MODULATIONS OF CALCIUM BINDING AND OF ENERGY COUPLING BY THE CALCIUM PUMP OF SARCOPLASMIC RETICULUM

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by

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TO MY PARENTS

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ABSTRACT

Calcium transport in isolated SR vesicles is tightly coupled to ATP hydrolysis with a stoichiometry of two Ca^{2+} ions per molecule of ATP hydrolysed. A mechanism of random binding of Ca^{2+} and ATP to the calcium pump has been postulated. Different substrates which support Ca^{2+} transport have, however, been shown to have differing Ca^{2+} concentration dependence of hydrolysis and of Ca^{2+} transport. In addition, it has been shown that the affinity of the calcium pump is greatly enhanced by the presence of the Ca^{2+}-chelating agent ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) (Berman, M.C. (1982) J. Biol. Chem. 257, 1953-1957). The mechanism of Ca^{2+} binding to the (Ca^{2+},Mg^{2+})-ATPase has been investigated in this study by determining the calcium dependence of Ca^{2+} transport and the effects of various substrates on this parameter. Ca^{2+} transport has been measured by means of the calcium-stat technique (Berman, M.C., and Aderem, A.A. (1981) Anal. Biochem. 115, 297-301), in the absence of chelating agents, using a calcium-specific electrode as end-point detector. Free Ca^{2+}-ion dependence of transport showed sigmoidal behaviour with high apparent cooperativity ($n_{H(Ca^{2+})} > 5$) and dissociation constant ($[Ca^{2+}]_{0.5}$) of 0.95 μM at 5 mM ATP and 5 mM $MgCl_2$. Inclusion of $CaEGTA^{2-}$, 100 μM, in the assay medium decreased both $n_{H(Ca^{2+})}$ and $[Ca^{2+}]_{0.5}$. $[Ca^{2+}]_{0.5}$ values increased from 1 to 5 μM in the order ATP > ITP > acetylphosphate > p-nitrophenylphosphate when transport was supported by these substrates. Increasing ATP, in the range 1 to 10 mM, at constant 1 mM $MgCl_2$ decreased $[Ca^{2+}]_{0.5}$ from 1.5 to 0.15 μM. Increasing MgATP in the range 1 to 10 mM did not have any significant effect on $[Ca^{2+}]_{0.5}$. Changes in the concentration of acetylphosphate and p-nitrophenylphosphate had no effect on $[Ca^{2+}]_{0.5}$ while the calcium chelators pyrophosphate and citrate decreased $[Ca^{2+}]_{0.5}$ 3- to 5-fold. The naturally occurring calcium-chelating proteins calmodulin and parvalbumin were without effect.

The effects of substrates on the apparent affinity of the calcium-stimulatory sites of the (Ca^{2+},Mg^{2+})-ATPase are correlated with their ability to bind Ca^{2+} and form a calcium-anion complex. These effects have been masked in previous studies by the presence of $CaEGTA^{2-}$. It

is suggested that the calcium chelating anion allows the pump to overcome thermodynamic barriers which exist at low external free calcium concentrations.

The calcium-stat and pH-stat techniques were extended to investigate the stoichiometry of the Ca^{2+} pump. Coupling ratios are conventionally measured by kinetic methods in which the rate of ATP-dependent Ca^{2+} transport is related to Ca^{2+}-dependent ATPase activity. We have developed an alternate method in which the stoichiometric amount of Ca^{2+} transported per pulse of ATP infused is measured by the calcium-stat method using either a Ca^{2+}-electrode or arsenazo III as end-point detectors. Maximum coupling ratios of 1.81 $\pm$ 0.13 occurred at pH 6.8, 25°C, and in the presence of saturating Ca^{2+} concentrations. In an analogous experiment, coupling was determined by a Ca^{2+}-pulse method in which the amount of ATP hydrolysed per pulse of Ca^{2+} is obtained by means of a pH-stat procedure. Under conditions of saturating ATP concentrations, a maximum coupling ratio of 1.78 $\pm$ 0.12 was obtained. Coupling ratios were constant between pH 5.5 and pH 7.2, but decreased at alkaline pH in a manner suggesting participation of an ionisable group, with an apparent pKa of 7.9, in the coupling process. The coupling ratio was unaltered between 6°C and 30°C at pH 6.8, but decreased at higher temperatures, falling to 0.4 at 42°C. Uncoupling by alkaline pH and high temperatures was reversible. The coupling process was Ca^{2+} dependent, with a $[Ca^{2+}]_{0.5}$ of 0.12 μM. A Hill coefficient of 2.0 for the calcium dependence of coupling, implies that both Ca^{2+}-transport sites have to be saturated for optimal coupling.

Ca^{2+} ions, which were transported into vesicles under conditions resulting in low coupling ratios, were retained as the calcium oxalate precipitate following complete hydrolysis of substrate. The first order rate constant for Ca^{2+} efflux (0.7 min^{-1}), into medium containing EGTA and of $^{45}Ca^{2+} \leftrightarrow {}^{40}Ca^{2+}$ exchange, following maximal loading with calcium oxalate, were independent of pH. In addition, sub-integral coupling ratios could not be accounted for by the presence of a population of unsealed vesicles. The observed changes in coupling ratio as a function of pH, temperature and of Ca^{2+} concentration, cannot readily by explained on the basis of a 'pump-leak' model. Rather, the

Ca^{2+}-ATPase of isolated sarcoplasmic reticulum vesicles appears to pump Ca^{2+} ions with variable stoichiometry, that is dependent upon its thermodynamic loading. It is suggested that variable stoichiometry, as a result of 'slippage', is an integral property of the Ca^{2+} pump. Variable stoichiometry is inconsistent with active transport models that either include a single reaction cycle, in which vectorial and scalar reactions are obligatorily coupled, or which propose a pump having two conformations with alternating affinities and orientations of Ca^{2+}-binding sites.

The following manuscripts have arisen directly or indirectly out of the work for this thesis:

1. A.M. Fourie, S. Meltzer, M.C. Berman, and A.I. Louw (1983) The Effect of Cardiotoxin on (Ca^{2+},Mg^{2+})-ATPase of the Erythrocyte and Sarcoplasmic Reticulum. Biochemistry International 6, 581-591.

2. Sybella Meltzer and Mervyn C. Berman (1984) Effects of pH, Temperature and Calcium Concentration on the Stoichiometry of the Calcium Pump of Sarcoplasmic Reticulum. J. Biol. Chem. (in press).

3. Sybella Meltzer and Mervyn C. Berman (1983) Determinations of Coupling Ratios of the Calcium Pump of Sarcoplasmic Reticulum by Pulse Methods. Submitted to Anal. Biochem.

ABBREVIATIONS

AcP	Acetylphosphate
AMPCPP	Adenosine 5'-(α, β methylene) triphosphate
AMPPNP	Adenosine 5'-(β, γ-imino) triphosphate
Arsenazo III	2,2'-(1,8-dihydroxy-3,6-disulfo-2,7-naphthalene-bis (azo))-dibenzenearsonic acid
$[Ca^{2+}]_{free}$	Free Ca^{2+} concentration
CCCP	Carbonyl cyanide-m-chlorophenylhydrazone
$C_{12}E_8$	Dodecyl octaethyl glycol monoether
CFC	Complex of fluorescamine with cycloheptaamylose
DMSO	Dimethylsulfoxide
dNPP	Dinitrophenylphosphate
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid
EP	Phosphoenzyme
ESR	Electron spin resonance
FAP	Furylacryloyl phosphate
FITC	Fluorescein isothiocyanate
Hepes	N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid
Mops	3-(N-morpholino) propanesulfonic acid
NTP	Nucleoside triphosphate
P_i	Inorganic phosphate
Pipes	1,4-piperazinediethane sulfonic acid
pNPP	p-nitrophenylphosphate
PP_i	Pyrophosphate
SDS	Sodium dodecyl sulphate
SR	Sarcoplasmic reticulum
TnC_{DANZ}	Dansylaziridine-labelled troponin C

CONTENTS

1.0 INTRODUCTION

1.1. STRUCTURE AND COMPOSITION OF THE SARCOPLASMIC MEMBRANES

The sarcoplasmic reticulum (SR) of striated muscle plays a central role in control of contractile activity. It acts as an intracellular sink for calcium during relaxation and releases Ca^{2+} during contraction. This highly differentiated endoplasmic reticulum is a self-contained network with a continuous hollow interior which surrounds each muscle fibril (Fig. 1) (Porter, 1961). The SR is fragmented at longitudinal intervals of one sarcomere in length by the transverse tubular or T-tubular system (Porter, 1961; Franzini-Armstrong, 1980). On each side of the T-tubules there are enlarged areas known as cisternae, with branched areas between the cisternae known as the longitudinal elements. The juncture of the three membranes (two terminal cisternae and one T-tubule) is referred to as the triad. The junction between the SR and the T-tubules is known as a junctional gap and has a width of 100-200 Å. Periodic densities, referred to as 'feet', cross the junctional gap to join the SR and T-tubular membranes (Franzini-Armstrong, 1980). Direct communication between the SR, the T-tubules and the feet have been postulated (Schneider and Chandler, 1973; Mathias et al., 1979).

The view has developed, starting with the experiments of Huxley and Taylor (1958) and Huxley and Straub (1958), that, under physiological conditions, contraction in skeletal muscle is triggered by depolarisation of the membranes of the T-tubules. This results in the release of Ca^{2+} into the myoplasm from its intracellular storage location, the SR, and thus activating the contractile proteins (Schneider and Chandler, 1973). (See Fuchs (1974) and Ebashi (1980) for a review on the possible mechanism involved in excitation-contraction coupling.)

SR membranes are fragmented when the muscle is homogenized, but reseal into smaller vesicles containing the Ca^{2+} transport system in the proper orientation, such that Ca^{2+} is transported into the vesicles (Nagai et al., 1960; Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1963). SR vesicles can be isolated by differential centrifugation of the homogenized muscle in an isotonic salt solution in the presence of sucrose (McFarland and Inesi, 1971), while contaminating actomyosin may be removed by solubilising with 0.6 M KCl (Martonosi, 1968). Isolated SR vesicles have a mean diameter of 1000 nm (Hasselbach and Elfvin, 1967;

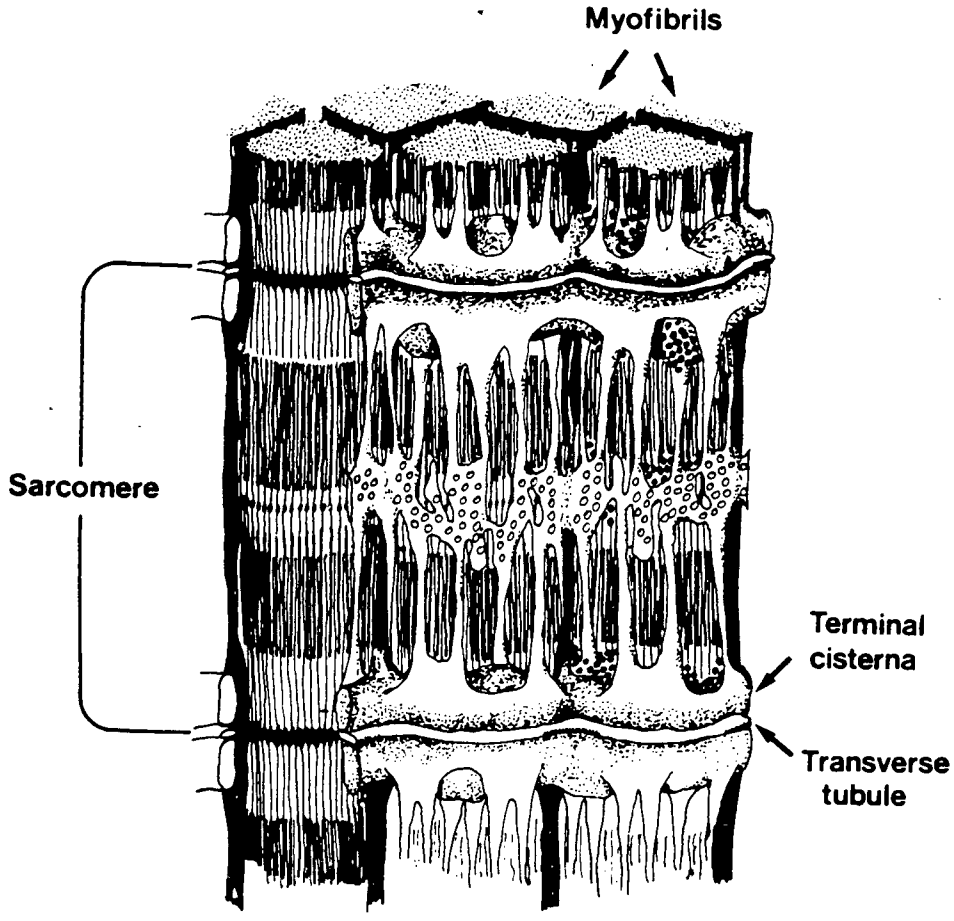


Figure 1: The sarcoplasmic reticulum surrounding the muscle fibrils (Lehninger, 1975).

Deamer and Baskin, 1969) and a volume of 4 µl/mg protein (Duggan and Maronosi, 1970; Diamond et al., 1980).

1.1.2. Composition of the Sarcoplasmic Reticulum Membrane

(a) Protein Composition

The SR membrane is composed of several intrinsic proteins of which the (Ca^{2+}, Mg^{2+})-ATPase (MacLennan, 1970), a proteolipid (MacLennan et al., 1972) and two glycoproteins (Michalak et al., 1980; Campbell and MacLennan, 1981) have been identified and purified. Two extrinsic proteins, calsequestrin (MacLennan and Wong, 1971) and a high affinity Ca^{2+}-binding protein (Ostwald and MacLennan, 1974) have also been characterised (Eletr and Inesi, 1972; Meissner et al., 1973; Meissner, 1973; Inesi and Scales, 1974). The latter proteins are loosely associated with the membrane and are easily extracted by mild treatment with detergents (Singer and Nicolson, 1972). Meissner (1975) found that SR vesicles separated into two different populations of high and low density on a sucrose-density gradient, and that the two populations had different protein compositions and different lipid-to-protein ratios. Electron microscope and calcium-binding studies suggested that these fractions were derived from different parts of the SR, such as the longitudinal sections and terminal cisternae (Meissner, 1975).

The (Ca^{2+}, Mg^{2+})-ATPase, which constitutes 60-90% of the total protein composition of SR vesicles, was first isolated as a proteolipid by MacLennan (1970) through the use of deoxycholate and salt fractionation. Its molecular weight, determined by gel electrophoresis, is approximately 100 000 daltons (McFarland and Inesi, 1971; Meissner and Fleischer, 1971) but controversy as to the exact value still exists (Le Maire et al., 1976; Møller et al., 1982). The purified ATPase catalysed ATP$\leftrightarrow$ADP exchange and was phosphorylated by (γ-^{32}P)-ATP in the presence of Ca^{2+} and Mg^{2+}. Several workers have isolated and purified the ATPase enzyme and determined its molecular weight, activity and extent of phosphorylation (For review, see MacLennan and Holland, 1975). X-ray diffraction, electron microscopy and tryptic digestion studies suggest that the ATPase enzyme is asymmetrically distributed across the membrane with more than half its mass exposed to the outside and very little mass at the inner surface of the membrane (Hasselbach and Elfvin, 1967; Dupont et al., 1973;

Worthington and Liu, 1973; Klip et al., 1980).

It is still uncertain whether the ATPase exists within the SR membrane as an oligomer or monomer. There is increasing evidence that the ATPase is self-associated in the membrane in oligomeric form (for review, see Ikemoto, 1982; Møller et al., 1982). Each ATPase peptide, however, seems to be able to perform the whole catalytic cycle of ATP hydrolysis and Ca^{2+} transport, as a functional monomer (Jørgenson et al., 1978; Møller et al., 1980; Dean and Gray, 1980). Detailed analysis of enzyme kinetics suggests an aggregational state of the membranous ATPase. This allows enzyme modulation by protein-protein interaction (Møller et al., 1980; Yates and Duance, 1976; Boyer et al., 1982).

The membrane-associated proteolipid was first isolated by MacLennan et al. (1972), free of sugar and phospholipid, and contained 150-200 nmols fatty acid per mg protein. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis indicated a molecular weight of 6 000 while amino acid analysis showed a molecular weight of 12 000. Although the function of this proteolipid is still unknown, Racker and Eytan (1975) showed that its presence in reconstitution studies greatly improved the efficiency of the Ca^{2+} pump, serving as a coupling factor. This is in accordance with Laggner and Graham (1976) who showed that the proteolipid reduced leakage in artificial phospholipid membranes.

The glycoprotein composition of SR vesicles has been analysed by Michalak et al. (1980) using concanavalin-A binding. Four glycoproteins of M_r 160 000, 63 000, 60 000 and 53 000 have been identified. The 63 000 dalton glycoprotein is calsequestrin. The 160 000-, 60 000- and 53 000 dalton glycoproteins are invarient constituents of SR vesicles. The 53 000 dalton glycoprotein is a transmembrane protein, largely exposed to the cytoplasmic surface of vesicles. Although the 160 000 dalton glycoprotein has not been extensively studied, initial experiments suggest that it has similar properties to that of the 53 000 dalton glycoprotein. Both intrinsic glycoproteins bind azido-ATP ($8\text{-}N_3\text{-}ATP$) and thus probably contain high affinity ATP-binding sites (Campbell and MacLennan, 1983). The physiological role of these glycoproteins is not known.

Calsequestrin, composing 5-19% of the total protein of SR, is an

extrinsic, acidic glycoprotein having a molecular weight of 63 000 (MacLennan and Wong, 1971; Michalak et al., 1980). It binds approximately 900 nmoles of Ca^{2+} per mg protein with $K_d = 4x10^{-5}$ M (MacLennan and Wong, 1971). It was originally suggested that calsequestrin serves to bind and store Ca^{2+} within the lumen of SR vesicles. Malan et al. (1975), however, showed that the different populations of SR vesicles, as originally isolated by Meissner (1975), displayed ATPase activity which was proportional to the relative content of ATPase and not of calsequestrin. SR vesicles, with higher calsequestrin content, were less able to maintain the Ca^{2+} gradient, in the presence of ATP, whilst Ca^{2+}-binding capacity was greater in the absence of ATP.

A second acidic protein, which has high affinity for Ca^{2+} ($K_d = 2.5 \times 10^{-6}$ M), but low capacity (16-22 nmols Ca^{2+} per mg protein), was isolated and characterised by Ostwald and MacLennan (1974) and Ikemoto et al. (1974). It has a molecular weight of 55 000 dalton and is known as the high-affinity or M_{55} protein. Considerable speculation as to whether calsequestrin and the M_{55} protein are located at the exterior or interior surface of SR membranes still exists (Duggan and Matonosi, 1970; MacLennan and Wong, 1971; Thorley-Lawson and Green, 1973; Stewart and MacLennan, 1974; MacLennan and Holland, 1975).

(b) Lipid Composition

The ATPase enzyme is surrounded by lipids comprised of 80% phospholipids and 20% neutral lipids, the latter being 95% cholesterol (Tada et al., 1978). It has been shown that, of the phospholipids, phosphatidylcholine (65-73%) and phosphatidylethanolamine (12-19%) are required for maximum transport activity (Knowles et al., 1976). Ca^{2+}-accumulating vesicles can be reconstituted from purified enzyme and phospholipids, suggesting that they are essential for activity (Racker, 1972; Meissner and Fleischer, 1974; Warren et al., 1974). Electron spin resonance (ESR) studies, using spin-labelled analogues of stearic acid and spin probes, attached to the ATPase protein, suggest that Ca^{2+}-ATPase activity is related to the fluidity of membrane lipids. Restriction of mobility of phospholipids by decreasing temperature (Inesi et al., 1973) and delipidation (Seelig and Hasselback, 1971) inhibits ATPase activity. Hidalgo and co-workers (1976) demonstrated that SR lipids

are involved in the decomposition of the phosphoenzyme during the ATPase reaction cycle. The role of phospholipids in the function of the (Ca^{2+}, Mg^{2+})-ATPase has recently been reviewed (Bennet et al., 1980).

The concept of an immobile lipid 'annulus' was introduced by Metcalf and Warren (1977), who showed that a decrease in lipid, by cholate extraction, from a molar ratio of approximately 115 phospholipids per ATPase (the physiological ratio) to 30 phospholipids per ATPase, did not cause loss of activity. The latter 30 phospholipids per ATPase protein are considered to be highly immobile and are referred to as a lipid annulus. Depletion of these lipids leads to enzyme inactivation.

(c) Proteolipid Fragmentation of the ATPase

The Ca^{2+}-ATPase has been systematically dissected to localize distinct domains to catalytic (ATPase) and ionophoric (Ca^{2+} translocation) functions (Fig. 2). Exposure to trypsin results in degradation of the 100 000 (100 K) dalton protein into two fragments of M_r = 55 000 (55 K) and 45 000 (45 K), with the subsequent appearance of fragments of M_r = 30 000 (30 K) and 20 000 (20 K) (Thorley-Lawson and Green, 1973; Inesi and Scales, 1974; Shamoo et al., 1977; Stewart and MacLennan, 1974; Stewart et al., 1976). The 55 K- and 45 K dalton fractions result from a single cleavage of the 100 K dalton ATPase enzyme, without loss of activity (Shamoo et al., 1976; Stewart et al., 1976). The 30 K and 20 K molecular weight fragments result from further cleavage of the 55 K dalton fragment. The 55 K- and 20 K dalton fragments have Ca^{2+}-ionophore activity, while the 45 K dalton fragment does not. The 30 K dalton fragment acts as a non-selective ionophore (Shamoo et al., 1976). The 20 K dalton fragment is further degraded by means of cyanogen bromide into fragments of molecular mass less than 2 000 dalton, which still exhibits ionophore activity. The M_r = 55 K and 30 K fragments contain the site of phosphorylation and the hydrolytic site of the enzyme, since both these fragments can be phosphorylated by (γ-^{32}P)-ATP (Thorley-Lawson and Green, 1973).

Stewart and co-workers (1976), by the use of antibodies against the various tryptic fragments obtained, were able to show that the 55 K

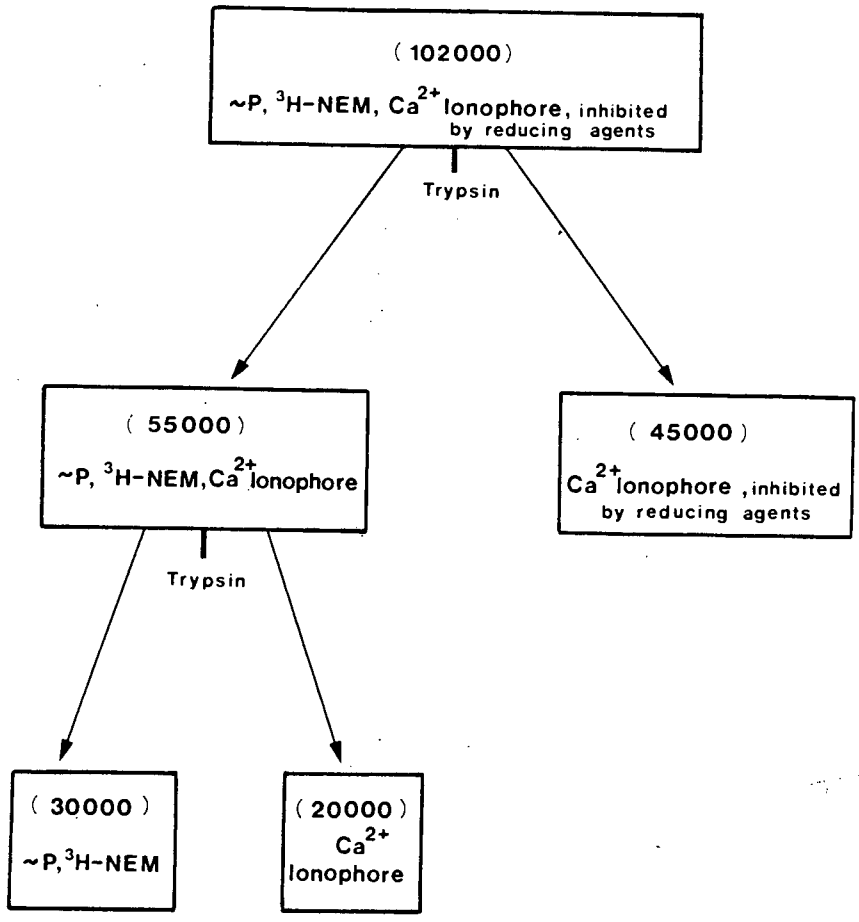


Figure 2: Localisation of functional assignments in tryptic digestion fragments of (Ca^{2+},Mg^{2+})-ATPase.

~P, phosphorylation site. ^{3}H-NEM, N-[^{3}H]ethylmaleimide binding sites (Shamoo and Murphy, 1979).

dalton fragment was largely exposed, while the 20 K- and 55 K dalton fragments were only partially exposed. Shamoo et al, (1977) have suggested that the ATPase enzyme has a hydrophobic channel, the 45 K dalton fragment, spanning the membrane. They have postulated that the 55 K dalton portion exists on the external surface and contains both the 30 K dalton phosphorylating site and the 20 K dalton ionophoric site, which acts as a 'gate' and is partially buried within the hydrophobic mouth of the 45 K dalton fragment. This would explain why separation of the 55 K dalton fragment from the 45 K dalton fragment does not lead to loss of activity. Further cleavage of the 55 K dalton fragment, to form the two fragments of M_r = 30 K and 20 K, results in uncoupling of ATPase activity from Ca^{2+} transport activity, since the gate no longer interacts with the hydrolytic site.

1.2. THE REACTION MECHANISM OF THE CALCIUM PUMP OF SARCOPLASMIC RETICULUM OF SKELETAL MUSCLE

The active transport of Ca^{2+} ions by SR vesicles isolated from rabbit skeletal muscle is tightly coupled to ATP hydrolysis by the membrane bound (Ca^{2+}, Mg^{2+})-ATPase enzyme (ATP phosphohydrolase, EC. 3.6.1.3). The γ-phosphate of ATP is transferred to the β-carboxyl group of an aspartate residue (Degani and Boyer, 1973) of the ATPase to form an acid-stable phosphorylated intermediate, EP (de Meis, 1972).

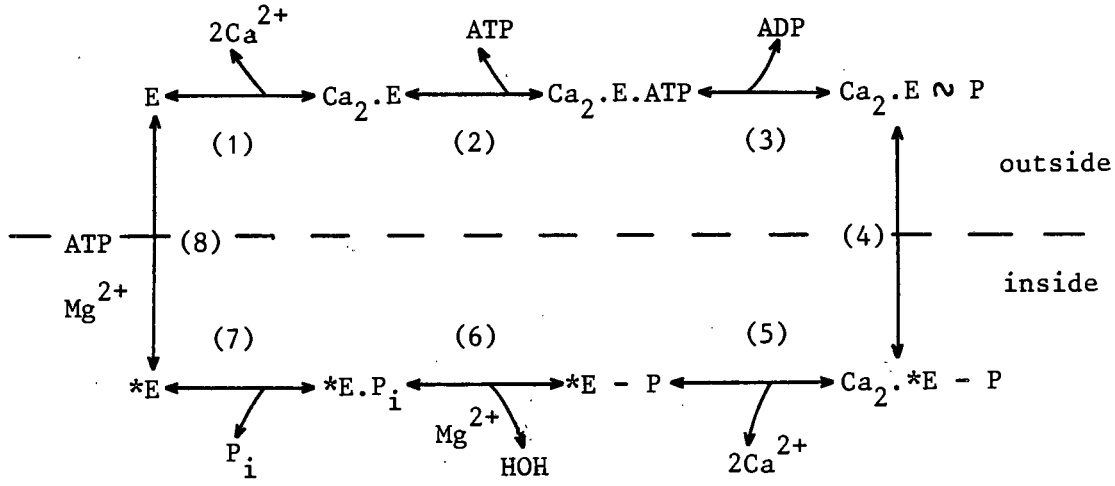
Two different types of Mg^{2+}-dependent ATPase activities are exhibited by the SR ATPase: Ca^{2+}-independent (basic) ATPase and Ca^{2+}-stimulated (extra) ATPase activities (Hasselbach and Makinose, 1961, 1962). The 'basic' ATPase activity is distinguished from the 'extra' ATPase activity in that it has a lower affinity for ATP (Weber et al., 1966; Yamamoto and Tonomura, 1967) and a different temperature coefficient and pH profile (Yamamoto and Tonomura, 1967). The Ca^{2+}-stimulated ATPase activity is inactivated by thiol reagents, while the basic activity is unaffected (Yamamoto and Tonomura, 1967; Inesi et al., 1967; Murphy, 1976). It is uncertain whether the two activities are due to two different enzymes (Seaydarian and Mommaerts, 1965; Fernandez et al, 1980) or due to different conformations of the same enzyme (Inesi et al., 1976; Froehlich and Taylor, 1976; Carvalho-Alves and Scofano, 1983; Bick et al., 1983).

The effect of Ca^{2+} on the (Ca^{2+},Mg^{2+})-ATPase is complex. Low free Ca^{2+} concentrations ($[Ca^{2+}]_{free}$) ($K_d \approx 1$ μM) result in an approximately five-fold stimulation of activity, while high concentrations (>0.1 mM) inhibit ATP hydrolysis (Yamamoto and Tonomura, 1967; Makinose, 1969; Inesi et al., 1970; Pang and Briggs, 1977). Ca^{2+} activation of ATPase activity displays non-Michaelis-Menten kinetics, showing positive cooperativity with a Hill coefficient $n_{H(Ca^{2+})}$ of approximately two (The and Hasselbach, 1972; Neet and Green, 1977). This is consistent with a coupling ratio of 2, which is the number of Ca^{2+} ions translocated across the SR membrane for every ATP molecule hydrolysed (Martonosi and Feretos, 1964; Weber et al., 1966; Yamada et al., 1970; Kurzmack and Inesi, 1977; Meltzer and Berman, 1983).

The ATP-concentration dependence of ATPase activity is biphasic, showing an apparent saturation at 50-100 μM MgATP and further activation in the millimolar range (Taylor and Hattan, 1979; Verjovski-Almeida and Inesi, 1979). This biphasic curve is interpreted as being due to a high affinity catalytic site and a low affinity regulatory or 'allosteric' site (Inesi et al., 1967; 1970; de Meis and de Mello, 1973; Froehlich and Taylor, 1975; Carvalho et al., 1976; Souza and de Meis, 1976; Neet and Green, 1977; Dupont, 1977; Taylor and Hattan, 1979; Verjovski-Almeida and Inesi, 1979).

There are three main steps involved in the active transport of Ca^{2+} ions across the SR membrane: (i) binding and recognition at the external surface by the high affinity Ca^{2+}-binding sites, (ii) translocation, and finally (iii) release from the low affinity binding sites on the other side of the membrane, into the vesicular lumen. This vectorial process is achieved by a conformational change, due to phosphorylation of the protein, converting the high affinity binding sites into low affinity sites (Ikemoto, 1974; 1975).

A composite reaction sequence, based on all the available evidence, to explain the vectorial coupling of ATP hydrolysis with Ca^{2+} transport in SR vesicles, has been proposed (de Meis and Boyer, 1978).



SCHEME I

The following data support this model:

(i) Ligand-mediated conformational changes of the (Ca^{2+}, Mg^{2+})-ATPase

The Ca^{2+}-dependent ATPase is represented by two conformations: E, when the Ca^{2+}-binding sites face the outer surface and has high affinity for Ca^{2+} (K_m = 0.1 - 0.3 μM) and *E, when it faces the inner surface and has low affinity for Ca^{2+} (K_m = 1 - 3 mM) (de Meis and Carvalho, 1974; Ikemoto, 1975; de Meis and Sorenson, 1975). Phosphorylation of the enzyme results in an approximate three orders of magnitude decrease in its affinity for Ca^{2+}, indicating that the conformational change (i.e. the interconversion of E to *E) and thus Ca^{2+} translocation, is mediated by phosphorylation (Ikemoto, 1975; 1976; Verjovski-Almeida and de Meis, 1977; Takakuwa and Kanazawa, 1981). The enzyme in the E form may be phosphorylated by nucleotide triphosphates (NTP) (step 3) but not by inorganic phosphate (P_i), while the *E form may be phosphorylated by P_i but not by NTP (step 7) (Masuda and de Meis, 1973; de Meis and Masuda, 1974). The hydrolytic cycle occurs on the exterior surface of the vesicles and it is assumed that phosphorylation by P_i (step 7) and NTP (step 2) occurs at a common site.

(ii) The phosphoenzyme intermediate

The (Ca^{2+}, Mg^{2+})-ATPase catalyses phosphate exchange between ADP and ATP (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1962; 1963).

This exchange exhibits the same dependence on Ca^{2+} concentration as ATP hydrolysis and Ca^{2+} transport, suggesting that it is a true reaction intermediate (Hasselbach and Makinose, 1961; Hasselbach, 1964; Degani and Boyer, 1973). One mole of phosphate is incorporated as an acid-stable intermediate, E-P, for every ATP molecule hydrolysed by the isolated ATPase (MacLennan, 1970). Maximal levels of phosphoenzyme, corresponding to 4 nmol/mg protein, are obtained under optimal conditions of saturating substrate concentrations, as well as by kinetic manipulations favouring formation of the E-P intermediate over breakdown (e.g. acid pH) (Watanabe et al., 1981). The phosphoryl group is covalently bound to a β-carboxyl of an aspartyl residue (Degani and Boyer, 1973; Bastide et al., 1973) to form an acylphosphoprotein similar to that formed by the (Na^+, K^+)-ATPase (Post et al., 1972). The phosphoprotein is alkali labile (Yamamoto and Tomomura, 1967; 1968; Makinose, 1969) and is hydrolysed by hydroxylamine to form the hydroxamate (Yamamoto and Tonomura, 1967; Makinose, 1969; Yamamoto et al., 1971).

Two types of EP intermediates in the enzyme reaction cycle have been described (Masuda and de Meis, 1973; de Meis and Tume, 1977; Takakuwa and Kanazawa, 1979; Yamada and Ikemoto, 1980). The one formed first has a high affinity for Ca^{2+} and corresponds to the transport site being available at the exterior surface of the vesicles. The second, formed subsequently, has low affinity for Ca^{2+} and corresponds to the transport sites facing the interior surface of the membrane (Yamada and Ikemoto, 1980). The two EP intermediates have been termed ADP-sensitive and ADP-insensitive, respectively, since only the ADP-sensitive intermediate reacts with ADP to form ATP through the back reaction (Shigekawa et al., 1978). The ADP-insensitive EP has been further resolved into a Mg^{2+}-sensitive and Mg^{2+}-insensitive form (Yamada and Ikemoto, 1980).

(iii) Sequential Ca^{2+} translocation, release and dephosphorylation

The conversion from an ADP-sensitive EP to an ADP-insensitive EP, is accompanied by a dramatic decrease in the Ca^{2+} affinity of the enzyme and probably corresponds to the Ca^{2+} translocation step (Yamada and Tonomura, 1972; Ikemoto, 1975; 1976; Verjovski-Almeida and de Meis, 1977). Calcium is rapidly transferred to a site inaccessible to external ethyleneglycol-

bis-(β-amino-ethyl ether) N,N'-tetraacetic acid (EGTA) (Dupont, 1980; Takakuwa and Kanazawa, 1981; Takisawa and Makinose, 1981) and is then released into the vesicular lumen. The acyl-phosphoenzyme reacts with water and inorganic phosphate is released to the exterior. The enzyme undergoes another conformational change, such that the high affinity Ca^{2+}-binding sites face the exterior and the cycle can be repeated (step 8). This step has been shown to be accelerated by millimolar concentrations of ATP (Froehlich and Taylor, 1976; Scofano et al., 1979; Verjovski-Almeida et al., 1978).

(iv) The ternary $E.P_i.Mg$ complex

A study of the effect of Mg^{2+} and P_i (step 7) on EP formation indicates an inter-dependent relationship with random binding of Mg^{2+} and P_i to *E (Kolassa et al., 1979). The kinetic parameters of this reverse reaction can be fitted to a model which presumes the formation of a ternary complex, $E.P_i.Mg$, prior to phosphoprotein formation, $E\text{-}P_i.Mg$ (Punzengruber, 1978; Lacapére et al., 1981). The fact that ADP-insensitive EP decomposition is accelerated by Mg^{2+} and K^+ (Yamada and Ikemoto, 1980) and that $P_i \leftrightarrow H_2O^{18}$ exchange is rapid (Ariki and Boyer, 1980) further substantiates the postulate of an acid-labile or non-covalent EP form. However, the proposed involvement of this EP intermediate in the reaction cycle, as proposed by Froehlich and Taylor (1975; 1976), has not been substantiated (Sumida et al., 1976).

(v) Reversibility of the catalytic cycle

The forward reaction of the (Ca^{2+},Mg^{2+})-ATPase is characterised by the hydrolysis of ATP, tightly coupled to the vectorial transport of Ca^{2+} ions. The electrochemical osmotic energy, derived from this Ca^{2+} ion gradient across the membrane, can be utilised for the synthesis of ATP from its precursors, ADP and P_i, which occurs concomitantly with activated Ca^{2+} efflux (Makinose and Hasselbach, 1971; Makinose 1971; 1972; Yamada and Tonamura, 1972). This process is dependent on the saturation of the internally located low affinity Ca^{2+}-binding sites (de Meis and Tume, 1977). The stoichiometry of 2 moles of Ca^{2+} released per mole of ATP synthesised is maintained (Hasselbach, 1978).

The reverse reaction is initiated experimentally by the addition of

EGTA, which chelates the Ca^{2+} bound to the external high affinity sites of the enzyme, and promotes the E to *E conformational transition (step 8). The *E conformation of the enzyme is competent to react with inorganic phosphate and Mg^{2+} to form a phosphoenzyme, *E-P, via an *E.P_i.Mg ternary complex (steps 7 and 6) (Puzengruber et al., 1978; Boyer et al., 1977). This conformation of the phosphoenzyme is insensitive to ADP (Yamada and Ikemoto, 1980). Binding of Ca^{2+} to the low affinity internal sites (step 5) results in the conversion of the phosphoenzyme to a form capable of phosphorylating ADP (step 4) (Masuda and de Meis, 1973; de Meis and Tume, 1977; Yamada and Ikemoto, 1980). Ca^{2+} is released from the high affinity binding sites to the external medium, with concomitant release of ATP.

(vi) The role of magnesium

The role of Mg^{2+} in the composite reaction sequence (Scheme I) has not as yet been completely elucidated. It has, however, been shown to effect several steps of the catalytic cycle:

(a) MgATP is the true substrate for hydrolysis by the (Ca^{2+}, Mg^{2+})-ATPase (Weber et al., 1966; Yamamoto and Tonomura, 1967; Meissner, 1973; Vianna, 1975);

(b) Mg^{2+} is probably involved in Ca^{2+} translocation (step 4), mediated by EP (Takakuwa and Kanazawa, 1981);

(c) Mg^{2+} is a prerequisite for dephosphorylation (step 6) and for phosphorylation from P_i (step 7) (Inesi et al., 1970; Kanazawa et al., 1971; Masuda and de Meis, 1973; Kanazawa and Boyer, 1973; Froehlich and Taylor, 1975; Garrahan et al., 1976; Takakuwa and Kanazawa, 1982). It was shown that, although Mg^{2+} that activates dephosphorylation is distinct from that bound to the substrate as MgATP, it has to bind to the Mg^{2+}-binding site located on the outer surface of the membrane before Ca^{2+} dissociates from ADP-insensitive EP (before step 5) (Takakuwa and Kanazawa, 1983).

More recent studies by Guillain et al. (1982) suggest that Mg^{2+} binding induces a state in the enzyme which allows ATP to have a regulatory role.

Isolated SR vesicles have, therefore, proven suitable for the study of the molecular mechanism of active Ca^{2+} transport, since the (Ca^{2+},Mg^{2+})-ATPase is a structurally simple unit, constituting 60-90% of the membranous protein, and it is solely responsible for translocation of Ca^{2+} ions by a process tightly coupled to ATP hydrolysis. Modulations of Ca^{2+} binding and of the coupling process have been investigated in this study. Ca^{2+} binding has been studied by determining the Ca^{2+}-dependence of Ca^{2+} transport, in the absence of EGTA. This Ca^{2+}-chelating anion has been shown to have significant effects on the apparent Ca^{2+} affinity of the calcium pump (Berman, 1982) and was found to promote thermodynamic efficiency at low Ca^{2+} concentrations. Preliminary results showed that the substrate ATP also enhances the affinity of the transport system, in the absence of EGTA (Meltzer et al., 1981; Berman, 1982). The results of this study, concerning the activating effects of high amounts of ATP and other substrates and anions on the Ca^{2+}-dependence of Ca^{2+} transport by the (Ca^{2+}, Mg^{2+})-ATPase, is presented in Section 2.0.

2.0. SUBSTRATE MODULATION OF THE CALCIUM AFFINITY OF THE CALCIUM PUMP OF SARCOPLASMIC RETICULUM

2.1. INTRODUCTION

2.1.1. CALCIUM-BINDING SITES OF THE (Ca^{2+}, Mg^{2+})-ATPase

Ca^{2+} transport and Ca^{2+}-dependent ATPase activity is absolutely dependent on Ca^{2+} binding to Ca^{2+}-specific binding sites. The Ca^{2+}-binding sites have been the subject of a considerable number of investigations.

Two types of Ca^{2+}-binding sites were initially demonstrated in SR vesicles, by means of equilibrium dialysis. The majority of sites (80-90%) showed relatively weak affinity for Ca^{2+} (K_d = 0.32 mM), were inhibited by high ionic strength and were non-specific (Cohen and Selinger, 1969; Chevallier and Butow, 1971). Two Ca^{2+}-specific binding sites, which differ in their affinity for Ca^{2+}, constituted the remaining sites. In the presence of 0.6 M KCl and 1 mM $MgCl_2$, approximately 10 nmol Ca^{2+} per mg SR protein were bound to sites of higher affinity (K_d = 1.3 μM) and 100 nmol Ca^{2+} per mg protein to the sites of lower affinity (K_d = 32 μM) (Chevallier and Butow, 1971).

Later, passive binding studies showed that the purified ATPase bound 10-15 nmol Ca^{2+} per mg SR protein (K_d = 0.5 - 2.5 μM) over a range of pH and ionic conditions, in the absence of ATP (Meissner 1973; Meissner et al., 1973). This suggests that the high affinity binding site of intact SR vesicles represents binding sites of the Ca^{2+}-ATPase and, since Ca^{2+} activation of Ca^{2+} transport, ATP hydrolysis and EP formation are in the same Ca^{2+} concentration range, activation must occur at these sites. Ikemoto (1974) characterised three Ca^{2+}-binding sites on the purified ATPase at 0°C and in the absence of ATP, with binding constants of 4×10^6 M^{-1} (α-site), 5×10^4 M^{-1} (β-site) and 1×10^3 M^{-1} (γ-site). ATP increased the affinity of all the sites but decreased the apparent capacity of the α- and β-sites. At 22°C, two α-sites but no β-site were detected (Ikemoto, 1975). Ca^{2+} binding to the α-site activated Ca^{2+} transport, while binding to the γ-site inhibited activity. Ca^{2+} binding at the β-site had no effect on enzyme activity (Ikemoto, 1975).

It is inherent in the scheme used to describe the reaction sequence of the (Ca^{2+}, Mg^{2+})-ATPase (Reaction Scheme I) that the external high

affinity Ca^{2+}-binding sites re-orientate and change their affinity upon phosphorylation and Ca^{2+} translocation. This is supported by the finding of Ikemoto (1975) that, in leaky vesicles, the α-sites decrease their affinity and capacity upon phosphorylation. In addition, externally bound Ca^{2+} becomes occluded upon EP formation, as evidenced by the fact that it becomes inaccessible to EGTA (Dupont, 1980; Takakuwa and Kanazawa, 1981; Takisawa and Makinose, 1981). This can be explained, at least partly, by re-orientation of the transport sites. Reversal of the Ca^{2+} pump, resulting in ATP synthesis, requires that either (i) SR vesicles are preloaded with Ca^{2+} or (ii) that the ATPase is phosphorylated by P_i, in the absence of Ca^{2+}, in leaky vesicles, and that Ca^{2+}, sufficient to saturate low affinity Ca^{2+}-binding sites, is added subsequently. This indirect evidence supports the concept of Ca^{2+}-binding site re-orientation, such that low affinity sites face the vesicular lumen. Additional low affinity Ca^{2+}-binding sites in the non-phosphorylated enzyme have been demonstrated by Hasselbach and Koenig (1980) from short term Ca^{2+}-binding and enthalpy studies. The role of these binding sites remains to be established.

A variety of probes have been used to investigate the re-orientation of Ca^{2+}-binding sites, both in the phosphorylated enzyme (Reaction Scheme I, step 4) and in the non-phosphorylated form of the enzyme, subsequent to EP hydrolysis (step 8). This conformational change from E∿P to *E-P, or from *E to E, respectively, has been studied by means of (i) electron spin resonance (ESR) studies of iodoacetamide labelled SR (Landgraf and Inesi, 1969; Coan and Inesi, 1976, 1977), (ii) sulfhydral group specific reagents (Murphy, 1976; Thorley-Lawson and Green, 1977; Anderson and Moller, 1977), (iii) fluorescent changes of the covalently attached fluorescent probe fluorescein isothiocyanate (FITC) or the hydrophobic fluorescent probe 8-anilino-1-naphthalene sulfonic acid (ANS^-) which binds directly to the enzyme (Arav et al., 1983) and (iv) intrinsic tryptophan fluorescence (Dupont, 1976; Dupont and Leigh, 1978; Guillain et al., 1980, 1981).

ESR studies have yielded conflicting results as to the meaning of the spectral changes (Laggner et al., 1981). Landgraf and Inesi (1969) originally found that spectral changes occurred upon binding of nucleotide in the absence of Ca^{2+} and Mg^{2+}. Other workers have observed changes

induced by ATP, only in the presence of these divalent cations (Nakamura et al., 1972) and hence have related this effect to phosphorylation of the ATPase. Coan et al. (1979) provided evidence suggesting that the conformational changes induced by the simultaneous binding of nucleotide and Ca^{2+} relate to changes in the Ca^{2+}-binding sites from high to low affinity upon phosphorylation of the ATPase. Other studies, however, have shown that conformational changes are obtained even with ATP analogues, which do not support Ca^{2+} transport (Coan and Inesi, 1977; Champeil et al., 1978).

