EXCITATION-CONTRACTION COUPLING AND CARDIAC CONTRACTILE FORCE

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EXCITATION-CONTRACTION COUPLING AND CARDIAC CONTRACTILE FORCE

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This book is dedicated to Kathy, Brian & Becky

PREFACE

The main aim of this monograph is to provide an overview of calcium regulation in cardiac muscle cells, particularly with respect to excitation-contraction coupling and the control of cardiac contractile force. It is my hope that this book will be useful to students of the cardiovascular system and muscle at all different levels and in different disciplines (such as physiology, biochemistry, pharmacology and pathophysiology). I also hope that it will find use for those studying developmental, comparative and disease processes as well as more integrative phenomenon. I kept several goals in mind in writing this monograph. First, it should be easily readable. Second, I chose to include numerous illustrations and tables to help integrate results from numerous investigators in practical formats and also present key figures from important papers. Thus, this monograph may serve as a resource of information for people working in the areas described herein. Third, the presentation is a very personal one, and I have necessarily drawn extensively on my personal experience in this field over the past 15 years. This, I think, helps maintain a certain continuity of thought from chapter to chapter. Fourth, I have made serious attempts to make each chapter "up to date", despite the breadth of topics covered. I have also tried to be equitable in choosing references while not intending to be comprehensive or exhaustive. Neither of these aims can be perfectly matched, and I apologize to the many investigators whose papers I have not cited, but should have.

While I thank all of my colleagues who make this a stimulating area in which to work, I would especially like to thank those who contributed by helpful discussions, providing original figures, sending preprints of manuscripts, and by commenting on drafts of individual chapters. These individuals include: S. Baudet, B.P. Bean, J.R. Berlin, J.H.B. Bridge, A. Fabiato, S. Fleischer, J.S. Frank, C. Franzini-Armstrong, M.M. Hosey, L.V. Hryshko, N. Ikemoto, L.R. Jones, W.J. Lederer, D.H. MacLennan, G. Meissner, M. Morad, K.D. Philipson, J.D. Potter, E. Ríos, R.J. Solaro, J.R. Sommer, J.G. Tidball, J. McD. Tormey, W.G. Wier, A. Williams, D.T. Yue.

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Finally, a very special thanks are due to my wife, Kathryn E. Bers, whose combination of patience and assistance have made this book possible.

Donald M. Bers

January 1991

INTRODUCTION

How is the heartbeat generated? What controls the strength of contraction of heart muscle? What are the links between cardiac structure and function? How does our understanding of movement in skeletal and smooth muscle and in non-muscle cells influence our thinking about the development of force in heart muscle? Are there important species differences in how contraction is regulated in the heart? While these important questions have been asked many times, exciting results in many areas of mammalian biology have set the stage for this refreshing new book on *Excitation-Contraction Coupling and Cardiac Contractile Force*. This informative and quantitative book always remains readable. Don Bers explains how contraction arises in heart and how it is controlled. Furthermore, he presents insightful and stimulating discussions of apparently disparate results that will inform and delight both students and "experts". In many ways, Don paints a modern "portrait" of how the heart works and in this picture he shows a close-up of the structural, chemical and physiological links between excitation and contraction.

The recent molecular investigations of excitation-contraction coupling in skeletal and heart muscle have brought together cell physiologists, molecular biologists and physicians in numerous research projects that form the background for this book. These new investigations have led to the explosion of information that would challenge the individual who only seeks to read the primary sources. Don simplifies our task by bringing much of this material together in a single coherent presentation. Exciting questions abound and this book introduces and/or lays the foundation for many of them. Some are stated explicitly by Don while others depend on Don's presentation and the reader's background. For example, the five questions below are among the ones that jump out at me. (1) In heart and skeletal muscle cells, the sarcolemma (SL) has been reported to have many more dihydropyridine receptors than functional calcium channels. Is this apparent excess real or an artifact? If it is real, what does this excess mean? (2) Another question also centers on the dihydropyridine receptor (DHP-R) which is the L-type calcium channel in heart and skeletal muscle. Recent cDNA sequence information along with investigations of structure and function using DHP-R chimeras from heart and skeletal muscle have suggested that a specific cytoplasmic domain or loop of the DHP-R can confer important properties on this receptor/channel. With the skeletal muscle loop in place, E-C coupling in skeletal muscle is "normal" (SL voltage-sensor-dependent calcium release) but when the cardiac loop is in place, the E-C coupling resembles that normally seen in heart (calcium-induced calcium-release). This raises the question of how this particular cytoplasmic loop normally interacts with the SR calcium release channel (i.e. ryanodine

receptor) in skeletal muscle. In heart muscle the question is whether there is any interaction at all between the cytoplasmic loop of the DHP-R and the SR calcium release channel. Furthermore, I must wonder if this interaction (if any exists) changes during calcium overload or during maneuvers that change the inotropic state of the heart muscle cell. (3) While calcium-induced calcium release (CICR) appears to be the dominant factor in explaining the link between excitation and contraction in heart muscle, a question lurks just below the surface. How is CICR modulated? What is the relationship between Ca influx and Ca release from the SR? It would appear that all elements of CICR can be modified by intracellular calcium, drugs and neurohormones -- including calcium channels, Na-Ca exchangers, Ca-ATPases, and calcium release channels. Furthermore the release process also seems subject to modulation by intra-SR calcium and may also involve calsequestrin. (4) Do the T-tubular membrane and the non-invaginated SL membrane participate in a similar manner in E-C coupling? Do they have similar densities of DHP-Rs? Do they possess the same CICR elements? (5) How can one make use of our knowledge of E-C coupling and Cellular Ca regulation to develop improved inotropic agents? Many questions are raised by the book, and each reader will undoubtedly focus on different ones. Although Don does not answer or directly address all of our questions, he provides an improved vantage point for us to view the issues important to each of us and to the field in general.

In his portrait of E-C coupling in heart, Don Bers presents many new findings commingled with "established" truth and paints a new picture of how contraction arises and is controlled in the heart. The picture is sharper and contains many new details. He assembles important measurements in new and useful tables, presents figures from recent and more classical publications and shows new figures to supplement his presentation. While integrating new observations with traditional "facts", Don is able to retain both the excitement of discovery and the inevitable controversy arising when important questions cannot be fully answered. This book, written by an active research scientist, therefore provides a critical state-of-the-art report on how the heart works as an electrically and chemically regulated contractile machine.

W.J. Lederer

Baltimore, Maryland

January 1991

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CHAPTER 1

MAJOR CELLULAR STRUCTURES INVOLVED IN EXCITATION-CONTRACTION COUPLING

Numerous cellular structures are involved with the process of excitation-contraction coupling (E-C coupling) in cardiac muscle cells. This chapter serves to introduce some of these components from an ultrastructural perspective and each general component will be discussed in greater detail in subsequent chapters.

Figures 1 and 2 are schematic drawings of the structure of amphibian skeletal muscle (Fig. 1) and mammalian ventricular muscle (Fig. 2) from a classic ultrastructural study by Fawcett and McNutt (1969; also based on the work of Peachey, 1965).

Despite differences between skeletal and cardiac muscle which will be discussed below, the general scheme of E-C coupling is similar. Electrical excitation of the surface membrane leads to an action potential which propagates as a wave of depolarization along the surface and along the transverse tubules (T-tubule). The depolarization of the T-tubule (or sarcolemma) overlying the terminal cisternae (or subsarcolemmal cisternae) of the sarcoplasmic reticulum (SR) induces the release of Ca from the SR. The details of how this sarcolemmal depolarization is able to induce SR Ca release will be the subject of much ensuing discussion. The Ca released from the SR then binds to the Ca-binding subunit of the thin filament protein troponin which serves to activate contraction. The situation *vis a vis* cellular Ca movements is also complicated (especially in heart muscle) by the presence of Ca channels and transport systems in the sarcolemmal membrane which may transport substantial quantities of Ca and may play an important role in mediating or modulating Ca release from the SR.

There is a recurring theme that skeletal muscle contraction depends critically and almost exclusively on Ca released from the SR with quantitatively insignificant Ca entry across the sarcolemma during a normal twitch. Cardiac muscle contraction, on the other hand, depends on both Ca entry across the sarcolemma as well as Ca release from the SR (and the relative importance may vary, e.g. Bers, 1985). While these conclusions are based mainly on physiological experiments to be discussed later, it is worth pointing out that ultrastructural differences (Figs. 1 and 2) also seem to be consistent with this conclusion. That is, skeletal muscle has a very extensive and well organized SR network with large capacious terminal cisternae abutting the narrow T- tubules. In contrast, cardiac muscle

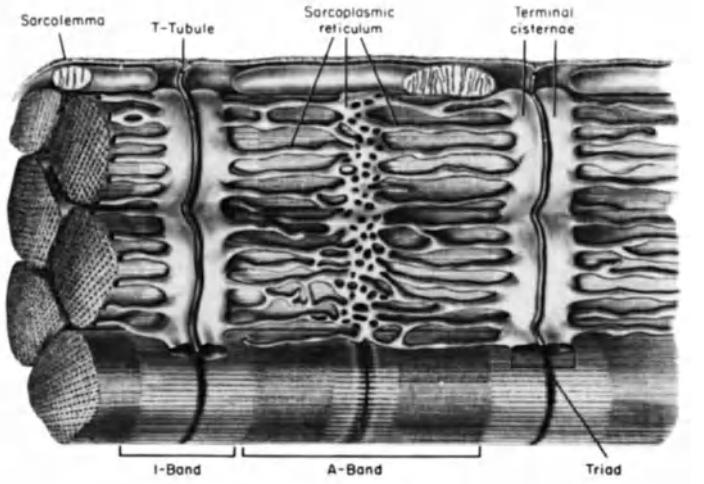


Figure 1. Schematic diagram of T-tubules and SR associated with several myofibrils in frog skeletal muscle. Each myofibril is surrounded by the meshwork of SR. The SR is greatly enlarged at the terminal cisternae where it comes into close contact from both sides with the relatively narrow T-tubule forming the *triad* at the Z-line. In mammalian skeletal muscle, T-tubules and triads are normally at the A-I band junction. (From Fawcett & McNutt, 1969, by copyright permission of the Rockefeller University Press).

typically has a more sparse and poorly organized SR system with smaller saccular enlargements at the cell surface and at junctions with the much larger diameter T-tubules (200 vs. 30-40 nm). In addition, cardiac myocytes are typically less than 20 μm thick whereas the diameter of skeletal muscle fibers is usually many times larger (e.g. 100 μm). The smaller diameter of heart cells would make diffusion from the extracellular space to the myocyte interior (and T-tubule matrix) more plausible with respect to diffusional limitations than would be the case for skeletal muscle. Thus, the structure of the cardiac myocyte is consistent with a more important, quantitative role of transsarcolemmal Ca fluxes.

SARCOLEMMA AND TRANSVERSE TUBULES

The surface sarcolemma is physically continuous with the membrane of the T-tubule and as such the two combine to form the permeability barrier between the inside of the cell and the extracellular medium. Thus from this functional perspective it is suitable to refer to the complex simply as "the sarcolemma". As a point of semantic distinction it seems reasonable to use the terms "surface sarcolemma" or "external sarcolemma" if one means to exclude the T- tubule when referring to sarcolemma.

The ultrastructural organization of the cardiac sarcolemma is important for several reasons. First, it is the site at which Ca enters (and leaves) the cell, so the localization of

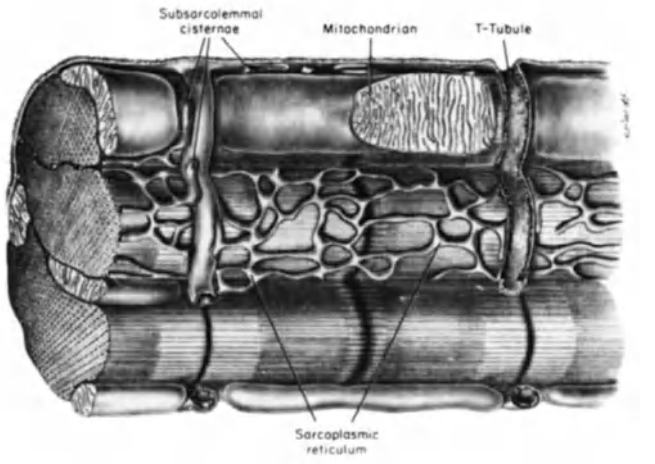


Figure 2. Schematic diagram of T-tubules and SR associated with the myofilaments in mammalian cardiac muscle. Compared to skeletal muscle, cardiac T-tubules are of much larger diameter and the SR is more sparse, lacking the dominant terminal cisternae, but includes junctional couplings with the external sarcolemma as well as the T-tubules. Mitochondria are plentiful and myofibrils are also more irregular in heart. (From Fawcett & McNutt, 1969, by copyright permission of the Rockefeller University Press).

the relevant transport systems is of functional importance. This is particularly the case because there may be differential distribution of ion channels, pumps, or other membrane characteristics. For example, Almers and Stirling (1984) point out that the density of Na channels (Jaimovitch *et al.*, 1976), delayed rectifier K channels (Kirsch *et al.*, 1977) and (Na + K) ATPase pump sites ((Venosa & Horowicz, 1981) are considerably lower in skeletal muscle T-tubules than on the surface sarcolemma, while the density of Ca channels is more than 4 times higher in T-tubules (Almers. *et al.*, 1981). Indeed, vesicles isolated from skeletal muscle T-tubules have extremely high densities of specific dihydropyridine binding sites indicative of Ca channels. Jorgensen *et al.* (1989) immunolocalized skeletal muscle dihydropyridine receptors in clusters, primarily in T-tubules. Brandt (1985) separated SR-associated sarcolemmal vesicles from rabbit heart which were presumed to represent a T-tubular fraction. This fraction demonstrated a high density of dihydropyridine binding sites (vs. muscarinic receptors) when compared to sarcolemma which was not associated with SR (i.e. presumed to be surface sarcolemma). However, Doyle *et al.*, (1986) found a lower density of dihydropyridine receptors (vs. saxitoxin receptors) in putative T-tubular fractions than in surface sarcolemma fractions from sheep or bovine hearts. The E-C coupling sites where the SR comes into close contact with the sarcolemma are also exclusively (in skeletal muscle) or predominantly (in mammalian cardiac muscle) in the T-tubules (Page & Surdyk-Droske, 1979).

I have compiled some quantitative ultrastructural data of surface areas of sarcolemma and SR components in several cardiac and skeletal muscle preparations (Table 1). Peachey (1965) reported that in frog skeletal muscle the T-tubular area is about 7 times the area of the external sarcolemma. In mammalian skeletal muscle the T-tubular area is somewhat less dominant, but still relatively large. There also seems to be a difference between fast (Vastus) and slow (Soleus) muscle which may have a functional correlate (Eisenberg, 1983). In mammalian ventricle about 30-50% of the sarcolemmal area is in the T-tubules and in mammalian atrium this fraction is < 15%. Bird, amphibian and reptilian hearts apparently lack T-tubules entirely. The ratio of external sarcolemma/cell volume is also inversely related to the cell diameter (i.e. 2/radius if one assumes a cylindrical shape). On this basis (as well as direct observations) the cell diameters in frog and lizard hearts and mammalian atria are smaller than those of mammalian ventricular muscle. For these smaller cells, T-tubules are less important for inward spread of activation (or Ca diffusion) than in mammalian ventricle or the even larger diameter cells in skeletal muscle. Cardiac Purkinje fibers are cells which are specialized for electrical conduction and the large cross-sectional area decreases the longitudinal internal resistivity and hence increases the rate of propagation of electrical impulses.

A major structural specialization of the sarcolemma is couplings with the SR (e.g. triads and diads). Figure 1 suggests that in skeletal muscle, most of the T-tubular membrane appears to be involved with junctional, triadic complexes with the SR (Peachey, 1965). The triad refers to the coupling of two SR terminal cisternae to either side of a T-tubule. In cardiac muscle these junctions are more apparent as diads and can occur either at the surface sarcolemma or with T-tubular membrane. In mammalian ventricular muscle Page and Surdyk-Droske (1979) found that 4-8% of the external sarcolemma is involved in junctional complexes whereas 20-50% of the T-tubular membrane is so involved. They also reported that rabbit ventricle had a smaller sarcolemmal fraction involved in SR junctions (4.6% of surface and 20.6% of T-tubule) compared to rat or mouse ventricle (6.5-7.7% of surface and 40-48% of T-tubule). This structural observation may be related to physiological results which have suggested that the contraction in rat ventricle depends on SR Ca more strongly than does the contraction in rabbit ventricle or frog ventricle (in which junctional couplings are also sparse and restricted to the external sarcolemma) (Fabiato and Fabiato, 1978; Bers *et al.*, 1981; Fabiato, 1982; Bers 1985 and see chapter 8). That is, rabbit and frog (but not rat) ventricular muscle twitch contractions can be supported largely by Ca entry from the extracellular space.

The sarcolemma also exhibits small pockets or caveolae, which are flask-shaped invaginations (50-80 nm in diameter) and contribute significantly (~10%) to the surface area of both surface and T-tubular sarcolemma (Levin & Page, 1980). It is not clear what function these caveolae serve, though it seems clear that they are neither pinocytotic nor reservoirs of membrane for recruitment during physical stress (Sommer & Johnson, 1979; Langer *et al.*, 1982).

TABLE 1

Cellular Elements: Surface Area/Cell Volume ($\mu m^2/\mu m^3$)

	SL					SR		
	Ext. SL	TT	% of SL in TT	Junct Ext SL	Junct TT	Junct SR	Free SR	Tot SR
Finch V[a]	0.56	0	0	-	-	0.18	0.57	0.75
Mouse V[a]	0.28	0.22	44%	-	-	0.20	0.64	0.84
Mouse V[b]	0.324	0.34	51%	-	-	0.22	0.65	0.84
Mouse LA[b]	0.65	0.10	13%	-	-	0.09	1.48	1.57
Mouse RA[b]	0.62	0.08	11%	-	-	0.06	1.63	1.69
GP A[c]	-	0.014	-	-	-	0.08†	2.21	2.29
GP V[c]	-	0.42	-	-	-	0.13†	1.83	1.96
Rat V[d]	0.27	0.07	21%	0.038	-	0.16	1.3	1.46
Rat V[e]	0.31	0.15	33%	0.023	0.069	0.19	1.0	1.19
Rabbit V[e]	0.33	0.23	41%	0.010	0.042	-	-	-
						Junct SL Tot	% of SL as diad Ext SL	% of SL as diad TT
Rabbit V[f]	0.35	0.25	42%	0.016	0.051	0.068	4.6%	21%
Rat V[f]	0.31	0.15	33%	0.023	0.069	0.093	7.7%	48%
Mouse V[f]	0.31	0.17	37%	0.020	0.070	0.090	6.5%	40%
Frog V[g]	1.19	0	0	-	-	0.014	0.26	0.27
Frog A[g]	1.32	0	0	-	-	0.018	0.44	0.46
Lizard V[g]	1.14	0	0	-	-	0.045	0.46	0.50
Lizard A[g]	1.25	0	0	-	-	0.056	0.89	0.94
Frog Sartorius[h]	0.04	0.28	88%	-	-	1.4	4	5.4
Frog Sartorius[i]	0.064	0.22	77%	-	-	0.54	1.5	2.0
GP soleus[j]	0.116	0.064	36%	-	-	0.24	0.73	0.97
GP white vastus[k]	0.097	0.146	60%	-	-	0.41	0.91	1.32
GP red vastus[l]	0.097	0.148	60%	-	-	0.33	0.65	0.98
Sheep Purkinje[m]	0.037	0.423*	92%*	-	-	-	-	-
Dog Purkinje[n]	0.096	0.041*	30%*	-	-	-	-	-

[a]Bossen *et al.*, 1978; Sommer & Johnson, 1979
[b]Bossen *et al.*, 1981
[c]Forbes & Van Niel, 1988
[d]Page *et al.*, 1971
[e]Page, 1978
[f]Page & Surdyk-Droske, 1979
[g]Bossen & Sommer, 1984
[h]Peachy, 1965
[i]Mobley & Eisenberg, 1975
[j]Eisenberg *et al.*, 1974
[k]Eisenberg & Kuda, 1975
[l]Eisenberg & Kuda, 1976
[m]Mobley & Page, 1972
[n]Eisenberg & Cohen, 1983

Ventricle (V), Atrium (A), Guinea Pig (GP), External sarcolemma (Ext SL), T-tubule (TT), Junctional (Junct), Total (Tot). Mammalian skeletal muscle can be classified into slow (soleus), fast/glycolytic (white vastus) and fast/oxidative/glycolytic (red vastus). *Intercellular clefts or folds for Purkinje fibers, which lack TT. † Excluding corbular SR.

The other major specialization of the sarcolemma is the region where cells are closely apposed end to end and known as the intercalated disk. The ends of cardiac muscle cells generally interdigitate (Fig. 3) and the classic work Sjöstrand *et al.*, (1958) described three differentiations in this region which are known as 1) the nexus or gap junction, 2) fascia adherens or intermediate junction and 3) macula adherens or desmosome. The fascia and macula adherens appear to be of central importance in the mechanical connection of one cell to the next and the macula adherens is sometimes likened to a spot-weld between cells. Intermediate filaments also appear to insert at the intercalated disk (Price and Sanger, 1983) and this emphasizes the potential mechanical function of this cytoskeletal structure.

The gap junctions are predominantly located on the regions of the intercalated disk which parallel the long axis of the cell (see recent reviews by Spray & Burt, 1990 and Severs, 1990). Page and Shibata (1981) estimated that gap junctions make up only 0.7 - 1% of the total sarcolemma area in rat and rabbit ventricle (~ 3% in dog Purkinje Strand, Eisenberg & Cohen, 1983). Chen *et al.*, (1989), however, demonstrate that these values in rat ventricle may underestimate the gap junctional area by 3-4 fold (based on comparison of classical cross sections vs. *en face* sections of the transverse cell borders). It is now clear that these junctions serve as the low resistance electrical pathways that allow the heart to function as an electrical syncytium. Revel and Karnovsky (1967) first demonstrated that the membranes of the two cells did not fuse, but were separated by a narrow 2 nm gap at the nexus and also observed hexagonal arrays, now known to be the functional unit. A general working model of the gap junction has evolved (e.g. Unwin & Zampighi, 1977; Makowski *et al.*, 1977) in which one hexameric unit with a central pore (or connexon) from each cell meet within the gap and form a pore which allows direct connection between the cytoplasms of the two cells. The diameter of the pore has been estimated to be 1.6-2.0 nm by the cell-to-cell diffusion of fluorescent tracers (Flagg-Newton *et al.*, 1979). The molecular weight of the monomers which make up the connexon differ in liver (mw ~ 28,000, Henderson *et al.*, 1979; Hertzberg and Gilula, 1979) lens (mw = 26,000, Goodenough, 1979) and cardiac cells (mw = 43,000, Kensler & Goodenough, 1980; Manjunath *et al.*, 1982; Beyer *et al.*, 1987) where the cardiac protein appears to have an extra polypeptide on the cytoplasmic side which may contribute to functional differences in channel properties (Manjunath & Page, 1985). Cardiac gap junctional permeability can be decreased by elevated $[Ca]_i$ (DeMello, 1975; Weingart, 1977) and intracellular acidification (Reber & Weingart, 1982) which would have the functional advantage of uncoupling cells which are damaged or metabolically compromised. While the interaction of these effects may be complex and synergistic (Burt, 1987), Spray *et al.*, (1982) concluded that the proton sensitivity is greater than the Ca sensitivity.

TABLE 2

Cellular Elements: As % of Cell Volume

	MF	Mito	Nuc	TT	Junct SR	Free SR	SR tot	SR:TT
Finch V[a]	57.3	34	-	0	0.21	0.62	0.83	-
Mouse V[a]	54.3	37.5	-	0.8	0.22	0.65	0.87	0.75
Mouse V[b]	54.3	37.5	-	0.95	0.22	0.65	0.87	0.92
Mouse L.A[b]	53.0	20.3	-	0.25	0.10	1.62	1.73	6.92
Mouse R.A[b]	52.6	19.8	-	0.20	0.06	1.69	1.76	8.80
Guinea Pig A[c]	43.2	17.9	3.8	0.08	0.46†	9.47	9.93	124
Guinea Pig V[c]	45.2	25.3	2.8	2.62	0.56†	7.37	7.93	3.03
Guinea Pig A[d]	41.4	14	4	-	0.5	1.7	2.2	-
Guinea Pig V[e]	50	25	-	~2	-	-	-	-
Rat V[f]	48.1	34	-	1.2	0.3	3.2	3.5	2.9
Rat V[g]	46.7	36	2	-	0.3	3.2	3.5	-
Frog V[h]	46.1	13.8	-	0	0.03	0.35	0.38	-
Frog A[h]	42.4	12.0	-	0	0.03	0.53	0.56	-
Lizard V[h]	50.1	25.8	-	0	0.09	0.6	0.69	-
Lizard A[h]	41.1	18.7	-	0	0.11	1.12	1.22	-
Frog V[i]	-	-	-	0	0.2	0.3	0.5	-
Frog Sartorius[j]	82.6	1.6	-	0.32	4.1	5	9.1	28.4
Guinea Pig								
Soleus[k]	86.7	4.9	0.9	0.14	0.9	2.2	3.1	22.1
White Vastus[l]	82.0	1.9	0.15	0.27	1.6	3.0	4.6	17.0
Red Vastus[m]	80.3	8.2	0.9	0.28	1.2	2.0	3.3	11.8
Sheep Purkinje[n]	23.4	10.3	1	0.23*	-	-	-	-

[a] Bossen *et al.*, 1978
Sommer & Johnson, 1979
[b] Bossen *et al.*, 1981
[c] Forbes & Van Niel, 1988
[d] Frank *et al.*, 1975
[e] Eisenberg *et al.*, 1985
[f] Page *et al.*, 1971
[g] Page, 1978
[h] Bossen & Sommer, 1984
[i] Page & Niedergerke, 1972
[j] Mobley & Eisenberg, 1975
[k] Eisenberg *et al.*, 1974
[l] Eisenberg & Kuda, 1975
[m] Eisenberg & Kuda, 1976
[n] Mobley & Page, 1972

Myofilaments (MF), Mitochondria (Mito), Nucleus (Nuc), Ventricle (V), Atrium (A), T-tubule (TT), Junctional (Junct). Mammalian skeletal muscle can be classified into slow (soleus), Fast/glycolytic (white vastus) and fast/oxidative/glycolytic (red vastus). *Intercellular clefts or folds for Purkinje fibers. † Excluding corbular SR. The SR volumes estimated by Forbes & Van Niel were acknowledged to be artifactually high due to a contrast effect.

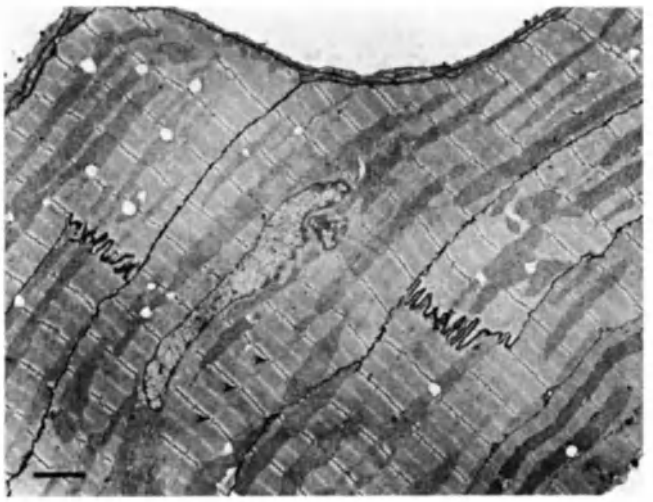


Figure 3. Finch cardiac muscle stained with colloidal lanthanum hydroxide as extracellular marker. Note the interdigitation of the cell ends and the lack of T-tubules. The arrowheads indicate structures identified at higher magnification as extended junctional SR. Bar = 2 μm. (From Sommer & Waugh, 1976 with permission).

The term sarcolemma has so far been used implicitly in reference to the phospholipid/cholesterol bilayer with the integral proteins (e.g. ion channels and pumps) which are "floating" in it. The sarcolemma, as such has clear regional specialization, (e.g. diads and gap junctions) where the bilayer surfaces are in close contact with special structures and where special proteins are located. The outer surface of muscle sarcolemma is also invested with a layer of material with abundant acidic mucopolysaccharides (Bennet, 1963). Frank *et al.*, (1977) have divided this glycocalyx (or "sweet husk") functionally, into surface coat (a less dense, 20 nm layer immediately adjacent to the sarcolemma) and an external lamina a more dense outer 30 nm layer at the interstitial interface). This glycocalyx is rich in sialic acid residues which may account for a substantial fixed negative charge in this region and explain the observation that pretreatment with neuraminidase decreases the labelling of the glycocalyx with cationic electron-dense markers (lanthanum, ruthenium red and colloidal iron, Frank *et al.*, 1977). Langer *et al.*, (1976) found that such neuraminidase treatment greatly increased cellular Ca exchange and suggested that sialic acid moieties in the surface coat might be important in regulating sarcolemma Ca permeability. Exposure of cells to Ca-free solution causes the external lamina to lift away from the surface coat (Fig. 4, Frank *et al.*, 1977). They attributed this to break down of sugar-Ca-sugar bridges and suggested that this might be involved in damage associated with readmission of Ca (i.e. the Ca paradox described by Zimmerman & Hülsmann, 1966, see also Frank *et al.*, 1982; Chapman & Tunstall, 1987; Bhojani & Chapman, 1990).

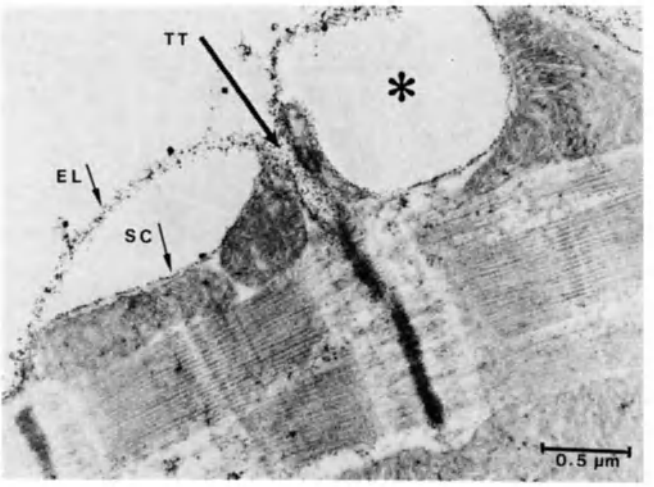


Figure 4. Rabbit ventricular muscle stained with colloidal iron. The muscle had been perfused in Ca-free solution for 20 min. Note the separation of the external lamina (EL) from the surface coat (SC) forming a bleb (*). The EL is anchored where it penetrates into the T-tubule (TT). (From Frank *et al.*, 1977, by permission of the American Heart Association, Inc.)

This separation of external lamina in Ca-free solution creates surface blebs which span from T-tubule to T-tubule, such that the external lamina remains anchored by its extension into the T-system. This also points to the fact that these surface layers remain associated with the sarcolemma in T-tubules in cardiac muscle. This contrasts with skeletal muscle where the glycocalyx does not appear to extend into the narrow T-tubules (Fawcett & McNutt, 1969).

THE EXTRACELLULAR SPACE

The contents of the extracellular space are important for understanding their possible direct participation in cardiac function (above), but also for correcting measurements made in intact preparations in terms of intracellular vs extracellular concentrations. Frank and Langer (1974) characterized the extracellular space in arterially perfused rabbit intraventricular septum and also measured extracellular space volume by both morphological and chemical means. They found that 59% of the extracellular space is vascular, 23% ground substance (resembling the glycocalyx material described above), 7% connective tissue cells, 6% empty space and 4% collagen. They also demonstrated that 36% of the cell circumference at its widest point is within 200 nm of a capillary. This close proximity to capillaries is illustrated in Fig. 5 and emphasizes the fact that in vascularly perfused ventricular muscle, the cell has rapid access to the vascular contents (rather than requiring a strictly series model for diffusion with an intervening interstitial compartment).

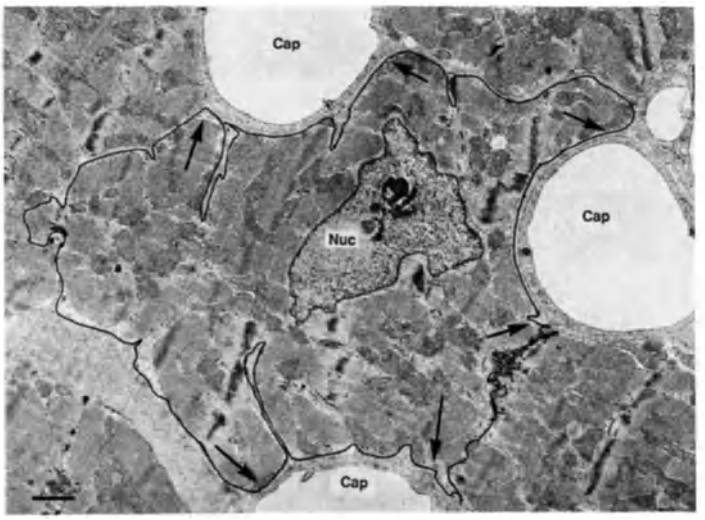


Figure 5. Rabbit interventricular septal cell in transverse section. Note that ~36% of the cell surface is within 200 nm of a capillary (cap), as indicated by the borders between arrowheads. Bar = 1 μm. (From Frank & Langer, 1974, by copyright permission of the Rockefeller University Press).

Frank and Langer estimated the extracellular space fraction of the total tissue morphometrically (27.6% including T-tubules) and chemically (35.7% using ^{14}C-sucrose and 36.2% using ^{35}S-sulfate). Lee and Fozzard (1975) reported a similar value (32.9%) for the ^{35}S-sulfate space in superfused rabbit papillary muscle. Bridge *et al.*, (1982) measured a similar value for the extracellular space in rabbit heart *in vivo* using ^{14}C-sucrose (0.303 ml/g) and CoEDTA (0.294 ml/g), but found much larger values in the isolated aqueous perfused intraventricular septum (0.51 ml/g for CoEDTA and 0.46 ml/g for ^{14}C-sucrose). While most extracellular space values for mammalian ventricle are in the 25-35% range, the higher value reported by Bridge *et al.*, (1982) for the intraventricular septum may reflect tissue edema due to the low oncotic pressure of the aqueous vascular perfusion. Thus, the extracellular space volume used to correct tissue contents to intracellular or extracellular contents should be measured under the same experimental conditions.

SARCOPLASMIC RETICULUM

The SR is an entirely intracellular, membrane bounded compartment which is not continuous with the sarcolemma. The main function of this organelle in muscle appears to be sequestration and release of Ca to the myoplasm. The volume of SR varies among cell types (e.g. Table 2) being most abundant in skeletal muscle, less abundant in mammalian heart and least abundant in frog ventricle. This may reflect functional differences in the

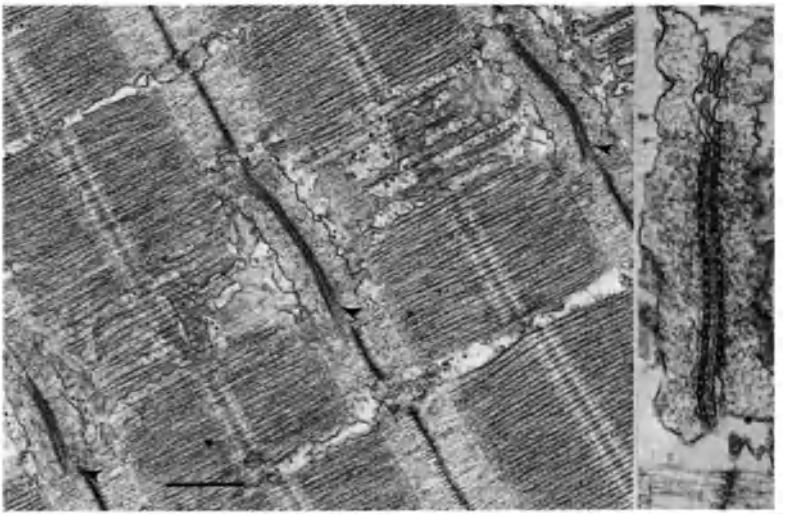


Figure 6. Tangential view of 3 triads from frog sartorius muscle (arrowheads) showing the periodic junctional "feet" at the site where the SR and T-tubule come into close contact. Bar = 0.5 μm and the inset is at 2.2 - fold higher magnification. (From Franzini-Armstrong, 1970, by copyright permission of the Rockefeller University Press).

relative importance of SR Ca in the activation of contraction (See Chapters 7 & 8). The SR in skeletal muscle is very highly organized. This allowed Winegrad (1965) to perform his classical autoradiographic study in which he identified the terminal cisternae as the site of Ca release from the SR. This was a very important conclusion because it indicated anatomical segregation of transport functions within the SR. Except for the junctions between the SR and sarcolemma, the SR membrane appears fairly homogeneous and contains mainly the SR Ca-ATPase pump protein (Stewart & MacLennan, 1974; Katz *et al.*, 1986) which is manifest as intramembrane particles ~8 nm in diameter and 3000-5000/μm^2 of SR membrane (Franzini-Armstrong, 1975). These particles are also observed in isolated SR vesicles and the density may be slightly lower in cardiac SR vesicles (Baskin & Deamer, 1969). Even the major part of the terminal cisternae appear to have Ca-pump protein. Thus, the vast majority of the surface of the SR is likely to function primarily to remove Ca from the cytoplasm. The relative area of the SR surface involved in couplings with the sarcolemma (Junct SR in Table 1) also varies substantially from one muscle type to another. Again, skeletal muscle has the greatest area of junctional SR, with mammalian heart being less and frog heart the least.

The junctions of SR with sarcolemma (surface or T-tubule, see Fig. 2) are highly specialized and feature bridging structures or spanning proteins which have been called "feet" by Franzini-Armstrong (1970, see Fig. 6). These "feet" structures have also been called pillars, spanning proteins, bridges and junctional feet. Similar structures are also seen in cardiac muscle at the junction of SR with either surface or T-tubular sarcolemma

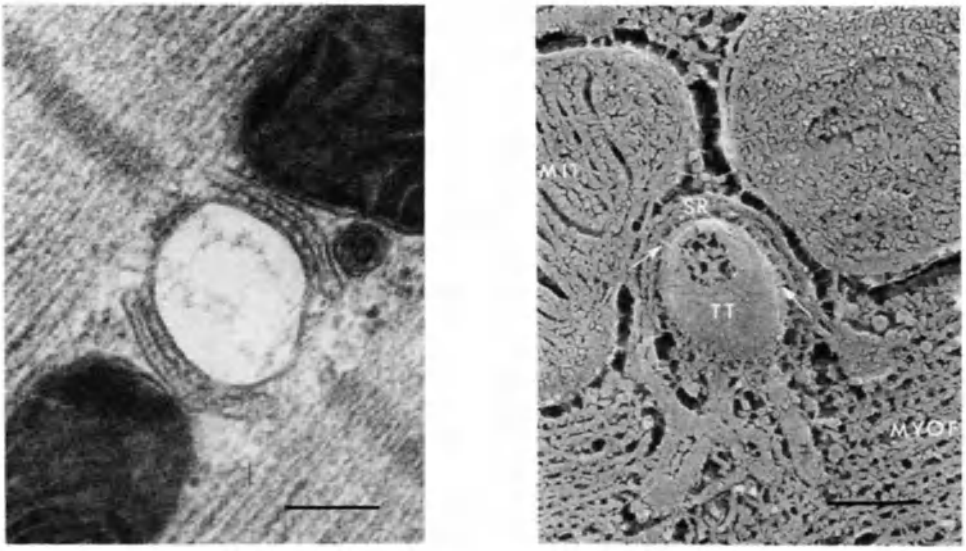


Figure 7. Rat papillary muscle in a thin section electron micrograph (left) and freeze-etched electron microscopy after ultra-rapid freezing without fixation (right). The junctional "feet" between the SR and T-tubule (TT) membranes can be seen to periodically span the gap. Bar=0.2 μm. (From Frank, 1990 with permission).

(see Fig. 7). Caldwell & Caswell (1982) provided the first biochemical evidence for a high molecular weight protein which could be the junctional feet. Based on their distinctive morphology and high affinity for the neutral plant alkaloid, ryanodine, these feet have recently been purified and identified as the Ca-release channel of the SR in skeletal muscle (Inui *et al.*, 1987a; Lai *et al.*, 1987) and cardiac muscle (Lai *et al.*, 1988; Inui *et al.*, 1987b). This protein is so large (mw = 560,000 for the monomer Takeshima, *et al.*, 1989), that the functional unit which is a tetramer can be examined at the electron microscopic level (Saito *et al.*, 1988; Wagenknecht *et al.*, 1989). These results (see Fig. 8 and page 112) are particularly exciting because there is the prospect of gaining physiologically useful information about the physical structure of the Ca channel pore directly from ultrastructural approaches.

In addition Block *et al.*, (1988) have demonstrated that these feet are organized in a distinct pattern on the SR underneath the T-tubular membrane and are matched by an organized array of particles in the T-tubule membrane which might be the sarcolemmal Ca channel protein (or dihydropyridine receptor, see Fig. 9). The potential implications of this arrangement for E-C coupling are in some respects self-evident, but will be considered more specifically in Chapter 7. The interior of the SR also contains a low affinity, high capacity Ca binding protein, calsequestrin (Ostwald & MacLennan, 1974), especially in SR derived from the terminal cisternae (Meissner, 1975). This protein may be important in increasing the Ca buffering capacity of the SR.

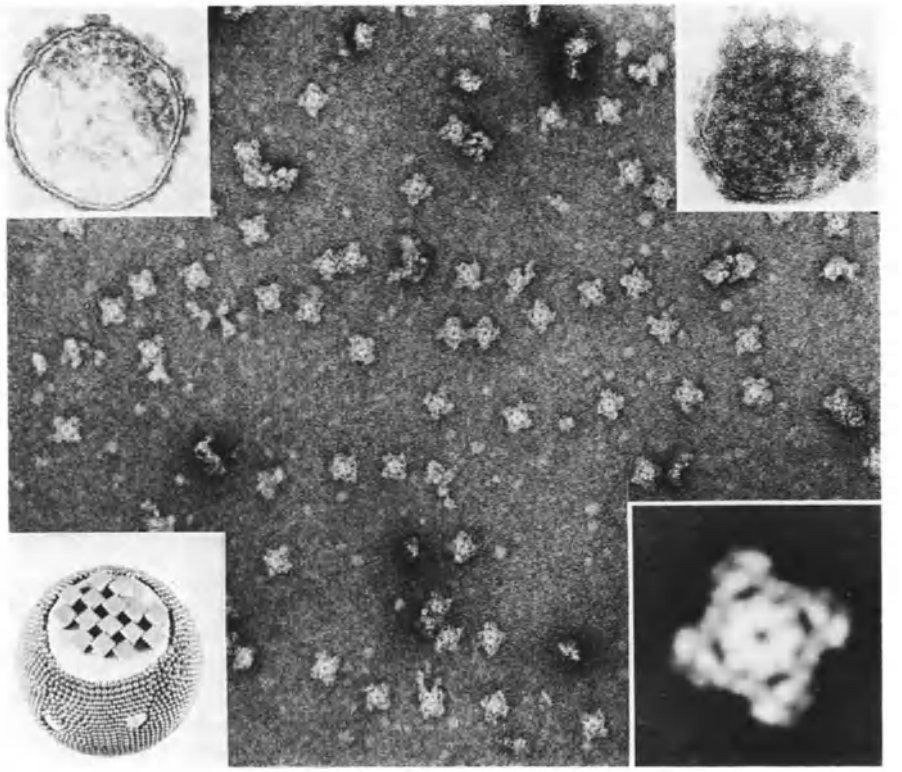


Figure 8. Morphology of the foot protein/ ryanodine receptor/ Ca release channel of skeletal SR. Center) Electron micrograph of negatively stained receptors showing the four-fold symmetry and square shape (~27 nm/side) and computer average of 240 images (bottom right). The foot protein can be seen to extend ~12 nm from the surface of the SR vesicle originating from the terminal cisternae (top left) and the same square array can be seen in tangential sections of these vesicles (top right). A model of the terminal cisternal membrane (bottom left) shows a junctional face membrane with foot structures and the remainder of the SR surface covered largely by the Ca-pump protein. (From Fleischer & Inui, 1989 by permission of Annual Reviews Inc.).

Sommer and colleagues have also described what seems to be junctional SR (including feet), which does not come into contact with any sarcolemma component. One of these specialized regions has been called extended junctional SR and is prominent in the interior of avian cardiac myocytes which lack T-tubules (Jewett *et al.*, 1971; Sommer & Johnson, 1979). This extended junctional SR occurs along the Z-disk in bird ventricle where T-tubule/SR junctions would be prominent in mammalian ventricle (see Table 1 and Fig. 3). Corbular SR (Dolber & Sommer, 1981) is functionally similar to extended junctional SR in that it exhibits the morphology of junctional SR, but is not in the vicinity of the sarcolemma. Corbular SR has been described in mammalian ventricle (Dolber &

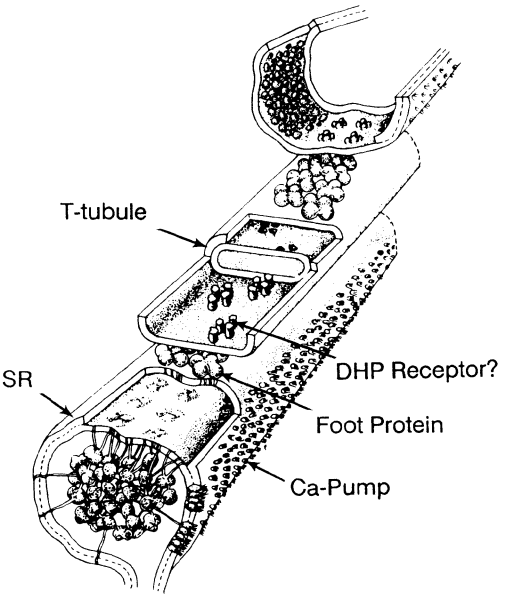


Figure 9. A three-dimensional reconstruction of the relative positions of key proteins at the triad. The SR is filled with calsequestrin and the non-junctional surface is covered with the Ca-pump protein. The fourfold foot protein is organized in two parallel rows and protrudes from the SR. A similar array of proteins (which could be dihydropyridine receptor/Ca channel proteins) exists in the T-tubular membrane, but the axis of fourfold symmetry is rotated and they lie only over alternating foot structures. (From Block *et al.*, 1988, by copyright permission of the Rockefeller University Press)

Sommer, 1984), atrium and Purkinje fibers (Sommer & Johnson, 1968; 1979) and also in chicken cardiac muscle (Jewett *et al.*, 1973; Jorgenson & Campbell, 1984). These "uncoupled" SR components with morphology like true junctional SR provides a functional challenge to models of E-C coupling. That is, what is the function of these SR components? Do they participate in Ca release during the activation of a normal contraction? Is the mechanism of release distinct from that of true junctional SR?

MITOCHONDRIA

About 35% of the volume of mammalian and avian ventricular muscle cells is occupied by mitochondria (see Table 2). The mitochondria are the site of oxidative phosphorylation and the tricarboxylic acid cycle and the large mitochondrial content in mammalian ventricle bespeaks the high demands on this organelle for energy supplied by aerobic metabolism. The mitochondrial fraction of cell volume is lower in mammalian atrial muscle and Purkinje fibers as well as amphibian and reptilian hearts and skeletal muscle (Table 2). Indeed, there is substantial variation in mitochondrial volume among

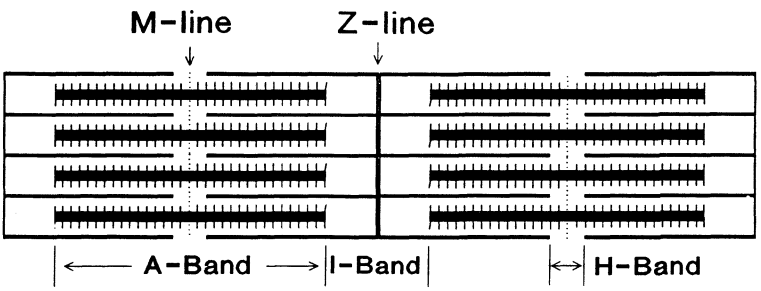


Figure 10. The organization of the sarcomere. The thin filaments meet at the Z-lines and the center of the thick filaments is known as the M-line. The I-band (or isotropic band) is the area where there are only thin filaments and the A-band (or anisotropic band) is the length of the thick filaments. The region of the thick filament where there is no overlap with thin filaments is known as the H-band (or H-zone).

different types of skeletal muscles that probably reflects differences in the oxidative capacity of those muscle types. The surface area of the folded inner mitochondrial membrane in rat left ventricle has been estimated to be 20 $\mu m^2/\mu m^3$ of cell volume (Page, 1978). This membrane is the site of control of transport of metabolites and ions (e.g. Ca and protons). The surface area is more than 10 times larger than that of the combined sarcolemmal and SR membranes, so a modest Ca transport by mitochondria could have a profound impact on overall cellular Ca regulation.

Cardiac mitochondria are usually cylindrical, although often flattened by the tight packing within the cells. The cristae within the cardiac mitochondrial are more tightly packed than those in hepatocytes, perhaps reflecting the intensive energy demands of the myocardium (Sommer & Johnson, 1979). A layer of mitochondria is often found just under the plasma membrane and also between adjacent myofibrils. As mitochondria are squeezed in everywhere in cardiac cells, it is not possible to assign any specific significance to the mitochondria distribution within the cell.

MYOFILAMENTS

The myofilaments occupy about half of the cell volume in mammalian ventricular myocardium (Table 2). In skeletal muscle this myofilament volume is larger and in atria and cells specialized for electrical conduction (Purkinje fibers) this value is smaller. The myofilaments are composed of the thick (or myosin) and thin (or actin) filaments as well as associated contractile and cytoskeletal components. The myofilaments run in bundles or fibrils which are less clearly defined in cardiac muscle than in skeletal muscle due to branching.

The term myofilaments often refers to the contractile machinery of the cell and indeed they represent the end effector responsible for transducing chemical energy into mechanical energy and work. The sarcomere is the fundamental contractile unit in striated muscle and is bounded by the Z line (Figure 10). The Z-lines appear to be the anchor points where intermediate filaments of the cytoskeleton are connected to actin filaments (Price & Sanger, 1983). From the Z-lines, the thin filaments project ~ 1 μm from each end toward the center of the sarcomere. The thick filaments are 1.6 μm long and centered on the M-line where thick filaments are connected to each other in a hexagonal array by radial crosslinks. The regulation of myofilament force development will be discussed further in Chapter 2.

OTHER CELLULAR CONSTITUENTS

Cardiac myocytes usually are mononucleate and also contain Golgi apparatus, lysosomes, lipofuscin granules and peroxisomes (Sommer & Johnson, 1979). Lipid droplets and β-glycogen granules are also present in cardiac myoplasm and are more abundant than in skeletal muscle. Cardiac myocytes also have a well developed cytoskeleton. This serves as a structure intimately associated with and supporting the physical organization of the contractile proteins. These cytoskeletal elements include intermediate filaments (e.g. desmin), which form a longitudinal and transverse network to anchor the myofilaments (Price & Sanger, 1983) as well as microtubules and microfilaments.

CHAPTER 2

MYOFILAMENTS: THE END EFFECTOR OF E-C COUPLING

When cytoplasmic [Ca] rises, the myofilaments are activated in a [Ca]-dependent manner, thereby transducing the chemical signal and chemical energy (ATP) into mechanical force or shortening. Under physiological conditions, skeletal muscle contractile force can be varied by summation of contractions, tetanus and recruitment of additional fibers. Cardiac muscle, on the other hand, functions as a syncytium such that each cell contracts at every beat. The heart must also relax between contractions. Thus, there is neither the practical possibility of recruitment of additional cells, nor summation, nor tetanization to alter the force of contraction to meet altered demands. Therefore, in cardiac muscle, the force of contraction is varied in large part by changes in the peak $[Ca]_i$ reached during systole.

For this reason I place considerable emphasis on the factors influencing the $[Ca]_i$ in subsequent chapters, often with implicit assumptions about the consequent effects on myofilament activation. However, since contraction is the physiological role of cardiac muscle, it is important to set out some fundamental characteristics of the contractile proteins and how the sensitivity of the myofilaments to Ca can be altered under physiologically relevant conditions.

MYOFILAMENT PROTEINS

The thick filament is composed largely of myosin but also contains some smaller proteins, such as C-Protein. Each myosin heavy chain (mw ~ 450,000) has a long (~120 nm) α-helical tail and a globular head (Fig. 11a). The tails of the myosin heavy chain form the main axis of the thick filament. The heads form the crossbridges which interact with the thin filaments, contain the site of ATP hydrolysis and have two light chains associated with each head. The myosin molecule is hexameric, composed of two heavy chains with their tails coiled around each other and two myosin light chains per heavy chain. Based on susceptibility to specific proteases, the myosin heavy chain has been broken down into light meromyosin (most of the tails) and heavy meromyosin which is further divided into subfragment 1 (S1, globular head and enzymatic activity) and subfragment 2 (S2, the

TABLE 3

Cardiac Contractile Proteins

	Molecular Weight†
Myosin Heavy Chain	200,000
Myosin Light Chain 1	26-28,000
Myosin Light Chain 2	18-20,000
Actin	41,700*
Tropomyosin	67,000*
Troponin T	38,000
Troponin I	23,500*
Troponin C	18,400*

† All values are from a review by Swynghedauw (1986) for mammalian ventricular muscle.
* Indicates molecular weights determined by sequence analysis.

residual tails). The central region of the thick filament is devoid of crossbridges, reflecting the tail-to-tail abutment of myosin molecules in this region.

The proteins of the thin filament and their interactions have recently been reviewed in detail by Zot and Potter (1987). The backbone of the thin filament is composed of two chains of the globular protein, G-actin which form a helical double-stranded F-actin polymer (Figure 11b). Tropomyosin is a long flexible protein which lays in the groove between the actin strands and spans about 7 actin monomers. Tropomyosin is also a double-stranded protein (coiled coil), mostly α-helical and the two strands may be connected by a disulfide bridge (Fig. 11C). At every seventh actin there is a troponin complex attached to tropomyosin. The troponin complex is made up of three subunits: troponin T (TnT, or the tropomyosin binding subunit), troponin C (TnC, or the Ca binding subunit), and troponin I (TnI, or the inhibitory subunit which can also bind to actin). The arrangement of these subunits is indicated in Figures 11C and 12. TnT is elongated and lays along tropomyosin over about 2-3 actin monomers (Ohtsuki, 1979; Flicker *et al.*, 1982). This arrangement may allow TnT to better control the position of tropomyosin. TnI interacts specifically with TnT and also binds specifically to actin. By binding to actin, TnI can prevent myosin from interacting with actin. TnI also interacts specifically with TnC and the characteristics of this interaction depend on whether Ca is bound to the high affinity Ca binding sites on TnC. The interaction between TnI and TnC is also strongly affected by Ca binding to the lower affinity Ca binding site and this effect is important in the physiologic regulation of contraction (see below).

Skeletal TnC has been extensively characterized and contains 4 Ca-binding sites, two Ca-specific sites ($K_{d(Ca)}$ = 200 nM in the troponin complex) and two sites at which Ca and Mg bind competitively, known as Ca-Mg sites ($K_{d(Ca)}$ = 2 nM; $K_{d(Mg)}$ = 25 μM in the troponin complex). It is notable that the affinity of these sites on TnC are different when the intact system is partly or wholly disassembled (e.g. in isolated TnC the Ca-specific site

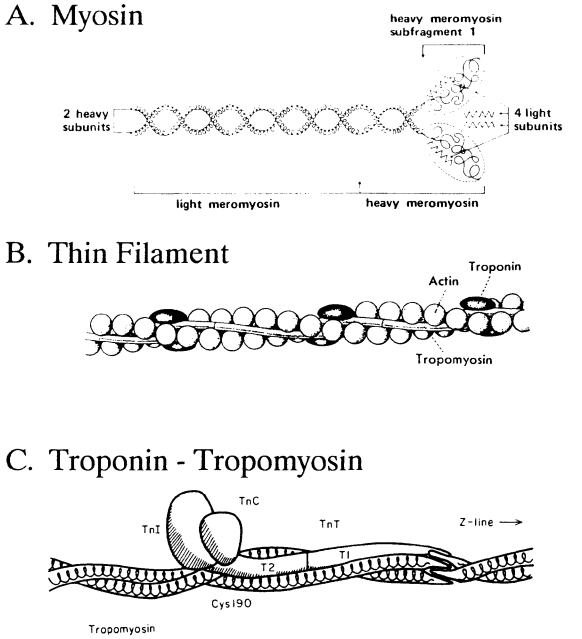


Figure 11. Myofilament proteins. A. The myosin molecule is ~160 nm in overall length with two globular heads (heavy meromyosin subfragment 1) and tails which exist as a coiled coil (From Katz, 1977 with permission). B. The thin filament, illustrating the relationship of the major constituents based on the classic diagram by Ebashi *et al.* (1969) and later modified (From Ebashi, 1974 with permission). C. The interaction of troponin subunits with the tropomyosin molecule. Note that the tropomyosin molecule is also predominantly a coiled coil (From Flicker *et al.*, 1982 with permission).

has ~10 times lower affinity for Ca than the whole troponin complex, see Table 4). The Ca-specific sites appear to be the sites which are responsible for regulating contraction (Zot and Potter, 1987).

