CALCIUM REGULATION OF CALCIUM TRANSPORT BY SARCOPLASMIC RETICULUM

By

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B.Sc.(Hons.), Liverpool Polytechnic, 1981
M.Sc. University of Alberta, 1986

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Interdisciplinary Studies

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

June 1991

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Department of INTERDISCIPLINARY STUDIES

The University of British Columbia
Vancouver, Canada

Date 2nd APRIL 1991

ABSTRACT

The sarcoplasmic reticulum (SR) of skeletal muscle is an intracellular membraneous network that, through the cyclical release and re-uptake of Ca^{2+} into and from, respectively, the cytoplasmic space, regulates myofilament shortening and, therefore, muscle contraction. SR derived from the terminal cisternae (HSR) demonstrates the property of Ca^{2+}-induced Ca^{2+} release. Upon attainment of a threshold intralumenal Ca^{2+} load, application of a small pulse of extralumenal Ca^{2+} stimulates the release of a pool of intralumenal Ca^{2+} via the ligand gated Ca^{2+} permeable pore of the Ca^{2+} release channel/ryanodine receptor complex. It was hypothesised that intralumenal Ca^{2+} regulates the opening of the release channel.

HSR vesicles were purified from skeletal and cardiac muscle by a novel technique. Structural characterisation of these membranes demonstrated an enrichment of harvested fractions in the Ca^{2+} release channel and the intralumenal Ca^{2+} binding protein, calsequestrin. In radiometric studies, skeletal HSR vesicles were shown to bind ryanodine with high capacity at both low and high affinity sites, with 2 fold stimulation of Ca^{2+} accumulation by the polyorganic cation Ca^{2+} channel blocker, ruthenium red. HSR vesicles passively loaded Ca^{2+}. Passive loading of HSR vesicles with Ca^{2+} was found to be non-linearly dependent upon the concentration of Ca^{2+} within the loading medium. This suggested the presence of 2 intralumenal Ca^{2+} binding sites with different affinities for Ca^{2+}. A spectroscopic dual-wavelength assay of Ca^{2+} release was developed that took advantage of peculiar spectral properties of the metallochromic Ca^{2+} sensitive dye Antipyrylazo III. In the presence of mM Mg·ATP and mM Mg2+ the initial fast phase of HSR Ca^{2+} was well resolved. Evidence was presented that initial rapid uptake was associated with high

affinity binding to an intralumenal Ca^{2+} compartment. Ca^{2+}-induced Ca^{2+} release was shown to occur with a threshold loading of intralumenal Ca^{2+}. The intralumenal Ca^{2+} threshold for Ca^{2+}-induced Ca^{2+} release was decreased in the presence of ryanodine. Ryanodine induced Ca^{2+} release was also dependent upon the amount of intralumenal Ca^{2+}. Ryanodine was also shown to inhibit sustained Ca^{2+}-induced Ca^{2+} release by apparent inhibition of the binding of Ca^{2+} to intralumenal sites. These results suggested that junctional state transitions of the Ca^{2+} channel and calsequestrin were interdependent.

Purified mM and mM Ca^{2+} activated neutral protease isoforms selectively cleaved the Ca^{2+} channel into 410 and 150kDa peptides with limited proteolysis. This was demonstrated in both HSR vesicles and the purified Ca^{2+} release channel. A novel 88kDa protein was also shown to be fragmented by both CANP isoforms. The identity of this prominent HSR associated protein remains obscure. CANP fragmentation of HSR protein elevated passive and active $^{45}Ca^{2+}$ loading in vesicles. This indicated that selective structural modification of the cytoplasmic portion of the release channel modified the comformational states of a intralumenal Ca^{2+} binding compartment in HSR vesicles. In spectroscopic studies, CANP proteolysis of HSR proteins increased the sensitivity to Ca^{2+} and ryanodine-induced Ca^{2+} release through decreases in the required intralumenal Ca^{2+} threshold for release. These functional alterations coincided with apparent single site cleavage of the release channel. Further proteolysis of the initial 410 and 150kDa peptides was without further significant effect upon function.

Based upon the hypothesis that primary sequences rich in proline (P), glutamate (E), aspartate (D), serine (S) and threonine (T) (PEST regions) are recognition sites for CANP binding to substrates, a search for PEST regions within the Ca^{2+} channel was undertaken. It was tentatively proposed that two

PEST regions near the N-terminal of the Ca^{2+} release channel may represent sites close to the CANP cleavage site.

The results of this work were discussed in relation to a possible role of Ca^{2+}-induced Ca^{2+} release in regulating the patterning of Ca^{2+} cytosolic transients. The frequency and amplitude of cytosolic Ca^{2+} transients appear to be important in regulating protein expression. The requirement of intralumenal Ca^{2+}-induced Ca^{2+} release may be a means by which the cyclical uptake and release of Ca^{2+} during muscle relaxation and contraction can be coordinated. This coordination may define the patterning of cytosolic Ca^{2+} transients. The increased sensitivity to Ca^{2+}-induced Ca^{2+} release by HSR after CANP treatment may represent a means by which the patterning of cytosolic Ca^{2+} transients can be altered to effect changes in protein synthesis.

Signature of thesis co-supervisor	Signature of thesis co-supervisor

ACKNOWLEDGEMENTS

I cannot begin to describe the gratitude that I have for my principle advisor and long time friend, Dr Angelo Belcastro. It is his support, imagination, and keen insights which have contributed immeasurably to my thinking and my work. I shall miss him and his wonderful family. I thank you.

I would like to extend my deepest thanks to Dr Sidney Katz for his support, his trust, his guidance, and his friendship. It has been a thoroughly rewarding experience working with you. Many thanks.

I wish to thank the members of my supervisory committee, Dr Angelo Belcastro, Dr Sidney Katz, Dr Peter Hochachka, and Dr David Godin. Thank you for having listened to me. Thankyou all for your support.

I would like to acknowledge the friendship and lively informative exchanges with Mr Bruce Allen and Dr Kevin Wang.
I learned much from these two talented people. It has been an honour working with you.

I would like to acknowledge the skilled technical assistance of Mr Anthony Borel with the ryanodine purification. In addition, I would like to thank Ms Cheryl Machan for skilled assistance with many aspects of this work.

I would like to thank all members of Dr Katz's laboratory for their friendship.

Many thanks to Dr T Kuo for the generous gift of the CANP antibodies.

I wish to thank all members of the Faculty of Pharmaceutical Sciences and staff and members of the School of Physical Education and Recreation at the University of British Columbia.

Finally, I wish to thank my wonderful wife, Jo-Anne, for having supported me throughout all the trials and tribulations of these graduate years. Thankyou for the typing of this manuscript. Thankyou for being a friend.

DEDICATION

To Jo-Anne, you have always been there for me

To my family, especially my parents.

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LIST OF ABBREVIATIONS

%	percent
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophate
APIII	Antipyrylazo III
ATP	adenosine 5'-triphosphate
β	beta
Ca^{2+}	calcium free ion
Ca^{2+}ATPase	calcium stimulated, magnesium dependent ATPase pump protein
CaM	calmodulin
CANP	calcium activated neutral protease
CBP	calcium binding protein
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate
CP	creatine phosphate
CPK	creatine phosphokinase
CPP	calcium pump protein
CRC	calcium release channel
δ	delta
ΔA	difference absorbance
DEAE	diethylaminoethyl
DTT	dithiothreitol
EGTA	ethyleneglycol bis(b-aminoethylether) N,N,N',N'-tetraacetic acid
g	gram

HEPES	4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid
HSR	heavy sarcoplasmic reticulum
IP_3	myoinositol 1,4,5 triphosphate
ISR	intermediate sarcoplasmic reticulum
K'	first order dissociation constant
K''	second order dissociation constant
K^+	potassium free ion
Ka	association constant
kDa	kilodalton
Km	michaelis menten constant
L	litre
μ	micro
m	milli
M	molar
mg	milligram
Mg^{2+}	magnesium free ion
min	minute
mol	mole
MOPS	morpholinopropanesulphonic acid
M_r	relative molecular mass
n	nano
Na^+	sodium free ion
p	pico
PAGE	polyacrylamide gel electrophoresis
Pi	inorganic phosphate
PIPES	1,4,-piperazinediethanesulphonic acid
pmol	picomole

PMSF	phenylmethylsulphonyl fluoride
pS	picosiemens
s	second
S	svedberg
SR	sarcoplasmic reticulum
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethyldiamine
TLCK	N-a-p-tosyl-L-lysine chloromethyl ketone
Tris	tris(hydroxymethyl)aminomethane
ω	omega

INTRODUCTION.

I. Role of Calcium in Living Organisms

The first discovery of the existence of calcium was made nearly two centuries ago by Humphry Davy in 1808. Early recognition of the biological importance of calcium (Ca^{2+}) is often attributed to the pioneering work of Ringer (1883) who discovered that constituents of tap water rather than distilled water during the preparation of physiological saline, activated the sustained beating of the frog heart. Ringer later determined that the constituent was Ca^{2+} and was an absolute requirement for contraction. Since that time it has become clear that Ca^{2+} is extensively distributed throughout the entire organism and performs a wide variety of diverse functions. These range from the regulation of cell division and growth (including formation and maintenance of the skeletal structures) to the regulation of synaptosomal and neuromuscular transmitter release (Rubin, 1970), stimulus-secretion coupling from endocrine cells (Douglas 1968), mast cell histamine release (Foreman, 1981) and excitation contraction coupling (Heilbrunn and Wiercinski, 1947; Hill, 1949; Marsh, 1952; Ebashi and Lipmann, 1962; Winegrad, 1965; Fabiato, 1983).

Vertebrate Ca^{2+} exists largely immobilised as hydroxyapatite in the presence of collagen matrix (i.e. bone). In relation, a small amount of residual Ca^{2+} is distributed between extracellular and intracellular compartments. Within the extracellular compartment total Ca^{2+} concentration ($[Ca^{2+}]$) is relatively constant with estimates ranging between 2-3 mM (Fabiato 1983, Carifoli, 1987) half of which exists within an ionised or unbound form. Intracellularly, the tissue specific distribution of Ca^{2+} is quite varied and is dependent upon the particular requirement for Ca^{2+} in mediating intracellular processes. Erythrocytes contain very little Ca^{2+} (20μM) (Long and Mouat, 1972). On the other hand, cardiac

muscle cells, which utilise Ca^{2+} in the regulation of contractile and oxidative processes, contain 1mM total Ca^{2+} accounting for total cell water (Fabiato, 1983). Estimated intracellular free [Ca^{2+}] in resting or relaxed cells, however, is maintained close to 0.1μM. The remainder of Ca^{2+} is either a) sequestered within intracellular compartments (e.g. endoplasmic reticulum, sarcoplasmic reticulum, mitochondria, and cell nuclei) or b) bound to a variety of intracellular Ca^{2+} binding proteins. Maintenance of 1,000-2,000 fold Ca^{2+} electrochemical gradients that necessarily results from compartmentation is achieved by a variety of energy dependent protein pumps (e.g. Ca^{2+}-ATPases) and cation antiporters (e.g. Na/Ca antiporters) which operate, ideally, in dynamic equilibrium with a multitude of other intracellular and extracellular processes.

Compartmentation of Ca^{2+} in this manner has two important consequences for the cell. The first is that calcium effects upon a variety of molecular and biochemical processes can be made to be selective, transient, and reversible (and therefore regulatory in nature) through the controlled release and re-uptake of Ca^{2+} into and from, respectively, the cytosol. The second is that Ca^{2+} effects can be tolerated within an enviroment of phosphate driven metabolism (Weber, 1976). Intracellular energy dependent processes utilise the free energy liberation from the hydrolysis of mainly ATP. At rest, Pi concentrations are as high as 1-2mM in the fast twitch skeletal muscle and 5mM in cardiac cells (Ackerman et al., 1980; Meyer et al., 1982). With repetitive contractions Pi levels may be 15 fold elevated (see Meyer et al., 1984). The high solubility product of various forms of apatite would lead to cytosolic calcium phosphate precipitation and eventually cell death in the absence of precise control of intracellular Ca^{2+} distribution.

In view of the precarious co-existence of phosphate and calcium metabolism it is remarkable that Ca^{2+} is central to the regulation of a multitude of cellular events in various cell types. Although the evolutionary choice of Ca^{2+} in this

regard is not completely clear (see Carafoli, 1987) it's suitability is vested in the complex electronic structure and binding chemistry of Ca^{2+} (Williams, 1976). The flexible coordination number (Ca^{2+} can bind between 6 to 8 electrons, usually from oxygen) and the relatively large size of Ca^{2+} permits tight protein binding with variable bonding distances. This reduces the configurational requirements of protein binding sites that for binding of the smaller more regularly shaped Mg^{2+} are more specific. Ca^{2+} can therefore bind to a wide variety of proteins with high affinity and specificity (in that it can exclude Mg^{2+}).

Compartmentation of Ca^{2+} within subcellular organelles provides both a source and sink of Ca^{2+}. It is the binding of cytosolic Ca^{2+} to proteins and the subsequent induction of conformational change that provides the basis by which Ca^{2+} modulates cellular function. Carafoli (1987) has catagorised these proteins into two groups. In the first group, intrinsic membrane proteins (see later), initially bind Ca^{2+} with various affinities and translocate it from the cytosol across compartment boundries (i.e. membranes) whereupon the protein is free to accept another Ca^{2+} ion. Proteins of the second group largely form the super family of calcium-modulated proteins (Kretsinger, 1975). The calci-forms of this group ($Kd_{(Ca)}=10^6M^{-1}$) are either active enzymes or modulate the function of other enzymes. The majority of these proteins (see Table 1) are evolutionary homologs and can be categorised into sub-families each containing between two to eight copies of a conserved structural region known as an E-F hand (Moncrief et al., 1990). This structural motif was first deduced from x-ray diffraction studies of parvalbumin (Moews and Kretsinger, 1975). As shown in Figure 1, 29 amino acids are arranged in a helix-loop-helix configuration with formation of a thermodynamically stable pocket upon interaction of paired E-F hand units. Ca^{2+} therefore functions as an intermediary signal or second messenger mediating the trafficking of

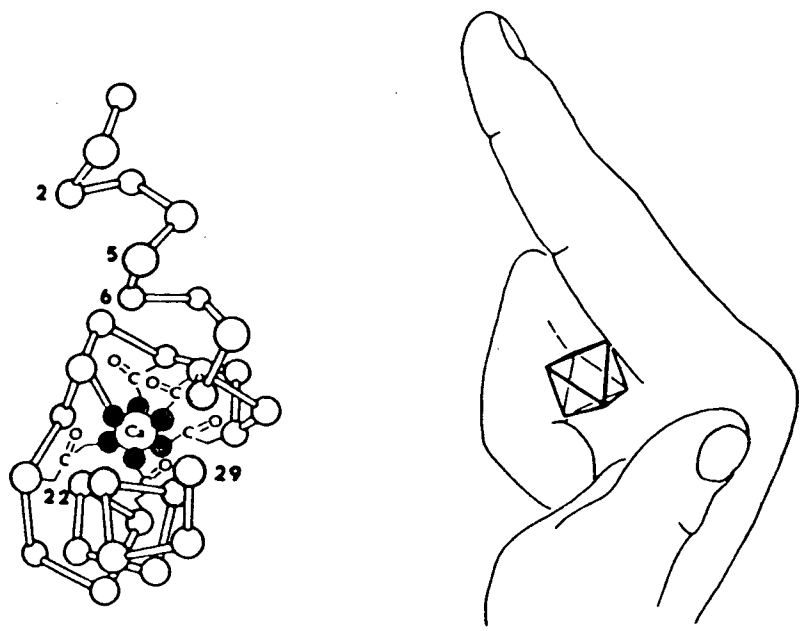
Table 1. Subfamilies of Calcium-binding proteins and unique EF-hand homologs.

	#Ca^{2+} binding EF-hand domains
Calmodulin	2-4
Troponin C	2-4
Essential light chain of myosin	1-3
Regulatory light chain of myosin	1
Sarcoplasmic calcium-binding protein	1-4
Calpain	2-4
Aequorin	3
Strongylocentrotus purpuratus ectodermal protein	3-4
Calbindin	4
Parvalbumin	2
α-actinin	0-2
S100	0-2
Calcineurin B (Bos)	4
Tropinin C (Astacus)	2
Calcium vector protein (Branchiostoma)	2
Caltractin (Chlamydomonas)	4
CDC31 (Saccharomyces)	2
10-kD protein (Tetahymena)	2
Eight-domain protein (Lytechinus)	7
Calcium-binding protein (Streptomyces)	4

Table adapted from Moncrief et al., 1990.

Fig. 1. The EF-hand or calmodulin fold. EF-hands consist of an α-helix (symbolised by the forefinger of a right hand), a loop around the Ca^{2+} ion (represented by the clenched middle finger), and a second α-helix (symbolised by the thumb). Amino acids 1-11 comprise the first α-helix; 19-29 the second. The stipled α-carbons-2, 5, 6, 9, 22, 25, 26, and 29- usually have hydrophobic side chains. (from Moncrief et al., 1990)

Figure 1.



biological information encoded in one form (e.g. receptor-ligand binding) through its conversion to another form (e.g. enzyme activation). This form of intra- and intercellular communication is known as stimulus-response coupling or biological signal transduction in which tight control of Ca^{2+} movements coupled to other biological processes is essential for cell viability.

II. Control of Calcium Movements

Regulation of the second messenger function of Ca^{2+} can be described in two ways. The first in terms of receptor mediated regulation of Ca^{2+} movements. The second in terms of specific devices controlling Ca^{2+} compartmentation.

1. Receptor Mediated Control

Ca^{2+} movements can be activated in two ways. In the first, binding of a ligand to a receptor molecule located on the surface of the plasma membrane induces a change in membrane potential (e.g. acetylcholine binding to acetylcholone receptor) and leads to i) activation of voltage dependent Ca^{2+} channels or ii) release of Ca^{2+} from sarcoplasmic reticulum (see later). In the second, more universal type, ligand-receptor binding activates several intracellular regulatory cascades with production of additional second messengers (e.g. 3',5'-adenosine (guanosine) monophosphate or cAMP (cGMP), myoinositol 1',4',5'-triphosphate (IP_3) and diacylglycerol (DAG).

The activity of a second messenger metabolic enzyme e.g. adenylate cyclase, guanylate cyclase or phosphoinositide phosphodiesterase (phospholipase C) is regulated by the ligand (agonist)-bound receptor via a family of coupling proteins, known as G-proteins, located within the membrane interior (see

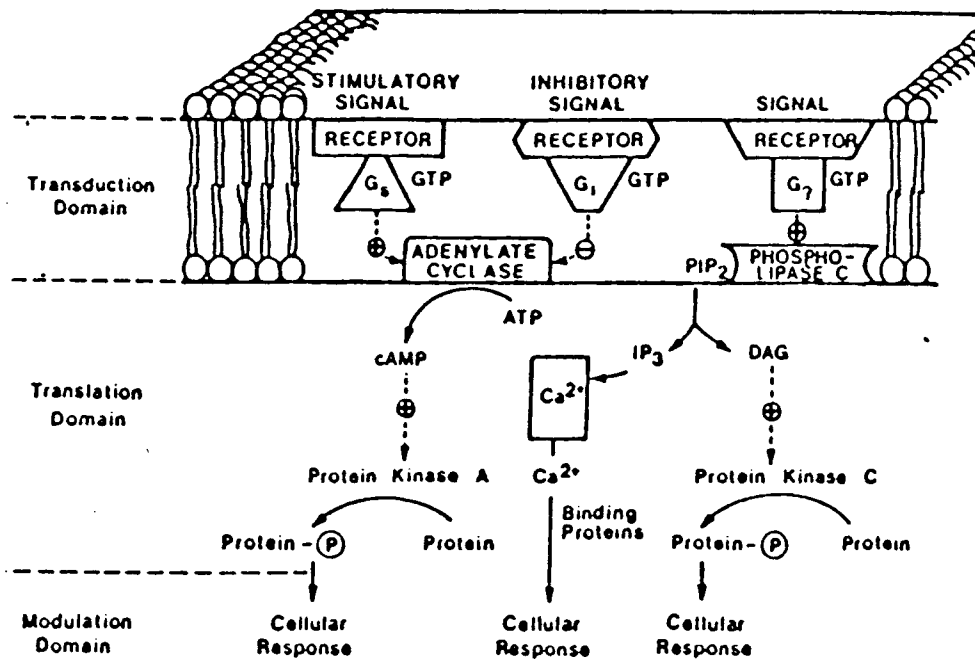
Gilman, 1987). The 3 components (receptor, G-protein, enzyme) constitute the transduction domain (see Figure 2). As heterotrimers with α, β and γ subunits, G-proteins are regulated by the cyclical binding (activation) and hydrolysis (deactivation) of GTP (to GDP + Pi) upon the α subunit. G-protein activation may then lead to activation or inhibition of the metabolic enzyme. Myocardial adenylate cyclase for example is activated by stimulating G-proteins (G_s) with production of cAMP by receptor binding of β-adrenergic agonists (epinephrine, norepinephrine, isoproterenol). Inhibition occurs upon muscarinic receptor binding of acetylcholine and activation of inhibitory G-proteins (G_i) (Josepheson and Speraklis, 1982). Similarly, production of IP_3 and DAG form the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C involves the intermediary action of G_s (Litosch and Fain, 1986, Taylor and Merrit, 1986; Fain et al., 1988).

a. Phosphorylation Effects

Production of the second messengers cAMP, cGMP, and DAG results in phosphorylation of specific cellular proteins and represents the most common consequence of second messenger effects. Of importance in the control of Ca^{2+} fluxes are phosphorylation of several proteins regulating Ca^{2+} transport. The myocardial SR Ca^{2+}-ATPase regulatory protein (phospholamban) and the sarcolemmal Ca^{2+}-ATPase are both phosphorylated and activated by cAMP-dependent protein kinase (Caroni and Carifoli, 1981, Neyses et al., 1985; Tada et al, 1975; James et al, 1989, Vittone et al., 1990). Similarly protein kinase C was shown to phosphorylate phospholamban (Movesian et al., 1984) and the Ca^{2+}-ATPase from erythrocytes (Smallwood et al., 1988). In addition both the dihydropyridine binding α_1 subunits of skeletal and cardiac muscle, L-type Ca^{2+} channels (165 and 185 kDa

Fig. 2. Schematic representation of signal cascades. The transduction domain (membrane) consists of receptors, G-proteins, and metabolic enzymes. The translation domain consists of signal cascades and the modulation domain consists of responses to second messenger effects.(from Mooibroek and Wang, 1988)

Figure 2.


STIMULATORY SIGNAL
INHIBITORY SIGNAL
SIGNAL
RECEPTOR
RECEPTOR
RECEPTOR
Transduction Domain
GTP
GTP
GTP
ADENYLATE CYCLASE
PIP2
PHOSPHO-LIPASE C
ATP
cAMP
IP3
DAG
Translation Domain
Ca2+
Protein Kinase A
Ca2+
Protein Kinase C
Binding Proteins
Protein - P
Protein
Protein - P
Protein
Modulation Domain
Cellular Response
Cellular Response
Cellular Response

respectively) and the 52kDa β-subunit have been shown to be phosphorylated by both protein-kinase C and cAMP activated protein kinase A (O'Callahan and Hosey, 1988, Röhrkasten et al., 1988, O'Callahan et al., 1988). Phosphorylation increased the mean open time of cardiac L-type channels (Reuter et al., 1982) and therefore increased the slow inward Ca^{2+} current upon t-tubule membrane depolarisation. Evidence suggests that cAMP dependent phosphorylation of cardiac and skeletal L-type channels may occur by direct activation of a closely associated G_S of adenylyl cyclase (Yatani et al., 1987, 1988).

b. Myoinositol Triphosphate Effects

Regulation of Ca^{2+} movements by IP_3 occurs independently of phosphorylation and involves the direct binding of IP_3 to receptors associated with Ca^{2+} channels that access a IP_3 sensitive Ca^{2+} pool (Berridge and Irvine, 1989). The location of this Ca^{2+} pool is within the endoplasmic reticulum and other related membrane structures (see later). The function of the IP_3 sensitive Ca^{2+} pool is unclear although identity of a GTP-activated IP_3 insensitive Ca^{2+} pool and its communication with the IP_3 sensitive Ca^{2+} pool in smooth muscle cells (Ghosh et al., 1989) suggests a complex IP_3 mediated control of intracellular Ca^{2+} fluxes. Such control mechanisms may mediate intracellular Ca^{2+} oscillations which appear to propagate in the form of waves (Berridge and Irvine, 1989). It is further unclear to what extent these oscillations are associated with the linked oscillations of free Ca^{2+} and glycolytic flux observed in skeletal muscle extracts and insulinoma cells (Corkey et al., 1988, Tornheim, 1988). Berridge and Irvine (1989) have suggested that information may be encoded in Ca^{2+} waves as a frequency modulated signal. This is quite different from amplitude modulatation of Ca^{2+} signals (transients) associated with Ca^{2+} release from the sarcoplasmic reticulum membranes to initiate muscle contraction.

2. Compartment Mediated Control of Calcium Movements

With the exception of erythrocytes, eukaryotic cells possess several Ca^{2+} transporting membrane systems that utilise the electrochemical energy stored within transmembrane Ca^{2+} gradients to perform a variety of specific Ca^{2+} activated and Ca^{2+} regulated functions intracellularly. Principle membrane systems include plasma membrane, mitochondria, endoplasmic reticulum and sarcoplasmic reticulum. In addition, membrane systems of the Golgi apparatus (Virk et al., 1985), lysosomes (Klempner, 1985), adrenal chromaffin cells (Burgoyne et al., 1989), and the nuclear envelope (Hashimoto et al., 1989) all demonstrate Ca^{2+}-ATPase activity. Recent studies also indicate the presence of IP_3 receptors upon the surface of nuclear membranes (Ross et al., 1989) suggesting the presence of concerted Ca^{2+} control mechanisms within this membrane system.

a. Mitochondria

The principle function of the mitochondria is the regulation of oxidative energy metabolism as first suggested by Lardy and Wellman (1952) and later confirmed by Chance and Williams (1955). However, earlier erroneous observations of the Ca^{2+} uptake capacity of the mitochondria (Vasington and Murphy, 1962) suggested that these organelles may participate as important regulators of cytosolic Ca^{2+} buffering. More recent X-ray microanalysis (Somlyo and Walz, 1985) indicated that under normal conditions the intramitochondrial Ca^{2+} pool would contribute little to the cytosolic Ca^{2+} pool. Under pathological conditions where cytosolic Ca^{2+} may be elevated, mitochondrial Ca^{2+} uptake is associated with matrix Ca^{2+} phosphate precipitation (Carafoli et al., 1965).

According to Carafoli (1987) such "matrix loading" may constitute a safety device for cellular Ca^{2+} control.

The principle function of intramitochondrial Ca^{2+} is believed to be the regulation of Ca^{2+}-sensitive dehydrogenases which catalyse rate limiting production of reducing equivalents during tricarboxylic acid cycle flux (Denton and McCormack, 1985, 1989; Hansford, 1985). Transmitochondrial Ca^{2+} flux (and therefore matrix $[Ca^{2+}]$) is regulated by two independent pathways. Ca^{2+} uptake is mediated by a low affinity (Km=30μM at 3mM Mg^{2+}) transporter and is driven by an inside negative membrane potential ($\Delta\Psi$) across the inner mitochondrial membrane established by H^+ efflux coupled to cytochrome mediated electron transfer. Ca^{2+} efflux occurs via a specific Na^+/Ca^{2+} exchanger which mediates electroneutral exchange of two Na^+ ions for one Ca^{2+} ion. The exchange is driven by an inwardly directed Na^+ gradient (10mM extramitochondrial Na^+) that is maintained by a Na^+/H^+ exchanger coupled to the inwardly directed proton electrochemical gradient ($\Delta\mu_{H+}$).

b. Plasma Membrane

The plasma membrane possess essentially 3 different types of Ca^{2+} handling devices: a Ca^{2+}-ATPase; a Na+/Ca2+ exchanger; and Ca^{2+} channels.

(i). Calcium ATPase

The Ca^{2+}-ATPase is a high affinity low capacity Ca^{2+} pump that extrudes cytosolic Ca^{2+} into the extracellular space. In erythrocytes and skeletal muscle membranes Km values for Ca^{2+} of 1μM or less have been reported (Caroni and Carafoli, 1981; Hidalgo et al., 1986) with 2-3 fold increased sensitivity in the presence of calmodulin. The Ca^{2+}-ATPase of plasma membranes appear to be similar within a variety of different excitable and non-excitable cells including

erythrocytes (Penniston, 1982), squid axons (Post et al., 1969), smooth muscle (White and Blostein, 1982), t-tubules (Hidalgo et al., 1986) and cardiac and skeletal sarcolemma (Caroni and Carafoli, 1981; Mickelson et al., 1985; Michalak et al., 1984). Plasma membrane Ca^{2+} pumps are important in the maintenance of low cytosolic Ca^{2+} levels. Carafoli (1987) has suggested that this function, other than in erythrocytes, may be supplemented by Na^+/Ca^{2+} exchange mechanisms owing to the low capacity Ca^{2+} handling characteristics (10-30nmol.mg protein^{-1}.min^{-1}). However, Hidalgo et al. (1986) argued that the relatively large surface area of t-tubule membranes implicates a major role for Ca^{2+} extrusion by cardiac and skeletal plasma membrane/t-tubule Ca^{2+} pumps.

(ii). Sodium-Calcium Exchange

The exchange of cytosolic Ca^{2+} for extracellular Na^+ is a carrier-mediated process which is especially prevalent in excitable tissues. Although Na^+/Ca^{2+} exchange is reversible (Reuter and Seitz, 1968) the main function of the carrier is the maintenance of low cytosolic Ca^{2+} levels. The electrogenic character of the exchanger ($3Na^+$ for $1Ca^{2+}$) was demonstrated in the earlier vesicle studies of intralumenal Na^+ dependent Ca^{2+} uptake with accumulation of the lipophilic cation tetraphenylphosphonium in response to generation of an inside negative $\Delta\Psi$ (Reeves and Sutko, 1980). The exchanger in contrast to the Ca^{2+}-ATPase is a low Ca^{2+} affinity high capacity exchanger of Ca^{2+} with reported Km values that vary widely in vesicle (1.5 to 140μM) and intact tissue (180-350μM) studies (see Reeves, 1985). In contrast, good agreement upon Ca^{2+} transport rates (5-30nmol.mg protein^{-1}.s_{-1}) by the exchanger (Reeves and Sutko, 1983; Philipson and Nishimoto, 1983; Caroni and Carafoli, 1983) provided estimates of transmembrane Ca^{2+} flux in intact tissue of 3-16pmol/cm^2/sec. A low site density was reported for the exchanger with turnover number estimated as high

as 1000 s^{-1} or more (Cheon and Reeves, 1988). Recently, the Na^+/Ca^{2+} exchanger from cardiac sarcolemma was cloned with cDNA encoding a protein of 970 residues (Nicoll et al., 1990). Na^+/Ca^{2+} was expressed when RNA, synthesised from cDNA, was injected into Xenopus oocytes. Structural similarity was noted between the exchanger and the Na^+/K^+ ATPase (Nicoll et al., 1990).

(iii). Calcium Channels

In excitable tissues an additional pathway for Ca^{2+} flux across the plasma membranes are a family of gated Ca^{2+} channels allowing the entry of extracellular Ca^{2+} into the cytosol in response to membrane depolarisation. These channels were originally categorised into low and high threshold Ca^{2+} conductance pathways based upon voltage dependent gating properties (Llinas and Yarom, 1981). More recently, in accordance with the nomenclature adopted by Nowycky et al. (1985), Ca^{2+} channels in neuronal and muscle plasma membranes are categorised as N,T,L, and P type channels (Llinas et al., 1989). In heart muscle L and T channels have been described (Bean, 1989). L-type channels can be distinguished by i) their dihydropyridine binding properties ii) large depolarisation requirement for activation iii) slow inactivation leading to long lasting Ca^{2+} currents and iv) relatively large conductances (~15-25 ps with 100mM Ba carrier current) (Bean 1985, Mitra and Morad 1986). Converse properties characterise T-type channels which unlike L-type channels contribute little to the main Ca^{2+} current (I_{Ca}) during membrane depolarisation. The L-type channel or dihydropyridine receptor is also preferentially localised to the T-tubules of skeletal muscle. (Hosey et al., 1989). However, less than 5% appear to function as Ca^{2+} channels (Schwartz et al., 1985).

It is now believed that skeletal muscle dihydropyridine receptors function as voltage sensors mediating responses of the SR ryanodine receptor to the t-tubule depolarisation (Leung et al., 1988; Catteral et al., 1989; Tanabe et al., 1989; Adams et al., 1989). N-type (high threshold) Ca^{2+} channels are found largely in neuronal tissue in addition to L- and T-types (Nowycky et al., 1985). A P-type Ca^{2+} channel has also been described in Purkinje cells (Llinas et al., 1989) and bears some resemblance to N-type Ca^{2+} channels.

c. Endoplasmic Reticulum and Related Structures

The endoplasmic reticulum (ER) of non-muscle cells share several of the Ca^{2+} transporting structural features of sarcoplasmic reticulum. Liver and pancreas ER demonstrate Ca^{2+}-ATPase activity with similar kinetic properties described for skeletal muscle SR (Bayerdorffer et al., 1984; Kraus-Freidmann al., 1985). In addition an intraluminal Ca^{2+} binding protein, calregulin, has been identified in supernatants of bovine liver-homogenates that although immunologically unrelated to SR calsequestrin share similar $^{45}Ca^{2+}$ binding, Stains-All staining and apparent molecular weight (63kDa) characteristics with the latter protein (Waisman et al., 1985; Khanna and Waisman, 1986; Damiani et al., 1988). A similar protein, chromogranin A, was identified in Ca^{2+} releasing chromaffin vesicles and microsomes isolated from bovine adrenal medulla (You and Albanesi, 1990).

The release of Ca^{2+} from ER is stimulated by binding of IP_3 to a specific receptor protein. Recent cloning and expression (Furuichi et al., 1989) of IP_3 receptor (P_{400} protein) with a relative molecular mass of 250,000 (Ross et al., 1989) reveals a remarkable sequence homology at the COOH region with the 565kDa ryanodine receptor in skeletal muscle (Mignery et al., 1989). Reconstruction of the P_{400} protein into vesicles demonstrated that the IP_3

receptor is likely the Ca^{2+} permeable pore mediating IP_3 induced Ca^{2+} release, in vivo (Ferris et al., 1989). Recent immunological studies suggest that the IP_3 Ca^{2+} sensitive pool is localised to the ribosomes bearing endoplasmic reticulum the nuclear envelope and portions of the smooth endoplasmic reticulum (Ross etal., 1989). The relationship between these subcellular fractions and the smooth surfaced IP_3 sensitive Ca^{2+} releasing "Calcisome" structures (Volpe et al., 1988) is unclear, however.

d. Sarcoplasmic Reticulum

The Sarcoplasmic Reticulum is an intracellular membrane structure in smooth, cardiac and skeletal muscle that regulates the pool of Ca^{2+} associated with the transient triggering of muscle contraction. From intralumenal stores, Ca^{2+} is transiently released and rapidly re-accumulated via a Ca^{2+} channel and a Ca^{2+}-ATPase, respectively. This structure is the focus of the current work and will be reviewed in more detail below.

III. Sarcoplasmic Reticulum Structure and Function

1. Historical Overview

The presence of an intracellular organelle with fine ultrastructure that closely associated with myofibrils was first described by Retzius (1890). Sarcoplasmic reticulum was originally prepared as a crude skeletal muscle particulate fraction by Kielley and Mayerhof (1948) who demonstrated the presence of Mg^{2+}-stimulated ATPase activity. Hill (1949) calculated that the presence of an internal Ca^{2+} store was necessary to account for rapid contraction of fast-twitch skeletal

muscle. Later, Marsh (1951,1952) demonstrated that "relaxing factor" isolated from muscle extracts stimulated relaxation of actomyosin. Kumagai et al. (1955) demonstrated that the "relaxing factor" was particulate rather than soluble and Ebashi (1958) showed that the relaxing activity of the factor correlated with the associated ATP hydrolytic activity. Concurrently, Hasselbach and Makinose (1961) and Ebashi and Lipmann (1962) demonstrated that the particulate fraction was vesicular in nature and contained an ATPase that in the presence of both Mg^{2+} and ATP could remove extravesicular Ca^{2+}. Description by electron microscopy of an endoplasmic reticulum in muscle was initiated by Porter and Palade (1957). This was extended to a 3-dimensional reconstruction from serial sections demonstrating a close association of the reticulum with the t-tubule region of skeletal muscle (Anderson-Cedergren, 1959). Later, Revel (1962) identified the presence of junctional processes linking the t-tubule to the terminal cisternae of the reticulum. Autoradiographic demonstration of calcium release from the terminal cisternae and calcium sequestration by the longitudinal reticulum (Winegrad, 1965) supported an earlier speculation (Huxley and Taylor, 1958) that muscle contraction was stimulated by events initiated from the triads of t-tubule/reticulum junction. It is now clear that the sarcoplasmic reticulum is a closed intracellular membranous network in close association with the myofibrils and t-tubular membranes of muscle. In response to t-tubule membrane depolarisation the SR regulates muscle contraction and relaxation through the cyclical release and re-uptake, respectively, of Ca^{2+} into and from the cytoplasmic space.

2. Morphology of SR

Sarcoplasmic Reticulum (SR) is an enclosed intracellular membrane system with two morphologically, and functionally distinct regions (Peachey and Franzini-Armstrong, 1983). The junctional region of SR (JSR) form cisternae in close proximity to the transverse tubular system (t-tubules). The free SR (FSR) forms the longitudinal and fenestrated regions of the reticulum connecting a pair of cisternae.

The freeze-fractured FSR is densely populated with 85-90 Å particles on the cytoplasmic fracture face. The membrane is asymmetric with hardly discernable indentations and few particles on the lumenal face (Deamer and Baskin, 1969; MacLennan et al., 1971; Franzini-Armstrong, 1975, 1980). The intramembraneous particles appear to represent the Ca^{2+} pump protein with 85 Å particle density well correlated to the rate of Ca^{2+} influx, the Ca^{2+} stimulated ATPase activity, and the relaxation rate of various muscles (Baskin, 1971; Rayns et al., 1975; Devine and Rayns, 1975; Martonosi, 1980). In fast twitch muscle 85 Å particle density was earlier estimated with unidirectional shadowing of freeze-fractured replicas at 3,000-5,000.μm^{-2} across the FSR (Franzini-Armstrong, 1975). More recently, rotary shadowing techniques estimated a 3 to 5 times higher particle density (Napolitano et al., 1983; Franzini-Armstrong and Ferguson, 1985). Slow twitch muscle particle density is approximately half that of fast twitch muscle (Jorgensen et al., 1983).

The Ca^{2+} pump protein is transmembranous (Hidalgo and Ikemoto, 1977) and protrudes from the cytoplasmic face as 40 Å particles (Inesi and Scales, 1974; Saito et al., 1978). The discrepent density distribution of the cytoplasmic and intramembranous particles was suggested to be due to formation of clustered ATPase oligomers within the membrane (Vanderkooi et al., 1977). This notion received earlier support from observation of cross-linked oligomers (200 and 400kDa) in solubilised membranes (Le Maire et al., 1976; Chyn and Martonosi,

1977). However, Gingold et al. (1981) suggested that the fluorescence energy transfer technique of Vanderkooi et al. (1977) provided an unreliable estimate of the oligomeric state of the Ca^{2+}-ATPase. In addition, Andersen et al. (1986) pointed out that oligomer formation in solubilised membranes depends upon the relative concentration of lipid, detergent and protein. Given the potential for methodological artifact, it remains uncertain to what extent intramembranous particles visualised from freeze-fracture replicas actually represent Ca^{2+}-ATPase moleclues (see Heegaard et al., 1990).

A pronounced morphological transition from the FSR to the JSR is observed with absence of 85 Å particles and appearance of larger and less densely packed particles on the freeze-fractured cytoplasmic leaflet (Martonosi, 1968) and immunoferritin labelled membranes (Jorgensen et al., 1983). The sharp transition between the FSR and JSR was suggested to create an intralumenal free diffusional barrier to Ca^{2+} (see Martonosi, 1984).

The close apposition of the JSR and the t-tubules forms the triad structure. Flattened surfaces of 2 cisternal sacs of the JSR flank the junctional surface of a single t-tubule. In the myocardium and invertebrate muscle, a single element of the JSR occasionally forms a junction or diad with a t-tubule (Martonosi and Beeler, 1983). The junctional gap between the t-tubule and the junctional face membrane (JFM) of JSR is approximately 100-200 Å with dense projections or "feet" connecting the cytoplasmic leaflets of the two membranous systems (Franzini-Armstrong, 1980). The feet are spaced with regular periodicity at 300 Å intervals and arranged in rows of 2 or 3 on each t-tubule face (Jewett et al., 1971). The "feet" are associated with the JSR and are attached to the fragmented JSR membrane (Campbell et al., 1980). Additional junction spanning structures or "bridges" were earlier identified after tannic acid staining and were suggested to represent phospholipid (Somlyo, 1979). Later, junctional spanning "pillars"

were observed the density of which reportedly increased with the contractile state of the muscle (Eisenberg and Eisenberg, 1982). Dulhunty (1989) has suggested that "feet", "bridges", and "pillars" represent different images of the same junctional spanning structure.

Recent evidence suggests that the junctional spanning material represents a complex of proteins including the dihydropydine receptor, the ryanodine receptor and, possibly, aldolase, phosphoglyceraldehyde dehydrogenase, and an, as yet, unidentified 95 kDa protein (Brandt et al., 1990; Kim et al., 1990). Additional features of the JSR/t-tubule region include (a) periodically spaced surface indentations on the cisternae close to the junctional gap (Dulhunty et al., 1983; Dulhunty and Valois, 1983) and (b) "tethers" which radially span the t-tubule lumen and are aligned with "feet" and calsequestrin (see later) of a pair SR cisternae. The function of the tethers is unclear although Dulhunty (1989) suggested they may function as Ca^{2+} buffers within the t-tubule lumen where total [Ca^{2+}] may be 28mM.

3. Protein Composition of SR

Mechanical disruption of muscle in salt or sucrose containing solutions fragments the SR membrane which forms sealed spherical vesicles or microsomes (mean diameter = 80-100nm). Meissner (1975) initially separated crude SR microsomes into populations of "light" and "heavy" SR vesicles. It is now well recognised that "light" SR (LSR) is composed largely of Ca^{2+}-ATPase (over 90% of the total protein) and is referable to the longitudinal and fenestrated SR. "Heavy" SR is derived from the terminal cisternae and in addition to the Ca^{2+}-ATPase (55-60% total protein) is (a) enriched in the electron dense acidic Ca^{2+} binding calsequestrin and (b) contains much of the "feet" protein, in

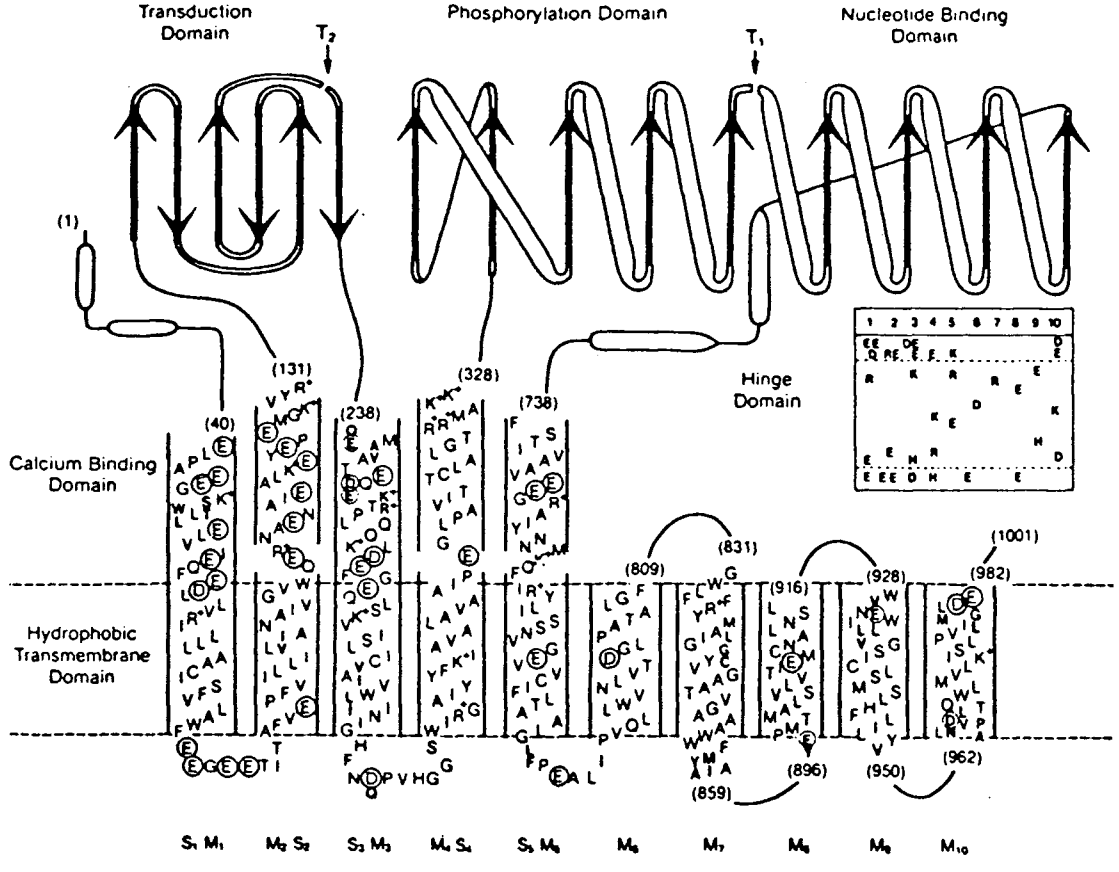
particular the ryanodine receptor. The SR proteins comprise, approximately, 65% of the microsomal dry weight (Inesi, 1981) and, in addition to the above, contain several other proteins. The best characterised of these include sarcalumenin, a 53 kDa glycoprotein, calreticulin, and a 55kDa multifunctional thyroid hormone binding protein.

a. Calcium-ATPase

Earlier studies showed that mild tryptic digest cleaves the Ca^{2+}-ATPase into 55kDA and 45kDa peptides; fragments A and B, respectively (Thorley-Lawson and Green, 1973; Inesi and Scales, 1974). Ca^{2+}-ATPase activity was preserved and both fragments remained associated with the membrane. Incorporation of ^{32}P into fragment A localised the site of ATP hydrolysis (Stewart et al., 1976). Extended digestion of fragment A resulted in 2 smaller subfragments; a 33kDa (A_1) fragment and a 22kDa (A_2) fragment. Fragment B remained intact. The ^{32}P label incorporated into the Ca^{2+}-ATPase prior to extended digest was recovered from fragment A_1. Stewart et al. (1976) sequenced fragment A_1 and identified the active site as a phosphoaspartate residue. Allen et al., (1980) deduced that the alignment of the fragments was A_2-A_1-B with A_2 possessing the N-Ac-Met terminal sequence. More recently, Garcia de Ancos and Inesi (1988) suggested that the A_1 and A_2 fragments form the Ca^{2+} binding domain of the Ca^{2+}-ATPase. Earlier, cDNA clones encoding two forms of rabbit skeletal muscle SR Ca^{2+}-ATPase were obtained (MacLennan et al., 1985). Consistent with tryptic digest studies secondary structural predictions suggested the protein possessed 3 cytoplasmic domains. MacLennan et al. (1985) identified 10 transmembrane helices linked to the bulky cytoplasmic domains via a penta-helical stalk (see Figure 3). One domain (B) was identified as the nucleotide binding site which is separate from the

Fig. 3. Structural diagram of the SR Calcium-ATPase protein (Calcium Pump). The structural prediction of the SR Ca^{2+}-ATPase is based upon hydrophobicity plots of the primary sequence. T_1 and T_2 refer to the typtic cleavage sites producing fragments A and B, and A_1 and A_2, respectively. S refers to the pentahelical stalk sectors; M refers to the membrane spanning segments (taken from Brandl et al., 1986).

Figure 3.


Transduction Domain
Phosphorylation Domain
Nucleotide Binding Domain
T2
T1
(1)
(40)
(131)
(238)
(328)
(738)
(809)
(831)
(859)
(896)
(916)
(928)
(950)
(962)
(982)
(1001)
Calcium Binding Domain
Hydrophobic Transmembrane Domain
Hinge Domain
S1 M1
M2 S2
S3 M3
M4 S4
S5 M6
M6
M7
M8
M9
M10

phosphoaspartyl site (A_1) located upon an adjoining domain. The third cytoplasmic domain (A_2) was suggested to be a transduction domain as cleavage at the second tryptic site (located within this domain) uncoupled Ca^{2+} transport from ATPase activity which remains intact. The penta-helical stalk was suggested as the high affinity Ca^{2+} binding site due to enrichment of amphipathic α helical segments with polar glutamate residues. Very little of the protein is located upon the lumenal face of the membrane. However, on the loop between the first and second transmembrane helices a group of 4 glutamic acid residues was suggested by MacLennan et al. (1985) to be the low affinity Ca^{2+} binding site. More recently Leberer et al. (1990) suggested that these residues may form a Ca^{2+} bridging structure with the acidic COOH domain of sarcalumenin (see below). Site directed mutagenesis of several polar transmembranous residues revealed important contributions of 3 glutamic acids, one aspartate, one asparagine and one threonine residue in putative transmembrane segments M4, M5, M6 and M8 to the formation of high affinity Ca^{2+} binding sites of the Ca^{2+}-ATPase (Clarke et al., 1990a). In addition, substitutions at Thr 181, Gly 182, and Glu 183 within the β-strand domain indicated that these residues were essential for E_1P to E_2P conformational transitions (Clarke et al., 1990b).

b. Ryanodine Receptor

Cadwell and Caswell (1982) initially suggested that the high molecular weight SR proteins (300-325kDa), resolved by SDS-PAGE, possibly represented the components of the "feet" or "pillar" structures that had been identified earlier (Franzini-Armstrong, 1970; Eisenberg and Eisenberg, 1982). Seiler et al. (1984) later showed that these proteins bound calmodulin, were phosphorylated by

cAMP and calmodulin, dependent protein kinases and were degraded by calcium activated neutral protease (CANP).

Kawamoto et al. (1986) were the first to purify the high molecular weight protein (~300kDa) from Zwittergent (3-14) detergent solubilised terminal cisternae vesicles and to recognise that protein dissolution of vesicles required moderate to high salt concentrations. With affinity purified antibodies the 300kDa (spanning) protein was localised by immulogold staining of the tissue sections and rotary shadowed isolated protein to the junctional face membrane between the terminal cisternae and the t-tubules. This was rapidly followed by reports demonstrating that the 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulphonate (CHAPS) solubilised high molecular weight protein bound ryanodine specifically in skeletal (Lai et al., 1987; Inui et al., 1987) and cardiac (Inui et al., 1987) muscle. Later, Imagawa et al.(1987) demonstrated that this protein , solubilised in digitonin, possessed single channel activity in planar bilayers consistent with it's identity with the Ca^{2+} permeable pore of the Ca^{2+} release channel. This was later confirmed by Lai et al. (1988a,b), Hymel et al. (1988), Smith et al. (1988), Anderson et al. (1989). Subsequent studies have shown that the functional ryanodine binding Ca^{2+} channel complex is a 30S quatrefoil structure composed of four identical 9S monomers (Lai et al., 1989). Each leaflet of the 30s particle represented a single ~450kDa protein that self associated cooperatively to form a Ca^{2+} conducting homotetramer.

Complimentary DNA for the rabbit form of the protein has been sequenced and expressed (Takeshima et al., 1989). These studies deduced a protein sequence of 5,037 amino acids and a molecular weight of 565,223. Later, Zorzato et al. (1990) obtained cDNA clones for human and rabbit forms of the ryanodine receptor in which the former encoded for a protein of fewer amino acids (5032) and lower molecular weight (563,584). Otsu et al. (1990) then obtained a cDNA

clone encoding the rabbit cardiac form of the release channel with slightly lower molecular weight than the skeletal form (4,969 residues; Mr=564,711). A 66% sequence homology between the skeletal and cardiac proteins was reported (Otsu et al., 1990). Major differences were noted in the presence of a highly acidic glutamate rich domain (residues 1872-1923) in the skeletal form which were not well conserved in the cardiac form. Imagawa et al. (1989) showed that the cardiac form was immunologically distinct from isoforms expressed in fast-twitch and slow-twitch muscle. In both skeletal and cardiac forms a regulatory domain was identified that contained potential binding sites for calmodulin and nucleotides (Zorzata et al., 1989; Otsu et al., 1990). These domains were found to lie within a protease-sensitive region of the receptor (Marks et al., 1990). Otsu et al. (1990) suggested that the dispersed sequence differences noted between the two proteins reflects the fact that they represent different gene products. Mackenzie et al. (1989) showed that the human cardiac form is derived from chromosome 1 as opposed to the skeletal form located on chromosome 19.

For both the cardiac and skeletal receptor protein secondary structural predictions revealed the presence of 12 potential transmembranous segments (Zorzato et al. 1989; Otsu et al., 1990). This is different from earlier predictions (Takeshima et al., 1989) of only 4 transmembrane segments. Negatively stained images of the purified receptor revealed the presence of protein "loops" surrounding a low density region or cavity (2-4nm) associated with each leaflet of the quatrefoil (Lai et al, 1989; Anderson et al., 1989). These were identified as "peripheral vestibules" (Wagenknecht et al., 1989). The apposition of each leaflet formed a "central channel" (Wagenknecht et al., 1989) of 12-14nm in diameter (Lai et al., 1989). Wagenknecht et al (1989) report apparent "radial channels" emanating from the central cavity and connecting with the "peripheral vestibules". These authors proposed that these structures may represent

pathways for Ca^{2+} release. However, Liu et al. (1989) argued that the cavities within each leaflet were too large to constitute ion channels. The pathway for Ca^{2+} release within the ryanodine receptor molecule remains, therefore, to be elucidated.

