REGULATION OF THE CALCIUM TRANSPORT ATPASE OF RAT HEART SARCOPLASMIC RETICULUM

By

RAJESH MAHEY

B.Sc., The Sunderland Polytechnic, England, 1983

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Division of Pharmacology and Toxicology

The Faculty of Pharmaceutical Sciences

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

October 1986

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Pharmacology and Toxicology (Fac. Pharmaceutical Sciences)

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date December 9, 1986

ABSTRACT

The sarcoplasmic reticulum Ca^{2+}-pumping ATPase is the primary system responsible for the removal of calcium from the sarcoplasm during relaxation of skeletal and cardiac muscles. Since the rat heart SR is used frequently in our laboratory to study the Ca^{2+}-transport defects in disease states, the Ca^{2+}-ATPase activity of this system was characterized. Calmodulin (CaM) and cAMP-dependent protein kinase (cAMP-PK) are known to regulate the dog cardiac SR Ca^{2+}-pump. The effects of these regulators on the rat heart SR Ca^{2+}-pump were studied. Studies were also carried out to investigate the effects of Triton X-100 on SR Ca^{2+}-ATPase activity and the regulation of this activity by CaM.

The rat heart SR Ca^{2+}-ATPase was stimulated in a concentration-dependent manner by both Ca^{2+} and Mg^{2+} in the complete absence of the other cation. Magnesium produced a concentration-dependent increase in the basal ATPase activity without affecting the maximal ATPase activity. This appeared to result in a gradual disappearance of the Ca^{2+} dependency of the ATPase activity. Addition of 100uM CDTA (trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid), in the absence of added magnesium, produced no effect on Ca^{2+} stimulation of ATPase activity. The results appear to indicate the presence of a low affinity non-specific divalent cation-stimulated ATPase.

At a constant Mg:ATP ratio, ATP stimulated the SR Ca^{2+}-ATPase activity in a concentration-dependent manner. Double-

reciprocal plots of the data suggest that the true substrate for rat heart SR Ca^{2+}-ATPase may be ATP and not Mg.ATP.

In the crude SR, CaM did not stimulate total or Ca^{2+}-stimulated ATPase activity over a range of Ca^{2+} and Mg^{2+} concentrations. CaM also failed to stimulate membrane phosphorylation over a range of Mg^{2+} concentrations. Furthermore, CaM did not produce a significant effect on calcium transport into SR vesicles. The catalytic subunit of cAMP-dependent protein kinase was also ineffective in stimulating membrane phosphorylation and Ca^{2+}-ATPase activity. Two CaM antagonists, trifluperazine and compound 48/80, did not affect the rat heart SR ATPase activity.

The ATPase activity in Triton-washed SR membranes appeared to be increased at low Triton concentrations. This effect was probably due to the removal of non-intrinsic proteins, leaky vesicles or altered membrane fluidity. At higher Triton X-100 concentrations, the ATPase activity was lost, probably due to loss of the phospholipid environment.

When SR membranes phosphorylated under conditions similar to those used for the ATPase assay were analysed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) followed by autoradiography, a single phosphorylated protein of 7,500-9,000 dalton was observed. This protein may represent the monomeric form of phospholamban. CaM, however, appeared to have no effect on the phosphorylation of this 7,500-9,000 dalton protein in either untreated or Triton-washed SR membranes. It is

speculated that the rat heart SR contains tightly bound CaM which cannot be removed by treatment with Triton X-100.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor, Dr. Sidney Katz for all the guidance and support throughout this study. I would like to thank my committee members, Drs. B.D. Roufogalis, J. Diamond, K. MacLeod. I would also like to thank Dr. D. Godin for substituting for Dr. B.D. Roufogalis in his absence.

I wish to thank Dr. M. Bridges, Dr. D. Jeffery and other members of Dr. Katz's laboratory for their technical assistance and moral support.

Finally, I wish to thank all members of the faculty, staff, and graduate student body in The Faculty of Pharmaceutical Sciences, U.B.C. for making this Masters program an enjoyable experience.

LIST OF ABREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
^{o}C	degrees Celsius
$C_{12}E_9$	polyoxyethylene 9-lauryl ether
Ca^{2+}	free ionized calcium
$(Ca^{2+}+Mg^{2+})$-ATPase	calcium-stimulated, magnesium dependent ATPase
CaM	calmodulin
cAMP	adenosine 3',5'-cyclic monophosphate
CDTA	trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
Ci	curie
cpm	counts per minute
dpm	disintegrations per minute
E_1	the high affinity state of the enzyme
E_2	the low affinity state of the enzyme
E-C	excitation-contraction
EGTA	ethyleneglycol bis-(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EP	phosphorylated intermediate of $(Ca^{2+}+Mg^{2+})$-ATPase
et al	and others
g	gram
xg	gravitational unit
$K_{0.5}$	concentration at half-maximal response

l	litre
m	milli
M	molar
μ	micro
mA	milliamperes
M.W.	molecular weight
min	minutes
n	nano
p	pico
PAGE	polyacrylamide gel electrophoresis
P_i	inorganic phosphate
PLB	phospholamban
PK	protein kinase
rpm	revolutions per minute
S.A	specific activity
SDS	sodium dodecyl sulphate
S.E.M.	standard error of mean
SL	sarcolemma
SR	sarcoplasmic reticulum
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
tris	tris(hydroxy)aminomethane
w/w	weight per unit weight

DEDICATION

To my mom and dad, for their love and patience.

INTRODUCTION

1. Role of Calcium in Biological Systems

a). Historical

Calcium (Ca^{2+}) plays a key role in many biological systems. It began with the serendipidous discovery of Sydney Ringer (1883) that a solution of sodium chloride and tap water was more efficacious in maintaining cardiac contractility than sodium chloride and distilled water; Ringer concluded that the minute amounts of calcium present in tap water antagonized the "injurious" effects of sodium.

An important first step in the elucidation of the role of calcium in biological systems was provided by Mines (1911) who found that calcium, strontium, or barium could reverse the gradual diminution in the response of frog gastrocnemius muscle perfused with 0.7% NaCl to electric stimulation. He concluded that calcium, strontium and barium had some special chemical property that enabled them to interact with some unknown constituent of the cell.

In addition to its importance in providing rigidity by being a major constituent of bone, calcium is involved in a diversity of functions in biological systems. These include contraction of all forms of muscle, the metabolic processes of glycogenolysis and gluconeogenesis, the mobilization and secretion of exocrine, endocrine and neurocrine products, the

transport and secretion of fluids and electrolytes and the growth of cells (Rasmussen, 1986).

b). Ca^{2+} as a Second Messenger

Calcium has been proposed as the universal intracellular messenger, linking events at the cell surface to functions in the cell interior. However, calcium affects different systems in different ways. For example, the manner by which Ca^{2+} couples excitation to contraction in skeletal muscle is very different from that in smooth muscle (Rasmussen, 1986). In brief cellular responses such as neurosecretion and skeletal muscle contraction, the magnitude and duration of response is a function of the magnitude and duration of the Ca^{2+} message. However, in sustained responses such as insulin secretion, smooth muscle contraction or aldosterone secretion, a more subtle and complex relationship exists between the intracellular free Ca^{2+} and the cellular response.

Two branches of the Ca^{2+} messenger system, with distinct temporal roles, have been proposed: a calmodulin (CaM) branch (Wang and Waisman, 1979) responsible for either brief responses or the initial phase of sustained responses and a C-kinase branch (Nishizuka, 1986) responsible for the sustained phase of cellular responses. The Ca^{2+} messenger system is intimately related to and modified by the cyclic AMP messenger system and the arachidonic acid cascade.

2. Regulation of Intracellular Ca^{2+}

The functional species of calcium is the small pool of free cytosolic Ca^{2+}. The resting intracellular free Ca^{2+} concentration is very low (0.1-0.3μM) in virtually all animal cells (Blinks et al, 1982). In contrast, the extracellular Ca^{2+} concentration is approximately 1mM (Rasmussen, 1986). Thus, there is a 4,000-10,000-fold gradient across the membrane. This gradient is maintained by i) a low permeability of the plasma membrane to Ca^{2+} (Borle, 1981), ii) a number of mechanisms to extrude Ca^{2+} from the cell, and iii) intracellular Ca^{2+} sequestering systems. Two types of active transport mechanisms are involved in extrusion of Ca^{2+} from the cell, namely the Ca^{2+}-ATPase pump (Penniston, 1983) and the Na^{+}-Ca^{2+} exchange system (Reeves, 1985). The relative importance of the two mechanisms in various cells is uncertain. Cytosolic free Ca^{2+} is also removed by two subcellular organelles, mitochondria and smooth endoplasmic reticulum (Becker et al, 1980). These organelles also serve as a pool of releasable Ca^{2+}. A limited buffering capacity is provided by the cytosolic binding proteins such as parvalbumin in skeletal muscle (Wnuk et al, 1982) and vitamin D-induced calcium binding protein in the intestinal columnar epithelial cells (Wasserman and Fullmer, 1982).

3. Sarcoplasmic Reticulum and Regulation of Intracellular Calcium

a). Morphology of SR

In skeletal and cardiac muscle, the SR Ca^{2+}-pump is the primary system involved in the rapid sequestration of Ca^{2+} from the sarcoplasm during relaxation (Shamoo and Ambudkar, 1984). SR can be related to the endoplasmic reticulum of the other cell types. As Porter and Pallade described, it "consists of membrane-limited vesicles, tubules and cisternae associated in a continuous reticulum structure which forms face-like sleeves around the myofibrils. It shows a definable organization which repeats with each sarcomere of the fibre so that the entire system is segmented in phase with the striations of the associated myofibrils" (Porter and Pallade, 1957). It is differentiated into specialized regions consisting of the junctional SR, mainly the terminal cisternae which form coupling with the transverse tubules and the longitudinal (free) SR. The transverse tubules run perpendicular to the longitudinal SR and form "triads" with the terminal cisternae. The volume of the tubular network has been estimated as 11-13% and 6-9% of the total fibre volume in the fast skeletal muscle and cardiac muscle, respectively (Peachey, 1965; Nayler, 1977). Terminal cisternae account for 5% of the total fibre volume in the fast skeletal muscle (Peachey, 1965). Assuming the intravesicular volume of 10 µl/mg SR protein, the SR Ca^{2+} concentration has

been estimated to be approximately 10mM (Nayler and Dresel, 1984).

The SR is composed of a lipid bilayer containing phosphatidylcholine (60-75%), phosphatidylethanolamine (10-20%), phosphatidylserine (5-10%), phosphatidylinositol (10%), and other phospholipids in smaller concentrations (MacLennan et al, 1971; Meissner and Fleisher, 1971). It also contains a number of proteins. The ATPase protein of M.W. 100,000 dalton (Meissner et al, 1973) accounts for 60-80% of total skeletal SR protein (deMeis and Inesi, 1982; MacLennan et al, 1978) and 35-40% of cardiac SR protein (Suko and Hasselbach, 1976). Calsequestrin, an acidic protein with M.W. of 55,000 dalton (Cala and Jones, 1983; Campbell et al, 1983), two high affinity calcium binding proteins (M.W. 53,000 and 160,000 dalton), a proteolipid of M.W. 12,000 dalton and a glycoprotein of 53,000 dalton (Campbell et al, 1983) are the other major protein constituents.

b) Excitation-Contraction Coupling in Cardiac and Skeletal Muscle

The depolarization of sarcolemma (SL) is the initial step in excitation-contraction (E-C) coupling. This depolarization results in the opening of the Ca^{2+} channels in the SL (Katz, 1985; Shamoo and Ambudkar, 1984) which allow the passive influx of Ca^{2+} down a concentration gradient. However, the amount of Ca^{2+} entering into the sarcoplasm during this process is not sufficient to account for the activation of contraction on a

beat-to-beat basis (Tada and Inui, 1983). Therefore, it is important that an intracellular pool of Ca^{2+} be mobilized. The SR is believed to provide most of the required Ca^{2+} for contraction.

The mechanism by which excitation results in the release of Ca^{2+} from the SR remains controversial. There is evidence for two different mechanisms: a) Ca^{2+}-induced Ca^{2+} release and b) depolarization-induced Ca^{2+} release. Ford and Podolsky (1972) have shown that during depolarization a small amount of the extracellular Ca^{2+} leaks into the myofilament space causing release of enough Ca^{2+} from the SR to raise the cytoplasmic Ca^{2+} levels to those required for activation of the myofilaments. Further evidence for this has been provided by Fabiato and Fabiato (1975). According to the latter theory, the depolarization is passed down the transverse tubules modifying the SR membrane which results in the release of Ca^{2+} from SR. This is shown by a direct alteration of the membrane potential of SR to cause Ca^{2+} release (Nakajima and Endo, 1973). Fabiato and Fabiato concluded that while depolarization-induced Ca^{2+} release is important in skeletal muscle, the Ca^{2+}-induced calcium release is important in cardiac muscle (Fabiato and Fabiato, 1977).

There is evidence to show that the site of Ca^{2+} release is the terminal cisternae (Huxley and Taylor, 1958; Winegrad, 1968). Huxley and Taylor showed that the local excitation of a single transverse tubule leads to a contraction which is

confined to the half sarcomere. Autoradiographic studies by Winegrad showed that Ca^{2+} is accumulated by the longitudinal SR and transported slowly to the terminal cisternae and released from that site upon excitation. Most of the SR Ca^{2+} has been shown to be located in the terminal cisternae (Winegrad, 1968). More recently, using a French press to dissociate transverse tubules from SR, Ikemoto and colleagues (1984) concluded that the transverse tubules play an important role in depolarization-induced Ca^{2+} release.

c). SR as the Relaxing Factor

Muscle relaxation results when the cytosolic Ca^{2+} is taken up by the SR, thus lowering the concentration of free Ca^{2+} to the resting levels of 10^{-8}-10^{-7}M (Blinks et al, 1982). The ability of the SR Ca^{2+}-transport to control intracellular free Ca^{2+} is reflected by its 5-10 times greater affinity for Ca^{2+} than that of the contractile proteins. Early studies by Ebashi showed that the binding of Ca^{2+} by a number of chelators correlated well with their relaxing effects (Ebashi, 1960) indicating that the relaxation is caused by removal of Ca^{2+} from the myofibril environment. He later suggested that the ATP-dependent Ca^{2+} binding capacity of "vesicular relaxing factor" is comparable to the relaxation produced by chelating agents (Ebashi, 1961). It was later shown that inhibition of Ca^{2+} accumulation in SR resulted in inhibition of relaxation (Ebashi and Lipman, 1962; Hasselbach and Makinose, 1962) and that agents that

augment Ca^{2+} uptake also potentiate the relaxing effect of the SR.

Inside the SR, Ca^{2+} is bound to calsequestrin (MacLennan and Wong, 1971; Campbell et al, 1983). Cardiac calsequestrin has a capacity to bind 650 nmoles of Ca^{2+}/mg protein (Cala and Jones, 1983), although the skeletal calsequestrin has been reported to bind up to 970 nmoles Ca^{2+}/mg protein (Cala and Jones, 1983; Campbell et al, 1983). The distribution of calsequestrin correlates well with the major Ca^{2+} stores of SR, i.e. the terminal cisternae (Jorgensen et al, 1979).

d). The SR Ca^{2+}-Transport ATPase

How is Ca^{2+} transported into the SR lumen? It was noted in earlier studies that Ca^{2+} transport into SR vesicles required ATP (Ebashi and Lipman, 1962; Hasselbach and Makinose, 1962). Hasselbach and Makinose were probably the first people to demonstrate that ATP hydolysis and Ca^{2+} transport were linked (Hasselbach and Makinose, 1962; Hasselbach, 1964). The enzyme system responsible for Ca^{2+} transport was named Ca^{2+}-dependent adenosine triphosphatase (Ca^{2+}-ATPase). This enzyme is capable of transporting two molecules of Ca^{2+} for each molecule of ATP hydrolyzed (Hasselbach, 1978; Tada et al, 1978; Weber, 1966). This 2:1 ratio has been supported by Ca^{2+} binding studies in soluble ATPase systems (Kosk-Kosicka et al, 1983) which showed that there were 8-10 nmoles high affinity Ca^{2+} binding sites per mg protein and only 4-5 nmoles of phosphoenzyme intermediate per

mg protein. Other studies have shown that the enzyme requires magnesium (Mg) for its activity and therefore, it is normally referred to as the (Ca^{2+}+Mg^{2+})-ATPase. The Mg^{2+} dependent activity (in absence of Ca^{2+}) is referred to as the 'basal' activity while the Ca^{2+}-stimulated activity is called the 'extra' ATPase activity (Hasselbach, 1964, 1978). Ca^{2+} can potentiate the basal activity by 7-8 fold (Hasselbach and Makinose, 1962). The actual process of Ca^{2+} transport is coupled to the extra ATPase activity (Hasselbach, 1978).

The ATPase molecules are asymmetrically embedded in the SR membrane (deMeis, 1981). Static diffraction studies show the ATPase molecule to be a tapered rectangle (Herbette and Blasie, 1980). The wider part of the rectangle, accounting for 62% of the mass, has been shown to be polar and extruding to the sarcoplasmic surface. The other part, accounting for 38%, is inside the lipid bilayer (deMeis, 1981; Herbette and Blasie, 1980). There are essentially no extensions to the lumen of the SR.

i). Structure of SR Ca^{2+}-Transport ATPase

Radiation-inactivation techniques indicate the molecular size of the Ca^{2+}-pump protein to be 213,000-229,000 dalton (Chamberlain et al, 1983). This molecular weight is consistent with a dimer. However, each monomer has been shown to be able to perform the entire catalytic cycle of ATP hydrolysis and Ca^{2+} transport (Moller et al, 1982). Although Martonosi had reported

partial purification of the ATPase with Ca^{2+} transport and ATPase activity (Martonosi, 1968), the first successful purification of the enzyme was made from skeletal muscle by MacLennan (1970). The enzyme has been fractionated into different protein fragments. A mild digestion with trypsin resulted in two protein fragments: the NH_2 terminal fragment of 55,000 dalton (fragment A) and the COOH terminal fragment (fragment B) of 45,000 dalton (Migala et al, 1973; Stewart and MacLennan, 1974; Thorley-Lawson and Green, 1973). Further digestion of ATPase resulted in a fragmentation of the 55,000 dalton peptide (60,000 dalton fragment of Thorley-Lawson and Green) into 30,000 (fragment A_1) and 20,000 dalton (fragment A_2) proteins (Stewart et al, 1976). All three fragments were tightly associated with one another and with the membrane (MacLennan et al, 1976; Thorley-Lawson and Green, 1975). Saito et al (1984) have recently reported a further trypsin cleavage of the A_1 fragment into A_{1a} and A_{1b} and the appearance of another fragment C (M.W. 27,000-28,000 dalton). Based on the observations in the fluorescein isothiocyanate binding studies, the 45,000 dalton peptide has been identified to be the nucleotide binding protein (Mitchinson, 1982). Dicyclohexylcarbodiimide binding studies have shown the 25,000 dalton fragment to contain the Ca^{2+} binding site (Pick and Racker, 1979). The 30,000 dalton fragment has been shown to contain the phosphorylation site (Thorley-Lawson and Green, 1973). The first cleavage of ATPase into A and B fragments produced no change in the ATP hydrolysis or Ca^{2+}

uptake activities (Stewart and MacLennan, 1974). However, the second cleavage caused a loss of the Ca^{2+} uptake activity without affecting ATP hydrolysis (Scott and Shamoo, 1982).

One major concern is the loss of activity in the solubilized enzyme. It was noted that the detergent removed the phospholipid environment of the enzyme which was very important for the activity (MacLennan, 1970). Therefore, attempts were made to reconstitute the solubilized enzyme by dialysis to remove the excess detergent and by adding exogenous lipids (MacLennan et al, 1971; Racker, 1972; Warren et al, 1974). This treatment resulted in the return of activity to original or higher levels. It has recently been reported that a minimum of 23 phospholipids per enzyme were required for enzyme activity (Hidalgo et al, 1986). However, Dean and Tanford (1978) have reported that the enzyme can be delipidated down to one mole of phospholipid per mole of ATPase without loss of activity.

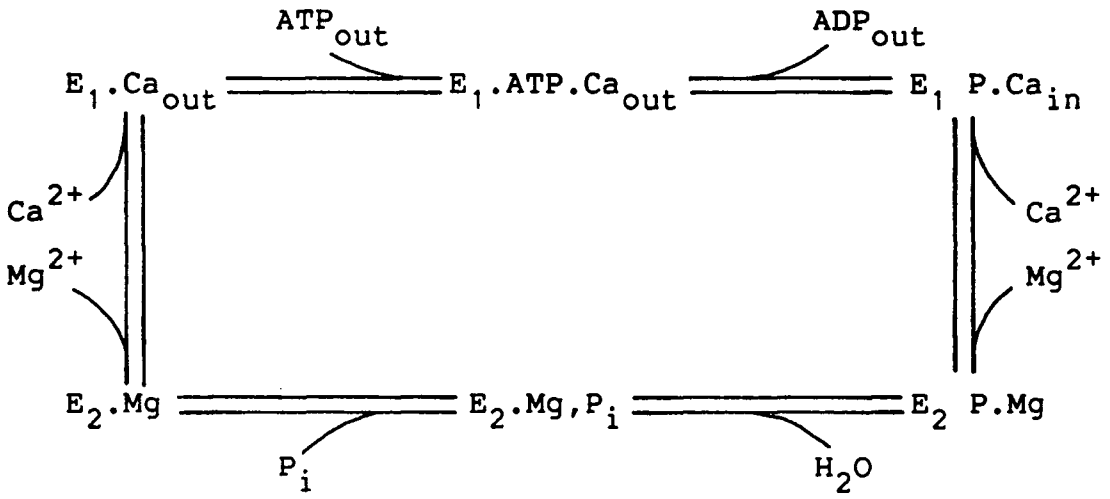
ii). The Ca^{2+} Transport Cycle

The mechanism of Ca^{2+} transport by the ATPase is now becoming clear. In a recent review, Haynes (1983) has proposed a Ca^{2+} transport cycle with the following 8 steps: a) binding of 2 Ca^{2+} and Mg-ATP to external sites of the enzyme with high affinity and random order, b) enzyme phosphorylation, c) inward translocation of the Ca^{2+}-laden sites, d) Ca^{2+} release to the SR lumen and ADP to the external medium (random order), e) binding of Mg^{2+} or a charge-stoichiometric amount of K^{+} plus H^{+} to the

translocator, f) dephosphorylation, g) the return of K^+ and H^+ from the translocator to the outside, h) dissociation of K^+ and H^+ from the translocator and completion of the cycle with step a). A time-resolved diffraction study has shown that Ca^{2+} transport results in a shift of electron density from the extra-vesicular surface of the SR membrane to the interior of the membrane (Herbette and Blasie, 1980). The high affinity Ca^{2+} binding proteins (step a) have been identified to be the 20,000 dalton tryptic fragments of ATPase (Pick and Racker, 1979). The binding of the first Ca^{2+} leads to conformational changes which result in an increased affinity for binding of the second Ca^{2+} (Inesi et al, 1980). After phosphorylation, the high affinity sites are converted to low affinity sites and release the Ca^{2+} into the lumen of the SR (Ikemoto, 1974).

The phosphorylated intermediate (EP) in step b) has been shown to be an acyl phosphate (Shigekawa et al, 1976) which can be hydrolysed by hydroxylamine (Makinose, 1969). The formation of the EP is Ca^{2+} dependent and Mg^{2+} causes dephosphorylation (Shigekawa et al, 1976).

A scheme proposed by Tada and Katz (1982) for the Ca^{2+} transport cycle incorporates these observations into the following sequence of steps:



E_1 in the above diagram represents the high affinity Ca^{2+} binding state of the enzyme while E_2 represents the low affinity state. The "in" and "out" refer to the inside and the outside of the SR vesicles, respectively.

e). Phospholamban and the Regulation of SR Ca^{2+}-Transport ATPase

In the early seventies, cAMP-dependent protein kinase (cAMP-PK) and cAMP were observed to produce a stimulation of Ca^{2+} transport and ATP hydrolysis in cardiac SR (Kirchberger et al, 1972). This stimulation was shown to be due to the phosphorylation of an SR protein (Kirchberger et al, 1974; Tada et al, 1974), later termed phospholamban (PLB) meaning 'phosphate receptor' (Tada et al, 1975). A recent study with a PLB-specific antibody provides direct evidence for the involvement of PLB as a regulatory protein of the SR Ca^{2+} pump (Suzuki and Wang, 1986).

It has also been reported that calmodulin (CaM), a calcium binding protein present in most cells (Cheung, 1980), stimulated

Ca^{2+} uptake (Katz and Remtulla, 1978; Lopaschuk et al, 1980) and ATPase activity (Lopaschuk et al, 1980) in the SR. Unlike the cAMP-PK stimulation, Ca^{2+} appears to be essential for the CaM-dependent stimulation. CaM stimulation is also thought to be via the phosphorylation of PLB (Kirchberger and Antonetz, 1982a; LePeuch et al, 1979). An endogenous kinase, called Ca^{2+}-CaM-dependent protein kinase (CaM-PK) is believed to be responsible for CaM-dependent phosphorylation of PLB (Davis et al, 1983; Kirchberger and Antonetz, 1982a; LePeuch et al, 1979). CaM-PK has, very recently, been partially purified and characterized (Molla and Demaille, 1986). It is identified as a 56,000 dalton protein with a broad substrate specificity.

Early observations indicated that the stimulatory effects of cAMP-PK and CaM on the SR Ca^{2+} transport system were additive (Katz, 1980; Lopaschuk et al, 1980), suggesting that different mechanisms were involved. Therefore, it was suggested that the PLB phosphorylation occurs at separate and distinct sites (Bilezikjian et al, 1981; Kranias et al, 1980). A recent report suggests the involvement of three sites, one phosphorylated by cAMP-PK and the other two by CaM-PK (Imagawa et al, 1986). One of the CaM-PK phosphorylated sites may be the same as the cAMP-PK phosphorylated site. Chiesi et al (1983) have reported evidence for the possibility of two functionally distinct proteolipids, one of which is selective for CaM and the other for cAMP-PK. Tada and Inui (1983) reported that the cAMP-PK-mediated phosphorylation of PLB can be observed in the absence

of any CaM-dependent phosphorylation. However, some workers have suggested that the CaM-PK system is required for the cAMP-PK-dependent phosphorylation (LePeuch et al 1979, Wray and Gray, 1977) and that cAMP can only amplify the CaM-dependent phosphorylation. Lindemann and Watanabe (1985), on the other hand, have suggested that both cAMP and Ca^{2+} are required for Ca^{2+}-CaM dependent phosphorylation.

It has recently been reported that protein kinase C (Ca^{2+}-phospholipid-dependent protein kinase) also stimulates the phosphorylation of PLB resulting in a 2-fold stimulation of Ca^{2+} transport (Movsesian et al, 1984). The site phosphorylated by this kinase appears to be different from the two sites phosphorylated by cAMP-PK and CaM-PK. Movsesian et al also reported a common site which could be phosphorylated by all three kinases.

The PLB content has been suggested to be 3% (Caponi et al, 1983), 4% (Tada et al, 1979) or 6% (LePeuch et al, 1979) of total SR protein. It appears to be an acidic protein (Tada and Inui, 1983) which can be extracted into an acidified chloroform-methanol mixture (Bidlack and Shamoo, 1980). However, recently, Jones and colleagues have reported that dephosphorylated PLB is strongly basic and rich in cysteine (Jones et al, 1985). This is in direct contrast to the previous reports which have shown little or no cysteine in PLB (see Tada and Inui, 1983). PLB appears to be strongly bound to the Ca^{2+}-ATPase molecule (Tada et al, 1975; LePeuch et al, 1980) with the phosphorylation sites exposed to the cytosol. The stoichiometry of association between

the two proteins is believed to be 1:1 (LePeuch et al, 1979). However, Wegener et al (1986) have recently questioned this 1:1 stoichiometric ratio and suggested a much smaller concentration of PLB. Kranias et al (1983) have reported the isolation of Ca^{2+}-ATPase essentially free of PLB, indicating only a loose association between the two proteins. The phosphorylated PLB displays characteristics of a phosphoester as shown by its stability in hot alkali and in hydroxylamine. The phosphorylation involves incorporation of the terminal phosphate group of ATP into a serine residue of PLB (Kirchberger et al, 1974).

Phospholamban has been reported to be a proteolipid with an apparent molecular weight of 20,000-25,000 dalton (Bidlack et al, 1982; Jones et al, 1985; Kirchberger and Antonetz, 1982b; Lamers and Stinis, 1980; Tada et al, 1975; Wegener and Jones, 1984; Wray and Gray, 1977). Reports have suggested PLB to be a dimer of 11,000 dalton (LePeuch et al, 1979), a trimer of 11,000, 8,000 and 4,000 dalton (Louis et al, 1982), a tetramer of 5,500 (Kirchberger and Antonetz, 1982b), a pentamer of 9,000 dalton (Wegener and Jones, 1984) or more recently, a pentamer of 5,000-6,000 dalton (Fuji et al, 1986; Jones et al, 1985). A partial amino acid sequence of PLB has recently been reported (Fuji et al, 1986).

The mechanism by which phosphorylation of PLB leads to stimulation of Ca^{2+} transport is not known. Possibilities include, a) acceleration of dephosphorylation of the phosphorylated intermediate of the Ca^{2+}-ATPase (Katz et al, 1985; Tada et

al, 1982), and b) conformational changes in the active site of the Ca^{2+}-ATPase which result in the translocation of the Ca^{2+} binding subunit from the outside to the inside (Tada et al, 1982). The stoichiometric relationship of 2:1 between the Ca^{2+} transported and the ATP hydrolysed is not altered during PLB-mediated stimulation of Ca^{2+} transport (Tada et al, 1974).