Intrinsic tryptophan fluorescence has, on the other hand, emerged as a reliable index of the *E to E transition induced by Ca^{2+} binding. The intensity of the fluorescence signal varies with enzyme states. These are, in order from lower to higher fluorescence intensity (i) the Mg^{2+} state produced in the presence of Mg^{2+} and EGTA, (ii) EP formed from P_i and (iii) the Ca^{2+} state produced by high affinity Ca^{2+} binding (Dupont, 1976; Dupont and Leigh, 1978; Guillain et al., 1980). This phenomenon has been used under both equilibrium (Dupont, 1976; Guillain et al., 1980; Verjovski-Almeida and Silva, 1981) and transient conditions (Dupont and Leigh, 1978; Guillain et al., 1981; Dupont, 1982) to study the mechanism of Ca^{2+} binding.

The effect of Ca^{2+} binding on the kinetics of the transition from the P_i reactive *E form to the ATP reactive E form of the Ca^{2+} pump was first studied by rapid quench techniques, in which the amount of EP formation was measured following the addition of both Ca^{2+} and ATP to a Ca^{2+}-depleted enzyme. Phosphorylation was slow compared to that of Ca^{2+}-pre-incubated vesicles. This indicated a slow conformational change of *E to E which precedes Ca^{2+} binding, since Ca^{2+} binding to high affinity sites was considered to be rapid (Souza and de Meis, 1976; Rauch et al., 1977; Vieyra et al., 1979). However, estimations of the *E$\leftrightarrow$E transition were made in the presence of ATP, which in itself was found to activate this transition, even at 5 μM (Guillain et al., 1980). Ca^{2+} dissociation and the E to *E transition was also found to be slow when monitoring the loss of ATPase activity (Rauch et al., 1978) or the appearance of reactivity towards P_i (Verjovski-Almeida et al., 1978) on addition of EGTA.

Earlier passive binding studies using $^{45}Ca^{2+}$ under equilibrium

conditions and Ca^{2+} activation of Ca^{2+} transport, ATP hydrolysis and EP formation, indicated a single class of two identical high affinity Ca^{2+}-binding sites per ATPase monomer (Chevallier and Butow, 1971; Meissner, 1973; Ikemoto, 1975; Vianna, 1975; Neet and Green, 1977). It was thus initially assumed that two Ca^{2+} ions are needed to bind to two identical non-interacting sites before enzyme activation occurs. Recent studies by Inesi and coworkers (Inesi et al., 1980), in which high affinity Ca^{2+} binding was measured, following equilibration of SR vesicles with Ca^{2+} buffers on chromatographic columns, showed a single class of high affinity sites (K_{app} = 2-3x10^6 M^{-1}) to the extent of two Ca^{2+} ions per ATPase phosphorylation site. Analysis of Ca^{2+} binding, as a function of Ca^{2+} concentration, demonstrated a cooperative mechanism of binding to two interacting sites, which differ in affinity by two orders of magnitude. The conversion of the lower affinity site ($K_d \simeq 10^{-5}$ M) to a high affinity site ($K_d \simeq 10^{-7}$ M) was accompanied by a conformational change that was detected on ESR spectroscopy of spin-labelled vesicles. Using kinetic measurements of enzyme phosphorylation by ATP, Inesi et al. (1980) were able to demonstrate that the conformational change, which is induced by Ca^{2+} (and ATP) is a relatively slow step, supporting the above rapid quench studies. Sequential binding of Ca^{2+}, as proposed by Inesi et al. (1980) can be represented as

$$E + Ca^{2+} \longleftrightarrow E.Ca \overset{slow}{\longleftrightarrow} {}'E.Ca + Ca^{2+} \longleftrightarrow {}''E.Ca_2$$

Cooperative binding was confirmed for SR vesicles by Watanabe et al. (1981), under equilibrium conditions, and by Verjovski-Almeida and Silva (1981), using intrinsic tryptophan fluorescence. In both studies it was shown that cooperativity was abolished in the $C_{12}E_8$ (dodecyl octaethyl glycol monoether) solubilised enzyme. Verjovski-Almeida and Silva (1981), however, found that when vesicles were solubilised in the presence of millimolar concentrations of Ca^{2+}, cooperativity was retained. Watanabe et al. (1981) showed that Ca^{2+} binding was highly pH dependent and that the Ca^{2+} affinity as well as the degree of cooperativity increases with a rise in pH. Cooperative equilibrium binding of Ca^{2+}, as modulated by pH, was analysed by Hill and Inesi (1982) by statistical mechanical treatment of a theoretical model, which consists of four equivalent interacting subunits, each having one Ca^{2+} or proton binding site. A similar tetrameric model was suggested independently

by Verjovski-Almeida and Silva (1981).

Initial intrinsic fluorescence studies by Dupont (1976) and Dupont and Leigh (1978) suggested that the rate of the Ca^{2+}-induced conformational change (k_{on}) was too slow to represent direct binding of Ca^{2+} and so these workers postulated a two-step binding process which included a slow isomerisation step such that

$$*E + Ca^{2+} \longleftrightarrow *E.Ca \overset{\text{slow}}{\longleftrightarrow} E.Ca$$

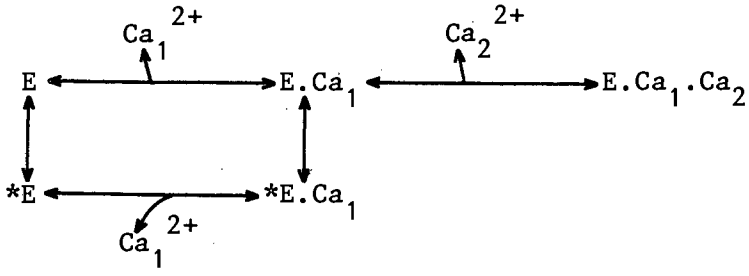
Guillain et al. (1980) measured the rate of change in intrinsic tryptophan fluorescence (k_{obs}) for both Ca^{2+} binding (k_{on}) and dissociation (k_{off}) as a function of $[Ca^{2+}]_{free}$. At pH 7.0 and in the presence of 100 mM KCl and 1-20 mM $MgCl_2$, k_{obs} first decreased and then increased as the Ca^{2+} concentration rose. They suggested that formation of the ECa_2 complex is controlled by an isomerisation step which occurs either before (decreasing k_{obs}) or after (increasing k_{obs}) Ca^{2+} binding, such that

$$\begin{array}{ccc} E & + Ca^{2+} \longleftrightarrow & E.Ca \\ \updownarrow & & \updownarrow \\ *E & + Ca^{2+} \longleftrightarrow & *E.Ca \end{array}$$

This scheme, however, neglects cooperativity of Ca^{2+} binding and has the problem that at zero free Ca^{2+}, the equilibrium between P_i-reactive and ATP-reactive conformations are not totally displaced towards one of these conformations. An important implication of this scheme, however, is that, in the absence of a Ca^{2+} gradient, *E does exhibit high affinity sites for Ca^{2+} binding.

The reaction scheme of Guillain et al. (1980) also could not explain the decrease in the rate of Ca^{2+} dissociation (k_{off}) with increasing Ca^{2+} concentration. This could only be explained by assuming that the two Ca^{2+}-binding sites are interacting and non-equivalent (Ikemoto et al., 1981; Dupont, 1982). Ikemoto and coworkers demonstrated non-equivalence of binding sites, based upon (i) the rate of dissociation of Ca^{2+} after addition of EGTA and (ii) upon the rate of EP formation in the presence of ATP, depending on whether Ca^{2+} was added to the enzyme

before or after ATP. Since both sites became indistinguishable in detergent, under conditions where the monomer predominates, they have suggested that nonequivalence of the two types of Ca^{2+}-binding sites in native SR is due to the existence of kinetically nonequivalent subunits. Dupont (1982), using subzero temperatures and glycerol solvents, was able to directly examine the rate of Ca^{2+} binding to high affinity sites over a range of Ca^{2+} concentrations by Millipore filtration, in the absence of ATP. In addition, Dupont studied the kinetics of Ca^{2+} exchange at constant $[Ca^{2+}]_{free}$. The results, from Ca^{2+}-binding studies, were consistent with sequential binding to two interacting sites. Ca^{2+} binding to sites of low apparent affinity ($K_d \approx 25$ μM) is fast. Occupation of these sites induces a slow conformational change, which increases its own apparent affinity and reveals a second site of high affinity. At equilibrium, Ca^{2+} removal from a fast exchanging site, by addition of EGTA, accelerates the rate of release of a slow exchanging site. The non-equivalence of the two classes of sites and their interaction can be represented by the reaction scheme



An equally plausible mechanism of Ca^{2+} binding can be proposed to account for all the above results (Dupont, 1982). In such a model, the asymmetry is only apparent, and is created by substrate binding, and an unfavourable pre-existing fast equilibrium exists between two different conformations of one of the Ca^{2+} sites. Champeil and coworkers (Champeil et al., 1983) studied the effect of Mg^{2+} on the Ca^{2+}-dependent transient kinetics by stopped flow fluorescence and by phosphorylation. They found that, in the absence of KCl and $MgCl_2$, at pH 7.0, the intrinsic fluorescence rise, after addition of Ca^{2+} to a Ca^{2+}-deprived enzyme, was monoexponential, whereas preincubation of the Ca^{2+}-deprived enzyme with Mg^{2+} induced a biphasic fluorescence rise at high Ca^{2+} concentrations. Since the rate constant of the slow phase was an increasing function of Ca^{2+} concentration, it was suggested that substrate binding takes place

before enzyme isomerisation and that Ca^{2+} sites are available on the *E conformation of the enzyme. Although this does not rule out the suggestion of Dupont (1982) of an unfavourable pre-existing equilibrium, which would account for the low apparent affinity for initial binding, it does suggest that in the presence of Mg^{2+}, where both a fast and slow phase of fluorescence change exists, one site for low affinity Ca^{2+} binding is permanently and readily accessible on the outer surface of SR vesicles. It is suggested that Ca^{2+}-binding sites are absent, in the absence of Mg^{2+}, and that their appearance is rate-limiting, whilst Mg^{2+} drives the enzyme towards a conformation capable of fast Ca^{2+} binding. Phosphorylation of the Ca^{2+} pump after simultaneous addition of Ca^{2+}, Mg^{2+} and ATP was faster when SR was originally Ca^{2+}-depleted in the presence of Mg^{2+}, suggesting that acceleration by ATP (and Ca^{2+}) of protein isomerisation is only fully expressed if the enzyme is preincubated with Mg^{2+}.

Competition by Mg^{2+} ions for the high affinity Ca^{2+}-binding sites has been previously suggested (Chevallier and Butow, 1971 ; Meissner, 1973; Vianna, 1975; Souza and de Meis, 1976; Scofano and de Meis, 1981). Additional Mg^{2+}-binding sites, distinct from the Ca^{2+}-specific binding sites, have also been suggested (Vianna, 1975; Makinose and Boll, 1979). Fluorescent changes, as the result of Mg^{2+} binding to the Ca^{2+}-ATPase, suggest that E.Mg is a different state to that resulting from Ca^{2+} binding to form the state, $E.Ca_2$ (Guillain et al., 1982).

The stoichiometry of Ca^{2+} binding to high affinity sites is still a matter of controversy. Most variations result from the use of different molecular weights for the ATPase, estimation of the percentage functional enzyme in any particular SR preparation and to inaccurate determinations of protein concentration (Møller et al., 1982; Watanabe et al., 1982). Although earlier binding studies were in favour of two Ca^{2+}-binding sites per ATPase monomer (Chevallier and Butow, 1971; Meissner, 1973; Ikemoto, 1975), more recent studies point to a stoichiometry of 1 mol per mol protein or 8 nmol per mg protein (Inesi et al., 1980; Watanabe et al., 1981; Verjovski-Almeida and Silva, 1981; Dupont, 1982). Although Verjovski-Almeida and Silva (1981) originally demonstrated loss of cooperative Ca^{2+}-dependent activation of ATPase activity in $C_{12}E_8$-solubilised vesicle (a finding supported by Watanabe et al., (1981)), later studies (Silva and Verjovski-Almeida, 1983) indicate that the

monomeric form binds Ca^{2+} cooperatively, with a stoichiometry of 2 mol per mol ATPase, while the dimeric form binds 1 mol per mol. This latter result is supported by the studies of Møller et al. (1980) and Murphy et al. (1982) who showed that the monomeric form of the enzyme displays a Ca^{2+}-dependence of activation of ATP hydrolysis which was cooperative (n_H = 1.8 - 1.9). These results suggest that the calcium sites interact intramolecularly. Thus, it is uncertain as to whether the stoichiometry of Ca^{2+} binding per ATPase polypeptide chain is one or two and further investigation is required to elucidate a correct working model for Ca^{2+} binding to non-equivalent interacting Ca^{2+} sites.

From the above, it appears that the two Ca^{2+}-binding sites that are obligatory during turnover of the catalytic cycle are indeed non-equivalent, and that the cooperativity previously noted in stimulation of transport, ATP hydrolysis and EP formation, is the result of binding of a first Ca^{2+} to convert the second site from a low to a high affinity site. In addition, the conformational change that is associated with this cooperative phenomenon has been localised to the *E⟷E transition (Reaction Scheme I, step 8).

Most of the above studies were conducted in EGTA buffers, which are used to remove medium contaminating Ca^{2+}. It has recently been suggested that Ca/EGTA buffers increase the apparent affinity of Ca^{2+}-binding sites by an order of magnitude (Sarkadi et al., 1971; Al-Jabore and Routogalis, 1981; Berman, 1982a). In addition, preliminary experiments suggest that ATP, also a Ca^{2+} chelator, decreases $K_{0.5}$ for Ca^{2+} activation of transport (Meltzer et al., 1981; Berman, 1982a). The effect of a range of Ca^{2+}-chelating anions on Ca^{2+} binding was further investigated in this study.

2.1.2. ATP BINDING AND SUBSTRATE REGULATION

The (Ca^{2+},Mg^{2+})-ATPase of SR shows complex ATP-dependence for a number of functions and parameters that reflect binding of ATP to specific sites. Evaluation of the ATP-binding parameters have been based either on passive binding or on kinetic studies. ATP binding studies require that measurements are carried out in the absence of Ca^{2+} and at low

temperatures, to minimise hydrolysis. Under these conditions, ATP binds as the Mg^{2+}-chelate (Inesi and Almendares, 1968; Meissner, 1973; Vianna, 1975) with an apparent K_d of 1-10 μM (Meissner, 1973; Pang and Briggs, 1977; Arav et al., 1983). Some binding studies indicate one binding site per molecule i.e. 6-8 nmol ATP per mg protein (1 mol per mol) (Meissner, 1973; Anderson et al., 1982; Clore et al., 1982; Murphy et al., 1982) while others indicate that the enzyme possesses two classes of ATP-binding sites and only binds 4 nmol ATP per mg protein with high affinity (Dupont, 1977). The second site has low affinity for ATP ($K_d \approx 1$ mM) (Dupont, 1977). Covalent modification of the active site by fluorescein isothiocyanate (FITC) shows biphasic kinetics for the inactivation of Ca^{2+} transport, indicative of two types of ATP-binding sites. Modification by 4 nmol FITC per mg protein resulted in complete inactivation of enzyme activity (Pick and Karlish, 1980; Pick and Bassilian, 1981; Pick, 1981).

The presence of two binding sites per ATPase correlates well with its complex enzyme kinetics. The ATP-dependence of Ca^{2+} transport and of ATP hydrolysis is biphasic. Saturation of the high affinity ATP-binding sites (K_d = 2-3 μM) at 50-100 μM is followed by secondary activation in the millimolar ATP concentration range (Froehlich and Taylor, 1975; Yates and Duance, 1976; Neet and Green, 1977; Taylor and Hattan, 1979). This complex behaviour has been ascribed to either (i) two families of active sites, (ii) negative cooperativity between two identical sites or (iii) coupling between a high affinity catalytic or a low affinity regulatory site.

Dupont (1977) has deduced from steady state kinetic experiments, using ATP analogues, that the high affinity site is the hydrolytic site and that its affinity is independent of medium free Ca^{2+}. The rate of ATP hydrolysis at this site was modulated by binding of ATP to the second low affinity site. The analogues adenosine 5'-(α,β methylene) triphosphate (AMPCPP) and pyrophosphate (PP_i), which are slowly hydrolysable, were as effective as ATP in activating the catalytic activity of the high affinity site, while ADP, adenosine 5'-(β,γ-imino) triphosphate (AMPPNP) and P_i, were ineffective. The low affinity site is non-specific and can be occupied by both purine and pyrimidine nucleotides (de Meis and de Mello, 1973; Taylor and Hattan, 1979) and by AMPCPP but

not by ADP and other non-nucleotide pseudosubstrates which support Ca^{2+} transport (Dupont, 1977; Taylor and Hattan, 1979).

The regulatory role and stimulation of ATP hydrolysis and Ca^{2+} transport at millimolar ATP concentrations has been ascribed to activation of the rate limiting *E to E interconversion (Step 8, Reaction Scheme I). The presence of this activating site has been used to explain the following experimental observations: (i) inhibition of catalysis by high Ca^{2+} concentrations in leaky vesicles is accompanied by an increase in EP formation from ITP but not from ATP as substrate (Souza and de Meis, 1976); (ii) ATP can overcome Ca^{2+} inhibition of medium $P_i \leftrightarrow HOH$ exchange (de Meis and Boyer, 1978); (iii) a greater rate of oxygen exchange is found with ITP than with ATP (de Meis and Boyer, 1978) and (iv) phosphorylation of the enzyme by P_i during steady state ATP cleavage, in the presence of Ca^{2+}, is greatly diminished as the concentration of ATP is increased from 0.1 to 5 mM (de Meis and Boyer, 1978; McIntosh and Boyer, 1983). Direct evidence that ATP can convert *E to E has been obtained from intrinsic fluorescence measurements. Guillain et al. (1981) have shown that 5 μM ATP increases k_{obs} for $*E \leftrightarrow E$ from 1.3 s^{-1} to 7 s^{-1}.

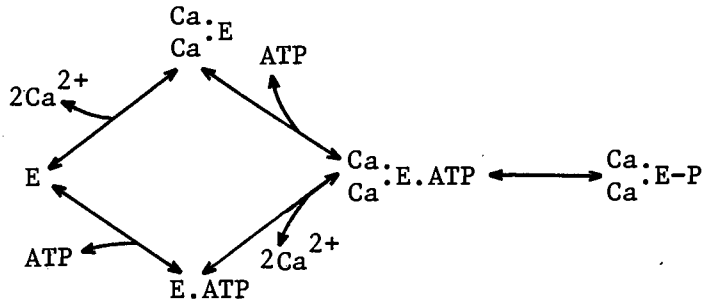
Evidence for modulation of other intermediates of the catalytic cycle by ATP binding to high and low affinity sites has been obtained by McIntosh and Boyer from $P_i \leftrightarrow HOH$ exchange measurements (McIntosh and Boyer, 1983; McIntosh, 1983). A rather striking feature is that ATP modulates Ca^{2+}-activated ATPase activity in three different concentration ranges; (i) 5-10 μM ATP accelerates the rate of EP dephosphorylation; (ii) a further increase in ATP, up to 100 μM, decreases the rate of EP hydrolysis and EP formation from P_i and (iii) millimolar ATP concentrations again increase the rate of EP hydrolysis, while the level of EP formed from P_i and the total medium $P_i \leftrightarrow HOH$ exchange decreases. The high affinity ATP modulation ([ATP] < 100 μM) was interpreted as resulting from ATP binding to catalytic sites before dephosphorylation while the low affinity (millimolar) ATP modulation is related to the secondary increase in initial velocity of ATP cleavage, with acceleration of $*E \leftrightarrow E$ transitions, in addition to effects on dephosphorylation. It is suggested that at physiological concentration of ATP all the intermediates of the catalytic cycle have bound ATP. In addition, steady state levels of EP are not changed at high ATP concentrations, suggesting

that both EP formation and hydrolysis is activated by ATP binding to low affinity sites (Froehlich and Taylor, 1976; Verjovski-Almeida et al., 1978).

It has been suggested by several workers that modulatory effects may be caused by protein-protein or subunit-subunit interaction (for review see Møller et al., 1982). A flip-flop model, in which one subunit of an associated dimer, upon ATP binding, assumes a conformation that does not transport Ca^{2+} but rather modulates catalysis of its neighbouring subunit, has been considered (Froehlich and Taylor, 1975, 1976; McIntosh, 1983). Pick (1981) has, in addition, followed the interconversion between different conformational states of FITC-labelled ATPase and has suggested that the two different ATP-binding sites may represent a single site on two different conformations of the enzyme. The enzyme may bind ATP, in the absence of Ca^{2+}, with low affinity, while, in the presence of Ca^{2+}, a conformational change may be induced, converting the low affinity binding site to high affinity. A similar result was obtained by Moczydlowski and Fortes (1981a & b) for the (Na^+,K^+)-ATPase. They confirmed the existence of only one binding site for the ATP analogue 2',3'-O-(2,4,6-trinitrocyclohexylienylidene) adenosine triphosphate (TNP-ATP) and concluded that the catalytic and regulatory ATP binding sites interconvert and do not co-exist independently. Strong evidence against such a mechanism is the finding of Møller et al. (1982), that activation of ATP hydrolysis by binding of ATP to low affinity sites persists in the $C_{12}E_8$-solubilised monomer.

2.1.3. POSSIBLE INTERACTION BETWEEN Ca^{2+}- AND ATP-BINDING SITES

The Ca^{2+} affinity of intact and leaky SR vesicles is the same in the presence and absence of ATP (Fiehn and Migala, 1971; Meissner, 1973), indicating that ATP does not create additional high affinity binding sites for Ca^{2+}. The passive Ca^{2+}-binding parameters, $[Ca^{2+}]_{0.5}$ and n_H, are unaffected by ATP, MgATP or MgITP (Yates and Duance, 1976; Vianna, 1975). Based on the experimental observations that neither ligand influences the affinity of the enzyme for the other (Kanazawa et al., 1971; Meissner, 1973) and that both Ca^{2+} and ATP bind to the Ca^{2+}-ATPase in the absence of each other, a mechanism of random binding has been postulated (Tada et al., 1978) (Reaction Scheme II)



REACTION SCHEME II

It was, however, shown by de Meis and de Mello (1973) that binding of substrate does modify the affinity of the Ca^{2+}-pump for Ca^{2+}, ATP being more effective than ITP and GTP in increasing the affinity. Ikemoto (1974) observed an increase in the affinity of all classes of Ca^{2+}-binding sites in the presence of ATP. A study by Murphy (1976) showed that a conformational change, resulting from ATP binding to spin-labelled ATPase, restricted one or two labelled residues which were not in the vicinity of the ATP-binding site, but near the Ca^{2+}-binding site. Scofano et al. (1979) found that the apparent K_m for ATP was lower when vesicles were preincubated with EGTA. These results, together with the fact that the Ca^{2+}-dependence of Ca^{2+} transport is different when supported by varying pseudosubstrates (see Section 2.1.5), contradict a mechanism of random binding.

The criterion for an ordered mechanism of binding to multisubstrate enzymes has been defined by Fersht (1977) as "the binding of the one substrate causing a conformational change, which increases the affinity of the enzyme for the other substrate" and more rigorously by Cornish-Bowden (1976) as "no binding site exists on the enzyme for one of the two substrates until the other is bound". The results of de Meis and de Mello (1973) are consistent with the definition of Fersht, indicating an ordered mechanism, while other results (Kanazawa et al., 1971; Fiehn and Migala, 1971; Meissner, 1973; Yates and Duance, 1976) support the definition of Cornish-Bowden and suggest random binding. Thus, although it is generally accepted that Ca^{2+} and ATP bind randomly (Reaction Scheme II) to the (Ca^{2+},Mg^{2+})-ATPase, there is now substantial experimental evidence for non-random binding and for interaction between ATP- and Ca^{2+}-binding sites.

2.1.4. SUBSTRATE SPECIFICITY OF THE (Ca^{2+}, Mg^{2+})-ATPase

The nucleotides ITP, GTP, CTP and dATP can, in addition to the natural substrate ATP, support active Ca^{2+} transport (Martonosi and Feretos, 1964a). The velocity of hydrolysis is graded in the following sequence: ATP (1.0) > ITP (0.8) > GTP (0.7) > CTP (0.55) > UTP (0.25), according to their rates relative to ATP (Makinose and The, 1965).

Nucleotide triphosphates can be replaced as energy donors by the pseudosubstrates acetylphosphate (AcP) (de Meis, 1969), p-nitrophenylphosphate (p NPP) (Inesi, 1971) and carbamylphosphate (Pucell and Martonosi, 1970). Two Ca^{2+} ions are transported per molecule of substrate hydrolysed with all substrates. The properties of the acylphosphoprotein is indistinguishable from that formed from ATP and can phosphorylate ADP stoichiometrically (Nakamura and Tonamura, 1978). The pseudosubstrates have different Ca^{2+}-concentration requirements for phosphorylation and the rate of Ca^{2+} transport and hydrolysis is less than that of ATP (Pucell and Martonosi, 1970; Inesi, 1971; Nakamura and Tonomura, 1977; Scofano et al., 1979; Kurzmack et al., 1981). This implies that an additional rate limiting step exists when Ca^{2+} transport is supported by the pseudosubstrates.

Secondary activation of substrate hydrolysis and of Ca^{2+} transport is only observed for ATP, ITP and GTP; the latter two at considerably higher concentrations (de Meis and de Mello, 1973; Taylor and Hattan, 1979). The pseudosubstrates, AcP (de Meis and Hasselbach, 1971; Pucell and Martonosi, 1970), pNPP (Inesi, 1971; Rossi et al., 1979) and furylacryloylphosphate (FAP) (Kurzmack et al., 1978; Rossi et al., 1979) do not show the secondary activation phenomenon and obey Michaelis-Menten kinetics in the steady state, consistent with one binding site.

Rossi et al. (1979), by using a range of pseudosubstrates to support Ca^{2+} transport, including pNPP, FAP, methylumbelliferylphosphate and dinitrophenylphosphate (dNPP), showed that the most efficient substrate is the one with the best leaving group. Ca^{2+} stimulation was characterised by positive cooperativity with Hill coefficients of 1.5, 2.1 and 1.85 and $[Ca^{2+}]_{0.5}$ of 0.5, 2.2 and 7.5 μM for the substrates ATP, pNPP and dNPP, respectively. In contrast to previous studies, a

coupling ratio of one was found for these pseudosubstrates. This difference between ATP and the pseudosubstrates was attributed to different rate limiting steps (Rossi et al., 1979).

The maximum concentration of phosphoenzyme formed with all substrates studied is half a mol per mol ATPase. One exception exists, in that AcP supports EP formation to the extent of one mol per mol (Friedman and Makinose, 1970; de Meis and de Mello, 1973). This apparently anomalous result has been ascribed to the fact that the acetate anion is a weaker phosphate acceptor than ADP.

The study of the reaction mechanism of the (Ca^{2+},Mg^{2+})-ATPase has been greatly facilitated by the use of artificial substrates. A greater insight into the precise stereochemistry of substrate interaction may, for example, be gained by the utilisation of alternate substrates and nucleotide analogues. The rationale for the use of pseudosubstrates in this study is based on the previously obtained variations in $[Ca^{2+}]_{0.5}$ with different pseudosubstrates, and they may thus be used to probe possible interaction between Ca^{2+}- and substrate-binding sites.

2.2. EXPERIMENTAL PROCEDURES

2.2.1. ISOLATION AND PURIFICATION OF SARCOPLASMIC RETICULUM VESICLES

Sarcoplasmic reticulum vesicles were prepared according to the method of Eletr and Inesi (1972), which includes a KCl extraction step, to remove contaminating actomyosin. SR was isolated from the white skeletal muscle of the hind legs of New Zealand white, crossed with a commercial hybrid-strain male rabbit. The entire isolation procedure was carried out at 0-4°C. Following excision, the tissue (200 g) was washed in 0.1 mM ethylenediamine-tetraacetic acid (EDTA), pH 7.0. The muscle was homogenised at maximum speed in a waring blender, for 15 sec every 5 min for a total of one hour, in 800 ml of medium A, consisting of 10 mM histidine, pH 7.0, 0.3 M sucrose and 0.1 mM EDTA. The pH was adjusted to 7.0 with a 5% (w/v) NaOH solution. The homogenate was centrifuged at 15 000 x g for 20 min in rotor no. JA 14 in a Beckman J2-21 centrifuge. The supernatant was filtered through glass wool and washed with medium A to remove low-density lipid aggregates. The filtrate was recentrifuged at 40 000 x g for 90 min in rotor no. 21 in a Beckman model L4 ultracentrifuge. The pellet was resuspended and incubated for 40 min in 100 ml of medium B, containing 10 mM histidine, pH 7.0 and 0.6 M KCl, to solubilise contaminating actomyosin. The suspension was centrifuged at 15 000 x g for 20 min in rotor no. JA 20 in a Beckman J2-21 centrifuge. The supernatant was recentrifuged at 78 000 x g for 60 min in rotor no. 30 in a Beckman model L2-65B ultracentrifuge. The final sediment was resuspended in 10 ml of buffer C, containing 10 mM imidazole, 0.3 M sucrose, pH 7.4. The stock solution of SR vesicles was stored at 0°C and assays were performed within four days. Calcium transport activity varied between 1.0 and 2.0 μmol Ca^{2+} per min per mg protein.

SR vesicles were separated into light and heavy vesicles according to the method of Fernandez et al. (1980). SR protein (10 mg) was loaded onto a discontinuous sucrose gradient of 25% (1.5 ml), 35% (4.0 ml), 40% (5.0 ml) and 50% (1.5 ml) sucrose (w/v) (1.09. 1.13, 1.16, 1.19 density in g/cm³ at 20°C, respectively) and centrifuged overnight (16 h) at 4°C in a SW 27.1 rotor in a Beckman L565 ultracentrifuge at 150 000 x g (24 000 r.p.m.). All sucrose solutions contained 20 mM Tris-maleate buffer, pH 7.0. Populations of SR vesicles that were concentrated at the interfaces between 35% and 40% sucrose (light) and between

40% and 50% sucrose (heavy) were collected (Fig. 3). The fractions were diluted approximately ten-fold with the Tris-maleate buffer and concentrated by centrifugation at 78 000 x g for 30 min in rotor no. 30 in a Beckman model L2-65B ultracentrifuge. The pellets were resuspended in buffer C. The percentage 'basal' activity was higher (three-fold) with the heavy fraction than with the light fraction. Under standard conditions, SR was not fractionated on a sucrose gradient, since this procedure did not have a significant effect on the overall activity.

Determination of protein concentration:

The protein concentration was determined by an automated Lowry method (Oosta et al., 1978), using the standard modules of the Technicon Autoanalyser I system. Folin-Ciocalteu phenol reagent was diluted 1 in 8 with water, to give maximum colour development. Alkaline copper tartrate solution was prepared by mixing 5 parts of 2% (w/v) Na-K tartrate, 5 parts of 0.6% (w/v) copper II sulphate and 90 parts of a solution composed of 4% (w/v) sodium carbonate in 0.2 M sodium hydroxide.

Standards, using bovine serum albumin (Sigma), in the range 10 to 100 µg/ml, was prepared by dilution in 0.15 M NaCl. The calibration curve was fitted by a quadratic equation. Suspensions of SR vesicles (10 µl containing 0.05 to 0.2 mg protein) were mixed with 10 µl of 10% sodium deoxycholate. After standing for 10 min to allow solubilisation, 0.15 M NaCl was added to give a final concentration of approximately 50 µg protein per ml. All protein measurements were performed in triplicate.

2.2.2. ATP-DEPENDENT CALCIUM TRANSPORT MEASUREMENTS

2.2.2.1. The $^{45}Ca^{2+}$/Millipore Filtration Method

The transport medium (0.5 ml) contained 50 mM 1,4-piperazinediethane sulfonic acid (Pipes), pH 6.8, 100 mM KCl, 0.02 to 0.1 mg/ml SR protein, 5 mM $MgCl_2$, 5 mM potassium oxalate, 0.5 mM EGTA and 0.5 mM $^{45}CaCl_2$ (3000 cpm/nmol). $[Ca^{2+}]_{free}$ was calculated to be 11.2 µM, using a stability constant, K_{CaEGTA}, of $10^{11.00}$ (Schwartzenbach et al., 1957). The reaction, at 20°C, was initiated by the addition of ATP (5 mM) and

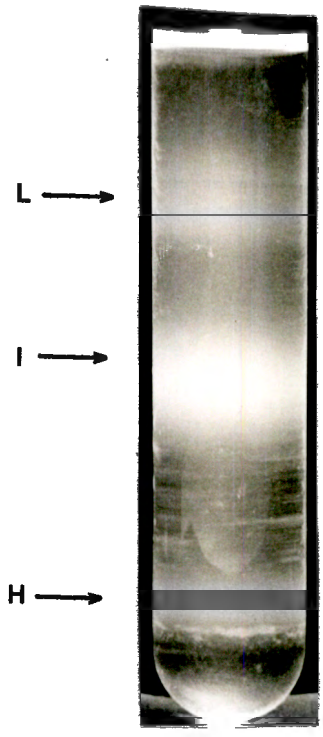


Figure 3: Fractionation of sarcoplasmic reticulum on a discontinuous sucrose density gradient.

SR protein (10 mg) was loaded onto a discontinuous sucrose gradient of 25% (1.5 ml), 35% (4.0 ml), 40% (5.0 ml) and 50% (1.5 ml) sucrose, in 20 mM Tris-maleate, pH 7.0, and centrifuged overnight at 4°C at 150 000 x g. L, light; I, intermediate; H, heavy vesicles.

terminated at various time intervals by filtering an aliquot (200 µl) through a Millipore filter (type HA, 0.45 µpore size) (Martonosi and Feretos, 1964a) and by washing with medium containing 20 mM imidazole, pH 6.0, 120 mM KCl and 5 mM $CaCl_2$. The filter discs were placed in 6 ml of Instagel (Packard) and assayed for radioactivity. The amounts of Ca^{2+} transported per mg protein were calculated from the radioactivity remaining on the filter and plotted as a function of time. The rate of Ca^{2+} transport was determined from the slope of this plot, which was linear over the first 60 sec.

2.2.2.2. A Potentiometric Calcium-Stat Method using a Calcium-Electrode as End-point Detector

Continuous recording of calcium transport by isolated SR vesicles was performed by the calcium-stat method, described by Berman and Aderem (1981). The reaction was conducted in a jacketed, magnetically stirred glass reaction vessel that was thermostatted by means of a circulating water bath at 20°C. A calcium-specific electrode (Radiometer type F2002), and a KCl reference electrode (Radiometer type K801) were connected to the titration assembly and served as end-point detector. Rates of addition of standardised $CaCl_2$, during Ca^{2+} transport, were monitored by means of an electrical transducer (10 turn, 10 Kohm Helipots and voltage source), connected to a potentiometric recorder (Housten Omniscribe). The voltage was also fed into an analogue-to-digital converter, which was interfaced with a Micronova Minicomputer (Data General), which was programmed in BASIC.

The reaction medium, 2 ml, at pH 7.2, contained 0.1 M KCl, 5 mM $MgCl_2$ and 5 mM potassium oxalate. The pH was maintained constant by means of a pH-stat, in the absence of buffer. ATP was added to a final concentration of 5 mM, unless otherwise stated. The Ca^{2+} concentration was adjusted as required and maintained constant during the course of the reaction by titration with $CaCl_2$. The reaction was initiated by the addition of SR vesicles (100-200 µg SR protein).

Fifty data points were recorded over a time interval of between 60-200 seconds. Initial rates of transport were derived from least-square fitting of the data to a non-linear binomial function.

An ATP regenerating system, consisting of 0.5 mM creatine phosphate and 5 U/ml creatine kinase, was included in the reaction medium, at low (<1 mM) ATP concentrations. Ca^{2+} transport was measured at 25°C.

Calibration of the calcium electrode:

The calcium electrode was calibrated, using unbuffered $CaCl_2$ in the range 10^{-2} - 10^{-4} M free Ca^{2+} and CaEGTA in the range 10^{-6} - 10^{-8} M free Ca^{2+} ($K_{CaEGTA} = 10^{11.00}$) (Schwartzenbach et al., 1957). All solutions were prepared in 0.1 M KCl, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.2. The inclusion of KCl was to ensure that all calibration solutions were at similar ionic strength.

The electrode potential, plotted as a function of the log of $[Ca^{2+}]_{free}$, showed that Nernstian behaviour was maintained (in the absence of Mg^{2+}) with a slope of 30.26 mV per 10-fold change in $[Ca^{2+}]_{free}$ (Fig. 4A). The electrode was standardised daily against a 10^{-6} M $CaCl_2$ solution, to eliminate errors due to a drift in electrode potential.

Selectivity measurements of interfering ions:

The potentiometric selectivity coefficient, $K_{A,B}$, defines the ability of an ion-selective electrode to distinguish between different ions in the same solution (IUPAC Recommendations for Nomenclature of Ion-Selective Electrodes, 1976). The potential of the cell, in a solution containing the primary ion, A, and the interfering ion, B, is given by the modified Nernstian equation:

$$E = E^\circ \pm \frac{2.303\ RT}{n_A F} \log_{10}(a_A + K_{A,B}\, a_B^{(n_A/n_B)})$$

where n_A and n_B are the charges on ions A and B and E° is the standard potential of the electrode.

The potentiometric coefficient, $K_{Ca^{2+},Mg^{2+}}$, was measured by the fixed interference method (Bailey, 1976; Simpson, 1980), in which solutions of constant Mg^{2+} and varying Ca^{2+} concentrations were prepared and their electrode potentials measured. All solutions contained 0.1 M KCl, 10 mM 3-[N-morpholino]propanesulfonic acid (MOPS), pH 7.0

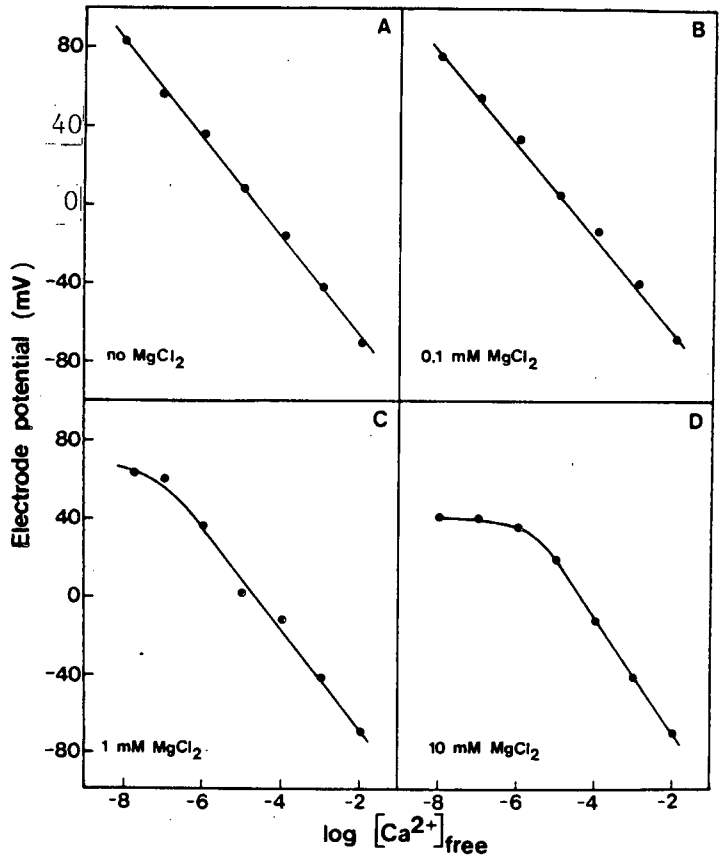


Figure 4: Relation between electrode potential and the logarithm of the free Ca^{2+} concentration.

Medium: 10 mM Mops, pH 6.8, 100 mM KCl, 1 mM EGTA and either no $MgCl_2$ (A), 0.1 mM $MgCl_2$ (B), 1 mM $MgCl_2$ (C) or 10 mM $MgCl_2$ (D). $CaCl_2$ was added to give the desired free Ca^{2+} concentration which was calculated as described in Experimental Procedures.

and 1 mM EGTA. A plot of the electrode potential, at 25°C, as a function of the log of free Ca^{2+} concentration, in the presence of 0, 0.1, 1.0 and 10.0 mM free Mg^{2+}, is shown in Fig. 4. The electrode response deviated from linearity at low ($<10^{-7}$ M)$[Ca^{2+}]_{free}$, when the free Mg^{2+} concentration was greater than 1.0 mM. The potentiometric coefficient, $K_{Ca^{2+}, Mg^{2+}}$, calculated from Fig. 4C and Fig. 4D has a value of 3.5×10^{-5} at 1.0 mM Mg^{2+} and 1.78×10^{-4} at 10 mM Mg^{2+}, respectively. Free Ca^{2+} concentrations were calculated, using the modified Nernstian equation.

Standardisation of $CaCl_2$ solutions:

The concentration of $CaCl_2$ solutions was determined by titration against standard 0.5 mM EDTA solution in 0.05 M sodium borate, pH 9.0, using the calcium electrode as end-point detector. $[Ca^{2+}]_{free}$ was maintained constant at 1 µM. $CaCl_2$ was also standardised by atomic absorption spectrophotometry with a Varion-Techtron Atomic Absorption Spectrophotometer (type AA). Absorption of emission from the Ca^{2+} lamp was determined at 422.7 nm in a nitrous oxide-acetylene flame. Calcium (20 µM) was added as an internal standard.

2.2.2.3. A Spectrophotometric Calcium-Stat Method using Arsenazo III as Calcium Indicator

A new, independent spectrophotometric calcium-stat, in which the free ionised Ca^{2+} concentration is measured using the Ca^{2+}-sensitive dye arsenazo III (2,2'-[1,8-dihydroxy-3,6-bisulfo-2,7-napthalene-bis (azo)]-dibenzenearsonic acid) as Ca^{2+} indicator, was developed, to measure Ca^{2+} transport, in the absence of EGTA. This spectrophotometric Ca^{2+}-stat has the advantage over the potentiometric Ca^{2+}-stat in that the response time of the arsenzo III signal ($t_{\frac{1}{2}}$ = 8 msec (Ogawa et al., 1980)) is much faster than that of the Ca^{2+}-electrode ($t_{\frac{1}{2}} \simeq$ 1 min (Meltzer and Berman, 1983)). Interference by Mg^{2+} ions can, in addition, be excluded by choosing a suitable wavelength pair at which absorbance change, resulting from formation of a Ca^{2+}-arsenazo III complex, can be measured. Arsenazo III is very stable (Kendrick et al., 1977) and therefore calibration procedures do not have to be repeated daily.

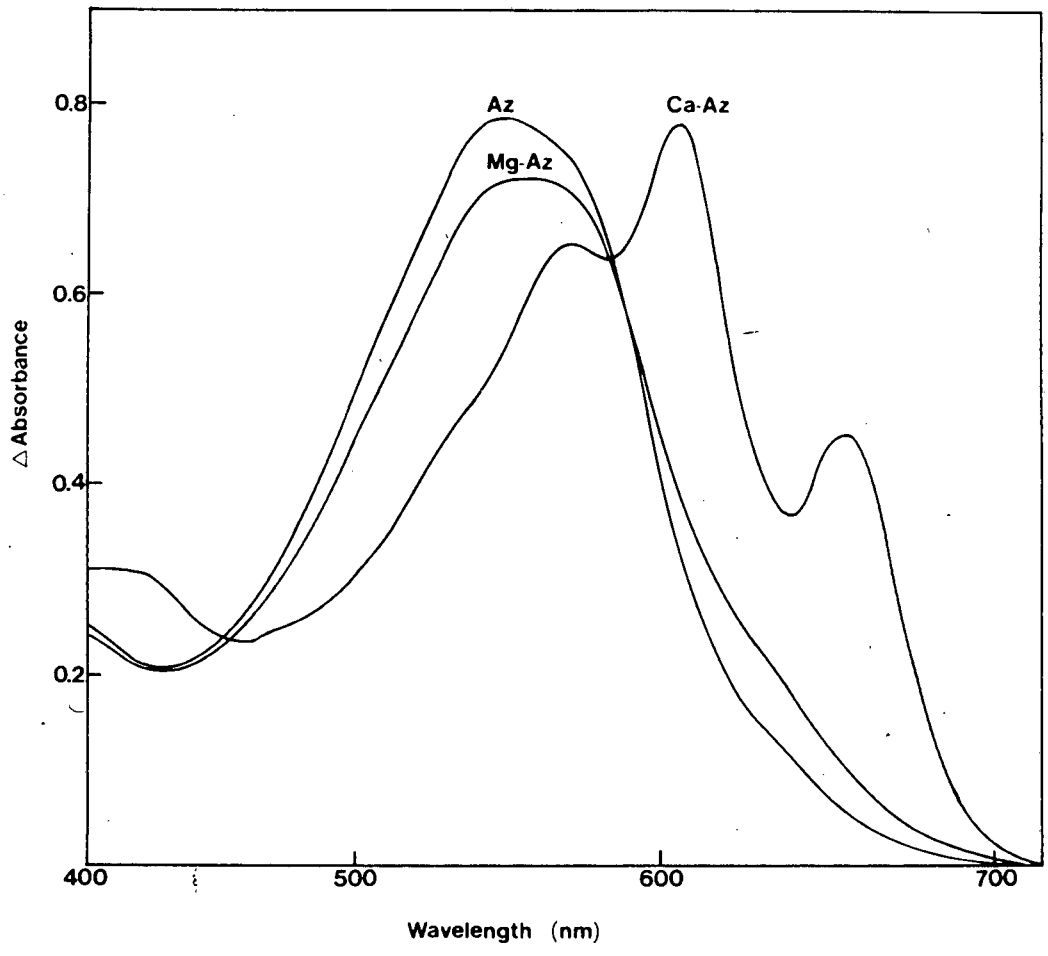


Figure 5: Absorption spectra of arsenazo III, Ca arsenazo III and Mg arsenazo III, ionic strength 0.05.

