $(Ca^{2+}-Mg^{2+})$-ATPase activity obtained following incubation
of boiled EDTA-treated fast skeletal SR extracts in the presence or absence
of TFP (60 μM).

Addition	$(Ca^{2+}-Mg^{2+})$-ATPase activity (nmoles/mg/min)	
	-TFP	+TFP
none	8.24	7.07
calmodulin (60 nM)	20.81	15.42
boiled + EDTA-treated fSR supernatant:		
10 μl	9.25	7.83
75 μl	17.65	14.60

$(Ca^{2+}-Mg^{2+})$-ATPase activity and preparation of supernatants were as
described in Methods. Result is typical of five experiments.

FIGURE 18

Effect of 60 nM calmodulin (CAM) and 60 μM trifluoperazine (TFP) in the presence of CAM on calcium-dependent ATPase activity of red cell membranes prepared according to the method of Niggli et al (1981). Ca^{++}-ATPase activity and free Ca^{++} concentration (10 μM) were determined as described in Methods. Result shown is the mean $\pm$ S.D. of three experiments.
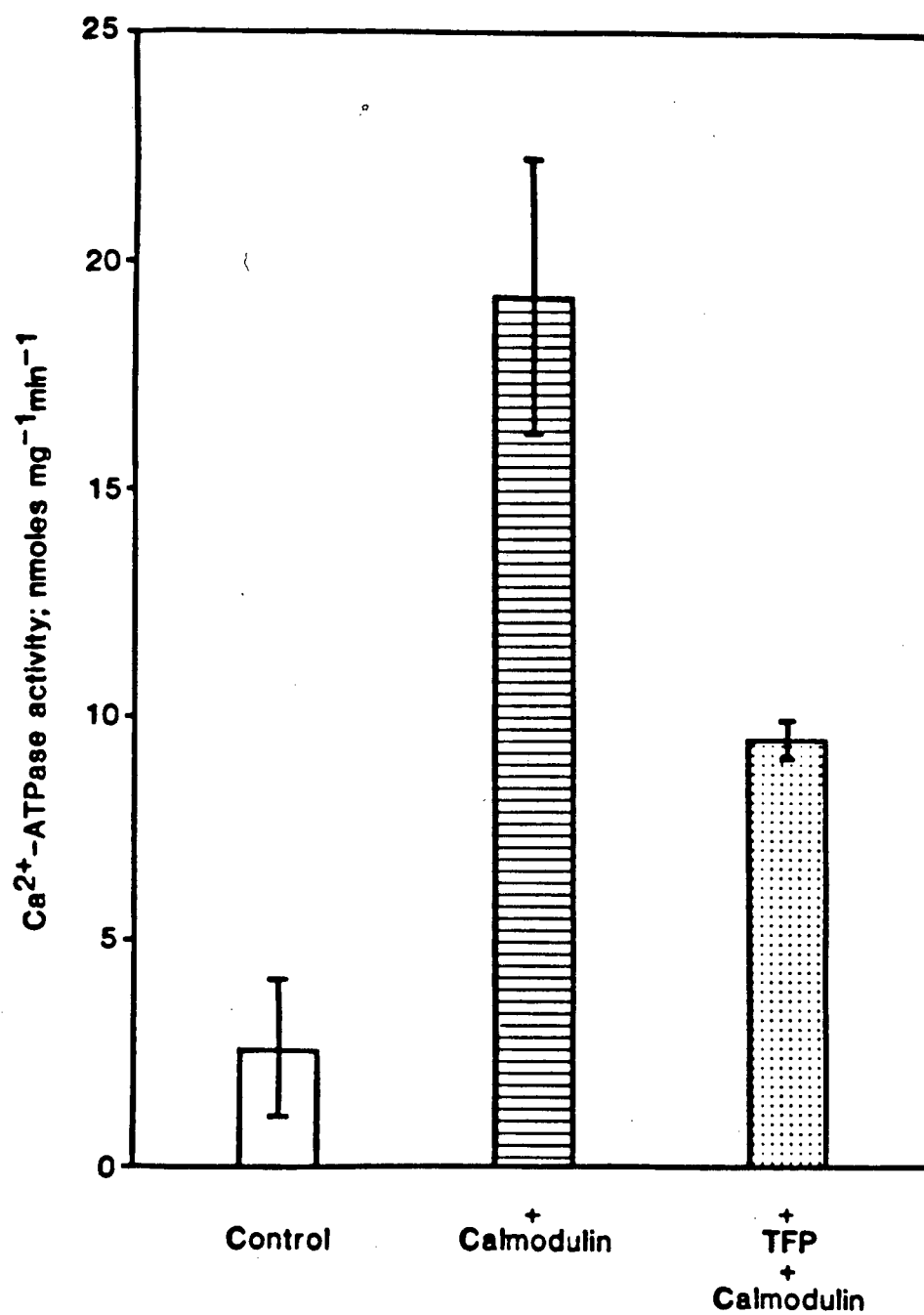
reverse this stimulation. This result was also observed in boiled + EDTA-treated extracts of sSR (not shown).

The level of inhibition of CAM stimulation of red cell Ca^{++}-ATPase by 60 μM TFP was rather low in comparison to other results found in the literature (e.g., Raess and Vincenzi, 1980). It was therefore decided to switch to a red cell membrane preparation that had been shown previously to contain a CAM-dependent (Ca^{++}-Mg^{++})-ATPase activity that was more markedly inhibited by TFP (Niggli et al, 1981) . As shown in Figure 18, the Ca^{++}-dependent ATPase activity of EDTA-washed membranes prepared according to this method was stimulated 4-6-fold by CAM; 60 μM TFP produced a 50% inhibition of this CAM-stimulated Ca^{++}-dependent ATPase activity. Not shown in Figure 18 however, is the marked inhibition (25-40%) by this concentration of TFP of Ca^{++}-ATPase activity in the absence of added CAM. In addition, stimulation by boiled + EDTA-treated extracts was still only inhibited by 30-45% (not shown).

It was clear, therefore, that before any conclusions could be drawn concerning the presence or absence of CAM in skeletal SR extracts, a more specific inhibitor of CAM stimulation was required. Compound 48/80, an agent that causes degranulation of mast cells (Goth, 1973) was recently shown by Gietzen et al (1983) to antagonize the CAM-induced stimulation of red cell Ca^{++}-ATPase without suppressing the "basal" activity (that Ca^{++}-ATPase activity present in the absence of added CAM). In our hands, compound 48/80 inhibited CAM-induced stimulation of the EDTA-washed red blood cell Ca^{++}-ATPase activity (IC50 = 3.0

μg/ml) by 60-70% but, unlike the findings of Gietzen et al (1983), we observed that the anti-calmodulin agent also inhibited basal ATPase activity to the same degree (Fig 19). Compound 48/80 (10 μg/ml) inhibited the stimulation of red cell Ca^{++}-ATPase activity induced by both boiled + EDTA-treated fSR and sSR extracts by 45% and 74%, respectively (Table 5).

As mentioned in the Objectives, the work of MacLennan (Campbell and MacLennan, 1982) had implied that harsh treatment such as boiling of the SR membranes was not necessary in order to liberate bound CAM. Rather, incubation of SR with 1 mM EGTA was all that was required. Our studies, therefore, attempted to replicate the work of MacLennan (1972) who demonstrated that EGTA treatment of the SR membranes resulted in a decreased Ca^{++}-uptake activity which could be restored by re-addition of the dialyzed supernatant (i.e. calmodulin). As shown in Figure 20, Ca^{++}-uptake activity in slow skeletal SR was significantly lower than that found in fast SR; the addition of CAM was unable to stimulate uptake activity in either fSR or sSR. Similar to the findings of MacLennan (1972), SR which had been previously washed in 1 mM EGTA demonstrated a reduced capacity to transport Ca^{++} compared to control. The exogenous addition of calmodulin (thought by Campbell and MacLennan (1982) to be depleted in this type of SR preparation) did not restore uptake activity but, rather, appeared to significantly decrease Ca^{++} transport in both EGTA-washed fSR and sSR.

To further determine whether CAM was present in the supernatant derived from 1 mM EGTA-washed SR, we incubated this

FIGURE 19

Concentration dependence of compound 48/80 on

calcium-dependent ATPase activity in red cell membranes (0.1

mg) prepared according to the method of Niggli et al (1981).

Calmodulin (60 nM)-stimulated (●————●) , basal (o————o)

Ca^{++}-ATPase activity, and free Ca^{++} concentrations (10 μM) were

determined as described in Methods. Result shown is a typical

experiment.
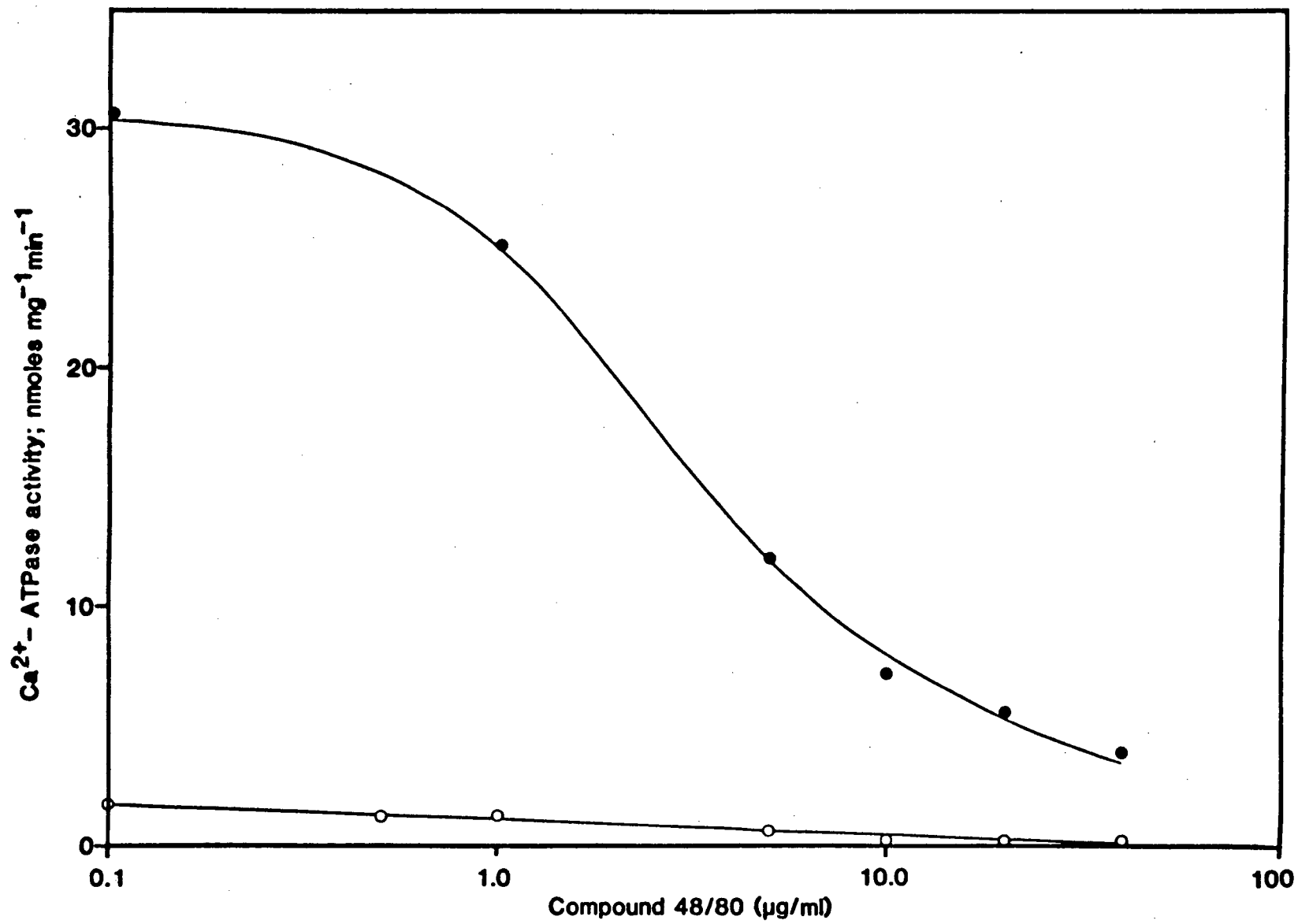
Table 5
Red Cell membrane Ca^{2+}-ATPase activity in the presence of boiled + EDTA-treated supernatants derived from fast and slow skeletal SR and the effect of compound 48/80 (10 µg/ml)

Addition	(Ca^{2+})-ATPase activity (nmoles/mg/min)		
	-48/80	+48/80	%inhibition
none	3.34	1.29	61
calmodulin (1 µM)	26.54	8.77	67
boiled + EDTA-treated fSR supernatant	7.48	4.13	45
boiled + EDTA-treated sSR supernatant	9.80	2.58	74

Measurement of Ca^{2+}-ATPase activity and preparation of supernatants were performed as described in methods. Results represent a typical experiment.