A [^{3}H]ryanodine binding protein (~400kDa) was recently isolated from crude rabbit brain membranes (McPherson and Campbell, 1990). This protein sedimented as an apparent homotetramer (30s) in sucrose gradients and was immunologically cross-reactive to monoclonal antibodies raised against the rabbit skeletal muscle (fast-twitch) isoform. The precise tissue distribution of this brain protein is uncertain. However, it raises the intriguing possiblity that a Ca^{2+} control mechanism similar to that found in muscle also operates in brain.

c. Calsequestrin

Calsequestrin, one of the major accessorial proteins of the HSR, is an acidic glycoprotein (Maclennan and Campbell, 1979) that binds Ca^{2+} with variable affinity that is dependent upon ionic strength (Kd=1mM at 100mM KCl) (MacLennan and Wong, 1971). Calsequestrin is localized within the terminal cisternae upon the lumenal surface (Hidalgo and Ikemoto, 1977) and is thought to be a major intralumenal Ca^{2+} buffer with a Ca^{2+} binding capacity of 40-50 mol Ca^{2+} per mol protein (Reithmeier et al., 1987). Jorgensen and Campbell (1984) showed that calsequestrin was also present in non-junctional regions of the SR (corbular SR) that appeared to be specialised regions of the fenestrated SR localised to the I-band of chicken ventricular muscle. The concentration of calsequestrin within the cisternae was estimated at 100mg per ml internal cisternal volume (Williams and Beeler, 1986) such that at saturation calsequestrin can bind 100mM Ca^{2+} (or 1000nmol.mg-1 protein). The cDNA for skeletal muscle calsequestrin encodes a protein of 367 residues with a molecular weight

of 42,445 (Zarain-Herzberg et al., 1988). The complete amino acid sequence has also been obtained for the cardiac isoform with a deduced molecular weight of 45,269 and 65% sequence homology with the skeletal form (Scott et al., 1988). Apparent molecular weight estimates derived from alkaline gels may be as high as 63,000 in skeletal muscle and 55,000 in cardiac muscle. This is attributable to the highly acidic character of this protein. Cozens and Reithmeier (1984) reported that calsequestrin is highly asymmetric with an extended structure created by the electrostatic repulsion amongst neighbouring negative charges. In addition, calsequestrin reportedly underwent large conformational transitions from an extended structure in the absence of Ca^{2+} to a compact, globular structure in it's presence (Cozens and Reithmeier, 1984). At physiological concentrations of calsequestrin, however, secondary structural transitions in solution were relatively invariant with alterations in Ca^{2+} and ionic environment (Williams and Beeler, 1986). On the other hand, Williams and Beeler (1986) showed that the crystalline structure of calsequestrin was sensitively dependent upon ionic conditions. Recent evidence suggests that calsequestrin may belong to a multigene family with remarkable homology to a putative laminin-binding protein, apartactin (Yazaki et al., 1990). In this regard Fliegel et al. (1987) identified the presence of a Ser-Glu-Glu sequence which is found in sarcalumenin, 53kDa glycoprotein, and the, recently identified, 165kDa histidine-rich calcium and low density lipoprotein binding protein (HCP) of sarcoplasmic reticulum (Hofmann et al., 1989) together with troponin C and calmodulin (Fliegel et al., 1990). This motif appears to be important in protein-protein interactions (Earnshaw, 1987).

d. Calreticulin

Earlier known as the 55kDa high affinity Ca^{2+} binding protein (HABP), calreticulin was found to bind 1 mol Ca^{2+}/mol protein with high affinity (Kd=3μM) and 25 mol Ca^{2+}/mol protein with low affinity (MacLennan et al., 1972; Ostwald and MacLennan, 1974). Recently, calreticulin was cloned with cDNA encoding for a protein of unexpectedly lower molecular weight (Mr=46,567) than was earlier determined from both acidic and alkaline gels (Fliegel et al., 1989). The carboxyl terminus of calreticulin is highly acidic and may represent the Ca^{2+} binding domain (Fliegel et al., 1989). The function of calreticulin is unknown although the KDEL (Lys-Asp-Glu-Leu) structural motif at the COOH terminal suggests that it may be associated with protein retention within the SR lumen (Fliegel et al., 1989, 1990). Calreticulin was shown to be localised to the lumen of the terminal cisternae and is structurally homologous to Calregulin, an ER associated protein (Fliegel at al., 1989).

e. 53kDa Glycoprotein

Using Laemmli (Laemmli, 1970) gels to separate and identify possible contaminants within the 55kDa band of Weber and Osborne gels (Weber and Osborn, 1969), Michalak et al. (1980) discovered a glycoprotein with an apparent molecular weight of 53,000. Unlike the glycoprotein of the Na^{+}+K^{+}-ATPase, the 53 kDa protein of purified SR did not form a tight association with the Ca^{2+}-ATPase. The 53 kDa glycoprotein was later purified and shown to be transmembranous owing to it's reactivity with concanavalin A in leaky vesicles and cycloheptaamylose-fluorescamine complex in sealed vesicles (Campbell and MacLennan, 1981). Recently, cDNA clones were obtained that encoded a protein of 453 residues and a molecular weight of 52,421 (Leberer et al., 1989). The protein was found not to regulate ATPase activity with little evidence of Ca^{2+} binding properties. The function of this protein remains unclear although the

COOH terminal is identical to that of the Ca^{2+} binding sarcalumenin (see below). This together with the co-localisation of the 53kDa protein with sarcalumenin led Leberer et al. (1990) to suggest that this protein may be involved with Ca^{2+} sequestration in non-junctional SR.

f. Sarcalumenin

Sarcalumenin is a 160kDA glycoprotein that was found to be immunologically related to the 53kDa glycoprotein (Leberer et al., 1989). cDNA clones obtained by Leberer et al. (1989) encoded an unprocessed protein of 889 residues, substantially less than would account for a protein of 160kDa. Transfection of COS-1 cells with the cDNA resulted in expression of the mature 160kDa protein. The COOH terminal was found to be highly acidic and to bind 400nmol Ca^{2+}/mg protein with intermediate affinity (Kd=300-600μM) depending upon ionic strength (Leberer et al., 1990).

g. 55kDa Thyroid Hormone Binding Protein

The presence of 55kDa multifunctional thyroid hormone binding protein (T_3BP) in cardiac and skeletal SR membranes was indicated from earlier studies (Fliegel et al., 1989). Anti-calreticulin antibodies were found reactive to the COOH terminus of T_3BP and enabled isolation of full length cDNA clones for T_3BP (Fliegel et al., 1990). The COOH terminus was found to highly acidic and contained a variant, RDEL (Arg-Asp-Glu-Leu), of the KDEL retention signal found in several heat shock and glucose regulated proteins.

4. SR Calcium Uptake

The function of SR in cardiac and skeletal muscle is to provide both a source and sink for Ca^{2+} that, upon binding to troponin-C, stimulates

myofilament shortening and therefore muscle contraction. The release of Ca^{2+} occurs from the terminal cisternae in response to t-tubule membrane depolarisation. Relaxation of muscle occurs via Ca^{2+}-ATPase mediated Ca^{2+} uptake into the longitudinal or FSR.

a. Ca^{2+} transport

Ca^{2+} transport by the Ca^{2+}-ATPase occurs with the binding of both the translocated Ca^{2+} ion and the Mg·ATP to specific sites located upon the cytoplasmic face of the enzyme (Weber et al., 1966). Ca^{2+} binding, which has been studied with native and reconstituted vesicles and purified Ca^{2+}-ATPase, occurs with high affinity in the absence of ATP (Meissner, 1973: Ikemoto, 1974; Chiu and Hayes, 1977). Three classes of Ca^{2+} binding sites were identified in the purified enzyme (Ikemoto, 1975). At $0^{o}C$ all three binding sites (α,β,γ classes) were expressed with association constants (Ka) and number of sites (n) per class as follows: α sites ($Ka=3x10^{6}M^{-1}$, n=1);β sites ($Ka=5x10^{4}M^{-1}$,n=1); γ sites ($Ka=10^{3}M^{-1}$, n=3). Above $22^{o}C$, however, the number of α sites increased to 2 with suppression of β site binding and maintenance of low affinity of γ site binding. Inesi et al. (1980) showed that Ca^{2+} binding to the high affinity sites of Ca^{2+}-ATPase is cooperative with a derived Hill coefficient of 1.82 and apparent Ka of $2.3x10^{6}M^{-1}$. Ca^{2+} binding to the external high affinity sites of the Ca^{2+}-ATPase induced a slow conformational changes in the protein (Pick and Karlish, 1980) and was considered to be one of the rate limiting steps of the catalytic cycle (Dupont, 1977; Pick and Karlish, 1982).

Mg·ATP is the true substrate for translocation of Ca^{2+} (Vianna, 1975). ATP binds to the transport enzyme with high affinity (Meissner, 1973) only as a Mg.nucleotide complex (Hasselbach et al. 1981). Hasselbach et al. (1981) suggested that Mg^{2+} functions both as an ionic cofactor and a charge

compensator for the polyphosphate residues of the unliganded nucleotide substrate. The resulting Mg-ATPase-enzyme complex is formed by random binding of the cofactor and substrate to the translocator. Ca^{2+} and Mg·ATP bind in a molar ratio of 2 as determined from Hill plot coefficients under optimal conditions (Tada et al., 1978). 8-9nmol Ca^{2+} can bind per mg of Ca^{2+}-ATPase with 4-4.5 nmol phosphoenzyme (EP) formed with each turnover of the pump (Inesi et al., 1982). Ca^{2+} and Mg·ATP binding was earlier thought to occur randomly as implied from the apparent lack of allosteric effect of the two binding sites (Vianna, 1975). However, Chiu and Haynes (1980) and Pick and Karlish (1982) showed that Ca^{2+} occupation of the high affinity sites prior to substrate binding is crucial for formation of the influx stabilized form of the enzyme. Inesi et al. (1980), and Martonosi and Beeler (1983) suggested that the active quaternary complex may occur with successive Ca^{2+} binding steps which precede and follow Mg·ATP binding.

Formation of the active quaternary complex leads to hydrolysis of ATP where γ phosphate is covalently attached to Asp 351 (De Ancos and Inesi, 1988) with formation of an acid stable, alkaline labile E_1P enzyme (see Figure 3). Ca^{2+} translocation is coincident conversion of an ADP-sensitive E_1P to an ADP-insensitive E_2P (Takisawa and Makinose, 1983). The energy of this isomerisation step is transferred into osmotic work and the creation of an inward Ca^{2+} gradient associated with reduction of Ca^{2+} affinity (Chiu and Haynes, 1980; Hasselbach et al., 1981). Following Ca^{2+} translocation E_2P decays with liberation of Pi and formation of E_2. The cycle is then repeated with binding of Ca^{2+} and Mg.ATP (Kawashima et al., 1990).

b. Calcium Pump Regulation

(i). Calcium

Under optimal conditions of Mg·ATP, pH and temperature, rapid Ca^{2+} influx in SR vesicles, is stimulated at Ca^{2+} concentrations above 0.01μM with maximal Ca^{2+} influx occurring in the presence of 1-10μM Ca^{2+} (Weber et al., 1963; Hasselbach, 1979). External Ca^{2+} concentrations above 29μM were found to inhibit Ca^{2+} accumulation (Chiu and Haynes, 1980). Although prior occupation of the external high affinity Ca^{2+} binding sites stabilizes the Ca^{2+}-ATPase in it's influx mode (Chiu and Haynes, 1980) rising Ca^{2+} concentrations form an inhibitory Ca-ATP complex (Kanazawa et al., 1971; Hasselbach et al., 1981). Ca-ATP, under particular conditions may serve as a substrate for the Ca^{2+}-ATPase (Wakabyashi and Shigekawa, 1984) although the partial catalytic reactions were considerably slower than with Mg·ATP as the substrate (Shigekawa et al., 1983).

Elevated intralumenal Ca^{2+} (~20mM), inhibits Ca^{2+} accumulation (Yamada et al., 1972). The inhibitory mechanism is uncertain although stimulation of Ca^{2+}-ATPase mediated Ca^{2+} efflux through low affinity site binding of Ca^{2+} may occur at elevated intralumenal Ca^{2+} (Hasselbach and Waas, 1982).

(ii). Magnesium

Magnesium regulation the of Ca^{2+}-ATPase is complex. The complex of Mg^{2+} and ATP is the physiological substrate for the Ca^{2+}-ATPase (Weber et al. 1966; Makinose and Boll, 1979) with the cation acting, possibly, as an ionic cofactor or change compensator for the nucleotide (Hasselbach et al., 1981).

Mg^{2+} is thought to remain bound or in an occluded state (EDTA inaccessible) upon initial EP formation (Hasselbach and Oetliker, 1983) and has been shown to activate the reversible transphosphorylation from ATP to the enzyme (Hasselbach et al., 1981). Mg^{2+} accelerated the E_1P to E_2P transition

(Shigekawa and Dougherty, 1978) and was essential for the phosphorylation of the Ca^{2+}-ATPase by Pi (Masuda and DeMeis, 1973) and enzyme dephosphorylation as monitored through Pi-H_2O-exchange (Kanazawa and Boyer, 1973). Mg^{2+} may act a single site although evidence is inconclusive (see Makinose and Boll, 1979; Yamada and Tonomura, 1972; Shigekawa et al., 1983; Takakuwa and Kanazawa, 1982)

Millimolar concentrations of Mg^{2+} (1-5mM) stimulate both Ca^{2+} influx and efflux, with Ca^{2+} influx inhibition at elevated Mg^{2+} (10mM) (Froelich and Taylor, 1976). Mg^{2+} inhibition may due to Mg^{2+} competition with Ca^{2+} for binding (Yamamoto et al., 1979; Haynes, 1983)

(iii). ATP

The vesicular form of the Ca^{2+}-ATPase is non-linearly dependent upon ATP for both Ca^{2+} transport and ATPase activity (Martonosi and Beeler, 1983). Mg·ATP, in addition to substrate provision (Hasselbach et al., 1981), stimulates a secondary activation of the Ca^{2+}-ATPase at higher substrate concentrations. Up to 100μM Mg·ATP, hydrolysis is stimulated with first order kinetics. Above 0.1mM Mg·ATP a secondary activation of ATP hydrolysis is observed (McIntosh and Boyer, 1983) with two distinct Km values of 2-3μM and 500 μM (Moller et al., 1980). The biphasic activation of the Ca^{2+}-ATPase indicates the presence of 2 classes of Mg·ATP binding sites (Hasselbach, 1979). The high affinity site is the catalytic site whereas the low affinity site has a regulatory function (Dupont, 1977). Chiu and Haynes (1980) and Haynes (1983) proposed that these effects were mediated through a single Mg·ATP binding site of variable affinity that was dependent upon cationic concentration.

5. SR Calcium Release

a. Overview

Muscle contraction in skeletal and cardiac muscle results from relief of troponin-I inhibition of myosin and actin interaction through Ca^{2+} binding to troponin-C (Leavis and Gergely, 1984). The source of Ca^{2+} is the terminal cisternae of the sarcoplasmic reticulum. In response to a depolarising action potential propagated along the t-tubule the SR releases Ca^{2+} via the Ca^{2+} permeable pore of the ryanodine receptor with a first order rate constant between 50 and 100 s^{-1} observed in frog skeletal muscle (Baylor et al., 1983; Melzer et al., 1984). However, the mechanism by which the electrical signal leads to the triggering of Ca^{2+} release remains elusive.

b. Triggering Mechanism of SR Calcium Release.

Two main hypotheses have been forwarded to account for the triggering of Ca^{2+} release in both skeletal and cardiac muscle. The first proposes that a mechanical coupling between the dihydropyridine receptor of the t-tubules and the ryanodine receptor of the terminal cisternae mediates a non-linear charge transfer across the t-tubule/SR junction. The second proposes that Ca^{2+} release is stimulated by transient local increases in the production of second messengers in response to t-tubule depolarisation (e.g. Ca^{2+} and/or IP_3).

(i). Charge Movement Triggered Calcium Release

The charge movement theory is based upon the earlier observation (Schneider and Chandler, 1973) of extra outward and inward currents measured after voltage pulses were applied to voltage clamped frog sartorius single muscle fibres. Three key features of the charge movement were that i) the total charge

movement during excitation and recovery was conserved but of opposite sign ii) the amount of charge was saturable and sigmoidally related to the voltage pulse and iii) the rate constants of the outward ("on" pulse) and inward ("off" pulse) charge transients were strongly voltage dependent (range 179-249 s^{-1} at 80mV). Charge movement was not, therefore, rate limiting and could account for the strong voltage dependence of SR Ca^{2+} release. In addition the results implicated movement of a fixed number of charges rather than movement of intra- of extracellular ions. Chandler et al. (1976) proposed that charge movement occurred between membrane bound charged groups which act either as a re-orientable dipole or can move in response to changes in membrane electric field. Schneider and Chandler (1973) calculated that the amount of charge movement was consistent with the density distribution (700 per μ^2) of the t-tubule/SR spanning feet protein (Franzini-Armstrong, 1970).

Later Rios and Brum (1987) implicated the involvement of the L-type Ca^{2+} channel/dihyropyridine receptor in mediating charge movement through demonstration of reduced charge movement and SR Ca^{2+} release in response to nifedipine at concentrations consistent with the affinity (kd=32nm) of the receptor for dihydropyridines. Similar effects upon contractility (Eisenberg et al., 1983) and charge movement (Hui et al., 1984) inhibition were also observed with A-600, a related dihydropyridine compound (Morad et al., 1983). Further evidence that the skeletal muscle receptor is the voltage sensor mediating intramembrane charge movement arises from demonstration that expression of skeletal muscle cDNA in dysgenic myotubes restores normal skeletal type E-C coupling (Tanabe et al., 1988, 1990; Adams et al., 1990). The critical regions appear to reside within the Ca^{2+} conducting α_1 subunit (Pelzer et al., 1990) upon the proposed cytoplasmic domain between repeats 2 and 3 of the protein (Tanabe et al., 1990). Fill et al. (1988,1990) also observed non-linear charge

movement and capacitance properties associated with the voltage dependence of the ryanodine receptor open time in channels incorporated into Mueller-Rudin bilayers. Futhermore, Ma et al. (1988) have demonstrated gap junction properties associated with the voltage dependence of the receptor open state probability. Such properties would be expected of a channel sensitive to "gating" currents. These studies provide some evidence toward a molecular basis for Schneider and Chandler's (1973) original hypothesis.

(ii). Calcium-Induced Calcium-Release

The phenomenon of regenerative or Ca^{2+}-induced Ca^{2+}-release (CICR) was first demonstrated by Ford and Podolsky (1970) and Endo et al. (1970) using skinned (split) fibre preparations from frog skeletal muscle. Numerous studies have since demonstrated the presence of this phenomenon in skinned fibres and isolated vesicles from both cardiac and skeletal muscle. Ca^{2+} release from skeletal and cardiac SR vesicles was maximally stimulated by 5-10μM Ca^{2+} in the presence of 5mM adenosine 5'-(β,γ-methylene) triphosphate (Meissner et al., 1986; Meissner and Henderson, 1987) with release rate constants (30-100 s^{-1}) compatible with physiological Ca^{2+} release. In the presence of nucleotides (AMP-PCP) and Mg^{2+}, channel mediated Ca^{2+} efflux was cooperatively dependent upon trigger Ca^{2+} (Meissner et al., 1986). In skeletal muscle the apparent affinity for trigger Ca^{2+} binding is approximately $10^5 M^{-1}$ (see Ikemoto et al., 1989). Amounts of Ca^{2+} release rather than release rate constants were a graded function of intralumenal Ca^{2+} loads (Kim et al., 1983).

Endo (1977) has consistently argued, based upon earlier observations the CICR may not constitute a physiological mechanism of Ca^{2+} release, in skeletal muscle at least, due to it's observation under apparently unphysiologically exaggerated conditions (elevated Mg^{2+}, high intralumenal Ca^{2+}). Fibre studies have

indicated (see Winegrad, 1982) that despite the regenerative nature of CICR, this form of Ca^{2+} release does not produce maximal contractions. Furthermore, Stanfield and Ashcroft (1982) concluded that inward Ca^{2+} currents were too slow to account for the rate of E-C coupling. However, Stephenson (1982) proposed that earlier fibre studies erroneously assumed tight control of myofilament space Ca^{2+} by bath Ca^{2+}-EGTA buffers. Without adequate control of Ca^{2+} within the immediate vicinity of the SR, particularly within unstirred layers, description of the physiological significance of CICR is limited.

The significance of CICR in cardiac muscle is more accepted due to the apparent increased sensitivity to trigger Ca^{2+} in this tissue with low Ca^{2+} pre-loading of the SR (Fabiato and Fabiato, 1975). Futhermore it was shown that an increase in free Mg^{2+} up to a presumed physiological level (3.16mM) actually potentiated the amplitude of SR Ca^{2+} release in skinned cardiac cells and cat caudofemoralis muscle (Fabiato, 1981, 1982). Fabiato (1983) argued that the gating stimulus is more a function of the rate of change of the free Ca^{2+} rather than the Ca^{2+} concentration per se. It was suggested that failure to account for this in previous studies with skeletal muscle fibres may explain why CICR has been poorly demonstrated in this model.

Assessment of the physiological role of CICR from studies upon vesicles depends critically upon estimates of intracellular free Mg^{2+}. Estimates using different metallochromic dyes varied between 200μM and 6mM free [Mg^{2+}] depending upon assignments of intracellular pH (Baylor et al., 1982). In vesicle studies (Meissner et al., 1986) Mg^{2+} concentrations approximating those found intracellularly (0.2-4mM) reduced significantly the first order rate constant (at 5μM Ca^{2+}) for CICR although rapid releases were still observed. The conditions under which releases were observed in vitro (22 ^{o}C) may have underestimated the true release rates in vivo at 37 ^{o}C.

More recent studies with intact cardiac cells using fluorometric dyes Indo-1 and Fura-2 demonstrated that the voltage dependent Ca^{2+} current (I_{Ca}) from L-type channels is well correlated with the transient rise of myoplasmic Ca^{2+} (Ca^{2+} transient) released from the SR (Callewaert et al., 1988; Barcenas-Ruiz and Wier, 1987; Beucklemann and Weir, 1988; Nabauer et al., 1989). These observations clearly support a mechanism of Ca^{2+} induced Ca^{2+} release in the heart. However, recent studies (Cannell et al., 1987; Cohen and Lederer, 1987, 1988; Lederer et al., 1989) have demonstrated that the voltage dependent peak Ca^{2+} transient precedes the voltage dependent I_{Ca} indicating stimulation of Ca^{2+} release prior to activation of trigger Ca^{2+} flux. These observations which support the involvement of charge movement have been criticised (see Fabiato, 1989) on the basis of potential methodological artifacts triggering cardiac SR Ca^{2+} release. Therefore, it remains unclear whether SR Ca^{2+} release from either cardiac or skeletal muscle involves a single triggering mechanism (i.e. CICR vs charge movement) or whether SR Ca^{2+} release is triggered by both mechanisms in a highly complex manner. Recently, Nesterov (1989) has postulated that an initial Ca^{2+} release from channels within the immediate vicinity of the t-tubule/SR junction is dependent upon ion or charge movements may initiate a regenerative or Ca^{2+} induced Ca^{2+} release from more laterally disposed Ca^{2+} channels.

c. Modification of Calcium Release

(i). Nucleotides

Rapid kinetic studies of Ca^{2+} release (Meissner et al., 1986; Morii et al., 1986; Moutin and Dupont, 1988) from SR vesicles passively loaded with Ca^{2+} demonstrated up to 25 fold increases in the release rate constant in the presence of adenine nucleotide (5mM AMP-PCP or ATP). In the cardiac SR, Ca^{2+}

triggered release rates were stimulated by over 2 orders of magnitude in the presence of nucleotide (Meissner and Henderson, 1987). The amount of Ca^{2+} triggered release of Ca^{2+} was relatively unaffected by nucleotide which stimulated release in the following order of effectiveness; AMP-PCP > cAMP > AMP > ADP > adenine > adenosine (Meissner, 1984). With depolarisation induced Ca^{2+} release, nucleotides elevated amounts of release without effects upon the first order rate constant (Kim et al., 1983; Ikemoto et al., 1984, 1985). The effects of nucleotides appear to be mediated by direct action upon the channel itself (Lui et al., 1989).

(ii). Extravesicular Calcium

Ca^{2+} release induced by t-tubule depolarisation was shown not to require extravesicular Ca^{2+} in both fibre studies (Endo and Nakajima, 1973; Stephenson, 1981) and vesicle studies (Ikemoto et al, 1985). However, the Ca^{2+} stimulation of depolarisation and Ca^{2+} induced release was shown to be similar (Ikemoto et al., 1985) exhibiting a bell shaped dependence upon extravesicular Ca^{2+} between pCa 6 to 5 in agreement with similar reports (Moutin and Dupont, 1988; Meissner et al., 1986). These observations indicated that both forms of release were mediated by the same Ca^{2+} channel (see Ikemoto et al., 1989).

(iii). Drugs

In numerous studies with whole cells, peeled fibres, vesicles and release channels incorporated into bilayers, a variety of different drugs have been shown to activate SR Ca^{2+} release directly.

Caffeine was earlier shown to stimulate Ca^{2+} release at sub-threshold cytosolic Ca^{2+} (Ogawa, 1970, Weber and Hertz, 1968) and to decrease the Ca^{2+} threshold for Ca^{2+}-induced Ca^{2+} release (see Endo, 1977). In both cardiac (Meissner and

Henderson, 1987) and skeletal vesicles (Katz et al., 1977; Kim et al., 1983; Su and Hasselbach, 1984; Rousseau et al., 1988) caffeine stimulated Ca^{2+} release with a reported Km value of 200 μM (Kim et al., 1983). Recent evidence suggested that a 170kDa glycoprotein may be a common receptor for Ca^{2+} and caffeine mediated effects (Rubtsov and Murphy, 1988). This is at variance with a direct effect of caffeine to increase the open state probability of isolated channels incorporated into bilayers (Lui et al., 1989).

Quercetin, an inhibitor of the Ca^{2+}-ATPase (Shoshan et al., 1980; Shoshan and MacLennan, 1981; Gilchrist et al., 1990) has been shown to stimulate (Palade et al., 1983) SR Ca^{2+} release (Palade, 1987; Ikemoto et al., 1983) at low concentration (Km ~3μM).

Doxorubicin (Adriamycin) was also shown to stimulate unidirectional Ca^{2+} efflux (8 fold) with inhibition of SR Ca^{2+} uptake and greater than 2 fold stimulation of Ca^{2+} dependent ATPase activity (Zorzato et al., 1985). Part of the cardiotoxic effects of doxorubicin (Caroni et al., 1981) may be due, in part, to its' action upon the SR Ca^{2+} release channel.

Another drug with toxic effects upon skeletal, cardiac and smooth muscle is the alkaloid ryanodine. Ryanodine exhibits complex inhibitory and stimulatory effects upon SR Ca^{2+} release channel function (Jenden and Fairhurst, 1969). It is now known that ryanodine binds to the release channel at both low and high affinity sites the expression of which is dependent upon assay conditions (see Lai and Meissner, 1989). Ryanodine binding affinity is elevated by micromolar Ca^{2+}, and millimolar concentrations of adenine nucleotides and caffeine (Lai and Meissner, 1989; Lai et al., 1989; Chu et al., 1990). Ryanodine binding was also dependent upon osmolarity with optimum binding observed in the presence of 1M NaCl (or KCl) (Pessah et al., 1987; Inui et al., 1987; Michilak et al., 1988; Chu et al., 1990; Ogawa and Harafuji, 1990).

The correspondence between ligand effects upon ryanodine binding and the Ca^{2+} releasing behaviour of the SR suggest that ryanodine binds preferentially to the open state Ca^{2+} channel (Pessah et al., 1986; Lai et al., 1989; Chu et al., 1990). Consequently, ryanodine binding to SR membranes and ryanodine effects upon Ca^{2+} fluxes may reflect Ca^{2+} channel conformation(s). The inhibitory effects of ryanodine upon Ca^{2+} uptake in sarcotubular fragments (crude SR) were first observed by Fairhurst and Jenden (1966) and Jenden and Fairhurst (1969). Fairhurst and Hasselbach (1970) observed that ryanodine (100μM) preferentially increased Ca^{2+} efflux from the heavy SR Ca^{2+} loaded fraction with increased Ca^{2+}-ATPase activity. More recently, studies upon junctional SR vesicles and Ca^{2+} channels incorporated into bilayer have demonstrated that with low micromolar concentrations, at least, ryanodine inhibits the ATP dependent uptake of Ca^{2+} (Lattanzio et al., 1987; Fleischer et al., 1985 and Inui et al., 1988) passive $^{45}Ca^{2+}$ loading of vesicles (Meissner, 1986) and maintains the open state of the high conductance Ca^{2+} channel (Nagasaki and Fleischer, 1988; Lai and Meissner, 1989). These effects have been attributed to high affinity ryanodine binding resulting in maintenance of open channel states (Fleischer et al., 1985). The Ca^{2+} releasing action of ryanodine was also observed by Hansford and Lakatta (1987) in cardiac myocytes.

Contrary effects of ryanodine upon SR function (i.e. inhibition of Ca^{2+} channel opening or Ca^{2+} release) have also been reported. Within smooth muscle preparations ryanodine (~10μM) was shown to diminish depolarisation (Marban et al., 1985; Sutko and Kenyon, 1983), K^+ and norepinephrine (Ashida et al., 1988; Iko et al, 1986), and caffeine and carbachol evoked contractures (Iino et al., 1988; Iko et al., 1986). These observations parallel similar ryanodine effects upon tension and Ca^{2+} transients in cardiac and purkinje fibres (Cannell et al., 1985; Wier et al., 1985) and skinned cardiac cells (Fabiato, 1985; Sutko et al., 1985).

Ryanodine also markedly stimulated Ca^{2+} accumulation and inhibited Ca^{2+} release in heavy SR vesicles derived from the terminal cisternae of cardiac and skeletal muscle (Jones and Besch, 1979; Jones et al., 1979; Chamberlain et al., 1983; Hasselbach and Migala, 1986; Meissner, 1986 and Lattanzio et al., 1987).

Meissner (1986) suggested the contrary effects of ryanodine on SR Ca^{2+} handling may reflect differential action of more than one ryanodine binding site. Moreover, the expression of these effects was dependent upon temperature, assay conditions and time of exposure to ryanodine (Meissner, 1986; Lattanzio et al., 1987). Although it is clear that Ca^{2+} influences the expression of the number of ryanodine binding sites (Lai et al., 1989) it is unclear whether this reflects an effect of Ca^{2+} upon extraluminal or intraluminal Ca^{2+} sites.

(iv). Magnesium

Numerous studies have shown that at millimolar (1-10mM free Mg^{2+}) concentrations (Meissner, 1984; Meissner et al. 1986; Meissner and Henderson 1987; and Gilchrist, et al., 1990) inhibits the release of Ca^{2+} from cardiac and skeletal SR vesicles. Similarly, Mg^{2+} reduced the open state probabilities of purified and vesicle associated channels incorporated into bilayers (Liu et al., 1989; Rousseau et al., 1986; and Smith et al., 1985). The mechanism by which Mg^{2+} acts remains unclear (Meissner et al., 1986) although the above studies indicate a direct effect upon the release channel.

(v). Anaesthetics

In skinned skeletal fibre studies procaine (Thorens and Endo, 1975; Ford and Podolsky, 1970) and tetracaine (Thorens and Endo, 1975) inhibited Ca^{2+} induced Ca^{2+} release and caffeine induced (Feinstein, 1963) contractures. Similar inhibition of CICR was observed in isolated cardiac and skeletal muscle vesicles

with SKF 525-A and procaine (Chamberlain et al., 1984) and tetracaine (Ohnishi, 1979; Antoniu et al., 1985; Morii et al., 1986; Meissner and Henderson, 1987).

Conversely, halothane has been shown to activate SR Ca^{2+} release directly at subthreshold extraluminal Ca^{2+} levels required for CICR (Ohnishi, 1979; Ohnishi et al., 1983; Kim et al, 1984, Palade, 1987) and to increase both the amount and rate constant for CICR from skeletal SR vesicles (Kim et al., 1984; Mickelson et al., 1986, 1988). In addition, low doses of halothane (0.47-1.89mM) reversibly stimulated SR Ca^{2+} efflux directly in chemically skinned rat tuberculae (Herland et al., 1990). The mechanism of anaesthetic mediated effects are unknown although a hydrophic interaction with some component of the release channel complex has been suggested (Chamberlain et al., 1984). The hypersensitivity of Malignant Hyperthermia (MH) patients to halothane and the decreased intraluminal Ca^{2+} requirement for both halothane induced and Ca^{2+} induced release of SR Ca^{2+} together with the probable mutation of the MH SR Ca^{2+} release channel (MacLennan et al., 1990; Knudson et al., 1990; Zorzato et al., 1990) suggests that halothane interacts with an important aspect of the triggering mechanism of the Ca^{2+} release channel.

(vi). Organic Polycations

Numerous studies have shown that ruthenium red ($[(NH_3)^5$ RuORu $(NH_3)^4$ ORu $(NH_3)^5]^{6+}$), which was originally shown to inhibit mitochondrial Ca^{2+} uptake through Ca^{2+} channel inhibition (Rahamimoff and Alnaes, 1973), stimulates Ca^{2+} accumulation in SR vesicles (see Ikemoto et al., 1989). The action of ruthenium red is via direct inhibition of Ca^{2+} release channel mediated Ca^{2+} efflux. In a detailed study Palade (1987) demonstrated that this and other polyamines including i) antibiotics (neomycin, gentamycin, streptomycin, clindamycin, kanamycin, tobramycin) ii) naturally occuring polyamines

(spermine, spermidine) and iii) basic proteins and polypeptides (polylysine, polyarginine, some histones, protamine) were effective in blocking the skeletal SR Ca^{2+} release channels. Similar inhibitory effects and of neomycin were reported in cardiac SR (Meissner and Henderson, 1987). More recently, complex effects of polylysine (Cifuentes et al., 1989) upon stimulation and inhibition of SR Ca^{2+} release at low (0.3μM) and high (30μM) concentrations, respectively, were observed. Cifuentes et al. (1989) demonstrated direct binding of polylysine to the release channel with variable binding to calsequestrin and/or a 100 kDa protein. Ruthenium red inhibited polylysine-induced Ca^{2+} release but did not displace the latter from the release channel. This suggests the presence of multiple classes of polyamine binding sites upon the channel.

(vii). Protons

Earlier studies showed that a rapid increase in extravesicular pH initiated a rapid release of Ca^{2+} from skeletal (Nakamura and Schwartz 1970, 1972) and cardiac SR (Nakamura and Schwartz, 1970). Similar results were reported with submaximally Ca^{2+} loaded (Ca^{2+}-ATPase mediated) vesicles (Dunnet and Naylor, 1979). Subsequent studies indicated that an inward protein transmembrane gradient (>0.2 pH units) rather than a finite extraluminal pH level was critical in demonstrating pH jump induction of Ca^{2+} release (Shoshan et al., 1981; MacLennan et al., 1982). Similar results have been demonstrated more recently (Meissner, 1984; Sumbilla and Inesi 1987) in both SR vesicles (Argaman and Shoshan-Barmatz 1988; Shoshan-Barmatz, 1988) and skinned fibres (Shoshan et al., 1981). The pH jump induced release of Ca^{2+} was inhibited by dicyclohexylcarbodiimide (DCCD) which evidently bound to the Ca^{2+} release channel with inhibition of ryanodine binding.

(viii). Reactive Group Modification

DCCD inhibition of pH induced Ca^{2+} release was suggested to involve free carboxyl residues of either glutamate or aspartate (Argaman and Shoshan-Barmatz, 1988). These putative carboxy residues which were reactive to acetic anhydride but not the more hydrophilic carboxyl reagents N-ethyl-5-phenlyisoxazolium-3'-sulphonate (WRK) and N-ethoxycarboxyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) were suggested to lie within a hydrophobic domain of the protein(s) regulating Ca^{2+} release. Studies from the same group (Shoshan-Barmatz, 1986) suggested that acetic or maleic anhydride may also react with positively charged amino residues located on the SR lumen to activate Ca^{2+} release.

Interest has also been generated in role of sulphydryl (SH) groups in the modification of SR Ca^{2+} release. Earlier studies demonstrated induction of Ca^{2+} release in skeletal SR by microtubular concentrations of heavy metals (Cu^{2+}, Hg^{2+}, Ag^{2+}) and para-chloromercuribenzenesulphonate (Abramson et al., 1982; 1983; Bindoli and Fleischer, 1983). Release was mediated by SH group modification (Salama and Abramson, 1984) and could be blocked by known inhibitiors (e.g. tetracaine, procaine and Ruthenium Red) of Ca^{2+} channel mediated Ca^{2+} efflux (Trimm et al., 1986).

Oxidation of SH groups to initiate Ca^{2+} release was found to be reversible upon addition of dithiothreitol in both skeletal and cardiac SR (Abramson et al., 1988; Zaida et al., 1989; Prabhu and Salama, 1990). SR vesicles fused with planner bilayers exhibited increased Ca^{2+} channel open probabilities with elevated choline permeability and Mg^{2+} efflux in intact SR vesicles (Nagura et al., 1988). Rapid kinetic studies of Ag^{2+} induced Ca^{2+} release (Moutin and Dupont, 1988; Moutin et al., 1989) estimated release rate constants, in the absence of Mg^{2+} (between 6 and $24s^{-1}$) were increased by the presence of KCl. SH

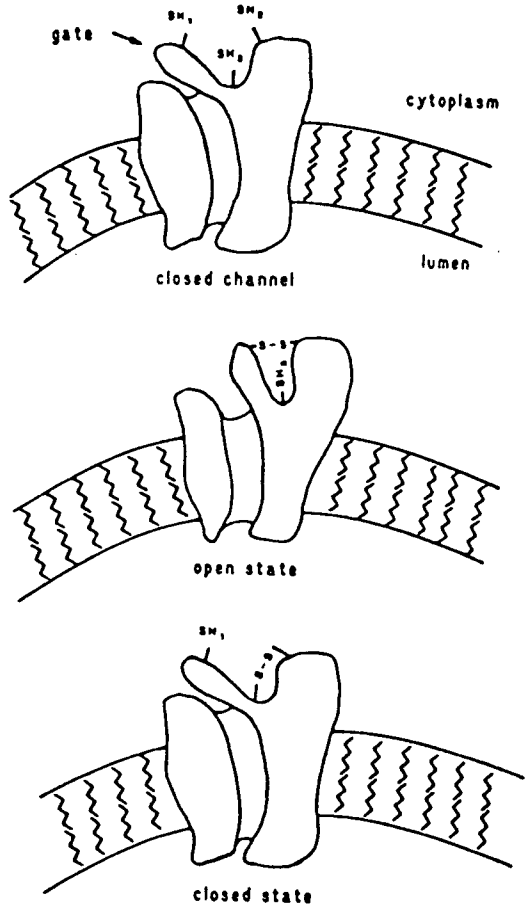
induced Ca^{2+} release was inhibited by the prior addition of nucleotide (Zaidi et al., 1989) while release rates were increased by nucleotides subsequent to SH group modification (Stuart and Abramson, 1988; Zaidi et al, 1989). These complex effects of nucleotide are difficult to reconcile with modification of a single reactive SH site on the SR. The site of action of thiol containing compounds in stimulating Ca^{2+} release is unclear. Concentrations of compounds (5-50μM) most effective in stimulating Ca^{2+} release (4,4'-dithiopyridine, 2,2'-dithiopyridine) inhibited [^{3}H]ryanodine binding to SR vesicles. This is inconsistent with the view that ryanodine binding is favoured in the open state of the Ca^{2+} release channel (Lai et al., 1989; Chu et al., 1990). Abramson and Salama (1989) suggested the SH effects may be mediated via a thiol-disulphide exchange trigger reaction (Figure 4). The site of thiol mediated effects may not be the ryanodine receptor, as earlier thought, but a novel 106kDa Ca^{2+} channel protein distinct from the Ca^{2+}-ATPase (Zaidi et al., 1990). However, the physiological triggering of Ca^{2+} release involving such a mechanism remains controversial. Brunder et al. (1988) found that in split fibres, concentrations of glutathione and DTT which inhibited heavy metal induced Ca^{2+} release did not inhibit Ca^{2+} transients induced by electrical stimulation.

(ix). Myoinositol Triphosphate

The potential role of IP_3 in mediating SR Ca^{2+} release is controversial. As described earlier IP_3 mobilises Ca^{2+} from several intracellular stores in a variety of cell types. The ER and/or ER associated structures are the main store of Ca^{2+}. Studies on skinned fibres from cardiac (Nosek et al., 1986) and skeletal muscle (Vergara et al., 1985; Volpe et al., 1985; Walker et al., 1987; Donaldson et al., 1987) and cardiac cells (Kentish et al., 1990) demonstrated either direct IP_3 activation of Ca^{2+} release or

Fig. 4. Gating of the Ca^{2+} release channel. A hypothetical model of Ca^{2+} release channel gating involving thiol-disulphide exchange between closely apposed thiol groups. (from Abramson and Salama, 1989).

Figure 4.


gate
cytoplasm
lumen
closed channel
open state
closed state

IP_3 potentiation of CICR (Movesian et al., 1985). However, Lea et al. (1986) using skinned frog muscle fibres, Movesian et al. (1985) using myocytes, Mikos and Snow (1987) using skeletal SR vesicles and Rousseau et al. (1986) using cardiac SR vesicles fused into bilayers did not observe IP_3 induction or potentiation of Ca^{2+} release or channel opening. SR containing vesicles (from frog muscle) fused into bilayers did exhibit IP_3 stimulation of Ca^{2+} channel open probability (Suarez-Ilsa et al., 1988). In this study the unexpected inhibition of channel activity by sub-micromolar ryanodine (200nm) suggests the presence of other membranes which may have been sensitive to IP_3.

(x). Calmodulin

Several proteins other than the Ca^{2+} release channel are known to be important in the regulation of Ca^{2+} release. Calmodulin, an ubiquitous E-F hand containing protein has been shown to inhibit the release of SR Ca^{2+} in passively and actively Ca^{2+} loaded skeletal vesicles (Plank et al., 1988; Meissner, 1986) and passively Ca^{2+} loaded cardiac vesicles (Meissner and Henderson, 1987). The effect of calmodulin appeared to be due to direct binding effects rather than due to calmodulin mediated phosphorylation of the Ca^{2+} channel or associated protein(s) (Plank et al., 1988). Calmodulin did not completely inhibit Ca^{2+} release (Meissner, 1986) and did not decrease Ca^{2+} channel conductance (Smith et al., 1989). Rather, calmodulin decreased single channel mean open times by accelerating the open to closed state transitions in cardiac and skeletal vesicles fused into bilayers (Smith et al., 1989). Schneider and Simon (1988) proposed a two step model for calmodulin mediated channel inactivation in which Ca^{2+} bound calmodulin could directly bind to the channel at an inactivation site. However, Smith et al., (1989) and Meissner (1986) demonstrated partial antagonism of calmodulin effects by nucleotides (5mM AMP-PCP) and elevated

cytosolic Ca^{2+} (50μM). Smith et al. (1989) proposed that calmodulin may function to counteract the effects of elevated cytosolic Ca^{2+} upon Ca^{2+} channel opening during sustained muscle activity

(xi). Intralumenal Calcium

In contrast to the well documented effects of extralumenal effectors and inhibitors of Ca^{2+} channel mediated Ca^{2+} release from the SR, relatively little is known of the role (if any) of intralumenal Ca^{2+} in regulating Ca^{2+} channel opening and closing. This has been largely a problem of access to the intralumenal compartment. The issue of how intralumenal Ca^{2+} might regulate Ca^{2+} release Ca^{2+} be traced back to the contrary views of Weber et al. (1966) and Ebashi and Endo (1968) in accounting for the distribution of intralumenal Ca^{2+} subsequent to Ca^{2+} uptake. Weber et al. (1966) suggested that intralumenal Ca^{2+} was largely bound to a low affinity Ca^{2+} binding site. Ebashi and Endo (1968) argued that in order to account for the rapid uptake of a large pool of cytosolic Ca^{2+} during relaxation (700nmoles/ml cytosol) high affinity Ca^{2+} binding sites located either in the Ca^{2+}-ATPase and/or intralumenal Ca^{2+} binding proteins. The corollary to this latter view was that saturation of this site would lead to back inhibition of the ATPase (see Endo, 1977). This is important since intralumenal Ca^{2+} inhibition was forwarded to account for the mechanism of Ca^{2+}-induced Ca^{2+} release. As Endo et al. (1981) noted "the ease of evoking Ca^{2+}-induced Ca^{2+} release is dependent on the level of loading of the SR".

The phenomenon of intralumenal threshold Ca^{2+} requirement for Ca^{2+} triggered release has been demonstrated in a limited number of studies with SR vesicles (see Ohnishi, 1979; Nelson and Nelson, 1990). The conclusion of these studies was that loading of SR to a critical level of Ca^{2+} regulated the sensitivity of the triggering mechanism to extralumenal Ca^{2+}. A similar phenomenon has

been observed with forms of spontaneous Ca^{2+} release (Palade et al., 1983). However, in Endo's (1977) view, Ca^{2+} loading to a threshold level would serve to inhibit the Ca^{2+}-ATPase and prevent accumulation of the trigger Ca^{2+}. Ca^{2+}-induced Ca^{2+} release would be strictly a function of extralumenal Ca^{2+} binding the sensitivity of which was presumed to be unaffected by intralumenal Ca^{2+} loads. This is quite different from the conclusions of later studies that the intralumenal Ca^{2+} threshold requirement for Ca^{2+}-induced Ca^{2+} release reflects a role of intralumenal Ca^{2+} in directly regulating this form of Ca^{2+} release.

Miyamato and Kasai (1979) demonstrated an assymetric distribution of Ca^{2+} binding sites in crude (unfractionated) SR vesicles. Intralumenally, 2 high affinity ($Kd=2.7x10^{-7}$ and $4.7x10^{-6}M$) and 2 low affinity ($Kd=1.05x10^{-3}$ and $3.8x10^{-2}M$) Ca^{2+} binding sites were identified. One low affinity site ($kd=1.05x10^{-3}$) was found to bind ~45 nmol Ca^{2+}.mg HSR^{-1} binding sites consistent with it's identity as calsequestrin (Cozens and Reithmeier, 1984). With a Kd of ~1mM and a physiological concentration of calsequestrin of ~2.6mM or 104mg/ml SR (Mr~40,000) at an internal volume of ~3µl per mg SR (Williams and Beeler, 1986) it can be calculated that about 90% of Ca^{2+} accumulated into the terminal cisternae (100-150nmol.mg^{-1} SR protein) would be bound to calsequestrin. Most of the releasable Ca^{2+} pool would then be derived from a bound store. Williams and Beeler (1986) showed that at physiological concentrations, calsequestrin undergoes little secondary structural changes as monitored by circular dichroism with manipulations in Ca^{2+} and ionic conditions contrary previous observations with dilute concentrations of calsequestrin (Cozens and Reithmeier, 1984). However, Ikemoto and Koshita (1988) reported that the rapid biphasic kinetics of arsenazo III difference absorbance during Ca^{2+} uptake paralleled the biphasic kinetics of intrinsic tryptophan fluorescence of calsequestrin upon Ca^{2+} binding.

These studies together with the observation that i) removal of intralumenal calsequestrin reversibly abolished caffeine induced Ca^{2+} release (Ronjat and Ikemoto, 1989) and ii) that fluoresence of DACM, incorporated into the Ca^{2+} release channel, suggested an important role for intralumenal Ca^{2+} in regulating structural states of the release channel indirectly through effects upon calsequestrin. Recent studies identified a critical binding region on calsequestrin (between Lys 86 and Lys 191) that interacted with the Ca^{2+} channel rich junctional face membranes of the SR cisternae (Collins et al., 1990). Collins et al. (1990) suggested that Ca^{2+} binding to calsequestrin may induce a coil-helix transition which may be directly or indirectly an important regulator of Ca^{2+} channel states.

(xii). Proteases

The extreme protease sensitivity of the ryanodine receptor was first recognised by Seiler et al., (1984) who showed that treatment of SR membranes with CANP led to extensive fragmentation of the Ca^{2+} channel with no discernable effect upon the remaining SR proteins. Prior to identification of the Ca^{2+} channel constituents Shoshan-Barmatz et al. (1987) reported that tryptic digest of SR vesicles uncoupled ATP hydrolysis from Ca^{2+} transport. They suggested the presence of an additional trypsin sensitive protein other than the Ca^{2+}-ATPase which regulated SR Ca^{2+} transport. Shoshan-Barmatz and Zarka (1988) demonstrated small increases in SR Ca^{2+} accumulation with low level trypsin exposure; conditions which led to formation of a 135kDa digest product retention of which correlated with maintenance of [^{3}H]ryanodine binding. Limited tryptic digest was also reported to stimulate the activation of Ca^{2+} efflux by cAMP, Hg^{2+}, and doxorubicin from passively Ca^{2+} loaded vesicles (Trimm et al., 1988). In ascribing a modified pathway for Ca^{2+} release these studies could

not discriminate between effects of trypsin upon the Ca^{2+}-ATPase and the Ca^{2+} release channel.

Chu et al. (1988) noted little effect of extensive tryptic fragmentation of the release channel upon functional properties. Moreover, no significant loss of electron dense material from the channel rich junctional face domain of cisternal vesicles was observed. Chu et al. (1988) suggested that this reflected the presence of mutiple non-covalent interactions maintaining the channel ultrastructure. Rapid kinetic studies of Ca^{2+} release from Ca^{2+} loaded vesicles revealed a bifunctional effect of trypsin exposure (Meissner et al., 1989). Mild tryptic digest resulted in slight increases in passive loading with little effect upon the rate of release. Extensive tryptic digest led to loss of Ca^{2+} retaining properties of vesicles and increase in the rate of Ca^{2+} release. These latter effects were paralled by loss of low affinity ryanodine binding and irreversible loss of channel activity from bilayer recordings (Meissner et al., 1989).

Recently, Rardon et al. (1990) reported the effects of millimolar Ca^{2+} sensitive CANP from smooth muscle upon cardiac and skeletal muscle SR. A discrete peptide pattern was observed with production of 315kDa and 150kDa products under mild proteolytic conditions. Extensive proteolysis removed the 315kDa product leaving an apparent 150kDa limiting digest product. Increases and decreases in single site [^{3}H]ryanodine binding were observed for the skeletal and cardiac vesicles, respectively. However, for both skeletal and cardiac SR CANP treatment resulted in loss of channel inactivation in planar bilayer recordings. Gilchrist et al. (1990b) reported a similar pattern of CANP mediated proteolysis in cardiac and skeletal SR vesicles. Comparison was made, however, between the effects of skeletal muscle micromolar and millimolar Ca^{2+} sensitive CANP after which subtle differences were noted in the production of peptides by each isoform. The effect of CANP was to elevate (15-20%) the amount of Ca^{2+}

retained after passive loading, to increase the rate of slow phase ATP dependent Ca^{2+} accumulation by vesicles and to in crease low and high affinity [^{3}H]ryanodine binding capacity. These results reflected an apparent modification of the sensitivity of channel opening to either intralumenal or extralumenal Ca^{2+}.

IV. Calcium Activated Neutral Protease.

Calcium Activated Neutral Protease (CANP) is an ubiquitous intracellular cysteine protease found within a variety of cells across a number of animal species (see Suzuki et al., 1984). The proteinase bears a remarkable homology to papain and is optimally active within a pH range of 7-8 (Murachi et al., 1979). In most tissues CANP exists in two isoforms. One isoform requires μM Ca^{2+} for activation (μCANP) whereas mCANP is activated by mM Ca^{2+} concentrations. m CANP is the predominant isoform is most mammalian tissues although μCANP is the only isoform found in erythrocytes (Wang et al., 1989). Both isoforms of CANP exist as heterodimers. The large subunit (80-82kDa) is the catalytic subunit whereas the small 29kDa subunit is believed to serve a regulatory function (Suzuki et al., 1987). The large subunit has four domains (I, II, III, IV). The catalytic domain (domain II) is the cysteine protease domain and shows a high degree of sequence homology to cathepsins B and H, Papain and Actinidin (Suzuki et al., 1987). Domain IV, at the -COOH terminal has been identified as a CaM like Ca^{2+} binding domain with 4 EF-hand structures. This region appears to define the Ca^{2+} dependence of CANP activation in each isoform. Sequence comparison between μ- and mCANP reveals increased hydrophobicity in domain IV of the μCANP isoform (Imajoh et al., 1988). The active sites of CANP are located at Cys-108 and His-265 and are found to be

conserved residues within the proteases listed above (Suzuki et al., 1984). Barret (1986), has proposed that nitrogens in the imidazole ring of His-265 initiates proton abstruction of the Cys-108 sulfer atom. The resultant thiyl radical then attacks the amide carbonyl group of the protein substrate. The function of the remaining domains remains obscure. Suzuki et al., (1987) have suggested that these domains may be important for interaction of the large subunit with the small subunit.

CANP exhibits the property of autolytic activation for expression of full activity. Suzuki et al. (1987) suggested that CANP activity may be repressed by interaction of domain II with domains I and III. Autolytic activation leads to production of a partially cleaved large subunit (~78-76kDa in erythrocyte μCANP) that exhibits an increased sensitivity for Ca^{2+} activation (Pontremoli and Melloni, 1986). However, further auto-proteolysis results in inactivation of CANP. Autolysis is also associated with cleavage of the small subunit with production of a 20kDa product from the 29kDa parent peptide (Suzuki et al., 1988; DeMartino et al., 1986; Croall, 1989).

1. Regulation of CANP

a. Phospholipid

Although CANP is often considered to exist largely within the cytosol (Suzuki et al., 1988) is has been proposed that autolytic activation of CANP occurs through plasma membrane association (Mellgren, 1987). The membrane/autolysis hypothesis was proposed through observation that both membrane associated isoforms of CANP rapidly autolyse in the presence of Ca^{2+} and that autolysis reduces the Ca^{2+} dependence of CANP activation. In particular, phosphatidyl inositol has been shown to markedly increase the Ca^{2+}

sensitivity of CANP (Cong et al., 1989). Imajoh et al. (1986) have shown that the hydrophobic glycine rich N-terminal region (domain V) of the small subunit is required for the membrane interaction and activation of the small subunit with membrane bound phosphatidyl inositol mediated the autolytic activation of CANP.

b. Calpastatin

Calpastatin is an endogenous protein inhibitor of CANP that exists as two distinct types. Liver calpastatin has a molecular weight of 107,000 while erythrocyte calpastatin exhibits a molecular weight of 70,000 (Murachi, 1983). One liver CANP molecule binds 4-10 molecules of CANP whereas erythrocyte CANP is associated with the binding of 3-5 CANP molecules. The mechanism of calpastatin inhibition of CANP involves a Ca^{2+} dependent association of calpastatin and CANP (Imajoh and Suzuki, 1985). Four internal repeat sequences within the primary structure of calpastatin appear to be important in the expression of inhibitory activity (Maki et al., 1987).