Solutions contained 40 μM arsenazo III, 100 mM KCl, 60 mM Pipes, pH 6.8.

Arsenazo III has several advantages over murexide for the determination of low concentrations of ionised Ca^{2+} in biological systems: (a) the molar extinction coefficient for the differential spectra maxima are about two fold greater than those for murexide and (b) the apparent association constant for arsenzao III, at biological pH and ionic strength, is about two orders of magnitude greater than that of murexide which greatly increases the differential absorbance change observed with a given Ca^{2+} concentration (in the physiological range of intracellular Ca^{2+} concentrations) (Dipolo et al., 1976; Johnson et al., 1983).

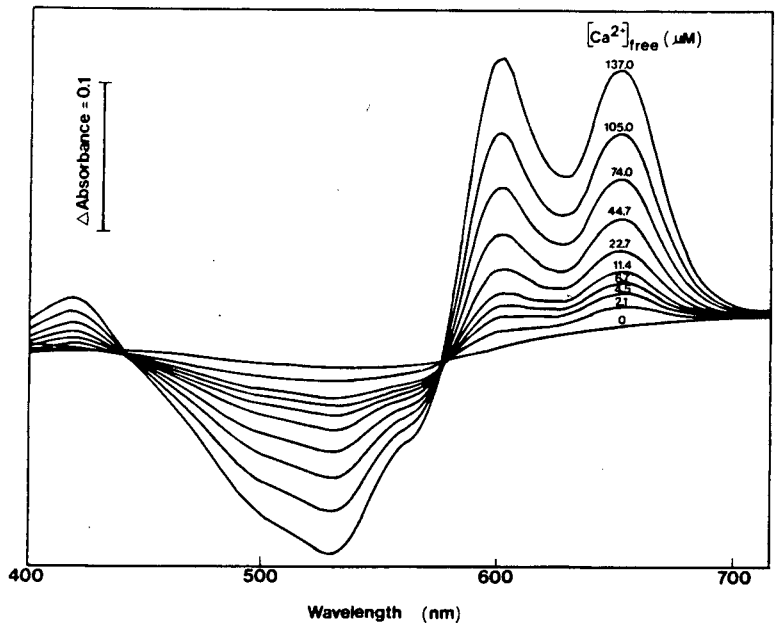
Although arsenazo III has high sensitivity to Ca^{2+}, it has the following disadvantages: (a) interference by Mg^{2+}, (b) uncertainty and variations in the Ca^{2+}-arsenazo III complex structure at different Ca^{2+}: arsenazo III ratios (Ogawa et al., 1980) and (c) arsenazo III can only be used over a limited pH range due to the presence of ionisable groups.

Purification:

Arsenazo III, obtained from Sigma, contained 2-4% (w/v) calcium. Ca^{2+} was removed by ion-exchange chromatography, as described by Dipolo et al. (1976). A solution of 1.2 mM arsenazo III was passed three times through a column of Chelex 100 (Biorad company), prewashed with 0.1 M sodium acetate, pH 6.5. The eluate of the third passage was used throughout the experiments. Since aqueous solutions of the dye have been reported to be stable for long periods of time, up to several years (Kendrick et al., 1977), the solution was stored, tightly stoppered, at room temperature.

Calibration procedure:

All absorbance measurements were made on an Aminco DW.2 dual wavelength spectrophotometer. The spectra of arsenazo III, Ca^{2+}-arsenazo III and Mg^{2+}-arsenazo III, at $\mu = 0.05$, are shown in Fig. 5. The difference spectra for the Ca^{2+} and Mg^{2+} chelates, in the presence of 40 μM arsenazo III, 0.1 M KCl, 60 mM Pipes, pH 6.8, are shown in Fig. 6. The Ca^{2+} difference spectra exhibit a broad minimum at about 530 nm, two maxima at 600 and 653 nm, and an isosbestic point at 575 nm. The difference spectra for Mg^{2+} binding by the dye has a broad minimum at



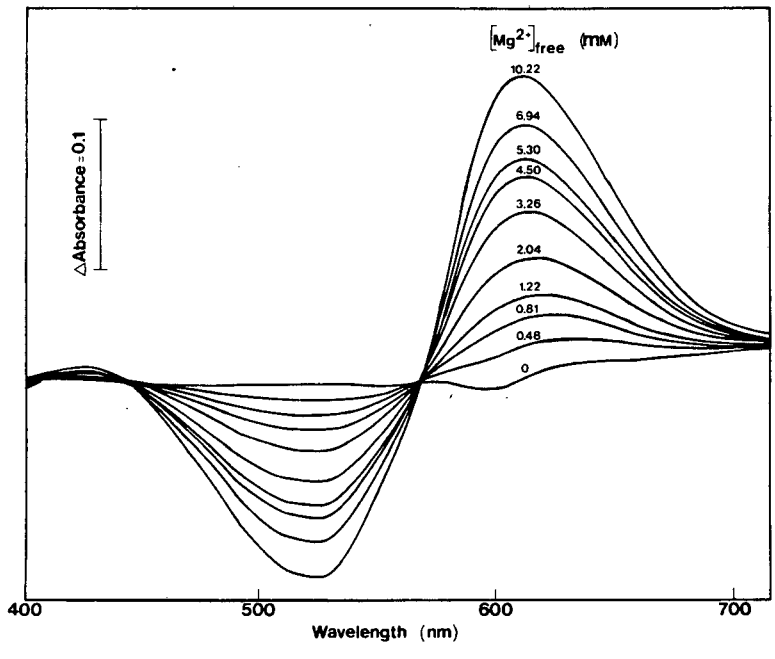


Figure 6: Differential absorption spectra of arsenazo III as a function of Ca^{2+} and Mg^{2+}.

The free cation concentration is indicated above each trace. Medium: 40 μM arsenazo III, 60 mM Pipes, pH 6.8, 100 mM KCl, 1 mM EGTA.

523 nm, a single maximum at 612 nm and an isosbestic point at 568 nm.

Although the difference spectra for the Ca^{2+} and Mg^{2+} chelates have their maxima at different wavelengths, Mg^{2+} interference is not negligible but can be minimised by choosing a suitable wavelength pair at which the difference signal for Mg^{2+} is small, while that for Ca^{2+} is large. The concentration of free Ca^{2+} in the presence of 1 mM EGTA, without added divalent cations, can be taken as zero, since a further addition of 1 mM EGTA does not further decrease the Ca^{2+} signal (Fig. 7). The influence of Mg^{2+} on the Ca^{2+} signal was investigated at the wavelength pairs 675-685 nm (Scarpa et al., 1978) and 660-685 nm (Dipolo et al., 1976; Thomas, 1979) (Fig. 7). The sensitivity of arsenazo III for Ca^{2+} over Mg^{2+} at the wavelength pair 675-685 nm was calculated to be 1500 : 1, while that at the wavelength pair 660-685 nm is 1200 : 1, using the calculated concentrations of free divalent cations, and the observed absorbance changes. The absorbance change resulting from 1 µM free Ca^{2+} is three times greater at the 660-685 nm wavelength pair, and, since this is the concentration range of importance in this study, all further studies were conducted at this wavelength pair.

Calibration of the absorbance change (660-685 nm) of Ca^{2+}-arsenazo III, with respect to ionised Ca^{2+}, is shown in Fig. 8. The calibration medium contained 60 mM Pipes, pH 6.8, 100 mM KCl, 1 mM $MgCl_2$, 40 µM arsenazo III, 1 mM EGTA and 0-1 mM $CaCl_2$. Under these conditions nearly all the added $CaCl_2$ is complexed by EGTA which is a much stronger buffer than arsenazo III, and hence the free Ca^{2+} concentration was calculated from the CaEGTA association constant only, which was taken as $10^{11.0}$ (Schwartzenbach et al., 1957). The calibration curve, relating absorbance changes of arsenazo III with varying calcium concentrations, showed deviations from linear behaviour and was distinctly S-shaped. At high Ca^{2+} concentrations, the deviation is readily explained by saturation of Ca^{2+} binding by arsenazo III. In the low $[Ca^{2+}]_{free}$ range (<3 µM), the deviation is explained by the formation of a 2:1 complex of arsenazo III with Ca^{2+} (Budesinsky, 1958; Thomas, 1979; Ogawa et al., 1980) and not a 1:1 complex, as is often assumed. All experiments were conducted at Ca^{2+} concentrations of less than 3 µM, and the calibration curve was described by a quadratic function of the form $A = a+b([Ca^{2+}]) + c([Ca^{2+}]^2)$. Typical values for a, b and c were 0.0083, 2.58 and 1.32, respectively,

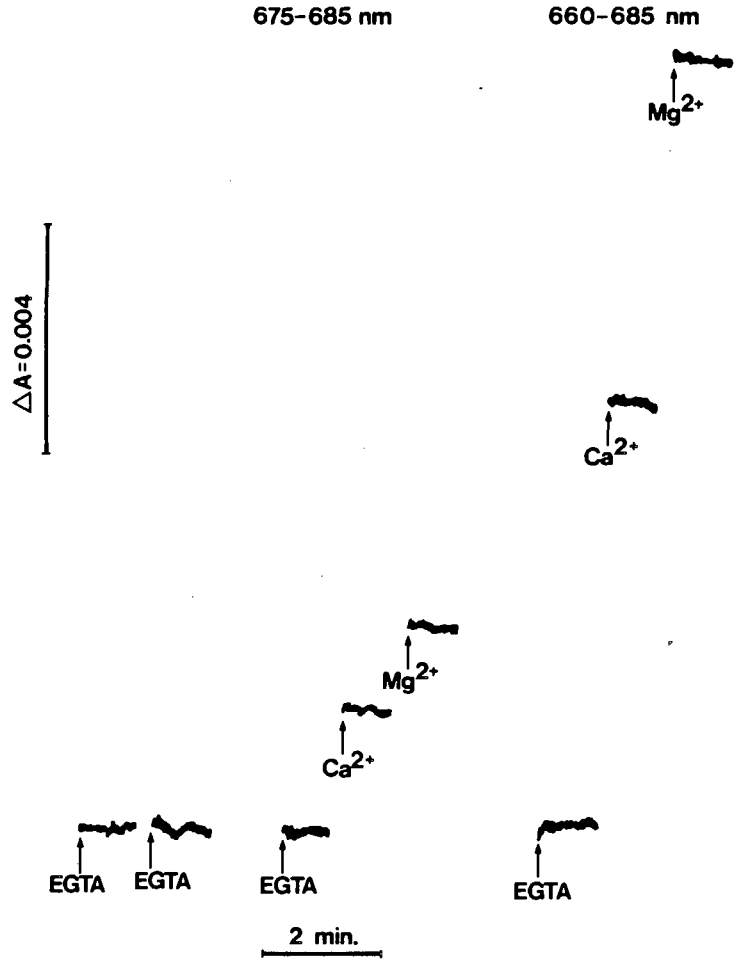


Figure 7: Effect of magnesium on calcium-arsenazo III absorption at different wavelength pairs.

Medium: 40 µM arsenazo III, 60 mM Pipes, pH 6.8, 100 mM KCl. EGTA (1 mM), $MgCl_2$ (1 mM) and $CaCl_2$ (0.67 mM) were added where indicated. $[Ca^{2+}]_{free}$ was 1.05 µM.

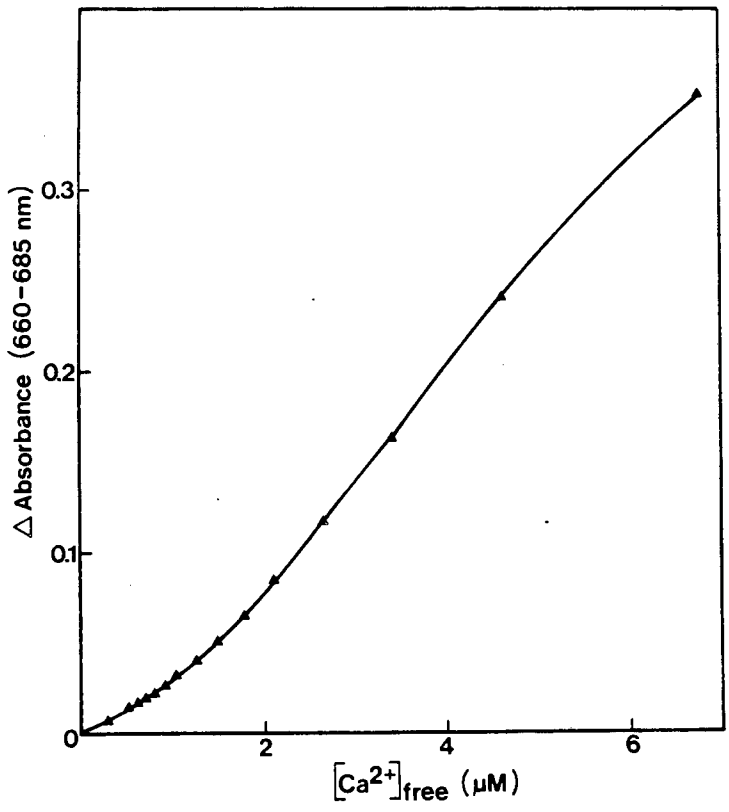


Figure 8: Calibration curve showing absorbance change of Ca^{2+}-arsenazo III with ionised Ca^{2+}.

Medium: 40 μM arsenazo III, 60 mM Pipes, pH 6.8, 1 mM EGTA, 100 mM KCl. Absorbance in the presence of 1 mM EGTA was taken as zero free Ca^{2+}. Total added Ca^{2+} was increased from 0 to 1 mM.

at a full scale deflection of 0.05 absorbance units.

Calcium transport measurements:

Calcium transport was measured by a Ca^{2+}-stat method analogous to that described in Section 2.2.2.2. The voltage output from the spectrophotometer, which was related to $[Ca^{2+}]_{free}$, was connected via an analogue-to-digital converter to an Apple II microcomputer, which was programmed to act as titrator. Calcium was infused into the reaction mixture, as a 10 mM $CaCl_2$ solution, from an Autoburette (Radiometer type ABU 80) that was controlled via a parallel interface (type BCD A/D-1). The volume of titrant, together with the Ca^{2+} concentration, was recorded in the graphics mode on a visual display unit (type 100G). The reaction medium, at 20°C, consisted of 100 mM KCl, 1 mM $MgCl_2$, 5 mM potassium oxalate, 60 mM Pipes, pH 6.8 and 0.05 - 0.1 mg/ml SR protein. The reaction was initiated by addition of 1 mM ATP. The solution in the cuvette (3 ml) was continuously stirred by means of a vibrating platinum loop (Aminco). Fifty data points, taken over a period of 140 seconds, were fitted to a binomial function and the initial rate of transport was calculated.

2.2.3. COMPUTATIONS

2.2.3.1. Derivation of the Concentration of Individual Species in Complex Reaction Mixtures

The concentration of free metal ions, free nucleotide species and metal-nucleotide complexes were calculated with the use of a computer programme written in BASIC for a HP-85 desktop computer (Hewlett Packard) (Appendix A), according to the method of Storer and Cornish-Bowden (1976). The equilibria considered, and the values used for the association constants, are shown in Table I. The conservation equations for the metal chelates and metal ions contain all their free and complexed forms shown in Table 1. Calcium and H^+ were not included when these were measured directly by the Ca^{2+}- and pH-electrodes, respectively. The total concentration of the species was used as initial estimates of the concentrations of the free species, since the programme does not allow a direct calculation of these. The free concentrations

were then refined by a reiteration procedure until the difference between two consecutive values was less than 0.1% (Appendix A).

2.2.3.2. Calculation of the Kinetic Parameters V_{max}, K_m, $K_{0.5}$ and n_H

Values for V_{max}, K_m, $K_{0.5}$ and n_H were obtained by non-linear regression analysis of the functions

$$v = V_{max} \frac{S^{n_H}}{K_m + S^{n_H}}$$

and $K_{0.5} = \exp (\log K_m/n_H)$

as described by Atkins (1973). The kinetic parameters were calculated by use of a computer programme written in BASIC for the HP-85 desktop computer. All graphs are computer drawn by fitting the data points to the equations above.

TABLE I

ASSOCIATION CONSTANTS USED IN OUR CALCULATIONS

Reaction	log K	Ref*
$H^+ + EGTA^{4-} \longleftrightarrow HEGTA^{3-}$	9.47	1
$H^+ + HEGTA^{3-} \longleftrightarrow H_2EGTA^{2-}$	8.85	1
$H^+ + H_2EGTA^{2-} \longleftrightarrow H_3EGTA^-$	2.66	1
$H^+ + H_3EGTA^- \longleftrightarrow H_4EGTA$	2.0	1
$Mg^{2+} + EGTA^{4-} \longleftrightarrow MgEGTA^{2-}$	5.21	1
$Mg^{2+} + HEGTA^{3-} \longleftrightarrow MgEGTA^-$	3.36	1
$Ca^{2+} + EGTA^{4-} \longleftrightarrow CaEGTA^{2-}$	10.97	1
$Ca^{2+} + HEGTA^{3-} \longleftrightarrow CaHEGTA^-$	3.79	1
$H^+ + ATP^{4-} \longleftrightarrow HATP^{3-}$	7.02	2
$H^+ + HATP^{3-} \longleftrightarrow H_2ATP^{2-}$	4.02	2
$Ca^{2+} + ATP^{4-} \longleftrightarrow CaATP^{2-}$	4.32	2
$Ca^{2+} + HATP^{3-} \longleftrightarrow CaHATP^-$	2.13	2
$Mg^{2+} + ATP^{4-} \longleftrightarrow MgATP^{2-}$	4.65	2
$Mg^{2+} + HATP^{3-} \longleftrightarrow MgHATP^-$	2.65	2
$K^+ + ATP^{4-} \longleftrightarrow KATP^{3-}$	1.11	2
$Ca^{2+} + AcP \longleftrightarrow CaAcP$ (pH 6.8)	0.89	3
$Mg^{2+} + AcP \longleftrightarrow MgAcP$ (pH 6.8)	0.76	3
$Mg^{2+} + pNPP \longleftrightarrow MgNPP$	2.22	4
$H^+ + PPi^{4-} \longleftrightarrow HPPi^{3-}$	9.0	2
$H^+ + HPPi^{3-} \longleftrightarrow H_2PPi^{2-}$	6.23	2
$Ca^{2+} + PPi^{4-} \longleftrightarrow CaPPi^{2-}$	5.46	2
$Mg^{2+} + PPi^{4-} \longleftrightarrow MgPPi^{2-}$	4.70	2
$H^+ + citrate \longleftrightarrow H\text{-citrate}$	5.68	2
$H^+ + H\text{-citrate} \longleftrightarrow H_2\text{-citrate}$	4.38	2
$Ca^{2+} + citrate \longleftrightarrow Ca\text{-citrate}$	3.55	2
$Mg^{2+} + citrate \longleftrightarrow Mg\text{-citrate}$	3.4	2
$H^+ + PO_4^{3-} \longleftrightarrow HPO_2^{2-}$	11.74	1
$H^+ + HPO_4^{2-} \longleftrightarrow H_2PO_4^-$	5.72	1
$Ca^{2+} + PO_4^{3-} \longleftrightarrow CaPO_4^-$	6.46	1
$Mg^{2+} + PO_4^{3-} \longleftrightarrow MgPO_4$	3.4	1

* 1. Martell and Smith, 1974; 1975
2. Vianna, 1975
3. Oestreich and Jones, 1966
4. Robbinson, 1969

2.3. RESULTS

2.3.1. THE CALCIUM-CONCENTRATION DEPENDENCE OF CALCIUM TRANSPORT IN THE PRESENCE AND ABSENCE OF EGTA

Calcium transport is conventionally measured essentially according to the method of Martonosi and Feretos (1964a) or to modifications thereof, in which the amount of radioactive calcium, transported into the SR vesicles, is measured as a function of time. The inclusion of oxalate, a freely permeable anion, ensures that the transported Ca^{2+} is precipitated within the vesicular lumen, thus 'clamping' internal $[Ca^{2+}]_{free}$ at a low value. EGTA, an impermeable anion (Weber et al., 1966), is a Ca^{2+}-selective chelating agent, and acts as a Ca^{2+} buffer such that $[Ca^{2+}]_{free}$ in the medium can be adjusted. An alternative to EGTA has been to utilise the Ca^{2+}-scavenging ability of isolated SR vesicles and to measure calcium transport by a calcium-stat technique (Berman and Aderem, 1981) using a Ca^{2+}-specific electrode as $[Ca^{2+}]_{free}$ detector.

The Ca^{2+}-dependence of Ca^{2+} transport was measured by the $^{45}Ca^{2+}$/Millipore filtration technique, in the presence of 0.5 mM EGTA, and by the calcium-stat technique in the absence of EGTA (Fig. 9). The saturation curves towards Ca^{2+} display the same maximum transport activity of 2.0 μmol $min^{-1}mg^{-1}$. The apparent affinity of the Ca^{2+}-transport sites in the absence of EGTA ($[Ca^{2+}]_{0.5}$ = 0.99 μM) is an order of magnitude lower than that measured in its presence ($[Ca^{2+}]_{0.5}$ = 0.1 μM). The Ca^{2+}-dependence of transport, in the absence of EGTA, shows extreme sigmoidal behaviour, such that a Hill coefficient, $n_{H(Ca^{2+})}$, of greater than five is required to describe the curve. In the presence of EGTA, $n_{H(Ca^{2+})}$ = 1.7 is in good agreement with that previously reported (Vianna, 1975; Tada et al., 1978).

It has previously been demonstrated in this laboratory (Berman, 1982a) that the species $CaEGTA^{2-}$ increased the apparent affinity of the Ca^{2+}-transport sites, with half maximal effect at approximately 20 μM. The effect of CaEGTA (100 μM) on the Ca^{2+}-dependence of Ca^{2+} transport, supported by the substrates ATP and acetyl phosphate is shown in Fig. 10. CaEGTA had no obvious effect on Ca^{2+} transport at saturating Ca^{2+} concentrations, but stimulated the calcium pump at limiting external $[Ca^{2+}]_{free}$, in the presence of both substrates. CaEGTA (100 μM)

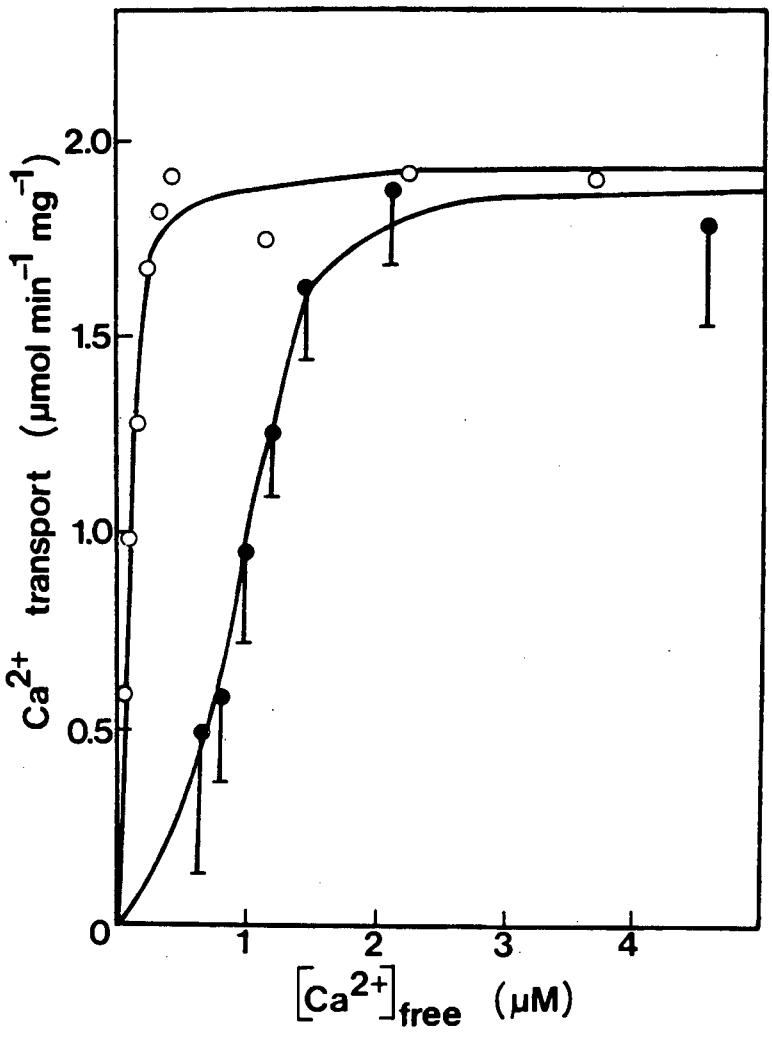


Figure 9: The Ca^{2+}-dependence of Ca^{2+} transport in the presence and absence of EGTA.

Calcium transport was measured by the $^{45}Ca^{2+}$ Millipore filtration technique (○) in the presence of 0.5 mM EGTA and by the calcium-stat technique (●) in the absence of EGTA in medium as described under Experimental Procedures.

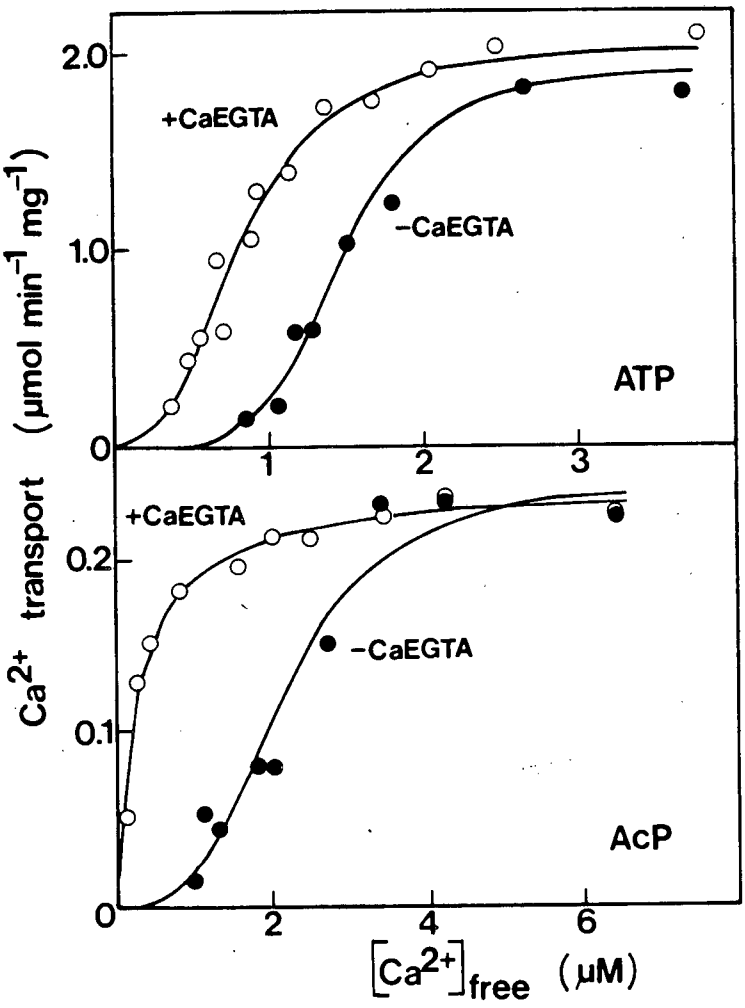


Figure 10: The effect of CaEGTA on the Ca^{2+}-dependence of Ca^{2+} transport supported by ATP and acetylphosphate.

Ca^{2+} transport was measured by the Ca^{2+}-stat method in medium (2 ml), at 20°C, containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, pH 7.2 and 5 mM of either ATP (top panel) or acetylphosphate (bottom panel). The reaction was initiated by addition of 100-200 µg SR protein. CaEGTA (100 µM) was added before the start of the reaction where indicated.

decreased $[Ca^{2+}]_{0.5}$ from 1.5 to 0.8 μM with ATP as substrate and from 2.2 to 0.7 μM with AcP as substrate. The Hill coefficient was lowered to approximately two in the presence of CaEGTA.

The (Na^+,K^+)-ATPase of lymphocyte plasma membranes is similarly activated by EGTA, a phenomenon which was attributed by Segel et al. (1981) to be due to zinc chelation rather than calcium chelation. They showed stimulation of ATPase activity by histidine, which chelates zinc but not Ca^{2+}. Stimulation of Ca^{2+} transport by the (Ca^{2+},Mg^{2+})-ATPase of SR vesicles, in the presence of 20 μM histidine, is shown in Fig. 11. Ca^{2+} transport, at $[Ca^{2+}]_{free}$ = 0.3 μM, increased from 0.13 to 0.97 μmol min^{-1} mg^{-1} on addition of 50 μM CaEGTA. Since EGTA activation of transport, in the present experiment, is evident at concentrations of histidine that would be expected to chelate any available free zinc, it is concluded that the effects are due to interaction of CaEGTA with the Ca^{2+}-transport site of the Ca^{2+}-ATPase.

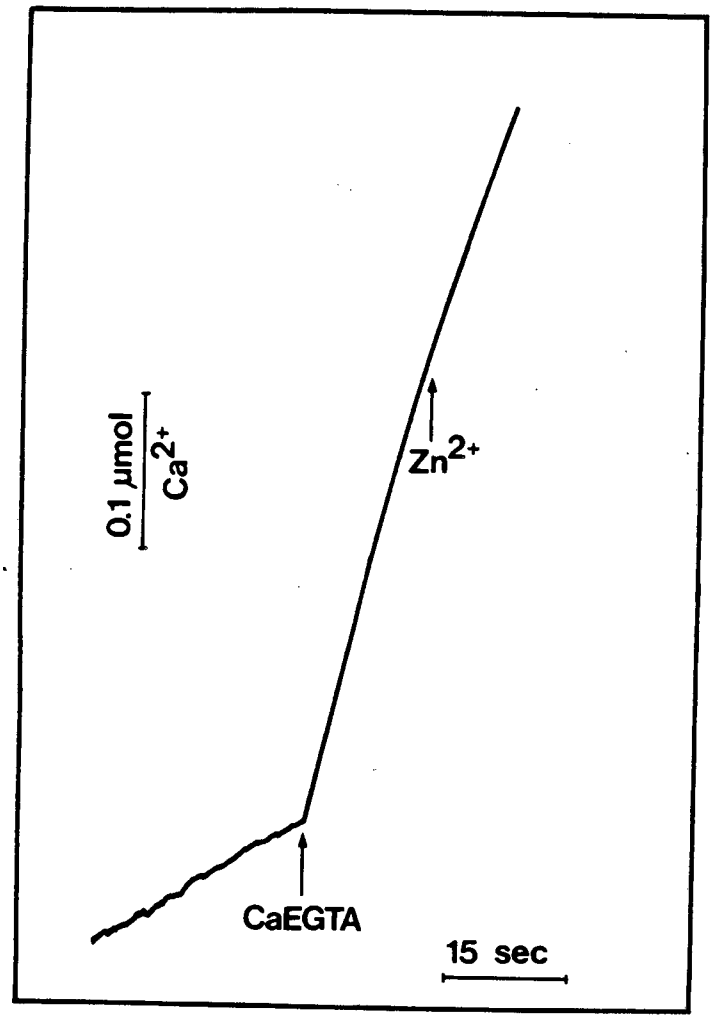


Figure 11: Stimulation of Ca^{2+} transport by CaEGTA in the presence of 20 mM histidine.

The reaction medium, at 20°C, contained 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 20 mM histidine, pH 7.2, 5 mM ATP and 100 µg SR protein. Ca^{2+} transport was monitored as described under "Experimental Procedures". CaEGTA, 50 µM, and $ZnCl_2$, 25 µM, were added where indicated.

2.3.2. EFFECTS OF SOLUBLE ATP-METAL COMPLEXES ON THE APPARENT CALCIUM AFFINITY OF CALCIUM TRANSPORT

The results described in Section 2.3.1 suggest that Ca^{2+} transport by SR vesicles is stimulated by the calcium complex of EGTA. The question arose as to whether there is a natural ligand present in the cytosol of muscle cells with the same function as EGTA *in vitro*.

MgATP, the true substrate for ATP hydrolysis (Vianna, 1975), in the range 1.0 to 7.5 mM, resulted in an increase in the maximum rate of Ca^{2+} transport, at 20°C, from 0.6 to 1.1 μmol min^{-1} mg^{-1} (Fig. 12), while the affinity of the enzyme for Ca^{2+} was unaffected ($[Ca^{2+}]_{0.5}$ = 0.8 μM).

The effect of various ATP complexes on the affinity of the (Ca^{2+},Mg^{2+})-ATPase for Ca^{2+} was investigated by increasing the total ATP concentration from 1.0 to 10.0 mM while maintaining the total Mg^{2+} concentration constant at 1.0 mM (Fig. 13). Under these experimental conditions, the maximum rate of transport, at saturating Ca^{2+} concentrations, decreased from 2.33 to 1.2 μmol min^{-1} mg^{-1}. These data can be explained on the basis that MgATP is the true substrate for ATP hydrolysis (Fig. 12) and that either free Mg^{2+}, which is required at an essential step in the catalytic cycle is decreased to limiting (micromolar) concentrations upon chelation by ATP (see below) or, as suggested by Vianna (1975), CaATP, which increased in concentration, inhibits MgATP binding.

Increasing the total ATP concentration from 1.0 to 10.0 mM, in the presence of 1.0 mM $MgCl_2$, resulted in a decrease in $[Ca^{2+}]_{0.5}$ from 1.05 to 0.15 μM (Fig. 13). The question arose as to which ligand species was responsible for this apparent increase in the enzyme's affinity for Ca^{2+}. There are four possible effectors, viz. MgATP, free ATP, CaATP and free Mg^{2+}. The concentration of MgATP remained practically constant (varied from 0.8 to 0.97 mM), when the total ATP concentration was increased, and was therefore excluded from further consideration. The relationship between free Mg^{2+}, CaATP and free ATP concentration and the enzyme's affinity for Ca^{2+} is shown in Fig. 14.

Increasing the total ATP from 1.0 to 10.0 mM, in the presence of

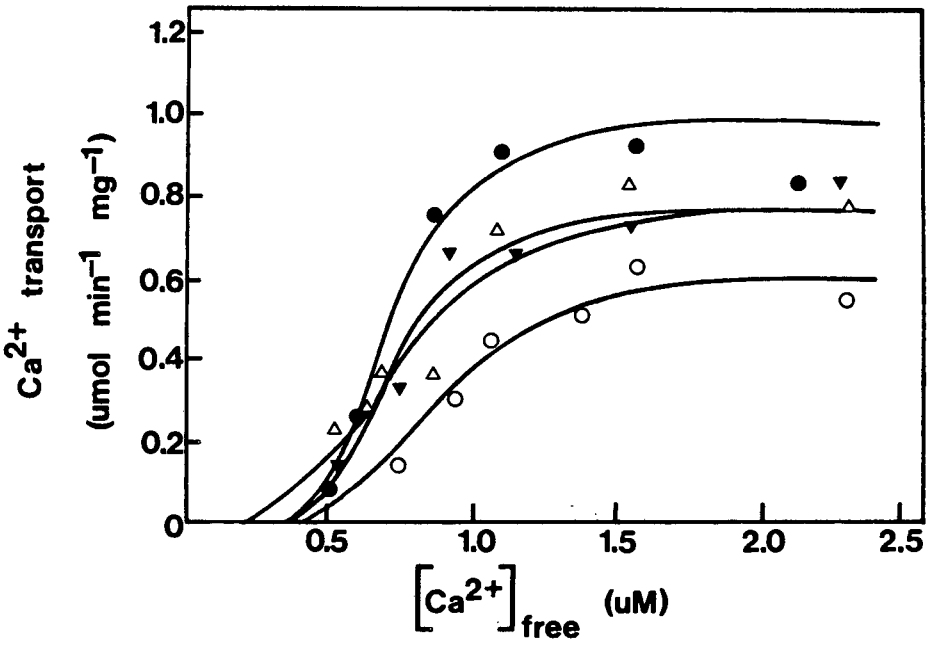


Figure 12: Effect of MgATP on the Ca^{2+} concentration dependence of Ca^{2+} transport.

Ca^{2+} transport was measured as in Fig. 7. The ATP concentration was either 1 mM (○), 3 mM (▼), 5 mM (△) or 7.5 mM (●). Equimolar concentrations of $MgCl_2$ was included in the reaction mixture.

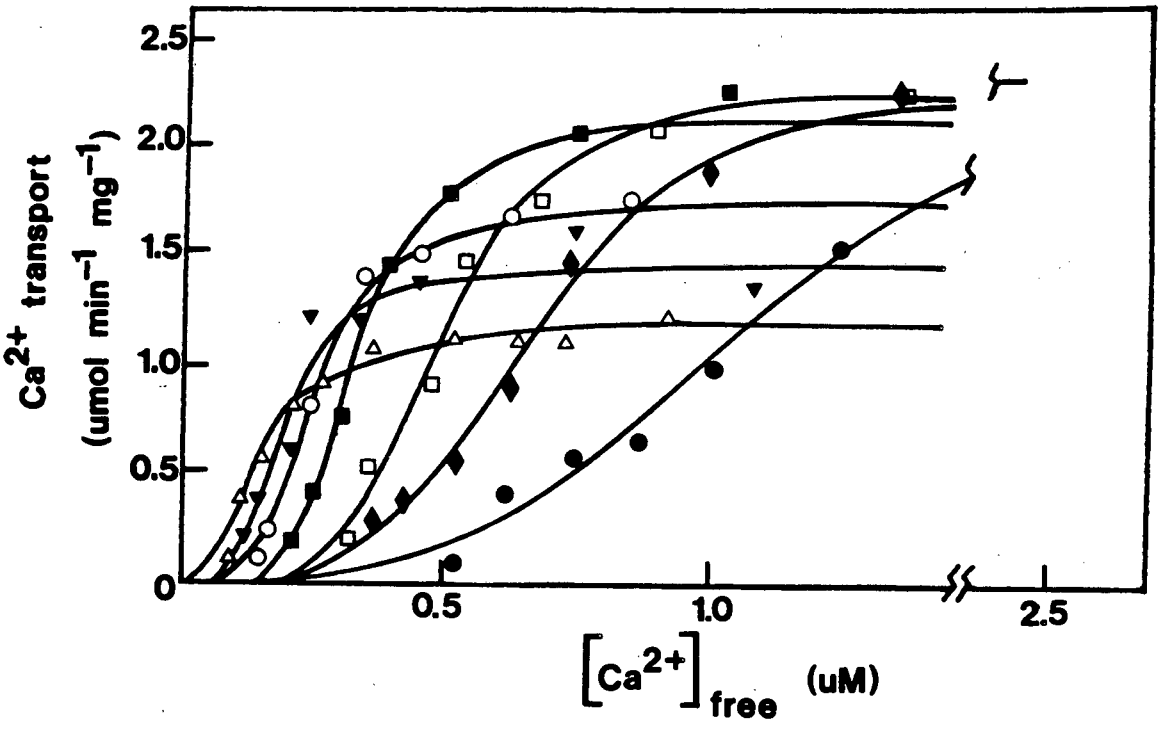


Figure 13: Effect of ATP concentration on the Ca^{2+}-concentration dependence of Ca^{2+} transport.

Ca^{2+} transport was measured as in Fig. 7. ATP concentrations were either 1 mM (●), 1.5 mM (♦), 2 mM (□), 3 mM (■), 5 mM (○), 7.5 mM (▼) or 10 mM (△). The total $MgCl_2$ concentration was constant at 1 mM.

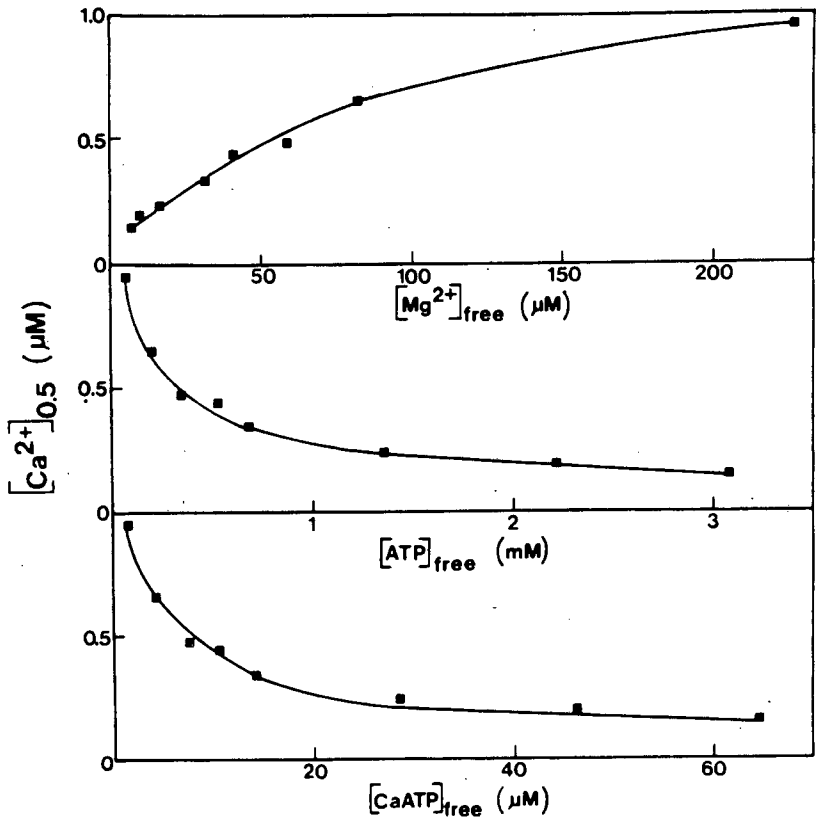


Figure 14: Relationship between $[Ca^{2+}]_{0.5}$ and the concentration of free Mg^{2+}, free ATP and CaATP.

$[Ca^{2+}]_{0.5}$ was obtained from Fig. 9. The concentration of free Mg^{2+}, free ATP and CaATP (at 1 μM free Ca^{2+}) was calculated as described under "Experimental Procedures".

1.0 mM Mg^{2+}, resulted in a decrease of free Mg^{2+} from 250 to 10 μM, and was associated with an increase in the affinity of the enzyme for Ca^{2+}, with $[Mg^{2+}]_{0.5}$ = 40 μM. The possibility existed that free Mg^{2+} was competing with Ca^{2+} for the Ca^{2+}-binding sites on the enzyme, thereby increasing its apparent affinity. An experiment was designed in which the free ATP and the CaATP concentration was varied, while the free Mg^{2+} concentration was kept constant at 7-8 μM, by buffering with pyrophosphate (Table II). Increasing free ATP from 0.3 to 3.0 μM and CaATP from 6.0 to 64.0 μM resulted in an increase in the affinity of the enzyme for Ca^{2+} ($[Ca^{2+}]_{0.5}$ decreased from 0.5 to 0.2 μM). A $[Ca^{2+}]_{0.5}$ value of 0.5 μM in the presence of 0.3 mM free ATP or 6 μM CaATP and that of 0.2 μM in the presence of 3 mM free ATP or 65 μM CaATP, is in good agreement with the extrapolated values from Fig. 14. At constant free ATP and CaATP concentration, varying the free Mg^{2+} concentration over the range (10 to 100 μM), at which maximum apparent change in affinity was observed (Fig. 14), had no effect on $[Ca^{2+}]_{0.5}$ (Table III).

The results therefore indicate that free Mg^{2+}, in the micromolar range, had no effect on the apparent affinity of the enzyme for Ca^{2+}, but that either free ATP, in the millimolar range ($[ATP]_{0.5}$ = 0.5 mM) or CaATP, in the micromolar range ($[CaATP]_{0.5}$ = 10 μM), modulates the apparent affinity of the (Ca^{2+},Mg^{2+})-ATPase for Ca^{2+}. Since it was not possible to vary the concentration of free ATP, without affecting the concentration of CaATP in mixtures containing ATP and Ca^{2+}, the possible effects of other substrates, that do not bind Ca^{2+} with high affinity, was studied.

TABLE II

Effect of ATP and CaATP on $[Ca^{2+}]_{0.5}$ at constant Mg^{2+} concentrations

Ca^{2+} transport was measured by the Ca^{2+}-stat technique in medium, at 20°C, containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM K-oxalate, pH 7.2 and either 1 mM or 10 mM ATP. Pyrophosphate (1 mM) was included where indicated. $[Ca^{2+}]_{0.5}$ and the concentration of free ATP, free Mg^{2+}, MgATP and CaATP was determined as described under "Experimental Procedures".

Substrate	$[Ca^{2+}]_{0.5}$ (μM)	$[ATP]_{free}$ (mM)	$[Mg^{2+}]_{free}$ (μM)	[MgATP] (mM)	[CaATP] (μM)
1 mM ATP + 1 mM PP_i	0.5	0.3	8.33	0.115	6.26
10 mM ATP	0.2	3.08	7.16	0.786	64.4

TABLE III

The effect of low Mg^{2+} concentrations on $[Ca^{2+}]_{0.5}$

Ca^{2+} transport was measured by the Ca^{2+}-stat technique in medium, at 25°C, containing 100 mM KCl, 10 μM ATP, 5 mM potassium oxalate, 20 mM Tris, pH 7.2, 1.0 mM creatine phosphate and 200 U/ml creatine kinase. $MgCl_2$ was varied from 10 to 100 μM. $[Ca^{2+}]_{0.5}$ and free Mg^{2+} was calculated as described under "Experimental Procedures".

$[Mg^{2+}]_{total}$ (μM)	$[Mg^{2+}]_{free}$ (μM)	$[Ca^{2+}]_{0.5}$ (μM)
10	8.8	1.00
50	46	0.79
100	94	0.84

2.3.3. SUBSTRATE DEPENDENCE OF THE APPARENT CALCIUM AFFINITY OF CALCIUM TRANSPORT

Ca^{2+} transport by isolated SR vesicles is supported by ATP as well as a number of pseudosubstrates (Makinose and The, 1965; De Meis, 1969; Pucell and Martonosi, 1971; Inesi, 1971). Maximum transport rates for various substrates, at 20°C, were 1.5 (ATP), 1.3 (ITP), 0.5 (AcP) and 0.04 (pNPP) µmol min^{-1} mg^{-1}. The Ca^{2+} concentration dependence of Ca^{2+} transport for these substrates is shown in Fig. 15. The $[Ca^{2+}]_{0.5}$ for the various substrates varied from 1 to 5 µM in the order ATP < ITP < AcP < pNPP. The data suggest that the substrate modulates the apparent affinity of the enzyme for Ca^{2+}.