Cardiac TnC differs significantly from skeletal TnC in that the cardiac isoform has only one Ca-specific binding site. With the affinities in Table 4, resting $[Ca]_i$ = 100 nM and $[Mg]_i$ = 1 mM, the cardiac Ca-Mg sites would be ~97% saturated (90% with Ca and 7% with Mg). Thus, these sites would always be nearly saturated. The Ca-specific site has a K_d ~500 nM and thus would be expected to respond to $[Ca]_i$ changes which are likely to occur physiologically (see below). Pan and Solaro (1987) measured Ca binding in detergent treated canine ventricle which was otherwise intact. They found a slightly lower affinity for the Ca-specific site of TnC during rigor compared to the isolated TnC complex in Table 4 (1.2×10^6 M^{-1}) and the affinity was further reduced ~3-4 fold by adding ATP to dissociate the rigor complexes.

TABLE 4

Ca and Mg Binding Affinities of Skeletal and Cardiac Troponin

	Ca Specific Sites Sites		Ca-Mg Sites		
	(n)	K_{Ca} (M^{-1})	(n)	K_{Ca} (M^{-1})	K_{Mg} (M^{-1})
Skeletal TnC	(2)	3.2×10^5	(2)	2.1×10^7	4×10^3
Skeletal TnC · TnI	(2)	3.5×10^6	(2)	2.2×10^8	4×10^4
Skeletal Tn	(2)	4.9×10^6	(2)	5.3×10^8	4×10^4
Cardiac TnC	(1)	2.5×10^5	(2)	1.4×10^7	7×10^2
Cardiac TnC · TnI	(1)	1.0×10^6	(2)	3.2×10^8	3×10^3
Cardiac Tn (reconstituted)	(1)	2.5×10^6	(2)	3.7×10^8	3×10^3
Cardiac Tn (native)	(1)	1.7×10^6	(2)	4.2×10^8	---

Values are from Holroyde *et al.* (1980) and Potter & Johnson (1982).

TnC can also bind other di- and trivalent cations (Fuchs, 1974). Kerrick *et al.* (1980) demonstrated that cardiac myofilaments could be activated equally well by Ca or Sr, but that skeletal muscle is much less sensitive to activation by Sr than Ca.

Cardiac muscle also exhibits different isoforms of several of the contractile proteins (e.g. myosin and myosin light chains) than fast skeletal muscle and can modulate the expression of various cardiac isoforms *in vivo* (Swynghedauw, 1986; Morkin, 1987). For example, there are two main isoforms of the cardiac myosin heavy chain (α, β), sometimes referred to as fast (α) and slow (β) based on the myosin ATPase rate or shortening rate. Three different dimers can form ($\alpha\alpha$, or V_1, $\alpha\beta$ or V_2 and $\beta\beta$ or V_3). There are species differences (e.g. rat ventricle is mostly V_1 and rabbit ventricle is mostly V_3), adaptational differences (rat ventricle shifts to V_3 during hypertrophy) and thyroid hormone can induce a switch from β- to α-myosin heavy chain production.

MECHANISM BY WHICH Ca ACTIVATES CONTRACTION

It seems quite clear now that the rise in cytoplasmic [Ca] is the event which activates the myofilaments and a fairly clear picture (though not complete) of the molecular basis for this regulation of the contractile machinery by Ca is developing, including mapping of peptide regions involved with changes in subunit interactions (Zot and Potter, 1987). Figure 12 is a schematic diagram which illustrates our current

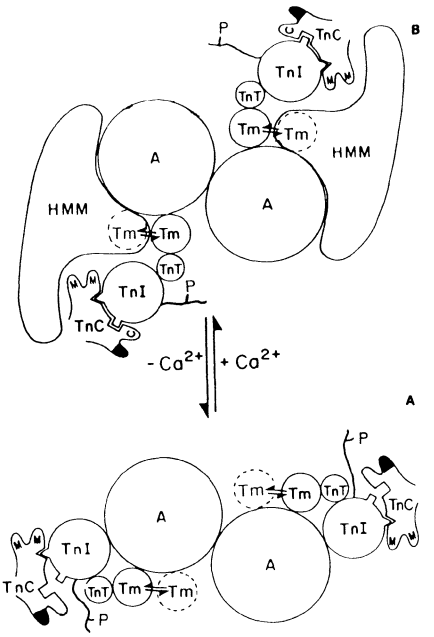


Figure 12. The Ca-dependent regulation of the acto-myosin interaction in cardiac muscle by troponin and tropomyosin (Tm). In the absence of Ca (bottom), TnI binds to actin thereby preventing myosin from interacting with actin. This also draws Tm out of the groove, thereby extending the myosin blocking effect to adjacent actin monomers (which lack TnI). In the presence of Ca (top), TnI binds more firmly to TnC and not to actin. In this case Tm is also not drawn out of the groove and the interaction of myosin and actin can occur (From Warber & Potter, 1986 with permission).

understanding of some of these interactions. At rest when $[Ca]_i$ is low, the Ca-specific sites of TnC are unoccupied. In this condition the interaction between TnI and TnC at a critical region of the TnI molecule is weak and this region of TnI appears to interact more strongly with actin. This would favor the configuration of the troponin-tropomyosin complex illustrated in the bottom of Figure 12 where the troponin-tropomyosin complex is shifted peripherally and more out of the axial groove of the actin filament. When $[Ca]_i$ rises, Ca binds to the Ca specific site of TnC. This may then strengthen the specific interaction of TnC with TnI and effectively destabilize the interaction of TnI with actin. This favors the more axial location of the troponin-tropomyosin complex and removes the steric hindrance to myosin interaction with actin and consequently allows force production and/or shortening. The tight interaction between TnT and tropomyosin is probably important in the transmission of this conformational change along the thin filament to the actin monomers which do not have associated TnI subunits.

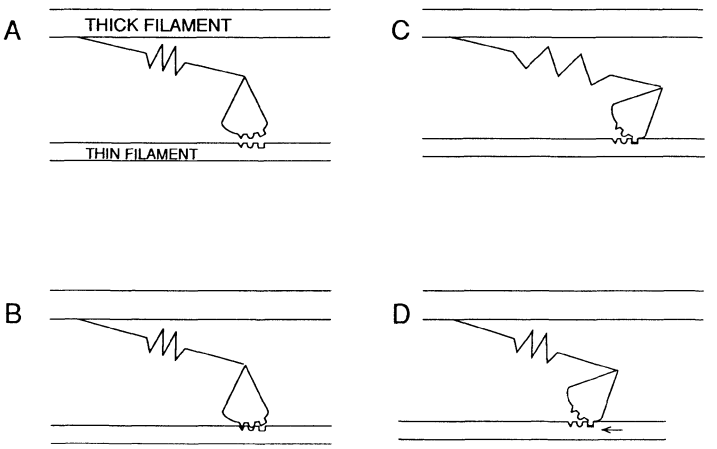


Figure 13. A mechanical model of active crossbridges based on the original diagram by Huxley and Simmons (1971). A. a detached crossbridge (e.g. M•ATP in Fig. 14). B. an attached crossbridge prior to developing force (e.g. A•M•ADP•P_i in Fig. 14). C. an attached crossbridge developing force stored in the elastic component (e.g. A•M•ADP* in Fig. 14). D. a crossbridge rotated and translated so the filaments "slide" relative to one another (e.g. A•M•ADP or A•M in Fig. 14).

The movement of tropomyosin deeper into the actin groove was first suggested by x-ray diffraction studies (Haselgrove, 1973; Huxley, 1973) and the structural and biochemical results seem to be in generally good agreement with this steric hindrance model of myofilament regulation. However, Chalovich *et al.* (1981) reported evidence suggesting that the regulatory proteins might directly block the acto-myosin ATPase reaction in the absence of Ca, without preventing interaction between myosin heads and actin. Thus, while there is much evidence in support of the steric hindrance model described above (and it is the dominant theory of Ca regulation of the myofilaments), some details of the mechanism are not completely resolved.

ACTO-MYOSIN ATPase

In the presence of sufficient Ca, myosin can interact with actin which greatly increases the ability of myosin ATPase to hydrolyze ATP and also allows transformation of chemical energy stored in ATP to mechanical energy and work. A physical model for this transduction known as the sliding filament theory, came from x-ray diffraction studies (Huxley, 1969) and mechanical perturbation studies (Huxley and Simmons, 1971) and is illustrated schematically in Figure 13. At rest the myosin heads (or crossbridges) extend from the thick filament perpendicular to the filament axis. Upon activation the crossbridge

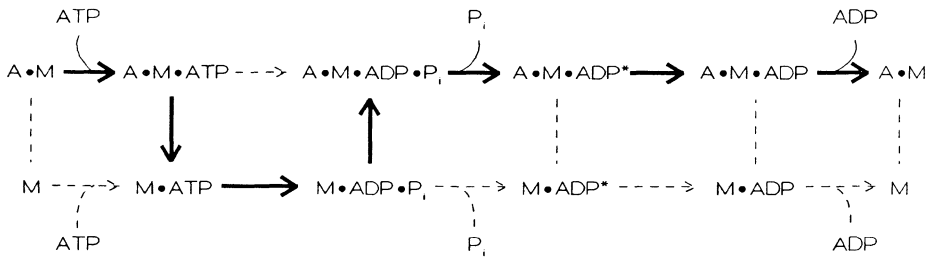


Figure 14. A chemical model of the steps in the crossbridge cycle (or acto-myosin ATPase) based on Goldman & Brenner (1987). The top row is the states in which actin (A) interacts with myosin (M). The heavy solid lines indicate the normal reaction pathway, but all the reactions can occur and are reversible. Two energetically different states of A•M•ADP are indicated by the inclusion of an asterisk on one.

can interact with the thin filament and force generation or relative filament movement is produced by a rotation of the myosin head (perhaps due to a series of stable states as indicated). Isometric force would be analogous to storing the potential energy of this myosin head rotation temporarily in an elastic component of the myosin molecule (Fig. 13C). Alternatively, the rotational movement can produce relative motion of the thick and thin filaments (i.e. shortening) if the muscle force exceeds the load (Fig. 13D).

The chemical steps involved in the crossbridge cycle have also been extensively characterized and correlated with such physico-mechanical schemes (e.g. Goldman, 1987; Brenner, 1987). The general chemical scheme is illustrated in Figure 14. At rest myosin (M) is mostly complexed with ATP (M•ATP) or in the rapidly equilibrated M•ADP•P_i where ATP is technically hydrolyzed, but the energy has not been used. As $[Ca]_i$ rises M•ADP•P_i can interact with actin and phosphate is rapidly released. The acto-myosin passes through at least two energetic states where ADP remains bound (A•M•ADP* and A•M•ADP) and these latter transitions may encompass the so-called "power stroke" or myosin head rotation. The affinity of myosin for actin increases along this series of steps and is strongest after ADP dissociates (i.e. A•M). However, at normal $[ATP]_i$, A•M binds ATP rapidly and this induces dissociation of actin and M•ATP. The cycle can then continue until $[Ca]_i$ declines, thereby stopping myofilament interaction (in the M•ADP•P_i state) or until ATP is depleted (i.e. rigor, with the cycle stopped in the A•M state). There may also be a small component of crossbridge cycling that occurs in the absence of elevated $[Ca]_i$ (Fabiato and Fabiato, 1975b; Goldman *et al.*, 1984; Reuben *et al.*, 1971).

THE LENGTH-TENSION RELATIONSHIP

The relationship between sarcomere length and the maximum force which can be developed is a central issue in the above physical models of muscle contraction. The

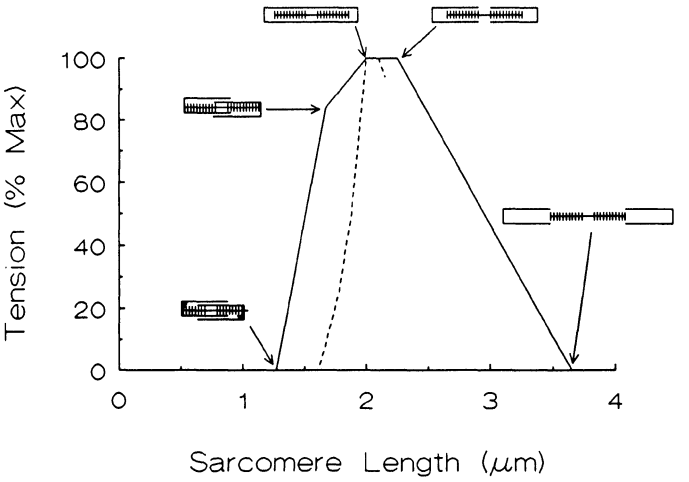


Figure 15. The length-tension relationship in frog skeletal muscle as described by Gordon *et al.* (1966) is indicated by the solid line and the inset diagrams. The length-tension relationship for cat cardiac muscle (described by Allen *et al.*, 1974) is also illustrated (broken curve).

relationship was described in detail by Gordon *et al.*, (1966) in frog skeletal muscle and their results are summarized in Fig. 15. The fundamental assumption is that the maximal force at any sarcomere length is determined by the degree of overlap of the thick and thin filaments (i.e. how many crossbridges can cycle). Thus, as the sarcomere is stretched from its optimal overlap (2 - 2.2 μm) the force gradually declines until it is 0 at the point where there is no longer overlap (~3.6 μm). At shorter sarcomere lengths (2.0 to 1.6 μm) the thin filaments cross over and may impede effective crossbridge formation. Finally, when the thick filament collides with the Z-line (~1.65 μm) the resistance to shortening is greatly increased and externally developed force drops steeply as the thick filament is compressed.

Cardiac muscle has a strong parallel elastic component which normally prevents cardiac sarcomeres from reaching the "descending limb" of the length-tension curve (e.g. sarcomere lengths greater than 2.3 μm). This is of course a good thing for the heart as it would result in progressive decrease in contraction and failure of cardiac output. Thus the heart normally functions along the "ascending limb" of the length-tension curve, so that one would expect increased contraction with increase in sarcomere length (or preload). This increase in fiber overlap is undoubtedly involved in the classic Frank-Starling law of the heart, whereby increased diastolic volume leads to increased systolic contraction. However, the length-tension relationship for cardiac muscle is considerably steeper than skeletal muscle for sarcomere lengths between 1.8 and 2.0 μm (Allen *et al.*, 1974, see broken curve in Fig. 15). Hibberd and Jewell (1982) and Kentish *et al.* (1986) demonstrated that the Ca sensitivity of the myofilaments was increased at longer

sarcomere lengths which would steepen the length-tension curve for a given [Ca]. This effect appears to be specific for cardiac TnC, since Babu *et al.*, (1988) demonstrated that reconstitution with skeletal TnC failed to produce the steep length dependence observed with native or reconstituted cardiac TnC. Allen and Kurihara (1982) also showed that a quick release and shortening of ferret ventricular muscle was accompanied by an apparent release of Ca from the myofilaments (using the Ca-sensitive photoprotein aequorin). In addition, at steady state at longer sarcomere lengths the SR Ca release is increased (Fabiato, 1980; Allen & Kurihara, 1982). Thus, there are multiple protective mechanisms which increase the hearts ability to contract as the heart is stretched and increased myofilament overlap, increased myofilament Ca sensitivity and increased SR Ca release may all contribute to the Frank-Starling law of the heart.

THE Ca SENSITIVITY OF THE MYOFILAMENTS

Cardiac myofilaments are activated by Ca in a graded manner and as such the relationship between free [Ca] and force development is of fundamental importance. Most of the experimental results on this issue have been obtained in cardiac muscle preparations which have had their sarcolemma removed or permeabilized and are called "skinned" preparations. Since myofilaments are sensitive to submicromolar [Ca] and contaminant [Ca] is usually several micromolar, Ca chelators such as EGTA are used to buffer [Ca] in the range of interest. The solutions used for these skinned fibers usually also contains ATP, Mg and some other solutes (e.g. ATP regenerating systems, pH buffer and KCl) and computer programs (e.g. Fabiato, 1988) are usually used to solve the multiple ion equilibria and prepare solutions of known free [Ca].

The pCa (-log[Ca]) values reported for the threshold [Ca] and [Ca] at half maximal force for mammalian cardiac muscle have been reported to be 5.9-7.0 and 4.7-6.0, respectively (Solaro *et al.*, 1974; Kerrick & Donaldson, 1975; Endo & Kitazawa, 1977; Fabiato & Fabiato, 1975a; 1978; McCLellen & Winegrad, 1978; Hibberd & Jewell, 1979; Fabiato, 1981a; Miller & Smith, 1984; Harrison & Bers, 1989a). While some portion of this large difference is due to species differences and particular experimental conditions (i.e. ionic strength, temperature, pH...), it is also possible that it reflects inaccuracies in calculated free [Ca]. This issue has been critically addressed by Bers (1982), Miller & Smith (1984) and Harrison & Bers (1989b) and the errors in [Ca] can be greater than 3-fold (i.e. 0.5 pCa units). These inaccuracies can be attributed to a) incorrect choice of (or use of) association constants, b) inappropriate modifications of the association constants for differences in ionic strength and temperature, c) small systematic errors in pH and d) overestimation of EGTA purity.

This complication makes it more difficult to compare quantitative details of Ca-sensitivity curves from one lab to another. While qualitative conclusions are probably valid, this may not always be the case. For example, based on results from 12°C and 22°C

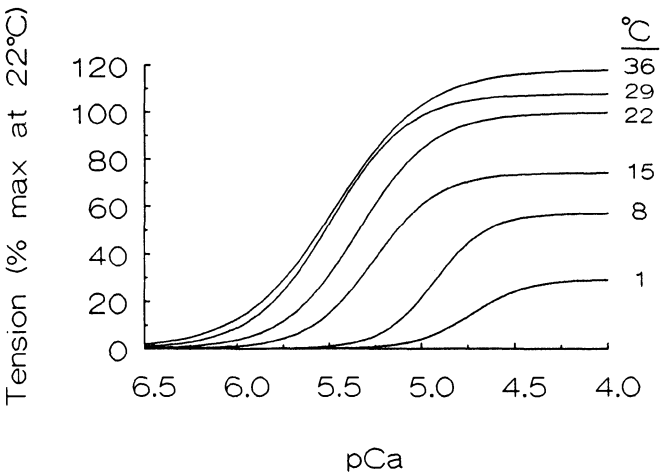


Figure 16. The influence of temperature on the Ca sensitivity of chemically "skinned" rabbit ventricular muscle (data from Harrison and Bers, 1989a has been redrawn). Both the Ca sensitivity and the maximum force are reduced at lower temperatures.

in mechanically skinned canine Purkinje cells, Fabiato (1985b) concluded that myofilament Ca sensitivity in cardiac muscle may be increased by cooling (as is the case in skeletal muscle, Stephenson & Williams, 1981; 1985; Godt & Lindley, 1982). However, a relatively minor adjustment of Fabiato's solution calculations, based on our studies of the influence of ionic strength and temperature on the affinity of EGTA for Ca (Harrison & Bers, 1987), greatly reduced the apparent temperature-induced shift reported by Fabiato (Harrison & Bers, 1989b). Furthermore, we found that over the temperature range 37°C to 1°C there was a progressive decrease in Ca sensitivity of the myofilaments with cooling in chemically skinned rabbit ventricular muscle (Fig. 16 and Table 5, Harrison & Bers, 1989a). Similar shifts were also reported for guinea-pig, rat and frog ventricle (Harrison & Bers, 1990a). We also found that much of the difference between the influence of temperature on cardiac and skeletal muscle could be attributed to the TnC type in the muscle, based on experiments where rat ventricular TnC was extracted and replaced by rabbit skeletal TnC or bovine cardiac TnC (Harrison & Bers, 1990b). These results raise an interesting thermodynamic difference between cardiac and skeletal muscle regulation at the level of TnC. That is, cooling decreases the Ca sensitivity of cardiac myofilaments, but increases the sensitivity of skeletal myofilaments (and largely due to the TnC type).

The foregoing also indicates that minor oversights in the calculations of free [Ca] in the complex solutions used in skinned fiber studies can lead to qualitative as well as quantitative errors. It is therefore useful to obtain an independent check of the free [Ca] in the solutions used whenever possible (e.g. with a Ca-selective electrode, Bers, 1982).

TABLE 5

The Influence of Temperature on Ca Sensitivity of Rabbit Ventricular Muscle.

Temperature (°C)	$pCa_{1/2}$	Hill Coefficient	Maximum Force (%)
36	5.47 ± 0.07	1.75 ± 0.1	118.5 ± 10.0
29	5.49 ± 0.07	2.06 ± 0.1	108 ± 4.6
22	5.34 ± 0.05	2.15 ± 0.6	100
15	5.26 ± 0.09	2.49 ± 0.2	74.3 ± 6.0
8	4.93 ± 0.06	3.06 ± 0.5	57.2 ± 7.0
1	4.73 ± 0.04	2.94 ± 0.6	29.3 ± 5.4

Data are from Harrison and Bers, 1989a.

With these potential caveats in mind some other factors which alter myofilament Ca sensitivity will be discussed below.

Skinned cardiac muscle fibers also exhibit hysteresis in the force-pCa relationship, such that Ca sensitivity is apparently greater when [Ca] is decreasing than when [Ca] is increasing (Harrison *et al.*, 1988). This hysteresis diminishes with increasing sarcomere length and is virtually eliminated at sarcomere lengths greater than 2.2 μm. This implies that for full relaxation to occur at shorter sarcomere lengths, the $[Ca]_i$ must be lower than would be supposed from force-pCa relationships obtained in the activating direction. From a functional standpoint, this may be an important consideration, since this may limit relaxation between contractions even if $[Ca]_i$ has reached a stable diastolic level.

Figures 17 shows superimposed pCa vs tension curves from several cardiac species. The data in Fig. 17A is from Fabiato (1982). Fabiato & Fabiato (1978b) previously reported comparative data under slightly different conditions (and with 3 abscissa calibrations, illustrating the difficulty of correctly calculating free [Ca] in these solutions). In that report they found that the Ca sensitivity of myofilaments from human atrium and ventricle were indistinguishable from those of rat and rabbit ventricle. Figures 17B and C are from Harrison and Bers (1990a) and Lues *et al.* (1988) respectively. The experimental conditions in the three panels are all somewhat different. The main point of these figures is that there are only minor differences in Ca sensitivity in different mammalian cardiac muscle preparations under the same conditions and the $pCa_{1/2}$ values for these skinned fiber preparations are typically between 5.2 and 5.7 and Hill coefficients between 1.9 and 2.5 at 22-29°C with ionic strength ~160 mM, pH~7.0 with 3-5 mM Mg ATP and 2-3 mM free Mg. These will serve as a base for the comparisons below.

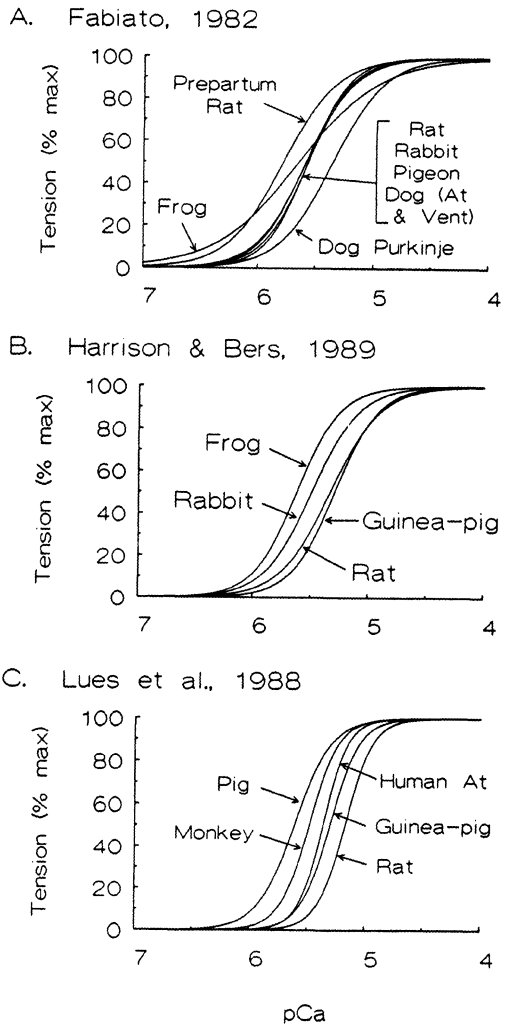


Figure 17. The myofilament Ca sensitivity in various cardiac muscle preparations. Data from A. Fabiato (1982), B. Harrison & Bers (1989a) and C. Lues *et al.* (1988) have been redrawn (based on curve fits to the Hill equation). All data were normalized to the maximum force under the prevalent conditions. The conditions are slightly different in all three panels. The major variations are (in mM):

	Temp.	pH	Γ	[EGTA]	[pH-buff]	[Mg]	[MgATP]
A.	22°C	7.1	160	10	30	3	3
B.	29°C	7.0	180	10	25	2.3	~5
C.	21°C	6.7	140	5	30	~2.5	~10

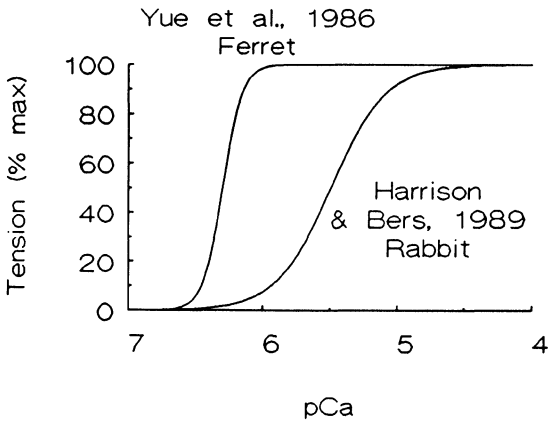


Figure 18. The myofilament Ca sensitivity determined in intact ferret ventricular muscle at 30°C (from Yue *et al.*, 1986) and in chemically skinned rabbit ventricular muscle at 29°C (from Harrison and Bers, 1989a). The curves are Hill curves based on the reported $pCa_{1/2}$ and n values.

FORCE-pCa RELATION IN INTACT CARDIAC MUSCLE

All of the foregoing and much of what will come below is based on experiments in muscles in which the sarcolemma has been disrupted so that the intracellular environment can be controlled. However, this has the substantial disadvantage that some cellular constituents which might affect myofilament Ca sensitivity may be lost. For example, Harrison *et al.*, (1986) found that carnosine and related compounds (imidazoles), which are abundant in skeletal muscle (Crush, 1970) and may total as much as 10 mM in cardiac muscle (O'Dowd *et al.*, 1988) significantly increase cardiac myofilament Ca sensitivity.

Yue *et al.* (1986) estimated the steady state force-pCa relationship in intact ferret ventricular muscle using aequorin to measure $[Ca]_i$ and ryanodine treatment to allow the heart muscle to be effectively tetanized. They found a mean $pCa_{1/2}$ of 6.3 and a mean Hill coefficient of 6.1. This is a remarkably steep curve and the half-maximum point is at a [Ca] (500 nM) considerably lower than almost any skinned fiber results. The myofilament force would go from less than 5% at 300 nM $[Ca]_i$ to 95% of maximum at 800 nM $[Ca]_i$. The specific factors responsible for this striking disparity (e.g. see Fig. 18) are not yet clear, but remains a challenge to those using skinned fiber preparations.

FACTORS WHICH INFLUENCE THE FORCE-pCa RELATIONSHIP

A large number of factors are known to modify the relationship between [Ca] and the force generated by cardiac myofilaments and these have been most extensively studied

in skinned cardiac muscle preparations (e.g. Table 6). As described above, both cooling and shorter sarcomere length decreases the Ca sensitivity and also the maximum force generated by the myofilaments (Hibberd & Jewell, 1982; Kentish *et al.*, 1986; Harrison and Bers, 1989a). Similarly, acidosis also decreases both the Ca sensitivity and maximum force generated by the myofilaments (Fabiato & Fabiato, 1978a, Blanchard & Solaro, 1984). This may be particularly important during pathological conditions such as hypoxia or ischemia where intracellular pH (pH_i) is known to decline (Jacobus *et al.*, 1982). An interesting note is that the Ca sensitivity of myofilaments from perinatal hearts is almost unaffected by acidosis (Solaro *et al.*, 1986, 1988). Their results suggested that the difference in pH sensitivity was due to a difference in either TnI or TnT (rather than TnC).

It has been argued that the intracellular acidosis accompanying early ischemia is not sufficient to explain the mechanical dysfunction (Jacobus *et al.*, 1982; Weiss *et al.*, 1984; Allen & Orchard, 1987) especially if one only considers the effect of pH_i on the myofilaments. Ischemia also results in decreases of creatine phosphate and ATP levels and increases of inorganic phosphate (PO_4) levels. While decreasing [ATP] (as MgATP) increases myofilament sensitivity, it also decreases the maximum force (Best *et al.*, 1977). Kentish (1986) demonstrated that the increase in PO_4 which accompanies the decline in high energy phosphates (ATP and creatine phosphate) during ischemia can dramatically decrease both Ca sensitivity and maximum force. The overall effect seems to be that the increase in PO_4 and to a somewhat lesser extent the decrease in pH_i in ischemia combine to produce a dramatic decrease in myofilament Ca sensitivity, which plays an important role in the early decline in mechanical function.

Increasing the free [Mg] also decreases myofilament Ca sensitivity (Fabiato & Fabiato, 1975b; Best *et al.*, 1977). Increasing ionic strength (but not osmolality) decreases both Ca sensitivity and maximum force of the myofilaments (Kentish, 1984). Imidazoles (e.g. carnosine and *N*-acetyl histidine) which are naturally occurring in cardiac muscle cells (O'Dowd *et al.*, 1988) increase myofilament Ca sensitivity (Harrison *et al.*, 1985). These compounds are chemically related to caffeine, which is often used in the study of cardiac muscle Ca metabolism because it can deplete the SR of Ca. However, caffeine and other methylxanthines are potent myofilament Ca sensitizers (Wendt & Stephenson, 1983; Fabiato, 1981b). These compounds (e.g. 3-isobutyl-1-methylxanthine) are also phosphodiesterase inhibitors, but the effect on the myofilaments is not mediated by cyclic AMP.

A number of newer inotropic agents are thought to function in part via their ability to inhibit cardiac phosphodiesterases and thereby elevate cyclic AMP (e.g. amrinone, milrinone, pimobendan and sulmazole). However, some of the newer inotropic agents also increase myofilament Ca sensitivity, notably pimobendan (or AR-L 115-BS, Fujino *et al.*, 1988), sulmazole (or UDCG-115-BS, Solaro & Rüegg, 1982), isomazole (Lues *et al.*, 1988), BM 14.478 (Freund *et al.*, 1987), MCI-154 (Kitada *et al.*, 1987), DPI 201-106 (Scholtysik *et al.*, 1985) and perhexiline & bepridil (Silver *et al.* 1985), but not milrinone (Fujino *et al.*,

TABLE 6

Factors which alter Cardiac Myofilament Ca Sensitivity

Factor	ref	Factor Change	Sensitivity Direction	$\Delta pCa_{\frac{1}{2}}$	Maximum Force Direction	Δ(%)
Temperature	a	36 → 22°C	dec	0.18	dec	16
		22 → 8°C	dec	0.20	dec	43
Sarcomere Length	b	2.0 → 2.35μm	inc	0.21	inc	34
	c	1.75 → 2.05μm	inc	0.25	inc	36
pH	d	7.0 → 6.6	dec	0.34	dec	10
pH	e	7.0 → 6.5	dec	0.29	dec	12
PO_4 (pH 7)	f	0 → 20mM	dec	0.37	dec	69
MgATP (1mM Mg)	g	0.03 → 4mM	dec	0.3	dec	31
Mg (4mM MgATP)	g	0.05 → 1mM	dec	0.4	inc	21
Mg (3mM MgATP)	h	0.3 → 3mM	dec	0.33	-	-
Ionic Strength (Γ)	i	0.1 → 0.2M	dec	0.3	dec	17
TnI Phosphorylation	j	0 → ~50%	dec	0.45	-	-
Carnosine	k	0 → 15mM	inc	0.2	inc	3
Caffeine	l	0 → 20mM	inc	0.32	dec	6
Sulmazole (AR-L115 BS)	m	0 → 1mM	inc	0.2	inc	7
Isomazole	n	0 → 1mM	inc	0.1	inc	~15
Pimobendan (UDCG-115-BS)	o	0 → 50μM	inc	~0.2	nil	

a) Harrison & Bers, 1989a
b) Hibberd & Jewell, 1982
c) Kentish *et al.*, 1986
d) Fabiato & Fabiato, 1978a
e) Blanchard & Solaro, 1984
f) Kentish, 1986
g) Best et al, 1977
h) Fabiato & Fabiato, 1975b
i) Kentish, 1984
j) Mope *et al.*, 1980
k) Harrison *et al.*, 1985
l) Wendt & Stephenson, 1983
m) Rüegg, 1986
n) Lues *et al.*, 1988
o) Fujino *et al.*, 1988

1988). Thus, the action of these drugs on the myofilaments contributes to their inotropic effect.

Ray and England (1976) and Solaro *et al.* (1976) demonstrated a cyclic AMP-dependent phosphorylation of cardiac TnI in response to β-adrenergic stimulation. This phosphorylation induced a decrease in myofilament Ca sensitivity in intact ventricular muscle(Okazaki *et al.*, 1990) and in skinned fibers which could be mimicked by cyclic AMP (McClellan & Winegrad, 1978; Holroyde *et al.*, 1979; Mope *et al.*, 1980; Herzig *et al.*, 1981). Phosphorylation of TnI (and the shift in Ca sensitivity) could also be reversed by cyclic GMP or cholinergic agonists (Mope *et al.*,1980; Horowits & Winegrad, 1983). For β-adrenergic stimulation to produce its well known inotropic effect, the amplitude of the intracellular Ca transient must more than compensate for the reduced myofilament Ca sensitivity (e.g. by enhancement of Ca-current and SR Ca release, see Chapters 4, 6 and 9).

The decline in myofilament Ca sensitivity induced by β-adrenergic stimulation is accompanied by an increased off-rate of Ca from TnC. In principal this could contribute to the faster relaxation of contractions observed in the presence of β-agonists. However, McIvor *et al.* (1988) demonstrated that the myofilament desensitization was not necessary for faster relaxation. That is, the acceleration of relaxation could be attributed entirely to the other actions of isoproterenol (e.g. increased SR Ca uptake rate, secondary to cyclic AMP dependent phosphorylation of phospholamban, Kirchberger *et al.*, 1974; Tada *et al.*, 1974; Lindeman & Watanabe, 1985). This could be true even though phosphorylation of TnI appears to occur at lower isoprenaline concentrations than phosphorylation of phospholamban (Karczewski *et al.*, 1990).

One of the myosin light chains (known as LC2, the 18,000 dalton, P-light chain or regulatory light chain) can also be phosphorylated by a Ca-calmodulin dependent protein kinase (Pires *et al.*, 1974). The phosphorylation of this regulatory light chain appears to be crucial in the activation of smooth muscle contraction (Aksoy *et al.*, 1976; Walsh *et al.*, 1983). However, the physiological role of this phosphorylation in cardiac muscle is not clear (England, 1984; Gevers, 1984). Phosphorylation of this regulatory light chain by myosin light chain kinase increases the Ca sensitivity of the myofilaments in skinned pig ventricular muscle (Morano *et al.*, 1985), but decreases the rate of myosin crossbridge cycling (Franks *et al.*, 1984). Silver *et al.* (1986) demonstrated that there are frequency dependent changes in the degree of phosphorylation of myosin light chains in rabbit ventricular muscle. Thus, while phosphorylation of myosin light chains occurs and modifies the acto-myosin ATPase, the details of this action and its physiological importance have not been unequivocally established.

Other myofilament sites may also be modified by phosphorylation (e.g. see Solaro *et al.*, 1987) and other factors which I have not discussed. Nevertheless, one simple conclusion is quite clear. The control of the myofilaments by $[Ca]_i$ is very complex. Furthermore, the simplifying assumption often used (e.g. in subsequent chapters) that the myofilaments respond to a given $[Ca]_i$ in a direct and simple black box manner is incomplete. However, such implicit assumptions make it considerably easier to discuss the equally complicated area of $[Ca]_i$ regulation in relation to cardiac muscle function. One must retain the perspective that the relationship between $[Ca]_i$ and contraction is not fixed and it is contraction which is the physiological function of cardiac muscle cells. Indeed, even when cellular cardiac physiologists talk about contraction, we usually mean quasi-isometric contractions in papillary muscles (or muscle strips) or unloaded contractions in single isolated myocytes rather than the more complicated *in vivo* contractions. Again, the simplifications made in the name of reductionist science must at least be borne in mind.

CHAPTER 3

POSSIBLE SOURCES AND SINKS OF ACTIVATOR CALCIUM

Ca REQUIREMENTS FOR THE ACTIVATION OF CONTRACTION

In order to consider the potential sources of Ca which could, in principle, contribute to the activation of myofilaments, we must first consider how much Ca is required. The Ca binding of the cardiac myofilaments and of homogenized ventricular muscle were measured by Solaro *et al.* (1974) and Pierce *et al.* (1985), respectively. Fabiato (1983) also calculated the buffering capacity based on many estimates of the Ca buffering site concentrations and affinities (e.g. for troponin C, calmodulin, phosphocreatine, ATP, outer SR surface, and the inner sarcolemmal surface). The results of these measurements and calculations are shown in Figure 19 (in units of μmol Ca/kg wet wt.). To convert these values to the units of μmol/liter cell water (excluding mitochondrial space), the values can simply be multiplied by 2.509 (Fabiato, 1983). Fabiato's curve is higher than the data of Solaro *et al.* (1974), as would be expected due to the consideration of additional cellular buffers. Fabiato's curve is lower than the data of Pierce *et al.* (1985). This might also be expected since Pierce *et al.* (1985) measured buffering in whole homogenate which may include additional sites, both appropriately (i.e. intracellular sites not included by Fabiato, 1983) and inappropriately (e.g. extracellular sites and non-myocyte sites). It may also be noted that the values from Pierce *et al.* (1985) are differences in total Ca based on an unknown basal amount bound at 0.25 μM Ca, taken as 0. Since this basal number is near diastolic [Ca] and we are mostly interested in changes in bound Ca associated with increases in [Ca] and activation, this *per se* is not a serious limitation.

In Figure 20, I have graphed the relationship between total Ca and tension based on Fabiato's (1983) estimations and indicated the free [Ca] which corresponds to certain points on the curve. For example, if resting free [Ca] is 125 nM (corresponding to about 4 μmol/kg wet wt.), an additional 30 μmol of Ca/kg wet wt. would need to be supplied for 30% of maximal force to be reached (and 50 μmol/kg wet wt. for 70% maximal force). Under normal experimental conditions, at 25 to 30°C, it seems probable that twitch contractions only reach about 30% of maximum force (Fabiato, 1981a; Harrison and Bers,

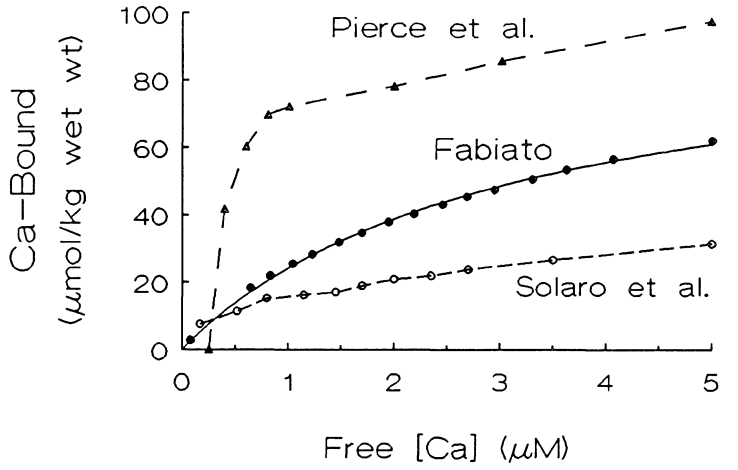


Figure 19. Ca buffering in cardiac muscle cells. The data are for Ca binding to isolated canine cardiac myofilaments (Solaro *et al.*, 1974), homogenized rabbit ventricular muscle (Pierce *et al.*, 1985) and calculations based on compiled binding data for troponin C, calmodulin, phosphocreatine, ATP and the cytoplasmic surface of the SR and sarcolemma (Fabiato, 1983). The results of Fabiato are conveniently described by the indicated single rectangular hyperbola (Ca bound = $95.65/\{1+(2.93\times10^{-6}/[Ca])\}$).

1989a). It may be expected that the fraction of maximal force reached at higher temperatures (e.g. 37°C), will be considerably smaller due to the hypothermic inotropy which is prevalent in cardiac muscle. That is, contractile force during a twitch increases up to 500% upon reducing temperature from 37°C down to 25°C, despite a decrease in the Ca sensitivity and maximal force developed by the myofilaments (Shattock and Bers, 1987; Harrison and Bers, 1989a). Thus, while the numbers may be somewhat imprecise with respect to the complexities of intracellular Ca buffering, they give us a rough estimate that 30-50 μmol of Ca/kg wet wt. must be supplied to the myofilaments to support a normal contraction. It is interesting that Alpert *et al.* (1989) arrived at a similar value (52 μmol/kg wet wt) by assuming that 87% of the tension-independent heat (or creatine phosphate consumption) in rabbit ventricular muscle was attributed to Ca pumping by the SR. This also emphasizes the energetic investment the cell makes in Ca regulation.

The forgoing discussion indicates that we do not know with quantitative precision how much Ca must be supplied during a cardiac contraction. This is further complicated by questions about the *in vivo* Ca sensitivity of the myofilaments (see page 29). Thus, the Ca which must be added to the cytoplasm to activate a cardiac contraction could vary even more widely (e.g. ~ 20-100 μmol/kg wet wt) and this is further complicated by kinetic considerations (Robertson *et al.*, 1981).

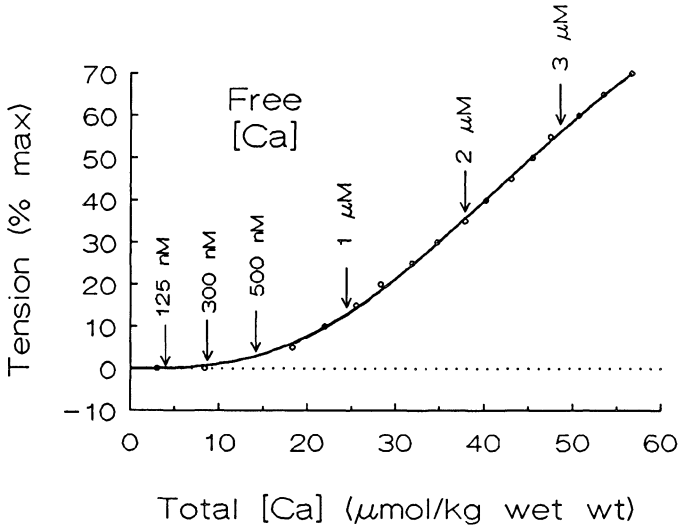


Figure 20. Dependence of cardiac tension on total cytosolic [Ca] including the intracellular Ca binding sites as in Fig 19 and based on the values assumed by Fabiato (1983). The arrows indicate certain associated free [Ca] concentrations along the curve. The smooth curve is fit to calculated values (Tension = $128/\{1+(73742/[\text{Ca-total}]^{2.82})\}$), where [Ca-total] is in μmol/kg wet wt. from Fabiato's data. Note that 1 μmol/kg wet wt. = 2.5 μmol/liter accessible cell volume (i.e. non-mitochondrial).

There is also some quantitative question about what the *normal* resting free [Ca] is in cardiac muscle (probably ~75-200 nM) and how high $[Ca]_i$ goes during a *normal* contraction (~0.5-3 μM, see e.g. Blinks, 1986). This is largely due to questions about the *in vivo* calibration of the Ca_i indicators which have been used (Ca microelectrodes, aequorin, quin2, fura-2 and indo-1). For example, Ca microelectrodes can overestimate $[Ca]_i$ because of imperfect impalements, fura-2 and indo-1 may underestimate $[Ca]_i$ because of binding of the indicator to intracellular constituents (Konishi *et al.*, 1988). The situation is even more problematic when cells are loaded with these indicators by incubation with their acetoxymethylester (AM) forms (rather than direct intracellular application of the free acid form). In this case, the cellular signals are complicated by the fluorescence of incompletely de-esterified forms of the indicator (Lückhoff, 1986) and intracellular compartmentalization of the indicator (e.g. ~50 % may be trapped in the mitochondria, Spurgeon *et al.*, 1990). With the other questions above, this places limitations on the quantitative detail with which we can currently understand intracellular Ca regulation.

Another consideration relevant to the potential contribution of cellular sites to Ca fluxes are the rates at which they can supply Ca to and remove Ca from the cytoplasm.

The peak of the Ca_i transient in mammalian cardiac muscle at ~30°C is generally reached in ≤ 30 msec. Thus, the rate of Ca rise in the cytoplasm must be ≥ 1 mmol/kg wet wt/sec (30 μmol/kg wet wt/30 msec). The rate of Ca removal from the cytoplasm might be about 10-fold slower, i.e. ~ 100 μmol/kg wet wt/sec. These very rough estimates are given just to indicate the general magnitudes that might be expected of sources or processes which contribute importantly to the Ca movements underlying contraction.

We can consider which cellular sites may have sufficient Ca to activate contraction (i.e., which sites are potential candidates from a quantitative perspective). One can readily calculate that the extracellular space which constitutes approximately 30% of the tissue volume (see Chapter 1), could supply more than enough Ca (2 mmol/l ECS × 0.3 l ECS/kg wet wt. = 600 μmol/kg wet wt.). Clearly, this would be more than enough Ca to activate contraction, but it would have to cross the sarcolemma as Ca influx. This estimate assumes that the total Ca concentration in the extracellular space is the same as that in the bulk solution. However, this is clearly not the case in the interstitial space where fixed negative charges are prevalent (see Chapter 1). Indeed, we have previously measured low affinity Ca binding to the external surface of the cardiac sarcolemma which appears largely bound to phospholipids (Bers & Langer, 1979; Philipson *et al.*, 1980). Isolated cardiac sarcolemmal vesicles bind approximately 80 nmol Ca/mg sarcolemmal protein and this can be extrapolated to approximately 700 μmol/kg wet wt. in the intact tissue under physiological ionic conditions (Bers & Langer, 1979). Thus, in principle there is more than enough Ca even bound along the sarcolemmal surface to fully activate contractions. Again, the critical question lies in the regulation of Ca influx (see below).

The inner surface of the sarcolemmal membrane (i.e. facing the cytoplasm), can also bind significant quantities of Ca under physiological intracellular conditions (Bers *et al.*, 1986). In isolated sarcolemmal vesicles Bers *et al.* (1986) and Mansier & Bers (1986) estimated this to be approximately 30 to 40 μmol/kg wet wt. at pCa 6.5. Thus, while these values for the inner sarcolemmal surface are an order of magnitude smaller, they are still of a magnitude which may be relevant to activation of myofilaments. In addition, the cytoplasmic surface of the SR and calmodulin and other soluble proteins, may each contribute ~30-50 μmol/kg wet wt to the total intracellular Ca buffering (based on values in Fabiato, 1983).

The Ca content of the SR has been estimated by numerous investigators and values vary (in μmol Ca/kg wet wt.): 170 (Solaro and Briggs, 1974); 100 to 300 (Dani *et al.*, 1979); 125 (Hunter *et al.*, 1981); ~160 (Levitsky *et al.*, 1981); >57 (Fabiato, 1983); 100 to 200 (Bers *et al.*, 1989). The general consensus, then, is that there is more than enough Ca in the SR to support a single contraction. Finally, the mitochondria have a tremendous capacity for accumulation of Ca (Carafoli & Lehninger, 1971). Nevertheless, there is a significant question as to the Ca content in the mitochondria under normal resting physiological conditions (Carafoli, 1987). However, because of the tremendous capacity of

the mitochondria as a Ca sink, their potential role in the supply of Ca per contraction, must be considered.

It may be useful at this point to note that the same sites considered above as potential Ca sources are also potential Ca sinks. The important question to be addressed from here is the mechanism by which Ca is transported to and from these sources and sinks.

Ca TRANSPORT ACROSS THE SARCOLEMMA

Ca in the extracellular space (including that bound to low affinity sites at the outer sarcolemmal surface and interstitium), must cross the sarcolemmal membrane. The two main routes by which Ca is known to enter the cell are by voltage-dependent Ca channels (or pores) and the Na/Ca exchange countertransport system. Since there is a very large electrochemical gradient favoring Ca entry at rest (e.g. ~200 mV) any type of leak could provide an additional route of Ca entry. Ca current will be discussed in Chapter 4.

There are also two mechanisms known to contribute to the efflux of Ca across the sarcolemma. These are the Na/Ca exchange and the sarcolemmal ATP-dependent Ca-pump (or Ca-ATPase). It may be pointed out at this stage that during any steady-state condition, the Ca influx and Ca efflux across the sarcolemma must be exactly the same over each cardiac cycle. Otherwise a net gain or loss of cell Ca will occur and a steady-state condition will not exist. The sarcolemmal Na/Ca exchange and Ca-pump will be discussed in more detail in Chapter 5.

Ca TRANSPORT BY THE SARCOPLASMIC RETICULUM

The SR has a well characterized ATP-dependent Ca-pump (or Ca-ATPase) that is distinct from the sarcolemmal Ca-pump. This is the main route by which Ca is accumulated by the SR. In the last few years it has become increasingly clear that Ca is released from the SR into the cytoplasm via a channel (known as the SR Ca-release channel). This protein also appears to be the ryanodine receptor and the foot protein observed ultrastructurally (see Chapter 1). The location and function of this protein suggest a critical role for the foot-protein/SR Ca-release channel in excitation-contraction coupling. These mechanisms of SR Ca transport will be discussed in more detail in Chapters 6 and 7.

GENERAL SCHEME OF Ca CYCLE IN CARDIAC MYOCYTE

It seems clear that transsarcolemmal Ca influx and SR Ca release play dominant roles in the rise of $[Ca]_i$ which activates contraction in the heart. It is useful at this point to

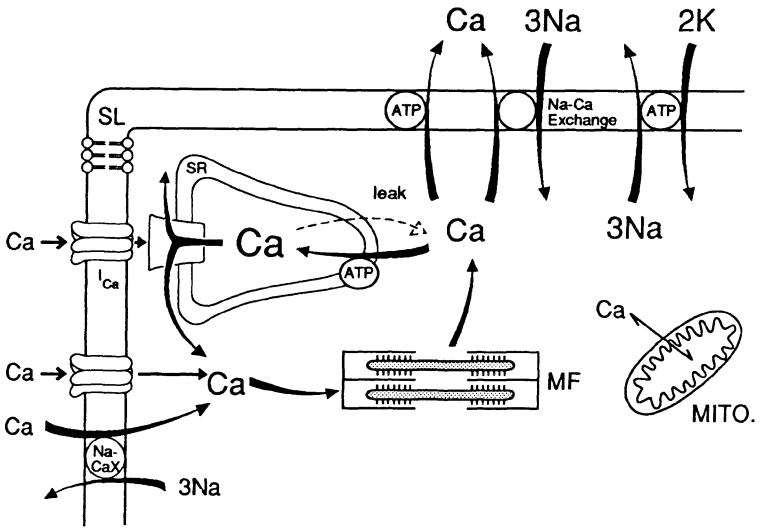


Figure 21. General scheme of Ca cycle in a cardiac myocyte. Ca can enter via Ca channels and Na/Ca exchange. Ca current may also control the SR Ca release by the SR Ca release channel/ ryanodine receptor/ foot protein. Ca is removed from the myofilaments (MF) and cytoplasm by the SR Ca-ATPase pump and the sarcolemmal Ca-ATPase pump and Na/Ca exchange.

present a simple working model of cellular Ca fluxes which can serve as a background for further discussion (Fig. 21).

During the cardiac action potential, Ca may enter the cell via sarcolemmal Ca channels (possibly more than one type) and there might also be Ca entry via Na/Ca exchange. This Ca which enters the cell may contribute directly to the activation of the myofilaments. Ca entry may also be involved in the activation of SR Ca release. This is the well known Ca-induced Ca-release process described in detail by Fabiato (1985a,b,c, see Chapter 7). Whether sarcolemmal Ca channels are, in fact, located directly over the SR Ca-release channel has not yet been proven, but enough indirect evidence (see Chapters 6 and 7) consistent with this arrangement makes it reasonable to include it in this working model. Whether it is Ca entry via this sarcolemmal Ca channel *per se* or some electro-mechanical coupling between the sarcolemmal Ca channel protein and the SR Ca-release protein is more controversial, especially in skeletal muscle and this will be discussed in Chapter 7. In any event, the myofilaments are activated by the combination of Ca influx and SR Ca release.

For relaxation to occur, Ca must be removed from the cytoplasm such that Ca will dissociate from TnC. At least three processes are involved in removing Ca from the cytoplasm. Ca may be transported either into the SR by the SR Ca-pump or out of the cell by either the sarcolemmal Ca-pump or Na/Ca exchange. These three transport systems are thus in competition for cytoplasmic Ca (see Chapter 5).

A small leak of Ca out of the SR into the cytoplasm which may be important at rest is also indicated in Fig. 21. Whether or not this leak actually occurs via the SR Ca-release channel is not important here, where the point is simply that there must be some finite rate at which Ca leaks down its ~10,000-fold concentration gradient (~1mM inside SR: ~100 nM in cytosol). This Ca in the cytoplasm is then subject to the competing Ca transport systems above. If all the Ca were re-accumulated by the SR there would be no net loss of SR Ca. However, if some of that Ca leaked by the SR is extruded from the cell by Na/Ca exchange or the sarcolemmal Ca-pump, it will represent a net loss of Ca from the SR and the cell. This is probably the basis for the cardiac muscle phenomenon known as rest decay (Allen *et al.*, 1976; Bers, 1985), wherein most mammalian cardiac muscle preparations exhibit smaller post-rest contractions after longer rest intervals (see Chapters 5, 6 and 8). Thus, the resting muscle may not be at a true steady-state with respect to Ca fluxes for a rather long time.

A similar type of leak must exist at the sarcolemma, since the electrochemical gradient between the extracellular space and cytoplasm is even greater than between the SR and cytoplasm (due to the negative membrane potential). Again, it matters little whether this leak is via sarcolemmal Ca channels or some less specific route. One can readily appreciate that such a Ca entry could shift the balance of cellular Ca fluxes.

It should be realized that the ability of the sarcolemmal membrane to maintain the intracellular Ca content within specific limits is absolutely essential. Even a small imbalance would lead to progressive gains or losses of cellular Ca which could overload the cell (and SR) or deplete the cell (and SR) of Ca. The intracellular compartments can only buffer the changes in cell Ca driven by the sarcolemma. In addition, the intracellular buffers are in a closed system and consequently are of finite capacity. In the following sections, I will discuss the largest potential buffering compartment for intracellular Ca (the mitochondria) and an interesting non-membrane bounded buffering site (the inner sarcolemmal surface). Both of these types of sites must be considered in a comprehensive model of cellular Ca fluxes.

MITOCHONDRIAL Ca TRANSPORT

Isolated mitochondria can accumulate very large amounts of Ca (Lehninger *et al.*, 1967; Carafoli & Lehninger, 1971), particularly in the presence of inorganic phosphate due to the precipitation of insoluble Ca-phosphate deposits known as matrix loading (Carafoli,

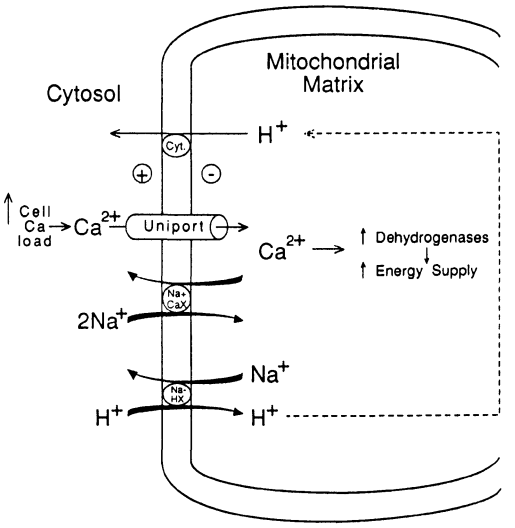


Figure 22. The Ca cycle across the inner mitochondrial membrane. Ca enters via a uniport, down an electrical gradient established by the proton pump. Ca can be extruded by a Na/Ca antiport and Na is extruded by Na/H exchange thereby completing the cycle. Elevated cytoplasmic [Ca] can lead to elevated mitochondrial [Ca] and increased activity of mitochondrial dehydrogenases.