2. Role of CANP

The intracellular role of CANP remains unclear. CANP appears to target specific substrates that are primarily located either at the plasma membrane, the cytoskeleton or within the cytosol. Localisation of CANP within these compartments has been deduced from several immunolocalisation studies within a variety of tissues (Murachi, 1989; Goll et al., 1989). It has been considered that localisation of CANP to plasma membranes may mediate proteolytic modification of membrane associated proteins in response to increases in local concentrations of Ca^{2+} close to Ca^{2+} channels (Belles et al., 1988; Goll et al., 1989). These responses are numerous. Mellgren (1987) has

proposed that CANP effects upon cytoskeletal structures may be directed toward the restructuring of membranes that would be necessary for membrane rapair and growth. In this regard, Mellgren (1987) has suggested that CANP may be involved in the remodelling of postsynaptic membranes resulting in proliferation of dendrite spines. It is this involvement of CANP which is thought to be involved with long term potentiation in hippocampal neurons after repetitive stimulation. CANP, therefore, appears to play an important role im memory retention. In many cases where the substrate is an enzyme limited CANP proteolysis leads to activation of function, prefering to cleave between the domains rather within (Suzuki et al., 1987). In this respect, Suzuki and Ohno (1990), have suggested that CANP may act as a "biotransformer" mediating alterations in function, rather than attenuation, in response to alterations in cellular metabolic activity and, ultimately, Ca^{2+} metabolism. It has become evident that the Ca^{2+} release channel of sarcoplasmic reticulum is also sensitive to limited CANP mediated proteolysis (Seiler et al., 1984). The central role of the Ca^{2+} release channel in regulation of intracellular Ca^{2+} levels in smooth and striated muscle implicates an interesting role for CANP in the regulation of Ca^{2+} metabolism in muscle.

V. Thesis Rational

Recent studies have demonstrated that regenerative HSR Ca^{2+} release is regulated through an intimate Ca^{2+} mediated structural and functional association of calsequestrin with the ryanodine receptor. Calsequestrin was shown to be attached to the junctional face of the cisternal lumen (Franzini-Armstrong et al., 1987) as filamentous strands aligned with the ryanodine

receptor/dihydropyridine complex and intra t-tubular "tether" proteins (Dulhunty, 1989). In the presence of mM Ca^{2+}, calsequestrin formed dense intralumenal aggregates adjacent to the junctional face of HSR vesicles (Saito et al., 1984). Circular dichroism studies with soluble calsequestrin also revealed Ca^{2+} dependent formation of globular structures from extended forms of the Ca^{2+} depleted protein (Cozens and Reithmeier, 1984). More recently, Collins et al. (1990) identified a critical Ca^{2+} channel interaction domain on calsequestrin (between Lys 86 and Lys 191) that was suggested to undergo a coil-helix transition upon Ca^{2+} binding. Functional studies revealed the relative intensity of the fluorescent conformational probe, DACM, incorporated into the Ca^{2+} release channel, was well correlated with Ca^{2+} binding to calsequestrin (Ikemoto et al., 1989). Additionally, removal of intralumenal calsequestrin reversibly abolished caffeine induced Ca^{2+} release (Ronjat and Ikemoto, 1989). This was consistent with an observed correlation between the intralumenal Ca^{2+} dependence of the caffeine induced Ca^{2+} release rate constant and the degree of Ca^{2+} binding to calsequestrin (Ikemoto et al., 1989). These studies suggest that Ca^{2+} occupied states of calsequestrin are important in the regulation of Ca^{2+} release. This implicates complex formation between the release channel and calsequestrin in which functional regulation may be, conceivably, reciprocal.

In many different myopathies (eg diabetes, muscular dystrophy, starvation, ischemia, chronic fatique, malignant hyperthermia) SR Ca^{2+} release is often impaired. The impairment is generally associated with increased Ca^{2+} channel leakiness which may contribute to elevated cytosolic Ca^{2+}. With malignant hyperthermia the impaired SR function is due to elevated sensitivity to Ca^{2+}-induced and caffeine-induced Ca^{2+} release due to molecular differences in Ca^{2+} channel structure arising from genetic mutation (O'Brien, 1990). In other cases defective Ca^{2+} release arises secondarily to primary defects of specific origin. In

addition, different myopathies are commonly characterised by disrupted energy metabolism, K^+ loss, thiol group oxidation, intracellular proton accumulation, vacuolation of triadic and mitochondrial membranes and disruption of myofibril ultrastructural organisation. Since the defect is often preserved through vesicle purification, release modification must arise from some structural or conformational alteration in the isolated membranes.

It is not clear to what extent a common basis for the specific secondary defect exists among the different myopathies. However, a common consequence of intracellular Ca^{2+} overload that may result in modification of SR Ca^{2+} release is the activation of Calpains (CANP). Although the specific mechanism for CANP activation is uncertain, it is clear that elevated cytosolic Ca^{2+} alone leads to intracellular proteolysis characteristic of the action of CANP. In vitro, CANP activation has been shown to result in limited proteolysis of the Ca^{2+} channel. The functional consequences of CANP action appear to be modified channel gating as determined from bilayer recordings (Rardon et al., 1990). How CANP structural modification of the channel affects the Ca^{2+} handling characteristics of the SR is unknown, however. It would be interesting for example to determine whether Ca^{2+} channel structural alterations results in alteration in channel/calsequestrin interaction and, possibly, intralumenal Ca^{2+} handling. Could the action of CANP upon the Ca^{2+} channel account for the alteration of SR Ca^{2+} handling observed in vivo? There is currently no direct evidence to support or refute this notion.

In the case of SR membranes, intralumenal Ca^{2+} accumulation has been shown to be associated with impaired Ca^{2+} release (Gonzales-Serratos et al., 1978) after prolonged contraction. However, it is not clear whether (a) release inhibition and cisternal Ca^{2+} accumulation results from channel impairment or (b) impaired release results from intralumenal Ca^{2+} accumulation. Preliminary

studies (Gilchrist et al., 1990) indicated that extralumenal ligand modification of Ca^{2+} release modifies the intralumenal Ca^{2+} dependence of Ca^{2+} release. Elevated Mg^{2+}, in addition to reducing the extralumenal Ca^{2+} sensivity of CICR, increased the intralumenal Ca^{2+} threshold for Ca^{2+} release (Gilchrist et al., 1990). These observations suggested that effector modification of Ca^{2+} channel states may modify the functional coupling to calsequestrin. This concept has received little attention but may be important in understanding the regulation of SR Ca^{2+} release. In this regard, the plant alkaloid ryanodine might be a potentially valuable probe of the relationship between Ca^{2+} channel states and intralumenal Ca^{2+} binding. Ryanodine binds specifically to the Ca^{2+} channel and mediates complex activatory and inhibitory effects upon HSR Ca^{2+} release (Meissner, 1986b; Lattanzio et al., 1987) that are dependent upon the existing state of the channel. Demonstration that dual effects of ryanodine depend upon the state of intralumenal Ca^{2+} loading would provide evidence that transitions between functional states of the channel and calsequestrin are reciprocally interdependent. If these events are linked, could structural modification of the channel by CANP alter the intralumenal Ca^{2+} depedence of Ca^{2+} release in a manner that may account for the altered Ca^{2+} handling and Ca^{2+} compartmentation associated with myopathy?

VI. Objectives of this study.

It is hypothesised that intralumenal Ca^{2+} within the terminal cisternaeregion of the SR is important in the regulation of the Ca^{2+} release channel. It is further hypothesised that structural and pharmacological perturbation of the release channel will alter the intralumenal Ca^{2+} dependence of SR Ca^{2+} release. The following objectives were established.

1) To purify skeletal and cardiac SR membranes referable to the terminal cisternae.

2) To develop a Ca^{2+} transport assay method that will probe intralumenal Ca^{2+} compartment.

3) To purify μ- and mCANP from skeletal muscle.

4) To investigate the role of intralumenal Ca^{2+} upon Ca^{2+} induced Ca^{2+} release.

5) To investigate the effect of ryanodine upon the intralumenal Ca^{2+} dependence of Ca^{2+} induced Ca^{2+} release.

6) To characterise the structural and functional effects of CANP upon HSR Ca^{2+} release.

MATERIALS AND METHODS

I. Materials

The chemicals and/or proteins were purchased from the following sources.

1. Sigma Chemical Co.

A23187

2-mercaptoethanol

ATP (Na^+ and TRIS salt)

Antipyrylazo III

Aprotinin

Bovine serum albumin

Bromophenol blue

Calmodulin-agarose

Casein

CHAPS

Citric acid

Copper sulphate

CPK

CP

EDTA

EGTA

Folin reagent

Formamide

Hepes

Imidazole

Isopropanol

Leupeptin

Magnesium chloride

Quercetin

PIPES

PMSF

Sodium tartrate

Stains-All

TEMED

TLCK

TPCK

Trichloroacetic acid

Tris base

Tris-HCl

Trypsin inhibitor (soybean, type IV)

Omega-aminohexyl-agarose

2. BDH Chemicals

Ammonium sulphate

Calcium chloride

Dimethyl dichlorosilane

Ethanol

Glacial acetic acid

Glycerol

Glycine

Hydrochloric acid

Magnesium chloride

Methanol

Potassium chloride

Potassium hydrogen phosphate

Potassium hydroxide

Sodium carbonate

Sodium chloride

Sodium hydroxide

SDS

Sucrose

3. Bio-Rad Laboratories

Acrylamide

Ammonium persulfate

Coomassie brilliant blue R-250

N,N'-methlene-bisacrylamide

SDS-PAGE high molecular weight standards

SDS-PAGE low molecular weight standards

4. Pharmacia

DEAE-sepharose CL-4B

Gel filtration molecular weight standards

5. LKB

Ultrogel AcA34

6. New England Nuclear

Aquasol (liquid scintillation fluid)

[^{3}H]Ryanodine (specific activity=60Ci.mmol^{-1})

[^{45}Ca]Calcium chloride (specific activity=125Ci.mmol^{-1})

7. Serdary Research Products

Phosphatidylcholine (Pig liver)

8. Progressive Agri Systems Inc.

Ryanodine

II. Methods

1. Isolation of HSR membranes.

HSR membranes were prepared from fast twitch rabbit skeletal muscle using the buffer systems described by Chu et al. (1986) with several modifications to their protocol. Back and hindlimb muscle was rapidly excised, trimmed of excess fat and connective tissue and placed in 0.9% saline on ice within 10 minutes. Muscle was cut and weighed into 20 gram portions and was immediately freeze-clamped in liquid N_2 between aluminium tongs into 20 gram discs, wrapped in aluminium foil and stored at -70^{o}C for up to 6 months. Frozen muscle was ground to a fine powder under liquid nitrogen using a porcelain pestle and mortar packed in ice. The powdered material was added to 5 volumes (w/v) of homogenisation buffer containing 300mM sucrose, 20mM imidazole, 0.5mM EGTA, 1mM DTT, 2mM PMSF, 10μM leupeptin (pH 7.4) and was thawed (0-5^{o}C) with constant stirring. The protein slurry was homogenised in a 125ml Waring blendor bottle at slow speed (15,000 rpm) for two 40 second bursts with a 40 second interval. The homogenates were combined and were centrifuged for 10 minutes in a Beckman JA-14 rotor (Beckman Instruments, Palo Alto, CA) at 11,500 rpm. The supernatant was decanted and filtered through 4 ply cheese-cloth and centrifuged at 45,000 rpm in a Beckman 50.2 rotor for 90 minutes. Pelleted material was suspended in homogenisation buffer to 40mg.ml^{-1} and centrifuged at 27,000 rpm (Beckman SW 28 rotor) for 16 hrs through a linear 25-45% (w/w) sucrose gradient containing 5mM imidazole, 1mM DTT, 2mM PMSF, 10μM leupeptin (pH 7.4). HSR membranes enriched in ryanodine receptor protein were collected from the 38-40% region, slowly diluted (1:4) to 10% sucrose with buffer containing 150mM KCl, and 20mM imidazole, pelleted as above, and resuspended in homogenisation buffer containing 0.01μM

leupeptin and 1mM DTT to a final concentration of 40-50mg.ml^{-1}. All procedures were conducted in the cold room and, typically, 200 grams (10 discs) of muscle were used per isolation.

Cardiac SR was prepared from frozen dog hearts using the same procedure with modifications. In addition to the protease inhibitors leupeptin and PMSF, all buffers contained 5μg.ml^{-1} aprotinin, 100μg.ml^{-1} TLCK, 100μg.ml^{-1} TPCK, and 50μg.ml^{-1} soyabean trypsin inhibitor. After sucrose density gradient fractionation of SR membranes, protein was diluted to 10% sucrose (as above) with the inclusion of 400mM KCl to remove accessorial protein. Membranes were incubated with constant stirring for 2 hrs at 4^{o}C and were pelleted and resuspended in homogenisation to ~30mg.ml^{-1}.

2. Ryanodine Receptor Purification.

Ten ml of skeletal muscle junctional SR (~25mg.ml) was thawed and diluted to a final concentration of 10mg.ml^{-1} in buffer containing 1M NaCl, 20mM tris-OH (pH 7.4), 2mM DTT, and 10μg.ml^{-1} leupeptin (at 22^{o}C). After 20 mins, protein was solubilised with addition of an equal volume of buffer containing 2% CHAPS, 1% asolectin, 20mM tris-OH (pH 7.4), and 2mM DTT. Ca^{2+} was added from stock solution to a final concentration of 200μM. After 30 mins solubilisation at room temperature, CHAPS-insoluble material was sedimented at 45,000 rpm in a Beckman 50.2 rotor for 30 mins. The soluble supernatant was decanted and diluted ten fold in buffer containing 50mM KCl, 20mM tris-OH (pH 7.4), 2mM DTT, 200μM Ca^{2+}, 5 μg.ml^{-1} leupeptin (4^{o}C). The insoluble pellet was resuspended in muscle homogenisation buffer to an approximate concentration of 1mg.ml^{-1}. Solubilised membranes were applied to a calmodulin (CaM)-agarose affinity column (10 cm x 1.6 cm), pre-equilibrated at cold cabinet temperature (4^{o}C) with 0.1% CHAPS, 0.05% asolectin, 20 mM Tris-OH (pH 7.4),

200μM Ca^{2+}, 5μg.ml^{-1}, 2mM DTT (column buffer), at a flow rate of 0.5 ml.min^{-1}. The column was washed exhaustively at 0.5 ml.min^{-1} with column buffer and protein was eluted across a 150 ml linear EGTA (10mM) gradient from 200μM Ca^{2+} at a flow rate of 0.5ml.min^{-1} with 4ml fractions collected. For SDS-PAGE analysis, aliquots (1ml) were added to 0.5ml of ice-cold TCA (10%) in an eppendorf microcentrifuge tube to preciptate protein. Protein was sedimented at 14,000 rpm in a Beckman microcentrifuge and pellets were resuspended directly in SDS-PAGE sample buffer.

3. Purification of CANP.

The purification of both micromolar and millimolar Ca^{2+} sensitive CANP (μCANP and mCANP, respectively) from frozen rabbit skeletal muscle was performed as described (Tan et al., 1988) with several modifications. Frozen muscle was ground as described for the preparation of HSR membranes and homogenised in low salt buffer (LSB) containing 2mM EDTA, 2mM EGTA, 50 mM HEPES, 1mM DTT (pH 7.4). Muscle homogenate was centrifuge at 11,500 rpm in a JA-14 rotor for 10 minutes. The supernatant was decanted, filtered across 4 ply cheese-cloth and made to 25% saturated ammonium sulphate by the addition of solid salt. The solution was constantly stirred in the cold room for 30 mins and precipitated material was sedimented in at 13,500 rpm for 30 mins in a Beckman JA-14 rotor. The supernatant was decanted and the solution made to 65% ammonium sulphate with a supplemental addition of salt. Stirring and sedimentation was repeated as above. After each addition of salt the pH was adjusted to 7.4 with the addition of tris base. Precipitated material was resuspended in 0.5 volumes (of starting muscle weight) of LSB and the suspended protein was dialysed four times at 2 hour intervals (4 ^{o}C) across 34mm diameter (3,500 mol. wt. cutoff) Spectraphor membrane tubing (Spectrum

Medical Industries, Los Angeles, CA) against 40 volumes of LSB followed by final overnight dialysis.

The ammonium sulphate free protein suspension was diluted with an equal volume of LSB and applied to a 200ml DEAE-Sepharose CL-4B anion exchange column (2.6cm x 35cm), pre-equilibrated with LSB, at 75ml.min^{-1}. After extensive washing (10 bed volumes) with LSB, protein was eluted (75ml.min^{-1}) with a 400ml salt gradient from zero to 400mM KCl in LSB (High salt buffer or HSB). Fractions were assayed for CANP activity (see later) and, characteristically, μCANP eluted between 120 and 180mM KCl while mCANP eluted later as a distinct peak between 250 and 330mM KCl (see results). The ionic strength of each fraction (10ml) was measured with a hand-held ion conductivity meter. The μ- and mCANP containing fractions were each pooled and separately applied at 75ml.hr^{-1} to a Phenyl-Sepharose CL-4B column (1.6cm x 25cm) pre-equilibrated with 200mM KCl in LSB (LSB-200). In the case of μCANP, the ionic strength was raised to an equivalent 250mM KCl with addition of HSB. The column was washed (5 bed vols) with LSB-200 and protein was eluted in 4ml fractions with LSB at 75ml.hr^{-1}. CANP fractions were pooled and loaded onto a ω-Hexylamine-agarose bi-functional affinity column (1.6cm x 12 cm) at 75ml.hr^{-1}. Protein was eluted with a 100ml linear gradient of LSB and HSB. Active CANP fractions were pooled and concentrated to 3ml in an Amicon N_2 pressure cell across YM-10 membranes (at 4^{o}C). Each CANP isoform was loaded onto an Ultrogel AcA 34 gel filtration column (1.6cm x 80cm), pre-equilibrated with LSB, at 6ml.hr^{-1}. The protein was eluted with LSB and collected in 3ml fractions. Purified CANP was concentrated in LSB to 100 μg.ml^{-1} in an Amincon N_2 pressure cell across YM-10 membranes (0-4 ^{o}C). When required, CANP was further concentrated to 1mg.ml^{-1} with Centricon

microconcentrators (PM-10 membranes) in 50mM HEPES, 0.25mM EGTA and 1mM DTT (pH 7.4, 0-4 ^{o}C).

4. Protein Determination.

Protein was assayed using a modification of the Lowry method (Lowry et al., 1951). 400 µl of protein solution (20-100µg.ml^{-1} in H_2O) was added to 3ml of solution containing 1.785% (w/v) Na_2CO_3, 0.893M NaOH, 0.893% SDS, 0.0089% $CuSO_4$, 0.0178% $NaC_4H_4O_6$ and was incubated for 10 mins. 300 µl of 0.5N Folin reagent was added to each tube to initiate colour development. After 45 mins, a blue colour was visualised and absorbance was determined spectroscopically at 750nM against a blank solution prepared identically to the above except for the addition of distilled-deionised water instead of protein. Relative protein concentration was determined from the aborbances of the standard curve using BSA (Fraction V) as the protein standard.

5. Assay of CANP Proteolytic Activity.

CANP activity was standardised to the proteolysis of casein substrate and was assayed at 37^{o}C in the presence of 2mg.ml^{-1} casein, 5mM DTT, 2.5mM Ca^{2+} (unless otherwise stated) and 50mM HEPES (pH 7.4). After 30 minutes, caseinolysis was quenched upon addition of an equal volume of ice cold 5% TCA. A unit of CANP activity is defined as the amount of TCA soluble product resulting in an absorbance increase of 1 unit at 280nm after CANP digest of casein substrate. Estimates of CANP activity were performed with triplicate determinations upon 3 serial dilutions of each isoform. Due to potentially significant non-linearities of this assay, resulting from substrate limitations, quantitative comparison of µ- and mCANP functional effects required restriction of difference absorbance measurements at 280nm (after background subtraction)

to less than 0.3 absorbance units. The Ca^{2+} dependence of CANP activity was determined as above except with the addition of 100μM EGTA (added in conjunction with CANP) and various concentrations of Ca^{2+} required to obtain desired free Ca^{2+}.

6. Antipyrylazo III Purification.

Antipyrylazo III (AP III) was purchased from Sigma (Sigma Chemical Co., St. Louis, MO) and purified by double re-crystallisation as described (Scarpa, 1978). In a typical preparation, 500mg AP III was added to 12.5ml 40% ethanol (4% dye solution w/v) in a borosilicate test tube and heated to 60 ^{o}C in a water bath for 1hr to solubilise the dye. The tube was removed from the water bath and the interior bottom of the tube was scratched with a glass pipette to initiate crystallisation. The solution was cooled and allowed to stand overnight in the dark at room temperature. Crystals of AP III were collected by filtration across Whatman (#3) filter paper and were dried in a warming oven (60 ^{o}C) for 3hrs. Dried crystals were harvested and weighed and the solubilisation/crystallisation/filtration procedure then repeated at the same ratio of dye and ethanol.

7. Double-Beam Spectroscopy.

Double beam difference spectra (between 640nm and 790nm) were obtained using a SLM Aminco DW2C spectrophotometer on-line with a SLM Aminco MIDAN II kinetic processor/controller. The optical chopper speed was 270 Hz with a monochromator bandpass set to 3nm and a light path of 1cm (3ml quartz cuvettes). The buffer for spectral records contained 300mM sucrose, 150mM KCl, 20mM PIPES (pH 7.0), 50μM AP III (Buffer A) and was titrated with concentrated stocks of Mg.ATP, Mg^{2+} and, Ca^{2+} (prepared in Buffer A) as described in the

figure legends. All cuvette solutions were thermostatically controlled (27°C) and continually mixed with a magnetic flea stirring accessory. Baseline spectral records were obtained with a full chart scale difference absorbance (ΔA) of 0.1 absorbance units at the slowest scanning rate (0.5nm per second). Baselines were collected and stored on-line by the processor and were automatically subtracted from subsequent dye:ligand difference spectral records.

8. Calculation of Calcium:Dye (CaD_2) Dissociation Constants.

The molar extinction coefficients (ε) of the Ca^{2+} free AP III (εD) and the Ca^{2+}:AP III complex (εCaD_2) were obtained using procedures described for Arsenazo III (Kendrick et al., 1977). All spectroscopic procedures were performed using a Shimadzu UV-160 spectrophotometer and 1ml quartz cuvettes. εD was found from the absorbance of the Ca^{2+}-free dye (Buffer A plus 200μM EGTA) read against a blank cuvette containing Buffer A prepared without addition of dye. For calculation of εCaD_2, the dye concentration in Buffer A was increased to 2mM AP III. Upon titration with μM Ca^{2+} (10-50μM) all of the Ca^{2+} would be bound to AP III and εCaD_2 could be found from the slope of ΔA vs $[Ca^{2+}]$ and was determined in the presence and absence of 1mM Mg^{2+}. The value for $[CaD_2]$ was obtained from $\Delta A=(\varepsilon CaD_2-2\varepsilon D).[CaD_2]$ as decribed (Rios and Schneider, 1981) and the free Ca^{2+} in the cuvette could then be obtained from $Ca_f=Ca_t-[CaD_2]$ where Ca_f=free Ca^{2+} and Ca_t=total Ca^{2+}. Apparent first order dissociation constants (K'_{CaD}) for CaD_2 in the absence and presence of 1mM Mg^{2+} were obtained by Ca^{2+} titration of Buffer A. The value was calculated from the negative reciprocal abscissa intercept obtained from double reciprocal plots of ΔA vs Ca_f. Each plot was a linear least-squares fit to the data. Second order dissociation constants (K''_{CaD}) were obtained, as

described (Abramcheck and Best, 1989), from the relation $K''_{CaD}=Ca_f.(D_t-2CaD_2)^2/CaD_2$ where D_t is the total [AP III] (50μM).

9. Spectroscopic Determination of HSR Calcium Transport.

Spectroscopic determinations of HSR Ca^{2+} uptake and Ca^{2+} release were monitored in the dual-wavelength mode of the SLM Aminco DW2C spectrophotometer. The recording wavelength pairs were 675-790nm and 720-790nm as indicated in the figure legends. HSR vesicles from freshly thawed stock (40 to 50 $mg.ml^{-1}$) were pre-incubated to final concentrations of 0.5 to 1$mg.ml^{-1}$ for 3 minutes in 2.75ml transport media containing 300mM sucrose, 100mM KCl, 20mM PIPES (pH 7.0), 50μM AP III (25°C) and, various concentrations of Ca^{2+} and Mg^{2+}, as indicated in the figure legends. Ca^{2+} uptake was initiated by addition of stock 50mM Mg.ATP to a final concentration of 1mM. Mg.ATP and other reagents were introduced to the sample compartment with a Unimetrics microlitre syringe through a customised polystyrene diaphragm that completely excluded the entry of external light upon initiation of and during Ca^{2+} transport. When required, ATP regeneration was supported by the inclusion of 5mM creatine phosphate (PC) and 12.5 $units.ml^{-1}$ creatine phosphokinase (CPK: EC 2.7.3.2; from rabbit skeletal muscle) during pre-incubation.

10. Determination of Calcium Stimulated ATPase Activity.

HSR vesicles were pre-incubated to a final concentration of 10$\mu g.ml^{-1}$ in 380μl buffer containing 300mM sucrose, 100mM KCl, 20mM PIPES (pH 7.0, at 23°C), 100μM EGTA, and various concentrations of Ca^{2+} and Mg^{2+} to obtain the desired free concentrations of divalent cation taking into account the presence of ATP (as indicated in the figure legends). ATPase activity was initiated upon

addition of 20µl Mg.ATP from 130mM stock. Assays were run in the presence and absence of 10µl A23187 and 50µM Ruthenium Red. Pilot studies indicated that linear reactions could be obtained for up to 10 mins without back inhibition of ATPase activity due to accumulation of ADP. ATPase activity was quenched upon addition of 200µl of 10% SDS (w/v).

11. Determination of Inorganic Phosphate.

Liberation of inorganic phosphate (Pi) resulting from Ca^{2+}-ATPase-mediated ATP turnover was determined spectrophotometrically by the method of Raess and Vincenzi (1980). After reactions were quenched with 200µl of 10% SDS, 200µl of 9% (w/v) ascorbic acid (in H_2O) and 200µl of 1.25 (w/v) ammonium molybdate in 6.5% H_2SO_4 (v/v) were added to initiate formatiom of a phosphomolybdate complex. Absorbance at 660nm was recorded after 30 mins of colour development. Absorbance blanks were prepared by addition of SDS to reaction tubes prior to addition of Mg.ATP. Absorbance values for the blanks were subtracted from all sample absorbances and the amount of Pi liberated was determined using K_2HPO_4 as a standard. Ruthenium red exhibited peak absorbance at around 600nm. Therefore, separate ruthenium red containing blanks and standard curves were also prepared.

12. Calcium Release from Passsively Loaded Vesicles.

HSR vesicles from frozen stock were freshly thawed and incubated at a concentration of 10mg.ml^{-1} for 24hrs at 4^{o}C in the presence of 300mM sucrose, 100mM KCl, 50mM HEPES, 10mM $^{45}Ca^{2+}$ (specific activity=10,000-15,000 dpm.nmol^{-1}), 5mM DTT (pH 7.4). Membranes were incubated for a further 2hrs at 23^{o}C and Ca^{2+} loading and Ca^{2+} release were assayed as follows: Five microlitres of incubated membranes were rapidly diluted 250 fold into 300mM

sucrose, 100mM KCl and 50mM HEPES containing either 10mM Mg^{2+}, 50μM ruthenium red and 100μM EGTA to inhibit Ca^{2+} release or 5mM EGTA and 4.9mM Ca^{2+} (free Ca^{2+}=10μM) to stimulate the release of intravesicular Ca^{2+}. 100μl aliquots of diluted membrane suspension were vacuum filtered across 13mm diameter 0.45μ nitrocellulose filters (HAWP type) pre-soaked in release inhibiting buffer and were washed with 5 volumes of the same cold buffer. A customised filtration manifold consisted of base units from 13mm diameter polypropylene Swinnex filter holders with each luer slip outlet inserted into the top luer lock inlet of stainless-steel Becton-Dickinson 3-way stop-cocks. Four to six such units were attached end to end to provide individual and consistent liquid flow control for each unit at each time point. Wetted filters, when placed on the polypropylene support screen, self-sealed with the applied vacuum and could thus be removed immediately after washing to minimise non-specific $^{45}Ca^{2+}$ leaks. Filters were air-dried and $^{45}Ca^{2+}$ retained upon the filters was determined by liquid scintillation in a Packard Tricarb 4530 liquid scintillation counter.

13. Assay of Calcium transport in CANP Treated HSR.

The effect of CANP upon HSR Ca^{2+} transport was evaluated by spectroscopic and radiometric methods.

a. Spectroscopic analysis:

Various amounts (20-30μl) of concentrated CANP (100-250U.ml^{-1}) suspended in 300mM sucrose, 250μM EGTA, 50mM HEPES (pH 7.4), and 1mM DTT were added directly to 2.75mg HSR membranes (50-60μl) in fixed CANP/HSR ratios (0.5-2.5U/mg HSR protein). Immediately after CANP addition, various volumes of Ca^{2+} (1.5-3μl) were added from stock to stimulate proteolysis (25^{o}C).

Proteolysis was quenched after various times upon addition of concentrated leupeptin (10mg.ml^{-1}) to a final concentration of 250μM. The HSR/CANP/Ca^{2+} mixture was then added directly to 2.75ml of AP III Ca^{2+} transport buffer in the cuvette and Ca^{2+} uptake and Ca^{2+} release were assayed as described earlier. Controls were run with leupeptin addition made prior to CANP addition.

b. Radiometric analysis:

HSR membranes (5mg.ml^{-1}) were proteolysed for 20 mins (23^{o}C) at a CANP/HSR ratio of 5U/mg in the presence of 300mM sucrose, 100mM KCl, 50mM PIPES (pH 7.4), 5mM $^{45}Ca^{2+}$ (specific activity=15,000 dpm.nmol^{-1}). Proteolysis was quenched with an equal volume buffer containing 300mM sucrose, 100mM KCl, 50mM PIPES (pH 7.4), and 5mM EGTA. Membranes were diluted 50 fold into Ca^{2+} transport buffer containing 300mM sucrose, 100mM KCl, 50mM PIPES, 5.5mM Mg^{2+}, 187.68μM $^{45}Ca^{2+}$ (specific activity=15,000 dpm.nmol^{-1}) and 50μM EGTA (pH 7.4 at 23^{o}C) in the presence or absence of 50μM ruthenium red. The supplemental Ca^{2+} and EGTA added with the protein resulted in final total concentrations of 237.68μM and 100μM, respectively. ATP was added to a final concentration of 6.107mM to initiate Ca^{2+} accumulation. Final free concentrations of Ca^{2+}, Mg.ATP and Mg^{2+} were 25μM, 5mM and 0.5mM, respectively. Aliquots (150ml) of uptake media were vacuum filtered as above at various time points and filters were washed with 5 volumes of cold Ca^{2+} ruthenium red/Mg^{2+} containing release inhibiting buffer with radioactivity determined as above (see earlier section).

14. [^{3}H]Ryanodine Purification.

Commercially available ryanodine was purchased from Agri-Systems International as a tan coloured powder (lot # 8E09). This batch which was

poorly soluble above 5mM, in 10% methanol, is known to contain non-UV absorbing impurities (Humerickhouse et al., 1989) and was further purified by flash chromatography (Still et al., 1978) as described (Ruest et al., 1985). Ryanodine (50mg) was dissolved in 3ml chloroform and was applied to a glass column (12mm x 150mm) packed with Silica Gel 60 (230-400 mesh). Ryanodine was eluted with 40ml $CHCl_3$/MeOH/40% aqueous CH_3NH_2 (90:10:1.5) and identified with formation of a brown chromophore on heated H_2SO_4 sprayed TLC plates (Whatman 250μM layer #4420 222). Fractions were pooled and vacuum dried with formation of a colourless translucent ryanodine residue which was suspended in distilled-deionised H_2O to a final concentration of 20mM. This procedure resulted in 50% recovery of the starting material weight. [^{3}H]ryanodine (60 Ci/mmol) was purchased from DuPont and used without further purification.

15. [^{3}H]Ryanodine Binding.

HSR vesicles (400μg.ml^{-1} final concentration) were incubated at 22^{o}C for 24 hours in media containing 300mM sucrose, 150mM KCl, 5mM AMP, 100μM leupeptin, 8nM-1mM [^{3}H]ryanodine and 50mM HEPES (pH 7.4). Stock [^{3}H]ryanodine was added to a final concentration of 8nM with increasing concentrations of total ryanodine made from the addition of unlabelled ryanodine. 100μl aliquots were vacuum filtered across 0.45μ (13mm diameter) nitrocellulose filters which were rinsed with 10 volumes of ice cold buffer containing 300mM sucrose, 150mM KCl and 50mM HEPES (pH 7.4). Specific binding was obtained by assuming non-specific [^{3}H]ryanodine binding to be a constant proportion of total binding relative to [^{3}H]ryanodine binding observed at 1mM ryanodine. Radioactivity was assayed by liquid scintillation methods as described above.

16. SDS-Polyacrylamide Gel Electrophoresis.

HSR proteins were electrophoretically resolved across 10% uniform and 5-15% or mostly 3-13% linear polyacrylamide gradient resolving slab gels [37.5:1 acrylamide/N,N'-methylenebis-(acrylamide)] essentially as described (Laemmli, 1971). The resolving gel contained 375mM tris-OH (pH 8.8), 0.1% (w/v) SDS, and a 0-25% (v/v) glycerol gradient. The stacking gel (2.5% acrylamide (w/v)) contained 125mM tris-OH (pH 6.8), and 0.1% (w/v) SDS. Gels were polymerised with 5µl TEMED per 15ml gel solution and 150µl, 45µl, and 9µl additions of 10% (w/v) ammonium persulphate to the stacking, low acrylamide, and high acrylamide gel solutions, respectively. Protein samples were digested for 10 mins at room temperature to a final concentration of $1mg.ml^{-1}$ in 187.5mM tris-OH, 2% SDS, 10% glycerol, 5% B-mercaptoethanol and 0.2% bromophenol blue. The tank buffer contained 250mM tris-OH (pH 8.3), 192mM glycine, and 0.1% SDS. Electrophoresis was performed using the Bio-Rad Protean II or the Hoeffer Mighty Small slab gel apparatus. Proteins were resolved overnight at 12.5mA/slab for large gels or 20mA/slab for mini-gels.

17. Staining of Proteins Resolved by SDS-PAGE.

a. Coomassie Blue.

Gels were placed directly into solvent containing 0.275% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA) in MeOH/glacial CH_3COOH/H_2O (5:1:5) and protein was stained for 1hr. Gels were destained briefly in MeOH/glacial CH_3COOH/H_2O (5:1:5) and then extensively with several changes of the above solvent (4:1:15).

b. Stains-All.

Ca^{2+} binding proteins in HSR fractions resolved by SDS-PAGE were identified by their staining properties with the carbocyanine dye, Stains-All (3,3'-Diethyl-9-Methyl-4,5,4',5'-Dibenzothia carbocyanine), using the modifications (Campbell et al., 1983) of the original procedure by King and Morrison (1976). After electrophoresis the gel was dialysed in 25% 2-propanol with several changes of solvent during a 24 hr period to remove SDS which precipitates the stain. 5ml of 0.1% (w/v) Stains-All formamide was added to 195ml of solution containing 15ml formamide, 50ml 2-propanol, 130ml 46mM tris-base (pH 8.8). The pH of the final stain solution was adjusted to 8.8. The gel was placed in the stain for at least 24 hours after which highly acidic proteins and glycoproteins were visualised as blue or purple staining proteins. Proteolipids and lipid stained yellow while the majority of remaining protein stained pink.

18.CANP Proteolysis of HSR Resolved by SDS-PAGE.

HSR membranes ($4mg.ml^{-1}$) were exposed to CANP at various CANP/HSR ratios (0.1-10units/mg) in buffer containing 300mM sucrose, 50mM HEPES (pH 7.4) and various free [Ca^{2+}] (5μM-20mM) for 20 minutes at 23°C. Proteolysis was quenched upon addition of an equal volume of identical buffer containing 50mM EGTA and 100μM leupeptin. Samples were diluted with an equal volume of 2 times concentrated SDS-PAGE sample buffer (see earlier) and incubated for 10 mins.

19. Detergent Solubilisation of HSR Membranes for Immunolocalisation of Membrane Associated CANP.

Freshly thawed HSR membranes were incubated for 10 mins on ice at a concentration of 5mg.ml^{-1} in 2.5 ml of 2M NaCl, 50mM HEPES, 500μM Ca^{2+} (pH 7.4) in the presence or absence of 200μM leupeptin. Membranes were solubilised by addition of an equal volume of buffer containing 3% CHAPS, 1% pig brain phosphatidylcholine (Serdary Research Laboratory), 50mM HEPES and 10mM DTT (pH 7.4). Solubilsed membranes were further incubated at 23^{o}C for 2.5 hrs and insoluble material was sedimented in 5ml Beckman G-max minicentrifugation tubes at 45,000 rpm for 35 mins in a Ti 50 rotor. Pelleted material was suspended in 50mM HEPES (pH 7.4) in the presence or absence of 50μM leupeptin. Soluble and insoluble material was assayed for protein (see earlier) and samples were prepared for SDS-PAGE

20. Immunostaining of HSR Membranes

Western transfer of proteins resolved by SDS-PAGE was performed using the buffer system of Towbin et al. (1979). Proteins were electroblotted onto 0.45μ nitrocellulose membranes at 250mA/gel at 100V for 2hrs. Membranes were then incubated for 2hrs in blocking buffer containing 5% fat-free skim milk in tris-buffered saline or TBS (0.5M NaCl, 20mM tris-OH pH 7.4 at 23^{o}C). Monospecific polyclonal Calpain antibodies were a generous gift from Dr. T.S. Kuo (Dept. Pathology, Wayne State Univ., Detroit, MI). Polyclonal antisera which was cross-reactive to both μ and mCANP was raised in rabbits against purified mCANP isolated from rat hearts. Membranes were incubated overnight in antisera containing blocking buffer and washed once in TBS for 10 mins and then twice for 10 mins in TBS containing 0.2% Tween 20 followed by one 10 min wash in TBS. Membranes were incubated with the secondary goat anti-rabbit antibody

conjugated with horseradish peroxidase for 2hrs in blocking buffer. Washing of membranes was then repeated as above and horseradish peroxidase was detected with 4-chloro-1-naphthol and hydrogen peroxide using the Bio-Rad Immuno-Blot Assay System (cat# 170-6534) in accordance with the manufacturer's instructions.

21. Calculation of free ion concentrations.

Free Ca^{2+}, Mg^{2+}, ATP, and Mg.ATP concentrations were calculated using a Fortran IV based hand-held calculator (Texas Instrument TI-59 programmable calculator) program as described (Fabiato and Fabiato, 1979). Absolute stability constants used, were those tabulated in the original article by Fabiato and Fabiato (1979).

22. Data Analysis.

Where appropriate, all data points from a single experiment represent means of triplicate observations. Data is expressed as means +/- standard error the mean (SEM). Unless otherwise indicated the data presented was derived from one entire experiment and was representative of highly reproducable observations from atleast three separate experiments. For relative quantitation of proteins, gels were scanned at 633nm with a LKB 2202 Ultroscan Neon-Helium Laser Densitometer (LKB Pharmacia, Uppsala, Sweden) with protein peak integration determined using a LKB 2221 Integrator/Recorder. Extent of CANP proteolysis of the high molecular weight calcium release channel was calculated from the absorbance ratio (R_{633}) at 633nm of the cleaved protein (A_{633}) versus control protein (A'_{633}) (ie. $R_{633}=A_{633}/A'_{633}$) where $1-R_{633}$ represents the fractional loss of the channel.

RESULTS

I. Protein purification

1. Purification of HSR membranes.

Figure 5 (lanes C and D) shows that membranes prepared by freeze/grinding of rabbit skeletal muscle results in membrane preparation enriched in 550kDa and 57kDa proteins. These proteins, the ryanodine receptor/Ca^{2+} release channel and calsequestrin, respectively, are localised to the junctional terminal cisternae of the SR as dissused in the introduction. The yields of the membranes harvested from skeletal muscle are shown in Table 2. An additional major protein was found at ~105kDa. This protein was present in all of the membranes obtained from the sucrose gradients and is likely the Ca^{2+}-ATPase (Ca^{2+} pump). Not well resolved in this figure (see figures 29 and 30) is the presence of an additional protein banding at ~95kDa which was more evident in proteins resolved in lane A. Proteins in lane A contained minor amounts of 550 and 57kDa protein and are possibly a mixture of lighter density membranes derived from t-tubules, sarcolemma, and longitudinal SR. Enzyme marker studies were not performed upon this fraction. Table 2 shows that protein in lane B was the major membrane fraction in the sucrose gradient and contained small amounts of 550 and 57kDa protein. These membranes were shown to release lower proportions of intralumenal Ca^{2+} contents after Ca^{2+} induced Ca^{2+} release than the fractions harvested from the 35-38% sucrose region of the gradient. The fractions from the 29-33% sucrose region are possibly a mixture of membranes derived from the longitudinal and cisternal SR and

Table 2: Yields and calcium release characteristics of fractionated crude SR microsomes.

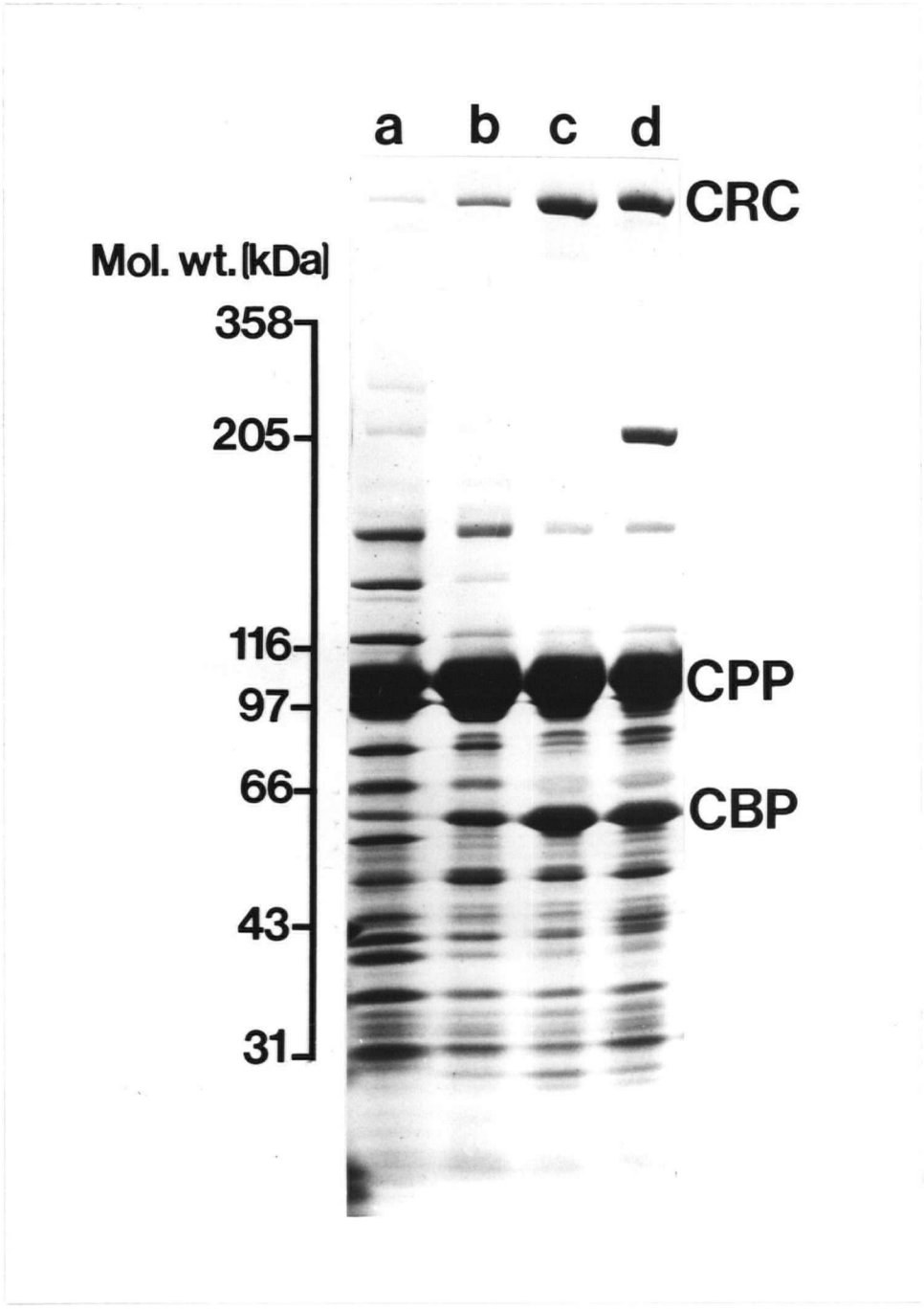
Sucrose Gradient Region (%)	Yield[a] (mg.g-1)	Ca^{2+} release[b] (%)
25-27	0.1 (5)	0
29-33	1.37+/-0.2 (29)	10
35-38	0.52+/-0.07 (29)	35-40
39-41	0.05+/-0.001 (4)	30-35

a. Values are averages (+/-S.D.) for membrane mg protein per gram muscle. Bracketted value = number of preparations.

b. Ca^{2+} release was assessed spectrophotometrically from the amount of Ca^{2+} release in actively loaded vesicles as described in Figure 21.

Fig. 5. Purification of HSR membranes from rabbit skeletal muscle. Membranes sedimenting in the 25-27% (lane a), 29-33% (lane b), 35-38% (lane c) and 39-41% (lane d) regions of the 25-45% linear sucrose gradient were separated and resolved by SDS-PAGE on a 3-13% linear acrylamide gradient. 25µl of membranes ($2mg.ml^{-1}$) were diluted by an equal volume of 2X sample buffer (see methods) and layered on top of a 2.5% acrylamide stacking gel. A constant current of 12mA was applied across the gel and the running time was 12-15 hrs. Proteins were stained in 0.275% (w/v) Coomassie Brilliant Blue R-250 and gels were destained in MeOH/glacial acetic acid/H_2O (5:1:5). The scale for protein molecular weight is on the left of the figure as determined by migration of the following protein standards: α_2 macroglobulin (dimer; 358kDa); myosin heavy chain (205kDa); β-galactosidase (116kDa); phosphorylase b (97kDa); bovine serum albumin (66kDa); ovalbumin (43kDa); carbonic anhydrase (31kDa). CRC, CPP, and CBP denote the position of the Calcium Release Channel (ryanodine receptor), the Calcium Pump Protein (Ca^{2+}-ATPase), and the Calcium Binding Protein (calsequestrin), respectively.

Figure 5.



will be referred to as intermediate SR or ISR.

Figure 6 shows that calsequestrin stains an intense blue colour with the carbocyanine dye, Stains-All, and provides a further indication that the protein fractions in lane C of Figure 5 are highly enriched in membranes derived from the terminal cisternae. The calsequestrin content of ISR was much reduced (lane B) and was present in minor amounts in the light membrane fractions. Also evident in the original coloured gels but poorly visualised in non-colour photographs was a lighter blue staining 165/175kDa doublet. This doublet was more evident in lanes C and D than lane B and was absent in lane A. The 105kDa Ca^{2+}-ATPase and the 550kDa ryanodine receptor stained pink in Stains-All stained gels and are seen in the black and white prints as lighter intensity bands. Densitometric scanning of several Coomassie Blue stained gels revealed a HSR 550/105/57kDa ratio of 1:5:2. These membranes are hereafter referred to as heavy SR or HSR and were used in all further structure/function studies. Membranes in lane D were also referable to HSR. In view of the low yield (see Table 2) and presence of contaminating myosin (205kDa) these membranes were not used in the study.

Figure 7 shows that HSR can be similarly purified from frozen rat (lane B) and dog (lane C) hearts by identical procedures used for rabbit skeletal HSR (lane A). The advantage with this method over conventional mincing of tissue is that isolated organs need not be used immediately but can be freeze clamped in liquid N_2 and stored at below -70^{o}C for at least six months. Inclusion of a cocktail of protease inhibitors was found to be essential to the maintenance of protein structural integrity, particularly in the case of the extremely protease sensitive ryanodine receptor (~500kDa protein in lanes B and C). Figure 6 also shows the presence of a 55kDa calequetrin isoform

Fig. 6. Stains-All staining of skeletal and cardiac HSR proteins resolved by SDS-PAGE. 50μg of skeletal microsomal protein harvested from 25-27% (lane A), 29-33% (lane B) and 35-38% (lane C) sucrose gradients were resolved on 3-13% linear gradient SDS-PAGE gels (see methods). Lane D contains 50μg of cardiac SR protein. The open arrow indicates the banding position (~57kDa) of skeletal calsequestrin (dark blue staining). The solid arrow indicates the banding position of cardiac calsequestrin (~53kDa). The single arrowhead denotes the position of a light blue staining 165/175kDa glycoprotein doublet in lanes B and C. The double arrowhead indicates the position of a 145/155kDa light blue staining glycoprotein doublet. Molecular weight markers are as indicated in Figure 5.

Figure 6

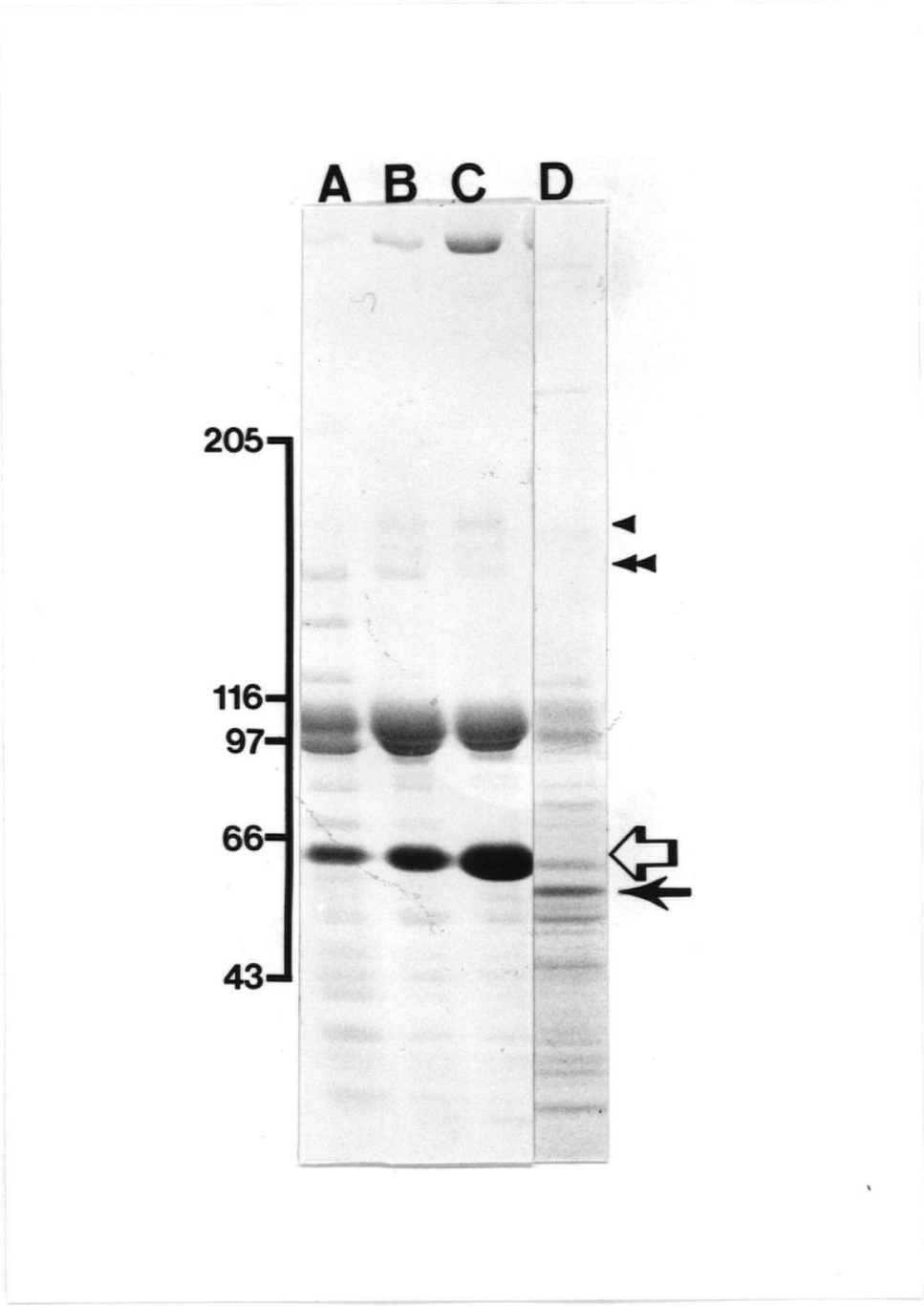
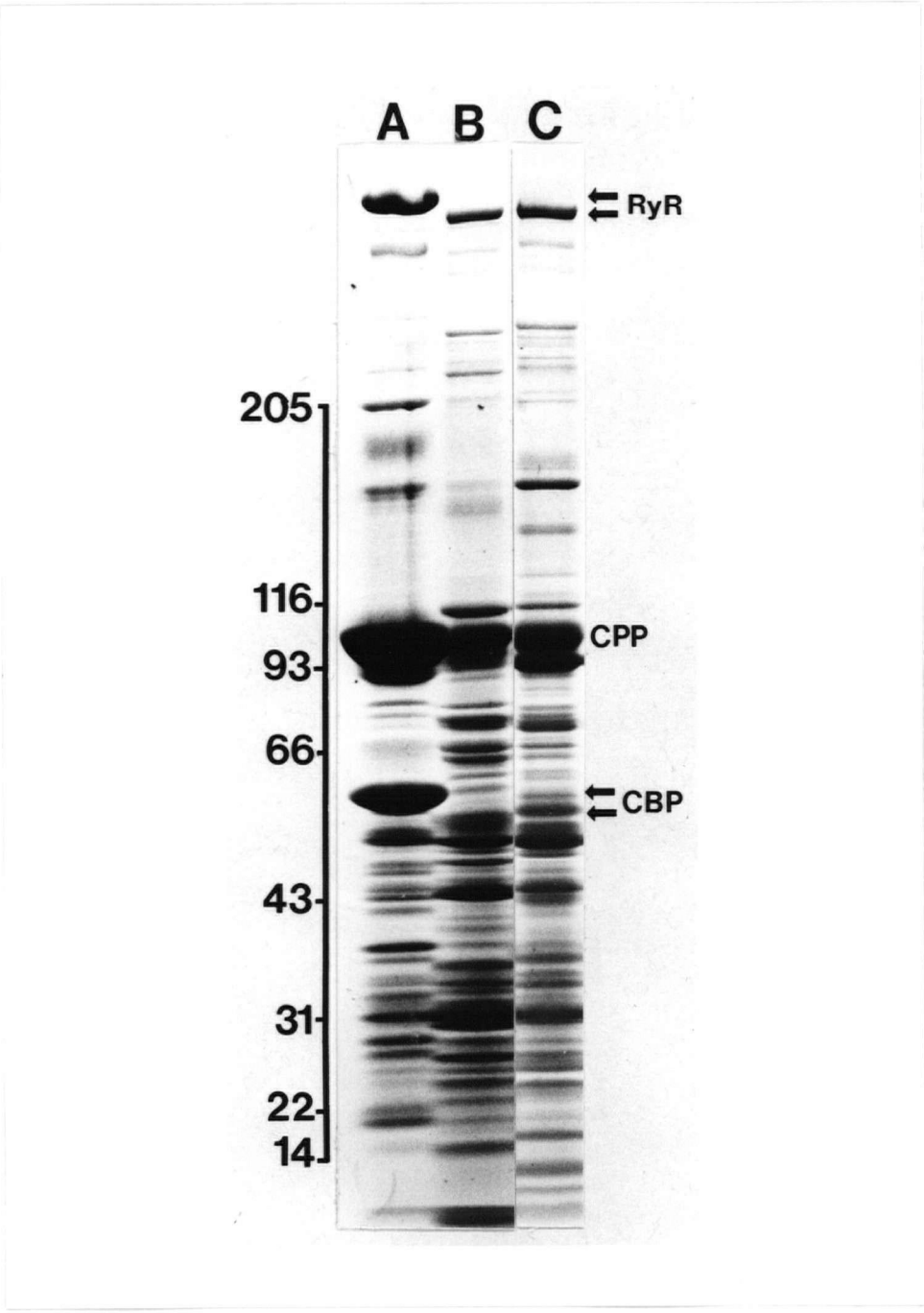


Fig. 7. SDS-PAGE comparison of skeletal and cardiac HSR. Comparison of rabbit skeletal HSR proteins (lane A) with rat (lane B) and canine (lane C) cardiac HSR proteins resolved on 3-13% linear gradient polyacrylamide gels (50μg per well). Molecular weight markers (kDa) are as indicated on the left of the figure. RyR = ryanodine receptor; CPP = Ca^{2+} pump protein (Ca^{2+}-ATPase); CBP = Ca^{2+} binding protein (calsequestrin). The arrows indicate the different molecular weights of RyR and CBP in skeletal (upper) and cardiac (lower) fractions.