The Ca^{2+}-concentration dependence of Ca^{2+} transport at varying pseudosubstrate concentrations was investigated. Increasing the concentration of AcP from 2.5 to 10.0 mM resulted in an increase in the rate of Ca^{2+} transport from 0.22 to 0.39 µmol min^{-1} mg^{-1} (Fig. 16A). Similarly, increasing the concentration of pNPP increased V_{max} (Fig. 16B). The Ca^{2+} affinity of the enzyme did not change significantly with change in substrate concentration, a fact that was correlated with their inability to form significant amounts of Ca^{2+}-chelating complexes.

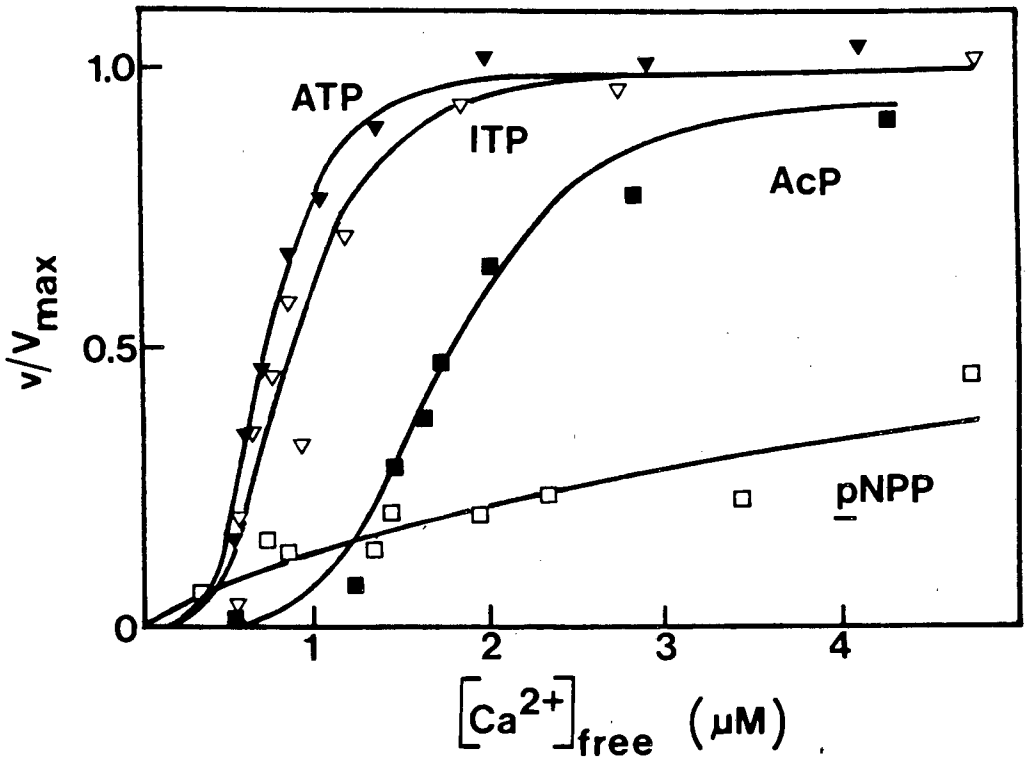


Figure 15: The effect of substrate on the Ca^{2+}-concentration dependence of Ca^{2+} transport.

Ca^{2+} transport was measured by the Ca^{2+}-stat method in medium (2 ml), at 20°C, containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, pH 7.2 and 5 mM of either ATP, ITP, acetylphosphate (AcP) or p-nitrophenylphosphate (pNPP). The reaction was initiated by the addition of 100-200 μg SR protein.

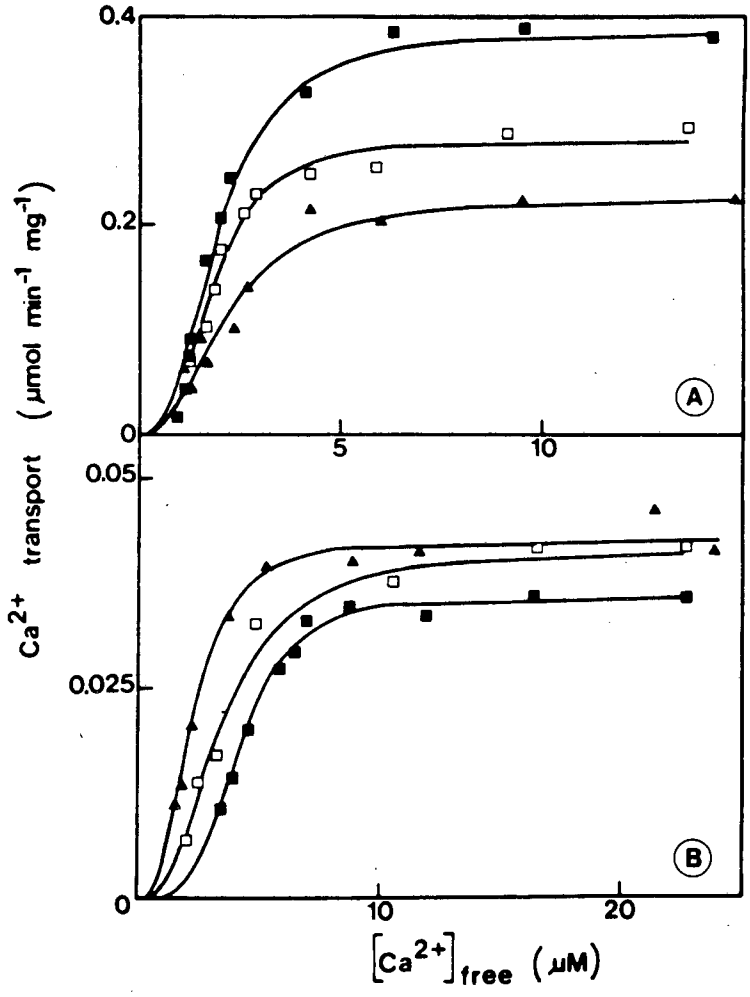


Figure 16: Effect of increasing concentrations of acetyl phosphate and p-nitrophenyl phosphate on the Ca^{2+}-concentration dependence of Ca^{2+} transport.

Ca^{2+} transport was measured by the Ca^{2+}-stat method in medium containing 100 mM KCl, 5 mM potassium oxalate, 5 mM $MgCl_2$, pH 7.2, and either A: 2.5 mM (▲), 5 mM (□) or 10 mM (■) acetylphposphate, or B: 10 mM (■), 20 mM (□) or 30 mM (▲) p-nitrophenyl phosphate.

2.3.4. THE EFFECT OF CALCIUM CHELATING ANIONS ON $[Ca^{2+}]_{0.5}$

We have shown that both EGTA and ATP modulate the apparent Ca^{2+} affinity of the Ca^{2+}-transport sites of the (Ca^{2+},Mg^{2+})-ATPase while AcP and pNPP are without effect. The ability of EGTA and ATP to enhance the affinity of the transport sites appeared to correlate with their affinity for Ca^{2+} to form a Ca^{2+}-chelate (Berman, 1982a). It was of interest to establish whether this is a general phenomenon or whether it only applies to specific Ca^{2+}-chelating anions.

Increasing the concentration of pyrophosphate from 0 to 5 mM, in the presence of 100 µM ATP, resulted in a decrease in $[Ca^{2+}]_{0.5}$ from 1.25 to 0.54 µM (Table IV). V_{max} increased from 0.65 µmol min^{-1} mg^{-1} in the absence of PP_i to 1.3 µmol $min^{-1}mg^{-1}$ in the presence of 5 mM PP_i. Increasing the concentration of citrate from 0 to 15 mM, under the same experimental conditions, similarly resulted in an increase in V_{max} from 0.9 to 1.7 µmol $min^{-1}mg^{-1}$ (Table V). The effect of citrate on $[Ca^{2+}]_{0.5}$ is complicated by the interfering effect of Mg^{2+} on the electrode potential (see Section 2.2.2.2). When ignored, $[Ca^{2+}]_{0.5}$ apparently decreased ten-fold from 1.17 to 0.18 µM. Increasing the citrate concentration however decreased $[Mg^{2+}]_{free}$ and thus the interfering effect. By correcting for Mg^{2+} interference (Table V), it is clear that citrate only decreases $[Ca^{2+}]_{0.5}$ three-fold, at concentrations above 5 mM.

Although it has previously been shown that pyrophosphate has an effect on the rate of Ca^{2+} transport (Dupont, 1977), the effect of citrate is unexplicable.

The results suggest that Ca^{2+} chelating anions enhance the apparent affinity of the Ca^{2+}-transport sites. It can be seen from Table IV and Table V, that this effect correlates with the concentration of the Ca^{2+}-chelate, which is in the range 5-20 µM.

TABLE IV

The effect of pyrophosphate on Ca^{2+}-transport kinetics

Ca^{2+} transport was measured by the Ca^{2+}-stat method in medium, at 25°C, containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM K-oxalate, 20 mM Tris, pH 7.2, 0.5 mM creatine phosphate, 5 U/ml creatine kinase and 100 mM ATP. $[Ca^{2+}]_{0.5}$ and $[CaP_2O_7^{2-}]$ was calculated as described under "Experimental Procedures".

$[P_2O_7^{4-}]$ (mM)	V_{max} (μmol min^{-1} mg^{-1})	$[Ca^{2+}]_{0.5}$ (μM)	$[CaP_2O_7^{2-}]$ (μM)
0*	0.65	1.25	0.0
1	0.90	0.78	1.0
3	1.25	0.68	3.9
5	1.14	0.53	8.3

* 0.1 mM ATP throughout

TABLE V

The effect of citrate on Ca^{2+}-transport kinetics

Ca^{2+} transport was measured as in legend to Table IV. $[Ca^{2+}]_{0.5}$ was calculated as described under "Experimental Procedures", using electrode potentials in which Mg^{2+} interference was both ignored ("uncorrected") and taken into consideration ("corrected") as discussed in text.

[Citrate] (mM)	V_{max} (μmol $min^{-1}mg^{-1}$)	$[Ca^{2+}]_{0.5}$ (μM)		$[Ca^{2+}$-citrate] (μM)
		'uncorrected'	'corrected'	
0*	0.90	1.17	0.49	-
1	1.12	1.04	0.54	0.3
3	1.53	0.70	0.51	1.5
5	1.62	0.56	0.51	4.3
7.5	1.68	0.35	0.34	10.6
10	1.72	0.26	0.26	18.4
15	1.51	0.18	0.18	35.0

* 0.1 mM ATP throughout

2.3.5. POSSIBLE MECHANISMS OF EGTA MODULATION OF THE CALCIUM PUMP

It was of interest to us to determine at which step in the overall reaction sequence these Ca^{2+}-chelates were exerting their effect. Both Ca^{2+} and ATP can mediate the conversion of the enzyme from a low Ca^{2+} affinity form (*E) to a high affinity form (E). The effect of ATP on this conformational transition manifests as a secondary activation of Ca^{2+} transport and of ATPase activity at ATP concentrations in excess of those required to saturate the high affinity ATP-binding sites (see Sections 2.1.1 and 2.2.2).

The ATP dependence of Ca^{2+} transport in the presence and absence of CaEGTA is shown in Fig. 17. Ca^{2+} transport measurements were determined at 25°C, in the presence of an ATP regenerating system, and at saturating $[Ca^{2+}]_{free}$ (4 μM). CaEGTA, 100 μM, had no effect on ATP activation of transport under these conditions. One might have expected that, if CaEGTA had a similar effect as ATP on activation of Ca^{2+} transport, that the biphasic curve obtained in Fig. 17 would have been abolished or that the low affinity regulatory site would have obtained higher affinity. It is concluded that CaEGTA does not exert its effect on Step 8 of Reaction Scheme I, i.e. the *E to E interconversion.

EGTA activation of Ca^{2+} transport at limiting $[Ca^{2+}]_{free}$ was explained by Berman (1982a) to occur via formation of a ternary complex of the form E.Ca.EGTA. We were interested to see whether the Ca^{2+} pump has EGTA binding sites which may facilitate Ca^{2+} binding. ^{14}C-labelled EDTA-binding was determined by the flow dialysis method (Colowick and Womack, 1969). No binding was detected in the presence or absence of Ca^{2+} and/or Mg^{2+}. EGTA and EDTA binding to the Ca^{2+} binding proteins calmodulin and parvalbumin and to bovine α-lactalbumin has, however, been reported by Haiech et al. (1980) and by Kronman and Bratcher (1983), respectively.

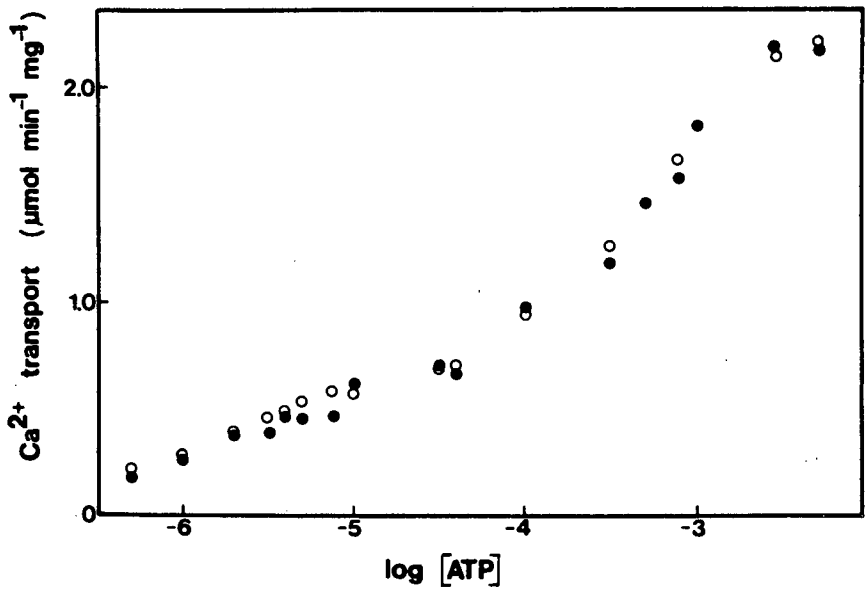


Figure 17: ATP dependence of calcium transport in the absence and presence of CaEGTA.

Ca^{2+} transport was measured by the Ca^{2+}-stat method in medium, at 25°C, containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, pH 7.2, 0.5 mM creatine phosphate, 5 U/ml creatine kinase and varying ATP from 0.5 µM to 7.5 mM. The free Ca^{2+} concentration was 4 µM both in the absence (●) and presence (O) of 100 µM CaEGTA. Transport was initiated by addition of 100 µg SR protein.

2.3.6. EFFECT OF CALCIUM PRECIPITATING ANIONS ON $[Ca^{2+}]_{0.5}$

The effect of Ca^{2+}-chelating anions on the apparent Ca^{2+} affinity of the Ca^{2+} pump observed above must be explained by changes in the medium or binding to the enzyme at the cytoplasmic surface, since the SR membrane is not permeable to these anions (Weber *et al.*, 1966). The possible effects of varying the internal Ca^{2+} concentration on the kinetic parameters of Ca^{2+} transport were investigated by varying the concentration of the Ca^{2+}-precipitating anions, oxalate and phosphate.

Increasing the concentration of oxalate from 1.0 to 5.0 mM decreases the free internal Ca^{2+} concentration from 20 to 4 μM (K_{sp} = 2×10^{-8} M) (Hasselbach and Makinose, 1963). This resulted in a concomitant increase in transport from 0.6 to 1.0 μmol $min^{-1} mg^{-1}$ and a decrease in $[Ca^{2+}]_{0.5}$ from 6 to 1 μM (Fig. 18A). Phosphate in the range 5 to 15 mM decreases the internal free Ca^{2+} concentration from 1.5 to 0.5 mM (K_{sp} = 7.5×10^{-6} M) (Hasselbach and Makinose, 1963) and decreased $[Ca^{2+}]_{0.5}$ from 3.5 to 2 μM. V_{max} was increased from 0.15 to 0.42 μmol min^{-1} mg^{-1} (Fig. 18B).

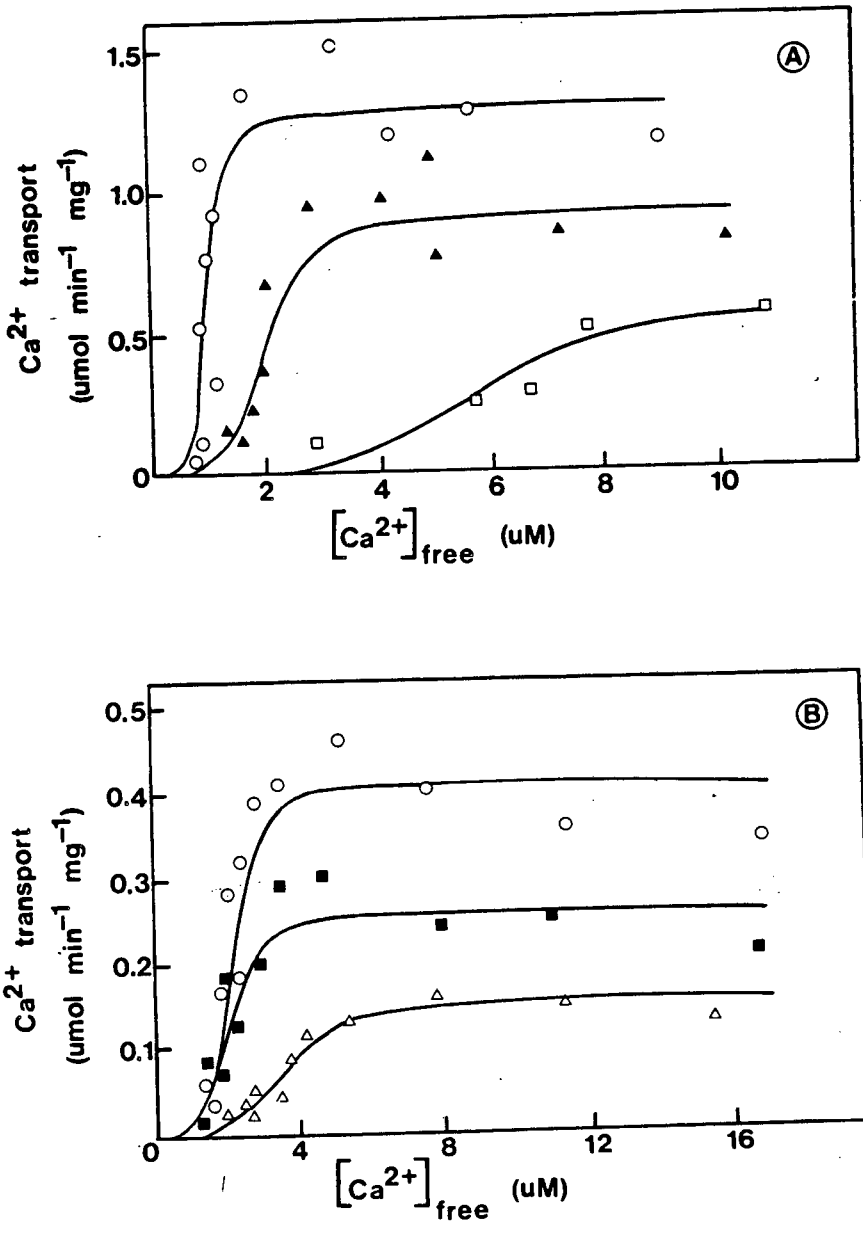


Figure 18: Effect of oxalate (A) and phosphate (B) on Ca^{2+} transport kinetics.

Ca^{2+} transport was measured by the Ca^{2+}-stat method in medium, at 20°C, containing 100 mM KCl, 5 mM $MgCl_2$, pH 7.2, 5 mM ATP and either A: 1 mM (□), 3 mM (▲) or 5 mM (○) oxalate or B: 5 mM (△), 10 mM (■) or 15 mM (○) phosphate. Ca^{2+} transport was initiated by addition of 100 µg SR protein.

2.3.7. EFFECT OF pH AND TEMPERATURE ON THE CALCIUM AFFINITY OF TRANSPORT SITES

Passive Ca^{2+} binding to the (Ca^{2+},Mg^{2+})-ATPase is highly pH dependent, exhibiting high affinity at alkaline pH and low affinity at acid pH (Watanabe et al., 1981). Analysis of the pH dependence of Ca^{2+} binding by Hill and Inesi (1982) indicated a tetramer composed of four equivalent and interacting subunits, together with competitive binding of one calcium and one proton per site.

The pH-dependence of the Ca^{2+} affinity of the Ca^{2+}-transport sites was investigated both in the presence and absence of CaEGTA. $[Ca^{2+}]_{0.5}$ (0.8 μM in the presence of 100 μM CaEGTA) and V_{max} (0.8 μmol $min^{-1}mg^{-1}$) was unaffected by increasing the pH from 6.5 to 7.5, in the presence or absence of CaEGTA (Fig. 19).

Temperature is a convenient parameter with which to induce perturbations in membrane structure, such as crystalline-liquid crystalline phase transitions of lipid moieties, or protein conformational changes. The occurrence of temperature induced transitions in SR vesicles is demonstrated by the nonlinear Arrhenius plots obtained for the rates of Ca^{2+} transport and Ca^{2+}-activated ATP hydrolysis (Deamer, 1973; Inesi et al., 1973; Davis et al., 1976). A change in their activation energies is obtained at around 20°C. We investigated the effect of temperature on Ca^{2+} transport in the presence of 5 mM ATP. Increasing the temperature in the range 14°C to 27°C, the range over which a break in the Arrhenius plot is obtained, increased the maximum rate of Ca^{2+} transport from 1.2 to 5.9 μmol $min^{-1}mg^{-1}$, while having no effect on $[Ca^{2+}]_{0.5}$ (Fig. 20).

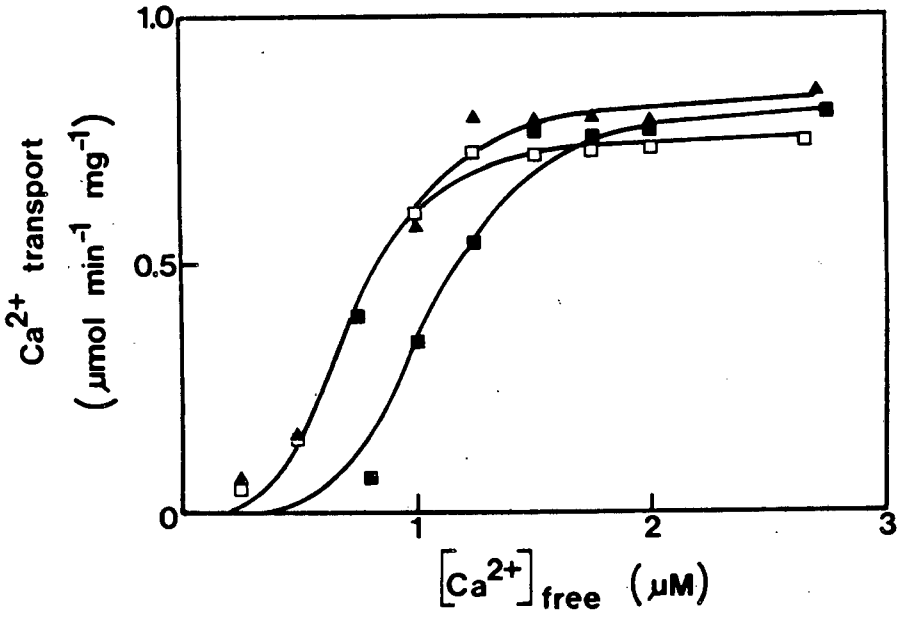


Figure 19: Effect of pH on Ca^{2+} transport.

Ca^{2+} transport was measured by means of the Ca^{2+}-stat method in medium, at 20°C, containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 5 mM ATP, 100 μM EGTA and 100 μg SR protein. The pH of the medium was adjusted to, and maintained constant at, pH 6.5 (□), 7.0 (■) or 7.5 (▲) by means of a pH-stat by titration with 10 mM NaOH.

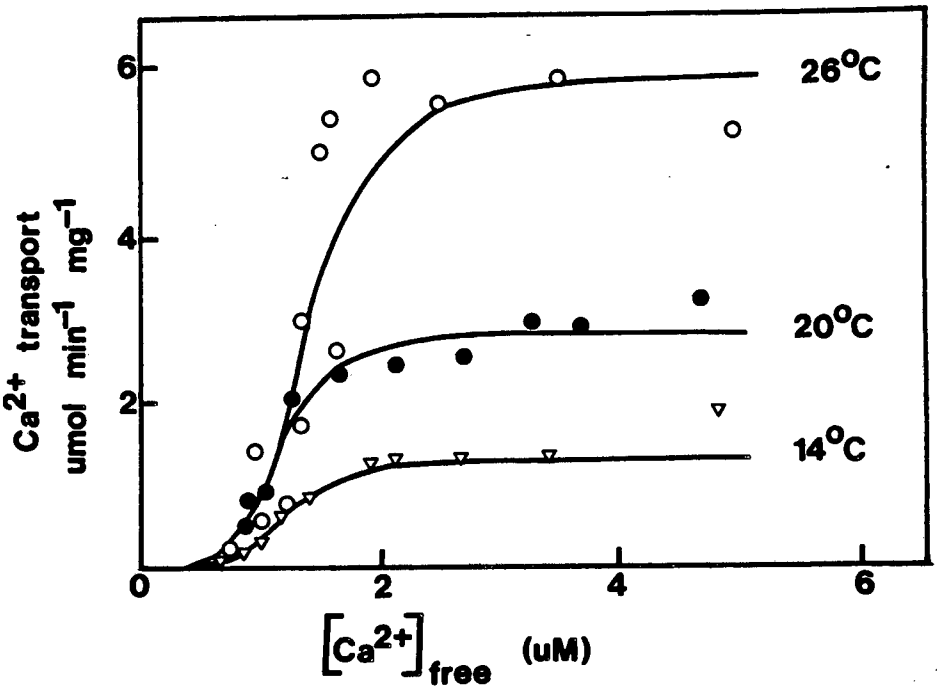


Figure 20: Effect of temperature on Ca^{2+} transport.

Ca^{2+} transport was measured as in Fig. 15. The temperature was adjusted to 14°C (▽), 20°C (●) and 27°C (○) by means of a circulating water bath.

2.3.8. EFFECT OF CALMODULIN AND PARVALBUMIN ON CALCIUM TRANSPORT

Most of the Ca^{2+} effects in eukaryotic cells are mediated by calmodulin, which, in the presence of micromolar Ca^{2+}, binds to and activates target enzymes such as myosin-light-chain kinase and human eyrthrocyte inside-out plasma membrane (Ca^{2+},Mg^{2+})-ATPase (Demaille, 1980). Calmodulin, a high affinity acidic Ca^{2+}-binding protein, increases the Ca^{2+} affinity of the (Ca^{2+},Mg^{2+})-ATPase of human erythrocytes (Sarkadi, 1980), as well as the maximum rate of transport (Waisman et al., 1981). Parvalbumin is a low molecular weight protein which binds Ca^{2+} with high affinity. While it is found in relatively high quantities in skeletal muscle, it has no known function (Kretszinger, 1976).

The possible effect of calmodulin and parvalbumin on SR (Ca^{2+},Mg^{2+})-ATPase was determined at limiting and saturating $[Ca^{2+}]_{free}$ (Fig. 21). The added calmodulin and parvalbumin was saturated with Ca^{2+} and thus, upon addition, caused a slight increase in $[Ca^{2+}]_{free}$. The titrator was thus switched off until the $[Ca^{2+}]_{free}$ dropped to the stat-point, after which titration was resumed. This is seen in Fig. 21 as a delay in Ca^{2+} transport upon the addition of both calmodulin and parvalbumin.

Calmodulin (7.5 µg/ml), at concentrations 100 times greater than that required for stimulation of the human erythrocyte membrane (Waisman et al., 1981), had no effect on the rate of Ca^{2+} transport, either at 0.4 µM (Fig. 21A) or at 3 µM (Fig. 21B) $[Ca^{2+}]_{free}$. Parvalbumin, 100 µg/ml, similarly had no effect on the rate of Ca^{2+} transport at limiting (Fig. 21C) and saturating (Fig. 21D) $[Ca^{2+}]_{free}$.

It is concluded that protein-bound Ca^{2+}, in the form of Ca^{2+}-calmodulin and Ca^{2+}-parvalbumin, do not modify the apparent affinity of the Ca^{2+} pump of SR vesicles, as was noted with low molecular weight complexes of the cation.

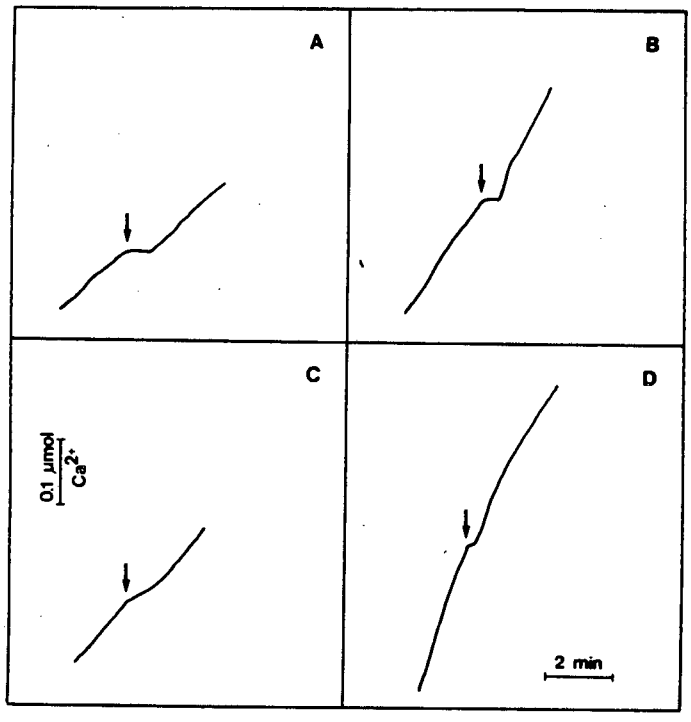


Figure 21: Effect of calmodulin and parvalbumin on Ca^{2+} transport.

The assay medium, 2 ml at 20°C, contained 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 5 mM ATP and 100 µg SR vesicles. Ca^{2+} transport was monitored as described under "Experimental Procedures". Calmodulin (7.5 µg/ml) (A and B) and parvalbumin (100 µg/ml) (C and D) were added where indicated. The free Ca^{2+} concentration was 0.5 µM in panels A and C, and 3 µM in panels B and D.

2.3.9. CALCIUM DEPENDENCE OF TRANSPORT AS MEASURED BY A SPECTROPHOTOMETRIC CALCIUM-STAT USING ARSENAZO III AS CALCIUM INDICATOR

A spectrophotometric calcium-stat, using arsenazo III as Ca^{2+} indicator, was used as an independent method to re-investigate the Ca^{2+}-dependence of Ca^{2+} transport in the absence of EGTA. All experiments were conducted at 20°C, pH 6.8 and in the presence of 1 mM $MgCl_2$, to minimise interference with the Ca^{2+} absorption signal. EGTA, 1 mM, was added after completion of the asssy to minimise uncertainties arising from baseline shifts. The effect of ATP concentration on the Ca^{2+}-dependence of Ca^{2+} transport is shown in Fig. 22. Increasing the concentration of ATP from 1 to 10 mM resulted in a decrease in V_{max} from 1.0 to 0.83 μmol min^{-1} mg^{-1} while $[Ca^{2+}]_{0.5}$ remained unchanged at 0.14 μM. This value for $[Ca^{2+}]_{0.5}$, found in the absence of CaEGTA, corresponded well with that found in the presence of EGTA (Fig. 9) and was ten-fold lower than that found in the absence of EGTA, using the Ca^{2+}-electrode as end-point detector (Fig. 10).

This anomaly was investigated by examining the continuous depletion of Ca^{2+} from the medium. A typical recording of the change in absorption during Ca^{2+} transport, in the presence of 1 mM ATP, 10 mM ATP and 5 mM AcP is shown in Fig. 23. The rate of Ca^{2+} depletion from the medium with 1 mM ATP (1.0 μmol min^{-1} mg^{-1}) was greater than with 10 mM ATP (0.76 μmol min^{-1} mg^{-1}) and 5 mM AcP (0.26 μmol min^{-1} mg^{-1}). Ca^{2+} transport continued until the Ca^{2+} concentration in the medium was depleted to limiting levels (<0.1 μM). This level was the same whether 1 mM ATP, 10 mM ATP or 5 mM AcP was used as substrate. Furthermore, the inclusion of CaEGTA, 100 μM, in the reaction medium had no effect on the kinetics of Ca^{2+} transport in the presence of 1 mM ATP (Fig. 23A). These results were in contrast to those similarly measured with the Ca^{2+}-electrode (Fig. 24), in which the Ca^{2+} concentration at which transport ceases, depended on both the substrate and its concentration. This phenomenon previously manifested as a change in apparent Ca^{2+}-affinity with varying substrates and varying ATP concentrations.

The effect of arsenazo III on the Ca^{2+}-dependence of Ca^{2+} transport was determined using the potentiometric calcium-stat (Fig. 25). Arsenazo III, at the concentration used to measure transport, 40 μM, had

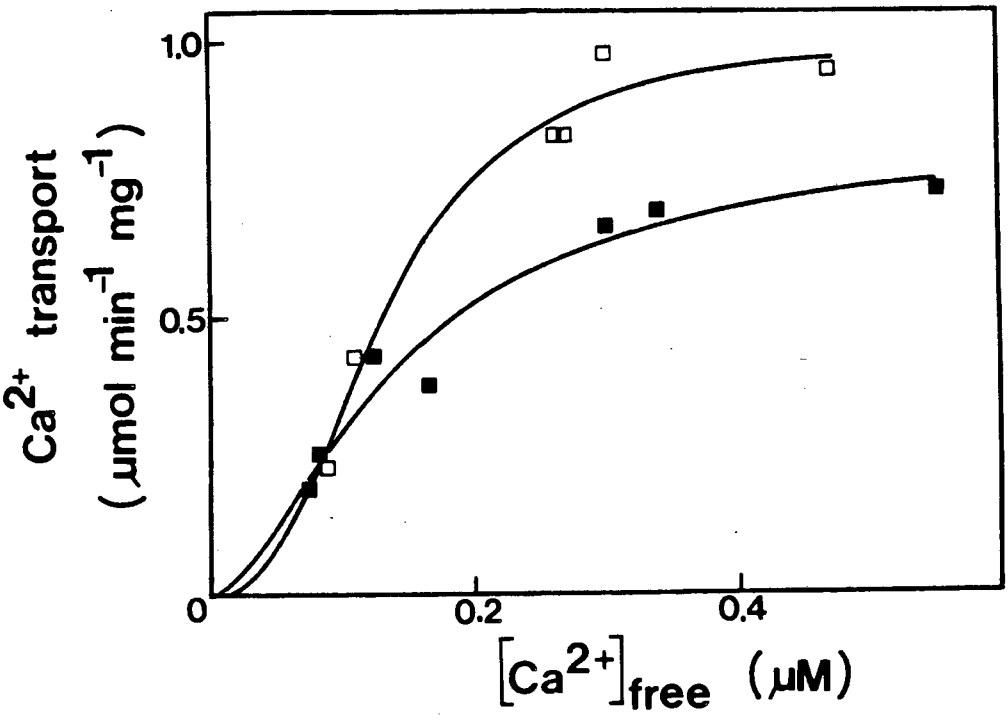


Figure 22: Effect of ATP concentration on free calcium ion dependence of calcium transport.

Calcium uptake by SR vesicles was measured by a spectrophotometric calcium-stat method in medium, at 20°C, containing 100 mM KCl, 5 mM potassium oxalate, 1 mM $MgCl_2$, 50 mM Pipes, pH 6.8, 40 µM arsenazo III and either 1 mM (□) or 10 mM (■) ATP. Transport was initiated with 100 µg SR protein.

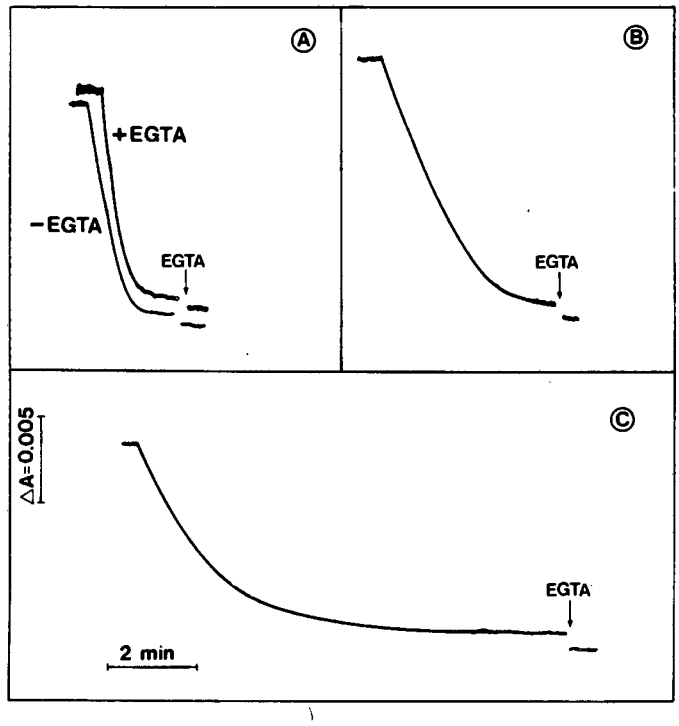


Figure 23: Effect of substrate on Ca^{2+} transport determined spectrophotometrically.

The assay medium, 3 ml at 20°C, contained 100 mM KCl, 5 mM potassium oxalate, 1 mM $MgCl_2$, 50 mM Pipes, pH 6.8, 40 µM arsenazo III and 100 µg SR protein. Transport was initiated by either 1 mM ATP (A), 10 mM ATP (B) or 5 mM AcP (C). EGTA was added where indicated.

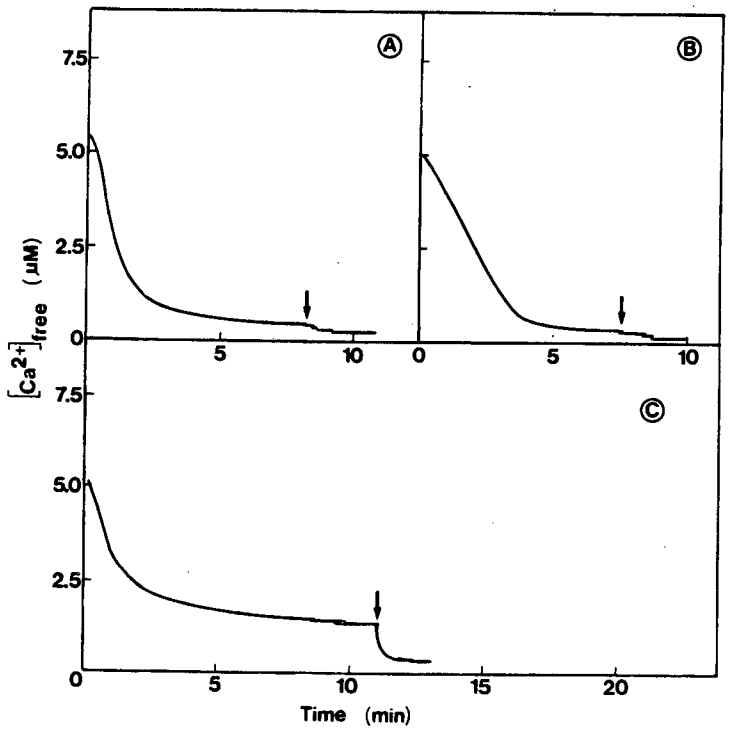


Figure 24: The effect of substrate on Ca^{2+} transport determined potentiometrically.

Ca^{2+} transport was measured, at 20°C, in medium containing 100 mM KCl, 5 mM potassium oxalate, 5 mM $MgCl_2$, 20 mM Pipes, pH 6.8 and 100 μg SR protein in a total volume of 2 ml. Transport was initiated with either 1 mM ATP (A), 10 mM ATP (B) or 5 mM AcP (C). EGTA, 1 mM, was added as indicated by the arrow.

no effect on $[Ca^{2+}]_{0.5}$ while 100 µM CaEGTA decreased $[Ca^{2+}]_{0.5}$ from 1.4 to 0.8 µM. Arsenazo III had an effect on the maximum rate of Ca^{2+} transport, decreasing it from 0.97 to 0.51 µmol min^{-1} mg^{-1}. A similar effect of arsenazo III on transport has previously been noted (Johnson et al., 1983). The reason for this inhibition was not further investigated but may be due to the arsonic acid group, which has been shown to enhance Ca^{2+} efflux (Hasselbach et al., 1972).

The results for $[Ca^{2+}]_{0.5}$ obtained in the absence of EGTA by the potentiometric Ca^{2+}-stat and by the spectrophotometric Ca^{2+}-stat vary considerably. It was apparent that the Ca^{2+}-electrode may be unreliable at $[Ca^{2+}]_{free} < 1$ µM. This was borne out using $^{45}Ca^{2+}$. Ca^{2+} transport was initiated in medium containing a known $^{45}Ca^{2+}$ specific radioactivity. On completion of Ca^{2+} transport, an aliquot of the medium was filtered through a Millipore and the radioactivity in the filtrate was related to the Ca^{2+} concentration of the medium. The Ca^{2+}-electrode potential was three times higher than that predicted from the radioactivity in the filtrate, suggesting that the electrode response at $[Ca^{2+}]_{free} < 1$ µM is inaccurate.

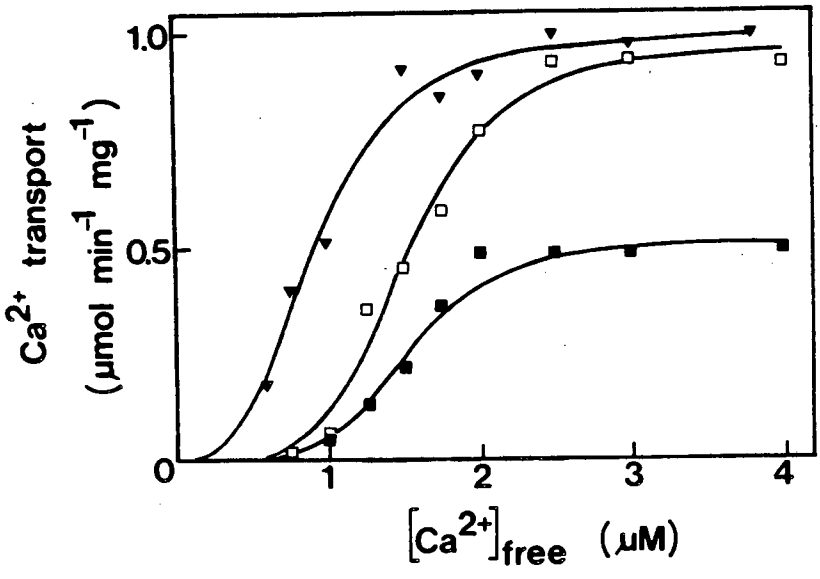


Figure 25: Effect of CaEGTA and arsenazo III on the Ca^{2+} dependence of Ca^{2+} transport measured by a potentiometric Ca^{2+}-stat.

Ca^{2+} transport was measured in medium, at 20°C, containing 100 mM KCl, 5 mM potassium oxalatè, 5 mM $MgCl_2$, 20 mM Pipes, pH 6.8 and 5 mM ATP. Transport was initiated by 100 µg SR protein, in the presence of 100 µM EGTA (▼), in the absence of CaEGTA (□) and in the presence of 40 µM arsenazo III (■).

2.4. DISCUSSION

The reaction scheme for the (Ca^{2+},Mg^{2+})-ATPase (Scheme I) incorporates random binding of Ca^{2+} and substrate to the enzyme and is based on studies which have failed to detect any effect of ATP on the parameters of Ca^{2+} binding and thus on the affinity of the enzyme for Ca^{2+} (Kanazawa et al., 1971; The and Hasselbach, 1972; Meissner, 1973). There is evidence, however, that the Ca^{2+} transport system exhibits characteristics similar to those described for allosteric enzymes. A study of the kinetic properties of the Ca^{2+}-dependent ATPase activity of SR showed that ATP (Yamamoto and Tonomura, 1967) and also ITP and GTP (de Meis and de Mello, 1973) modify the Ca^{2+} affinity of the enzyme. The Ca^{2+}-concentration dependence of Ca^{2+} transport varies depending on the substrate hydrolysed (Friedman and Makinose, 1970; de Meis and Hasselbach, 1971; Rossi et al., 1979), further indicating that the affinity of the enzyme for Ca^{2+} is determined by substrate binding. No satisfactory explanation has been given for these anomalies and a mechanism of random binding of Ca^{2+} and ATP is still generally accepted. The present study, in which Ca^{2+}-stimulation of transport has been used as an indication of saturation of Ca^{2+}-binding sites, supports a mechanism whereby binding of one Ca^{2+} ion to the enzyme is governed by the substrate hydrolysed or by a Ca^{2+}-chelating species.