1987). A typical mitochondrial Ca uptake of 100 nmol/mg mitochondrial protein would correspond to 7000 μmol Ca/kg wet wt. (assuming ~70 mg mitochondrial protein/g wet wt, Scarpa & Graziotti, 1973) and can store ~20 times more (Carafoli, 1975). While this is a potentially enormous capacity, it appears that under physiological conditions anticipated *in vivo*, mitochondria are likely to contain 1-2 nmol Ca/mg protein (70-140 μmol/kg wet wt, Carafoli, 1987). In a situation perhaps closer to *in vivo* conditions, Fry *et al.* (1984a) showed that mitochondrial Ca uptake was not appreciable in hyperpermeabilized isolated cardiac myocytes until cytoplasmic [Ca] exceeded 1 μM. Their estimates can be extrapolated to give similar values as above in isolated mitochondria. This is still a relevant amount with respect to the requirements of myofilament activation.

Figure 22 illustrates the Ca cycle of the inner mitochondrial membrane (e.g. see Crompton, 1985). Ca enters via a uniport system down a large electrochemical gradient (~-180 mV) set up by proton extrusion linked to the passage of electrons down the respiratory chain. This uniporter is blocked competitively by physiological $[Mg]_i$ (Nicholls & Ackerman, 1982) and also potently by ruthenium red (Moore, 1971) and lanthanides (Mela, 1969; Reed & Bygrave, 1974). Ca entry via the uniport pathway exhibits a sigmoid dependence on [Ca] and under physiologic ionic conditions has a K_m > 30 μM for Ca. Thus, at the $[Ca]_i$ associated with the cardiac cycle (0.1-1 μM) the influx pathway will be at a relatively low level. The ability of mitochondria to accumulate Ca led Lehninger (1974)

and Carafoli (1975) to speculate that mitochondria may be involved in removing Ca from the cytoplasm during cardiac relaxation. Harrison and Miller (1984) demonstrated a fast phase of relaxation of a caffeine-induced contraction in skinned rat ventricular muscle which was sensitive to ruthenium red and azide. They inferred that the mitochondria could accumulate sufficient Ca to be quantitatively important in cardiac relaxation. However, in intact cardiac muscle Bers and Bridge (1989) showed that when both the SR Ca uptake and sarcolemmal Na/Ca exchange were inhibited, relaxation was slowed by more than an order of magnitude and was often incomplete. Thus, it is unlikely that mitochondria can compete effectively with the SR Ca-pump and sarcolemmal Na/Ca exchange under physiological conditions and probably do not contribute quantitatively to normal cardiac relaxation.

The main route of Ca extrusion from the mitochondria is via an electroneutral (i.e. 2:1) Na/Ca exchange (or antiport, Crompton *et al.*, 1976). The stoichiometry of this exchanger could also be > 2 Na:Ca (Crompton, 1985). The [Na] dependence of this Na/Ca antiporter is sigmoidal with half-maximal Ca extrusion at ~5-8 mM Na which means that this system will be quite sensitive to changes of intracellular [Na] in the physiological range (Crompton *et al.*, 1976; Fry *et al.*, 1984b). While variations in bulk cytoplasmic [Na] during the cardiac cycle are probably insufficient to cause rapid release of mitochondrial Ca, large changes in [Na] could induce substantial mitochondrial Ca release *in vitro* (Crompton *et al.*, 1976). The mitochondrial Na/Ca antiporter is particularly active in heart, somewhat less in skeletal muscle and least active in certain non-excitable cells (e.g. liver and renal cortex; Crompton *et al.*, 1978; Crompton, 1985). There is also a Na-independent extrusion of Ca from mitochondria which is less prominent in heart, but more so in tissues lacking the Na/Ca antiport activity (e.g. liver and kidney; Crompton, 1985). The inner mitochondrial membrane also has an active Na/H exchange system (Mitchell & Moyle, 1967) which may be the means by which Na is extruded from the matrix and also completes the cycle. In this way the energy for Ca extrusion via Na/Ca exchange depends also on the proton movement during respiration and the consequently negative intramitochondrial potential.

While there was early interest in the potential role of mitochondria in *mediating* cardiac Ca fluxes during the contraction-relaxation cycle (e.g. Lehninger, 1974; Carafoli, 1975), emphasis has shifted around the other way in recent years. That is, mitochondrial Ca fluxes may be working to regulate intramitochondrial processes. There are three mitochondrial matrix enzymes which are activated by Ca in the low μM range (pyruvate dehydrogenase, α-oxoglutarate dehydrogenase and the NAD-dependent isocitrate dehydrogenase, Denton & McCormack, 1980, 1985, 1990; Hansford, 1985, 1987). Thus, increases in mitochondrial Ca via the above mechanisms would occur when cytosolic [Ca] is relatively high and the energy demands are also high (e.g., contractile activation and Ca pumping). In this way, the rise in cytoplasmic (and mitochondrial) [Ca] can increase oxidative metabolism and thereby increase ATP production to meet increased demands. A potentially interesting twist on this is that cellular Ca loading is often secondary to cellular

Na loading, via sarcolemmal Na/Ca exchange (e.g. when the Na-pump is inhibited by digitalis). In this case the increase of mitochondrial Ca which *could* stimulate oxidative metabolism may be minimized by the elevation of $[Na]_i$ which would deplete the mitochondria of Ca. Thus, energy supply may not go up to meet demands and the cytoplasmic Ca load will be more severe. This might favor more force production, but could also elevate diastolic $[Ca]_i$ and compromise cardiac relaxation.

Several recent estimates have indicated that under physiological conditions there is probably only a small [Ca] gradient across the inner mitochondrial membrane, with internal [Ca] being slightly lower than cytoplasmic [Ca] (Moreno-Sanchez & Hansford, 1988; McCormack *et al.*, 1988). Based on the trans-mitochondrial potential (-180 mV) Ca would be at equilibrium when mitochondrial [Ca] is 10^6 times the cytoplasmic value (i.e. 100 mM-1 M!). Ca is thus far from equilibrium and considerable energy is required to extrude Ca from mitochondria up this electrochemical gradient. While the [Na] gradient may be the immediate source of energy, the Na gradient is created by the proton gradient. Thus the true energy source is respiration and the protonmotive force it generates.

It should be noted that mitochondria use the same pool of energy to phosphorylate ADP to ATP as to drive Ca uptake (i.e. the protonmotive force). Energized, isolated mitochondria have been shown to take up Ca rather than make ATP (Vercesi *et al.*, 1978). This would obviously be a dangerous situation *in vivo*, but it appears that at physiological $[Mg]_i$ the uniport is inhibited strongly enough that mitochondrial energy is preferentially used to make ATP (Sordahl, 1975).

It is well known that heart mitochondria can accumulate massive amounts of Ca under pathological conditions such as ischemia (Reimer & Jennings, 1986) and this may serve as an important safety device for heart cells. Cellular Ca overload is a common early component of cell injury in many cell types (Shanne *et al.*, 1979) and could quickly become disastrous in heart cells since high cytosolic [Ca] would keep energy consumption by the myofilaments and Ca-ATPases high (not to mention triggered arrhythmias). Sustained contracture could also worsen the situation by decreasing local blood flow by vascular compression. If the mitochondria can temporarily compensate for a cellular Ca load by taking up large amounts of Ca, permanent cell damage might be avoided. Unfortunately it is a double-edged sword, since Ca accumulation by the mitochondria diminishes ATP production and eventually compromises the mitochondria. Thus the survival of the cell might depend on whether the mitochondria can survive a given degree of Ca loading.

While it seems clear from the forgoing that mitochondrial Ca transport is important in the regulation of intramitochondrial dehydrogenases and in coping with cellular Ca overload, its relevance to the normal contraction-relaxation cycle is not so clear. An extremist point of view would be that the mitochondria respond passively to the fluctuations in cytoplasmic Ca during the cardiac cycle. But with the capacity of the mitochondrial Ca transport one must consider how even such a "passive response" might

affect the cytoplasmic Ca transient. Crompton (1985) has modeled the response of cardiac mitochondria to the phasic changes in Ca during the cardiac cycle. For a cytoplasmic [Ca] change from ~200 nM to ~2 μM and back, intramitochondrial [Ca] increased from ~1.03 to 1.05 μM and according to the buffering capacity assumed for the mitochondria this would correspond to ~0.02 nmol/mg mitochondrial protein or ~1.4 μmol/kg wet wt. This is a relatively small amount in relation to the Ca movements involved in the activation of contraction at these $[Ca]_i$ values (~5%, see Fig. 20). This value for mitochondria is rather small mainly because of the relatively slow kinetics of the transport processes, since at a steady state $[Ca]_i$ of 2 μM the mitochondrial $[Ca]_i$ would be very much higher (Crompton, 1985).

In conclusion, it seems that mitochondria play a very minor role in Ca movements during contraction and relaxation. However, with slower increases in "mean" cytoplasmic [Ca] the mitochondrial Ca transport may play a critical role in increasing metabolism to meet increased metabolic demands or in more severe cellular Ca overload may store massive amounts of Ca to protect the cytoplasm from very high Ca levels.

Ca AT THE INNER SARCOLEMMAL SURFACE

Lüllman and Peters (1977, 1979) proposed that Ca bound to specific sites at the inner sarcolemmal surface could play an important role in the activation of the myofilaments. They proposed the existence of membrane potential (E_m)-sensitive Ca binding sites on the inner sarcolemmal surface (Fig. 23a). They suggested that negatively charged phospholipids, (e.g. phosphatidylserine) served in this role. Post *et al.* (1988) recently demonstrated that the negatively charged phospholipids (phosphatidylserine and phosphatidylinositol) comprise 7.2% of the sarcolemmal phospholipids and are exclusively on the inner sarcolemmal leaflet. Using their value for total phospholipid content (1.4 μmol/mg sarcolemmal protein) this amounts to 100 nmol/mg sarcolemmal protein which could participate in Ca binding at the inner sarcolemmal surface. Further extrapolation to whole tissue (assuming 120 mg homogenate protein/g tissue and 10-20-fold sarcolemmal purification) indicate that this could amount to as much as 500-1000 μmol/kg wet wt.

According to the model of Lüllman and Peters (1977, 1979) these sites would possess a high affinity (K_d ~ 10 nM) at resting E_m and be almost fully saturated even at diastolic $[Ca]_i$ levels (~100 nM). In this model (Fig. 23a) depolarization increases the proton activity within the sarcolemma in the vicinity of the internal high affinity binding sites. This decrease of local pH would decrease the affinity of the sarcolemmal Ca binding sites and Ca would be liberated to the cytosolic medium in an amount that could activate the myofilaments. The processes would be reversed during relaxation and the binding sites would regain their high affinity for Ca. From a biophysical standpoint this is an intriguing hypothesis.

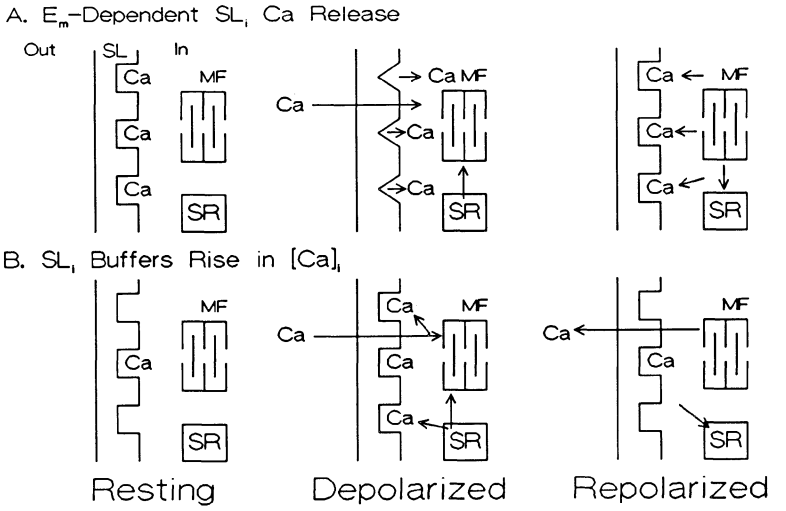


Figure 23. Two possible roles of high affinity Ca binding sites on the inner sarcolemmal surface during a cycle of depolarization and repolarization. A. According to Lüllmann & Peters (1977, 1979) depolarization would decrease the affinity of these sites causing Ca release (and rebinding upon repolarization). B. Membrane sites may serve mainly to buffer the rise in [Ca] which occurs due to other processes during the contraction.

Another role which high affinity sites on the inner sarcolemmal leaflet could play is as a simple buffer of intracellular [Ca] changes (Fig. 23b). At diastolic $[Ca]_i$ levels these sites may be partially saturated. Upon depolarization internal [Ca] rises and these sarcolemmal sites would become more fully saturated. In this way these sites would simply add to the cellular Ca buffering capacity.

Mansier and Bers (1984) measured Ca binding to isolated cardiac sarcolemmal vesicles which were largely inside-out in their orientation over a free [Ca] range from 100 nM to 100 μM and estimated two apparent K_d values (100 nM and 56 μM). They were, however, unable to demonstrate any E_m-dependence to the measured Ca binding and the total number of sites could not be well measured. Frankis and Lindenmayer (1984) and Bers *et al.* (1986) using different sarcolemmal vesicle preparations measured ~8 - 25 nmol Ca binding/mg sarcolemmal protein at 300-5000 nM free [Ca].

Bers *et al.* (1986) also measured a small, but significant decrease in Ca binding (2 nmol/mg sarcolemmal protein) to isolated cardiac sarcolemmal vesicles at pCa = 6.5 upon membrane depolarization from -80 to 0 mV (expressed in cellular orientation), using KCl gradients and the K-selective ionophore valinomycin to establish E_m. They extrapolated this value to whole tissue (8 μmol/kg wet wt.) and indicated that this might be a lower limit,

since only sealed vesicles can sustain an E_m. If the fraction of leaky vesicles (37%) was corrected for, this value would increase to 13 μmol/kg wet wt. In the sarcolemmal preparation used only about 20% of the vesicles were inside-out and sealed, so if these E_m-sensitive sites were only exposed in inside-out vesicles they could amount to ~40 μmol/kg wet wt. (or higher, since the binding assay as used tends to underestimate Ca binding).

Such internal sarcolemmal Ca binding sites could in principle release Ca to the myoplasm upon depolarization, but the normal rise in $[Ca]_i$ which occurs during the contraction would tend to **increase** Ca bound to these sites. Thus, in the experiments of Bers *et al.* (1986), the decrease in Ca bound due to depolarization from -80 to 0 mV at pCa = 6.5 was more than offset by an increase in Ca binding at pCa = 5.3. Although the mixed population of vesicle orientation makes such extrapolations equivocal, it may be that depolarization simply decreases the ability of these sites to buffer increases in $[Ca]_i$. Physiological $[Na]_i$ (5 - 15 mM) and $[Mg]_i$ (0.5 - 3 mM) also decrease Ca binding in these sarcolemmal preparations (Frankis & Lindenmayer, 1984; Bers *et al.*, 1986). Akera *et al.* (1976) estimated that the [Na] at the inner sarcolemmal surface (in a compartment near the sarcolemma, 0.1% of cell volume) could vary over tens of mM due to Na channel current and hence modulate the (Na+K)-ATPase during the cardiac cycle. Although such local functional [Na] changes may not be that large, they might occur and would tend to displace Ca from the aforementioned sarcolemmal Ca sites (or might also enhance Ca entry via Na/Ca exchange).

Experiments with intact cells provide convincing evidence against a quantitative role for these inner sarcolemmal Ca binding sites in excitation-contraction coupling. When extracellular Ca is removed very quickly (< 1 sec) so that intracellular [Ca] and stores are not appreciably altered, depolarization does not produce measurable contraction or rise in $[Ca]_i$ (Rich *et al.*, 1988; Näbauer *et al.*, 1989). Thus, in the absence of Ca influx, depolarization does not lead to a significant Ca release from anywhere. In the end then it would seem that the inner sarcolemmal sites may serve mainly as additional sites for intracellular Ca buffering.

INTRACELLULAR Ca BUFFERING

Our more recent estimates of Ca buffering by sites on the inner sarcolemmal surface at physiological $[Ca]_i$ (Bers *et al.*, 1986 and above) are considerably higher than used by Fabiato (1983) in his model of intracellular Ca buffering (Figs 19 & 20). At that time Fabiato had to extrapolate from our measurements at much higher [Ca] (K_d = 100 μM, B_{max} = 447 μmol/kg wet wt, Bers & Langer, 1979; Bers *et al.*, 1981) which did not allow identification of higher affinity sites which may be more relevant at physiological [Ca]. Table 7 shows affinities, numbers of total sites and estimated amounts of Ca bound to these various sites at both pCa 7 and pCa 6 (selected to approximate diastolic and peak $[Ca]_i$ during contractions) and the difference (Δ). Both of the values of inner sarcolemmal

Ca binding are indicated along with other values included by Fabiato (1983). Passive Ca binding to the outer surface of mitochondria are also included. These sites are of low affinity, but the large mitochondrial surface area makes them rather plentiful. Higher affinity mitochondrial Ca binding sites had also been reported (Carafoli & Lehninger, 1971), but those values may be incorrect, due to incomplete deenergization of the mitochondria (Ackerman *et al.*, 1974).

The inclusion of these additional binding sites increases the apparent buffer capacity of the intracellular milieu from the values reported by Fabiato (1983) closer to the values measured by Pierce *et al.* (1985, see Fig 19). For example, as indicated in Table 7, increasing $[Ca]_i$ from 0.1 μM to 1 μM would require the addition of 81 vs 21 μmol/kg wet wt. This range bespeaks the imprecision with which the true intracellular Ca buffering power is currently known. Converting these values for the cytoplasmic volume, Ca buffer capacities of 53 - 200 μM/pCa unit are obtained. These values are considerably lower than estimates of cellular pH buffering in mammalian cardiac muscle (20-90 mM/pH unit, Ellis & Thomas, 1976; Wallert & Fröhlich, 1989; Bountra *et al.*, 1990). This may be relevant because intracellular acidosis can increase $[Ca]_i$ probably by competing with Ca at intracellular binding sites (Bers & Ellis, 1982) and changes in $[Ca]_i$ can lead to intracellular acidosis (Vaughan-Jones *et al.*, 1983).

The manner of extrapolation of Ca binding to whole tissue deserves some comment. In extrapolation of our Ca binding to isolated sarcolemmal vesicles we divide by the purification factor (i.e. the amount by which sarcolemmal marker enzymes are enriched). This purification is typically ~30-fold which gives a value of ~1 mg sarcolemmal protein/30 mg homogenate protein. This factor *is* clearly the correct way to extrapolate from the material which we have isolated and call sarcolemma, but this fraction is not purely sarcolemma (i.e. there is some contamination). It is commonly assumed that ~2% of the cell protein is plasma membrane and thus the limiting purification would be 50-fold. Thus the numbers I have quoted above for sarcolemmal Ca binding might overestimate true sarcolemmal Ca binding by ~60%. Nevertheless, it might be reasonable, in the present context, to include the component due to unknown contamination which might not otherwise be tabulated. Fabiato (1983) used the assumption that there are 2.1 mg of SR protein/g wet wt (Levitsky *et al.*, 1981). With 120 mg homogenate protein/g wet wt, this would imply a 60-fold purification, which seems plausible. If anything, this might underestimate the relative SR Ca binding, since there is more SR membrane surface than sarcolemma membrane in mammalian ventricular muscle (see table 1). Scarpa and Graziotti (1973), estimated that there are ~80 mg mitochondrial protein/g wet wt of heart. This would imply that mitochondria could only be purified ~1.5-fold (assuming 120 mg protein/g wet wt). On the other hand, it is simple to find ~3-fold enrichment of mitochondrial markers in a crude fraction. Thus, the content of mitochondrial Ca binding sites above might be overestimated by as much as a factor of 2. Again the bottom line is that the numbers for cytoplasmic Ca buffering are not known with great precision.

TABLE 7

Passive Intracellular Ca Buffers

	K_d (μM)	Ca-Bound (μmol/kg wet weight) B_{max}	at pCa=7	at pCa=6	ΔCa
Inner Sarcolemma [a]	0.18	64	22	54	32
Outer SR [b]	1	19	1.7	9.5	8
Outer Mitochondria [c]	100	3500	3.5	35	31
Troponin C [b]	2	28	1.3	9.3	8
Calmodulin total [bde]	0.1-1	10	0.2	1.2	1
ATP [be]	200	1196	0.05	0.5	0.5
Phosphocreatine [be]	71,400	4780	~0.01	~0.1	0.1
Total of above	--	--	29	110	81.6
Inner Sarcolemma [b]	100	447	0.4	4.4	4
Outer SR [b]	1	19	1.7	9.5	8
Troponin C [b]	2	28	1.3	9.3	8
Calmodulin total [bde]	0.1-1	10	0.2	1.2	1
ATP [be]	200	1196	0.05	0.5	0.5
Phosphocreatine [be]	71,400	4780	~0.01	~0.1	0.1
Total [bf]	--		3.7	25	21.6

[a] Bers *et al.*, 1986 and unpublished data.
[b] Fabiato, 1983; where values were compiled from several sources
[c] Carafoli & Lehninger, 1971; Langer *et al.*, 1982
[d] Calmodulin results are from four classes of binding sites which also exhibit specific affinities for Mg and K and these characteristics were accounted for using the constants compiled by Fabiato, 1983 and Haiech *et al.*, 1981.
[e] Binding was calculated assuming [K] = 140 mM and [Mg] = 1 mM.
[f] Includes the lower sarcolemmal binding and not the mitochondria surface.

A further complication in considering the role of these buffering sites in the cell during the normal cardiac cycle is that [Ca] is constantly changing and the kinetics and spatial distribution of these sites becomes important. Robertson *et al.* (1981) presented a kinetic model for Ca binding in cardiac and skeletal muscle including binding to troponin (Ca-specific and Ca-Mg sites), myosin, calmodulin and parvalbumin (for skeletal). At the current limit of resolution, it appears that cellular [Ca] changes relatively uniformly during a normal contraction in the cell, although there is spatial non-uniformity during spontaneous contractions in Ca-overload (Wier *et al.*, 1987) and presumably at the microscopic level around the mouths of Ca channels (e.g. Simon & Llinás, 1985).

This chapter has provided a general framework and some quantitative estimates regarding some of the processes which are involved in the regulation of intracellular Ca in cardiac muscle cells. This should set the scene for a more detailed discussion of some of

these key mechanisms and their interrelationships in the subsequent chapters. In the next chapter I will discuss Ca influx via Ca current. Chapter 5 will include discussion of Ca influx and efflux mediated by the sarcolemmal Ca-pump and Na/Ca exchange. Chapter 6 will focus on Ca uptake and release from the SR and Chapter 7 will examine the mechanism of E-C coupling. The final two chapters (8 & 9) will emphasize integrative aspects of how these processes may interact in the intact muscle cell. This should provide a clearer picture of how these processes interact under physiological conditions and also in different inotropic states.

CHAPTER 4

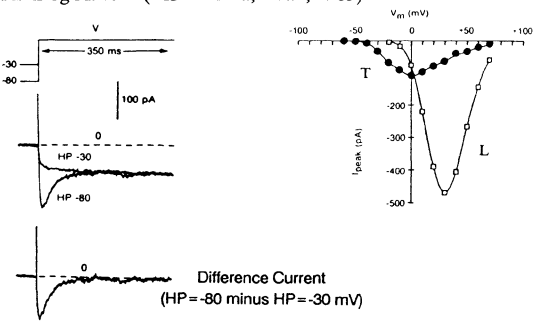
Ca INFLUX VIA SARCOLEMMAL Ca CHANNELS

Since the time of Ringer (1883) it has been known that extracellular [Ca] is important in cardiac muscle contraction. Cardiac Ca currents were first characterized as "slow inward current" or I_{si}, since several tens of msec were required for peak current to be achieved (e.g. Rougier *et al.*, 1969; Mascher & Peper, 1969; Beeler & Reuter, 1970; Ochi, 1970). Since the advent of the patch-clamp technique it has become clear that Ca current (I_{Ca}) can reach a peak value in ~2-3 msec after a depolarization (e.g. see Fig. 24). Thus the moniker of slow inward current seems inappropriate, although some have retained the moniker I_{si} and consider it the *secondary* inward current in deference to Na current which activates still faster than I_{Ca}. The inward I_{Ca} during the normal cardiac action potential contributes to the action potential plateau and is also involved in the activation of contraction (directly and/or indirectly).

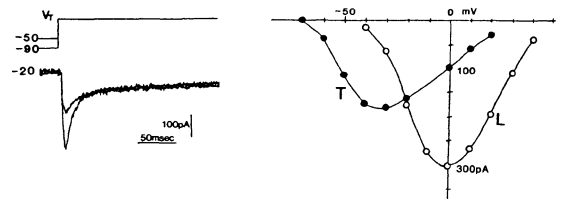
Ca CHANNEL TYPES

Hagiwara *et al.* (1975) were probably the first to demonstrate two classes of Ca channels in a single cell type. Nowycky *et al.* (1985) characterized three types of Ca channels in dorsal root ganglion cells, giving them names which have been generally adopted in the present nomenclature. L type Ca channels are characterized by a Large conductance (~25 pS in 110 mM Ba), Long lasting openings (with Ba as the charge carrier), sensitivity to 1,4-dihydropyridines (DHPs) and activation at Larger depolarizations (i.e. at more positive E_m). T type channels are characterized by a Tiny conductance (~8 pS), Transient openings, insensitivity to DHPs, and activation at more negative E_m. N type Ca channels are Neither T nor L, are predominantly found in Neurons, and are intermediate in conductance and voltage dependence. This classification is useful, but is clearly an oversimplification since a spectrum of Ca channel types with more subtle differences is now emerging from results in different cell types, even among channels considered to be L type. For example, ω-conotoxin strongly inhibits neuronal L type Ca channels, but not cardiac or skeletal muscle L type channels (McCleskey *et al.*, 1987). In addition the activation and inactivation kinetics in skeletal muscle L- type channels are ~10-fold slower than in cardiac muscle (Bean, 1989).

A. Dog Atrium (115 mM Ba; Bean, 1985)



B. Dog Purkinje Fiber (2 mM Ca; Hirano et al., 1989)



C. Several Species (Total I_{Ca})

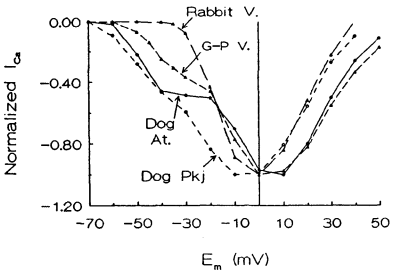


Figure 24. Voltage-dependence of whole cell Ca current in several cell types illustrating two types (L & T) of Ca channels. **A.** Ba currents (with 115 mM Ba) induced by depolarizations to various test potentials from holding potentials (HP) of -80 or -30 mV. The E_m protocol is shown in the top trace, the currents in the middle trace and the difference between these currents in the bottom panel. The peak I_{Ba} activated from -30 mV is attributed to L-type Ca channels (□) and the additional transient difference current activated from -80 mV (•) is attributed to T-type Ca channels (from Bean, 1985, with permission). **B.** Similar protocol to A., but in dog Purkinje fiber cell and with 2 mM Ca as charge carrier (from Hirano *et al.*, 1989, with permission). The shifts along the voltage axis can be ascribed to differences in surface potential in 2 mM Ca *vs* 115 mM Ba (see Fig. 27 and page 58). **C.** Total I_{Ca} from several species (HP = -80, -90 or -100 mV), where the hump at ~-40 mV is due to T-type current and differs among the tissues studied. Dog Purkinje (from Hirano *et al.*, 1989) and rabbit ventricle (G.M. Briggs & D.M. Bers, unpublished) are in 2 mM Ca and dog atrium (from Bean, 1985) and guinea-pig ventricle (from Mitra & Morad, 1986) are with 5 mM Ca, but are shifted by -10 mV to compensate for surface potential differences.

TABLE 8

Properties of Cardiac Ca^{2+} Channel Types

	L	T
Activation range		
5 mM Ca^{2+}	Positive to -30 mV	Positive to -60 mV
110 mM Ba^{2+}	Positive to -10 mV	Positive to -50 mV
Inactivation		
Range	Positive to -40 mV	-90 to -60 mV
Ca^{2+} dependent	Yes	No
Voltage dependent	Slow	Fast
Tail deactivation	Fast	Slow
Conductance		
110 Ba^{2+}	25 pS	8 pS
110 Ca^{2+}	8 pS	8 pS
150 Na^{+}, EDTA	80 pS	50 pS
Mean open time	Typically < 1 ms	Short, 1-2 ms
Kinetics (Ba)	Multiple bursts/pulse	1 burst per pulse, (inactivation)
Pharmacological sensitivity:		
Dihydropyridines	Yes	No
Cd^{2+}	High	Low
Ni^{2+}	Low	High
Isoproterenol	Yes	No
Excised patch	Loses Activity	Retains Activity

This tabulation is from Hess (1988) with values mostly obtained at room temperature.

Cardiac muscle appears to contain both L and T type Ca channels (e.g. see Fig. 24 & Table 8), but not N type channels (Bean, 1985, 1989; Nilius *et al.*, 1985). T channels appear to be quite prominent in certain atrial cells (e.g. canine, Bean, 1985) and Purkinje cells (Hirano *et al.*, 1989), but are less prominent in most ventricular cells (e.g. guinea-pig). T channel current also seems to be absent in bullfrog ventricle and both ventricle and atrium of calf and rabbit (Bean, 1989, Gonzalez-Rudo *et al.*, 1989). The functional significance of the T channels is not known, though they might be involved in pacemaker activity (Hagiwara *et al.*, 1988).

Since L channel inactivation is Ca dependent (Kokubun & Irisawa, 1984, Lee et al, 1985) L type I_{Ca} is transient and the kinetics can be confused with T type I_{Ca}. Therefore, L and T channels can be best distinguished when Ba is the charge carrier since L channel inactivation is then rather slow (e.g. Fig 24A). Where T current is prominent, it can also be resolved by the difference in E_m-dependence (Fig 24B). In these cell types the current-voltage relationship (from holding potentials of -80 or -90 mV) also shows a prominent "shoulder" at negative E_m, particularly when compared to tissues with less prominent T current (Fig 24C).

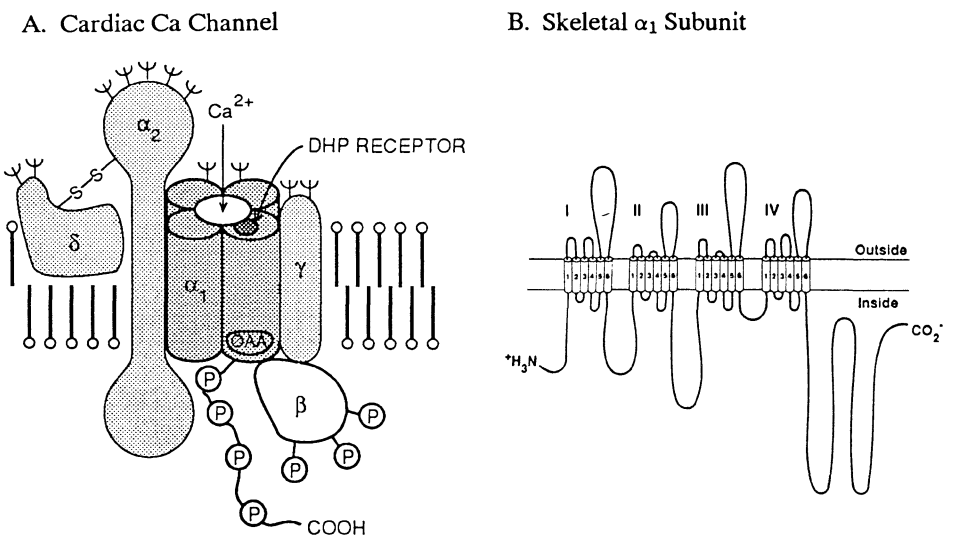


Figure 25. **A.** Schematic diagram of the subunit structure of the cardiac L-type Ca channel, indicating sites of potential phosphorylation (P), glycosylation, ligand binding and disulfide links (based on a diagram generously supplied by M. Hosey). **B.** Diagram of the α_1 subunit of the skeletal muscle L-type Ca channel showing the 4 homologous transmembrane domains (I-IV), each containing 6 probable membrane spanning α-helices (S1-S6) (from Catterall, 1988, with permission).

The L type Ca channel appears to be responsible for the vast majority of Ca entry in ventricular muscle. It is interesting that the preponderance of L over T type I_{Ca} is in cell types which have more extensive T-tubules (see Table 1). This might be expected if in heart (as in skeletal muscle) most of the L type Ca channels are in T-tubular membrane (see pg 3). Unless otherwise specified, when Ca channels are discussed below, the L type Ca channel will be the default assumption for cardiac myocytes.

BIOCHEMICAL CHARACTERIZATION OF L TYPE Ca CHANNELS

L type Ca channels are often characterized by their sensitivity to DHPs. Most DHPs act as Ca channel blockers or antagonists (e.g. nifedipine, nisoldipine and nitrendipine), while some DHPs act as Ca channel agonists (e.g. (-)Bay K 8644, but not (+)Bay K 8644). The agonist agents appear to greatly prolong the open time of the channel (see Fig. 30). The DHP-sensitivity of L type Ca channels and high density of DHP receptors in skeletal muscle T-tubules has helped allow extensive biochemical characterization of this channel, which has contributed to our understanding of the cardiac channel (Fig 25). The L type channel has been isolated from skeletal muscle (Curtis &

TABLE 9

Biochemical Characterization of the L-type Ca

	α_1 (sk)	α_1 (card)	α_2 (sk)	α_2 (card)	β (sk)	γ (sk)	δ (sk)
Mass (kDa)							
Mobility							
Reduced	~170	~190	140	140	52-65	30-33	24-33
Non-reduced	~170	~190	165	170	52-65	30-33	-
DNA-Sequence	212	243	125	ND	52	26	ND
Stoichiometry	1	ND	1	ND	1	1	1
Disulfide Links	-	-	to δ	to δ	-	-	to α_2
Membrane Domains							
Transmembrane	++	++	+		-	+	+
Intracellular	++	++	?		++	?	-
Extracellular	++	++	++		-	++	+
Glycosylation	-		++		-	+	+
Phosphorylation	+		-		+	-	-
Dihydropyridine site	+	+	-	-	-	-	-
Phenylalkylamine site	+	+					

Compiled after Campbell *et al.*, 1988 and results from other references cited in the text. (sk = skeletal, card = cardiac.)

Catterall, 1984; Borsotto *et al.*, 1984; Flockerzi *et al.*, 1986) and is composed of 5 subunits (α_1, α_2, β, γ and δ, see Fig. 25 and Table 9). The α_1, α_2, β and γ subunits of the skeletal channel have been sequenced (Tanabe *et al.*, 1987; Ellis *et al.*, 1988; Ruth *et al.*, 1989; Jay *et al*, 1990; Bosse *et al.*, 1990) as well as the α_1 subunit of the cardiac channel (Mikami *et al.*, 1989). The α_1 subunit appears to bear the main known functional characteristics of these channels. That is, the α_1 subunit bears the DHP, phenylalkylamine and benzothiazepine receptors (Galizzi *et al.*, 1986; Sharp *et al.*, 1987; Sieber *et al.*, 1987; Vaghy *et al.*, 1987; Striessnig *et al.*, 1990) is phosphorylated by cAMP- and Ca-calmodulin-dependent protein kinases (Curtis & Catterall, 1985; Hosey *et al.*, 1986, 1987; Imagawa *et al.*, 1987; Nastainczyk *et al.*, 1987) and protein kinase C (O'Callahan *et al.*, 1988) and also exhibits E_m-dependent Ca current when a cell line lacking the other subunits was transfected with complementary DNA coding for the α_1 subunit (Perez-Reyes *et al.*, 1989). Co-expression of the cardiac α_1 and skeletal α_2 subunits in *Xenopus* oocytes increased the I_{Ca} measured in comparison to the cardiac α_1 subunit alone (Mikami *et al.*, 1989), suggesting a possible modulatory role for other subunits. At the moment, the function of the other subunits is not known.

The cardiac α_1 subunit is larger than the skeletal muscle protein by ~20 or 30 kDaltons (Chang & Hosey, 1988; Mikami *et al.*, 1989) and this could explain some of the functional differences in cardiac *vs* skeletal I_{Ca}. The molecular weights of both purified α_1

subunits are also substantially lower than indicated by the amino acid sequences. The differences could be due to proteolysis *in vivo* or during purification.

There is striking homology between the α_1 subunit of the DHP receptor and the tetrodotoxin-sensitive Na channel (Tanabe *et al.*, 1987; Noda *et al.*, 1986). The similarity is particularly notable in that both proteins appear to have four internal homologous repeated units (I-IV) or domains, each of which contains six α-helical putative membrane spanning segments (S1-S6). These four domains are thought to be arranged in a square array around a central pore which could serve as the ion channel (see Fig. 25, Noda *et al.*, 1984; Tanabe *et al.*, 1987). Each S4 transmembrane span also bears a positively charged amino acid (arginine or lysine) at almost every third position with intervening nonpolar amino acids. These positively charged S4 spans may serve as the voltage sensor for the channel and be involved in regulation of channel gating (Catterall, 1986, 1988). A screwlike rotation of each S4 span (60° or 5Å outward) could result in the net transfer of 1-2 net charges through the membrane field. This could provide the apparent 6 charges/Na channel measured as gating current (Armstrong, 1981). The apparent conservation of channel structures should accelerate the elucidation of the molecular basis and regulation of channel gating and ion transport.

Ca CHANNEL SELECTIVITY AND PERMEATION

McCleskey and Almers (1985) determined that the narrowest point in the Ca channel pore must have a diameter of ~6 Å since it is permeable to tetramethylammonium. Thus, the Ca channel must exert selectivity by means other than exclusion by size. Hagiwara *et al.* (1974) observed that Ba current through Ca channels was greater than Ca current, but that the Ba current was more susceptible to block by Co. They concluded that this was due to competition at a binding site within the channel with a weaker affinity for Ba than for Ca, such that Co would prevent Ba current more effectively. This also explains the higher control Ba *vs* Ca current. Since Ba doesn't "stick" in the channel as much, it can go through more quickly.

Hess *et al.* (1986) assessed the selectivity of cardiac Ca channels by measuring reversal potentials of I_{Ca} in asymmetric salt solutions (see Table 10). They also reported single channel conductances for the permeant ions, which shows, for example, that Na current through the Ca channel is very large and as might be expected Na does not compete very well for a Ca-selective site in the pore. Indeed, Na current through cardiac and skeletal Ca channels is blocked by Ca with a $K_d \sim 1$ μM [Ca] (see Fig. 26 and Almers & McCleskey, 1984; Almers *et al.*, 1984, Hess & Tsien, 1984). Ca current, on the other hand, is not measured until nearly mM [Ca] and saturates with a K_d ~14 mM (Hess *et al.*, 1986). This disparity suggests that more than one Ca binding site is in the Ca channel.

TABLE 10

Selectivity of Cardiac L-type Ca Channel

Ion	P_{ion}/P_{Cs}	Single-channel Conductance (pS)†	Unhydrated Radius (Å)
Ca	4200.0	9.0	0.99
Sr	2800.0	9.0	1.13
Ba	1700.0	25.0	1.35
Li	9.9	45.0	0.60
Na	3.6	85.0	0.95
K	1.4	-	1.33
Cs	1.0	-	1.69
Mg	~0	-	0.65

*Selectivities as permeability ratios based on measurements of reversal potentials by Hess *et al.*, 1986 and values are from Tsien *et al.*, 1987. †From Hess *et al.*, 1986 with 110 mM divalent and 150 mM monovalent (the latter at pH 9.0 to limit proton block of the channel, Prod'hom *et al.*, 1987).

The anomalous mole fraction effect is a related phenomenon which is also strongly indicative of more than one site in the channel (though the conditions necessary to demonstrate this effect unequivocally may be restricted, Yue & Marban, 1987; Friel & Tsien, 1989). This effect is where the current through a Ca channel is paradoxically smaller in mixtures of Ca and Ba (at constant total [Ca]+[Ba]) than in either ion alone (Hess & Tsien, 1984).

These types of experiments led Almers & McCleskey (1984) and Hess & Tsien (1984) to propose a model for Ca channel permeation which includes 2 Ca selective sites in the permeation pathway (Fig. 26C). The potential energy ($\Delta G/RT$) for a Ca or Na ion as it goes through an unoccupied Ca channel are indicated by the solid (Ca) and dashed curves (Na). The two energy wells correspond to 2 Ca binding sites. Occupancy of one of the sites by Ca would change the energy profile (due to its two positive charges) such that a Na ion entering the other site would not dwell long due to electrostatic repulsion. Thus, Na current is blocked by μM [Ca]. Single occupancy by Ca would also decrease the affinity for Ca at the other site from $K_d \sim 0.5$ μM to ~10 mM If another Ca ion does enter the other site, the repulsive forces are strong, but there is a 50% chance that the first Ca ion will leave the pore (even at $E_m = 0$). With the usual strong inwardly directed electrochemical driving force for Ca entry ($E_{Ca} \sim +120$ mV), this will result in a rapid inward Ca current. In this way double occupancy of the channel sites overcomes the anticipated slow off-rate expected of a high affinity site and allows both rapid ion flux through the channel and high divalent cation selectivity. Indeed, the high Ca flux rates do not occur until mM [Ca], where double occupancy of the channel is expected with this model (see Fig. 26B). Yue

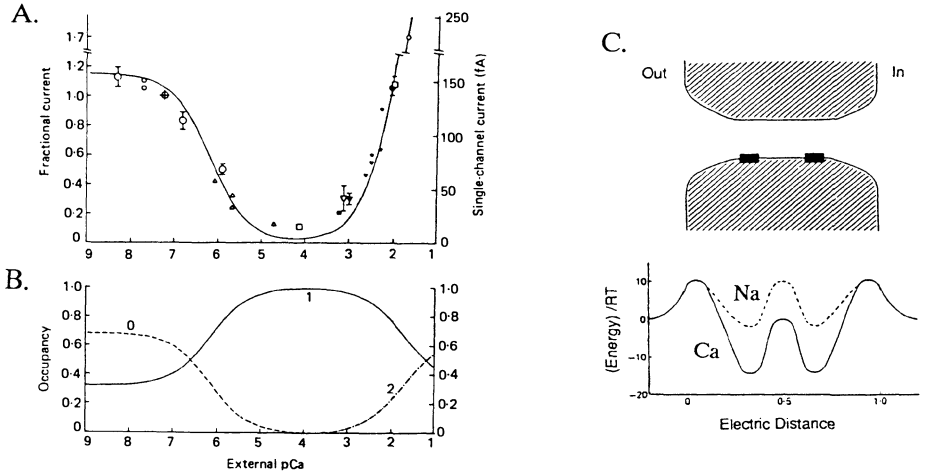


Figure 26. Ca channel permeation. **A.** Increasing $[Ca]_o$ blocks Na current through the Ca channel at $K_d \sim 1$ μM. At higher $[Ca]_o$ (mM) the current is carried by Ca ions. **B.** fractional occupancy of the Ca channel by none (0), one (1) or two (2) ions. Ca current increases when the channel is occupied by 2 Ca ions. **C.** Schematic diagram of the Ca channel, showing two binding sites and the energy profile for a single Ca or Na ion as they traverse the channel where the abscissa is the electrical distance across the membrane (from Almers & McLeskey, 1984, with panel C. as modified by Tsien *et al.*, 1987, with permissions).

and Marban (1990) have even suggested that more than 2 Ca binding sites in the permeation pathway may be required to explain certain Ca channel properties.

This sort of model is also consistent with the flickering block of Ca channels by protons (Prod'hom *et al.*, 1987) and certain multivalent cations (Lansman *et al.*, 1986). This flickering block may reflect transient binding of these competing ions to the sites in the pore and has allowed estimates of on- and off-rates. The block of Ca channels by other multivalent ions can also indicate the selectivity of the sites in the channel. Certain ions may also block the channel, but still pass through. The voltage dependence for the relief of block can indicate whether the ion can be "forced" through the channel, or must exit from the same side it entered (Lansman *et al.*, 1986). It is interesting that Mg can readily permeate skeletal muscle Ca channels, but is almost impermeant in the cardiac channel (Almers & Palade, 1981; Hess *et al.*, 1986). Extracellular Mg also depresses contraction and Ca current much more strongly in rat than rabbit ventricle (Shine, 1979; Hall *et al.*, 1990). In symmetrical solutions the Ca channel exhibits a very linear current-voltage relationship (Rosenberg *et al.*, 1986) indicating that the permeation pathway is functionally symmetrical. These characteristics and tissue differences place certain constraints on models of ion permeation such as above and may also help to develop more explicit molecular models.

NUMBERS OF Ca CHANNELS

Schwartz *et al.* (1985) estimated that there are 30 - 50 times as many specific DHP receptors in frog skeletal muscle as functional L type Ca channels. In isolated mammalian ventricular myocytes, the density of L type Ca channels has been estimated from single channel and whole cell I_{Ca} measurements to be 3-5/μm^2 (McDonald *et al.*, 1986; Tsien *et al.*, 1983). We typically measure specific DHP binding to isolated adult rabbit and ferret ventricular myocytes to be ~150 fmol/mg protein (as did Green *et al.*, 1985 and Kokubun *et al.*, 1986 in rat myocytes). In rabbit ventricular homogenate the receptor density is somewhat lower (~90 fmol/mg homogenate protein), but this value can be more directly extrapolated to a surface density. Assuming 120 mg protein/cm^3, 25% extracellular space and a surface to volume ratio of 0.6/μm (including T-tubules, Table 1), this would correspond to ~20 DHP receptors/μm^2. This is in the same range as estimates of Ca channel density based on non-linear charge movement in cardiac myocytes thought to represent gating of the Ca channel (3.7-5.5 nC/μF, Field *et al.*, 1988; Bean & Rios, 1989; Hadley & Lederer, 1989). Assuming that 6 elementary charges move across the membrane field when a channel opens, these "gating currents" would correspond to 37-57 channels/μm^2.

The estimate of Ca channel density (N) based on whole cell Ca current (I_{Ca}) density and the single channel current (i_{Ca}) uses the relationship $N = (I_{Ca}/(i_{Ca} \times p_o))$. The channel density calculated depends critically on the open channel probability (p_o). The value of p_o assumed from single channel records must represent the true mean open probability of all functional Ca channels. If the relatively high p_o (~0.8) used for the whole cell I_{Ca} by McDonald *et al.* (1986) was too high, the channel density estimated would be too low. It seems likely that p_o must be substantially lower than this because whole cell I_{Ca} can be increased several-fold in the presence of Bay K 8644 which is known to increase p_o (Hess *et al.*, 1984; Bean & Rios, 1989). Lew *et al.* (1991) found the density of DHP receptors to be the same as that of L-type Ca channels (~15/μm^2) in rabbit ventricular myocytes. They obtained an overall p_o = 0.03, which might more closely reflect the true mean open probability of all functional Ca channels (including those which are relatively inactive). Thus, there may not be any great discrepancy between the density of cardiac DHP receptors and L-type Ca channels. This might also be true for skeletal muscle Ca channels if the average p_o at the peak of the whole cell current is much less than 1.0 (the value assumed by Schwartz *et al.*, 1985)

Ca CHANNEL GATING

Cardiac I_{Ca} is rapidly activated by depolarization, reaching a peak in ~2-7 msec, depending on the temperature and E_m (see Fig. 24). Ca channel activation seems to depend primarily on E_m, but as for most voltage sensitive channels, is also sensitive to changes in surface potential (e.g. Wilson *et al.*, 1983; Hille, 1984). The surface potential

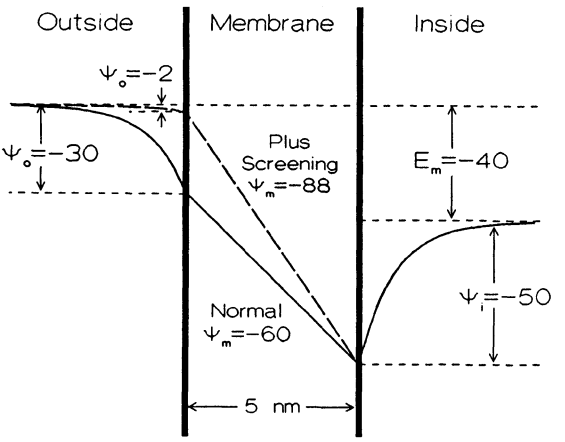


Figure 27. The influence of surface charge screening on the trans-bilayer potential (ψ_m). At higher extracellular divalent ion concentration, external surface charge is screened thereby decreasing ψ_o and making ψ_m more negative for the same E_m. Thus E_m must be more depolarized for the channel to sense the same ψ_m and become activated.

arises from fixed negative charges on the membrane surface (McLaughlin, 1977, 1989). Figure 27 illustrates the effect that changing surface potential (ψ_o and ψ_i) can have on the electric field within the membrane (ψ_m), which may be what the voltage sensors of the channel respond to. I have selected to consider the situation near the threshold of I_{Ca} activation (~E_m=-40) in a physiological medium (2 mM Ca and ~150 mM ionic strength) and used Gouy-Chapman theory of the diffuse double layer (Grahame, 1947). The values for the surface potentials are somewhat arbitrarily chosen, but would correspond to surface charge densities of ~1 elementary charge/ 100-300 Å^2 (which roughly corresponds to expected surface densities of acidic phospholipids and sugars). This is also similar to the value estimated by Kass & Krafte (1987) from divalent cation-induced shifts of Ca channel gating in cardiac Purkinje fibers (i.e. 1/250 Å^2). The ψ_i was assumed to be larger, since negatively charged phospholipids are preferentially on the inner sarcolemmal leaflet in cardiac muscle (Post *et al.*, 1988) and the divalent cation concentration is lower inside the cell than out. Divalent cations are especially effective in screening surface charge since they are concentrated by the negatively charged surface and may also bind to the negatively charged sites to neutralize them (McLaughlin *et al.*, 1981; Bers *et al.*, 1985).

Given these conditions, the "normal" state at E_m=-40 in Fig. 27 would correspond to a trans-bilayer potential of ψ_m=-60. If extracellular divalent cation concentration is increased such that the external surface potential (ψ_o) is nearly abolished then the trans-bilayer potential is considerably more polarized (ψ_m=-88) for the same E_m=-40. Thus a greater E_m depolarization will be required for the channel to "see" the same trans-bilayer field and hence threshold for activation. This is probably the reason the current-voltage

relationship with 115 mM Ba in Fig. 24A is shifted to more depolarized E_m compared with 2 mM Ca in Fig. 24B. This effect also readily explains the increased excitability of cells in low divalent cation solutions. That is, the threshold potential for channels would be shifted more negative (closer to the resting E_m).

The effect of the surface potential decays exponentially over a few Debye lengths (one Debye length ~ 1 nm). The surface potential has clear effects on channel gating, but might also alter Ca channel conductance by virtue of the surface Ca concentrating effect of the negative potential. However, Coronado & Affolter (1986) demonstrated that the single channel conductance was relatively insensitive to the surface potential. Thus, in contrast to the "gating sensor", the opening into the permeation pathway may be relatively shielded from ψ_o effects (due to elevation above the bilayer surface and/or a charge-free disc around the pore of about 20 Å).

Inactivation of Ca channels is time-dependent and also depends on both E_m and $[Ca]_i$ (Lee *et al.*, 1985; Kass & Sanguinetti, 1984; Hadley & Hume, 1987). The E_m dependence is subject to the same surface charge effects described above for activation (i.e. both activation and inactivation are shifted to more positive E_m at higher $[Ca]_o$, Hirano *et al.*, 1989). The Ca-dependence of inactivation is illustrated by the markedly slower inactivation when current through the Ca channel is carried by either Ba or Na instead of Ca (see Figs. 24 and 65). Hadley & Hume (1987) demonstrated that inactivation of monovalent cation current through the Ca channel was entirely E_m-dependent (although incomplete, Fig. 28A) and that there was an additional component of inactivation when Ca was the charge carrier. This additional component also had an E_m-dependence like the peak I_{Ca} (i.e. maximal at E_m ~ 0 mV).

The steady state activation and inactivation curves for I_{Ca} in cardiac Ca channels are typified by the results of Cohen & Lederer (1988) in Fig. 28B for both neonate and adult rat ventricle. The variable which reflects the activation of the Ca channel (d_∞) starts increasing at ~-30 mV and is half maximal at ~-5 mV. The inactivation variable (f_∞) begins decreasing at ~-40 and is half-complete at -20 mV in adult and -7 mV in neonate. The shaded area of overlap indicates that a steady state, non-inactivating component of I_{Ca} occurs and that this component is larger in the neonate. This non-inactivating current is known as the Ca "window" current and it can be appreciated that this window current is most significant around plateau levels of the action potential and is more prominent in the neonate than adult rat (as is the action potential plateau). Guinea pig ventricle also demonstrates a larger window current and action potential plateau than adult rat ventricle (Josephson *et al.*, 1984). It can also be seen that the main difference in adult rat ventricle in Fig. 28B is that the inactivation curve (f_∞) in the adult rat is shifted to more negative potentials than in the neonate. Cohen & Lederer (1988) also showed that the adult rat f_∞ curve became similar to the neonatal f_∞ curve when the SR Ca release in adult was inhibited by caffeine or ryanodine or when the $[Ca]_i$ transient was buffered by intracellular EGTA or BAPTA (all of which had little effect on the inactivation in the neonate). These

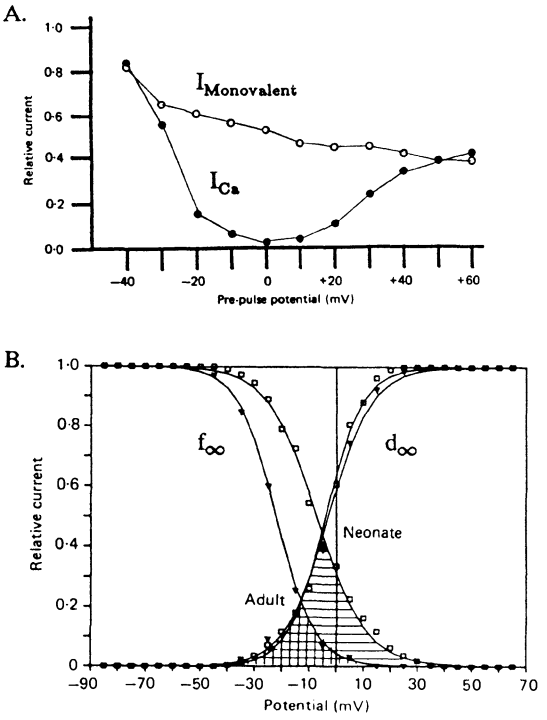


Figure 28. Inactivation of cardiac Ca channels. **A.** Peak monovalent cation and Ca current through Ca channels (at -10 mV) after 500 msec pulses to the indicated potentials in a guinea-pig ventricular myocyte. The $I_{monovalent}$ inactivation is attributed to voltage-dependence and the difference in the degree of inactivation with Ca is attributed to Ca-dependent inactivation (from Hadley & Hume, 1987, with permission). **B.** Steady state activation (d_∞) and inactivation (f_∞) of I_{Ca} in adult (▽) and neonatal (□) rat ventricular myocytes as described by Cohen & Lederer (1988, reproduced with permission). The shaded areas indicate potentials at which the channel activated and where inactivation is incomplete (i.e. a steady state I_{Ca} or Ca "window" current).

results indicate that SR Ca release and $[Ca]_i$ transients can be expected to have major effects on the I_{Ca} (i.e. SR Ca release can contribute to I_{Ca} inactivation). Furthermore, these effects may be expected to differ in species (or under conditions) where the SR Ca is more prominent (e.g. adult rat *vs* neonatal rat, guinea-pig or rabbit ventricle, see also Chapter 8).

The Ca-dependent inactivation of I_{Ca} appears to depend on Ca_i and as such may provide a sort of feedback control to limit further Ca entry. A remarkable feature of the Ca_i-dependent inactivation is that it is still readily apparent even when $[Ca]_i$ is fairly heavily buffered by EGTA or BAPTA, such that there is no sign at all of contraction. This suggests that Ca entering via the Ca channel must be exerting this inactivating effect,

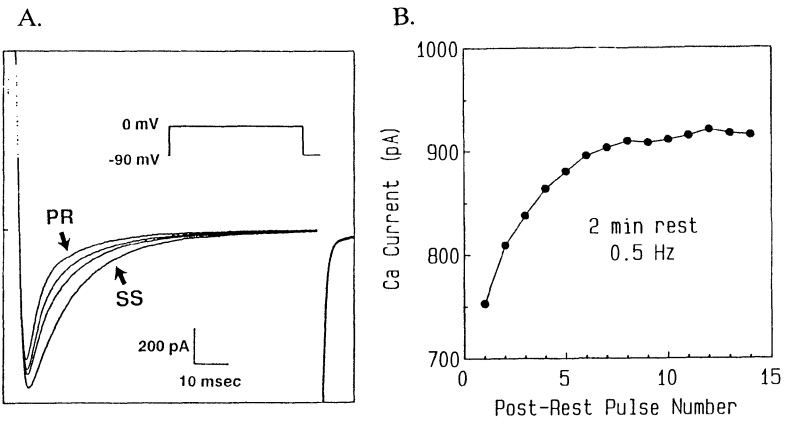


Figure 29. Recovery of I_{Ca} after a 2 min rest period in ferret ventricular myocyte. At left, the I_{Ca} traces at the first post-rest pulse (PR) and the second, third and steady state (SS) pulses are shown. The post-rest I_{Ca} "staircase" at 0.5 Hz is illustrated at right. It may be noted that when the holding potential is more depolarized (~-40 mV) a declining I_{Ca} "staircase" is observed (from L.V. Hryshko and D.M. Bers, unpublished).

locally and perhaps directly, at the channel, prior to mixing with the cytoplasmic Ca pool. This is consistent with the observation that the Ca_i-dependent inactivation is much faster than the intrinsically E_m-dependent component at $E_m \sim 0$ mV.