Figure 7



in cardiac HSR, albeit to a lesser degree of enrichment than in skeletal HSR (compare lanes C an D).

2.Ryanodine receptor purification

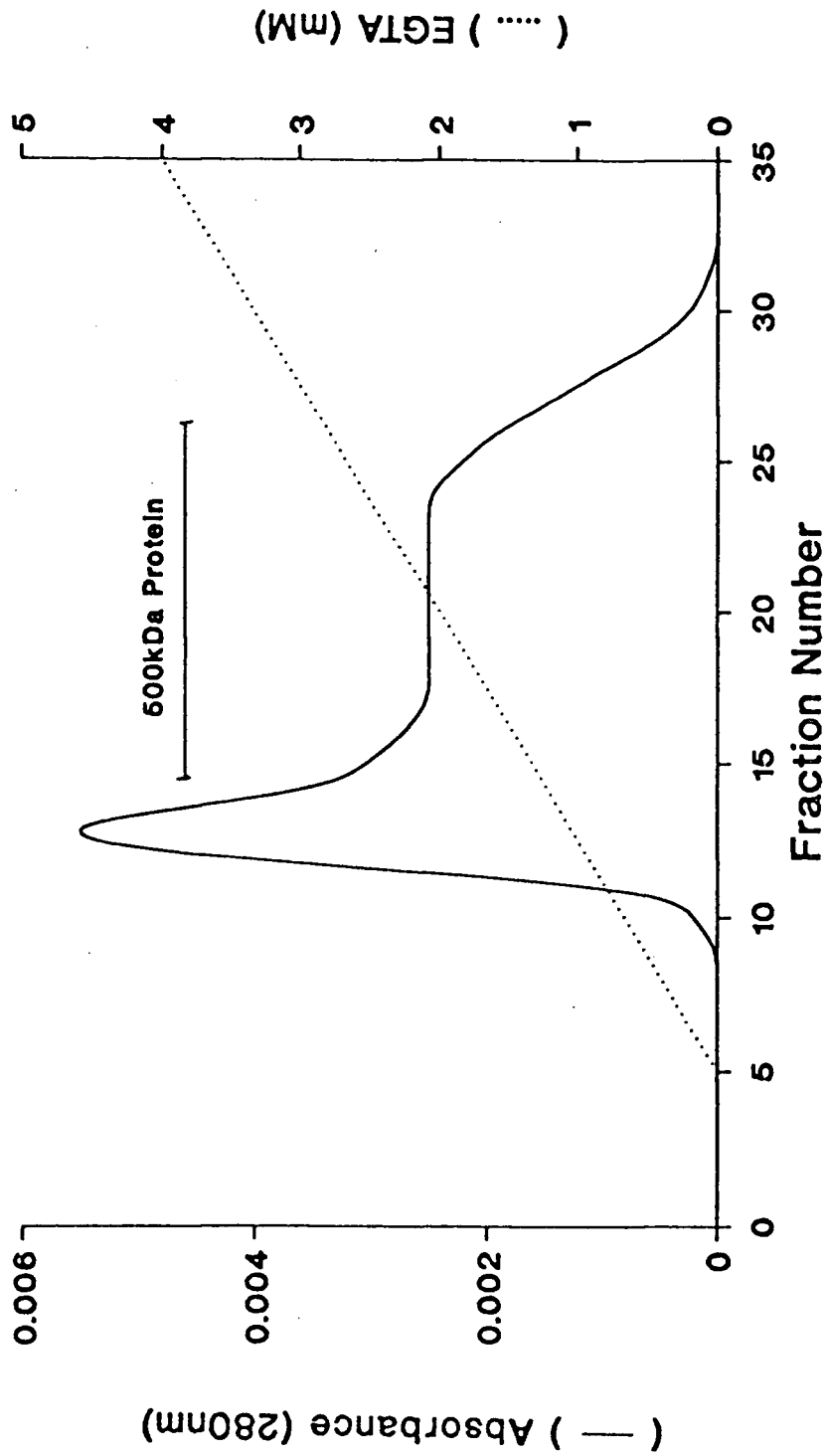
The ryanodine receptor was purified to examine the effect of CANP upon the solubilised form of this intrinsic membrane protein. The isolation protocol was based upon the premise that the ryanodine receptor is a calmodulin (CaM) binding protein (Seiler et al., 1984). Figure 8 shows the elution profile of CHAPS solubilised proteins bound to the CaM-agarose affinity column. Protein eluted with an initial sharp peak corresponding to the protein in lanes 11-14 of Figure 9. This elution was followed by a broad shoulder elution of purified ryanodine receptor protein (lanes 15-26). These latter fractions were pooled and used for further analysis. It is noteworthy that although the unbound or flow through fraction in lane D of figure 9 shows that most of the ryanodine receptor associated with the column, the yield of purified protein was very low for a single step procedure (~1%). This was observed in over 15 preparations. Most of the protein appeared to "bleed" off the column during the wash stage. Therefore, although specific Ca^{2+} dependent binding to CaM was observed, this binding appeared to be weak under the conditions employed.

3. Purification of CANP

During preliminary studies of CANP effects upon HSR protein structure and function, μCANP (a generous gift from Dr. K. Wang) purified from the cytosolic fraction of human erythrocytes, was used. In addition, commercially available mCANP was also used. The questionable purity and cost of the latter preparation and the undetermined species and

Fig. 8. Calmodulin (CaM)-agarose affinity chromatography of CHAPS solubilised HSR protein. 500ml of solubilised HSR membranes (~250μg) in 100mM KCl, 20mM tris-OH (pH 7.4), 200uM Ca^{2+}, 5μg.ml^{-1} leupeptin, 0.1% CHAPS, 0.05% asolectin and 2mM DTT were loaded onto a CaM-agarose column (10cm x 1.6cm) at 30ml.hr^{-1}. The column was pre-equilibrated in the above buffer and protein was eluted after extensive washing with a linear (0-5mM) EGTA gradient (dotted line) in the same buffer. 4ml fractions were collected and the absorbance (A280) traces (solid line) were obtained from a flow through UV-monitor. Protein in 1ml fractions was precipitated with addition or 0.5ml ice-cold 10% (w/v) TCA. Sedimented protein was suspended in SDS-PAGE sample buffer and resolved across 5-15% acrylamide gradient gels.

Figure 8


(——) Absorbance (280nm)
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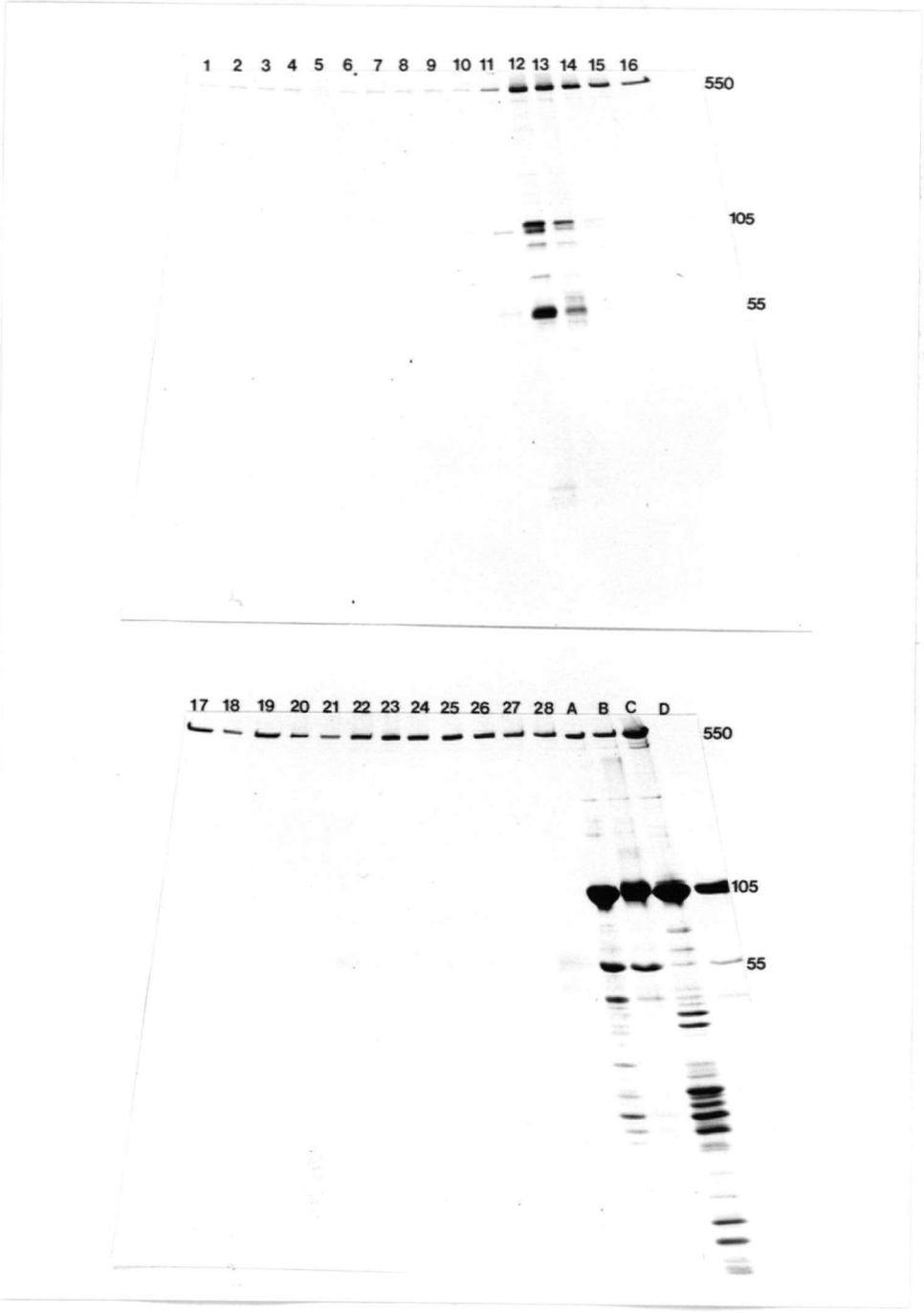
0.004
0.002
0
0
5
10
15
20
25
30
35
Fraction Number
600kDa Protein
(.....) EGTA (mM)
5
4
3
2
1
0

Fig. 9. SDS-PAGE resolution of CaM-agarose affinity purified HSR proteins. 1ml of protein collected in each fraction tube after Ca^{2+}/EGTA gradient elution (as described in Figure 8) was denatured in 3.3% (V/V) TCA on ice. Sedimented protein was resuspended in 50µl SDS-PAGE sample buffer (see methods) and resolved on 5-15% linear gradient SDS-PAGE gels. Protein was stained with Coomassie-brilliant blue as described in the methods. Each lane (1-27) corresponds to each fraction obtained in Figure 8. Lanes A to D correspond to untreated HSR, CHAPS soluble fraction, CHAPS insoluble fraction and flow through (unbound to CaM column) fractions respectively. The positions of the Ca^{2+} channel, Ca^{2+}-ATPase and calsequestrin are indicated to the right of the Figure at 550, 105 and 55kDa, respectively.

Figure 9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550
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17 18 19 20 21 22 23 24 25 26 27 28 A B C D
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105
55

tissue specific effects of CANP emphasized the need to purify endogenous CANP in the present study. Both μCANP and mCANP was purified from the cytosolic fraction of rabbit skeletal muscle homogenates using standard chromatographic procedures (Figures 10-13). Both isoforms were conveniently well separated after the first ion-exchange column step (Figure 10) and could be further purified individually. The co-elution of calpastatin, the endogenous Calcium Activated Protease Inhibitor (CDPI), resulted in a masking of μCANP activity. As shown in Table 3, CDPI was separated from μCANP during phenyl-sepharose chromatography with a 1.5 fold elevation of total μCANP activity. It is of interest to note that the decreased retention of μCANP vs mCANP upon hydrophilic ion-exchange columns is opposite to their respective interactions with the hydrophobic phenyl-sepharose column. This was consistently observed and may reflect the decreased overall hydrophobicity of mCANP vs μCANP (Imajoh et al., 1988) particularly within domain IV, the Ca^{2+} binding region. Figure 14 shows the protein profile after each purification step. Significant purification was acheived after the phenyl-sepharose and gel-permeation steps. Lanes D and D' show the μ- and mCANP fractions used in this study with the respective 82 and 80kDa large subunits prepared intact and un-autolysed. Co-purifying with each CANP fraction, in addition to 30kDa small subunits, were single proteins of different molecular weights. Whether these proteins represented contaminants of specific CANP binding proteins was unclear and was not investigated further. However, each was present in stoichiometric amounts in every CANP fraction obtained after gel-filtration.

II. Functional Characterisation of HSR membranes

Fig. 10. DEAE-sepharose CL-4B anion exchange chromatography of ammonium sulphate precipitated u- and mCANP from rabbit skeletal muscle homogenates. Protein from 25-65% ammonium sulphate was loaded onto the column after extensive dialysis. After column washing the protein was eluted with a linear salt gradient (0-400mM KCl) in LSB (see methods). The separate elution profiles of μ- and mCANP are as indicated by the horizontal bars. Co-elution of calpastatin (CDPI) with μCANP masked the activity of CANP and created the apparent double elution peak in this region. Protein absorbance at 280nm ( ) and CANP activity ( ) was measured for each tube. The elution profile was obtained after isolation of CANP from 300g skeletal muscle and is representative of 4 preparations.

Figure 10

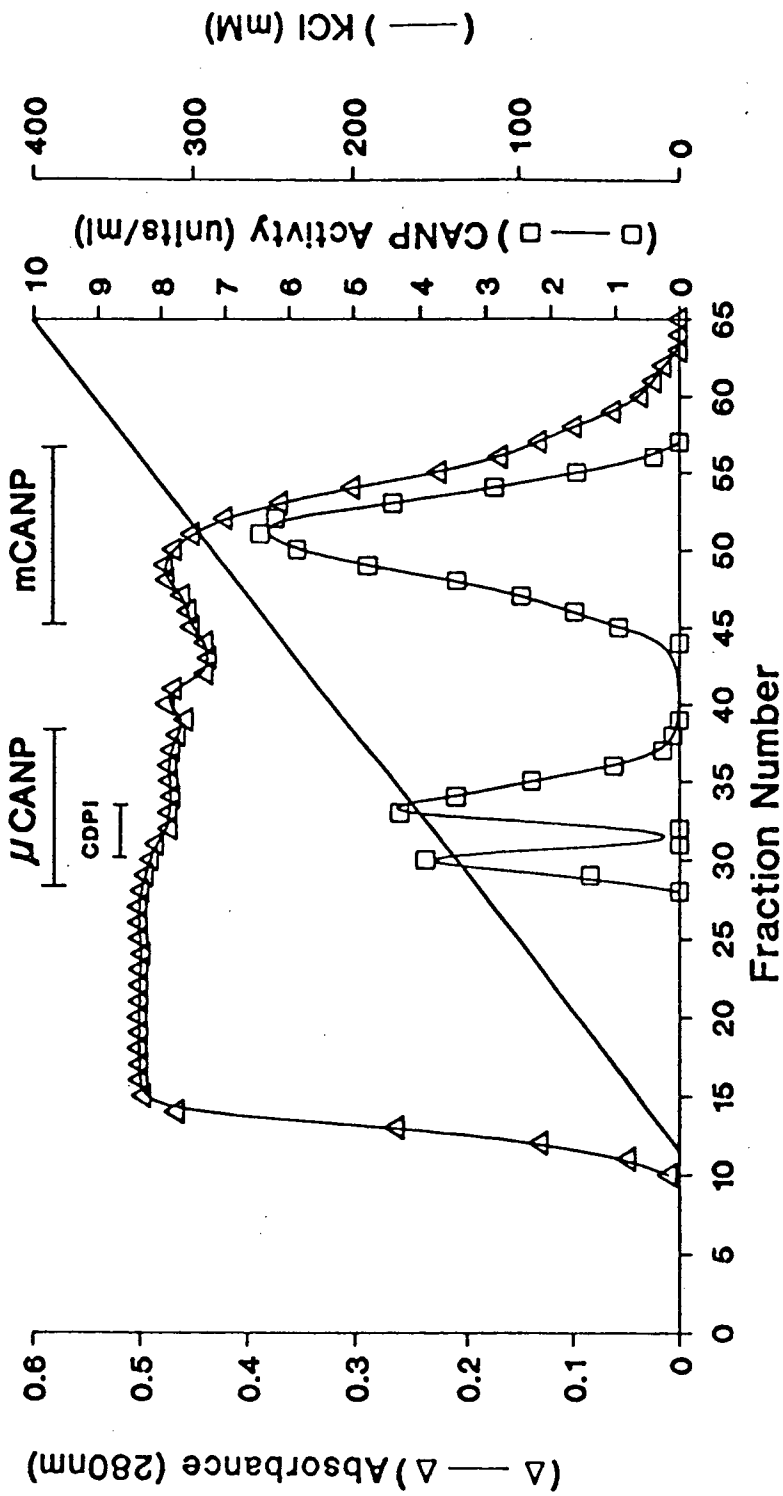

(Δ —— Δ) Absorbance (280nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Fraction Number
μCANP
CDPI
mCANP
(□ —— □) CANP Activity (unit/ml)
10 9 8 7 6 5 4 3 2 1 0
(——) KCl (mM)
400 300 200 100 0

Fig. 11. Phenyl-sepharose CL-4B chromatography of DEAE separated μ- and mCANP. In (A) ionic strength of the pooled DEAE-sepharose fraction μCANP was elevated to 250mM KCl with HSB (see methods) and loaded onto a phenyl-sepharose column pre-equilibrated with LSB-200. The column was washed with LSB-200 and CANP was eluted with LSB. CANP activity ( ) and absorbance at 280nm ( ) were determined as described in the methods. In (B) ionic strength of the pooled DEAE-sepharose mCANP fraction was not adjusted and protein was loaded directly onto the column. All other procedures were as described for μCANP.

Figure 11

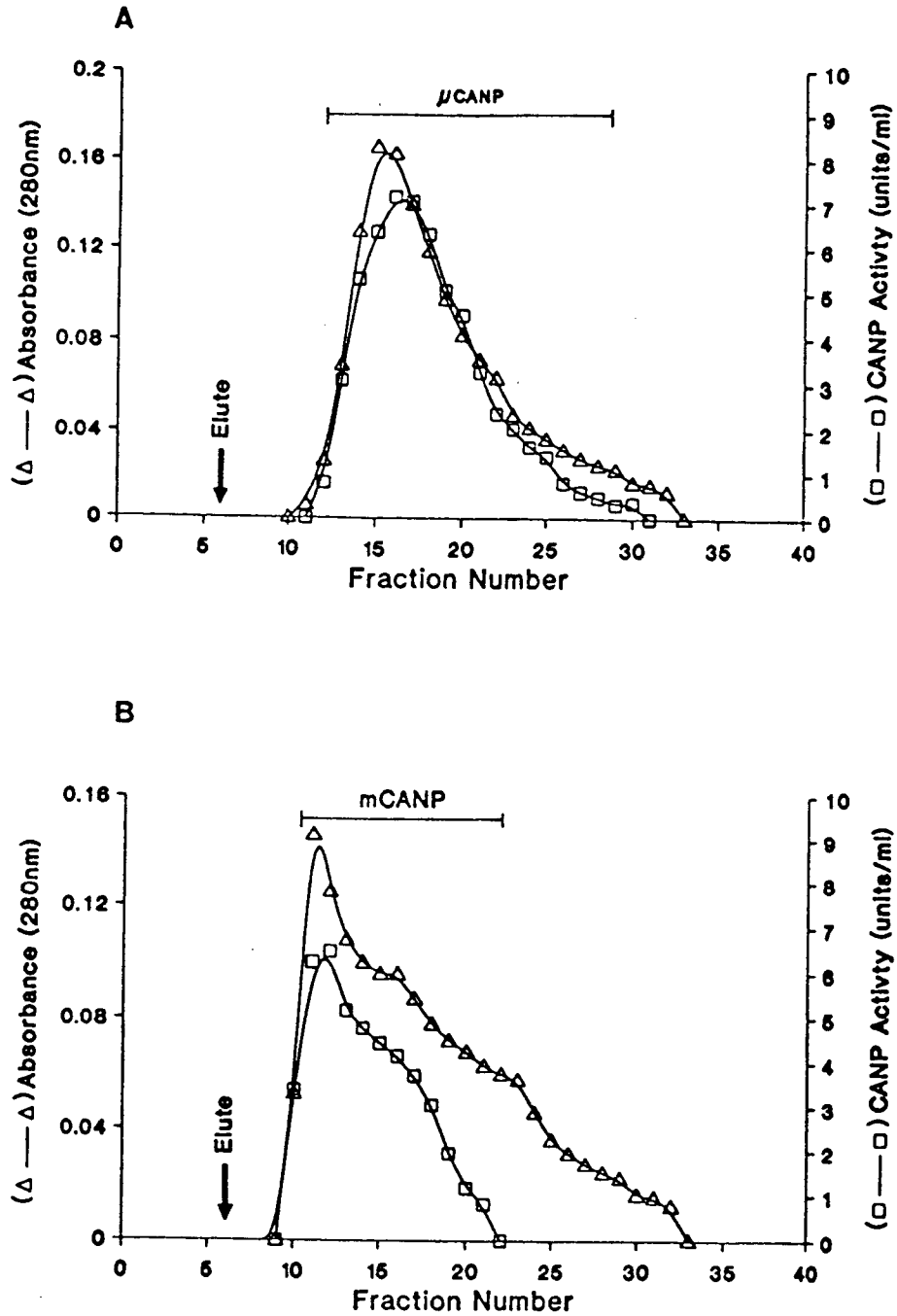

A
μCANP
Elute
(Δ — Δ) Absorbance (280nm)
(□ — □) CANP Activty (units/ml)
Fraction Number
B
mCANP
Elute
(Δ — Δ) Absorbance (280nm)
(□ — □) CANP Activty (units/ml)
Fraction Number

Fig. 12. Omega-hexylamine-agarose chromatography of phenyl-sepharose CL-4B isolated μ- and mCANP. In (A) the pooled μCANP fractions from the phenyl-sepharose column were loaded onto an ω-hexylamine column bifunctional column (1.6cm x 12cm) pre-incubated with LSB as described in the methods. After extensive washing with LSB the protein was eluted across a 0-400mM KCl (180ml) linear salt gradient in LSB. Absorbance at 280nm ( ) and μCANP activity ( ) was monitored for each fraction (4ml) as described in the methods. Each tube was measured for ion conductivity to identify the KCl gradient as shown. In (B) the corresponding elution for mCANP is shown. Identical procedures to that described for μCANP were adopted.

Figure 12

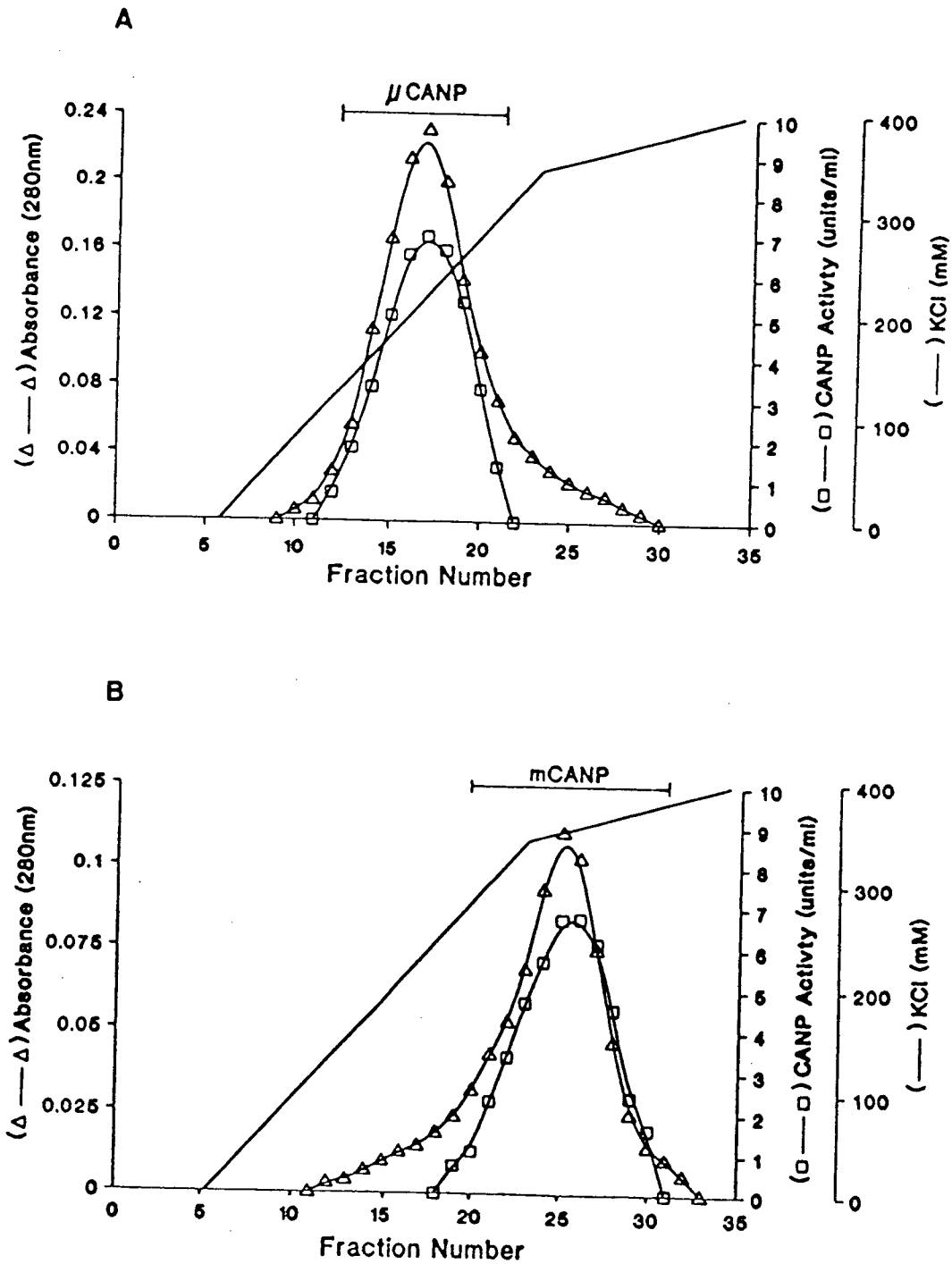

A
μCANP
(Δ — Δ) Absorbance (280nm)
(□ — □) CANP Activty (units/ml)
(—) KCl (mM)
Fraction Number
B
mCANP
(Δ — Δ) Absorbance (280nm)
(□ — □) CANP Activty (units/ml)
(—) KCl (mM)
Fraction Number

Fig. 13. Gel permeation (Ultrogel AcA 34) chromatography of ω-hexylamine-agarose isolated CANP. In (A) protein eluting from the ω-hexylamine column were pooled and concentrated to 3ml in an Amicon N_2 pressure cell across YM-10 membranes on ice. For each column run 1.5ml of μCANP was loaded onto the column (1.6cm x 80cm) at $6ml.hr^{-1}$. The column was pre-equilibrated with LSB which was also used as the elutant. Protein was collected in 3ml fractions each of which was monitored for absorbance at 280nm ( ) and activity ( ). In (B) identical procedures to that in (A) were employed for mCANP gel filtration. The void volume as determined from prior Blue Dextran-200 chromatography was 60ml.

Figure 13

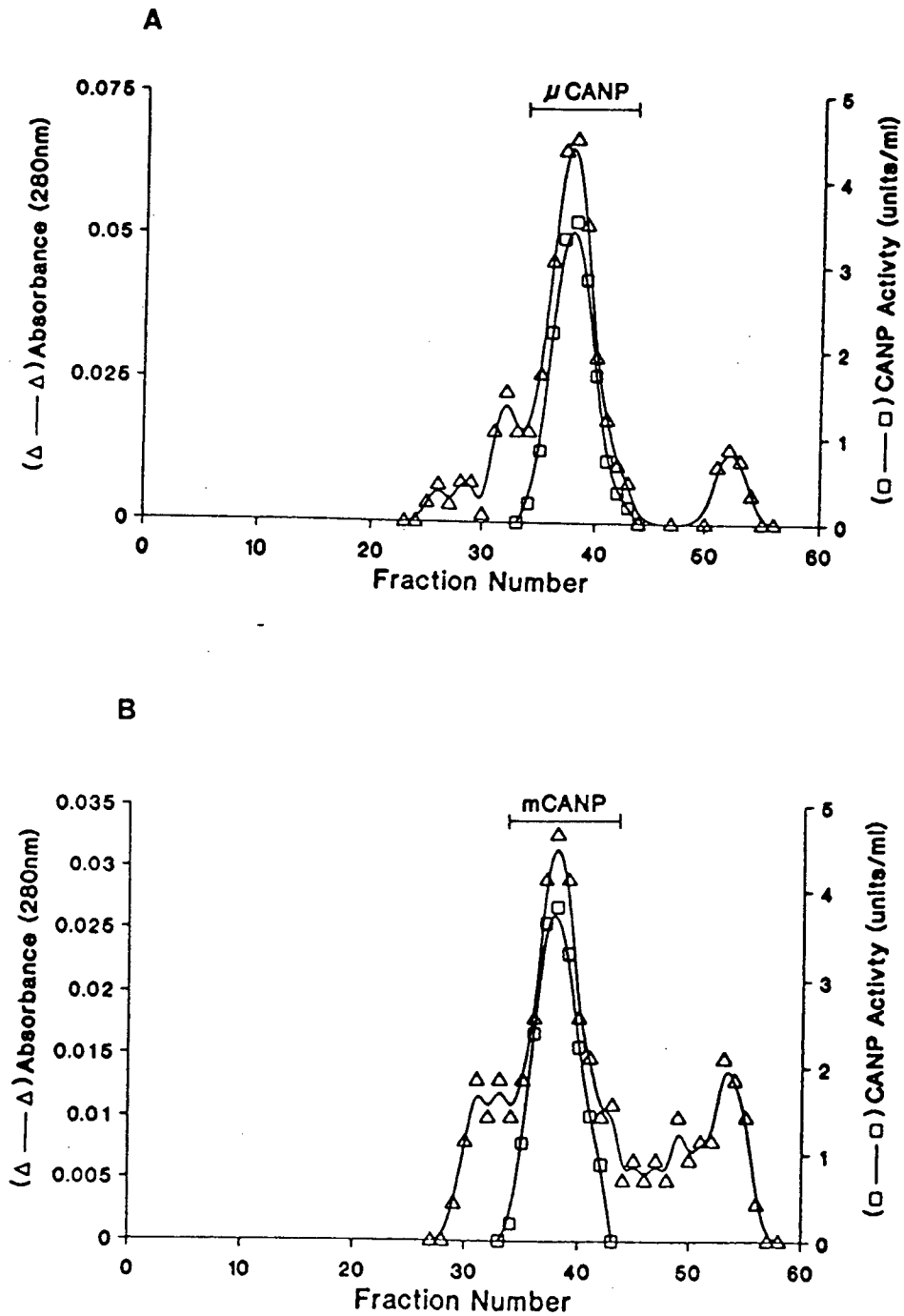

A
μCANP
(Δ — Δ) Absorbance (280nm)
(□ — □) CANP Activty (units/ml)
Fraction Number
B
mCANP
(Δ — Δ) Absorbance (280nm)
(□ — □) CANP Activty (units/ml)
Fraction Number

Fig. 14. Purification scheme of CANP purification followed by SDS-PAGE. CANP containing fractions were pooled after DEAE-sepharose CL-4B (lanes A, A'), phenyl-sepharose (lanes B, B'), ω-hexylamine (lanes C, C'), and AcA 34 Ultragel gel permeation (lanes D, D') column chromatography. 25μl of each pool was dissolved in 25μl of 2x sample buffer (see methods) and applied directly to the sample wells of Hoeffer Mighty Small electrophoresis gels. The resolving gel was 10% acrylamide and the stacking gel was 2.5% acrylamide. Proteins were stained with Coomassie-R250. Molecular weight marker proteins ($M_r x10^3$) on the left of the panel are as indicated in the legend to Figure 5. 82, 80, and 30kDa proteins refer to large subunits of μCANP and mCANP and the small subunit, respectively.

Figure 14

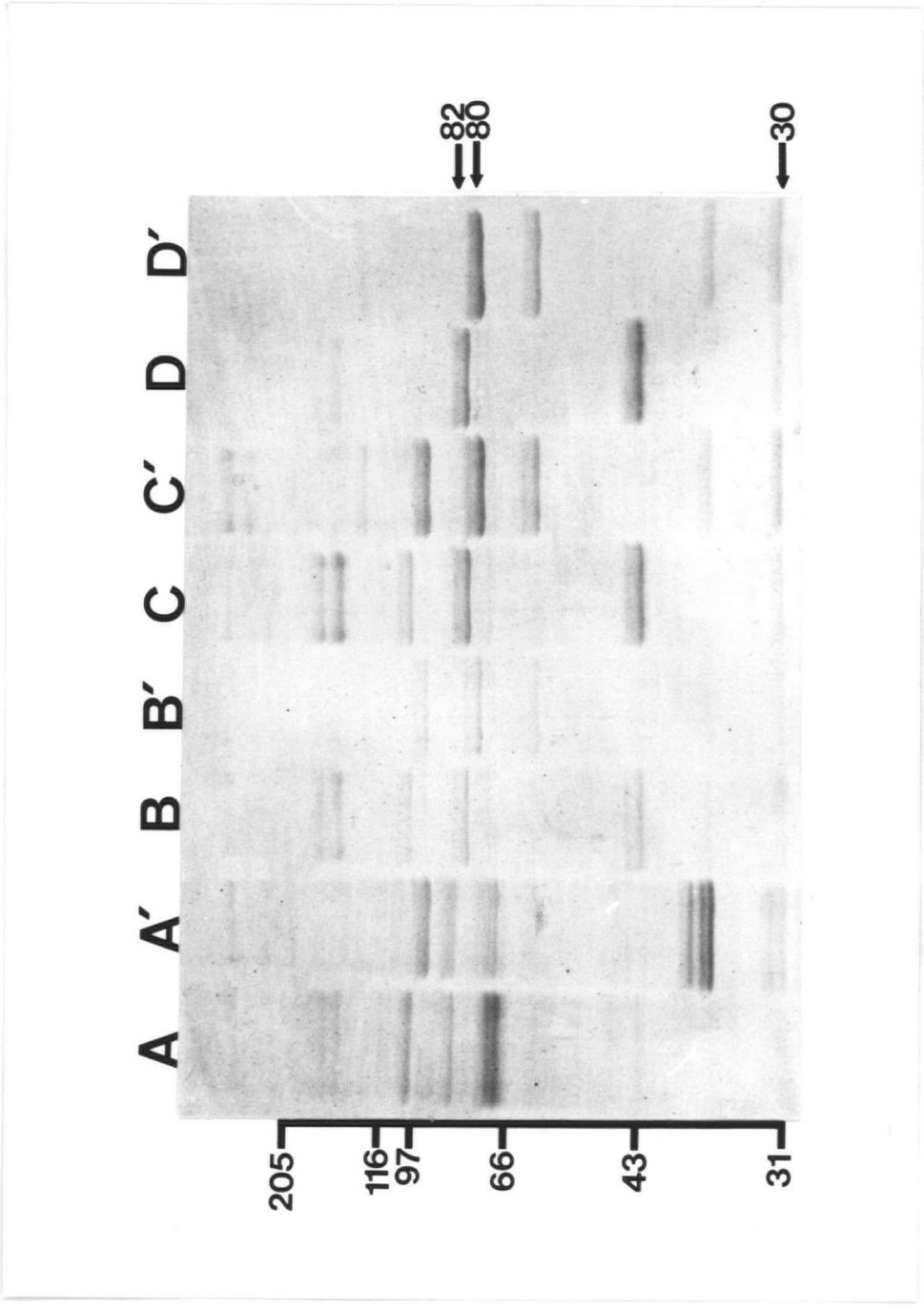


Table 3: Purification of μ- and mCANP from rabbit skeletal muscle

Fraction	Total Protein (mg)	Specific Activity (Units.mg^{-1})	Purification (fold)
Dialysate	15,000	0.0209	1.0
DEAE-sepharose(m)[a]	240	1.84	8.8
DEAE-sepharose(μ)	210	0.8	3.8
Phenyl-sepharose(m)	40	5.5	263.0
Phenyl-sepharose(μ)	36	6.56	313.0
ω-hexylamine(m)	18	11.36	544.0
ω-hexylamine(μ)	15	10.9	522.0
Ultragel-AcA34(m)	4	32.5	1555.0
Ultragel-AcA34(μ)	3	35.0	1674.0

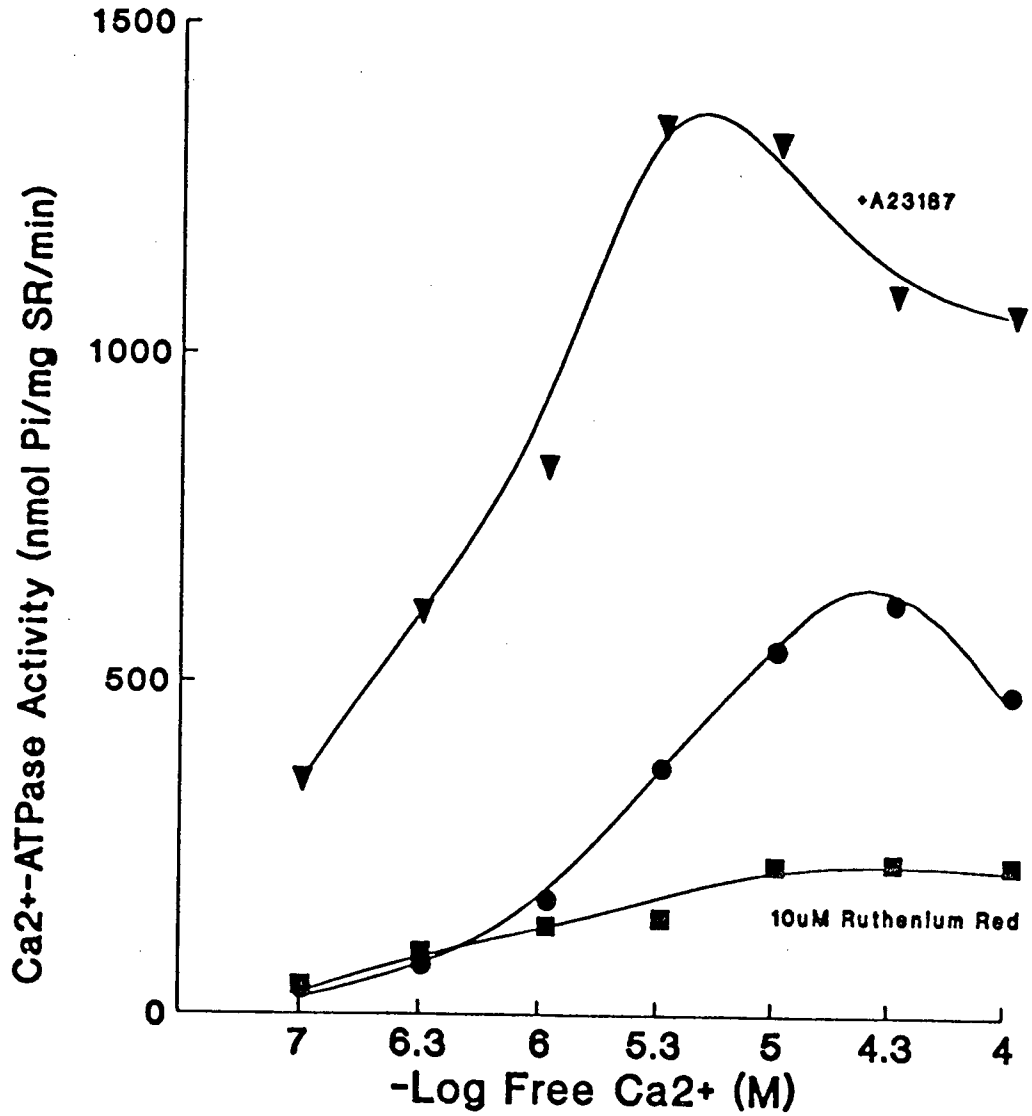
a. Bracketted symbols (μ, m) refer to μCANP and mCANP, respectively.

1. Ca^{2+} dependent activation of membrane bound Ca^{2+}-ATPase activity.

The procedures used in this study for isolating HSR membranes were novel in that frozen rather than fresh muscle was used as a source of tissue. It was important, therefore, to determine whether HSR membrane integrity and the functional properties of the Ca^{2+} pump were preserved. Figure 15 shows that in the presence various free Ca^{2+} concentrations, peak Ca^{2+} stimulated ATPase activity (650nmol Pi.mg $HSR^{-1}.min^{-1}$) was observed at 40-50μM free Ca^{2+} in native membranes. A classic bell shaped Ca^{2+} activation profile was observed with inhibition of ATPase activity between 50 and 100μM Ca^{2+}. In the presence of 10μM ruthenium red, a blocker of the Ca^{2+} channel, a 2.8 fold suppression of ATPase activity was observed with a dramatic reduction of the Ca^{2+} activation profile of ATPase activity. This result indicates that expression of ATPase activity is in part, determined by a Ca^{2+} leak pathway which in these preparations is likely the Ca^{2+} release channel. On the other hand, a 2-fold stimulation of peak ATPase activity (1375nmol Pi.mg $HSR^{-1}.min^{-1}$) was observed at ~5μM free Ca^{2+} upon addition of 10μM of the Ca^{2+} ionophore A23187. The ionophore uncouples ATPase activity from intralumenal Ca^{2+} regulation and expresses solely the extralumenal Ca^{2+} regulation of ATPase activity. In this regard it is interesting that in the presence of ionophore the pCa_{50} is reduced (0.6μM) whereas in its absence the observed pCa_{50} was much higher (4μM). These differential effects of the Ca^{2+} channel blocker and the Ca^{2+} ionophore indicated that the HSR Ca^{2+}-ATPase was under tight control of both extralumenal Ca^{2+} and intralumenal Ca^{2+} and that non-specific membrane permeability to Ca^{2+} in these membranes was very small.

Fig. 15. Calcium dependent stimulation of HSR calcium stimulated ATPase activity. HSR vesicles ($10\mu g.ml^{-1}$) were assayed for ATPase activity in the presence of 300mM sucrose, 100mM KCl, 20mM PIPES (pH 7.0), 1.25mM free Mg^{2+} and 0.1 to 100μM free Ca^{2+} ($22^{o}C$). ATPase turnover was stimulated by addition of 5mM free Mg·ATP and reactions were quenched by addition of SDS (see methods). Reactions were run in the presence and absence of 10μM Ruthenium Red and 10μM A23187. Stability constants used for calculation of total Mg^{2+}, Ca^{2+} and ATP required for desired free concentrations were obtained from a calculator program described by Fabiato and Fabiato (1979). Ca^{2+} stimulated ATPase activity was obtained by subtraction of ATPase activity observed in the presence of 100uM EGTA. This was less than 10% of the total activity at pCa 4.3. Data points are means of 4 observations from a single experiment (sem less than +/-5%). The experiment was repeated on 3 separate occassions with identical results.

Figure 15

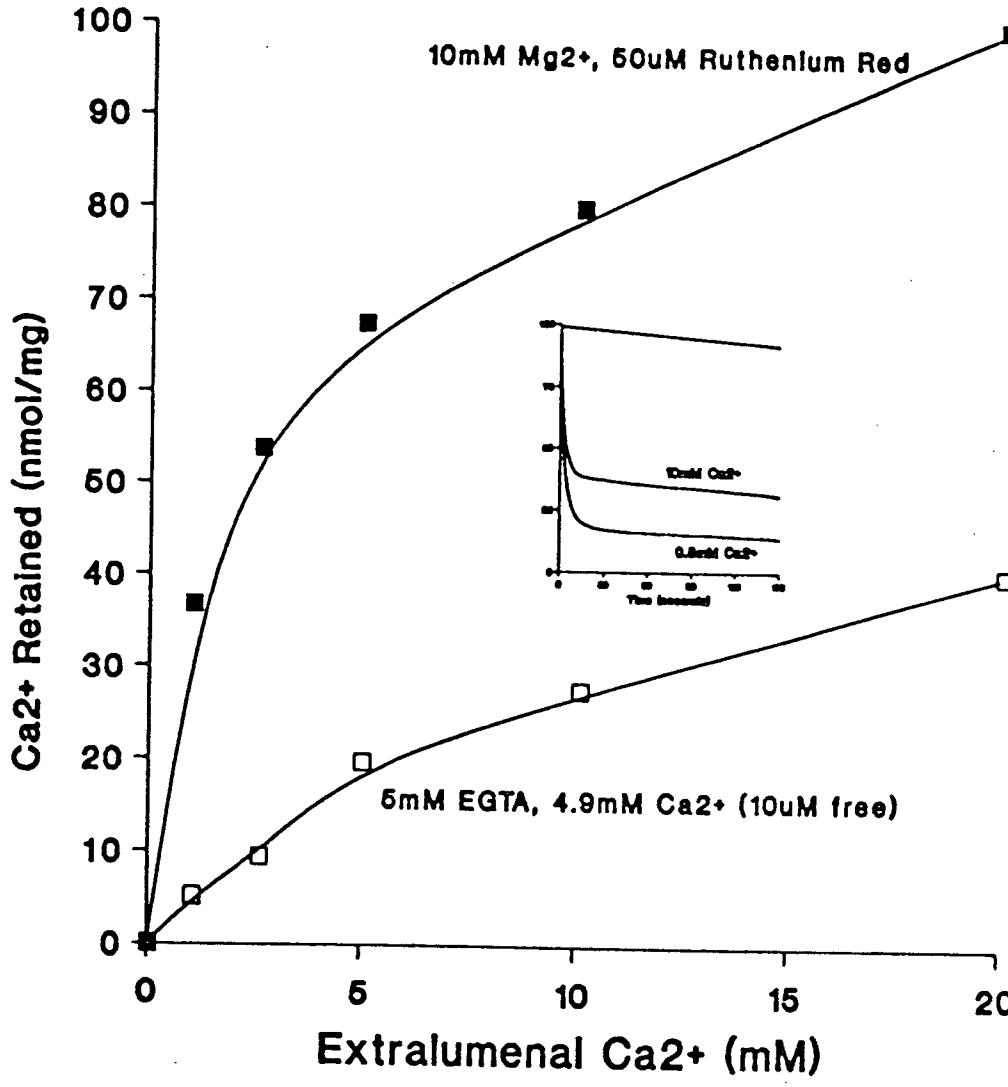

1500
1000
500
0
Ca2+-ATPase Activity (nmol Pi/mg SR/min)
+A23187
10uM Ruthenium Red
7
6.3
6
5.3
5
4.3
4
-Log Free Ca2+ (M)

2. Passive Ca^{2+} loading and Ca^{2+} release.

In Figure 16 the Ca^{2+} release characteristics of HSR vesicles was examined as a function of intralumenal Ca^{2+} load. HSR vesicles were incubated with various concentrations of Ca^{2+} and aliquots of the loaded vesicles were rapidly diluted into iso-osmotic media containing either (a) Ca^{2+} channel blockers, ruthenium red (50μM) and elevated Mg^{2+} (10mM), designed to inhibit Ca^{2+} release or (b) 10μM free Ca^{2+} (5mM EGTA, 4.9mM Ca^{2+}) to stimulate Ca^{2+} release. Figure 16A shows that intralumenal Ca^{2+} loading was steeply dependent upon extralumenal Ca^{2+} up to ~2mM Ca^{2+}. Between 2mM and 10mM Ca^{2+} the increase in the Ca^{2+} retained was much more gradual and non-saturating. The total amount of Ca^{2+} released within 15 seconds increased with elevated intralumenal Ca^{2+} load. However, as shown in Figure 16B, the fractional Ca^{2+} release was greatest at low intralumenal Ca^{2+} (86% at 500μM extralumenal Ca^{2+}) and progressively decreased with increased intralumenal Ca^{2+} (60% at 10mM extralumenal Ca^{2+}). It is worth noting at this point that the design of the filtration device allowed rapid removal of filters from support grids during time dependent filtration assays. Preliminary investigations showed that when filters remained on support grids, as in the case when a 12 port Millipore filtration drum was used, the Ca^{2+} contents of the vesicles would leak out. Because of this a large constant error was found between the first filter (lowest counts) and the twelfth filter (highest counts). In most cases with the customised filtration device, the within sample standard error of counting at each point was less than 2.5%. This apparatus was, therefore, used in all radiometric determinations of ligand binding and Ca^{2+} flux kinetics performed in this study.

Fig. 16. Effects of varying extralumenal calcium upon HSR calcium loading and calcium-induced calcium release. 10mg.ml^{-1} vesicles were passively loaded in various amounts of $^{45}Ca^{2+}$ as indicated. After maximum loading, vesicles were rapidly diluted (250 fold) into iso-osmotic release quench buffer (filled squares) of release stimulating buffer (open squares). Aliquots were rapidly removed at 15, 30, 60, 120 second intervals and filters were rinsed with quench buffer (see methods). The data represent Ca^{2+} retained by the vesicles 15 seconds after dilution. The inset compares the Ca^{2+} release profile after loading in 0.5mM (A) and 10mM (B) Ca^{2+}. The ordinate represents % maximum Ca^{2+} loaded and the abscissa represents time (seconds). Each data point is a mean of 3 observations from a single experiment (sem less than +/-5%). The entire experiment was repeated twice with similar results.

Figure 16


10mM Mg2+, 50uM Ruthenium Red
5mM EGTA, 4.9mM Ca2+ (10uM free)
Ca2+ Retained (nmol/mg)
Extralumenal Ca2+ (mM)
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20

3. Spectroscopy of HSR Ca^{2+} transport.

a. Ca^{2+}:AP III difference spectral characteristics.

Antipyrylazo III (AP III) is a Ca^{2+} sensitive metallochromic dye (1:2 Ca^{2+}:AP III binding stoichiometry) that is linearly responsive to Ca^{2+} over a Ca^{2+} concentration range appropriate for the study of HSR Ca^{2+} transport (Scarpa et al., 1979). Ca^{2+}:dye (CaD_2) difference absorbance (ΔA) is very sensitive, however, to optical interferences from Mg^{2+} and ATP. The use of AP III in dual wavelength studies is a potentially powerful means of investigating HSR Ca^{2+} transport. It was important, therefore, to investigate the limitations and spectral characteristics of this dye and to establish conditions for the accurate quantitation of HSR Ca^{2+} fluxes.

(i). Mg^{2+} effects upon CaD_2:AP III difference absorbance.

The CaD_2 difference spectrum between 640nm and 790nm is shown in Figure 17A (trace a). Absorbance maxima (λ_{max}) were observed at 652nm and 716nm with an isosbestic point at the 790nm reference wavelength. Table 4 also shows that with the purified dye there was good agreement between apparent first order (K'_{CaD}=257μM) and second order (K''_{CaD}=24789μM^2) dissociation constants, fixed by the relationship $K'_{CaD}=K''_{CaD}/2D$ where D is the total dye concentration (Rios and Schneider, 1981). Addition of Mg^{2+} to sample cuvette solutions containing 50μM Ca^{2+} decreased dye:ligand ΔA particularly at wavelengths above 700nm with formation of a single λ_{max} at 685nm (Figure 17A). Part of the Mg^{2+} effect is via a reduction of the CaD_2 stability constant with formation of a 1:1 complex (MgD) with the pure dye (Baylor et al., 1982). The presence of Mg^{2+} resulted in 1.24 and 1.26 fold increases, respectively, in K'_{CaD} and K''_{CaD}. Isosbestic points for the MgD spectra were recorded

Fig. 17. Double beam spectroscopy of AP III:divalent cation ΔA spectra. (A). Effect of Mg^{2+} upon difference spectra in the presence of Ca^{2+}. Trace a is the Ca^{2+}:AP III difference spectrum obtained with 50μM Ca^{2+} and 50μM AP III. Mg^{2+} was added in 500μM increments (traces b to g) to a final concentration of 3mM. (B). Mg^{2+}:AP III difference spectra. Mg^{2+} was added in 1mM increments (traces a to e) to 5mM (trace e). (C) Effect of Tris-ATP (traces b to e) upon difference spectra in the presence of 5mM Mg^{2+} (trace a). Nucleotide was added in 250μM increments to a final concentration of 1mM. Both reference and sample cuvettes contained basic solution (see **Methods**). Ca^{2+}, Mg^{2+} and nucleotide were added to the sample cuvette from stock (2.5mM, 100mM and 50mM, respectively) prepared in basic solution. Spectra (between 640 and 790 nm) were obtained after baseline subtraction. Scales for ΔA are as indicated with positive and negative absorbance positioned above and below, respectively, the horizontal baseline in each figure.