FIGURE 20

Calcium-uptake activity of skeletal fast SR (fSR), slow SR (sSR), or 1 mM EGTA-washed fSR and sSR in the presence of calmodulin (0.24 μM). Ca^{++}-uptake activity, preparation of SR, and free Ca^{++} concentrations were as described in Methods. Result shown is the mean $\pm$ S.D. of three experiments.
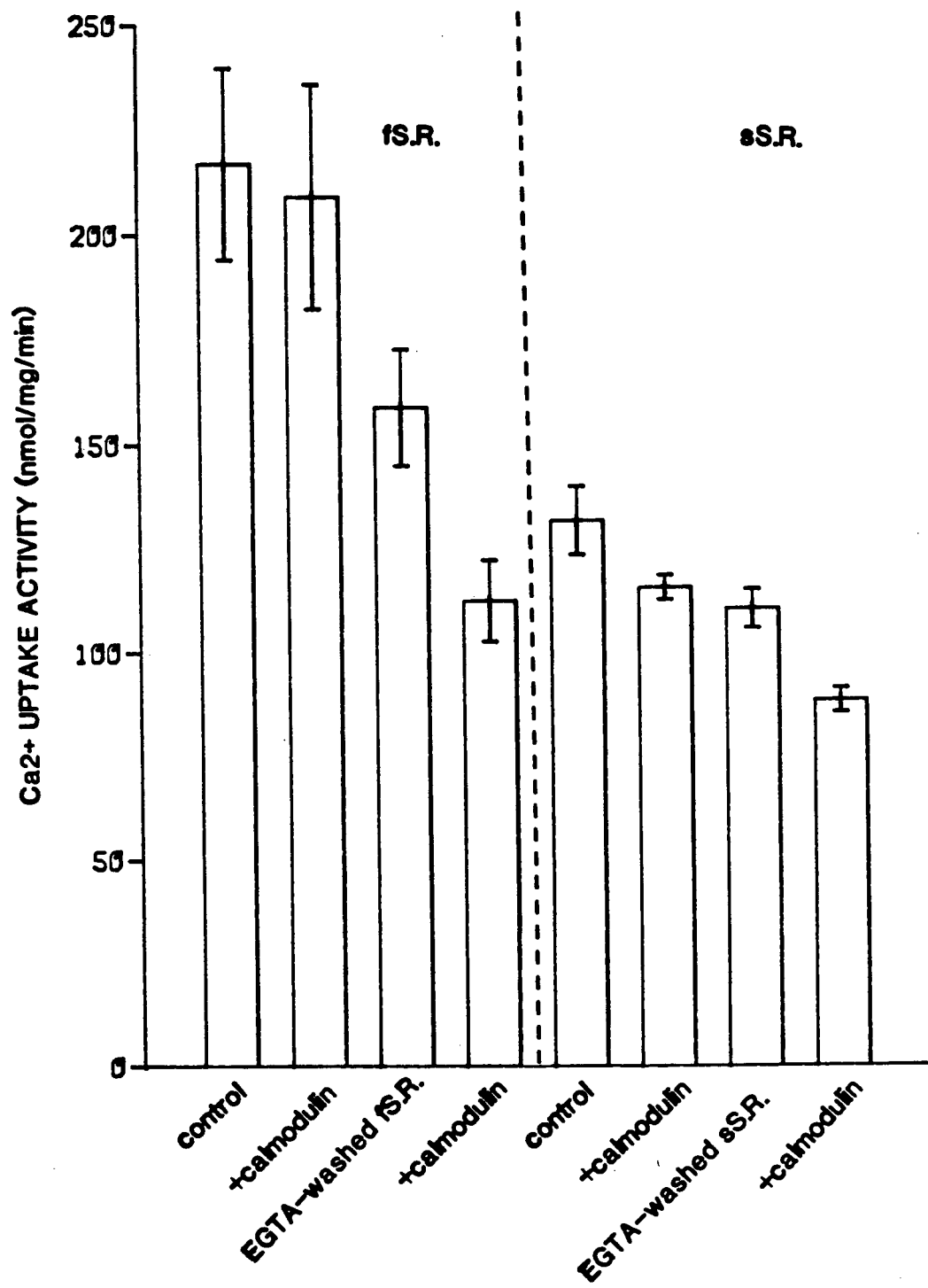
extract with red cell (Ca^{++}-Mg^{++})-ATPase in the presence and

absence of TFP. In addition, the SR pellet derived following

washing with 1 mM EGTA was subjected to boiling in the presence

(and absence) of 0.2 mM EDTA to determine whether further

amounts of CAM could be liberated. As shown in Table 6 (b), the

boiled + EDTA treated supernatants of 1 mM EGTA-washed fSR and

sSR were able to stimulate Ca^{++}-ATPase activity, and this

stimulation could be inhibited by the addition of TFP. In

addition, supernatants derived from 1 mM EGTA-washed SR,

particularly those from fSR , were able to stimulate

Ca^{++}-ATPase activity with the stimulation inhibited by TFP.

(ii) Direct measurements of calmodulin by radioimmunoassay

In order to directly determine the presence of CAM in the

skeletal SR extracts we undertook an estimation of CAM content

using an ^{125}I-radioimmunoassay. As shown in Table 7, untreated

fSR vesicles contain low levels of CAM. Boiling, and

particularly boiling in the presence of EDTA, released more CAM

into the supernatant. We found that boiled + EDTA-treated sSR

yielded more CAM/mg starting protein than similar treatment of

fSR (Table 7b) although the CAM content of both fSR and sSR

ranged broadly from preparation to preparation. When we

examined the supernatants of boiled + EDTA-treated SR that had

previously been washed in 1 mM EGTA, it was observed that at

least two-thirds of the levels of CAM were liberated as in SR

that did not receive the EGTA treatment. Supernatants from

EGTA-washed fSR consistently showed 0 or trace levels of CAM

Table 6
Measured levels of (Ca^{2+})-ATPase in the presence of TFP (60 μM) and
(a) supernatants of 1 mM EGTA-washed fast and slow SR vesicles boiled in
the presence of 0.2 mM EDTA, or (b) EGTA-washed fast and slow SR supernatants.

Addition	(Ca^{2+})-ATPase activity (nmoles/mg/min)	
	-TFP	+TFP
(a)		
none	20.89	15.93
boiled + EDTA-treated supernatant of 1 mM EGTA-washed fSR	34.34	24.07
boiled + EDTA-treated supernatant of 1 mM EGTA-washed sSR	27.26	17.35
(b)		
1 mM EGTA-washed fSR supernatant	70.10	58.06
1 mM EGTA-washed sSR supernatant	27.97	19.11

Ca^{2+}-ATPase activity and preparation of supernatants were performed as
described in Methods. Results represent a typical experiment.

Table 7
Radioimmunoassay for calmodulin in various preparations of fast and slow rabbit skeletal SR.

Condition	ng calmodulin/mg SR protein
untreated fSR microsomes	2.1
(a) fSR supernatant derived from microsomes which were:	
i) boiled	18.0
ii) boiled following 1 mM EGTA wash	12.2
iii) boiled + EDTA-treated	28.5
iv) boiled + EDTA-treated following 1 mM EGTA-wash	19.0
v) washed in 1 mM EGTA	0
(b) sSR supernatants derived from microsomes which were:	
i) boiled + EDTA-treated	64.9
ii) boiled + EDTA-treated following 1 mM EGTA wash	20-75
iii) washed in 1 mM EGTA	0-8

Supernatants derived from SR and calmodulin levels were determined as described in Methods. Results are typical of at least two experiments.

while those derived from sSR often showed moderate levels of CAM. Recently, it has been found that radioimmunoassay-derived levels of CAM vary dramatically depending on whether or not the samples are heated (NEN technical bulletin, 1983). We therefore compared the unboiled supernatants from 1 mM EGTA-washed SR to an unboiled "standard" CAM and found 0 levels of CAM in fSR supernatants whereas sSR supernatants showed rather high levels (data not shown).

(iii) SDS-PAGE

Another method employed to determine whether CAM was present in extracts was the analysis of samples by SDS-PAGE. As shown in Figure 21, a standard CAM sample (80 ng) typically ran as a doublet of 19-21 Kdaltons and maintained this molecular weight even if subjected to boiling in the presence or absence of EDTA. All of the boiled + EDTA-treated supernatants revealed protein bands that might be indicative of CAM, since CAM has been reported in the literature to variably migrate between 15.5 and 21.5 Kdaltons on SDS-PAGE (Table 8). The only protein band that consistently ran with our standard CAM sample was the 19.5 Kdalton protein from boiled + EDTA-treated, 1 mM EGTA-washed fSR supernatant.

It was of interest that the supernatants derived from 1 mM EGTA-washed fSR showed bands of 16.5 and 21.5 Kdaltons on SDS-PAGE, suggesting the presence of CAM, whereas the EGTA-washed sSR supernatant did not show any bands below 35 Kdaltons (Figure 21). The multitude of bands in the EGTA-washed

FIGURE 21

Sodium-dodecyl sulfate polyacrylamide (12.5%) gel
electrophoretic protein bands of various supernatants derived
from either boiled + EDTA-treated or 1 mM EGTA-washed skeletal
SR. Samples shown are:

(a) calmodulin standard

(b) boiled + EDTA-treated fSR supernatant

(c) boiled + EDTA-treated sSR supernatant

(d) boiled + EDTA-treated supernatant derived from 1 mM
    EGTA-washed fSR

(e) boiled + EDTA-treated supernatant derived from 1 mM
    EGTA-washed sSR

(f) 1 mM EGTA-washed fSR supernatant

(g) 1 mM EGTA-washed sSR supernatant

Preparation of supernatants and SDS-PAGE were as described in
Methods. Result shown is a typical electrophoretogram.

MW Standards

200,000
116,250
92,500
66,200
45,000
31,000
21,500
14,400

(a) (b) (c) (d) (e) (f) (g)
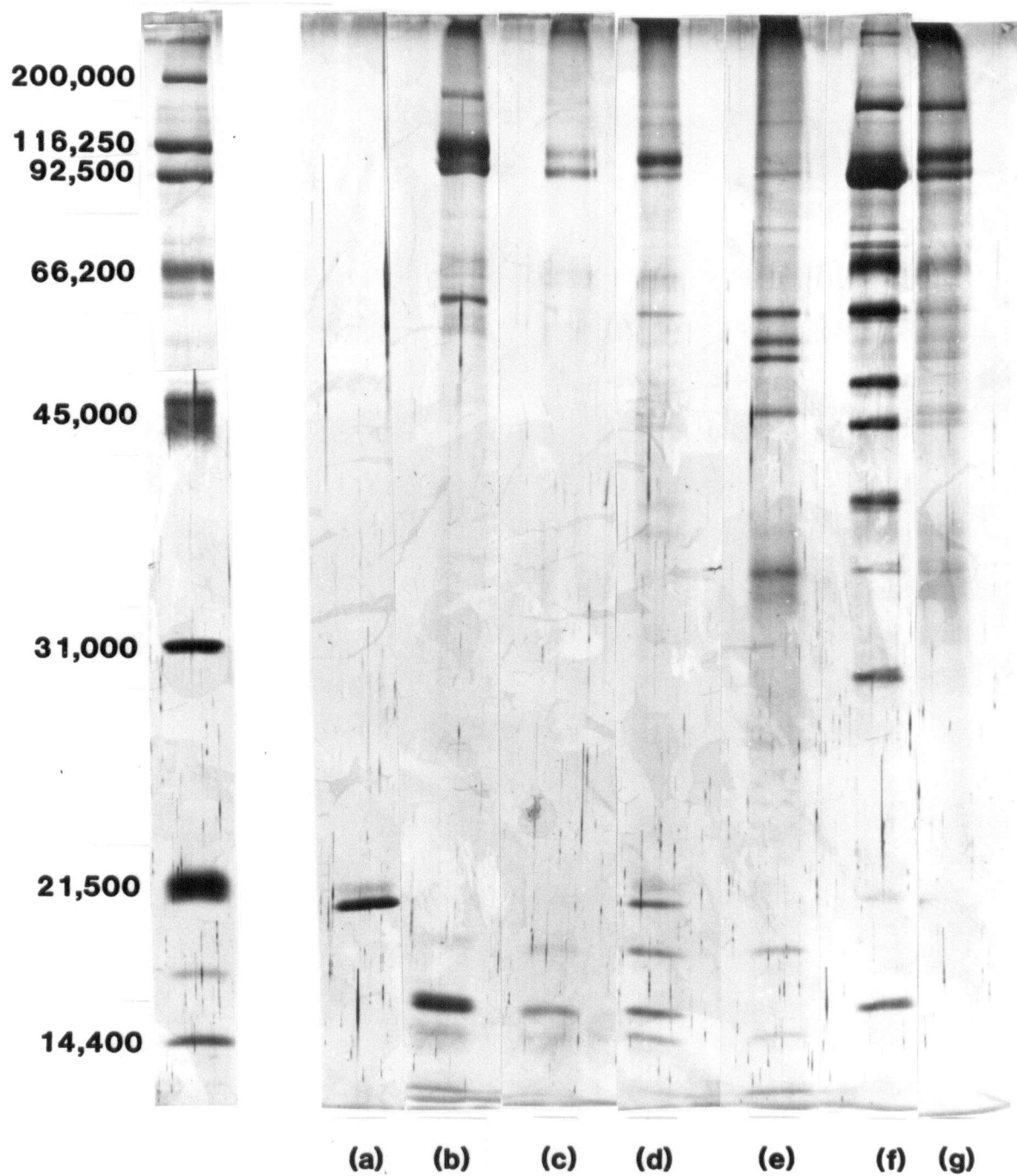
Table 8
Molecular weight of some prominent protein bands below 20 KDaltons in rabbit skeletal SR extracts as shown in Fig.21.

Condition	Prominent bands (MW x 1000)
calmodulin standard	19.5
boiled + EDTA-treated fSR supernatant	15.8, 16.5, 18.5
boiled + EDTA-treated sSR supernatant	15.8, 16.5, 18.5
boiled + EDTA-treated fSR supernatant following 1 mM EGTA wash	15.5, 16.0, 18.0, 19.5
boiled + EDTA-treated sSR supernatant following 1 mM EGTA wash	15.5, 18.0
supernatant from 1 mM EGTA-washed fSR	16.5, 19.5
supernatant from 1 mM EGTA-washed sSR	--

SDS-PAGE and preparation of supernatants were performed as described in Methods. Results shown are typical of at least three preparations.

fSR supernatant, however, made us suspect the presence of contaminating SR membrane proteins. We therefore centrifuged all of the extracts (100,000 xg for 30 min) and then re-examined their ability to stimulate Ca^{++}-ATPase activity: there was no change in the ability of the boiled + EDTA-treated extracts to stimulate ATPase activity but the supernatants from 1 mM EGTA-washed fSR were no longer able to stimulate this activity. In addition, SDS-PAGE revealed that both the 16.5 and 21.5 kdalton bands present in the latter supernatant prior to ultracentrifugation were missing following this procedure, suggesting that the marked stimulation observed with the supernatant derived from 1 mM EGTA-washed fSR was most likely due to SR membrane contamination.