Intracellular Ca can also exert a potentiating effect on Ca channels which was first suggested by Marban & Tsien (1982) in multicellular preparations and more recently documented in isolated cardiac myocytes (Lee, 1987; Argibay *et al.*, 1988; Bers & Hess, 1988; Fedida *et al.*, 1988a,b; Tseng, 1988; Gurney *et al.*, 1989; Hryshko & Bers., 1990). This effect is readily demonstrable as an I_{Ca} "staircase" after a rest with voltage steps to ~0 mV from physiological holding potentials (-70 to -90 mV, Fig. 29). When trains are resumed with holding potentials about -40 mV such that a "negative staircase" of I_{Ca} is observed (Hryshko & Bers, 1990), the I_{Ca} staircase is probably obscured by the slow recovery of Ca channels from inactivation at -40 mV. The increase in I_{Ca} depends on Ca entry via the channel (i.e. it is not seen with Ba as the charge carrier) and is associated with a decrease in the rate of I_{Ca} inactivation. The I_{Ca} enhancement occurs over several pulses, suggesting a cumulative effect, yet it is still readily apparent when $[Ca]_i$ is buffered with 10 mM EGTA (though it can be suppressed by 20 mM BAPTA, a faster Ca chelator). It seems probable that Ca entry via Ca channels acts locally (such that intracellular EGTA does not readily prevent it), and that a second messenger is involved such as a phosphorylation of the channel (accounting for the cumulative effect over several pulses, Zygmunt & Maylie, 1990). At this point the possibility of a second messenger has not been directly evaluated.

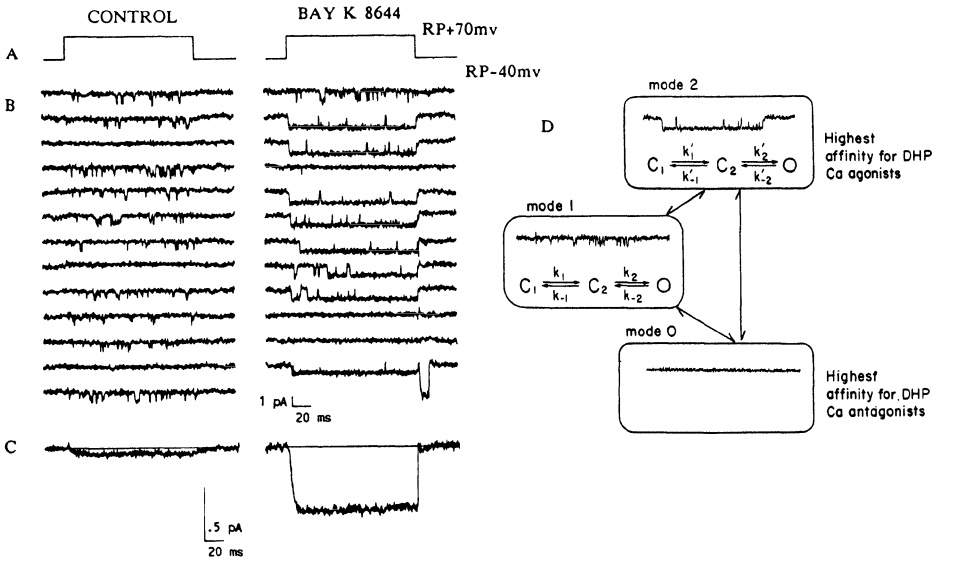


Figure 30. Effect of the Ca channel agonist Bay K 8644 on Ca channel gating from single channel patch clamp recordings. **A.** Voltage clamp protocol referenced to the "resting potential" (RP) ~-60 mV. **B.** Single sweeps in the absence (left) and presence (right) of 5 μM Bay K 8644. **C.** Average current from all single sweeps. **D.** Model of Ca channel gating "modes" proposed by Hess et al. (1984). The transitions between modes is slow compared to the gating within a mode (indicated as C_1, C_2 and O for two closed and one open state). (All panels are from Hess et al., 1984, with permission).

This facilitatory effect of Ca entry on subsequent I_{Ca} is kinetically distinct and can coexist with the Ca_i-dependent inactivation described above.

AMOUNT OF Ca ENTRY VIA Ca CHANNELS

Since it is clear that cardiac contraction depends on the extracellular [Ca], it is important to consider the amount of Ca entry via I_{Ca} with respect to the Ca requirements for myofilament activation. Peak I_{Ca} of about 1-2 nA is fairly typical in mammalian ventricular myocytes of about 30 pL volume. Accurate integration of the amount of Ca entry during a voltage clamp pulse is somewhat complicated by difficulty in assessing the component of the steady state current which is the non-inactivating (or window) Ca current. Using either a linear leak subtraction (using small hyperpolarizing pulses) or replacement of Ca with an impermeant ion (e.g. Co or Mg) to correct for leak current during 100-200 msec voltage clamp pulses to ~0 mV, I typically find values for integrated I_{Ca} which would add 15-25 μM Ca to the cell (e.g. similar to that reported by Isenberg, 1982). Correcting for 25% extracellular space, this corresponds to 10-18 μmol/kg wet wt. Estimates of Ca entry based on extracellular Ca depletions also yield similar values (Bers,

1983). According to Fabiato's (1983) estimates of intracellular Ca buffering (Fig. 20), this would only be sufficient by itself to raise $[Ca]_i$ from ~125 nM to ~500 nM and activate only about 4-5% of maximal force. Thus, it would seem that Ca entry via I_{Ca} would not normally be sufficient to activate cardiac muscle contractions. However, it should be noted that there are numerous quantitative assumptions in the calculations above which may prevent this conclusion from being unequivocal (e.g. intact vs skinned myofilament Ca sensitivity, see pages 29,34). Indeed, contractions in rabbit, guinea-pig and frog ventricle are only slightly depressed by agents known to block normal SR Ca release (ryanodine and caffeine, Sutko & Willerson, 1980; Sutko & Kenyon, 1983; Bers, 1985). Thus, it is not entirely clear to what extent Ca influx via I_{Ca} participates in direct activation of the myofilaments (or to the induction of SR Ca release and/or reloading). The interplay between these systems will be addressed in Chapter 8.

MODULATION OF CARDIAC Ca CHANNELS BY AGONISTS AND ANTAGONISTS

A hallmark of L type Ca channels is their sensitivity to DHPs (e.g. nifedipine, nitrendipine, nimodipine, nisoldipine, PN 200-110, Bay K 8644, azidopine and iodipine). Indeed, the specific binding of DHPs to the α_1 subunit of the L type Ca channel was important in the isolation of the protein. Most of these DHPs decrease I_{Ca} and are known as "Ca channel blockers" or "Ca channel antagonists." Some DHPs, notably (-) Bay K 8644, (+) S-202-791 and CGP 28392 known as "Ca channel agonists", greatly increase I_{Ca} by increasing the duration of single Ca channel openings, without altering the single channel conductance (Brown *et al.*, 1984; Hess *et al.*, 1984; Kokubun & Reuter, 1984; Kokubun *et al.*, 1986). Bay K 8644 can increase channel open times from ~0.5 msec to ~20 msec and Hess *et al.* (1984) suggested that Bay K 8644 binding to the Ca channel stabilized a state of the channel, called "mode 2" in which long stable openings occur (see Fig. 30). The channel can still switch to the normal state (mode 1) in the presence of Bay K 8644 where the openings are shorter and indistinguishable from the control conditions. The mode 2 type openings can also be seen under control conditions, but they are quite rare (Hess *et al.*, 1984; Yue *et al.*, 1990). Thus, Ca agonists can greatly increase the likelihood of these long lasting, mode 2 openings. Ca antagonists DHPs, on the other hand, inhibit I_{Ca} apparently by favoring a mode of channel gating (mode 0) characterized by the channel being unavailable to open. While this modal model of Ca channel gating is widely used, its applicability has recently been challenged (Lacerda & Brown, 1989).

Early experiments indicated that the affinity of cardiac microsomes for nitrendipine was some 1000 times higher than the concentrations required to block I_{Ca} (Bellemann *et al.*, 1981; Lee & Tsien, 1983). This was subsequently explained by the voltage dependence of DHP binding to Ca channels. For example Bean (1984) demonstrated that depolarization of E_m from -80 mV to -10 mV in ventricular myocytes decreased the

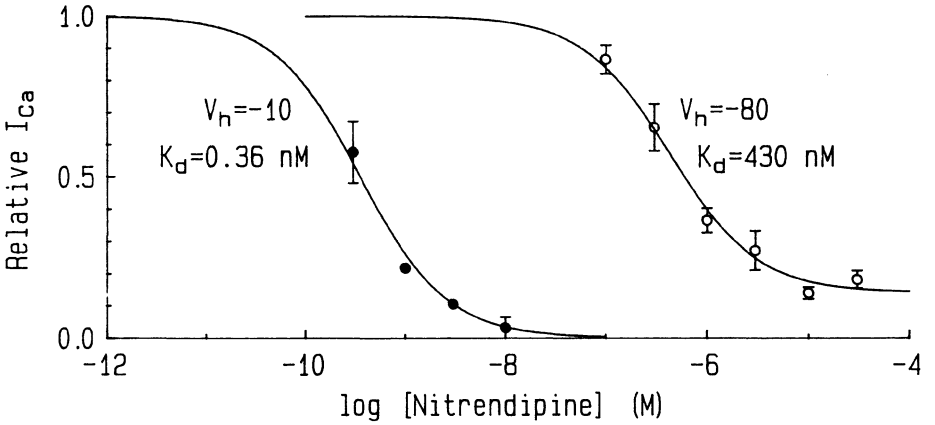


Figure 31. Different I_{Ca} blocking effectiveness of nifedipine in canine cardiac myocytes depends on holding potential (V_h). The apparent K_d for I_{Ca} block decreased by 1200-fold when V_h was depolarized from -80 to -10 mV (curves are fit to original data taken from Bean, 1984). Incomplete block when V_h = -80 might be due to a fraction of I_{Ca} which is not through L-type Ca channels.

apparent K_d for I_{Ca} inhibition by nitrendipine from ~700 nM to 0.36 nM (see Fig. 31). Similar results were found by Sanguinetti & Kass (1984) and both concluded that DHPs bind preferentially to the inactivated state of the channel. This conclusion was supported by ^{3}H-DHP binding experiments in isolated sarcolemmal vesicles (Schilling & Drewe, 1986) and myocytes (Green *et al.*, 1985; Kokubun *et al.*, 1986). These results fit well with the modulated receptor hypothesis described by Hondeghem & Katzung (1977) and Hille (1977) to explain the voltage- and use-dependent block of Na channels by local anesthetics.

Sanguinetti & Kass (1984) also compared DHPs (and verapamil) which are neutral at physiological pH (nitrendipine and nisoldipine) with ones that bear a net charge at pH = 7.4 (verapamil and to an intermediate degree, nicardipine). The charged ligands appeared to block the channel only when it was in the open state (i.e. requiring voltage pulses) and as such are strictly *use-dependent.* These charged ligands may need for the channel to open to gain access to the receptor site. The neutral ligands, on the other hand appear able to block I_{Ca} whether the channel is in the open or inactivated state (i.e. at depolarized holding potentials without requiring pulses) and as such are more strictly *voltage-dependent* than use-dependent. The more hydrophobic nature of these ligands may allow them to gain access to the receptor site even when the channel is inactivated. Chester *et al.* (1987) suggested that the DHPs act by first partitioning into the membrane bilayer and then approaching the DHP receptor site by lateral diffusion. Valdivia & Coronado (1988) concluded that the DHP receptor is in the lipid phase adjacent to the external end of the Ca channel. Arena & Kass (1989) concluded that the charged group of DHPs bound to the receptor are accessible to external protons (whether the channel is open or not). This would be consistent with the receptor being at the external end of the

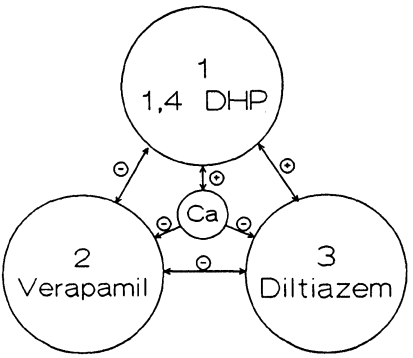


Figure 32. Functional interrelationship between receptor sites on the L-type Ca channel (after Glossmann et al., 1984, 1985). For example, site #1 (dihydropyridine receptor) occupancy increases diltiazem binding and Ca binding, but inhibits phenylalkylamine (ΦAA) binding (and these three effects are reciprocal).

channel. Hescheler *et al.* (1982) demonstrated that the binding site for phenylalkylamines (another class of Ca channel ligands, see below) is at the inner sarcolemmal surface since impermeant ligands were ineffective from the outside.

The voltage-dependence of the DHPs compared to the use-dependence of verapamil may explain the greater efficacy of the DHPs as vasodilators and the relative lack of effect on cardiac muscle at therapeutic levels. That is, since resting vascular smooth muscle is usually at more depolarized levels of E_m and can undergo long further depolarizations, DHPs will interact preferentially with these smooth muscle Ca channels rather than cardiac Ca channels which do not spend long enough times at depolarized enough potentials to be blocked by therapeutic concentrations of DHPs. Of course this also explains why the cardiac effects observed with Ca antagonists are more pronounced in pacemaker cells which have relatively depolarized diastolic E_m level. This effect also contributes to the antiarrhythmic effect of Ca channel blockers.

At least three classes of drugs interact specifically with the L type Ca channel: 1) DHPs (above), 2) phenylalkylamines (ΦAA, such as verapamil, D600, D888 and D890) and 3) benzothiazipines (e.g. diltiazem), and these sites in turn interact allosterically (Fig. 32, Glossmann *et al.*, 1984, 1985). DHP and benzothiazipine binding reciprocally stimulate binding at the other site and ΦAA binding reciprocally inhibits DHP binding and benzothiazipine binding. Ca (and other divalent cations) stimulates DHP binding, but depresses ΦAA and benzothiazipine binding. It has also been argued that there are two distinct DHP receptors, one for Ca antagonists and one for Ca agonists (Kokubun *et al.*, 1986; Brown *et al.*, 1986). The stereoisomers of two well known Ca agonists appear to act as pure Ca antagonists [(-)-R-202-791 and (+) Bay K 8644, Williams *et al.*, 1985; Franckowiak *et al.*, 1985; Kokubun *et al.*, 1986] A further complication is that one stereoisomer which is a Ca agonist [(+)-S-202-791], switches from an I_{Ca} agonist at

negative test potentials to an I_{Ca} antagonist at $E_m \sim 0$ mV (along with a change from allosteric enhancement of PN200-110 binding to competitive inhibition, Kokubun *et al.*, 1986; Kamp *et al.*, 1989). Even nitrendipine, a DHP known as a Ca antagonist, can increase I_{Ca} activated from negative holding potentials (Brown *et al.*, 1986). Thus, while the precise nature of the DHP receptor(s) is not entirely clear, this group of compounds are valuable tools in understanding the regulation of Ca channels. Diphenylbutylpiperidine neuroleptics such as fluspiriline and pimozide may also bind to the Ca channel at an independent receptor (Gould *et al.*, 1983; Galizzi *et al.*, 1986).

MODULATION OF CARDIAC Ca CHANNELS BY ADRENERGIC AGENTS

Agonists for β-adrenergic receptors have been known to increase Ca current for some time (Reuter, 1967; Rougier *et al.*, 1968). A central theme has been that the Ca channel is phosphorylated by cyclic AMP-dependent protein kinase (see Figure 33 e.g., see Sperelakis & Schneider, 1976; Reuter & Scholz, 1977; Tsien, 1977 and see Tsien *et al.*, 1986; Hosey & Lazdunski, 1988 and Hartzell, 1988 for recent reviews). Support for this β-adrenergic pathway comes from experiments showing that the effect can be mimicked by intracellular cAMP, cAMP analogs and phosphodiesterase inhibitors (Tsien *et al.*, 1972; Tsien, 1973; Vogel & Sperelakis, 1981; Cachelin *et al.*, 1983; Nargeot *et al.*, 1983 Kameyama *et al.*, 1985), direct activation of adenylate cyclase by forskolin (Wahler & Sperelakis, 1985; Hescheler *et al.*, 1986) or non-hydrolyzable GTP analogs (Josephson & Sperelakis, 1978) and intracellular application of the catalytic subunit of the cyclic AMP-dependent protein kinase (Osterrieder *et al.*, 1982; Brum *et al.*, 1983). Dephosphorylation of the Ca channel by protein phosphatases (1 and 2A) also abolish the increase in I_{Ca} seen with isoprenaline (Hescheler *et al.*, 1987a), but do not decrease basal I_{Ca}. The latter finding also indicates that phosphorylation of the Ca channel is not **required** for channel activity in cardiac myocytes, in contrast to Ca channels in some other cell types which must be phosphorylated to function (Eckert & Chad, 1984; Armstrong & Eckert, 1987).

Thus, the biochemical pathway important in this effect has become clear. Occupation of the β-adrenergic receptor by an agonist leads to the activation of a GTP binding protein (G protein) known as G_s which, in turn, activates adenylate cyclase, hence producing cyclic AMP. The increase in cyclic AMP leads to the dissociation of the regulatory and catalytic subunits of the cyclic AMP-dependent protein kinase (PKA). The catalytic subunit of PKA phosphorylates several proteins including the L-type Ca channel. Recently, a more rapid parallel pathway has been defined by which β-adrenergic agonists can increase I_{Ca} and which may be physiologically important (Yatani *et al.*, 1987; Yatani & Brown, 1989, Pelzer *et al.*, 1990). These investigators showed that G_s can exert a direct effect to increase Ca channel activity, bypassing the more established, but slower, cyclic AMP-dependent pathway (Fig. 33). Several other receptor-activated channels, such as the

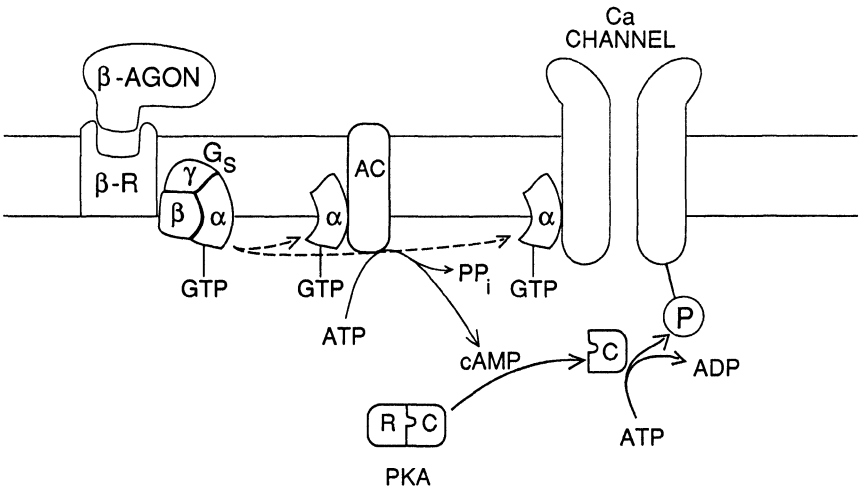


Figure 33. Dual pathways for activation of Ca channels by β-adrenergic stimulation. The "classic" pathway is via stimulation of adenylate cyclase (AC, via activation of the GTP binding protein G_s), increased [cAMP] and phosphorylation of the Ca channel by the catalytic subunit (C) of the cAMP-dependent protein kinase (PKA, where R is the regulatory subunit of PKA). A novel pathway is via a direct effect of the activated α subunit of G_s on the Ca channel (β-agon = β-agonist and β-R= β receptor, based on a diagram by Brown & Birnbaumer, 1988).

muscarinic activated potassium channel in cardiac muscle, also appear to work via a direct G protein modulation (e.g. Codina *et al.*, 1987 and see review by Brown & Birnbaumer, 1988).

Although the biochemical pathway seems clear, the molecular means by which Ca channel gating is modified by β-agonists (via phosphorylation or G proteins) is somewhat less clear. It is helpful to consider the relationship between whole cell I_{Ca} (I) and single channel current (i):

$$I = N_f \times p_o \times i$$

where N_f is the number of functional channels in the cell and p_o is the probability that the channel will be open. There is no change in the single channel conductance, so i is not changed (Reuter *et al.*, 1982). Single channel recording indicated that p_o was increased by β-agonists or dibutyryl-cAMP (Cachelin *et al.*, 1983; Brum *et al.*, 1984). Based on ensemble fluctuation analysis of whole cell I_{Ca}, Bean *et al.* (1984) concluded that β-adrenergic stimulation also increased the average number of functional Ca channels per cell. This fit well with the biochemical information, such that phosphorylation might "activate" previously "dormant" Ca channels. However, Bean *et al.* (1984) could not directly distinguish changes in p_o from N_f, but depended on the conclusion that the increase in p_o from single channel records was insufficient to explain the increase in I_{Ca}. It seems unlikely that isoprenaline increases N_f for two reasons: 1) "new" channels do not appear in

membrane patches with β-stimulation (as would be expected if dormant channels become functional). 2) The outward current via Ca channels at very positive potentials is not increased by β-stimulation, as would be expected if there are more channels (Bean *et al.*, 1984). The increase in I_{Ca} at more negative potentials (around $E_m = 0$) may be due to a phosphorylation-dependent shift in the E_m dependence of I_{Ca} activation (d_∞) toward more negative potentials (Bean, 1990). This shift brings the E_m-dependence of I_{Ca} activation closer to the E_m-dependence of Ca channel gating current (which is not much shifted by β-stimulation, Bean, 1990). Bean suggested that β-stimulation may increase I_{Ca} by making the coupling between the charge movement and opening of the Ca channel more efficient.

In single channel records β-stimulation decreases the time that the channel stays closed (mode 0, Cachelin *et al.*, 1983; Brum *et al.*, 1984) at a particular voltage. This agrees with the more macroscopic description above. Yue *et al.* (1990) have also shown that β-adrenergic agonists (and 8-bromo-cAMP) can increase the probability that the channel will be in a mode where its probability of opening is higher (mode 1 *vs* mode 0 or 0_a, another mode with very sparse openings) and also where long-lasting openings occur (mode 2) similar to those observed with Bay K 8644 (see Fig. 30). Thus, the increase in I_{Ca} induced by β-adrenergic stimulation (and channel phosphorylation) is probably entirely due to an increase in p_o of the channel. This increased p_o is mediated either by a change in the E_m dependence of activation or a shift toward modes of channel gating where longer openings are favored.

Other hormones are known to modify cardiac Ca currents, although activation of cardiac α-adrenergic receptors does not alter I_{Ca} (Hescheler *et al.*, 1988). Histamine, acting at H_2 receptors can also increase cardiac I_{Ca} by activating adenylate cyclase and the cAMP cascade (Hescheler *et al.*, 1987b; Levi & Alloatti, 1988). Cardiac I_{Ca} can also be inhibited by acetylcholine acting at muscarinic receptors (Fischmeister & Hartzell, 1986; Hescheler *et al.*, 1986). This inhibitory effect is much more prominent when I_{Ca} has been enhanced via β-adrenergic agonists or cAMP and is probably due to an acetylcholine-induced and G-protein-mediated (G_i) inhibition of adenylate cyclase (and cAMP). These studies indicate that acetylcholine can decrease basal I_{Ca} in a direct manner (i.e. not via alteration in cyclic nucleotide levels, Hartzell, 1988). Acetylcholine also stimulates guanylate cyclase resulting in elevated cGMP (Watanabe & Besch, 1975; Flitney & Singh, 1981). As suggested by Flitney & Singh (1981), this elevated cGMP stimulates cyclic nucleotide phosphodiesterase providing a second mechanism whereby muscarinic cholinergic activation may reduce cAMP. Atrial natriuretic factor (ANF) prevents cAMP dependent enhancement of cardiac I_{Ca} in a similar manner to acetylcholine. That is, ANF activates a G protein (G_i) which inhibits adenylate cyclase and also activates guanylate cyclase, thereby stimulating cAMP breakdown (Anand-Srivastava and Cantin, 1986; Cramb *et al.*, 1987, Gisbert & Fischmeister, 1988). Adenosine also decreased I_{Ca} only after it had been enhanced via stimulation of the adenylate cyclase system and again this may be mediated by activation of G_i which inhibits adenylate cyclase (Belardinelli & Isenberg,

1983; West *et al.*, 1986). Angiotensin II increases I_{Ca}, but apparently via stimulation of protein kinase C rather than cAMP-dependent protein kinase (Allen *et al.*, 1988; Dösemeci *et al.*, 1988). Phorbol esters and protein kinase C were shown to increase I_{Ca} in *Aplysia* neurons (DeReimer *et al.*, 1985) and also in cardiac myocytes (Dösemeci *et al.*, 1988; Lacerda *et al.*, 1988, but see Walsh & Kass, 1988).

In conclusion, I would like to emphasize the central role of I_{Ca} in cardiac E-C coupling and overall Ca regulation and contraction. The kinetics and amplitude of the I_{Ca} are critical factors in controlling the amount of Ca released by the SR (see Chapter 7). Ca which enters as I_{Ca} may also contribute directly to the activation of the myofilaments as well as to the replenishment of SR Ca stores (see Chapter 8). The amount of Ca influx via I_{Ca} must be extruded from the cell during the same cardiac cycle (e.g. via Na/Ca exchange) for a steady state to exist. Any uncompensated Ca influx could constitute a progressive Ca load for the cell. Due to the high conductance of these ion channels, a relatively small number of Ca channels which fail to inactivate could lead to substantial Ca gain. This, of course, can compromise relaxation and contraction and even be arrhythmogenic (see Chapters 5 & 9).

CHAPTER 5

Na/Ca EXCHANGE AND THE SARCOLEMMAL Ca-PUMP

THE SARCOLEMMAL Ca-PUMP

The two known mechanisms responsible for extrusion of Ca from cardiac myocytes are the sarcolemmal Ca-ATPase pump and Na/Ca exchange. A plasma membrane Ca-pump was first reported in erythrocytes (Schatzmann, 1966) and has since been demonstrated in many other cells (Schatzmann, 1982, 1989). The red cell plasma membrane Ca-pump has been most extensively characterized and appears closely related to that in other tissues. The purified protein is 138 kDa and is no more homologous with the SR Ca-pump protein than it is with the (Na+K)-ATPase or proton pump (Niggli *et al.*, 1981; Verma *et al.*, 1988). A central stretch of ~80 kDa is all that is required for Ca transport and a 30 kDa stretch at the carboxy terminal contains a 10 kDa regulatory calmodulin binding domain (James *et al.*, 1988). One Ca ion seems to be transported per ATP hydrolyzed (Rega & Garrahan, 1986) and Ca extrusion by this pump appears coupled to proton influx (1Ca:1H, Kuwayama, 1988). The turnover rate of plasma membrane Ca-pumps may approach ~1000/min with $K_m(Ca) \leq 1$ μM (Schatzmann, 1989).

The cardiac sarcolemmal Ca-pump was first described in vesicle studies by Caroni & Carafoli (1980). They also demonstrated a stimulatory effect of cAMP-dependent phosphorylation (~3-fold) and calmodulin on the pump (Caroni & Carafoli 1981a,b). They found a $K_m(ATP) \sim 30$ μM, $K_m(Ca) = 0.3$ μM and $V_{max} = 31$ nmol/mg protein/min in the presence of endogenous calmodulin *vs.* $K_m(Ca) = 11$ μM and $V_{max} = 10$ nmol/mg protein/min in calmodulin depleted preparations. Dixon & Haynes (1989) compared the cardiac sarcolemma Ca-pump activated by calmodulin, cAMP-dependent protein kinase or both (see Table 11). They found that calmodulin had a profound effect on the $K_m(Ca)$ and V_{max}, while smaller affects were observed with cAMP-dependent protein kinase.

The maximum rate at which Ca might be extruded from cardiac myocytes by this Ca-pump can be estimated from these V_{max} values. Assuming 1 mg sarcolemmal fraction/30 mg ventricular protein (30-fold sarcolemmal marker enrichment) and 120 mg ventricular protein/g wet wt, 36 nmol/mg protein/min corresponds to 2.4 μmol/kg wet

wt/sec. This is a modest rate of Ca transport compared to that which occurs during the cardiac cycle particularly compared to Ca transport by sarcolemmal Ca channels (Chapter 4) and the Na/Ca exchange (below). Indeed, if relaxation of cardiac muscle were solely dependent on the sarcolemmal Ca-pump, it would likely take 20-40 sec for relaxation even at the V_{max} rate for the pump. Thus, while the sarcolemmal Ca-pump can have a high affinity for $[Ca]_i$, the transport rate is too slow for it to be important to Ca fluxes during the cardiac cycle. It might, however be more important in slow longer term extrusion of Ca by the cell (but see the end of this chapter).

TABLE 11
Kinetic Properties of the Cardiac Sarcolemmal Ca-Pump

	V_{max} (nmol/mg pn/min)	K_m(Ca) (nM)	n (Hill)
Basal	1.7 ± 0.3	1800 ± 100	1.6 ± 0.1
+cAMP-dependent Protein Kinase	3.1 ± 0.5	1100 ± 100	1.7 ± 0.1
+Calmodulin	15.0 ± 2.5	64 ± 1.4	3.7 ± 0.2
+ Both	36.0 ± 6.5	63 ± 1.7	3.7 ± 0.1

Values are taken from Dixon & Haynes (1989).

Na/Ca EXCHANGE

Von Wilbrandt & Koller (1948) proposed that the site of action of Ca on the heart was likely to be at the cell membrane and that contractions depended on the ratio $[Ca]_o/[Na]_o$. Lüttgau & Niedergerke (1958) suggested that Na and Ca compete for an anionic site (R^{2-}) responsible for bringing Ca into the cell, consistent with a Ca/Na^2 model. Repke (1964) and Langer (1964) suggested that intracellular Na might be linked to Ca influx in some way. This helped explain the basis of digitalis inotropy and the "staircase" or positive force-frequency relationship in cardiac muscle (where increasing frequency leads to increased contractile force). Reuter & Seitz (1968) in heart and Baker *et al.* (1969) in squid giant axon were the first to document the presence of a Na/Ca exchange counter transport system. In the ensuing 20 years much work has been done to characterize this system and determine its role in cardiac Ca regulation (see recent reviews by Philipson, 1985b, 1990; Reeves, 1985, 1990; Reeves & Philipson, 1989; Sheu & Blaustein, 1986; Eisner & Lederer, 1985, 1989; Blaustein, 1989b).

Fundamental Characterizations in Sarcolemmal Vesicles

Much important fundamental information about Na/Ca exchange in cardiac sarcolemma comes from studies of isolated cardiac sarcolemmal vesicles (**SLV**). The general strategy for studying Na/Ca exchange in SLV is illustrated in Figure 34. Reeves &

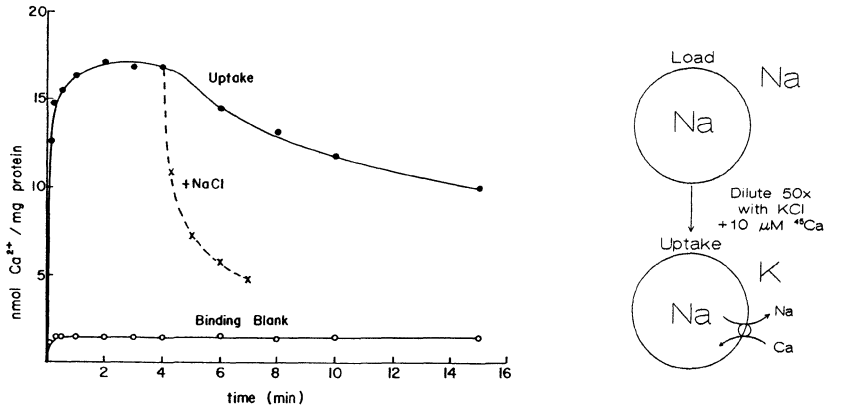


Figure 34. The measurement of Na_i-dependent ^{45}Ca uptake in isolated cardiac sarcolemmal vesicles. Vesicles are pre-equilibrated with 140 mM Na and then diluted 50× into a solution with ^{45}Ca and 140 mM KCl or in the case at left 280 mM sucrose + 50 μM $CaCl_2$. Parallel samples diluted into the same [Na] serve as blanks. Samples are filtered and rinsed with 0.5 mM $LaCl_3$ or quenched with a La solution. After uptake has occurred, addition of 50 mM NaCl induced a rapid efflux of Ca, indicative of the reversibility of the Na/Ca exchange system (left panel is from Bers et al., 1980, with permission).

Sutko (1979) were the first to demonstrate Na/Ca exchange in SLV by measuring ^{45}Ca uptake into a relatively crude preparation of SLV preloaded passively with Na and diluted into a Na-free medium. Pitts (1979) made early estimates that the Na/Ca exchange stoichiometry was 3:1 (Na:Ca). Several studies demonstrated that Na/Ca exchange is sensitive to transmembrane potential (since more positive intravesicular potential increased Ca uptake) and is also capable of generating a voltage gradient (Bers *et al.*, 1980; Reeves & Sutko, 1980; Philipson & Nishimoto, 1980; Caroni *et al.*, 1980). These results were consistent with an electrogenic Na/Ca exchange (i.e. >2Na:1Ca), but the stoichiometry was most clearly demonstrated by Reeves & Hale (1984) using a thermodynamic approach. Their measurements of the [Na] gradient required to prevent net Ca transport at various membrane potentials indicated a stoichiometry of 3Na:1Ca. This stoichiometry is completely consistent with a wide array of other studies and is now generally accepted as *the* true stoichiometry of Na/Ca exchange in cardiac muscle (e.g. Reeves & Philipson, 1989).

It has been surprisingly difficult to separate inside-out and right-side out cardiac sarcolemmal vesicles to study the symmetry of the Na/Ca exchange. Philipson & Nishimoto (1982a) and Philipson (1985a) circumvented this limitation by comparing Na/Ca exchange in vesicles which were loaded with Na only by the action of the sarcolemmal (Na+K)ATPase pump (i.e. inside-out vesicles) with that in the whole population of SLV. They found a similar K_m(Ca) (~25 μM) and a similar Na-dependence

of Ca efflux (K_m~30 mM) in both populations, suggesting a relatively symmetrical exchanger. However, Na appears to inhibit Ca binding to the external side of the exchanger more effectively than at the internal side (Philipson, 1985a). Thus the Na/Ca exchange system can work in either direction and is fairly, but not completely symmetrical. The asymmetry seems more pronounced in intact cells where Miura & Kimura (1989) estimated $K_m(Ca_i)$ to be 0.6 μM and $K_m(Ca_o)$ to be 140 μM.

There is general agreement that the K_m(Na) of the Na/Ca exchanger is ~30 mM in SLV and in intact cardiac cells (Reeves & Philipson, 1989). There is less agreement about the K_m(Ca). In isolated SLV the reported values of K_m(Ca) range from 1.5-200 μM, although most values are in the 20-40 μM range (Reeves & Philipson, 1989). Estimates of $K_m(Ca_i)$ from intact cells have suggested values around 1 μM (e.g. Miura & Kimura, 1989). The lower $K_m(Ca_i)$ in intact cells could be due to an effect of ATP, which has been shown to increase the [Ca] sensitivity of Na/Ca exchange in squid axon from ~12 to 1-3 μM (DiPolo & Beaugé, 1988) and ATP also increased Na/Ca exchange in excised cardiac membrane patches (Hilgemann, 1990). ATP stimulation of the Na/Ca exchange in SLV has been observed by Caroni & Carafoli (1983), but not by others (Reeves & Philipson, 1989). Non-hydrolyzable ATP analogs do not stimulate Na/Ca exchange (Hilgemann, 1990), but ATPαS which may thiophosphorylate the exchanger produces a large stimulation of Na/Ca exchange in nerve (DiPolo & Beaugé, 1988). Thus, ATP-dependent stimulation is thought to be due to a regulatory phosphorylation of the exchanger (rather than an allosteric effect of ATP or use of ATP as an energy source for transport, Reeves, 1990).

Ca_i also appears to increase Ca influx via Na/Ca exchange in an allosteric manner with a $K_m(Ca_i)$~20 nM (i.e. from the Na side, Reeves & Poronnik, 1987; Miura & Kimura, 1989) and Hilgemann (1990) demonstrated that this $K_m(Ca_i)$ depends on the presence of ATP. Reeves & Poronnik (1987) also found a Ca_i dependence for Ca uptake in SLV via Na/Ca exchange, but much higher [Ca] appeared to be required. The Na/Ca exchanger can also produce Ca/Ca exchange which is greatly facilitated by the presence of monovalent cations (Philipson & Nishimoto, 1981; Slaughter *et al.*, 1983). The latter authors also proposed a symmetrical working model of the exchanger, in which an *A site* could bind either one Ca ion or two Na ions and a *B site* could bind monovalent cations only (e.g. the third Na when 3Na ions are being exchanged for Ca. This site could also bind Li, Na, Rb or K on the side where Ca binds to the *A Site*). While this simple model has been useful in clarifying competitive aspects of the Na/Ca exchange process, more detailed kinetic and thermodynamic models have also been described (DiFrancesco & Noble, 1985; Johnson & Kootsey, 1985; Läuger, 1987; Hilgemann, 1988).

Table 12 lists some factors which alter cardiac sarcolemmal Na/Ca exchange. Increased Na/Ca exchange after proteolysis might be due to removal of some regulatory portion of the molecule and Philipson *et al.* (1988) found that this stimulation was associated with a conversion of a putative Na/Ca exchange protein from 120 to 70 kDa.

Table 12

Factors That Alter Sarcolemmal Na/Ca Exchange

Enhancers of Na/Ca Exchange

E_m	Depolarization → ↑ Ca entry	a,b,c
pH	Alkalosis	d
ATP	mM ATP (↓ K_m(Ca))	e,f,g,h
$[Ca]_i$	~40 nM → ↑ Ca entry (regulatory)	f,g,i,j
Proteinase	Trypsin, Chymotrypsin, Pronase, Papain, Ficin → ↑ Exchange (↓K_m(Ca))	k
Phospholipase	Phospholipase C & D → ↑ Exchange	l
Anionic Amphiphiles	SDS, Lauric acid → ↑ Exchange (↓K_m)	m,n
Redox Modification	$FeSO_4$ & DTT, Glutathione	o
Lipids	Cholesterol, Unsaturated/doxyl lipids → ↑ Exchange	q,r,s
Ca Chelator	EGTA, EDTA, CDTA (↓K_m)	p

Inhibitors of Na/Ca Exchange

pH	↓ pH → ↓ Exchange	d
Inorganic Cations	$La^{3+} > Nd^{3+} > Tm^{3+} \sim Y^{3+} > Cd^{2+} \gg Sr^{2+} > Ba^{2+} \sim Mn^{2+} \gg Mg^{2}$	t
Cationic Amphiphiles	Dodecylamine → ↓ Exchange (↓V_{max})	m
Adriamycin (doxorubicin)		u
Chlorpromazine		u
Tetracaine, Dibucaine, Ethanol		v,w,x
Verapamil	(K_i = 200 μM)	y
Quinidine		z,aa
Polymyxin B		k
Quinacrine	(K_i = 50 μM)	bb
Bepridil	(K_i = 30 μM)	cc
Dichlorobenzamil	(K_i = 4 - 17 μM)	dd,ee
Harmaline	(K_i = 250 μM)	ff
Methylation		gg

a) Bers et al., 1980, b) Caroni et al., 1980, c) Philipson & Nishimoto, 1980, d) Philipson et al., 1982, e) DiPolo & Beaugé, 1987, f) DiPolo & Beaugé, 1988, g) Hilgemann, 1990, h) Caroni & Carafoli, 1983, i) Miura & Kimura, 1989, j) Reeves & Poronnik, 1987, k) Philipson & Nishimoto, 1982b, l) Philipson & Nishimoto, 1984, m) Philipson, 1984, n) Philipson et al., 1985, o) Reeves et al., 1986, p) Trosper & Philipson, 1984 q) Philipson & Ward. 1985, r) Philipson & Ward, 1987, s) Vemuri & Philipson, 1987, t) Trosper & Philipson, 1983, u) Caroni et al., 1981, v) Allen & Baker, 1989, w) Michaelis et al., 1987, x) Michaelis & Michaelis, 1983, y) Van Amsterdam & Zaagzma, 1986, z) Ledvora & Hegvary, 1983, aa) Mentrard et al., 1984, bb) De la Peña & Reeves, 1987, cc) Garcia et al., 1988, dd) Kaczarowski et al., 1985, ee) Bielefeld et al., 1986, ff) Suleiman & Reeves, 1987, gg) Vemuri & Philipson, 1988.

Hilgemann (1990) suggested that proteolysis during preparation of SLV could be responsible for the apparent loss of the regulatory effects of ATP and Ca_i in these SLV.

The Na/Ca exchange is remarkably sensitive to alteration of the lipid bilayer. Exchange is increased by reconstitution with acidic phospholipids, the addition of exogenous negatively charged amphiphiles or phospholipase cleavage of native phospholipids to yield negatively charged membrane lipids. Certain anionic head groups are more stimulatory for Na/Ca exchange than others and several cationic amphiphiles are inhibitory (Philipson, 1984). The effects of charged amphiphiles and phospholipids does not appear to be simply due to surface charge effects (e.g. concentrating Ca near the exchanger). Indeed, Na/Ca exchange is virtually unaffected by changes in surface charge or surface [Ca] (Bers *et al.*, 1985). We used the organic divalent cation, dimethonium to screen surface charge and thereby modify surface [Ca]. At low ionic strength, dimethonium strongly decreased surface charge, surface [Ca] and Ca binding to sarcolemmal phospholipids, but did not affect Na/Ca exchange. Thus, the negatively charged amphiphiles may enhance Na/Ca exchange via a more specific interaction with the Ca binding site on the Na/Ca exchanger.

The stimulatory effect of negatively charged amphiphiles on exchange activity is enhanced if the lipophilic portion of the molecule also disorders the bilayer, especially toward the center of the bilayer (e.g. by unsaturated bonds or inclusion of doxyl- groups, Philipson & Ward, 1987). Optimal reconstitution of exchange activity requires acidic phospholipids and ~20% cholesterol with other related sterols unable to substitute (Vemuri & Philipson, 1988).

The pathophysiological consequences of the above properties are not yet clear. However, during cardiac ischemia, the declining pH would be expected to decrease Na/Ca exchange, production of fatty acids (e.g. arachidonate, see Chien *et al.*, 1984; Philipson & Ward, 1985) would increase Na/Ca exchange and redox stimulation might also stimulate Na/Ca exchange. These latter effects might limit the depression of Na/Ca exchange during ischemia and help minimize cardiac Ca overload. On the other hand SLV from ischemic myocardium exhibit reduced Na/Ca exchange (Bersohn *et al.*, 1982) and fatty acids also increase passive Ca permeability in SLV (Philipson & Ward, 1985) so that the net result in ischemia is still unclear.

Ca chelators, such as EGTA stimulate Na/Ca exchange by reducing $K_m(Ca)$ (e.g. from ~20 to ~1 μM, Trosper & Philipson, 1984) and similar effects have been reported for the plasma membrane Ca pump (Schatzmann, 1973; Sarkadi *et al.*, 1979). This might account for the lower $K_m(Ca)$ estimated in intact cells, perfused with EGTA buffers than in SLV where $K_m(Ca)$ determinations have generally been made without Ca chelators. It is not yet known whether the Ca affinity of the exchanger is also increased by the related Ca chelators which are used as fluorescent Ca indicators in intact cells (e.g. indo-1 and fura-2). This would complicate the use of these indicators in the study of Na/Ca exchange.

The Na/Ca exchanger can be inhibited by a number of drugs (Table 12), but none of these agents is very potent as an inhibitor. Even the most potent inhibitors among them are not very selective. That is, they inhibit other ion transport systems and channels at even lower concentrations than those required to inhibit Na/Ca exchange (Kaczorowski *et al.*, 1989). For example, dichlorobenzamil inhibits sarcolemmal Ca channels, and related amiloride derivatives are potent inhibitors of Na/H exchange, Na-coupled sugar and amino acid transport, Na, K & Ca channels, and cholinergic and adrenergic receptors This lack of a specific inhibitor (ligand) of Na/Ca exchange has been a serious limitation, both to the isolation of the protein and characterization of the physiological action of the Na/Ca exchanger.

The Na/Ca exchanger protein has been reported to have a molecular weight of 33 (Soldati *et al.*, 1985; Longoni & Carafoli, 1987), 82 (Hale *et al.*, 1984) and 220 kDa (Hale *et al.*, 1988). Philipson *et al.* (1988) identified 70 and 120 kDa proteins as the Na/Ca exchanger and suggested that the smaller protein may be a proteolytic fragment of the larger. Under non-reducing conditions only one band (~160 kDa) was present. In further studies using monoclonal antibodies, Vemuri *et al.* (1990) have indicated that the 70 and 120 kDa proteins weakly cross react with antibodies to the rod Na/Ca exchanger (below). They also identified a 160 kDa protein, from which the 120 and 70 kDa protein may be derived due to endogenous protease. This larger protein may correspond to the 150 kDa protein identified as the Na/Ca exchanger by Durkin *et al.* (1990). Ambesi et al (1990) also identified 70 and 120 kDa proteins as the cardiac Na/Ca exchanger.

The cardiac Na/Ca exchanger has recently been cloned by Nicoll *et al.* (1990) and consists of 970 amino acids (MW = 108 kDa) including 12 putative transmembrane domains and one large cytoplasmic hydrophilic domain (between the 6th and 7th transmembrane spans, see Fig. 35). The structure has 3 potential glycosylation sites on the extracellular side, one of which appears to be glycosylated. This glycosylation may account for the higher apparent molecular weights of the exchanger on polyacrylamide gels (120 and 170 kDa). The protein is highly acidic and bears a gross similarity to the (Na+K) ATPase having a 23 amino acid stretch with 48% identity. On the large cytoplasmic domain (520 amino acids) there is a calmodulin binding domain and a potential phosphorylation site which could be a substrate for calmodulin or cAMP-dependent protein kinase.

Cardiac muscle is one of the richest sources of Na/Ca exchange activity compared to other tissues (see Reeves & Philipson, 1989). However, Na/Ca exchange activity about 10-fold lower has been reported in smooth and skeletal muscle SLV (Slaughter *et al.*, 1989; Donoso & Hidalgo, 1989; Blaustein, 1989a). Important Na/Ca exchange activity is also found in retinal rod outer segments and in brain synaptosomes. The synaptosomal Na/Ca exchanger has been isolated and has a molecular weight of 70 kDa (Barzilai *et al.*, 1984, 1987) and the rod Na/Ca exchanger has been isolated (M_r=220 kDa, Cook & Kaupp, 1988; Nicoll & Applebury, 1989). Barzilai & Rahamimoff (1987) reported a stoichiometry

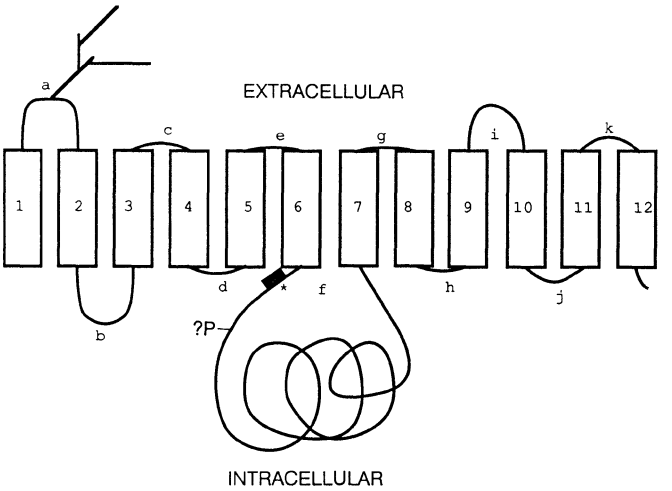


Figure 35. Working model of the Na/Ca exchanger based on the work of Nicholl *et al.* (1990) indicating 12 membrane spanning segments and possible site of glycosylation (a), phosphorylation (?P) and calmodulin binding (*). The figure was generously provided by K. D. Philipson and D. A. Nicholl.

of 5Na:1Ca, but this is not widely accepted (Reeves & Philipson, 1989). Yau & Nakatani (1984) first reported a stoichiometry of 3Na:1Ca for the rod Na/Ca exchanger. Interestingly, the larger rod Na/Ca exchanger also appears to require K and in fact transports K along with Ca with an overall stoichiometry of 4Na:(1Ca+1K) (Schnetkamp *et al.*, 1989; Cervetto *et al.*, 1989). This contrasts with the heart Na/Ca exchanger.

Na/Ca Exchange Current in Myocytes

Horackova & Vassort (1979) were the first to report ionic current in cardiac muscle which may have been attributable to Na/Ca exchange, but the interpretation was not unequivocal. With the advent of whole cell voltage clamp and internal dialysis more definitive measurements could be made. Kimura *et al.* (1986) demonstrated Ca_o activation of an outward current only in the presence of Na_i (30 mM) and Ca_i (73 nM) and with all other known ionic currents blocked (including the Na-pump). They followed this early report with detailed characterizations of the Na/Ca exchange current in ventricular myocytes (Kimura *et al.*, 1987; Miura & Kimura, 1989). Figure 36 shows an example from Kimura *et al.* (1987) where exchange current was activated by 140 mM Na_o only after $[Ca]_i$ was increased from nominally 0 to 430 nM.

Mechmann & Pott (1986) demonstrated that spontaneous Ca release from the SR activated a Na_o-dependent inward current identified as Na/Ca exchange current. This was really a variation on an experiment done by Clusin *et al.* (1983) where caffeine-induced SR Ca release activated a Na_o-dependent inward current suggested to be Na/Ca exchange. These depolarizing Na/Ca exchange currents raise the question of the arrhythmogenic

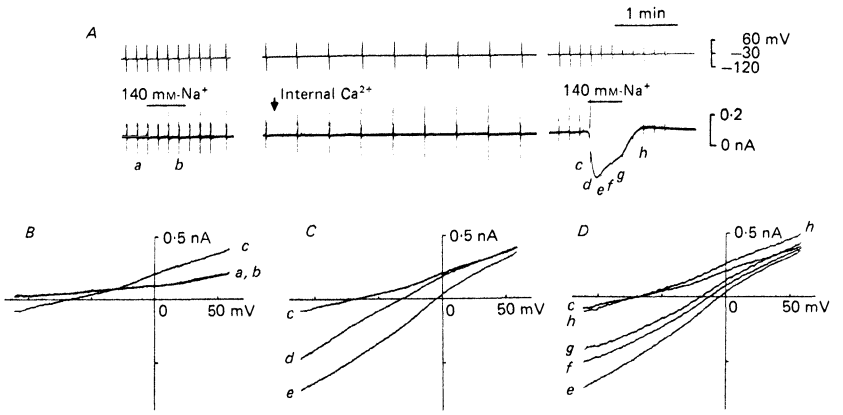


Figure 36. Na/Ca exchange current recording in a dialyzed and voltage-clamped guinea-pig ventricular myocyte. **A)** Slow recording of E_m (top) and $I_{Na/Ca}$ where the spikes are from ramp depolarizations used to generate the current voltage relationships in **B, C** and **D** at the times indicated (a-h). The bars in **A** refer to changing the extracellular solution from 140 mM LiCl to 140 mM NaCl ($[Ca]_o$ was 1 mM throughout). At the arrow $[Ca]_i$ was increased from nominally Ca free to 430 nM with 42 mM EGTA and 140 CsCl throughout. Ouabain, Ba, Cs, D600 and tetraethylammonium were used to inhibit other ionic currents. Application of Na_o stimulated $I_{Na/Ca}$ only after $[Ca]_i$ was raised. The gradual decline in $I_{Na/Ca}$ (d-g) was supposed to be due to local Ca depletions (from Kimura et al., 1987, with permission).

currents associated with cellular Ca overload. This relates to the observation of "transient inward current" (I_{ti}) associated with after-contractions in Na_i-loaded Purkinje fibers (Lederer & Tsien, 1976). When the Na-pump is inhibited by cardioactive steroids (e.g. ouabain) the cells gain Ca due to Na/Ca exchange and then exhibit spontaneous SR Ca release. It can be difficult to distinguish between an inward Na/Ca exchange current or inward current via a Ca_i-activated non-selective channel (e.g. Colquhoun *et al.*, 1981). Both of these currents may be involved and the relative contributions to the arrhythmogenic I_{ti} is still not entirely clear, but under certain conditions Kimura (1988) has estimated that about 15% of I_{ti} is Ca_i activated non-selective current and 85% is Na/Ca exchange current ($I_{Na/Ca}$) and similar conclusion was reached by Fedida *et al.* (1987b).

Hume & Uehara (1986a,b) have studied another manifestation of $I_{Na/Ca}$, that is the so called "creep" currents first described by Eisner & Lederer (1979). Depolarizing voltage clamp pulses in Na_i loaded cells are associated with a declining (creeping) outward current (probably due to a declining Ca entry via Na/Ca exchange). Repolarization also produces an inward tail current which slowly declines as Ca is extruded via Na/Ca exchange current. These creep currents depend on [Na] and [Ca] gradients and are suppressed by La and dichlorobenzamil in a manner consistent with $I_{Na/Ca}$ (Bielefeld *et al.*, 1986; Hume, 1987).

V_{max} vs. Ca Requirements and Site Density

To appreciate the contribution of Na/Ca exchange to cellular Ca fluxes it is valuable to consider the rate at which this system can transport Ca. In SLV Ca transport rates of ~25 nmol/mg protein/sec are typically observed (Reeves & Philipson, 1989). Assuming 30-fold purification of SLV and 120 mg tissue protein/g wet wt tissue this would be ~100 μmol/kg wet wt/sec. $I_{Na/Ca}$ of ~300 pA have been recorded by several investigators in mammalian ventricular myocytes (typically ~30 pL in volume). This also corresponds to ~100 μmol/kg wet wt/sec. Miura & Kimura (1989) estimated the maximum $I_{Na/Ca}$ in guinea-pig ventricular myocytes to be 3 μA/μF. Assuming 1 μF/cm^2 and a cell surface:volume ratio of 0.5 μm^2/μm^3 (Page, 1978) this is 150 μmol/kg wet wt. Recently Li & Kimura (1990) and Hilgemann (1990) reported a maximum peak $I_{Na/Ca}$ in guinea-pig ventricular myocytes and excised patches which could be about ten times higher with high $[Na]_i$ (~30 μA/μF). Whether or not these higher transport rates (e.g. with 100-140 mM $[Na]_i$) are physiologically important, even the lower numbers (~3 μA/μF) indicate that Na/Ca exchange has the capacity to transport a large amount of Ca (more than enough in 1 sec to activate or relax a substantial contraction, e.g. compare with chapter 3). The 300 pA $I_{Na/Ca}$ is 10-30% of the peak I_{Ca} in ventricular myocytes under normal circumstances (e.g. 1-3 nA) and the Na/Ca exchange does not inactivate rapidly as does I_{Ca}. Thus, it is becoming increasingly clear that Na/Ca exchange can contribute importantly to Ca fluxes during the cardiac cycle (see also Chapters 8 & 9).

Cheon & Reeves (1988) estimated the site density of the Na/Ca exchanger to be 25 pmol/mg protein (or 75/μm^2 in reconstituted proteoliposomes) and with a V_{max} of 25 nmol/mg protein/sec this also provided an estimate of the turnover number (1000/sec). If this turnover rate holds for the Na/Ca exchanger in isolated cells discussed in the previous paragraph (3 μA/μF), those exchange activities would correspond to 187 exchangers/μm^2. These values are 10-20-fold higher than the density of dihydropyridine receptors and functional Ca channels (see page 57). If the higher $I_{Na/Ca}$ values (30 μA/μF) are used, the Na/Ca exchanger density would be similar to that of the sarcolemmal Na-pump (see page 175).