Figure 17

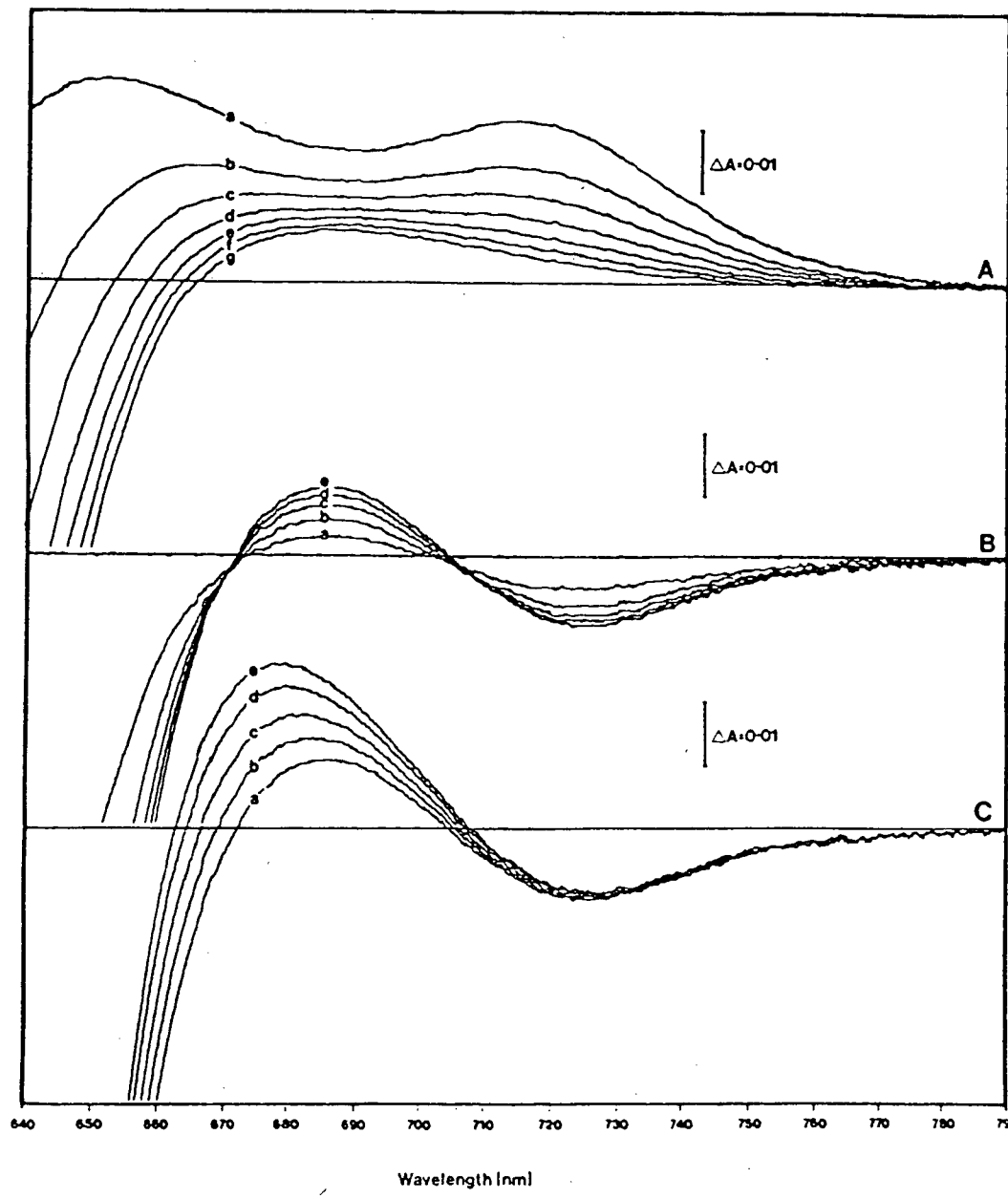

a
b
c
d
e
f
g
△A=0·01
A
△A=0·01
B
△A=0·01
C
640
650
660
670
680
690
700
710
720
730
740
750
760
770
780
790
Wavelength (nm)

Table 4. Calcium:APIII Dissociation constants.

εCaD_2[a] ($mM^{-1}.cm^{-1}$)	First Order Dissociation (μM)	Second Order Dissociation (μM^2)
8.33	257	24789
7.14	318	31234

a. εD, the molar extinction coefficient for the Ca^{2+}, Mg^{2+} free dyes was calculated at 0.7 ($mM^{-1}.cm^{-1}$).

at 670nm and 705nm with an observed λ_{max} at 685nm (Figure 17B) that coincides with λ_{max} recorded at 3mM Mg^{2+} in the presence of Ca^{2+} (Figure 17A). The MgD difference spectrum was itself modified by the addition of ATP (tris salt) with elevated absorbance below 720nm and shift of λ_{max} to 675nm (Figure 17C).

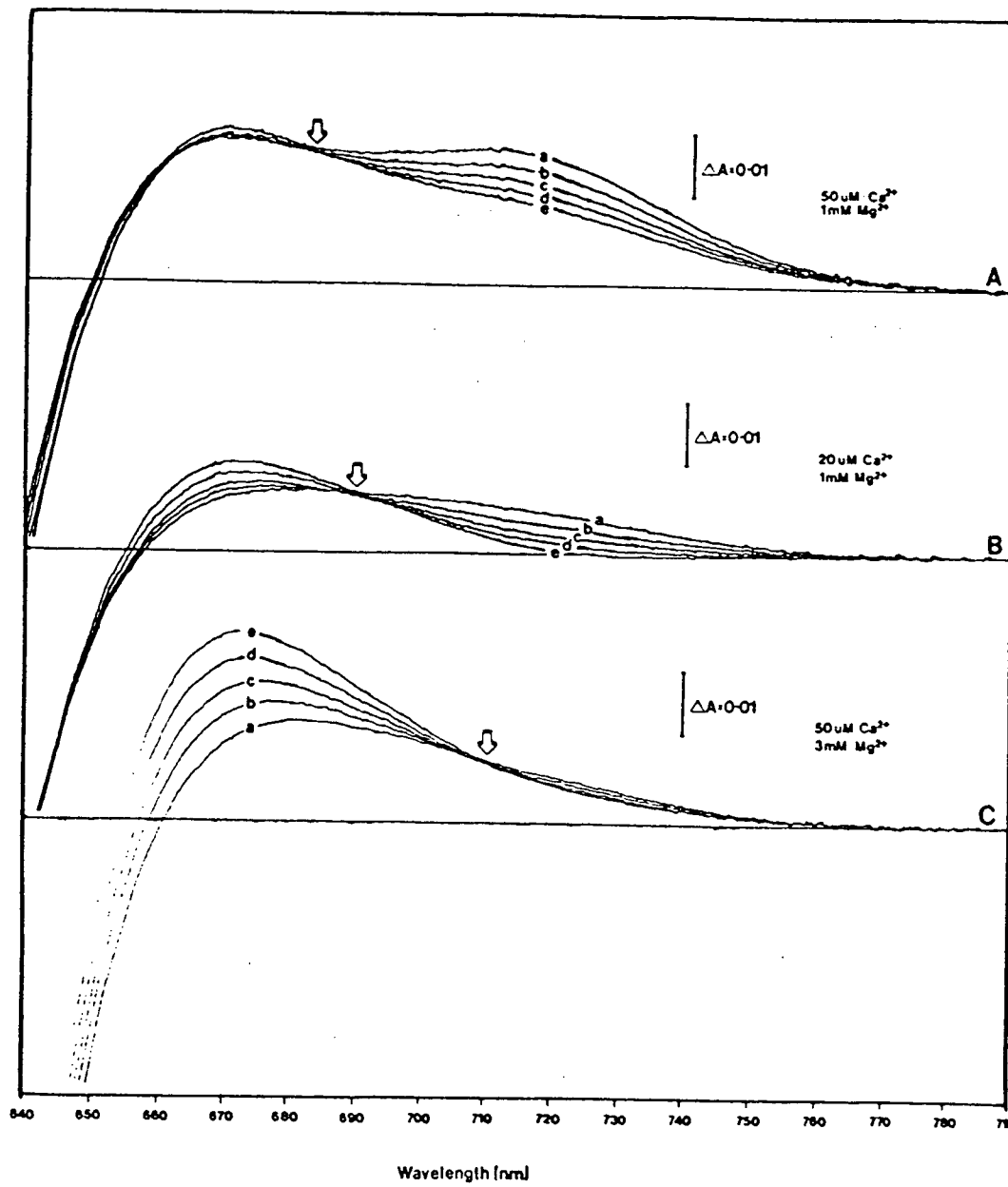
(ii). Mg.ATP effects upon divalent cation:AP III difference absorbance.

In the presence of both Ca^{2+} and Mg^{2+}, nucleotide addition altered the spectral waveform in a manner that was dependent upon the relative proportion of divalent cation. At high Ca^{2+} (50μM) and 1mM Mg^{2+}, Mg.ATP addition decreased ΔA above 700nm with small effects at 670nm (Figure 18A). At low Ca^{2+} (20μM) and 1mM Mg^{2+} the addition of Mg.ATP decreased and increased ΔA at 700nm and 685nm respectively (Figure 18B). Nucleotide addition with both elevated Mg^{2+} and Ca^{2+} modified the ΔA spectra (Figure 18C) in a manner similar to that observed in the absence of added Ca^{2+} (Figure 17C). Within the range of divalent cations defined by Figure 18 the addition of up to 1mM Mg.ATP led to the appearance of cross-over points (open arrows) which intersected the spectra obtained prior to the addition of nucleotide. The cross-over point is not a true isosbestic point since the position was dependent upon the relative concentrations of divalent cation. Spectral modification was similar with tris-ATP addition although definition of the cross-over point was enhanced by Mg.ATP. Formation of cross-over points was also observed in the presence of creatine phosphate at slightly different wavelengths.

b. Spectroscopic resolution of Ca^{2+} uptake and release.

Fig. 18. Effect of Mg.ATP upon AP III:divalent cation difference spectra. Nucleotide was added in 250μM increments (traces b-c) to 1mM final concentration (trace e) in the presence of (A). 50μM Ca^{2+}, 1mM Mg^{2+} (B). 20μM Ca^{2+}, 1mM Mg^{2+} (C). 50μM Ca^{2+}, 3mM Mg^{2+}. All other conditions and figure descriptions were as described in the legend to Figure 17.

Figure 18


△A=0·01
50uM Ca2+
1mM Mg2+
A
a
b
c
d
e
△A=0·01
20uM Ca2+
1mM Mg2+
B
a
b
c
d
e
△A=0·01
50uM Ca2+
3mM Mg2+
C
a
b
c
d
e
840
850
860
870
880
890
700
710
720
730
740
750
760
770
780
790
Wavelength [nm]

(i). Wavelength pair selection.

The appropriateness of the divalent cation and nucleotide concentrations defined by Figure 18 for the study of HSR Ca^{2+} transport, by dual-wavelength spectroscopy, was convenient as sample wavelengths could be set to cross-over points with elimination of ATP induced artifacts upon initiation of Ca^{2+} uptake with greater than 500μM nucleotide. In Figure 19A the decrease in ΔA due to ATP was estimated by pre-incubation of HSR vesicles ($1mg.ml^{-1}$) with the Ca^{2+} ionophore A23187. Addition of 1mM Mg.ATP (solid arrows), reduced ΔA by 0.008 to 0.009 absorbance units, consistent with the decrease observed in the absence of protein (see Figure 18A). After subtraction of the ΔA artifact a substantial residual component of the rapid decrease in ΔA was observed in the absence of ionophore. In Figure 19B, Mg.ATP addition to HSR, pre-incubated with A23187, was without effect upon ΔA at 675nm in accord with Figure 18A. At this sample wavelength Ca^{2+} uptake was clearly resolved into 3 kinetically distinguishable phases: a fast initial phase (a, $>650nmol.mg^{-1}.min^{-1}$); a slow secondary phase (b, $40nmol.mg^{-1}.min^{-1}$) and; an intermediate tertiary phase (c, $85nmol.mg^{-1}.min^{-1}$). After accumulation of essentially all extralumenal Ca^{2+} (including ~10μM contaminating Ca^{2+}) a steady state was reached. Repetitive addition of 3μM free Ca^{2+} (open arrows) to the medium triggered a rapid release of intralumenal Ca^{2+} ($35nmol.mg^{-1}$). The secondary and tertiary phases of uptake have been attributed to Ca^{2+} release channel opening and closing, respectively, and are modified by initial extravesicular Ca^{2+} load (Morii et al., 1985).

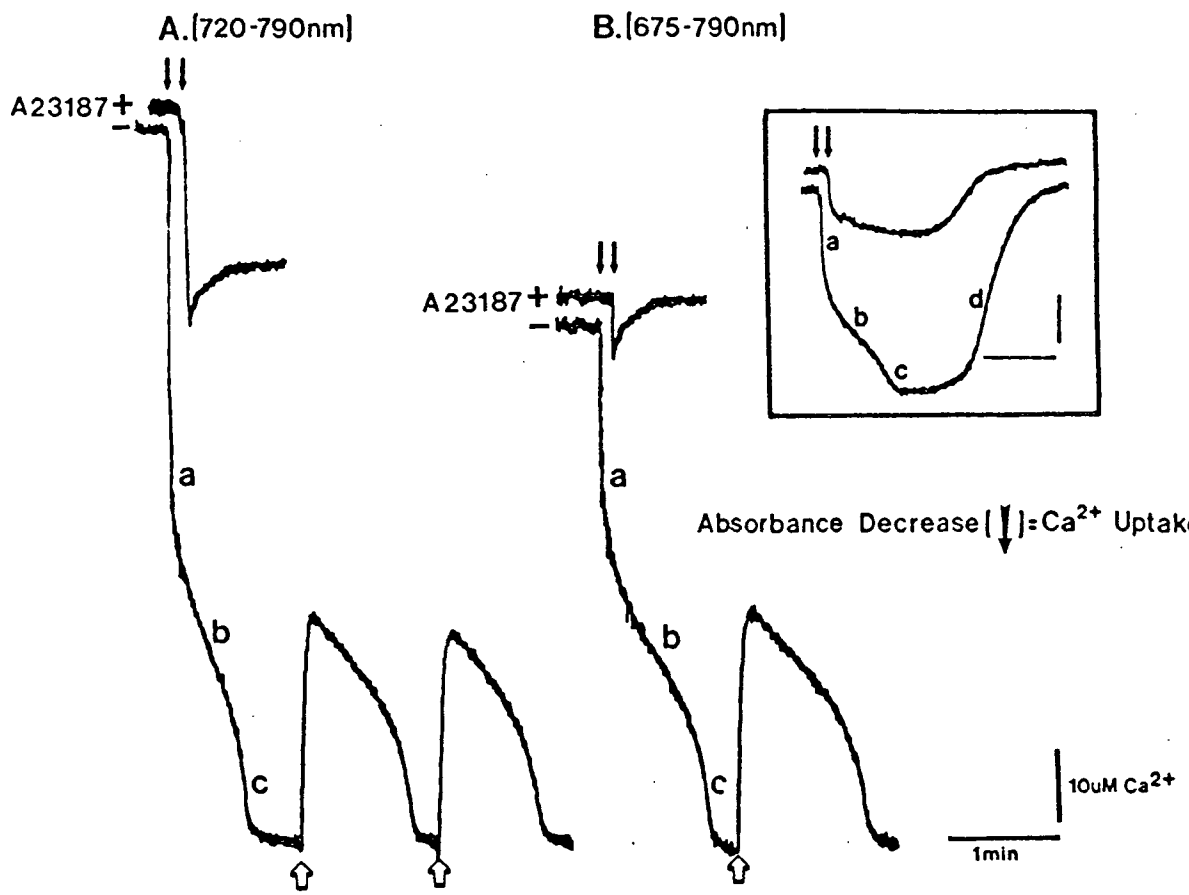
(ii). Mg^{2+} effects upon HSR Ca^{2+} uptake and release.

A prediction of Figure 18C is that elevated Mg^{2+} (>3mM) would eliminate the ATP absorbance artifact at the customary

Fig. 19. Effect of wavelength pair upon spectroscopic resolution of initial HSR calcium uptake. Wavelength pairs were (A). 720-790nm and (B). 675-790nm. Ca^{2+} uptake was initiated by the rapid addition of 1mM Mg.ATP (solid arrows) in the presence of 1mM Mg^{2+}, 12.5 units.ml^{-1} CPK and, 5mM CP under conditions otherwise described in **Methods**. In both A and B vesicles were pre-incubated for 1 minute in the presence (+) or absence (-) of the Ca^{2+} ionophore, A23187 (10μM), prior to the addition of Mg.ATP. At the indicated times (open arrows) Ca^{2+} release was induced by the direct addition of 3μM Ca^{2+} to the cuvette. Downward excursions of the traces (absorbance decrease) denote Ca^{2+} uptake and upward excursions (absorbance increase) denote Ca^{2+} release. The scales for time (abscissa) and ΔCa^{2+} (ordinate) are as indicated. The component phases of uptake and release of Ca^{2+} are indicated on the traces (see text for explanation).

Inset: Shows the resolution of initial Ca^{2+} uptake at 682.5-790nm wavelength pair compared to Ca^{2+}-ATPase inhibition by Quercetin. Vesicles were pre-incubated in the presence (+) and absence (-) of 100μM Quercetin under conditions described above except for the absence of ATP regeneration. 1mM Mg.ATP addition (solid arrows) initiated Ca^{2+} uptake. The vertical and horizontal bars denote scales for ΔCa^{2+} and time with the same dimensions as for A and B.

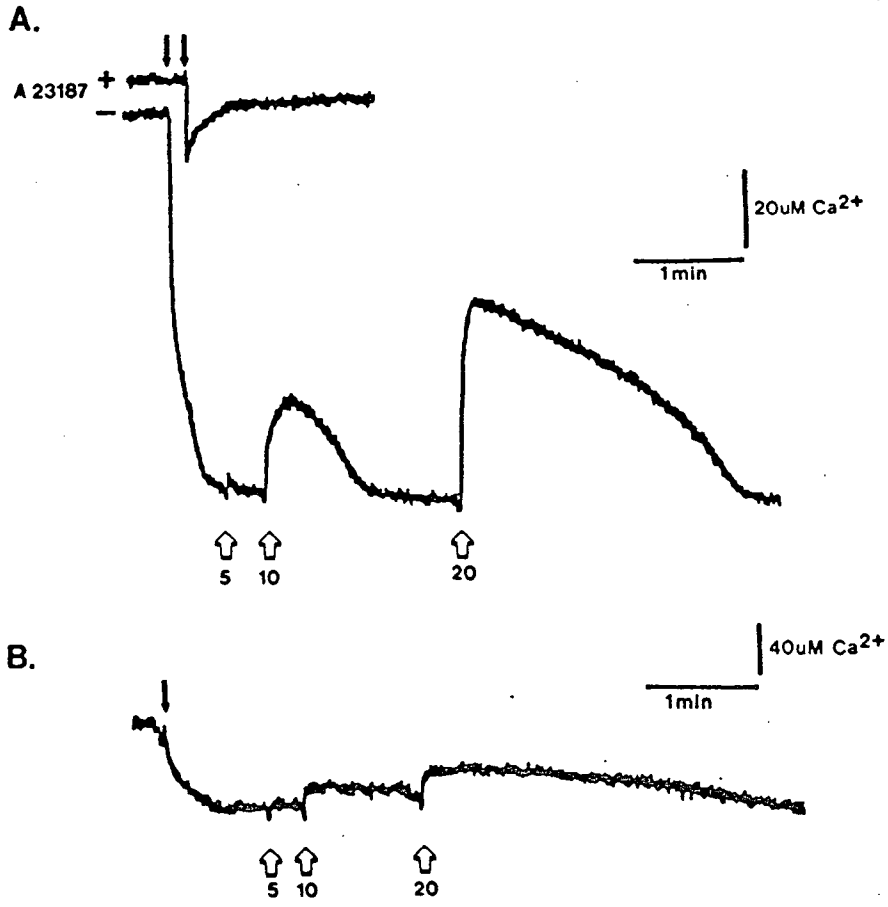
Figure 19


A.[720-790nm]
B.[675-790nm]
A23187+
−
a
b
c
d
Absorbance Decrease[ ]=Ca2+ Uptake
10uM Ca2+
1min

720-790nm dual-wavelength pair. As shown in Figure 20A, the absorbance artifact was significantly reduced when vesicles were pre-incubated in the presence of 3mM Mg^{2+} prior to addition of nucleotide and is completely eliminated at 10mM total Mg^{2+} (Figure 20B). However, loss of both the secondary and tertiary phases of uptake was also observed with elevated Mg^{2+}. Additionally, Ca^{2+}-induced Ca^{2+} release was less sensitive to trigger Ca^{2+} (Figure 20A). Ca^{2+} release was not triggered by 5μM free Ca^{2+} and 10μM free Ca^{2+} was required to minimally stimulate Ca^{2+} release. With 20μM free trigger Ca^{2+}, 25-30nmol $Ca^{2+}.mg^{-1}$ was released. Mg^{2+}, therefore, appeared under these assay conditions to decrease the Ca^{2+} sensitivity of Ca^{2+}-induced Ca^{2+} release in agreement with rapid kinetic studies of $^{45}Ca^{2+}$ release from passivley loaded HSR vesicles (Meissner et al., 1986). A 60% reduction in the rate of Ca^{2+} re-uptake, after 20μM Ca^{2+} addition, was also observed when compared to the lower Mg^{2+} condition in Figure 19 where the sum of added and released Ca^{2+} was comparable (~40-50nmol $Ca^{2+}.mg^{-1}$). At 10mM Mg^{2+} (Figure 20B) the initial rate of Ca^{2+} uptake was much reduced and Ca^{2+}-induced Ca^{2+} release was completely abolished with a greatly extended re-uptake of added Ca^{2+}. These data indicate that Mg^{2+} may also inhibit Ca^{2+} channel closing subsequent to release, although discrimination between this possibility and Ca^{2+}-ATPase inhibition was not pursued. It was also evident that Mg^{2+} elevation decreased the sensitivity of measurement. Under the conditions defined by Figure 19, at both wavelength pairs, Δ10μM Ca^{2+} produced a ΔA of 0.0025 absorbance units. At 10mM total Mg^{2+}, Δ50μM Ca^{2+} resulted in a ΔA of only 0.0024 absorbance units.

Fig. 20. Effect of elevated magnesium upon HSR calcium uptake. Assay conditions were as described in Fig. 3 except vesicles were pre-incubated in (A). 3mM Mg^{2+} in the presence (+) and absence (-) of 10μM A23187 and, (B). 9mM Mg^{2+} without A23187 treatment. Ca^{2+} uptake was initiated by 1mM Mg.ATP addition (solid arrows) in the presence of ATP regeneration (see **Methods**). Ca^{2+} release was induced by addition of 5, 10 and 20μM Ca^{2+} as indicated (open arrows). The horizontal and vertical bars denote scales for time and ΔCa^{2+} as indicated. The direction of Ca^{2+} uptake and release are as described in the legend to Fig. 19.

Figure 20



c. Intralumenal Ca^{2+} dependence of HSR Ca^{2+} release.

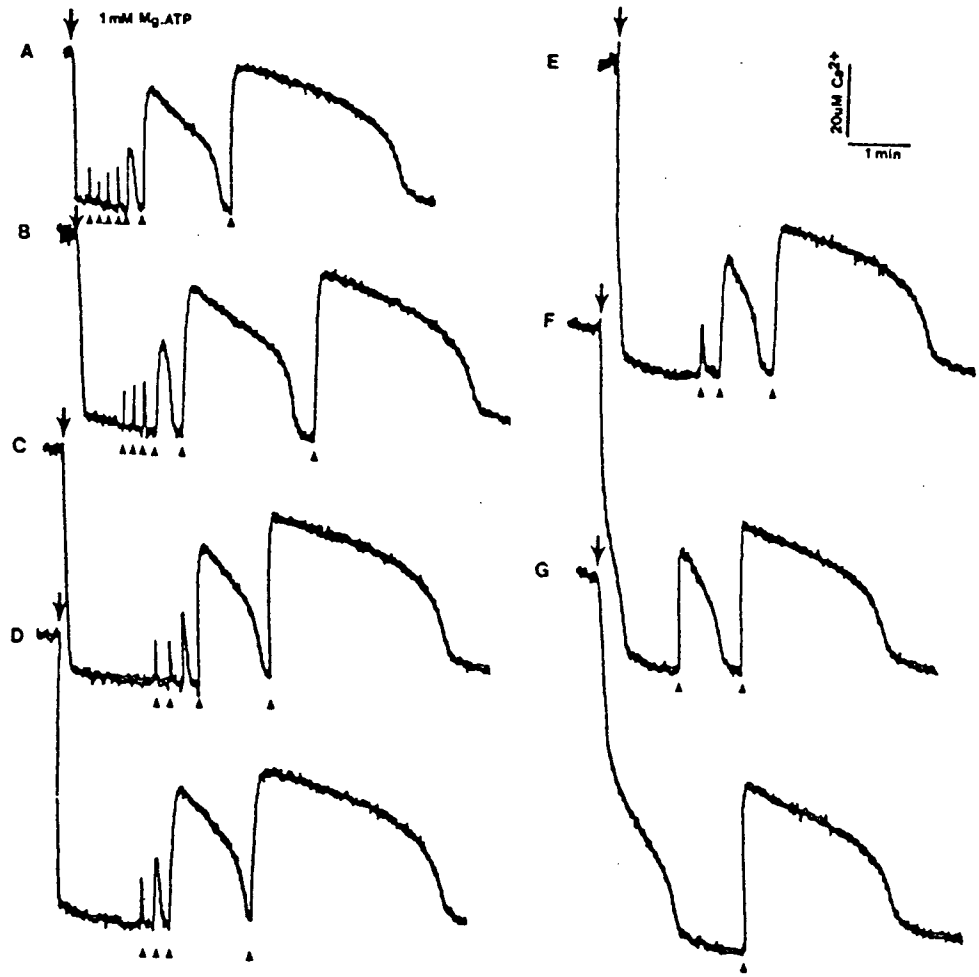
(i). Ca^{2+}-induced Ca^{2+} release.

The foregoing spectroscopic studies demonstrated that the initial phase of HSR Ca^{2+} uptake could be well resolved in the presence of 1mM Mg^{2+} by selection of a 675-790nm dual-wavelength pair. Subsequent spectroscopic studies of HSR Ca^{2+} transport were, therefore, performed in transport media containing 300mM sucrose, 100mM KCl, 20mM PIPES (pH 7.0), 1mM Mg^{2+}, 50μM AP III, 12.5 units.ml^{-1} CPK, 5-20mM CP, and various concentrations of Ca^{2+}. In Figure 21 HSR vesicles (1mg.ml^{-1}) were incubated for 1 minute in transport buffer containing 5mM CP. In traces A to G initial extravesicular Ca^{2+} was incremented by 10μM Ca^{2+} from 40μM Ca^{2+} (trace A) to 100μM Ca^{2+} (trace G). Trace A shows that 10μM pulses of Ca2+ (~5μM free) added subsequent to the initial uptake were rapidly accumulated (820 nmol.mg HSR^{-1}.min^{-1}) up to an intraluminal Ca^{2+} load of 80 nmoles.mg^{-1}. Further addition of Ca^{2+} led to partial stimulation of Ca^{2+} release (Ca^{2+}-induced Ca^{2+} release) that progressively increased to maximal Ca^{2+} release observed after the accumulation of 100nmoles.mg^{-1} Ca^{2+}. Subsequent traces (B to D) show that as initial Ca^{2+} was incremented by 10μM the amount of pulse Ca^{2+} required to elicit Ca^{2+}-induced Ca^{2+} release was reduced by a corresponding amount with maintenance of the initial fast phase of Ca^{2+} accumulation.

After 90 and 100nmoles.mg^{-1} accumulated Ca^{2+}, the release of Ca^{2+} was followed by a biphasic reaccumulation of Ca^{2+} that was resolved into an initial slow phase (5-10nmol.mg^{-1}.min^{-1}) and a secondary intermediate phase (30-40nmol.mg^{-1}.min^{-1}). In traces E to G, increasing initial Ca^{2+} appearance of a slow phase of Ca^{2+} accumulation followed by a faster intermediate phase of uptake. These data indicate that threshold filling of an intralumenal Ca^{2+}

Fig. 21. Intralumenal calcium requirement for calcium-induced calcium release. Ca^{2+} uptake was monitored by dual-wavelength spectroscopy of Ca^{2+}:APIII difference absorbance at a 675nm sample wavelength and a 790nm reference wavelength. HSR membranes ($1mg.ml^{-1}$) were pre-incubated (1minute) at 25°C in transport buffer containing 300mM sucrose, 100mM KCl, 20mM PIPES (pH 7.0), 1mM Mg^{2+}, 5mM CP, 12.5 $units.ml^{-1}$ CPK and 40(A), 50(B), 60(C), 70(D), 80(E), 90(F), and 100μM Ca^{2+} (G). Uptake was initiated by the addition of 1mM Mg·ATP as indicated (arrows) and 10μM Ca^{2+} pulses were made to the sample cuvette (arrowheads). The scales for time (minutes) and Ca^{2+} are as indicated with a downward deflection of the traces representing uptake.

Figure 21



pool is stimulates Ca^{2+} channel opening during (a) Ca^{2+} triggering of Ca^{2+} release and (b) Ca^{2+} uptake when the extralumenal Ca^{2+} load just matches or exceeds a specific intralumenal Ca^{2+} sink.

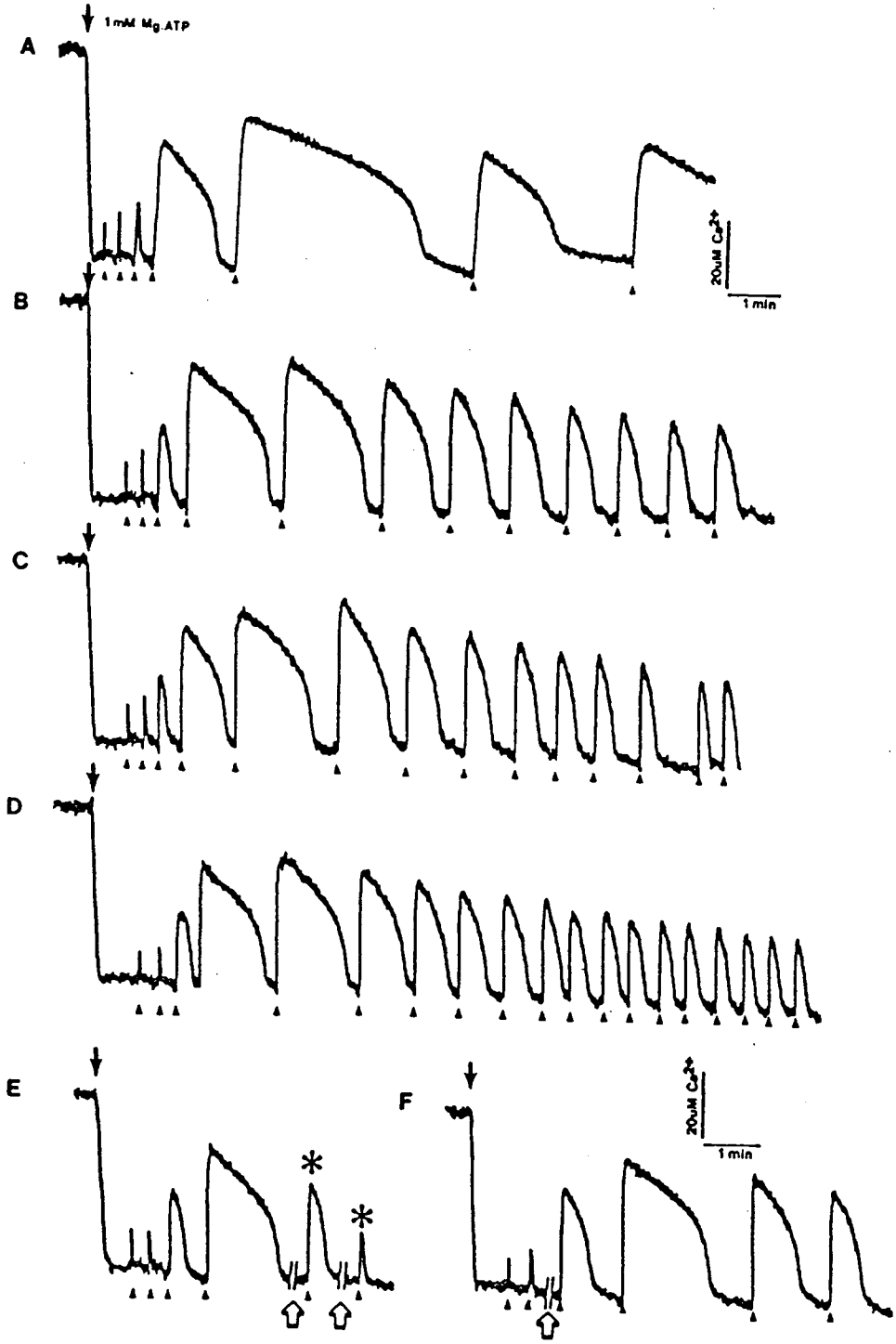
(ii). Repetitive triggering of Ca^{2+}-induced Ca^{2+} release.

It was of interest to examine the effect of increasing intralumenal Ca^{2+} upon Ca^{2+}-induced Ca^{2+} release. Figure 22 (trace A) shows that repetitive addition of Ca^{2+} to trigger Ca^{2+} release led to a gradual decline in the amount of released Ca^{2+} and an increase in the rate of slow phase Ca^{2+} reuptake. HSR vesicles eventually failed to reaccumulate all extralumenal Ca^{2+} with continued application of Ca^{2+} pulses. This was followed by further spontaneous Ca^{2+} release and appeared to be due to depletion of ATP.

With supplementation of phosphocreatine up to 20mM the extent of submaximal and maximal Ca^{2+} release triggered by 10μM total Ca^{2+} was unaffected and demonstrated a similar dependence upon intralumenal Ca^{2+} load. However, the rate of slow phase Ca^{2+} reaccumulation was markedly elevated with shortening of Ca^{2+} channel open time. Although released Ca^{2+} could be completely reaccumulated upon induction of release, the amount of released Ca^{2+} declined progressively with successive Ca^{2+} pulses. It was reasoned that this may be due to either intralumenal Ca^{2+} overload or some other time dependent phenomenon. Placement of a 5 minute interval after the initial maximal Ca^{2+} release (trace E, open arrow) showed that the next Ca^{2+} triggered release was much reduced (asterisk) and equivalent to the magnitude of release that would have been observed had Ca^{2+} been added during that time (compare with trace D). With a further 5 minute interval Ca^{2+} release was effectively abolished. However, placement of the interval prior to submaximal and maximal release (trace F) had little effect upon these and

Fig. 22. Effects of creatine phosphate concentration upon HSR calcium uptake and release. Ca^{2+} uptake was monitored by dual-wavelength spectroscopy of Ca^{2+}:APIII difference absorbance at a wavelength pair of 675-790nm. In (A) to (F) Ca^{2+} uptake by HSR vesicles ($1mg.ml^{-1}$) was initiated upon addition of 1mM Mg·ATP (solid arrows) to the sample cuvette containing 60μM Ca^{2+} in APIII Ca^{2+} transport buffer (see Figure 21). Ca^{2+} transport was supported by an ATP regenerating system consisting of 12.5 $units.ml^{-1}$ CPK and 5mM (A), 10mM (B), 15mM (C) and 20mM (D,E,F) CP. After accumulation of all extravesicular Ca^{2+} in the initial Ca^{2+} load, 10μM total Ca^{2+} aliquots were added as indicated (arrowheads). In (E) and (F) the open arrows denote a 5 minute interval during which no Ca^{2+} was added. Each condition (A to F) was repeated at least 3 times.

Figure 22



subsequent releases. This suggested that events associated with Ca^{2+} channel opening resulted either in (a) a time dependent inactivation of Ca^{2+}-induced Ca^{2+} release or (b) that the compartment of intralumenal threshold Ca^{2+} was depleted.

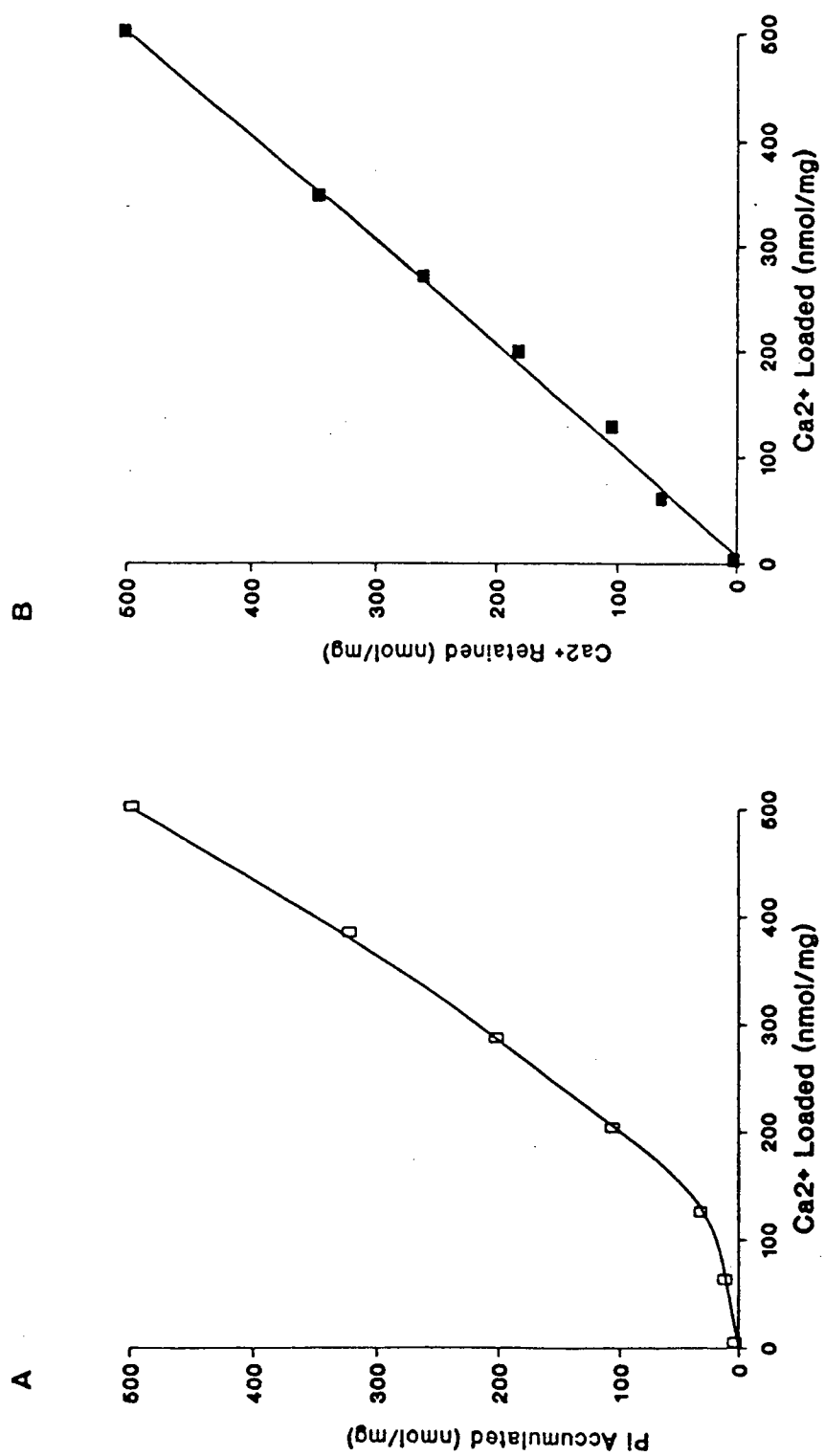
4. Filtration studies of Ca^{2+} release from actively loaded vesicles.

a. Pi accumulation during active Ca^{2+} transport.

The first hypothesis from the preceding was considered unlikely since preliminary studies showed that, at 2-4 fold lower protein concentrations, repetitive Ca^{2+}-induced Ca^{2+} release could be sustained without loss of Ca^{2+} release. The second hypothesis was tested with the view that excess Pi generated from CP hydrolysis may accumulate intralumenally and establish a competitive Ca^{2+} binding compartment. Under identical conditions to that described in Figure 22, vesicles were filtered at intervals corresponding to the addition and accumulation of known amounts of Ca^{2+}. In one set of experiments with Ca^{2+} (Figure 23A), filters were assayed for Pi while in a parallel set of experiments (Figure 23B) filters were assayed for $^{45}Ca^{2+}$. Accumulated $^{45}Ca^{2+}$ correlated as expected, with that added. Accumulation of Pi, however, occurred in a biphasic manner with a steeprise of Pi accumulation observed after the accumulation of 150nmol Ca^{2+}.mg HSR^{-1}. Upon accumulation of 500nmoles $Ca^{2+}.mg^{-1}$ intravesicular Pi contents were identical suggesting a 1:1 binding stoichiometry of Ca^{2+} and phosphate. Back extrapolation of the second Pi curve reveals a phosphate free Ca^{2+} site of 125nmol.mg^{-1}. This value is close to the intralumenal Ca^{2+} threshold for Ca^{2+}-induced Ca^{2+} release.

Fig. 23. Calcium and inorganic phosphate (Pi) retention by HSR vesicles undergoing ATP dependent calcium accumulation. In (A) HSR vesicles (1mg.ml^{-1}) vesicles were pre-incubated with 60uM total $^{45}Ca^{2+}$ and Ca^{2+} uptake was initiated upon addition of 1mM Mg·ATP in the presence of 300mM sucrose, 100mM KCl, 20mM PIPES (pH 7.0), 1mM Mg^{2+}, 12.5 units.ml^{-1} CPK, 20mM CP (2ml volume). After 3 minutes 3x100ul aliquots were withdrawn and immediately filtered and rinsed. The procedure was repeated after addition of 120nmol $^{45}Ca^{2+}$ to the remaining vesicles. The Ca^{2+} addition/filtration procedure was continued up to a total of six successive 120nmol $^{45}Ca^{2+}$ additions. In (B) the experimental protocol was replicated except for the addition of $^{40}Ca^{2+}$. After rinsing, filters (13mM HAWP type) were then placed into 600uL of 6.7% (w/v) SDS and incubated overnight. Determination of Pi was performed as described in the methods. The data represent means of 4 observations from 2 sets of experiments (sem less than +/-10%).

Figure 23


A
Pi Accumulated (nmol/mg)
0
100
200
300
400
500
Ca2+ Loaded (nmol/mg)
0
100
200
300
400
500
B
Ca2+ Retained (nmol/mg)
0
100
200
300
400
500
Ca2+ Loaded (nmol/mg)
0
100
200
300
400
500

b. Ca^{2+} release.

The possibilty that alterations in the extralumenal compartment were responsible for the inhibition of Ca^{2+} release was then tested through dilution of actively Ca^{2+} loaded vesicles into media that either inhibited or stimulated Ca^{2+} release. Figure 24 shows that at low (60μM) Ca^{2+} loaded states, 10μm extralumenal Ca^{2+} triggered a large release of intralumenal Ca^{2+} comparable to that observed with passivley loaded vesicles (see Figure 16). With elevated ATP dependent Ca^{2+} loading in the presence of 20mM CP, Ca^{2+} release was markedly inhibited. It is noteworthy that direct measurements of pH during Ca^{2+} transport showed that the inhibition of Ca^{2+} release was not associated with proton accumulation due to Pi release. This may reflect the fact that CP hydrolysis is a proton consuming reaction and would be expected to counter proton release during ATP hydrolysis. The data from Figures 22 to 24 indicate that intralumenal Pi accumulation may, under the conditions employed in this study, directly or indirectly inhibit Ca^{2+} release. During the filtration studies above, it was observed that the region of the filter occupied by the membranes was particularly white indicating formation of Ca^{2+}:Pi preciptation. This suggests that Pi may compete with calsequestrin for Ca^{2+} binding and would implicate an important role for the Ca^{2+}:calsequestrin compartment in mediating Ca^{2+} channel opening.

5. Ryanodine effects upon Ca^{2+} uptake and Ca^{2+} release.

a. Ca^{2+} uptake.

In Figure 25A the effect of vesicle pre-incubation with ryanodine prior to addition of Mg.ATP was examined. In the presence of 1μM-10μM ryanodine rapid Ca^{2+} uptake was

Fig. 24. Effect of elevated (20mM) creatine phosphate (CP) upon calcium-induced calcium release from actively loaded vesicles. HSR (1mg.ml^{-1}) were initially loaded with 60μM total $^{45}Ca^{2+}$ (SA=50,000dpm.nmol^{-1}) in the presence of 300mM sucrose, 100mM KCl, 20mM PIPES (pH 7.0), 20mM CP, 12.5 units.ml^{-1} CPK, 1mM Mg^{2+} (2ml volume). Ca^{2+} uptake was initiated by addition of 1mM Mg·ATP. After 3 minutes 2x5μl aliquots were withdrawn and rapidly diluted (250 fold) into either iso-osmotic Ca^{2+} release buffer containing 4.9mM Ca^{2+} and 5mM EGTA or quench buffer contianing 10mM Mg^{2+}, 50μM ruthenium red and 100μM EGTA (therefore 20μl total). 200μL aliquots from either quench (open symbols) or release (filled symbols) buffers were rapidly filtered across 0.45μ nitrocellulose filters at the indicated times. The filters were then washed with 5 volumes of quench buffer. An amount of Ca^{2+} was then added to the actively accumulating vesicles which raised the intralumenal Ca^{2+} by an additional 60nmol.mg^{-1}. The loss of protein due to aliquoting and the dilution of remaining protein were both accounted for. After a further 3 minutes 2x5μl aliquots (total=4x5μl) were withdrawn and the dilution and filtration procedures described above to either induce or inhibit release were repeated. Vesicles were therefore loaded to 60 ( ), 120 ( ), 180 ( ), 240 ( ) and 300 ( ) nmol.mg^{-1}. The data are means of 4 observations from 2 sets of experiments.

Figure 24

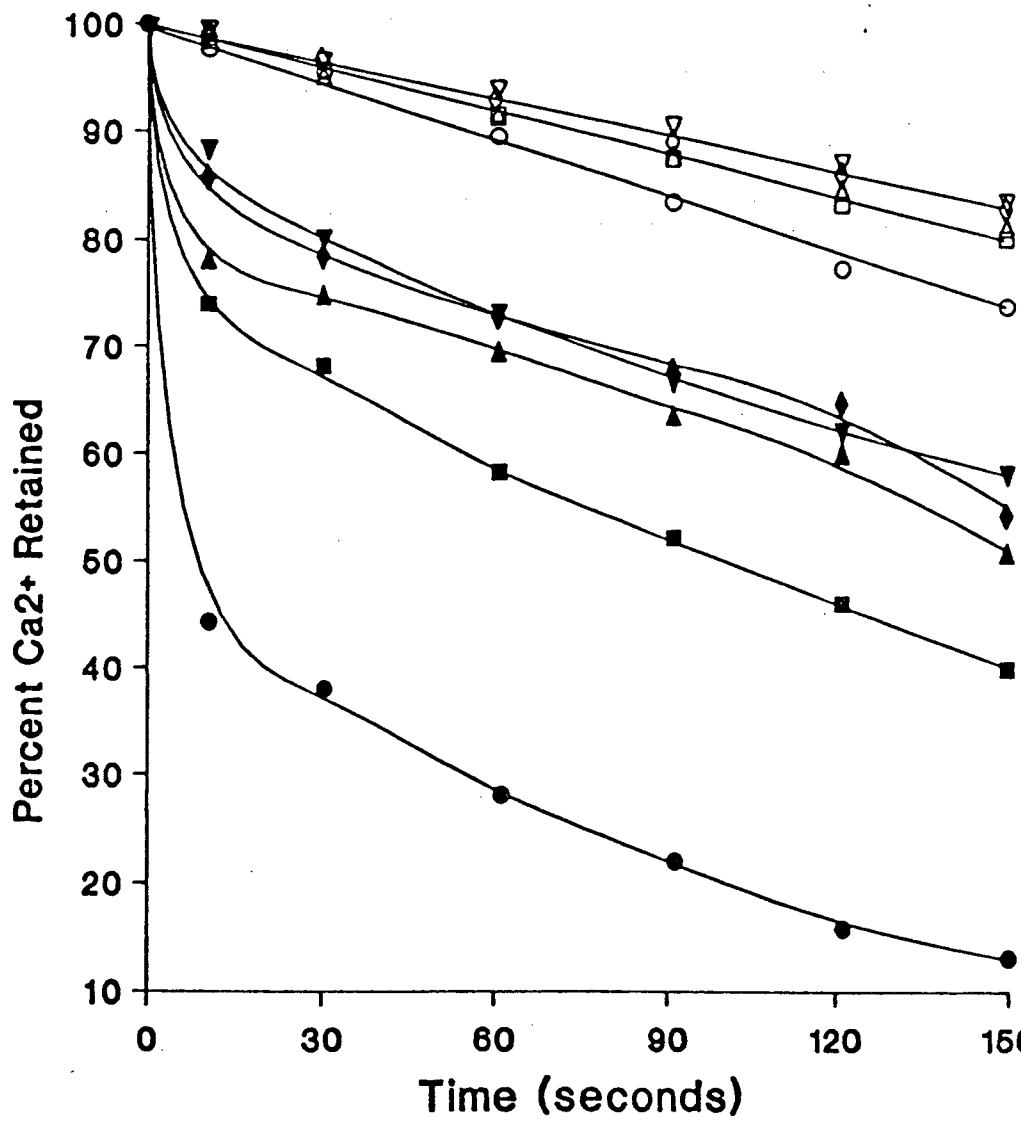
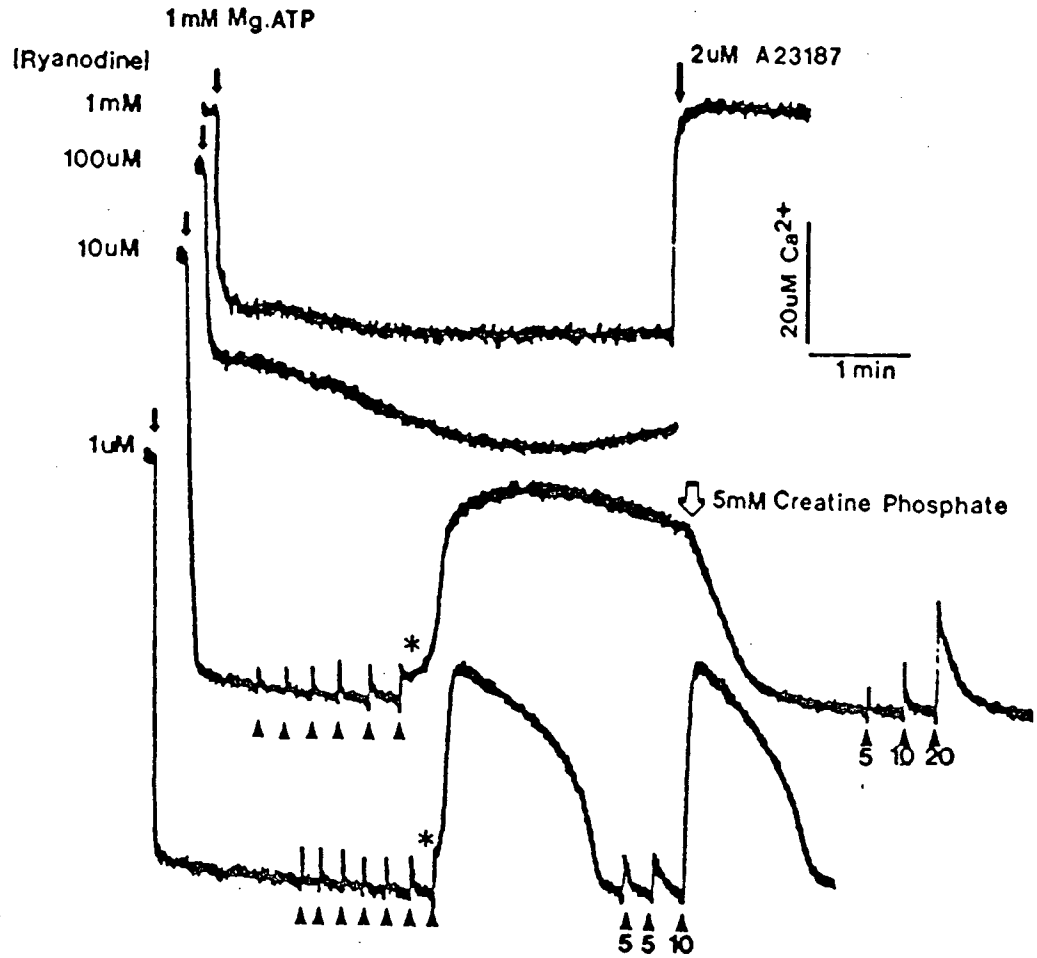

100
90
80
70
60
50
40
30
20
10
Percent Ca2+ Retained
0
30
60
90
120
150
Time (seconds)

Fig. 25. Ryanodine effects upon HSR calcium uptake. Ca^{2+} transport was assayed as described (see Figure 21). 1mg.ml^{-1} vesicles were pre-incubated for 5 minutes in the presence of 60μM Ca^{2+} and various concentrations of ryanodine as indicated. Ca^{2+} uptake was initiated by addition of 1mM Mg·ATP to the cuvette and unless otherwise indicated 5μM Ca^{2+} pulses (arrowheads) were added to the cuvette during the steady state.

Figure 25


1mM Mg.ATP
[Ryanodine]
1mM
100uM
10uM
1uM
2uM A23187
20uM Ca2+
1 min
5mM Creatine Phosphate
5 10 20
5 5 10

observed with slight decreases in the intralumenal Ca^{2+} requirement for Ca^{2+} induced Ca^{2+} release. Vesicles released 30nmoles Ca^{2+}.mg-1 in response to 5μM trigger Ca^{2+} (~3μM free) although Ca^{2+} release frequently followed a delay (asterisk) subsequent to addition of 5μM trigger Ca^{2+}. The total amount of Ca^{2+} released at low μM ryanodine was unaltered. Pre-incubation of vesicles with elevated ryanodine (100μM-1mM) resulted in 65% loss of Ca^{2+} accumulating ability. The accumulated Ca^{2+} could then be released upon application of Ca^{2+} ionophore.

At 1μM ryanodine, vesicles could accumulate the released Ca^{2+} and remained sensitive to subsequent Ca^{2+} triggering of Ca^{2+} release. At ryanodine concentrations greater than 1μM released Ca^{2+} was not accumulated in the presence of 5mM initial CP. Further addition of 5mM CP stimulated a slow Ca^{2+} reaccumulation. However, vesicles were insensitive to repeated Ca^{2+} triggering of Ca^{2+} release.

b. Intralumenal Ca^{2+} threshold for Ca^{2+}-induced Ca^{2+} release.