C) SR Calcium Transport in a Chronic Disease State: Experimentally-induced Diabetes

Table 9 shows body weights, serum glucose, and serum insulin levels of streptozotocin-induced diabetic rats 120 days after the induction of diabetes. Body weight and serum insulin levels at time of sacrifice were significantly lower in diabetic rats than in controls. Another index of diabetes, elevated levels of serum glucose, were also observed in these diabetic animals.

ATP-dependent tris oxalate-facilitated Ca^{++}-uptake was measured in skeletal muscle microsomal preparations enriched in SR from control and chronically diabetic rats (Figure 22). At all free Ca^{++} concentrations tested (0.1-2.0 μM), the rate of

Table 9
Measurement of body weight, serum glucose, and serum insulin levels of control and diabetic rats. Numbers in parentheses indicate the number of animals. Results are expressed as the mean ± S.D.

		Control	Diabetic
A)	Body weight (g) (at time of sacrifice)	488 ± 48 (5)	339 ± 25 (6)*
B)	Serum Glucose (mg %)	133 ± 15 (5)	650 ± 111 (6)*
C)	Serum insulin (µU/ml)	40 ± 7 (5)	17 ± 3 (5)*

* Significantly less than control, $p < 0.05$.

FIGURE 22

Effect of chronic diabetes (120 days) on skeletal SR

calcium-uptake at various calcium concentrations. Ca^{++}-uptake

and free Ca^{++}concentrations were determined as described in

Methods in SR preparations derived from control (□————□) and

streptozotocin-treated (o————o) rats. Result shown is the

mean ± S.D. of 4 control and 5 diabetic animals.
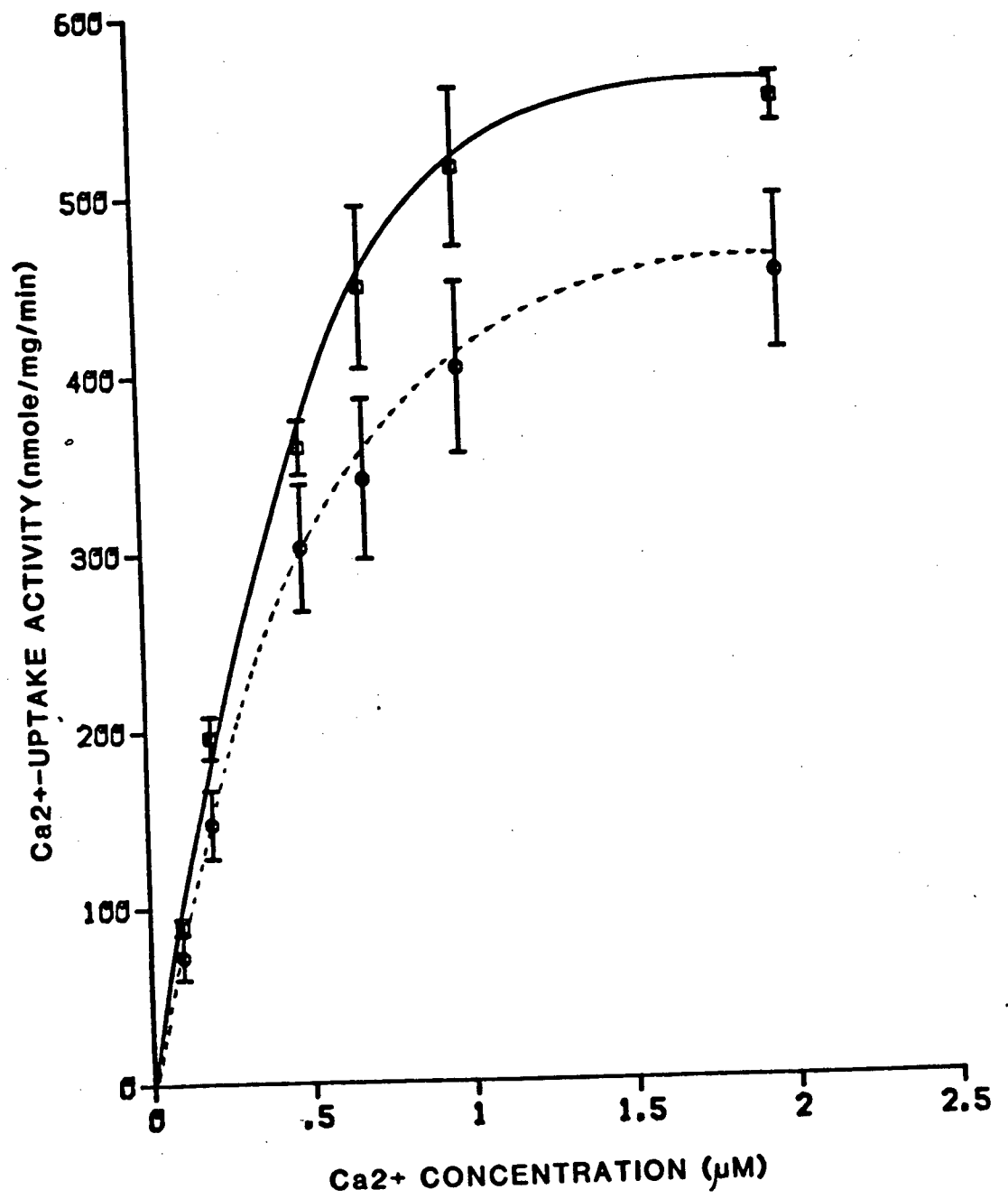
Ca^{++}-uptake was significantly reduced ($p < 0.05$) in the SR preparations obtained from diabetic rats compared to controls.

Levels of carnitine and long chain acylcarnitine were measured in microsomal preparations enriched in SR prepared from control and chronically diabetic rats (Table 10). Preparations derived from diabetic animals contained significantly elevated ($p < 0.05$) levels of both free carnitine and long chain acylcarnitines compared to controls.

Table 10
Levels of carnitine and long chain acylcarnitines in cardiac microsomal
preparations enriched in sarcoplasmic reticulum from control and three month
diabetic rats.

Condition	Metabolite	Tissue levels (nmole carnitine/mg SR)
control (n = 5)	(i) acid-soluble carnitine	3.34 ± 0.55
	(ii) fatty acylcarnitine	1.01 ± 0.20
diabetic (n = 5)	(i) acid-soluble carnitine	5.31 ± 1.92*
	(ii) fatty acylcarnitine	1.72 ± 0.35*

*significantly elevated compared to control $p < 0.05$

Results are expressed as the mean ± S.D. Preparation and measurement of
metabolites are as described in Methods. Diabetes was induced by a single
i.v. injection of 50 mg/kg streptozotocin.

DISCUSSION

Calcium is a crucial ion in the modulation of excitation-contraction coupling in both skeletal and cardiac muscle. The sarcoplasmic reticulum membrane plays a critical role since it is the principal calcium-sequestering membrane system responsible for muscle relaxation. In skeletal muscle it probably functions as well as the store of "activator calcium"; whether the SR also functions as the sole site of activator calcium in mediating cardiac contractility remains controversial. The significance of precise regulation of calcium fluxes is perhaps best exemplified by a variety of disease states, such as Duchenne Muscular Dystrophy and Malignant Hyperthermia, in which Ca^{++} regulation, perhaps at the level of the SR, is thought to be deficient.

This study has been an attempt to examine the function and regulation of SR in normal and diseased tissue, in the hope of elucidating the unique position that the SR occupies in each case. Accordingly, I shall discuss each aspect in turn.

I Regulation of a Purified Cardiac Sarcoplasmic Reticulum Preparation

In contrast to the extensive system present in skeletal muscle, SR is more scarce in cardiac muscle, with surface membranes (SL and transverse tubule) and mitochondria constituting a higher percentage of the total membrane mass

(Fawcett and McNutt, 1969). The vast majority of studies on cardiac SR structure and function have been performed on crude microsomal preparations which have been shown to be contaminated by sarcolemmal and mitochondrial membranes (Besch et al, 1976). Accordingly, we attempted to purify dog cardiac SR by Ca-oxalate loading followed by sucrose density gradient centrifugation as described by Jones et al (1979). The Ca-oxalate precipitate that forms inside the SR vesicles increases the vesicle density relative to other vesicles that either do not transport Ca^{++} or do so at a slower rate. Subjected to sucrose density gradient centrifugation, the loaded vesicles are driven to the bottom of the centrifugation tube where they can be collected.

Two markers of enzyme activity expressed exclusively by membranes other than SR were used to determine the degree of SR vesicle contamination: cytochrome c oxidase activity for mitochondrial membranes and Na^+,K^+-ATPase activity for sarcolemmal membranes.

Mitochondrial membrane content, as measured by cytochrome c oxidase activity, was found to be decreased three-fold only in those preparations in which Ca-oxalate loading was performed in the presence of the mitochondrial Ca^{++}-uptake inhibitor, sodium azide. In their original procedure, Jones et al (1979) neglected the inclusion of this inhibitor and did not report the degree of mitochondrial membrane contamination of their purified SR preparation. Levitzki et al (1976), however, did include sodium azide in their Ca-oxalate loading procedure for

the purification of pigeon heart SR and noted a 3.7-fold decrease in cytochrome (a + a_3) activity compared to crude SR.

The extent of sarcolemmal membrane contamination was determined by measurement of "patent" and "latent" ouabain-inhibitable Na^+,K^+-ATPase activity measured in the absence and presence of detergent, respectively. ATP and ouabain are thought to bind to opposite sides of the transmembrane enzyme (Perrone and Blostein, 1973), such that sealed right-side out or inside-out vesicles would only present one face for binding. The presence of detergent (e.g. Triton X-100) allows the membrane to become "leaky" and therefore potentially available to both ATP and ouabain, such that the "latent" enzymic activity is now expressed. As shown in Table 2, the values of "patent" Na^+,K^+-ATPase activity were similar in both crude and purified SR preparations. Incubation of both types of vesicles with 0.05% Triton X-100 resulted in a marked elevation of "latent" activity in the crude preparation only, suggesting a discrimination of the two types of preparations with respect to content of sarcolemmal Na^+,K^+-ATPase enzyme sites. Purified sarcolemmal vesicles have Na^+,K^+-ATPase activities estimated between 2000 and 3000 nmoles/mg/min (Jones and Besch, 1979; Chamberlain et al, 1983) suggesting that a value of 88 nmoles/mg/min in the purified preparation represented an SL contamination of 3-5%, slightly higher than the upper limit of 2% indicated by Jones and Besch (1979), but still below the 9% SL contamination reported in the purified SR preparation of Chamberlain et al (1983).

A comparison of protein composition between crude and purified cardiac SR microsomes using SDS-PAGE revealed similar polypeptide profiles as has been noted by Jones et al (1979). Since differences in protein banding patterns have been found between SR and SL (Jones et al, 1979), it would seem that the degree of SL contamination in our crude SR preparation was not significant enough to alter the protein patterns observed in the purified preparation. The marked exception between crude and purified SR was the absence in the purified preparation of a 95,000 MW protein, of unknown identity. We have speculated that the missing protein may be phosphorylase b or phosphorylase b kinase. Recently, Narahara and Green (1983) have confirmed the selective loss of a 96,000 MW protein in frog sartorius muscle induced to contract upon electrical stimulation or Ca^{++} infusion, and have suggested a Ca^{++}-activated neutral protease directed towards phosphorylase kinase may be responsible. The large increase in intracellular Ca^{++} concentration occurring during Ca-oxalate loading may indeed precipitate activation of such a protease and so be responsible for the noted disappearance of the 95 kdalton protein.

Calcium uptake and $(Ca^{++}-Mg^{++})$-ATPase activity was increased 3-5-fold in the purified preparation compared to crude. The Ca^{++}-uptake effect appears to be one on Vmax, as the Km for Ca^{++} was similar in both preparations. These enhancements as a result of purification have previously been reported in SR derived from pigeon heart (Levitzki et al, 1976)

and dog heart (Jones et al, 1979). The decreasing rate of calcium uptake with time seen with purified SR (Figure 9) is probably a form of "back-inhibition" (Weber et al, 1966); inhibition of Ca^{++} uptake and ATPase activity that occurs as increasingly elevated levels of Ca^{++} in the vesicle lumen begin to exert negative feedback on further Ca^{++} transport. Normally, oxalate acts as a Ca^{++} "sink" or buffer such that a Ca-oxalate salt of low solubility forms in the vesicle. As Ca^{++} enters, it forms a Ca-oxalate precipitate which ensures a low free Ca^{++} concentration, thus preventing "back-inhibition" from occurring. Loading the vesicles with Ca-oxalate, however, may have exceeded the capacity of the anion to maintain a low and constant free Ca^{++} concentration.

Although the velocity of both the Ca^{++}-uptake and Ca^{++}-ATPase reactions was increased in the purified preparation of SR, the degree of stimulation by cAMP-PK and CAM was similar to that noted in crude SR. It is well established, for example, that cAMP-PK stimulates Ca^{++}-uptake and Ca^{++}-ATPase in crude cardiac SR 2-3-fold (Kirchberger et al, 1972; Tada et al, 1975). Stimulation by cAMP-PK of the purified SR preparation resulted in a 2 and 2.5-fold stimulation of Ca^{++}-ATPase activity at 0.2 and 1.0 μM free Ca^{++} concentrations, respectively (Figure 12) while Ca^{++}-uptake was stimulated by 100% at 0.2 μM free Ca^{++} and 22% at 1.0 μM free Ca^{++} (Figure 11). The low levels of Ca^{++}-uptake stimulation by cAMP-PK, particularly at the higher free Ca^{++} concentrations, are contrary to the findings in the literature in crude SR, and may

be due to the already maximal levels of Ca^{++}-uptake activity achieved (350 nmoles/mg/min) at 1.0 μM free Ca^{++}.