In addition to the PLB pathway, CaM has been postulated to act directly on Ca^{2+}-ATPase molecules without the mediation of PLB (Katz, 1980). This conclusion was reached from the observation that CaM stimulated the dephosphorylation of the calcium-dependent phosphoprotein intermediate of the ATPase (EP) and thus the turnover rate of calcium transport (Katz, 1980). More recently, our laboratory has reported that CaM stimulated Ca^{2+}-ATPase activity under conditions where no augmentation of PLB phosphoprotein formation was seen (Katz et al, 1985). Louis and Maffitt (1982) have also observed that at Ca^{2+} concentrations where CaM-PK is inactive, CaM stimulation of Ca^{2+}-ATPase activity is present.

In ^{125}I-CaM binding experiments, Louis and Jarvis observed that the major product containing the ^{125}I label was a 40,000 dalton protein with a minor component of 120,000 dalton (Louis and Jarvis, 1982). They suggested that the 40,000 dalton component may represent a 1:1 complex of PLB and ^{125}I-CaM. It was also postulated that the 120,000 dalton component was due to a 1:1 cross-link between the 100,000 dalton Ca^{2+}-ATPase and ^{125}I-CaM. This would indicate a direct interaction of CaM with

the ATPase. A direct interaction between the two proteins is also indicated by the observations of Mas Oliva et al (1983) who reported that for the optimum effect of CaM, approximately equivalent molar concentrations of CaM and Ca^{2+}-ATPase were required.

Although the evidence presented above appears convincing, some reports in the literature contradict the hypothesis of a direct effect of CaM on SR Ca^{2+}-ATPase. Using the labelled CaM gel overlay techniques, Molla et al (1985) detected at least 7 CaM-binding proteins in SR vesicles. However, none of these was believed to be the Ca^{2+}-ATPase. In addition, Caroni and Carafoli (1981) have found that, unlike sarcolemmal Ca^{2+}-ATPase, the SR Ca^{2+}-ATPase failed to bind to CaM affinity chromatography columns.

OBJECTIVES OF THE PRESENT STUDY

Most of the work on cardiac SR Ca^{2+}-ATPase has been done using dogs as the experimental model. However, since our laboratory is interested in a number of disease models of rat heart, including diabetes and thyroid hormone alterations, we decided to conduct our studies on rat cardiac SR preparations. Therefore, the knowledge gained in this study may be pertinent to the disease models.

In initial experiments, we assayed the Ca^{2+}-ATPase activity in rat heart SR under identical conditions to those used for dog heart. A high "basal" ATPase activity was observed in these experiments with little or no reproducible Ca^{2+}-dependent ATPase activity. On reviewing the literature, it was discovered that Penpargkul (1979) had reported this unusually high basal ATPase activity in rat heart SR. Recently, Velema et al (1985) reported that in the absence of chelators, Mg^{2+} (0.45mM and 5.0mM) caused an apparent loss of Ca^{2+}-dependency of the rat heart sarcolemmal Ca^{2+}-ATPase. They concluded that Mg^{2+} was not essential for SL Ca^{2+}-ATPase activity. Therefore, in this study, we investigated the effects of Mg^{2+} on SR ATPase activity.

As discussed in the INTRODUCTION, dog cardiac SR Ca^{2+}-ATPase is regulated by CaM, cAMP-PK and protein kinase C. Although its physiological role is not known, CaM has been suggested to be important in the beat to beat regulation of cardiac Ca^{2+}-transport (Kirchberger and Antonetz, 1982a) or in certain pathological conditions (Tada et al, 1983). The role of

CaM and cAMP-PK in the regulation of rat cardiac SR Ca^{2+}-transport has not been elucidated. The effects of these potential regulators on SR Ca^{2+}-ATPase activity and SR membrane phosphorylation were therefore investigated in the current study.

Detergent treatment of SR membranes has been shown to solubilize regulatory proteins and alter ATPase activity. In this present investigation, Triton-washed membranes were studied for possible alterations in the Mg^{2+} and Ca^{2+} components of ATPase activity and membrane phosphorylation.

MATERIALS AND METHODS

A. MATERIALS

a). Radiochemicals:

γ-^{32}P-ATP (10-40 Ci/mmole) was purchased from Amersham and $^{45}CaCl_2$ (10-40 mCi/mg calcium) was purchased from Amersham or New England Nuclear.

b). Reagents:

The following chemicals were purchased from Sigma Chemical Company:

adenosine triphosphate (disodium),

adenosine triphosphate (tris),

bovine serum albumin,

Bromophenol Blue,

catalytic subunit of cyclic AMP dependent protein kinase,

CDTA,

compound 48/80,

EGTA,

glycerol,

glycine,

L-histidine,

hydroxylamine,

magnesium chloride,

2-mercaptoethanol,

sodium azide,

sucrose,

tetraethyl-methylenediamine (TEMED),

trichloroacetic acid (crystalline)

trichloroacetic acid (100% solution),

trifluoperazine,

Tris-base,

Tris-hydrochloride,

Tris-maleate,

Tris-oxalate,

Triton X-100.

The following chemicals were purchased from BDH Biochemicals: calcium chloride, sodium bicarbonate, sodium dodecyl sulphate, sodium hydroxide and potassium chloride.

The following electrophoresis chemicals were obtained from Bio-Rad: acrylamide, N,N'-methylene-bis acrylamide, ammonium persulphate, Coomassie Brilliant Blue R-250 and high and low molecular weight standards. The dye reagent for the protein assay was also purchased from Bio-Rad.

Gamma-globulin was obtained either from Calbiochem or Sigma Chemical Co.

Cello-seal^{R} and Scinti-Verse^{TM} II were obtained from Fisher Scientific Co. Aquasol^{R} was from New England Nuclear. Potassium phosphate (monobasic) was purchased from Amechem and sodium chloride was from Chemonics Scientific. Glacial acetic acid was

from Mallinckrodt Chemical Works Ltd, while activated charcoal was purchased either from BDH Biochemicals or Fisher Scientific Co.

B. METHODS

1. Preparation of Cardiac Microsomes Enriched in Sarcoplasmic Reticulum

Method 1. In initial studies, rat cardiac microsomes enriched in SR were prepared by a modification of the method of Sumida et al (1978). The entire procedure was carried out at 4°C. Wistar rats of both sexes (250-300 grammes) were killed by stunning and dislocation of the neck. The hearts were immediately dissected out and placed in ice cold 0.9% NaCl. The ventricles were trimmed of atria, blood vessels, fat and connective tissue, cut into small pieces and homogenized in 25 ml 10mM Tris-maleate, pH 6.8 with a teflon pestle by 5 passes at 1500 rpm. The homogenate was centrifuged in 50 ml Beckman centrifuge tubes at 4,000xg for 10 minutes. The supernatant was passed through four layers of cheese cloth and centrifuged at 15,000xg for 20 minutes. The second supernatant was again passed through cheese cloth and the filtrate centrifuged at 40,000xg for 90 minutes. The resulting pellet was resuspended in 10 ml 10mM Tris-maleate, pH 6.8 containing 0.6M KCl and centrifuged in 15 ml Corex^{R} tubes at 40,000xg for 110 minutes. The final pellet, enriched in SR, was resuspended in a medium containing 10mM Tris-maleate and 40% sucrose, quick frozen in liquid nitrogen and stored at -80°C.

Method 2. In the later studies, the SR vesicles were prepared by the method of Jones et al (1979) with slight modifica-

tions. The entire procedure was carried out at 4°C. The hearts were isolated and trimmed as before and homogenized in Beckman 50 ml centrifuge tubes in 15 ml 10mM $NaHCO_3$, pH 7.4 for 3x15 seconds using a Polytron homogenizer (Kinematica PT 10-35) at a setting of 4.0. The homogenate was diluted to 25 ml and centrifuged at 500xg for 5 minutes. The supernatant was filtered through four layers of cheese cloth and centrifuged at 7,000xg for 15 minutes. The pellet was discarded and the supernatant was centrifuged at 31,000xg for 30 minutes. The pellet was resuspended in 10 ml 30mM histidine-Cl, pH 7.0, containing 0.6M KCl and centrifuged in 15 ml Corex^R tubes at 31,000xg for 30 minutes. The resulting pellet, enriched in SR, was resuspended in a medium containing 0.25M sucrose, 0.3M KCl and 0.1M Tris base, pH 7.2. These resuspended SR vesicles were quick frozen in liquid nitrogen and stored at -80°C, prior to use, normally within one week.

2. Preparation of "Triton-washed" SR Membranes

The SR vesicles were diluted in the final resuspending medium (0.25M sucrose, 0.3M KCl and 0.1M Tris base, pH 7.2) containing different Triton X-100 concentrations to give the required detergent/protein ratio and incubated at 4°C for 60 minutes. This mixture was centrifuged at 105,000xg for 60 minutes to separate the solubilized proteins from the pellet. This 105,000xg pellet was termed the "Triton-washed" membranes.

3. Measurement of calcium uptake into SR vesicles

ATP-dependent calcium transport into cardiac SR vesicles was measured by a modification of the method of Tada et al (1974). The reaction medium contained 40mM histidine-Cl, pH 6.8, 110mM KCl, 5mM $MgCl_2$, 2.5mM Tris-oxalate, 5mM NaN_3 and 5mM ATP. The vesicles (approx. 40 µg) were preincubated with the medium for 6.5 minutes at 30°C and the reaction was started by the addition of the desired $CaCl_2$ solutions (containing $^{45}CaCl_2$; 200,000 dpm/sample). The desired free Ca^{2+} concentrations were maintained by addition of EGTA (ethylene glycol bis(β-aminoethyl ether)-N,N' tetraacetic acid) and determined by a Fortran program (see below). The reaction was terminated after 5 minutes by filtering a 0.41 ml aliquot of the reaction mixture through a 0.45 um Millipore filter (HA 45, Millipore^R Co.). The filter was washed twice with 20 ml of 40mM Tris-HCl, pH 7.2, dried and counted in Aquasol^R using a TRI-CARB^R 4530 Scintillation counter (Packard).

The rate of Ca^{2+} uptake by SR vesicles, expressed in nanomoles calcium transported per mg protein per minute, was calculated using the following formula:

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

where:

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

total counts = total ^{45}Ca counts (dpm) added to each tube

blank counts = ^{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 microsomal proteins were incubated in the presence of $CaCl_2$ (5min)

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

4. Assay of $(Ca^{2+}+Mg^{2+})$-ATPase Activity in SR Vesicles

$(Ca^{2+}+Mg^{2+})$-ATPase activity was measured by a modification of the method of Katz and Blostein (1975). SR vesicles (25 µg/ml) were preincubated for 5 minutes at 30°C in a medium containing 40mM histidine-Cl, pH 6.8, 100mM KCl, 5mM NaN_3, 100µM EGTA, 0-2mM $MgCl_2$ and $CaCl_2$ to give the desired free Ca^{2+} as determined by a Fortran program (see below). Whenever CaM, catalytic subunit of cAMP dependent protein kinase, trifluoperazine (TFP) or compound 48/80 were used, they were included in the medium. The reaction was started by the addition of 500µM ATP (containing ^{32}P-ATP; 200,000 dpm/sample). The reaction was terminated after 2.5 min (except in the time-course experiments) with ice cold "TCA stop solution" containing 5% trichloroacetic acid (TCA), 5mM Na_2ATP and 2mM KH_2PO_4. A suspension of activated charcoal (0.15 g per ml) was added to the samples to adsorb

unhydrolyzed ATP. The samples were shaken in an Eppendorf^{R} Shaker 5432 (Brinkman Instruments) for 5 minutes followed by centrifugation at 1500xg for 5 minutes. An aliquot of the supernatant, containing liberated ^{32}Pi was counted in Aquasol^{R} or Scinti-Verse^{TM} II.

Ca^{2+}-dependent ATPase activity was calculated by subtracting the "basal" activity (in absence of Ca^{2+}) from the "total" activity (in presence of Ca^{2+}).

The ATPase activity, expressed in nmoles ATP hydrolysed per mg protein per minute, was calculated by the following formula:

$$\text{ATPase Activity} = \frac{(\text{sample counts} - \text{background}) \times \text{dilution factor}}{\text{S.A.} \times \text{mg protein per sample} \times \text{reaction time in minutes}}$$

where:

$$\text{dilution factor} = \frac{\text{reaction volume} + \text{volume stop solution} + \text{volume charcoal}}{\text{volume counted}}$$

$$\text{S.A.} = \text{specific activity} = \frac{\text{total cpm added to each sample}}{\text{nmoles ATP in each sample}}$$

sample counts = ^{32}Pi counts (cpm) obtained per sample

background = counts (cpm) obtained from scintillation fluid alone

5. Phosphorylation of Rat Cardiac SR Vesicles

The conditions for phosphorylation were similar to those used for the ATPase assay. SR membranes (50 µg) were preincubated for 10 minutes at 30°C in a medium containing 40mM histidine-Cl, pH 6.8, 100mM KCl, 200 or 500µM $MgCl_2$, 5 mM NaN_3, 100µM EGTA and 40 µM $CaCl_2$ (0.5µM free Ca^{2+}). CaM (3 µg/ml) or catalytic subunit of cAMP-dependent protein kinase (125 Units per tube) were added to the desired reaction tubes. The reaction was initiated by the addition of 200µM ATP (containing ^{32}P-ATP; $2x10^6$ dpm/sample) and was terminated after 1 minute with TCA stop solution (5% TCA, 5mM Na_2ATP and 2mM KH_2PO_4). Bovine serum albumin (0.125% final concentration) was added to the tubes. The tubes were mixed by vortexing and centrifuged at 850xg for 5 min at 4°C. The supernatant was discarded. The pellet was resuspended in 0.5 ml of either 0.6M hydroxylamine/0.8M sodium acetate, pH 5.2 or 0.6M NaCl/0.8M sodium acetate, pH 5.2 (control). After a 10 minute incubation at room temperature, 1 ml ice cold 15% TCA was added to the samples and the membranes pelleted at 850xg for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 5% TCA, applied to glass fibre filters (Whatman GF/A) and washed with 30 ml 5% TCA solution. The filters were dried and counted for ^{32}Pi in Aquasol^R or Scinti-Verse^{TM} II.

The amount of ^{32}P bound, expressed in pmoles ^{32}P bound per mg protein, was calculated as follows:

$$^{32}P \text{ bound} = \frac{(\text{sample counts} - \text{background})}{\text{specific activity X mg protein per sample}}$$

where:

$$\text{specific activity} = \frac{^{32}P \text{ counts per picomole of ATP}}{\text{total ATP in picomoles}}$$

$$\text{total counts} = \text{total } ^{32}P \text{ counts added to each tube}$$

sample counts = ^{32}P counts (cpm) obtained per sample

6. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Autoradiography of Phosphorylated Cardiac SR Proteins

Gel Preparation: Polyacrylamide slab gels (12%) were cast according to the method of Laemmli and Favre (1973), using a 5% stacking gel. The "separating" gel contained 12% acrylamide-bis acrylamide mixture (30:0.8 w/w), 375mM Tris-HCl buffer, pH 8.8, 0.1% sodium dodecyl sulphate (SDS), 3.45% glycerol, 0.15 mg/ml ammonium persulphate and 0.03% tetraethyl-methylenediamine (TEMED). After mixing, the gel solution was immediately added to the gel chamber. About 1.0 ml of distilled water was carefully layered on top to keep the surface of the gel from drying and the gel was allowed to polymerize for 2 hours or more at room temperature.

The "stacking" gel consisted of 5% acrylamide-bis acryl-

amide mixture (30:0.8 w/w), 315mM Tris-Cl buffer, pH 6.8, 0.1% SDS, 0.4 mg/ml ammonium persulphate and 0.136% TEMED. The distilled water was removed from the separating gel. The stacking gel solution was mixed and very carefully added to the top of the stacking gel. A teflon comb was inserted into the stacking gel and the gel was allowed to polymerize at room temperature for at least one hour.

Sample Preparation and Electrophoresis: When phosphorylated SR membranes were to be analysed on SDS-PAGE followed by auto-radiography, the incubation conditions were identical to those above except higher protein concentrations (40-100 µg) were used and the specific activity of ^{32}P-ATP was increased ($4x10^6$ cpm/sample). The reaction was stopped with 1.0 ml ice-cold 15% TCA solution. The samples were then centrifuged at 1500xg for 10 min and the supernatant discarded. The pellet was resuspended in 25 ul of sample buffer containing 43mM SDS, 1.25M urea, 0.125mM dithiothreitol, 12.5mM Tris-HCl, pH 6.8 and a small amount of Bromophenol Blue. Samples were vortexed, neutralized to pH 7.0 with 2M Tris base and 60 µl of each sample was applied to separate wells in the gel.

The running buffer contained 0.25M Tris buffer, pH 8.3, 1.92M glycine and 1% SDS. The gels were run overnight at room temperature under a constant current of 10mA (PS 1200 DC Power Supply, Hoefer Scientific Instruments) per slab. The protein standards used for estimation of molecular weight (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), soyabean trypsin inhibitor (21,500) and lysozyme (14,400).

Staining, Destaining and Autoradiography: The gels were stained in a mixture containing methanol, acetic acid, water (5:1:5) and 0.25% Coomassie Brilliant Blue R-250 for 30 minutes at room temperature. The gels were then destained in a mixture of methanol, acetic acid and water (5:1:5) for one hour (3 changes) and then in methanol:acetic acid:water (4:1:15) until the background became clear. The gels were fixed in acetic acid:glycerol:water (10:3:87) for 30 minutes and then in 70% methanol for 20 minutes. The gels were immediately dried under vacuum at 80°C for 90 minutes and exposed to X-ray film (Kodak Min-R) using an intensifier screen (Cronex Lightning Plus, Du3 pont) for 7-14 days at -80°C. The films were developed to visualize the phosphorylated bands.

7. Protein Assay

The protein contents of the cardiac SR-enriched microsomal preparations were determined by the method of Bradford (1976) as simplified by Bio-Rad (Bio-Rad Protein Assay, Instruction Manual). Bovine gamma-globulin was used as the standard to yield protein ranges of 4-20 μg (micro assay) or 20-100 μg (macro assay). A full protein concentration curve was produced for each assay. In the initial macro assays the gamma-globulin or sample

protein was diluted to 100 ul in distilled water. The diluted dye reagent (5 ml of 5-fold diluted) was added, and the tubes were read at 595 nm in a spectrophotometer. The protein concentrations were determined by extrapolation from the standard curves. In the later experiments, the procedure was modified so that the entire assay could be performed in disposable cuvettes. For this, 1 volume of dye reagent concentrate was diluted with 3 volumes of distilled water instead of a 1:4 dilution and 3 ml of this diluted dye reagent was used, instead of 5 ml, to develop colour. The rest of the procedure was the same as before. The micro assay was carried out in the exact manner as described in the Bio-Rad Manual.

8. Determination of Free Calcium Concentrations

Free calcium concentrations were determined using the Fortran program "CATIONS" written by Goldstein (1979). Association constants for cations and ligands were obtained from Martell and Smith (1979, 1982) and were corrected for ionic strength, pH and temperature according to the methods described by these authors. The corrected log association constants of chelating ligands (in order of first to fourth proton association) used were: 9.440, 8.820, 2.770 and 2.110 for EGTA; 12.345, 6.210, 3.640 and 2.530 for CDTA; 6.635, 4.125, 0.0 and 0.0 for ATP. Association constants for monoprotonated species were calculated according to the procedure of Blinks et al. (1982). Log association constants for unprotonated and monoprotonated

cation-ligand complexes (in order) were: 5.341 and 3.631 for Mg-EGTA; 11.114 and 0.0 for Mg-CDTA; 4.114 and 2.128 for Mg-ATP; 10.762 and 5.222 for Ca-EGTA; 13.109 and 0.0 for Ca-CDTA; 3.758 and 1.923 for Ca-ATP.

9. Statistical Analysis

Whenever two means were compared, the statistical analysis was performed using Student's unpaired t-test. The null-hypothesis was rejected only if the two means were significantly different at a 0.05 or 0.01 significance level.

RESULTS

1. Characterization of Rat Heart Sarcoplasmic Reticulum Calcium Transport ATPase

The rat heart SR Ca^{2+}-transport ATPase has not been well characterized. Therefore, in our initial experiments we determined the optimum assay conditions.

a). Effect of SR Protein Concentration on ATPase Activity

To determine the optimum protein concentration for the assay, the effect of a range of protein concentrations (5-100 mg/ml) was investigated. The ATPase activity increased with protein concentration in an almost linear manner up to a concentration of 35 µg/ml and then started to plateau (figure 1). A protein concentration of 25 µg/ml was selected for use in future experiments. This choice was made since this concentration produced activity in the linear part of the curve.

b). Time-Course for ATPase Activity

The time-course of ATP hydrolysis was studied in crude SR preparations. The results using SR prepared by method I are shown in figure 2. The increase in ATPase activity with time showed linearity at least up to 4 minutes. A reaction time of 2.5 minutes was chosen for further experimentation. At this reaction time and SR protein concentration, the ATP hydrolytic

Figure 1.

Effect of changing protein concentrations on (Ca^{2+}+Mg^{2+})-ATPase activity. The ATPase activity, at 200μM added $MgCl_2$ and 7.0μM free Ca^{2+}, was determined as described in Methods. The results shown represent the mean of observations from two different SR preparations prepared and assayed on different days.

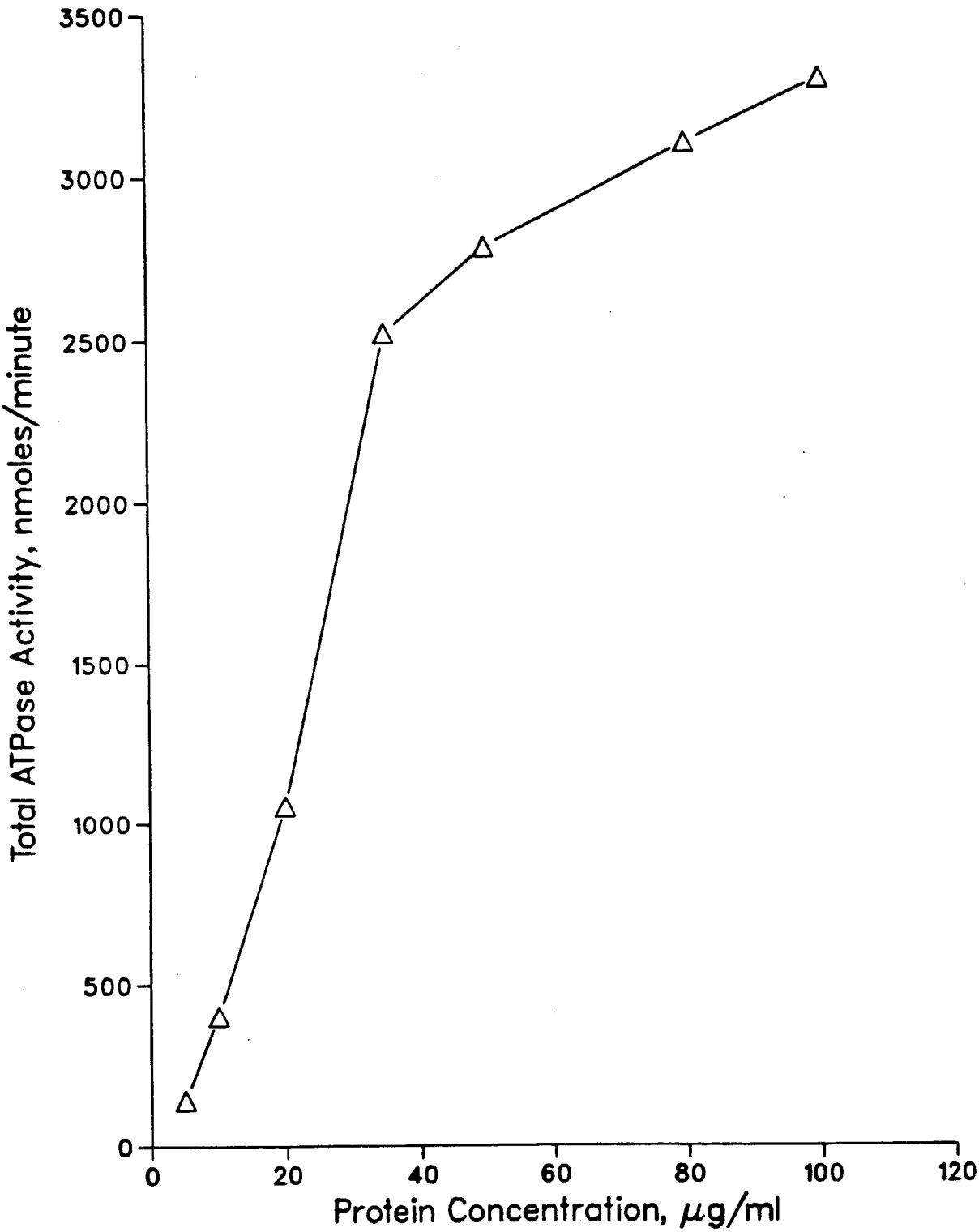
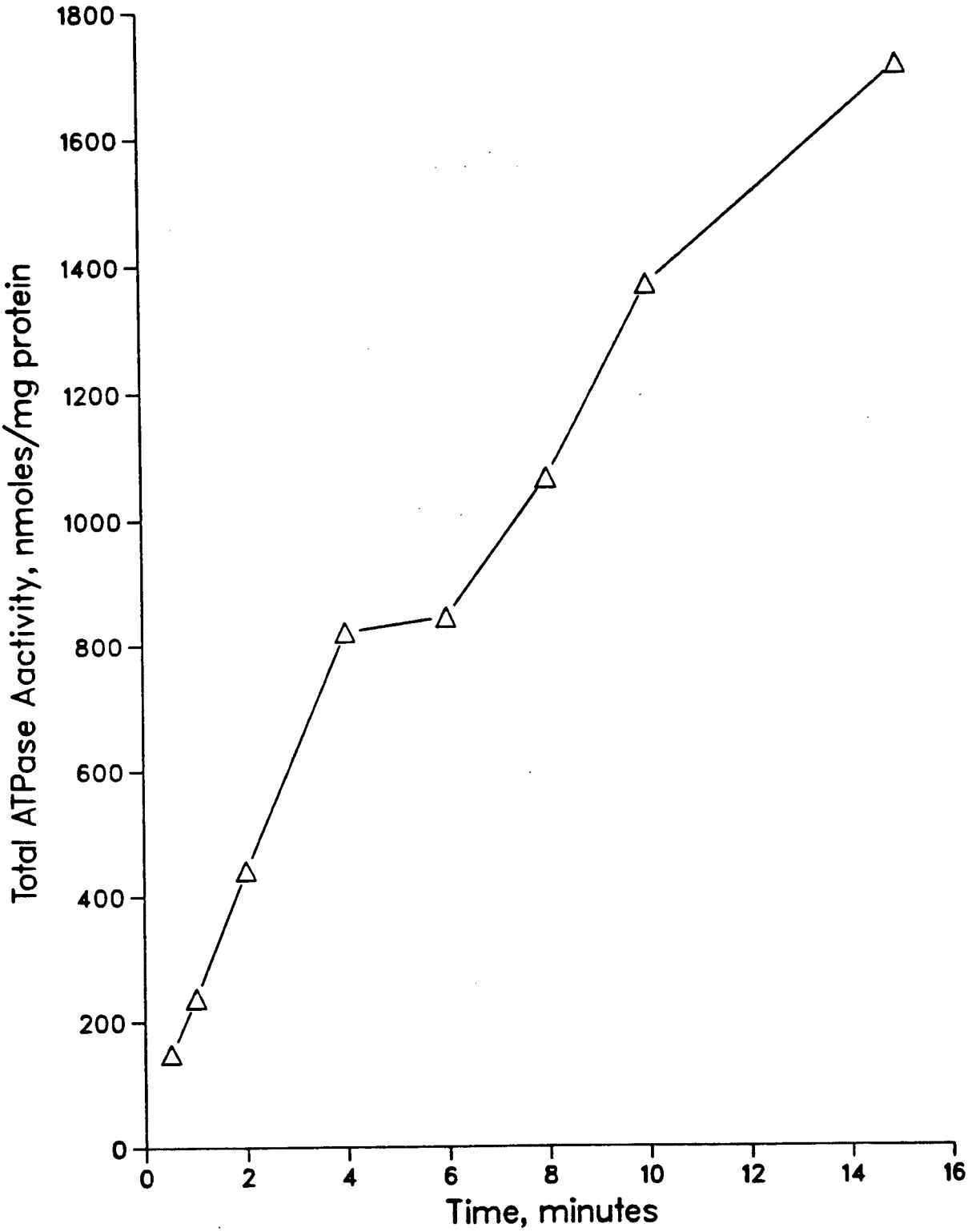

3500
3000
2500
2000
1500
1000
500
0
Total ATPase Activity, nmoles/minute
0
20
40
60
80
100
120
Protein Concentration, μg/ml

Figure 2.

Time-course of (Ca^{2+}+Mg^{2+})-ATPase activity in the rat heart SR preparation. The ATPase activity, at 200μM added $MgCl_2$ and 7.0μM free Ca^{2+}, was determined as described in Methods. The results shown represent the mean of observations from two different SR preparations prepared and assayed on different days.