Figure 26A shows the effects of ryanodine addition after complete uptake of medium Ca^{2+} upon Ca^{2+}-induced Ca^{2+} release. As in Figure 21, Ca^{2+} pulses were added until release was observed. Increasing ryanodine resulted in a decrease in the intralumenal Ca^{2+} threshold of Ca^{2+}-induced Ca^{2+} release. At 2mM ryanodine, a delayed onset spontaneous Ca^{2+} release resulted without Ca^{2+} triggering. The rates of initial Ca^{2+} release in the presence of ryanodine (953nmol Ca^{2+}.mg HSR^{-1}) were not measurably different from controls (971nmol Ca^{2+}.mg HSR^{-1}). However, the magnitude of Ca^{2+} triggered release increased with elevated ryanodine despite decreased vesicle Ca^{2+} loading. As in figure 25 low ryanodine resulted in an initial fast Ca^{2+} release followed by a slower release phase. At high ryanodine concentrations

Fig. 26. Effect of ryanodine upon calcium stimulation of calcium release. In (A) Ca^{2+} uptake in HSR vesicles ($1mg.ml^{-1}$) was monitored as described in Fig 21. Vesicles were pre-incubated in the presence of 60uM Ca^{2+} for 1 minute and uptake was initiated by the addition of 1mM Mg·ATP to the cuvette. After accumulation of 60nmol Ca^{2+}.mg HSR^{-1} various concentrations of ryanodine were added (open arrow) prior to addition of 5μM Ca^{2+} pulses (arrowheads). In (B) Ca^{2+} uptake was as in (A) except for the presence of 20mM CP. Ryanodine (5 μM) was added either prior to addition of 10μM Ca^{2+} pulses (arrowheads) in (a) or prior to partial stimulation of Ca^{2+} release in (b). Each trace was repeated twice with identical consequences for repetitive triggering of Ca^{2+} release.

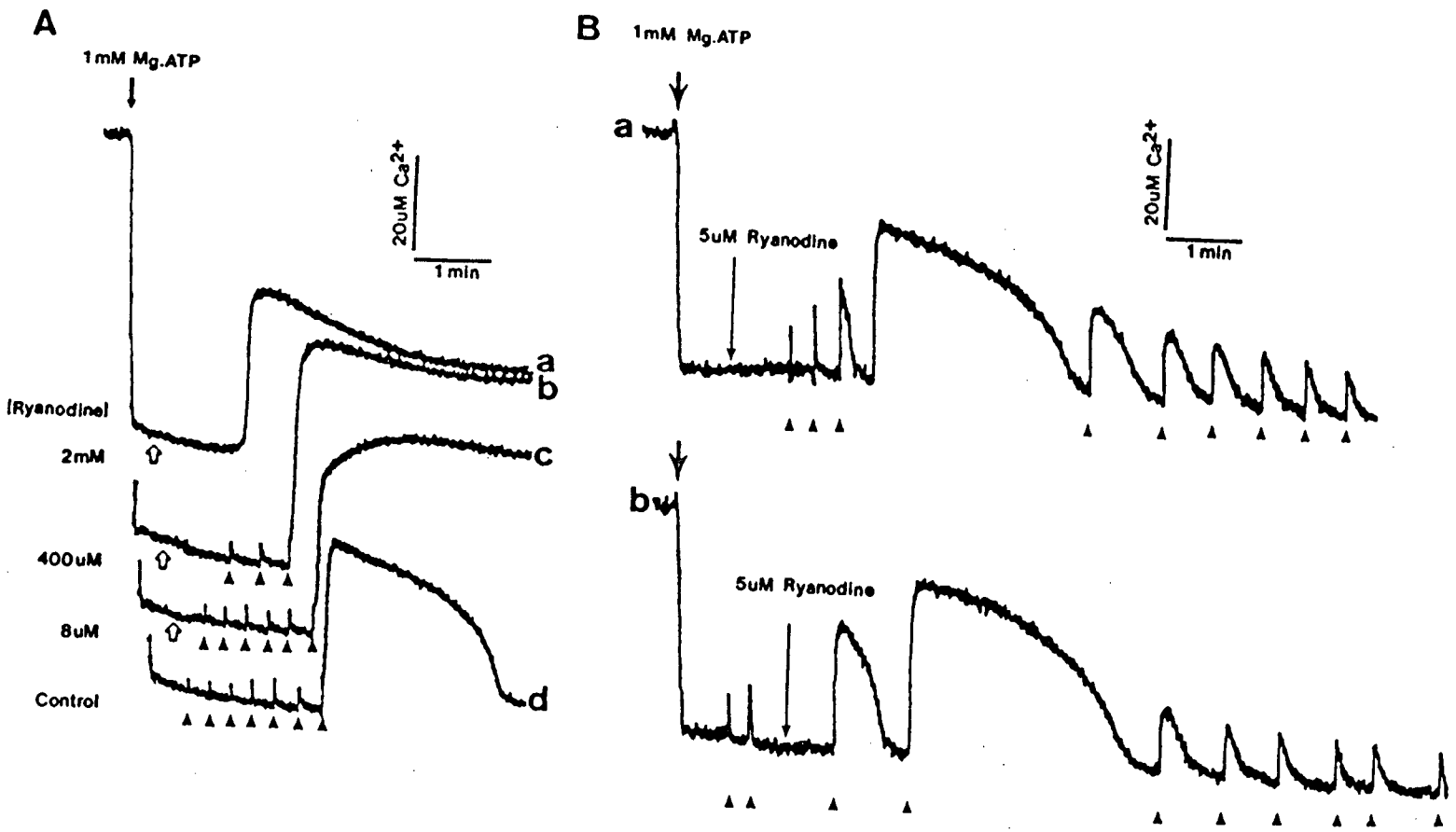

A
1mM Mg.ATP
20uM Ca2+
1 min
a
b
[Ryanodine]
2mM
c
400uM
8uM
Control
d
B
1mM Mg.ATP
a
5uM Ryanodine
20uM Ca2+
1 min
b
5uM Ryanodine

Figure 26

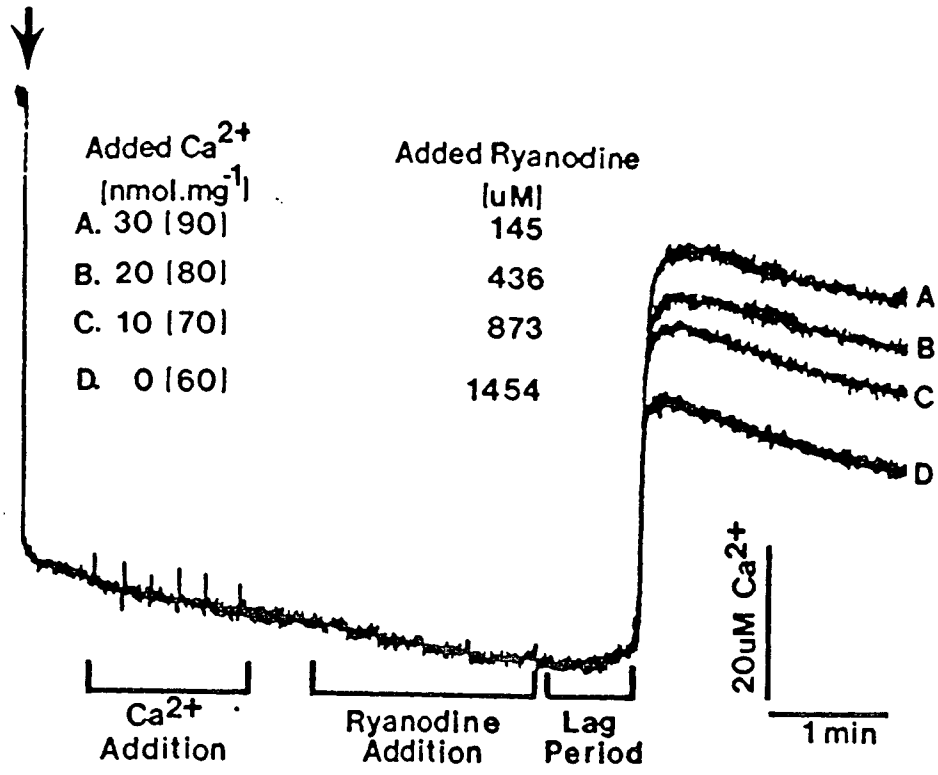
(100μM-2mM) the initial fast phase was immediately followed by a transient reaccumulation, the rate of which increased with elevated ryanodine. A further 10-15nmol Ca^{2+}.mg HSR^{-1} of Ca^{2+} re-uptake was followed in several experiments during the subsequent 45 minutes beyond that shown. Figure 26B shows that with CP supplementation prior to initiation of Ca^{2+} uptake the released Ca^{2+}, after addition of ryanodine, was reaccumulated with a much slower time course than in the absence of ryanodine (compare with trace D, Figure 22). This is consistent with μM ryanodine effects upon maintenance of the open channel state (Meissner, 1986). However, despite re-accumulation of released Ca^{2+}, subsequent Ca^{2+} releases were markedly inhibited and the uptake of added Ca^{2+} also markedly slower. This suggests that the releasable pool of intralumenal Ca^{2+} was also modified either directly or indirectly by ryanodine. However, these effects of ryanodine appear to attend formation of the open channel state since ryanodine addition prior to the maximal Ca^{2+} release did not modify the rate or the extent of maximal Ca^{2+} release. On the other hand, addition of ryanodine at more Ca^{2+} loaded states increased submaximal Ca^{2+} releases.

c. Intralumenal Ca^{2+} threshold for ryanodine-induced Ca^{2+} release.

The intralumenal Ca^{2+} sensititity of ryanodine induced Ca^{2+} release was then examined in figure 27. At defined Ca^{2+} loads, ryanodine was added in 5μL aliquots at 30 second intervals until gradual upward excursions of the absorbance traces were observed during the lag period. The predictability of Ca^{2+}-induced Ca^{2+} release under these assay conditions permitted Ca^{2+} loading of vesicles with pulse Ca^{2+} up to one 5μM addition less than that required to induce release. The amount of ryanodine induced Ca^{2+} release increased with the intralumenal Ca^{2+} load. This was

Fig. 27. Effect of intralumenal calcium load upon ryanodine induced calcium release. HSR vesicles (1mg.ml^{-1}) were allowed to accumulate 60nmol Ca^{2+}.mg protein^{-1} upon addition of 1mM Mg·ATP (arrow) as in Fig. 21. As indicated at the bottom of the trace, fixed amounts of Ca^{2+} was added as a series of 5μM Ca^{2+} pulses to achieve a desired level of filling. This was followed by addition of ryanodine in 5μl aliquots (from 20mM stock at 30 second intervals). Ryanodine addition was terminated upon observation of a definite progressive rise in the absorbance trace during the lag period. In the centre of the trace are shown the amounts of ryanodine added to induce release at the corresponding intralumenal Ca^{2+} load. The Ca^{2+} release traces to the right are labelled according to the loading scheme shown. The release traces from conditions B to D are superimposed upon the entire trace obtained under loading condition A.

Figure 27


Added Ca^{2+}
[nmol.mg^{-1}]
A. 30 [90]
B. 20 [80]
C. 10 [70]
D. 0 [60]
Added Ryanodine
[uM]
145
436
873
1454
A
B
C
D
20uM Ca^{2+}
1 min
Ca^{2+} Addition
Ryanodine Addition
Lag Period

different from ryanodine augmentation of Ca^{2+} induced Ca^{2+} release (see Figure 26A) which may reflect mixed effects of ryanodine induced release and ryanodine stimulation of Ca^{2+} induced release. The ryanodine triggering of Ca^{2+} release at 25°C required a minimum 125μM ryanodine and was immediately followed by partial re-uptake of released Ca^{2+}. At 30°C, a two fold decrease in the amount of ryanodine was required to induce Ca^{2+} release (not shown).

III. CANP effects upon HSR structure and function.

1. CANP proteolysis of HSR proteins.

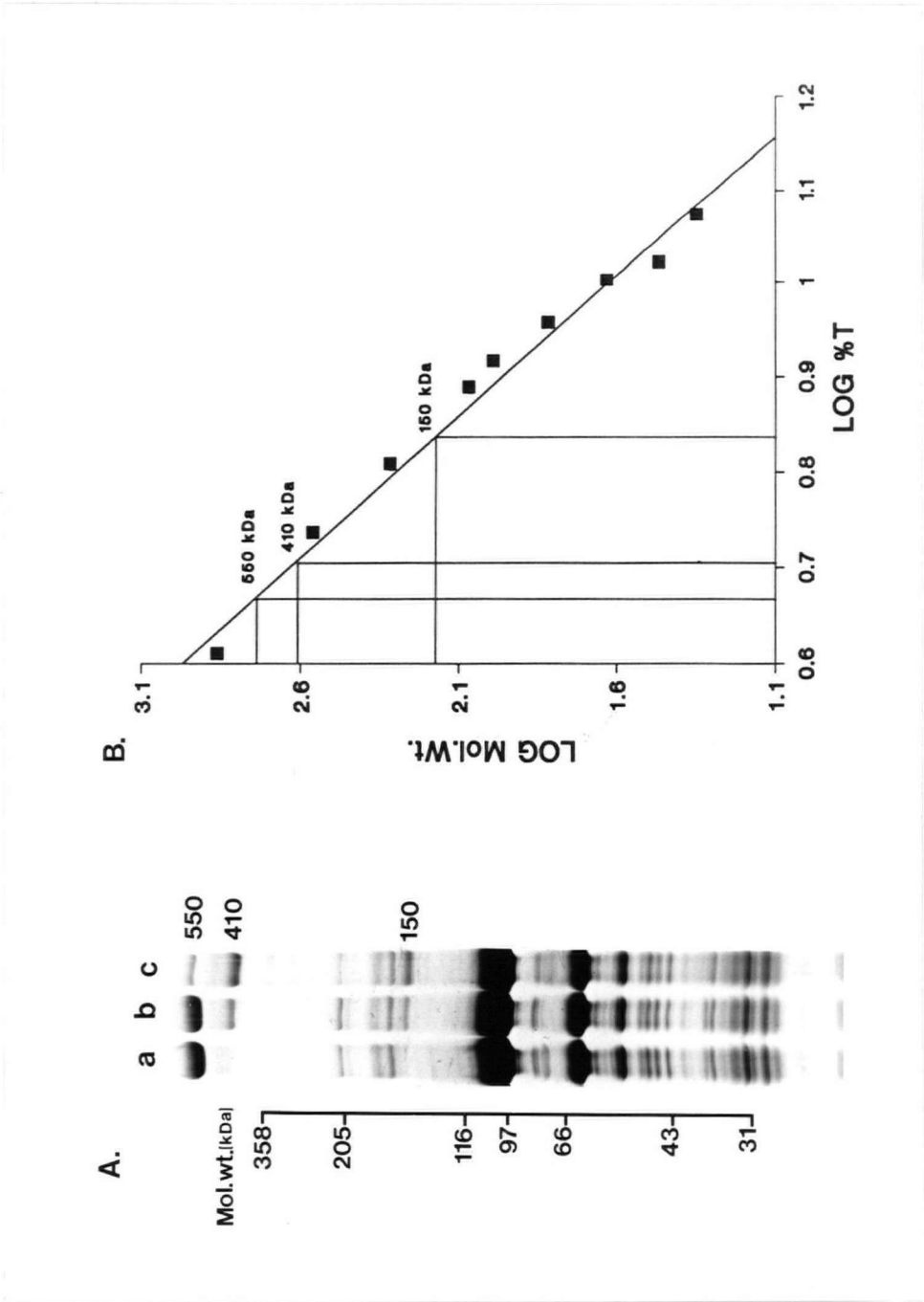
In order to study the effects of CANP upon HSR Ca^{2+} transport, in particular Ca^{2+} release, it was important to characterise the effects of CANP upon HSR protein structure.

a. Endogenous CANP effects.

Preparation of membranes without leupeptin addition to isolation buffers resulted in the appearance of a second high molecular weight peptide (Figure 28A, lane B). This common observation has been attributed to the action of CANP (Seiler et al., 1984). The inclusion of 2.5mM Ca^{2+} in sucrose density gradients during purification of the post 45,000 rpm crude microsomal pellet led to further fragmentation of the Ca^{2+} release channel in HSR fractions (Figure 28A, lane C). This intially suggested that CANP co-purifies with the HSR and/or the crude microsomal fraction. With 3-13% gels the size of the Ca^{2+} release channel estimated from linear log/log plots of size (m.w.) versus %T (Figure 28B) was 550 kDa. This is very close to the actual size (565 kDa) of the Ca^{2+} release channel determined from

Fig. 28. Endogenous CANP effects upon HSR protein structure. In (A) HSR membranes were isolated (see methods) in the presence (lane a) and absence of 5μM leupeptin (lanes b and c) and resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on linear gradient (3-13%) gels. In lane c 1mM Ca^{2+} was included in the 25-45% sucrose density gradient buffers for the 16 hr sedimentation to equilibrium centrifugation step. On the left of the panel are positions of molecular weight marker proteins (α_2-macroglobulin (dimer), 358 kDa; myosin heavy chain, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 31 kDa). To the right of the panel are the estimated molecular weights (kDa) of the intact calcium release channel (550) and proteolytic fragments (410, 150). These estimates were interpolated from the least squares fit in (B) of log molecular weight (Mol. Wt.) versus log acrylamide concentration (%T) as described (). The filled squares mark the positions of the above standards with the inclusion of (a) the α_2-macroglobulin tetramer (725 kDa) observed under non-reducing conditions and (b) soyabean trypsin inhibitor (21 kDa).

Figure 28


A.
a b c
550
410
150
Mol.wt.(kDa)
358
205
116
97
66
43
31
B.
LOG Mol.Wt.
3.1
2.6
2.1
1.6
1.1
660 kDa
410 kDa
150 kDa
0.6
0.7
0.8
0.9
1
1.1
1.2
LOG %T

the cDNA primary sequence (Takeshima et al., 1989). From this plot the size of the high molecular weight proteolytic fragment was found to be 410 kDa. Coincident with appearance of the 410 kDa peptide was production of a 150 kDa peptide (Figure 28, lanes B and C).

b. Exogenous CANP effects.

Observation of endogenous CANP mediated proteolysis of the Ca^{2+} release channel prompted examination of the Ca^{2+} dependence of proteolysis of HSR proteins by two forms of skeletal muscle CANP differentially sensitive to micromolar and millimolar concentrations of Ca^{2+} (μCANP and mCANP, respectively). Figures 29A and 30A shows that a major HSR substrate for both forms of CANP was the 550 kDa ryanodine receptor/Ca^{2+} release channel. As with endogenous CANP (Figure 28A) limited proteolysis of the 550 kDa peptide by both μCANP and mCANP resulted in the appearance of 410 and 150 kDa digest products (Figures 29A and 30A, respectively). Production of these peptides appeared to represent an initial proteolytic stage. Distinct differences in the Ca^{2+} sensitivity of μ- and mCANP mediated 550 kDa fragmentation were readily apparent. Maximal Ca^{2+} activation of 550 kDa proteolysis was observed at 500 μM and 2.5 mM free Ca^{2+} for μ- and mCANP respectively. Figure 31 shows that the Ca^{2+} dependence of μ- and mCANP proteolysis of the 550 kDa peptide was similar to the corresponding profile observed for caseinolysis. Half maximal proteolysis of casein and the 550 kDa peptide was observed at 50 and 75 μM Ca^{2+}, respectively, for μCANP, whereas with mCANP the corresponding half-maximal values were 750 and 760 μM Ca^{2+}, respectively. Additionally, μCANP mediated 550 kDa fragmentation was inhibited at elevated (>1mM) Ca^{2+} (Figure 29A lanes I to K) as observed with caseinolysis (Figure

Fig. 29. Calcium and protease dependence of μCANP mediated proteolysis of HSR proteins. In (A) 4mg.ml^{-1} HSR membranes were exposed to 1 unit CANP/mg HSR^{-1} for 20 minutes at 23°C. The following free Ca^{2+} concentrations corresponding to lanes B to K, respectively, were employed to stimulate CANP activity: 5μM (B), 10μM (C), 50μM (D), 100μM (E), 250μM (F), 500μM (G), 1mM (H), 2.5mM (I), 5mM (J), and 10mM (K). Lane A was a control (zero added μCANP) and each lane contained 60μL protein. In (B) 4mg.ml^{-1} membranes were exposed to 0.1, 0.5, 1.0, 2.5, and 5 Units μCANP/mg HSR (Lanes A to E, respectively) for 20 minutes in the presence of 1mM free Ca^{2+}. In (A) the amount of total Ca^{2+} added to obtain desired free Ca^{2+} concentrations was adjusted to account for the amount of EGTA containing μCANP with previously determined activity. In (B) various admixtures of EGTA were made with various amounts of μCANP to maintain a constant EGTA concentration. To the left of Figure 29A is the molecular weight scale as described in the legend to Figure 5. To the right of (A) and (B) are the substrates (550 and 88kDa) and digest products (410, 338, 286, 175, 160, 150, 137 kDa) of CANP mediated proteolysis. The open arrow to the right of Figure 29B indicates the position of μCANP.

Figure 29

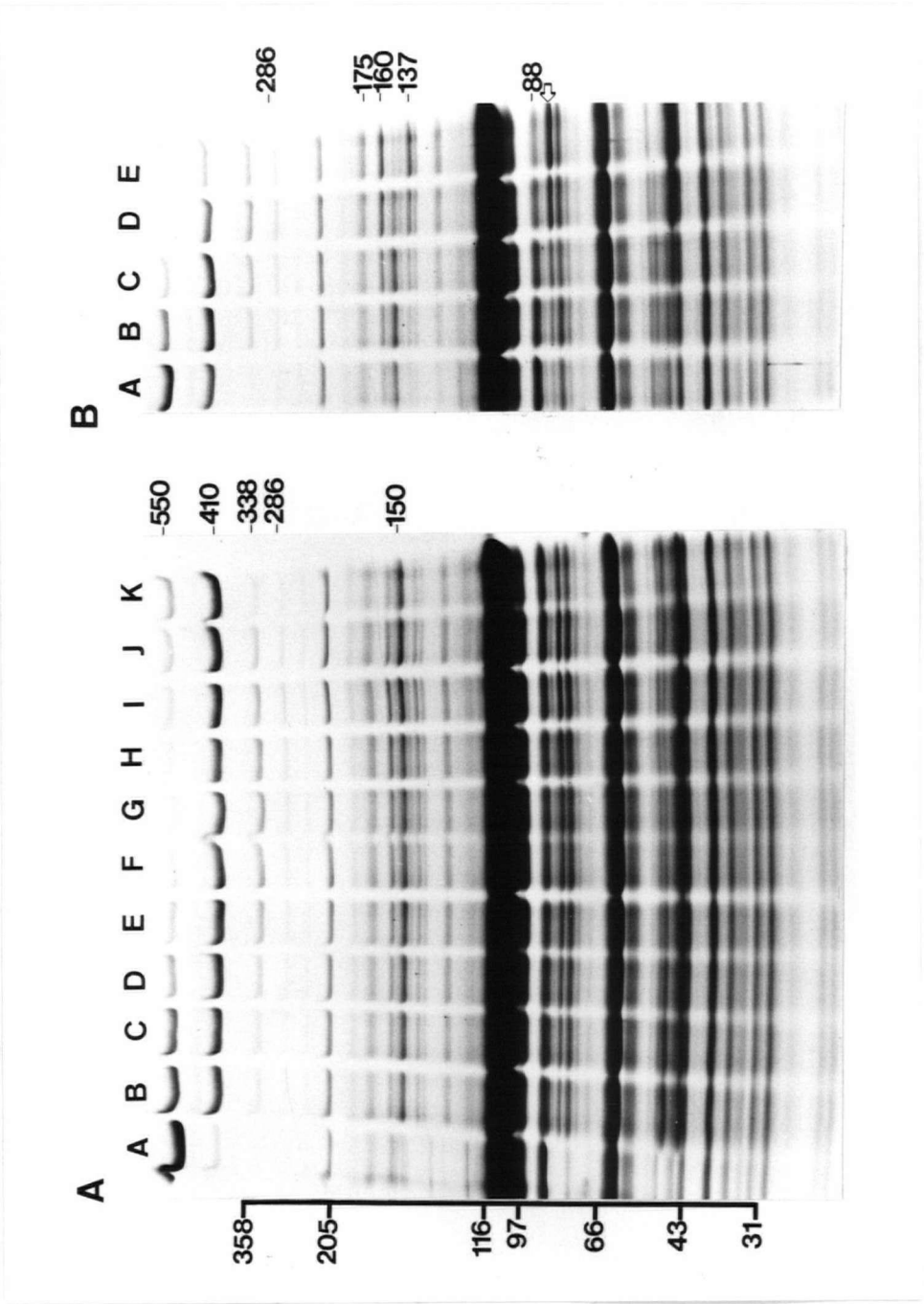

A
A B C D E F G H I J K
550
410
338
286
150
358
205
116
97
66
43
31
B
A B C D E
286
175
160
137
88

Fig. 30. Calcium and protease dependence of mCANP mediated proteolysis of HSR proteins. For both (A) and (B) identical experimental conditions to that employed for μCANP mediated proteolysis of HSR proteins. For description of this figure see legend to Figure 29.

Figure 30

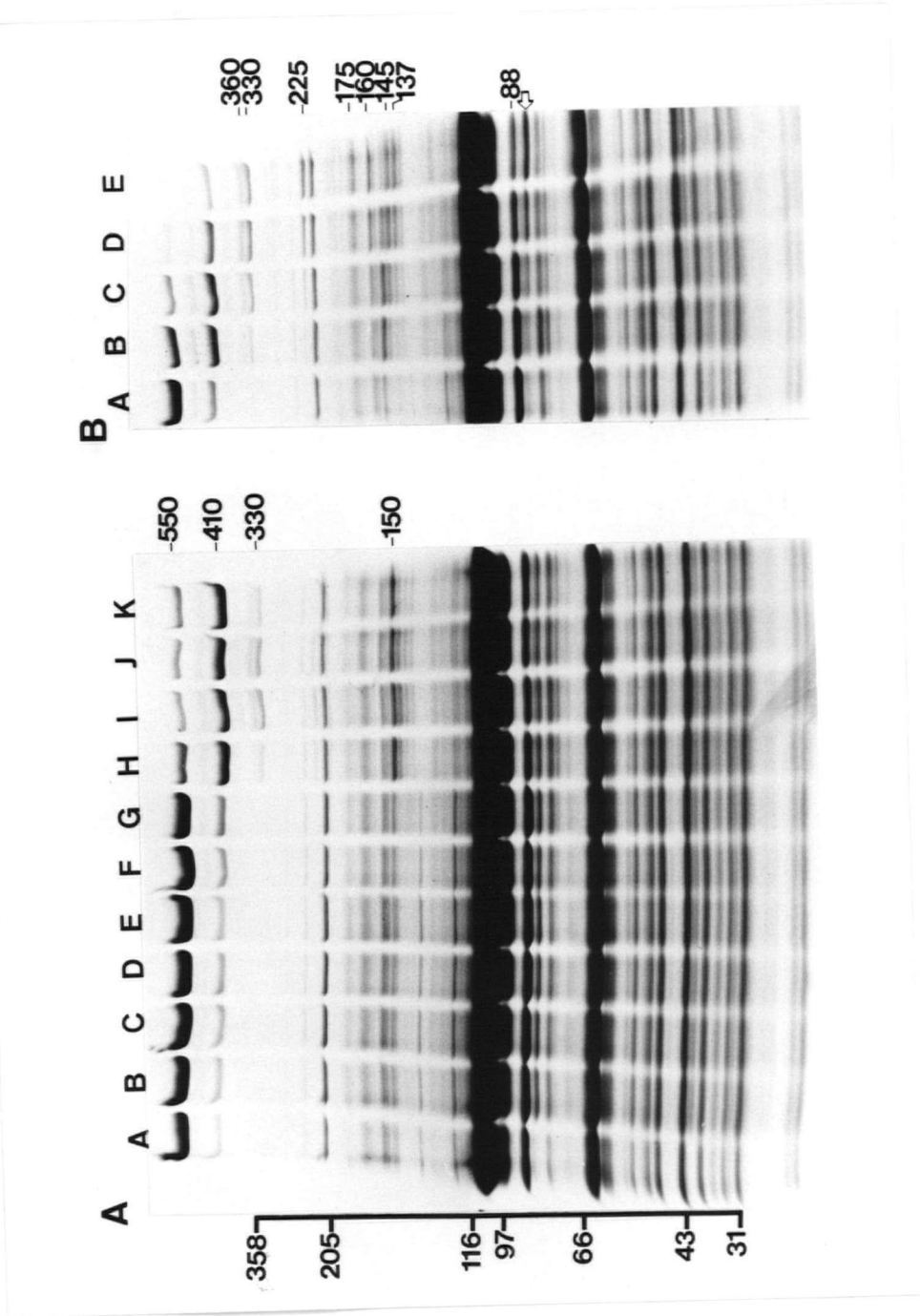
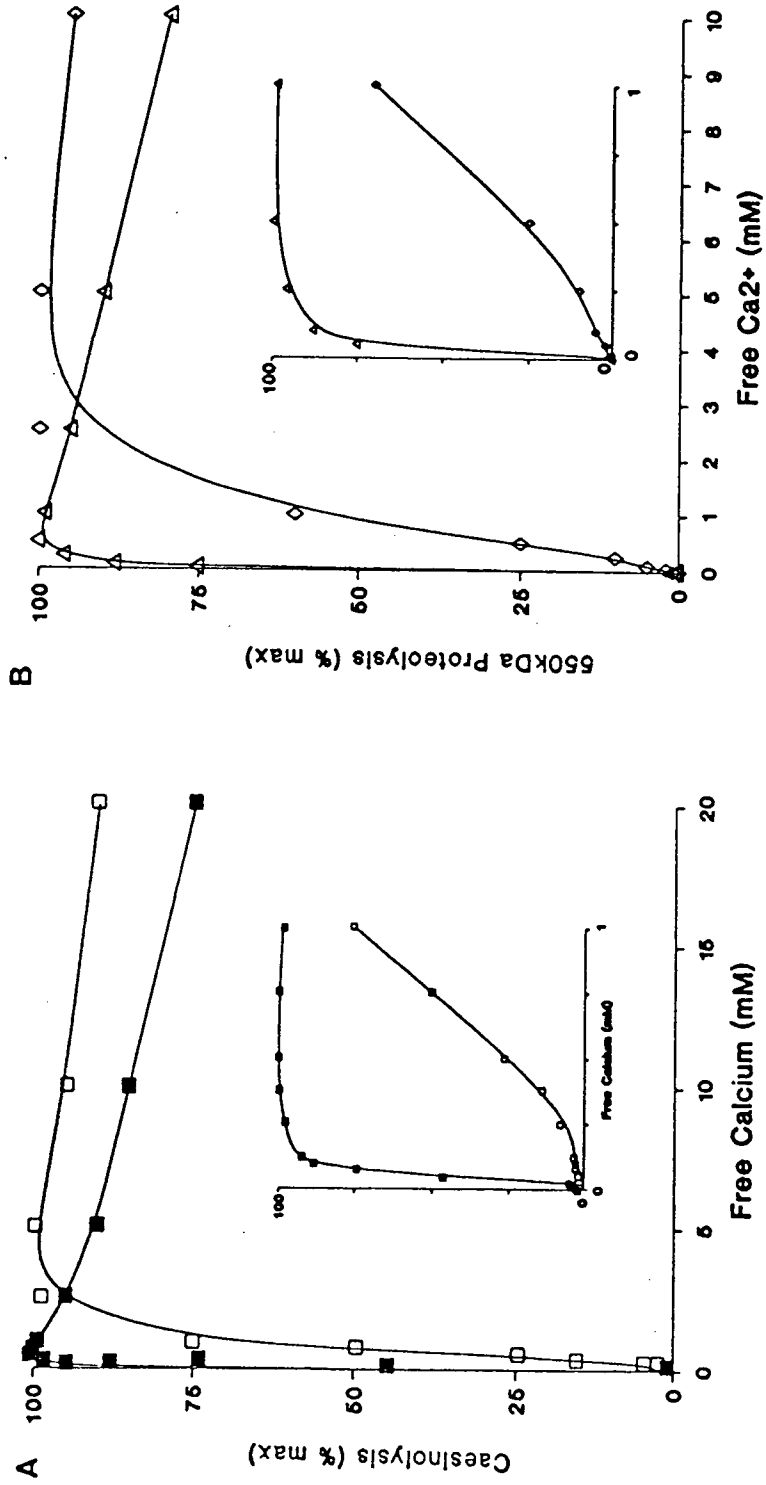

A
A B C D E F G H I J K
550
410
330
150
358
205
116
97
66
43
31
B
A B C D E
360
330
225
175
160
145
137
88

Fig. 31. Comparison of the calcium dependence of CANP mediated proteolysis of (A) casein and (B) the skeletal muscle ryanodine receptor. In (A) both μ- and mCANP were adjusted to 6 units.ml^{-1} in 1mM EGTA, 50mM HEPES (pH7.4) from a previous determination of activity (see methods). Caseinolysis was assayed in the presence of 2mg.ml^{-1} casein, 5mM DTT, 50mM HEPES (pH 7.4) at 30^{o}C, and various total concentrations of Ca^{2+} required to obtain desired free Ca^{2+} concentrations in the presence of 100μM EGTA (added with CANP). The apparent Ca^{2+} /EGTA stability constant was 1.552 x 10^{7} M^{-1}. Caseinolysis was initiated upon addition of CANP as a 50μL aliqout to a final 500μL reaction volume. CANP activity was determined as described in the methods. In (B) CANP proteolysis of the 550kDa channel and electrophoretic resolution of proteins was identical to that described in Figure (x) upon a separate HSR preparation with free Ca^{2+} varied between 5μM and 10mM Ca^{2+}. Coomassie blue stained 550kDa protein was densitometrically scanned and proteolysis calculated (see methods) from the fractional loss of 550 KDa protein at 1mM and 5mM Ca^{2+} for μ- and mCANP, respectively. The insets in (A) and (B) show the Ca^{2+} dependence of CANP mediate caseinolysis and 550kDa proteolysis, respectively, over a narrower range of Ca^{2+}. Data points are means of 3 observations from a single experiment (sem less than +/-5%). Data are representative of 3 separate experiments in (A) and five gels in (B).

Figure 31


B
650kDa Proteolysis (% max)
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10
Free Ca2+ (mM)
A
Caesinolysis (% max)
100
75
50
25
0
0 5 10 15 20
Free Calcium (mM)

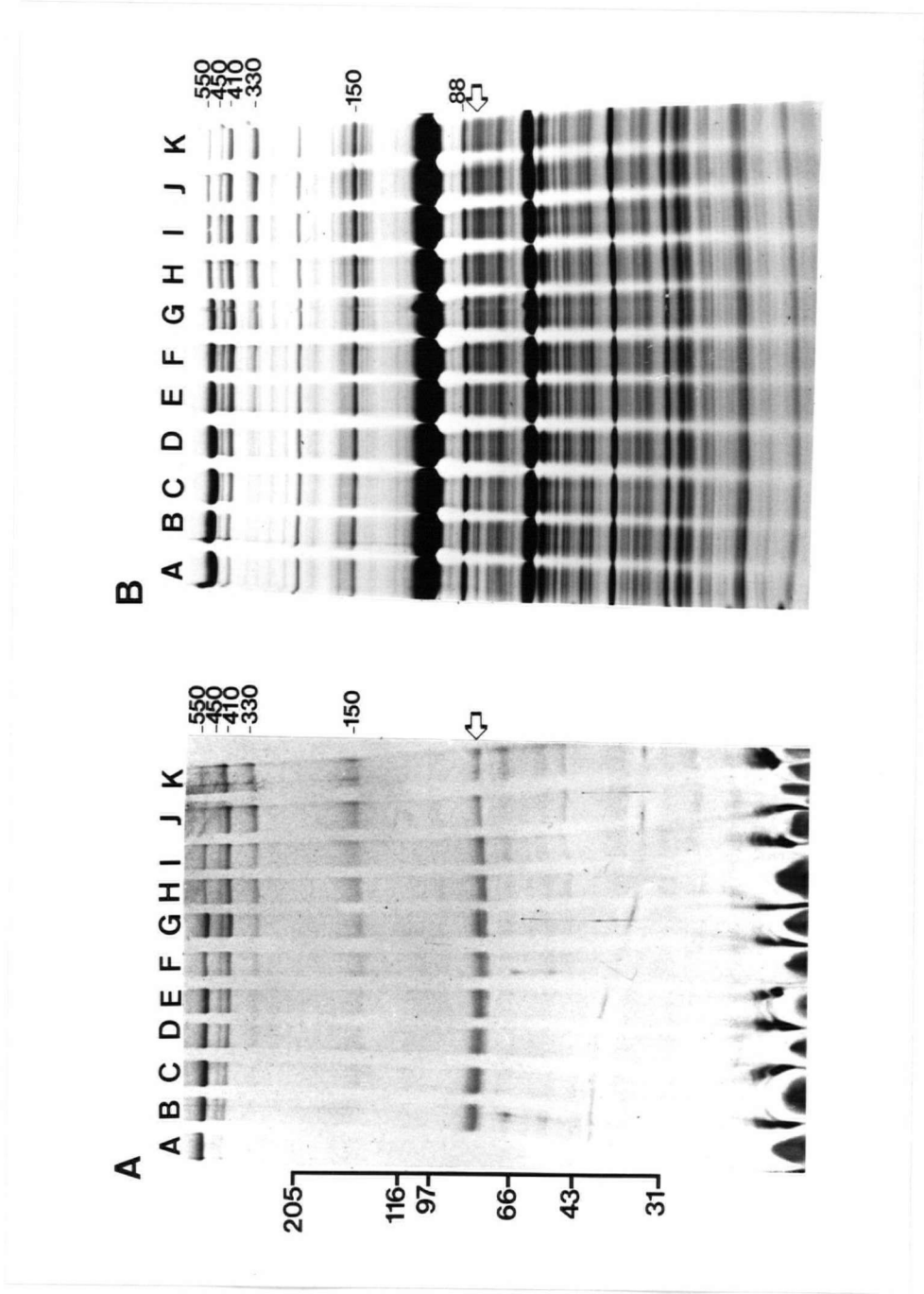
31A). On the other hand, mCANP proteolysis of both casein and the 550 kDa substrates was relatively unaffected at high Ca^{2+} (Figure 31B).

With elevated Ca^{2+} activation or increased CANP/HSR ratios (Figures 29B and 30B) a secondary stage fragmentation of the release channel was evident. For both CANP isoforms, formation of major 330, 175 and 165kDa peptides appeared to coincide with loss of the 410 kDa product. Isoform specific digest products for μCANP (286kDa) and mCANP (360, 225kDa) were also observed. Secondary stage fragmentation by both CANP isoforms was also associated with further fragmentation of the 150 kDa peptide. Again, however, subtle differences were observed between the pattern of μ- and mCANP mediated fragmentation of this peptide. Extensive mCANP digestion resulted in sequential production of 145, 137, and 130 kDa peptides. The latter two peptides were not further degraded at higher CANP/HSR ratios (at 10 and 20 units mg^{-1}; data not shown) and appeared to represent limiting digest products. With μCANP, however, production of the 145 kDa peptide was never observed with direct fragmentation of the 150 kDa parent peptide to a limiting 137 kDa product. In addition to the 550kDa protein, proteolysis of a major 88kDa protein was also observed. The 88 kDa protein was cleaved in a similar Ca^{2+} and protease concentration dependent manner as seen with the 550 kDa peptide (Figures 29 and 30). The identity of this HSR associating protein and it's contribution to the regulation of HSR Ca^{2+} transport is unknown.

Figure 32 shows that the proteolytic pattern of the solubilised ryanodine receptor is identical to that observed with the native vesicle associated form of the protein. With μCANP from human erythrocytes the time dependent production of the 410 and 150kDa major peptides within the two proteins was indistinquishable. This indicates that the effects of CANP upon Ca^{2+} channel structure are highly

Fig. 32. Comparison of the effects of μCANP upon the CHAPS solubilised and vesicular 550kDa protein. In (A) 20ml (~80μg protein) of the pooled purified Ca^{2+} channel fractions obtained from CaM-agarose affinity chromatography were proteolysed at 25°C for various times by direct addition of 5mM Ca^{2+} (estimated 2.5mM free Ca^{2+} with ~2.5mM EGTA) and 25μ Units human erythrocyte μCANP. 2ml aliquots were denatured in 3.3% (V/V) TCA on ice after 0.5 (lane b), 1 (lane c), 2 (lane D), 5 (lane E), 7 (lane F), 10 (lane G), 20 (lane H), 30 (lane I), 45 (lane J), and 60 minutes (lane K). Lane A represents the control (without μCANP addition). Sedimented material was resuspended in 50μl sample buffer and layered onto 5-15% gradient SDS-PAGE gels as in Figure 9. In (B) 500μg HSR protein was exposed to CANP under identical conditions of CANP and time of proteolysis as in (A). Each lane in (B) corresponds to the same time of proteolysis as in (A). Proteolysis was stimulated by addition of 2.5mM Ca^{2+} and aliquots containing 50μg protein were added directly to 2xSDS-PAGE sample buffer to quench proteolysis. 100μl samples containing 50μg protein were resolved by SDS-PAGE as above. Proteins were stained with Coomassie-R250 (see methods). The open arrows in (A) and (B) indicated the positions of CANP (~82kDa). To the right of (A) and (B) are molecular weights ($M_r x10^3$) of substrates (550, 88) and proteolytic products (450, 410, 330, 150).

Figure 32


A
A B C D E F G H I J K
-550
-450
-410
-330
-150
205
116
97
66
43
31
B
A B C D E F G H I J K
-550
-450
-410
-330
-150
-88

selective and restricted to the cytosolic domain of the protein. It is of interest to note that a transition peptide (~450kDa) was often observed in 5-15% gels for both μ- and mCANP. With 3-13% gels, this peptide was only faintly resolved.

Figure 33 shows that the ryanodine receptor of canine cardiac HSR membranes was also specificly fragmented by μ- and mCANP with production of 360 and 155kDa limited digestion products at 360 and 155kDa. Each peptide was further fragmented to 300kDa and 150kDa products, respectively. Eventual loss of high molecular weight protein was observed after extensive proteolysis with accumulation of minor products at ~175kDa. The 150kDa peptide was not further degraded, however, and appeared to be a limiting peptide. This pattern of fragmentation was similar to the skeletal HSR although no subtle differences between μ- and mCANP effects were observed.

2.Immunolocalisation of CANP to HSR membranes.

Observation of endogenous CANP mediated proteolysis of the 550kDa peptide during purification of HSR membranes (Figure 28, lanes B and C) prompted investigation of the possible association of this protease with HSR. In the right panel of Figure 34, the 4 membrane fractions harvested after 25-45% sucrose density gradient centrifugation were immunoblotted with a polyclonal anti-calpain antibody, cross-reactive to μ- and mCANP. For reference, the complementary Coomassie-Blue stained fractions shown in Figure 5 are reproduced here (Figure 34A). These experiments revealed the presence of a 90 kDa protein reactive to the antibody. This protein was present in all the fractions although more abundant in the lighter density membrane fractions.

In preliminary studies of HSR solubilisation with CHAPS it was observed that the 550 kDa protein, present in the CHAPS insoluble fraction, was selectively fragmented when resuspended after sedimentation in buffer devoid of

Fig. 33. CANP proteolysis of cardiac HSR proteins. Under identical conditions to that defined for skeletal HSR proteolysis in Figures 29B and 30B, cardiac HSR membranes were treated with μCANP (A) and mCANP (B). In (A) and (B) lane A corresponds to control, untreated HSR. Lanes B to E in (A) correspond to treatment with 0.1, 0.5, 1.0 and 2.5 units CANP.mg HSR^{-1}, respectively. In (B) lanes B to D correspond to treatment with 0.5, 1.0 and 2.5 $units.mg^{-1}$, respectively. CRC, CPP AND CBP have the same meanings as in Figure 5. Positions of peptides ($M_r x10^3$) are indicated to the right of each panel.

Figure 33

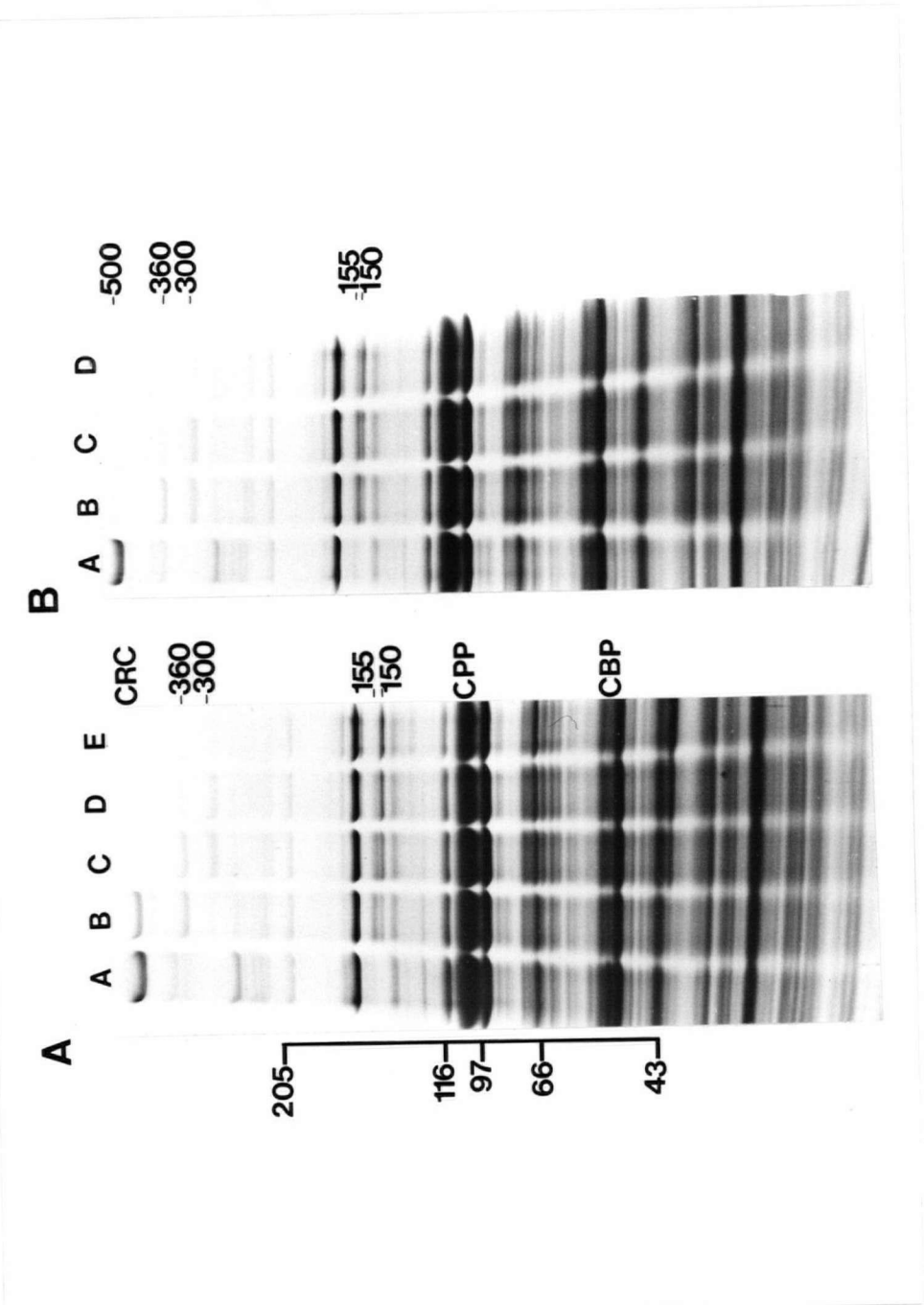

A
A B C D E
CRC
-360
-300
155
150
CPP
CBP
205
116
97
66
43
B
A B C D
-500
-360
-300
155
150

Fig. 34. Immunostaining of SR microsomal protein reactive to CANP polyclonal antisera. Microsomes obtained from the 45,000 rpm centrifugation step were separated by density gradient centrifugation (see methods) into 4 discrete bands at 25-27%, 29-33%, 35-38% and 39-41% sucrose. In (A) 50 μg protein of each fraction (lanes a to d, respectively) was resolved by SDS-PAGE (3-13% gradient gels) and stained with Coomassie brilliant blue R-250. Identity of the calcium release channel/ryanodine receptor (CRC), the calcium pump protein/Ca^{2+} ATPase (CPP) and the calcium binding protein/calsequestrin (CBP) is as indicated on the right of the panel. Western blots of protein (50 μg per lane) from complementary gels were transfered onto 0.45μ nitrocellulose membranes and were incubated with CANP polyclonal antisera for 24 hrs (see methods). After washing, blots were incubated for a further 2 hrs with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase. Staining of horseradish peroxidase after reaction with 4-chloro-1-naphthol and hydrogen peroxide permited identification of a 90 kDa SR/t-tubule associated protein (open arrow) reactive to CANP polyclonal antisera. Fractionation of proteins in (A) was representative of the results of over 30 separate membrane isolations. Immunoblots in (B) were reproduced with 4 separate membrane preparations with identical results.

Figure 34

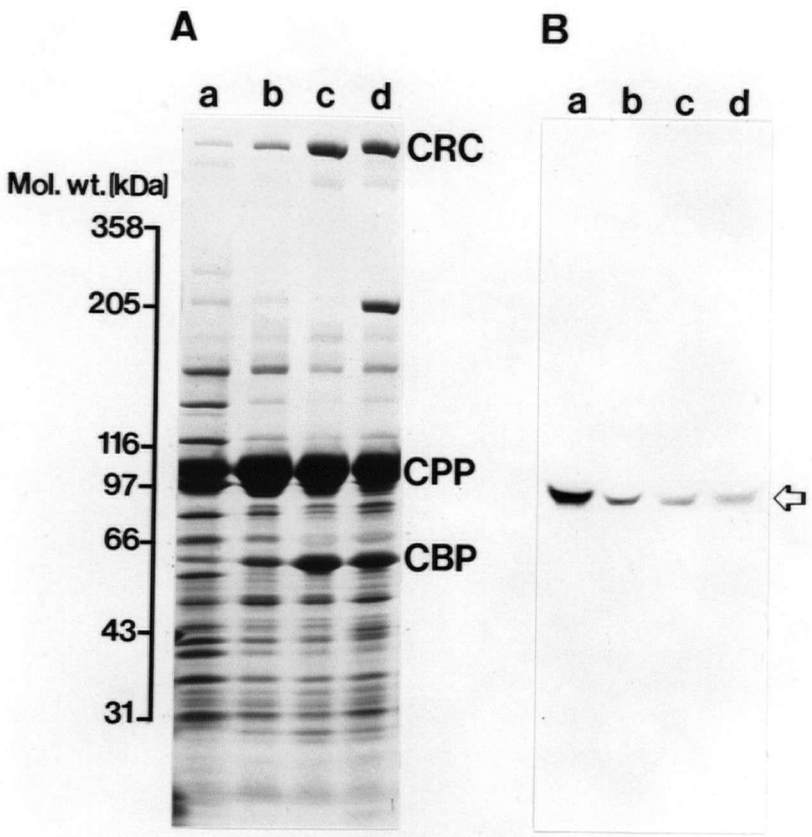
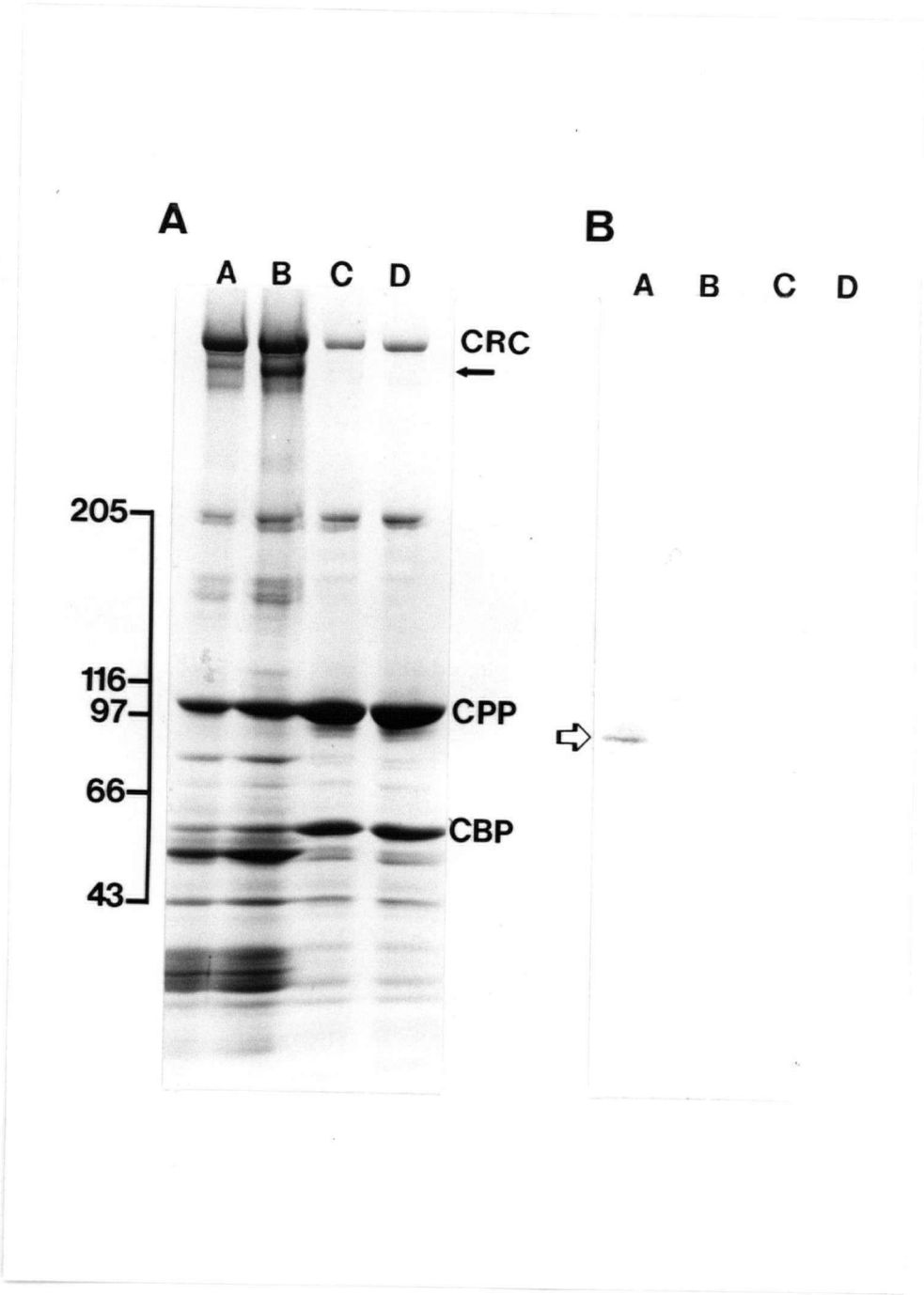


Fig. 35. Immunostaining of CHAPS solubilised protein reactive to CANP polyclonal antisera. HSR membranes were solubilised in 1.5% CHAPS: 0.5% phosphatidylcholine as described in the methods. In (A) membranes were solubilised in the presence (lanes A and C) and absence (lanes B and D) of 100μM leupeptin. 50μg of CHAPS insoluble (lanes A and B) and CHAPS soluble (lanes C and D) were resolved across 3-13% SDS-PAGE gradient gels and stained with Coomassie-R250 (see methods). In (B) complementary gels were run as in (A) and proteins were immunoblotted with CANP polyclonal antisera as in Figure 35. The solid arrow indicates the position of the 410kDa proteolytic cleavage product. The open arrow indicates the position (90kDa) of the immunoreactive protein in lane A.