We have confirmed, by means of a calcium-stat method, that there is a difference in both the rate of Ca^{2+} transport and the apparent affinity of the enzyme for Ca^{2+} in the presence of different substrates (Fig. 15). Maximum rates of Ca^{2+} transport are greater with ATP and ITP than with AcP and pNPP, even though it is presumed that under saturating substrate conditions the enzyme has maximum levels of EP formation. A possible explanation is that either the rate of EP formation is limiting with pseudosubstrates (i.e. the hydrolysed product is a poor leaving group) (Rossi et al., 1979) or else that there is another rate limiting step, subsequent to EP formation, which is promoted by ATP only (Verjovski-Almeida and Inesi, 1979). The difference in the affinity of the Ca^{2+}-transport sites with varying substrates (Fig. 15) cannot be explained in terms of non-interacting substrate and metal binding sites. If we assume that the Ca^{2+}-binding sites are identical in the presence of AcP and ATP, for example, then at 2 μM free Ca^{2+}, these sites should be virtually saturated and higher concentrations of Ca^{2+} would not activate transport in the presence of AcP. Differences in apparent affinities

can therefore only be due to modification of the Ca^{2+}-binding sites by the substrate, since saturation of these sites alone are not solely responsible for modulation of the substrate cycle.

It has recently been demonstrated in this laboratory that, as opposed to previous studies measured in the presence of the Ca^{2+}-chelating agent EGTA, the calcium-stat method for determining Ca^{2+} transport, in the absence of EGTA, leads to higher $[Ca^{2+}]_{0.5}$ values and Hill coefficients while V_{max} remained unchanged (Berman, 1982a). This was confirmed in this study with both ATP and AcP as substrates (Fig. 10). In addition, the Ca^{2+}-dependence of Ca^{2+} transport gave $[Ca^{2+}]_{0.5}$ values ten-fold higher, when determined by the calcium-stat method, in the absence of EGTA, as opposed to the conventional Millipore filtration technique, in the presence of EGTA (Fig. 9). Berman (1982a) suggested that the species exerting these effects was $CaEGTA^{2-}$ and suggested that the enzyme may recognise the Ca^{2+} ion of the CaEGTA complex, by formation of a ternary complex, E.Ca.EGTA. The question arose as to whether there is an endogenous Ca^{2+}-chelator with properties similar to that of CaEGTA, that increases the Ca^{2+}-affinity of the (Ca^{2+},Mg^{2+})-ATPase such that stimulation can occur at approximately 10^{-8} M Ca^{2+}, the cytosolic Ca^{2+} concentration during relaxation. Can the differences found with the pseudosubstrates, discussed above, be explained by this <u>in vivo</u> effector?

The present study shows that increasing the free ATP and CaATP concentration resulted in an increase in the apparent affinity of the (Ca^{2+},Mg^{2+})-ATPase for Ca^{2+} (Fig. 13). At constant 1 mM $MgCl_2$, an increase in ATP from 1 to 10 mM reduced $[Ca^{2+}]_{0.5}$ from 1.05 to 0.15 µM. The concentration of MgATP remained practically constant and was therefore excluded as the modulating species. We do, however, confirm that MgATP is the true substrate for ATP hydrolysis (Vianna, 1975), since an increase in MgATP concentration resulted in an increase in V_{max} (Fig. 12). The possibility that the modulation was effected by free Mg^{2+}, competing with Ca^{2+} for its binding sites, was excluded by the observation that, on the one hand, varying the free Mg^{2+} concentration in the presence of constant concentrations of free ATP and CaATP (Table III) did not result in a change in $[Ca^{2+}]_{0.5}$, while, on the other hand, varying the free ATP and CaATP concentration in the presence of

constant Mg^{2+} (Table II), resulted in a 5-fold increase in the affinity of the enzyme for Ca^{2+}. Furthermore, the affinity of the (Ca^{2+},Mg^{2+})-ATPase for Ca^{2+} (0.35 µM) has been found to be approximately 30 000 times higher than that for Mg^{2+} (10.6 mM) (Yamada and Tonomura, 1972), suggesting that it is unlikely that Mg^{2+}, in the µM range, would compete with Ca^{2+} for the Ca^{2+}-binding sites. It was therefore evident that either free ATP, liganded to a low affinity ($[ATP]_{0.5}$ = 0.4 mM) site or CaATP, bound to a high affinity ($[CaATP]_{0.5}$ = 10 µM) site was able to modulate the affinity of the (Ca^{2+},Mg^{2+})-ATPase for Ca^{2+}. It was not possible to distinguish between these two species, since the ratio of ATP : CaATP in any given mixture, at 1 µM free Ca^{2+}, will always be 40:1 and cannot be varied.

Evidence that CaATP may actually be the modulating species is presented. (i) The Ca^{2+}-chelating anions, pyrophosphate and citrate, both increased the Ca^{2+} affinity of the pump at a Ca^{2+}-chelator concentration of 5-20 µM (Table IV and V) which was in the same range as that of CaATP (Fig. 14) and CaEGTA (Berman, 1982a). (ii) Acetyl phosphate and p-nitro-phenyl phosphate, substrates which only bind Ca^{2+} weakly at pH 7.2, did not modulate the affinity of the enzyme, while stimulating Ca^{2+} transport at saturating Ca^{2+} concentrations (Fig. 16). Also, $[Ca^{2+}]_{0.5}$ values, obtained in the presence of AcP and pNPP, are higher than that with ATP and ITP as substrate. (iii) Low ATP concentrations (1-10 µM), which resulted in saturation of the catalytic site, did not result in a change in $[Ca^{2+}]_{0.5}$ (data not shown).

The ability of substrates to modulate the apparent affinity of the Ca^{2+} pump is therefore positively correlated with their ability to form Ca^{2+}-chelates. These chelates appear to activate the pump in a manner similar to that of CaEGTA (Berman, 1982a), by forming a ternary complex with the enzyme. Of particular interest is the finding by Vianna (1975) that the SR membrane displays an affinity for CaATP at least 8-times higher than that for MgATP but, while MgATP is hydrolysed, CaATP is not. It has, however, recently been demonstrated that the complex of Ca^{2+} with ATP may serve as a substrate for enzyme phosphorylation (Yamada and Ikemoto, 1980; Shigekawa et al., 1983). Shigekawa and coworkers (1983) found that the reaction sequence with CaATP as substrate is basically the same as that observed when MgATP

serves as a substrate, although the rate constants for each reaction step differs significantly for these two types of substrates. It has also been suggested that CaATP is the substrate, at low ATP concentrations, of the Ca^{2+}-ATPase of human erythrocyte membranes, with a K_m for CaATP of 0.011 μM (Graf and Penniston, 1981).

The effect of EGTA buffers on the apparent Ca^{2+} affinity has been observed for other ATPases. It was originally shown by Schatzmann (1973) that the free Ca^{2+} concentration required for half-maximum activation of the (Ca^{2+},Mg^{2+})-ATPase of Ca^{2+}-loaded resealed ghosts of red cells is about two orders of magnitude smaller in a CaEGTA buffer, than in an unbuffered medium. Comparative studies by Sarkadi et al. (1979) in inside-out red cell membrane vesicles revealed similar effects by CaEGTA buffers. They suggested that the red cell membrane ATPase has at least two different binding sites for Ca^{2+}; one of high affinity for free Ca^{2+} and the second of lower affinity which can be increased to higher affinity in the presence of a chelator. Similarly, the apparent Ca^{2+} affinity of the (Ca^{2+},Mg^{2+})-ATPase in human erythrocyte membranes and in the solubilised enzyme increased with increasing concentrations of EGTA used to buffer free Ca^{2+} (Al-Jabore and Roufogalis, 1981). Analysis of their kinetic data showed that a low Ca^{2+} affinity state of the enzyme was converted to a high affinity state by the species $CaEGTA^{2-}$. EGTA did not mimic the effect of calmodulin since, whereas calmodulin increased both V_{max} and the affinity, EGTA only had an effect on the latter parameter. The effect of EGTA was also evident in the presence of calmodulin. No effect of calmodulin or parvalbumin was found on $[Ca^{2+}]_{0.5}$ of the sarcoplasmic reticulum (Ca^{2+},Mg^{2+})-ATPase (Fig. 21).

A study on the regulation of intracellular Ca^{2+} by Baker (1978) revealed that the Na^+-dependent movement of Ca^{2+} across the membrane of squid axons is influenced by ATP and that ATP could not be replaced in this action by non-hydrolysable analogues. The presence of ATP was found to increase both the apparent affinity of the internal Ca^{2+}-binding site for Ca^{2+} and the apparent affinity of the external Na^+-binding site for Na^+, without altering the maximum rate of transport. No explanation was given to account for this observation.

Passive binding studies, using $^{45}Ca^{2+}$ under equilibrium conditions, previously indicated a single class of two high affinity Ca^{2+}-binding sites per ATP monomer (Watanabe et al., 1981). More recent studies, which have examined the kinetics of Ca^{2+} binding, indicate that the two Ca^{2+}-binding sites that are involved in the catalytic cycle may, in fact, be non-identical (Inesi et al., 1980; Guillain et al., 1980; Guillain et al., 1981; Dupont, 1982) and that the cooperativity, previously noted in the stimulation of both Ca^{2+} transport and ATPase activity, is the result of binding of Ca^{2+} to the first Ca^{2+} site and conversion of the second site from low to high affinity. This is achieved by a slow conformational change, localised to the *E to E transition, which is accelerated by both Ca^{2+} and ATP (See Section 2.1.1 and 2.1.2). The results, presented here, suggest that Ca^{2+}-chelates can similarly convert a low affinity Ca^{2+}-binding site to one of high affinity. Stimulation of Ca^{2+} transport by free Ca^{2+} is observed even in the presence of chelating anions, indicating that the ligand only affects one of the Ca^{2+}-binding sites. Taken together, the results may be explained in terms of a two-state model for the enzyme; *E, which has low affinity and E, which has high affinity for Ca^{2+}. The Ca^{2+}-chelate, CaX, initially binds to the low affinity conformation of the enzyme to form a ternary complex, *E.CaX. Following a conformational change, a second free Ca^{2+} ion binds to the high affinity site. It is presumed that the anion dissociates from the enzyme at some later stage in the catalytic cycle. Anion-facilitated Ca^{2+} binding may thus be represented as follows:

$$*E \overset{CaX}{\rightleftharpoons} *E.CaX \rightleftharpoons E.CaX \overset{Ca^{2+}}{\rightleftharpoons} E.CaX.Ca \overset{X}{\rightleftharpoons} E\ Ca_2$$

where X is a chelating anion. Free ATP cannot increase the affinity of the enzyme for Ca^{2+} but can only increase the rate of the *E$\rightleftharpoons$E transition which will result in an increase in the population of enzyme in the E conformation. This will manifest as an increase in V_{max} only. This will explain why CaEGTA had no effect on the ATP-dependence of Ca^{2+} transport, since saturating concentrations of Ca^{2+} were used and only the maximum rate of transport was determined (Fig. 17).

Positive cooperativity is, by classical definition, attributed to interaction between identical sites. A Hill coefficient of greater

than five, as obtained in this study and in a previous study in the absence of EGTA (Berman, 1982a), would imply at least five identical Ca^{2+}-binding sites. This is incompatible with previously obtained results, in which the presence of two high affinity binding sites and a Hill coefficient of two (Ikemoto et al., 1975; Inesi et al., 1980) has been demonstrated. This high apparent positive cooperativity may be due to a number of factors. Firstly, an energy barrier, which manifests as a high Hill coefficient and a region of zero Ca^{2+} transport, may exist. The anion may be required to overcome such a barrier and facilitate the binding of the first Ca^{2+} ion. Berman (1982a+b) has envisaged a mechanism whereby CaEGTA promotes thermodynamic efficiency by conserving its potential binding energy in some unspecified conformation of the ternary complex E.Ca.EGTA, and releasing it upon dissociation of EGTA.

Urban et al. (1978) have studied the interaction of K^+ and Na^+ across gramicidin channels in terms of a two-site model with preferential residency at the openings of the port. Transfer of cations across the channel is restricted to an energy barrier which they have related to the absence of a negatively charged ionic species towards the centre of the channel. The ion-activity dependence of the probabilities of occupation of both sites was of particular interest (Fig. 26). This probability was vanishing low at cation concentrations below 10^{-4} M but steeply rose above this value. An alternative model, based on Urban and coworkers suggestions, has been proposed for Ca^{2+} binding (Berman, 1982b), whereby anions might, by binding to a site inside the calcium channel, diminish the energy barrier between sites at the ends of the ionophoric pore within the ATPase (Fig. 27). This mechanism would explain why the effect of ATP concentration on the apparent affinity of the (Ca^{2+},Mg^{2+})-ATPase for Ca^{2+} has not previously been observed in the presence of CaEGTA buffers.

A second reason for the observed high Hill coefficients in the absence of EGTA may be due to the existence of a balance between influx and efflux of Ca^{2+}, governed by the ratio of $[Ca^{2+}]_{in}$ / $[Ca^{2+}]_{out}$, at low Ca^{2+} concentrations. All measurements were made in the presence of 5 mM oxalate which would be expected to clamp intervesicular Ca^{2+} to 4 μM (Hasselbach and Makinose, 1963). Since transport is abolished

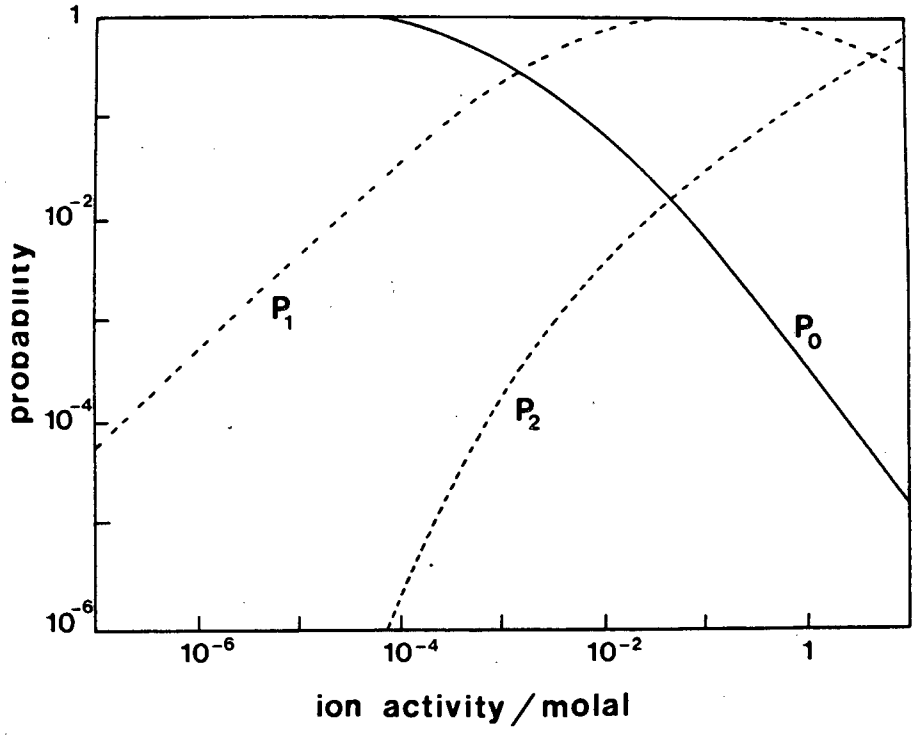


Figure 26: Cation concentration-dependence of probabilities of occupancy of zero (P_o), one (P_1) or two sites (P_2), in a two-site pore model of the gramicidin channel (Urban et al., 1978).

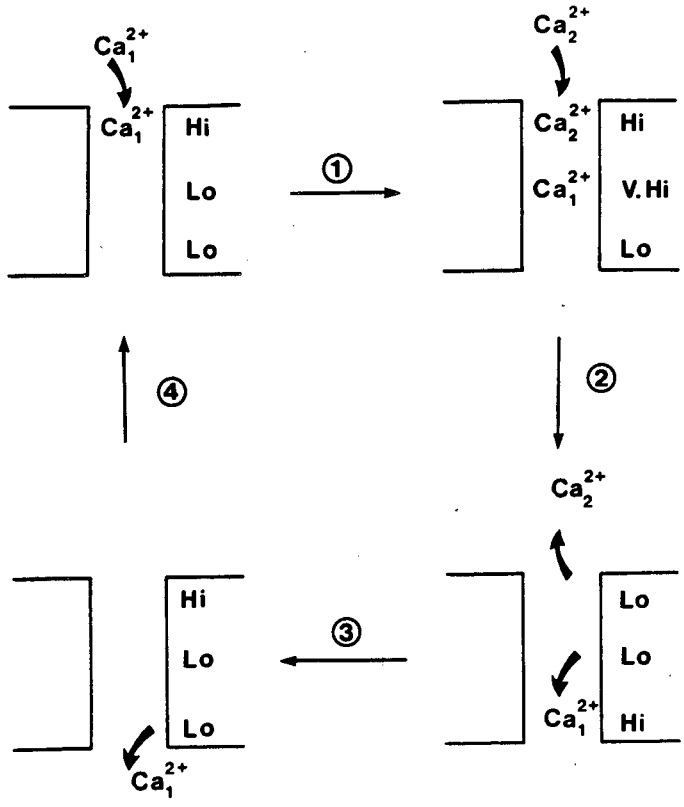


Figure 27: A model for active calcium transport through the Ca^{2+} pump of SR ATPase.

The model has three Ca^{2+}-binding sites and the sites at the entrance and exit of the channel alternate between high (Hi) and low (Lo) affinity. A third site, situated towards the centre of the channel, can exist in a very high affinity form (V.Hi), perhaps related to a hydrophobic environment or be supplied by an anionic prosthetic species such as $EGTA^{4-}$ and ATP^{4-}. This model allows varying affinity of the pump depending on occupancy of the latter site (Berman, 1982b).

below approximately 0.5 μM, in the absence of EGTA, it would appear that the pump is thermodynamically inefficient at $[Ca^{2+}]_{in}$ / $[Ca^{2+}]_{out}$ < 10 and that CaEGTA or CaATP promotes this efficiency. Supporting this possibility is the ability of oxalate and phosphate to decrease $[Ca^{2+}]_{0.5}$ (Fig. 18). Increasing the oxalate concentration results in a decrease in $[Ca^{2+}]_{in}$ and thus a decrease in Ca^{2+} efflux. This may be seen as an apparent increase in transport at low $[Ca^{2+}]_{out}$, and thus of a decrease in $[Ca^{2+}]_{0.5}$. However, both oxalate and phosphate are Ca^{2+}-chelating anions and thus may exert their effect in a manner similar to that of EGTA and ATP.

An increase in apparent affinity of the Ca^{2+}-transport sites of the (Ca^{2+},Mg^{2+})-ATPase of SR vesicles, in the presence of Ca^{2+}-chelating anions has been demonstrated in this study. The Ca^{2+}-dependence of Ca^{2+} transport was measured by a potentiometric Ca^{2+}-stat method, using a Ca^{2+}-specific electrode as end-point detector. The data appear to fit in with those found in other energy transducing systems (Schatzmann, 1973; Sarkadi et al., 1979; Al-Jabore and Roufogalis, 1981) but is in contrast to that found when arsenazo III was used as Ca^{2+}-indicator. Theoretically, both should yield the same value for $[Ca^{2+}]_{0.5}$ and possible explanations for this anomaly are presented.

(i) Ca-EGTA buffers were used to allow calibration of the Ca^{2+}-specific electrode in the range of Ca^{2+} concentration corresponding to the skeletal muscle pCa, which varies from 5 to 8 and is lower than contaminating Ca^{2+}. It was found that, in the absence of Mg^{2+}, calibration at $[Ca^{2+}]_{free} < 10^{-5}$ M and $[Ca^{2+}]_{free} > 10^{-4}$ M was continuous and obeyed Nernstian behaviour (Fig. 4). It is possible, however, that at very low $[Ca^{2+}]_{free}$ ($< 10^{-6}$ M), a critical total Ca^{2+} concentration is required and that this Ca^{2+} may be in the form CaEGTA or a Ca^{2+}-chelating complex. Since the electrode membrane used in this study was an ion-exchanger, the anion may facilitate Ca^{2+} exchange across the electrode membrane. This phenomenon has not previously been noted.

(ii) Arsenazo III has the disadvantage that, due to its high affinity for Ca^{2+}, it acts as a sink for Ca^{2+} in solution (Johnson et al., 1983). Calibration of the arsenazo III absorption signal is also conducted using

Ca-EGTA buffers. EGTA is a much stronger buffer than arsenazo III and it was, therefore, assumed that all the added Ca^{2+} was complexed to EGTA and that $[Ca^{2+}]_{free}$, calculated from the CaEGTA equilibrium constant alone, was that seen by arsenazo III. In the absence of EGTA, however, at 5 μM Ca^{2+} and 40 μM arsenazo III, 76% of the total Ca^{2+} is complexed to arsenazo III (Johnson et al., 1983) so that only the remaining free arsenazo III will give an absorption change with change in $[Ca^{2+}]_{free}$. Uncertainties may arise in the determination of the Ca^{2+}-dependence of the Ca^{2+} transport sites, at low Ca^{2+} concentrations, and a $[Ca^{2+}]_{free}$ which is actually lower than the true value will be assumed. Since arsenazo III is a Ca^{2+}-chelating anion of considerable affinity (pK_a = 4.7) (Scarpa et al., 1978; Dipolo et al., 1976), it could have an effect on $[Ca^{2+}]_{0.5}$, by formation of the Ca-arsenazo III complex, in a manner similar to the effect of CaEGTA and CaATP. Arsenazo III (40 μM), however, had no effect on $[Ca^{2+}]_{0.5}$ as determined by the potentiometric Ca^{2+}-stat (Fig. 25). The concentration of Ca-arsenazo III in the reaction medium was approximately 30 μM and is therefore in the range where it should have exerted its effect if it could have replaced CaEGTA in increasing the apparent Ca^{2+} affinity of the Ca^{2+}-transport sites.

There appear to be uncertainties in the measurements of $[Ca^{2+}]_{free}$, with and without chelating agents, in the low ranges. It is premature at this stage to claim that ATP or any other naturally occurring anion is biologically active in vivo, under physiological conditions. A more reliable method for the determination of $[Ca^{2+}]_{free}$ in the nanomolar range is needed. The Ca^{2+}-electrode itself may be a useful model in the future to study the interaction of Ca^{2+} with Ca^{2+} binding sites. In view of these uncertainties, a different approach was investigated to look at modulations of the Ca^{2+} pump. The effects of varying parameters on the stoichiometry of the pump was investigated in the second part of this thesis. The methodology which had been developed in the first section has been extended and is the basis for this new line of approach.

3.0. ENERGY COUPLING BY THE CALCIUM PUMP OF SARCOPLASMIC RETICULUM

3.1. INTRODUCTION

Active transport of Ca^{2+} across the sarcoplasmic reticulum is a vectorial process, which utilises the free energy released by the scalar reaction of ATP hydrolysis to transport Ca^{2+} against a concentration gradient. The free energy of hydrolysis, under standard conditions, is approximately 14 kcal/mol (Hasselbach and Waas, 1982; Tanford, 1983a) and is in good agreement with the estimated Ca^{2+} gradient of 2 000 - 100 000 (Weber and Herz, 1966; Makinose and The, 1965) achieved under experimental conditions. This is supported by the finding of Jeacocke (1982), who showed that chemiosmotic equilibrium is virtually achieved in the relaxed state of muscle.

It is generally assumed that two Ca^{2+} ions bind and are transported for each turnover of the enzyme, during which a single molecule of ATP is hydrolysed. A coupling ratio (Ca^{2+}/ATP) of two has been obtained under steady state conditions (Hasselbach and Makinose, 1963; Martonosi and Feretos, 1964b; Weber et al., 1966; Sumida et al., 1978), as well as under transient state conditions, after the initial burst of Ca^{2+} translocation (Kurzmack and Inesi, 1977; Verjovski-Almeida et al., 1978). This is in agreement with passive binding studies, which identify two high affinity Ca^{2+}-binding sites per pump unit (Inesi et al., 1980; Verjovski-Almeida and Silva, 1981), and positive cooperativity, with a Hill coefficient of approximately two, for Ca^{2+} stimulation of both transport, and of ATPase activity (The and Hasselbach, 1972; Vianna, 1975; Neet and Green, 1977). In addition, Watanabe et al. (1981) have demonstrated two high affinity Ca^{2+}-binding sites per phosphorylation site, and positive cooperativity between these sites, in both SR vesicles and in the purified ATPase. A strict correlation between the time of Ca^{2+} uptake and Ca^{2+}-dependent substrate hydrolysis has also been found for the pseudosubstrates pNPP (Inesi, 1971), AcP (Friedman and Makinose, 1970) and the nucleotides triphosphates that support Ca^{2+} transport (Makinose and The, 1965).

Addition of ADP and P_i to calcium loaded vesicles results in efflux of calcium and synthesis of ATP (Makinose and Hasselbach, 1971; Panet and Selinger, 1972; Deamer and Baskin, 1972; Yamada et al., 1972). A coupling ratio of two is observed for this reverse reaction, when conditions are chosen, such that the reaction runs unidirectionally to completion. The stoichiometry of the calcium efflux-dependent ATP

synthesis is thus identical with that of active calcium accumulation.

Larger and smaller, and sometimes variable, coupling ratios have been described. In native SR vesicles, under optimal conditions, coupling ratios vary in the range 1.5 to 2.1. It was originally thought that variable stoichiometries occurred, depending on the transmembrane gradient for calcium ions (Weber et al., 1963; Hasselbach and Makinose, 1963) but later studies showed that the (Ca^{2+},Mg^{2+})-ATPase has a fixed stoichiometry of two under conditions of low free external ($[Ca^{2+}]_{free}$) Ca^{2+} concentrations (<0.1 μM) and low ATP concentrations (0.2 μM), in the presence and absence of oxalate (Weber et al., 1966).

Values of one for the Ca^{2+}/ATP ratio have been obtained by several workers, under standard, optimal conditions, in the steady state (Hasselbach and Makinose, 1963; Yamada et al., 1970; Katz et al., 1980; Waas and Hasselbach, 1981). Rossi et al. (1979) showed, by means of steady state kinetics, that the psuedosubstrates pNPP, dNPP, methylumbelliferylphosphate and FAP support Ca^{2+} transport with a coupling ratio of one, while the stoichiometry of the pump, using ATP as substrate, could be varied between zero and two depending on the substrate concentration and pH of the medium. Fassold et al. (1981) measured initial Ca^{2+} transport:phosphoprotein ratios formed from GTP hydrolysis, since EP formation is slower in the presence of this nucleotide. They found that, while saturation of the high affinity Ca^{2+}-binding and transport sites, as well as the GTP-binding sites, resulted in a Ca^{2+} transport:EP ratio of two, variations in the concentration of the two ligands yielded ratios between 2 and 6. It was presumed that every ATPase molecule has two Ca^{2+}-binding sites and that transport:EP ratios of 4 to 6 would require the formation of dimers and trimers. In such a model, phosphorylation of one ATPase molecule induced translocation in neighbouring non-phosphorylated units. Van Winkle and co-workers (1981) have shown that, while skeletal SR can hydrolyse GTP with a concomitant membrane translocation of Ca^{2+}, cardiac SR could not. In cardiac SR, stable Ca^{2+}-dependent acyl phosphate is not formed from GTP and therefore cardiac SR cannot utilise the free energy of hydrolysis of this substrate to support Ca^{2+} translocation.

There is a marked difference in pH sensitivity between Ca^{2+} transport

and Ca^{2+}-dependent ATPase activity. The pH optimum for ATP-dependent Ca^{2+} accumulation ranges from 6.2 - 6.8, and decreases as the medium becomes more alkaline (Duggan, 1977; Tate et al., 1981). In contrast, Ca^{2+}-dependent ATPase activity exhibits a pH profile with maximum activity at more alkaline pH (7.2 - 8.0), at which Ca^{2+} accumulation is inhibited (Shigekawa et al., 1976; Duggan, 1977; Tate et al., 1981). The stoichiometry of the pump has, in addition, been shown to be temperature dependent, such that a maximum coupling ratio of two is obtained between 0-35°C, in both the forward and reverse direction, but decreases as the temperature is further raised to above 40°C (Inesi et al., 1973; Davis et al., 1976; Vale and Carvalho, 1980).

From the above and numerous other studies it can be concluded that, although the ratio of Ca^{2+} ions translocated per mol of ATP hydrolysed is generally two, variable stoichiometry is obtained under certain conditions. Coupling ratios of greater than two have generally been attributed to experimental artifacts, such as Ca^{2+}-oxalate precipitation in the external medium or that Ca^{2+} transport ceases before ATP hydrolysis, resulting in an underestimation of ATPase activity (Hasselbach, 1981). Deviations from the optimal value of 2.0 have, on the other hand, been explained on the basis of leaky vesicles, or alternatively, SR vesicles may not all be uniformly sealed (Hasselbach, 1981; Berman, 1982b).

An essential prerequisite for observing tight coupling is a low passive calcium permeability of the SR membrane. SR vesicles have been shown to release Ca^{2+} passively, either when the Ca^{2+} pump is blocked (Makinose and Hasselbach, 1965), during Ca^{2+} influx (Jilka and Martonosi, 1977; Nakamura and Tonomura, 1978), or under conditions where there is a large Ca^{2+} gradient across the membrane i.e. in the presence of EGTA (Feher and Briggs, 1982; 1983). Alkaline pH is thought to increase membrane permeability, since a sudden increase in pH results in release of previously accumulated Ca^{2+}, in the absence of oxalate (Nakamaru and Schwartz, 1972; Dunnet and Nayler, 1979). Duggan and Martonosi (1970) showed that SR vesicles are impermeable to inulin and dextran in the range pH 7.0 to 9.0 while readily permeable to urea and sucrose in the same pH range. At 40°C, inulin penetration, however, occurred at pH 7.0, in the presence of EGTA. This would suggest that

alkaline pH and high temperatures increase the membrane permeability, at least to small molecules. Berman (1982b), however, showed that the rate of passive efflux is approximately 33 nmol min^{-1} mg^{-1} i.e. 19-fold less than could explain sub-optimal coupling ratios on the basis of increased membrane permeability. Furthermore, efflux rates reduce in the presence of ATP and Mg^{2+}, and in the presence of Ca^{2+}-precipitating anions, which lower the free internal Ca^{2+} concentration and thus the Ca^{2+} gradient i.e. under conditions of active Ca^{2+} transport (Hasselbach, 1981). It thus seems unlikely that a coupling ratio of less than two can be accounted for solely by assuming the existence of passive leaks through lipid bilayers.

Procedures and chemical modifications of SR membranes have been reported to uncouple Ca^{2+} transport from ATPase activity (Table VI). Certain of these conditions are readily explained on the basis of increased membrane permeability. These include ageing, ether and alcohol treatment, and EGTA treatment under alkali conditions. Other conditions leading to uncoupling e.g. mild tryptic digestion, EGTA treatment at neutral pH, and mild acid treatment, cannot be explained on the basis of increased passive Ca^{2+} permeability, and appear to be due to 'intramolecular' perturbations of the Ca^{2+}-pump. Micromolar concentrations of Ca^{2+}, similar to that which stimulates ATPase activity, protects against uncoupling by EGTA treatment at neutral pH and since no increased permeability of SR membranes can be demonstrated, it is evident that the uncoupling process may be intramolecular in nature. This raises the question as to whether the Ca^{2+} pump has a fixed stoichiometry, where passive leaks only can account for coupling ratios of less than two or whether the pump can 'slip' or operate under suboptimal conditions, with variable stoichiometry.

Experimental determinations of coupling ratios under standard optimal conditions thus give values that approach 2.0. These have generally been determined by kinetic methods and are based upon initial rates of Ca^{2+} transport and of Ca^{2+}-dependent ATPase activity, in the presence of oxalate. Ca^{2+}-dependent ATPase activity is taken as the difference between total ATPase activity, in the presence of saturating levels of Ca^{2+}, and Ca^{2+}-independent or 'basal' activity, determined in the presence of excess EGTA. There are uncertainties regarding the nature

TABLE VI

Procedures resulting in uncoupling of Ca^{2+} transport from ATPase activity in sarcoplasmic reticulum membranes

Procedure	Conditions	Ref.
Aging	0°C, 2-4 days	Ebashi & Lipmann, 1962
Tryptic digestion	1:20 trypsin:membrane protein, 32°C, 15 s	Stewart & MacLennan, 1974 Shamoo et al., 1977
Diethyl ether	7.5% (v/v), 20 min room temp.	Inesi et al., 1967; Fiehn & Hasselbach, 1969
n-Alcohols	400 mM n-butanol, 15 min, room temp.	Hara & Kasai, 1977
Mild acid conditions EGTA, neutral	pH 5.6, 37°C, 5 min 0.5 mM EGTA, pH 7.0, 2 min	Berman et al., 1977 McIntosh & Berman, 1978
EGTA, alkaline	1 mM EGTA, pH 8.0 - 9.0, 30 min	Duggan & Martonosi, 1970
Halothane	10 mM halothane, 37°C, 2 min	Diamond & Berman, 1979
CFC*	0.5 mg/ml CFC, 5 mM ATP, pH 7.2, 37°C, 60 min	Hidalgo, 1980
Fluorescamine labelling	2-8 mol fluorescamine/ 10^5 g of SR protein, 22°C	Hidalgo et al., 1982

* CFC: complex of fluorescamine with cycloheptaamylose

of basal activity i.e. whether it represents a different enzyme, or a different form of the (Ca^{2+},Mg^{2+})-ATPase. Evidence for the former is based on findings that basal activity could be abolished when SR vesicles are solubilised (Yamamoto and Tonomura, 1967; McFarland and Inesi, 1970; Walter and Hasselbach, 1973), and that the insoluble fraction, following extraction with Triton X-100, contained approximately half of the basal activity, which was inhibited by specific inhibitors of mitochondrial ATPase (Fernandez et al., 1980). Previous reports that basal activity was not associated with formation of an acid-stable phosphorylated intermediate, have been cited as additional evidence that basal and Ca^{2+}-dependent activities are independent (Tada et al., 1978; de Meis and Vianna, 1979). Carvalho-Alves and Scofano (1983) have, however, recently demonstrated formation of a Ca^{2+}-independent, acid-stable phosphoenzyme, to the extent of 1 nmol mg^{-1} protein at low pH, in the absence of KCl and in the presence of 30% dimethylsulphoxide (DMSO). Conversion of basal activity to Ca^{2+}-stimulated activity of the same enzyme has been suggested on the basis of temperature and detergent effects on both activities (Inesi et al., 1976). From comparisons of ATPase and GTPase activities, Bick et al. (1983) have concluded that Ca^{2+}-independent activity is an alternate hydrolysis cycle. Alonso et al. (1981) studied ATP and P_i binding to SR in the steady state by depositing SR vesicles on to Millipore filters and continuously perfusing with medium containing (γ-^{32}P)-ATP and/or (2,8-^{3}H)-ATP. They showed that, following perfusion, a measurable amount of P_i was released by acids in the absence but not in the presence of Ca^{2+} and suggested that a single population of SR ATPase units change their mode of operation in response to Ca^{2+}. The question arises, therefore, whether subtraction of basal activity, in the measurement of stoichiometry, is justified.

Determination of Ca^{2+}/ATP by kinetic methods requires determination of Ca^{2+} transport and ATPase activity by two independent methods and experimental difficulties arise in optimising the two assays. In summary, the uncertainties in the determination of the true mechanistic stoichiometry of the Ca^{2+} pump include (i) whether or not to correct for basal ATPase activity; (ii) contribution from passive leakiness; and (iii) possibility of presence of populations of unsealed vesicles.

In this section of the present study, we have extended the Ca^{2+}-stat and pH-stat procedures, developed in the previous section, for the measurement of Ca^{2+}/ATP values by ATP- and Ca^{2+}-pulse techniques, respectively. The effect of a variety of conditions on the stoichiometry of the calcium pump has been investigated. The results obtained cannot be explained by a simple 'pump-leak' model, and on the basis of these findings, it is suggested that variable stoichiometry may be an integral property of the calcium pump of sarcoplasmic reticulum.

3.2. EXPERIMENTAL PROCEDURES

3.2.1. DETERMINATION OF COUPLING RATIOS BY AN ATP PULSE METHOD

The coupling ratio of the calcium pump of SR is defined as the ratio of Ca^{2+} ions transported to ATP hydrolysed. In the present study, 'pulse' methods, as opposed to previously described kinetic methods, have been employed to determine coupling ratios, under a variety of conditions. These are extensions of the Ca^{2+}-stat and pH-stat techniques, developed in the previous section. In the ATP-pulse method, a known aliquot of ATP is added and the amount of Ca^{2+} that is subsequently transported is determined by the Ca^{2+}-stat method.

A pulse of MgATP was infused, in the standard procedure, at a constant rate of 45 nmol min^{-1}, from a 100 µl Hamilton syringe into medium at 25°C, containing 50 mM Pipes, pH 6.8, 100 mM KCl, 1 mM $MgCl_2$, 5 mM potassium oxalate and 0.06 - 0.2 mg/ml SR protein. P^1, P^5-di(adenosine-5')-pentaphosphate (AP_5A) (Boehringer), a myokinase inhibitor (Lienhard and Secemski, 1973), was included, at a concentration of 25 µM, throughout. The free Ca^{2+} ion concentration was monitored by means of a Ca^{2+}-selective electrode (Radiometer Type F2002), or spectrophotometrically, using arsenazo III as Ca^{2+} indicator.

Calcium selective electrode method - The free Ca^{2+} concentration of the reaction medium was maintained constant by means of a Ca^{2+}-stat assembly, similar to that previously described by Berman and Aderem (1981)(Section 2.2.2.2), with minor modifications. The Ca^{2+}-electrode and calomel reference electrodes were connected via a standard pH-meter (Radiometer Type PHM 82) and an analogue-to-digital converter to an Apple II microcomputer. Calcium was infused into the reaction mixture, as a 10 mM $CaCl_2$ solution, from an Autoburette (Radiometer Type ABU 80), that was controlled via a parallel interface (Type BCD A/D-1). The volume of titrant, together with the free Ca^{2+} concentration were displayed on a visual display unit. The Ca^{2+}-electrode was calibrated as described in Section 2.2.2.2.

Arsenazo-III method - Monitoring of free Ca^{2+}, using arsenazo III as indicator, was performed, using an Aminco DW-2 dual wavelength spectophotometer, at the wavelength pair, 660 minus 685 nm. Free Ca^{2+} concentrations were maintained constant by means of a Ca^{2+}-stat arrangement

similar to that described above (Fig. 28) (see Section 2.2.2.3). The reaction temperature was controlled by means of a circulating water bath. Dry nitrogen was blown through the cuvette chamber, at temperatures below 10°C.

$^{45}CaCl_2$ method - ATP-pulse experiments were, in addition, conducted in medium in which the free Ca^{2+} concentration was adjusted by means of $^{45}Ca^{2+}$/EGTA buffers. The transport medium contained, in addition to that above, 10 mM EGTA and varying concentrations of $^{45}CaCl_2$. Free Ca^{2+} concentrations were calculated, taking into account all species involved in the equilibria between H^+, Mg^{2+}, Ca^{2+} and EGTA, using association constants given in Table I. MgATP, to a total of 227 nmol, was infused at a constant rate of 45 nmol min^{-1} into the medium. The amount of Ca^{2+} transported per pulse of ATP was obtained by determining the amount of $^{45}Ca^{2+}$ retained by the vesicles on Millipore filters (Type HA 0.45 µM). The free Ca^{2+} concentration did not vary by more than 14% during transport, even at the lowest Ca^{2+} concentration tested.

3.2.2. DETERMINATION OF COUPLING RATIOS BY THE CALCIUM PULSE METHOD

In the Ca^{2+}-pulse method, analogues to the ATP-pulse method, a known amount of Ca^{2+} was added to medium containing excess ATP and the amount of ATP hydrolysed was monitored by means of pH-stat determination of excess H^+ liberated.

ATP hydrolysis was monitored by means of a pH-stat procedure, in medium at 25°C, under nitrogen, containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 5 mM ATP, pH 6.8 and 0.15 to 0.25 mg/ml SR protein. The pH of the reaction medium was monitored on a standard pH-meter (Radiometer Type PHM 82), by means of a glass electrode (Radiometer Type G 2949 C), in combination with a calomel reference electrode, connected to a pH-titrator (Radiometer Type TTT 60), set in the pH-stat mode. CO_2-free NaOH (10 mM) was infused from an Autoburette and the volume recorded on the Apple II graphics system. Calcium chloride, 10 mM, that was pulsed into the reaction mixture, did not result in significant pH changes. Counter transport of H^+ does not contribute to Ca^{2+}/ATP values, since it only occurs during the

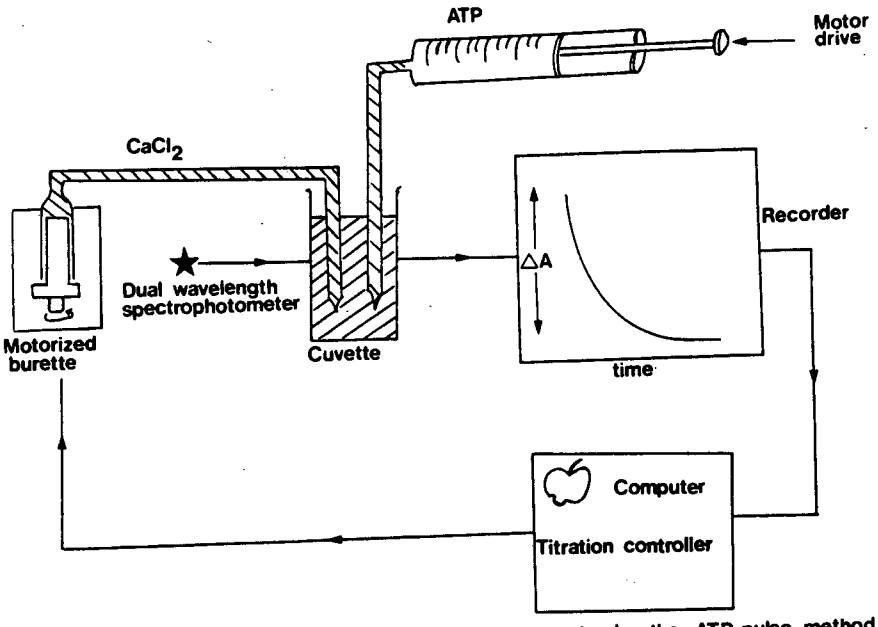


Figure 28: Diagrammatic representation of the spectrophotometric Ca^{2+}-stat for determining Ca^{2+}/ATP by an ATP-pulse method.

first few seconds of Ca^{2+} transport (Madeira, 1980; Chiesi and Inesi, 1980). Carbonyl cyanide-m-chlorophenylhydrazone (CCCP) (Calbiochem), a H^+ ionophore (Heytler and Prichard, 1962), had no effect on H^+/ATP or Ca^{2+}/ATP values.

The stoichiometric amount of protons liberated per mole of ATP hydrolysed was determined at pH 6.8, in medium containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 25 µM AP_5A, 0.3 to 0.5 mg/ml aged SR (14 days at 4°C), and at a free Ca^{2+} concentration, which was maintained constant by means of a Ca^{2+}-stat assembly, of 5 µM. The stoichiometric amount of H^+ liberated per mole of standardised MgATP was determined by means of the pH-stat assembly and was found to be 0.50 ± 0.02 (n=10) at pH 6.8 (Fig. 29).

Standardisation of sodium hydroxyde - CO_2-free NaOH was prepared by diluting the appropriate amount of saturated NaOH with weakly acid boiled water. The solution was kept in a tightly stoppered bottle, containing a soda lime trap. The solutions of NaOH was standardised against 0.100 N HCl (BDH Chemicals, Poole, UK), using the pH-stat assembly.

3.2.3. ASSAY OF ATPase ACTIVITY BY LIBERATION OF $^{32}P_i$ FROM γ-$^{32}P_i$-ATP

Mg^{2+}-ATPase activity (EC 3.6.1.4) and (Ca^{2+},Mg^{2+})-ATPase activity (EC 3.6.1.5) was assayed by measuring the release of $^{32}P_i$ from (γ-^{32}P)-ATP. The $^{32}P_i$ was converted to phosphomolybdate and extracted by the method of Avron (1960), as modified by de Meis and Carvalho (1974).

The assay mixture contained 20 mM Pipes, pH 6.8, 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 5 mM EGTA, 5 mM $CaCl_2$ and 0.5 mg/ml SR protein. The reaction was initiated by the addition of 5 mM (γ-^{32}P)-ATP (1000 cpm/nmol) and terminated at various time intervals by mixing an aliquot (0.1 ml) of the assay medium with 0.1 ml of 1 N perchloric acid (PCA). After 2 min incubation on ice, ammonium molybdate (50 µl of 0.06 M ammonia molybdate in 0.01 N HCl) was added, followed by 2 ml of a mixture of isobutyl alcohol and benzene (1:1, v/v). The solution was vigorously stirred for 45 sec in a vortex mixer and centrifuged at 3000 rpm for 1 min. An

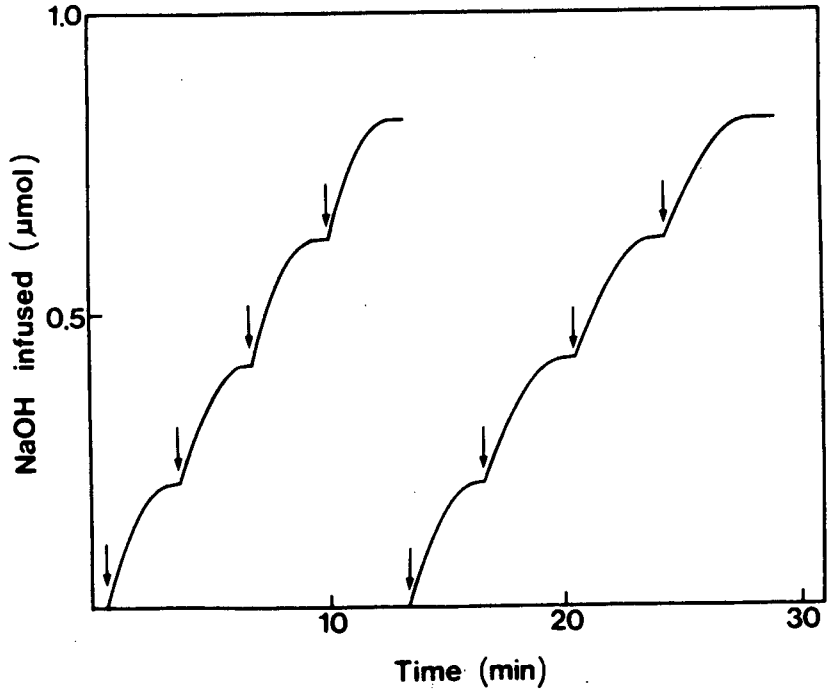


Figure 29: Determination of the stoichiometric amount of H^+ liberated per mol of ATP hydrolysed.