Total ATPase Aactivity, nmoles/mg protein
1800
1600
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
Time, minutes

activity was less than 15% and thus below the Pi accumulation that might produce product inhibition and increased levels of non-enzymatic hydrolysis.

c). Effect of Ouabain, Vanadate and Sodium Azide on ATPase Activity

As shown in table I, ouabain was found to have no effect on ATPase activity under our experimental conditions. Vanadate (2μM) produced an 8.5% inhibition of the ATPase activity, while 5mM sodium azide, a mitochondrial Ca^{2+}-transport inhibitor, produced a 19% inhibition of ATPase activity. In view of these results showing some possible mitochondrial contamination, sodium azide was routinely added to the reaction medium.

d). Study of Calcium Transport

The SR preparation used was able to support oxalate-facilitated Ca^{2+} transport indicating that the preparation is vesiculated to some degree. This was shown in time-course studies (figure 3) and a protein concentration curve (figure 4) for Ca^{2+} uptake. Calcium was accumulated in a linear manner for at least 15 minutes incubation time at 30°C (figure 3). This accumulation was linear up to 80 μg/ml protein.

2. Effect of Magnesium on the ATPase Activity in Rat Heart SR

a). Effect of Magnesium on Ca^{2+}-Activation of ATPase Activity

Table I

The effect of sarcolemmal and mitochondrial inhibitors on total ATPase activity. The results represent mean of observations from two different experiments.

Inhibitor	ATPase activity (nmoles/mg/min)	Percent Inhibition
None	327.18	--
1mM Ouabain	342.77	--
5mM Sodium azide	264.56	19.1
2µM Sodium vanadate	299.40	8.5

Figure 3.

Time-course of calcium uptake activity in rat heart SR vesicles. Calcium uptake, at 2μM free Ca^{2+}, was measured as described in Methods. The results shown represent the mean of observations from two individual SR preparations prepared and assayed on different days.

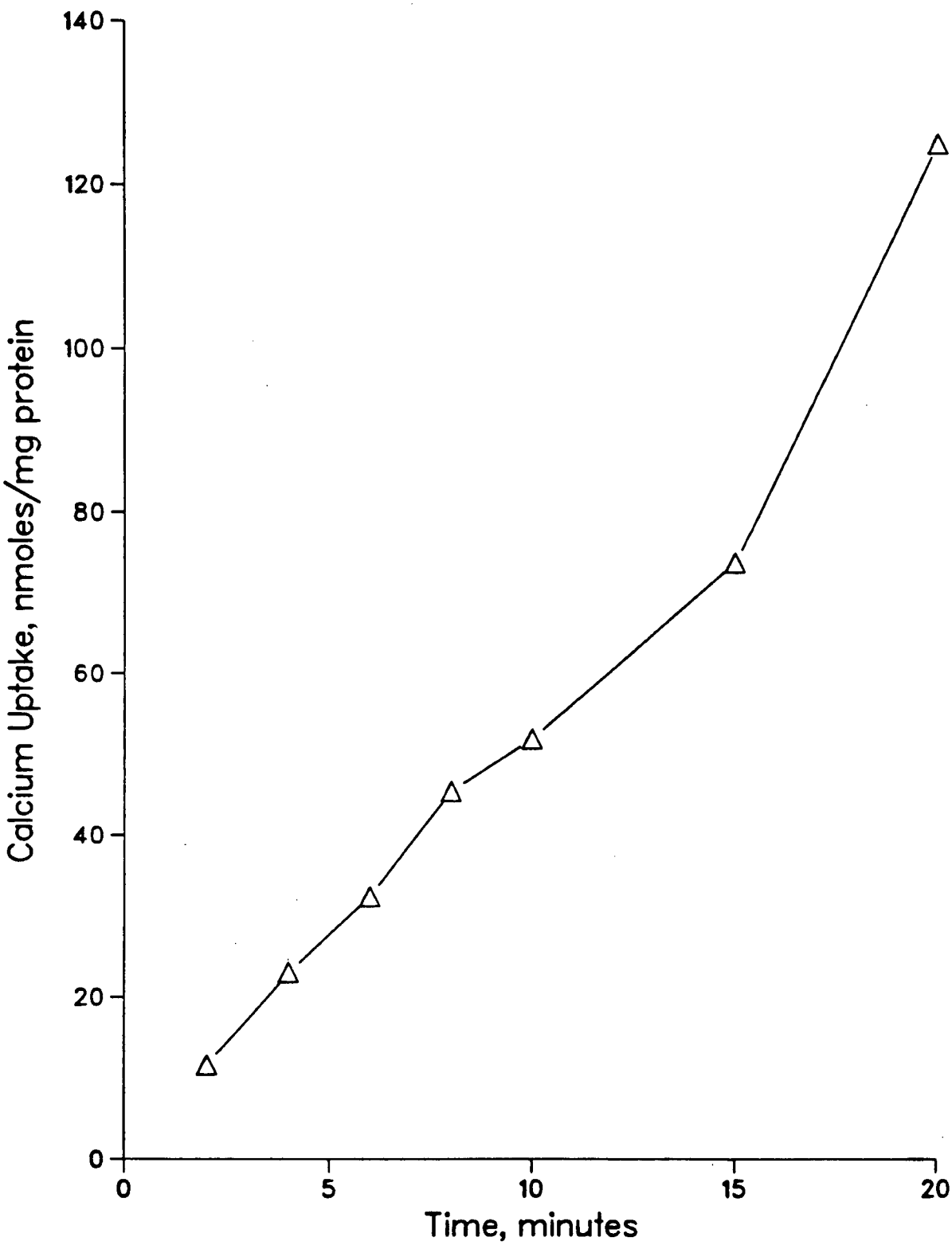
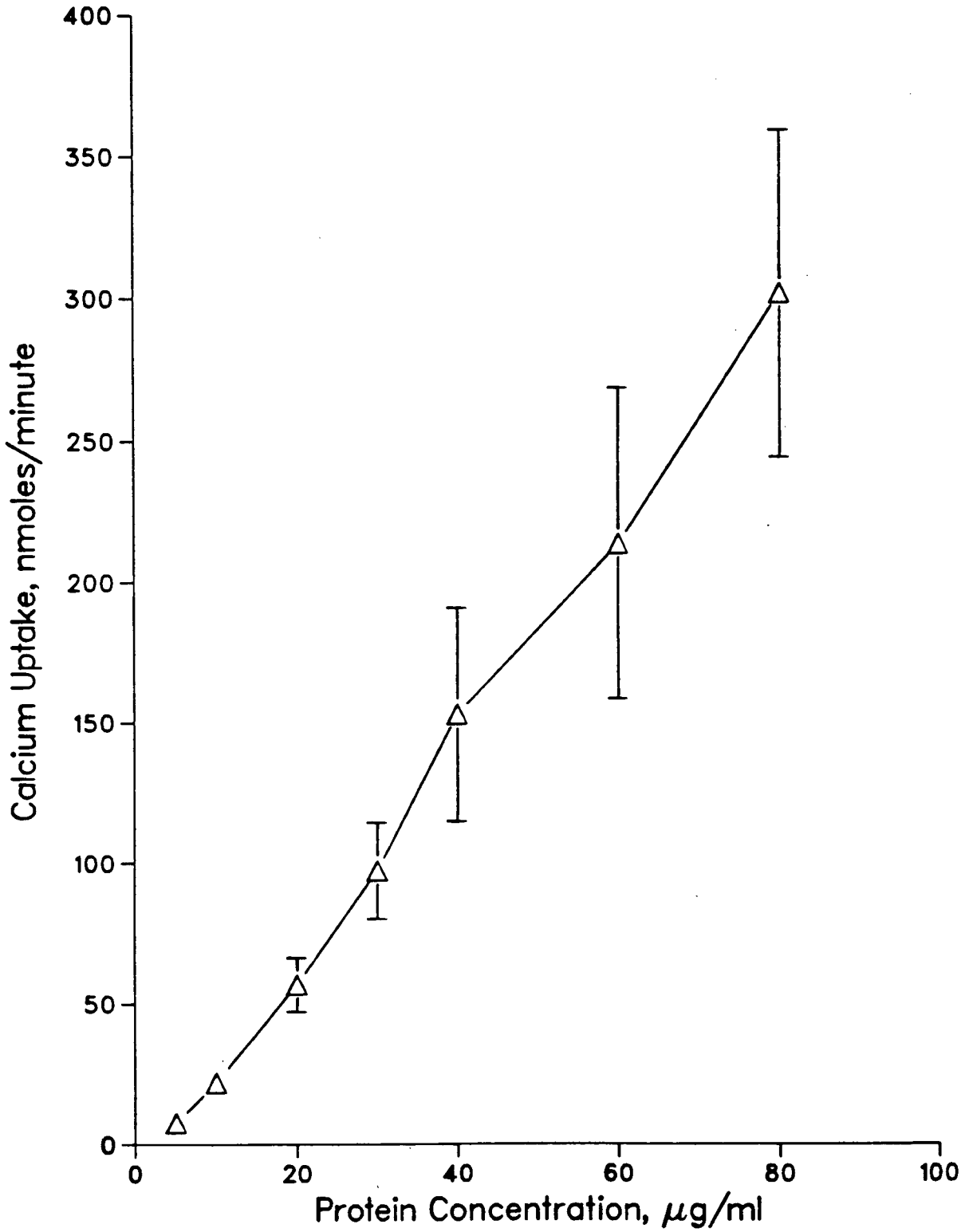

140
120
100
80
60
40
20
0
Calcium Uptake, nmoles/mg protein
0
5
10
15
20
Time, minutes

Figure 4.

Effect of changing protein concentrations on calcium uptake activity. Calcium uptake activity, at 2μM free Ca^{2+}, was determined as described in Methods. The results shown represent the mean ± S.E.M. of observations from at least three different SR preparations prepared and assayed on different days.


400
350
300
250
200
150
100
50
0
Calcium Uptake, nmoles/minute
0
20
40
60
80
100
Protein Concentration, μg/ml

In early experiments we observed a high basal ATPase activity in our preparations and were unable to detect a reproducible Ca^{2+}-dependent ATPase activity (data not shown). We, therefore, decided to determine the effect of magnesium on the Ca^{2+}-dependent component of ATPase activity. Figure 5a shows the Ca^{2+} activation of ATPase activity at different magnesium concentrations (0-2mM). The ATPase was found to be activated by Ca^{2+} in the complete absence of added magnesium. Concentrations of Mg (total added magnesium) up to 10μM had little or no effect on this activation. However, higher Mg produced a concentration-dependent increase in the basal ATPase activity and the total ATPase activity at the lower Ca^{2+} concentrations without any significant effect on the maximal ATPase activity at 300μM free Ca^{2+}. For example, 80μM Mg increased the basal ATPase activity 11-fold as compared to zero Mg, while the total ATPase activity at 300μM free Ca^{2+} was not increased. These observations can be interpreted as a concentration-dependent inhibition of the Ca^{2+} dependent ATPase activity by Mg. This is illustrated in figure 5b which shows the effect of Ca^{2+} on Ca^{2+}-dependent ATPase activity at different Mg concentrations. The Ca^{2+} concentration curves shifted to the right and downward with increasing Mg concentration. At 350μM Mg, the Ca^{2+} dependency appeared to be almost completely lost.

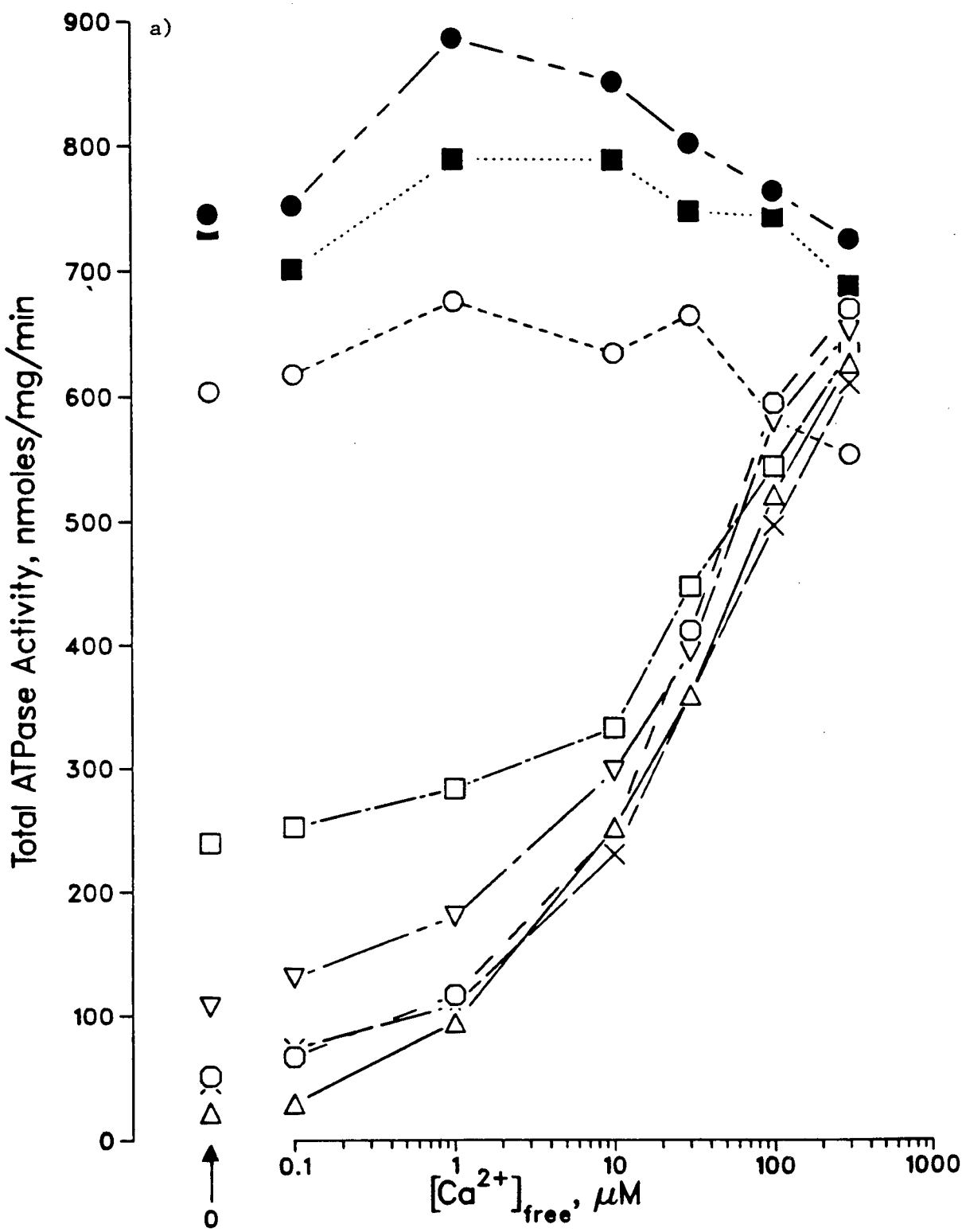
To determine whether the Ca^{2+} activation at zero Mg was due to endogenous free Mg, 100μM CDTA was included in the reaction medium. As seen in figure 6, CDTA did not inhibit this activa-

Figure 5.

a). Effect of magnesium on Ca^{2+} activation of $(Ca^{2+}+Mg^{2+})$-ATPase activity. The ATPase activity was assayed in the absence (△), or presence of 1.0μM (○), 10μM (⊗), 20μM (▽), 80μM (□), 350μM (○), 1mM (●) or 2mM (■) $MgCl_2$.

b). Effect of magnesium on Ca^{2+}-dependent ATPase activity. The ATPase activity was assayed in the absence (△), or presence of 1.0μM (○), 20μM (▽), 80μM (□), or 350μM (○) $MgCl_2$. The Ca^{2+}-ATPase activity was calculated by subtracting the basal activity (in presence of Mg^{2+} but no Ca^{2+}) from the total ATPase activity (in presence of Mg^{2+} and Ca^{2+}).

The ATPase activity was determined as described in Methods. The results shown represent the mean of observations from three individual SR preparations prepared and assayed on different days.


a)
900
800
700
600
500
400
300
200
100
0
0
Total ATPase Activity, nmoles/mg/min
0.1
1
10
100
1000
[Ca2+]free, μM

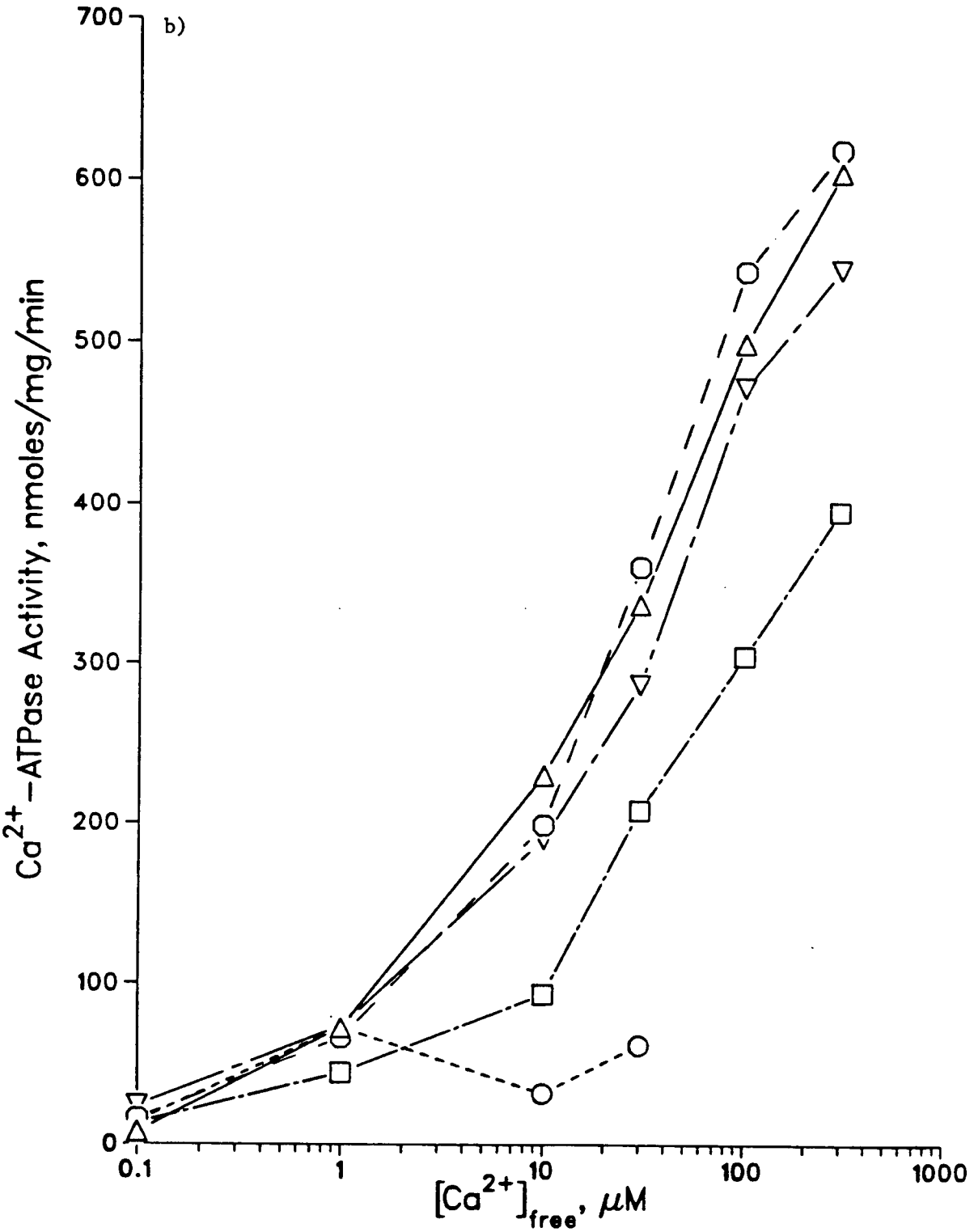
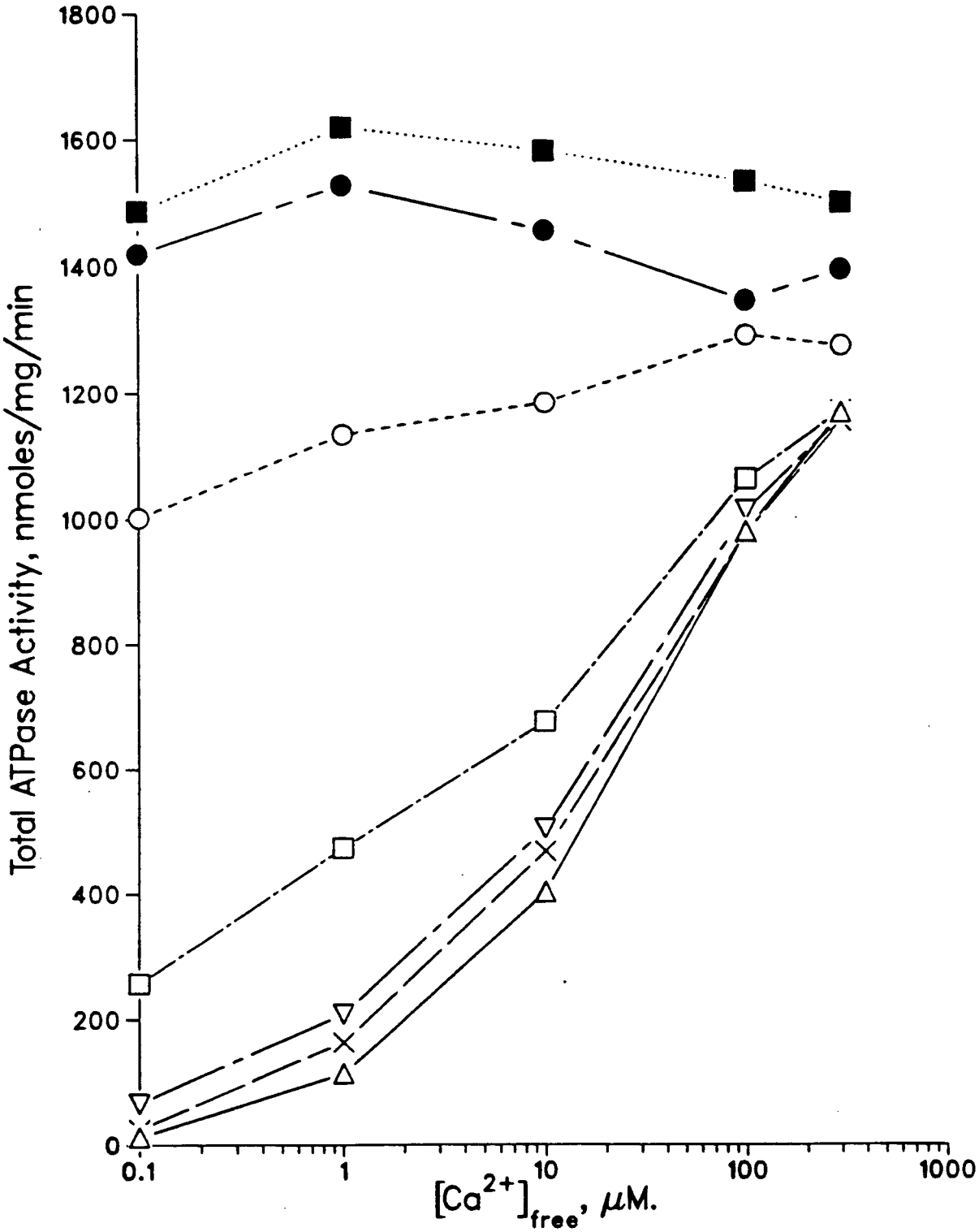

b)
700
600
500
400
300
200
100
0
Ca^{2+}-ATPase Activity, nmoles/mg/min
0.1
1
10
100
1000
$[Ca^{2+}]_{free}$, μM

Figure 6.

Effect of magnesium on (Ca^{2+}+Mg^{2+})-ATPase activity in the presence of 100μM CDTA. The ATPase activity was assayed in the absence (△), or presence of 10μM (×), 20μM (▽), 80μM (□), 350μM (○), 1mM (●) or 2mM (■) $MgCl_2$. The ATPase activity was determined as described in Methods. The results shown represent the mean of observations from two individual SR preparations prepared and assayed on different days.


1800
1600
1400
1200
1000
800
600
400
200
0
Total ATPase Activity, nmoles/mg/min
0.1
1
10
100
1000
[Ca2+]free, μM.

tion of ATPase activity. Under these conditions, increasing Mg concentrations resulted in a similar pattern of ATPase activity as seen in the absence of CDTA (figure 5a).

b). Role of Magnesium as a Co-Substrate

At zero free Ca^{2+}, when the Mg to ATP ratio was kept constant, the ATPase activity increased in a hyperbolic fashion (figure 7a). The addition of 0.8μM free Ca^{2+} resulted in stimulation of this activity as shown by a leftward movement of the curve. An increase in free Ca^{2+} to 7.0μM had little further effect on the ATPase activity. A double-reciprocal plot of ATPase activity vs. ATP concentration produced straight lines at all three Ca^{2+} concentrations (figure 7b). However, when the reciprocal of ATPase activity was plotted against the reciprocal of the concentration of the Mg.ATP complex, a non-linear graph was obtained (figure 7c).

3. Effect of Regulators on Rat Heart SR ATPase Activity

a). Effect of Calmodulin and C-subunit on ATPase Activity in SR Vesicles

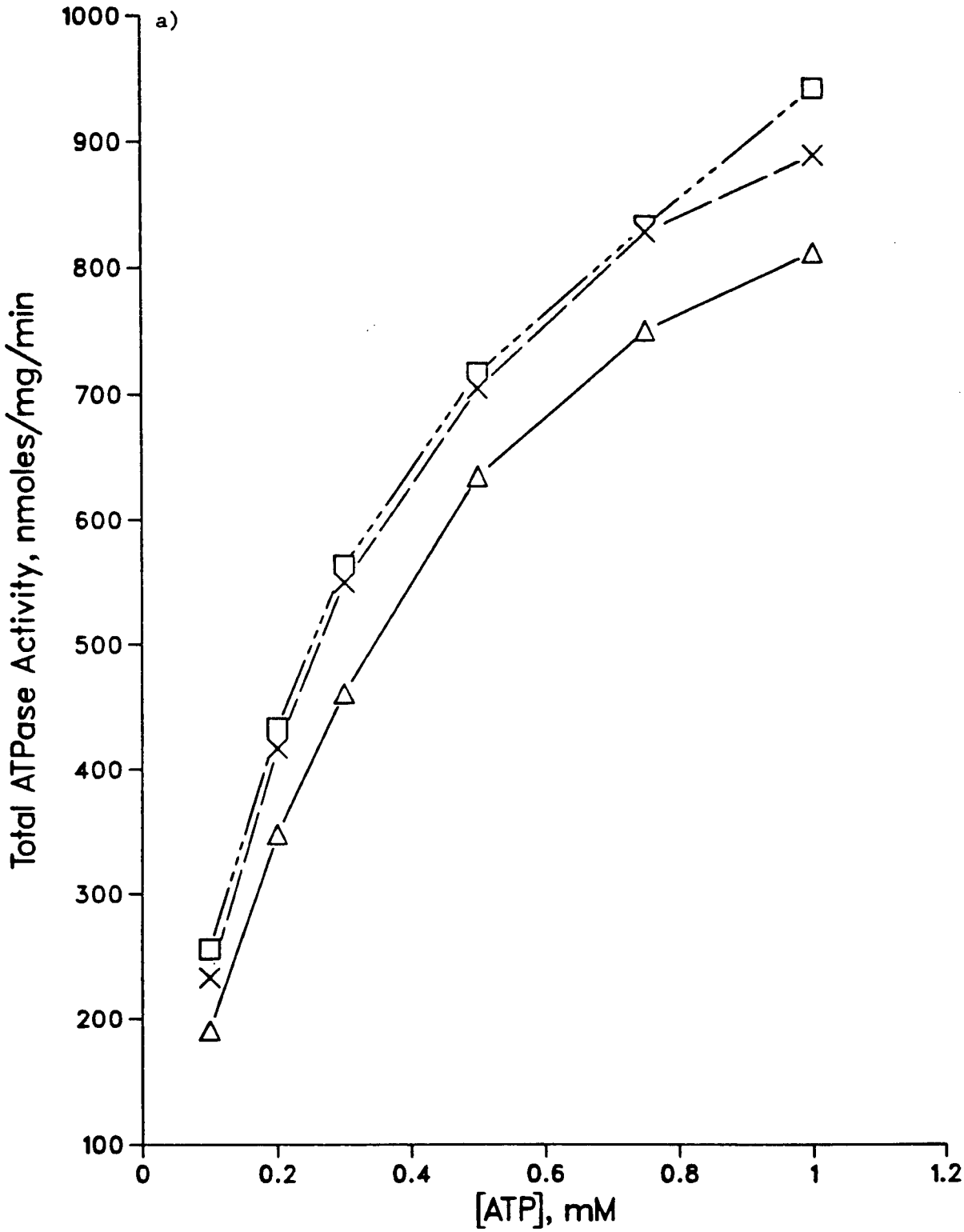
The effect of CaM on ATPase activity was investigated at various Ca^{2+} and Mg concentrations. We were unable to show a stimulatory effect of CaM at zero (figure 8a), 10μM (figure 8b), 50μM (figure 8c) and 500μM (figure 8d) Mg and with all free Ca^{2+}