Figures 11 and 12 show that, similar to the findings reported with crude SR (Kirchberger and Antonetz, 1982b; Plank et al, 1983), stimulation by CAM of Ca^{++}-uptake and Ca^{++}-ATPase activity was greater at low free Ca^{++} (0.2 μM free: 70-80% stimulation) than at the higher free Ca^{++} concentration studied (1.0 μM free: 40-45% stimulation). It was often noted, however, that 1 μM CAM was unable to markedly stimulate Ca^{++}-uptake activity at either free Ca^{++} concentration. Although preparation lability was initially suspected, the results obtained for CAM-dependent phosphorylation (see below) made us suspect a more subtle cause.

The effect of both cAMP-PK and CAM present together was examined only with respect to Ca^{++}-ATPase activity. Similar to the results of Lopaschuk et al (1980) obtained in crude SR, the presence of both regulators resulted in an additive stimulation compared to each regulator alone, at 0.2 μM free Ca^{++} concentration (Figure 12). We observed, though, a lack of additive stimulation at 1.0 μM free Ca^{++}, which, as noted above, may have been due to maximal activation of the Ca^{++} pump at this higher free Ca^{++} concentration.

A comparison of the two preparations of SR with respect to membrane phosphorylation revealed that incubation of either purified or crude SR vesicles with cAMP-PK or the catalytic (C) subunit of PK resulted in similar profiles. Typically, the highest levels of phosphorylation were attained in the presence

of cAMP-PK, both in crude and purified SR. The autoradiograms for both preparations were also identical (Figure 14): proteins of 98, 57, 51, and 44 Kdaltons increased incorporation of phosphate with time, a 68 Kdalton protein became dephosphorylated by two minutes, whereas a 9-11 Kdalton protein, assumed to be phospholamban (LePeuch et al, 1979), became maximally phosphorylated by two minutes; the latter time-course was in agreement with the findings of LaRaia and Morkin (1974) and Manalan and Jones (1982). Phosphorylation by C subunit followed a similar time-course to that observed with cAMP-PK (t 1/2 = 20-30 sec) but demonstrated a higher degree of total phosphorylation in the purified preparation compared to crude (Figures 13a, 13b).

Two marked differences, however, were apparent when an autoradiogram comparing the time-course of phosphorylation by cAMP-PK or C subunit were examined (Figure 14). First, many of the proteins phosphorylated either in crude or purified SR by cAMP-PK (e.g. 98K, 68K, 51K) were not phosphorylated in the presence of C subunit, suggesting the impurity of the cAMP-PK preparation, and recommending the use of C subunit in further work. A 56 kdalton protein was phosphorylated by both cAMP-PK and C subunit, particularly markedly in the former case, and may represent autophosphorylation of the regulatory subunit of cAMP-PK (Erlichman et al, 1974). Since the C subunit is purportedly purified from regulatory subunit, it is likely that the 56 Kdalton phosphorylation in purified SR represents autophosphorylation of endogenous cAMP-PK.

The second difference noted between cAMP-PK and C subunit phosphorylation in purified and crude SR was the variability in phospholamban phosphorylation latency (Figure 14). Phospholamban was phosphorylated intensely almost immediately (10 sec) by C subunit and by 30 sec achieved maximal levels whereas phosphorylation by cAMP-PK was relatively slow in developing, attaining maximal levels of phosphorylation by 2 minutes. The difference in phospholamban phosphorylation velocity between these two essentially identical regulators may, in part, be due to the presence of impurities in the cAMP-PK preparation which may either physically or temporally impede access of the catalytic subunit to phospholamban kinase.

Differences between the crude and purified preparations of SR became apparent when we investigated the response to calmodulin-dependent phosphorylation. In the purified preparation calmodulin-dependent phosphorylation was inhibited by 50% compared to crude, despite the findings of enhanced cAMP-dependent phosphorylation in the former preparation (Figures 13a, 13b). These differences were mirrored in autoradiograms of crude and purified preparations of SR phosphorylated in the presence of CAM (Figure 15). Both the intensity and time to complete phosphorylation of phospholamban were markedly diminished in purified SR. Many of the extraneous protein bands phosphorylated in the presence of CAM in crude SR (Katz et al, 1983) were also missing in purified SR. Nevertheless, the two preparations were alike in that CAM-dependent phosphate incorporation was both slower and lower

than that observed with cAMP-PK or C subunit incubation. The somewhat lower levels of CAM-dependent total phosphorylation may reflect the sub-obtimal Ca^{++} concentrations (2 μM free) used. Tada and Katz (1982) report that maximal phospholamban phosphorylation (comparable to that observed with cAMP-PK) was achieved with a free Ca^{++} concentration between 5 and 10 μM.

Taken together, these results suggest that a purification of cardiac SR by Ca-oxalate loading was successful in that Ca^{++}-dependent processes (e.g. Ca^{++} transport and ATPase activities) associated with SR were enhanced while contamination by organelles other than SR was decreased. Nevertheless, a number of findings of this work lead to some doubt regarding the utility of this method of purification. First, the identity and function of the missing 95,000 MW band in the purified preparation remains unaccounted for. Second, the noted reduction in CAM-dependent phosphorylation and inconsistency of CAM-stimulated Ca^{++}-uptake activity in purified SR suggests a disturbance of CAM-mediated regulation in this preparation. The possibility exists that loading of the vesicles with Ca-oxalate may activate Ca^{++}-dependent proteolytic activity which could be reponsible for both the degradation of the 95 Kdalton protein and the inactivation of the active site or other accessory proteins crucial for the proper functioning of CAM-mediated events in SR. Another possibility may be the selective loss through "purification" of the CAM-dependent kinase which to date remains unidentified (Chiesi and Carafoli, 1983). Regardless of the mechanism,

purification by Ca-oxalate loading has recently come under sharp criticism as a means of SR vesicle separation. The objections include: (1) Ca-oxalate loading altering the property of enzymes located in the vesicle membrane (Velema and Zaagsma, 1981), (2) only 7-10% of cardiac vesicles actually becoming loaded (Van Winkle and Entman, 1979), (3) such a procedure appearing to select a specific subpopulation of cardiac SR vesicles (Jones and Cala, 1981), and, (4) contamination by T-tubules not being eliminated (Kawamoto and Baskin, 1983).

An aspect of cardiac SR purification that has not been addressed is an explanation as to why Ca^{++}-dependent activities are enhanced compared to crude SR. Kawamoto and Baskin (1983) loaded chicken skeletal muscle SR with Ca-phosphate and found an enrichment of a 100 kdalton protein which they assumed to be the $(Ca^{++}-Mg^{++})$-ATPase. A selective enrichment of ATPase protein was not observed in this study (Figure 8) and is in agreement with the lack of Ca^{++} pump protein enrichment observed in SR purified either in the presence (Levitzki et al, 1976; Jones and Besch, 1979) or absence (Chamberlain et al, 1983) of Ca-oxalate. The enhanced Ca^{++}-uptake activity, then, is probably due to the isolation of more sealed, right-side out vesicles which are capable of Ca^{++} transport, as opposed to the somewhat haphazard collection of sealed and unbroken vesicles present in cruder preparations.

II Studies examining the presence of calmodulin in fast and

slow-twitch skeletal muscle SR

Unlike the calmodulin-dependent system regulating the cardiac SR Ca^{++} pump, the role that CAM plays in the regulation of skeletal SR Ca^{++} transport remains to be elucidated.

There is little doubt that CAM is present in skeletal muscle although compared with other vertebrate tissues, its concentration is extremely low (Klee and Vanaman, 1982). In fast skeletal muscle, the CAM concentration has been estimated as 10-20 μM (Grand et al, 1979), 40% of which is thought to be the δ subunit of phosphorylase kinase. The amount of CAM in slow skeletal muscle has not been reported, but the contribution by phosphorylase kinase is likely negligible since the level of this glycolytic enzyme is 20-50-fold lower than that found in fast skeletal muscle. For comparison, it is estimated that the concentration of calmodulin in cardiac muscle is 10 μM, and between 3-5 μM in the cytosol of red blood cells (Scharff and Foder, 1981). Grand and Perry (1979) have subsequently modified their estimate of CAM content in skeletal muscle by stating that the vast majority of CAM-like activity is, in reality, troponin C. These determinations of tissue CAM content are indirect since they are typically made by boiling tissue fractions, and then comparing the stimulatory effect obtained on red blood cell phosphodiesterase activity with the stimulation produced by a known weight of pure CAM (Chiesi and Carafoli, 1982).

Our work has examined the claim that CAM is either tightly bound to the SR membrane and only removable by such harsh

treatment as boiling (Chiesi and Carafoli, 1982) or that it is somewhat less tightly bound and extractable by Ca^{++} chelation (Campbell and MacLennan, 1982). The dichotomy of "tight" and "loose" binding of CAM to cellular constituents is not new. Although CAM is generally regarded as a soluble protein, a considerable amount of CAM activity has been found to be associated with particulate fractions of mammalian tissues even after extensive washing of the particulate fraction with EGTA (Sobue et al, 1981; Kakiuchi et al, 1982). The terms "EGTA-extractable" and "EGTA-nonextractable" (or "particle-associated") have therefore been introduced. Kakiuchi et al (1982), in addition, have found that, similar to the "latency" of Na^+, K^+-ATPase activity expressed following treatment of SR vesicles with detergent (Besch et al, 1976), particle-associated CAM activity is also "latent" to some degree and can be unmasked by the presence of nonionic detergents.

Our studies have found that, unlike the claims of Campbell and MacLennan (1982), a considerable amount of CAM remains bound to both fSR and sSR even after extensive washing of the SR pellet with 1 mM EGTA. This determination was made using three independent lines of investigation. First, we examined the ability of the supernatants derived from EGTA-washed fast and slow SR to stimulate CAM-depleted red cell $(Ca^{++}-Mg^{++})$-ATPase activity. It was found that such supernatants were unable to stimulate ATPase activity if care was taken to ensure the removal of insoluble material (i.e. SR

membranes). The prevalence of contaminating SR membranes was particularly evident when we employed the second method of CAM identification, that of SDS-PAGE. As shown in Figure 21, EGTA-washed fSR supernatant that had not been subjected to high-speed (100,000 x g) centrifugation to thoroughly remove insoluble material showed a number of protein bands indiginous to the SR membrane. Following the removal of such material, the two proteins which could be attributed to CAM (see below), those of 16.5 and 19.5 Kdaltons, were no longer seen in the electrophoretogram.

As briefly mentioned above, skeletal muscle tissue contains a significant amount of troponin C which has a molecular weight (20 Kdaltons) similar to CAM. Thus, the presence of a protein migrating in the 16-20 Kdalton region of extract samples is not a reliable indication of the presence of CAM. In addition, the CAM standards used in these studies migrated between 19 and 21 Kdaltons regardless of whether they contained exogenously added Ca^{++} or chelator (EGTA). It is well known that CAM exhibits marked variation in its SDS-PAGE mobility depending on whether it is run in the presence of Ca^{++} (MW: 15,800 $\pm$ 700 daltons) or EGTA (MW: 18,200 $\pm$ 200 daltons) (Klee and Vanaman, 1982). As mentioned above, we did not see this change in mobility. An explanation may be that the Ca^{++} or EGTA must not only be present in the sample but also in the gel matrix and running buffer before the change in mobility is observed (Dr. A. Molla, personal communication). Thus we relied heavily on the third and most direct means of CAM

identification, that of ^{125}I-CAM radioimmunoassay (RIA).

As shown in Table 7, it was found that washing SR, particularly fSR, in the presence of EGTA was incapable of liberating enough CAM to be detectable by RIA, although SDS-PAGE revealed (Figure 21) that the EGTA-washed extracts contained membrane fragments. How was it possible, then, that the RIA for CAM was below the sensitivity of the electrophoretogram? The answer may lie with the fact that intact fSR membrane vesicles show very little CAM by RIA (Table 7), possibly due to the inaccesibility of the CAM present in the SR vesicles to the anti-CAM antibody of the RIA detection system.

The results cited above allow for the conclusion that, indeed, the CAM present in skeletal muscle may be EGTA-nonextractable. In order, then, to demonstrate the "latent" activity of CAM in these vesicles harsher treatment was required. We therefore boiled the SR vesicles in either the presence or absence of EDTA, either prior or following EGTA-washing, and again determined by the three independent lines of investigation whether or not CAM was present. Boiling of skeletal SR membranes, particularly in the presence of 0.2 mM EDTA, and isolation of supernatant resulted in (i) a dose-dependent stimulation of CAM-depleted red blood cell (Ca^{++}-Mg^{++})-ATPase activity (Table 4), (ii) the presence, on SDS-PAGE, of a number of protein bands ranging between 15.5 and 19.5 Kdaltons, the acceptable migration range of CAM (Table 8), and (iii), measureable levels of CAM as determined by ^{125}I-CAM

RIA (Table 7). All of the above were found regardless of whether the SR vesicles were pre-washed in 1 mM EGTA. CAM levels, as measured by RIA, were decreased by 33%, however, in the fSR vesicles pre-washed with EGTA (Table 7). This latter point is somewhat puzzling since one would expect to find this presumably EGTA-extractable CAM in the supernatant and so to be RIA-detectable (which it was not).