Ca Entry Via Na/Ca Exchange and Contraction

When $[Na]_o$ is decreased around intact cardiac muscle cells, large contractions occur and are attributed to Na/Ca exchange (e.g. Chapman & Tunstall, 1980; Chapman & Rodrigo, 1983). The force of contraction can also be strongly influenced by $[Na]_i$. An increase of intracellular Na activity (aNa_i) by as little as 1 mM can lead to more than a doubling of the twitch contraction (Im & Lee, 1984; Eisner *et al.*, 1984). These authors found that tension was proportional to $(aNa_i)^6$ and $(aNa_i)^3$, respectively. Of course, net Ca fluxes by the Na/Ca exchanger after reduction of the [Na] gradient may be due to increased Ca influx, decreased Ca efflux or both. Whether Ca entry via Na/Ca exchange

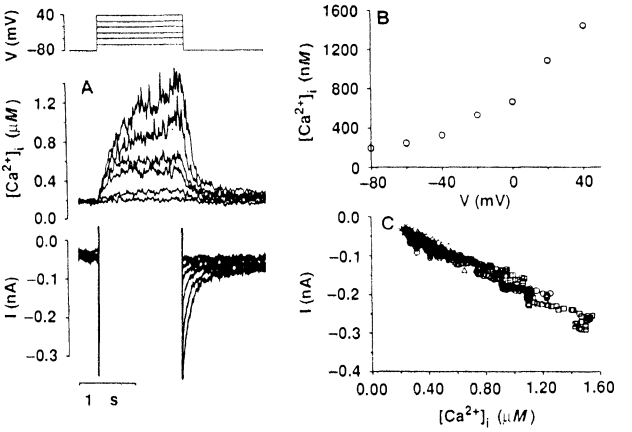


Figure 37. $[Ca]_i$ assessed by fura-2 fluorescence and $I_{Na/Ca}$ during depolarizations in a guinea-pig ventricular myocyte (**A**). The [Na] in the dialyzing pipette was 7.5 mM. Outward currents during the depolarization were off-scale. **B** and **C** show the E_m dependence of the Ca_i transient and the $[Ca]_i$-dependence of the "tail" current observed upon repolarization to -80 mV. Other ionic currents are blocked by Cs, tetraethylammonium, verapamil and ryanodine (from Barcenas-Ruiz et al., 1987, with permission).

can contribute appreciably to force development under more physiological conditions is less clear from studies of this type.

It now appears that Ca entry via Na/Ca exchange *can* contribute quantitatively to the direct activation of the myofilaments, since contractions and Ca_i transients can be activated by action potentials and long depolarizing voltage clamp pulses even when both sarcolemmal Ca channels and SR Ca release are inhibited (e.g. Eisner *et al.*, 1983; Cannell *et al.*, 1986; Hume & Uehara, 1986b; Barcenas-Ruiz *et al.*, 1987; Bers *et al.*, 1988). Barcenas-Ruiz *et al.* (1987) and Beuckelmann & Wier (1989) have reported $[Ca]_i$ transients and $I_{Na/Ca}$ during depolarizing pulses where all other known currents and SR Ca release were blocked. However, with only 7 mM Na in the dialyzing pipette, very large or very long depolarizations were required (see Figure 37). Interestingly, these studies showed a linear dependence of $I_{Na/Ca}$ on $[Ca]_i$ up to nearly 2 μM, suggesting that $K_m(Ca)$ for the exchanger is higher than 2 μM.

In intact rabbit ventricular muscle, Bers *et al.* (1988) showed that action potential activated contractions can be abolished by blocking Ca channels with nifedipine. However, if $[Na]_i$ is elevated (e.g. to 15-20 mM) large twitch contractions can still be elicited in the presence of nifedipine (see Fig. 97). We concluded that Ca entry via Na/Ca exchange does not normally contribute significantly to the activation of contraction, but can if $[Na]_i$ is elevated by inhibition of the Na-pump. We also presented indirect evidence suggesting

that, with elevated aNa_i, Ca entry via Na/Ca exchange might induce Ca release from the SR, but Cannell *et al.* (1987) presented evidence suggesting that this may not occur when $[Na]_i$ is only ~7 mM. Leblanc & Hume (1990) have also presented some more direct evidence that Ca entry via Na/Ca exchange may induce SR Ca release. In this case, they suggest that tetrodotoxin-sensitive Na entry may increase the subsarcolemmal $[Na]_i$ thereby activating Ca entry and SR Ca release. Though additional work is required to clarify the possible role of Na/Ca exchange in SR Ca release, it seems likely that Ca influx via Na/Ca exchange can only contribute quantitatively to contraction when $[Na]_i$ is high.

Thermodynamic Considerations

If there is more energy in the inward [Na] gradient (for 3 Na ions) than in the inward [Ca] gradient, Ca extrusion via this coupled transporter is thermodynamically favored (Mullins, 1979). That is

$$n(E_{Na} - E_m) > 2(E_{Ca} - E_m) \tag{5.1}$$

where n is the coupling ratio and E_{Ca} and E_{Na} are the equilibrium potentials for Ca and Na ($E_x = (RT/zF) \log ([X]_o/[X]_i)$. Then, for $n=3$, the potential at which the gradients are equal ($E_{Na/Ca}$) is the potential at which the $I_{Na/Ca}$ reverses. Hence,

$$E_{Na/Ca} = 3E_{Na} - 2E_{Ca} \tag{5.2}$$

Thus, whenever E_m is more positive than $E_{Na/Ca}$ Ca entry via the exchanger is favored and when E_m is negative to $E_{Na/Ca}$ Ca extrusion is favored.

Figure 38 illustrates how $E_{Na/Ca}$ may be expected to change during the action potential in ventricular muscle under normal conditions. The shape of the $E_{Na/Ca}$ curve is dictated by the $[Ca]_i$ transient (which will alter E_{Ca}). During diastole Ca extrusion is favored. During the action potential, there is a period where Ca influx via the exchanger is favored (shaded portion in Figure 38). It can be appreciated that the precise length of this period is rather sensitive to changes in peak $[Ca]_i$, the time course of the $[Ca]_i$ transient, the aNa_i and the shape of the action potential. This figure illustrates the dynamic, yet delicate balance of Ca fluxes mediated by the Na/Ca exchanger.

While the simple thermodynamic consideration above can be sufficient to predict the direction of Ca transport by Na/Ca exchange and the "driving force" ($E_{Na/Ca}$-E_m), the amplitude of the $I_{Na/Ca}$ may also be subject to kinetic limitations. For example, in Figure 38A the driving force for Ca extrusion via Na/Ca exchange during diastole is large, but the net Ca extrusion will be limited by the low diastolic $[Ca]_i$. Quantitative models of $I_{Na/Ca}$ incorporating these considerations have been described (e.g. DiFrancesco & Noble, 1985; Hilgemann, 1989; Beuckelmann & Wier, 1989). The net $I_{Na/Ca}$ can be considered as the difference between unidirectional fluxes on the system.

$$I_{Na/Ca} = K_{Na/Ca}(\text{Ca influx} - \text{Ca efflux}) \tag{5.3}$$

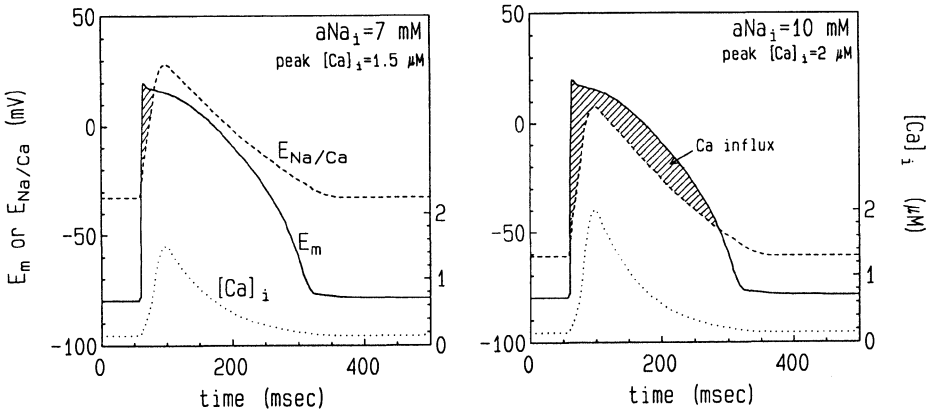


Figure 38. Schematic diagram of estimated changes in $E_{Na/Ca}$ during an action potential in rabbit ventricular muscle. When E_m is positive to $E_{Na/Ca}$, Ca influx via the Na/Ca exchanger is thermodynamically favored (shaded areas). When E_m is negative to $E_{Na/Ca}$ Ca extrusion is favored. Resting $[Ca]_i$=150 nM, $[Ca]_o$=2 mM and aNa_o=110 mM for both traces and aNa_i and peak $[Ca]_i$ are as indicated. The $[Ca]_i$ trace reaches a peak 40 msec after the action potential begins (after Bers, 1987b).

where $K_{Na/Ca}$ is a scaling factor which includes the rate constant of the exchanger at $E_m = 0$. The unidirectional Ca fluxes can be expected to depend on E_m as well as the concentration of the exchanger bound to extracellular Ca ($[E_oCa]$) and 3 intracellular Na ions ($[E_iNa_3]$). Thus, for Ca influx (and by analogy for Ca efflux)

$$\text{Ca influx} = k_i[E_oCa][E_iNa_3]\exp(E_mF\gamma/RT) \tag{5.4}$$

$$\text{Ca efflux} = k_o[E_iCa][E_oNa_3]\exp(E_mF(1-\gamma)/RT) \tag{5.5}$$

where k_i and k_o are intrinsic rate constants at $E_m = 0$ and γ is a partition parameter representing the fractional position of the energy barrier within the membrane electric field. The value of $[E_oCa]$ will depend on the $[Ca]_o$, $K_m(Ca)$, E_o and also on $[Na]_o$ and K_i for Na_o interacting with the exchanger. For example,

$$[E_oCa]/[E_o\text{-tot}] = 1/(1+(K_m(Ca)/[Ca]_o) \tag{5.6}$$

$$[E_oCa]/[E_o\text{-tot}] = [Ca]_o / \{[Ca]_o + K_m(Ca)(1 + [Na]_o/K_i)^2\} \tag{5.7}$$

and similar relations could be used for $[E_iNa_3]$, $[E_iCa]$ and $[E_oNa_3]$.

The detailed relationship of these kinetic parameters to the actual $I_{Na/Ca}$ is highly dependent on the specific details of the model of Na/Ca exchange utilized (e.g. simultaneous, consecutive, one step, two step, number of E_m-sensitive steps and species, Eisner & Lederer, 1985; Läuger, 1987; Hilgemann, 1989). Nevertheless, some simple

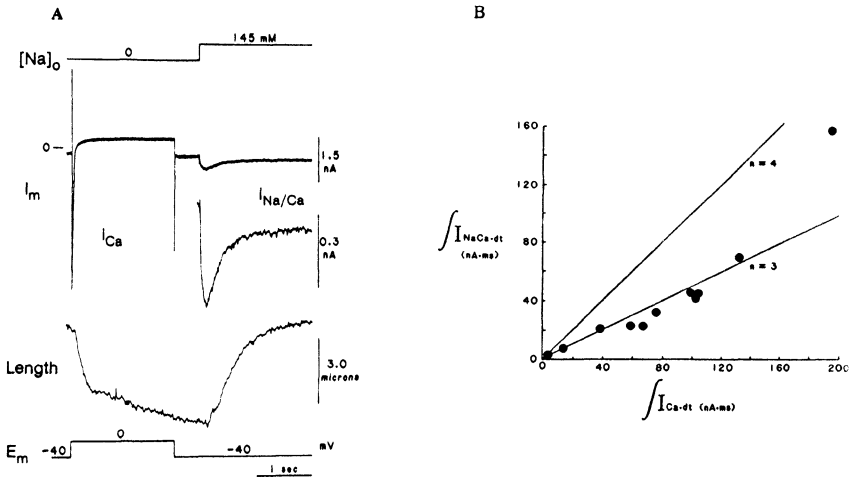


Figure 39. Ca entry via Ca current and Ca efflux via Na/Ca exchange. A) A voltage clamped guinea-pig ventricular myocyte was equilibrated with 10 mM caffeine to prevent SR Ca uptake and release. The I_{Ca} associated with the 2 sec depolarizing pulse in Na-free solution activated a contraction. The contraction relaxed only after $[Na]_o$ was rapidly returned, activating Ca extrusion via Na/Ca exchange (or $I_{Na/Ca}$). **B)** The integral of $I_{Na/Ca}$ is plotted as a function of the integrated I_{Ca}. The $\int I_{Ca}$ is roughly twice the $\int I_{Na/Ca}$ as would be expected for a Na/Ca exchange coupling ratio of n=3. (from Bridge et al., 1990, with permission).

kinetic models have been successful in reproducing certain aspects of measured $I_{Na/Ca}$ (Beuckelmann & Wier, 1989; Miura & Kimura, 1989) and the results of Hilgemann (1989) and Li & Kimura (1990) are most consistent with a simultaneous exchange mechanism. Egan *et al.* (1989) measured outward $I_{Na/Ca}$ by repolarizing during action potentials in guinea-pig ventricular myocytes and used the current to predict the time course of the Ca_i transient. They obtained reasonable Ca_i transients and also concluded that outward $I_{Na/Ca}$ can contribute significantly to repolarization during the normal ventricular action potential.

Returning to the thermodynamic considerations above, it seems probable that under normal conditions the Na/Ca exchanger serves mainly as a means of Ca extrusion and that Ca current is the main means of Ca entry into cardiac myocytes. The functional relationship of these two pathways is illustrated in Figure 39 from Bridge *et al.* (1990). A guinea-pig ventricular myocyte was voltage clamped using a dialyzing pipette containing Na-free, 130 mM CsCl to prevent Ca entry via Na/Ca exchange and K currents, respectively. Caffeine (10 mM) was also present to suppress SR Ca uptake. Depolarization in the absence of Na_o activated inward Ca current and cell shortening.

Repolarization by itself did not induce relaxation until Na_o was added using a quick solution switch. This relaxation was associated with an inward current expected of Ca extrusion via Na/Ca exchange. Integration of the nifedipine-sensitive inward Ca current and the putative $I_{Na/Ca}$ (Figure 39B) showed twice as much I_{Ca} as $I_{Na/Ca}$, consistent with all Ca entry via I_{Ca} and all extrusion via a 3Na:1Ca Na/Ca exchange. This experiment demonstrates the ability of I_{Ca} to supply Ca to support contraction and Na/Ca exchange to extrude Ca from the cell to produce relaxation.

COMPETITION BETWEEN Na/Ca EXCHANGE, THE SARCOLEMMAL Ca-PUMP AND SR Ca-PUMP DURING RELAXATION AND AT REST

Philipson and Ward (1986) extrapolated results from isolated SLV under quasi-physiological conditions and concluded that Na/Ca exchange and the sarcolemmal Ca-ATPase pump were likely to extrude Ca from the cell at comparable rates (~1.2 μmol/kg wet wt/sec). Barry and Smith (1984) suggested that a Na_o-independent mechanism (possibly the sarcolemmal Ca-pump) was responsible for the major fraction of ^{45}Ca efflux from cultured chick heart cells. These initial studies were complicated by unknown Ca-Ca exchange and Barry *et al.* (1986) later concluded that this Na_o-independent component of ^{45}Ca efflux was only ~20% of the Ca efflux via Na/Ca exchange.

There are at least 3 mechanisms which may be quantitatively involved in removal of Ca from the cytoplasm: 1) the SR Ca-pump, 2) the sarcolemmal Ca-pump and 3) Na/Ca exchange. We have more directly tested the competition among these systems for cytoplasmic Ca in intact cells (both during relaxation and in resting cells, Bers & Bridge, 1989; Bers *et al.*, 1989,1990; Hryshko *et al.*, 1989c).

Rapid cooling of cardiac muscle to ~0-1°C in < 1 sec leads to the apparent release of all of the Ca in the SR while simultaneously inhibiting Ca transport mechanisms (Bers *et al.*, 1989, and see Chapter 6). This results in the slow activation of a contracture at this temperature and the amplitude of this rapid cooling contracture (RCC) can be useful as an index of the amount of Ca which was in the SR at the time of cooling. I will discuss this aspect of RCCs in more detail in Chapter 6. Rewarming the muscle re-activates Ca transport systems (e.g. SR and sarcolemmal Ca pumps and Na/Ca exchange) allowing relaxation to occur. We have taken advantage of the cold-immobilized Ca transporters to change the bathing medium prior to relaxation and while $[Ca]_i$ is high.

Figure 40 shows the timecourse of relaxation in a normal Tyrode's (**NT**), when Na/Ca exchange was prevented by removal of extracellular Na and Ca (**0Na**), when SR Ca accumulation was prevented by 10 mM caffeine (**Caff**) and when both interventions are combined (**0Na+Caff**), thereby preventing Na/Ca exchange and SR Ca accumulation. When Na/Ca exchange is blocked the relaxation half-time ($t_{1/2}$) is slowed by ~30%. This

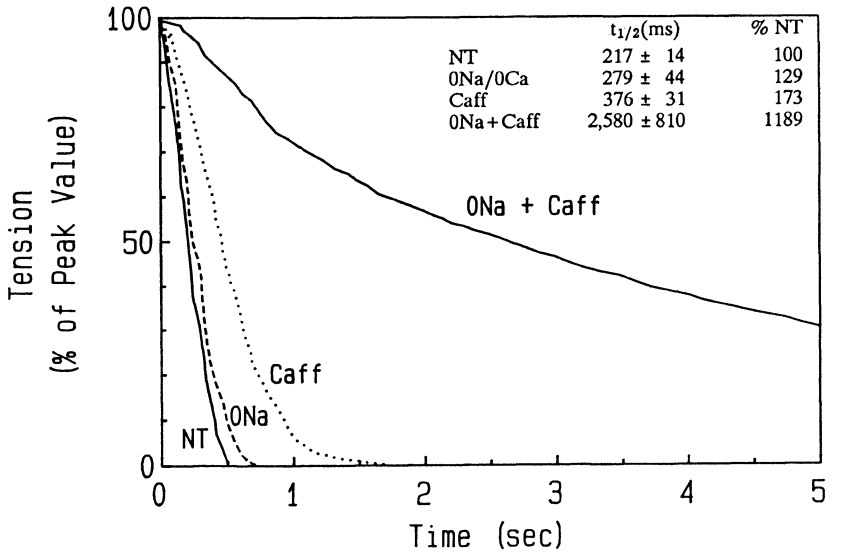


Figure 40. Na/Ca exchange and SR Ca pump compete for $[Ca]_i$ during the timecourse of relaxation in rabbit ventricular muscle after RCCs. During the time when the muscle is at ~1°C the superfusion solution was changed from a normal Tyrode's (**NT**) to either a Na-free, Ca-free (**0Na**), a NT with 10 mM caffeine (**Caff**) or a Na-free, Ca-free solution with 10 mM caffeine (**0Na + Caff**). Half-times of relaxation and percents of the NT value for pooled results are shown in the inset (from Bers & Bridge, 1989, with permission of the American Heart Association).

suggests that Na/Ca exchange may contribute about 30% to the removal of Ca from the cytoplasm during relaxation. Inhibition of SR Ca uptake slows relaxation by more (~70%), but quantitative interpretation of this result is complicated by the action of caffeine to increase myofilaments Ca sensitivity (that is, the sensitizing effect would exaggerate the slowing due to prevention of SR Ca uptake). When both systems are blocked relaxation is slowed almost 1100%. This result indicates that either the SR Ca pump or the sarcolemmal Na/Ca exchange (to a somewhat lesser extent) can produce relaxation at practical rates. However if both systems are blocked so that relaxation must depend on the sarcolemmal Ca-ATPase pump (and possibly other systems), relaxation is extremely slow (taking more than ~20 sec). Thus Na/Ca exchange and the SR Ca-pump compete during relaxation and are the only major routes by which Ca is removed from the cytoplasm during relaxation.

In Figure 41 we also examined the voltage-dependence of SR-mediated and Na/Ca exchange-mediated relaxation by altering the [K] gradient (Bers & Bridge, 1989). When relaxation was mediated by Na/Ca exchange (in the presence of caffeine) relaxation was highly voltage-dependent (see also Bridge *et al.*, 1988). This is expected on thermodynamic

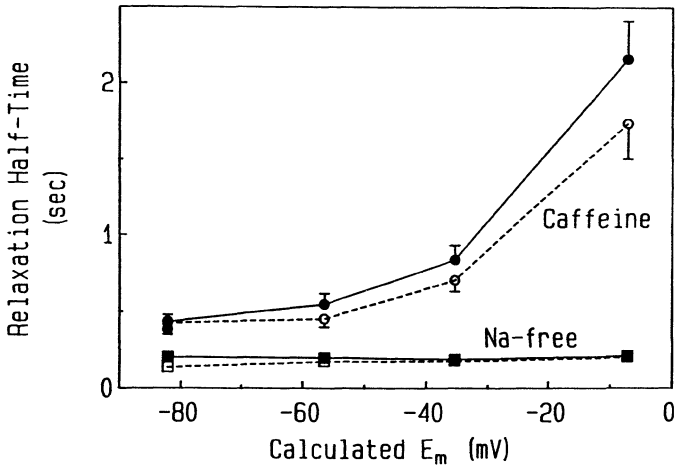


Figure 41. Voltage dependence of relaxation when Na/Ca exchange is prevented by Na-free, Ca-free solution (**Na-free**) and when SR uptake is prevented by 10 mM caffeine (**Caff**). Membrane potential was altered by changing $[K]_o$ during the RCC and rewarming-induced relaxation. The open symbols and were when the experiments were repeated in the presence of 10 μM nifedipine to block Ca current which could occur at the depolarized potentials. In caffeine experiments $[Na]_o$ was reduced to 40 mM and $[Ca]_o$ to ~40 μM to maintain a constant $[Na]^3/[Ca]$ ratio and thus constant $E_{Na/Ca}$ (from Bers & Bridge, 1989, with permission of the American Heart Association).

grounds, since Ca extrusion via Na/Ca exchange is favored by more negative E_m (see above). Nifedipine (broken lines) accelerated relaxation somewhat at depolarized E_m, suggesting the possibility of a small Ca "window current" at these potentials which could slow relaxation. In contrast to the results with Na/Ca exchange, relaxation mediated by the SR (in Na-free, Ca-free solution) was entirely independent of voltage. Crespo *et al.* (1990) recently reached similar conclusions, by comparing the slow phase of $[Ca]_i$ decline in ryanodine-treated guinea-pig ventricular myocytes (attributed to Na/Ca exchange) with the rapid $[Ca]_i$ decline in rat ventricular myocytes (attributed primarily to SR Ca uptake).

Figure 42 shows another type of experiment demonstrating the contribution of Na/Ca exchange to relaxation in an isolated rabbit ventricular myocyte (Hryshko *et al.*, 1989c). We used paired RCCs such that the second RCC could be used to assess the fraction of the SR Ca resequestered by the SR during relaxation from the first RCC. For example, if all of the Ca released from the SR at the first RCC were resequestered during relaxation, the second RCC should be the same size. This is the case in Figure 42B where the cell was bathed in Na-free, Ca-free solution during cooling and rewarming. However if Na/Ca exchange was allowed to compete with the SR (Figure 42A), the second RCC was

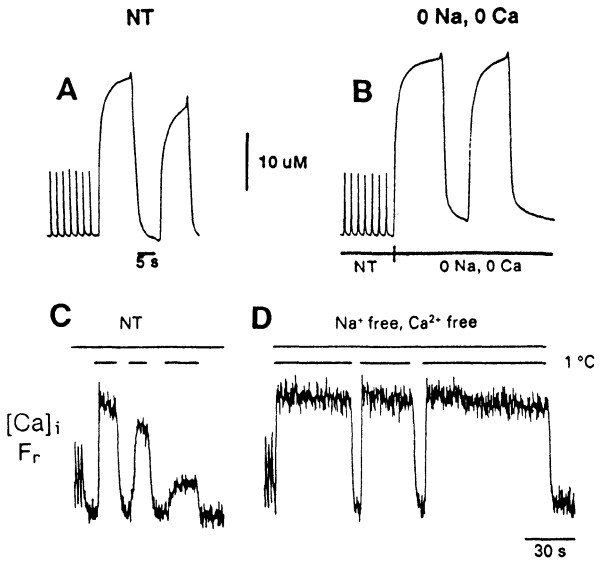


Figure 42. Rapid cooling contractures (RCCs) used to assess the resequestration of Ca by the SR. **A)** Paired RCCs induced after a train of electrically evoked twitches in a rabbit ventricular myocyte. The second RCC may be smaller because some of the Ca released during the first RCC is not resequestered by the SR during that relaxation. **B)** When Na/Ca exchange is prevented from extruding Ca during rewarming (by using a Na-free, Ca-free solution), the SR may resequester all of the Ca released at the first RCC (since the second RCC is as large as the first). **C** and **D)** A similar protocol in a guinea-pig ventricular myocyte except that Ca_i transients were measured during the RCCs (using indo-1) and three successive RCCs were used instead of two (from Hryshko et al., 1989c (top) and Bers et al., 1989 (bottom), with permission).

reduced to ~75% of the first RCC. This again implies that Na/Ca exchange extrudes an amount of Ca responsible for ~25% of developed tension in the presence of an intact SR.

Figure 42C & D shows a similar experiment in a guinea-pig ventricular myocyte except that $[Ca]_i$ is recorded using the fluorescent Ca indicator indo-1 and three RCCs are done in rapid succession, instead of two (Bers *et al.*, 1989). Again there was no decline in the Ca_i transient when RCCs and rewarmings were in Na-free, Ca-free solution (Figure 42D), but there was a sequential decline when extracellular Na and Ca were present (allowing Na/Ca exchange to compete with the SR, Figure 42C). In this case Na/Ca exchange reduced the amplitude of the second and third Ca_i transients by 36% and 51% (with respect to the preceding RCC).

Figure 43 shows an experiment in which the Na_o-dependent component of relaxation was examined in a voltage clamped rat ventricular myocyte where $[Ca]_i$ was measured using indo-1 (loaded into the cell via the patch clamp pipette, Bers *et al.*, 1990). The cell was depolarized from -50 to +15 mV for 50 msec (top) or 1 sec (bottom) in the

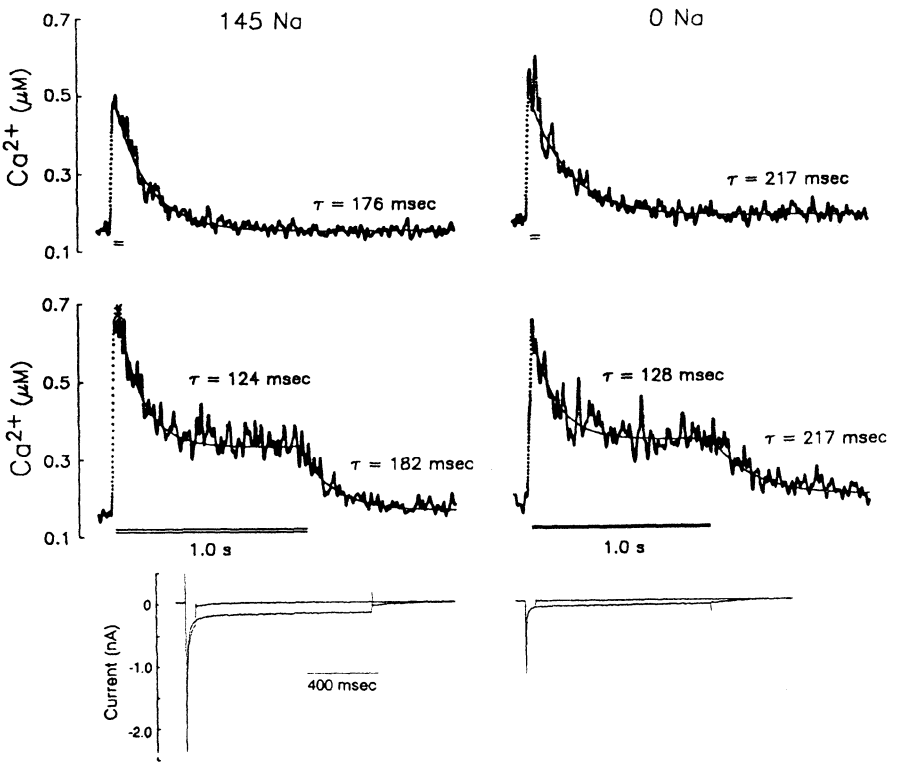


Figure 43. The effect of Na_o on the decline of $[Ca]_i$ in a rat ventricular myocyte with depolarizing pulses from -50 mV to +15 mV for 50 msec (top) or 1 sec (bottom). The dialyzing patch-clamp pipette contained ≤ 0.5 mM Na to prevent Ca influx via Na/Ca exchange and 70 μM indo-1 to assess $[Ca]_i$. During the long pulses and after repolarization, the $[Ca]_i$ was fit with a monoexponential decline (curves superimposed and time constants, τ indicated). During the long depolarizations there may be a small residual Ca influx via Ca channels (see current records at bottom). (from Bers et al., 1990, with permission).

presence (left) or absence (right) of 145 mM Na_o. After repolarization to -50 mV the decline of $[Ca]_i$ for both short and long pulses was monoexponential and the time constant (τ) was slowed by ~20% in the absence of Na_o. During the long pulses at +15 mV the $[Ca]_i$ also declined along a monoexponential, but was relatively insensitive to $[Na]_o$. This is consistent with the voltage dependence expected of Na/Ca exchange, where Ca extrusion is less favored at more positive E_m (such that Ca is preferentially pumped into the SR).

Figures 40-43 demonstrate that the Na/Ca exchange can compete with the SR Ca-pump during the relaxation of cardiac contraction with the Na/Ca exchanger being responsible for ~20 - 30% of the decline in $[Ca]_i$ or force during cardiac relaxation.

Despite the general similarity of the numbers it should also be appreciated that this fraction will vary depending on certain variables such as the action potential configuration, the resting $[Ca]_i$ and aNa_i, peak $[Ca]_i$, the timecourse of the Ca_i transient and the state of the SR and the Na/Ca exchanger itself (see Figure 38). For example, the long depolarization at +15 mV in Figure 43 (bottom left) biases the competition between the SR Ca-pump and the Na/Ca exchange in favor of the SR. Indeed, the next contraction after that one was relatively larger reflecting the higher SR Ca load. On the other hand, if the cell and SR are already Ca loaded, the decline of $[Ca]_i$ can still be accelerated by Na_o at +15 mV (see Fig. 10 in Bers *et al.*, 1990).

We have also taken advantage of RCCs to assess the diastolic efflux of Ca from cardiac myocytes. This works well for the following reasons. During rest there must be some finite rate of leak of Ca from the SR lumen to the cytoplasm. Ca in the cytoplasm is then subject to either extrusion from the cell (via Na/Ca exchange or the sarcolemmal Ca-pump) or resequestration by the SR (for the moment ignoring other potential Ca sinks). If all of this Ca is resequestered by the SR, then the Ca content of the SR would stay constant at rest. If some of this Ca is extruded from the cell, then the SR Ca content would progressively decline during rest. A decline in the first post-rest contraction occurs as a function of rest duration in most mammalian ventricular muscle preparations. This process is known as rest decay (Allen *et al.*, 1976) and is paralleled by a gradual loss of SR Ca during rest assessed by RCCs (Bridge, 1986; Bers *et al.*, 1987, 1989; Bers, 1989). The loss of SR Ca is also apparent as a loss of cellular Ca measured either as the loss of ^{45}Ca during rest (Janczewski & Lewartowski, 1986) or as the accumulation of extracellular Ca during rest assessed by extracellular Ca microelectrodes (Bers & MacLeod, 1986; MacLeod & Bers, 1987; Bers, 1987b). Thus the decline in the SR Ca content during rest is a reflection of the Ca efflux from the cell. If Ca is not being extruded from the cell, the SR Ca load (and RCCs) should remain high and if large amounts of Ca are extruded from the cell, the SR Ca content (and RCCs) should fall concomitantly.

Sutko *et al.* (1986) demonstrated that the rest decay of contractions was dependent on the transsarcolemmal [Na] gradient. This would be expected if the Na/Ca exchange were important in the Ca extrusion underlying the process of rest decay, but does not allow assessment of the relative roles of Na/Ca exchange and the sarcolemmal Ca-pump. Several investigators have suggested that the sarcolemma Ca-pump may be especially important in diastolic Ca efflux, due to its high affinity ($K_m(Ca) \sim 60$ nM, see Table 11) compared to that for Na/Ca exchange (e.g. $K_m(Ca) \sim 1$ μM or higher).

Figure 44 illustrates the process of rest decay in guinea-pig ventricular myocytes. RCCs were induced at various times after the last stimulated twitch and Ca_i transients (using indo-1 fluorescence) and shortening of the myocyte were recorded (Bers *et al.*, 1989). With longer rest intervals in normal Tyrode's (NT) the amplitude of the RCC and accompanying Ca_i transient are progressively smaller. When the rest period is in Na-free, Ca-free solution there is very little decline in the amplitude of the post-rest RCCs and Ca_i

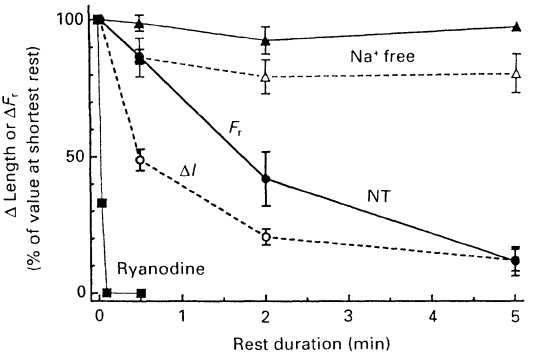


Figure 44. The influence of rest duration on the amplitude of shortening (Δl) or Ca_i transients (F_r) during RCCs in guinea-pig ventricular myocytes. Prior to the rest period the cells were stimulated at 0.5 Hz. $[Ca]_i$ was measured with indo-1. The solution during the rest period was changed from the normal Tyrode's (NT) to a Na-free, Ca-free solution for the RCCs on the "Na-free" curve. The more rapid decline of the shortening NT curve can be attributed to different manner in which the fluorescence signal and the myofilaments depend on [Ca]. Ryanodine was also equilibrated with cells (■) and did not prevent RCCs, but greatly accelerated the rate of rest decay (see Chapter 6) (from Bers et al., 1989, with permission).

transients even after 5 min of rest. This is consistent with Na/Ca exchange being the main means by which Ca is extruded from the resting cell. Indeed, the Na-free solution which is used to prevent Na/Ca exchange is also Ca-free to prevent Ca gain by Na/Ca exchange in the absence of Na_o. Under these conditions the sarcolemmal Ca-pump should be even better able to extrude Ca into the Ca-free solution. Even so, there is practically no rest decay in the absence of Na/Ca exchange. This result demonstrates that in intact cells, Ca efflux during diastole (as during relaxation) is mainly via Na/Ca exchange with the sarcolemmal Ca-pump making at most a very small contribution.

If 20-30% of the Ca removed from the cytoplasm during relaxation of a steady state contraction is extruded from the cell via Na/Ca exchange, it is to be expected that Ca influx should comprise a similar fraction of the Ca required to support contraction. This would be a requirement of the steady state. This is also in reasonable agreement with estimates of Ca entry via Ca current (~10 μmol/kg wet wt, see page 62) and the amount of Ca required to activate the myofilaments (30-50 μmol/kg wet wt, see page 34). The Na/Ca exchange would have to operate for 60-100 msec near V_{max} (3 μA/μF or 150 μmol/kg wet wt/sec, see page 80) or for longer at a lower rate to produce this amount of efflux. Of course these values are approximate and temporal overlap of Ca influx via I_{Ca} and efflux via Na/Ca exchange puts restrictions on this simple argument.

In conclusion, it is evident that the Na/Ca exchange system is very important in myocardial Ca regulation. The fundamental characteristics of this system are becoming increasingly clear (e.g. its 3:1 Na:Ca stoichiometry and molecular regulation). Na/Ca exchange is the main means by which Ca is extruded from the cell, during both relaxation and diastole. By comparison, the sarcolemmal Ca-pump seems relatively unimportant in cardiac muscle (particularly because net Ca movement in either direction via Na/Ca exchange would appear able to overwhelm this pump). Indeed, Na/Ca exchange can compete with the powerful SR Ca-pump for cytoplasmic Ca and thereby contribute to relaxation. The Na/Ca exchange can also mediate Ca influx sufficient to activate contraction, but this probably does not occur under normal physiological conditions (where the main role of the Na/Ca exchange seems to be to extrude Ca from the cell). In fact, Na/Ca exchange must extrude as much Ca as enters the cell via Ca current in each cardiac cycle, in order for a steady state to be achieved. As we have seen, the amount of Ca entry (via I_{Ca}) and extrusion via Na/Ca exchange may be about 20-30% of that required to activate a "normal" steady state contraction. Finally, since the Na/Ca exchange is the main means by which the cell extrudes Ca, anything which prevention of this Ca extrusion will increase cellular Ca loading and can lead to Ca overload. These issues will be addressed further in Chapter 9.

CHAPTER 6

SARCOPLASMIC RETICULUM Ca UPTAKE, CONTENT AND RELEASE

SR Ca-PUMP

Kielley & Meyerhoff (1948) first described a Mg-activated ATPase in a microsomal fraction from muscle. Ebashi (1961; Ebashi & Lipmann, 1962) and Hasselbach & Makinose (1961) later identified this as the membrane associated Ca-ATPase or "relaxing factor" in muscle responsible for lowering cytoplasmic [Ca]. This Ca-pump has been the subject of intensive study since that time (see recent reviews: Ikemoto, 1982; Hasselbach & Oetliker, 1983; Tanford, 1983; Martonosi & Beeler, 1983; Inesi, 1985, 1987; Fleischer & Tonomura, 1985; Entman & Van Winkle, 1986; Schatzmann, 1989).

Skeletal and cardiac muscle SR vesicles isolated on sucrose density gradients can be separated into two types of vesicles (Meissner, 1975; Campbell *et al.*, 1980; Jones & Cala, 1981; Saito *et al.*, 1984). A heavy SR fraction is obtained at higher density and contains terminal cisternae and the Ca-release channel (or ryanodine receptor) as well as the Ca-pump. At lower density, a light SR fraction is obtained in which most of the protein (~90%) is the Ca-pump (Meissner, 1975; Campbell, 1986; Fleischer & Inui, 1989). The light SR fraction probably originates from the longitudinal SR and this implies a functional distinction in different SR regions. That is, Ca is pumped into the SR along the longitudinal tubules and is released from the terminal cisternae, where the SR "foot" processes spans the gap from the SR to the sarcolemma (see Chapter 1). The release process will be discussed below and I will focus initially on the SR Ca-pump. However, it should be noted that many characteristics of the Ca-pump have been studied in mixed populations of SR vesicles (heavy and light). In heavy SR vesicles Ca uptake can be "short circuited" by open Ca-release channels (which seems to be the usual state of SR release channels in isolated vesicles). Ca uptake in cardiac heavy SR vesicles can be dramatically increased by inclusion of agents which block the Ca release channel (e.g. ruthenium red or Mg, Meissner & Henderson, 1987).

Two skeletal SR Ca-pump proteins have been sequenced and cloned and exhibit 84% amino acid identity (MacLennan *et al.*, 1985; Brandl *et al.*, 1986). One of these is

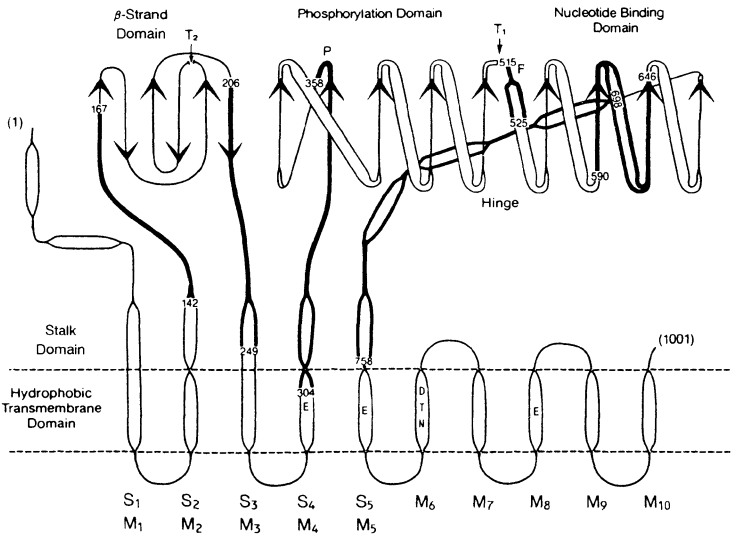


Figure 45. Schematic diagram of the skeletal muscle SR Ca-pump indicating predicted secondary structure. Most of the protein is on the cytoplasmic side of the SR membrane including β-strand, phosphorylation, nucleotide binding, stalk domains and a Hinge. Ten transmembrane spans (M_1-M_{10}) are indicated and 6 amino acids on M_4, M_5, M_6 and M_8 which are important in Ca-binding and are indicated by their 1 letter codes (E, glutamate 309, 771, 908; D, aspartate 800; T, threonine 799; N, asparagine 796). In the membrane these transmembrane spans may be arranged in a cylinder to form an ion channel through the SR bilayer. (Updated form of model diagram from McLennan *et al.*, 1987, with permission).

from fast twitch muscle (molecular weight = 110,331) and the other from slow twitch muscle (having 4 less amino acids) and the latter is apparently the same as the cardiac Ca-pump (Brandl *et al.*, 1986, 1987; MacLennan *et al.*, 1987). Figure 45 illustrates the proposed secondary structure of the SR Ca-pump proposed by MacLennan's group, indicating where ATP binds (nucleotide binding domain), where phosphorylation occurs (aspartate-351), a β-strand domain and a hinge region. The protein appears to have 10 membrane spanning regions (M_1-M_{10}), where M_1-M_5 each have an additional α-helical "stalk" regions on the cytoplasmic side. The crucial high affinity Ca binding sites were initially proposed to reside in the highly anionic stalk region (Brandl *et al.*, 1986). However, more recent results suggest that the important Ca binding sites are not in the stalk, but within the transmembrane domains (M_4-M_6 and M_8, Clarke *et al.*, 1989a,b).

The transport reaction starts with two Ca ions and one ATP molecule binding to high affinity binding sites on the cytoplasmic side of the pump (see Fig. 46). The terminal phosphate of ATP is then transferred to aspartate-351 on the Ca-pump and the Ca ions are then "occluded" (which simply means that they cannot be readily released to, or competed with on the cytoplasmic side). Considering the results of Clarke *et al.* (1989a,b) one may

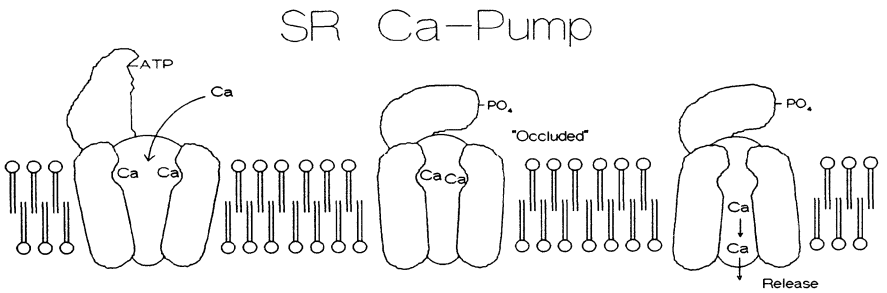


Figure 46. Simplified transport scheme for the SR Ca-pump. Starting from the left, 2 Ca ions bind with high affinity, then the bound ATP is used to phosphorylate aspartate-351 and modify the conformation of the protein so that the Ca ions are occluded. Finally, the Ca ions are released to the interior of the SR due to a decreased Ca affinity and the availability of a pathway into the SR.

suppose that Ca binds to sites in the transmembrane regions (M_4-M_6 & M_8) which could form a channel and that the phosphorylation alters the structure such that Ca cannot return to the cytoplasmic side from which it came (occlusion). The phosphorylation also reduces the Ca affinity and Ca may then be released to the lumen of the SR via the channel. This reduction in affinity is important for the rapid release of Ca into the SR lumen where ambient [Ca] is higher.

Two Ca ions are transported by the Ca-pump for each ATP molecule consumed in both skeletal and cardiac SR (Tada *et al.*, 1982), though lower stoichiometries are often reported for the *in vitro* cardiac Ca-pump. The lipid environment is also important for this enzyme and removal of the ~30 lipid molecules associated with the SR Ca-pump decreases Ca-dependent ATPase activity (Hidalgo *et al.*, 1976).

The number of SR Ca-pumps in guinea-pig ventricle was estimated from phosphoenzyme formation by Levitsky *et al.* (1981, 5.9 μmol/kg wet wt). The maximum turnover rate for the cardiac SR Ca-pump estimated from data of Shigekawa *et al.* (1976) in dog and Levitsky *et al.* (1981) in guinea-pig are about 10 - 15 Ca ions/pump/sec. This is similar to the turnover rate of the skeletal muscle SR Ca-pump and Shigekawa *et al.* (1976) attributed the slower Ca-pumping of cardiac SR to ~4-fold lower pump site density and lower Ca affinity.

Regulation of the Cardiac SR Ca-pump by Phospholamban

A major difference between the cardiac and skeletal muscle Ca-pumps is that the former is regulated by the phosphorylation of the protein phospholamban (Tada & Katz, 1982). Phospholamban exists as a homopentamer (apparent total molecular weight ~ 22,000) and the amino acid sequence has been deduced from cDNA (Fujii *et al.*, 1987). The monomer is 6080 Da (52 amino acids) and it exhibits one hydrophobic and one hydrophilic domain. Simmerman *et al.* (1986) proposed a structural model of the pentamer

which could have a hydrophilic pore through the SR membrane with phosphorylation sites on the cytoplasmic surface. Kovacs *et al.* (1988) showed evidence that dephosphorylated phospholamban can form Ca-selective channels in lipid bilayers. It is not yet clear whether or how this ionophoric property might be related to the function of phospholamban in cardiac SR.

Phospholamban can be phosphorylated by cAMP-dependent protein kinase at serine-16 (Kirchberger *et al.*, 1974; Tada *et al.*, 1974; Simmerman *et al.*, 1986). This phosphorylation stimulates the pump by lowering the K_m(Ca) and has been observed in intact perfused hearts in response to catecholamines (Le Peuch *et al.*, 1980; Kranias & Solaro, 1982; Lindeman *et al.*, 1983). This stimulation of Ca uptake rate by catecholamines appears to be the main means by which β-adrenergic agonists accelerate relaxation in the heart (McIvor *et al.*, 1988). It may also be supposed that catecholamines will bias the competition between the SR Ca-pump and the sarcolemmal Na/Ca exchange in favor of the former limiting Ca extrusion from the cell via the exchanger (see Chapters 5 & 9). In combination with the potent stimulation of sarcolemmal Ca current by β-adrenergic agonists (see Chapter 4), this increase in SR Ca-pumping could result in a substantial increase in SR Ca content available for release.

Phospholamban can also be phosphorylated at threonine-17 by Ca-calmodulin dependent protein kinase (Le Peuch *et al.*, 1979; Simmerman *et al.*, 1986) and can also be phosphorylated by protein kinase C (Ca/phospholipid dependent, Iwasa & Hosey, 1984; Movesian *et al.*, 1984). These kinases can increase Ca pumping but it is not clear whether phosphorylation by these means has physiological importance. For example, Wegener *et al.* (1989) demonstrated a more gradual Ca-calmodulin-dependent phosphorylation of threonine-17 in response to β-adrenergic stimulation in intact heart. On the other hand Rogers *et al.* (1990) found that protein kinase C decreased SR Ca uptake in permeabilized myocytes.

Hicks *et al.* (1979) proposed that phospholamban functions as an inhibitor of the cardiac Ca-pump and that phosphorylation remove this inhibitory effect (i.e. decreasing the K_m(Ca) to a value similar to that of the skeletal muscle Ca-pump where phospholamban is lacking, Inui *et al.*, 1986). James *et al.* (1989) provided evidence that phospholamban interacts with the Ca-pump (near the phosphorylation site of the Ca-pump) only when phospholamban is dephosphorylated. While the molecular details of the interaction of phospholamban with the Ca-pump are not yet resolved, this regulatory derepression appears to be the general mechanism of action.

Regulation of the SR Ca-pump by Ca, pH, ATP and Mg

The K_m(Ca) of the cardiac SR Ca ATPase is typically 1-5 μM, but can be decreased 3-5 fold by phosphorylation of phospholamban (Tada *et al.*, 1974; Shamoo *et al.*, 1985). ATP seems to bind to two sites, a high affinity site ($K_d \sim 1$ μM) which is the substrate site and a second lower affinity site ($K_d \sim 200$ μM) which serves a regulatory role (deMeis &

Vianna, 1979; DuPont, 1977; Verjovski-Almeida & Inesi, 1979). When cellular ATP levels fall during ischemia, there may be some decline in SR Ca-pumping and slowing of relaxation due to the allosteric effect, but [ATP] would have to be very low to prevent ATP binding to the substrate site. The actual substrate for the Ca-pump is probably MgATP, but other nucleotides can also be used (Tada *et al.*, 1978). However, the GTPase activity of the cardiac Ca-pump has different characteristics (e.g. very low Ca sensitivity, Tate *et al.* 1985, 1989).

The SR Ca-pump is also sensitive to pH. Shigekawa *et al.* (1976) found a broad pH optimum around 8, which was more alkaline than that observed for skeletal muscle. Fabiato & Fabiato (1978a; Fabiato, 1985e) found that the Ca uptake by cardiac SR in the mechanically skinned cell was progressively decreased as pH was decreased from 7.4. Thus, acidosis associated with ischemia may be expected to depress the rate of SR Ca-pumping and thus slow relaxation as is observed during acidosis. Of course acidosis can also reduce the Ca sensitivity of the myofilaments, Ca current, Na/Ca exchange etc. (see Chapter 9).

It should also be noted that there is a broad range of values reported for the optimum pH and this may depend on the assay conditions (e.g. [Ca], oxalate, [Mg], temperature, [ATP], phosphorylation state, the type of SR vesicles...). Indeed, this consideration is valid for the [Ca] and [ATP] dependence as well and it is not clear that we know enough to accurately mimic the intracellular physiological conditions *in vitro* to determine the *in vivo* Ca-pump dependence on [Ca], [Mg], [ATP], etc. The situation is further complicated by the Ca release channel which will alter **net** Ca transport by the Ca-pump and the gating of this channel is also affected by [Ca], ATP, Mg and pH (see below). For example, Fabiato & Fabiato (1978a) reported that at pH 7.4 the Ca uptake by the SR was optimal at pCa = 7.75, since under their particular conditions higher [Ca] induced Ca release.

These considerations make it somewhat difficult to extrapolate the function of the SR Ca-pump quantitatively to the level of Ca regulation in the intact cell. Indeed, many of the characteristics described for isolated systems (such as the SR vesicles) *in vitro* will need to be examined again in more intact systems (such as cardiac myocytes) to determine how the regulation is altered in the cell where conditions are different and other competing or interacting systems are present. Two of the things we really want to know about the SR Ca-pump in this context are how fast can it pump Ca out of the cytoplasm (with respect to relaxation) and how much Ca can be pumped and stored in the SR under physiological conditions (such that it is available for release).

SR Ca Uptake Capacity

The maximal Ca content of the SR in mammalian cardiac muscle (in the absence of precipitating anions) has been estimated by numerous investigators and values vary (in μmol Ca/kg wet wt.): 170 (Solaro and Briggs, 1974); 100 to 300 (Dani *et al.*, 1979); 125

(Hunter *et al.*, 1981); ~160 (Levitsky *et al.*, 1981); >57 (Fabiato, 1983); 258 (Bridge, 1986; 100 to 200 (Bers *et al.*, 1989, and see Chapman *et al.*, 1983 for other values). Thus, there would appear to be sufficient Ca in the SR to activate the myofilaments (see Chapter 3). On the other hand, there is very little direct assessment of the SR Ca content in intact cells. There is also little information concerning the fraction of SR Ca released during a twitch.

If one extrapolates the apparent total [Ca] within the SR from these values assuming a tissue density of 1.06 g/ml, 30% extracellular space (200 μmol Ca/kg wet wt. = 275 μmol Ca/liter cell, see also pages 9 & 33) and the fraction of cell volume occupied by the SR (~2-3 %, see page 7) the implied intra-SR [Ca] would be ~9-14 mM! If this intra-SR Ca were all free, this would mean that the Ca-pump would have to maintain a [Ca] gradient of ~100,000. Most of the Ca in the SR is probably bound such that the free [Ca] in the SR is only ~1 mM (see below). For one ATP to provide sufficient energy for transporting 2 Ca ions up this chemical gradient requires $\Delta G = 2RT \ln([Ca]_{SR}/[Ca]_{cyt})$ or ~ 10 kcal/mol. This value of ΔG is close to the value of ΔG_{ATP} = 14 Kcal/mol estimated in cardiac muscle by Allen *et al.* (1985a). Thus, this [Ca] gradient would seem to be close to the thermodynamic limit for a very efficient Ca-pump.

Calsequestrin

Apparently the intra-SR [Ca] is buffered to a significant extent by calsequestrin. Calsequestrin was first described in skeletal muscle SR by MacLennan & Wong (1971). Meissner (1975) suggested that calsequestrin was primarily localized in the terminal cisternae of the SR. Calsequestrin in cardiac muscle was first identified and purified by Campbell *et al.* (1983) and Jorgensen & Campbell (1984) provided evidence that cardiac calsequestrin is localized to junctional SR (and corbular SR). The primary structure of cardiac calsequestrin has been deduced by cDNA cloning (MW = 45,269, Scott *et al.*, 1988) and is only 63% homologous with the fast twitch skeletal muscle calsequestrin (MW = 42,435, Fliegel *et al.*, 1987). As with the SR Ca-pump and phospholamban, "cardiac" calsequestrin is also expressed in slow twitch skeletal muscle (Scott *et al.*, 1988).

Cardiac calsequestrin is highly acidic and each molecule binds ~35-40 Ca ions (or ~900 nmol Ca/mg protein, Mitchell *et al.*, 1988) with an apparent $K_m(Ca)$ ~ 500 μM. Assuming the maximum yield of isolated cardiac calsequestrin reported by Campbell (1986, 40 mg/kg wet wt) represents a true yield of 25-50% of whole heart calsequestrin, this would correspond to 72 - 150 μmol of Ca binding sites per kg wet wt. With the assumptions about tissue density, extracellular space volume and SR volume from above, this would correspond to 5 - 11 mM of Ca binding sites in the SR. If calsequestrin makes up 15-25% of the SR protein in heart (as is the case for skeletal muscle, Campbell, 1986) these numbers could be several-fold higher. Thus, these sites could buffer a substantial fraction of the Ca taken up by the SR with an appropriately low affinity.

Calsequestrin appears to be attached to the junctional face membrane in the terminal cisternae of the SR (e.g. Franzini-Armstrong *et al.*, 1987) and when Ca binds to

calsequestrin the shape of the molecule changes (Ikemoto *et al.*, 1972, 1974; Cozens & Reithmeier, 1984). Ikemoto *et al.* (1989) have even proposed that calsequestrin is involved in the regulation of the Ca release channel in the SR, but additional work will be needed to clarify this possibility.

In addition to the Ca-pump and calsequestrin there is a third major SR protein, the 53,000 Dalton glycoprotein. This glycoprotein is co-distributed with the Ca-pump protein and was thought to modulate the pump, but Leberer *et al.* (1989a) cloned this glycoprotein (MW = 52,421) and found that it did not alter Ca-pump function in cotransfection studies. The function of this major glycoprotein remains unknown, but it is interesting that it is identical to the carboxy-terminal half of a minor glycoprotein (160,000 MW, sarcalumenin, Leberer *et al.*, 1989b, 1990), in which the amino-terminal half is highly acidic like calsequestrin and binds ~35 mol Ca/mol protein. The 53,000 Dalton glycoprotein itself did not bind Ca appreciably. There is another minor SR protein which binds Ca, known as the high affinity Ca-binding protein (or calreticulin, MW = 46,567, Fliegel *et al.*, 1989b). Calreticulin binds 1 Ca with high affinity and ~25 Ca with low affinity (MacLennan *et al.*, 1972; Ostwald & MacLennan, 1974) and is present in both muscle and non-muscle cells (Fliegel *et al.*, 1989a,b). All three of these proteins (53,000 and 160,000 Da glycoproteins and calreticulin) appear to be in the SR lumen, but their functions are not yet clear. Several other minor SR proteins are described by Campbell (1986).

SR Ca Uptake Rate and Relaxation

From the above it seems clear that the SR can accumulate sufficient Ca to activate the myofilaments (though the mechanism of Ca release will be discussed later). The other key issue is whether the SR can remove Ca from the cytoplasm rapidly enough to explain relaxation. In general, estimates of the rate of cardiac SR Ca transport in vesicles are lower than one would anticipate for relaxation (e.g. Chapman *et al.*, 1983). For example, maximal rates of Ca uptake by cardiac SR (at [Ca] $\geq$ 10 μM) are typically ~150 μmol/kg wet wt/sec (Solaro & Briggs, 1974; Will *et al.*, 1976; Feher *et al.*, 1988a,b). Feher *et al.* (1988b) measured the maximal rate of Ca uptake by the SR in dog ventricular homogenate and isolated SR taking precautions to block leakage of Ca out of heavy SR vesicles by including a very high concentration of ryanodine (see below) and to keep transport at V_{max} by using oxalate to precipitate Ca inside the vesicles. At 200 μM Ca the uptake rate was 174 μmol/kg wet wt/sec (and only 62 μmol/kg wet wt/sec in the absence of ryanodine). At lower values of [Ca] likely during contraction and relaxation (0.1 - 1 μM) the uptake rates are generally several - fold too low to account for the removal of Ca anticipated during relaxation (~250 μM Ca/kg wet wt/sec or ~50 μM Ca/kg wet wt in ~200 msec, e.g. Levitsky *et al.*, 1981).