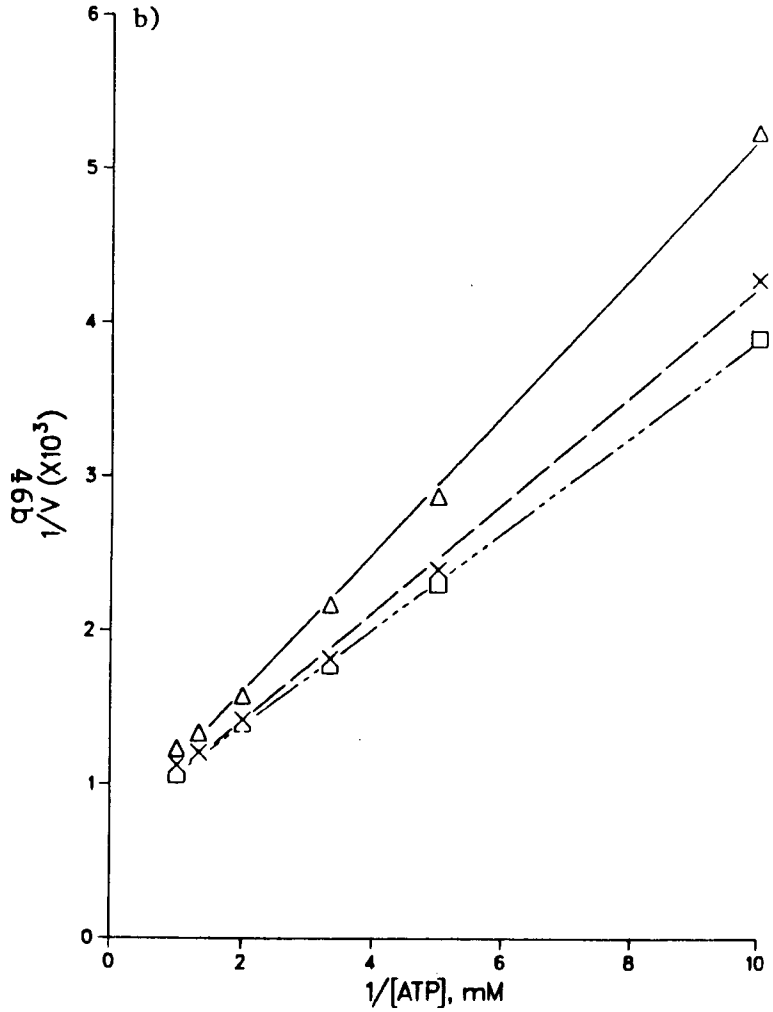
Figure 7.

a). Effect of changing ATP concentrations on (Ca^{2+}+Mg^{2+})-ATPase activity. The ATPase activity was assayed in the absence (△), or presence of 0.8μM (×) or 7μM (□) free Ca^{2+}. The concentration of $MgCl_2$ was adjusted to give a 1:1 ratio of Mg:ATP. The ATPase activity was determined as described in Methods. The results shown represent the mean of observations from two individual SR preparations prepared and assayed on different days.

b). Double-reciprocal plot of ATP hydrolysis versus ATP concentration.

c). Double-reciprocal plot of ATP hydrolysis versus Mg.ATP concentration.


a)
1000
900
800
700
600
500
400
300
200
100
Total ATPase Activity, nmoles/mg/min
0
0.2
0.4
0.6
0.8
1
1.2
[ATP], mM


b)
1/V (X10³)
1/[ATP], mM
0
1
2
3
4
5
6
0
2
4
6
8
10

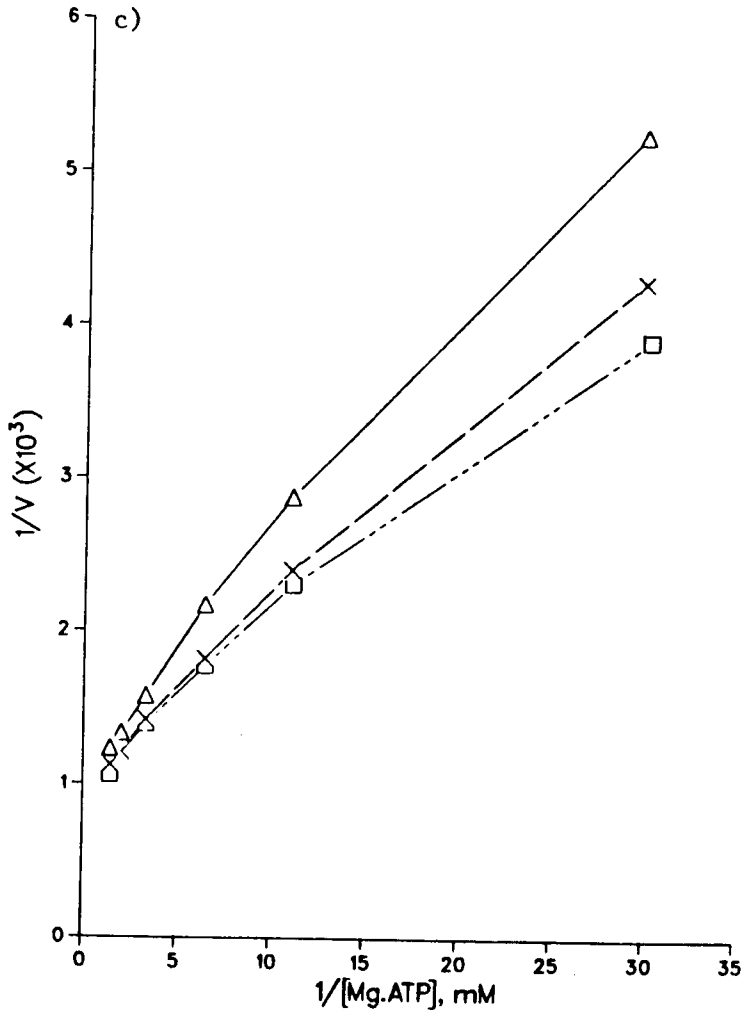
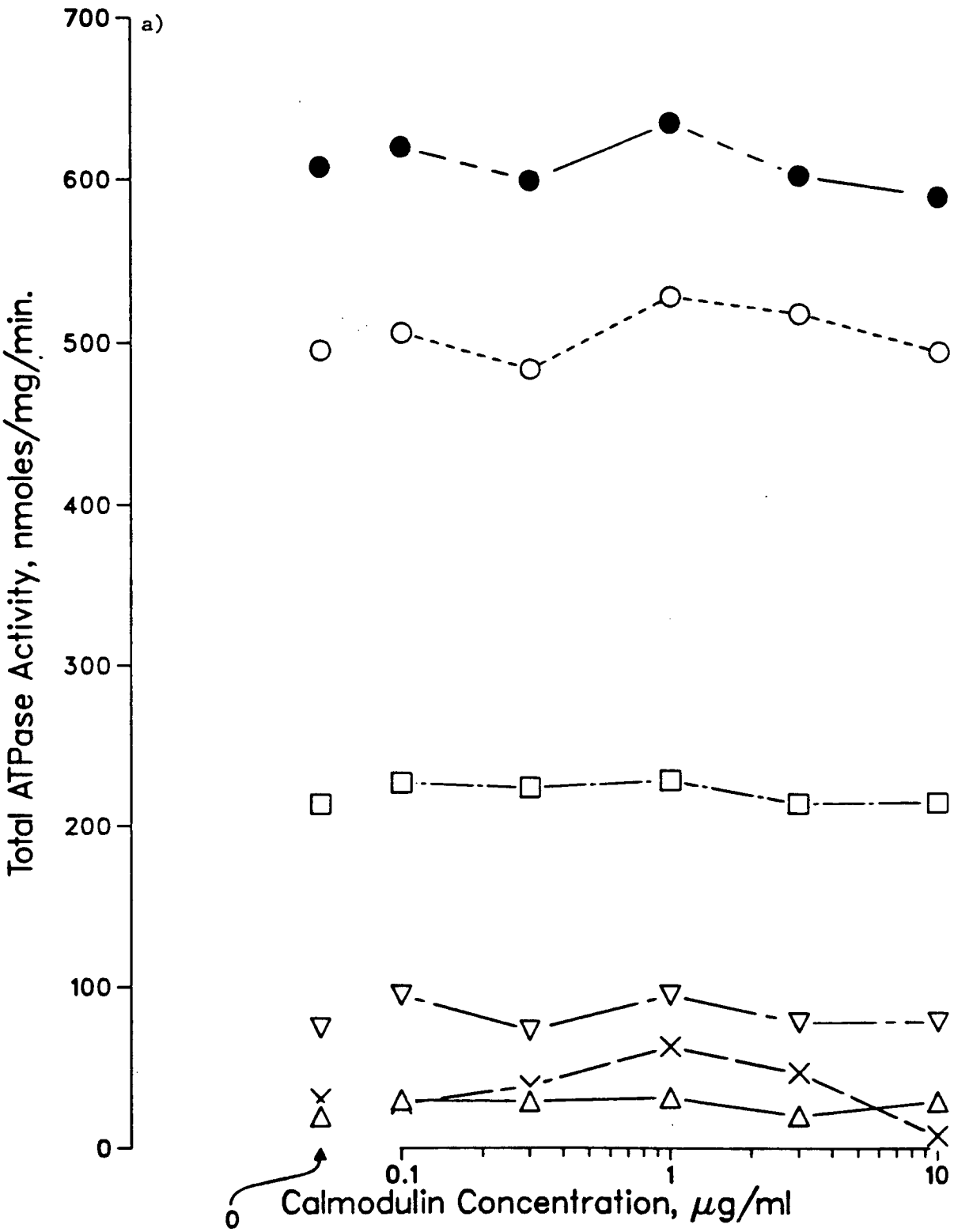
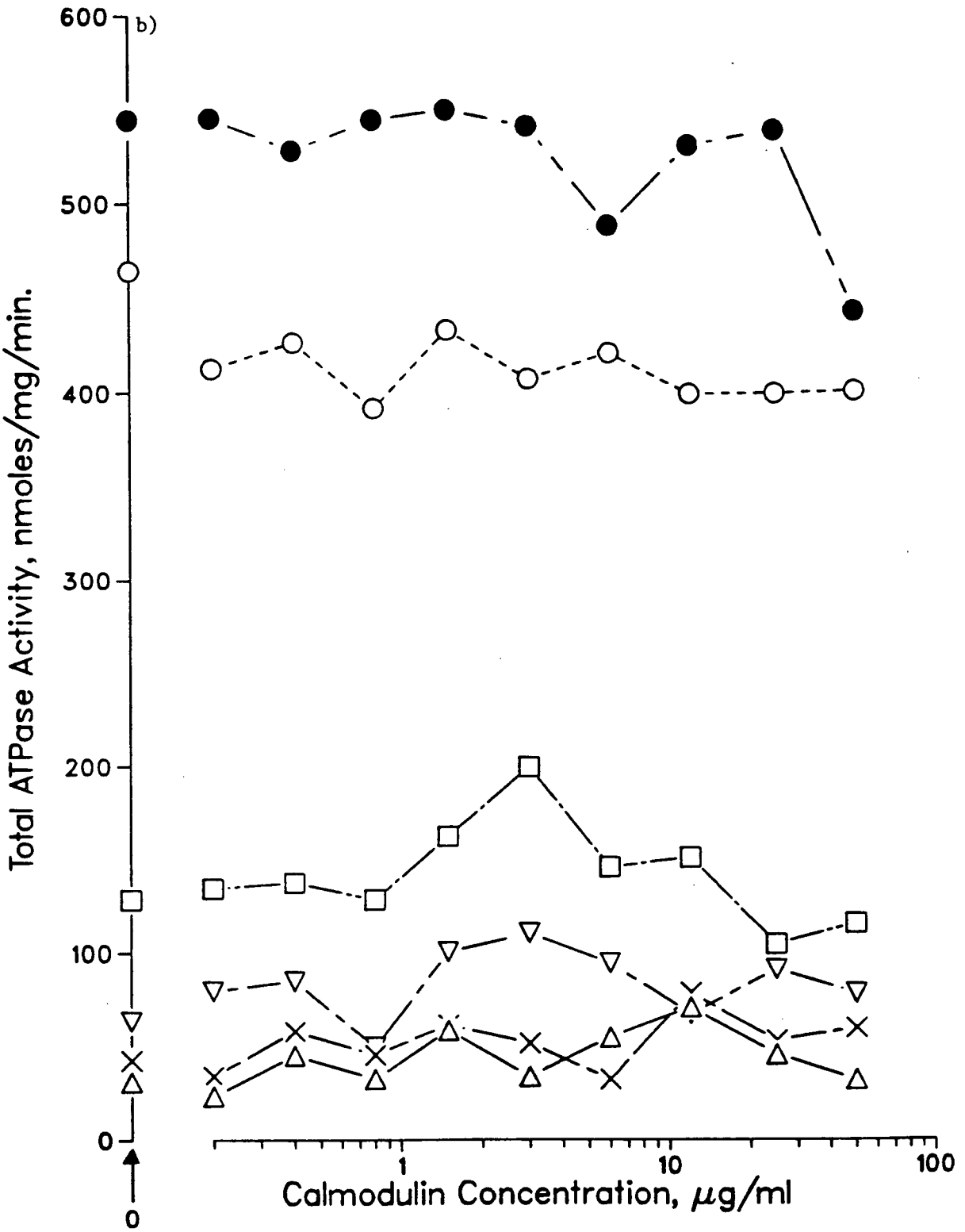
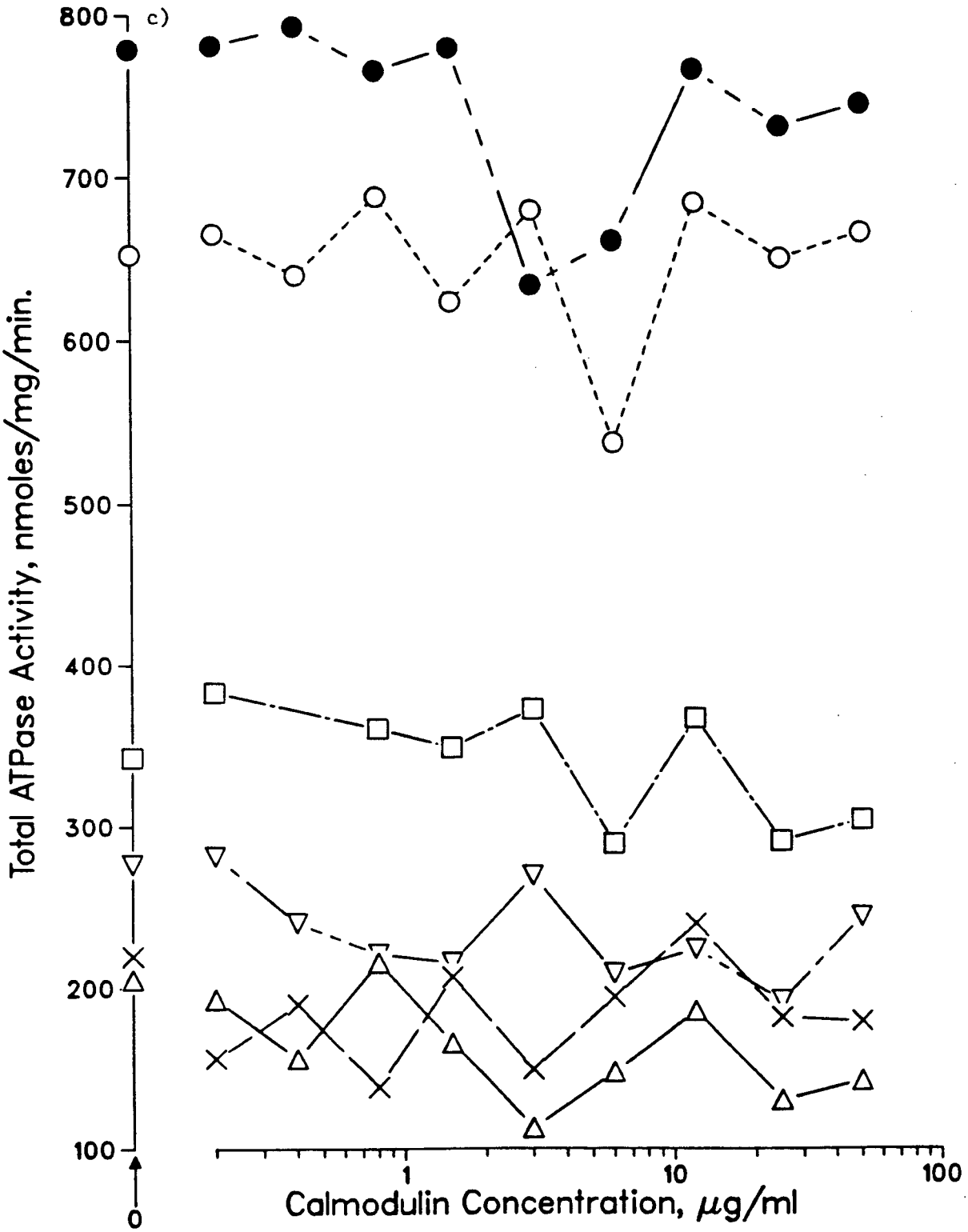

c)
1/V (X10³)
1/[Mg.ATP], mM
0
1
2
3
4
5
6
0
5
10
15
20
25
30
35

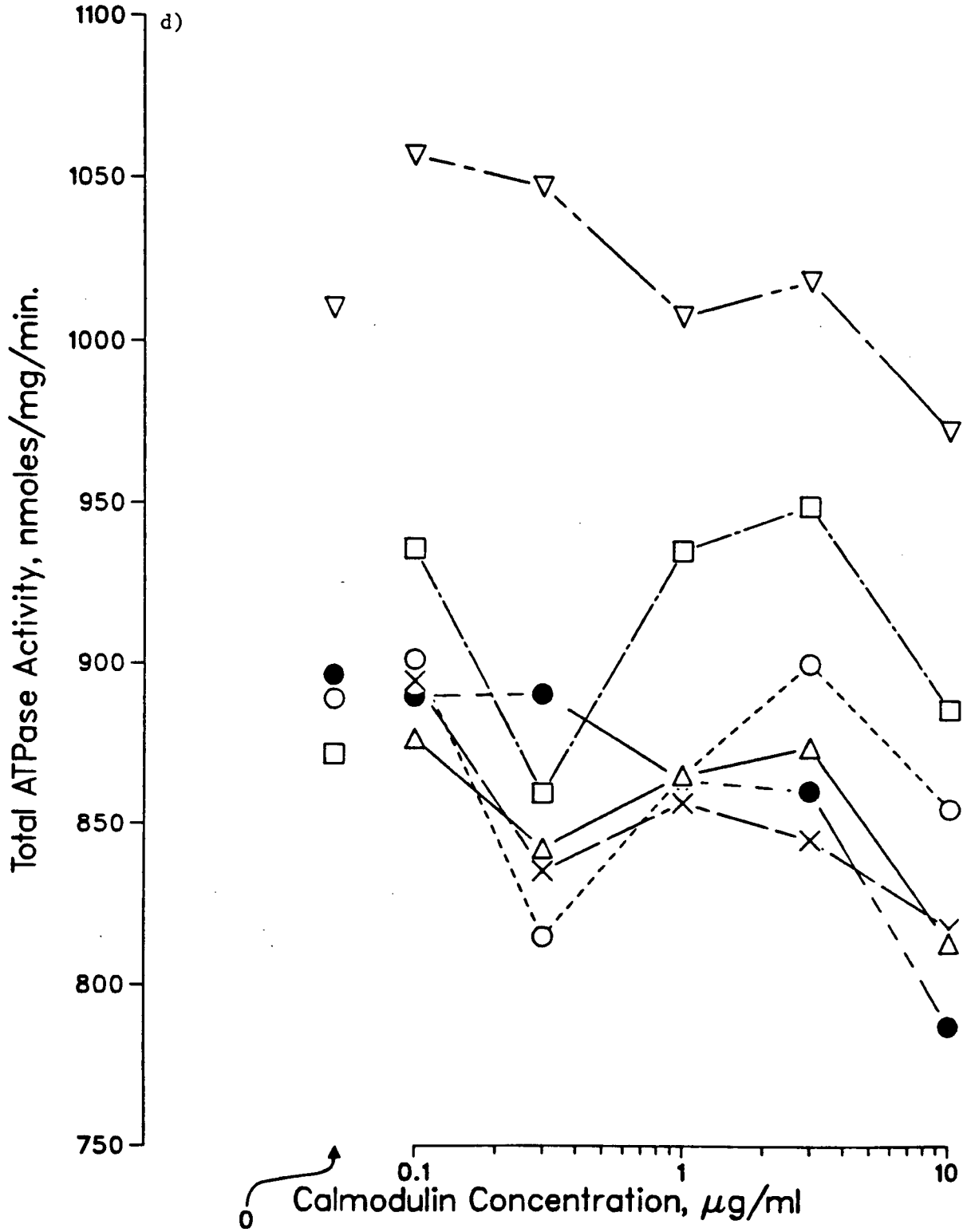
Figure 8.

Effect of calmodulin on $(Ca^{2+}+Mg^{2+})$-ATPase activity in rat heart SR. The ATPase activity was measured as described in Methods, in the absence (a) or presence of 10μM (b), 50μM (c) or 500μM (d) $MgCl_2$. The activity was assayed at zero (△), 0.1μM (×), 1.0μM (▽), 10μM (□), 100μM (○) and 300μM (●) free Ca^{2+}.


a)
700
600
500
400
300
200
100
0
Total ATPase Activity, nmoles/mg/min.
0
0.1
1
10
Calmodulin Concentration, μg/ml


b)
600
500
400
300
200
100
0
0
Total ATPase Activity, nmoles/mg/min.
1
10
100
Calmodulin Concentration, μg/ml


c)
800
700
600
500
400
300
200
100
0
Total ATPase Activity, nmoles/mg/min.
1
10
100
Calmodulin Concentration, μg/ml


d)
1100
1050
1000
950
900
850
800
750
Total ATPase Activity, nmoles/mg/min.
0
0.1
1
10
Calmodulin Concentration, μg/ml

concentrations studied.

The effect of cAMP-dependent protein kinase (cAMP-PK) on the ATPase activity of another regulator of the cardiac SR Ca^{2+}-pump was also investigated. Catalytic subunit of cAMP-PK (625 units/ml) appeared to inhibit the ATPase activity at high Ca^{2+} concentrations with little or no effect at low Ca^{2+} concentrations (figure 9).

b). Effect of Calmodulin on Calcium Uptake

To determine whether CaM stimulated Ca^{2+}-transport in the rat heart SR, uptake of $^{45}Ca^{2+}$ into SR vesicles was studied in the absence and presence of 3 µg/ml CaM. Under our experimental conditions, CaM did not produce a significant effect on Ca^{2+} uptake (figure 10).

c). Effects of Calmodulin Inhibitors on ATPase Activity

To investigate whether the lack of response to CaM was due to the presence of endogenous CaM, we studied the effect of CaM antagonists on ATPase activity. TFP had no effect on basal ATPase activity, but produced a slight inhibition of total ATPase activity at concentrations up to 100µM (figure 11). At higher concentrations, the total activity declined towards the basal levels. Another CaM antagonist, Compound 48/80 (0-100 µg/ml) failed to inhibit the basal and the total ATPase activities (figure 12).

Figure 9.

Effect of the catalytic-subunit of cAMP-dependent protein kinase on $(Ca^{2+}+Mg^{2+})$-ATPase activity. The ATPase activity, in the absence (△) or presence (×) of catalytic-subunit (625 units/ml), was measured, at 10μM added $MgCl_2$ and 10μM free Ca^{2+}, as described in Methods. The results shown represent the mean of observations from two individual preparations prepared and assayed on different days.

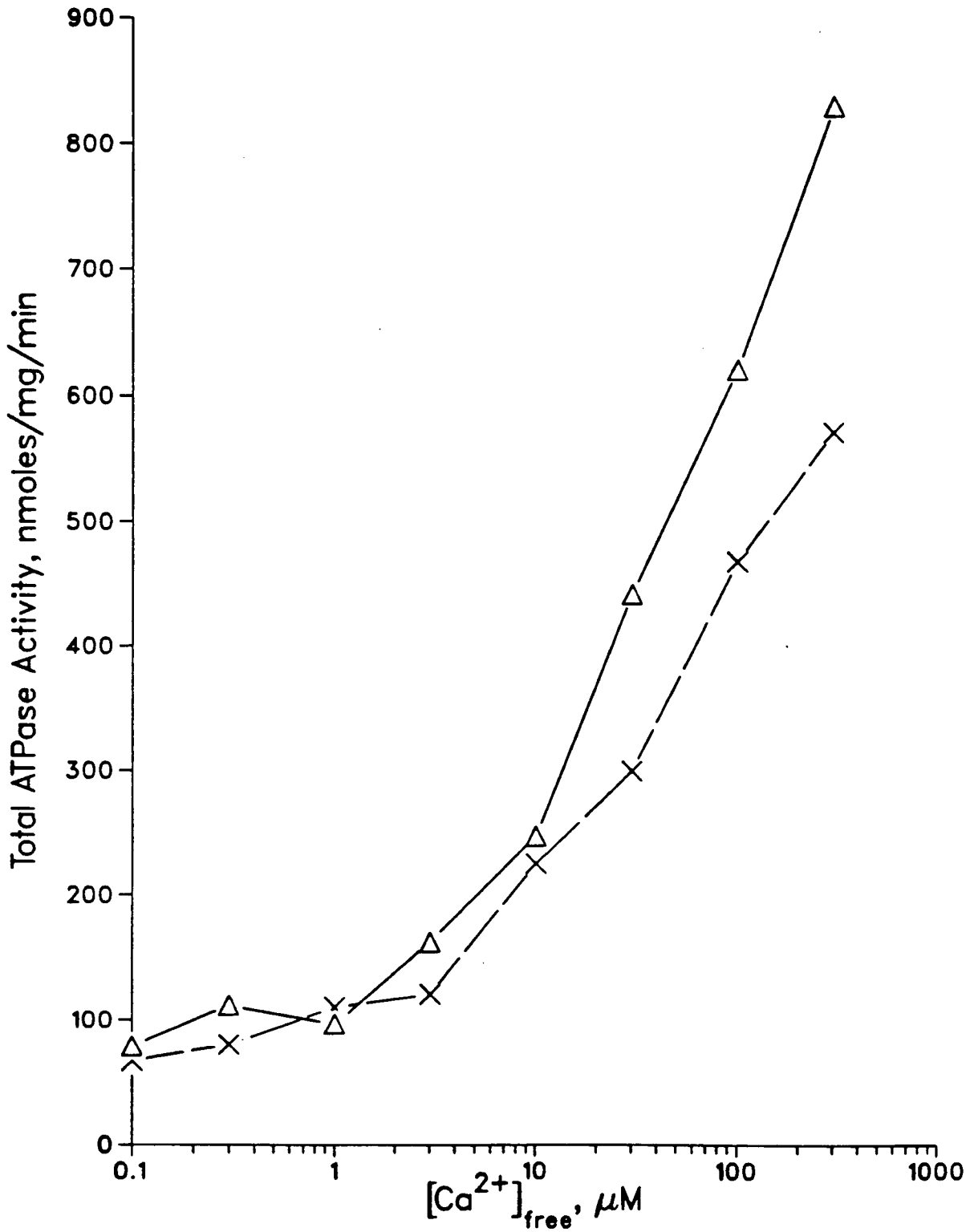

900
800
700
600
500
400
300
200
100
0
Total ATPase Activity, nmoles/mg/min
0.1
1
10
100
1000
[Ca2+]free, μM

Figure 10.

Effect of calmodulin on calcium uptake by SR vesicles. The uptake activity was assayed in the absence (△) and presence (X) of 3 μg/ml calmodulin, using the procedure described in Methods. The results shown represent the mean ± S.E.M. of observations from three individual preparations prepared and assayed on different days.

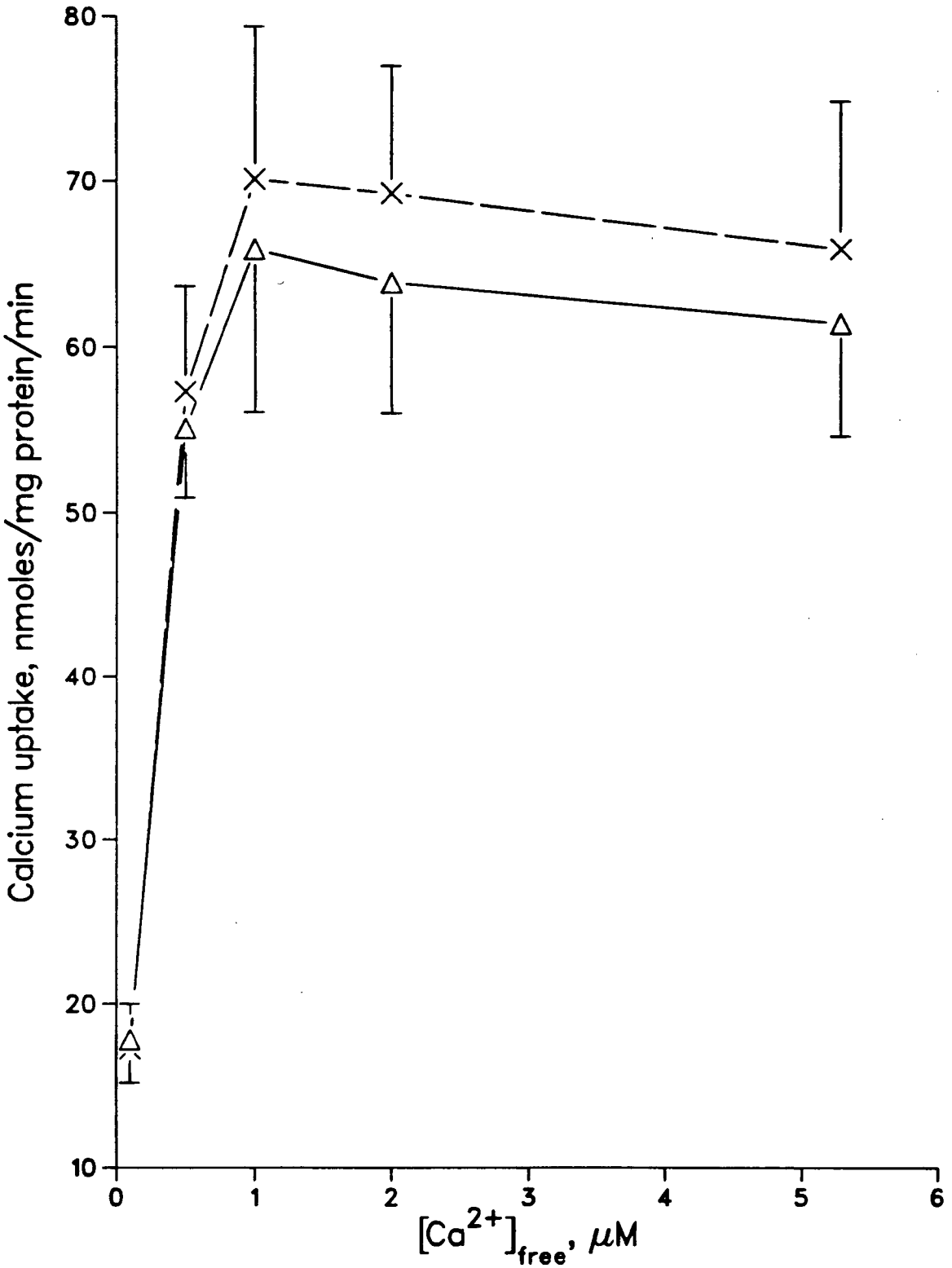

80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
Calcium uptake, nmoles/mg protein/min
[Ca2+]free, μM

Figure 11.