Most of the results obtained with fSR mirrored those obtained with sSR with one notable exception: the CAM content of sSR, as determined by RIA, was higher (per mg protein) than in fSR. This could reflect either endogenous CAM concentration differences or dissimilarities in the degree to which CAM is bound to the respective SR membranes. Although this aspect of skeletal muscle biochemistry has not yet been addressed in the literature, our data would sugggest the latter possibility on the basis of our finding of EGTA-extractable CAM in the supernatants of EGTA-washed sSR and not fSR (Table 7b). In addition, we were able to record a value of 85 ng/ mg SR protein in EGTA-washed sSR supernatant when an unboiled CAM standard was used; EGTA-washed fSR supernatant had 0 levels of CAM. The use of the unboiled standard was based on the recent discovery (NEN technical bulletin, 1983) that boiling of CAM uncovered more immunoreactive sites for the anti-CAM antibody. Our previous use of a "boiled" standard CAM for an unboiled supernatant sample could result in a gross underestimation of the actual amount of CAM present in the sample. The high level of EGTA-extractable CAM derived from sSR is further indirect

evidence that perhaps the protein is less avidly bound to the sSR membrane than to the fSR.

This study also demonstrated an inability of the anti-CAM agents, TFP and Compound 48/80, to restrict their inhibitory action to CAM-stimulated Ca^{++}-ATPase activity. Non-specific effects unrelated to interactions with CAM made conclusions regarding the presence or absence of CAM in skeletal muscle SR untenable. Unlike other reports in the literature (Gietzen et al, 1980; Raess and Vincenzi, 1980) documenting a 50% inhibitory potency of less than 20 uM TFP on CAM-stimulated red cell (Ca^{++}-Mg^{++})-ATPase activity, and similar to the findings of Levin and Weiss (1980) in rat erythrocytes, we were unable to show a significant decrease in activity below 50 μM TFP. Initial studies performed on red cell membranes hemolyzed in the presence of distilled, de-ionized water demonstrated poor TFP anti-CAM potency. Subsequent studies on membranes prepared according to Niggli et al (1981) utilized hypotonic tris-Cl, pH 7.4 (in the presence of 1 mM EDTA) as the hemolyzing agent, and were much more amenable to a TFP-inhibition of CAM-stimulated ATPase activity (Figure 18). It is not clear why the anti-CAM potency of TFP should vary with membrane preparation, but it may be that the poor buffering capacity of distilled water, used in the hemolysis step of the former preparation, may be partly responsible.

Unlike the findings of Gietzen et al (1983), Compound 48/80 exhibited inhibition of CAM-independent Ca^{++}-ATPase activity. The concentration of Compound 48/80 which exhibited

- 122 -

50% inhibition of CAM-stimulated Ca^{++}-ATPase activity (IC50) was 3.0 μg/ml, almost a 4-fold higher concentration than the value of 0.85 μg/ml obtained by Gietzen et al (1983). Differences in membrane preparative techniques or commercial preparations of Compound 48/80 may be responsible for the noted differences.

These studies therefore demonstrated that CAM is bound tightly to both fSR and sSR (although perhaps more tightly to fSR) and remains largely (greater than 2/3) EGTA-nonextractable. It is of interest that Diamond et al (1980) reported that rabbit fSR vesicles possess a class of tightly-bound calcium ions inaccessible to EGTA located, presumably, in a hydrophobic "pocket" in, or in close proximity to the ATPase. It is tempting to speculate that perhaps the EGTA-nonextractable CAM may be associated with such tightly bound Ca^{++} ions, and perhaps exert its regulatory effect in close proximity to the ATPase.

A major unresolved question concerns the nature of the stimulator that MacLennan (1972) observed that was able to restore the depressed Ca^{++}-uptake activity following EGTA-washing of fSR vesicles, and that Campbell and MacLennan (1982) found able to enhance, in a CAM-like manner, phosphorylation in SR vesicles purportedly depleted of CAM by treatment with 1 mM EGTA. With reference to the first finding of MacLennan (1972), supernatant (either untreated or following boiling) derived from EGTA-washed fSR were able to restore the depressed Ca^{++} transport activity of the washed vesicles. Once

the supernatant was dialyzed for only 4 hours, however, its ability to restore this activity was almost completely lost. One may therefore tentatively suggest that the restoration of activity was perhaps due to the presence of high EGTA concentrations (i.e. Ca-EGTA complexes) which have been shown by Berman (1982) to markedly stimulate the $(Ca^{++}-Mg^{++})$-ATPase activity of rabbit skeletal SR.

A similar explanation for the enhanced CAM-like phosphorylation of EGTA-washed SR vesicles observed by Campbell and MacLennan (1982) is not warranted. In their study, the supernatant derived from EGTA-washed vesicles was boiled for 5 minutes, centrifuged (100,000 x g) to remove insoluble material, and dialyzed. It is our contention, however, that the initial wash in the high EGTA concentration results in suspension of membrane fragments (which indeed requires a centrifugation of 100,000 x g to sediment). The boiling procedure that Campbell and MacLennan (1982) then undertake prior to centrifugation, may release bound CAM present in the contaminating SR membranes suspended in the supernatant. The resulting CAM, purported to have originated from EGTA washing of skeletal SR, is therefore more likely to have been derived from boiling of suspended SR membranes. As this study has shown, a remnant of the original CAM (less than 1/3) may be released by EGTA treatment, but, by far, the majority of CAM remains EGTA-inaccessible and removable only by harsher treatment.

III The Regulation of Skeletal SR in a Disease

State: Chronic, Experimentally-Induced Diabetes.

This study demonstrated both a significant decrease in Ca^{++}-uptake activity at all free calcium concentrations tested, and a significant increase of acid-soluble and long-chain acylcarnitine levels in microsomes enriched in SR derived from the skeletal muscle of diabetic rats, as compared to controls. These findings paralleled the results obtained in diabetic rat heart SR (Lopaschuk et al, 1983), and suggest a generalized pathophysiological mechanism affecting muscle tissue in the diabetic animal.

The defect in both Ca^{++}-uptake activity and levels of long-chain and free carnitine in diabetic rat heart SR has been linked to the noted diminished ability of isolated working hearts from diabetic rats to respond to increasing preload or afterload (Vadlamudi et al, 1982; Ingebretson et al, 1980). It is believed that such an alteration in heart function may be due to the accumulation of cellular long-chain acylcarnitines which have been shown to be potent inhibitors of SR Ca^{++} transport (Adams et al, 1979). The finding of a decreased Ca^{++}-uptake in diabetic rat skeletal muscle may also have a correlate in the noted clinical complaint of muscle weakness in many diabetic patients (Ellenberg, 1976). Current belief suggests that muscle weakness in diabetes may be the end result of axonal degeneration, also thought responsible for the clinical findings of decreased conduction velocity, demyelination, and altered axonal transport (Thomas et al,

1982). The decrease in skeletal muscle SR Ca^{++}-uptake may play an as yet unrecognized role in the etiology of such neuromuscular dysfunction.

Whether elevated levels of carnitine derivatives are primarily responsible for the decrease in Ca^{++}-uptake activity is unknown. A number of alterations in the skeletal muscle of chronically diabetic rats have been noted; of particular relevance is a significant decrease in both ATP concentration and pH in diabetic rat soleus muscle compared to controls (Moore et al, 1983). Both decreased levels of ATP (Weber et al, 1966) and H+ concentration (Mandel et al, 1982) have been shown to depress Ca^{++}-uptake activity.

The elevated levels of long-chain acylcarnitines observed may be of serious consequence to the SR membrane. The effect of such detergents range from incorporation and consequent alteration of the lipid bilayer at low concentrations, to irreversible loss of membrane phospholipids and physical disruption of the membrane at high concentrations leading, perhaps, to uncontrolled Ca^{++} flux, and ultimately cell death (Katz, 1982). The latter sequence of events may or may not be responsible for the noted muscle weakness in diabetes. However, the potency of the acylcarnitine derivatives for inhibition of membrane-bound enzymatic activity is undeniable, and thus their role is of relevance for future investigation of other pathophysiological mechanisms in the diabetic state.

BIBLIOGRAPHY

Adams RJ, Cohen DW, Gupte J, Johnson D, Wallick ET, Wang T, Schwartz A (1979) In Vitro effects of palmitylcarnitine on cardiac plasma membrane Na,K-ATPase, and sarcoplasmic reticulum Ca++-ATPase and Ca++-transport. J Biol Chem; 254:12404-12410.

Bell GH, Emslie-Smith D, Paterson CR. Textbook of Physiology, 10th ed. N.Y.:Churchill-Livingstone (1980):334-335.

Berman MC (1982) Stimulation of calcium transport of sarcoplasmic reticulum vesicles by the calcium complex of ethylene glycol bis (-aminoethyl ether)-N,N'-tetraacetic acid. J Biol Chem; 257:1953-1957.

Besch Jr HR, Jones LR, Watanabe AM (1976) Intact vesicles of canine cardiac sarcolemma. Evidence from vectorial properties of Na+,K+-ATPase. Circ Res; 39;586-595.

Boland R, de Boland AR, Ritz E, Hasselbach W (1983a) Effect of 1,25-dihydroxycholecalciferol on sarcoplasmic reticulum calcium transport in strontium-fed chicks. Calcif Tissue Int; 35:190-194.

Boland R, Mathews C, deBoland AR, Ritz E, Hasselbach W (1983b) Reversal of decreased phosphorylation of sarcoplasmic reticulum calcium transport ATPase by 1,25-dihydroxycholecalciferol in experimental uremia. Calcif Tissue Int; 35:195-201.

Bond GH, Clough DL (1973) A soluble protein activator (Mg++ + Ca++)-dependent ATPase in human red cell membranes. Biochim Biophys Acta; 323:592-599.

Borchman D, Simon R, Bicknell-Brown E (1982) Variation in the lipid composition of rabbit muscle sarcoplasmic reticulum membrane with muscle type. J Biol Chem: 257:14136-14139.

Bornet EP, Entman MC, vanWinkle WB, Schwartz A, LeHotay DC, Levey GS (1978) Cyclic AMP-induced enhancement of calcium accumulation by the sarcoplasmic reticulum with no modification of the sensitivity of the myofilaments to calcium in skinned fibre from a fast skeletal muscle. Biochim Biophys Acta; 539:253-260.

Bowman WC, Zaimis E (1958) The effects of adrenaline, noradrenaline and isoprenaline on skeletal muscle contractions in the cat. J Physiol; 144:92-107.

Bray BF, Rayns DG (1976) A comparative freeze-etch study of the sarcoplasmic reticulum of avian fast and slow muscle fiber. J Ultrastruct Res; 57:251-259.

Campbell KP, Franzini-Armstrong C, Shamoo AE (1980) Further characterization of light and heavy sarcoplasmic reticulum vesicles. Biochim Biophys Acta; 602:97-116.

Campbell KP, MacLennan DH (1982) A calmodulin-dependent protein kinase system from skeletal muscle sarcoplasmic reticulum. Phosphorylation with a 60,000 dalton protein. J Biol Chem; 257:1238-1246.

Campbell KP, MacLennan DH, Jorgensen AO, Mintzer MC (1983) Purification and characterization of calsequestrin from canine cardiac SR and identification of the 53,000 dalton glycoprotein. J Biol Chem; 258:1197-1204.

Caroni P, Carafoli E (1981) The Ca++-pumping ATPase of heart sarcolemma. Characterization, calmodulin dependence and partial purification. J Biol Chem; 256:3263-3270.

Caswell AH, Baker SP, Boyd H, Potter LT, Garcia M (1978) Beta-adrenergic receptor and adenylate cyclase in transverse tubules of skeletal muscle. J Biol Chem; 253:3049-54.

Caswell AH, Lau YH, Brunschwig J-P (1976) Ouabain-binding vesicles from skeletal muscle. Arch Biochem Biophys; 176:417-430.

Chafouleas JG, Dedman, JR, Munjaal, RP, Means, AR (1979) Calmodulin. Development and application of a sensitive radioimmunoassay. J Biol Chem; 254:10262-10267.

Chamberlain BK, Levitsky DO, Fleischer S (1983) Isolation and characterization of canine cardiac sarcoplasmic reticulum with improved Ca++ transport properties. J Biol Chem; 258:6602-6609.

Chapman RA (1979) Excitation-contraction coupling in cardiac muscle. Prog Biophys Mol Biol; 35:1-52.

Cheung WY (1970) Cyclic 3'5'-nucleotide phospodiesterase. Demonstration of an activator. Biochem Biophys Res Commun;38:533-538.

Chiesi M, Carafoli E (1982) The regulation of Ca++ transport by fast skeletal muscle sarcoplasmic reticulum. Role of calmodulin and the 53,000 dalton glycoprotein. J Biol Chem; 257:984-991.

Chiesi M, Carafoli E (1983) Role of calmodulin in skeletal muscle sarcoplasmic reticulum. Biochemistry; 22:985-993.

Close RJ (1972) Dynamic properties of mammalian skeletal muscle. Physiol Rev; 52:129-198.

Cullen MJ, Appleyard ST, Bindoff L (1979) Morphologic aspects of muscle breakdown and lysosomal activation. Ann NY Acad Sci; 317:440-464.

Cullen MJ, Fulthorpe JJ (1975) Stages in fibre breakdown in

Duchenne muscular dystrophy. An Electron-microscope study. J Neurol Sci; 24:179-200.

Curry CB, Barton JF, Francis MJO, Smith R (1974) Calcium uptake by sarcoplasmic reticulum of muscle vitamin D deficient rabbits. Nature; 249:83-84.

Dean WL, Tanford C (1978) Properties of a delipidated, detergent-activated Ca++-ATPase. Biochemistry; 17:1683-1690.

Dedman JR, Potter JD, Jackson RL, Johnson JD, Means AR (1977) Physiochemical properties of rat testis Ca++-dependent regulator protein of cyclic nucleotide phosphodiesterase. J Biol Chem; 252:8415-8422.