This may be a case where studies in isolated vesicles can provide only a rough estimation for extrapolation to the intact cell. Whether the cardiac SR can pump Ca rapidly enough to produce relaxation may be better studied in more intact systems (taking

advantage of the knowledge gained with isolated SR vesicles e.g. about caffeine sensitivity of SR Ca uptake, Weber & Herz, 1968). The experiment shown in Figure 40 (page 86) demonstrates that the SR Ca-pump is the main means by which $[Ca]_i$ is reduced during cardiac muscle relaxation. Relaxation was only slowed by ~29% when Na/Ca exchange was prevented, but if SR Ca accumulation was also prevented by inclusion of caffeine, relaxation was slowed dramatically (i.e. by >1000%, Bers & Bridge, 1989). Thus, while Ca extrusion via Na/Ca exchange may contribute to relaxation, the SR Ca-pump appears to be the dominant means of removing Ca from the cytoplasm during normal relaxation (see pages 80-85). It should be noted that the rate of cardiac muscle relaxation is also modified by mechanical factors such as load dependence, which are discussed in detail in a recent review by Brutsaert & Sys (1989), but will not be discussed here.

ASSESSMENT OF SR Ca CONTENT IN INTACT CARDIAC MUSCLE

The Ca content of the SR which is available for release is clearly an important determinant of contractile state, but is a difficult parameter to measure in intact muscle or myocytes. SR related Ca fluxes have been extensively studied in isolated SR vesicles and to a lesser extent in *skinned* cardiac muscle preparations. However, few experimental approaches provide information concerning the amount of Ca in the SR of intact cardiac muscle or myocytes.

Electron Probe Microanalysis

Electron probe microanalysis has been used to directly assess the Ca content of cardiac junctional SR (**jSR**, Wheeler-Clark & Tormey, 1987; Jorgensen *et al.*, 1988; Wendt-Gallitelli & Isenberg, 1989; Moravec & Bond, 1990). These studies have indicated Ca contents in the junctional SR (under non-Ca-depleting conditions) of ~15 mmol/kg dry wt. Under conditions expected to deplete the SR of Ca (e.g. long rest periods, ryanodine or freeze clamping during the rising phase of a contraction) values of 3-7 mmol/kg dry wt were reported. Appropriate extrapolation of these values to either mmol Ca/liter junctional SR or μmol/kg wet wt heart must take certain technical limitations into account (e.g. dispersion of the X-ray beam into other regions and geometry of junctional SR within the depth of section, ~200 nm). At the simplest level, assuming 4 kg wet jSR/kg dry jSR, a jSR density of 1.1 g/cm^3 and 0.3% of cell volume as jSR or terminal cisternae (see Table 2, page 7), 15 mmol/kg dry jSR would correspond to ~4 mM total [Ca] in the jSR or ~12 μmol/kg wet wt heart. This latter value is about 1/10 of the SR Ca content estimated by biochemical or physiological approaches above and about 25-40% of the Ca required to activate a normal contraction (see page 34). The 4 mM value also seems low for the total jSR [Ca] based on calculations above, especially since calsequestrin is located in this region of the SR. Furthermore, only ~1/2 to 3/4 of this Ca appears to be lost when the SR is Ca depleted (Walsh & Tormey, 1989; Moravec & Bond, 1990). Therefore, either electron

probe microanalysis methods significantly underestimate the amount of jSR Ca or the quantity of Ca localized specifically to the jSR is insufficient to explain activation of contraction. While electron probe microanalysis is probably the most direct and absolute method of measurement of SR Ca content, it is technically difficult, tissue destructive and clear extrapolations to intact tissue are still problematic.

Caffeine-Induced Contractures

One can also assess SR Ca by less direct means which can be done "on line" with intact, contracting cardiac muscle or myocytes. Two such approaches use the rapid application of caffeine or cold solution (~1°C) to induce SR Ca release at a desired time and the amplitude of the resulting contracture or $[Ca]_i$ transient can be used as an index of the SR Ca available for release at that time. An inherent limitation with these indirect approaches is that the actual amount of Ca released (in μmol/kg wet wt) is not easy to determine since the Ca released quickly equilibrates with the intracellular buffers (see Chapter 3). However, the ability to use these approaches reproducibly in the same muscle or myocyte makes them very sensitive means of assessing changes in SR Ca content. Of course whenever tension is being measured to assess SR Ca load, one must be careful that an intervention under study does not alter myofilament Ca sensitivity (e.g. as is the case for β-adrenergic agonists).

The use of caffeine-induced contractures in this manner was first described by Endo (1975b) in mechanically skinned skeletal muscle fibers and Fabiato (1985b; Fabiato & Fabiato, 1975a,1978a) has used this approach extensively in mechanically skinned cardiac myocytes. This approach has also been used in intact cardiac muscle and myocytes (Chapman & Léoty, 1976; Bers, 1987a; Smith *et al.*, 1988; O'Neill *et al.* 1990a). Figure 47 shows caffeine-induced contractures in an isolated rat ventricular myocyte, where the cell has been loaded with the fluorescent Ca indicator, indo-1. Indo-1 fluorescence is strongly quenched by caffeine, but in a wavelength-independent manner so that the fluorescence ratio used to estimate $[Ca]_i$ is unaffected. O'Neill *et al.* (1990a) have turned this potential problem into an advantage by using the quenching of fluorescence as an indicator of intracellular [caffeine]. Thus, the top trace shows the rapid rise in $[Ca]_i$ upon application of caffeine, the second trace shows myocyte shortening and the third trace shows the change in $[caffeine]_i$. The $[Ca]_i$ rise is transient and the decline may represent Ca extrusion from the cell via Na/Ca exchange. Indeed, under these conditions Clusin *et al.* (1983) and Callewaert *et al.* (1989) recorded inward currents thought to be $I_{Na/Ca}$ and Bers and MacLeod (1986) recorded a rise in extracellular [Ca] due to Ca extrusion from the cell. This interpretation is consistent with the results shown in Fig. 40, where relaxation of cooling contractions in the presence of caffeine depended almost exclusively on Na/Ca exchange.

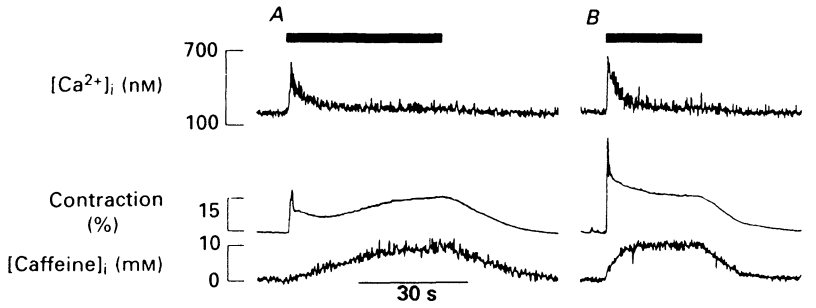


Figure 47. Caffeine-induced contractures in an isolated rat ventricular myocyte. 10 mM caffeine was applied during the period indicated by the bar above each panel, but the perfusion rate was slower in A. The top panel shows $[Ca]_i$ estimated from the ratio of indo-1 fluorescence at two wavelengths (the indicator was loaded by preincubation with the acetoxymethylester form of indo-1). The second trace is myocyte shortening as a percentage of resting cell length and the bottom trace is the intracellular [caffeine] estimated by quenching of indo-1 fluorescence at a single wavelength (from O'Neill *et al.*, 1990, with permission).

The biphasic nature of the contraction in Fig. 47A reflects a complicating aspect of caffeine-induced contractures, namely sensitization of the myofilaments to Ca (Fabiato, 1981b; Wendt & Stephenson, 1983; Eisner & Valdeolmillos, 1985). The second component of the contraction develops along the same timecourse as the rise in $[caffeine]_i$ and is likely to be due primarily to this myofilament sensitizing effect. Figure 47B is the same as 47A except that the application of caffeine was much more rapid, causing the two components of the contraction to merge creating a contraction with a phasic and a tonic component. O'Neill *et al.* (1990) compared the [caffeine] dependence of these two phases. The induction of SR Ca release was apparently maximal at ≥2.5 mM caffeine ($K_{1/2}$ ~ 1 mM) and the sensitizing effect appeared to increase linearly with [caffeine] up to 50 mM.

This is a useful approach, especially in isolated myocytes where diffusional limitations are minimal. The other effects of caffeine such as myofilament sensitization (above) and phosphodiesterase inhibition (Butcher & Sutherland, 1962) which can increase cAMP and cAMP-dependent protein kinase can complicate interpretations. The fact that the Ca released is immediately subject to transport systems such as Na/Ca exchange (above) is also a limitation. Finally, due to the transient nature of the $[Ca]_i$ rise (Fig. 47) diffusional barriers in multicellular preparations mean that the $[Ca]_i$ transient will occur at different times throughout the preparation. This lack of synchrony can prevent significant contractions from being observed. Indeed, we find that caffeine contracture amplitude is inversely related to the diameter of the multicellular preparation.

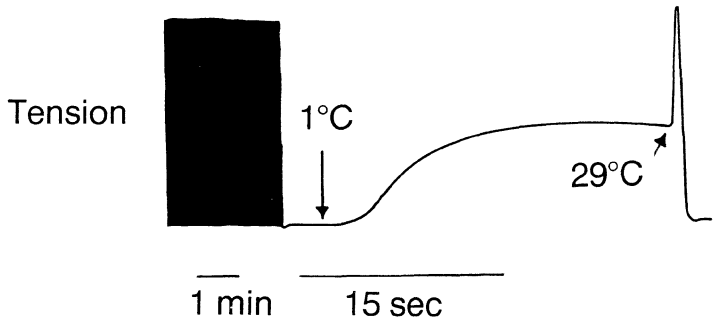


Figure 48. A rapid cooling contracture (RCC) in rabbit ventricular muscle. Steady state electrical stimulation (0.5 Hz) was terminated and 2 sec later (↓) the superfusate was switched from a 29°C control solution to a similar solution at 1°C. The surface temperature of the muscle decreased rapidly ($t_{\frac{1}{2}}$ ~ 100 msec). After the RCC had reached a maximum value the solution was switched back to 29°C (↗), which induced a "rewarming spike" (due to increased myofilament sensitivity) and rapid relaxation.

Rapid Cooling Contractures

Rapid cooling of mammalian cardiac muscle (to ~ 1°C) results in a contracture which is attributable to SR Ca release. Rapid cooling contractures (RCCs) were first described in skeletal muscle fibers by Sakai (1965), but were only observed after pretreatment with modest concentrations of caffeine (0.3-1 mM). This contrasts with mammalian cardiac muscle where RCCs are readily induced under normal conditions in the absence of caffeine (Kurihara & Sakai, 1985; Bridge, 1986). In mammalian cardiac muscle caffeine only inhibits RCCs (with a $K_{\frac{1}{2}}$ ~ 1 mM) and RCCs can be abolished by 5-10 mM caffeine (Bers *et al.*, 1987; Bers & Bridge, 1988).

Figures 48 and 49A show typical RCCs in rabbit ventricular muscle and isolated guinea-pig ventricular myocytes after stimulation was terminated. Rapid cooling from 30°C to 0-1°C induces a rapid release of the available SR Ca to the cytoplasm. This Ca then activates a contracture (slowly at 1°C), the amplitude of which is indicative of the amount of Ca available for release from the SR at the time of cooling. During the time at 0-1°C ion transport mechanisms (e.g. Na/Ca exchange and Ca-pumps) are inhibited so $[Ca]_i$ declines relatively slowly. Upon rewarming, a "rewarming spike" is observed on the tension record, but not on the $[Ca]_i$ trace. Thus, this "rewarming spike" is due to a rapid increase in myofilament Ca sensitivity induced by rewarming (in agreement with skinned fiber results, see page 26 and Harrison & Bers, 1989a). Rewarming also reactivates the ion transport mechanisms (such as the SR Ca-pump and Na/Ca exchange) which were inhibited by the cold. Reactivation of these processes causes the muscle to relax quickly and changing solution composition during the cold can allow evaluation of the transport mechanisms responsible for relaxation (see pages 85-88).

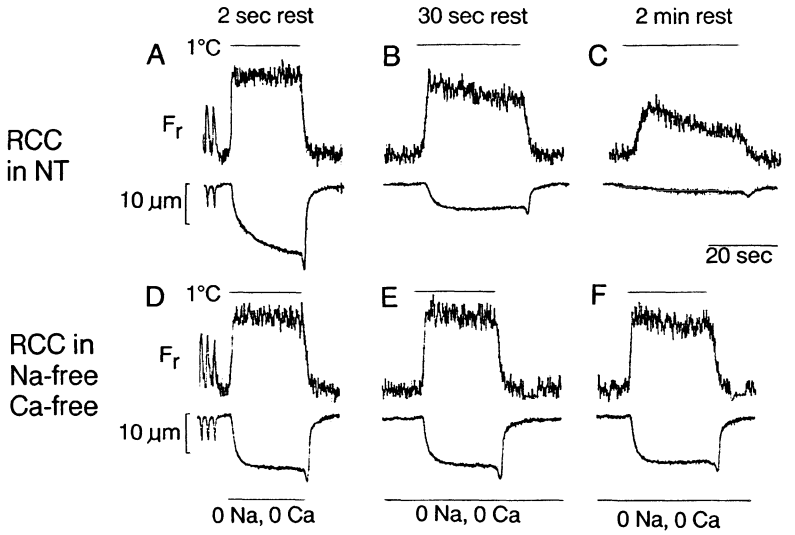


Figure 49. RCCs in an isolated guinea-pig ventricular myocyte. Shortening (in μm) and $[Ca]_i$ (F_r) during the last stimulated twitches (at 0.5 Hz in **A** and **D**) and during RCCs induced 2 sec after the last twitch (**A** and **D**), 30 sec after termination of another train of pulses (**B** and **E**) and after a similar 2 min rest (**C** and **F**). In **A-C** the superfusate during rest and RCCs was NT. In **D-F** the superfusate during rest and RCCs was Na-free and Ca-free, with 500 μM EGTA). The horizontal bar indicates the time during which the superfusate was at 1°C (from Bers *et al.*, 1989, with permission).

The surface of the muscle in Fig. 48 was cooled below 5°C in < 300 msec, reaching a stable temperature of ~1°C in about 1.5 sec. We estimate that the core of a 400 μm diameter muscle will be cooled to less than 5°C in < 2 sec. Thus, in comparison to the application of caffeine which would take minutes to equilibrate throughout a muscle of this size, cooling is almost synchronous. Additionally, during the time the muscle is at 0-1°C membrane Ca transport systems should be largely inhibited. Therefore, the rise in $[Ca]_i$ during RCCs will be less transient in nature than that during caffeine-induced contractures. This makes RCCs of greater practical utility in multicellular preparations.

The rate of rise of $[Ca]_i$ during an RCC can be very rapid (see Figs. 42 & 49). This Ca is apparently from the SR since RCCs can be abolished by depleting the SR with caffeine, ryanodine and long rest periods (Bers *et al.*, 1987). RCCs also do not depend on Ca entering the cell since the amplitude of RCCs are unaffected by the $[Ca]_o$ during the RCC (Bers *et al.*, 1989 and see Fig. 49A *vs* D) and large RCCs are observed even after long times in Ca_o-free solution (Fig. 49D-F). Sitsapesan *et al.* (1990, 1991) have also shown that cooling the cardiac Ca-release channel (incorporated in a bilayer) from 24 to 5°C dramatically increases the probability of the channel being open (from p_o = 0.004 to 0.35 at 100 nM Ca).

Figure 49A shows that the fluorescence ratio signal (F_r) indicative of $[Ca]_i$ is much higher during the RCC than during a normal twitch. Since cooling also slightly decreases the Ca affinity for indo-1, we concluded that only a fraction of the SR Ca available for release is in fact released at a normal twitch. It also seemed that the intracellular indo-1 was sometimes saturated by the Ca released during RCCs (Bers *et al.*, 1989). We estimated that for $[Ca]_i$ to 90-95% saturate indo-1 ($[Ca]_i$ ~ 13 - 32 μM based in *in vitro* calibrations) the SR would have to release ~100 - 200 μmol Ca/kg wet wt (based on intracellular Ca buffering estimates of Fabiato, 1983, Chapter 3). Since this is similar to other estimates of maximal SR Ca capacity, it seems likely that rapid cooling can release all of the available SR Ca.

RCCs measured as isometric contractile force are generally smaller than twitch contractions (Fig. 48), despite the high $[Ca]_i$ reached during the RCC (Bers *et al.*, 1989). This is expected based on the decreased Ca sensitivity and maximal force at 1°C (see Fig. 16 & Table 5 and Harrison & Bers, 1989a). When RCCs are measured as unloaded shortening in isolated myocytes or multicellular preparations they can be considerably larger than the amplitude of the twitch (Fig. 49 and Hryshko *et al.*, 1989c). The duration of $[Ca]_i$ elevation during the RCC in unloaded conditions prolongs the active state and may allow the myocyte or muscle to shorten progressively during the RCC (where the force needs to be only large enough to overcome passive viscoelastic properties and restoring forces, Niggli, 1987).

RCCs are also reproducible and can thus be used as an "on line" means of assessing SR Ca content at different times in intact cardiac muscle or myocytes. For example, Fig. 49A-C shows that with increasing periods of rest (2 sec - 2 min) under control conditions the amplitude of the RCC gradually decreases. This process is known as rest decay (see pages 90-91 and Chapter 8). If Ca extrusion from the cell via Na/Ca exchange is prevented during the rest by using a Na-free, Ca-free solution, then this decline in SR Ca content is also prevented (Fig. 49D-F). The $[Ca]_i$ does gradually decline during long RCCs as does force or shortening. This probably reflects the incomplete inhibition of Ca transport systems in the cold. Indeed, spontaneously relaxation of RCCs at 3-5°C is much faster than at 0-1°C.

We also used RCCs to assess the effect of ryanodine on SR Ca content in intact cardiac muscle and myocytes (Bers *et al.*, 1987, 1989; Bers & Bridge, 1989; Hryshko *et al.*, 1989c). After equilibration with 100-500 nM ryanodine, RCCs of similar amplitude could still be measured, but only immediately after a contraction or series of contractions (see Fig. 50). The SR Ca content appeared to decline with a half-time of ~1 sec which is ~100 times faster than the normal half-time of rest decay in rabbit ventricular muscle (~ 2 min). We interpreted these results to indicate that ryanodine did not prevent the SR from accumulating Ca when $[Ca]_i$ was high, but that it greatly accelerated the rate at which that Ca leaked back into the cytoplasm. This agrees with our observation that ryanodine does not slow relaxation greatly until $[Ca]_i$ is low (Bers & Bridge, 1989). This would be a simple

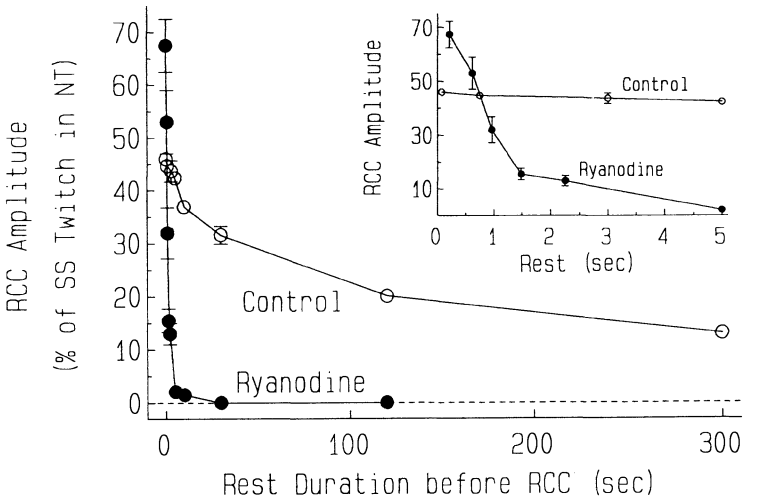


Figure 50. Rest decay of SR Ca content assessed by RCCs in rabbit ventricular muscle in control conditions and after equilibration with 100 nM ryanodine. Stimulation at 0.5 Hz was terminated and the muscle cooled after the rest periods indicated on the abscissa. The inset shows an expanded time scale for the first 5 sec of rest (redrawn using original data from Bers *et al.*, 1987).

pump-leak consideration. That is, when $[Ca]_i$ is high (and SR [Ca] is low) the rate of Ca-pumping exceeds the rate of ryanodine-induced leak. However as $[Ca]_i$ declines (and SR [Ca] rises) the leak becomes greater and the SR loses Ca. Furthermore, if other mechanisms can keep this Ca leak from elevating cytoplasmic [Ca] (e.g. Ca extrusion by Na/Ca exchange), the SR will be rapidly depleted and the Ca from the SR will be lost from the cell. These results agree with demonstrations that ryanodine causes the SR Ca release channel to be "locked" in a submaximal open state (see below, Rousseau *et al.*, 1987). They are also in perfect agreement with our results using another indirect means of assessing SR Ca content, namely extracellular Ca-selective microelectrodes (see below, Bers & MacLeod, 1986; MacLeod & Bers, 1987).

Cumulative Extracellular Ca Depletions

Under steady state conditions the amount of Ca which enters the cell at one beat must be extruded prior to the next beat, otherwise a progressive Ca loading will occur. However, in non-steady state conditions, such as when stimulation is terminated, there may be a net loss of cell Ca (rest decay). Conversely, when stimulation is resumed after a rest there may be a net uptake of Ca by the cell as it recovers toward steady state and the SR becomes refilled. We have used double barreled Ca-selective microelectrodes during these perturbations to monitor changes of extracellular [Ca] that are indicative of net cellular Ca

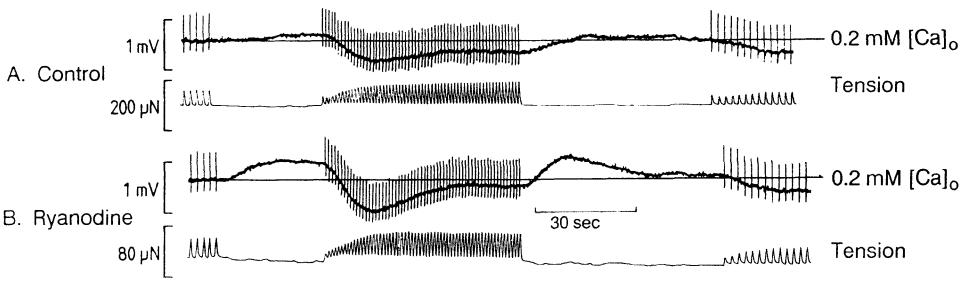


Figure 51. Extracellular Ca depletions (upper traces) and tension in rabbit ventricular muscle under control conditions (0.2 mM $[Ca]_o$) and after equilibration with 1 μM ryanodine (B). In both panels the muscle was stimulated at 0.5 Hz (steady state) at the beginning, 1 Hz for ~ 1 min in the middle and again at 0.5 Hz at the end with 0.5 and 1 min rest periods. The spikes on the $[Ca]_o$ trace are stimulus artifacts. Extracellular [Ca] was measured with a double-barrelled Ca-selective microelectrode (from Bers & MacLeod, 1986, with permission of the American Heart Association).

uptake and loss (e.g. Figure 51). The control traces in Fig. 51 show that when stimulation is stopped there is a small slow increase in $[Ca]_o$ indicative of net Ca extrusion during rest. When stimulation is resumed a depletion of $[Ca]_o$ occurs indicating a net Ca uptake by the cells. These cumulative cellular Ca losses and gains which occur during rest decay or resumption of contractions appear to be mainly due to changes in SR Ca. Evidence for this includes that Ca_o depletions can be inhibited by caffeine and require transsarcolemmal Ca flux, since they can be blocked by Co and nifedipine (Bers & MacLeod, 1986). In addition, larger Ca_o depletions are observed after long rest intervals when the SR is thought to have lost all of its Ca (MacLeod & Bers, 1987). Since the SR is then empty, a larger net Ca uptake is required to return it to steady state (hence the larger Ca_o depletion).

If the muscle is equilibrated with ryanodine (Fig 51B), the termination of stimulation is accompanied by a large and rapid Ca extrusion from the cell. This fits with the interpretation of ryanodine action above from RCCs. That is, during the trains of pulses the SR may be able to accumulate large amounts of Ca, since the Ca_o depletions in the presence of ryanodine are just as large as control and are still caffeine sensitive (MacLeod & Bers, 1987; Bers & MacLeod, 1986). When stimulation is terminated the large efflux of Ca (increased $[Ca]_o$ in Fig. 51B) may reflect the extrusion of SR Ca from the cell by Na/Ca exchange. Apparently $[Ca]_i$ remains reasonably low during this period of active Ca efflux since resting force is not elevated.

When the transsarcolemmal [Na] gradient is reduced, Ca_o accumulations and depletions can also be prevented (Bers & MacLeod, 1986; Bers, 1987b). This can be understood by considering that the decreased [Na] gradient will limit Ca extrusion by Na/Ca exchange. If Na/Ca exchange is unable to extrude sufficient Ca from the cell during rest to deplete the SR of Ca, there will be no Ca_o accumulation during rest and no

Ca_o depletion with resumption of stimulation back to steady state (i.e. the SR doesn't lose Ca during rest, so it doesn't need more to be reloaded to steady state).

The intriguing possibility that there might be some sort of direct pathway for Ca from the inside of the SR to the extracellular space (that is activated by ryanodine) has been raised by several investigators over the years (e.g. Sutko *et al.*, 1985; Bers & MacLeod, 1986). However, a passive pathway such as a channel does not seem plausible, since that would require a downhill electrochemical gradient for Ca from the SR to the extracellular space. If the potential within the SR is about -80 mV (i.e. cytoplasmic), the [Ca] in the SR would have to be > 1 M for this to be the case. As we have seen above, the [Ca] in the SR cannot possibly be that high. Indeed, the loss of SR Ca induced by ryanodine can be greatly slowed by inhibiting Ca extrusion by Na/Ca exchange (Bers & Christensen, 1990). Thus both the cytosol and Na/Ca exchange are in the pathway by which SR Ca gets out of the cell, even with ryanodine.

It may seem surprising that measuring $[Ca]_o$ works as a useful means of monitoring SR Ca content. However, this approach has been quite useful since it provides a rapid and sensitive "on line" assessment of net Ca flux across the sarcolemma, providing a substantial advantage over ^{45}Ca flux measurements. On the other hand, extracellular Ca depletion measurements are difficult to extrapolate in absolute units of μmol/kg wet wt as can be more readily done with radiotracer studies. A lower limit estimate of Ca uptake into the SR from this approach is 26 μmol/kg wet wt (MacLeod & Bers, 1987), but this does not include the Ca which would have derived from the large number of low affinity binding sites in the extracellular space. Hilgemann *et al.* (1983, Hilgemann & Langer, 1984) also studied cumulative Ca_o depletions in cardiac muscle using extracellular Ca indicator dyes. The measurement of $[Ca]_o$ changes with Ca microelectrodes and optical indicators have also been used to assess transsarcolemmal Ca fluxes at single contractions (Bers, 1983, 1985, 1987b; Dresdner & Kline, 1985; Pizarro *et al.*, 1985; Hilgemann, 1986a,b; Shattock & Bers, 1989).

Direct Chemical and Radiotracer Techniques

Direct chemical measurements (e.g. atomic absorption spectrometry) and ^{45}Ca flux measurements can provide values that are most easily extrapolated in units of μmol Ca/kg wet wt. However, the precise intracellular location of the Ca and temporal resolution of these approaches is limited. Long rest periods (through rest decay) were associated with Ca losses of 200, 258 and 390 μmol/kg wet wt in rabbit ventricle and ~1000 μmol/kg wet wt in guinea-pig ventricle (Pytkowski *et al.*, 1983; Lewartowski *et al.*, 1984; Bridge, 1986; Lewartowski & Pytkowski, 1987; Pierce *et al.*, 1987; Pytkowski, 1989). These values are only somewhat higher than the estimates cited earlier (except for the guinea-pig value), but part of these decreases could come from other compartments than the SR. Pytkowski (1989) also measured the caffeine-induced decrease in ^{45}Ca content in rabbit heart (170 μmol/kg wet wt), which was only 44% of the rest dependent loss of ^{45}Ca. Langer *et al.*

(1990) have also estimated a caffeine-sensitive fraction of cellular Ca content in rat ventricle (~100 μmol/kg wet wt) which accounted for 21% of the Ca in an "intermediate compartment" of ^{45}Ca washout ($t_{1/2}$ = 3-19 sec). Unfortunately, interpretations using this general approach are complicated by contributions of non-SR compartments and the observation that caffeine did not decrease the ^{45}Ca content in guinea-pig ventricle (Lewartowski & Pytkowski, 1987).

In conclusion, it is not possible to quote the absolute Ca content of the SR, but it seems that ~200 μmol/kg wet wt is a reasonable estimate for the maximal SR Ca content of mammalian ventricular muscle (though the accuracy of this value should probably be thought of as ± 50%). Unfortunately the Ca requirements for activation of contraction are not known very precisely either, but from the discussion in Chapter 3, a reasonable estimate was thought to be ~30 - 50 μmol/kg wet wt. Despite the equivocal nature of these estimates, it would seem that the SR might be able to hold sufficient Ca to supply a few contractions. Put another way, it would seem that only a fraction of the SR Ca may need to be released for a normal contraction. Another conclusion from this section is that some of the less direct means of assessing SR Ca content (such as caffeine-induced contractures, RCCs and Ca_o depletions) can provide sensitive "on line" information about changes in SR Ca content. However, these indirect approaches are less helpful in assessing the absolute value of SR Ca content.

SR Ca RELEASE CHANNEL OR RYANODINE RECEPTOR

The isolation and molecular identification of the SR Ca-release channel was greatly accelerated by the recognition that ryanodine was a selective and specific ligand for this channel. Ryanodine is a neutral plant alkaloid which was found to produce irreversible contracture of skeletal muscle, but progressive decline in cardiac muscle contraction (Jenden & Fairhurst, 1969; Hajdu & Leonard, 1961; Sutko & Willerson, 1980). An explanation for these different effects can be drawn from preceding discussions. Ryanodine may open the SR release channel in both skeletal and cardiac muscle (see below). In cardiac muscle the Ca lost from the SR can be extruded from the cell by the powerful Na/Ca exchange system in the sarcolemma, thereby "unloading" the SR and the cell of Ca so that contractions are depressed. In skeletal muscle where Na/Ca exchange activity is much lower and the SR Ca content is higher, much of the Ca from the SR may remain in the cytoplasm, activating a contracture which may consume the cellular ATP before the $[Ca]_i$ can be reduced (thereby making the contracture irreversible).

At low concentrations (1 nM - 1 μM) ryanodine accelerates Ca loss from heavy SR vesicles, but can also slow that efflux at very high concentrations (>100 μM, Nayler *et al.*, 1970; Fairhurst & Hasselbach, 1970; Jones *et al.*, 1979; Jones & Cala, 1981; Fabiato, 1985d; Meissner, 1986a). Smith *et al.* (1985, 1986a) demonstrated that the Ca release from these heavy SR vesicles from skeletal muscle could be attributed to a high conductance Ca

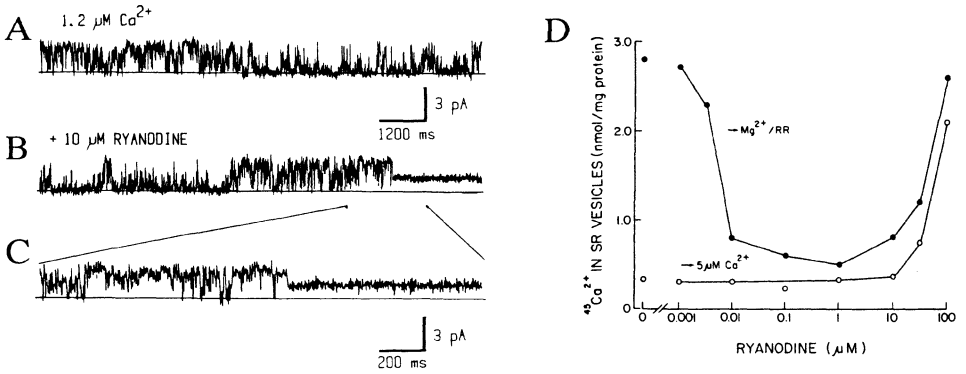


Figure 52. The influence of ryanodine on the SR Ca-release channel. **A-C.** Recording from a single cardiac Ca release channel incorporated in a lipid bilayer (currents are shown as upward deflections, from Rousseau *et al.*, 1987, with permission). The *cis* (cytoplasmic) side of the membrane contained 2.5 μM free [Ca] and the *trans* side contained 50 mM Ba as the charge carrier through the channel (pH 7.4, 0 mV holding potential). Ryanodine was added 1 min before panel **B** was recorded. **C.** shows the transition from normal gating to a stable subconductance state on an expanded time scale. **D.** Dependence of ^{45}Ca efflux from skeletal muscle SR vesicles on [ryanodine]. Vesicles were passively loaded with ^{45}Ca (100 μM) and incubated for 45 min with ryanodine. They were then diluted into a Mg + ruthenium red (RR) medium, which blocks efflux (unless pretreated with 5 nM - 30 μM ryanodine) or diluted into 5 μM Ca medium which quickly emptied the vesicles (except at high [ryanodine]). Ryanodine causes SR Ca release at 5 nM to ~30 μM, but blocks release at 100 μM (from Meissner, 1986a, with permission).

channel which they incorporated into lipid bilayers (i.e. the channel was similarly modulated by [Ca], ATP, ruthenium red and Mg). Rousseau *et al.* (1986, 1987) demonstrated similar channels in heart and also showed that ryanodine induced a long lived subconducting state of the SR Ca-release channel, (Fig. 52). That is, ryanodine (at least up to 10 μM) appears to "lock" the Ca release channel into an open, but lower than normal conducting state. The unitary conductance of the cardiac SR Ca release channel is ~75 pS (with 53 mM Ca on the *trans* or luminal side and 0.5 - 2.5 μM Ca on the *cis* or cytoplasmic side) and ryanodine decreased the conductance by ~ 50% (Rousseau *et al.*, 1986, 1987). The SR Ca release channel (like the sarcolemmal Ca channel) can also conduct monovalent ions in the absence of Ca and the selectivity for Ca is lower for the release channel (P_{Ca}/P_K ~ 6 *vs* 3000 for the sarcolemmal Ca channel, Smith *et al.*, 1988 and see Table 10, page 55). It may also be noted that the conductance of the SR Ca release channel is ~ 10 times higher than that of the sarcolemmal Ca channel under similar conditions and the rate of Ca transport by one Ca release channel is 6×10^5 times greater than the rate of Ca transport by one SR Ca-pump.

The high affinity effects of ryanodine on SR vesicles coupled with observations from more intact preparations (e.g. Sutko *et al.*, 1985) led to the use of ryanodine as a

specific ligand in binding studies with SR vesicles (Pessah *et al.*, 1985; Fleischer *et al.*, 1985; Alderson & Feher, 1987; Imagawa *et al.*, 1987; Inui *et al.*, 1987a; Lattanzio *et al.*, 1987; Meissner & Henderson, 1987). The affinity of the receptor for ryanodine is dependent on [Ca] and the presence of nucleotides (e.g. ATP), but in the conditions typically used by the investigators above K_d values of 4 - 36 nM are found and the numbers of sites in SR preparations vary from 0.5 - 14 pmol/mg protein. In intact cardiac ventricular myocytes or whole heart homogenates we typically measure specific ryanodine binding of ~300 fmol/mg protein, which corresponds to ~36 nmol/kg wet wt. This is very similar number of dihydropyridine receptors estimated in Chapter 4, but much smaller than the ~6000 nmol/kg wet wt for the SR Ca pump (see above).

Ryanodine has been used as a specific ligand allowing the purification of the ryanodine receptor from skeletal muscle (Inui *et al.*, 1987a; Campbell *et al.*, 1987; Imagawa *et al.*, 1987; Lai *et al.*, 1987,1988a) and cardiac muscle (Inui *et al.*, 1987b; Lai *et al.*, 1988b) with estimated molecular weights ~320-450 kDa for a monomer. The purified ryanodine receptor was also incorporated into bilayers and retained the Ca channel characteristics from the native SR (Imagawa *et al.*, 1987; Lai *et al.*, 1988a; Hymel *et al.*, 1988; Smith *et al.*, 1988).

The purified protein appears to be a homotetramer, based on its quatrefoil appearance (Saito *et al.*, 1988; Lai *et al.*, 1988a; Wagenknecht *et al.*, 1989, see also Figs. 8-9 and 53), gel permeation chromatography (Inui *et al.*, 1987a) and stoichiometry of high affinity ryanodine binding (Lai *et al.*, 1988a, 1989). Image processing allowed Wagenknecht *et al.* (1989) to reconstruct a detailed three-dimensional architecture of the ryanodine receptor/Ca release channel (Fig. 53). The complex is ~27 nm along each side and ~14 nm tall, which correspond reasonably well with the width and length of the junctional "feet" observed in intact muscle (Block *et al.*, 1988). The overall evidence now seems compelling that this molecular entity is the ryanodine receptor, the SR Ca-release channel and the "foot" process observed in electron micrographs (see Chapter 1).

The reconstruction of the ryanodine receptor in Fig. 53 is intriguing because it suggests an interesting possible pathway (or channel) for Ca flux. That is, Ca might enter the "base plate" (BP) from the lumen of the SR at the central "channel" (CC) and traverse to the peripheral vestibules (PV) via radial channels (RC) about halfway through the depth of the complex. While it is probably too soon to adopt a rigid interpretation from images of this type, the structural information provided is remarkable.

The skeletal ryanodine receptor (MW = 565,223 Da) has been cloned (Takeshima *et al.*, 1989; Marks *et al.*, 1989; Zorzato *et al.*, 1990) and Otsu *et al.* (1990) and Nakai *et al.* (1990) have recently cloned the cardiac ryanodine receptor which appears to be the product of a separate gene (564,711 Da). The proteins are 66% identical and of similar molecular weight, but the cardiac protein is 63 amino acids shorter (with more heavy amino acids). The tetrameric nature of the ryanodine receptor *in vivo* implies a 2,260,000

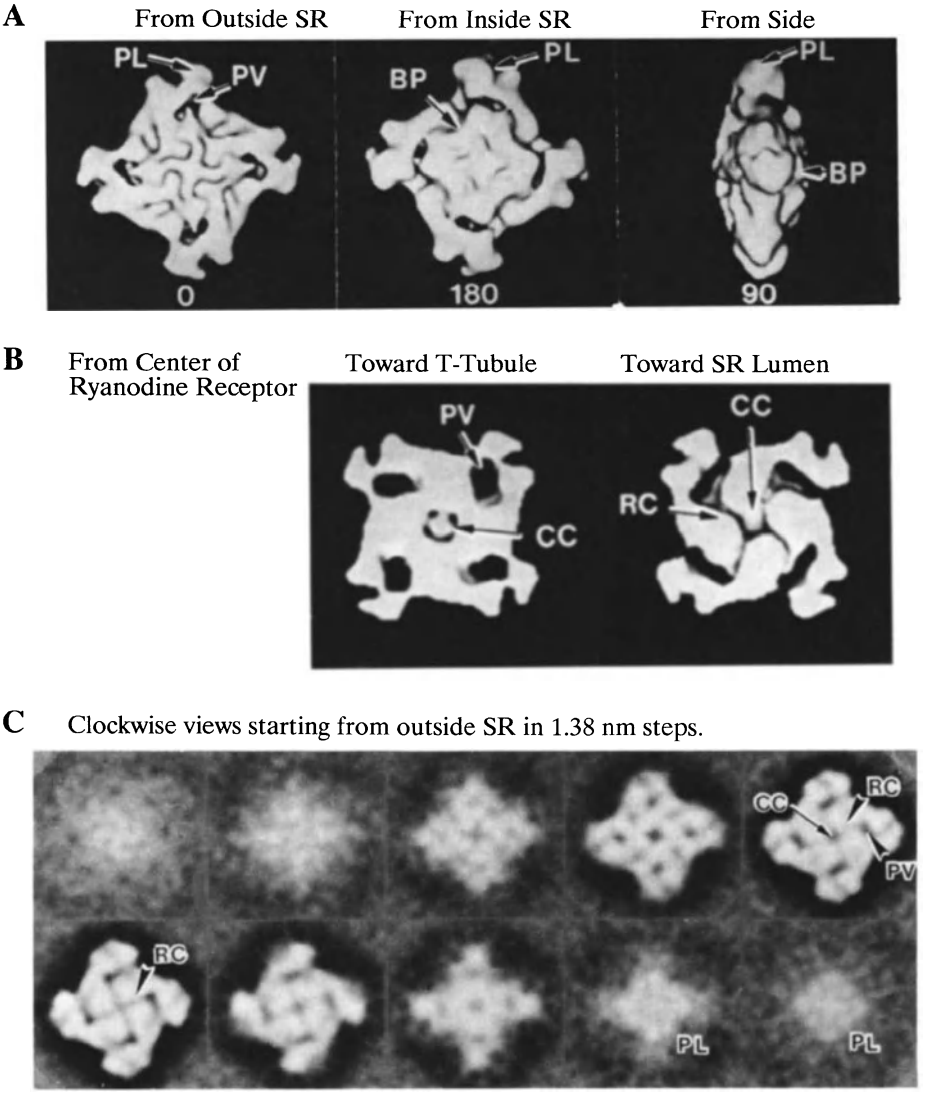


Figure 53. Three-dimensional reconstruction of the ryanodine receptor/foot protein or junctional channel complex (from Wagenknecht *et al.*, 1989, with permission). **A.** Surface representations of the reconstruction viewed from three different angles. **B.** Complementary halves of the reconstruction after slicing it in half to reveal views from the center toward the T-tubule and the SR lumen. **C.** Sections through the three dimensional reconstructed volume, starting from the T-tubule side and going toward the SR lumen in 1.38 nm steps (clockwise). The complex is 27 × 27 nm at its widest cross-section and 14 nm deep.

Da structure. The large size of this homotetramer has helped to identify it ultrastructurally as the junctional foot process. It also makes it somewhat more challenging to predict the secondary and tertiary structure of the protein. Takeshima *et al.* (1989) originally indicated only 4 transmembrane spanning domains at the carboxyl end and a modulator binding region a modest distance toward the amino terminal from that region. Otsu *et al.* (1990) have estimated a larger number of transmembrane spans (~12/ monomer) and have suggested a different nucleotide binding and phosphorylation site and several potential calmodulin binding sites. The cardiac (but not the skeletal) ryanodine receptor can also be phosphorylated by cAMP-dependent protein kinase (Takasago *et al.*, 1989). It is reasonable to expect that our understanding of the structure of this molecule will evolve rapidly in the next few years.

Regulation of Ca Release by the SR

The regulation of SR Ca release has been studied extensively by measuring Ca fluxes in isolated heavy SR vesicles (using ^{45}Ca or Ca indicators) and Ca release channels incorporated into artificial bilayers.

Figure 54 demonstrates that Ca activates the cardiac SR Ca release channel at submicromolar concentrations (although much higher [Ca] is required to activate the skeletal SR Ca release channel, Rousseau & Meissner, 1989; Rousseau *et al.*, 1986; Smith *et al.*, 1986a). Caffeine (at millimolar concentrations) also activates both the cardiac and skeletal release channel (Rousseau & Meissner, 1989; Rousseau *et al.*, 1988). The channel is inhibited by mM Mg and μM ruthenium red, even after caffeine activation (see Fig. 54 and Rousseau & Meissner, 1989). The skeletal muscle Ca release channel is more sensitive to block by Mg and ruthenium red (Rousseau *et al.*, 1986). ATP (and other adenine nucleotides also activate the cardiac SR Ca release channel at mM levels, but only if [Ca] is high enough to partially activate the channel (Rousseau *et al.*, 1986). Thus, Ca seems to be required for activation of the cardiac Ca release channel, whereas the skeletal Ca release channel can be activated to a similar (although submaximal) extent by either ATP or Ca alone (Rousseau *et al.*, 1986). The Ca release channel can also be blocked by decreasing pH (Ma *et al.*, 1988; Rousseau & Pinkos, 1990). Calmodulin (1 - 4 μM) appears to inhibit the Ca release channel in both cardiac and skeletal muscle (but only with [Ca] as high as 50 μM) by interacting directly with the release channel, rather than by activating a protein kinase (Meissner & Henderson, 1987; Smith *et al.*, 1989).

From the foregoing it is clear that there are similarities, but also differences in the cardiac and skeletal muscle SR Ca release channels. This may not be surprising since they are related, but different proteins and since the normal signal for Ca release in E-C coupling may be quite different (see Chapter 7, Fig. 71). For example, if Ca-induced Ca-release is the normal mode of Ca release in cardiac, but not in skeletal muscle, then the high degree and strict dependence of Ca release channel gating on [Ca] in cardiac muscle may be expected. On the other hand, the regulation of the isolated Ca release channel may

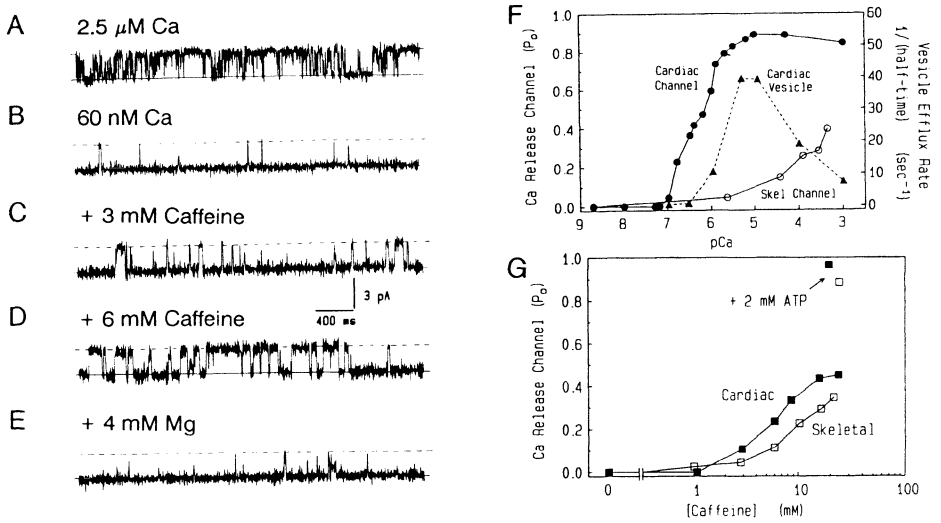


Figure 54. The influence of Ca, caffeine and Mg on cardiac and skeletal muscle Ca release channels incorporated into lipid bilayers. **A-E.** Records are from a single cardiac Ca release channel and show that reduction of *cis* [Ca] reduces channel opening (**B**), that caffeine activates the channel at low [Ca] (**C & D**) and that Mg blocks the channel (**E**). Current was carried by 50 mM Ca on the *trans* side. **F.** Ca dependence of channel open probability (P_o) as in A-E (for cardiac and skeletal Ca release channel) or of the rate of ^{45}Ca efflux from cardiac SR vesicles (as described in Fig. 51). **G.** Influence of caffeine on cardiac and skeletal Ca release channels in bilayers with ~60 nM *cis* Ca and ~50 mM *trans* Ca as charge carrier. Addition of 2 μM ATP caused the channels activated by caffeine to become almost fully open (P_o almost 1). (A-E and cardiac channel data in F & G are from Rousseau & Meissner, 1989, with permission and other data were digitized from Meissner & Henderson, 1987; Smith *et al.*, 1986a; Rousseau *et al.*, 1988).

be different from the channel *in situ*. For instance, elevated [Ca] activates the isolated channel in a steady state manner, while in "skinned" cells the Ca release channel appears to open and inactivate in a time- and [Ca]-dependent manner (Fabiato, 1985b).

For the most part, results from Ca flux studies with isolated heavy SR vesicles agree with those from Ca channel current measurements in bilayers described above. Caffeine has long been known to accelerate Ca efflux from SR vesicles and prevent Ca accumulation by heavy SR vesicles (e.g. Weber & Herz, 1968; Blaney *et al.*, 1978; Meissner & Henderson, 1987). Indeed caffeine-induced contractures can be used to assess SR Ca content in intact cardiac muscle and skinned fibers (see section above). Other methylxanthines and imidazoles can also cause release of Ca from cardiac SR (Chapman & Tunstall, 1988). Of course it is not expected that caffeine will release the fraction of Ca accumulated by light SR vesicles which lack Ca release channels.

In cardiac SR vesicles the rate of Ca efflux is also stimulated by μM Ca, mM ATP and inhibited by mM Mg ($K_{1/2}$ = 0.3 mM), μM ruthenium red, reduction of pH and calmodulin (Meissner & Henderson, 1987). This study demonstrated maximal Ca efflux with a time constant of ~10 msec (with 10 μM Ca and 5 mM AMP-PCP, an ATP analog). This is orders of magnitude faster than most previous reports of Ca-induced Ca-release in cardiac SR vesicles (e.g. Chamberlain *et al.*, 1984a,b; Seiler *et al.*, 1984). The rate of SR Ca release reported by Meissner & Henderson (1987) is of the order required to activate the myofilaments in the cell. One of the difficulties in extrapolating results from vesicles or planar bilayers is that they have rarely been done with combinations of [ATP], [Mg] and [Ca] in the physiological range expected in the cell. Meissner & Henderson (1987), however, showed that inclusion of 3 mM free Mg and 5 mM Mg-AMP-PCP shifted the Ca-dependence of Ca efflux rate in cardiac SR vesicles to higher [Ca]. In this case, Ca release was not appreciably stimulated until [Ca] reached 10 μM and continued to increase toward its maximal rate at nearly 1 mM (half-maximal rate at ~100 μM). It is reasonable that $[Ca]_i$ could reach this level if the regulatory site is near the mouth of the sarcolemmal Ca channel (see Chapter 7), but these conditions may still be short of physiological. For example, intracellular [Mg] is probably lower than 3 mM in cardiac muscle, which would increase the Ca sensitivity of the release channel.

Low concentrations of ryanodine ($\leq$30 μM) open the cardiac SR Ca release channel in either vesicles or bilayers to a stable subconducting state (Fig. 52) where the channel no longer responds to Ca, ATP, Mg or ruthenium red (Rousseau *et al.*, 87; Meissner, 1986a). This probably corresponds to the occupation of the high affinity ryanodine sites mentioned above (K_d ~ 10 nM). At very high ryanodine concentrations (>100 μM) the Ca release channel appears to be locked in a closed state (see Fig 52, Meissner, 1986a; Lai *et al.*, 1989). This may result from ryanodine binding to low affinity sites. Many of the above studies of ryanodine binding in heart have revealed low affinity sites with an apparent K_d in the μM range (e.g. Inui *et al.*, 1987b). Lai *et al.* (1989) demonstrated that there is a maximum of 1 high affinity ryanodine binding site per tetramer, depending on the [Ca] and [ATP]. They further demonstrated that there were three additional low affinity ryanodine sites per tetramer (K_d = 1-5 μM ryanodine) which exhibit negative cooperativity. Under conditions where no high affinity ryanodine binding is observed (e.g. low [Ca]), all four sites per tetramer are of the low affinity type.

Table 13 shows some factors that alter SR Ca release (including those discussed above). Halothane is of interest since it is used clinically as an inhalation anesthetic and its use has been associated with malignant hyperthermia. In genetically predisposed people (and pigs) halothane can induce sustained skeletal muscle contracture which results in an uncontrolled rise in body temperature that can be fatal. Endo *et al.* (1983) Nelson (1983) and Kim *et al.* (1984) reported greater halothane and Ca sensitivity of SR Ca release in malignant hyperthermia susceptible human and pig skeletal muscle. Mickelson *et al.* (1988, 1990; Fill *et al.*, 1990) have shown faster SR Ca release and differences in the ryanodine

Table 13

Factors Which Alter Ca Release from the SR

	Effective Concentration	Muscle Type	Reference
Enhancers of Ca Release			
Ca	0.3-10 μM	hrt	M & H'87; Rousseau & Meissner,1989
Caffeine	~1-10 mM	hrt	Fabiato, 1983; O'Neill et al., 1990; Rousseau & Meissner, 1989
ATP (or AMP-PCP)	1-5 mM	hrt	Rousseau et al., 1986; M & H'87
Ryanodine	0.01-30 μM	hrt/sk	Rousseau et al., 1987; Meissner, 1986a
Sulmazole (AR-L 115BS)	1 mM	hrt	Williams & Holmberg, 1990
Halothane	~0.5 mM	hrt/sk	Ohnishi, 1979; Su & Kerrick, 1979 Palade, 1987b
Doxorubicin	7-25 μM	sk	Zorzato et al., 1985; Nagasaki & Fleischer, 1989
Quercetin	10-300 μM	sk	Kirino & Shimizu, 1982; Palade, 1987b
Bromo-eudistomin D	~5 μM	sk	Nakamura et al., 1986
Sulfhydryl reagents			
$AgNO_3$	0.1-15 μM	sk	Salama & Abramson, 1984
Ag^+ or Hg^{2+}	10-25 μM	hrt	Prabhu & Salama, 1990
Cu^{2+}/Cysteine	2-10 μM	sk	Trimm et al., 1986
$Ins(1,4,5)P_3$	10-30 μM	hrt	Fabiato, 1990
	10-20 μM	sk	Volpe et al., 1985
	0.5 μM	sm	Walker et al., 1987
Inhibitors of Ca Release			
Mg	1-3 mM	hrt	M & H'87; Fabiato, 1983
Ruthenium red	10 μM	hrt	M & H'87
Ryanodine	>100 μM	hrt/sk	Meissner, 1986a; Jones et al., 1979; Lai et al., 1989
Dantrolene	2 μM	sk	Danko et al., 1985
Calmodulin	1 μM	hrt	Smith et al., 1989; M & H'87
Tetracaine, procaine	0.1, 1 mM	sk	Palade, 1987a; Antoniu et al., 1985
Neomycin, gentamycin	60-200 nM	sk	Palade, 1987c
Spermine, spermidine	20-200 μM	sk	Palade, 1987c

M & H'87 is Meissner & Henderson, 1987; hrt=heart, sk=skeletal and sm=smooth muscle. This table is based on tables compiled by Fleischer & Inui (1989) and more extensive tables by Palade (1987b, Palade et al., 1989) which were focused on skeletal muscle SR vesicles. This table is intended to focus on cardiac SR Ca release where data is available.

receptor in skeletal muscle from pigs with malignant hyperthermia (providing a possible clue to the molecular basis of the disease). Dantrolene is a skeletal muscle relaxant which is used clinically to curtail the contracture during the malignant hyperthermic contractures above. Dantrolene blocks SR Ca release, but interestingly has only relatively weak effects on cardiac muscle (Ellis *et al.*, 1976; Van Winkle, 1976; Danko *et al.*, 1985).

Other SR Channels Related to Ca Release

Although the foregoing discussion paints a compelling picture for the identity of the ryanodine receptor/ foot protein as *the* SR Ca release channel, there have been reports of

smaller conductance channels associated with the ryanodine receptor in skeletal and cardiac muscle which are not sensitive to Ca, Mg or ATP (Smith *et al.*, 1986b; Suarez-Isla *et al.*, 1986; Rardon *et al.*, 1989). Zaidi *et al.* (1989) and Hilkert *et al.* (1990) have also identified a 106 kDa protein which they find exhibits similar sensitivity to Ca, Mg, ATP and ruthenium red (but different ryanodine sensitivity) as the ryanodine receptor/ foot protein described above. It is not yet clear how these other reported channel activities are related to the function of the SR in intact muscle.

Inositol(1,4,5)-trisphosphate ($Ins(1,4,5)P_3$) has been well documented to induce Ca release from internal stores in non-muscle cells (Berridge, 1987; Berridge & Galione, 1988; Berridge & Irvine, 1989). The role of $Ins(1,4,5)P_3$ in the activation of SR Ca release in muscle will be discussed in Chapter 7. The $Ins(1,4,5)P_3$ receptor has been isolated from neural tissue (Supattapone *et al.*, 1988) and smooth muscle (Chadwick *et al.*, 1990). The neural $Ins(1,4,5)P_3$ receptor has been cloned (MW = 313 kDa) and may contain up to ~ 7 transmembrane domains and there are certain sequence homologies with the ryanodine receptor (Furuichi *et al.*, 1989; Mignery *et al.*, 1989). Chadwick *et al.* (1990) also showed that the smooth muscle $Ins(1,4,5)P_3$ receptor has the same quatrefoil structure (25 × 25 nm) as the skeletal muscle ryanodine receptor (although the apparent molecular weight is considerably smaller) and they suggested a similar tetrameric arrangement of 224,000 Da monomers. The neural and smooth muscle $Ins(1,4,5)P_3$ receptor appear to be similar, but the latter has a higher $Ins(1,4,5)P_3$ affinity and lower binding stoichiometry (K_d = 2.4 nM *vs* ~80 nM, Supattapone *et al.*, 1988; Chadwick *et al.*, 1990). $Ins(1,4,5)P_3$ activation of a smooth muscle SR Ca channel has been shown in both SR vesicles and after incorporation into lipid bilayers (Watras & Benevolensky, 1987; Ehrlich & Watras, 1988). The purified $Ins(1,4,5)P_3$ receptor from brain has also been reconstituted in lipid vesicles, indicating that the protein has both the $Ins(1,4,5)P_3$ site and the Ca release channel it regulates (Ferris *et al.*, 1990). Furthermore, the release induced by $Ins(1,4,5)P_3$ is enhanced by ATP. While $Ins(1,4,5)P_3$ is capable of inducing SR Ca release in skeletal, smooth and cardiac muscle (Volpe *et al.*, 1985; Walker *et al.*, 1987; Fabiato, 1990), the clearest role for this Ca release pathway in E-C coupling appears to be in smooth muscle (see Chapter 7).