Effect of trifluoperazine on the basal (Δ) and total (X) ATPase activity in the rat heart SR. The ATPase activity, at 10μM added $MgCl_2$ and 10μM free Ca^{2+}, was measured as described in Methods. The results shown represent the mean of observations from two individual preparations prepared and assayed on different days.

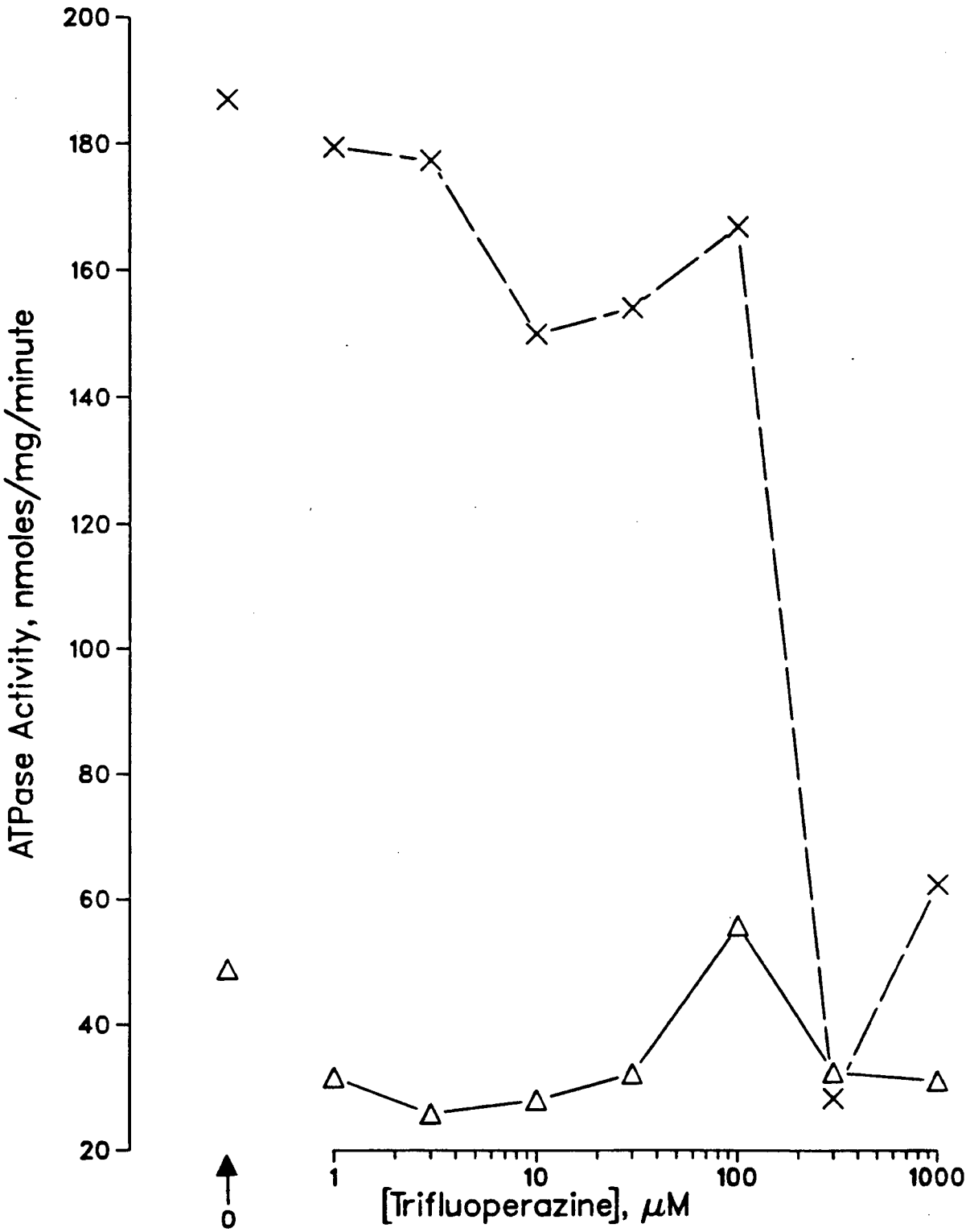
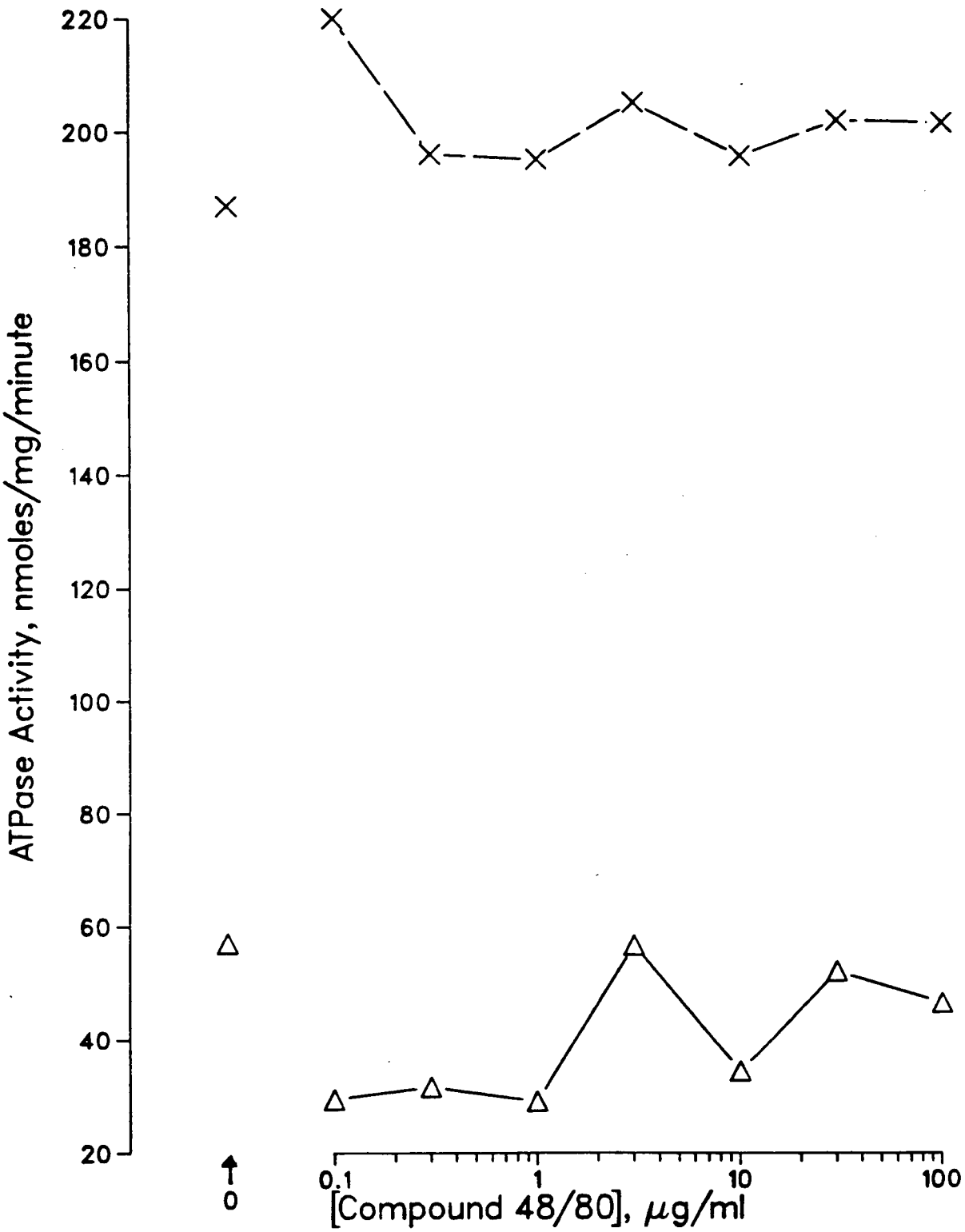

200
180
160
140
120
100
80
60
40
20
0
ATPase Activity, nmoles/mg/minute
1
10
100
1000
[Trifluoperazine], μM

Figure 12.

Effect of compound 48/80 on the basal (Δ) and total (X) ATPase activity in the rat heart SR. The ATPase activity, at 10μM added $MgCl_2$ and 10μM free Ca^{2+}, was measured as described in Methods. The results shown represent the mean of observations from two individual preparations prepared and assayed on different days.


220
200
180
160
140
120
100
80
60
40
20
0
ATPase Activity, nmoles/mg/minute
0.1
1
10
100
[Compound 48/80], μg/ml

4. Effect of Triton Treatment on ATPase Activity and the Regulation of This Activity

a) Effect of Triton X-100 on SR ATPase Activity

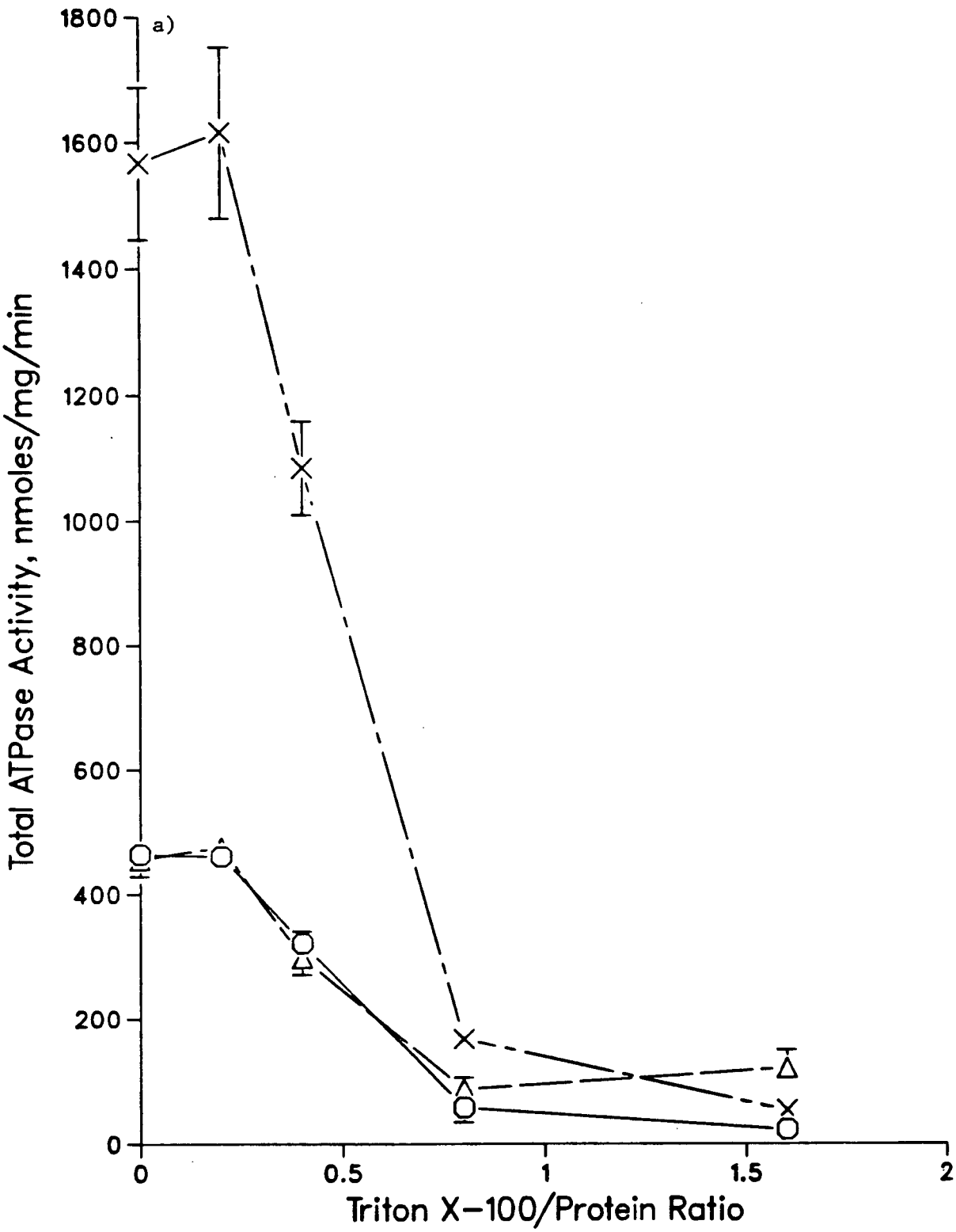
The effect of three different Mg concentrations on ATPase activity was studied in Triton-washed membranes. All three Mg concentrations produced a similar pattern of total ATPase activity (figure 13a); the activity was higher at 500μM Mg compared to zero and 10μM Mg. There was a small, but insignificant increase in activity with a detergent/protein ratio of 0.2 (0.005% Triton at a protein concentration of 0.3125 μg/ml). The activity declined sharply at detergent/protein ratios higher than 0.2. At a detergent/protein ratio of 0.8, the activity was 10.7% (500μM Mg), 19.0% (10μM Mg) and 12.4% (zero Mg) of the ATPase activity in the control membranes. The decline in ATPase activity with Triton was faster at 500μM added Mg than zero and 10μM Mg.

The Ca^{2+}-dependent ATPase activity was highest at zero Mg and lowest at 500μM Mg (figure 13b). While the total ATPase activities at zero and 10μM added Mg were almost identical (figure 13a), the Ca^{2+}-dependent ATPase activity at 10μM Mg was lower than at zero added Mg (figure 13b). This decrease was significant in control membranes and in Triton-washed membranes at a Triton/protein ratio of 0.2. The decline of Ca^{2+}-dependent activity with Triton was fastest at zero Mg and slowest at 500μM Mg.

Figure 13.

Effect of magnesium on Total (a) and Ca^{2+}-dependent (b) ATPase activity in the Triton-washed SR membranes.

The Triton-washed membranes were obtained as described in Methods. The ATPase activity, at 10μM free Ca^{2+}, was assayed in the absence (○) or presence of 10μM (△) or 500μM (×) $MgCl_2$. The results shown represent the mean ± S.E.M. of observations from at least three individual preparations prepared and assayed on different days.


a)
1800
1600
1400
1200
1000
800
600
400
200
0
Total ATPase Activity, nmoles/mg/min
0
0.5
1
1.5
2
Triton X-100/Protein Ratio

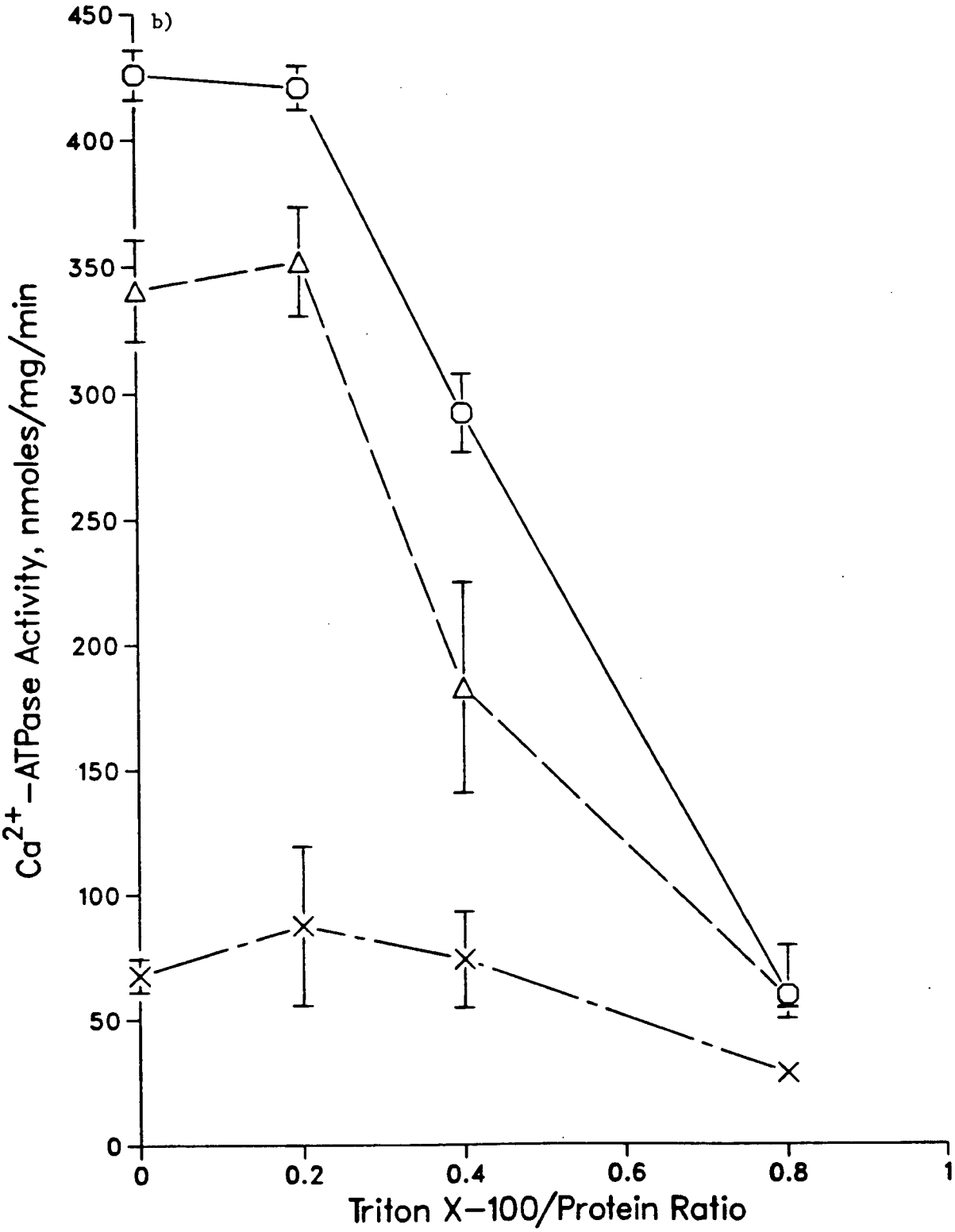

b)
450
400
350
300
250
200
150
100
50
0
0
0.2
0.4
0.6
0.8
1
Ca2+ -ATPase Activity, nmoles/mg/min
Triton X-100/Protein Ratio

Figure 14a compares the total ATPase activity of Triton-washed membranes and 105,000xg supernatant fractions. The total ATPase activity of the solubilized fractions declined with increasing Triton concentration reaching 9.3% of controls at a detergent/protein ratio of 1.6. On the other hand, Ca^{2+}-dependent ATPase activity of the supernatant fraction from membranes treated with low Triton concentration (detergent/protein of 0.2) was stimulated 2.4-fold; this activity declined to 23% of control at a detergent/protein ratio of 1.6 (figure 14b).

Table II shows the recovery pattern of the ATPase activity as calculated by multiplying the specific ATPase activity with the total protein recovered. The pellet from membranes treated with low detergent concentrations showed the highest recovery of both total and Ca^{2+}-dependent ATPase activity. The recovery declined after a detergent/protein ratio of 0.4, reaching about 10% of control at a detergent/protein ratio of 1.6. The supernatant fraction showed a high recovery of Ca^{2+}-dependent activity at a detergent/protein ratio of 0.2 and a high recovery of both total and Ca^{2+}-dependent activities at high detergent/protein ratios i.e. 0.8.

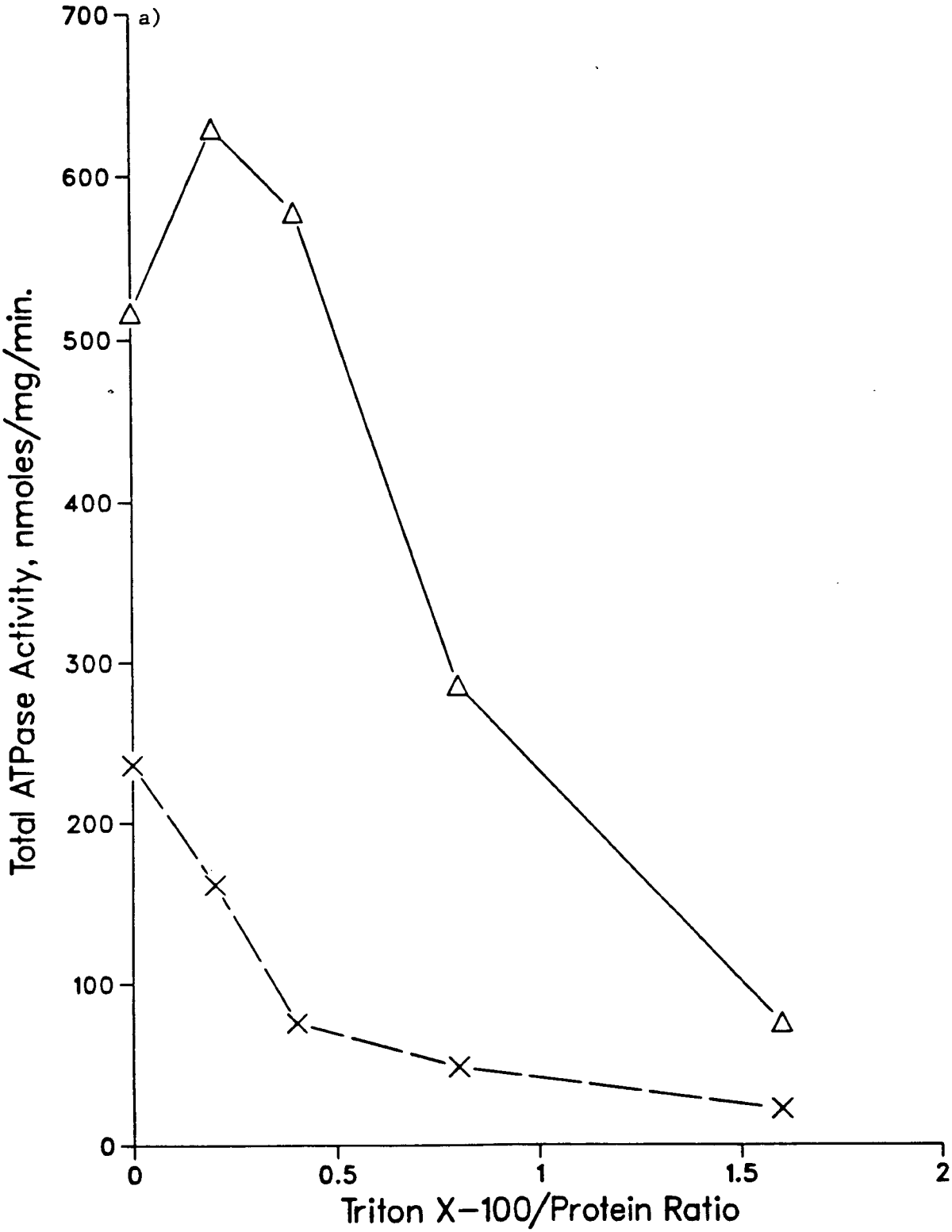
b). Effect of Calmodulin on ATPase Activity in Triton-washed Membranes

When CaM was added to the reaction medium, in the presence of 10μM free Ca^{2+} it failed to alter the total or Ca^{2+}-dependent ATPase activity of Triton-washed membranes (figure 15a and 15b).

Figure 14.

The levels of Total (a) and Ca^{2+}-dependent (b) ATPase activity in 105,000xg fractions of Triton-treated SR membranes in the presence of increasing concentrations of Triton X-100.

The fractions were obtained as described in Methods. The ATPase activity, in the pellet (△) and the supernatant (X) fractions, was measured as described in Methods. The $MgCl_2$ concentration in the assay medium was 200μM and the free Ca^{2+} concentration was kept constant at 10μM. The results shown represent the mean of observations from two individual preparations prepared and assayed on different days.


a)
700
600
500
400
300
200
100
0
Total ATPase Activity, nmoles/mg/min.
0
0.5
1
1.5
2
Triton X-100/Protein Ratio

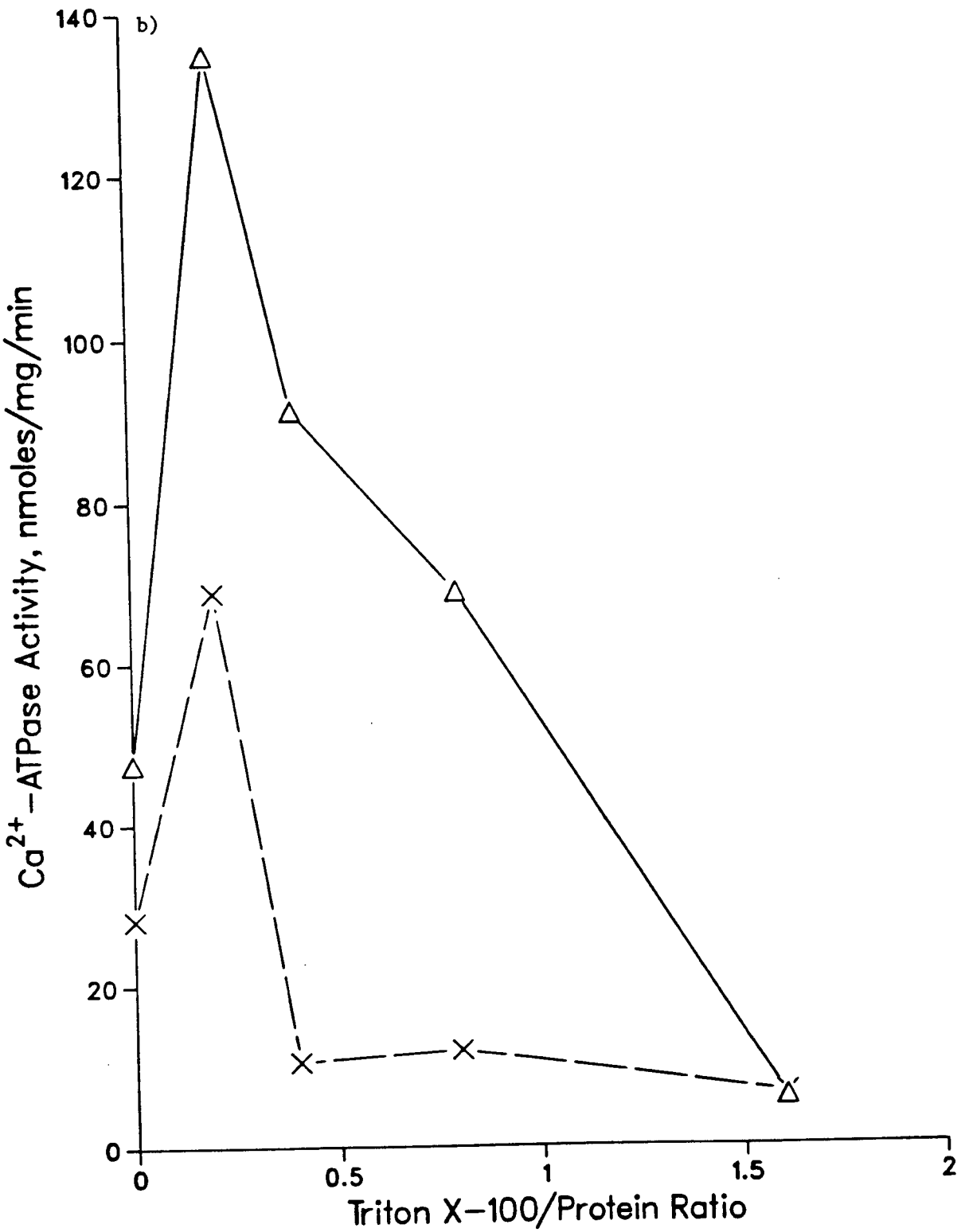

b)
140
120
100
80
60
40
20
0
0
0.5
1
1.5
2
Ca2+-ATPase Activity, nmoles/mg/min
Triton X-100/Protein Ratio

Table II

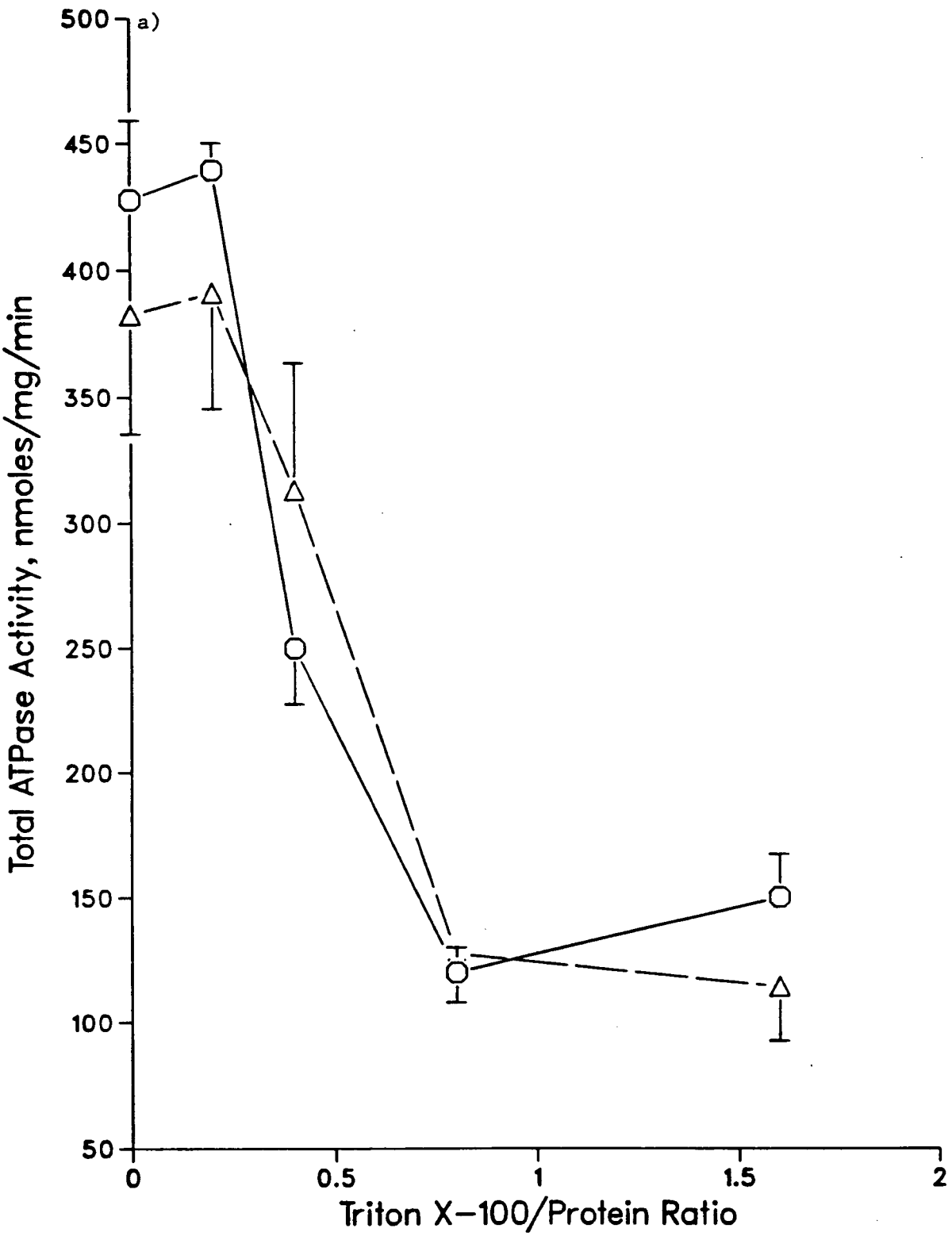
The recovery of ATPase activity* in the 105,000xg centrifugation fractions of Triton-treated SR membranes. The data represent mean of observations from 2 separate preparations.