DeFoor PH, Levitzki D, Biryukova T, Fleischer S (1980) Immunological dissimilarity of the calcium pump proteins of skeletal and cardiac muscle sarcoplasmic reticulum. Arch Biochem Biophys; 200:196-205.

deMeis L (1971) Allosteric inhibition by alkali ions of the Ca++ uptake and adenosine triphosphatase activity of skeletal muscle microsomes. J Biol Chem; 246:4764-4773.

deMeis L. The Sarcoplasmic Reticulum. Madison:John Wiley & Sons (1981).

deMeis L, Vianna AL (1979) Energy interconversion by the Ca++-dependent ATPase of the sarcoplasmic reticulum. Annu Rev Biochem; 48:275-292.

Dhalla NS (1976) Involvement of membrane systems in heart failure due to intracellular calcium overload and deficiency. J Mol Cell Cardiol; 8:661-667.

Dhalla NS, Pierce GN, Panagia V, Singal PG, Beamish RE (1982) Calcium movements in relation to heart function. Basic Res Cardiol; 77:117-139.

Dhalla N, Sulakhe P (1973) Calcium transport by the subcellular particles of the skeletal muscle of genetically dystrophic hamster. Biochem Med; 7:159-168.

Diamond EM, Norton KB, McIntosh DB, Berman MC (1980) Tightly bound calcium of adenosine triphosphatase in sarcoplasmic reticulum from rabbit skeletal muscle. J Biol Chem; 255:11351-11356.

DiMauro S, Rowland LP (1976) Urinary excretion of carnitine in Duchenne muscular dystrophy. Arch Neurol; 33:204-205.

Ebashi S, Masaki T, Tsukui T. Cardiac contractile proteins. Advances in Cardiology, vol. 12, R. Reader (ed). Basel:S.

Karger (1974):59-69.

Ebashi S, Sugita M. Current Topics in Nerve and Muscle
Research, A.J. Aquayo & G. Karpati (ed).
Amsterdam-Oxford:Excerpta Medica (1979):73-84.

Eisenberg Br, Kuda AM (1976) Discrimination between fiber
populations in mammalian skeletal muscle by using
ultrastructural parameters. J Ultrastruct Res; 54:76-88.

Ellenberg M (1976) Diabetic neuropathy: clinical aspects.
Metabolism; 25:1627-1655.

Endo M (1977) Calcium release from the sarcoplasmic reticulum.
Physiol Rev; 57:71-108.

Endo J, Hasselbach W (1976) Characterization of cardiac
sarcoplasmic reticulum ATP-ADP phosphate exchange and
phosphorylation of the calcium transport adenosine
triphosphatase. Eur J Biochem; 64:123-130.

England PJ (1975) Correlation between contractions and
phosphorylation of the inhibitory subunit of troponin in
perfused rat heart. FEBS Lett; 50:57-60.

England PJ (1976) Studies of the phosphorylation of the
inhibitory subunit of troponin during modification contraction
in perfused rat heart. Biochem J; 160:295-304.

Entman ML, Keslensky SS, Chu A, VanWinkle WB (1980) The
sarcoplasmic reticulum-glycogenolytic complex in mammalian
fast-twitch skeletal muscle. J. Biol Chem; 255:6245-6252.

Entman ML, Levey GS, Epstein SE (1969) Mechanism of action of
epinephrine and glucagon on the canine heart. Evidence for
increase in sarcotubular calcium stores mediated by cyclic
3',5'-AMP. Circ Res; 25:429-438.

Erlichman G, Hirsch AH, Rosen OM (1971) Interconversion of
cyclic nucleotide-independent forms of a protein kinase from
beef heart. Proc Natl Acad Sci USA; 68:731-735.

Erlichman J, Rosenfield R, Rosen OM (1974) Phosphorylation of
cyclic adenosine 3',5'-monophosphate-dependent protein kinase
from bovine cardiac muscle. J Biol Chem; 249:5000-5003.

Ettienne EM, Swartz K, Singer RH (1980) Increased turnover of
proteins from the sarcoplasmic reticulum of dystrophic chicken
muscle cells in tissue culture. J Biol Chem; 256:6408-6412.

Fabiato A, Fabiato F (1977) Calcium release from sarcoplasmic
reticulum. Circ Res; 40:119-129.

Fabiato A, Fabiato F (1978) Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. J Physiol; 276:233-255.

Fabiato A, Fabiato F (1978) Cyclic AMP-induced enhancement of the calcium accumulation by the sarcoplasmic reticulum with no modification of the sensitivity of the myofilaments to calcium in skinned fibre from a fast skeletal muscle. Biochem Biophys Acta; 539:253-260.

Fawcett DW, McNutt NS (1969) The ultrastructure of the cat myocardium. I. Ventricular papillary muscle. J Cell Biol; 42:1-45.

Fein FS, Kornstein LB, Strobeck JE, Capasso JM, Sonnenblick EH (1980) Altered myocardial mechanics in diabetic rats. Circ Res; 47:922-933.

Feuvray D, Idell-Wenger JA, Neely JR (1979) Effects of ischemia in rat myocardial function and metabolism in diabetes. Circ Res; 4:322-329.

Fiske CH, Subbarow YJ (1925) The colorimetric determination of phosphorus. J Biol Chem; 66:375-400.

Fleckenstein A, Janke J, Doring HJ, Leder O. Myocardial fiber necrosis due to intracellular Ca overlaod - a new principle in cardiac pathophysiology. Myocardial Biology, N.S Dhalla & G. Rona (ed). Baltimore:Univ. Park (1974):563-580.

Foder B, Scharff O (1981) Decrease of apparent calmodulin affinity of erytrocyte (Ca++-Mg++)-ATPase at low Ca++ concentrations. Biochim Biophys Acta; 649:367-376.

Frank JS, Langer GA, Nudd LM, Seraydarian K (1977) The myocardial cell surface, its histochemistry and the effect of sialic acid and calcium removal on its structure and cellular ionic exchange. Circ Res; 41:702-714.

Fritz IB (1959) Action of carnitine on long chain fatty acid oxidation by liver. Am J Physiol; 197:297-304.

Froelich JP, Taylor EW (1975) Transient state kinetic studies of sarcoplasmic reticulum adenosine triphosphatase. J Biol Chem; 250:2013-21.

Froelich JP, Taylor EW (1976) Transient state kinetic effects of calcium ion on sarcoplasmic reticulum adenosine triphosphatase. J Biol Chem; 251:2307-2315.

Furukawa T, Peter JB (1978) The muscular dystrophies and related disorders. 1. The muscular dystrophies. JAMA; 239:1537-1542.

Gallant EM, Godt RE, Gronert GA (1979) Role of plasma membrane defect of skeletal in malignant hyperthermia. Muscle and Nerve; 2:491-494.

Gietzen K, Adamczyk-Engelmann P, Wuthrich A, Konstantinova A, Bader H (1983) Compound 48/80: highly specific and powerful inhibitor of calmodulin-regulated functions. Mol Pharmacol; in press.

Gietzen K, Mansard A, Bader H (1980) Inhibition of human erythrocyte Ca++ transport ATPase by phenothiazines and butyrophenones. Biochem Biophys Res Comm; 94:674-681.

Goth A. Histamine release by drugs and chemicals. Histamine and Antihistamines, Vol. 1, International Encyclopedia of Pharmacology and Therapeutics, Sect. 74, M. Shacter (ed). Oxford:Pergamon Press (1973):25-43.

Grand RJA, Perry SV (1979) Calmodulin-binding proteins from brain and other tissues. Biochem J; 183:285-295.

Grand RJA, Perry SV, Weeks RA (1979) Troponin C-like proteins (calmodulins) from mammalian smooth muscle and other tissues. Biochem J; 177:521-529.

Gronert GA (1980) Malignant hyperthermia. Anesthesiology; 53:395-423.

Gronert GA, Heffron JJA, Taylor SR (1979) Skeletal muscle sarcoplasmic reticulum in porcine malignant hyperthermia. Eur J Pharmacol; 58:179-187.

Guyton AC. The coronary circulation and ischemic heart disease. Textbook of Medical Physiology, 6th ed. Toronto:WB Saunders Co. (1981):302.

Hanna S, Kamamoto R, McNamee M, Baskin RJ (1981) Enzymatic activity of dystrophic chicken sarcoplasmic reticulum. Biochim Biophys Acta; 643:41-54.

Hartweg D, Bader H (1983) Studies on protein composition of cardiac sarcoplasmic reticulum membranes.
Naunyn Schmiedebergs Arch Pharmacol;322(Supp):R25.

Hasselbach W (1964) Relaxation and the sarcotubular calcium pump. Fed Proc Fed Am Soc Exp Biol; 23:909-912.

Hasselbach W, Makinose M (1961) (German) Biochem Z; 333:518-528.

Hasselbach W, Makinose M (1962) ATP and active transport. Biochem Biophys Res Commun; 7:132-136.

Hasselbach W, Makinose M (1963) (German) Biochem Z; 339:94-111.

Heffron JJA (1979) Functional change in sarcoplasmic reticulum and mitochondria of diseased muscle. Biochem Soc Trans; 7:767-768.

Heinberg KW, Matthews C, Ritz E, Augustin J, Hasselbach W (1976) Active Ca transport of sarcoplasmic reticulum during experimental uremia. Eur J Biochem; 61:207-213.

Hicks MJ, Shigekawa M, Katz AM (1979) Mechanism by which cyclic adenosine 3'5' monophosphate-dependent protein kinase stimulates calcium transport in cardiac sarcoplasmic reticulum. Circ Res; 44:384-391.

Hidalgo C, Fernandez JC, Allen PD, Ryan JF, Sreter FA (1982) Isolation and characteriazation of sarcoplasmic reticulum from malignant hyperthermic swine; 37:137a.

Hidalgo C, Ikemoto N, Gergely J (1976) Role of phospholipids in the calcium-dependent ATPase of the sarcoplasmic reticulum. J Biol Chem; 251:4224-4232.

Hill LF, Lumb GA, Mauer EB, Stanbury SW (1973) Indirect inhibition of the biosynthesis of 1,25 dihydroxycholecalciferol in rats treated with a diphosphonate. Clin Sci; 44:335-347.

Hillis LD, Braunwald E (1977) Myocardial Ischemia. N Engl J Med; 296:971-978, 1034-1041, 1093-1096.

Ho M-M, Scales DJ, Inesi G (1983) The effect of trifluoperazine on the sarcoplasmic reticulum membrane. Biochem Biophys Acta; 730:64-70.

Huxley AF, Taylor RE (1958) Local activation of striated muscle fibers. J. Physiol; 144:426-441.

Idell-Wenger JA, Neely JR. Regulation of uptake and metabolism of fatty acid by muscle. Disturbances in Lipid and Lipoprotein Metabolism, J.M. Dietschy, A.M. Gott Jr, J.A. Ontko (ed). Baltimore:American Physiological Society (1978):269-283.

Ikemoto N (1974) The calcium binding sites involved in the regulation of the purified adenosine triphosphatase. J Biol Chem; 249:649-651.

Inesi G (1972) Active transport of calcium ion in sarcoplasmic membranes. Ann Rev Biophys Bioeng; 1:191-210.

Inesi G, Coan JA, Coan CR (1976) Two functional states of sarcoplasmic reticulum ATPase. Biochemistry; 15:5393-5398.

Inesi G, Kurzmack M, Coan C, Lewis DE (1980) Cooperative calcium binding and ATPase activation in sarcoplasmic reticulum vesicles. J Biol Chem; 255:3025-3031.

Inesi G, Maring E, Murphy AJ, McFarland BH (1979) A study of the phosphorylated intermediate of sarcoplasmic reticulum ATPase. Arch Biochem Biophys; 138:285-294.

Ingebretson CG, Moreau P, Hawelu-Johnson C, Ingebretson W (1980) Performance of diabetic rat hearts: effects of anoxia and increased work. Am J Physiol; 239:H614-H620.

Jones LR, Besch Jr HR (1979) Calcium handling by cardiac sarcoplasmic reticulum. Tex Rep Biol Med; 39:19-35.

Jones LR, Besch Jr HR, Fleming JW, McConnaughey MM, Watanabe AM (1979) Separation of vesicles of cardiac sarcolemma from vesicles of cardiac sarcoplasmic reticulum. Comparative biochemical analysis of component activities. J Biol Chem; 254:530-539.

Jones LR, Besch HR, Watanabe AM (1977) Monovalent cation stimulation of Ca++ uptake by cardiac membrane vesicles. J Biol Chem; 252:3315-3323.

Jones LR, Cala SE (1981) Biochemical evidence for functional heterogeneity of cardiac sarcoplasmic reticulum vesicles. J Biol Chem; 256:11809-11818.

Jorgensen AO, Kalnins V, MacLennan DH (1979) Localization of sarcoplasmic reticulum in rat skeletal muscle by immunofluorescence. J Cell Biol; 80:372-384.

Jorgensen AO, Shen ACY, MacLennan DH, Tokuyasu KT (1982) Ultrastructural localization of the Ca++, Mg++-dependent ATPase of sarcoplasmic reticulum in rat skeletal muscle by immunoferritin labelling of ultra-thin frozen sections. J Cell Biol; 92:409-416.

Kakiuchi S, Yasuda S, Yamazaki R, Teshima Y, Kanda K, Kakiucki R, Sobue K (1982) Quantitative determinations of calmodulin in the supernatant and particulate fractions of mammalian tissues. J Biochem (Tokyo); 92:1041-1048.

Kakiuchi S, Yamazaki R (1970) Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain. Biochem Biophys Res Commun; 41:1104-1110.

Kanazawa T, Yamada S, Yamamoto T, Tonomura Y (1971) Reaction mechansim of the Ca++-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. Vectorial Requirements for calcium and magnesium ions of three partial reactions of ATPase: formation and decompostion of a phosphorylated

intermediate and ATP-formation from ADP and the intermediate. J Biochem (Tokyo); 70:95-123.