H^+ production was measured by means of a pH-stat assembly in medium, at pH 6.8, containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 25 µM AP_5A, 0.3 - 0.5 mg/ml aged SR and at a free Ca^{2+} concentration of 5 µM. MgATP, 455 nmols, was added where indicated.

aliquot (1.2 ml) was removed from the organic phase and a further extraction, following the addition of 0.5 mM KH_2PO_4 and 1 ml of isobutanol : benzene, was performed. An aliquot (1 ml) of the second extraction was added to the first and any carry-over of (γ-^{32}P)-ATP into the organic phase was removed by the further addition of 1 ml of 0.5 M HCl. Following vigorous vortexing and centrifugation, an aliquot (1.5 ml) of the organic phase was removed, mixed with 10 ml of Instagel (Packard) liquid scintillation fluid and assayed for radioactivity.

Control experiments, to determine the amount of phosphate released by non-enzymatic means, were carried out by adding (γ-^{32}P)-ATP to the reaction mixture after the addition of 1 N PCA. This background value, which was never more than 5% of the total P_i liberated, was subtracted. A further series of control experiments proved that less than 0.1% $^{32}P_i$ remained in the aqueous phase. The amount of $^{32}P_i$ released was plotted as a function of reaction time and the ATPase activity (μmol P_i released/min/mg SR protein) was calculated from the slope of the curve.

3.2.4. DETERMINATION OF ATP CONCENTRATION

(a) NADPH-linked Assay

MgATP was standardised by monitoring NADPH formation in the presence of glucose, hexokinase and glucose-6-phosphate dehydrogenase (G6PDH), according to the method of Bergmeyer (1965). The reaction mixture, 3.00 ml at 25°C, contained 100 mM imidazole, pH 6.7, 20 mM glucose, 2 mM EDTA, 10 mM magnesium acetate, 10 μM AP_5A, 2.0 mM NADP, 30 mM creatine phosphate, 2.5 U/ml hexokinase and 1.5 U/ml G6PDH. MgATP (10 - 30 μl) was added to give a final concentration of 20 - 40 μM. The reaction was complete within 10 minutes and the absorbance was measured at 340 nm in a Varian Techtron (Model 635) spectrophotometer. The concentration of ATP was determined using an extinction coefficient for NADPH of 6.28×10^{-6} M^{-1} cm^{-1} (Bergmeyer, 1965).

(b) Firefly Luciferin-Luciferase Method

The ATP-dependent luciferin-luciferase enzyme, extracted from the

firefly, Photinus Pyralis, gives rise to bioluminescence, which is the basis for a sensitive and specific assay of ATP (Hastings, 1975). A Lumitran ATP photometer Model L3-000 (New Brunswick Scientific) was used for the measurement of luminescence according to the method of Stanley and Williams (1969).

Desiccated firefly lanterns, 20 mg (Sigma) were homogenised in a teflon homogeniser (Thomas, type AA) for 5 min with 2 ml of an ice-cold buffer, containing 0.1 M sodium arsenate and 40 mM magnesium sulphate, pH 7.4. The homogenate was centrifuged at 15 000 x g for 30 min at 2°C in a Beckman Model J2-21 centrifuge. The clear pale supernatant was decanted and stored on ice. The luciferase extract was usually prepared 12 - 15 hours before use, to allow the endogenous ATP to be hydrolyzed, decreasing blank values, and assays were carried out within 24 hrs of preparation.

Samples were pipetted into scintillation vials containing equal volumes of arsenate buffer (0.1 M sodium arsenate, 40 mM magnesium sulphate, pH 7.4), phosphate buffer (0.01 M potassium phosphate, 4 mM magnesium sulphate, pH 7.4) and distilled water, in a final volume of 1.0 ml. An aliquot of the luciferase preparation (40 µl) was added and the vial was placed in the counting well of the photometer and assayed within 5 sec over a period of 30 sec. The sample (30 µl of approximately 1 µM ATP) was added as soon as possible after the initial assay, and reassayed. An internal standard of 30×10^{-12} moles of ATP (30 µl of 1 µM ATP, made up in the phosphate buffer) was added after the sample had been assayed to correct for any unusual quenching of bioluminescences by the sample. The concentration of the sample was calculated and corrected for the internal standard, which did not differ by more than 15% from the standard curve.

A standard curve was prepared by assaying six successive aliquots of 30×10^{-12} moles of ATP, in a similar manner to that described above. A plot of light emitted versus ATP concentration was linear over the concentration range 30×10^{-12} moles ATP/ml to 1.8×10^{-10} moles ATP/ml (Fig. 30).

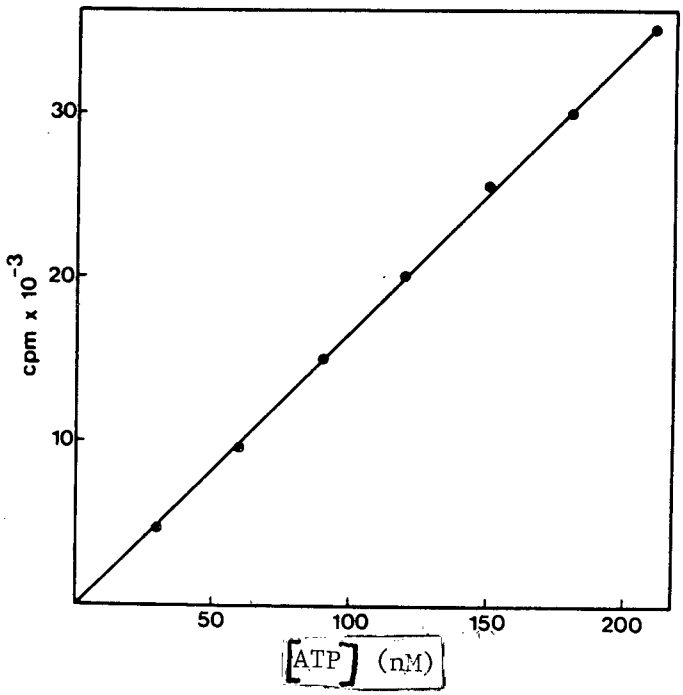


Figure 30: Linearity of luciferin-luciferase ATP assay in the range 30 - 210 nM ATP.

A series of ATP standard concentrations were added to vials containing the assay buffer and 30 µl of the luciferin-luciferase extract, as described in "Experimental Procedures". The amount of light emitted (cpm) was determined over a period of 30 s following an initial 5 s delay.

3.2.5. PURIFICATION OF VESICLES THROUGH LOADING WITH CALCIUM PHOSPHATE

A homogeneous population of SR vesicles, devoid of leaky membranes and 'inside-out' vesicles, was isolated according to the method of Bonnet et al. (1978). SR vesicles were loaded with calcium phosphate in medium containing 30 mM phosphate, pH 7.0, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 150 mM KCl, 3 mM ATP and 0.1 mg/ml SR protein, at 20°C. After 10 minutes of loading, the vesicles were immediately centrifuged at 2°C in a Beckman J2-21 centrifuge, with a JA 20 rotor, for 30 minutes at 6 000 x g. The protein pellet was resuspended in 30 mM Pipes, pH 7.0, 5 mM MgCl, 150 mM KCl. Light vesicles, which remained in the supernatant during centrifugation, were re-concentrated by centrifugation at 78 000 x g for 30 min, 0°C, in rotor no. 30 in a Beckman model L2-65 B ultracentrifuge.

Vesicles were unloaded by inducing Ca^{2+} efflux, according to the method of Hasselbach et al. (1972), with minor modifications. The pellet selected, as described above, was resuspended to a concentration of 2-4 mg/ml SR protein. EGTA, 2 mM, and 5 mM potassium arsenate were added to the vesicle suspension at room temperature. Ca^{2+} efflux, determined in a series of pilot experiments, continued for 5 min, and was stopped by addition of 2 mM $CaCl_2$ which, in addition, prevented inactivation by EGTA (McIntosh and Berman, 1978). Ca^{2+} transport is not affected by arsenate (5 mM) (Hasselbach et al., 1972).

3.2.6. FLUORESCENT MONITORING OF CALCIUM TRANSPORT USING DANSYLAZIRIDINE-LABELLED TROPONIN C

Dansylaziridine-labelled troponin C (TnC_{DANZ}) undergoes a greater than 2-fold fluorescence enhancement with Ca^{2+} binding to Ca^{2+}-specific regulatory sites of troponin C (Johnson and Schwartz, 1978). TnC_{DANZ} has been shown to provide a convenient means by which Ca^{2+} uptake and release in SR can be monitored (Johnson and Schwartz, 1978).

TnC_{DANZ} was obtained from Dr J.D. Johnson (University of Cincinatti College of Medicine, Cincinatti). Fluorescent measurements were conducted in an Aminco SPF-500 Fluorimeter, used in the ratio mode. Excitation was at 340 nm and emission was monitored at 520 nm. The

transport medium, at 25°C, contained 50 mM Pipes, 100 mM KCl, 5 mM potassium oxalate, 1 mM $MgCl_2$, 1.3 μM TnC_{DANZ} and 0.06 mg/ml SR protein. The pH of the medium was varied from 6.8 to 8.0 (TnC_{DANZ} fluorescence is independent of pH (Johnson and Schwartz, 1978)). Transport was initiated by addition of 45 nmols of ATP.

3.2.7. $^{45}Ca^{2+}$ EXCHANGE MEASUREMENTS

$^{45}Ca^{2+} \leftrightarrow {}^{40}Ca^{2+}$ exchange was determined, at 25°C, at both pH 6.8 and pH 7.8. At pH 6.8, the medium contained 50 mM Pipes, 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 0.5 mM EGTA and 0.5 mM $CaCl_2$. At pH 7.8, the medium consisted of 50 mM Hepes, 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 5 mM EGTA and 5.1 mM $CaCl_2$. Transport was initiated by addition of 5 mM ATP to medium containing 0.05 mg/ml SR protein. After steady state was reached (5 min), carrier-free $^{45}Ca^{2+}$ tracer was added and the amount of radioactivity retained on the Millipore filters was determined. In the Ca^{2+} transport assay, $^{45}Ca^{2+}$ was present from the start of the reaction. The Ca^{2+}/EGTA buffer mixtures were chosen such that the free Ca^{2+} concentration did not decrease to sub-saturating concentrations during the course of Ca^{2+} uptake.

3.2.8. DETERMINATION OF CALCIUM EFFLUX BY THE FLOW DIALYSIS METHOD

Calcium efflux was measured by using the flow-dialysis method of Tenu et al. (1976). The flow dialysis cell was as described by Colowick and Womack (1969), with a lower chamber volume of 0.1 ml. This chamber was perfused with a flow rate of 2 ml/min and the response time of the cell was checked to be approximately 30 sec. The medium consisted of a 20 mM Tris/HCl solution, pH 7.4, containing 5 mM $MgCl_2$, 0.15 M KCl, 1 mg/ml SR protein and 10^{-4} M $^{45}CaCl_2$. The reaction was initiated by the addition of 5 mM ATP. Fractions were collected at 20 s intervals and aliquots of each fraction were assayed for radioactivity in a liquid scintillation spectrometer.

3.3. RESULTS

3.3.1. DETERMINATION OF COUPLING RATIOS

3.3.1.1. Kinetic Method

Coupling ratios are conventionally measured by kinetic methods and are based upon initial rates of Ca^{2+} transport and of Ca^{2+}-dependent ATPase activity, in the presence of oxalate.

The time course of Ca^{2+} transport, total ATPase activity and Ca^{2+}-independent ATPase activity at 20°C and pH 6.8 is shown in Fig. 31. Ca^{2+} transport showed saturation kinetics with a maximum loading capacity of 3000 - 4000 nmoles/mg SR protein and an initial rate of transport equal to 1330 nmol $min^{-1}mg^{-1}$. Ca^{2+}-dependent ATPase activity was taken as the difference between the total ATPase activity (875 nmol $min^{-1}mg^{-1}$), determined in the presence of saturating Ca^{2+} concentration (11 μM), and Ca^{2+}-independent or 'basal' ATPase activity (128 nmol $min^{-1}mg^{-1}$), determined in the presence of excess EGTA. The coupling ratio was 1.78 and is in good agreement with previously reported values obtained kinetically (Tada et al., 1978; de Meis and Vianna, 1979).

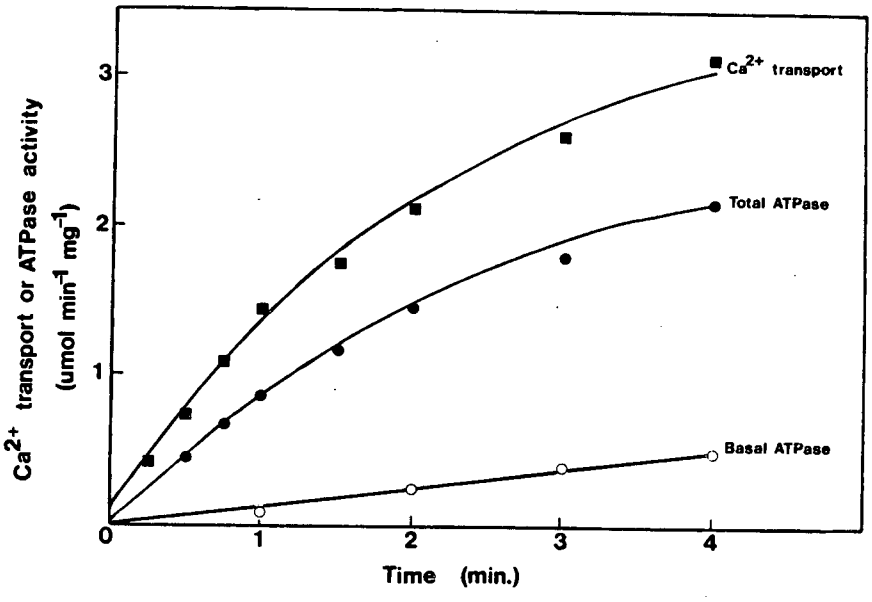


Figure 31: Time course of Ca^{2+} transport, total ATPase and Ca^{2+}-independent (basal) ATPase activity.

Ca^{2+} transport was determined by the $^{45}Ca^{2+}$ Millipore filtration technique as described under "Experimental Procedures". ATPase activity was assayed by measuring release of $^{32}P_i$ from (γ-^{32}P) ATP. The $^{32}P_i$ was converted to phosphomolybdate and extracted as described under "Experimental Procedures". Assays were performed separately in medium containing 50 mM Pipes, pH 6.8, 100 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 5 mM potassium oxalate and 0.4 mg/ml SR protein. $CaCl_2$, 5 mM, was added, except when basal activity was determined.

3.3.1.2. ATP-Pulse Method

Typical assays of the determination of Ca^{2+}/ATP ratios by the ATP-pulse method are shown in Fig. 32, where free Ca^{2+} concentration has been monitored by a Ca^{2+} electrode (Fig. 32a) or by means of the arsenazo III method (Fig. 32b). Endogenous $[Ca^{2+}]_{free}$ in the reaction mixtures amounted to 2-3 µM. Free Ca^{2+} concentrations were allowed to 'stat' at 5 µM and 3 µM respectively for the two methods, by infusion of standardised $CaCl_2$ solution. Successive aliquots of 135 nmoles of ATP (as standardized MgATP) were added at a constant rate of 45 nmol/min over 3 minutes. Activation of transport resulted in a decrease in $[Ca^{2+}]_{free}$ in the medium and activation of infusion of $CaCl_2$ from the Autoburette. During ATP infusion, $[Ca^{2+}]_{free}$ decreased from 5 to 3.65 µM, when assayed by the Ca^{2+}-electrode method, reaching a minimum after approximately 2.5 minutes. Prolonged infusion of ATP at the same rate, for up to 5 min, did not decrease the free Ca^{2+} concentration further whilst the Ca^{2+}-stat was operative. Following cessation of infusion, $[Ca^{2+}]_{free}$ rose within 2 min to the 'stat' value, when titration of $CaCl_2$ ceased. The relatively slow responses are due to the limitations imposed by the Ca^{2+}-electrode ($t_{\frac{1}{2}}$ for jump from 6 µM to 11 µM = 1 min), necessitating relatively wide settings of the proportional band (10 mV, which was equivalent to a difference of 2.7 µM from the stat value of 5 µM).

The response time of the arsenazo III method was much more rapid ($t_{\frac{1}{2}}$ = 8 msec (Ogawa et al., 1980). This resulted in closer control of the free Ca^{2+} concentration during ATP infusions, such that free Ca^{2+} did not decrease by more than 0.3 µM below the stat point. This allowed narrower settings of the proportional band (ΔA = 0.01, equivalent to a change of 1.34 µM free Ca^{2+}).

ATP concentrations, monitored during the pulse of MgATP, did not rise to detectable levels ($\ll$0.2 µM). This is an indication of the high affinity of the Ca^{2+} pump for ATP (Tada et al., 1978; Vianna, 1975; Pang and Briggs, 1977; Arav et al., 1983). Following complete substrate hydrolysis, the Ca^{2+} concentration tended to drift above the set-point by 0.5 - 1.0 µM with the Ca^{2+}-electrode method and by approximately 0.05 µM with the arsenazo III method. Stabilisation was achieved after approximately 2-3 minutes. This can be explained either on the

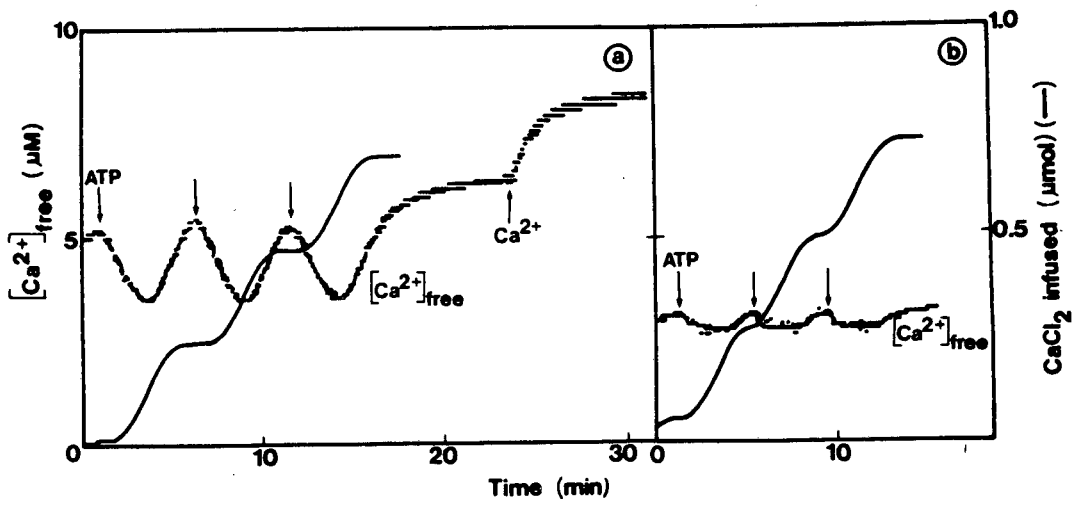


Figure 32: Typical titration curves for the determination of coupling ratios by the ATP-pulse method, using a Ca^{2+}-specific electrode (a) or arsenazo III (b) as Ca^{2+}-indicators.

The reaction media contained 50 mM Pipes, pH 6.8, 100 mM KCl, 5 mM potassium oxalate, 25 μM AP_5A, 1 mM $MgCl_2$ and 0.08 mg/ml SR protein. The reaction volumes were 4.0 ml and 3.0 ml in (a) and (b), respectively. Arsenazo III, 40 μM, was included in (b). Calcium transport was initiated by infusion of 135 nmol aliquots of ATP. $CaCl_2$, 50 nmols, was added where indicated.

basis of an over-titration, resulting from the slow response times of the Ca^{2+} detectors, or due to slow leakage from the loaded vesicles. However, since the Ca^{2+} concentration returned to identical levels following repeated additions, it was presumed that the amount of Ca^{2+} infused between subsequent additions, represented a true reflection of the amount of Ca^{2+} transported during the pulse.

Efflux of Ca^{2+} following the ATP pulse, was quantified in a separate experiment (Fig. 33). Ca^{2+} transport during ATP infusion was monitored as described above. After complete hydrolysis of added ATP, and when the free Ca^{2+} concentration had reached equilibrium, an aliquot of 140 nmol of Ca^{2+} was added to the reaction medium, increasing the free Ca^{2+} concentration from 6.1 to 13.6 μM. Efflux of Ca^{2+} that resulted in an increase in medium free Ca^{2+} by 1.1 μM, in this typical experiment, therefore represented 20 nmol Ca^{2+}, i.e. 5% of the total Ca^{2+} transported during hydrolysis of the aliquot of ATP. The possible contribution of slow leakage of Ca^{2+} from loaded vesicles was derived from the arsenazo III experiments because of its more rapid response time. Here the system stabilises within 1 min and no significant drift in Ca^{2+} concentration in the medium was detected during the ensuing 15 min. It is concluded that no significant efflux of transported Ca^{2+} occurred in the post-pulse period with the arsenazo III method and that the upward drift obtained with the Ca^{2+}-electrode was merely a reflection of the slow response of the detector.

The possible effects of ADP and P_i, formed during ATP hydrolysis, on Ca^{2+}/ATP ratios was considered. ADP (1 mM) and P_i (5 mM), at concentrations that result in reversal of the catalytic cycle (de Meis and Carvalho, 1974; Beil *et al.*, 1977; Yamada and Ikemoto, 1980), had no significant effect on measured Ca^{2+}/ATP values when added separately or simultaneously to the assay medium (data not shown).

The effects of additions of successive pulses of ATP are shown in Fig. 34. Maximum Ca^{2+}/ATP ratios were noted with the 2nd and 3rd additions, up to a total load of 2000 nmol Ca^{2+}/mg SR protein, and declined thereafter. The stoichiometry was slightly lowered during the first pulse, which may be related to the fact that a certain amount of Ca^{2+} is required to be transported in order that the internal free

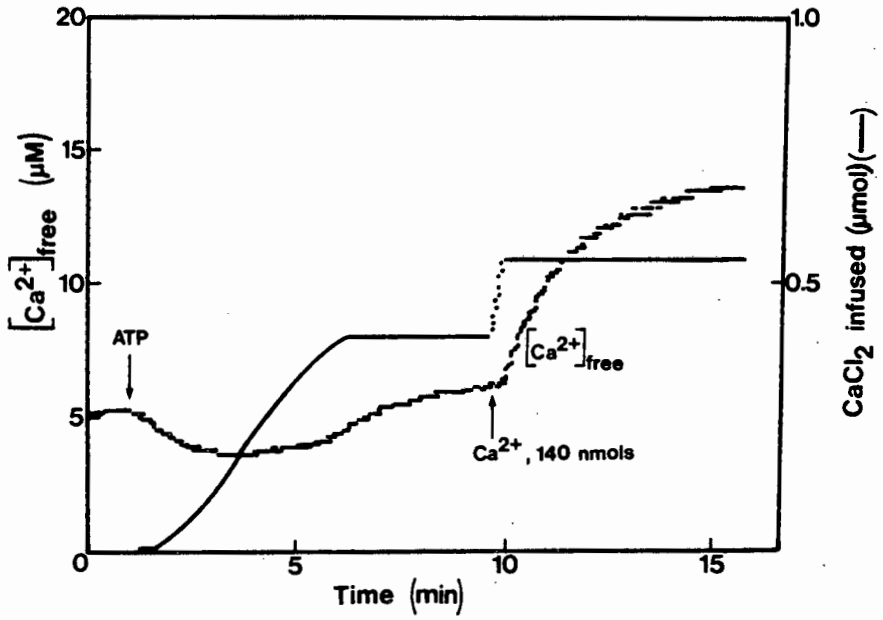


Figure 33: Calcium efflux in the 'post-pulse' period.

Ca^{2+} transport was measured by means of a Ca^{2+}-electrode, as in Fig. 32a. Following infusion of 218 nmol ATP, 398 nmol of Ca^{2+} was transported. After complete hydrolysis of substrate and equilibration of the Ca^{2+} concentration, a further aliquot of 140 nmol of Ca^{2+} was added, as indicated.

Ca^{2+} concentration exceeds the solubility product for calcium oxalate precipitation. The decline at higher Ca^{2+} loads has previously been noted by the kinetic method, where maximum loading of approximately 3000 - 4000 nmol Ca^{2+}/mg SR protein is observed in the presence of 5 mM potassium oxalate (Fig. 31). The range of Ca^{2+} loading over which constant Ca^{2+}/ATP ratios were observed, depended on the protein concentration, being higher at higher levels. In subsequent ATP-pulse experiments, Ca^{2+}/ATP ratios were measured, following an initial aliquot of MgATP, as an average of 2-4 successive additions of ATP, such that the total loading of Ca^{2+}, as calcium oxalate, was less than 50% of the maximum capacity. Consideration was given to the effect of the size of the pulse on the observed stoichiometry. Larger pulse sizes limited the number of aliquots required to fill the vesicles. This restricted the use of the method to lower and upper limits of an ATP pulse of 50 and 500 nmoles, respectively.

The coupling ratio was found to be 1.81 $\pm$ 0.13 for the Ca^{2+}-electrode method and 1.84 $\pm$ 0.12 for the arsenazo III method (n = 10) under standard conditions of pH 6.8, 25°C and saturating calcium concentrations.

Hasselbach (1981) has explained observed Ca^{2+}/ATP ratios of less than two in terms of leaky or unsealed vesicles. Bonnet et al. (1978) have described a procedure for purification of intact vesicles that contain correctly orientated pump units in sealed vesicles, based upon their selection by active loading with calcium phosphate and low speed sedimentation. Coupling ratios were determined in freshly isolated native SR and compared to values obtained on a population of vesicles that were either able or unable to be pelleted after loading with calcium phosphate crystals. The unloading of calcium phosphate-loaded vesicles was induced by arsenate and EGTA (Hasselbach et al., 1972). The possible effects of this procedure on measured coupling ratios were also determined. The results are shown in Table VII. The unloading procedure appeared to have no effect on measured coupling ratios. The pelletable calcium phosphate-loaded vesicles showed an increase in Ca^{2+}/ATP values, which were, however, still significantly less than 2.0. The non-pelletable vesicles showed a significantly lower Ca^{2+}/ATP ratio. Calcium phosphate pelletable vesicles represent approximately 20% of

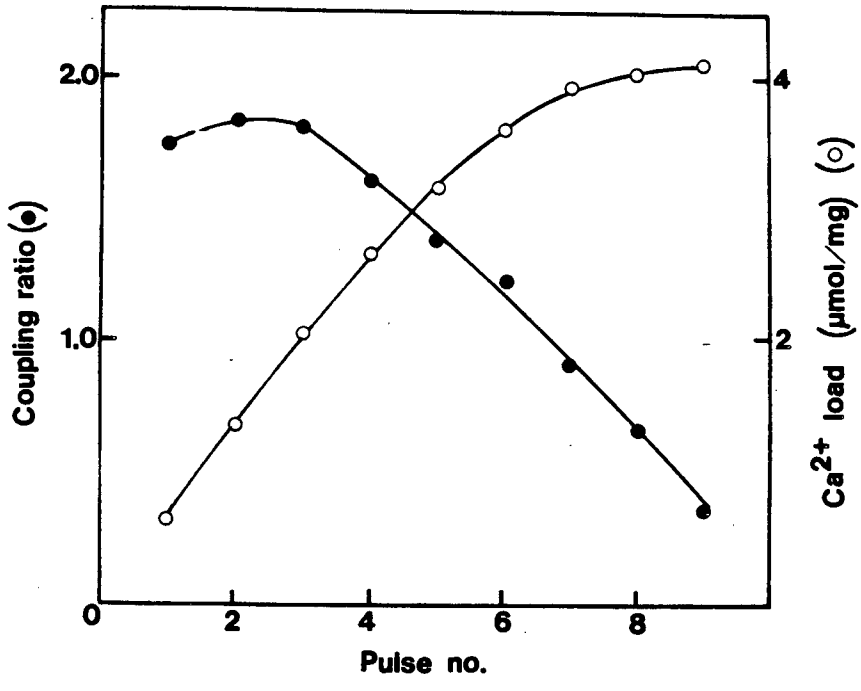


Figure 34: Effect of Ca^{2+} loading on measured ATP-dependent coupling ratios.

Coupling ratios were determined as described in Fig. 32b, using arsenazo III as Ca^{2+}-indicator, by infusion of multiple pulses of 135 nmol ATP into medium containing 0.09 mg/ml SR protein.

the total SR protein. The recovery of transport units following the loading procedure cannot be calculated with certainty since Ca^{2+}-dependent ATPase activity in open vesicles will be stimulated to varying degrees by lack of constraints from high intravesicular Ca^{2+} concentrations. It is concluded that the preparation of native SR vesicles, employed in this study, does contain a fraction of open vesicles, but that in a selected fraction of sealed vesicles, less than theoretical Ca^{2+}/ATP values are observed.

TABLE VII

Effect of purification of vesicles through their loading with calcium phosphate on experimentally determined coupling ratios

SR vesicles were loaded with calcium phosphate in medium containing 30 mM phosphate, pH 7.0, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 100 mM KCl, 3 mM ATP and 0.1 mg/ml SR protein. Following 10 min of incubation at 20°C, the suspension was centrifuged at 6000 x g for 30 min at 0°C. The protein pellet was resuspended in 30 mM Pipes, pH 7.0, 5 mM $MgCl_2$, 150 mM KCl, at a protein concentration of 2-4 mg/ml. Ca^{2+} efflux was induced by addition of 5 mM potassium arsenate and 2 mM EGTA. Coupling ratios were determined as described in Fig. 31a (n = 6).

	Ca^{2+}/ATP
Control SR	
Untreated	1.61 ± 0.05
Arsenate and EGTA treated	1.64 ± 0.06
Calcium phosphate loaded SR	
Pelletable vesicles	1.78 ± 0.04*
Non-pelletable vesicles	1.01 ± 0.06*

* $P < 0.01$ (loaded vesicles versus mean control value)

3.3.1.3. Ca^{2+}-Pulse Method

In analogous experiments to those just described, Ca^{2+}/ATP ratios were obtained by measuring ATP hydrolysis following infusion of a pulse of Ca^{2+}. Results of a typical assay are shown in Fig. 35. ATP hydrolysis at pH 6.8 was monitored by the pH-stat technique in medium containing 5 mM MgATP. Endogenous Ca^{2+} was rapidly transported into the SR vesicles in less than 30 secs and ATP was hydrolysed at a rate that was similar to the basal rate obtained in the presence of 1.0 mM EGTA. $CaCl_2$ was pulsed into the reaction medium at a rate of 1000 nmol/min, causing an increase of 2-3 μM free Ca^{2+}, which then declined rapidly to previous levels ($\ll$0.05 μM), when infusion was discontinued. Following complete Ca^{2+} uptake from the transport medium, the rate of ATP hydrolysis returned to that seen initially and the amount of ATP hydrolysed, due to Ca^{2+} stimulation, could be calculated. Ca^{2+}/ATP ratios were found to be 1.79 $\pm$ 0.15 under standard conditions of pH 6.8, 25°C and saturating ATP concentrations.

The size of the Ca^{2+} pulse depended on the amount of SR protein in the reaction medium, such that a capacity of 2000 nmol/mg was not exceeded. Multiple pulses of smaller size gave similar Ca^{2+}/ATP values, and the number of pulses possible depended on the amount of SR protein. Protein concentration had no effect on the measured Ca^{2+}/ATP ratios by the Ca^{2+} pulse method.

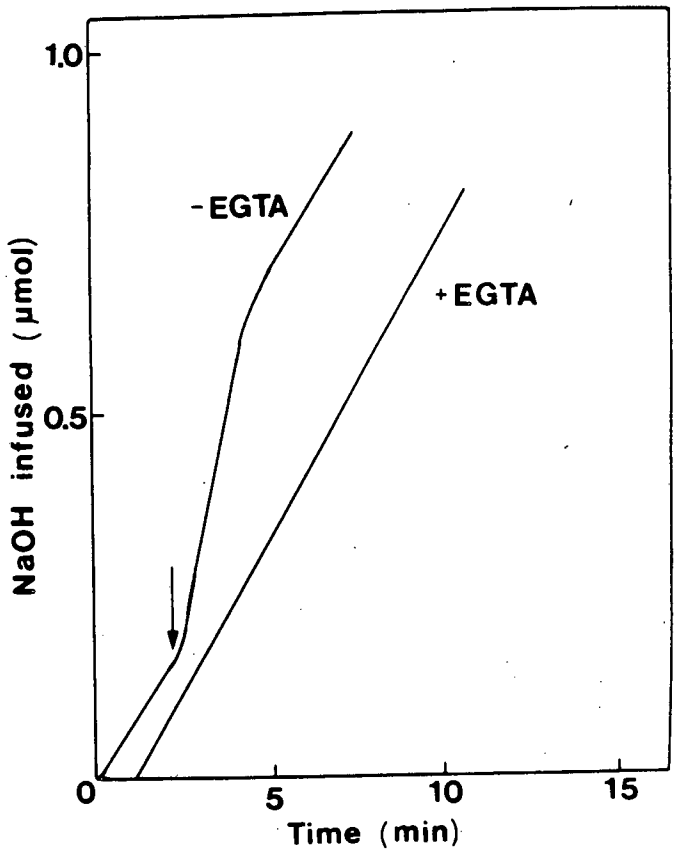


Figure 35: Typical titration curves for the determination of coupling ratios by the Ca^{2+}-pulse method.

ATP hydrolysis was monitored by means of a pH-stat, in medium, at 25°C and under nitrogen, containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 5 mM ATP, pH 6.8, 0.15 mg/ml SR protein and in the presence and absence of 1.0 mM EGTA. $CaCl_2$, 1000 nmols, was added where indicated.

3.3.1.4. Combined Infusion of Ca^{2+} and ATP

Variable ratios of Ca^{2+}:ATP were continuously infused at a rate of 45 nmol/min into medium in which $[Ca^{2+}]_{free}$ was monitored using arsenazo III as Ca^{2+}-indicator (Fig. 36). The concentration of MgATP in the infusion mixture was constant at 1.8 mM while that of $CaCl_2$ was varied between 2.9 and 4.5 mM to give Ca^{2+}:ATP ratios in the range 1.61 to 2.5. At low Ca^{2+}:ATP values, $[Ca^{2+}]_{free}$ increased at the start of infusion and sharply declined after a Ca^{2+} concentration of 2-3 μM had been reached. This initial increase is presumably due to the approximately 10-fold difference in the affinity of the Ca^{2+} pump for Ca^{2+} and ATP, such that ATP hydrolysis, and therefore Ca^{2+} transport, does not achieve a steady rate until the ATP concentration in the reaction medium has increased to approximately 10 μM. Since the ratio of Ca^{2+}:ATP was less than the true coupling ratio, this initial increase was followed by a sharp decrease in Ca^{2+} concentration. At Ca^{2+}:ATP values higher than the true coupling ratio, the Ca^{2+} concentration continually increased with time, while at Ca^{2+}:ATP values corresponding to the true stoichiometry of the pump, the Ca^{2+} concentration remained constant following the initial increase.

The inset to Fig. 36 shows a plot of the rate of change in absorbance, following the initial increase, against Ca^{2+}:ATP ratio infused. An intercept of 2.13, where there was no change in absorbance, following the initial rise, can be taken to represent the stoichiometry of the Ca^{2+} pump under steady state conditions in these experiments.

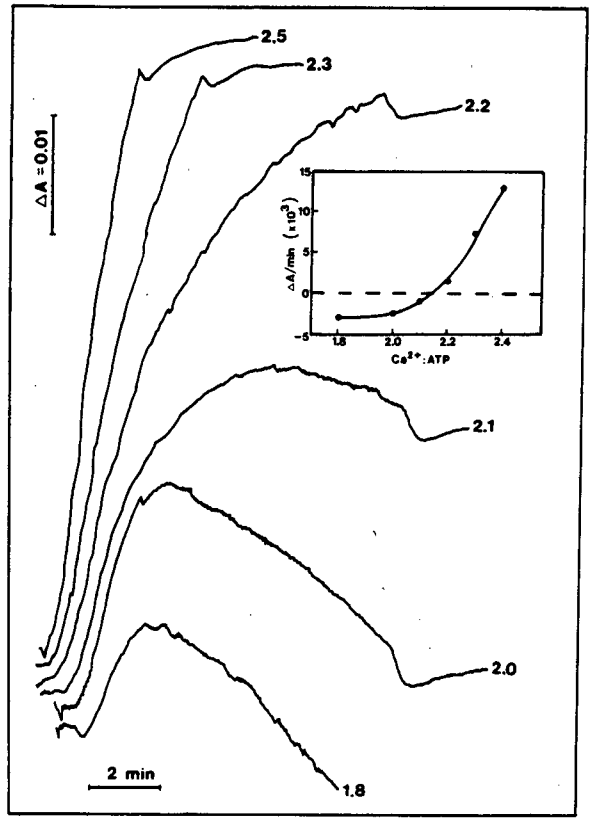


Figure 36: Absorbance changes during infusion of variable ratios of Ca^{2+}:ATP.

The medium contained 20 mM Pipes, pH 6.8, 100 mM KCl, 5 mM potassium oxalate, 1 mM $MgCl_2$, 40 µM arsenazo III and 0.105 mg/ml SR protein. Variable ratios of Ca^{2+}:ATP were infused at a rate of 45 nmol/min. The concentration of MgATP in the mixture was constant at 1.8 mM while that of $CaCl_2$ was varied between 2.9 and 4.5 mM. The inset shows a plot of the rate of change in absorbance, following the initial increase, against the ratio of Ca^{2+}:ATP infused, as described in text.

3.3.2. THE EFFECT OF pH ON THE STOICHIOMETRY OF CALCIUM TRANSPORT

The effects of varying pH in the range 5.5 to 9.0 on measured Ca^{2+}/ATP ratios, as determined by the Ca^{2+}-electrode method, are shown in Fig. 37. The Ca^{2+}/ATP ratio is relatively constant at approximately 1.6 - 1.7 between pH 5.5 and pH 7.0. At higher pH values, Ca^{2+}/ATP ratios, determined from the average of the second and third pulses, decreased with an apparent pK_a of 7.9. These findings are in agreement with previous observations by kinetic methods that showed maximum transport at pH 6.8, whilst ATPase activity only reached a maximum at pH 7.2 - 8.0, implying dissociation of transport from ATPase activity under alkaline conditions (Duggan, 1977; Tate et al., 1981). It was previously shown in this laboratory (Berman et al., 1977) that mild acid conditions (pH 5.5 at 37°C) cause uncoupling of Ca^{2+} transport. However, inactivation of transport is highly temperature dependent (E_a = 31 kcal mol^{-1} between 24° and 43°C) (Berman et al., 1977). At 25°C and under conditions of the present assay, uncoupling was minimal (<2%) at pH 5.5 and 25°C (Fig. 37).

It was noted that under the more alkaline conditions tested, the first pulse of ATP resulted in a lower Ca^{2+}/ATP ratio than subsequent pulses (Fig. 38), whereas at pH 6.8, the first, second and third pulses gave similar coupling ratios (see Fig. 32). This suggests that loading of SR vesicles with calcium oxalate modifies the measured coupling ratio under alkaline conditions. A plot of the Ca^{2+}/ATP ratio as a function of pH, determined from the first pulse, shows that transport into calcium-oxalate free vesicles is apparently more sensitive to uncoupling under alkaline conditions, with an apparent pK_a of 7.6 (Fig. 37).

It has previously been demonstrated by Tate et al. (1981) that the effects of alkaline conditions on Ca^{2+} transport are complex. These authors showed that preincubation of SR under alkaline conditions led to uncoupling, but if the same preparation was assayed at pH 6.8, full coupling was observed. These experiments were repeated and confirmed by continuous monitoring of Ca^{2+} transport by means of the Ca^{2+} electrode (Fig. 39). Incubation of SR vesicles at pH 8.0 for 5 min had no

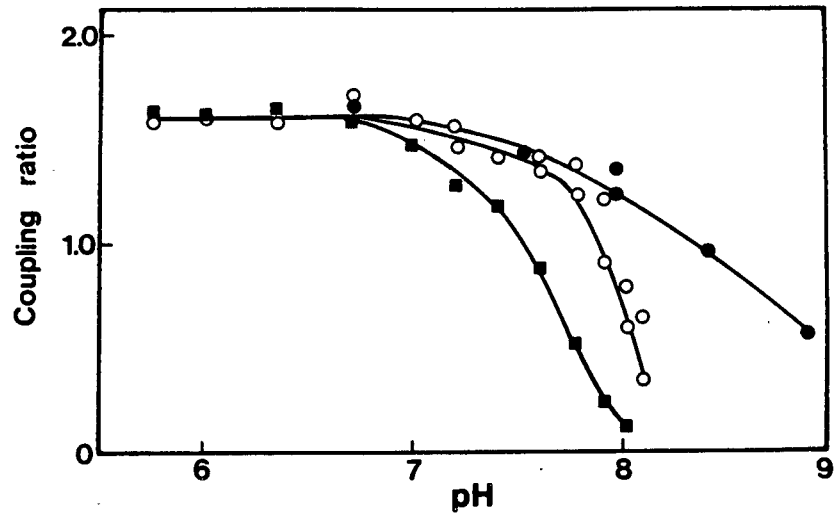


Figure 37: Effects of pH and of calcium oxalate loading on measured coupling ratios.

Coupling ratios were determined as described in Fig. 32a, using the Ca^{2+}-electrode as end-point detector. Ca^{2+}/ATP ratios were calculated at constant pH from the first pulse (■) and from the average of the second and third pulses (○). In addition, Ca^{2+}/ATP ratios were determined at varying pH, following initial loading of SR vesicles with a pulse of 135 nmols of ATP at pH 6.8 (●).

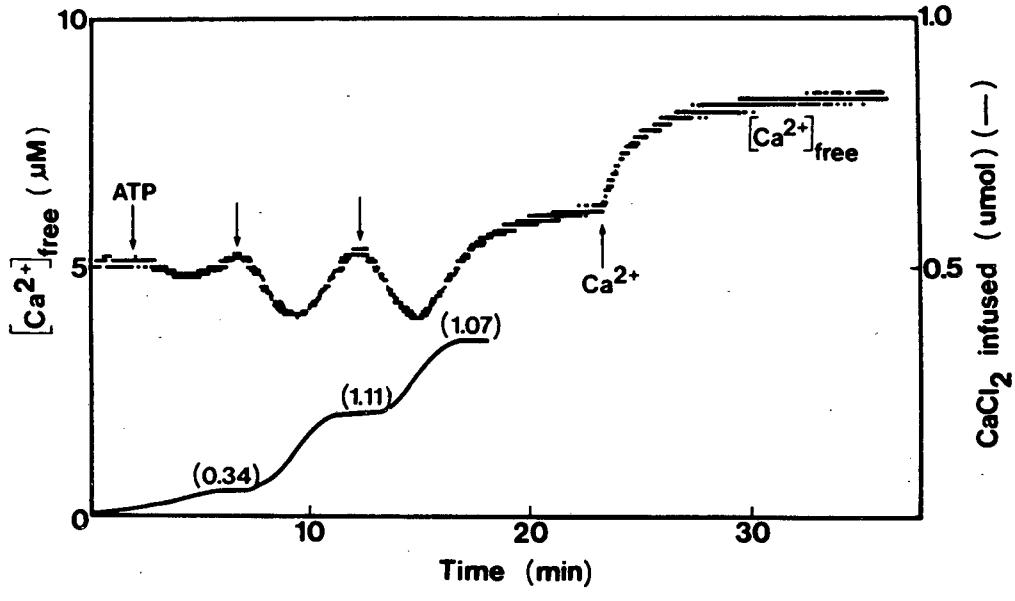


Figure 38: Titration curves for the determination of Ca^{2+}/ATP at alkaline pH.

Reaction conditions were as described in Fig. 32a, except that the pH was adjusted to 7.9. ATP, 135 nmols, and $CaCl_2$, 50 nmols, were added where indicated. The values in brackets are the calculated Ca^{2+}/ATP ratios for each successive pulse.

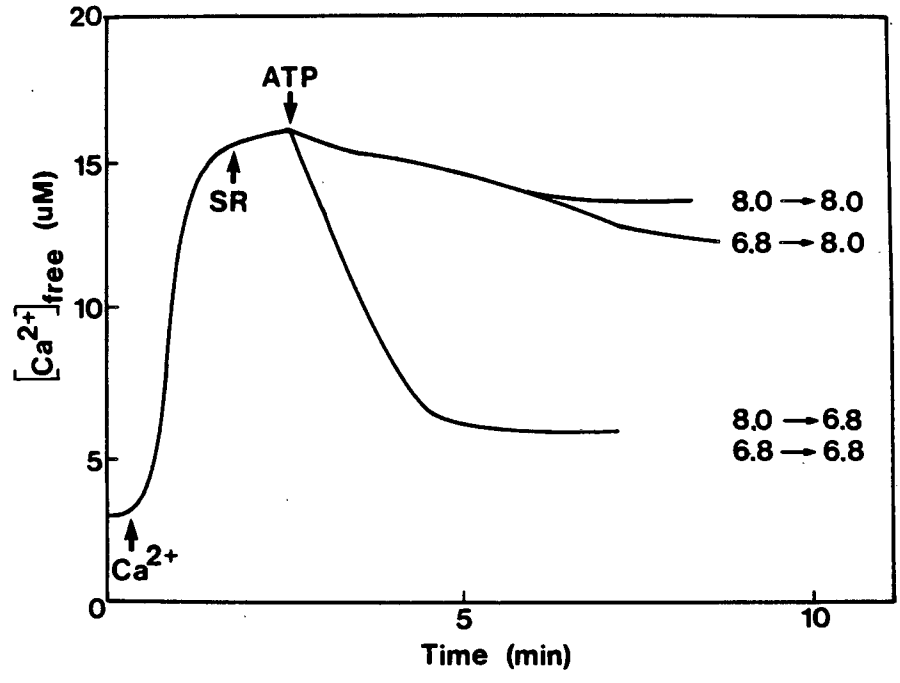


Figure 39: Reversibility of alkaline pH inhibition of Ca^{2+} transport. Ca^{2+} transport was obtained by monitoring depletion of medium Ca^{2+} by means of a Ca^{2+}-electrode. The reaction medium contained 20 mM Pipes, 100 mM KCl, 5 mM potassium oxalate, 1 mM $MgCl_2$ and 25 µM AP_5A. SR (0.06 mg/ml) was added following addition of $CaCl_2$ to give a free Ca^{2+} concentration of approximately 16 µM. Following a 2 min incubation at the pH indicated before the arrow, the pH was changed, by addition of 10-20 µl of NaOH or HCl, to the pH indicated after the arrow. Transport was initiated by addition of 182 nmols ATP.

significant effect on Ca^{2+} transport when assayed at pH 6.8. Conversely, incubation of SR at pH 6.8 did not prevent alkaline uncoupling at pH 8.0. Tate et al. (1981) reported that Ca^{2+} ions protected against inactivation with half-maximal protection at 8-15 μM at pH 7.4 - 7.6. The effects of initial loading of the vesicles with calcium-oxalate at pH 6.8 and subsequent determination of Ca^{2+}/ATP ratios under more alkaline conditions are also shown in Fig. 37. In calcium oxalate-loaded vesicles, considerable protection against alkaline uncoupling was observed, such that at pH 8.4, Ca^{2+}/ATP was 0.8 and significant ATP-dependent Ca^{2+}-uptake was still observed up to pH 8.9 (Ca^{2+}/ATP = 0.4). It is apparent that transport processes into empty and partially calcium-oxalate loaded vesicles exhibit different susceptibility to alkaline uncoupling.