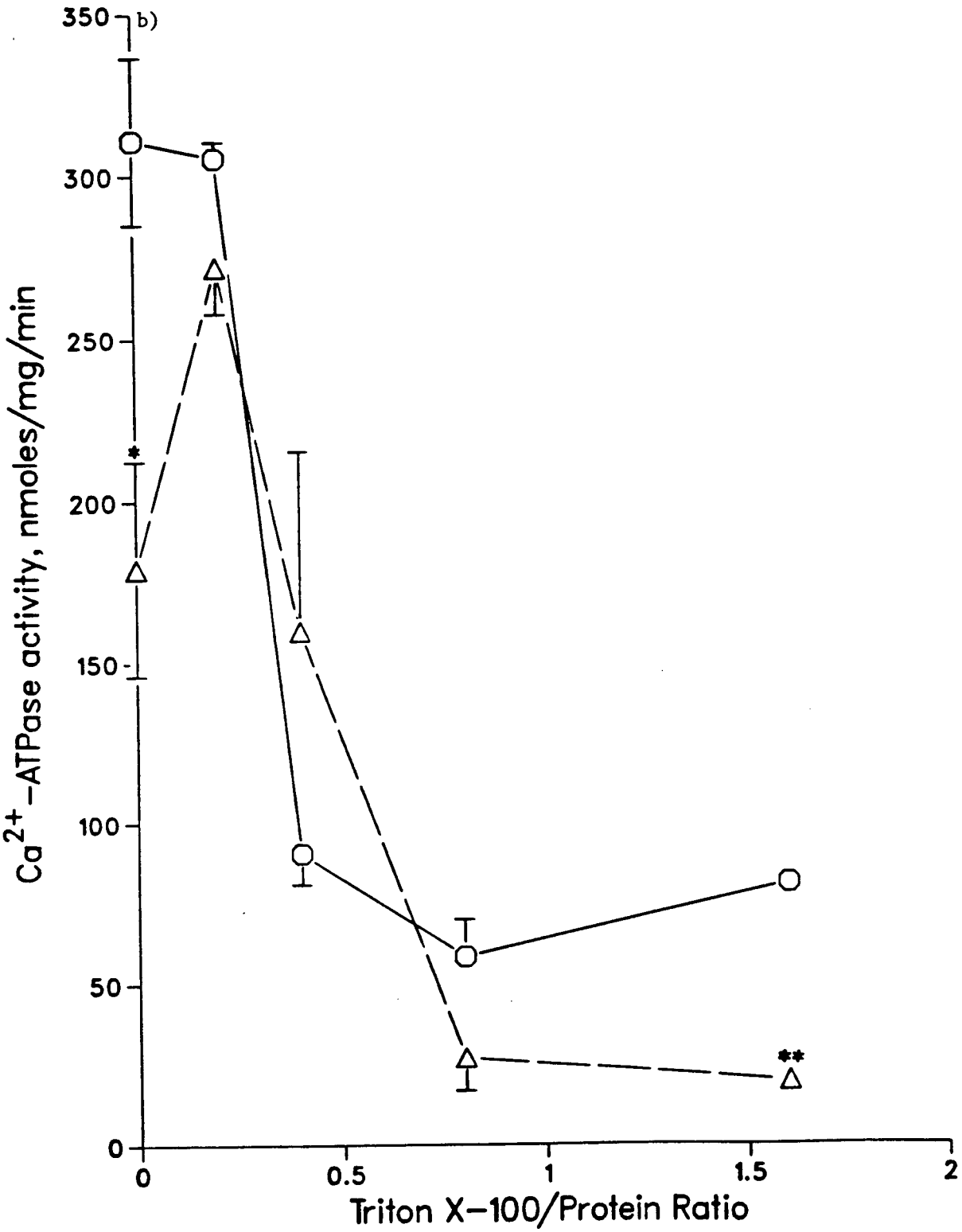
Detergent/protein ratio	Pellet		Supernatant	
	Total	Ca^{2+}-dep	Total	Ca^{2+}-dep
0	142.56	12.90	7.56	0.82
0.2	170.20	37.39	8.12	3.30
0.4	177.17	27.46	6.76	0.98
0.8	67.86	15.12	10.18	2.52
1.6	12.74	1.20	6.96	2.10

*The ATPase recovery was calculated by multiplying the specific activity with the total protein recovered and is expressed in micromoles per min

Figure 15.

Effect of calmodulin on Total (a) and Ca^{2+}-dependent (b) ATPase activity in Triton-washed SR membranes. The Triton-washed membranes were obtained as described in Methods. The ATPase activity was assayed, at 10μM added $MgCl_2$ and 10μM free Ca^{2+}, in the absence (○) or presence (△) of 3 μg/ml calmodulin, as described in Methods. The results shown represent the mean ± S.E.M. of observations from three individual preparations prepared and assayed on different days. Asterisk indicates significantly different from controls (no calmodulin) at $p < 0.05$ (*) or $p < 0.01$ (**).


a)
500
450
400
350
300
250
200
150
100
50
Total ATPase Activity, nmoles/mg/min
0
0.5
1
1.5
2
Triton X-100/Protein Ratio


b)
350
300
250
200
150
100
50
0
*
**
Ca^{2+}-ATPase activity, nmoles/mg/min
0
0.5
1
1.5
2
Triton X-100/Protein Ratio

CaM produced a significant inhibition of the Ca^{2+}-dependent ATPase activity in the control membranes and Triton-washed membranes at the 1.6 Triton/protein ratio (figure 15b). However, at intermediate Triton/protein ratios, CaM did not produce a significant effect on this ATPase activity.

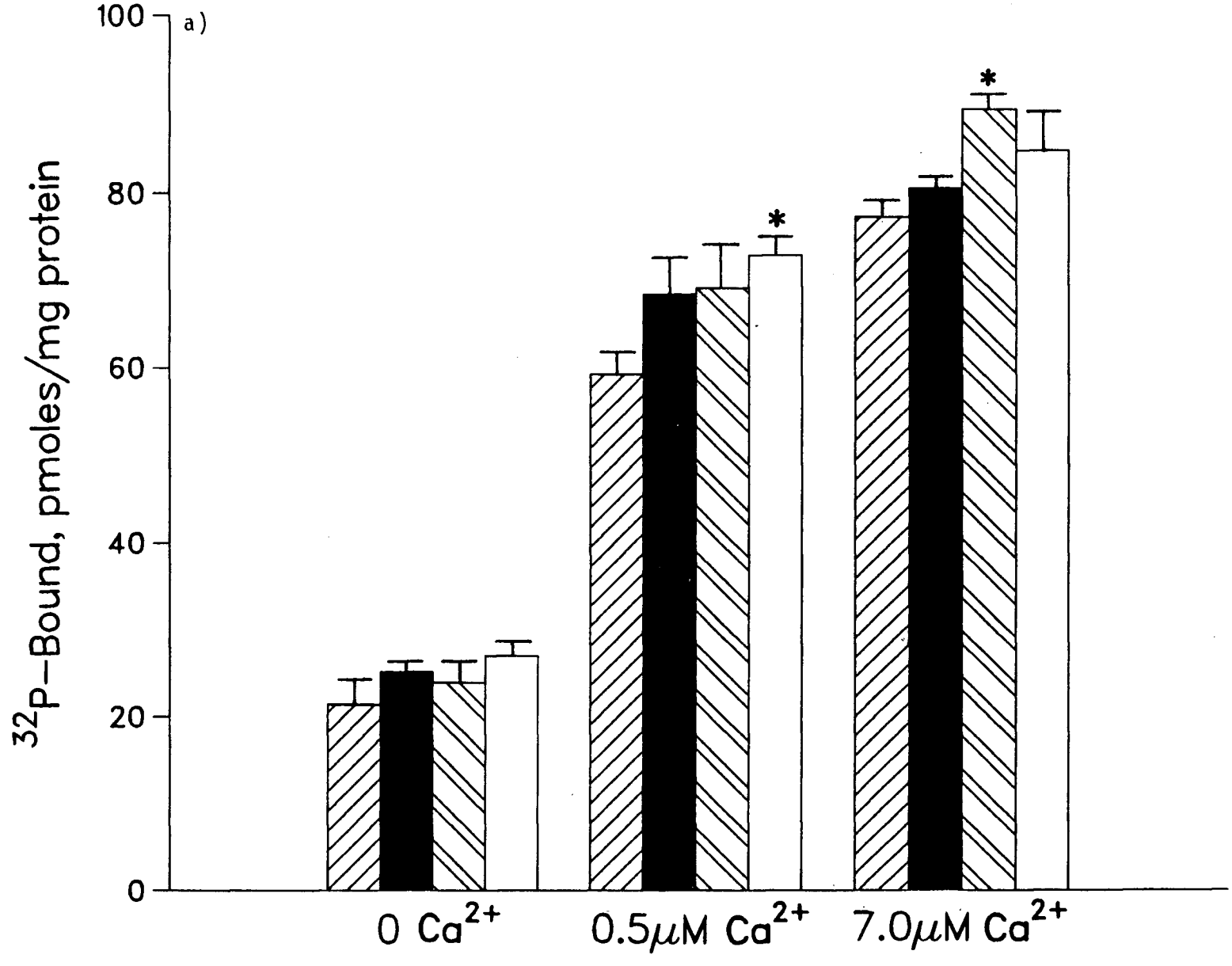
5. Studies on Phosphorylation of SR Membranes With and Without Triton Treatment

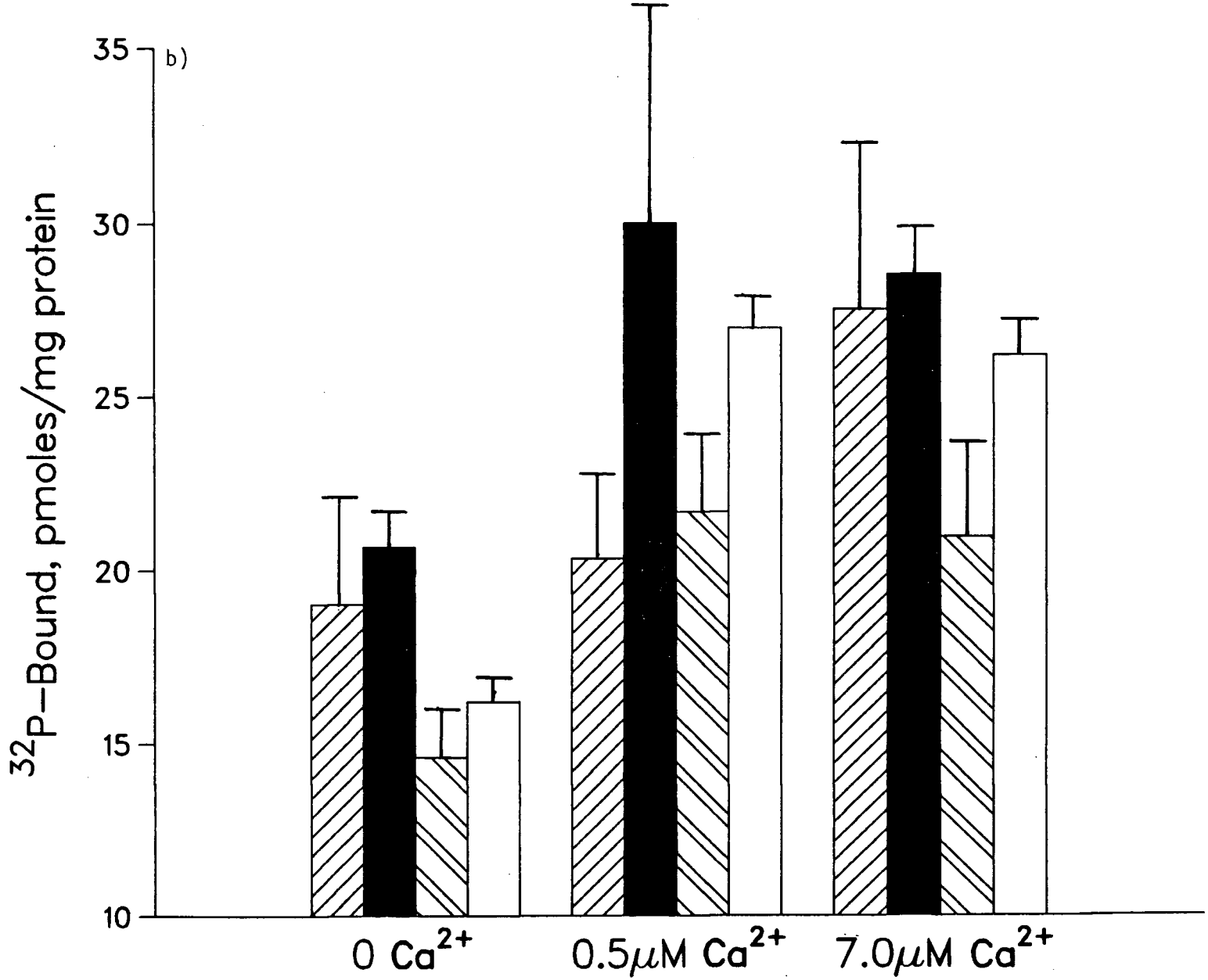
a). CaM and C-subunit-Dependent Phosphorylation of SR Membranes

Effects of CaM and C-subunit on SR membrane phosphorylation were investigated in the absence and presence of hydroxylamine to determine the effects of the two regulators on total (acyl and serine phosphorylation) and serine phosphorylation, respectively. Both CaM (3 μg/ml) and C-subunit (625 units per ml) had slight effects on total phosphorylation at zero Ca^{2+}. At 0.5μM and 7.0μM Ca^{2+}, both regulators stimulated ^{32}P incorporation into SR membrane proteins (figure 16a). However, only the C-subunit stimulated phosphorylation at 7.0μM free Ca^{2+} at significantly different levels from the controls. When both CaM and C-subunit were present, the stimulation was additive at zero and 0.5μM free Ca^{2+}, while at 7.0μM free Ca^{2+}, the combination resulted in an intermediate level of phosphorylation. However, only at 0.5μM Ca^{2+} was this phosphorylation significantly different from control. Membrane phosphorylation was stimulated by 0.5μM and 7.0μM free Ca^{2+} both in the absence and presence of the regulators.

Figure 16.

Effect of calmodulin and the catalytic subunit of cAMP-dependent protein kinase on phosphorylation of rat heart SR in the absence (a) or presence (b) of 0.6M hydroxylamine. Phosphorylation of SR membranes was measured, at zero, 0.5μM or 7.0μM free Ca^{2+} in the absence of a regulator (▨) or in the presence of 3 μg/ml calmodulin (■), 625 units/ml C-subunit (▧) or both (□). The concentration of $MgCl_2$ was 200μM. The amount of ^{32}P-incorporation was determined as described in Methods. The results shown represent the mean ± S.E.M. of observations from three individual preparations prepared and assayed on different days. Asterisk indicates significantly different from controls (no regulator present) at $p < 0.05$ (*).


a)
100
80
60
40
20
0
^{32}P–Bound, pmoles/mg protein
*
*
0 Ca^{2+}
0.5μM Ca^{2+}
7.0μM Ca^{2+}


b)
35
30
25
20
15
10
^{32}P–Bound, pmoles/mg protein
0 Ca^{2+}
0.5μM Ca^{2+}
7.0μM Ca^{2+}

In the presence of 0.6M hydroxylamine, 3 μg/ml CaM produced no effect on ^{32}P incorporation at zero and 7.0μM free Ca^{2+} (figure 16b). Although this concentration of CaM showed some stimulation of hydroxylamine-insensitive phosphorylation at 0.5μM free Ca^{2+}, it was not statistically significant. C-subunit appeared to inhibit hydroxylamine-insensitive phosphorylation at zero and 7.0μM free Ca^{2+}. However, this inhibition was not statistically significant.

b). Effect of Magnesium on Phosphorylation of SR Membranes

Figure 17 shows the effect of changing Mg concentration on the phosphorylation of SR membranes in the presence and absence of 0.6M hydroxylamine. ^{32}P incorporation was stimulated by Mg in a concentration-dependent manner both in the absence and presence of hydroxylamine. Hydroxylamine-insensitive phosphorylation appeared to be more sensitive than total phosphorylation as shown by stimulation at lower Mg concentrations. CaM had no effect on either total or hydroxylamine-insensitive phosphorylation.

c). Phosphorylation of Triton-washed SR Membranes

Quantitation: CaM-dependent phosphorylation of Triton-washed SR was studied in the presence and absence (NaCl control) of 0.6M hydroxylamine. In the absence of hydroxylamine (figure 18a), ^{32}P incorporation into Triton-washed membranes decreased

Figure 17.

Effect of magnesium on phosphorylation of SR membrane proteins in the absence (△, ×) and presence (□, ○) of 0.6M hydroxylamine. Incorporation of ^{32}P into SR membrane proteins, in the absence (△, □) or presence (×, ○) of 3 μg/ml calmodulin was measured at 0.5μM free Ca^{2+}, as described in Methods. The results shown represent the mean ± S.E.M. of observations from three individual preparations prepared and assayed on different days.

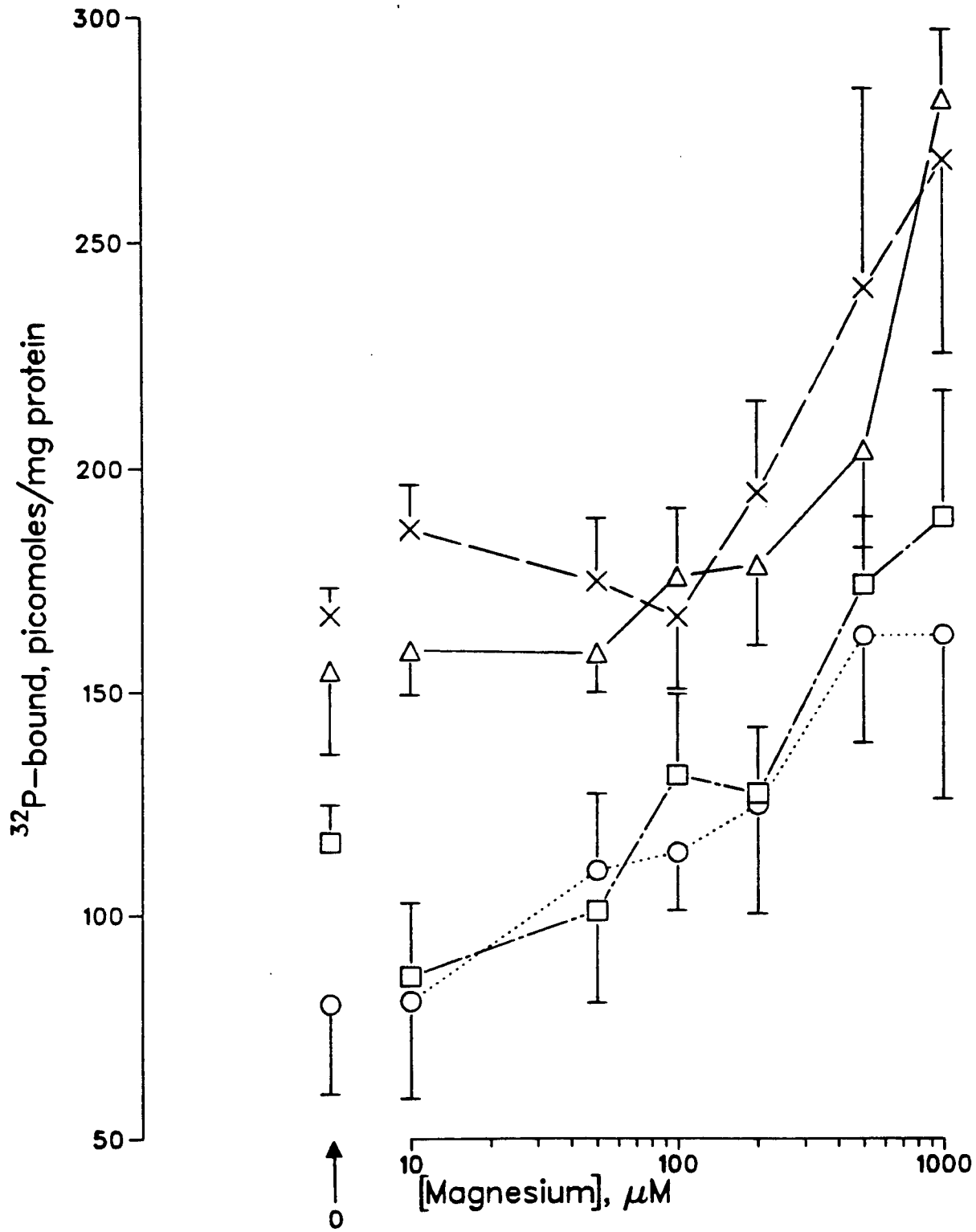
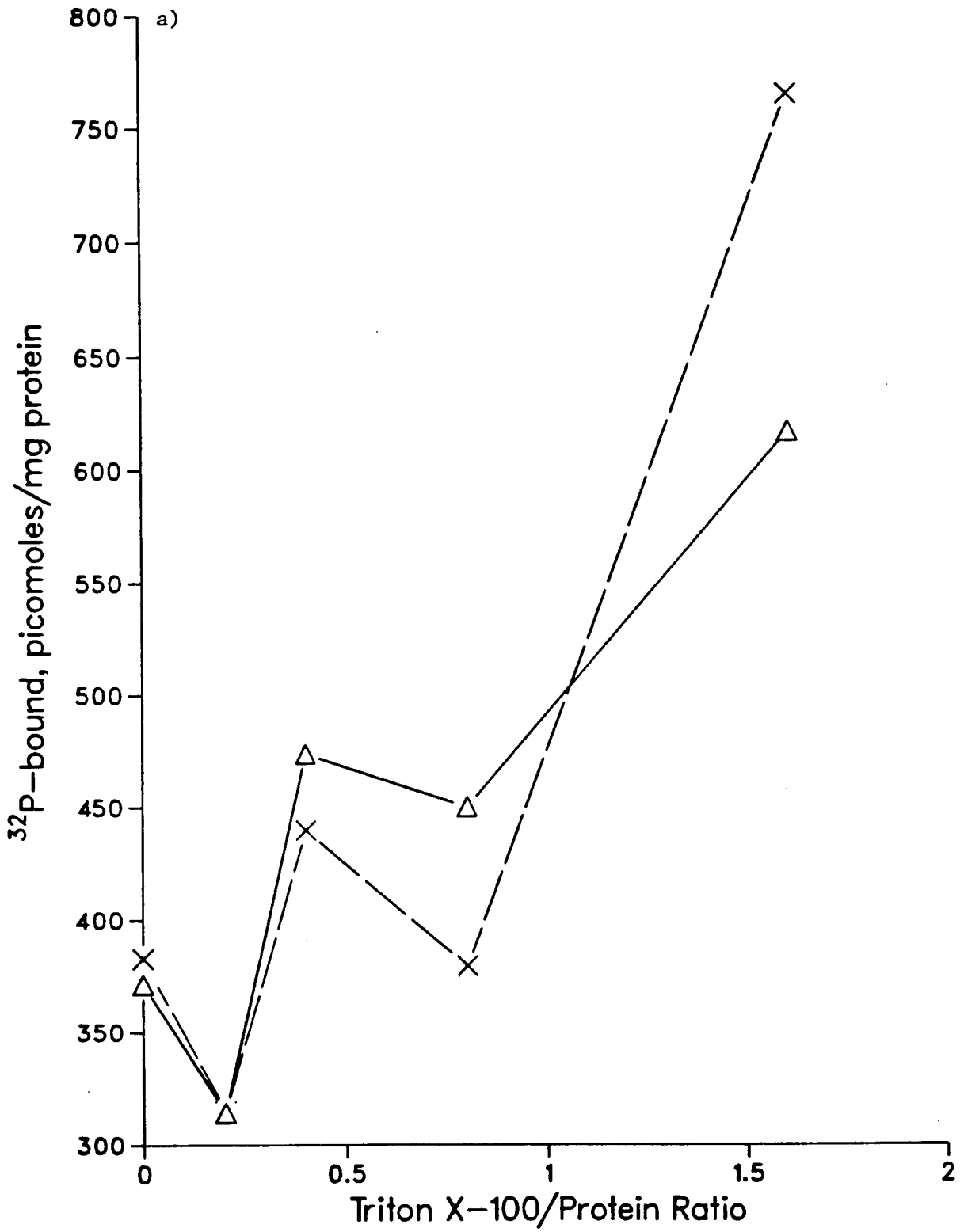
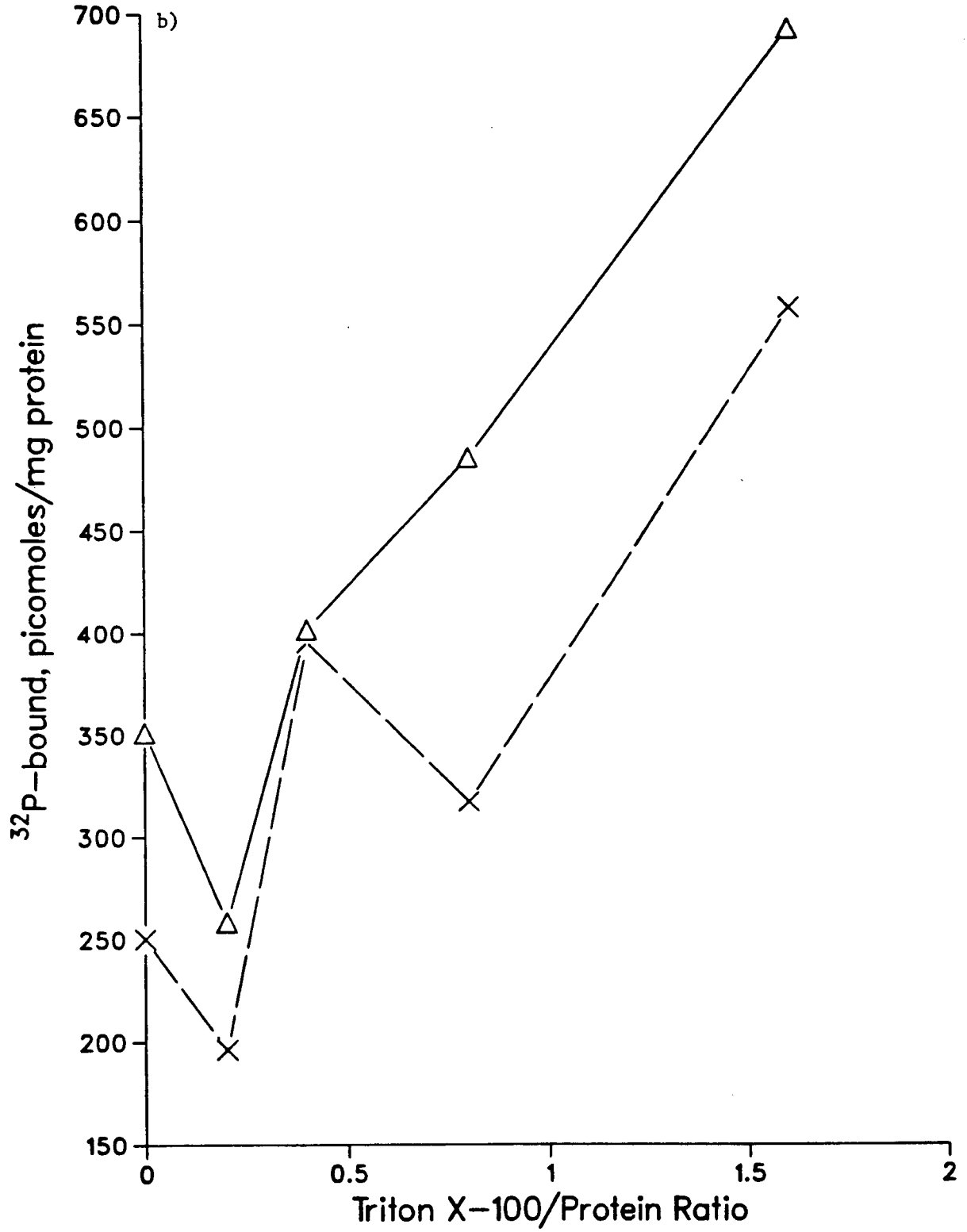

300
250
200
150
100
50
^{32}P–bound, picomoles/mg protein
0
10
100
1000
[Magnesium], μM

Figure 18.