Katz AM (1975) Congestive heart failure. Role of altered myocardial cellular control. N Engl J Med; 293:1184-1191.

Katz AM (1982) Membrane-derived lipids and the pathogenesis of ischemic myocardial damage. J Molec Cell Cardiol; 14:627-632.

Katz AM, Messineo FC (1981) Lipid-membrane interactions and the pathogenesis of ischemic damage in the myocardium. Circ Res; 48:1-16.

Katz AM, Tada M, Kirchberger MA (1975) Control of calcium transport in the myocardium by the cyclic AMP-protein kinase system. Adv Cyclic Nucleotide Res; 5:453-472.

Katz S (1980) Mechanism of stimulation of calcium transport in cardiac sarcoplasmic reticulum preparations by calmodulin. Ann NY Acad Sci; 356:267-278.

Katz S, Blostein R (1975) Ca++-stimulated membrane phosphorylation and ATPase activity of the human erythrocyte. Biochim Biophys Acta; 388:314-324.

Katz S, Remtulla MA (1978) Phosphodiesterase protein activator stimulates calcium transport in cardiac microsomal preparations enriched in sarcoplasmic reticulum. Biochem Biophys Res Commun; 83:1373-1379.

Katz S, Richter B, Eibschutz B (1983) Characterization of calmodulin-dependent and cyclic-AMP-dependent protein kinase stimulation of cardiac sarcoplasmic reticulum calcium transport. Poster presented at the International Society for Heart Research, London, England.

Katz S, Wong APG. Regulation of calcium transport in microsomal preparation enriched in sarcoplasmic reticulum from rabbit skeletal muscle. Calmodulin and Intracellular Ca++ Receptors, S. Kakiucki, H. Hidaka, A.R. Means (ed). N.Y:Plenum Press (1982):36.

Kawamoto RM, Baskin RJ (1983) Calcium transport, ATPase activity, and lipid compostion in sarcoplasmic reticulum isolated from isogenic lines of normal and dystrophic chickens. Biochim Biophys Acta; 732:620-626.

Kirchberger MA, Antonetz T (1982a) Phospholamban: dissociation of the 22,000 molecular weight protein of cardiac sarcoplasmic reticulum into 11,000 and 5,500 molecular weight forms. Biochem Biophys Res Commun; 105:152-156.

Kirchberger MA, Antonetz T (1982b) Calmodulin-mediated

regulation of calcium transport and (Ca++ + Mg++)-activated ATPase activity in isolated cardiac sarcoplasmic reticulum. J Biol Chem; 257:5685-5691.

Kirchberger MA, Antonetz T (1982c) Stimulation by calmodulin of (Ca++ + Mg++)-activated ATPase activity in fast skeletal muscle sarcoplasmic reticulum vesicles: possible role of a phospholamban-like protein. Biophys J; 37:137a.

Kirchberger MA, Tada M (1976) Effects of adenosine 3',5'-monophosphate dependent protein kinase on sarcoplasmic reticulum isolated from cardiac and slow and fast contracting skeletal muscles. J Biol Chem; 251:725-729.

Kirchberger MA, Tada M, Katz AM (1974) Adenosine 3',5'-monophosphate-dependent protein kinase-catalyzed phosphorylation reaction and its relationship to calcium transport in cardiac sarcoplasmic reticulum. J Biol Chem; 249:6166-6173.

Kirchberger MA, Tada M, Repke DI, Katz AM (1972) Cyclic adenosine 3',5'-monophosphate-dependent protein kinase stimulation of calcium uptake by canine cardiac microsomes. J Mol Cell Cardiol; 4:673-680.

Klee CB, Vanaman TC (1982) Calmodulin. Adv Protein Chem; 35:213-321.

Kranias EG, Bick R, Schwartz A (1980) Phosphorylation of a 100,000 dalton component and its relationship to calcium transport in sarcoplasmic reticulum from rabbit skeletal muscle. Biochim Biophys Acta; 628:438-450.

Kranias EG, Bilezikjian LM, Potter JD, Piascik MT, Schwartz A (1980) The role of calmodulin in regulation of cardiac sarcoplasmic reticulum phosphorylation. Ann NY Acad Sci; 356:279-291.

Kranias EG, Samaha FJ, Schwartz A (1983) Mechanism of the stimulation of Ca++-dependent ATPase of skeletal muscle sarcoplasmic reticulum by protein kinase. Biochim Biophys Acta; 731:79-87.

Kranias EG, Solaro RJ (1982) Phosphorylation of troponin I and phospholamban during catecholamine stimulation of rabbit heart. Nature; 298:182-184.

Laemmli VK, Farre M (1973) Maturation of the head of bacteriophage T4: I. DNA packaging events. J Molec Biol; 80:575-599.

Langer GA, Frank JS (1972) Lanthanum in heart cell culture: effect on calcium exchange correlated with its localization. J

Cell Biol; 54:441-455.

Langer GA, Frank JS, Philipson KD (1982) Ultrastructure and calcium exchange of the sarcolemma, sarcoplasmic reticulum and mitochondria of the myocardium. Pharacol Ther; 16:331-376.

LaPorte DC, Wierman BM, Storm DR (1980) Calcium-induced exposure of a hydrophobic surface on calmodulin. Biochemistry; 19:3814.

LaRaia PJ, Morkin E (1974) Adenosine 3',5'-monophosphate-dependent membrane phosphorylation. A possible mechanism for the control of microsomal calcium transport in heart muscle. Circ Res; 35:298-306.

LeMaire M, Lind KE, Jorgensen KE, Roigaard H, Moller JV (1978) Enzymatically active Ca++ ATPase from sarcoplasmic reticulum membranes, solubilized by nonionic detergents. Role of lipid for aggregation of the protein. J Biol Chem; 253:7051-7060.

LePeuch CJ, Haiech J, DeMaille JG (1979) Concerted regulation of cardiac sarcoplasmic reticulum calcium transport by cyclic adenosine monophosphate-dependent and calcium-calmodulin dependent phosphorylation. Biochemistry; 18:5150-5157.

LePeuch CJ, LePeuch DAM, DeMaille JG (1980) Phospholamban, activator of the cardiac sarcoplasmic reticulum pump. Physiochemical properties and diagonal purification. Biochemistry; 19:3368-3373.

Levin RM, Weiss B (1979) Binding of trifluoperazine to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. Mol Pharmacol; 13:690-697.

Levin RM, Weiss B (1980) Inhibition by trifluoperazine of calmodulin-induced activation of ATPase activity of rat erythrocyte. Neuropharmacology; 19:169-174.

Levitsky Do, Aliev MK, Kuzman AV, Levchenko TS, Smirnov VN, Chazov EI (1976) Isolation of calcium pump system and purification of calcium ion-dependent ATPase from heart muscle. Biochim Biophys Acta; 443:468-484.

Levitsky DO, Benevolensky DS, Levchenko TS, Kuzman AV. The cardiac relaxing system. Advances in Myocardiology, Vol. 3 (1982):393-405.

Lopaschuk GD, Katz S, McNeill JH (1983) The effect of alloxan- and streptozocin-induced diabetes on calcium transport in rat cardiac sarcoplasmic reticulum. The possible involvement of long chain acylcarnitines. Can J Physiol Pharmacol; 61:439-448.

Lopaschuk G, Richter B, Katz S (1980) Characterization of

calmodulin effects on calcium transport in cardiac microsomes enriched in sarcoplasmic reticulum. Biochemistry; 19:5603-5607.

Louis CF, Maffitt M (1982) Characterization of calmodulin-mediated phosphorylation of cardiac muscle sarcoplasmic reticulum. Arch Biochem Biophys; 218:109-118.

Louis CF, Maffitt M, Jarvis B (1982) Factors that modify the molecular size of phospholamban, the 23,000-dalton cardiac sarcoplasmic reticulum phosphoprotein. J Biol Chem; 257:15182-15186.

Lowry OH, Rosebrough NJ, Farr AC, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem; 193:265-275.

Luff AR, Atwood HR (1971) Changes in the sarcoplasmic reticulum and transverse tubular system of fast and slow skeletal muscle of the mouse during postnatal development. J Cell Biol; 51:369-383.

Lullmann H, Peters T (1977) Plasmalemmal calcium in cardiac excitation-contraction coupling. Clin Exp Pharmacol Physiol; 4:49-57.

Luthra MG (1982) Trifluoperazine inhibition of calmodulin-sensitive CA++-ATPase and calmodulin insensitive (Na+ + K+)-and Mg++-ATPase activities of human and rat red blood cells. Biochim Biophys Acta; 692:271-277.

McGarry JD, Foster DW (1976) An improved and simplified radioisotopic assay for the determination of free and esterified carnitine. J Lipid Res; 17:277-281.

MacLennan DH (1970) Purification and properties of adenosine triphosphatase from sarcoplasmic reticulum. J Biol Chem; 245:4508-4518.

MacLennan DH. Partial resolution and reconstitution of the Ca++ transport system of sarcoplasmic reticulum. Myocardial Biology, Vol. 4, N.S. Dhalla (ed). (1972):507-517.

MacLennan DH, Seeman P, Iles GH, Yip CC (1971) Resolution of enzymes of biological transport. 2. Membrane formation by adenosine triphosphatase of sarcoplasmic reticulum. J Biol Chem; 246:2702.

MacLennan DH, Wong PTS (1971) Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. Proc Nat Acad Sci USA; 68:1231-1235.

Manalan AS, Jones LR (1982) Characterization of the intrinsic cAMP-dependent protein kinase activity and endogenous

substrates in highly purified cardiac sarcolemmal vesicles. J Biol Chem; 257:10052-10062.

Mandel F, Kranias EG, deGende AG, Sumida M, Schwartz A (1982) The effect of pH on the transient-state kinetics of Ca++-Mg++-ATPase of cardiac sarcoplasmic reticulum. A comparison with skeletal sarcoplasmic reticulum. Circ Res; 50:310-317.

Martonosi A (1968) Sarcoplasmic reticulum. VI. Microsomal Ca++ transport in genetic muscular dystrophy of mice. Proc Soc Exp Biol Med; 127:824-828.

Martonosi A. Biochemical and clinical aspects of SR function. Curent Topics in Membrane Transport Vol. 3, F. Bonner & A. Kleinzeller (ed). NY:Academic Press (1972):83-197.

Matthews C, Heinberg KW, Ritz E, Agostini B, Fritzsche J, Hasselbach W (1977) Effect of 1,25-dihydroxycholecalciferol on impaired calcium transport by the sarcoplasmic reticulum in experimental uremia. Kidney Int; 11:227-235.

Maunder CA, Yarom R, Dubowitz V (1977) Electron-microscope x-ray microanalysis of normal and diseased human muscle. J Neurol Sci; 3:323-334.

Meissner G (1975) Isolation and characterization of two types of sarcoplasmic reticulum vesicles. Biochim Biophys Acta; 389:51-68.

Meissner G, Conner G, Fleischer S (1973) Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca++-pump and Ca++-binding proteins. Biochim Biophys Acta; 298:246-269.

Meissner G, Fleischer S (1971) Characterization of sarcoplasmic reticulum from skeletal muscle. Biochim Biophys Acta; 241:356-378.

Meissner G, Fleischer S (1973) Ca++-uptake in reconstituted sarcoplasmic reticulum ATPase. Biochemistry; 15:5293-5298.

Messineo FC, Pinto PB, Katz AM. Effects of palmitic acid and palmitylcarnitine on calcium sequestration by rabbit skeletal sarcoplasmic reticulum vesicles. Advances in Myocardiology. Vol. 3 (1982). NY:Plenum Press, 407-415.

Migala A, Agostini B, Hasselbach W (1973) Tryptic fragmentation of the calcium transport system in the sarcoplasmic reticulum. Z Naturforsch; 28:178-182.

Moller JV, Anderson JP, LeMaire M (1982) The sarcoplasmic reticulum Ca++-ATPase. Molec Cell Biochem; 42:83-107.

Moore RD, Munford JW, Pillsworth TJ,Jr (1983) Effects of streptozotocin diabetes and fasting on intracellular sodium and adenosine triphosphate in rat soleus muscle. J Physiol (London); 338:277-294.

Morrissey JH (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem; 117:307-310.

Nakamura Y, Schwartz A (1972) The influence of hydrogen ion concentration on calcium binding and release by skeletal muscle sarcoplasmic reticulum. J Gen Physiol; 59:22-32.

Narahara HT, Green JD (1983) Selective loss of a plasma membrane protein associated with contraction of skeletal muscle. Biochim Biophys Acta; 730:71-75.

Nelson TE. Excitation-contraction coupling: a common etiologic pathway for malignant hyperthermic susceptible muscle. Second International Symposium on Malignant Hyperthermia, J.A. Aldrete & B.A. Britt (ed). NY:Grune & Stratton (1978):23-36.

NEN Technical Bulletin (1983). Catalog No. NEK-018.

Niggli V, Adunyah ES, Penniston JT, Carafoli E (1981) Purified (Ca++-Mg++)-ATPase of the erythrocyte membrane. Reconstitution and effect of calmodulin and phospholipids. J Biol Chem; 256: 395-401.

Niggli V, Ronner P, Carafoli E, Penniston JT (1979) Effects of calmodulin on the (Ca++ + Mg++)ATPase partially purified from erythrocyte membranes. Arch Biochem Biophys; 198:124-130.

Noble D. Initiation of the Heartbeat. Oxford:Clarendon Press (1975).

Oberc MA, Engel WK (1977) Ultrastructural localization of calcium in normal and abnormal skeletal muscle. Lab Invest; 36:566-577.

Oliver MF. Metabolism of the normal and ischaemic myocardium. Developments in Cardiovascular Medicine. C.J. Dickinson & J. Marks (ed). Lancaster:MTP Press (1978):145ff.