Figure 35



leupeptin (Figure 35A, lane B). The soluble fraction was apparently resistant to endogenous proteolysis regardless of the presence or absence of leupeptin (compare lanes C and D). It was then investigated whether a specific localisation of CANP to HSR membranes might account for these observations. Immunoblots of membranes resolved by SDS-PAGE revealed localisation of immunoreactivity toward the anti-calpain antibody solely in the pelleted fraction prepared in the presence of leupeptin (Figure 35B). Loss of immunostaining and 550 kDa proteolysis of the pelleted material in non-leupeptin containing buffers is consistent with autolytic fragmentation of CANP.

3. CANP effects upon HSR function.

a. Passive Ca^{2+} loading and Ca^{2+}-induced Ca^{2+} release.

The functional effects of both μ- and mCANP digest of HSR proteins were examined through studies of Ca^{2+}-induced Ca^{2+} release from vesicles passively loaded with Ca^{2+} (Figure 36 and Table 5). For both isoforms, Ca^{2+} loading was elevated by 15% with mild CANP treatment as determined after rapid dilution of Ca^{2+} loaded vesicles into Ca^{2+} release inhibiting media containing the Ca^{2+} channel blockers ruthenium red and Mg^{2+}. Extensive CANP treatment with eventual production of limiting digest products did not markedly stimulate further increases in HSR Ca^{2+} loading. Much of the modified Ca^{2+} handling appeared to be associated with initial stage production of the 410 and 150 kDa peptides. The elevation of HSR Ca^{2+} loading after CANP digest was accompanied by 30-35% increases in fractional Ca^{2+} release with identical amounts of Ca^{2+} remaining after release. A similar elevation of Ca^{2+} loading and Ca^{2+} release was also observed with CANP treatment of cardiac HSR

Fig. 36. Calcium loading and calcium induced calcium release and mCANP treated HSR membranes. HSR vesicles (10mg.ml^{-1}) were incubated at 22^{o}C in the presence of 300mM sucrose, 150mM KCl, 5mM DTT, 10mM $^{45}Ca^{2+}$, 50mM HEPES (pH 7.4) and either 0.25, 1, or 5 units.mg^{-1} HSR for 20 minutes. Proteolysis was quenched upon addition of 100μM leupeptin. Control experiments were performed in the presence of 5 units.mg^{-1} CANP with prior addition of 100μM leupeptin. Membranes were subsequently incubated at 4^{o}C for 24 hrs followed by a further 2 hr incubation at 22^{o}C. Aliquots (5μl) were rapidly diluted (250 fold) and mixed into iso-osmotic media (300mM sucrose, 150mM KCl, 50mM HEPES, pH 7.4) containing, in addition, either 5mM EGTA and 4.9mM Ca^{2+} (10μM free Ca^{2+}) or 10mM Mg^{2+}, 50μM ruthenium red and 100μM EGTA (Quench buffer). At the indicated time intervals 200μl aliquots were withdrawn and vacuum filtered across 13mm diameter 0.45μ nitrocellulose filters which were rapidly washed with 5 volumes of Quench buffer. Filters were immediately removed from their support grids, air dried and assayed for radioactivity by liquid scintillation counting. The data represent means (standard error less than +/-5%) of triplicate determinations from a single experiment representative of 4 independent experiments conducted on separate HSR preparations using 3 separate CANP preparations.

Figure 36

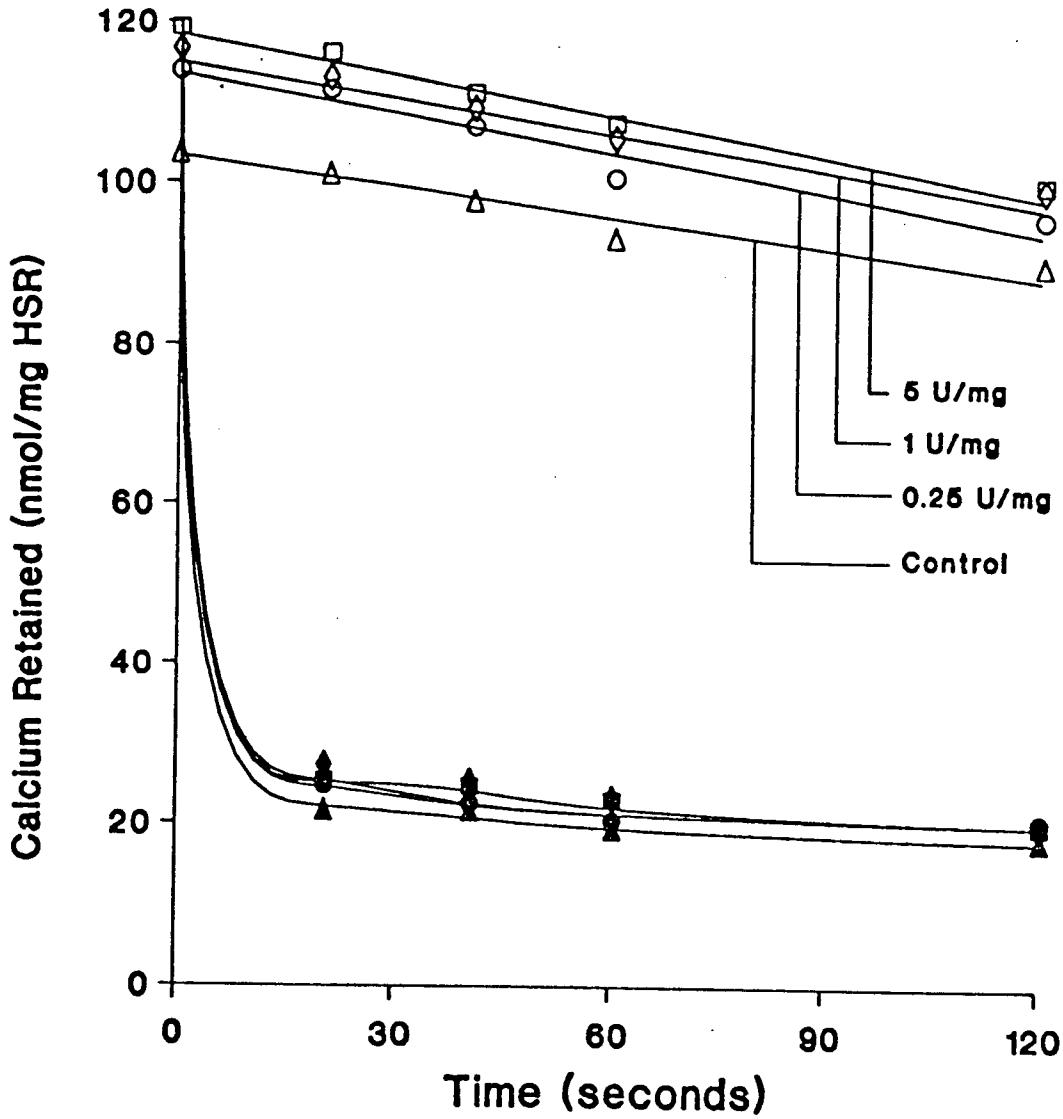

120
100
80
60
40
20
0
0
30
60
90
120
Calcium Retained (nmol/mg HSR)
Time (seconds)
5 U/mg
1 U/mg
0.25 U/mg
Control

Table 5. Calcium release from μCANP treated vesicles.

CANP/HSR RATIO (Units.mg HSR^{-1})	Ca^{2+} Retained[b] (nmol Ca^{2+}.mg HSR^{-1})	Ca^{2+} Released (% max.)
Control[a]	103+/-1.7	77.7
0.25	115.5+/-2.7	80.1
1.0	117.9+/-2.2	80.6
5.0	119.3+/-1.9	80.9

a. Control vesicles were treated with leupeptin prior to addition of 5 Units CANP.mg HSR^{-1}

b. Determinations represents averages (+/-sem) from triplicate observations in a single experiment.

(Figure 37).

b. ATP dependent calcium accumulation.

Consistent with the above effects was the observation of similar increases in Ca^{2+}-ATPase mediated Ca^{2+} accumulation under conditions of Ca^{2+} (25μM free) and pH (7.4) designed to promote the open state of the channel (Figure 38). These absolute increases, which were not due to modified Ca^{2+}-ATPase activity determined in the presence of A23187 (Table 6), were preserved in the presence of ruthenium red.

c. Spectroscopic studies of CANP effects upon HSR Ca^{2+} transport.

The above findings of a possible alteration of an intralumenal Ca^{2+} compartment (Figures 37 and 38) were investigated further with dual-wavelength spectroscopy of HSR Ca^{2+} uptake and release.

(i). Ca^{2+} uptake.

With limited exposure of HSR membranes to mCANP, the rates of both the secondary slow phase and tertiary fast phase of Ca^{2+} accumulation were slightly elevated (Figure 39B). With prolonged CANP treatment, the rate of the slow phase accumulation was doubled with a 50% shortening of the time required to accumulate all extravesicular Ca^{2+}. Similar effects were observed with endogenous CANP (Figure 39A).

(ii). Ca^{2+} release.

Figure 40 shows that with extensive μ- and mCANP treatment the amount of intralumenal Ca^{2+} required to trigger the release of Ca^{2+} uptake was decreased slightly by 15%. Similarly, the amount of ryanodine required to induce

Fig. 37. Effect of μCANP and mCANP upon passive calcium loading and release by cardiac HSR vesicles. Vesicles ($10mg.ml^{-1}$) were exposed to 5mM $^{45}Ca^{2+}$ and 0.5 $U.mg^{-1}$ CANP for 30 mins. Proteolysis was quenched by addition of leupeptin to a final concentration of 100μM. Control ( ) vesicles were treated similarly to μCANP ( ) and mCANP ( ) except that leupeptin was added prior to CANP. Vesicles were further incubated for 20hrs at $4^{o}C$. Vesicles were diluted 250 fold into either 50μM ruthenium red containing media to obtain the zero time $^{45}Ca^{2+}$ loading by back extrapolation, or 4.9mM Ca^{2+}/5mM EGTA buffer (10μM free Ca^{2+}) to induce $^{45}Ca^{2+}$ release.

Figure 37

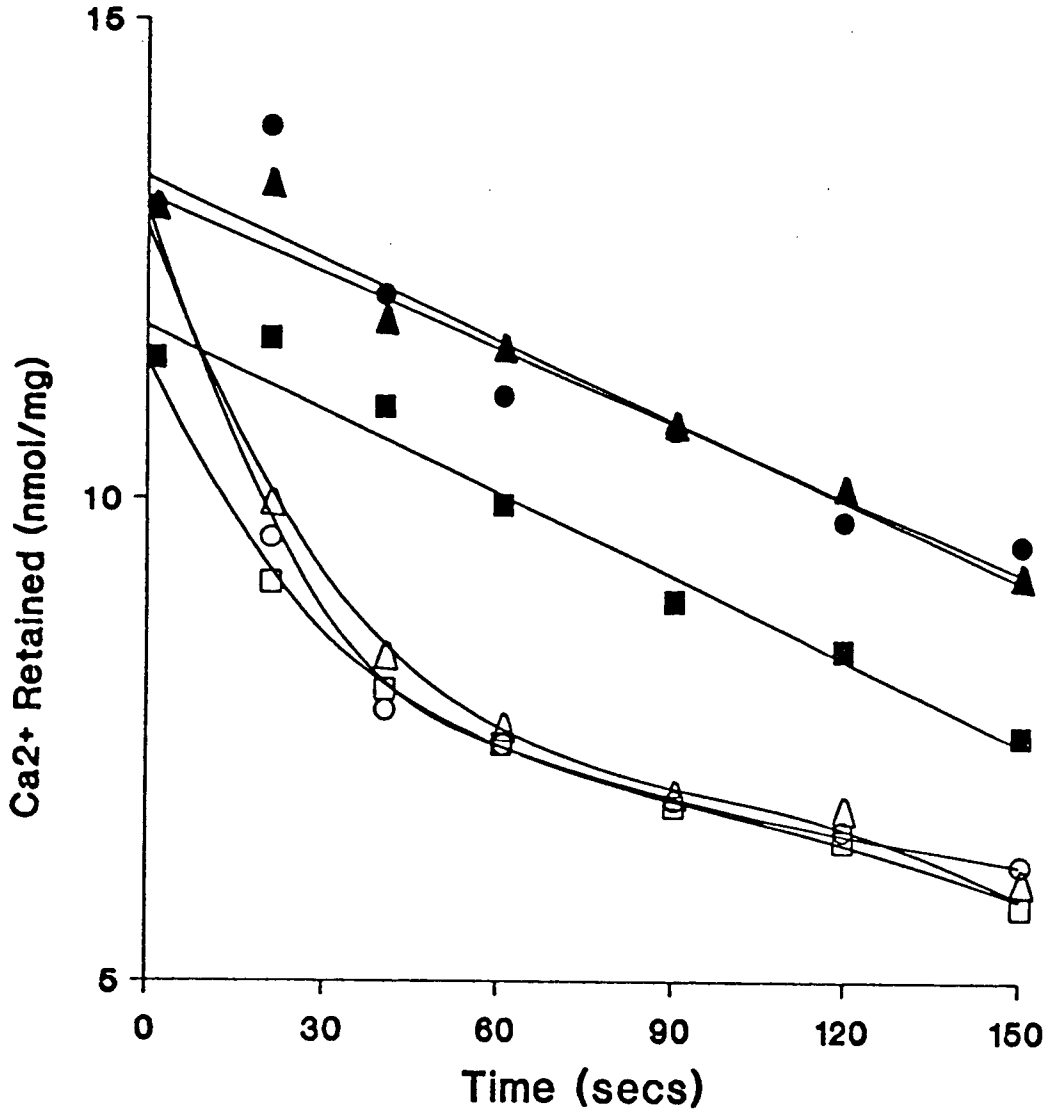


Fig. 38. Effect of CANP upon ATP dependent calcium uptake by HSR vesicles. 5mg.ml^{-1} HSR was exposed to both μ- ( ) and mCANP ( ) as described in methods. Vesicles were diluted into iso-osmotic transport buffer and Ca^{2+} uptake was initiated upon addition of Mg.ATP (5mM free in the presence of 25μM free $^{45}Ca^{2+}$ and 500uM free Mg^{2+}. The effects of CANP upon Ca^{2+} uptake were compared to controls ( ) in the presence (filled symbols) and absence (open symbols) of 50μM ruthenium red. Aliquots (150μl) were filtered at the indicated times and radioactivity determined by liquid scintillation methods.

Figure 38

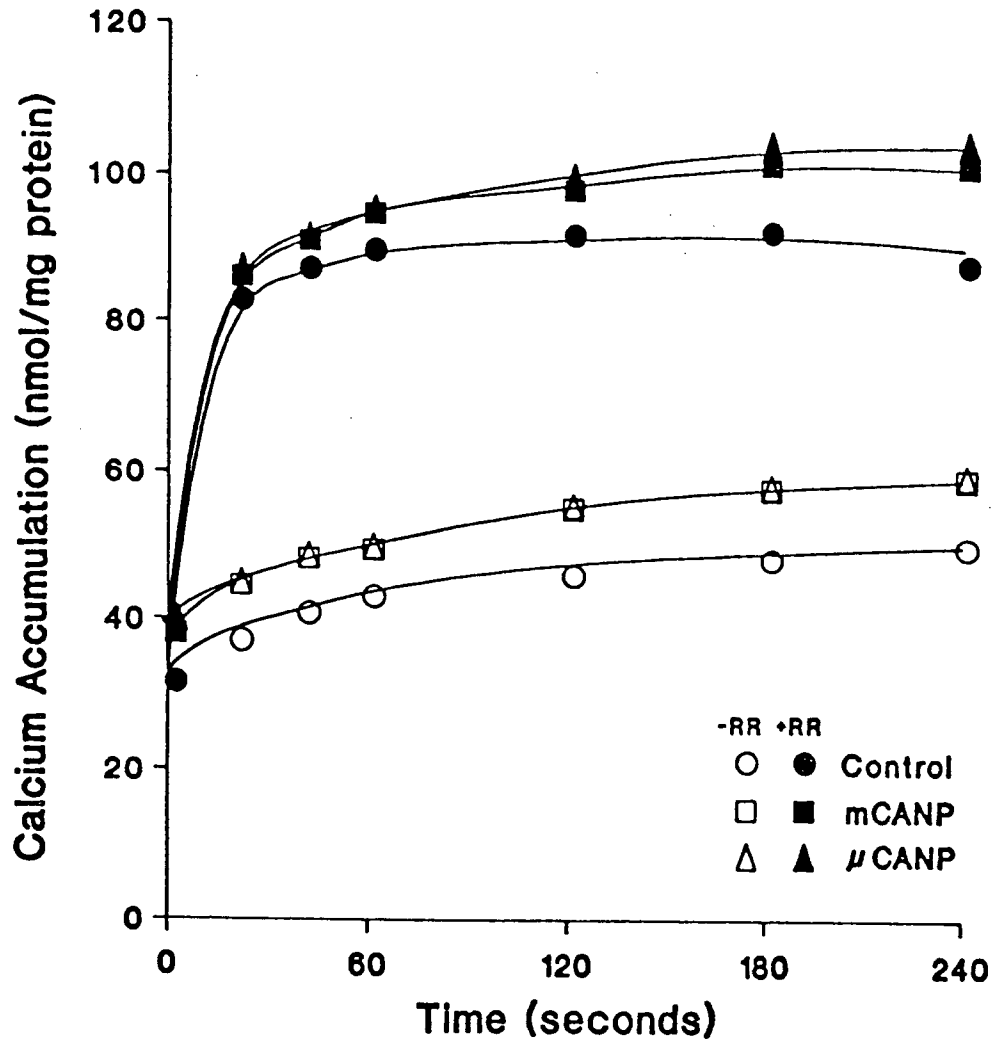

120
100
80
60
40
20
0
Calcium Accumulation (nmol/mg protein)
0
60
120
180
240
Time (seconds)
-RR +RR
Control
mCANP
μCANP

Table 6. Ca^{2+}-stimulated ATPase activity of CANP treated HSR membranes.

	Ca^{2+}-ATPase (nmol Pi.mg HSR^{-1}.$min.^{-1}$)[a]	
	-A23187	-A23187
μCANP	635+/-43	1391+/-109
mCANP	641+/-50	1384+/-81

a. Ca^{2+}-ATPase activity was determined at 25μM free Ca^{2+} in the presence of 500μM free Mg^{2+}. Activity observed in the presence of 500μM EGTA was subtracted from the total activity to yield Ca^{2+} stimulated activity.

Fig. 39. Effect of CANP mediated proteolysis of HSR upon calcium uptake. In (A) 0.5mg.ml^{-1} HSR vesicles isolated in the absence (trace a) or presence (trace b) of leupeptin (Leu) were incubated in the presence of transport buffer containing 60μM Ca^{2+} and 5mM CP as described in Figure x. Ca^{2+} uptake was initiated by addition of 1mM Mg·ATP. In (B) 1.375mg HSR (40mg.ml^{-1}) were pre-treated at 25^{o}C with 1.375 units mCANP in the presence of 300mM sucrose, 100mM KCl, 50mM HEPES (pH 7.4), 5mM DTT, 2.5mM Ca^{2+} for 2 minutes (trace b) and 30 minutes (trace c). Proteolysis was terminated upon addition of leupeptin to a final concentration of 100μM. In trace a (control) leupeptin was added prior to the addition of mCANP. Vesicles were added to the cuvette and pre-incubated for 20 minutes to allow efflux of passively loaded Ca^{2+}. Ca^{2+} uptake was initiated by 1mM Mg·ATP addition and monitored by dual-wavelength spectroscopy as in (A) above.

Figure 39

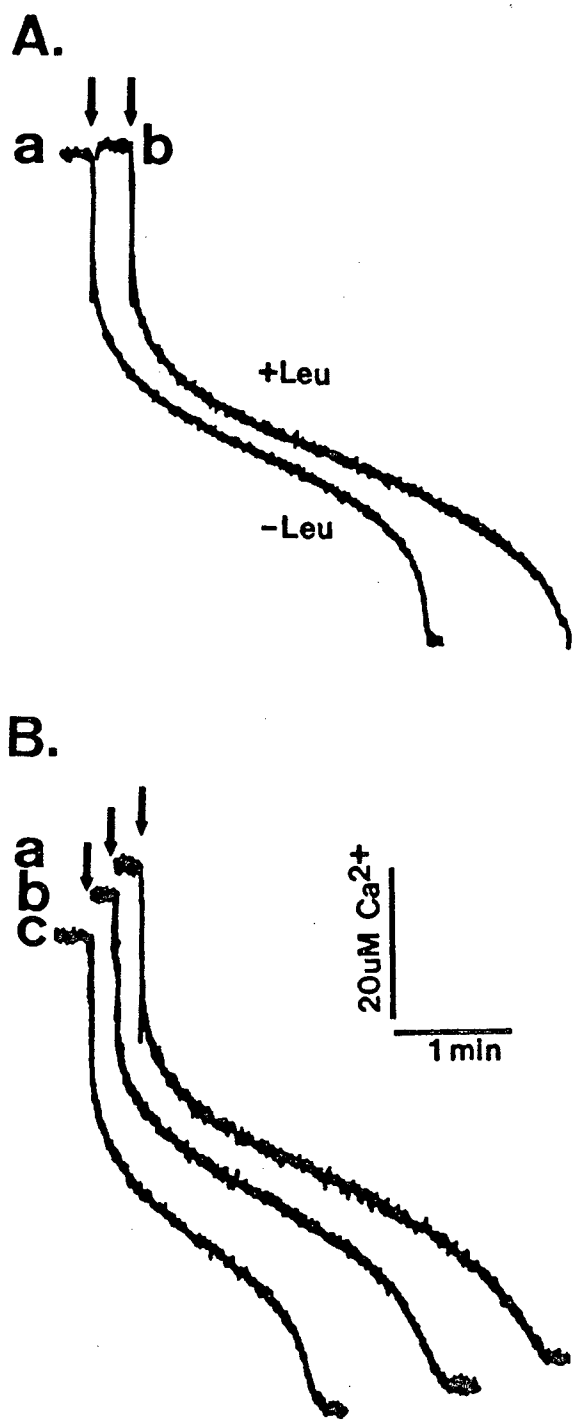
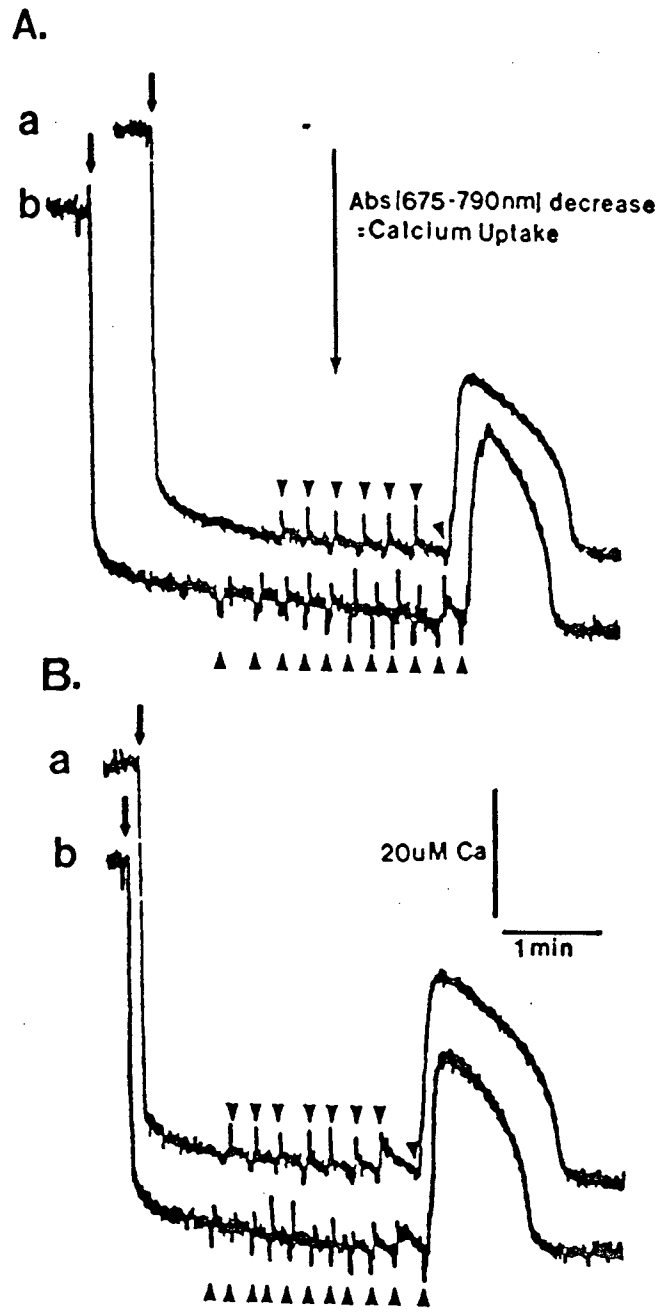


Fig. 40. Effects of CANP upon intralumenal calcium dependence of calcium induced calcium release. 2.75mg HSR protein were exposed to 2.75 units μCANP (A) and mCANP (B) in the presence of 2.5mM Ca^{2+} for 20 minutes at 25°C. The composition of the proteolysis buffer was otherwise, identical to that described in Figure 39B. Ca^{2+} uptake was assayed as described in Figure 21 with addition of 5μM Ca^{2+} pulses (arrowheads) to stimulate Ca^{2+} release. The final EGTA concentration in the cuvette was ~3-5μM. In (A) and (B) controls (trace b) were treated identically to CANP proteolysed HSR (trace a) except for addition of 100μM leupeptin prior to addition of CANP. Solid arrows indicate addition of 1mM Mg·ATP.

Figure 40



Ca^{2+} release was also decreased (Table 7). The amount of Ca^{2+} released in this assay system was unaltered although the rate of Ca^{2+} re-accumulation was decreased. These effects were only observed after extensive CANP mediated proteolysis of the channel. Although these were small changes in HSR function the titration of intralumenal Ca^{2+} sites in this manner was highly predictable.

4. CANP effects upon [^{3}H]ryanodine binding.

In view of the specific CANP effects upon Ca^{2+} channel structure it was appropriate to examine these effects upon ryanodine binding. CANP mediated fragmentation of the release channel upon [^{3}H]ryanodine binding are shown in Figure 41. Incubation of membranes in the presence of 5mM AMP, 50μM free Ca^{2+} and 8nM-2.5μM [^{3}H]ryanodine revealed the presence of both low and high affinity ryanodine binding sites. Under conditions which promoted complete fragmentation of the intact 550kDa peptide by both CANP isoforms, total [^{3}H]ryanodine binding was elevated by 25%. Scatchad analysis revealed that both low and high affinity B_{max} values were elevated after both μ and mCANP treatment (Figure 42). These data show that [^{3}H]ryanodine binding affinity was unaffected at either site by CANP treatment of HSR membranes.

Table 7. Effect of CANP proteolysis upon ryanodine induced calcium release.

Added Ca^{2+b}	Added Ryanodine (μM)[b]		
($nmol.mg^{-1}$)	Control	μCANP[a]	mCANP[a]
60	144[c]	106	108
80	396	288	324
100	1440	1260	1188

a. Proteolysis was conducted under identical conditions to that described in Figure 40.

b. The experimental protocol for Ca^{2+} and ryanodine addition was performed as described in Figure 27.

c. Observations are averages of two determinations at each Ca^{2+} loading.

Fig. 41. Effect of μCANP and mCANP upon ryanodine binding to HSR membranes. HSR vesicles (20 $mg.ml^{-1}$) were exposed to CANP (1 $U.mg^{-1}$ HSR) for 20 minutes at room temperature (23 ^{o}C) in the presence of 300mM sucrose, 150mM KCl, 5mM Ca^{2+}, 5mM DTT, 50mM HEPES (pH 7.4). Proteolysis was quenched upon addition of leupeptin to a final concentration of 100μM. Control experiments were performed with the addition of leupeptin prior to the addition of CANP. Aliquots (10μl) of the above mixture were diluted fifty fold into media containing 300mM sucrose, 150mM KCl, 5mM AMP, 8nM-1mM [^{3}H]ryanodine and 50mM HEPES (pH 7.4) and membranes were incubated at 23 ^{o}C for 24 hours. Final protein and Ca^{2+} concentrations were 400$\mu g.ml^{-1}$ and 100μM (50μM free) respectively. Effects of μCANP ( ) and mCANP ( ) HSR upon specific [^{3}H]ryanodine binding are compared to controls ( ). At 1mM [^{3}H]ryanodine total binding for control, μCANP and mCANP were 23.1, 23.5 and 23.8 $nmol.mg^{-1}$ HSR, respectively ($p>0.05$). Specific binding was obtained by assuming non-specific [^{3}H]ryanodine binding to be a constant proportion of total binding. The above data were means of triplicate observations in a single experiment and are representative of three independent experiments conducted on different HSR preparations (less than 5% standard error).

Figure 41

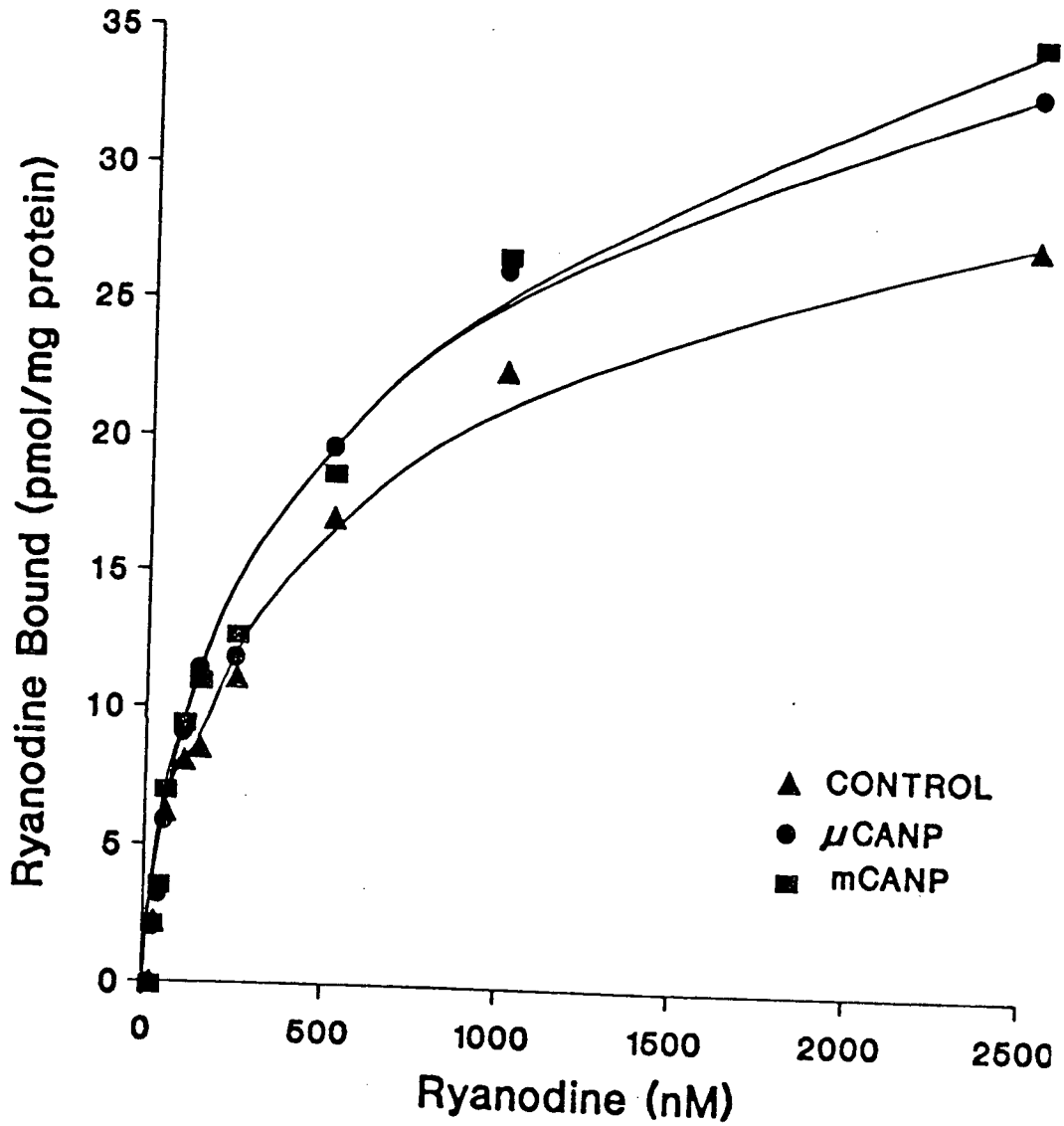

Ryanodine Bound (pmol/mg protein)
35
30
25
20
15
10
5
0
0
500
1000
1500
2000
2500
Ryanodine (nM)
CONTROL
μCANP
mCANP

Fig. 42. Scatchard analysis of ryanodine binding to HSR membranes. Data from Fig. 41 was replotted as a scatchard diagram. The table in the figure summarises the maximum [^{3}H]ryanodine binding at each low and high affinity site.

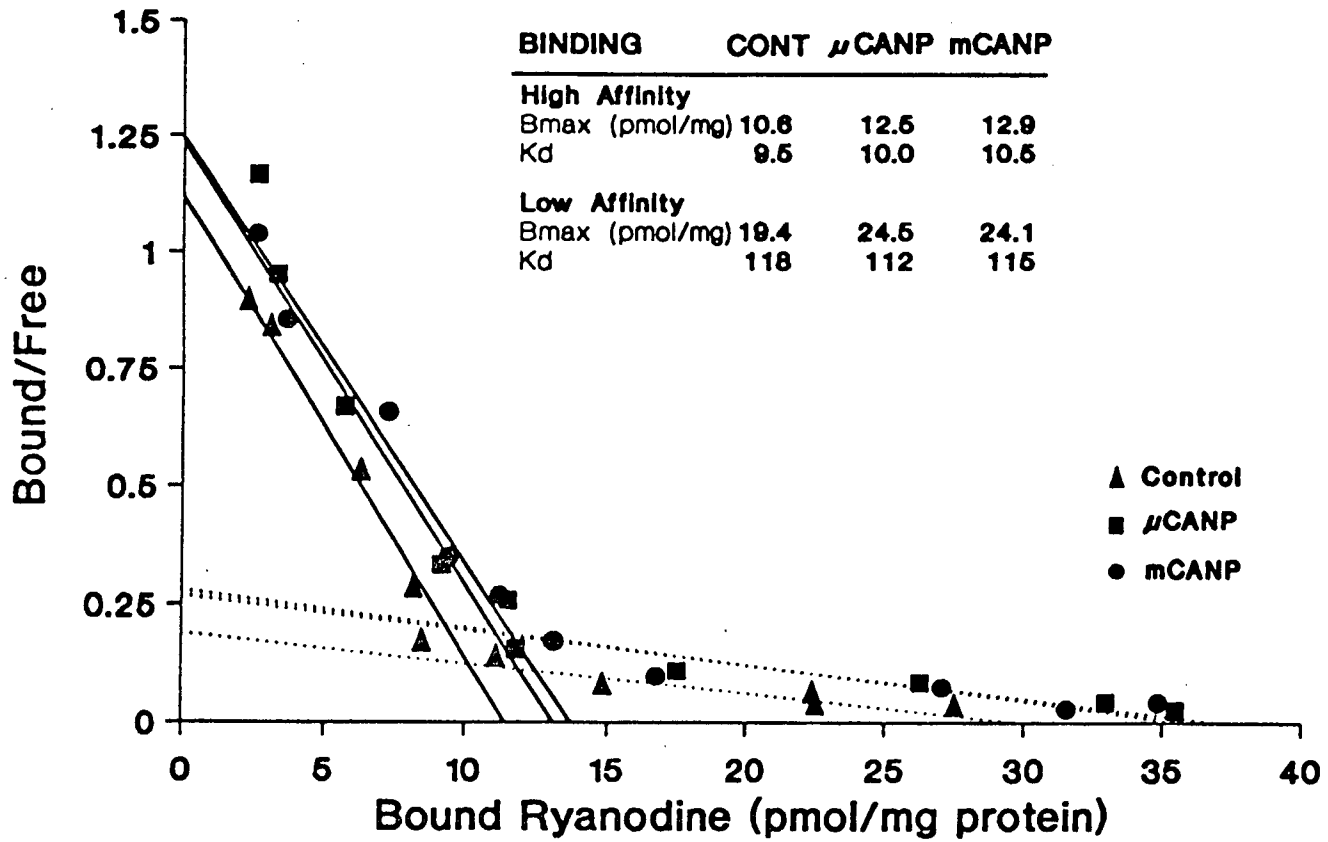

BINDING CONT μCANP mCANP
High Affinity
Bmax (pmol/mg) 10.6 12.5 12.9
Kd 9.5 10.0 10.5
Low Affinity
Bmax (pmol/mg) 19.4 24.5 24.1
Kd 118 112 115
Control
μCANP
mCANP
Bound/Free
Bound Ryanodine (pmol/mg protein)
1.5
1.25
1
0.75
0.5
0.25
0
0
5
10
15
20
25
30
35
40

Figure 42

DISCUSSION

The major hypothesis within this study was that intralumenal Ca^{2+} within the terminal cisternae of sarcoplasmic reticulum is an important regulator of the Ca^{2+} release channel. It was envisioned that the binding of Ca^{2+} to calsequestrin might facilitate the timing of Ca^{2+} channel opening and closing in order to coordinate the movements of Ca^{2+} into and out of the SR during isotonic contraction of muscle. Considering that the release channel and the Ca^{2+}-ATPase are both regulated by the same cytosolic effectors within a similar concentration range (e.g. μM Ca^{2+}, mM Mg·ATP, mM Mg^{2+}), such a signalling mechanism might be necessary. Within this scheme, the conformational changes that would be associated with Ca^{2+} release channel opening and closing would be coordinated with structural changes in calsequestrin associated with the binding and dissociation of Ca^{2+}. It was then hypothesised that structural modification of the Ca^{2+} release channel by Ca^{2+} activated neutral proteases during episodes of cytosolic Ca^{2+} overload would lead to alteration in the association of Ca^{2+} with calsequestrin or some other intralumenal Ca^{2+} binding compartment. In exploring these hypotheses the following objectives were established. (a) to obtain a highly purified membrane preparation, referable to the terminal cisternae of the SR, (b) to develop a Ca^{2+} transport assay that allowed investigation of the intralumenal Ca^{2+} compartment, (c) to obtain highly purified preparations of CANP, (d) to assess the effect of intralumenal Ca^{2+} upon Ca^{2+} release channel function and (e) to assess the effect of pharmacological and structural manipulation of the Ca^{2+} release channel upon intralumenal Ca^{2+} dependence of Ca^{2+} release.

I. HSR structural characterisation

In pursuit of the first objective Figures 5 and 6 show that with the novel freezing/grinding method of tissue disruption, highly purified HSR membrane preparations could be obtained. The isolated HSR membranes were enriched in 550kDa and 57kDa proteins identified, respectively, as the Ca^{2+} release channel/ryanodine receptor (Lai et al., 1987; Inui et al., 1987; Imagawa et al., 1987) and calsequestrin (Maclennan and Campbell, 1979). The amount of calsequestrin within these preparations was approximately 20%, as deduced by densitometric scanning of Coomassie blue stained gels. This value is, likely, an underestimate, as calsequestrin binds this dye relatively poorly in view of its high acidity (Reithmeier et al., 1987). In isolated SR, Williams and Beeler (1986) estimated that calsequestrin constituted 30-33% of the total protein within the terminal cisternae. With Stains-All staining, the amount of evident 57kDa protein was much increased in relation to the amount of 105kDa protein (Ca^{2+}-ATPase). Calsequestrin therefore appeared to represent a major protein within these preparations.

The amount of 550kDa protein was estimated at 7-10% of the total SR protein from densitometry. These membranes were shown to bind a total of 30pmol ryanodine.mg HSR^{-1} (Figure 42) with identification of low and high affinity ryanodine binding sites. These results are consistent with similar values (32-33pmol.mg^{-1}) reported by Lai et al. (1989) who also observed dual site binding of ryanodine. It is difficult, however, to make strict comparisons of data obtained from reported ryanodine binding studies since a survey of the literature reveals a diversity of findings. Ryanodine, reportedly, induces both activation and inhibition of Ca^{2+} channel function (see Meissner, 1986; Lattanzio et al., 1987). Observation of these contrary effects appears to depend upon assay conditions. In many cases, studies report the existence of a single high affinity ryanodine binding site (e.g. Imagawa et al., 1987; Mickelson et al., 1990; Pessah et al., 1987;

Chu et al., 1990). It has been shown that assays conducted in the presence of high salt (1M KCl or NaCl) result in expression of a low affinity ryanodine binding site (McGrew et al., 1989; Lai et al., 1989; Meissner et al., 1989). In the present study [^{3}H] ryanodine binding studies were conducted in the presence of 150mM KCl. This is a substantially lower and more physiological ionic strength and represents conditions under which most other laboratories report single high affinity [^{3}H] ryanodine. The basis for these differences is unclear. A curious finding was that expression of low affinity [^{3}H] ryanodine receptor sites was related to the stage of development and ryanodine receptor content in dystrophic and normal chicken pectoralis muscle in the presence of physiological salt (Pessah and Scheidt, 1990). Whether this can be taken to indicate that expression of ryanodine binding site affinity is also a function of receptor density and architecture within the membrane is unclear. However, observation of high capacity ryanodine binding at both low and high ryanodine binding sites in the present study was taken as indicative of the enrichment of this microsomal preparation in terminal cisternae membranes.

II. HSR functional characterisation

The above observations upon the structural properties of the isolated HSR membranes were consistent with the functional properties expected of terminal cisternae derived fractions. As shown in Figure 15, Ca^{2+}-stimulated ATPase activity at pH 7.0 was markedly suppressed (2.8 fold) by the presence of ruthenium red (10μM), an organic polycation blocker of the Ca^{2+} release channel in HSR membranes (Palade, 1987a). This is reflected in the 1.8-2 fold elevation in $^{45}Ca^{2+}$ accumulation at pH 7.4 (Figure 38). Conversely, the Ca^{2+} ionophore stimulated the Ca^{2+}-ATPase activity. However, A23187 also increased the apparent Ca^{2+} sensitivity of Ca^{2+}-stimulated ATPase in these membranes. The

basis for this was not experimentally pursued. This effect may reflect uncoupling of indirect Ca^{2+} channel regulation of ATPase activity. As indicated earlier, the Ca^{2+}-ATPase and the Ca^{2+} release channel are both activated within a similar concentration range of Ca^{2+} (see Gould et al., 1987). In the absence of channel modification, ATPase activity would be activated by both (a) a Ca^{2+} activated leak pathway (open channel) and (b) a Ca^{2+} activated ATPase. In the presence of A23187, Ca^{2+} activation of the major leak pathway would be diminished with expression of, solely, the Ca^{2+} activated ATPase. The absolute values reported here for ATPase activity, in the presence and absence of ruthenium red and A23187, are markedly (1.5-3 fold) lower than that reported in a comprehensive study of HSR function (Chu et al., 1986). In addition, Chu et al. (1986) observed only 1.1 to 1.45 fold A23187 stimulation of ATPase activity between 5 and 0.1mM Mg^{2+}, for junctional terminal cisternae membranes. Conversely, ruthenium red inhibited ATPase activity by only 16-24%.

It is appropriate, at this point, to comment upon the procedures for preparation of the membranes used in this study. Conventional protocols commonly employ mincing of fresh muscle in preparation for homogenisation. In this study muscle was ground to a fine, electrostatically charged powder under liquid nitrogen. Conventional homogenisation techniques were then employed utilising buffer systems and centrifugation protocols described by Chu et al. (1986). It is possible that membranes, in this case, were freeze fractured, rather than sheared. This appears to result in retention of much of the junctional protein in these membranes, which were obtained with high yield (see Table 2). In addition to the 550kDa and 57kDa proteins, these membranes also demonstrated the marked presence a ~95kDa protein (see Figures 5, 6, 29, 30), which may be referable to the junctional protein identified by Brandt et al. (1990). These membranes, therefore, appeared to be highly enriched in

membrane derived from the terminal cisternae and were used throughout the remainder of this study.

III. CANP characterisation

As discussed earlier, in order to investigate CANP effects upon HSR structure and function it was essential to obtain a purified endogenous source of this protease. Isolation of purified m- and mCANP from rabbit skeletal muscle was achieved by standard chromatographic techniques (Figures 10 to 13). The presence of an additional protein associated with each isoform (Figure 14, lanes D and D') was curious. For each isoform, the additional protein was present in stoichiometric amounts with the large subunit during gel permeation chromatography. Subsequent repeated phenyl-sepharose chromatography did not remove this protein. Both fractions were differentially sensitive to Ca^{2+}, as expected, with observed pCa_{50} values of 50mM and 750mM Ca^{2+} with casein substrate for μ- and mCANP respectively, (Figure 31A). These are consistent with similar values reported by Cong et al. (1989) for bovine skeletal muscle. However, the reported Ca^{2+} activation characteristics of both μ- and mCANP are varied depending upon the source of tissue and the species. Chicken gizzard mCANP was half maximally activated by 150μM Ca^{2+} (Hathaway et al., 1982), whereas Suzuki et al. (1981) reported a pCa_{50} of 400μM for chicken skeletal muscle mCANP. This variation was instrumental in identifying the need to purify endogenous CANP.

Significant variation in reported Ca^{2+} activation characteristics CANP purified by different laboratories using the same tissue is also evident. With bovine cardiac mCANP, Clark et al. (1986) reported half maximal activation by 300μM Ca^{2+}, whereas Tan et al. (1988) reported a value of 1mM. These differences may be due to varying degrees of autolytic activation of Ca^{2+}

sensitivity. This may depend upon the method of isolation. It is also evident that the method of assaying CANP significantly affects the estimated activity. With casein substrate, it was observed (Gilchrist and Belcastro, 1990; unpublished observations) that the estimate of CANP activity depended non-linearly upon CANP concentration. This effect was not due to substrate limitations, since increasing concentrations of casein did not increase caseinolysis. This peculiar behaviour of CANP is under current investigation (Gilchrist, Machan and Belcastro). The significance of this, to the present study, was that comparison of μ- and mCANP effects required several repeated determinations of caseinolysis. Concentrations of CANP were adjusted from a previous determination in order to obtain identical absorbance values (A_{280}) at each of 4 serial dilutions. CANP activity was then estimated from the average determination. This effect was earlier reported (Tan et al., 1988 cf Table 2), but the significance of this was ignored. The difficulty in obtaining quantitative estimates of CANP for comparative purposes was further exacerbated by the peculiar loss of CANP activity upon protease concentration, both in N_2 pressure cells and centrifugation filtration (Gilchrist and Belcastro, 1990; unpublished observations). This phenomenon was also reported by Kenessey et al. (1989) with brain mCANP. This does not appear to be due to autolytic inactivation of CANP, since the large subunits of purified μ- and mCANP fractions (see Figure 14) remained intact after concentration for gel permeation chromatography. This concentration dependent behaviour of CANP may involve hydrophobic site mediated aggregation of CANP. The distinct elution peak after phenyl-sepharose chromatography (Figure 11) was only observed with concentrated CANP preparations. With dilute CANP suspensions, CANP eluted much later and with a broad elution profile.

IV. CANP effects upon HSR structure

In preliminary studies, it was found that the effectiveness of μ- and mCANP in fragmenting HSR protein was particularly variable. Recognition of the above problem allowed a clear comparison of μ- and mCANP effects. It was shown that both isoforms of CANP fragmented the channel in an identical protease concentration dependent manner (Figures 29A and 30A). As reported earlier (Seiler et al., 1984), both the intact 550kDa protein and the 410kDa digestion product were good CANP substrates. Subtle differences were evident, however, in the fragmentation pattern. In particular 360 and 225kDa mCANP specific peptides were observed whereas mCANP digestion resulted in production of a faint 286kDa peptide. Both proteases resulted in production of high molecular weight limiting peptides at 175, 165, and 137kDa. In this regard, a two stage fragmentation pattern was evident with both proteases. The primary stage could be identified with production of 410 and 150kDa peptides from the 550kDa parent protein. The sum molecular weight of these peptides (560kDa) suggests that initial cleavage of the ryanodine receptor by CANP was initiated at a single specific site. This was particularly evident in Figure 30A (lanes H to K) where production of the 150kDa peptide coincided with appearance of the 410kDa peptide. This was also seen in Figure 28A, after endogenous proteolysis of skeletal HSR and exogenous CANP treated cardiac HSR (Figure 33).

Secondary stage fragmentation was coincident upon complete fragmentation of the intact 550kDa peptide. In the case of mCANP this stage was marked by the appearance of (a) a 225kDa fragment from presumably, the 410kDa fragment and (b) a 137kDa limiting peptide that resulted from progressive fragmentation of the 150kDa peptide through a 145kDa product. With μCANP the fragmentation of the 150kDa protein resulted in abrupt production of the 137kDa peptide. This was always observed with endogenous μCANP. With exogenous

human erythrocyte μCANP, fragmentation of the 150kDa fragment resembled the gradual pattern of endogenous mCANP proteolysis (Figure 32). This suggests that CANP species differences may determine substrate selectivity to a small degree. However, Figure 32 shows that the major cleavage products (410, 330, 150kDa) are identical between CANP species and isoforms (compare Figures 29, 30, 32). Furthermore, an identical rate and pattern of fragmentation was obtained with the CHAPS (0.1%) solubilised and vesicular ryanodine receptor. The conditions under which this was observed appear to be dependent upon the concentration of CHAPS detergent. At higher CHAPS concentrations (1-1.5%), normally employed to solubilise the channel (see Lai et al., 1989), CANP was inactivated (D. Croall; personal communication).

After the completion of the experimental work, Rardon et al. (1990) published similar observations of chicken gizzard mCANP fragmentation of the ryanodine receptor. Production of 350 and 315kDa peptides was observed after limited proteolysis with a limiting 150kDa peptide after extensive proteolysis. The high molecular weight fragments, resolved on their 5% acrylamide gels are likely equivalent to the 410 and 330kDa peptides observed in the present study using 3-13% gradient gels. Within these gels, linear log/log plots of %T (% acrylamide) versus M_r revealed that good estimates of the size of high molecular weight components could be obtained (Figure 29B). Several differences in the results of the 2 studies were, however, evident. Rardon et al. (1990) could not attribute initial limited proteolysis to a single site cleavage and did not observe further fragmentation of the 150kDa peptide. It is unclear whether these differences can be attributed to the different sources of CANP used in each study. It is interesting that the chicken gizzard mCANP employed by Rardon et al. (1990) was, earlier, reported to be half maximally activated by 150μM Ca^{2+} (Hathaway

et al., 1982). In the present study, rabbit skeletal muscle mCANP was half maximally activated by 750μM Ca^{2+} (Figure 31).

A novel contribution of this study the was demonstration of striking differences in Ca^{2+} sensitivity of μ- and mCANP mediated 550kDa fragmentation (Figures 20,30). The Ca^{2+} sensitive fragmentation for each isoform, obtained from densitometry of Coomassie-Blue stained gels, was identical to the observed with Ca^{2+} activated caseinolysis (Figure 31). The importance of this is that the Ca^{2+} stimulation of proteolysis was due to Ca^{2+} activation of CANP, rather than Ca^{2+} induced conformational changes in 550kDa substrate sensitivity to proteolysis. Belcastro, Machan and Gilchrist (1990; submitted) have shown that CANP substrate proteolytic sensitivity in myofibrils was increased by Ca^{2+} induced protein conformational changes. In addition, the present study identified a second 88kDa substrate that was cleaved in a similar Ca^{2+} and CANP concentration dependent manner to the 550kDa protein (Figures 29, 30). The identity of this HSR associating protein is unknown. However, a recent study showed a similar peptide to be phosphorylated in a Ca^{2+} /calmodulin-dependent manner (Chu et al., 1990). It is possible that this protein may be associated with the feet structures spanning the junctional gap between the junctional face membranes of the cisternae and the t-tubules. The content of this protein was quite variable between membrane preparations and could, in some preparations, be resolved as a doublet (see Figures 5, 28).

In a recent study, Kim et al. (1990) showed that the connections between the t-tubules and the terminal cisternae in "weak triads" could be broken by mCANP with an increase in the buoyant density of the HSR membranes in sucrose density gradients. The same group (Brandt et al., 1990) reported production of 270, 250, 220 and 190kDa mCANP digest products observed after immunoblotting of terminal cisternae enriched membranes with a polyclonal

antibody raised against the ryanodine receptor. The molecular weight estimates for their fragments are different from that reported here and direct comparison with the results of their study to the present is difficult. Moreover, Brandt et al. (1990) did not observe fragmentation of a ~80-90kDa protein although this may have been precluded by the resolution and reprographic quality of their gels.

The following observations led to the proposal that CANP may directly associate with HSR membranes: (a) the ryanodine receptor was a good substrate for CANP (Figures 29, 30); (b) the ryanodine receptor was partially degraded when HSR membranes were isolated in the absence of leupeptin (Seiler et al., 1984; Figure 28A; lane B); (c) CANP binds hydrophobically to plasma membranes (Murachi, 1989; Mellgren et al., 1987; Suzuki et al., 1987) and subcellular organelles (Gopalakrishna and Barsky, 1986); (d) calpastatin, an endogenous inhibitor protein of CANP (Mellgren, 1988), is localised to the SR (Mellgren, 1987); (e) prolonged incubation of HSR/t-tubule membranes in sucrose density gradients lacking leupeptin resulted in further fragmentation of the ryanodine receptor (Figure 29A, lane C). In order to test this hypothesis, the 4 major membrane fragments obtained from sucrose density gradients (Figure 34A) were immunoblotted (Figure 34B) with an antiCANP polyclonal antibody, a generous gift from Dr. T. Kuo. Immunoreactivity toward a ~90kDa protein was observed to be strongest in the light membrane fraction which was likely derived from the t-tubule/triad region. Curiously, ISR fractions (lane C) were more immunoreactive than HSR. This might indicate the presence of undissociated t-tubule/HSR membranes within this fraction. A preliminary observation, during earlier isolations of the ryanodine receptor, was that the CHAPS soluble fraction was apparently resistant to endogenous proteolysis in the absence of protease inhibitors. Conversely, the ryanodine receptor in the CHAPS insoluble pellet was highly susceptible to endogenous proteolysis. This suggested a specific

compartmentation of CANP within HSR membranes. The experiment was repeated and the hypothesis confirmed. Figure 35B (lane A) shows immunoreactivity toward a protein present in the leupeptin containing CHAPS insoluble pellet (open arrow). In the absence of protease inhibitor (lane B) the ryanodine receptor was cleaved (solid arrow) and immunoreactivity was absent. The latter was presumably due to CANP autolysis. Of interest is the enrichment of the CHAPS insoluble pellet in 165 and 88kDa protein relative to the CHAPS soluble membranes. These proteins were relatively more abundant in the light density membranes (Figure 5, lane A) which were of presumed t-tubule origin. The recent observation that CANP dissociates the "weak" triads (Kim et al., 1990) and the evident association of CANP with junctional membranes observed in this study would suggest that CANP may perform a specific role, in vivo, in targetting specific substrates. Observation of a ~90kDa CANP isoform is novel as the molecular weight of the purified cytosolic form and the cDNA encoded form of the large subunit for each CANP isoform from several different tissues were reported to be between 80,000 and 82,000 (Imajoh et al., 1988). However, Spalla et al. (1985) reported a molecular weight of 92,000 for μCANP purified from the hearts of hypertensive rats. It is possible that the immunoreactive 90kDa peptide observed in this study may be a novel isoform of CANP.