Effect of calmodulin on phosphorylation of Triton-washed SR membranes in the absence (a) or presence (b) of 0.6M hydroxylamine. The incorporation of ^{32}P into SR membrane proteins, in the absence (△) or presence (×) of 3 μg/ml calmodulin, was measured at 500μM $MgCl_2$ and 0.5μM free Ca^{2+}, as described in Methods. The results shown represent the mean of observations from three individual preparations prepared and assayed on different days.


a)
800
750
700
650
600
550
500
450
400
350
300
^{32}P–bound, picomoles/mg protein
0
0.5
1
1.5
2
Triton X–100/Protein Ratio


b)
700
650
600
550
500
450
400
350
300
250
200
150
0
0.5
1
1.5
2
^{32}P–bound, picomoles/mg protein
Triton X–100/Protein Ratio

slightly at 0.2 Triton/protein ratio and increased at higher detergent/protein ratios. However, none of these changes were significant when compared with membranes that were not treated with Triton. CaM had no significant effect on the amount of ^{32}P incorporated.

The hydroxylamine-insensitive phosphorylation showed a similar pattern (figure 18b). Again, higher Triton concentrations appeared to increase the hydroxylamine-insensitive phosphorylation. However, this increase was not significantly different from membranes washed in control buffer. CaM appeared to produce an inhibition of phosphorylation, but this inhibition was not significant.

Autoradiography: Gel electrophoresis followed by autoradiography of the phosphorylated membranes showed that a low molecular weight protein (Mr approx. 9,000) was phosphorylated in crude SR membranes (figure 19, lane 1). CaM (3 µg/ml) had no effect on the amount or the migration properties of this protein (figure 19, lane 2). The amount of phosphorylation of Triton-washed membranes appeared to increase with Triton concentrations up to 0.4 mg Triton/mg protein (figure 19, lanes 3, 5 and 7). No phosphorylation was observed in the supernatant fraction of these low Triton-treated membranes (figure 19, lanes 4, 6 and 8). However, at 0.8 and 1.6 Triton/protein ratios (figure 23, lanes 10 and 12, respectively), a protein of 7,500 dalton was phosphorylated in the supernatant fractions, while the 9,000 dalton protein in Triton-washed membranes gradually disappeared (lanes 9 and 11).

Figure 19.

^{32}P-Autoradiogram of SDS-polyacrylamide gel electrophoretogram of SR and Triton-washed membrane proteins phosphorylated with ^{32}P-ATP. The phosphorylation (at 500μM $MgCl_2$ and 0.5μM free Ca^{2+}), electrophoresis and autoradiography were carried out as described in Methods. Results shown are typical of two different experiments. The lanes represent:

1 - crude SR in absence of CaM,

2 - crude SR in presence of 3μg/ml CaM,

3 - membranes washed in zero Triton,

4 - supernatant from zero Triton-treated membranes,

5 - membranes washed in 0.2 mg Triton/mg protein,

6 - supernatant from membranes treated with 0.2 mg Triton/mg protein,

7 - membranes washed in 0.4 mg Triton/mg protein,

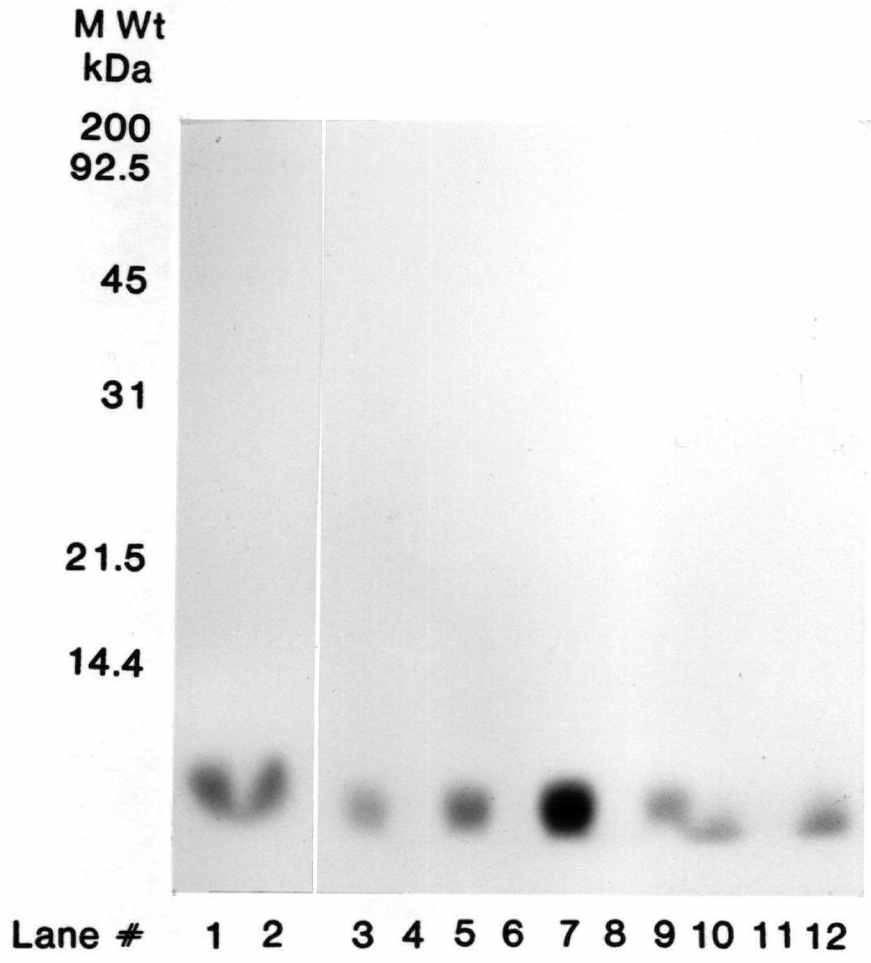
8 - supernatant from membranes treated with 0.4 mg Triton/mg protein,

9 - membranes washed in 0.8 mg Triton/mg protein,

10 - supernatant from membranes treated with 0.8 mg Triton/mg protein,

11 - membranes washed in 1.6 mg Triton/mg protein,

12 - supernatant from membranes treated with 1.6 mg Triton/mg protein,


M Wt
kDa
200
92.5
45
31
21.5
14.4
Lane # 1 2 3 4 5 6 7 8 9 10 11 12

DISCUSSION

It is now well established that the contractile process in skeletal and cardiac muscle is terminated by the active removal of free calcium from the sarcoplasm via the sarcoplasmic reticulum calcium pump. Most of the early knowledge in this area came from work with skeletal muscle. More recently, differences between the skeletal and cardiac SR Ca^{2+}-pump have been shown. For example, the SR from the two sources display a different affinity for Ca^{2+} ($K_{0.5}$ of 4.7μM for cardiac and 1.3μM for skeletal SR; Shamoo and Ambudkar, 1984). In addition, the ratio of Ca^{2+}-pump protein to total protein is different (approx. 40% in cardiac -- Suko and Hasselbach, 1976; and 60-80% in skeletal SR -- deMeis and Inesi, 1982). There also appears to be a marked difference in the regulation of the Ca^{2+}-pump and the role of the protein, phospholamban. The majority of the previous studies have been done on dog cardiac muscle SR. It is normally assumed that cardiac SR from all species would be similar with respect to the Ca^{2+}-pumping ATPase. However, our initial experiments with rat heart SR proved that this is not the case. The assay of Ca^{2+}-ATPase activity in rat heart SR using conditions identical to those used with dog heart SR revealed a high basal ATPase activity with little or no Ca^{2+}-dependent activity (data not shown). This prompted us to investigate the effect on this activity of the modification of our assay parameters; in particular, the requirement of SR Ca^{2+}-ATPase for Mg^{2+} and its regulation by CaM was investigated.

1. The Requirement of Mg^{2+} for SR Ca^{2+}-Transport Activity

a). The Role of Mg^{2+} in Ca^{2+}-Transport

The rat heart SR preparation used in our studies appears to be very active. Protein concentrations as low as 25 µg/ml (5 µg per tube) produced significant ATP hydrolysis at short reaction times (2.5 min). This is also substantiated by the fact that the 100,000 dalton protein corresponding to the Ca^{2+}-ATPase molecule appears to be a very small percentage of the total SR protein in our preparation (data not shown). The amount of protein used in our assays is very small compared to that used in other studies (Kirchberger and Antonetz, 1982a; Levitsky et al, 1976; Lopaschuk et al, 1980).

In our preparation, the Ca^{2+}-ATPase could be fully activated by Ca^{2+} in the complete absence of added Mg^{2+}. This observation could not be explained in light of the currently accepted view that Mg^{2+} is involved in the transport cycle of the SR Ca^{2+} pump. Mg^{2+} is thought to be involved in two ways: 1) the "true substrate" for the ATPase enzyme is thought to be Mg.ATP (Makinose and Boll, 1979; Vianna, 1975) and 2) free Mg^{2+} is thought to be essential for dephosphorylation of the enzyme (Kanazawa et al, 1971; Makinose and Boll, 1979; Souza and deMeis, 1976).

Vianna (1975) reported that if Mg.ATP is the true substrate, the double-reciprocal plot between ATP (when Mg:ATP ratio is 1:1) and ATPase activity would be non-linear. However,

when our data, showing the dependence of ATP hydrolysis on ATP (at constant Mg:ATP ratio of 1:1), was plotted on a double-reciprocal plot, a linear graph was obtained. Furthermore, a similar plot of ATPase activity vs. Mg.ATP was non-linear. These results indicate that in rat heart SR Ca^{2+}-ATPase ATP may be the true substrate instead of Mg.ATP.

The rat cardiac SR ATPase appeared to be stimulated in a concentration-dependent manner to maximal activity by Mg^{2+} or Ca^{2+}, in the complete absence of the other cation. Thus, when both cations were present, any increase in ATPase activity due to increased Mg^{2+} resulted in a loss of Ca^{2+}-dependent activity. These results explain the high basal activity and the absence of Ca^{2+}-dependent activity in our initial experiments where 500 μM Mg^{2+} was routinely used. A high basal ATPase activity in rat heart SR has previously been reported (Nayler et al, 1975; Penpargkul, 1979). However, an explanation for this observation was not provided. Nayler et al (1975) compared the rat SR ATPase activity with that of guinea-pig and found an almost 3-fold difference in the basal ATPase activities of the two species. Rat heart has also been reported to differ from other species with respect to its Ca^{2+} accumulating activity and contraction and relaxation characteristics (Penpargkul, 1979). This appears to be a species difference and not a phenomenon of the rat heart SR, since Velema et al (1985) have reported very similar results in rat heart sarcolemmal preparations. They found that Ca^{2+} was able to activate the sarcolemmal Ca^{2+}-ATPase in the absence of

added Mg^{2+}; upon addition of 0.45mM or 5 mM free Mg^{2+} the Ca^{2+} dependency was completely lost. No chelator system was used in these experiments; therefore, trace amounts of endogenous Mg^{2+} may have been present. In the presence of a chelator, Mg^{2+} produced a variable effect on the Ca^{2+} affinity depending on the chelator and the Ca^{2+} concentrations used. The reason for not observing the same effect in the presence of chelators may be the high concentration of chelators (2mM) used in their study. At such a high concentration, chelators may have their own effect on the ATPase activity.

The requirement of Mg^{2+} in the Ca^{2+} transport cycle of the SR Ca^{2+}-pump has recently been questioned. The findings of Velema et al (1985) as well as ours indicate that Mg^{2+} is not required for ATP hydrolysis by rat heart SR and SL Ca^{2+}-ATPases. Similar conclusions were drawn by Shigekawa et al (1983) in purified rabbit skeletal muscle SR Ca^{2+}-ATPase; using Ca.ATP as a substrate instead of Mg.ATP, they showed that Mg^{2+} was not required for the turnover of the Ca^{2+}-pump ATPase. In their studies, ATP hydrolysis could be observed with Ca.ATP as substrate with no Mg^{2+} present. ATP hydrolysis and conversion of E_1P to E_2P was slower with Ca.ATP compared to Mg.ATP. The conversion of E_1P to E_2P is suggested to be one of the Mg^{2+}-requiring steps in the Ca^{2+} transport cycle (Shigekawa and Dougherty, 1978). Ca.ATP and Mg.ATP appear to act as competitive inhibitors of each other (Shigekawa et al 1983; Vianna, 1975). Yamamoto et al (1985) have shown that in $C_{12}E_8$-

solubilized SR ATPase, ATP can be hydrolysed without going through the Mg^{2+}-dependent E_1P --> E_2P step. They suggested that E_1P may be directly hydrolysed to E and P_i. However, Shigekawa et al (1983) reported that in the presence of Ca.ATP, the conversion of E_1P to E_2P does take place, but slowly, leading to a slow turnover rate. These obsevations support our results which show a decreased Ca^{2+} dependence of ATPase activity with increasing Mg^{2+} concentrations (see figure 3b).

If the E_1P --> E_2P conversion is indeed stopped or slowed down, Ca^{2+}-transport would be interrupted since this is an essential step for delivering bound Ca^{2+} to the interior of the SR (Tada and Katz, 1982). A recent report by Costa and Madeira (1986) suggests that Mg^{2+} is required for the coupling of ATP hydrolysis to Ca^{2+} transport, while ATP hydrolysis is independent of Mg^{2+}. It should be mentioned, though, that these authors did not clearly explain their methodology and did not indicate the source of the SR preparation used. In addition, the Mg^{2+} concentrations used (0.2-50mM) in their study appeared to be high.

Mg^{2+} has been proposed as the counter-transported cation during calcium uptake by SR (Kanazawa et al, 1971). However, in their studies, Chiesi and Inesi (1980) showed that Mg^{2+} was not counter-transported. Ueno and Sekine (1978) observed Ca^{2+} transport in rabbit skeletal muscle SR in the absence of Mg^{2+}. Salama and Scarpa (1985) provided further evidence for this by showing that Mg^{2+} was not released from SR vesicles during active Ca^{2+} transport.

b). The Non-Specific Ca^{2+} (or Mg^{2+})-ATPase

An alternative explanation for the competitive nature of Mg^{2+} and Ca^{2+} stimulation of rat heart SR ATPase, can be provided. There is a strong possibility that we have been studying a low affinity non-specific divalent cation-stimulated ATPase which has been shown to be present in plasma membrane preparations of different tissues (Iwasa et al, 1982; Lotersztajn et al, 1981; Pershadsingh and McDonald, 1980; Verma and Penniston, 1981). Lotersztajn and colleagues showed that the basal ATPase activity in native rat liver plasma membranes was dramatically increased by 5μM Mg^{2+} and that 50μM Mg^{2+} resulted in a complete loss of Ca^{2+} stimulation. Verma and Penniston reported that in their initial experiments with rat corpus luteum ATPase using 6mM Mg^{2+}, Ca^{2+} did not produce a stimulation above the basal activity. These results are explained by the presence of a non-specific divalent cation-stimulated ATPase (also called Mg^{2+}-ATPase). This ATPase has also been demonstrated in rat kidney cortex (Parkinson and Radde, 1971), rat adipocyte plasma membranes (Pershadsingh and McDonald, 1980), and rat osteosarcoma plasma membranes (Murray et al, 1983). Furthermore, a high affinity Ca^{2+}-ATPase has been isolated from plasma membrane preparations of most of these tissues (Iwasa et al, 1982; Lotersztajn et al, 1981; Verma and Penniston, 1981). The 'non-specific' ATPase present appears to hinder the activity of the high affinity ATPase when studied in the native plasma

membranes under high Mg^{2+} conditions (Lotersztajn et al, 1981). The high affinity Ca^{2+}-ATPase is probably the Ca^{2+}-pumping enzyme (Verma and Penniston, 1981). The latter ATPase from all three tissue sources showed maximal stimulation of ATPase activity at 0.5-1.0μM free Ca^{2+} and no requirement for exogenous Mg^{2+}. However, small amounts of Mg^{2+} were required for this activity. This requirement is normally satisfied by endogenous Mg^{2+} as shown by a complete inhibition of the ATPase activity by CDTA (Pershadsingh and McDonald, 1980; Verma and Penniston, 1981). In our studies, however, 100μM CDTA failed to inhibit the ATPase activity. One explanation may be that the concentration of CDTA used in our study was too low to chelate all the Mg^{2+} present in the reaction medium.

Therefore, it is possible that in our study, the activity of the high affinity Ca^{2+}-pumping ATPase was masked by a low affinity non-specific ATPase. Further investigation utilizing lower free Ca^{2+} concentrations (0-1.0μM) and no added Mg^{2+} would be necessary to determine this possibility. From the above discussion it appears that the non-specific ATPase may be species-specific and not tissue-specific since all the studies discussed above used rat tissues. A similar ATPase has also been reported in rat liver endoplasmic reticulum (Kraus-Friedman, Satellite symposium of the 30th congress of IUPS, Seattle, 1986). Furthermore, Ca^{2+}-ATPases from other species, of both plasma membrane and SR origin, have been shown to be active in the presence of high Mg^{2+}.

The ATPase activities reported here were completely insensitive to ouabain, indicating that the ATP hydrolysis was not due to Na^+/K^+-ATPase activity. Vanadate (2μM) produced an 8.5% inhibition of the ATPase activity. This concentration of vanadate has been shown to almost completely inhibit the sarcolemmal Ca^{2+}-ATPase, while inhibiting only 2% of the SR Ca^{2+}-ATPase (Caroni and Carafoli, 1981). Therefore, it appears that the sarcolemmal contamination of our SR preparation was minimal. The inhibitory effect of sodium azide indicated that the major contaminant of our SR preparation was mitochondria. However, since 5mM sodium azide was present in all our assay media, the mitochondrial ATPase made little contribution to the total activity.

A similar non-specific ATPase has been reported in rat pancreatic plasma membranes (Ansah et al, 1984; Forget and Heisley, 1976; Hamlyn and Senior, 1983; Hurley et al, 1984). However, this ATPase has been identified to be a diphosphohydrolase due to its ability to hydrolyse all nucleotide di- and tri-phosphates. The non-specific ATPase described above, however, is more selective towards nucleotide triphosphates (Parkinson and Radde, 1971).

2. Regulation of SR Ca^{2+}-ATPase and Ca^{2+} Transport by CaM

One of the major differences between skeletal muscle and cardiac SR is that cardiac SR Ca^{2+}-transport is regulated by cAMP-dependent protein kinase, CaM (Tada and Inui, 1983) and

protein kinase C (Movsesian et al, 1984). cAMP-mediated regulation is thought to be responsible for the inotropic effects of catecholamines (Tada and Katz, 1982). The physiological relevance of CaM and C-kinase-mediated regulation is not known. CaM regulation may be operational in Ca^{2+}-overload conditions (Tada et al, 1983) or beat to beat control of Ca^{2+} transport (Kirchberger and Antonetz, 1982a). The second part of our study was concerned with an investigation of the mechanism of regulation of Ca^{2+}-transport in rat heart SR.

In our studies, CaM from two different commercial sources failed to stimulate the ATPase activity at low and high Mg concentrations (0-500μM) and different Ca^{2+} concentrations. Furthermore, CaM did not produce a significant effect on transport of $^{45}Ca^{2+}$ into SR vesicles. This, together with the lack of stimulation of total and hydroxylamine-resistant membrane phosphorylation by CaM at a range of Mg concentrations, provides convincing evidence that the rat heart SR Ca^{2+}-ATPase cannot be stimulated by exogenous CaM. This conclusion points to two possibilities: a) there is no endogenous CaM-PK and/or PLB present in rat heart or b) this preparation contains large quantities of endogenous CaM. In our studies, the C-subunit of cAMP-PK produced no stimulation of hydroxylamine-insensitive membrane phosphorylation or Ca^{2+}-ATPase activity at low free Ca^{2+} concentrations and a small inhibition at higher free Ca^{2+} concentrations. This, and the lack of CaM stimulation, may indicate a complete absence of the PLB regulatory system in rat

heart SR and supports the first possibility.

The lack of inhibition by the two CaM antagonists, TFP and compound 48/80, may indicate absence of endogenous CaM. The inhibitory effect of TFP at high concentrations may be due to its non-specific effects on the membrane.

Under the conditions of our experiments, more than 50% of the phosphorylation monitored was insensitive to hydroxylamine treatment, indicating that the phosphoprotein formed was not an acyl phosphate. Phosphorylation of a 9,000-11,000 dalton protein has previously been shown to account for more than 90% of the hydroxylamine-insensitive phosphoprotein formed under similar assay conditions (Plank et al, 1983). The autoradiography results (figure 19) in our studies indicate an apparent molecular weight of the phosphorylated protein to be 7,500-9,000 dalton. Similar proteins have been suggested to be monomers of PLB (Louis et al, 1982; Wegener and Jones, 1984). If this hydroxylamine-insensitive phosphorylation was due to incorporation of ^{32}P into PLB or a similar regulatory protein, it must be fully stimulated by endogenous CaM, since it was observed both in the absence and presence of exogenously added CaM. An explanation for the conflict between this finding and the lack of inhibition by CaM antagonists may be that the endogenous CaM was tightly bound as recently demonstrated in rat liver (Gazzotti et al, 1985) and calf heart sarcolemma (Caroni et al, 1983) and that this membrane-bound CaM was occluded from the CaM antagonists.

3. Effect of Detergent Treatment on ATPase Activity and its Regulation

At low concentrations (0.2 mg Triton/mg protein), Triton appeared to stimulate the ATPase activity in Triton-washed membranes. These results are consistant with the findings of Hidalgo et al (1986) who observed a 10-20% increase in the ATPase activity in membranes sedimented after treatment with 0.1 mg deoxycholate/mg protein. This increase was probably due to the removal of non-intrinsic proteins and leaky vesicles. An alternate explanation may be that the detergent stimulates the ATPase activity directly by binding to it (McIntosh and Davidson, 1984) and/or altering membrane fluidity (LeMaire et al, 1983). In addition, the recovery of ATPase activity also showed an elevation at 0.2 Triton/protein ratio. This may be due to aggregation of membrane vesicles (Helenius et al, 1979) leading to precipitation of more protein than the control membranes during centrifugation. The loss of ATPase activity at higher Triton concentrations appears to be due to solubilization of proteins and removal of the phospholipid environment (Hidalgo et al, 1986). The increased recovery of ATPase activity in the solubilized fraction at high Triton/protein ratios is basically a reflection of higher protein recovery, since the specific activities in the 105,000xg supernatant fractions declined with increasing Triton concentrations.

Again, the Ca^{2+}-dependent ATPase activity decreased with increasing Mg^{2+} concentrations in the Triton-washed membranes.

While the Ca^{2+}-dependent ATPase activity at 10μM added Mg was lower than at zero Mg, the total activities at the two Mg concentrations were almost identical. This indicates that the effect of Mg on ATPase activity may be a competitive one, i.e. Mg may be producing its effect by occupying Ca^{2+}-binding sites. Hence, the additive effect of the two cations was the same as that of Ca^{2+} alone, while in the presence of Mg, the Ca^{2+}-dependent effect was reduced. Triton appeared to diminish this effect of Mg on Ca^{2+}-dependent ATPase activity, since the activity appeared to merge as the Triton concentration was increased. This may be due to the enzyme becoming more specific for Ca^{2+}, which may result from selective removal or inactivation of Mg^{2+}-ATPase activity. This is in parallel with the findings that the purified SR Ca^{2+}-ATPase contains a lower percent basal ATPase activity as compared to the membrane bound enzyme (Nakamura et al, 1983).

As discussed earlier, CaM did not stimulate either the Ca^{2+}-ATPase activity or membrane phosphorylation in rat heart SR. We, hence, studied the effect of CaM on Triton-washed membranes to investigate whether Triton X-100 treatment removed endogenous CaM and/or exposed CaM-binding sites. CaM (3 μg/ml) was once again found to be without effect. This may indicate that endogenous CaM is not removed by Triton X-100 suggesting that if it is present, it is tightly bound to the SR membranes.

The increase in phosphorylation seen both in the autoradiograms and in the quantitative phosphorylation experiments with

the 0.4 Triton/protein ratio, may be due to exposure of the phosphorylation sites by the detergent. The appearance of the phosphorylated protein in the supernatant fraction at higher Triton/protein ratios indicate solubilization of this protein with other SR proteins.

In summary, the rat heart SR appears to have a different profile of ATPase activity to other SR systems that have been studied, including the dog heart SR. Ca^{2+} and Mg^{2+} were equally effective at stimulating the ATPase activity. Similar Ca^{2+}(or Mg^{2+})-ATPases have been demonstrated in other rat tissues indicating that this ATPase is species specific. This enzyme is not exclusive to plasma membrane since our studies were carried out in SR vesicles which were relatively free of sarcolemmal contamination. In addition, the regulation of rat heart SR Ca^{2+}-ATPase appears to be different from other cardiac systems since CaM and C-subunit did not stimulate the ATPase activity, membrane phosphorylation or Ca^{2+} uptake.

Our results point to the possibility that the rat heart SR preparations used in this study contain tightly-bound endogenous CaM. This would, probably be best confirmed by direct determinations of CaM using radioimmuno assay. Alternatively, CaM may be extracted from SR vesicles by boiling and the effects of the boiled extracts studied on a CaM-sensitive system such as erythrocyte Ca^{2+}-ATPase following a procedure similar to that of Eibschutz et al (1984) or Gazzotti et al (1985). Finally, phosphorylation of a protein with a similar apparent molecular

weight to monomeric PLB was seen in rat heart SR membranes. However, there is no concrete evidence that this protein is PLB, since it cannot be phosphorylated by exogenous CaM or C-subunit. Recently reported PLB-specific monoclonal antibodies (Suzuki and Wang, 1986) could be used to determine whether the 7,500-9,000 dalton protein is in fact the monomer of PLB.

SUMMARY AND CONCLUSIONS

1. Our results show that rat heart SR is different from skeletal muscle and dog cardiac SR. It possesses an ATPase activity which can be stimulated by either Ca^{2+} or Mg^{2+}. This ATPase appears to be similar to the non-specific divalent-cation stimulated ATPase that has been reported in a number of plasma membrane preparations from different rat tissues. This non-specific ATPase may not be the Ca^{2+} transporting ATPase, but may be masking the activity of the latter system. If the system is indeed similar to that of rat plasma membranes, it may be possible to study the high affinity Ca^{2+} transporting ATPase at low Ca^{2+} concentrations in the absence of magnesium.
2. Experiments with various Mg and ATP concentrations indicate that the substrate for the rat heart SR Ca^{2+} (or Mg^{2+})-ATPase may be free ATP and not the Mg.ATP complex.
3. CaM failed to stimulate the ATPase activity and phosphorylation of SR proteins. CaM antagonists were ineffective at inhibiting ATPase activity. However, phosphorylation of a 7,500-9,000 dalton protein was observed even in the absence of exogenous CaM. Therefore, it is speculated that the rat heart SR membranes contain tightly-bound endogenous CaM.
4. The Ca^{2+}-specificity of ATPase appeared to increase in Triton-washed membranes with increasing Triton concentrations. This may indicate a selective removal or inactivation of the Mg^{2+}-ATPase activity. Triton X-100 had no effect on

the regulation of rat heart SR Ca^{2+}-ATPase by CaM.

BIBLIOGRAPHY

Ansah, T-A., Molla, A. and Katz, S. (1984). Ca^{2+}-ATPase activity in pancreatic acinar plasma membranes. Regulation by calmodulin and acidic phospholipids. J. Biol. Chem., 259, 13442-13450.

Becker, G.L., Fiskum, G. and Lehninger, A.L. (1980). Regulation of free Ca^{2+} by liver mitochondria and endoplasmic reticulum. J. Biol. Chem., 255, 9009-9012.