Penpargkul S, Fein F, Sonnenblick EH, Scheuer J (1981) Depressed cardiac sarcoplasmic reticular function from diabetic rats. J Mol Cell Cardiol; 13:303-309.

Perrone JR, Blostein R (1973) Asymmetric interaction of inside-out and right-side-out erythrocyte membrane vesicles with ouabain. Biochim Biophys Acta; 291:680-689.

Pette D, Heilmann C (1977) Transformation of morphological, functional, and metabolic properties of fast-twitch muscle as induced by long-term electrical stimulation. Basic Res Cardiol; 72:247-253.

Pette D, Heilmann C (1979) Some characteristics of sarcoplasmic reticulum in fast and slow-twitch muscles. Biochem Soc Trans; 4:765-767.

Pick U, Racker E (1979) Inhibition of the (Ca++)ATPase sarcoplasmic reticulum by dicyclohexylcarbodimide: evidence for location of the Ca++ binding site in a hydrophobic region. Biochemistry; 18:108-113.

Pitts BJR, Tate CA, VanWinkle B, Wood JM, Entman MC (1978) Palmitylcarnitine inhibition of the calcium pump in cardiac sarcoplasmic reticulum: a possible role in myocardial ischemia. Life Sci; 23:391-402.

Pointon JJ, Francis MJO, Smith R (1979) Effect of vitamin D deficiency on sarcoplasmic reticulum function and troponin C concentration of rabbit skeletal muscle. Clin Sci; 87:257-263.

Prozialeck WC, Weiss B (1982) Inhibition of calmodulin by phenothiazines and related drugs: structure-activity relationships. J Pharmacol Exp Ther; 222:509-516.

Raess BV, Vincenzi FF (1980) Calmodulin activation of red blood cell (Ca++ + Mg++)-ATPase and its antagonism by phenothiazines. Mol Pharmacol; 18:253-258.

Randle PJ (1978) Interaction of metabolism and the physiological role of insulin. Rec Prog Horm Res; 22:1-44.

Rich TL, Langer GA (1975) A comparison of excitation-contraction coupling in heart and skeletal muscle: an examination of calcium induced calcium release. J Mol Cell Cardiol; 7:747-765.

Ringer B (1883) A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. J Physiol (Lond); 4:29-42.

Robbins SL, Cotran RS. Pathologic Basis of Disease. Toronto:W.B. Saunders Co. (1979):648-652.

Roc JH. Exploratory Concepts in Muscular Dystrophy and Related Disorders, A.T. Milkorat (ed). (1967):299-304.

Rosen OM, Erlichman J (1975) Reversible autophosphorylation of a cyclic 3',5'-AMP-dependent protein kinase from bovine cardiac muscle. J Biol Chem; 250:7788-7794.

Roufogalis B. Specificity of trifluoperazine and related phenothiazines for Ca++ binding proteins. Calcium and Cell Function, Vol. III, NY:Academic Press (1982):129-159.

Salmons S, Vrbova G (1969) The influence of activity on some contractile characteristics of mammalian fast and slow muscles. J Physiol (Lond); 201:535-549.

Schwartz A, Entman MC, Kaniike K. Lane LK, VanWinkle WB, Bornet EP (1976) The rate of calcium uptake in sarcoplasmic reticulum of cardiac muscle and skeletal muscle. Effects of cyclic-AMP-dependent protein kinase and phosphorylase b kinase. Biochim Biophys Acta; 426:57-72.

Schwartz A, Wood JM, Allen JC, Bornet EP, Entman MC, Goldstein MA, Sordahl LA, Suzuki M (1973) Biochemical and morphologic correlates of cardiac ischemia. 1. Membrane systems. Am J Cardiol; 32:46-61.

Shenolikar S, Cohen PTW, Cohen P, Nairn AC, Perry SV (1979) The role of calmodulin in the structure and regulation of phosphorylase kinase from rabbit skeletal muscle. Eur J. Biochem; 100:329-337.

Shigekawa M, Akowitz AA (1979) On the mechanism of Ca++ dependent adenosine triphosphatase of sarcoplasmic reticulum. J Biol Chem; 254:4726-4729.

Shigekawa M, O'Dougherty, Katz AM (1978) Reaction mechanism of Ca++-dependent ATP hydrolysis by skeletal muscle sarcoplasmic reticulum in the absence of added alkali metal salts. J Biol Chem; 253:1442-1450.

Shigekawa M, Finegan JM, Katz AM (1976) Calcium transport ATPase of canine cardiac sarcoplasmic reticulum. A comparison with that of rabbit fast skeletal muscle sarcoplasmic reticulum. J Biol Chem; 251:6894-6900.

Shigekawa M, Pearl CJ (1976) Activation of calcium transport in skeletal muscle sarcoplasmic reticulum by monovalent cations. J Biol Chem; 251:6947-6952.

Smith R, Stern G (1969) Muscular weakness in osteomalacia and hyperparathyroidism. J Neurol Sci; 8:511-520.

Sobue K, Yamazaki R, Yasuda S, Kakiuchi S (1981) Identity of the particulate form of calmodulin with soluble calmodulin. FEBS Lett; 129:215-219.

Solaro RJ, Moir AJG, Perry SV (1976) Phosphorylation of the inhibitory component of troponin and the inotropic effect of adrenaline in perfused rabbit heart. Nature; 262:615-616.

Sommer JR, Waugh RA (1976) The ultrastructure of the mammalian cardiac muscle cell with special emphasis on the tubular membrane systems. A review. Am J Pathol; 82:192-232.

Sorenson MM, deMeis L (1977) Effects of anions, pH and magnesium on calcium accumulation and release by sarcoplasmic reiculum vesicles. Biochim Biophys Acta; 465:210-223.

Sreter FA (1963) Cell water, sodium and potassium in stimulated red and white mammalian muscles. Am J Physiol; 205:1290-1294.

Sreter FA (1969) Temperature, pH and seasonal dependence of Ca-uptake and ATPase activity of white and red muscle microsomes. Arch Biochem Biophys; 134:25-33.

Sreter FA, Ikemoto N, Gergely J. Studies on the fragmented sarcoplasmic reticulum of normal and dystrophic mouse muscle. Exploratory Concepts in Muscular Dystrophy and Other Related Disorders, A.F. Milhorat (ed). Amsterdam:Exerpta Medica (1967):289-298.

Stanburg SW (1965) Muscular disorders of metabolic bone disease. Manch Med Gaz; 45:16-19.
Stearns SB (1980) Carnitine content of skeletal muscle from diabetic and insulin-treated diabetic rats. Biochem Med; 24:33-38.

Steenbergen C, Deleeuw G, Rich T, Williamson JR (1977) Effects of acidosis and ischemia on contractility and intracellular pH of rat heart. Circ Res; 41:849-858.

Stewart PS, MacLennan DH, Shamoo AE (1971) Resolution of enzymes of biological transport. 1. Isolation and characterization of tryptic fragments of adenosine triphosphatase of sarcoplasmic reticulum. J Biol Chem; 251:712-719.

Sumida M, Wang T, Mandel F, Froelich JP, Schwartz A (1978) Transent kinetics of Ca++ transport of sarcoplasmic reticulum: a comparison of cardiac and skeletal muscle. J Biol Chem; 253:8772-8777.

Sutherland EW, Rall TW (1960) The relation of adenosine 3',5' phosphate and phosphorylase to the actions of catecholamines and other hormones. Pharmacol Rev; 12:265-299.

Sutherland EW, Robinson GA, Butcher RW (1968) Some aspects of the biological role of adenosine 3',5'-monophosphate (cyclic AMP). Circulation; 37:279-306.

Tada M, Kadoma M-A, Ineri M, Yamada M, Ohmori F. Ca++-dependent ATPase of the sarcoplasmic reticulum. Transport and Bioenergetics in Biomembranes, R. Sato & Y. Kagaua (ed).

NY:Plenum Press (1982):137-164.

Tada M, Katz AM (1982) Phosphorylation of the sarcoplasmic reticulum and sarcolemma. Annu Rev Physiol; 44:401-423.

Tada M, Kirchberger MA, Katz AM (1975) Phosphorylation of a 22,000 dalton component of the cardiac sarcoplasmic reticulum by adenosine 3',5'-monophosphate-dependent protein kinase. J Biol Chem; 250:2640-2647.

Tada M, Kirchberger MA, Repke DI, Katz AM (1974) The stimulation of calcium transport in cardiac sarcoplasmic reticulum by adenosine 3',5'-monophosphate-dependent protein kinase. J Biol Chem; 249:6174-6180.

Tada M, Ohmori F, Kinoshita N, Abe H (1978) Cyclic AMP regulation of active calcium transport across membranes of sarcoplasmic reticulum--role of 22,000 dalton protein phospholamban. Adv Cyclic Nucleotide Res; 9:355-369.

Tada M, Ohmori F, Yamada M, Abe H (1979) Mechanism of the stimulation of Ca++-dependent ATPase of cardiac sarcoplasmic reticulum by adenosine 3',5'-monophosphate-dependent protein kinase. Role of the 22,000 dalton protein. J Biol Chem; 254:319-326.

Tada M, Yamamoto T, Tonomura Y (1978) Molecular mechanism of active calcium transport by sarcoplasmic reticulum. Physiol Rev; 58:1-79.

Takagi A, Schotland DL, Rowland LP (1973) Sarcoplasmic reticulum in Duchenne muscular dystrophy. Arch Neurol; 28:380-384.

Thomas PK, Ward JD, Watkins PJ. Diabetic neuropathy. Complications of Diabetes, 2nd ed, H. Keen & J. Jarrett (ed) London, England:Edwin Arnold (1982):109-136.

Thorley-Lawson DA, Green NM (1973) Studies on location and orientation of proteins in sarcoplasmic reticulum. Eur J Biochem; 40:403-413.

Vadlamudi RVSV, Rodgers RL, McNeill JH (1982) The effect of chronic alloxan- and streptozotocin-induced diabetes on isolated rat heart performance. Can J Physiol Pharmacol; 60:902-911.

VanWinkle WB, Entman ML (1979) Comparative aspects of cardiac and skeletal muscle sarcoplasmic reticulum. Life Sci; 25:1189-1200.

Velema J, Zaagsma J (1981) Purification and characterization of cardiac sarcolemma and sarcoplasmic reticulum from rat

ventricle muscle. Arch Biochem Biophys; 212:678-688.

Verjovski-Almeida S, Inesi G (1979) Rapid kinetics of calcium ion transport and ATPase activity in the sarcoplasmic reticulum of dystrophic muscle. Biochim Biophys Acta; 558:119-125.

Vincenzi FF (1981) Calmodulin pharmacology. Cell Calcium; 2:387-409.

Walton JN, Gardner-Medwin D. Disorders of Voluntary Muscle, J.N. Walton (ed). London:Churchill-Livingstone (1974):561-613.

Wang C, Fleischer S, Saito A (1979) Correlation of ultrastructure of reconstituted sarcoplasmic reticulum membrane vesicles with variation in phospholipid to protein ratio. J Biol Chem; 254:9209-9219.

Wang T, Grassi de Gende AO, Schwartz A (1979) Kinetic properties of calcium adenosine triphosphatase of sarcoplasmic reticulum isolated from cat skeletal muscles. A comparison of caudofemoralis (fast), tibilalis (mixed) and soleus (slow). J Biol Chem; 254:10675-10678.

Wang T, Grassi de Gende AO, Tsai L-I, Schwartz A (1981) Influence of monovalent cations on the Ca++-ATPase of sarcoplasmc reticulum isolated from rabbit skeletal and dog cardiac muscles. An interpretation of transient-state kinetic data. Biochim Biophys Acta; 637:523-529.

Warren GB, Toon PA, Birdsall NJM, Lee AG, Metcalfe JC (1974) Reversible lipid titrations of the activity of pure adenosine triphosphatase-lipid complexes. Biochemistry; 13:5501-5507.

Weber A (1971) Regulatory mechanisms of the calcium transport system of fragmented rabbit sarcoplasmic reticulum. I. The effect of accumulated calcium on transport and adenosine triphosphate hydrolysis. J Gen Physiol; 57:50-63.

Weber A, Herz R, Reiss I (1966) Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. Biochem Z; 345:329-369.

Wharton DC, Tzagoloff A (1967) Cytochrome oxidase from beef heart mitochondria. Methods Enzymol; 45:245-250.

Wohlfart B, Noble MIM (1982) The cardiac excitation-contraction cycle. Pharmacol Ther; 16;1-43.

Wolff DJ, Poirier PG, Brostrom CO, Brostrom MA (1977) Divalent cation binding properties of bovine brain Ca++-dependent regulator protein. J Biol Chem; 252:4108-4117.

Woods DS, Sorenson MM, Eastwood AB (1978) Duchenne dystrophy:

abnormal generation of tension and Ca++ regulation in single skinned fibers. Neurology; 28:447-457.

Yamada S, Tonomura Y (1972) Reaction mechanism of the Ca++-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. VII. Recognition and release of Ca++ ions. J Biochem; 72:417-425.

Yamamoto T, Takisawa H, Tonomura Y (1979) Reaction mechanisms for ATP hydrolysis and synthesis in the sarcoplasmic reticulum. Curr Top Bioenerg; 9:179-236.

Yamamoto T, Tonomura Y (1967) Reaction mechanism of the Ca++-dependent ATPase of sarcoplasmic reticulum from skeletal mucle. I. Kinetic studies. J Biochem (Japan); 62:558-575.

Yamamoto T, Tonomura Y (1968) Reaction mechanism of the Ca++-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. II. Intermediate formation of phosphoryl protein. J Biochem (Japan); 64:137-145.

Zubrzycka-Gaarn E, Korczak B, Osinska H, Sarzala MG (1982) Studies on sarcoplasmic reticulum from slow-twitch muscle. J Muscle Res Cell Motility; 3:191-212.