V. HSR calcium release

The earlier observations that (a) Ca^{2+}-induced Ca^{2+} release from the SR lumen required a threshold intralumenal Ca^{2+} (Ohnishi, 1979) and (b) tetanised frog and rat muscle (Gonzalez-Serratos et al., 1979; Sembrowich et al., 1983) accumulated elemental Ca^{2+} within the cisternal region of the SR in association with loss of contractile function, suggested that intralumenal Ca^{2+} was critical for the regulation of Ca^{2+} release. In the case of the latter, it was not clear

whether failure of some step in excitation contraction-coupling caused cisternal Ca^{2+} accumulation, or whether the accumulation of Ca^{2+} impaired it's release. However, the observation of reduced force production concomittant with sarcoplasmic Ca^{2+} imbalances ("Ca^{2+} overload") suggested that the alteration of SR Ca^{2+} release may also be related to Ca^{2+} activated CANP proteolysis of the release channel. In light of the suggested functional association of calsequestrin with the release channel (Meszaros and Ikemoto, 1985; Meszaros et al., 1987) it was proposed that CANP mediated structural modifications of the channel may alter intralumenal Ca^{2+} handling by, possibly, calsequestrin. CANP was, therefore, used as a physiological tool to probe the relationship between the release channel and the intralumenal Ca^{2+} compartment.

In this study, HSR vesicles were chosen as an in vitro model for the study of Ca^{2+} release since the relationship between intralumenal Ca^{2+} and Ca^{2+} release could be studied in isolation whilst retaining much of the structural and functional characteristics of the SR in vivo (see Ikemoto et al., 1989). Meissner (1984) has shown that HSR vesicles, passively loaded with Ca^{2+}, rapidly release ~75% of intralumenal Ca^{2+} within 75ms upon rapid dilution into media containing 5mM AMP-PCP and 4μM trigger Ca^{2+}. The observed Ca^{2+} efflux rate constant was $90s^{-1}$ under these conditions. In the absence of nucleotide the rate constant was reduced by almost 2 orders of magnitude (~$1.5s^{-1}$) with only a slight reduction in the amount of total Ca^{2+} released. The latter conditions were comparable to those of Figure 16 where 65-80% of vesicular Ca^{2+} contents within 15 seconds when Ca^{2+} loaded vesicles were rapidly diluted into iso-osmotic media containing 10μM free Ca^{2+}. This assay system has been highly successful in characterising the rapid kinetic properties of the Ca^{2+} release channel in both cardiac and skeletal SR (Meissner, 1986a; Meissner and Henderson, 1987). Meissner (1984, 1986a) concluded that the rate of Ca^{2+} release from HSR vesicles

was consistent with the rate observed, in vivo. In the present study, quantitation of the initial rapid kinetics of Ca^{2+} release was precluded by the manual methods employed. Figure 16A also shows that the amount of Ca^{2+} retained by the vesicles was non-linearly dependent upon the concentration of Ca^{2+} in the loading medium. Up to ~60nmol Ca^{2+}.mg HSR^{-1}, the vesicular Ca^{2+} loading was steeply dependent upon extralumenal Ca^{2+}. With elevated Ca^{2+} in the loading medium, (>2mM) vesicular Ca^{2+} retention increased more gradually. This was different from that reported earlier by Morii and Tonomura (1983) who showed that Ca^{2+} retention was proportional to the Ca^{2+} concentration in the loading buffer, up to 20mM. It should be noted, however, that their reported ~20nmol Ca^{2+}.mg SR^{-1} retained in the presence of 10mM Ca^{2+} is 5 fold lower than that reported here. The additional presence of 5mM Mg^{2+} in their loading buffer may have resulted in competition for intralumenal Ca^{2+} binding sites and thus lower Ca^{2+} retention.

In parallel with the non-linear Ca^{2+} dependence of Ca^{2+} loading, observed here, was the decrease in the fractional Ca^{2+} release expressed as a percentage of Ca^{2+} retained (Figure 16). This is also contrary to Morii and Tonomura (1983) who reported that fractional Ca^{2+} release was independent of intralumenal Ca^{2+} retention. The basis for this discrepancy is unclear. A curious observation was a concomittant increase in the amount of Ca^{2+} remaining in the vesicles, after induction of Ca^{2+} release (Figure 16A), when Ca^{2+} in the loading medium was increased. This was also unclear, although it may be related to an effect of Ca^{2+} toward vesicle aggregation. In the experiments described in Figure 28A (lane C), increasing the Ca^{2+} concentration in the sucrose gradient to 10mM led to formation of an undispersed, compact disc of all unfractionated HSR membranes (Gilchrist, Katz and Belcastro, unpublished observations). However, Meissner (1986a) has shown that with up to 62mM Ca^{2+} in the loading medium, the rates

of initial Ca^{2+} release progressively increased. This indicates that the Ca^{2+} releasing function of vesicles is not significantly impaired by high Ca^{2+} loads, both intralumenally and extralumenally.

Observation of non-linear Ca^{2+} loading in the present study would appear to indicate the presence of two intralumenal Ca^{2+} binding compartments with differing Ca^{2+} affinities. It is proposed that a higher affinity Ca^{2+} compartment of intermediate capacity is expressed at low intralumenal Ca^{2+} loading (<60nmol.mg^{-1}). Saturation of this compartment appears to be followed by filling of a lower affinity Ca^{2+} compartment. The size of the putative higher affinity compartment is consistent with its identity as calsequestrin. Although, sarcolumenin, a 160kDa glycoprotein, localised to the cisternae (Leberer et al., 1989) reportedly binds Ca^{2+} with intermediate affinity (kd=300-600μM) and high capacity (400nmol.mg^{-1}) the low content of this protein in these membranes (see Figures 5 and 6) precludes significant Ca^{2+} binding contribution from this protein. By elimination, calsequestrin would likely represent both the relatively high and low intralumenal Ca^{2+} binding compartments. This is consistent with the observation that sepharose-immobilised calsequestrin exhibits mutiple Ca^{2+} binding affinity (Charuk et al., 1990) as evidenced from non-linear scatchard analysis.

An additional method for the study of Ca^{2+} release is dual-wavelength spectroscopy of Ca^{2+} binding metalochromic dyes. This method, first developed by Ohnishi and Ebashi (1963), takes advantage of the fact that, at a sufficiently high intralumenal Ca^{2+} sink to extralumenal Ca^{2+} load ratio, SR vesicles can accumulate all extravesicular Ca^{2+} with Ca^{2+} release studied through subsequent rapid addition of various effectors to the cuvette. Scarpa et al. (1978) first showed that the metalochromic dye, Antipyrylazo III (APIII) is suitable for the study of SR function. APIII binds Ca^{2+} with a 2:1 (APIII:Ca^{2+}) stoichiometry

(Rios and Schneider, 1981) and exhibits a linear CaD_2 difference absorbance over a 10-60μM Ca^{2+} concentration range.

A disadvantage of this method is the substantial artifactual absorbance shift that is observed at the customary 720-790nm wavelength pair upon the addition of high ATP (>500μM) in the presence of low Mg^{2+} (Rubtsov and Murphy, 1988, Morii et al., 1985). Consequently, the initial phase of Ca^{2+} accumulation has been poorly resolved when SR Ca^{2+} transport is initiated by the addition of millimolar ATP. An alternate strategy that eliminates the ATP absorbance artifact at 720nm is the inclusion of excess Mg^{2+} (10mM) in assay media. This has been adopted when Murexide (Inesi and Scarpa, 1972), Arsenazo III (Herbette et al., 1981) and, Antipyrylazo III (Scarpa et al., 1978) were employed to monitor Ca^{2+} transients at the respective wavelength pairs. In view of the effects of elevated Mg^{2+} upon (a) HSR Ca^{2+} release (Meissner and Henderson, 1987) and (b) divalent cation:AP III difference spectra (Figures. 17 and 18) it was of interest to determine the general appropriateness of this strategy.

As shown in Figure 20A, the absorbance artifact was significantly reduced when vesicles were pre-incubated in the presence of 3mM Mg^{2+} prior to addition of nucleotide and was completely eliminated at 10mM total Mg^{2+} (Figure 20B). This effect is predicted by the spectral scans shown in Figures 17C and 18C. However, loss of both the secondary and tertiary phases of uptake was also observed with elevated Mg^{2+}. This may reflect Mg^{2+} inhibition of Ca^{2+} release channel opening subsequent to the initial fast phase and probably accounts for Mg^{2+} stimulation of HSR Ca^{2+} uptake reported earlier (Watras, 1985). Additionally, Ca^{2+}-induced Ca^{2+} release was less sensitive to trigger Ca^{2+} (Figure 20A). Ca^{2+} release was not triggered by 5μM free Ca^{2+} and 10μM free Ca^{2+} was required to minimally stimulate Ca^{2+} release. With 20μM free trigger Ca^{2+}, 25-30nmol $Ca^{2+}.mg^{-1}$ was released. Mg^{2+}, therefore, appears to

decrease the Ca^{2+} sensitivity of Ca^{2+}-induced Ca^{2+} release in agreement with rapid kinetic studies of $^{45}Ca^{2+}$ release from passivley loaded HSR vesicles (Meissner et al., 1986). A 60% reduction in the rate of Ca^{2+} re-uptake, after 20μM Ca^{2+} addition, was also observed when compared to the lower Mg^{2+} condition in Figure 19 where the sum of added and released Ca^{2+} was comparable (~40-50nmol $Ca^{2+}.mg^{-1}$). At 10mM Mg^{2+} (Figure 20B) the initial rate of Ca^{2+} uptake was much reduced and Ca^{2+}-induced Ca^{2+} release was completely abolished with a greatly extended re-uptake of added Ca^{2+}. These data indicate that Mg^{2+} may also inhibit Ca^{2+} channel closing subsequent to release, although the alternative possibility, that of Ca^{2+}-ATPase inhibition (see McWhirter et al., 1987), was not investigated. It was also evident that Mg^{2+} elevation decreased the sensitivity of measurement. Under the conditions defined by Figure 19, at both wavelength pairs, Δ10μM Ca^{2+} produced a ΔA of 0.0025 absorbance units. At 10mM total Mg^{2+} (Figure 20B) Δ50μM Ca^{2+} resulted in a ΔA of only 0.0024 absorbance units.

Clearly, elevation of assay Mg^{2+}, in order to eliminate ATP absorbance artifacts during dual-wavelength spectroscopy of Ca^{2+}:AP III ΔA changes, is an inappropriate general strategy for HSR Ca^{2+} transport studies. Selection of new wavelength pairs in accordance with the influence of ATP upon dye:ligand difference spectra (Figure 18) facilitates study of HSR Ca^{2+} uptake and release at relatively high levels of ATP and intracellularly appropriate concentrations of Ca^{2+} and Mg^{2+}. In addition, resolution of initial rapid uptake of Ca^{2+} is improved without loss of measurement sensitivity.

The triphasic Ca^{2+} uptake kinetic profile of HSR was first reported by Chu et al. (1983) who observed that this phenomenon was peculiar to the presence of PIPES buffer and an ATP regeneration system in Ca^{2+} transport media. Numerous studies have since confirmed that original observation using PIPES

(Morii et al., 1985; Rubtsov and Murphy, 1988; Gilchrist et al., 1990), and MOPS (Plank et al., 1988). In a detailed study, Morii et al. (1985) observed that the rate of the slow phase was decreased in the presence of effectors which stimulated Ca^{2+} release (e.g. adenine nucleotides). It was suggested that the slow phase resulted from the opening of the Ca^{2+} channel and therefore an increase in net Ca^{2+} efflux. The subsequent fast phase was attributed to channel closure involving a multi-state transition between channel conformations. The mechanism for this remains obscure. Interestingly, Morii et al. (1985) also showed that as the amount of initial Ca^{2+} was increased at constant protein concentration, the rate of the slow phase decreased. Conversely, at a fixed Ca^{2+}, increased protein concentration led to diminuition of channel opening with increased rates of slow phase Ca^{2+} accumulation. These results were also obscure and remained unexplained. In the present study, it was considered that these results reflected regulation by a saturable intralumenal Ca^{2+} compartment. If intralumenal Ca^{2+} regulated Ca^{2+} channel opening, then at a sufficiently large Ca^{2+} sink that exceeded the Ca^{2+} load, Ca^{2+} channel opening should not be observed. Furthermore, by incrementally loading the vesicles with Ca^{2+} subsequent to initial uptake it should be possible to identify the size of the intralumenal pool. This hypothesis was tested and confirmed in Figure 21.

Ca^{2+} induced Ca^{2+} release did not occur until a finite concentration of intralumenal Ca^{2+} had been obtained (Figure 21). Furthermore, the threshold was independent of whether Ca^{2+} was added prior or subsequent to Ca^{2+} uptake. Observation of triphasic kinetics at higher initial Ca^{2+} loads (traces F and G) also coincided with observation of partial stimulation of Ca^{2+} release upon accumulation of 80-90nmol Ca^{2+}.mg HSR^{-1}. It should be noted that with addition of lower (5μM) Ca^{2+} pulses (control trace, Figure 26A) partial stimulation of Ca^{2+} release prior to maximal Ca^{2+} release was not observed.

The intralumenal Ca^{2+} requirement for maximal Ca^{2+} induced Ca^{2+} release was unaffected. This suggests Ca^{2+} channel opening may be regulated interdependently by both intralumenal and extralumenal Ca^{2+}. Therefore, in this assay system, where a dynamic redistribution of Ca^{2+} occurs (i.e. as extralumenal Ca^{2+} is removed, intralumenal Ca^{2+} increases), observation of the slow phase will depend upon how Ca^{2+} is distributed between extralumenal and intralumenal Ca^{2+} pools at anytime during uptake. This, in turn, is determined by the ratio of the Ca^{2+} sink to the Ca^{2+} load. This, however, does not account for tertiary rapid uptake.

The effect of further loading of vesicles with pulse Ca^{2+}, upon Ca^{2+} induced Ca^{2+} release, was investigated. Figure 22 (trace A) shows that vesicles failed to reaccumulate all extralumenal Ca^{2+} subsequent to induction of release. Eventually, spontaneous Ca^{2+} release occurred similar to that observed in Figure 19 (phase d of the inset). The magnitude and the extent of release was variable. However, addition of further CP, in preliminary studies, showed that the released Ca^{2+} could be reaccumulated. This had been demonstrated earlier in similar studies (Watras and Katz, 1984) and indicated that ATP had been exhausted as suggested (McWhirter et al., 1987). Supplementation of uptake media with up to 20mM CP led to sustained uptake of pulsed Ca^{2+}. As indicated in Figure 23A, this also resulted in stoichiometric accumulation, at high Ca^{2+} loads, of phosphate that, presumably, was generated from CP hydrolysis. This phenomenon was associated with loss of Ca^{2+} induced Ca^{2+} release. A possible explanation for this would be that apatite formation diminished the available pool of intralumenal releasable Ca^{2+}. A similar loss of pH induced Ca^{2+} release was also observed with Ca^{2+}:oxalate loaded vesicles (Entman et al., 1978).

Earlier studies showed that elevated CP in the presence of a regenerating system inhibited spontaneous Ca^{2+} release from both light SR (longitudinal SR) and triad membranes (Palade et al., 1983). Palade et al. (1983) also reported (observations not shown) that the presence of CP, alone, inhibited Ca^{2+} release. The presence of elevated CP did not inhibit the initial maximal Ca^{2+} release in the present study, (Figure 22A traces D to F; and Figure 22B). Furthermore, Ca^{2+} release inhibition, due to accumulation of creatine, was also not indicated since release inhibition was sustained in parallel filtration studies (Figure 24) in the absence of elevated creatine or CP. Figure 23A indicated the presence of an intralumenal phosphate free Ca^{2+} compartment (~125nmol.Ca^{2+}.mg HSR^{-1}) that approximated the amount of Ca^{2+} accumulated prior to maximal Ca^{2+} release (Figure 21). This suggests that during initial Ca^{2+} uptake, Pi, generated from ATP turnover was not accessible to the intralumenal Ca^{2+} pool. This may indicate that, prior to maximal release, either (a) phosphate was membrane impermeable or (b) Ca^{2+} was bound with high affinity to presumably, calsequestrin. The former possibility appears unlikely since small amounts of phosphate (10-15nmol Pi.mg HSR^{-1}) were detected upon filters prior to release. The second possibility seems more likely, therefore. This would suggest that maximal Ca^{2+} induced Ca^{2+} release attends conformational transition of calsequestrin from a relatively high or intermediate Ca^{2+} affinity state to a state of relatively low Ca^{2+} binding affinity.

This suggestion is consistent with similar observations by Miyamoto and Kasai (1979) who observed two intralumenal low affinity Ca^{2+} binding sites with dissociation constants of $1.05x10^{-3}$M and $3.8x10^{-2}$M and maximal binding capacities of ~45 and 153nmol.Ca^{2+}.mg SR^{-1}, respectively. This approximates the ~160nmol Ca^{2+}.mg HSR^{-1} observed close to intravesicular Ca^{2+} saturation after loading in 62mM Ca^{2+} (Meissner, 1984). The higher affinity compartment

was suggested by Miyamoto and Kasai (1979) to be calsequestrin while the identity of the lower affinity compartment was obscure.

Time dependent loss of releasable Ca^{2+} (Figure 22A, trace E) might then reflect competition for Ca^{2+} between 2 Ca^{2+} pools. The first pool would be Ca^{2+}:Pi. The second pool would be Ca^{2+}:calsequestrin. In the case of Ca^{2+}:Pi, an apparent dissociation constant, assuming pH 7.0 intralumenally, of $3.018x10^{-1}M$ can be calculated for the equilibrium complex using absolute stability constants and the computational program described by Fabiato (1979). Assuming a high affinity Ca^{2+} binding site for calsequestrin during initial Ca^{2+} uptake (Kd<1mM), then Ca^{2+} will be largely associated with this protein. With decreased affinity of calsequestrin for Ca^{2+}, during Ca^{2+} release, the equilibrium would shift toward the Pi pool where-upon apatite formation occurs at a sufficiently high concentration of the Ca^{2+}:Pi equilibrium complex, as evidenced in Figure 23A. With each Ca^{2+} uptake and release cycle, less Ca^{2+} might then be available for subsequent release due to incomplete filling of the releasable Ca^{2+} pool. Unlike Ca^{2+} release from passively loaded vesicles in the present assay system, Ca^{2+} release is a net result of Ca^{2+} efflux and Ca^{2+} influx. It is not clear what proportion of the entire releasable pool in these vesicles that the ~35nmol Ca^{2+}.mg HSR^{-1}, observed in Figures 21 and 22, actually represents. In Figure 16A ~60nmol Ca^{2+}.mg HSR^{-1} was released at loading level of 100nmol Ca^{2+}. In vivo, the exchangable SR Ca^{2+} pool during muscle contraction can be calculated at ~70nmol.mg HSR^{-1}, assuming an SR volume of 10µl.mg $protein^{-1}$ and 100µl SR.ml $muscle^{-1}$ (see Endo, 1977). This is close to the ~60nmol Ca^{2+}.mg HSR^{-1} released at a loading level of 100nmol Ca^{2+}.mg HSR^{-1} in Figure 16A. However, Ca^{2+} could also be released at low Ca^{2+} loads (Figure 16), contrary to the notion of an intralumenal Ca^{2+} threshold requirment for Ca^{2+} induced Ca^{2+} release observed in Figure 21 and, in vivo (see Endo, 1977). The discrepancy between

Ca^{2+} released in passively vs actively loaded vesicles may implicate a role for the Ca^{2+}- ATPase in the regulation of Ca^{2+} release as suggested (Meszaros and Ikemoto, 1985a,b; Ikemoto et al., 1989). This has been suggested to be mediated by filling of calsequestrin with Ca^{2+} (Ikemoto et al., 1989).

In the present study, the data from Figure 21 is consistent with regulation of Ca^{2+} release by intralumenal Ca^{2+} bound to calsequestrin. Furthermore, loss of Ca^{2+} release with elevated intralumenal Pi (Figure 22A), is consistent with the converse property; that depletion of this pool inhibits Ca^{2+} release. It is appropriate to comment on results published by Nelson and Nelson (1990) after completion of the experimental results reported here. These authors showed, similarly, that Ca^{2+} induced Ca^{2+} release, in HSR vesicles, required an intralumenal Ca^{2+} threshold. Nelson and Nelson (1990) observed that increasing trigger Ca^{2+} was required to induce release, subsequent to observation of initial maximal release. Nelson and Nelson (1990) suggested that this represented the presence of a second extralumenal Ca^{2+} trigger site. This was observed with 10μM Ca^{2+} pulses as employed in Figure 21. Figure 25 shows a similar apparent loss of extralumenal Ca^{2+} sensitivity for Ca^{2+} induced Ca^{2+} release. Although this was observed with low (1μM) ryanodine this was also commonly observed in control samples (Gilchrist, Katz and Belcastro, unpublished observations). Nelson and Nelson (1990) reported, under almost identical assay conditions to Figure 22, an intralumenal Ca^{2+} threshold for ~70nmol Ca^{2+}.mg HSR^{-1} after 7x10nmol Ca^{2+} additions to elicit maximal release in a 1ml volume (therefore, 10μM Ca^{2+} pulses). Subsequent additions were made with 0.5-2μM total Ca^{2+} in the cuvette until a secondary release was observed. Nelson and Nelson (1990) suggested that this reflected the presence of a second Ca^{2+} trigger site. The notion of a second extralumenal Ca^{2+} trigger site is refuted in the present study on the basis that (a) less Ca^{2+} was added by

Nelson and Nelson (1990) to induce secondary releases (2μM) than primary releases (10μM) and (b) the loss of Ca^{2+} induced Ca^{2+} release correlates with inorganic phosphate precipitation when ATP regeneration is employed.

In light of the foregoing evidence that intralumenal Ca^{2+} regulates Ca^{2+} release, it was of interest to determine the effect of ryanodine binding to the release channel upon intralumenal Ca^{2+} loading. In accord with earlier observations (Jenden and Fairhurst, 1967; Fairhurst and Hasselbach, 1970; Fleischer et al., 1985; Meissner, 1986b; Lattanzio et al., 1987), ryanodine evidently maintained the open state of the channel with loss of Ca^{2+} uptake (Figure 25). Under the short pre-incubation conditions employed, elevated ryanodine (1mM) did not appear to result in channel closure and increased Ca^{2+} retention as observed (Feher et al., 1988) and was unaffected by prolonged pre-incubation. The hypothesis that ryanodine effects might depend upon Ca^{2+} channel states was tested in the following way. In Figure 25, the channel, prior to addition of Mg.ATP, was presumed to be open. This was deduced from the preliminary observation that vesicles isolated without a final 100mM KCl washing released ~85-90nmol Ca^{2+} .mg HSR^{-1} when added to 100mM KCl containing APIII reaction media. The source of this Ca^{2+} was presumably calsequestrin and the release was observed through a steady increase in the absorbance signal during pre-incubation.

Addition of Mg.ATP without Ca^{2+} addition produced absorbance traces almost identical to that observed in Figure 19 (Gilchrist, Katz, Belcastro; unpublished observations). In order for Ca^{2+} to be released, the channel must, therefore, have been open. Addition of ryanodine after Ca^{2+} uptake with the channel, presumably, closed, led to an increased intralumenal Ca^{2+} sensitivity of Ca^{2+} induced Ca^{2+} release with failure to reaccumulate Ca^{2+} in the presence of 5μM CP. At higher concentrations of ryanodine (2mM) spontaneous Ca^{2+}

release occurred with partial reaccumulation of Ca^{2+}. These results, as in Figure 25, suggested that ryanodine maintained or locked the channel in an open state. Figure 25 and Figure 26B also showed that supplementation of the uptake medium with CP enabled accumulation of released Ca^{2+}. Further triggering of Ca^{2+} release was attenuated, though, despite vesicle loading with Ca^{2+}. This was independent of whether CP was added prior to uptake or after release. In this instance Pi measurements of parallel filtration studies were not performed. It is unclear, therefore, whether this facilitation of uptake was due to Ca^{2+}:Pi precipitation, altough Figures 22 to 24 suggest that this is likely. The curious loss of subsequent Ca^{2+} release indicates that ryanodine also precludes replenishment of the intralumenal releasable pool of Ca^{2+}. This may reflect the mechanism by which ryanodine has been shown to deplete the intralumenal Ca^{2+} stores in arterial smooth muscle (Ashida et al., 1988; Ito et al., 1986; Iino et al., 1988) and cardiac purkinje fibres (Marban and Weir, 1985). In addition, these results may account for observation of ryanodine inhibition of Ca^{2+} release (Fabiato, 1985), Ca^{2+} oscillations (Lakatta et al., 1985) and after contractions (Sutko and Kenyon, 1983) in cardiac cells. Depletion of intracellular cardiac Ca^{2+} pools, identified as SR, was also demonstrated, in radioisotope perfusion studies of Ca^{2+} distribution, by prior administration of ryanodine (Hunter et al., 1983). The depletion of intralumenal SR Ca^{2+} pools may, therefore represent an event subsequent to ryanodine stimulation of Ca^{2+} release as suggested by Figures 25 and 26A. This effect of ryanodine, to inhibit the reaccumulation of Ca^{2+}, is not due to inhibition of the Ca^{2+}-ATPase (Besch, 1985). Thus, it appears that ryanodine inhibition of Ca^{2+} release may be due to an inability of SR to replenish intralumenal Ca^{2+} stores for subsequent release. Inhibition of Ca^{2+} release by ryanodine may therefore reflect a coupling between the functional states of the Ca^{2+} channel and calsequestrin. As indicated by Figure 26B,

ryanodine may serve to maintain calsequestrin in a low affinity Ca^{2+} binding state via maintenance of the open channel state. The interdependence between Ca^{2+} channel states and the extent of HSR Ca^{2+} preloading was clearly demonstrated in Figure 27. Ryanodine induced Ca^{2+} release was always preceded by a delay followed by a rapid (~1100nmol Ca^{2+}.mg HSR^{-1}.min^{-1}) release as observed earlier (Palade, 1987b). As shown in Figure 27, the rate of Ca^{2+} release was not detectably different at any level of Ca^{2+} loading. However, discrimination between rates of Ca^{2+} release could not be precisely determined since the rate was too fast. Conversely, the amount of Ca^{2+} release was dependent upon the extent of Ca^{2+} preloading. Furthermore, the amount of ryanodine required to induce release decreased with elevated Ca^{2+} loading.

The observation of ryanodine stimulation of Ca^{2+} release brings into question the state of the channel required for ryanodine binding. Evidently, ryanodine must bind to the channel in a presumed closed state in order for ryanodine to elicit Ca^{2+} release. Observation of ryanodine induced Ca^{2+} release from preloaded vesicles always required high concentrations of ryanodine (>125μM). On the other hand, loss of Ca^{2+} accumulation in Ca^{2+} depleted states (i.e. ryanodine addition prior to uptake) was observed at lower concentrations of ryanodine (100μM). The findings of Figures 25 to 27 are at variance with other reports, both in terms of the effect of ryanodine and the amount of ryanodine used to demonstrate these effects. In the first instance, several studies have shown that ryanodine (>300μM) closes the channel in planar bilayer studies (Nagasaki and Fleischer, 1988; Lai and Meissner, 1989) and increases Ca^{2+} uptake in vesicle studies (Jones et al., 1979; Jones and Besch, 1979; Seiler et al., 1984; Hasselbach and Migala, 1987; Feher et al., 1988). In one other study (Fairhurst, 1974), 300μM ryanodine was shown to decrease Ca^{2+} uptake by skeletal SR vesicles. However, under similar assay conditions from the same

group Fleischer et al. (1985) and McGrew et al. (1989) showed that similar concentrations of ryanodine (400nM) was without effect upon and increased, respectively, Ca^{2+} accumulation in skeletal SR vesicles. The difference between the assays was that (i) longer pre-incubation times with ryanodine in the latter report were employed and (ii) vesicles were isolated in the absence of protease inhibitors in the earlier report.

As shown by Meissner (1986b), the activatory and inhibitory effects of ryanodine were modified by the incubation times of ryanodine with HSR vesicles. Prolonged incubation (>30minutes) inhibited Ca^{2+} efflux at 100μM ryanodine whereas short incubations (<30 minutes) activated Ca^{2+} efflux at the same ryanodine concentration. In the present study, 1mM ryanodine effects (Figure 25) were independent of incubation times. The only case where inhibitory effects of ryanodine upon channel opening might be indicated is after ryanodine induced Ca^{2+} release (Figure 26A and 27) where partial re-uptake was observed. At low concentrations of ryanodine (Figure 26A) the Ca^{2+} released after Ca^{2+} triggering was not partially reaccumulated. This may reflect differential effects of ryanodine at high affinity (activatory) and low affinity (inhibitory) binding sites (Figure 42). In most cases, though, the effect of ryanodine was to either inhibit uptake in Ca^{2+} depleted vesicles or to stimulate ryanodine and Ca^{2+} induced Ca^{2+} release in actively loaded vesicles.

The results of the present study clearly demonstrate that ryanodine effects are significantly dependent upon the amount of HSR intralumenal Ca^{2+}. Below a loading level of ~60nmol Ca^{2+}.mg HSR^{-1}, addition of ryanodine did not stimulate release. Above 60nmol Ca^{2+}.mg HSR^{-1}, increasing intralumenal Ca^{2+} loading decreased the amount of ryanodine required to induce release. With the suggestion, earlier, of two classes of intralumenal Ca^{2+} binding sites, it is possible that subtle conformational changes may occur within the channel at

different levels of loading. This may occur beyond an intralumenal load of ~50-60nmol Ca^{2+}.mg HSR^{-1} with formation of a lower affinity intralumenal Ca^{2+} site that i), "primes" the channel for opening and ii), "primes" the intralumenal releasable Ca^{2+} pool for release. This remains a speculative view and must await further studies.

VI. CANP effects upon HSR Calcium release

In parallel studies to the above the functional effects of the channel selective protease, CANP (Figures 29, 30) were investigated. Prior to the recognition that the high molecular weight protein doublet visualised on SDS-PAGE gels actually represented the CANP cleaved form of the native channel, many studies had already been conducted upon the characterisation of HSR Ca^{2+} uptake and release. General agreement prevailed that HSR vesicles contained a ligand gated Ca^{2+} channel that was activated by nucleotides (Smith et al., 1985), μM Ca^{2+} (Meissner, 1984) and inhibited by Mg^{2+} (Meissner, 1984) and ruthenium red (Fleischer, 1985). Indeed, much of what is now known concerning the regulation of Ca^{2+} channel events (i.e. channel opening and Ca^{2+} release) was established from studies in which the channel was proteolytically modified by CANP. In preliminary spectroscopic studies, it was apparent that addition of leupeptin to isolation buffers "decreased" the rate at which Ca^{2+} could be accumulated (Gilchrist, Katz, and Belcastro; unpublished observations). It should be pointed out that these studies employed lower concentrations of protein (0.25 to 0.5mg.ml^{-1}) with similar concentrations of Ca^{2+} to that employed in Figure 19. At these concentrations, the slow phase of uptake (<1nmol Ca^{2+}.mg HSR^{-1}.$min.^{-1}$) was greatly extended. As discussed earlier, this behaviour reflected the dependence of channel opening upon the Ca^{2+} sink:Ca^{2+} load ratio. These studies were repeated in Figure 39A and with 0.5mg.HSR protein.ml^{-1} with

observation of faster accumulation of Ca^{2+} uptake during the secondary (slow) and tertiary (intermediate) phases. These were small changes but were always observed when the effect of protease inhibition was evaluated. With extensive exogenous protease treatment of HSR vesicles, the slow phase Ca^{2+} uptake was elevated 2-fold (Figure 39B). Consistent with the hypothesis that intralumenal Ca^{2+} was important in the regulation of channel opening, it was proposed that CANP proteolysis of the channel may modify the intralumenal Ca^{2+} compartment. This was tested in Figures 36, 37 and 38. In each case, μ- and mCANP (i) elevated passive Ca^{2+} loading and fractional Ca^{2+} release in skeletal (Figure 36 and Table 5) and cardiac HSR (Figure 37) and (ii) increased ATP dependent Ca^{2+} accumulation in skeletal HSR in the presence and absence of ruthenium red. Much of the increase in intralumenal Ca^{2+} retention was associated with limited proteolysis (ie production of 410 and 150kDa peptides). Extended proteolysis produced only minor further modification. This suggests that modified Ca^{2+} handling by HSR vesicles was associated with initial cleavage of the 550kDa peptide to 410 and 150kDa products. The associated increase in the fractional release of Ca^{2+} is interesting since Figure 16 shows that increased Ca^{2+} loading decreased the fractional release of Ca^{2+}. This suggested that CANP increased the intralumenal releasable pool of Ca^{2+}. As shown in Figures 41 and 42, extended CANP proteolysis also resulted in small increases in low and high affinity [^{3}H]-ryanodine binding. This indicated that the intralumenal Ca^{2+} sensitivity of ryanodine- and Ca^{2+}-induced release may be increased in CANP treated SR. These hypothesis were tested using the assays developed in Figures 21 and 27. Extended CANP proteolysis (a) decreased the amount of Ca^{2+} loading required for Ca^{2+} induced Ca^{2+} release and (b) increased the time for reaccumulation of released Ca^{2+} (Figure 40). These were reflected in a decrease in the amount of ryanodine required to stimulate

ryanodine induced Ca^{2+} release. These results suggest that the increased sensitivity to Ca^{2+}- and ryanodine-induced Ca^{2+} release may be related to elevated intralumenal binding of Ca^{2+}. This is a tentative conclusion, however. Although these were consistent observations it is not clear, why CANP treated vesicles passively and actively loaded more Ca^{2+} (Figures 36-38), yet required less intralumenal Ca^{2+} for the induction of Ca^{2+} release (Figure 40 and Table 7). An interesting finding is that after induction of release, the time course of Ca^{2+} reaccumulation during the slow phase was longer after CANP treatment. This suggests a more sustained channel opening and may reflect loss of channel inactivation observed by Rardon et al. (1990) using vesicles incorporated into bilayers. Rardon et al. (1990) also observed elevated (single site) ryanodine binding in CANP treated skeletal HSR. In CANP treated cardiac HSR, ryanodine binding was depressed although CANP-mediated channel inactivation was observed with both HSR types. These observed effects of CANP show similarities to and differences from the effects of trypsin and chymotrypsin upon channel function. With limited trypsin digest of skeletal HSR vesicles, increases were observed in Ca^{2+} loading, channel open time (Meissner et al., 1989) and $^{45}Ca^{2+}$ uptake (Chu et al., 1988; Shoshan-Barmatz and Zarka, 1988). Concomittant loss of high affinity ryanodine binding was observed (Meissner et al., 1989; Shoshan-Barmatz and Zarka, 1988; Chu et al., 1988) subsequent to tryptic digest. With extended trypsin digestion, Meissner et al. (1989) demonstrated loss of channel function as determined from bilayer recordings and loss of passive $^{45}Ca^{2+}$ loading. These effects of trypsin were markedly different from the effects of CANP reported in the present study and by Rardon et al. (1990). These differences appear to reflect the selectivity of cleavage sites for CANP, in contrast to trypsin. A documented feature of CANP is that it tends to cleave between functional domains rather than within (Suzuki and Ohno,

1990). In this regard, Suzuki and Ohno (1990) suggested that CANP fulfills a regulatory role in which the regulation is considered unidirectional. Rather than fulfilling a digestive role, CANP may act as a "biotransformer" (Suzuki and Ohno, 1990). The absence of obvious impairment of channel function after CANP treatment, may be consistent with this. It is unlikely, therefore, that CANP cleaves within the V8 and Achromobacter endoprotease sensitive modulator region (residues 2619-3016) of the Ca^{2+} channel (see Marks et al., 1990). A single site cleavage model, as proposed earlier, for limited CANP proteolysis, would predict cleavage at either residues ~1360 or ~3670 (assuming 110 daltons per residue and a 150kDa digest product) of the 5032-5037 residue protein (see Takeshima et al., 1989; Zorzato et al., 1990).

In a recent study, it has been suggested (Wang et al., 1989) that CANP effects may be mediated by binding to recognition sites rich in proline (P), glutamate (E), aspartate (D), serine (S), and threonine (T) residues (PEST regions). This was based upon the earlier observation (Rogers et al., 1986), that several proteins with short half-lives contain one or more PEST sequences. It was suggested that potential phosphorylation of serine and threonine residues may generate a highly charged hydrophilic loop region (Rechsteiner, 1987) that may facilitate Ca^{2+} dependent binding of CANP (Wang et al., 1990). The additional observation was that many CaM binding proteins are good CANP substrates and that many CANP substrates contain one or more PEST sequences. The fact that the release channel is a CaM binding protein (Seiler et al., 1984) suggested that a search for PEST sequences might reveal potential CANP cleavage sites. Table 8 lists the predicted PEST sequences in the channel protein obtained from the "PEST-FIND" computer program (see methods). Nine "good" PEST sequences were obtained, 2 of which were located close to residue 1360. These two regions are separated by

Table 8. PEST regions in the calcium release channel sorted by score.

Residue #		PEST	Sequence
From	To	score[a]	[ ]
1865	1894	33.08	MIEPEVFTEEEEEEEEEEEEEEEEEDEEE
2042	2061	13.63	CGIQLEGEEEEPEEETSLSS
4447	4473	8.01	PEGAGGLGDMGDTTPAEPPTPEGSPIL
3288	3310	4.76	GPEAPPPALPAGAPPPCTAVTSD
4582	4649	2.39	VSDSPPGEDDMEGSAAGDLA/../FLEESTGYMEPALWCLSLLH
4866	4885	1.56	SEDEDEPDMLCDDMMTCYLF
1355	1371	.68	EGTPGGTPQPGVEAQPV
1303	1323	.21	CTAGATPLAPPGLQPPAEDEA
4231	4294	.13	MELFVAFCEDTIFEMQIAAQ/../EGAAGAEGAAGTVAAGATAR

a. A PEST score of > 0 was indicative of a good PEST region.

~50 residues and may represent the sites of cleavage from production of the ~450 and 410kDa peptides seen clearly in Figure 2. This is a tentative proposal, though. A corollary to the apparent requirment of higher order structures for site recognition by CANP was that CANP binding and cleavage sites may be determined by tertiary structure rather than strictly primary structure (Wang et al., 1989).

VII. Contribution of this work to the existing literature.

The physiological mechanism for the triggering of Ca^{2+} release from the SR cisternae of skeletal muscle in response to t-tubule depolarisation is unknown. Two hypotheses have been forwarded to account for this triggering. The first suggests that a channel gating voltage sensor within the junctional membranes may respond to a movement of charge between the t-tubules and the SR (Schneider and Chandler, 1973). The second proposes that a flux of cytosolic Ca^{2+} may elicit SR Ca^{2+} release through direct binding to a trigger site associated with the Ca^{2+} release channel complex (see Endo, 1977; Fabiato, 1989). The source of this Ca^{2+} may be (i) L-type Ca^{2+} channels located within the t-tubules (ii), bound to specific site on the cytoplasmic leaflet of the t-tubule membrane (Frank, 1982) or (iii) derived from an initial depolarisation induced release of Ca^{2+} which elicits a further regenerative Ca^{2+} induced Ca^{2+} release from the more laterally disposed Ca^{2+} channels. (Ford and Podolsky, 1970; Nesterov, 1988). In this work, the role of intralumenal Ca^{2+} in regulating Ca^{2+} induced Ca^{2+} release has been examined. It has been shown that observation of Ca^{2+} induced Ca^{2+} release is dependent upon a critical level of intralumenal Ca^{2+} loading. This has been shown to occur under "physiologically appropriate" conditions of free Mg^{2+} (~1mM). In addition, preliminary observations have shown that the effects of the alkaloid ryanodine upon Ca^{2+} release are also

sensitively dependent upon the level of intralumenal Ca^{2+} loading. Indirect evidence has been presented that different levels of intralumenal Ca^{2+} loading may sensitize the SR Ca^{2+} channel to extralumenal Ca^{2+} effectors. Thus, as Endo (1981) now acknowledges, Ca^{2+} induced Ca^{2+} release is a real and perhaps physiologically relevant observation, albeit requiring different pre-conditions to the depolarisation induced Ca^{2+} release (see Ikemoto et al., 1989).

Little is known of the potential role of and cellular requirement for a regenerative type Ca^{2+} release that is dependent upon critical intralumenal Ca^{2+} loading of the cisternal region. It could be argued, however, that such a mechanism may confer the advantage that the release of Ca^{2+} is well coordinated with it's uptake from the cytosol. In this way, release would not occur until a finite pool of cytosolic Ca^{2+} had been redistributed back into the SR. The evidence for this is currently lacking. Taken further, such a mechanism may confer an autoregulatory character to SR Ca^{2+} release. As Williams (1990) has pointed out, the spatial distribution of Ca^{2+} pumps and channels within the cell confers a kinetic property to the regulation of intracellular Ca^{2+} handling. Rather that Ca^{2+} signalling existing as a consequence of simply the binding constant between Ca^{2+} and its site of action, Williams (1990) has suggested that the intracellular cycling of Ca^{2+} between Ca^{2+} pumps, effector sites and Ca^{2+} channels establishes a homeostatic level within which Ca^{2+} metabolism is coordinated and kinetically integrated with energy metabolism. Changes in the regulation of Ca^{2+} handling resets the level of homeostasis in the cell. In the quest to understand the mechanism of SR Ca^{2+} release it is, perhaps, somewhat overlooked that the regulation of SR Ca^{2+} handling may also be important in the control of protein expression and growth coupled to the metabolic demands of the cell. The possibility that the Ca^{2+} release channel is important in this regard is suggested from the enhanced expression of the ryanodine receptor in neonate

cardiac tissue (Michalak, 1988; see also Michalak, 1985) and in dennervated fast twitch skeletal muscle (Zorzato, et al., 1989). In this regard, Martonosi (1982) has suggested that the pattern of cytosolic Ca^{2+} transients determines the expression of SR protein, in particular the Ca^{2+}-ATPase. This may be similar to the frequency modulated Ca^{2+} signal in a variety of cells that is initiated by IP_3 and propagated by regenerative Ca^{2+} induced Ca^{2+} release (Berridge, 1990). Conceivably, the requirement of intralumenal Ca^{2+} saturation for Ca^{2+} release may represent a means by which the patterning of Ca^{2+} transients can be regulated. This is similar to the view that intralumenal Ca^{2+} in Ca^{2+} and IP_3 sensitive releasable Ca^{2+} pools, in a variety of cell types are regulated by the saturation of intralumenal Ca^{2+} compartments (Irvine, 1990). Irvine postulated a "Quantal" release of Ca^{2+} from these structures that involved the saturation of intralumenal Ca^{2+}. The quantal nature of SR Ca^{2+} release was also evident in the present study.

Within this scheme, variations in the frequency and amplitude of cytosolic Ca^{2+} transients may be tolerable within limits determined, in part, by the metabolic state of the cell. Asynchrony between Ca^{2+} and Pi metabolism may lead to apatite formation within the SR lumen. As also shown in this study, Pi accumulation by the SR either directly or, as suggested, indirectly through competition for the releasable Ca^{2+} pool, leads to inhibition of Ca^{2+} release. This may represent part of the mechanism of muscle fatigue after intense muscle contraction, consistent with earlier observations (Gonzalez Serratos et al., 1978; Somylo et al., 1978, 1981). Within skeletal muscle, such an event may actually constitute a safety device when muscle activity approaches the limits of muscle capabilities. The question then is what are those limits, how are they set and can they be changed? Nesterov (1985) has argued that the morphofunctional characteristics of muscle across a wide variety of species are well correlated to

the use of either sodium or Ca^{2+} electrochemical gradients across plasma membranes, in triggering muscle contraction. It was suggested that reduction of functional activity leads to a decline in the dependence upon transsarcolemmal Na^{+} and an increased dependence upon Ca^{2+} gradients. In devervated muscle, a similar transformation may occur with increased synthesis of a different isoform (perhaps cardiac) of the channel as tentatively suggested by Zorzato et al. (1989). These would be long term adaptations and presumably would evoke a different pattern of intracellular Ca^{2+} signalling. It seems likely that the molecular defect in the malignant hyperthermia Ca^{2+} release channel accounts for the hypertrophied state of skeletal muscle in animal models (Pietrain pigs) possessing the defect. This hypertrophy, possibly, results from the alteration of Ca^{2+} signalling from a channel that is evidently more sensitive to Ca^{2+}-induced Ca^{2+} release (Nelson, 1983, 1984; Mickelson et al., 1985, 1986, 1988; O'Brien, 1990). In the short term, could the patterning of Ca^{2+} signals be modified within the same structures? Data from the present study suggests that the effect of CANP may increase the sensitivity of the Ca^{2+} release channel to Ca^{2+} triggered release. Within this scheme, activation of CANP in response to a variety of stressors may initiate limited proteolysis of the channel and possibly, sever structural connections within the "weak triads" (Brandt et al., 1990). This may lead to loss of electrogenic Ca^{2+} release and increased regenerative Ca^{2+} release. It should be stressed that these are tentative suggestions. It is not known whether, in vivo, Ca^{2+} channels may adapt functionally in response to CANP or whether, as suggested by Rardon et al. (1990), Ca^{2+} channels are disfunctional and fail to inactivate. The apparent localisation of CANP to the t-tubule/SR junction and the selective cleavage of the release channel, as indicated in the present study, would support the hypothesis that CANP may play an important

role in modification of excitation-contraction coupling during pathophysiological states.

VIII. Summary.

The purpose of this study was to investigate the relationship between intralumenal Ca^{2+} loading by HSR vesicles and Ca^{2+} release. It was anticipated that structural and pharmacological intervention of Ca^{2+} channel operation would provide insight into the interaction between Ca^{2+} channel states and Ca^{2+} loaded states of calsequestrin. Heavy sarcoplasmic reticulum (HSR) membranes were purified from rabbit fast twitch skeletal muscle and canine hearts. Structural characterisation of these membranes revealed an abundance of 565kDa ryanodine receptor and 57kDa calsequestrin. The enrichment of both proteins indicated that these membrane preparations were derived from the terminal cisternae.

Skeletal HSR membranes bound ryanodine with high capacity at both low and high affinity sites. Ca^{2+} transport and Ca^{2+}-ATPase activities were activated and inhibited, respectively in the presence of the Ca^{2+} channel blocker ruthenium red. In active Ca^{2+} transport assays skeletal HSR membranes released 35-40% of their intralumenal Ca^{2+} contents in response to the application of extralumenal trigger Ca^{2+}. A dual-wavelength spectroscopic method was developed that permitted the study of initial ATP dependent Ca^{2+} uptake by HSR vesicles without optical artifacts arising from the addition of nucleotide. This technique permitted accurate estimate of the initial rate of HSR Ca^{2+} uptake as a function of the initial Ca^{2+} load. It was shown that the appearance of multiphasic Ca^{2+} uptake kinetics at elevated extralumenal Ca^{2+} loads depended upon the relative distribution of Ca^{2+} between intralumenal

Ca^{2+} binding sites and the extralumenal space. These studies also showed that HSR vesicles required a threshold filling of intralumenal Ca^{2+} for observation of Ca^{2+} induced Ca^{2+} release (CICR). The plant alkaloid ryanodine was then employed to investigate the dependence of intralumenal Ca^{2+} regulation of Ca^{2+} release upon Ca^{2+} channel states. It was demonstrated that ryanodine decreased the intralumenal Ca^{2+} threshold requirement for Ca^{2+} induced Ca^{2+} release. In addition, ryanodine induced Ca^{2+} release was shown to be dependent upon the intralumenal Ca^{2+} load. These studies suggested that state transitions between the ryanodine receptor and calsequestrin were functionally linked and reciprocally interdependent.

The effects of specific structural modification of the release channel by Ca^{2+} activated neutral protease (CANP) upon HSR Ca^{2+} handling was then investigated. Isoforms of CANP requiring micromolar (μCANP) and millimolar (mCANP) concentrations of activating Ca^{2+} were isolated from the cytosolic fractions of rabbit skeletal muscle homogenates. These CANP isoforms selectively cleaved the purified and vesicular form of the 565kDa Ca^{2+} release channel (the major substrate) into 410 and 150kDa fragments during limited proteolysis. Evidence suggested that CANP proteolysis was initiated with single site cleavage. Extended proteolysis resulted in secondary fragmentation of the initial peptide products with formation of a 135kDa limiting peptide. Primary structural analysis of the Ca^{2+} release channel revealed the presence of several regions that were rich in proline (P), glutamate (E), aspartate (D), threonine (T), and serine (S) (PEST) residues. PEST sequences have been suggested to act as substrate recognition sites for CANP proteolysis (Wang et al., 1989). Two PEST rich sequences 1300 to 1350 amino acids down from the N-terminal which were tentatively assigned as possible initial CANP cleavage sites.

Extensive CANP mediated fragmentation of the 565kDa peptide resulted in subtle changes of HSR functional properties. Ryanodine binding at both low and high affinity binding sites was elevated after proteolysis. CANP treated membranes demonstrated elevated intralumenal Ca^{2+} binding and fractional Ca^{2+} release after passive Ca^{2+} loading. In addition, CANP proteolysis increased the intralumenal Ca^{2+} sensitivity of actively Ca^{2+} loaded vesicles to Ca^{2+}-induced and ryanodine induced Ca^{2+} release. These studies suggest that CANP cleaves close to a domain on the Ca^{2+} channel that is important in the regulation of intralumenal HSR Ca^{2+} handling.

The potential pathophysiological importance of the action of CANP upon HSR membranes and Ca^{2+} channel operation was then studied through attempts to identify a structural association of CANP with HSR membranes. Immunolocalisation studies revealed that CANP co-purified with HSR membranes and was specifically associated with a CHAPS insoluble HSR membrane fraction. Greater immunoreactivity was, however, observed with light membrane fractions referable to the t-tubules. These studies indicated that CANP may be localised to the t-tubule/cisternal region of skeletal muscle in close association with the Ca^{2+} release channel.

The function of CANP, in vivo, remains unclear. In the present study CANP was shown to elicit subtle changes in Ca^{2+} handling by HSR membranes, in vitro. The observed functional effects of CANP were discussed in relation to a potential role of regenerative HSR Ca^{2+} release in mediating Ca^{2+} signalling during adaptation. One potential advantage of regenerative Ca^{2+} release is that it may confer an autoregulatory property to the coordination of HSR Ca^{2+} uptake and release. Given a finite intracellular pool of Ca^{2+} that is cyclically exchanged between the cytosol and the SR lumen it is conceivable that the Ca^{2+} release pool must be replenished before the next release event is triggered. It

was proposed that such a mechanism may also govern the patterning of cytosolic Ca^{2+} transients which appear to be an important aspect of gene expression. The evidently subtle changes in the Ca^{2+} handling brought about by CANP may adjust the patterning of intracellular Ca^{2+} signals without significant impairment of the Ca^{2+} release mechanism. It was suggested that CANP may sever structural connections between "weak triads" in order to establish a greater dependence upon regenerative rather than electrogenic triggering of Ca^{2+} release. Such a mechanism may constitute part of the process by which muscle cells respond to both acute and chronic changes in contractile behaviour.

IX. Conclusions.

1. Purified heavy sarcoplasmic reticulum membranes were isolated from rabbit fast twitch skeletal muscle and canine hearts. In both cardiac and skeletal SR, membranes were characterised by the presence of structural components (ryanodine receptor and calsequestrin) which indicated that these preparations were enriched in membranes derived from the terminal cisternae.

2. Functional characterisation of skeletal HSR showed that the Ca^{2+} release channel structure bound ryanodine with high capacity at both low and high affinity sites. Ca^{2+} transport and Ca^{2+}-ATPase activities were activated and inhibited, respectively in the presence of the Ca^{2+} channel blocker ruthenium red.

3. Ca^{2+} loading studies in skeletal HSR vesicles indicated the presence of 2 intralumenal Ca^{2+} binding sites with apparently different binding affinities.

4. A spectroscopic method for the assay of Ca^{2+} transport was developed that allowed for the improved resolution of the initial rapid phase of HSR Ca^{2+} uptake in the presence of physiologically appropriate concentrations of Mg^{2+}.

5. The above spectroscopic techniques demonstrated (i), that HSR vesicles required a threshold filling of intralumenal Ca^{2+} for observation of Ca^{2+} induced Ca^{2+} release (ii), that ryanodine decreased the intralumenal Ca^{2+} threshold requirment for Ca^{2+} induced Ca^{2+} release and (iii) ryanodine induced Ca^{2+} release was shown to be dependent upon the intralumenal Ca^{2+} load.

6. Isoforms of calpain (CANP) requiring micromolar (μCANP) and millimolar (mCANP) concentrations of Ca^{2+} were isolated from the cytosolic fractions of rabbit skeletal muscle homogenates.

7. These CANP isoforms were found to selectively cleave the purified and vesicular form of the 550kDa Ca^{2+} release channel into 410 and 150kDa fragments during limited proteolysis.

8. A novel 88kDa CANP substrate was identified in HSR membranes and was cleaved in a Ca^{2+} and protease dependent manner similar to the 550kDa Ca^{2+} release channel.

9. Proteolysis of the release channel i) increased intralumenal Ca^{2+} loading and fractional Ca^{2+} release in passively loaded cardiac and skeletal HSR, ii) increased the sensitivity of actively loaded skeletal HSR to Ca^{2+}-induced and ryanodine induced Ca^{2+} release.

10. Immunolocalisation studies indicated that CANP may be localised to the t-tubule/cisternal region of skeletal muscle.

11. Analysis of the primary structural regions of the Ca^{2+} release channel that were rich in proline (P), glutamate (E), aspartate (D), threonine (T), and serine (S) (PEST) residues, indicated the presence of 2 PEST rich sequences 1300 to 1350 amino acids down from the N-terminal which were tentatively assigned as possible CANP cleavage sites.

12. The regulation of HSR Ca^{2+} induced Ca^{2+} release by intralumenal Ca^{2+} and its modification by CANP was discussed in relation to a potential role of regenerative HSR Ca^{2+} release in mediating Ca^{2+} signalling during adaptation.

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