Bidlack, J.M., Ambudkar, I.S. and Shamoo, A.E.(1982). Purification of phospholamban, a 22,000-dalton protein from cardiac sarcoplasmic reticulum that is specifically phosphorylated by cyclic AMP-dependent protein kinase. J. Biol. Chem., 257, 4501-4506.

Bidlack, J.M. and Shamoo, A.E. (1980). Adenosine 3',5'-monophosphate-dependent phosphorylation of a 6000 and 22000 dalton protein from cardiac sarcoplasmic reticulum. Biochim. Biophys. Acta, 632, 310-325.

Bilezikjian, L.M., Kranias, E.G., Potter, J.D. and Schwartz, A. (1981). Studies on phosphorylation of canine cardiac sarcoplasmic reticulum by calmodulin-dependent protein kinase. Circ. Res., 49, 1356-1362.

Blinks, J.R., Weir, W.G., Hess, P. and Prendergest, F.G. (1982). Measurement of calcium concentration in living cells. Prog. Biophys. Molec. Biol., 40, 1-114.

Borle, A.B. (1981). Control, modulation , and regulation of cell calcium. Rev. Physiol. Biochem. Pharmacol., 90, 13-153.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248-254.

Cala, S.E. and Jones, L.R. (1983). Rapid purification of calsequestrin from cardiac and skeletal muscle sarcoplasmic reticulum vesicles by Ca^{2+}-dependent elution from Phenyl-Sepharose. J. Biol. Chem., 258, 11932-11936.

Campbell, K.P., MacLennan, D.H., Jorgensen, A.O. and Mintzer, M.C. (1983). Purification and characterization of calsequestrin from canine cardiac sarcoplasmic reticulum and identification of the 53,000 dalton glycoprotein. J. Biol. Chem., 258, 1197-1204.

Caponi, J-P., Rinaldi, M.L., Guilleux, F. and Demaille, J.G. (1983). Isolation of cardiac membrane proteolipids by high

pressure liquid chromatography. A comparison of reticular and sarcolemmal proteolipids, phospholamban and calciductin. Biochim. Biophys. Acta, 728, 83-91.

Caroni, P. and Carafoli, E. (1981). The Ca^{2+}-pumping ATPase of heart sarcolemma. Characterization, calmodulin dependence, and partial purification. J. Biol. Chem., 256, 3263-3270.

Caroni, P., Zurini, M., Clark, A. and Carafoli, E. (1983). Further characterization and reconstitution of the purified Ca^{2+}-pumping ATPase of heart sarcolemma. J. Biol. Chem., 258, 7305-7310.

Chamberlain, B.K., Berenski, C.J., Jung, C.Y. and Fleischer, S. (1983). Determination of the oligomeric structure of the Ca^{2+} pump protein in canine sarcoplasmic reticulum membranes using radiation inactivation analysis. J. Biol. Chem., 258, 11997-12001.

Cheung, W.Y. (1980). Calmodulin plays a pivotal role in cellular regulation. Science, 207, 19-27.

Chiesi, M., Gasser, J. and Carafoli, E. (1983). Phospholamban of cardiac sarcoplasmic reticulum consists of two functionally distinct proteolipids. FEBS Lett.,160, 61-66.

Chiesi, M. and Inesi, G. (1980). Adenosine 5'-triphosphate dependent fluxes of manganese and hydrogen ions in sarcoplasmic reticulum vesicles. Biochemistry, 19, 2912-2918.

Costa, A.G.D. and Madiera, V.M.C. (1986). Magnesium and manganese ions modulate Ca^{2+} uptake and its energetic coupling in sarcoplasmic reticulum. Arch. Biochem. Biophys., 249, 199-206.

Davis, B.A., Schwartz, A., Samaha, F.J. and Kranias, E.G. (1983). Regulation of cardiac sarcoplasmic reticulum calcium transport by calcium-calmodulin-dependent phosphorylation. J. Biol. Chem., 258, 13587-13591.

Dean, W.I. and Tanford, C. (1978). Properties of a delipidated, detergent-activated Ca^{2+}-ATPase. Biochemistry, 17, 1683-1690.

deMeis, L. (1981). The Sarcoplasmic Reticulum. Transport and Energy Transduction (ed. E.E. Bittar). John Wiley & Sons, New York.

deMeis, L. and Inesi, G. (1982). The transport of calcium by sarcoplasmic reticulum and various microsomal preparations. In: Membrane Transport of Calcium (ed. E. Carafoli). Academic Press, London. pp.141-186.

Ebashi, S. (1960). Calcium binding and relaxation in the actinomyosin system. J. Biochem., 48, 150-151.

Ebashi, S. (1961). Calcium binding activity of vesicular relaxing factor. J. Biochem., 50, 236-244.

Ebashi, S. and Lipman, F. (1962). Adenosine triphosphatase-linked concentration of calcium ions in a particulate fraction of rabbit muscle. J. Cell Biol., 14, 389-400.

Eibschutz, B., Wong, A.P.G., Lopaschuk, G.D. and Katz, S. (1984). The presence and binding characteristics of calmodulin in microsomal preparations enriched in sarcoplasmic reticulum from rabbit skeletal muscle. Cell Calcium, 5, 391-400.

Fabiato, A. and Fabiato, F. (1975). Contractions induced by a calcium triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cell. J. Physiol. (London), 249, 469-495.

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

Ford, L.E. and Podolsky, R.J. (1972). Intracellular calcium movements in skinned muscle fibres. J. Physiol. (London), 223, 21-33.

Forget, G. and Heisler, S. (1976). Calcium-activated ATPase activity in a plasma membrane rich preparation of rat pancreas. Clin. Exp. Pharmacol. Physiol., 3, 67-72.

Fuji, J., Kadoma, M., Tada, M., Tada, H. and Sakiyama, F. (1986). Characterisation of structural unit of phospholamban by amino acid sequencing and electrophoretic analysis. Biochem. Biophys. Res. Commun., 138, 1044-1050.

Gazzotti, P., Flura, M. and Gloor, M. (1985). The association of calmodulin with subcellular fractions isolated from rat liver. Biochem. Biophys. Res. Commun., 127, 358-365.

Goldstein, D. (1979). Calculation of the concentration of the free cations and cation-ligand complexes in solutions containing multiple divalent-cations and ligands. Biophys. J., 26, 235-242.

Hamlyn, J.M. and Senior, A.E. (1983). Evidence that Mg^{2+}- or Ca^{2+}-activated adenosine triphosphatase in rat pancreas is a plasma-membrane ecto-enzyme. Biochem. J., 214, 59-68.

Hasselbach, W. (1964). Relaxing factor and relaxation of muscle. Prog. Biophys. Mol. Biol., 14, 169-222.

Hasselbach, W. (1978). The reversibility of the sarcoplasmic reticulum calcium pump. Biochim. Biophys. Acta, 515, 23-53.

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

Haynes, D.H. (1983). Mechanism of a Ca^{2+} transport by Ca^{2+}-Mg^{2+}-ATPase pump: Analysis of major states and pathways. Am. J. Physiol., 244, G3-G12.

Helenius, A., McCaslin, D.R., Fries, E. and Tanford, C. (1979). Properties of detergents. Methods Enzymol., 56, 734-749.

Herbette, L. and Blasie, J.K. (1980). Static and time resolved structural studies on isolated sarcoplasmic reticulum membranes. In: Calcium Binding Proteins: Structure and Function (Eds. F.L. Siegal, E. Carafoli, R.H. Kretsinger, D.H. MacLennan, R.H. Wasserman). Elsevier, New York-Amsterdam-Oxford. pp.115-120.

Hidalgo, C., Fuente, M.D.L. and Gonzalez, M.E. (1986). Role of lipids in sarcoplasmic reticulum: A higher lipid content is required to sustain phosphoenzyme decomposition than phosphoenzyme formation. Arch. Biochem. Biophys., 247, 365-371.

Hurley, T.W., Becker, J.K. and Martinez, J.R. (1984). High affinity, calcium-stimulated adenosine triphosphatase activity in the particulate fraction of rat pancreatic acini. J. Biol. Chem., 259, 7061-7066.

Huxley, A.F. and Taylor, R.E. (1958). Local activation of striated muscle fibres. J. Physiol. (London), 144, 426-441.

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

Ikemoto, N., Antoniu, B. and Kim, D.H. (1984). Rapid calcium release from the isolated sarcoplasmic reticulum is triggered via the attached transverse tubular system. J. Biol. Chem., 259, 13151-13158.

Imagawa, T., Watanabe, T., and Nakamura, T. (1986). Subunit structure and multiple phosphorylation sites of phospholamban. J. Biochem., 99, 41-53.

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

Iwasa, Y., Iwasa, T., Higashi, K., Matsui, K. and Miyamoto, E. (1982). Demonstration of a high affinity Ca^{2+}-ATPase in rat liver plasma membranes. Biochem. Biophys. Res. Commun., 105,

488-494.

Jones, L.R., Besch, H.R., Jr., Fleming. J.W., McConnoughey, M.M. and Watanabe, A.M. (1979). Separation of vesicles of cardiac sarcolemma from vesicles of canine sarcoplasmic reticulum. Comparative biochemical analysis of compartment activities. J. Biol. Chem, 254, 530-539.

Jones, L.R., Simmerman, H.K., Wilson, W.W., Gurd, R.N. and Wegener, A.D. (1985). Purification and characterization of phospholamban from canine cardiac sarcoplasmic reticulum. J. Biol. Chem., 260, 7721-7730.

Jorgensen, A.O., Kalnins, V. and MacLennan, D.H. (1979). Localization of sarcoplasmic reticulum proteins in rat skeletal muscle by immunofluorescence. J. Cell Biol., 80, 372-384.

Kanazawa, T., Yamada, S., Yamamoto, T. and Tonomura, Y. (1971). Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. V. Vectorial requirements for calcium and magnesium ions of three partial reactions of ATPase: Formation and decomposition of a phosphorylated intermediate and ATP-formation from ADP and the intermediate. J. Biochem., 70, 95-123.

Katz, A.M. (1985). Basic cellular mechanisms of action of the calcium-channel blockers. Am. J. Cardiol., 55, 2B-9B.

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

Katz, S., Richter, B. and Eibschutz, B. (1985). Characterization of calmodulin-dependent and cyclic-AMP-dependent protein kinase stimulation of cardiac sarcoplasmic reticulum calcium transport. In: Advances in Myocardiology (eds. N.S. Dhalla, D.J. Hearse), vol 6. Plenum Press, New York. pp.233-247.

Katz, S. and Blostein, R. (1975). Ca^{2+}-stimulated membrane phosphorylation and ATPase activity of human erythrocyte. Biochim. Biophys. Acta, 388, 314-324.

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

Kirchberger, M.A. and Antonetz, T. (1982a). Calmodulin-mediated regulation of calcium transport and (Ca^{2+}+Mg^{2+})-activated ATPase activity in isolated cardiac sarcoplasmic reticulum. J. Biol. Chem., 257, 5685-5691.

Kirchberger, M.A. and Antonetz, T. (1982b). 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, M.A., Tada, M. and Katz, A.M. (1974). Adenosine 3',5'-monophosphate-dependent protein kinase-catalysed phosphorylation reaction and its relationship to calcium transport in cardiac sarcoplasmic reticulum. J. Biol. Chem., 249, 6166-6173.

Kirchberger, M.A., Tada, M., Repke, D.I. and Katz, A.M. (1972). Cyclic adenosine 3',5'-monophosphate-dependent protein kinase stimulation of calcium uptake by canine cardiac microsomes. J. Mol. Cell. Cardiol., 4, 673-680.

Kosk-kosicka, D., Kurzmack, M. and Inesi, G. (1983). Kinetic characterisation of detergent-solubilized sarcoplasmic adenosinetriphosphatase. Biochemistry, 22, 2559-2567.

Kranias, E.G., Bilezikjian, L.M., Potter, J.D., Piascik, M.T. and Schwartz, A. (1980). The role of calmodulin in regulation of cardiac sarcoplasmic reticulum phosphorylation. Ann. N.Y. Acad. Sci., 356, 279-291.

Kranias, E.G., Nakamura, J. and Schwartz, A. (1983). Phospholamban, the regulator of the cardiac sarcoplasmic reticulum calcium pump, does not copurify with the Ca^{2+}-ATPase enzyme. Biochim. Biophys. Acta, 749, 62-68.

Laemmli, U.K. and Favre, M. (1973). Maturation of the head of bacteriophage T4: I. DNA packaging events. J. Molec. Biol., 80, 575-599.

Lamers, J.M.J. and Stinis, J.T. (1980). Phosphorylation of low molecular weight proteins in purified preparations of rat heart sarcolemma and sarcoplasmic reticulum. Biochim. Biophys. Acta, 624, 443-459.

LeMaire, M., Kwee, S., Andersen, J.P. and Moller, J.V. (1983). Mode of interaction of polyoxyethyleneglycol detergents with membrane proteins. Eur. J. Biochem., 129, 525-532.

LePeuch, C.J., Guilleux, J-C and Demaille, J.G. (1980). Phospholamban phosphorylation in the perfused rat heart is not solely dependent on β-adrenergic stimulation. FEBS Lett., 114, 165-168.

LePeuch, C.J., Haiech, J. and Demaille, J.G. (1979). Concerted regulation of cardiac sarcoplasmic reticulum calcium transport by cyclic adenosine monophosphate-dependent and calcium-calmodulin-dependent phosphorylation. Biochemistry,

18, 5150-5157.

Levitsky, D.O., Aliev, M.K., Kuzmin, A.V., Levchenko, T.S., Smirnov, V.N. and Chazov, E.I. (1976). Isolation of calcium pump system and purification of calcium ion-dependent ATPase from heart muscle. Biochim. Biophys. Acta, 443, 468-484.

Lindemann, J.P. and Watanabe, A.M. (1985). Phosphorylation of phospholamban in intact myocardium. Role of Ca^{2+}-calmodulin-dependent mechanisms. J. Biol. Chem., 260, 4516-4525.

Lopaschuk, G., Richter, B. and Katz, S. (1980). Characterization of calmodulin effect on calcium transport in cardiac microsomes enriched in sarcoplasmic reticulum. Biochemistry, 19, 5603-5607.

Lotersztajn, S., Hanoune, J. and Pecker, F. (1981). A high affinity calcium-stimulated magnesium dependent ATPase in rat liver plasma membranes. Dependence on an endogenous protein activator distinct from calmodulin. J. Biol. Chem., 256, 11209-11215.

Louis, C.F. and Jarvis, B. (1982). Affinity labelling of calmodulin-binding components in canine cardiac sarcoplasmic reticulum. J. Biol. Chem., 257, 15187-15191.

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

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

McIntosh, D.B. and Davidson, G.A. (1984). Effects of nonsolubilizing and solubilizing concentrations of Triton X-100 on Ca^{2+} binding and Ca^{2+}-ATPase activity of sarcoplasmic reticulum. Biochemistry, 23, 1959-1965.

MacLennan, D.H. (1970). Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. J. Biol. Chem., 245, 4508-4518.

MacLennan, D.H., Khanna, V.K. and Stewart, P.S. (1976). Restoration of calcium transport in the trypsin-treated (Ca^{2+}+Mg^{2+})-dependent adenosine triphosphatase of sarcoplasmic reticulum exposed to sodium dodecyl sulfate. J. Biol. Chem., 251, 7271-7274.

MacLennan, D.H., Seeman, P., Iles, G.H. and Yip, C.C. (1971). Resolution of enzymes of biological transport. 2. Membrane formation by adenosine triphosphatase of sarcoplasmic reti-

culum. J. Biol. Chem., 246, 2702-2710.

MacLennan, D.H. and Wong, P.T.S. (1971). Isolation of a calcium sequestering protein from sarcoplasmic reticulum. Proc. Natl. Acad. Sci., USA, 68, 1231-1235.

MacLennan, D.H., Zubrzycka, E., Jorgensen, A.O. and Kalnins, V.I. (1978). Assembly of the sarcoplasmic reticulum. In: The Molecular Biology of Membranes (eds. S. Fleischer, Y. Hatefi, D.H. MacLennan, A. Tzagolo). Plenum Publishing Corporation, New York. pp.309-320.

Makinose, M. (1969). The phosphorylation of the membrane protein of sarcoplasmic vesicles during activated calcium transport. Eur. J. Biochem., 10, 74-82.

Makinose, M. and Boll, W. (1979). The role of magnesium on the sarcoplasmic calcium pump. In: Cation Flux Across Biomembranes (eds. Y. Mukohata, L. Packer). Academic Press, New York. pp.89-100.

Martell, A. and Smith, R. (1974). "Critical Stability Constants", vol 1. Plenum Press, New York.

Martell, A. and Smith, R. (1982). "Critical Stability Constants", vol 5. Plenum Press, New York.

Martonosi, A. (1968). Sarcoplasmic reticulum: IV. Solubilisation of microsomal adenosine triphosphatase. J. Biol. Chem., 243, 71-81.

Mas Oliva, J., deMeis, L. and Inesi, G. (1983). Calmodulin stimulates both adenosine 5'-triphosphate hydrolysis and synthesis catalyzed by a cardiac calcium ion dependent adenosinetriphosphatase. Biochemistry, 22, 5822-5825.

Meissner, G., Conner, G.E. and Fleischer, S. (1973). Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{2+} pump and Ca^{2+} binding protein. Biochim. Biophys. Acta, 298, 246-269.

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

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

Mines, G.R. (1911). On the replacement of calcium in certain neuro-muscular mechanisms by allied substances. J. Physiol. (London), 42, 251-266.

Mitchinson, C., Wilderspin, A.F., Trinnaman, B.J. and Green, N.M. (1982). Identification of a labelled peptide after stoichiometric reaction of fluorescein isothiocyanate with the Ca^{2+}-dependent adenosine triphosphatase of sarcoplasmic reticulum. FEBS Lett., 146, 87-92.

Molla, A., Caponi, J.P. and Demaille, J.G. (1985). Cardiac sarcoplasmic-reticulum calmodulin-binding proteins. Biochem. J., 226, 859-865.

Molla, A. and Demaille, J.G. (1986). Characterisation and partial purification of cardiac sarcoplasmic reticulum phospholamban kinase. Biochemistry, 25, 3415-3424.

Moller, J.V., Andersen, J.P. and LeMaire, M. (1982). The sarcoplasmic reticulum Ca^{2+}-ATPase. Mol. Cell. Biochem., 42, 83-107.

Movsesian, M.A., Nishikawa, M. and Adelstein, R.S. (1984). Phosphorylation of phospholamban by calcium-activated, phospholipid-dependent protein kinase. Stimulation of cardiac sarcoplasmic reticulum calcium uptake. J. Biol. Chem, 259, 8029-8032.

Murray, E., Gorsky, J.P., and Penniston, J.T. (1983). High-affinity Ca^{2+}-stimulated and Mg^{2+}-dependent ATPase from rat osteosarcoma, 6, 527-533.

Nakajima, Y. and Endo, M. (1973). Release of calcium induced by 'depolarisation' of sarcoplasmic reticulum membrane. Nature (New Biol.), 246, 216-218.

Nakamura, J., Wang, T., Tsai, L-I. and Schwartz, A. (1983). Properties and characterization of a highly purified sarcoplasmic reticulum Ca^{2+}-ATPase from dog cardiac and rabbit skeletal muscle. J. Biol. Chem., 258, 5079-5083.

Nayler, W.G. (1977). The plasma membrane and sarcoplasmic reticulum of muscle. In: Mammalian Cell Membrane, vol 3 (eds. G.A. Jamieson, D.M. Robinson). Butterworths, London. pp.147-171.

Nayler, W.G. and Dresel, P.E. (1984). Ca^{2+} and the sarcoplasmic reticulum. J. Mol. Cell. Cardiol., 16, 165-174.

Nayler, W.G., Dunnet, J. and Berry, D. (1975). The calcium accumulating activity of subcellular fractions isolated from rat and guinea pig heart muscle. J. Mol. Cell. Cardiol., 7, 275-288.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. Science, 233, 305-312.

Parkinson, D.K. and Radde, I.C. (1971). Properties of a Ca^{2+}- and Mg^{2+}-activated ATP-hydrolyzing enzyme in rat kidney cortex. Biochim. Biophys. Acta, 242, 238-246.

Peachey, L.D. (1965). The sarcoplasmic reticulum and transverse tubules of the frog sartorius. J. Cell Biol., 25, 209-231.

Penpargkul, S. (1979). Effects of adenine nucleotides on calcium binding by rat heart sarcoplasmic reticulum. Cardiovasc. Res., 13, 243-253.

Pershadsingh, H.A. and McDonald, J.M. (1980). A high affinity calcium-stimulated magnesium-dependent adenosine triphosphatase in rat adipocyte plasma membranes. J. Biol. Chem., 255, 4087-4093.

Pick, U. and Racker, E. (1979). Inhibition of the (Ca^{2+})ATPase from sarcoplasmic reticulum by dicyclohexylcarbodiimide: Evidence for location of the Ca^{2+} binding site on a hydrophobic region. Biochemistry, 18, 108-113.

Plank, B., Pifl, C., Hellmann, G., Wyskovsky, W., Hoffmann, R. and Suko, J. (1983). Correlation between calmodulin-dependent increase in the rate of calcium transport and calmodulin-dependent phosphorylation of cardiac sarcoplasmic reticulum. Eur. J. Biochem., 136, 215-221.

Porter, K.R. and Pallade, G.E. (1957). Studies on the endoplasmic reticulum III. Its form and distribution in striated muscle cells. J. Biophys. Biochem. Cytol., 3, 269-300.

Racker, E. (1972). Reconstitution of a calcium pump with phospholipids and a purified Ca^{2+} adenosine triphosphatase from sarcoplasmic reticulum. J. Biol. Chem., 247, 8198-8200.

Rasmussen, H. (1986). The calcium messenger system, part 1. New Engl. J. Med., 314, 1094-1101.

Reeves, J.P. (1985). The sarcolemmal sodium-calcium exchange system. In: Current Topics in Membrane Transport, Volume 25. (ed. F. Bronner). Academic Press, Orlando. pp.77-127.

Ringer, S. (1883). A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. J. Physiol. (London), 4, 29-42.

Saito, K., Imamura, Y. and Kawakita, M. (1984). Effect of temperature and added ligands on the susceptibility of Ca^{2+}, Mg^{2+}-adenosine triphosphatase of the sarcoplasmic reticulum to trypsin. J. Biochem., 95, 1297-1304.

Salama, G. and Scarpa, A. (1985). Magnesium permeability of sarcoplasmic reticulum. Mg^{2+} is not countertransported

during ATP-dependent Ca^{2+} uptake by sarcoplasmic reticulum. J. Biol. Chem., 260, 11697-11705.

Scott, T.L. and Shamoo, A.E. (1982). Disruption of energy transduction in sarcoplasmic reticulum by trypsin cleavage of $(Ca^{2+}+Mg^{2+})$-ATPase. J. Memb. Biol., 64, 137-144.

Shamoo, A.E. and Ambudkar, I.S. (1984). Regulation of calcium transport in cardiac cells. Can J. Physiol. Pharmacol., 62, 9-22.

Shigekawa, M. and Dougherty, J.P. (1978). Reaction mechanism of Ca^{2+}-dependent ATP hydrolysis by skeletal muscle sarcoplasmic reticulum in the absence of added alkali metal salts. III. Sequential occurence of ADP-sensitive and ADP-insensitive phosphoenzymes. J. Biol. Chem., 253, 1458-1464.

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

Shigekawa, M., Wakabayashi, S. and Nakamura, H. (1983). Reaction mechanism of Ca^{2+}-dependent adenosine triphosphatase of sarcoplasmic reticulum ATP hydrolysis with CaATP as a sustrate and role of divalent cation. J. Biol. Chem., 258, 8698-8707.

Souza, D.O.G. and deMeis, L. (1976). Calcium and magnesium regulation of phosphorylation by ATP and ITP in sarcoplasmic reticulum vesicles. J. Biol. Chem., 251, 6355-6359.

Stewart, P.S. and MacLennan, D.H. (1974). Surface particles of sarcoplasmic reticulum membranes. Structural features of the adenosine triphosphatase. J. Biol. Chem., 249, 985-993.

Stewart, P.S., MacLennan, D.H. and Shamoo, A.E. (1976). Isolation and characterisation of tryptic fragments of the adenosine triphosphatase of sarcoplasmic reticulum. J. Biol. Chem., 251, 712-719.

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

Sumida, M., Wang, T., Mandel, F., Froelich, J.P. and Schwartz, A. (1978). Transient kinetics of Ca^{2+} transport of sarcoplasmic reticulum. A comparison of cardiac and skeletal muscle. J. Biol. Chem., 253, 8772-8777.

Suzuki, T. and Wang, J.H. (1986). Stimulation of bovine cardiac sarcoplasmic reticulum Ca^{2+} pump and blocking of phospholam-

ban phosphorylation and dephosphorylation by a phospholamban monoclonal antibody. J. Biol. Chem., 261, 7018-7023.

Tada, M. and Inui, M. (1983). Regulation of calcium transport by the ATPase-phospholamban system. J. Mol. Cell. Cardiol., 15, 565-575.

Tada, M., Inui, M., Yamada, M., Kadoma, M-A, Kuzuya, T., Abe, H. and Kakiuchi, S. (1983). Effects of phospholamban phosphorylation catalysed by adenosine 3':5'-monophosphate- and calmodulin-dependent protein kinases on calcium transport ATPase of cardiac sarcoplasmic reticulum. J. Mol. Cell. Cardiol., 15, 335-345.

Tada, M., Kadoma, M-A, Inui, M., Yamada, M. and Ohmori, F. (1982). Ca^{2+}-dependent ATPase of sarcoplasmic reticulum. In: "Transport and Bioenergetics in Biomembranes" (eds. R. Sato, Y. Kagawa). Plenum Press, New York. pp. 137-164.

Tada, M. and Katz, A.M. (1982). Phosphorylation of the sarcoplasmic reticulum and sarcolemma. Ann. Rev. Physiol., 44, 401-423.

Tada, M., Kirchberger, M.A. and Katz, A.M. (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, M.A., Repke, D.I. and Katz, A.M. (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., Yamada, M., Ohmori, F., Kuzuya, T. and Abe, H. (1979). Mechanism of cyclic AMP regulation of active calcium transport by cardiac sarcoplasmic reticulum. In: Calcium Flux Across Biomembranes (eds. Y. Mukohata, L. Packer). Academic Press, New York. pp.179-190.

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

Thorley-Lawson, D.A. and Green, N.M. (1973). Studies on the location and orientation of proteins in the SR. Eur. J. Biochem., 40, 403-413.

Thorley-Lawson, D.A. and Green, N.M. (1975). Separation and characterisation of trypsin fragments from the adenosine triphosphatase of sarcoplasmic reticulum. Eur. J. Biochem., 59, 193-200.

Ueno, T. and Sekine, T. (1978). Study on calcium transport by

sarcoplasmic reticulum vesicles using fluorescent probes. J. Biochem., 84, 787-794.

Velema, J., Blom, J.J. and Zaagsma, J. (1985). Comparison of (Ca^{2+}-Mg^{2+})-ATPase and Ca^{2+}-ATPase in rat cardiac sarcolemma. Int. J. Biochem., 17, 1091-1096.

Verma, A.K. and Penniston, J.T. (1981). A high affinity Ca^{2+}-stimulated and Mg^{2+}-dependent ATPase in rat corpus luteum plasma membrane fractions. J. Biol. Chem., 256, 1269-1275.

Vianna, A.L. (1975). Interaction of calcium and magnesium in activating and inhibiting the nucleotide triphosphatase of sarcoplasmic reticulum vesicles. Biochim. Biophys. Acta, 410, 389-406.

Wang, J.H. and Waisman, D.H. (1979). Calmodulin and its role in the second messenger system. Curr. Top. Cell. Regul., 15, 47-107.

Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974). Reconstitution of a calcium pump using defined membrane components. Proc. Natl. Acad. Sci., USA, 71, 622-626.

Wasserman, R.H. and Fullmer, C.S. (1982). Vitamin D-induced calcium binding protein. In: Calcium and Cell Function, vol II (ed. W.Y. Cheung). Academic Press, New York. pp.175-216.

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

Wegener, A.D. and Jones, L.R. (1984). Phosphorylation-induced mobility shift in phospholamban in sodium dodecyl-sulfate-polyacrylamide gels. J. Biol. Chem., 259, 1834-1841.

Wegener, A.D., Simmerman, H.K.B., Liepnieks, J. and Jones, L.R. (1986). Proteolytic cleavage of phospholamban purified from canine cardiac sarcoplasmic reticulum vesicles. Generation of a low resolution model of phospholamban structure. J. Biol. Chem., 261, 5154-5159.

Winegrad, S. (1968). Intracellular calcium movements of frog skeletal muscle during recovery from tetanus. J. Gen. Physiol., 51, 65-83.

Wnuk, W., Cox, J.A. and Stein, E.A. (1982). Parvalbumins and other soluble high affinity calcium binding proteins from muscle. In: Calcium and Cell Function, vol II (ed. W.Y. Cheung). Academic Press, New York. pp.243-278.

Wray, H. and Gray, R.R. (1977). Cyclic AMP stimulation of

membrane phosphorylation and Ca^{2+}-activated, Mg^{2+}-dependent ATPase in cardiac sarcoplasmic reticulum. Biochim. Biophys. Acta, 461, 441-459.

Yamamoto, T., Yantorno, R.E. and Tonomura, Y. (1985). Oligomeric form of the Ca^{2+}, Mg^{2+}-dependent ATPase in sarcoplasmic reticulum. In: Structure and Function of Sarcoplasmic Reticulum (eds. S. Fleischer, Y. Tonomura). Academic Press, Orlando. pp.163-177.