The permeability of the SR membrane to monovalent ions is very high (Meissner, 1986b). High conductance K- and anion-selective channels exist in the SR membrane (Coronado & Miller, 1979, 1980; Coronado *et al.*, 1980; Hals *et al.*, 1989). Also, there do not appear to be any appreciable concentration gradients of monovalent ions between the inside of the SR and the cytoplasm (Somlyo *et al.*, 1977a,b; Somlyo & Somlyo, 1986). This has two important functional consequences. First, it implies that there is no membrane potential between the cytoplasm and the interior of the SR (which has implications for certain possible models of E-C coupling). Second, it allows Ca release to proceed rapidly with monovalent fluxes compensating quickly for the divalent charges (Ca) leaving the SR. Otherwise, the rate of Ca release could be limited, in part by the development of a large negative intra-SR potential (which would oppose further Ca flux from the SR).

In conclusion, it is clear that the SR can accumulate sufficient Ca and release it fast enough to be important in cardiac muscle contraction. Indeed, a great deal is now known about how the SR Ca-pump and SR Ca release channel work in isolated systems (such as SR vesicles and in bilayers). I have also discussed how SR Ca uptake can be assessed in relatively intact preparations. A major question which has not been addressed is the regulation of SR Ca release in the intact cell (i.e. E-C coupling). This is a complex question, but also a very important one since Ca release from the SR is probably the largest source of Ca contributing to the activation of the myofilaments in mammalian cardiac muscle. E-C coupling will be the topic of the next chapter.

CHAPTER 7

EXCITATION-CONTRACTION COUPLING

Since the classic experiments of Ringer (1883) demonstrated that the frog heart would not contract in the absence of extracellular Ca, it has been clear that Ca_o is critical in muscle contraction. This has been confirmed repeatedly and some modern day extensions of this fundamental observation are illustrated in Figs. 55 and 56. Figure 55A shows that when Ca_o is removed quickly from the medium around a rat ventricular myocyte contractions are immediately abolished (in < 1 sec, Rich *et al.*, 1988). In contrast, Figure 55B shows that skeletal muscle can contract for many minutes in the complete absence of extracellular Ca (Armstrong *et al.*, 1972). Figure 56 shows the voltage dependence of several parameters during voltage-clamp experiments with isolated guinea-pig myocytes. The E_m-dependence of contraction (shortening) and the Ca_i transient are very similar to the E_m-dependence of I_{Ca} in guinea-pig and other cardiac preparations (McDonald *et al.*, 1975; London & Krueger, 1986; Cannell *et al.*, 1987; Beuckelmann & Wier, 1988;

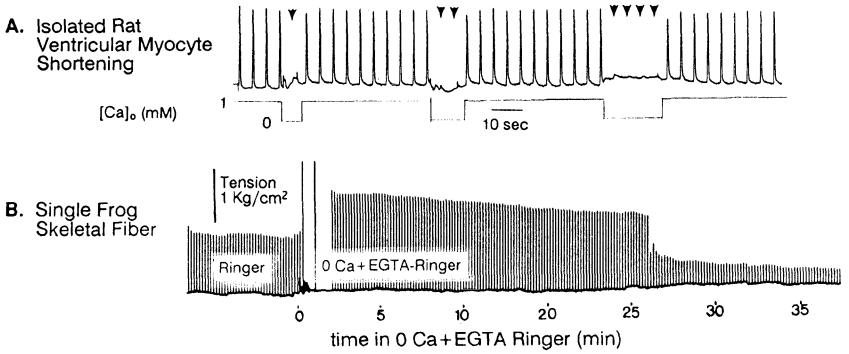


Figure 55. Ca_o-free solution abolishes contractions immediately (< 1 sec) in an isolated cardiac myocyte (**A.**), but does not decrease contraction in a single skeletal muscle fiber (**B.**) for >25 min. **A.** $[Ca]_o$ was changed by a rapid solution switching device between stimuli. The cell was stimulated at a continuous frequency of 0.2 Hz and arrowheads indicate stimulations in Ca_o-free solution (from Rich *et al.*, 1988, with permission). **B.** A single frog skeletal fiber stimulated at 0.1 Hz except during the switch to a Ca_o-free solution containing 1 mM EGTA. The eventual decline in force after ~26 min was attributed to gradual membrane depolarization (from Armstrong *et al.*, 1972, with permission).

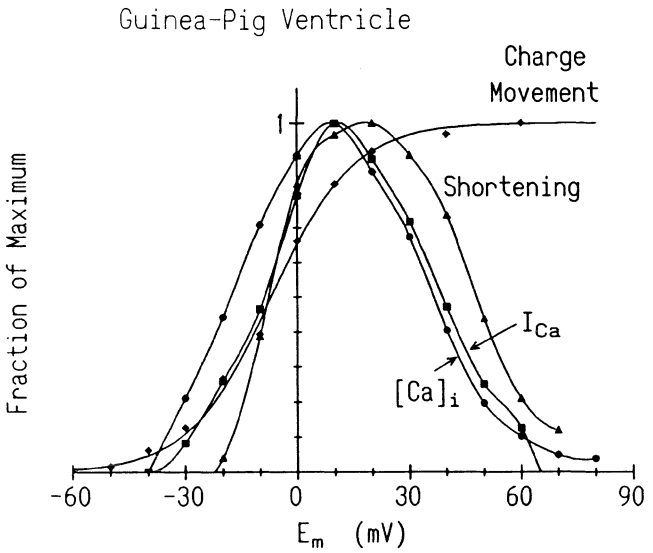


Figure 56. Voltage dependence of Ca current (I_{Ca}), change in $[Ca]_i$, myocyte shortening and intramembrane charge movement in isolated guinea-pig ventricular myocytes. Data were taken from Beuckelmann & Wier, 1988 (I_{Ca} and $[Ca]_i$ using fura-2), Hadley & Lederer, 1989 (charge movement) and Bers, Bridge & Spitzer, unpublished (shortening). Values were normalized to their maximal value (0.9 nA for I_{Ca}, 459 nM for $\Delta[Ca]_i$, 5 nC/μF for charge movement and 12 μm for shortening). Charge movement was fit with $Q=Q_{max}/(1+\exp[-(E_m-V^*)/k])$where Q_{max} was set to 1, V^*=-7 mV and k=11 mV. Holding potentials were -50 or -40 mV and test pulses were 20 msec (for charge movement) and 200-300 msec for the other curves.

Callewaert *et al.*, 1988; duBell & Houser, 1989). This is also true for the E_m-dependence of an intrinsic birefringence signal in cardiac muscle thought to be associated with SR Ca release (Maylie & Morad, 1984). This bell shaped E_m-dependence is strikingly different than the sigmoid E_m-dependence of the intramembrane charge movement in heart (Field *et al.*, 1988; Bean & Ríos, 1989; Hadley & Lederer, 1989) that is thought to be involved in skeletal muscle E-C coupling (Schneider & Chandler, 1973). Figure 57 shows that the parameters associated with contraction in skeletal muscle ($[Ca]_i$, tension and birefringence) have an E_m-dependence like that of the intramembrane charge movement and not like I_{Ca}. Indeed, since skeletal muscle Ca channels activate so slowly (time to peak at ~22°C is ~200 msec *vs* ~5 msec for cardiac I_{Ca}) it is doubtful if appreciable I_{Ca} flows during a normal twitch (Sanchez & Stefani, 1978,1983; Gonzalez-Serratos *et al.*, 1982).

Thus, there appears to be a fundamental difference in E-C coupling between cardiac and skeletal muscle despite qualitative similarities discussed in preceding chapters. It appears that the Ca required for the activation of the myofilaments in skeletal muscle is

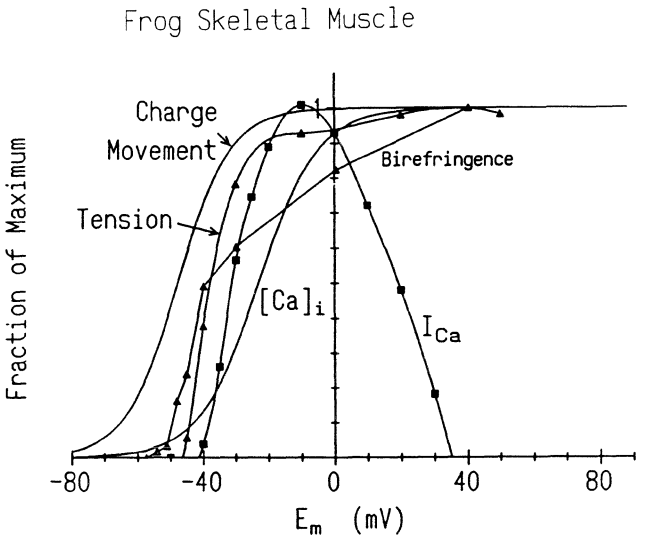


Figure 57. Voltage dependence of Ca current (I_{Ca}), change in $[Ca]_i$, isometric tension, birefringence and intramembrane charge movement in frog skeletal muscle fibers. Data were taken from Miledi *et al.*, 1977, ($[Ca]_i$ using arsenazo III), Chandler *et al.*, 1976a (charge movement), Caputo *et al.*, 1984 (tension), Baylor & Chandler, 1978 (birefringence) and Sanchez & Stefani, 1983 (I_{Ca}). Values were normalized to their maximal value (~2 μM for $\Delta[Ca]_i$, 21.5 nC/μF for charge movement, ~3 kg/cm^2 for tension and 110 μA/cm^2 for I_{Ca}). Charge movement was fit with $Q=Q_{max}/(1+\exp[-(E_m-V^*)/k]$ where Q_{max} was set to 1, V^*=-47.7 mV and k=8 mV. The original $[Ca]_i$ data was fit with the same equation, but with V^*=-23 mV and k=9 mV. Holding potentials were between -100 and -75 mV and test pulses were 100 msec for charge movement, tension and birefringence, 10 msec for $[Ca]_i$ and 1.8 sec for I_{Ca}.

released from within the cell and specifically from the SR. Furthermore, the Ca release from the SR appears to be related to the intramembrane charge movement. This more intrinsically E_m-dependent E-C coupling mechanism will be discussed in greater detail below. In cardiac muscle there are substantial transsarcolemmal Ca fluxes (see Chapters 4 & 5) and Ca influx via I_{Ca} may be more centrally involved in E-C coupling. While Ca influx may activate the myofilaments directly and serve to load the SR with Ca, it may also trigger the release of Ca from the SR. This is the so called Ca-induced Ca-release theory and will be discussed in more detail below.

Since Ca entry leads to Ca release in this mechanism, it is sometimes difficult to distinguish unequivocally between *direct* effects of Ca entry and the SR Ca release induced by the Ca entry. The relative dependence of cardiac contractions on Ca influx and SR Ca release will be directly addressed in Chapter 8. Three lines of evidence indicate that Ca release from the SR can contribute significantly to activation of contraction in cardiac

muscle: 1) the inhibition of cardiac muscle contractions by agents which affect SR Ca (caffeine and ryanodine), 2) interpretation of force-frequency relationships and 3) the quantitative estimates of Ca entry via I_{Ca} (see page 62) and requirements for myofilament activation (page 34). Thus it is important to consider the mechanism controlling SR Ca release in cardiac muscle.

As described at the end of Chapter 6, inositol(1,4,5)-trisphosphate (Ins(1,4,5)P_3) can serve as a chemical messenger which is capable of inducing Ca release from internal stores in many cell types. While the case for a physiological role of the Ins(1,4,5)P_3 pathway is stronger in smooth muscle, Ins(1,4,5)P_3 can induce SR Ca release in cardiac and skeletal muscle and this will also be discussed in some detail below.

The following sections will discuss three mechanisms by which SR Ca is released with focus on the muscle types in which they are most prominent (i.e. depolarization-induced Ca release in skeletal muscle, Ca-induced Ca-release in cardiac muscle and Ins(1,4,5)P_3-induced Ca release in smooth muscle). However, it should be borne in mind that there is evidence supporting all three mechanisms in all three tissues. In particular, I will include discussion of depolarization- and Ins(1,4,5)P_3-induced Ca release in cardiac muscle.

DEPOLARIZATION-INDUCED Ca RELEASE OR CHARGE-COUPLED Ca RELEASE

The activation of skeletal muscle is strongly E_m-dependent (Hodgkin & Horowicz, 1960). Schneider & Chandler (1973) described the E_m-dependence of an intramembrane charge movement thought to be related to activation. This charge movement is recorded as an outward current upon depolarization where all known ionic currents are blocked and linear capacitance current is subtracted (see Fig. 58B). It is thought to be membrane delimited because the same amount of charge moves back upon repolarization. This charge movement can be broken down into several components by voltage-clamp protocols and pharmacological agents, and one component (Q_γ) may be most closely related to SR Ca release (Hui, 1983). However, the identity and the roles of these components of charge movement are still controversial (e.g. Q_γ might be a result of Ca release rather than a cause, see reviews by Caillé *et al.*, 1985 and Huang, 1988).

Chandler *et al.* (1976a,b) followed up the initial study of Schneider & Chandler (1973) and proposed a physical "plunger" model by which the charge movement in the T-tubule membrane might activate Ca release from the SR (Fig. 58A). In this model, a charged particle $+Z_1$ (valence = +3) would move across the T-tubule membrane, pulling a long plunger (spanning the T-tubule-SR gap) out of the SR allowing Ca to be released to the cytoplasm. The release mechanism could then move more slowly to a refractory state when $-2Z_2$ (total valence more negative than -3) pulls $+Z_1$ back to the position where the

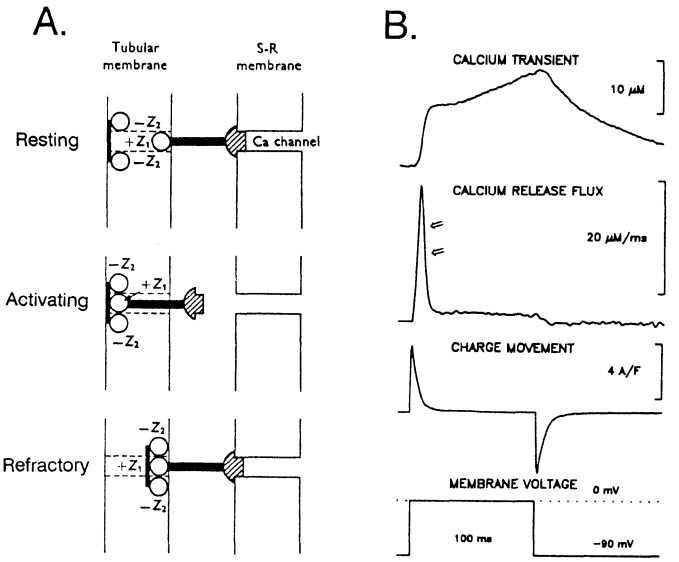


Figure 58. Intramembrane charge movement and SR Ca release. **A.** Hypothetical model of how intramembrane charge movement could regulate SR Ca release (from Chandler *et al.*, 1976b, with permission). The "plunger" blocking the SR Ca release channel is pulled out by the voltage-dependent movement of three positive charges ($+Z_1$) across the membrane electrical field (Activating). With maintained depolarization, the slower moving anionic groups (two $-Z_2$, with total charge magnitude > 3) gradually allow the SR release channel to close in a refractory state. **B.** Intracellular Ca transient, calculated Ca release flux and intramembrane charge movement in response to a depolarizing voltage clamp pulse in frog semitendinosus fiber (from Ríos & Pizarró, 1988, with permission). The charge movement may activate the SR Ca release channel, which in turn partially inactivates during the long pulse (arrows).

channel is plugged. Of course this is one of many possible models, but illustrates some general features one might expect.

Melzer *et al.* (1987) developed a means to estimate the timecourse of the Ca release flux from the SR of skeletal muscle based on the measured Ca_i transient and assumptions about Ca removal from the cytoplasm. Figure 58B shows a Ca_i transient, the calculated Ca release flux and the intramembrane charge movement during a voltage-clamp pulse in a frog skeletal muscle fiber (Ríos & Pizarró, 1988). Schneider & Simon (1988) showed that the rapid decline in Ca release (arrows in Fig. 58B) depended on $[Ca]_i$. This could be a related phenomenon to the Ca_i-dependent inactivation of sarcolemmal Ca channels (Chapter 4) and of cardiac SR Ca release channels (Fabiato, 1985b; Meissner & Henderson, 1987). The Ca release flux in skeletal muscle is also immediately and completely turned off by repolarization. Interestingly, when skeletal muscle is treated with a low concentration of caffeine, the shut-off of Ca release is not as tightly coupled to

repolarization (Simon *et al.* 1989; Klein *et al.*, 1990). What makes this particularly interesting is that low caffeine concentrations make skeletal SR Ca release more like that in cardiac muscle (in terms of [Ca] sensitivity and RCCs, Endo, 1975b; Rousseau *et al.*, 1988; Sakai, 1965; Konishi *et al.*, 1985). Indeed, Ca-induced Ca-release from the SR has been observed in skeletal muscle (Fabiato, 1984). Thus, while Ca release in skeletal muscle SR appears to be under tight E_m control, there is some indication that this Ca release can be affected by Ca in a manner qualitatively similar to Ca-induced Ca-release (Stephenson, 1981; Fabiato, 1984; Donaldson *et al.*, 1989).

There have been many reports over the years suggesting an important role for Ca_o in skeletal muscle E-C coupling (e.g. Frank, 1980), despite the results of Armstrong *et al.* (1972, Fig. 55B). Brum *et al.* (1988a,b) studied the effects of low $[Ca]_o$ on E-C coupling in frog skeletal muscle in detail. They concluded that the effects of low $[Ca]_o$ could be attributed to effects on the T-tubular voltage sensor (or charge movement) rather than on Ca fluxes *per se*.

Eisenberg *et al.* (1983) showed that skeletal muscle could be paralyzed by the sarcolemmal Ca channel antagonist, D600, using a specific protocol of cooling and depolarization. Hui *et al.* (1984) showed that charge movement was also inhibited under these conditions. Dihydropyridine Ca channel antagonists (nifedipine and PN200-110) can also inhibit charge movement as well as contraction, particularly in partially depolarized skeletal muscle (Lamb, 1986; Lamb & Walsh, 1987; Ríos & Brum, 1987). Ríos & Brum (1987) suggested that the dihydropyridine receptors are the voltage sensors for skeletal muscle E-C coupling (and thus are also the locus of the intramembrane charge movement). Since intramembrane charge movement has most often been associated with ion channel gating, the dihydropyridine receptor, which may function as a Ca channel, is a good candidate.

Two other lines of investigation make this an especially appealing possibility. First, the ryanodine receptor has been identified as both the SR Ca release channel and the "foot" process bridging the T-tubule - SR gap (see Chapter 6), as would be required for a mechanical coupling such as described by Chandler *et al.* (1976b, see Fig. 58A). Second, Block *et al.* (1988) showed that tetrameric arrays of particles in the T-tubule membrane (which could be dihydropyridine receptors) directly overlie the ryanodine receptor/foot protein/SR Ca release channel in an organized pattern (see Figs. 9 and 59). Figure 59 shows the organization of these arrays. The larger stippled circles represent ryanodine receptor monomers with their tetrameric units tilted with respect to the axis along which pairs of tetramers are aligned. The black dots represent the T-tubule particles which may well be dihydropyridine receptors, although their identification is still equivocal. These T-tubule particles are present only at alternating tetrads. The close apposition of the putative dihydropyridine receptor and the SR Ca release channel makes the charge coupled, mechanical type of model envisioned by Schneider & Chandler (1973) quite plausible (though of course many details must still be evaluated). It is also possible that some other

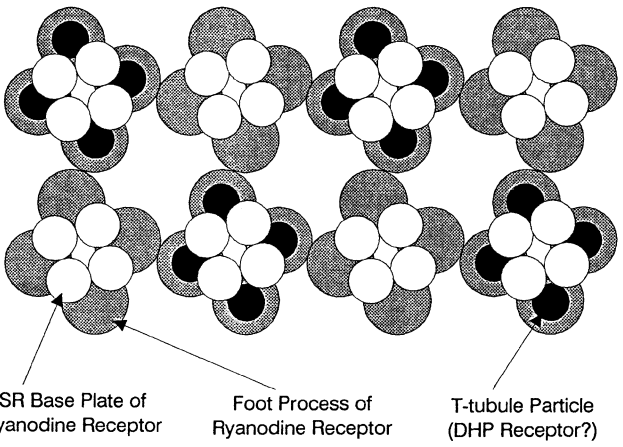


Figure 59. Schematic diagram of the spatial relationships between the components at the SR - T-tubule junction in skeletal muscle. The "foot" process of the ryanodine receptor (shaded) spans the gap between the SR membrane (in which the "base plate" of the ryanodine receptor is imbedded, white) and the T-tubule membrane in which the dihydropyridine receptor may be imbedded (black). Note that the putative dihydropyridine receptor tetramers (made up of 4 α_1 subunits) overlie the ryanodine receptor tetramers at alternating "feet" (redrawn after Block *et al.*, 1988).

E-C coupling mechanism is involved in activating the other half of the SR Ca release channels which are not directly under a putative dihydropyridine receptor, e.g. Ca-induced Ca-release (Block *et al.*, 1988; Ríos & Pizarró, 1988). The Ca released from one SR Ca release channel (induced by T-tubule depolarization) might activate an adjacent SR Ca release channel (which cannot participate in the same charge-coupled release process). Of course at this point this is purely speculation.

I should reiterate that in skeletal muscle Ca entry does not have to occur to activate Ca release. Any of group Ia and IIa elements of the periodic table can support charge movement and contraction (Ca > Sr > Mg > Ba >> Li> Na > K > Rb > Cs, Pizarró *et al.*, 1989). The relative affinities were also strikingly similar to those reported for the cardiac sarcolemmal L-type channel (see Table 10, page 55), with the notable exception of Mg, which does not seem to permeate the cardiac L-type channel (Hess *et al.*, 1986), but can permeate the skeletal muscle L-type Ca channel (McCleskey & Almers, 1985). It is possible that one of these ions must occupy the dihydropyridine receptor/Ca channel structure for the charge movement to occur, but that ionic current flow is not required. As

we will see below, this is in striking contrast to results in cardiac muscle, where Ca entry appears to be an absolute requirement for SR Ca release (see Fig. 65).

Murine Muscular Dysgenesis: A Model System

Muscular dysgenesis (*mdg*) is an autosomal recessive genetic mutation in mice that results in failure of E-C coupling in skeletal muscle (e.g. Klaus *et al.*, 1983). As such it has proven to be a valuable disease model in which to study E-C coupling (Adams & Beam, 1990). Both dihydropyridine receptors and the α_1 subunit of the Ca channel were lacking in skeletal muscle from *mdg* mice (Pinçon-Raymond *et al.*, 1985; Knudsen *et al.*, 1989). The slow Ca current and intramembrane charge movement were also found to be absent in these skeletal muscles (Beam *et al.*, 1986; Beam & Adams, 1990; Shimahara *et al.*, 1990). Normal E-C coupling, charge movement and I_{Ca} could also be restored in *mdg* myotubes by injection of cDNA encoding the normal skeletal dihydropyridine receptor (Tanabe *et al.*, 1988; Adams *et al.*, 1990). These results provide additional evidence to support the hypothesis that the dihydropyridine receptor is the voltage sensor which produces the charge movement that appears to be integral to skeletal muscle E-C coupling. It also provides evidence that the intramembrane charge movement and Ca current are both associated with the same dihydropyridine receptor molecule.

Tanabe *et al.* (1990a,b) also showed that if cDNA encoding the cardiac (rather than skeletal) dihydropyridine receptor was injected into dysgenic myotubes, Ca current and E-C coupling were again restored. However, in this case the Ca current was more like that observed in cardiac muscle (i.e. with faster activation and inactivation kinetics) and E-C coupling was also more like cardiac muscle (e.g. contractions were quickly abolished in the absence of extracellular Ca). Using chimeric cDNA they also found that replacing a single region of the cardiac dihydropyridine receptor with the skeletal counterpart (the cytoplasmic loop between domains II & III, see Fig. 25) was sufficient to cause E-C coupling to be skeletal muscle-like. Thus, it would seem that the different dihydropyridine receptors in cardiac and skeletal muscle are sufficient to explain a major difference in E-C coupling in these muscle types. That is, in skeletal muscle the dihydropyridine receptor appears to cause release by virtue of the charge movement (and whatever mechanical effect that produces), with the I_{Ca} being incidental. In cardiac muscle the Ca entry via the Ca channel (or dihydropyridine receptor) appears to be the critical event, although charge movement may still be important (but in this case because it may reflect the gating of the Ca channel).

Does the Dihydropyridine Receptor Contact the Ryanodine Receptor?

The molecular picture suggested by much of the physiological and ultrastructural results described previously is some variation of the simple model proposed by Chandler *et al.* (1976b, Fig. 58A). That is, the dihydropyridine receptor is the voltage sensor and provides the physical impetus (by charge movement across the membrane field) that is

transferred to the ryanodine receptor and causes the Ca release channel to open. The simplest version of such a scheme would be if the dihydropyridine receptor and ryanodine receptor were the only two molecules involved or required. However, attempts to demonstrate biochemically that these proteins physically interact (e.g. crosslinking studies) have not yet been successful. These studies have raised the possibility that some additional protein or factor might be interposed (e.g. see Fig. 63).

Caswell *et al.* (1979) demonstrated that junctions between SR and T-tubule vesicles (*triads*) broken apart by French Press treatment could be reformed by treatment with cacodylate buffer. Ikemoto *et al.* (1984) showed that *triads* reformed in this way also had restored depolarization-induced release of Ca from the SR (presumably due to T-tubule depolarization, see below). *Triad* reformation was also promoted by GAPD (glyceraldehyde 3-phosphate dehydrogenase, Corbett *et al.*, 1985; Caswell & Corbett, 1985) and aldolase was also reported to bind to the junctional foot protein and be released by Ins(1,4,5)P_3 (Thieleczek *et al.*, 1989). It may be noted that the glycolytic enzymes, GAPD and aldolase, are both cationic and bind to negatively charged proteins and membranes. Chadwick *et al.* (1988) also reported a 71,000 Da T-tubular protein which interacted with the ryanodine receptor. Brandt *et al.* (1990) and Kim *et al.* (1990) recently reported that both the dihydropyridine receptor and ryanodine receptor interact with GAPD, aldolase and also a novel 95,000 Da protein. They proposed a model of the triadic junction in which GAPD and the 95,000 Da protein couple the dihydropyridine receptor to the ryanodine receptor (Kim *et al.*, 1990). Whether any of these proteins are in fact directly involved in the process of E-C coupling is not known.

There is also indirect evidence in cardiac muscle to suggest that there is functional coupling between the sarcolemmal Ca channel and the SR Ca release channel. Bay K 8644 (a dihydropyridine Ca channel agonist) can induce depletion of SR Ca content in resting canine and ferret ventricular muscle (Hryshko *et al.*, 1989a,b, 1990). Bay K 8644 also increases ryanodine binding to isolated cardiac myocytes and skeletal muscle triads, but not in SR vesicles (qHryshko *et al.*, 1990). Anderson *et al.* (1990) have also reported that in skeletal muscle triads, ryanodine enhanced the binding of PN200-110 (a dihydropyridine Ca antagonist). Cohen & Lederer (1988) also showed that ryanodine could shift the E_m-dependence of sarcolemmal Ca channel inactivation in rat ventricular myocytes in which Ca transients were blocked by high intracellular EGTA concentration. These results may be explained by some functional communication between the dihydropyridine receptor and the ryanodine receptor. Whether these effects are mediated through some intermediary protein is not clear and further study is required.

Direct SR Depolarization

Peachey & Porter (1959) first raised the possibility that depolarization of the T-tubule may induce depolarization of the SR membrane itself, thereby inducing Ca release. This hypothesis was tested in mechanically skinned skeletal muscle fibers using ionic

substitution (Costantin & Podolsky, 1967; Nakajima & Endo, 1973). In this approach, a relatively impermeant anion (e.g. propionate, gluconate or methanesulfonate) is replaced by a permeant one (e.g. Cl) or a permeant cation (e.g. K) is replaced by a relatively impermeant one (e.g. choline, Tris, Li or Na). In either case, a diffusion potential is set up so that after the solution switch the inside of the SR would tend to become more negative. If the inside of the intact SR were positively polarized with respect to the myoplasm (i.e. as is the extracellular space), such a depolarization could simulate a physiological signal. This type of depolarization-induced Ca release has been reported (see Endo, 1985 for a review). However, there are two major factors which make this mechanism seem unlikely especially in terms of physiological relevance.

First, in mechanically skinned fibers, the T-tubules apparently seal off and in the presence of ATP (required in the skinned fiber solution) re-establish the normal sarcolemmal ion gradients. Thus the ionic substitution will depolarize the T-tubule membrane, such that the Ca release could still depend on charge movement and electromechanical coupling as described above. A compelling argument for this explanation is that such depolarization-induced Ca release in skinned fibers can be prevented by inhibition of the sarcolemmal Na-pump (so that the T-tubule cannot become polarized, Donaldson, 1985; Stephenson, 1985; Volpe & Stephenson, 1986). This argument also holds for heavy SR preparations which may include intact T-tubule-SR junctions. Second, as described at the end of Chapter 6, the monovalent cation permeability of the SR to physiological ions is very high and there are no appreciable ionic gradients between the SR and the cytoplasm (Somlyo *et al.*, 1977a,b; Meissner, 1986b; Somlyo & Somlyo, 1986). This makes it unlikely that there is a potential across the SR membrane.

Fabiato (1985f) also tested direct SR depolarization-induced Ca release in skinned pigeon cardiac myocytes which lack T-tubules, but exhibit Ca-induced Ca-release. He found no evidence for direct SR depolarization-induced Ca release in cardiac muscle. Thus a direct SR depolarization does not seem to be a tenable model.

Does Charge-Coupled Ca Release Work in Cardiac Muscle?

While charge-coupled SR Ca release appears to be functional in skeletal muscle, several lines of evidence suggest that it is not operational in cardiac muscle. 1) The E_m-dependence of $[Ca]_i$ and contraction follow I_{Ca} and not charge movement (Fig. 56). 2) Ca entry seems to be an absolute requirement in cardiac muscle (Figs. 55A & 65). 3) Depolarization does not induce SR Ca release by itself and does not appear to modify Ca-induced Ca-release from the SR (Figs. 64, 65 & 67). 4) Ca release from corbular and extended junctional SR in cardiac muscle is physically too distant for charge coupled Ca release (if, indeed Ca release from corbular SR occurs physiologically). 5) We don't yet know if the same exquisitely organized architecture apparent in skeletal muscle (Fig. 59) actually exists at SR-sarcolemmal junctions in heart.

Ca-INDUCED Ca-RELEASE

Ca-Induced Ca-Release in Skeletal Muscle

Ca-induced Ca-release from the SR was first described by Endo *et al.* (1970) and Ford & Podolsky (1970) in skinned skeletal muscle fibers. This phenomenon, where a small elevation of $[Ca]_i$ leads to a larger Ca release from the SR clearly exists in both cardiac and skeletal muscle SR (e.g. see Chapter 6), but the major question is really whether it occurs under physiological circumstances. For example, Endo (1975a, 1977) argued that Ca-induced Ca-release was only demonstrable in skeletal muscle fibers with an unphysiologically low [Mg], required heavy Ca loading of the SR and very high [Ca] to induce release (e.g. 100 μM Ca with 0.9 mM Mg, or 10 μM Ca with 50 μM Mg, see also Fig. 54) at which point the high $[Ca]_i$ could activate tension directly. Fabiato (1984, 1985g), however, demonstrated Ca-induced Ca-release in skeletal muscle with the SR loaded at 100 nM Ca and ~3 mM free [Mg], with Ca release induced by a very rapid increase of [Ca] to 200-600 nM. As described in the preceding sections, there is strong evidence supporting a charge-coupled Ca release in skeletal muscle. However, Ca-induced Ca-release may still be physiologically relevant. In the case of skeletal muscle, the Ca which might activate Ca release may come from the SR (secondary to charge coupled release from adjacent release channels, Ríos & Pizarró, 1988) or from sites on the inner T-tubular surface or sarcolemma upon depolarization (Frank, 1980; Bers *et al.*, 1986).

Ca-Induced Ca-Release in Mechanically Skinned Cardiac Muscle

In an elegant and formidable series of studies Fabiato & Fabiato have extensively characterized Ca-induced Ca-release in mechanically skinned single cardiac myocytes (Fabiato & Fabiato, 1973, 1975a,b, 1978a,b, 1979; Fabiato 1981a, 1983, 1985a-c). In the most recent extensive experimental series, Fabiato (1985a-c) used mechanically skinned canine Purkinje fibers since these cells do not have T-tubules (which could reseal and complicate interpretations). These experiments were done in the presence of 5 μM calmodulin (which slightly increases the Ca released) and ~3 mM free Mg. Fabiato (1985a) found that the largest Ca-induced Ca-release was observed in 1-3 mM Mg although the threshold [Ca] for induction of Ca-release is then higher than when lower [Mg] is used (Fabiato, 1983). He was able to apply solutions of various [Ca] at various rates (as fast as ~1 msec) to these skinned cells and measure the SR Ca release with both aequorin luminescence and force recordings. In this model of E-C coupling a small amount of Ca entry via I_{Ca} (simulated by the rapid Ca application) induces the release of Ca from the SR. Since Ca-induced Ca-release implies a positive feedback, one might suppose that the Ca release would proceed to completion (as released Ca would cause more and more Ca to be released). However, a remarkable feature of Ca-induced Ca-release is that the amount of Ca released is graded with the amount of "trigger" Ca (Fabiato, 1983, 1985b). Indeed, at higher [Ca] the Ca-induced Ca-release is inhibited or inactivated (see below).

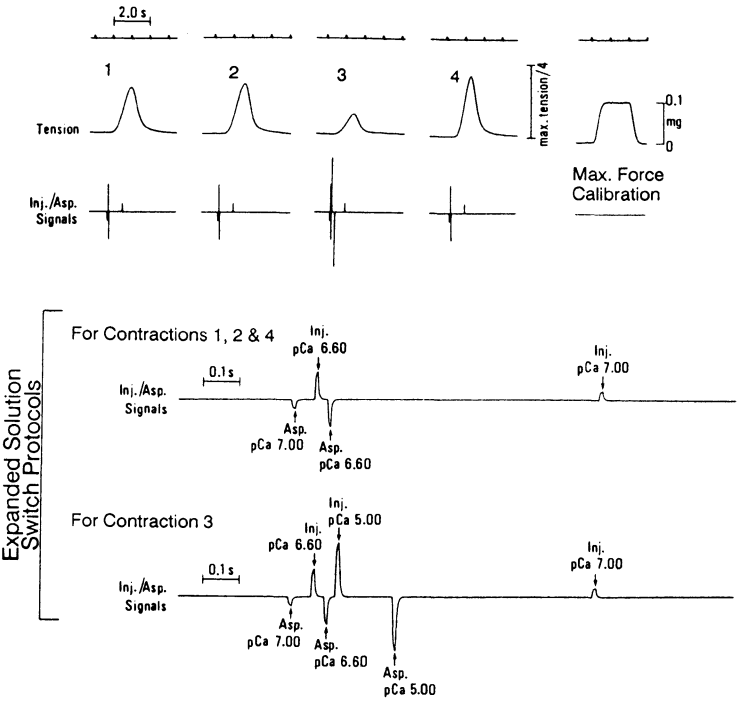


Figure 60. Contraction in a mechanically "skinned" canine cardiac Purkinje cell, illustrating the Ca-induced Ca-release process as studied by Fabiato (1985b). The rapid application and removal of experimental solutions is indicated by the injection/aspiration (Inj./Asp.) signals (note expanded time scale at bottom). During the initial period in pCa 7.00 buffer the SR accumulates Ca. Then the pCa 7.00 solution is withdrawn and a pCa 6.60 solution is injected around the cell and re-aspirated ~30 msec later. This small and brief increase in [Ca] surrounding the skinned cell induces the release of Ca from the SR and a contraction such that the peak myoplasmic [Ca] at the first tension trace is pCa 5.76. For the third contraction only, the [Ca] around the skinned cell was raised to pCa 5.00 for ~150 msec immediately after Ca release was initiated by pCa 6.60. This extra elevation of [Ca] led to a smaller contraction and peak [Ca] (pCa 5.92) and indicates that higher [Ca] can inactivate Ca release from the SR (from Fabiato, 1985b, with permission).

Figure 60 is an experiment illustrating the approach used by Fabiato (1985b). It also shows that high [Ca] can inactivate Ca-induced Ca-release. In contractions 1, 2 & 4, the 100 nM Ca (pCa 7) solution used to load the SR is withdrawn (Asp.) and pCa 6.6 solution is briefly applied to activate SR Ca release (note that this [Ca] is insufficient to directly activate contraction, Fig. 17A). In the third contraction a higher [Ca] (10 μM, pCa 5) is injected during the early rising phase of contraction for ~150 msec, producing a smaller contraction. This indicates that the higher [Ca] inactivates the usual Ca-induced

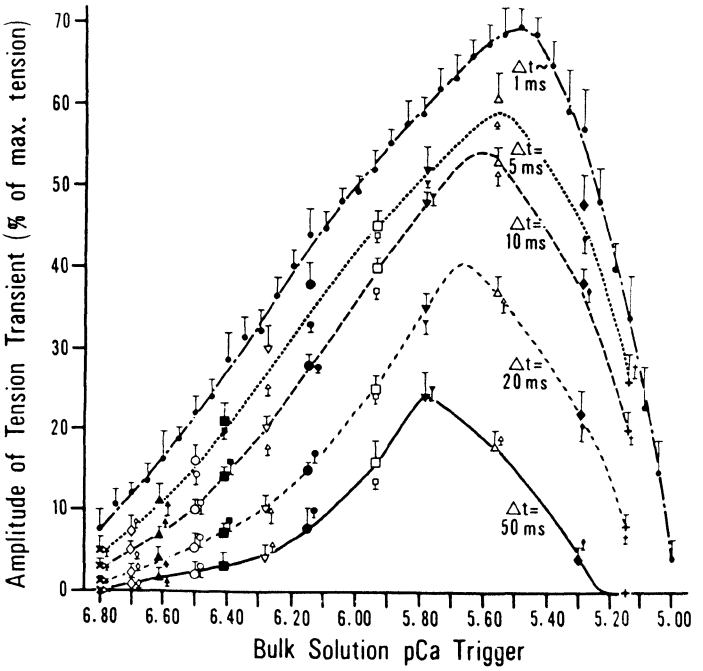


Figure 61. Relationship between the free [Ca] used as a trigger for SR Ca release (Bulk Solution pCa Trigger) and the amplitude of the contraction resulting from the Ca-induced Ca-release from the SR. The experiments were done much like the control contractions in Fig. 60, except that different pCa solutions were used in place of the pCa 6.60 solution and the time taken to increase [Ca] to the desired "trigger" concentration was varied from 1 to 50 msec. It can be seen that the Ca release depends on both the [Ca] of the trigger and the rate at which the [Ca] changes around the SR with a decline in Ca release at higher [Ca] (from Fabiato, 1985b, with permission).

Ca-release. The amplitude of the Ca-induced Ca-release depends on both the [Ca] used to induce Ca-release, but also the rate at which the [Ca] changes around the SR.

Figure 61 shows the "trigger" [Ca] dependence of the tension transient produced by Ca-induced Ca-release (Fabiato, 1985b). Data is shown for several different durations taken to reach the indicated "trigger" [Ca]. It can be appreciated that the Ca released depends on both the "trigger" [Ca] and the time taken to reach that [Ca]. At high (or supraoptimal) "trigger" [Ca] the SR Ca release was inhibited. Ca-induced Ca-release also appeared to exhibit a refractory period where a second Ca release could not be induced. This was not the case for caffeine-induced Ca-release. Fabiato (1985b) likened this to the inactivation (and recovery from inactivation) described for sarcolemmal Ca channels. He even showed evidence that some steady state inactivation existed at pCa 7.2 (63 nM),

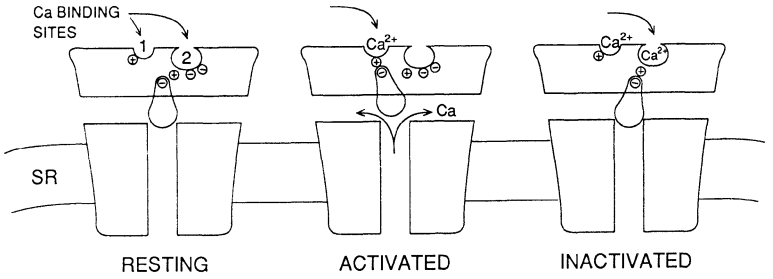


Figure 62. Diagram of a possible scheme for Ca-induced Ca-release from cardiac SR consistent with the model described by Fabiato (1985b). From the resting state (channel closed), Ca may bind rapidly to a relatively low affinity site (1), thereby activating the Ca release channel. Ca may then bind more slowly to a second higher affinity site (2) moving the release channel to an inactivated state. As cytoplasmic [Ca] decreases, Ca would be expected to dissociate from the lower affinity activating site first and then more slowly from the inactivating site to return the channel to the resting state.

which could be removed by a few seconds at pCa 7.9 (analogous to steady state inactivation of sarcolemmal Ca channels at depolarized potentials).

On the basis of these findings Fabiato (1985b) proposed a model where Ca binds to an activating site with a high *on rate* (but modest affinity) and also binds to a second inactivating site which has a higher affinity, but a slower association constant (~0.7 sec at pCa 7.2). Thus, when $[Ca]_i$ increases rapidly the activation site is occupied and SR Ca release occurs. The inactivation site will bind Ca more slowly and then turn off Ca release. Rapid application of very high [Ca] can still produce inactivation more directly since binding to the inactivation site is expected to be proportional to the product of the *on rate* and the [Ca]. Thus the very high [Ca] can partially overcome the limitation of the slow *on rate*. A schematic drawing of this sort of model is shown in Fig. 62. Although the appropriateness of this model has not been extensively tested, it fits with Fabiato's observations and may provide a practical working hypothesis.

Ca-Induced Ca-Release: Support from Intact Cardiac Myocytes.

Figure 63 puts the model above into the context of an intact cell (with a sarcolemma) and as a working hypothesis places the sarcolemmal Ca channel (or dihydropyridine receptor) directly above the SR Ca release channel (or ryanodine receptor). Thus Ca entry via I_{Ca} may have ready access to the activation (and inactivation sites) of the SR Ca release channel. The similar E_m-dependence of I_{Ca}, contraction and Ca_i transient in cardiac myocytes, as illustrated in Fig. 56, provide support for Ca-induced Ca-release as the important mechanism of E-C coupling in cardiac muscle (London &

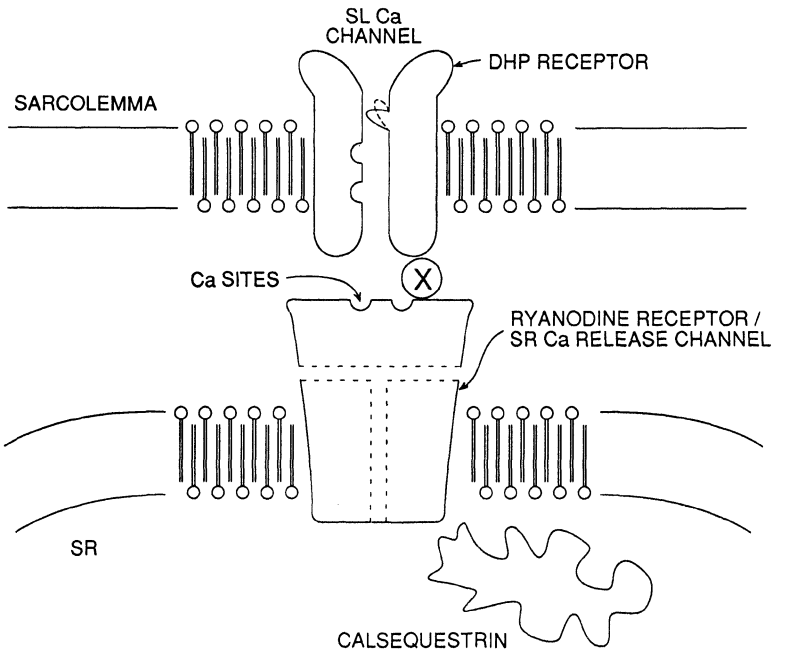


Figure 63. Schematic diagram of the possible functional arrangement of proteins involved in E-C coupling. The sarcolemmal Ca channel (or dihydropyridine receptor) may overlie the ryanodine receptor/Ca release channel. In skeletal muscle some direct mechanical link (perhaps via X) seems important, but has not been clearly identified yet. In cardiac muscle, the importance of such a physical link is unclear. However, Ca entry via sarcolemmal Ca channels may have preferential access to Ca binding sites on the SR release channel which might be involved in activation and inactivation of the SR Ca release channel.

Krueger, 1986; Cannell *et al.*, 1987; Beuckelmann & Wier, 1988; Callewaert *et al.*, 1988; duBell & Houser, 1989). However, this could simply have stemmed from a coincidental E_m-dependence of E-C coupling and I_{Ca}. Further support comes from the observation of I_{Ca} "tail transients" (Fig. 64, Cannell *et al.*, 1987; Beuckelmann & Wier, 1988). These occur when a cell which was voltage clamped beyond the reversal potential for Ca (e.g. $E_m = +100$ mV, where Ca channels are open, but no inward Ca current flows) is clamped back to a negative value of E_m (e.g. -50 mV) where Ca channels deactivate. However, as the Ca channels are closing, a large but short-lived inward Ca current flows through open Ca channels (tail current) and induces a Ca_i transient and contraction (at a resting E_m). This seems likely to reflect Ca-induced Ca-release, although a complex and unusual E_m-dependence could still explain this (Cannell *et al.*, 1987). These "tail Ca_i transients" are not observed in skeletal muscle, where voltage appears to control SR Ca release more directly.

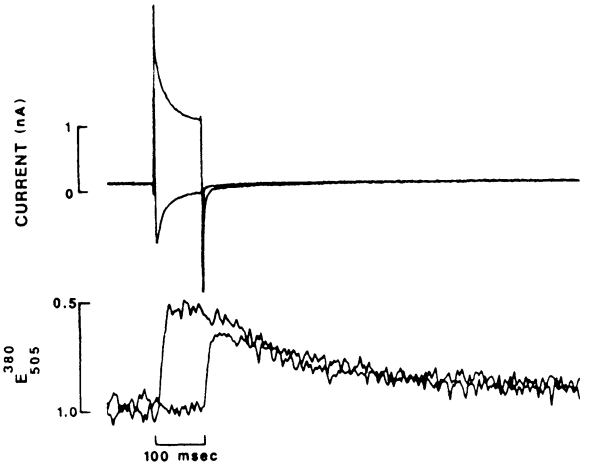


Figure 64. Activation of SR Ca release by repolarization in a rat ventricular myocyte (a "tail transient"). Current (top traces) and $[Ca]_i$ (lower Fura-2 fluorescence traces) in response to depolarization to either +10 mV (lower current trace and upper $[Ca]_i$ trace) or +100 mV (upper current and lower $[Ca]_i$ trace). The depolarization to +100 mV (i.e. beyond E_{Ca}) produces no inward I_{Ca} or Ca_i transient. Repolarization from +100 mV, however, produces a transient Ca current through channels opened by the depolarization (tail current) and a large contraction, which may result from Ca-induced Ca-release secondary to that Ca entry (from Cannell *et al.*, 1987, with permission).

Näbauer *et al.* (1989) provided compelling evidence for Ca-induced Ca-release in mammalian cardiac muscle. Figure 65 shows E_m, membrane current (i_m) and fura-2 fluorescence change (ΔF, indicative of $[Ca]_i$) in a rat ventricular myocyte. The first pulse shows a control depolarization, a large I_{Ca} and Ca_i transient. For the second pulse, 4 mM EGTA has been added to the solution to decrease $[Ca]_o$ to submicromolar levels. Under these conditions the Ca channel carries a large Na current (see Chapter 4) which inactivates very slowly compared to I_{Ca}. There is no Ca_i transient associated with this pulse despite the facts that 1) ionic current flowed through the Ca channel, 2) there was presumably charge movement associated with Ca channel activation and 3) there was plenty of SR Ca (as evidenced by the large caffeine-induced Ca_i transient). They also showed that Ba current could not induce SR Ca release. Thus, E-C coupling cannot be due to Ca channel charge movement or current *per se*. Ca entry appears to be an absolute requirement for induction of Ca release in cardiac muscle.

Two new photolabile Ca chelators (Nitr-5, Adams *et al.*, 1988 and DM-nitrophen, Kaplan & Ellis-Davies, 1988) in which Ca affinity is decreased upon illumination have made it possible to produce rapid increases in $[Ca]_i$ in intact cells. Kentish *et al.* (1990) used Nitr-5 to demonstrate Ca-induced Ca-release in saponin skinned cardiac muscle for comparison with Ins(1,4,5)P_3-induced Ca-release (see below). Valdeolmillos *et al.* (1989)

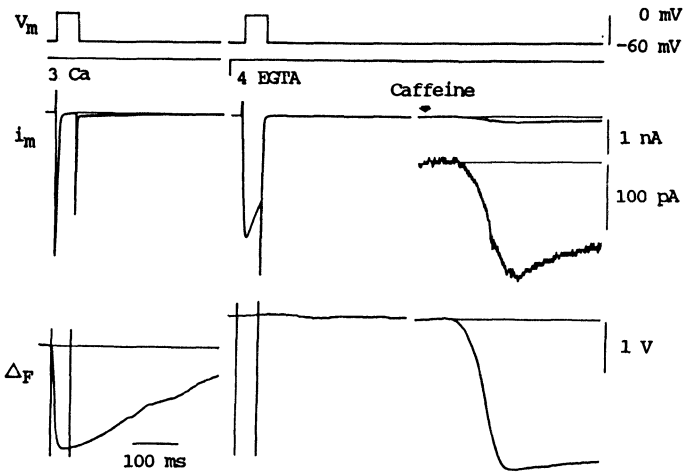


Figure 65. Ca entry is required for induction of SR Ca release in rat ventricular muscle. At left, depolarization activates a large Ca_i transient (measured by fura-2 fluorescence, where downward deflection indicates increasing $[Ca]_i$). After removal of Ca from the superfusate and addition of 4 mM EGTA, depolarization activates Na current through Ca channels (with characteristically slow inactivation). Na current through the Ca channel does not induce SR Ca release. Subsequent application of 5 mM caffeine induces a large Ca_i transient, indicating that there was plenty of SR Ca available for release in the center panel. Caffeine also activated a small inward current which could be due to Na/Ca exchange (from Näbauer *et al.*, 1989, with permission).

demonstrated that photolysis of Ca-Nitr-5 induced ryanodine-sensitive contractions in rat ventricular myocytes which persisted in the presence of 10 mM Ni (which would block Ca influx via I_{Ca} and Na/Ca exchange). These contractions probably reflect Ca-induced Ca-release, but E_m was not measured. Näbauer & Morad (1990) and Niggli & Lederer (1990) performed this type of photolysis experiment in isolated myocytes under voltage clamp. The contractions were largely suppressed by caffeine or ryanodine and were observed at constant E_m (excluding any possibility that the Ca release was depolarization-dependent). Niggli & Lederer (1990) also showed that the Ca-induced Ca-release was the same whether E_m was -100, 0 or +100 mV, suggesting that Ca-induced Ca-release in cardiac muscle is not modified by E_m (see also below). Näbauer & Morad (1990) also tried to reproduce Fabiato's experiment (shown in Fig. 60) by increasing $[Ca]_i$ with a flash during the rising phase of the contraction. They did not see any evidence of inactivation of SR Ca release with elevated $[Ca]_i$. They saw only enhancement of contraction. It is possible that the $[Ca]_i$ reached after DM-nitrophen photolysis was not high enough or for long enough to produce inactivation. Ca-dependent inactivation of SR Ca release channel is not observed in bilayer recordings (Rousseau & Meissner, 1989) but can be seen in SR vesicles (see Fig. 54, Meissner & Henderson, 1987).

Challenges to Ca-Induced Ca-Release in Cardiac Muscle

One challenge raised above is a clear demonstration of inactivation of Ca release. It is clear that for contractions to be graded (e.g. as they are with I_{Ca} amplitude) some sort of inactivation of Ca release is required to prevent Ca release from always going to completion. Certainly refractoriness exists where SR Ca is available for release (e.g. assessed by RCC) and I_{Ca} has recovered, but not much Ca is released (see Chapter 8). Feher & Fabiato (1990) raised the possibility that calmodulin might be important in mediating Ca_i-dependent inactivation of Ca release (although this inactivation was still observed in skinned fiber experiments such as Figs. 60 & 61 without added calmodulin, Fabiato, 1985b).

Two factors may make it difficult to demonstrate Ca_i-dependent inactivation of SR Ca release in intact cells. First, it is not possible to know how high $[Ca]_i$ goes in the restricted space around the Ca-release channel and for how long it is maintained. This is true for the photolysis experiments of Näbauer & Morad, (1990) as well as when I_{Ca} serves

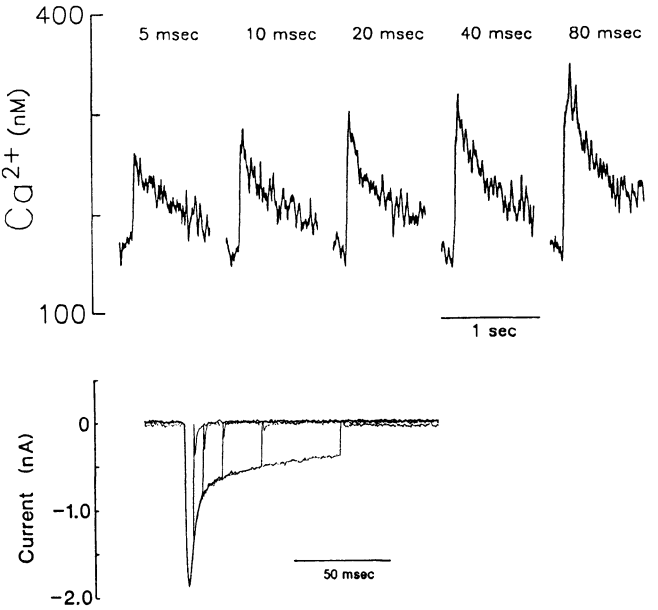


Figure 66. Duration dependence of Ca_i transients in a rat ventricular myocyte. The cell was loaded with the fluorescent Ca indicator, indo-1 via the patch clamp pipette. Test pulses to 0 mV (from -50 mV) for the indicated durations were given after a series of 5 conditioning pulses to 0 mV to ensure that the SR Ca load was the same at each test pulse. The lower panel shows superimposed Ca currents (leak subtracted) which were recorded during the Ca_i transients in the upper panel. The Ca_i transients increase in amplitude despite a constant peak I_{Ca} (from Bers *et al.*, 1990, with permission).

as the "trigger" for Ca release. In the latter case, since sarcolemmal Ca channels are strongly inactivated by local $[Ca]_i$, this may limit the rise in local $[Ca]_i$ (such that the descending limb in Fig. 61 is not easily reached). Second, slower elevation of $[Ca]_i$ also increases SR Ca loading. With higher SR Ca load, a reduction in the fraction of SR Ca release might still result in an increased absolute amount of Ca release. In conclusion, it would seem that this sort of inactivation of Ca release must occur, but it has not been demonstrated clearly in intact myocytes.

Another challenge to the hypothesis of Ca-induced Ca-release raised by Cannell *et al.* (1987, and see Fig. 66) is that the rise in $[Ca]_i$ can be curtailed by repolarization even after the peak of I_{Ca} has occurred. They suggested that this might reflect an intrinsic E_m-dependence of the Ca-induced Ca-release process. That is, that repolarization turned off SR Ca release (as in skeletal muscle). Another possibility is that repolarization hastened Ca extrusion via Na/Ca exchange, thereby limiting the rise of $[Ca]_i$. However, this possibility seems unlikely since Bers *et al.* (1990) demonstrated that this duration dependence of the Ca_i transient was still apparent when Na/Ca exchange had been eliminated (by using Na-free intracellular and extracellular solutions).