Experiments were devised to determine the extent of calcium-oxalate loading that is required to afford protection against alkaline conditions. The extent of calcium-oxalate loading was varied by the amount of ATP infused during the first pulse. The magnitude of subsequent pulses was maintained constant (135 nmol ATP). The results are shown in Fig. 40. It is apparent that the stoichiometry of the pump at alkaline pH is dependent on the initial load, such that a maximum coupling ratio of 1.0, at pH 7.9, was obtained subsequent to an initial pulse of greater than 136 nmol ATP. This corresponds to an initial calcium oxalate load of approximately 350 nmol/mg i.e. approximately 10% of the maximum load. Partial loading with calcium oxalate at pH 6.8 also afforded protection, but in this case complete protection was obtained at the smallest pulse (18 nmol) of ATP used that resulted in measurable Ca^{2+} transport.

It is concluded that the pH dependence of transport depends on several factors and that transport measured in the pH range 6.0 to 7.0 is relatively unaffected by prior low proton concentrations. However, the uncoupling observed under alkaline conditions had a pH profile that suggested participation of a single ionisable group. The permeability, as determined by efflux of Ca^{2+} from calcium oxalate loaded vesicles, was not enhanced at high pH values that resulted in low coupling ratios (Fig. 38). The tracings of $[Ca^{2+}]_{free}$, following hydrolysis of ATP, appear to be similar at pH 6.8 (Fig. 32) and at pH 7.9 (Fig. 38).

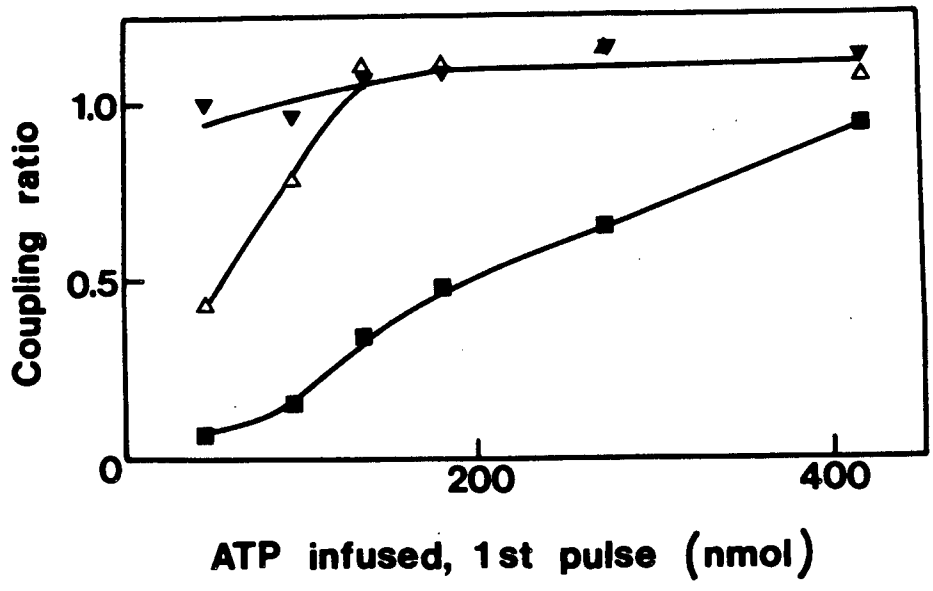


Figure 40: Effect of calcium oxalate loading on measured coupling ratios under alkaline conditions.

Coupling ratios were determined as described in Fig. 32a, at pH 7.9. Varying amounts of ATP were infused for the first pulse, with constant ATP (135 nmols) for the second and third pulses. Ca^{2+}/ATP ratios were calculated for the first (■), second (△) and third pulse (▼).

The response time of the Ca^{2+}-electrode is slow and an overtitration was observed, following complete hydrolysis of ATP (Fig. 38). This may mask any efflux that may have occurred in the post-pulse period. We therefore used the fluorescent derivative of troponin C (dansylaziridine-labelled troponin C or TnC_{DANZ}) (Johnson and Schwartz, 1978) to measure $[Ca^{2+}]_{free}$. TnC_{DANZ} fluorescence is unaffected by pH and serves as a good indicator of Ca^{2+} in the range 3.0 - 70 μM. The results are shown in Fig. 41. Ca^{2+} transport, at the pH indicated, was initiated by addition of 45 nmol ATP. The amount of Ca^{2+} transported, for the pulse of ATP, decreased with increasing pH. Following hydrolysis of ATP, the extent of Ca^{2+} efflux was monitored. It is evident that there is no significant difference in the rate or amount of Ca^{2+} effluxing at various pH values that could account for the variations in stoichiometry.

Passive Ca^{2+} efflux from SR vesicles has previously been shown to be inhibited by micromolar concentrations of Ca^{2+} (Chiesi and Inesi, 1979). The estimates of permeability, outlined above, were conducted in the presence of 5-6 μM free Ca^{2+}. Ca^{2+} efflux was, therefore, measured into medium containing EGTA. The results, shown in Fig. 42, indicate that the rates of efflux are similar at both pH 6.8 and 7.9, with rate constants of 0.7 min^{-1} and 0.71 min^{-1}. These values are also similar to that obtained by Feher and Briggs (1982) (k_{obs} = 0.8 min^{-1}) from vesicles, passivly loaded by equilibration with 20 mM $CaCl_2$, in the absence of oxalate.

The efflux experiments reflect the permeability of sealed vesicles, but will not detect the presence of unsealed vesicles. The effects of pH on the fraction of sealed vesicles in the total population of SR vesicles were determined. Following active loading with calcium phosphate at pH 6.8, the fraction of SR vesicular protein that sedimented, following low speed centrifugation, was 14%. Under alkaline conditions (pH 7.8) that resulted in low Ca^{2+}/ATP ratios, the fraction of pelletable vesicles actually increased to 16%. Similar findings have been reported by Bonnet et al. (1978). The opening of SR vesicles under alkaline conditions, therefore, does not appear to account for the observed low stoichiometry of the Ca^{2+} pump.

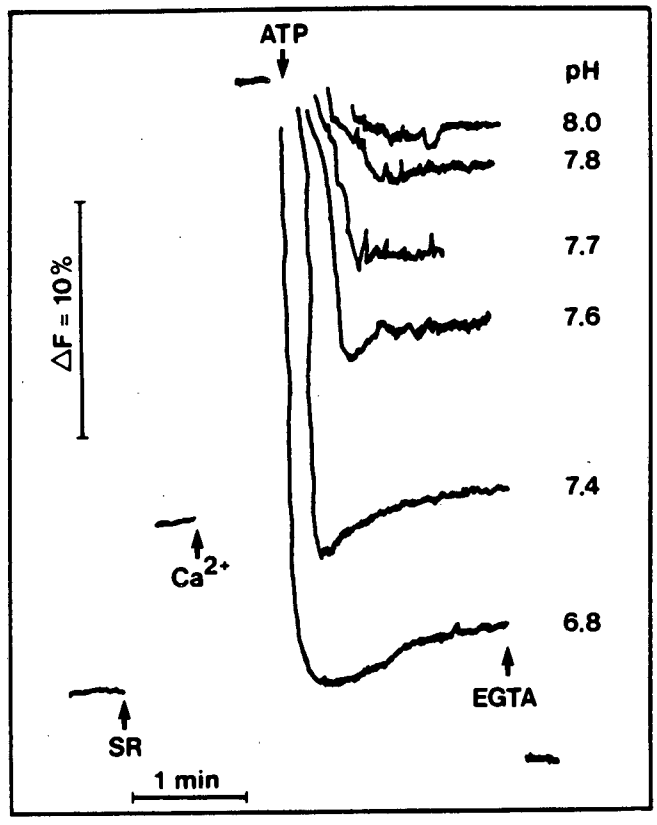


Figure 41. Effect of pH on Ca^{2+} transport monitored fluorometrically using TnC_{DANZ}.

Ca^{2+} transport was determined in medium containing 50 mM Pipes, 100 mM KCl, 5 mM potassium oxalate, 1 mM $MgCl_2$, 1.3 µM TnC_{DANZ}, 0.06 mg/ml SR protein and an initial Ca^{2+} concentration of 15 µM. Transport was initiated by addition of 45 nmols ATP to medium at the pH indicated alongside the curves. $CaCl_2$, 15 µM, and EGTA, 1 mM, was added where indicated.

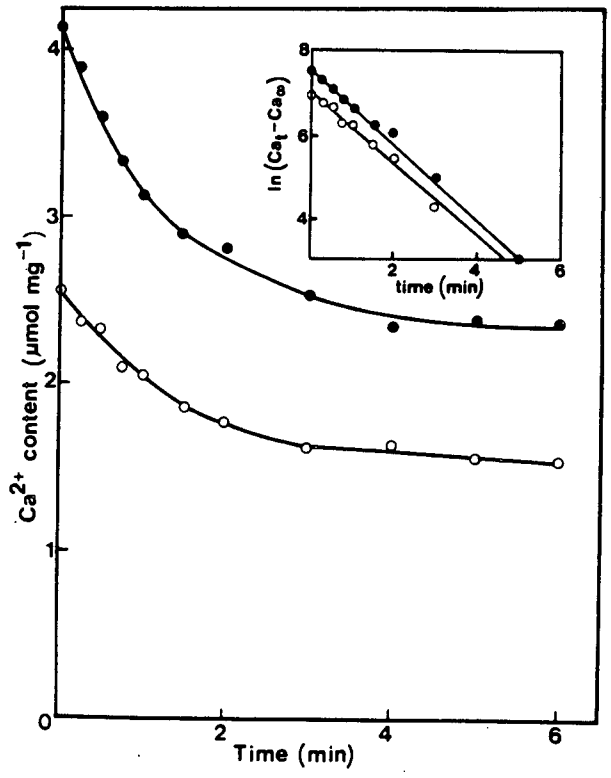


Figure 42: Effect of pH on Ca^{2+} efflux.

SR vesicles were actively loaded with $^{45}Ca^{2+}$-oxalate by infusion of 100 mM $^{45}CaCl_2$ into medium containing 50 mM Pipes, at pH 6.8 and 7.9, 100 mM KCl, 5 mM potassium oxalate, 1 mM $MgCl_2$, 5 mM ATP and 0.1 mg/ml SR protein. The infusion was continued until Ca^{2+} uptake ceased and $[Ca^{2+}]_{free}$ reached 10 μM. Ca^{2+} efflux was initiated by addition of 5 mM EGTA to the suspension of vesicles that had been loaded at pH 6.8 (●) and pH 7.9 (○). The inset shows a semilogarithmic plot of the data where Ca_t and Ca_∞ refer to the Ca^{2+} content of SR vesicles at time t and infinity, respectively.

$^{45}Ca^{2+}$ exchange into SR vesicles that have reached maximum loading in the presence of excess ATP has been used as an index of permeability on the basis that under these conditions the influx of $^{45}Ca^{2+}$ is equivalent to the sum of all efflux pathways (Takenaka et al., 1982). This method has been employed to determine the possible effects of pH on the permeability of SR vesicles to Ca^{2+}. The data of Fig. 43 show decreased exchange at pH 7.8 as compared to pH 6.8 i.e. alkaline pH appears to decrease rather than increase permeability. It should be noted that the $^{45}Ca^{2+}$ exchange method does not distinguish between simple passive diffusion through membrane lipids, as apposed to an efflux pathway through the Ca^{2+} pump itself. However, this approach provides further evidence that under alkaline conditions, that result in uncoupling, membrane permeability is not increased.

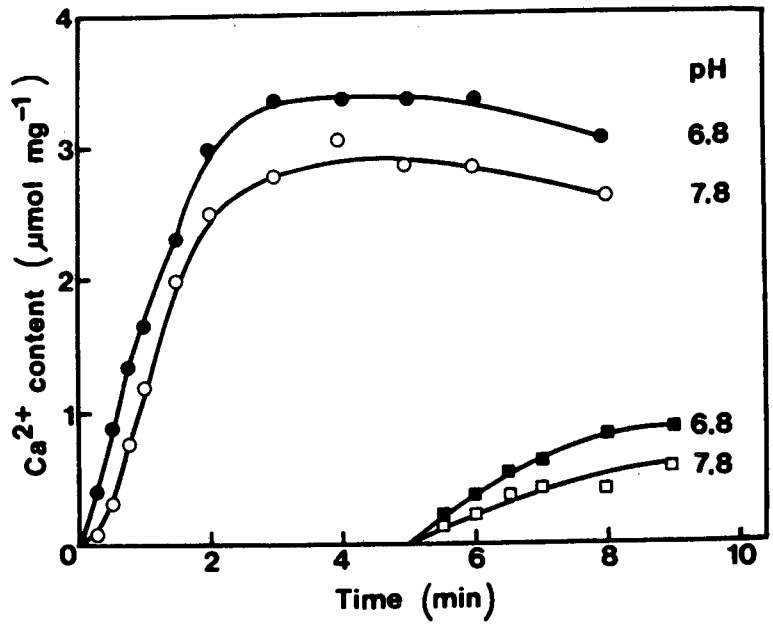


Figure 43: Effect of pH on Ca^{2+} exchange.

Ca^{2+} transport (●,○) was determined, at 25°C, at pH 6.8 (closed symbols), in medium containing 50 mM Pipes, 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 0.5 mM EGTA and 0.5 mM $^{45}CaCl_2$, and at pH 7.8 (open symbols) in medium containing 50 mM Hepes, 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 5 mM EGTA and 5.1 mM $^{45}CaCl_2$. Transport was initiated by addition of 5 mM ATP to medium containing 0.05 mg/ml SR protein. $^{45}Ca^{2+}$ exchange (■,□) was determined by addition of carrier-free $^{45}Ca^{2+}$ tracer at t = 5 min to medium containing $^{40}CaCl_2$ only.

3.3.3. EFFECTS OF TEMPERATURE

The temperature dependence of Ca^{2+}/ATP ratios was determined using arsenazo III as Ca^{2+}-indicator (Fig. 44). The possible effect of temperature on end-point measurements was considered. The change in the absorbance for zero calcium (1 mM EGTA) and the calibration curve, in the range 10-40°C, did not alter by more than 0.002 absorbance units, which is equivalent to a change of approximately 0.3 μM Ca^{2+}. Since coupling was assayed at constant temperature and $[Ca^{2+}]_{free}$, this will have negligible effects on the measured coupling ratios. The rate of Ca^{2+} transport and ATPase activity is highly dependent on temperature (McIntosh and Berman, 1978), so that the rate of ATP infusion was varied accordingly in order to prevent accumulation of ATP in the medium and thus minimise the contribution from basal ATPase activity. The rates of ATP infusion were set at 10 nmol/min at 7°C, 22 nmol/min at 15°C and 45 nmol/min above 25°C, to a total amount of 136 nmols of ATP. The results of the average Ca^{2+}/ATP values, obtained from the 2nd and 3rd pulse of ATP, are shown in Fig. 44a. A constant coupling ratio was obtained in the range 6°C to 29°C. A value of 1.70 ± 0.075 was obtained for all the data points in this temperature range. Sumida and Tonomura (1974) have previously suggested that the coupling ratio declines to unity at low temperature. This conclusion was based on kinetic determinations of ATPase activity and of Ca^{2+} transport, using the $^{45}Ca^{2+}$ method. Their experiments were conducted in the absence of $MgCl_2$. Their conclusion was supported by the findings of Ikemoto (1975) that the number of high affinity binding sites on the ATPase declined from two at 22°C down to one at 0°C. The reasons for the discrepancy between their data and those shown in Fig. 44a are not obvious, although it should be noted that in spite of a low transport stoichiometry, Sumida and Tonomura (1974) found that the activation of E-P formation was still positively cooperative at 0°C, with a Hill coefficient of two. The relative contribution of basal activity at low temperatures is increased, since the Ca^{2+}-dependent ATPase activity has a higher temperature coefficient than that of basal activity (Inesi et al., 1976). This, however, would tend to lower the apparent coupling ratios, i.e. the true stoichiometry would be higher than observed.

The coupling ratio decreased sharply above 30°C, was half maximal

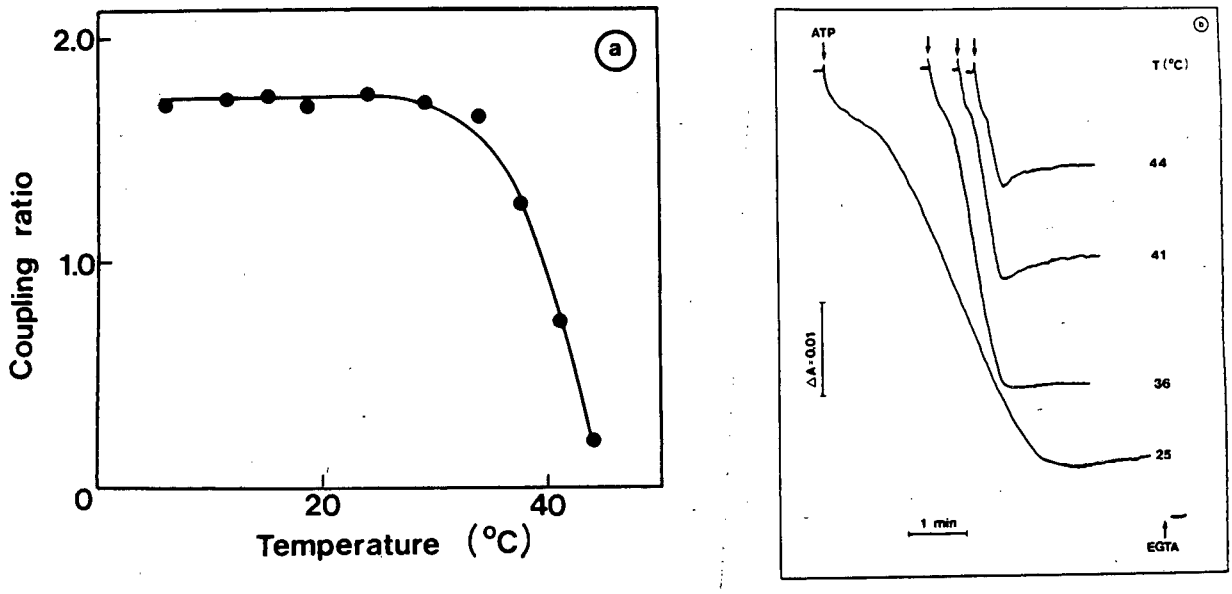


Figure 44: Temperature dependence of coupling ratio.

In (a) Ca^{2+}/ATP ratios were determined at various temperatures as described in Fig. 32b, using arsenazo III as Ca^{2+}-indicator. Data plotted are the average of the second and third 135 nmol pulse of ATP. In (b) the dual wavelength absorption changes due to medium $[Ca^{2+}]_{free}$ were continuously recorded at various temperatures from medium that contained 0.06 mg/ml SR protein. ATP, 55 nmols, and EGTA, 1 mM, were added where indicated.

at 40°C, and transport was virtually abolished at 46°C (Fig. 44a). Uncoupling of Ca^{2+} transport in the same range of elevated temperature has been reported previously (Inesi et al., 1973; Davis et al., 1976). For this reason, transport has conventionally been measured in the range of 20-30°C. Hasselbach, in a recent review (1981), has concluded that values approach the true stoichiometry of the Ca^{2+}-pump, which is two, and that the lowered values attained at the higher temperatures are due to increased permeability.

In the present studies, there was no change in medium Ca^{2+} following complete ATP hydrolysis, under conditions that gave maximum coupling, compared to those at temperatures that resulted in coupling ratios of less than 0.2, at equal degrees of loading, suggesting that increased membrane permeability was not significant. This was further investigated in experiments reported in Fig. 44b, in which the $[Ca^{2+}]_{free}$ of the medium was monitored, using arsenazo III, following addition of ATP without added Ca^{2+}, at various temperatures. It can be seen that a fixed amount of ATP (55 nmols) resulted in the transport of decreasing amounts of Ca^{2+}, as assessed from the depletion of medium Ca^{2+} at higher temperatures. Following hydrolysis of ATP, the extent of Ca^{2+} efflux from the loaded vesicles was followed. It is evident that there are no significant differences in the permeability of the membrane to Ca^{2+} that might account for the variable amounts of Ca^{2+} transported at different temperatures.

We have previously shown that Ca^{2+} transport becomes irreversibly uncoupled at elevated temperatures in the absence of free Ca^{2+} and that micromolar $[Ca^{2+}]_{out}$ protect against this inactivation (McIntosh and Berman, 1978). The reversibility of the effects of elevated temperatures was therefore investigated. Following hydrolysis of an initial pulse of ATP (90 nmols) at 39°C that gave a Ca^{2+}/ATP ratio of 0.93, the temperature of the cuvette was rapidly cooled (within 5 min) to 25°C. A further pulse of ATP yielded a Ca^{2+}/ATP ratio of 1.65. The effects of elevated temperatures are thus reversible.

3.3.4. CALCIUM CONCENTRATION DEPENDENCE OF STOICHIOMETRY

Initial experiments with both the Ca^{2+}-electrode and spectrophotometric ATP pulse methods suggested that Ca^{2+}/ATP ratios decreased in the range 0.1 - 0.3 μM Ca^{2+} from values observed at concentrations of free Ca^{2+} that saturate high-affinity Ca^{2+}-binding sites (3-5 μM). However, both of these end-point detectors are unsatisfactory in the submicromolar range, due to slowness of response and lack of sensitivity, respectively. The pulse method was, therefore, adapted to the measurement of Ca^{2+}/ATP ratios using the $^{45}Ca^{2+}$/EGTA method. $[Ca^{2+}]_{free}$ was varied by altering the ratio of total Ca^{2+} to EGTA, as described in Methods. The procedure employed was to infuse 220-230 nmol ATP at a fixed rate of 45 nmol min^{-1}. Lower amounts of ATP (90 nmols), equivalent to that used in the Ca^{2+}-stat methods, gave similar results at saturating Ca^{2+} concentrations. The higher amounts of ATP were, however, employed to give increased precision when low Ca^{2+}/ATP values were being measured. The results are shown in Fig. 45a. The inset shows the Ca^{2+} content of the vesicles at various $[Ca^{2+}]_{free}$ with time. This illustrates that as the $[Ca^{2+}]_{free}$ is lowered, the rate of Ca^{2+} transport decreases and that the total amount of Ca^{2+} transported, for a fixed amount of ATP, also decreases. At all concentrations of Ca^{2+} tested, the added ATP was more than 99% hydrolysed when the Ca^{2+} content of the vesicles became constant. This period ranged from 5 min (i.e. immediately after cessation of infusion) at 3 μM Ca^{2+} to 25 min at 0.05 μM Ca^{2+}. Furthermore, no decrease in the Ca^{2+} content, due to efflux, occurred following the hydrolysis of the pulse of ATP at 0.1 μM Ca^{2+}, and higher concentrations. However, due to the slow kinetics of Ca^{2+} transport and of ATP hydrolysis at the lowest concentration of Ca^{2+} tested (0.05 μM Ca^{2+}), the possibility of slow efflux could not be excluded.

Ca^{2+}/ATP ratios were constant in the range 0.5 to 3 μM but declined at lower $[Ca^{2+}]_{free}$ concentrations to reach 0.1 at 0.05 μM Ca^{2+} (Fig. 45a). The Ca^{2+} dependence of the coupling ratio suggests participation of a high affinity Ca^{2+}-binding site. The average value of three such sets of experiments were $[Ca^{2+}]_{0.5} = 0.12 \pm 0.03$ μM and $n_{H(Ca^{2+})} = 2.0 \pm 0.4$.

The calculation of stoichiometry neglects the contribution of basal

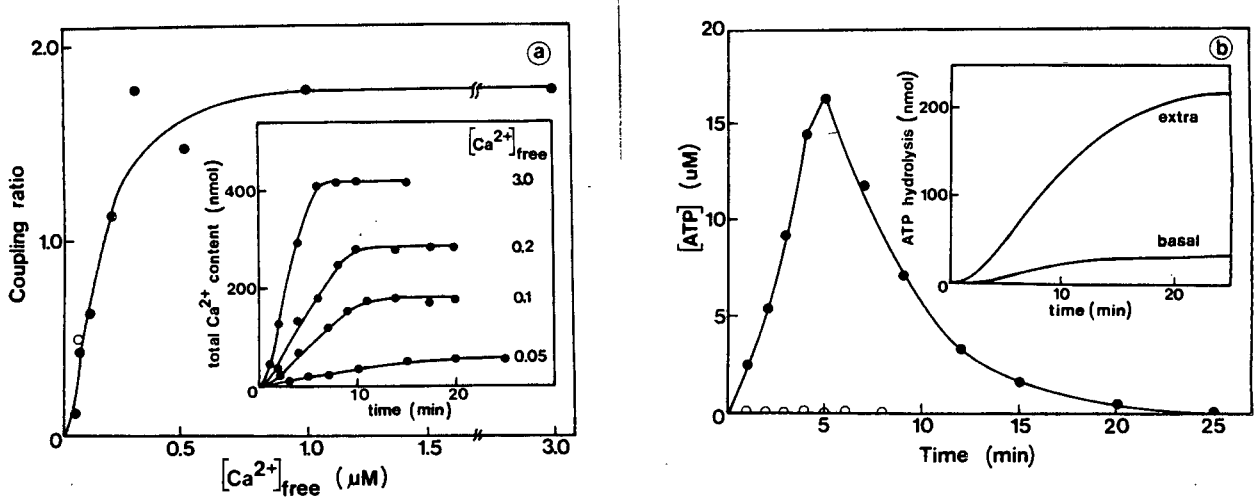


Figure 45: Ca^{2+}-dependence of measured Ca^{2+}/ATP ratios and the relative contribution of Ca^{2+}-independent (basal) ATPase activity.

The reaction medium contained 50 mM Pipes, pH 6.8, 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 25 μM AP_5A, 0.1 mg/ml SR protein, 10 mM EGTA and varying $^{45}CaCl_2$ in the range 0.8 - 8.4 mM to give the calculated Ca^{2+} free indicated. In (a) the coupling ratio was determined from the amount of Ca^{2+} transported per 227 nmol pulse of ATP (●). The inset shows the kinetics of Ca^{2+} transport during infusion of the pulse of ATP, at the calcium concentrations (μM) indicated alongside the curves. In (b) ATP concentrations were assayed by the luciferin/luciferase method, during infusion of a 227 nmol pulse of ATP over a period of 5 min, at 3 μM (○) and 0.075 μM (●) free Ca^{2+}. The inset shows the simulation, as described in the text, of the amounts of ATP hydrolyzed by Ca^{2+}-independent (basal) and by Ca^{2+}-dependent (extra) pathways. The value for Ca^{2+}/ATP at 0.075 μM Ca^{2+} free, corrected for basal ATPase activity (○), is plotted in (a).

activity, on the assumption that at the low ATP concentrations employed, ATP hydrolysis would be predominatly through extra ATPase activity. However, the possible extent of contribution of basal activity at low $[Ca^{2+}]_{free}$ would be expected to be higher than at saturating $[Ca^{2+}]_{free}$. The ATP concentrations were assayed in the reaction medium during and following cessation of infusion of the pulse of ATP. The results are shown in Fig. 45b. At 3 μM Ca^{2+}, ATP concentrations did not rise above 0.2 μM, but at 0.075 μM free Ca^{2+}, it rose steadily to 16 μM and declined exponentially thereafter, when infusion was discontinued. The contribution of basal to total ATPase activity was calculated by the numerical integration method (Fig. 45b, inset), using values for V_{max} and K_m of Ca^{2+}-dependent and Ca^{2+}-independent ATPase activity, as reported by Inesi et al. (1976). At 0.075 μM Ca^{2+}, 88% of the total infusion of 227 nmols of ATP would be hydrolysed by the Ca^{2+}-dependent pathway and the remainder by basal ATPase activity. On the assumption that basal and Ca^{2+}-dependent ATPase activities are carried out by independent activities, the determined values for Ca^{2+}/ATP of 0.44 at 0.075 μM Ca^{2+} should be corrected to 0.49 to allow for basal activity.

3.3.5. EFFECTS OF DETERGENTS AND CALCIUM IONOPHORES

The results obtained above are consistent with a view that uncoupling of Ca^{2+} transport from ATPase activity can occur under various conditions, in which increased passive membrane permeability is not a contributing factor. It may be relevant, however, that the above experiments were conducted in the presence of 5 mM oxalate, with resulting low $[Ca^{2+}]_{in}$ that would minimise measured efflux rates to insignificant levels, even if permeability were increased. In addition, the dissolution of calcium oxalate crystals is known to be slow (Tomazic and Nancollas, 1979). For these reasons, the effects of detergent and of Ca^{2+}-ionophore on the release of Ca^{2+} from calcium oxalate-loaded vesicles and on Ca^{2+}/ATP values were studied.

The effect of the non-ionic detergent, Triton X-100, on the amount of Ca^{2+} transported per fixed pulse of ATP, as determined by the arsenazo III method, is shown in Fig. 46. Detergent was added either before (Fig. 46a), or after (Fig. 46b) a pulse of 68 nmols of ATP. Triton X-100, in the range 0.005 - 0.02% (w/v) (0.55 - 2.22 mg/mg SR protein) decreased the Ca^{2+}/ATP ratio from 1.76 to <0.1. Following hydrolysis of ATP (Fig. 46a), the $[Ca^{2+}]_{free}$ in the medium increased to a greater extent at intermediate concentrations of detergent, suggesting an increased efflux of Ca^{2+}, but this efflux appeared to be relatively slow compared to active Ca^{2+} uptake. The effects of addition of detergent after active transport of Ca^{2+} is shown in Fig. 46b. Virtually all of the Ca^{2+} could be released at 0.01% Triton X-100. However, the effectiveness of detergent on Ca^{2+} release appeared to be less than its effects when added prior to transport. These experiments show (a) that the dissolution of calcium oxalate crystals is relatively rapid, when the SR vesicles are solubilized at high detergent concentrations, and (b) that the effects of detergents appear to be greater if permeability is altered prior to initiation of Ca^{2+} uptake.

The effect of the Ca^{2+}-ionophore, A23187, on Ca^{2+} transport, as determined by the arsenazo III method, is shown in Fig. 47. Ionophore was added either before (Fig. 47a) or after (Fig. 47b) a pulse of 90 nmols ATP. A23187, in the range 8×10^{-9} M to 3×10^{-8} M, decreased the amount of Ca^{2+} transported for that pulse of ATP, while the amount

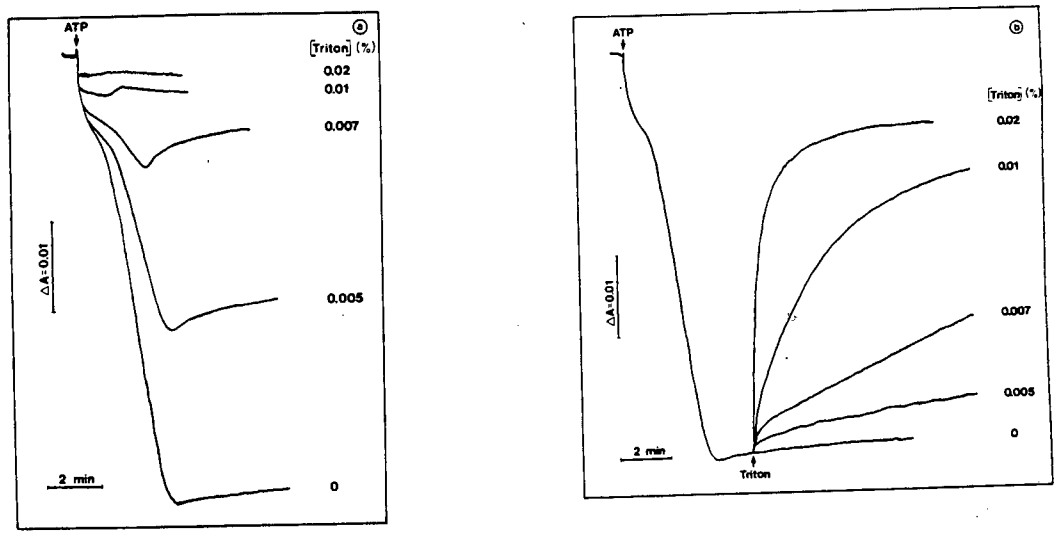


Figure 46: Effect of detergent on Ca^{2+} transport.

Ca^{2+} transport was assayed at 25°C as described in Fig. 44b. Triton X-100, to the final concentration indicated, was added either before (a) or after (b) transport was initiated by addition of 68 nmols ATP.

that effluxed, following hydrolysis of ATP, increased (Fig. 47a). This efflux, however, appeared small and slow compared to the rate of Ca^{2+} uptake. The effect of addition of ionophore on Ca^{2+} efflux, after initiation of Ca^{2+} transport, is shown in Fig. 47b. Virtually all the Ca^{2+} was released by 1 μM A23187. It is, however, evident that the amount of ionophore required to result in appreciable amounts of efflux are approximately 100-fold greater than that required to inhibit transport, when added before initiation. This phenomenon has previously been described by Katz et al. (1977) who examined the ability of calcium-precipitating anions to modify calcium release from SR vesicles that was made permeable by the ionophore X537A. They explained their results in terms of constraints imposed by a fixed low $[Ca^{2+}]_{in}$ on permeable vesicles. These constraints restrict Ca^{2+} release after Ca^{2+} permeability is increased. This constraint is however not solely due to the fact that the precipitate within the vesicles is slow to dissolve because destruction of the membranes with lipid solvents caused rapid release from calcium oxalate loaded vesicles (Fig. 46; Katz et al., 1977).

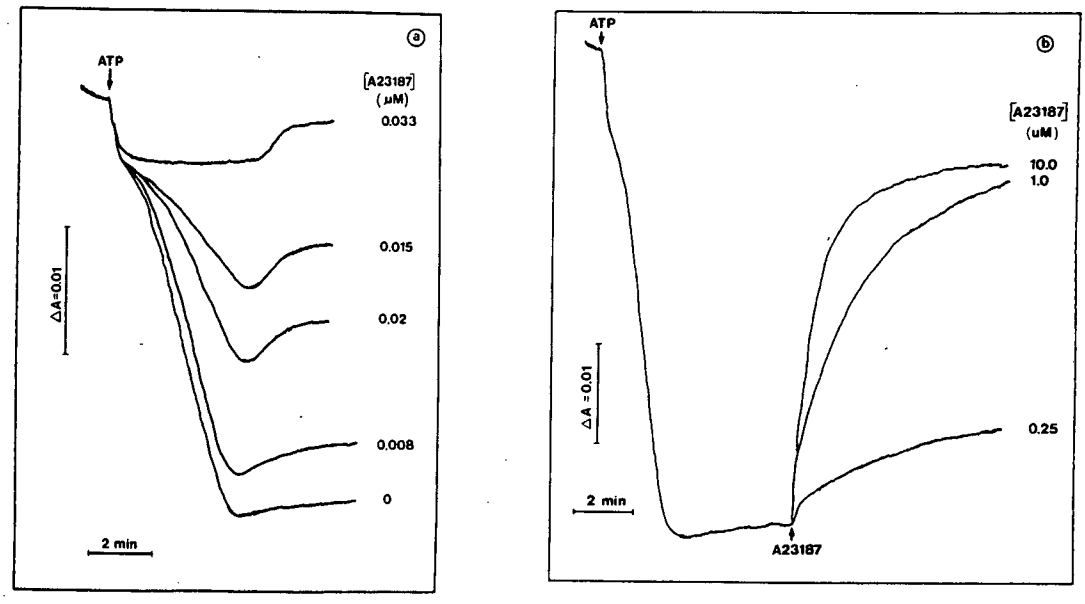


Figure 47: Effect of ionophore on Ca^{2+} transport.

Ca^{2+} transport was assayed at 25°C as described in Fig. 44b. A23187, to the final concentration indicated, was added either before (a) or after (b) transport was initiated by addition of 68 nmols ATP.

3.3.6. THE EFFECT OF CARDIOTOXIN ON THE (Ca^{2+},Mg^{2+})-ATPase OF SARCOPLASMIC RETICULUM

Toxic effects of cardiotoxins of snake venom are believed to be mediated by their effects on cell membranes (Lee, 1979). The mechanism of these effects is uncertain and several hypotheses have been proposed. SR membranes have been used as model targets for testing some of these. One view is that the hydrophobic portion of the toxin penetrates into the hydrophobic regions of the membrane, disrupting its structure and rendering the membrane phospholipids accessible to attack by phospholipase A_2, a contaminant of most cardiotoxin preparations (Klibansky et al., 1968). This would result in leakiness and a decrease in net Ca^{2+} uptake.

We have examined the effect of the purified cardiotoxin Haemochates hemachatus DLF (a kind gift of Dr A.I. Louw, C.S.I.R., Pretoria), containing no measurable phospholipase A_2 activity (Louw and Carlsson, 1979), on ATP-dependent calcium transport of isolated SR. Some of these results have been reported (Fourie et al., 1983).

The effects of the addition of cardiotoxin to the reaction medium, after the initiation of Ca^{2+} transport, were studied. No significant effect was noted up to concentrations of 1 µg cardiotoxin per 5 µg SR protein, in agreement with findings by Shiau et al. (1978). However, preincubation of cardiotoxin with SR at room temperature, prior to assay of transport, did have significant effects on Ca^{2+} transport (Fig. 48). Inhibition was complete within the first minute of pre-incubation of SR vesicles with toxin. Cardiotoxin, 7 µg, was found to decrease Ca^{2+} transport by 50-60% and inhibition could not be further increased by higher toxin concentrations up to 150 µg/ml ($\simeq 20 \times 10^{-6}$ M), by longer pre-incubation times or by higher incubation temperatures. ATPase activity, on the other hand, was found to be increased by only 10% (data not shown), when transport was maximally inhibited.

The data indicate that approximately 50% of transport activity was inhibited by cardiotoxin, whilst the remainder appears to be resistant to its effects. The possibility that these two transport

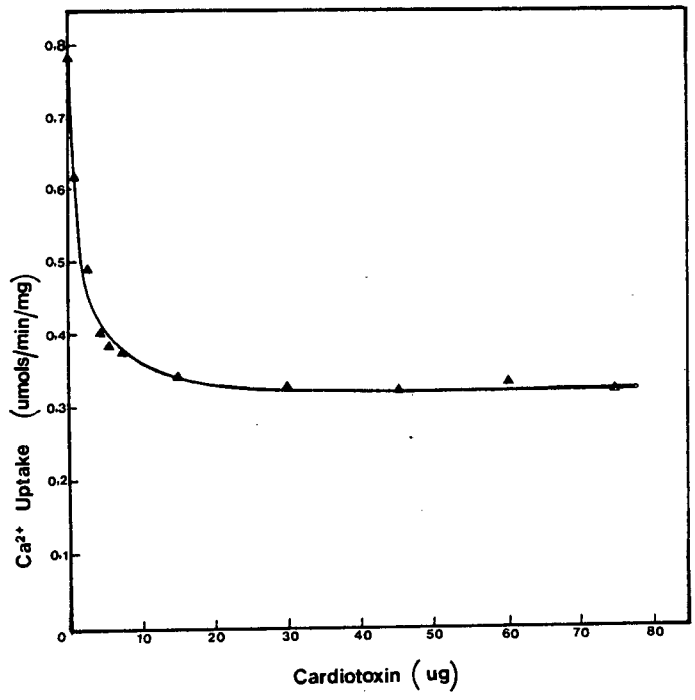


Figure 48: The effect of cardiotoxin on the rate of Ca^{2+} transport by SR vesicles.

The uptake medium (2 ml) at 20°C contained 100 mM KCl, 20 mM histidine, pH 7.2, 5 mM $MgCl_2$, 5 mM potassium oxalate and 5 mM ATP. The reaction was initiated by the addition of 105 µg SR protein, preincubated at room temperature for 5 min with varying concentrations of cardiotoxin. Ca^{2+} transport was measured by the Ca^{2+}-stat method.

activities resided in different populations of SR vesicles was investigated by calcium oxalate loading. SR vesicles, which had been maximally inhibited with cardiotoxin, were separated into fractions by density gradient centrifugation, according to the method of Fernandez et al. (1980) (Fig. 3). Toxin-treated SR vesicles took up half the amount of Ca^{2+} (2.23 μmol mg^{-1} protein) of control, untreated vesicles (4 μmol mg^{-1} protein). When transport was maximally inhibited, both heavy and light SR fractions were loaded with calcium oxalate and sedimented to the bottom of the sucrose gradient (Fig. 49), indicating that there is no population of these vesicles in the cardiotoxin treated SR that is unable to transport calcium.

It has previously been suggested that toxins act by increasing the rate of Ca^{2+} efflux from SR vesicles by increasing the permeability of the membrane to Ca^{2+} or by affecting the general stability of the SR vesicular membranes (Lau et al., 1974; Fassold et al., 1976; Nayler et al., 1976; Kim et al., 1978). The effect of cardiotoxin on Ca^{2+} efflux from SR vesicles was, therefore, investigated by the flow dialysis technique (Tenu et al., 1976). The results, shown in Fig. 50, indicate that Ca^{2+} taken up by cardiotoxin-treated SR did not efflux for up to 6 min following addition of ATP, neither did cardiotoxin cause efflux from $^{45}Ca^{2+}$ loaded untreated SR. These findings were further investigated by experiments reported in Fig. 51, in which medium calcium was monitored using arsenazo III following addition of ATP. It can be seen that the rate of depletion of Ca^{2+} from the transport medium, on addition of 36 nmols ATP, decreased as the concentration of cardiotoxin increased. The rate of Ca^{2+} transport was maximally inhibited by 40% at 0.1 mg cardiotoxin per mg SR protein. In addition, the amount of Ca^{2+} transported per 36 nmol pulse of ATP decreased with increasing cardiotoxin, whereas the extent of Ca^{2+} efflux from loaded vesicles was independent of the cardiotoxin concentration. It is evident from Fig. 51 that there are no significant effects of cardiotoxin on membrane permeability and that cardiotoxin decreases the stoichiometry of the Ca^{2+} pump. Ca^{2+}/ATP ratios were therefore determined by the ATP-pulse method, using arsenazo III as Ca^{2+}-indicator. SR vesicles were incubated with cardiotoxin (0.1 mg/mg SR protein) at room temperature for 5 min prior to initiation of Ca^{2+} transport. In the absence of cardiotoxin, Ca^{2+}/ATP ratios were

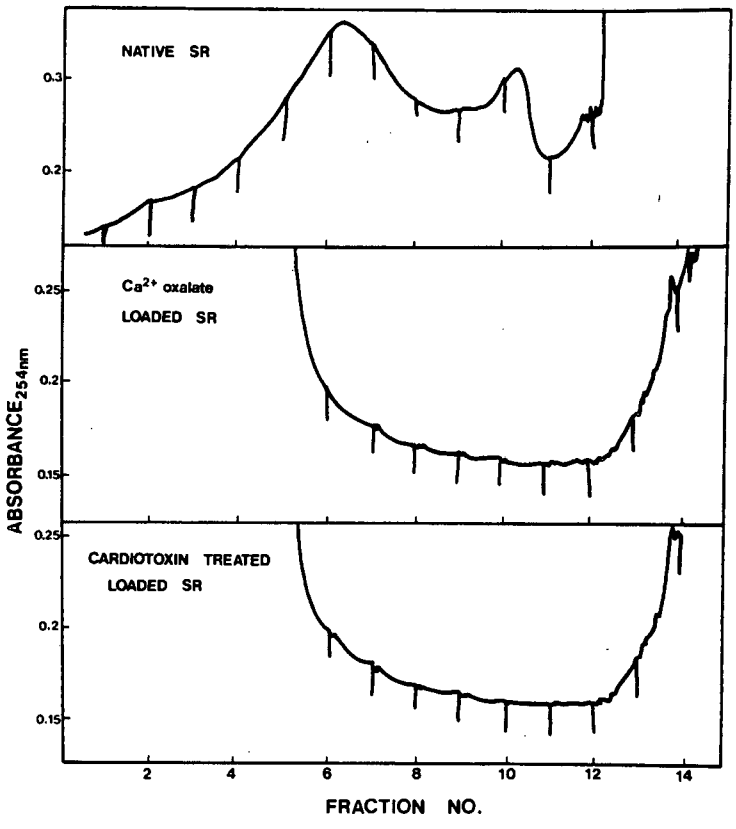


Figure 49: Absorption profile, at 254 nm, of a continuous sucrose gradient.

The gradient of 25 - 40% (w/w) sucrose was prepared in a 20 mM Tris-acetic acid buffer, pH 7.4 and centrifuged for 20 hrs at 2°C in a SW 27.1 Ti rotor at 27 000 rpm. The cardiotoxin treated and untreated SR vesicles were loaded with calcium oxalate. Unloaded vesicles were also separated into light and heavy fractions.

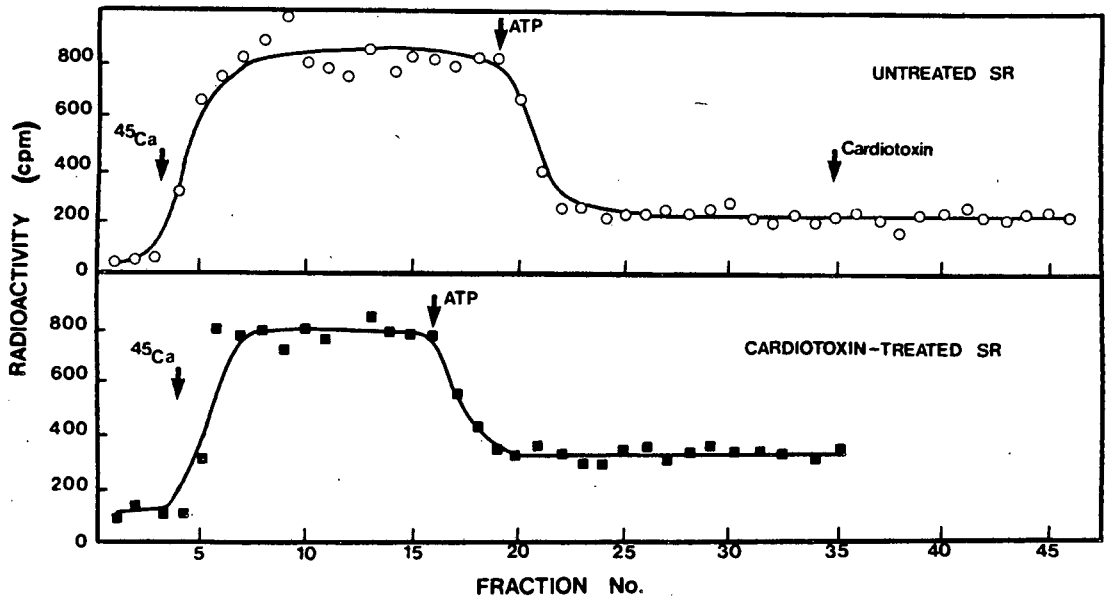


Figure 50: The effect of cardiotoxin on Ca^{2+} uptake and release.

Cardiotoxin, 0.14 mg/ml, was added to vesicles (1 mg/ml) before (Cardiotoxin treated SR) or after (untreated SR) initiation of transport. Ca^{2+} uptake was measured by the flow dialysis technique.

1.57 ± 0.04 (n=6). Incubation of SR with cardiotoxin decreased Ca^{2+}/ATP by 40% to a value of 1.02 ± 0.04 (n=6).

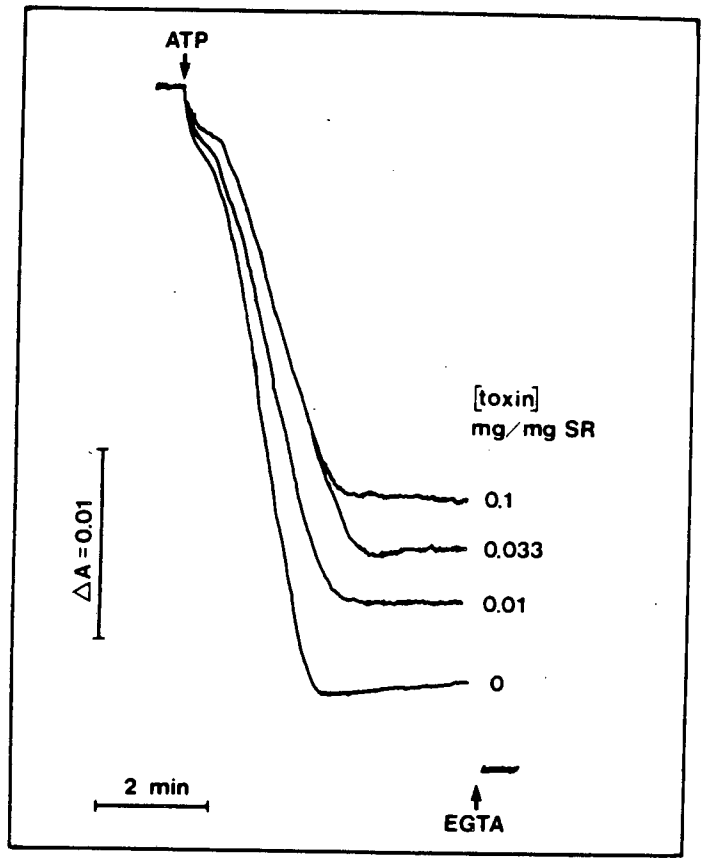


Figure 51: Effect of cardiotoxin on Ca^{2+} transport.

Ca^{2+} transport was assayed at 25°C as described in Fig. 44b. SR was incubated for 5 min at room temperature with cardiotoxin, at the concentration indicated, before initiation of transport by addition of 36 nmols ATP.

3.4. DISCUSSION

Previous experiments to determine the stoichiometry of the Ca^{2+} pump of SR have generally been based on kinetic methods that relate the initial rates of Ca^{2+} transport to Ca^{2+}-dependent ATPase activity (Fig. 31). Ca^{2+}-dependent ATPase activity is taken as the difference between total ATPase activity, at saturating Ca^{2+} concentrations, and Ca^{2+}-independent activity, determined in medium containing excess EGTA, such that $[Ca^{2+}]_{free}$ is less than 10^{-9} M. Apart from experimental difficulties in optimising transport and catalytic assays, there is uncertainty regarding the nature of basal activity (see Introduction, Section 3.1). The results reported here were derived by alternative non-kinetic approaches, which are solely dependent on standardisation of $CaCl_2$ and of MgATP in order to measure coupling ratios. Determination of Ca^{2+}/ATP values by the ATP- and Ca^{2+}-pulse methods have the advantage that they are continuous assays, where both the amount of Ca^{2+} transported and ATP hydrolysed is determined in a single experiment. During pulsed addition of ATP, as employed in the present studies, ATP concentrations did not increase above 1 μM, at saturating Ca^{2+} concentrations, and since basal activity has a K_m in the millimolar range (Inesi et al., 1976), its contribution to ATP consumption is negligible (< 0.5% of total ATPase activity). In the Ca^{2+} pulse assay (Fig. 35), it was assumed that basal activity continues at a constant rate during Ca^{2+}-dependent ATP hydrolysis. On the other hand, if it is assumed that basal activity is converted to extra activity, the calculated stoichiometry, in a typical experiment, would be 1.54 as opposed to 1.80.

An additional advantage is that the stat methods allow an estimation of the extent of passive leakage through the SR membrane. For the ATP-pulse method, this is obtained from continuous monitoring of $[Ca^{2+}]_{free}$, following ATP hydrolysis, in the 'post-pulse' period (Fig. 33). It is generally assumed that passive leakage is only dependent on the amount of Ca^{2+} in the intravesicular lumen (Feher and Briggs, 1982) and does not depend on enzyme turnover. Thus, if Ca^{2+}/ATP values of less than 2.0 are due to permeable vesicles, passive efflux should continue at a constant rate in the post-pulse period, when Ca^{2+} transport had ceased, until all the Ca^{2+}, except that bound to internal binding sites, has effluxed (Feher and Briggs, 1982). Monitoring of $[Ca^{2+}]_{free}$ in the post-pulse period showed that less than 5%

of the total Ca^{2+} retained by the vesicles, effluxed (Fig. 33)

Rates of hydrolysis of ATP in the Ca^{2+}-pulse method were not increased significantly after the pulse of Ca^{2+} had been transported and were similar to that in the presence of EGTA (Fig. 35). It can be calculated that if 1% of the infused Ca^{2+} continually effluxes, the steady-state level of free Ca^{2+} would be approximately 1 μM, leading to continuous ATP hydrolysis at near maximal rates, after the calcium pulse. Simple passive efflux through permeable lipid bilayers of SR vesicles, therefore, cannot account for non-integral coupling ratios.

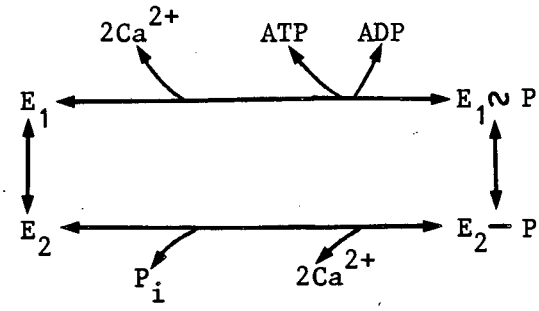
The stoichiometry of the Ca^{2+} pump varies as a function of external $[Ca^{2+}]_{free}$ (Fig. 45a), in a manner suggesting participation in the coupling process of Ca^{2+}-binding sites, having a dissociation constant of 0.12 μM. This value is similar to that for passive Ca^{2+} binding (Watanabe et al., 1981), for Ca^{2+} stimulation of Ca^{2+} transport and for Ca^{2+}-dependent ATPase activity (The and Hasselbach, 1972; Vianna, 1975; Neet and Green, 1977). A Hill coefficient of 2.0, obtained in the present study, suggests that at least two Ca^{2+}-binding sites require to be saturated, in order to obtain maximum coupling. A possible explanation for lowered Ca^{2+}/ATP values at subsaturating $[Ca^{2+}]_{free}$ is increased membrane leakiness, resulting in Ca^{2+} efflux, either down a concentration gradient or through a Ca^{2+} conductance channel that is closed by micromolar $[Ca^{2+}]_{out}$ (see below). The lowered stoichiometry cannot be explained on the basis of passive efflux, since Ca^{2+} transported, following infusion of ATP, was retained by the vesicles (Fig. 45b). These studies were conducted in the presence of 5 mM oxalate, such that $[Ca^{2+}]_{in}$ should not exceed 5 μM (Hasselbach and Makinose, 1963). On this basis, a change in $[Ca^{2+}]_{out}$ from 0.5 μM to 0.1 μM, the range in which uncoupling was noted, would only increase the Ca^{2+} gradient from 4.5 to 4.9 μM. Feher and Briggs (1982) have shown that passive Ca^{2+} efflux is dependent on Ca^{2+} load. Using their derived first order rate constant of 0.8 min^{-1} for efflux, and a vesicular volume of 4 μl mg^{-1} (Diamond et al., 1980), passive efflux can be calculated to occur with a rate of 0.014 - 0.016 nmol min^{-1} mg^{-1}. During pulsed Ca^{2+} transport that continued for 10 min at 0.1 μM Ca^{2+} (Fig. 45a, inset), the total amount of Ca^{2+} that effluxes passively can be calculated to be between 0.14 and 0.16 nmol mg^{-1} SR protein i.e. 0.03 - 0.05% of the net Ca^{2+} transported.

Ca^{2+} transport has been shown to be biphasic (Weber et al., 1966; Entman et al., 1973; Mesmier and Hasselbach, 1975; Madeira, 1982). Initial oxalate-independent transport, to a maximum vesicular capacity of 50 - 100 nmol Ca^{2+}/mg SR (Madeira, 1982), is followed by an oxalate-dependent phase. The oxalate-independent phase corresponds in magnitude to the total amount of Ca^{2+} transported in the absence of Ca^{2+} precipitating anions. This phase is highly pH dependent and is found to last longer at alkaline pH (Madeira, 1982). The kinetics of calcium oxalate precipitation are slow and exhibit a lag phase that is overcome by 'seeding'. Feher and Briggs (1980) have suggested that the process does not reach equilibrium during active transport. Taken together, these findings imply that $[Ca^{2+}]_{in}$ increases initially to a maximum of approximately 10-20 mM, before calcium oxalate precipitation is initiated. Following such 'seeding', $[Ca^{2+}]_{in}$ decreases but might well remain above the theoretical concentration, inferred from the solubility product. Only once Ca^{2+} transport has ceased will $[Ca^{2+}]_{in}$ approach this equilibrium value. The amount of Ca^{2+} that effluxes through passive pathways may therefore vary during the course of oxalate-dependent transport, being maximum during the oxalate-independent phase. The rate of passive efflux at a $[Ca^{2+}]_{in}$ of 20 mM can be calculated, as described above, to be 64 nmol min^{-1} mg^{-1}. Assuming that this phase lasts for 15 s, at pH 6.8 (see Fig. 46), the amount of Ca^{2+} that effluxes would be approximately 11% of that transported for a pulse of 68 nmols of ATP (assuming a stoichiometry of 2:1). This readily accounts for the observed subintegral coupling ratio of 1.7, obtained in that experiment. At higher pH values, this process lasts longer (1 min at pH 7.6 and 4 min at pH 7.9) so that the amount that effluxes during this phase would be 256 nmol mg^{-1} i.e. no net Ca^{2+} transport will be observed with a small pulse of ATP at pH 7.9. The pulse of ATP, at alkaline pH, therefore needs to be relatively large to give a high $[Ca^{2+}]_{in}$ that is maintained for a long enough period to initiate calcium oxalate precipitation. The extrapolated value (350 nmol mg^{-1}) for the ATP pulse that yields a maximum Ca^{2+}/ATP ratio at pH 7.9 (Ca^{2+}/ATP = 1.0), is of a similar magnitude (Fig. 40). It is concluded that efflux of Ca^{2+} during the oxalate-independent phase of Ca^{2+} uptake could account for the higher susceptibility of Ca^{2+} transport into empty vesicles at more alkaline pH values. The observed lowered stoichiometry at alkaline pH for Ca^{2+} transport into

vesicles containing calcium oxalate crystals with subsequent pulses of ATP cannot, however, be explained on this basis. In addition, subintegral coupling ratios, under optimal conditions of pH 6.8, 25°C, can only occur on the basis of passive Ca^{2+} efflux if the internal $[Ca^{2+}]_{free}$ remains high (between 10 and 20 mM) during Ca^{2+} transport.

We have confirmed previous observations of lowered stoichiometry at alkaline pH and high temperatures (Inesi et al., 1973; Davis et al., 1976; Duggan, 1977; Tate et al., 1981). Contribution of increased membrane leakiness or simple passive efflux of Ca^{2+} through lipid bilayers has, however, been shown to be negligible. In retrospect, it is difficult to reconcile increased leakiness of the lipid bilayer with, for example, alteration of pH in the range 7.2 to 7.9, since no ionisable group with a corresponding pKa exists on phospholipid head groups. Also, it is difficult to accept the original proposal of increased membrane permeability to account for suboptimal and non-integral coupling ratios, in view of the observations that (i) the permeability of lipid bilayers to Ca^{2+} is low (Vanderkooi and Martonosi, 1971), even though the incorporation of the ATPase enzyme into such bilayers increase their permeability somewhat (Jilka et al., 1975); (ii) it has been demonstrated that the main pathway for Ca^{2+} during passive efflux is through the pump protein (Jilka et al., 1975; Chiu and Haynes, 1977; 1980; Palade et al., 1983; Sorenson, 1983), and Ca^{2+}, in the micromolar range, blocks this efflux (Meissner and McKinley, 1976; Jilka and Martonosi, 1977; Chiesi and Inesi, 1979) and (iii) Ca^{2+} exchange under steady state conditions has been shown to occur via the Ca^{2+}-ATPase, with little, if any, through 'lipid channels' (Waas and Hasselbach, 1981; Takakuwa and Kanazawa, 1981; Takenaka et al., 1982). $Ca^{2+}_{out} \leftrightarrow Ca^{2+}_{in}$ exchange, at equilibrium, following maximal active loading of SR vesicles in the absence of oxalate, occurs with a rate of approximately 200-400 nmol min^{-1} mg^{-1} (Takenaka et al., 1982; Lau, 1983), which is approximately 10-fold greater than the rate of passive efflux from Ca^{2+}-loaded vesicles (20-60 nmol min^{-1} mg^{-1}) (Takenaka et al., 1982; Feher and Briggs, 1982; Sorenson, 1983). These findings suggest that, if there is leakage, it is associated with the Ca^{2+} ATPase, either at the lipid-protein interface, or through the pump protein itself.

The calcium transport cycle is fully reversible and two Ca^{2+} molecules efflux for every ATP synthesized (Tada et al., 1978; De Meis and Vianna, 1979). In the generally accepted scheme of Carvalho et al. (1976), shown in abbreviated form in Scheme III, forward and reverse movements of Ca^{2+} through the pump are linked obligatorily to the reaction $E_1 \sim P \leftrightarrow E_2\text{-}P$.



Scheme III

However, exchange studies have revealed that multiple reversals of individual partial reactions of the catalytic cycle occur during a single turnover (Barlogie et al., 1971; Makinose, 1971; de Meis and Boyer, 1978; Waas and Hasselbach, 1981; McIntosh and Boyer, 1983). Takenaka et al. (1982) have shown that the rate of ATP $\leftrightarrow$ ADP exchange (1500 nmol min^{-1} mg^{-1}) is 4-fold greater than that of $Ca^{2+}_{out} \leftrightarrow Ca^{2+}_{in}$ exchange, under conditions in which $P_i \leftrightarrow$ ATP exchange is minimal. Chiesi and Wen (1983) have recently shown an ATP-induced Ca^{2+} release phenomenon that is mediated by E-P (Scheme III) formation and that is not linked to ATP synthesis from ADP. These lines of evidence suggest that partial scalar reactions of the transport cycle are not mandatorily linked to vectorial transfer of Ca^{2+} ions.

A number of possible mechanisms may account for observed Ca^{2+}/ATP ratios of less than 2.0, and its variability as a function of pH, temperature and $[Ca^{2+}]_{out}$. These are represented in diagramatic form in Fig. 52. A 'pump-leak' model (Fig. 52a) may be excluded since no leakage of Ca^{2+} from SR vesicles was observed in the post ATP-pulse period. In the 'independent gated channel' model (Fig. 52b), the Ca^{2+} pump has a fixed stoichiometry of 2:1 and outward fluxes of Ca^{2+} occur via a channel that is independent of the forward flux. In order to explain the Ca^{2+} dependence of Ca^{2+}/ATP ratios we have to postulate

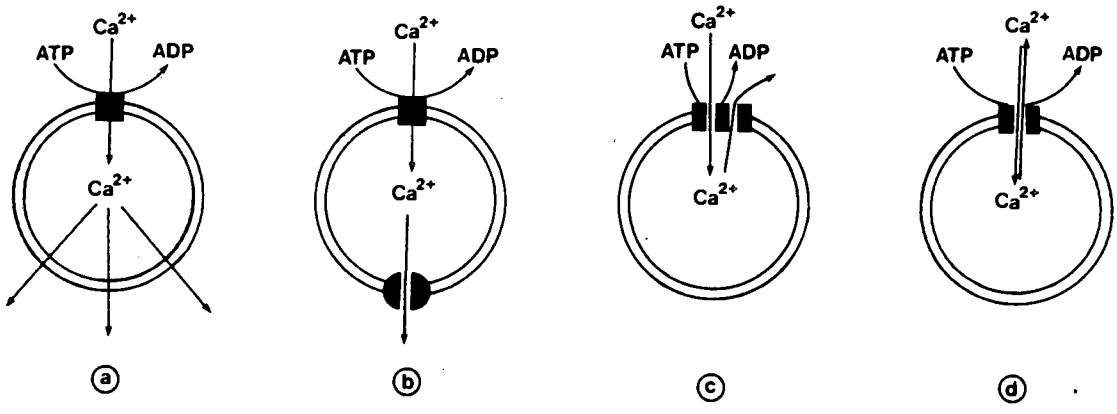


Figure 52: Possible mechanisms for observed variable stoichiometry of calcium transport by sarcoplasmic reticulum.

(a) Pump-passive leak, (b) pump-gated channel, (c) pump-facilitated efflux and (d) pump slippage models.

that the independent efflux channel is opened by $[Ca^{2+}]_{out}$ in the range 0.05 - 0.3 μM, and is shut by $[Ca^{2+}]_{out}$ > 0.5 μM. The variable coupling obtained with pH, according to this model, implies that the gating for the outward flux channel is controlled by a mechanism involving an ionizable group having a pK_a of 7.9. This efflux pathway may be related to that described by Shoshan and coworkers (1981), in which the Ca^{2+}-release channel is presumed to be gated by a pH gradient across the SR membrane, acid outside. Feher and Briggs (1983) eliminated the possibility of a carrier for Ca^{2+} or a gated calcium channel to account for the reverse flux observed at equilibrium loading, based on its dependence for Ca^{2+}_{out}, ATP and ADP.

A third (Fig. 52c) and fourth (Fig. 52d) possible models have in common that both forward and reverse calcium fluxes during active transport occur through the Ca^{2+}-ATPase. Such models are based on the evidence presented above, which indicates that the main flux of Ca^{2+}, at equilibrium, occurs through the pump protein (Waas and Hasselbach, 1981; Takenaka et al., 1982; Feher and Briggs, 1983). In addition, since no efflux was observed following ATP hydrolysis, lowered Ca^{2+}/ATP values observed under alkaline pH, high temperature and low $[Ca^{2+}]_{free}$ are manifestations of a process that is intimately related to turnover of the pump.

The phenomenon represented in Fig. 52d, in which reverse fluxes of Ca^{2+} is through the same channel as forward fluxes, is mechanistically linked to the concept of slippage, where the overall scalar and vectorial events are dissociated under conditions of thermodynamic loading, compared to unloaded conditions.

The occurrence of molecular slippage during active transport, if validated, may help to support or eliminate some of the models that are currently being considred. A model in which the Ca^{2+} pump has fixed and integral stoichiometry presumes that there is a single dominant reaction cycle, and that fluxes between alternate cycles are negligible. An alternative approach to active transport and energy transduction arises from the non-equilibrium thermodynamic approach, originally formulated by Kedem and Caplan (1965) and extended by other workers (Stucki, 1980; Pietrobon et al., 1981; Westerhoff et al., 1983). This approach assumes there is no a-priori fixed

stoichiometry and that varying contributions by minor cycles may be significant under different conditions. No reaction mechanism, but rather thermodynamically possible reaction pathways, is specified (Hill, 1977). Berman (personal communication) has analysed active transport by the (Ca^{2+},Mg^{2+})-ATPase of SR according to the Hill diagram method (Hill, 1977) (Fig. 53). The model involves a number of independent reaction cycles (Fig. 53b), one for each of the ligands Ca^{2+}, H^{+} and ATP (Fig. 53a). Thus, there are six possible reaction cycles, two of which couple ATP hydrolysis to Ca^{2+} transport (cycles f, h). Cycle h is presumed to be the dominant or forward (physiological) cycle corresponding to Scheme I. The partial flux diagrams for cycle a is shown in Fig. 53c and is obtained by adding to the cycle the maximum number of lines, without forming an additional cycle or closed pathway. The overall behaviour of the system can be described by the non-equilibrium thermodynamic approach where the fluxes of the three components are functions of their conjugate and non-conjugate driving forces according to the coefficients of the exponential force terms. The equations are non-linear and the coefficients obtained are rate constants of the individual reactions. According to this approach, the stoichiometries of elementary steps remain fixed, but the macroscopic stoichiometry, which is a function of the dominance of the main cycle, could theoretically vary from zero to maximum, depending on the relative rate constants and ligand concentrations.

Coupling between transport and catalytic events is maintained by a number of interlocking mechanisms that allows the reaction sequence to proceed to completion without the occurrence of side reactions (Jencks, 1980; Hammes, 1983). Jencks(1980) has defined certain mechanistic rules that need to be obeyed for coupling, such that the reaction is unidirectional and back reactions are minimised. The main concept that emerges is that no ATP hydrolysis occurs without concomitant Ca^{2+} transport and no reverse flux of Ca^{2+} occurs without ATP synthesis. This is postulated to be brought about by the enzyme existing in two different conformations; E_1 has high affinity outward orientated sites for Ca^{2+} and is phosphorylated by ATP only, while E_2 has inward-orientated low affinity Ca^{2+}-binding sites and is reactive towards P_i only (Scheme III). E-P remains inaccessible to water until Ca^{2+} translocation has occurred. Free-energy transfer from ATP to the

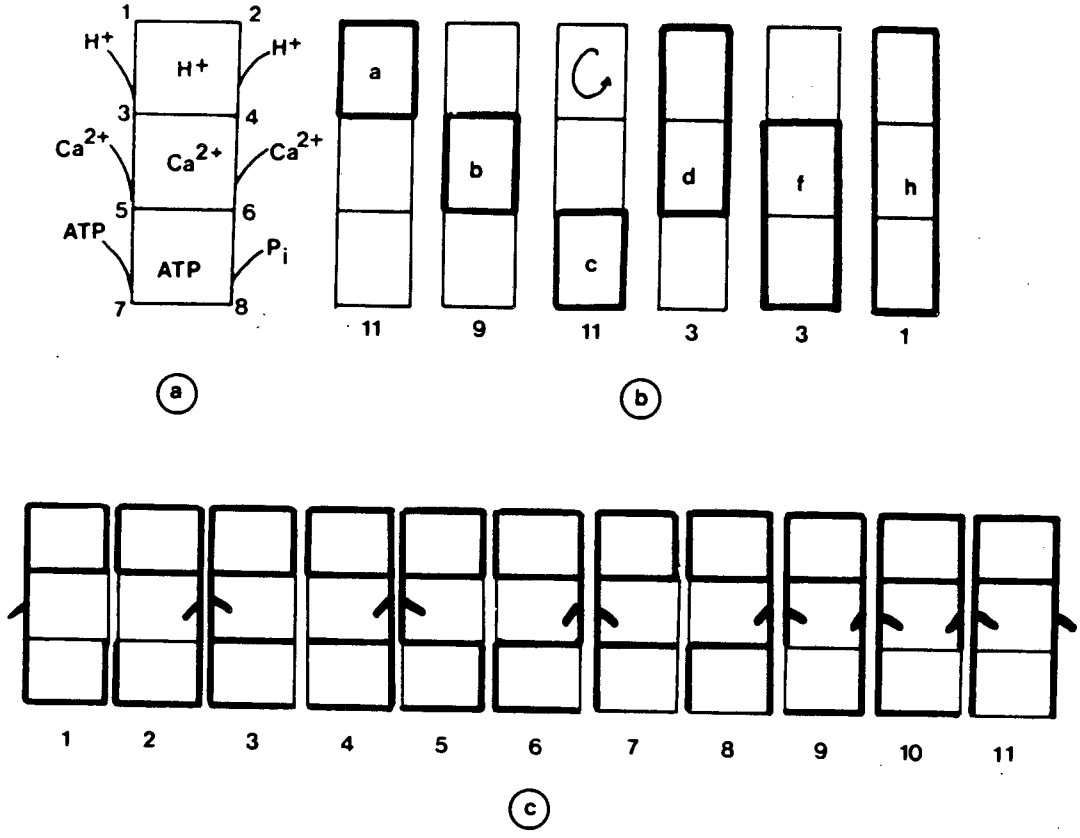


Figure 53: Analysis of active transport by the (Ca^{2+},Mg^{2+})-ATPase according to the Hill diagram method.

(a) Diagram for the model shown in Scheme III in which H^+ fluxes have been incorporated. In (b) the six cycles have been indicated. The numbers below each cycle refer to the number of flux diagrams per cycle. In (c) the eleven flux diagrams for cycle (a) are represented.

enzyme occurs through the phosphorylated intermediate which has a high energy level (Inesi, 1981). The translocation step is an energy-transducing step and utilises the free energy of hydrolysis to accomplish the conformational change in affinity and orientation of the calcium-enzyme complex. The net free energy change for each step is small to allow for comparable concentrations of intermediates and significant rates of forward and reverse reactions (Inesi, 1981; Hill and Eisenberg, 1981). This is in accordance with the theory of Jenks (1980) in which he conceives a system having no single 'power stroke' but where dissipation of energy is distributed over the whole catalytic cycle. On the other hand, Tanford (1983a) favours a mechanism whereby the energy available from ATP hydrolysis is utilised in the translocation step.

According to the above formalism for coupling of Ca^{2+} transport to ATP hydrolysis, variations in stoichiometry can occur by partial violations of Jenks' rules. Tanford (1983b) envisages a system in which alternations between the two conformational states of the enzyme are linked to switches in accessibility of Ca^{2+}-binding sites from outside to inside and to synchronous change in their affinities. These could involve either movement of the binding site itself, with the transported species remaining attached, or to rearrangement within the site (the alternating-access model) (Fig. 54). Tanford considers that a calcium channel, whose entrance and exits are accessible to opposite faces of the membrane, is unlikely on thermodynamic and kinetic grounds. Dupont (1983) has recently suggested a scheme in which free energy transduction between ATP and calcium is based on the transfer of solvation water between the acyl-phosphate bond and the bound calcium ions. He proposes that the two major conformational states of the enzyme, E and *E, as seen by intrinsic tryptophan fluorescence, do not exist during steady state catalysis, but that, in the presence of moderate ATP concentrations, the enzyme undergoes no sizeable conformational change. He showed that the E∿P to *E-P transition and phosphoryaltion by P_i was associated with a significant reduction in polarity of the active site accompanied by liberation of a large number of water molecules. It is proposed that the lost water is transferred to the hydration shell of Ca^{2+} that is bound to high affinity sites, resulting in a modification of the bonds formed by the

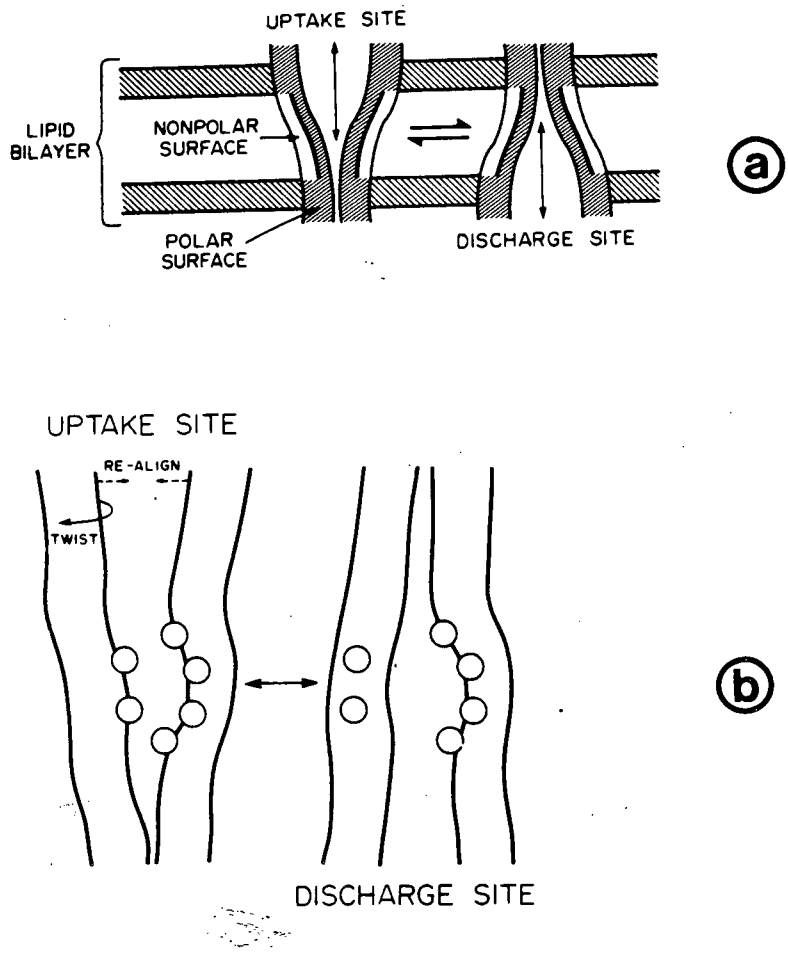


Figure 54: (a) Schematic representation of alternating access model for translocation of a single ion. Only the transmembrane portion of the protein is indicated. The width of the access openings is exaggerated.

(b) Hypothetical model for change in chemical potential of bound cation occurring in synchrony with translocation (Tanford, 1982). The circles are chelating groups, attached to transmembrane helices (Tanford, 1983a).

transported ion and a lowering of its affinity. In such a model uncoupling may result if the transfer of water to Ca^{2+}, following ATP hydrolysis, is deterred. Berman (1982b) has previously considered a model for active transport, in which a channel, containing multiple Ca^{2+}-binding sites, whose affinities vary synchronously between at least three states, results in vectorial and uphill transport (Fig. 27). In terms of such a model, variable stoichiometry may be a function of probabilities of forward versus reverse movements of Ca^{2+} ions from one site to another during change in state. The macroscopic stoichiometry of the overall process may thus vary without violation of the stoichiometry of elementary sequential steps of the reaction cycle.

Evidence for variable coupling has been shown to occur in other energy transducing systems. Pietrobon et al. (1981) examined the effects of antimycin A and funiculosin, two inhibitors which block electron transfer in the b-c_1 complex, on electron flow and electrochemical potential difference of H^+ ions in mitochondria, at static head (state 4). Their data did not comply with the "nature of leaks" and they interpreted their results in terms of redox-driven H^+-pumps that were not tightly coupled and molecular slipping within the pump at static head. Westerhoff et al. (1983) has used 'mosaic non-equilibrium thermodynamics' to distinguish between pump slippage and proton leakage and showed that both mechanisms were operative in mitochondria. Data obtained by Warncke and Slayman (1980) for the protonmotive ATPase of the plasma membrane of Neurospora have been interpreted as indicating a sharp change in stoichiometry under altered metabolic conditions. With regard to the literature on the plasmalemmal (Na^+, K^+)-ATPase, there is a wide measure of agreement that the stoichiometry is three Na^+ ions extruded and two K^+ ions taken up per molecule of ATP hydrolysed (Chapman, 1982). However, studies of isotopic Na^+ efflux and K^+ influx in dialysed squid axon have been interpreted to suggest that the stoichiometry of the reaction is variable, depending on the intracellular ionic composition (Mullins and Brinley, 1969). Eddy (1980) has examined the reason why accumulation of solutes (S) such as glucose and amino acids which are accumulated against a concentration gradient, coupled to symport of H^+ or Na^+ across the plasmalemma of prokaryotic and eukaryotic systems, varies as a function of $[S]_{out}$. Thus, at equilibrium, the ratio $[S]_{in}$/

$[S]_{out}$ if found to be large when $[S]_{out}$ is low and decreases as $[S]_{out}$ increases. Eddy (1980) has defined criteria to differentiate between 'external leaks' and symport pumps with 'slip' (represented diagrammatically in Fig. 55), and, based on these, has suggested that proton gradient-driven uptake of certain hexoses in Chlorella vulgaris involves slippage, rather than external leaks. Mitochondrial and chloroplast uncouplers have, in addition, been shown to have effects on energy coupling which cannot be explained solely by the fact that they confer H^+ conductance in membranes (Lee et al., 1969; Nicholls, 1974), but rather that proton pumps slip i.e. at static head, back diffusion of H^+ appears to be essentially through the proton channel of the ATPase with little contribution from ohmic leaks.

Chapman (1982) has shown how the stoichiometry of active Na^+,K^+ transport can vary when measuring flux ratios, depending on the ionic composition of the reaction medium. He proposed that, instead of a single reaction being catalysed by the membrane (Na^+,K^+)-ATPase, there is a series of two or more such reactions, each with its own fixed stoichiometry, but with each individual reaction occurring at different net rates, depending on the ionic composition. Variability in flux ratios could arise due to differing rate constants of different individual steps, and partial steps may occur, without the whole reaction cycle going to completion. Chapman postulates that both the ions must enter the reaction cycle at the same step (preferably before the first rate limiting step) and must come off together (perferably after the last rate limiting step) for maximum coupling to be obtained. In the measurement of unidirectional fluxes, findings of variable stoichiometry, as demonstrated by Mullins and Brinley (1969), is an inevitable consequence of a complex reaction mechanisms for a process of fixed overall stoichiometry (Chapman, 1982).

Stucki (1980) has extended the phenomenological theory of linear energy converters and applied it to oxidative phosphorylation. He has introduced the concept of conductance matching, which permits oxidative phosphorylation to operate with optimal efficiency. The necessary and sufficient condition for optimal efficiency was shown to be

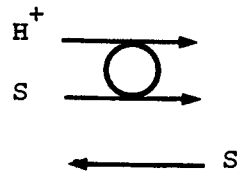
$$L_{33} / L_{11} = \sqrt{1 - q^2}$$

I Equilibrium model

(n is the effective number of equivalents of H^+ transferred per equivalent of S)

The steady state of S across the membrane is an equilibrium state in which the net proton current through the symport is zero. The steady state of S is not affected by increasing the number of pump molecules participating.

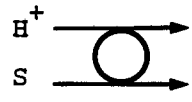
II Pump with external leak



A proton current occurs through the symport in the steady state of S. Increasing the pump activity relative to the leak increases the steady state level of $[S]_i$

III Pump with slip

The symport functions either as



or as

A proton current occurs through the symport in the steady state of S. The steady-state distribution of S is not dependent on the number of pump molecules participating, provided that the proton gradient across the cell membrane is constant.

Figure 55: Three models of the relations between the co-substrate and substrate of a proton symport, illustrating different modes of coupling of their flows across the cell membrane (Eddy, 1980).

where L_{11} is the phenomenological conductance of phosphorylation (rate of ATP synthesis), L_{33} is the phenomenological conductance of load (conductance of all ATP utilising processes) and q is the degree of coupling of oxidative phosphorylation driven by respiration. Thus the efficiency is zero if either a load with zero conductance or a load with infinite conductance is attached to oxidative phosphorylation. By maximising the net rate of ATP production, developed phosphate potential and efficiency, the degree of coupling, q, is 0.97. Although L_{11} is probably constant *in vivo*, L_{33} will fluctuate depending on metabolic activity. The degree of coupling will thus also vary in order to satisfy the condition of conductance matching. Fluctuations in L_{33} may however be prevented *in vivo* by other ATP-utilising reactions (thermodynamic buffering). This, however, will not be the case in an *in vitro* study and variable coupling may result so that optimal efficiency may be realised.

The physiological function of SR is (1) the rapid uptake of Ca^{2+} and (2) the maintenance of low cytosolic $[Ca^{2+}]_{free}$. The first results in rapid relaxation while the second allows switch off of the myosin ATPase during relaxation. The SR has a small volume, compared to that of the cytosol, resulting in a high resting $[Ca^{2+}]_{in}$ and high Ca^{2+} gradients. Assuming a stoichiometry of 2 Ca^{2+} ions transported by the Ca^{2+} pump for every ATP molecule hydrolysed, the reaction sequence may be written as

$$2\ Ca^{2+}_{out} + ATP \longleftrightarrow 2\ Ca^{2+}_{in} + ADP + P_i$$

The equilibrium constant, K, is given by

$$K = \left(\frac{[Ca^{2+}]_{in}}{[Ca^{2+}]_{out}}\right)^2 \times \frac{[ADP] \times [P_i]}{[ATP]}$$

and

$$\frac{[Ca^{2+}]_{in}}{[Ca^{2+}]_{out}} = \left(\frac{[ATP] \times K}{[ADP][P_i]}\right)^{\frac{1}{2}}$$

In the resting state, ATP = 5×10^{-3} M, ADP = P_i = 1×10^{-3} M (Akerboom et al., 1978). Thus

$$\frac{[Ca^{2+}]_{in}}{[Ca^{2+}]_{out}} \approx 5 \times 10^5$$

This is similar to the Ca^{2+} gradient obtained in vivo in which $[Ca^{2+}]_{out}$ = 1-5 nM and $[Ca^{2+}]_{in}$ = 5-20 mM, resulting in a gradient of 10^6 : 1 (Hasselbach, 1981).
More generally

$$\frac{[Ca^{2+}]_{in}}{[Ca^{2+}]_{out}} = \left(\frac{[ATP]xK}{[ADP][P_i]}\right)^{1/n}$$

Thus, decreasing the value of n, the coupling ratio, results in higher values at $[Ca^{2+}]_{in} / [Ca^{2+}]_{out}$. The present data, we believe, are sufficient grounds to raise the possibility of 'slippage' of the Ca^{2+} pump of SR under physiological conditions. High Ca^{2+}/ATP ratios under free flow conditions result in maximum initial rates of relaxation in intact muscle. Decreased stoichiometry at near static head conditions favours minimal levels of $[Ca^{2+}]_{free}$ in the sarcoplasm and hence of myosin ATPase activity.

It is apparent from the discussion above that existing models for the Ca^{2+} pump of SR, which are generally based on mechanisms having integral stoichiometry, cannot account for the experimental findings of variable coupling. Experimental evidence has been cited which suggests that deviations from the dominant reaction cycle may occur under certain conditions. It is suggested that the concept of molecular slippage be included in models for active transport, since variable stoichiometry may be an integral property of pump proteins; a concept that is not only important for the mechanism of Ca^{2+} transport in sarcoplasmic reticulum, but as a basic phenomenon of energy transduction.

The physiological significance of molecular slippage as a basic phenomenon of energy transduction needs to be investigated. We have

speculated that variable coupling plays a role in the SR Ca^{2+} pump of muscle cells where Ca^{2+} uptake varies between a state of free flow and static head. In addition, we have shown that the effects of Haemachates hemachatus cardiotoxin on SR cannot be explained on the basis of increased membrane leakiness. The results suggest that its action is directly on the ATPase enzyme resulting in intramolecular uncoupling of transport from ATPase activity. This supports the concept of Vogt et al. (1970) and Ng and Howard (1980) who showed that increased membrane leakiness cannot account for the effects of neurotoxins and myotoxins on SR. Cardiotoxin may be used in future studies as a tool to deliberately change the stoichiometry of the Ca^{2+} pump from 2:1 to 1:1.

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APPENDIX A

For a given combination of ligands and metals, the concentrations of the free metal ions, free ligands and metal-ligand complexes can be calculated according to the method of Storer and Cornish-Bowden (1976). As a representative example, consider the standard reaction medium which contained ATP, KCl and $MgCl_2$, at a given pH and a known free Ca^{2+} concentration. Using the appropriate equilibria applicable to this system (Table 1), the conservation equations for free ATP^{4-}, Mg^{2+} and K^+ can be written as follows:

(i) $$[ATP^{4-}]_T = [ATP^{4-}]_f + [HATP^{3-}] + [H^2ATP^{2-}] + [CaATP^{2-}]$$
$$+ [CaHATP^-] + [MgATP^{2-}] + [MgHATP^-] + [KATP^{3-}]$$

$$[ATP^{4-}]_T = [ATP^{4-}]_f \, (1 + K_1[H^+] + K_1K_2[H^+]^2 + K_3[Ca^{2+}]$$
$$+ K_1K_4[H^+][Ca^{2+}] + K_5[Mg^{2+}] + K_1K_6[H^+][Mg^{2+}] + K_7[K^+])$$

(ii) $$[Mg^{2+}]_T = [Mg^{2+}]_f + [MgATP^{2-}] + [MgHATP^-]$$

$$[Mg^{2+}]_T = [Mg^{2+}]_f \, (1 + K_5[ATP^{4-}] + K_1K_6[H^+][ATP^{4-}])$$

(iii) $$[K^+]_T = [K^+]_f + [KATP^{3-}]$$

$$[K^+]_T = [K^+]_f \, (1 + K_7[ATP^{4-}])$$

where K_1 to K_7 are the association constants corresponding to the equations in Table 1.

A listing of the computer programme written in BASIC for the HP-85 desk top computer (Hewlett Packard) is given:

```
10  REM TO CALCULATE CONC OF SPECIES IN SOLUTION
20  DISP "[ATP] TOTAL (M)"
30  INPUT A1
40  DISP "[MG2+] TOTAL (M)"
50  INPUT A3
```

```
 60  DISP "[K+] = 0.1 M"
 70  A4 = 0.1
 80  DISP "PH = ?"
 90  INPUT A6
100  B6 = 10^-A6
110  DISP "[CA2+] FREE (M)"
120  INPUT B2
130  REM ASSOCIATION CONSTANTS
140  REM HATP
150  K1 = 10471285
160  REM HHATP
170  K2 = 10471
180  REM CAATP
190  K3 = 20893
200  REM CAHATP
210  K4 = 135
220  REM MGATP
230  K5 = 44668
240  REM MGHATP
250  K6 = 447
260  REM KATP
270  K7 = 13
280  REM INITIAL VALUES = TOTAL
290  B1 = A1
300  B3 = A3
310  B4 = A4
320  B7 = A1
330  REM SETUP TERMS
340  C1 = K1 x B6
350  C2 = K1 x K2 x B6^2
360  C3 = K3 x B2
370  C4 = K1 x K4 x B6 x B2
380  C5 = K5 x B3
390  C6 = K1 x K6 x B6 x B3
400  C7 = K7 x B4
410  C8 = K5 x B1
420  C9 = K1 x K6 x B6 x B1
430  C0 = K7 x B1
```

```
440  REM CONSERVATION EQUATIONS
450  REM EQUATION FOR ATP
460  B1 = A1/(1 + C1 + C2 + C3 + C4 + C5 + C6 + C7)
470  REM EQUATION FOR MG2+
480  B3 = A3/(1 + D8 + D9)
490  REM EQUATION FOR K+
500  B4 = A4/(1 + CO)
510  DISP B1, B3
520  DISP B4
530  IF ABS ((B1 - B7) x 100/B1) < 10^-1 THEN 560
540  B7 = B1
550  GOTO 350
560  DISP " FREE [CA2+] = "; B2; "M"
570  DISP " FREE [ATP] = "; B1; "M"
580  DISP " FREE [MG2+] = "; B3; "M"
590  DISP "[MGATP] = "; K5 x B3 x B1; "M"
600  DISP "[CAATP] = "; K3 x B2 x B1; "M"
610  END
```

In standard uptake medium, containing 5 mM ATP, 5 mM MgCl, 100 mM KCl, pH 6.8 and at a free Ca^{2+} concentration of 1.0 µM, the concentration of the various species will be as indicated below.

Species	Concentration
Free Ca^{2+}	1.0 µM
Free ATP	0.16 mM
Free Mg^{2+}	0.61 mM
MgATP	4.3 mM
CaATP	3.25 µM