The possibility that Ca-induced Ca-release has intrinsic E_m-dependence also seems unlikely based on three types of experiments. First, the similar E_m-dependence of I_{Ca} and Ca_i transients or contractions, makes it seem unlikely (see Fig. 56). However, substantial Ca_i transients can be observed with almost immeasurable I_{Ca} (Cannell *et al.*, 1987). Figure 67 shows another way to look at this type of result. If SR Ca release depends on Ca entry

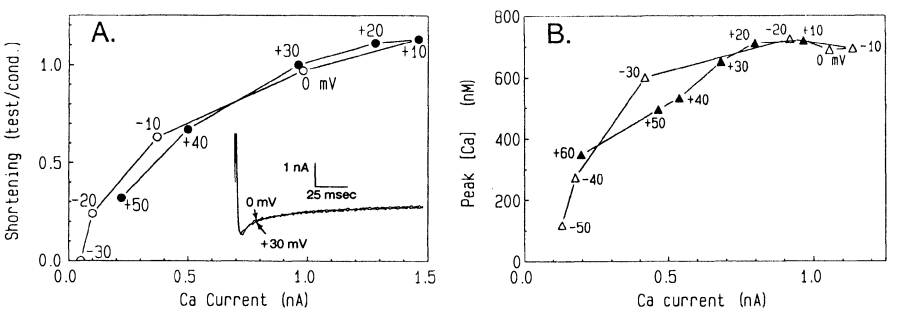


Figure 67. The Ca current dependence of shortening in guinea-pig ventricular myocyte (**A.**) and of Ca_i transient amplitude (**B.**) in rat ventricular myocyte. Conditioning pulses preceded the 200 msec test pulses to different E_m to ensure similar SR Ca content at the start of each test pulse. If SR Ca release was E_m-dependent one might expect "hysteresis" in these curves such that the same I_{Ca} would produce larger contraction (or peak $[Ca]_i$) at a more positive test potential. The lack of such "hysteresis" indicates that there is no apparent E_m-dependence of SR Ca release. The inset in **A** shows two raw current records offset to compensate a small difference in leak current (results are **A.** from unpublished experiments of D.M. Bers, J.H.B. Bridge & K.W. Spitzer and **B.** from Cannell *et al.*, 1987).

via I_{Ca} (independent of E_m *per se*), then one would expect the Ca_i transient or contraction for a given I_{Ca} to be the same at different voltages. That is what is observed in Fig. 67. If there were a substantial E_m-dependence, these curves should have a marked "hysteresis" (with higher $[Ca]_i$ or shortening at more positive E_m). Of course, the kinetics of the I_{Ca} will differ, especially at the extremes of E_m, but the inset shows that these effects can be small at E_m near 0 mV. A marked "hysteresis" of this sort can be seen when intracellular [Na] is elevated (Isenberg *et al.*, 1988). However, the higher $[Ca]_i$ observed at positive E_m in this case can be directly attributed to E_m-dependent Ca entry via Na/Ca exchange. A second argument against E_m-dependence of Ca-induced Ca-release is that Niggli & Lederer (1990) found the same contraction at -100, 0 and +100 mV when contraction was initiated by photolysis of Ca-Nitr-5 (see above). Third, in the type of experiment shown in Fig. 66, Cleeman & Morad (1991) showed that the Ca_i transient was equally well curtailed when the Ca entry via I_{Ca} was stopped by repolarization to -40 mV (deactivating I_{Ca}) or by further depolarization to +100 mV (exceeding the reversal potential for I_{Ca}). Together these results strongly suggest that Ca-induced Ca-release is not directly modulated by E_m.

The challenge is then to explain the voltage-clamp duration-dependence of the Ca_i transient within the context of the Ca_i-dependent activation and inactivation discussed above. Although difficult to test quantitatively, it is easy to imagine that SR Ca release in Fig. 66 is deactivated because local $[Ca]_i$ at an activator site decreases when the sustained component of Ca influx is stopped (by repolarization). Such a scheme would also require that the Ca which enters via I_{Ca} has preferential access to the sites which control SR Ca release (compared to the Ca which is released from the SR, Bers *et al.*, 1990). A more quantitative consideration of Ca-induced Ca-release in the junctional region would require much more detailed information about the physical locations of the key sites and about the diffusional limitations in this region. If Ca entry via Na/Ca exchange can also activate SR Ca release (Leblanc & Hume, 1990; Bers *et al.*, 1988) another degree of complexity is introduced (since this would imply that Ca entry via Ca channels may not be a particularly preferred pathway for induction of SR Ca release).

It may be of value at this point to consider at least in a general way the $[Ca]_i$ which might be expected in the vicinity of the inner mouth of a sarcolemmal Ca channel. This can be easily done using the solution to the diffusion equation for radial dependence of [Ca] from a point source in a hemisphere (Crank, 1975):

$$[Ca]_{rt} = [Ca]_{t0} + \frac{q}{2\pi Dr} \{erfc(\frac{r}{2\sqrt{Dt}})\} \qquad (7.1)$$

where $[Ca]_{t0}$ and $[Ca]_{rt}$ are the initial $[Ca]_i$ and the $[Ca]_i$ at a radial distance r, from the channel mouth at time t after a current q is activated and D is the diffusion coefficient.

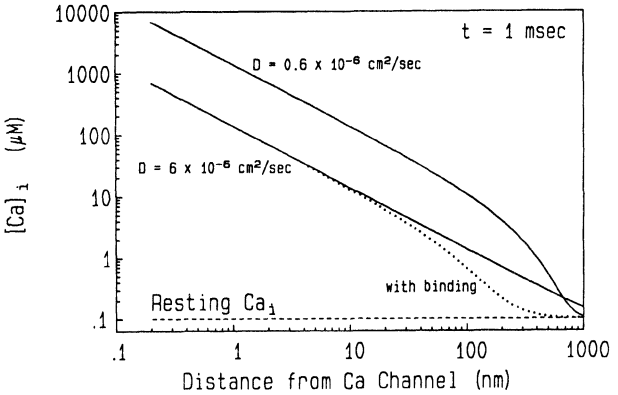


Figure 68. Predicted $[Ca]_i$ as a function of radial distance from the inner mouth of a Ca channel after 1 msec of current flow (0.1 pA). The two solid curves are simply plots of Eqn. 7.1 for q = 0.1 pA, $[Ca]_{to}$ = 100 nM, t = 1 msec and D = 6 and 0.6 x 10^{-6} cm^2/sec. The dotted curve is an estimate of how the lower solid curve would be altered by inclusion of instantaneous, linear Ca binding to the inner sarcolemmal surface (i.e. Ca bound = $[Ca]_i$ x 2.45 μm, corresponding to ~5 nmol Ca/mg sarcolemmal protein at 10 μM $[Ca]_i$). Numerical methods were as described by Bers & Peskoff, 1991, for external Ca binding and Ca_o depletion. For the inner sarcolemmal sites, the linear approximation of sarcolemmal Ca binding is poor because of the large range of $[Ca]_i$ expected, which means that the effect of Ca binding will be overestimated for $[Ca]_i$ > 10 μM and underestimated for $[Ca]_i$ < 10 μM.

Figure 68 shows the $[Ca]_i$ as a function of radial distance from the inner mouth of a sarcolemmal Ca channel at t = 1 msec. Two different values of D are shown. The lower curve (D = 6 x 10^{-6} cm^2/sec) uses D corresponding to Ca in free aqueous solution and the upper curve could result from restricted diffusion in the junctional region. A third curve (dotted) indicates the general way in which Ca binding to the inner sarcolemmal surface would tend to shift one of the curves (based on Bers & Peskoff, 1991). The main points are that [Ca] can rise to very high values near the inner mouth of the Ca channel (of radius ~ 0.2 nm) and can still be very high at the SR side of the junctional gap (r ~ 14 nm). Presumably, the Ca binding regulatory sites are between these extremes. For r < 10 - 20 nm the Ca_i accumulation is virtually to its steady state value by 1 msec (i.e. the complementary error function term in eq. 7.1 approaches 1). This means that Ca diffusion away from this region is "keeping up" with Ca entry via the channel. It is thus implicit that $[Ca]_i$ in this region will decrease very rapidly when I_{Ca} stops flowing. This may explain the rapid deactivation of SR Ca release when Ca influx is stopped by early repolarization. Of course this is a grossly oversimplified consideration, since it does not include the kinetics or locations of Ca binding sites in the cell. Suffice it to say that $[Ca]_i$ near sarcolemmal Ca channels can be very high, but since an individual channel may only be open for 0.5 - 1 msec (see Table 8), the actual [Ca] near a single channel will be changing rapidly.

Spontaneous SR Ca Release and Cyclic Contractions

Fabiato & Fabiato (1972) demonstrated that elevation of [Ca] in skinned cardiac myocytes could induce cyclic contractions. Such cyclic contractions are also observed in intact myocytes and muscles and are sometimes manifest as contractile waves or as a series of aftercontractions (Kass *et al.*, 1978; Orchard *et al.*, 1983; Stern *et al.*, 1983; Wier *et al.*, 1983; Allen *et al.*, 1984b; Kort & Lakatta, 1984; Capogrossi *et al.*, 1986a,b; Mulder *et al.*, 1989; Backx *et al.*, 1989; Takamatsu & Wier, 1990). In general these phenomena are observed under conditions where cells are expected to become heavily Ca loaded (e.g. high $[Ca]_o$, low $[Na]_o$, high stimulation frequency, long depolarizations, Na-pump inhibition and damaged sarcolemmal permeability barrier). The SR may spontaneously release Ca when it reaches some critical loading level. This suggests that SR Ca release may also be regulated by the intra-SR [Ca] (or the trans SR [Ca] gradient). Lakatta's group have argued that these spontaneous Ca releases are physiologically relevant and contribute to diastolic tone in the heart (Stern *et al.*, 1983; Capogrossi *et al.*, 1986). Fabiato (1985b) has argued strongly that these cyclical Ca releases are via a mechanism different from Ca-induced Ca-release, partly because they can be observed at [Ca] at which Ca-induced Ca-release is inactivated (e.g. pCa 4.5). He also argued that they represent a purely pathological state of Ca overload. Indeed, the lack of spontaneous contractions is often used as a criteria for healthy isolated cardiac myocytes.

Propagated waves of contraction and elevated $[Ca]_i$ travel at 0.1 - 10 mm/sec, which is too slow for electrical depolarization to sweep across the cell (Mulder *et al.*, 1989; Stern *et al.*, 1988). These waves have often been interpreted in the following way. A wave is initiated at some site where the SR is overloaded with Ca (due to local damage or other causes). Then the Ca released from that first site could "trigger" Ca release at an adjacent SR Ca release site (via Ca-induced Ca-release) and so on down the cell. It is also possible that Ca-induced Ca-release is not involved *per se*. If all SR regions are heavily Ca loaded and one region reaches its limit first (dumping its Ca to the cytoplasm), the additional Ca uptake in neighboring regions may cause those regions to release Ca. Theoretical models cannot clearly rule out either of these possibilities (Takamatsu & Wier, 1990). O'Neill *et al.* (1990b) provided evidence that the normal systolic rise in $[Ca]_i$ cannot propagate through rat ventricular myocytes which were not Ca overloaded. This supports Fabiato's contention that these waves and spontaneous Ca releases are non-physiological. When the cell (and SR) are not loaded near their limit, Ca release from one site can be pumped into the SR in adjacent regions without "overloading" them. This would also be of protective value, since Ca_i waves from Ca overloaded cells may be prevented from entering healthy cells. Whether the [Ca] within the SR or the trans-SR [Ca] gradient are responsible for activation of release during cyclical contractions is not yet clear.

Spontaneous contractions in the heart can lead to asynchrony of cellular contractions and consequent reduction in overall contractile force (Allen *et al.*, 1985b; Cannell *et al.*, 1985; Eisner & Valdeolmillos, 1986; Bers & Bridge, 1988). Cells which have

just experienced a spontaneous contraction at the time that the ventricular action potential arrives will be relatively refractory (in terms of SR Ca release) leading to lower force development. In addition, these refractory cells would provide a series compliance which can be stretched by normally activated cells as they shorten. Thus, the mechanical consequences of such spontaneous contractions can seriously compromise cardiac contractility. The spontaneous SR Ca release and aftercontraction also induce membrane depolarization (attributable to inward current via Na/Ca exchange or Ca-activated non-selective channels, see Chapters 5 & 9). These depolarizations and afterdepolarizations can lead to triggered arrhythmias (see Chapter 9 and Witt & Rosen, 1986). This combination of mechanical and electrophysiological dysfunctions emphasizes the danger of cellular Ca overload and the importance of the delicate balance of cellular Ca.

In conclusion, it is clear that Ca-induced Ca-release is a very important functional means by which SR Ca release is controlled in cardiac muscle, although many quantitative questions remain to be resolved. Ca-induced Ca-release might also be important in skeletal muscle E-C coupling, but that is less clear and the source of "trigger" Ca would be different.

INS(1,4,5)P_3-INDUCED Ca RELEASE

Ins(1,4,5)P_3 Induced Ca Release in Smooth Muscle

Inositol(1,4,5)-trisphosphate (Ins(1,4,5)P_3) can induce Ca release from the endoplasmic reticulum of many cell types (Berridge, 1987; Berridge & Galione, 1988; Berridge & Irvine, 1989). In smooth muscle Ins(1,4,5)P_3 induces SR Ca release in both skinned fibers and SR vesicles (Suematsu *et al.*, 1984; Carsten & Miller, 1985; Smith *et al.*, 1985; Somlyo *et al.*, 1985; Yamamato & van Breemen, 1985; Watras & Benevolensky, 1987). Ins(1,4,5)P_3-activated Ca channels were also described in SR vesicles from smooth muscle (Ehrlich & Watras, 1988). The Ins(1,4,5)P_3 activated Ca release in smooth muscle appears to be large enough and fast enough to explain adrenergic agonist induced E-C coupling (Somlyo *et al.*, 1985, 1988; Walker *et al.*, 1987).

One of the difficulties in assessing this hypothesis in isolated muscle preparations by application of Ins(1,4,5)P_3 is overcoming diffusional limitations such that the Ins(1,4,5)P_3 concentration rises very quickly at the SR surface (and is also not degraded on its way to the SR). Figure 69 illustrates an experiment which overcomes these limitations (Somlyo *et al.*, 1988). In this experiment a permeabilized smooth muscle is activated by laser photolysis of "caged" Ins(1,4,5)P_3 and "caged" phenylephrine (an α_1-adrenergic agonist). The release of ~1 μM Ins(1,4,5)P_3 activated contraction with a 0.4 sec lag. This latency is less than when the muscle was activated by release of ~5 μM phenylephrine (1.8 sec). These results are consistent with the relatively slow activation of contraction in smooth muscle and additional steps being involved with phenylephrine activation (e.g.

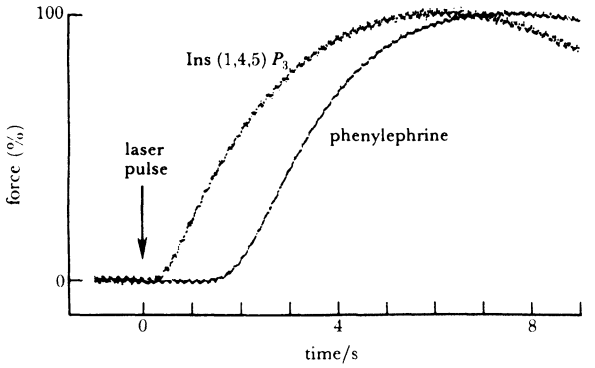


Figure 69. Activation of contraction in smooth muscle strips by photolysis of "caged" Ins(1,4,5)P_3 or "caged" phenylephrine (from Somlyo *et al.*, 1988, with permission). The guinea-pig portal vein strip had been depolarized by 143 mM KCl for the phenylephrine experiment and permeabilized by 50 mg/ml saponin for the Ins(1,4,5)P_3 experiment. About 10% of the 50 μM phenylephrine and 10 μM Ins(1,4,5)P_3 were released by the 50 nsec laser pulse.

receptor activation of phospholipase C which cleaves phosphatidylinositol 4,5-bisphosphate, or PIP_2 into Ins(1,4,5)P_3 and diacylglycerol). Smooth muscle also has a well developed metabolic pathway for production of Ins(1,4,5)P_3 (coupled to adrenergic receptors and G-proteins) and also for degradation of Ins(1,4,5)P_3 (Sasaguri *et al.*, 1985; Saida & Van Breeman, 1987; Walker *et al.*, 1987; Somlyo *et al.*, 1988). Heparin, which blocks the Ins(1,4,5)P_3 receptor (Worley *et al.*, 1987), can also inhibit phenylephrine and Ins(1,4,5)P_3-induced contractions in smooth muscle (Kobayashi *et al.*, 1988, 1989). Thus, it seems likely that Ins(1,4,5)P_3-induced SR Ca release is physiologically important in smooth muscle.

Ca-induced Ca-release has also been reported in smooth muscle (Saida, 1982) and there is a ryanodine sensitive Ca pool in vascular smooth muscle (Ito *et al.*, 1986; Hwang & van Breemen, 1987; Ashida *et al.*, 1988; Kanmura *et al.*, 1988). It is of interest that Iino *et al.* (1988) reported that guinea-pig smooth muscle appears to have two intracellular Ca pools: one which is both Ins(1,4,5)P_3 and caffeine/ryanodine sensitive and a second which is only Ins(1,4,5)P_3 sensitive. Thus, Ins(1,4,5)P_3-induced Ca-release and Ca-induced Ca-release (or even a skeletal muscle type E_m-dependent Ca-release) mechanisms may coexist in SR regions in smooth muscle. At this point the importance of Ins(1,4,5)P_3-induced Ca-release in smooth muscle E-C coupling seems compelling (e.g. Somlyo *et al.*, 1988; van Breemen & Saida, 1989; Somlyo & Himpens, 1989; Somlyo & Somlyo, 1990). However, it is not known what role non-Ins(1,4,5)P_3 linked mechanisms such as Ca-induced Ca-release might play in intact smooth muscle.

$Ins(1,4,5)P_3$ Induced Ca Release in Skeletal Muscle

The possibility that $Ins(1,4,5)P_3$ is involved in skeletal muscle E-C coupling was raised by observations that application of $Ins(1,4,5)P_3$ could induce SR Ca release in skinned skeletal muscle fibers and SR vesicles (Vergara *et al.*, 1985; Volpe *et al.*, 1985; Donaldson *et al.*, 1987). In support of this hypothesis are observations that 1) $Ins(1,4,5)P_3$ production is increased during a tetanus (Vergara *et al.*, 1985), 2) that the enzymes required for $Ins(1,4,5)P_3$ synthesis and degradation are present (Vergara *et al.*, 1985, 1987; Hidalgo *et al.*, 1986; Hidalgo & Jaimovich, 1989; Varsanyi *et al.*, 1989; Lagos & Vergara, 1990) and 3) that $Ins(1,4,5)P_3$ can activate ryanodine-sensitive SR Ca release channels in lipid bilayers (Suarez-Isla *et al.*, 1988; Liu *et al.*, 1989). These latter observations might be indicative of similarities between the ryanodine receptor and $Ins(1,4,5)P_3$ receptor (see page 117). It has also been suggested that the effect of $Ins(1,4,5)P_3$ on skeletal muscle is [Ca] and E_m-dependent (Volpe *et al.*, 1986; Donaldson *et al.*, 1988).

Other results have challenged the possibility that $Ins(1,4,5)P_3$ is important in skeletal E-C coupling. Several reports indicated that $Ins(1,4,5)P_3$ does not induce SR Ca release in skeletal muscle (Scherer & Ferguson, 1985; Lea *et al.*, 1986; Mikos & Snow, 1987; Palade, 1987c). Using photolysis of "caged" $Ins(1,4,5)P_3$ (as in Fig. 69), Walker *et al.* (1987) showed that $Ins(1,4,5)P_3$-induced contractions were too small and much too slow to be physiologically relevant. In addition, SR Ca release turns off abruptly with repolarization (Simon *et al.*, 1989), whereas degradation of the $Ins(1,4,5)P_3$ signal would be too slow by several orders of magnitude (Somlyo *et al.*, 1988). The enzymes and substrates involved in rapid turnover of $Ins(1,4,5)P_3$ are present in amounts which are at the very limit or below that which would make this mechanism feasible (Walker *et al.*, 1987; Somlyo *et al.*, 1988; Hidalgo & Jaimovich, 1989; Varsanyi *et al.*, 1989). Blinks *et al.* (1987) found that $Ins(1,4,5)P_3$ injection only induced contractions in skeletal muscle when intact cells were detubulated. They suggested that $Ins(1,4,5)P_3$ could induce depolarization of sealed off T-tubules, which would not occur in intact fibers. Thus, the Ca- and E_m-dependence above could be rephrased such that $Ins(1,4,5)P_3$ may alter the Ca- and E_m-dependence of SR Ca release. Finally, blocking the $Ins(1,4,5)P_3$ receptor with intracellular heparin produced no effect on skeletal muscle E-C coupling (Pape *et al.*, 1988) in contrast to the profound depression seen in smooth muscle (Kobayashi *et al.*, 1988). In conclusion, the role of $Ins(1,4,5)P_3$ in skeletal muscle E-C coupling remains somewhat controversial. However, it seems unlikely that $Ins(1,4,5)P_3$ is centrally involved in E-C coupling in skeletal muscle.

$Ins(1,4,5)P_3$ Induced Ca Release in Cardiac Muscle

Hirata *et al.* (1984) first showed that $Ins(1,4,5)P_3$ induced a slow release of Ca from cardiac SR vesicles. However, Movesian *et al.* (1985) found no effect of $Ins(1,4,5)P_3$ in isolated cardiac SR or in myocytes permeabilized by saponin. Nosek *et al.* (1986) found that $Ins(1,4,5)P_3$ potentiated spontaneous and caffeine-induced Ca-release in cardiac

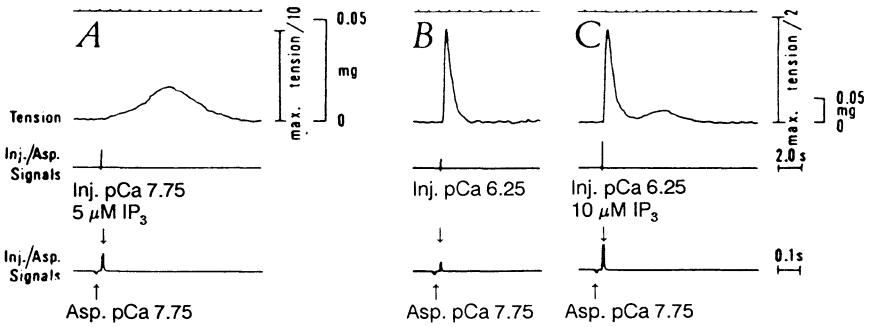


Figure 70. Ins(1,4,5)P_3 and Ca-induced Ca-release from cardiac SR in a mechanically skinned rat ventricular myocyte (from Fabiato, 1986b, with permission). Rapid application of 5 μM Ins(1,4,5)P_3 induced a relatively small and slow contraction (**A**), compared to the contraction induced by a rapid increase in [Ca] from pCa 7.75 to 6.25 (i.e. via Ca-induced Ca-release in **B**). Note difference in tension scale between **A** and **B**. Simultaneous application of these two stimuli produced a contraction in which the major part was not much different than with Ca-induced Ca-release alone (**C**).

muscle. Recently Vites & Pappano (1990) showed that Ins(1,4,5)P_3 induced contractions in small skinned multicellular preparations from chick heart which were caffeine- and ryanodine-sensitive. Fabiato (1986a,b, 1990) showed that rapid application of Ins(1,4,5)P_3 to very small skinned rat myocytes induced SR Ca release that was much smaller and slower than that induced by Ca-induced Ca-release (see Fig. 70). Kentish *et al.* (1990) activated contraction in skinned rat ventricular muscle by photolysis of "caged" Ca and "caged" Ins(1,4,5)P_3. They also found that very high concentrations of Ins(1,4,5)P_3 could induce SR Ca release but that the rate and extent was much lower than for Ca-induced Ca-release.

These studies led to the conclusion that Ins(1,4,5)P_3 is not the primary mechanism responsible for Ca release from cardiac SR. On the other hand these effects of Ins(1,4,5)P_3 may be physiologically important in the modulation of Ca-induced Ca-release from the SR. For example Ins(1,4,5)P_3 may increase the Ca sensitivity of Ca-induced Ca-release (Nosek *et al.*, 1986; Suarez-Isla *et al.*, 1988). Activation of cardiac α_1-adrenergic and muscarinic receptors has also been reported to increase Ins(1,4,5)P_3 production as well as contractile force (Gilmour & Zipes, 1985; Brown & Jones, 1986; Poggioli *et al.*, 1986; Jones *et al.*, 1988; Otani *et al.*, 1998; Scholz *et al.*, 1988). Thus, increased intracellular Ins(1,4,5)P_3 may be an important physiological means of modulating cardiac contractile force in response to hormones and pharmacological agents. While it seems likely that Ca-induced Ca-release is the primary mechanism of E-C coupling in cardiac muscle, it must be acknowledged that a significant role for an Ins(1,4,5)P_3-dependent mechanism cannot be unequivocally excluded.

OTHER POSSIBLE E-C COUPLING MECHANISMS

Based on initial observations that alkalosis decreased Ca uptake in SR vesicles Nakamura & Schwartz (1970) proposed that alkalinization could be a physiologically relevant mechanism for SR Ca release. The SR Ca release channel can be activated by increasing pH (Ma *et al.*, 1988), but Fabiato (1985e) also showed that increasing pH increased SR Ca accumulation. Fabiato (1985e) also found that dissipation of a pH gradient across the SR in skinned fibers failed to induce SR Ca release, contrary to the proposal of Shoshan *et al.* (1981). Thus, it seems unlikely that pH change is the primary physiological activator for SR Ca release. On the other hand the pH sensitivity of SR Ca uptake and release (as well as other processes) can be expected to modify SR Ca content, Ca release and contraction (Orchard & Kentish, 1990).

Early observations of Na effects on Ca uptake in SR vesicles raised the possibility that local elevation of $[Na]_i$ during the cardiac action potential might directly induce Ca release from the SR (Palmer & Posey, 1967; Vassort, 1973; Caillé *et al.*, 1979). Studies in isolated cardiac SR vesicles and skinned fibers, however indicate that replacement of K with Na produces only modest decreases in SR Ca (Jones *et al.*, 1977; Fabiato, 1986c). It is possible that these phenomena are related to the displacement of bound Ca at the inner sarcolemmal surface by mM Na (Frankis & Lindenmayer, 1984; Bers *et al.*, 1986, see page 44). Certainly Na entry via Na current during the action potential would elevate $[Na]_i$ near the sarcolemma (Akera *et al.*, 1976). This Na could displace membrane bound Ca elevating $[Ca]_i$ in this region. While this might modify the Ca-induced Ca-release it is clearly insufficient to induce Ca release by itself (since contractions are abolished in the absence of Ca_o despite maintained Na entry, Figs. 55A & 65).

Heavy metals (Ag^+, Hg^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}) and sulfhydryl oxidation can induce rapid Ca release in isolated SR vesicles (Abramson *et al.*, 1983, 1987; Salama & Abramson, 1984; Trimm *et al.*, 1986; Prabhu & Salama, 1990). Although there is no evidence to indicate that this latter mechanism is responsible for normal SR Ca release, in pathological conditions, where redox potentials may be changed, these effects might bias the normal mechanism of E-C coupling.

SUMMARY

From the forgoing discussions it seems that the central mechanism of E-C coupling is different in skeletal, cardiac and smooth muscle. In a greatly simplified manner we can use the 3 muscle types as models of the 3 major mechanisms of SR Ca release (i.e. Ca-induced in heart, depolarization-induced in skeletal and $Ins(1,4,5)P_3$-induced in smooth muscle, see Figs. 71 & 72). It should be emphasized that this is an oversimplification since there is some evidence in support of every permutation of mechanism and muscle type.

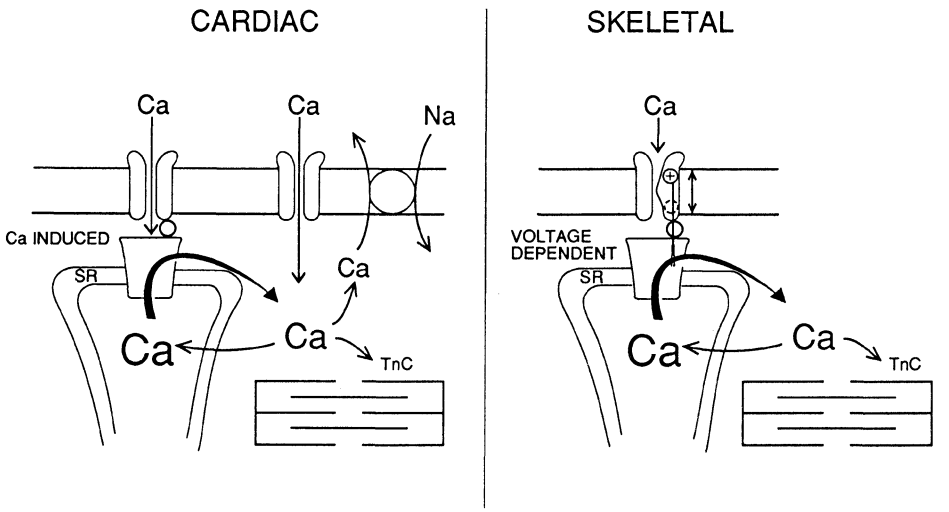


Figure 71. Mechanisms of E-C coupling in cardiac and skeletal muscle for which evidence is strongest. In cardiac muscle Ca entry via I_{Ca} can activate contraction both directly and also by inducing Ca release from the SR. The existence and importance of a physical link between the sarcolemmal and SR Ca channels in cardiac muscle is unclear. In the steady state the amount of Ca which enters the cell via I_{Ca} is probably extruded via Na/Ca exchange. In skeletal muscle the physical link between the sarcolemmal Ca channel (or dihydropyridine receptor) appears to be critical in electromechanical coupling (where sarcolemmal depolarization induces opening of the SR Ca release channel). Transsarcolemmal Ca fluxes do not appear to be important in skeletal muscle E-C coupling and Ca cycles mainly between the SR and the cytoplasm.

For example, depolarization-induced (or charge-coupled) Ca-release appears to be the crucial process in skeletal muscle. However, there are twice as many SR/ryanodine receptor tetrads as T-tubule/dihydropyridine receptor tetrads in skeletal muscle and Ca-induced Ca-release may be involved in activating some release channels (i.e. those lacking T-tubule tetrads). Ins(1,4,5)P_3 can also induce Ca release in skeletal muscle under some circumstances, although the physiological role for this pathway is not yet clear. In cardiac muscle Ca-induced Ca-release appears to be the crucial E-C coupling mechanism. Ins(1,4,5)P_3 may also modulate cardiac Ca release and there is some evidence for a functional direct link between the sarcolemma and the SR. Whether this junction is physiologically important (other than for bringing the SR Ca release channel near the sarcolemmal Ca channel) is not known. In smooth muscle the strongest evidence supports Ins(1,4,5)P_3-induced Ca release as the key E-C coupling process. On the other hand, smooth muscle contains ryanodine receptors and there is also evidence for Ca-induced Ca-release.

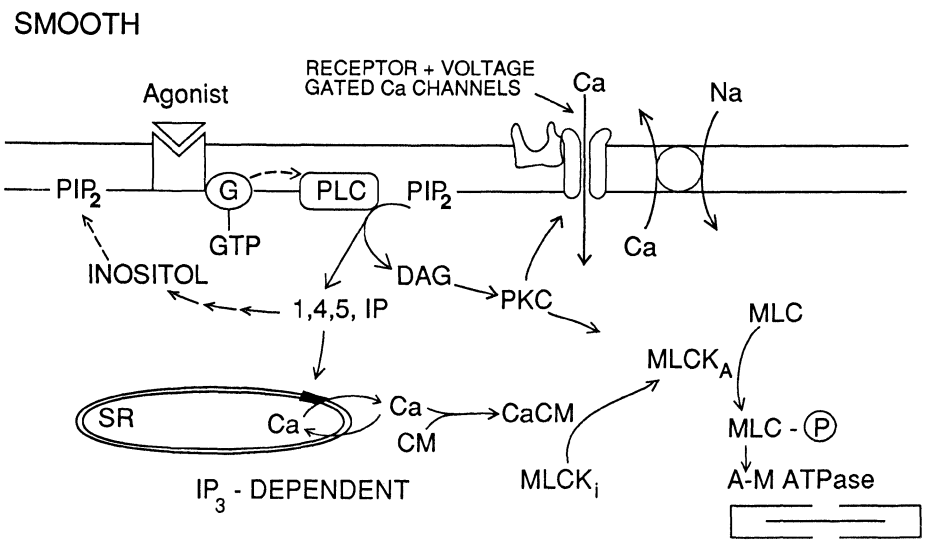


Figure 72. Ins(1,4,5)P_3 appears to have a more important role in E-C coupling in smooth muscle. A receptor agonist (e.g. α_1-adrenergic) activates a receptor and a GTP-binding protein coupled to that receptor. This activates phospholipase C (PLC), resulting in the production of Ins(1,4,5)P_3 and diacylglycerol (DAG). Ins(1,4,5)P_3 can then stimulate Ca release from the SR and DAG can activate protein kinase C (PKC) which can modify the contractile proteins and ion channels. The Ca-sensitive molecular "switch" allowing actin and myosin to interact is also different in smooth muscle. That is, Ca plus calmodulin (CM) activates a myosin light chain kinase (MLCK) to phosphorylate a myosin light chain (MLC) which allows actin and myosin to interact. This contrasts with the more direct role of Ca via troponin C (TnC) in striated muscle.

As illustrated in Figure 72, the process of E-C coupling and the Ca-dependent activation of the myofilaments in smooth muscle is rather different from that previously discussed for striated muscle (Somlyo *et al.*, 1988; Kamm & Stull, 1989; Somlyo & Himpens, 1989; van Breemen & Saida, 1989; Somlyo & Somlyo, 1990). In this case of pharmacomechanical coupling the process is started by a receptor agonist (e.g. α_1-adrenergic agonist) rather than an action potential as in striated muscle. The agonist occupied receptor activates a GTP binding protein which stimulates phospholipase C to cleave phosphatidylinositol-(4,5)-bisphosphate (PIP_2) into Ins(1,4,5)P_3 and 1,2-diacylglycerol (DAG). Ins(1,4,5)P_3 can induce SR Ca release and DAG can also effect many other cellular processes by activating protein kinase C. The situation in smooth muscle is also complicated by the presence of both receptor- and voltage- gated channels and substantial Na/Ca exchange activity. The presence of sarcolemmal Ca channels in cells with large surface:volume ratio raises the possibility of direct activation via Ca influx. Since ryanodine receptors are present and Ca-induced Ca-release can occur (not shown) in addition to Ins(1,4,5)P_3-induced Ca release, Ca regulation in smooth muscle can be quite

complex. In addition, the myofilaments in smooth muscle are activated by Ca in a manner distinct from that in striated muscle. Ca first binds to calmodulin to activate a myosin light chain kinase. Phosphorylation of myosin light chains then stimulates the actin-activated myosin ATPase activity. Several chemical reactions must occur along the E-C coupling pathway which can account for slow onset of force development in smooth muscle compared to striated muscle. Without going further than this simplified scheme for smooth muscle, it can already be appreciated that the regulation of Ca and contractile force is quite different than in striated muscle.

CHAPTER 8

CONTROL OF CARDIAC CONTRACTION BY SR Ca RELEASE AND SARCOLEMMAL Ca FLUXES

In the preceding chapters the importance of both Ca current and SR Ca release in E-C coupling have been discussed and it was suggested that Ca from both sources could contribute to the activation of contraction. A major goal of this chapter is to clarify how these two sources of Ca may differ in particular cardiac muscle preparations and under different experimental situations. I will also discuss how the transsarcolemmal Ca fluxes and the SR Ca fluxes interact in a simple framework of overall Ca regulation in cardiac muscle.

As concluded in Chapter 7, Ca entry via Ca channels is essential in the "triggering" of SR Ca release in E-C coupling. However, the Ca which enters via Ca channels also contributes to the elevation of $[Ca]_i$ during contraction. As mentioned earlier, it is indeed sometimes difficult to distinguish unequivocally between the direct effects of Ca entry and the effect of Ca entry on SR Ca release. For example, an intervention which increases I_{Ca} might increase contraction and the Ca_i transient, but one cannot readily determine how much of the effect is due to the increase in Ca entry itself, an increase in the fraction of SR Ca release by the I_{Ca} or even an increase in SR Ca load. One cannot simply block Ca entry to study the contribution of SR Ca to contraction, because Ca-induced Ca-release will also be blocked. On the other hand, one can inhibit the SR Ca release to study the activation of contraction by I_{Ca} in the absence of an SR contribution. This can be done by exposure of cardiac muscle preparations to caffeine or ryanodine, which can be expected to inhibit SR Ca release (see below). This approach is particularly valuable, since it seems clear that the SR is capable of releasing enough Ca to activate the myofilaments (see page 109), but it is less clear from quantitative extrapolations that I_{Ca} can supply enough Ca for activation of contraction (see page 62).

SPECIES, REGIONAL AND DEVELOPMENTAL DIFFERENCES

Figure 73 shows the effects of pre-equilibration with 10 mM caffeine or 100 nM ryanodine on steady state twitch contractions in several cardiac muscle preparations. It can

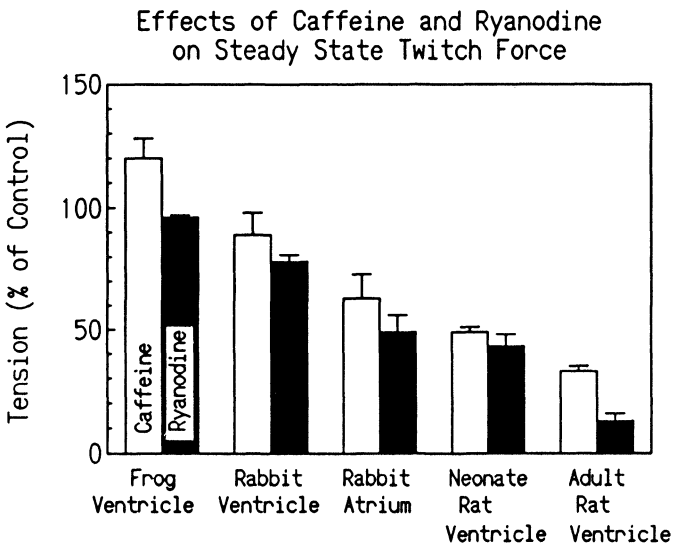


Figure 73. The effect of caffeine (10 mM) or ryanodine (100 nM) on steady state twitch contractions (0.5 Hz at 30°C or 23°C for frog) in various cardiac muscle preparations (Data is from Bers, 1989 and unpublished observations).

be seen that these agents depress twitch tension development to varying extents in different cardiac muscle preparations. This variation is apparent among different species (frog *vs* rabbit *vs* rat), at different stages of development, (neonatal *vs* adult rat) and regionally in the heart (rabbit ventricle *vs* atrium). While caffeine is well known to inhibit net SR Ca uptake by making the SR extremely leaky to Ca (and thereby preventing Ca uptake, Weber & Herz, 1968), it has many side effects. For example, it increases myofilament Ca sensitivity (e.g. Wendt & Stephenson, 1983 and see Chapter 2) and increases Ca influx (Blinks *et al.*, 1972; Bers, 1983; Tseng, 1988). Additionally, caffeine inhibits phosphodiesterases at the mM concentrations required for the effects on SR (Butcher & Sutherland, 1962) and can thus elevate cyclic AMP. Ryanodine is much more specific in its interaction with the SR (K_d = nM), but its mechanism of action seems to be somewhat more complex (Sutko *et al.*, 1985; Bers *et al.* 1987 and see Chapter 6 and below). The concentration dependence of these agents does not appear to vary in different tissues, despite the difference in maximal effect (Sutko & Willerson, 1980; Shattock & Bers, 1987). Therefore, the sensitivity of tension to depression by caffeine and ryanodine does seem, indeed, to be indicative of the relative requirement for SR Ca release in the activation of the myofilaments.

However, the fact that twitch contractions in rabbit ventricular muscle are only decreased by 20-30% by ryanodine does not mean that under normal conditions the SR

contributes only 20-30% of the Ca required for myofilament activation. It simply means that in the absence of a functional SR, Ca influx can supply sufficient Ca to activate a nearly normal amplitude contraction. Under normal conditions, the Ca released by the SR raises $[Ca]_i$ (decreasing the gradient for Ca influx) and contributes to inactivation of sarcolemmal Ca current and also shortens the action potential by activating outward K current (all of which would limit Ca influx). In the presence of ryanodine some of the Ca entering the cell may also be transiently accumulated by the SR (see pages 105-107), thereby decreasing the apparent effectiveness of a given Ca entry to activate contraction. Indeed, in the presence of ryanodine the SR may be able to accumulate a similar amount of Ca as under control conditions, albeit only transiently (Bers *et al.*, 1987; MacLeod & Bers, 1987). This transient uptake of Ca by ryanodine-treated SR may be especially important during post-rest contractions. At these post-rest contractions the SR is Ca-depleted, such that the net accumulation of Ca by the SR may more closely keep pace with Ca influx (which is also reduced during post-rest depolarization, see Fig. 29, page 61). This also explains the profound depression of post-rest contractions by ryanodine, which has been a hallmark of this agent since some of the earliest studies (e.g. Hajdu & Leonard, 1961). Caffeine may prevent the SR from accumulating Ca altogether (e.g. since rapid cooling contractures are abolished even at very short rest intervals, Bers *et al.*, 1987). Caffeine also does not depress post-rest contractions to nearly the extent that ryanodine does (Bers, 1985). Thus, in the presence of caffeine (*vs* ryanodine) Ca entry may have more direct access to the myofilaments (since the SR cannot accumulate appreciable Ca). Ryanodine usually prolongs action potential duration and at higher concentrations (or longer exposure) can result in recovery of twitch contraction despite a Ca-depleted SR (Sutko & Willerson, 1980; Bers, 1985; Lewartowski *et al.*, 1990). It is possible that when a larger fraction of SR release channels are occupied by ryanodine, the net effect is more like that observed with caffeine (i.e. the SR may no longer function as a transient Ca buffer when a larger fraction of channels are "locked" in an open state, see page 110).

There are also ultrastructural correlates with these pharmacological dissections. For example, the most highly-developed SR network occurs in adult rat ventricle, somewhat less in rabbit atrium and ventricle, and is by far the most sparse and poorly developed in frog ventricle (see Chapter 1). Furthermore, the small diameter of frog ventricular myocytes (and hence high surface/volume ratio) makes Ca entry a more plausible mechanism for activation in frog. Therefore, the virtual insensitivity of frog ventricle to ryanodine is not especially surprising and in frog ventricular muscle it is possible that the myofilaments can be activated entirely by Ca entering from outside the cell at each contraction (Morad & Cleeman, 1987; Chapman, 1983). As a side point, it may be worth noting that most of the side effects of caffeine mentioned above would tend to increase contractile force. Thus, in each case in Fig. 73, caffeine depresses tension by a smaller amount than does ryanodine. This is particularly apparent in frog ventricle, where ryanodine has almost no effect and caffeine increases twitch contractions.

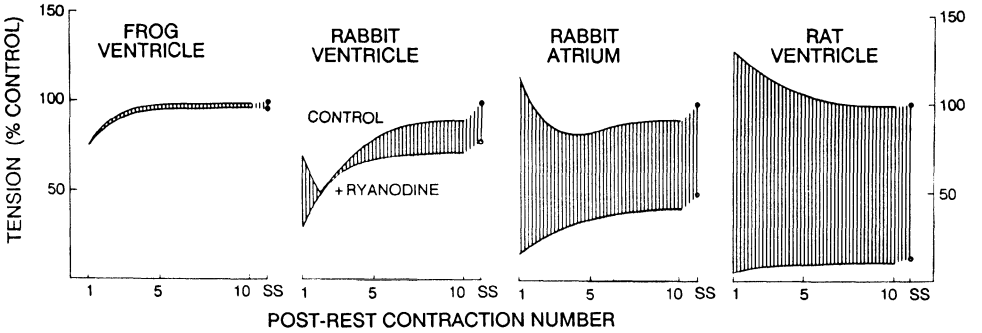


Figure 74. Recovery of twitch tension after a 30 sec rest in frog, rabbit and rat ventricle and rabbit atrium in the absence (top) and presence (bottom) of 100 nM ryanodine. Steady state twitch tension is indicated by the points at the end of each curve (SS). The shaded region is indicative of the relative SR contribution at a given beat (redrawn from Bers, 1985).

Fabiato & Fabiato (1978a; Fabiato, 1982) also reported substantial variation in the Ca-induced Ca-release characteristics among different cardiac muscle preparations which agrees with the above interpretation. For example, Ca-induced Ca-release could not be observed in frog or prenatal rat ventricle, but was most prominent in adult rat ventricular myocytes. While the developmental transition of relative SR dependence is perhaps most dramatic in rat ventricle, it is also readily apparent in other mammalian cardiac preparations (Penefsky, 1974; Maylie, 1982; Seguchi *et al.*, 1986). This also agrees with ultrastructural results which indicate that the T-tubule/SR system is gradually developing from the prenatal period through the first few weeks of life, albeit at different rates in different species (Scheibler & Wolff, 1962; Legato, 1979; Olivetti *et al.*, 1980; Hoerter *et al.*, 1981; Page & Beucker, 1981; Penefsky, 1983; Goldstein & Traeger, 1985). This also agrees with biochemical characterizations and Ca transport studies, where the total Ca uptake by SR vesicles increases over this developmental period in rat, rabbit and sheep ventricle (Nayler & Fassold, 1977; Nakanishi & Jarmakani, 1984; Mahony & Jones, 1986; Pegg & Michalak, 1987).

An interesting twist on this variation is that hibernating chipmunk ventricle appears to function more like rat ventricle during hibernation, but more like rabbit ventricle when not hibernating (Kondo & Shibata, 1984; Kondo, 1986, 1988). Thus, during hibernation, the chipmunk may rely entirely on intracellular cycling of Ca, which might conserve energy.

Consideration of results from several sources allows a rough sequencing of cardiac muscle preparations from most to least SR reliant (V, ventricle; A, atrium): calf Purkinje fiber > adult rat V > dog V ~ ferret V > neonate rat V ~ rabbit A > cat V > rabbit V ~ guinea-pig V > neonate rabbit V > fetal V (human, cat & rabbit) > trout V > frog V ~ toad V (Penefsky, 1974; Sutko & Willerson, 1980; Sutko & Kenyon, 1983; Bers, 1985;

Malécot *et al.*, 1986; Seguchi *et al.*, 1986; El-Sayed & Gesser, 1989). In a given species, atrial muscle seems always to be more dependent on SR Ca release (and has more active Ca-induced Ca-release, Fabiato, 1982). This may be partly due to the shorter action potential duration in atrial muscle such that less Ca can enter via Ca channels (or Na/Ca exchange). Of course this sequence is only approximate and should only serve as a general index. This is particularly true since the relative importance of the SR Ca release also varies under different experimental conditions in individual preparations (i.e., with changes in frequency, drugs, or other interventions). Figure 74 shows the effect of ryanodine on post-rest contractions in several cardiac preparations. Ryanodine makes all the recoveries back to steady state monotonically increasing and the shaded area emphasizes that the contribution of SR Ca to contractile activation may vary from beat to beat (as well as at steady state in different preparations). It is of obvious interest to know where adult human ventricular muscle fits in this general sequence. There is limited data available, but Cooper & Fry (1990) found that in some ways human ventricle resembled ferret ventricle but had ryanodine-sensitivity that was somewhat less than guinea-pig ventricle under their conditions. Fabiato & Fabiato (1978a) found the threshold for Ca-induced Ca-release in human ventricle to fall between that in rabbit and cat ventricle.

BIPHASIC CONTRACTIONS

If Ca influx and SR Ca release can both lead to activation of the myofilaments, conditions might be found where these two components are separable during a single contraction. Indeed, biphasic contractions have been reported under a variety of experimental conditions, especially when cAMP is likely to be increased or when the SR is relatively Ca-depleted (Braveny & Sumbera, 1970; Coraboeuf, 1974; Allen *et al.*, 1976; Beresewicz & Reuter, 1977; Seibel *et al.*, 1978; Bogdanov *et al.*, 1979; Endoh *et al.*, 1982; King & Bose, 1983; Reiter *et al.*, 1984; Honoré *et al.*, 1986, 1987; Malécot *et al.*, 1986). The first component is generally attributed to SR Ca release and the second to Ca influx.

Figure 75 shows an example of biphasic contractions induced by addition of the cardiotonic drug milrinone. Milrinone increases Ca current and appears to exert a mild caffeine-like action on the SR (Malécot *et al.*, 1986; Rapundalo *et al.*, 1986) Initially contractions are increased in amplitude and shortened in duration, which may be due to the enhancement of I_{Ca}. Over the next few minutes the fast peak decreases and a second component becomes increasingly apparent. We attributed this to the depressant action of milrinone on SR Ca uptake and release, such that the larger Ca entry via I_{Ca} could activate contraction directly (but more slowly). Figure 75B shows steady state biphasic contractions in the presence of milrinone (M). The early component could be suppressed by either caffeine or ryanodine. The second component was relatively unaffected by ryanodine, but was enhanced by caffeine. Since caffeine increases myofilament Ca sensitivity and I_{Ca},

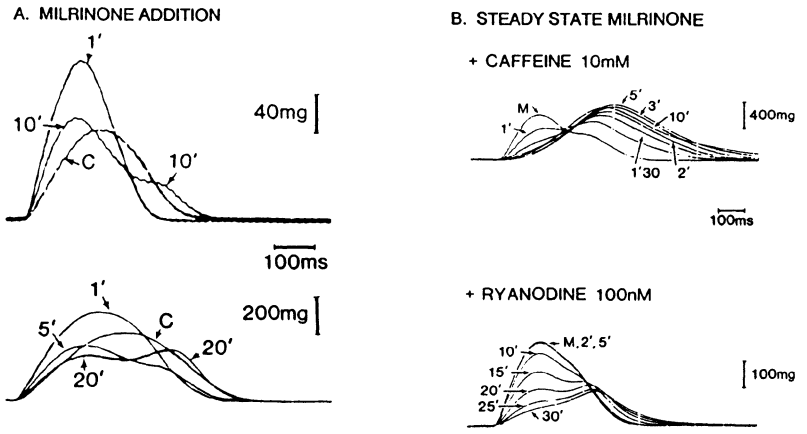


Figure 75. Biphasic contractions in ferret ventricular muscle induced by 240 μM milrinone (at 28°C and 0.5 Hz). **A.** Addition of milrinone to two different muscles, where C is the control and the numbers refer to the time after switching to milrinone containing solution (in min). **B.** Steady state biphasic contractions in milrinone (M) and changes in contractions after addition of 10 mM caffeine (top) or 100 nM ryanodine (bottom) for the times indicated. Both of these agents depress the first phase of contraction and caffeine also increases the second component. (from Malécot *et al.*, 1986, with permission of the American Heart Association)

these results are entirely consistent with the first component of the biphasic contractions being due to SR Ca release and the second component being due to Ca influx.

This slow second component of contraction may also be related to the very slow onset of contraction which is observed upon stimulation after a very long period of rest (rested state contraction) when the SR is thought to be Ca-depleted (Allen *et al.*, 1976; Seibel *et al.*, 1978; Reiter, 1988). These investigators have attributed this long latent period to the Ca entering via I_{Ca} being completely sequestered by the SR and then released after some mandatory time after the Ca has moved from "uptake sites" to "release sites". This delayed gating (or non-inactivating state) of the Ca release channel would constitute another challenge to the mechanisms of E-C coupling discussed in Chapter 7. Certainly, if the SR is Ca-depleted after a long rest, some fraction of the Ca entering will be accumulated by the SR before reaching the myofilaments. On the other hand, the cellular geometry and diffusional limitations imply that direct myofilament activation by Ca influx will be slow compared to activation by SR Ca release. Thus, it is not entirely clear for these slow contractions whether Ca necessarily passes through the SR or whether they are slow mainly due to the buffering action of the SR and the longer diffusion time required to activate the myofilaments via Ca influx.

A second, slower phase of contraction or "tonic phase" can be readily seen when depolarization is prolonged by voltage clamp pulses in both frog and mammalian heart (see

Figs. 43 & 82, Morad & Trautwein, 1968; Braveny & Sumbera, 1970; Goto *et al.*, 1971; Léoty & Raymond, 1972; Coraboeuf, 1974; Horackova & Vassort, 1976, 1979; Chapman & Tunstall, 1981; Chapman, 1983; Eisner *et al.*, 1983; Isenberg & Wendt-Gallitelli, 1989). These tonic contractions are very sensitive to the [Na] and [Ca] gradients and are more prominent at more positive potentials. These reports indicate that Ca entry via Na/Ca exchange is primarily responsible for the tonic component of contractions. Tonic contractions during long depolarizations might also be due to a small non-inactivating component of Ca current. Evidence for this comes from experiments where tonic $[Ca]_i$ elevation was observed during long depolarizations under conditions where Na/Ca exchange was inhibited by removal of both intracellular and extracellular Na (Bers *et al.*, 1990, see Fig. 43). This Ca "window" current will however decline at more positive potentials (approaching the Ca channel reversal potential). Thus, tonic phases of cardiac contraction may be attributed mainly to Ca entry via Na/Ca exchange, but Ca entry via Ca channels may also contribute at less positive potentials.

REST DECAY AND REST POTENTIATION

In this section I will discuss some of the dynamic changes which occur in SR Ca content in different cardiac muscle preparations and how these changes are related to changes in contraction and transsarcolemmal Ca fluxes. For much of this discussion I will focus on comparison of rabbit and rat ventricular muscle, which are representative species where cellular Ca fluxes are markedly different. Guinea-pig ventricle appears to behave in a manner very similar to rabbit ventricle. Dog and ferret ventricle share some features with rat ventricle, but differ in others.

Early Electrical and Mechanical Restitution

Immediately after a contraction has been activated some time is required before another contraction of the same amplitude can be activated. This early phase of recovery of contraction is often termed mechanical restitution and is analogous to a relative refractory period. Several systems probably contribute to this finite period of restitution. Both sarcolemmal Ca channels and SR Ca release channels must recover from inactivation and some finite time is required for Ca pumped into the longitudinal SR to be "available" for release from the junctional SR. Full recovery of contractile force in mammalian ventricle generally requires ~1 - 2 sec depending on temperature, species and other conditions (Gibbons & Fozzard, 1975; Edman & Jóhannsson, 1976; Wolfhart, 1979; Lipsius *et al.*, 1982; Yue *et al.*, 1985; Wier & Yue, 1986).

Ca current appears to recover considerably faster than contraction. Typically I_{Ca} recovers from inactivation in ~100-500 msec, but again this is dependent on temperature, species and particularly membrane potential (being faster at more negative potentials, Gibbons & Fozzard, 1975; Isenberg & Klöckner, 1982; Kass & Sanguinetti, 1984;

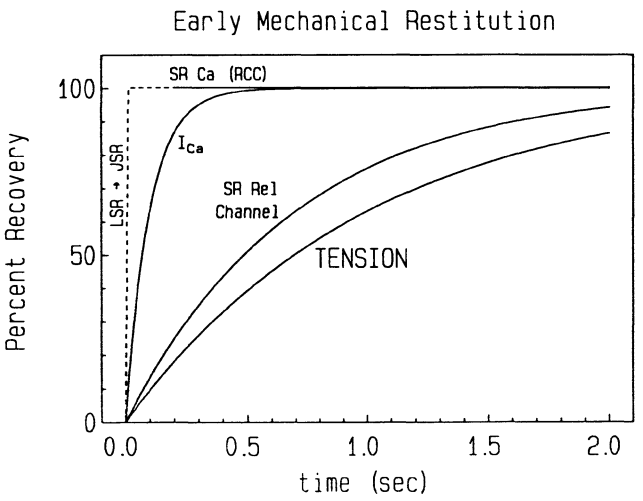


Figure 76. Recovery times for several factors contributing to early mechanical restitution in ventricular muscle. The time constants are only rough estimates for movement of Ca from longitudinal to junctional SR (LSR → JSR, τ=2 msec), for recovery from inactivation of I_{Ca} (τ=100 msec, e.g. Tseng,1988) and of the SR Ca release channel (τ=0.7 sec, Fabiato, 1985b), and for recovery of tension (τ=1 sec). The availability of SR Ca for release appears to be complete in ~200 msec, based on RCCs (Bers *et al.*, 1987). This is a greatly simplified figure merely to show the relative timecourses. The individual τ values are condition and tissue dependent. Additionally, both I_{Ca} and tension recovery often exhibit overshoot (Tseng, 1988; Wolfhart & Noble, 1982).

Josephson *et al.*, 1984; Lee *et al.*, 1985; Fischmeister & Hartzell, 1986; Hadley & Hume, 1987; Fedida *et al.*, 1987; Tseng, 1988; Argibay *et al.*, 1988). While slower components of Ca channel recovery (of the order 1-2 sec) have also been reported (Kass & Sanguinetti, 1984; Hadley & Hume, 1987), the consensus is that under comparable conditions (e.g. physiological E_m) I_{Ca} recovers considerably faster than force. There is also an overshoot in I_{Ca} recovery that is due to a facilitatory effect of Ca entry at the preceding pulse (see page 61). Thus, only a part of the early mechanical restitution can be readily ascribed to the recovery of Ca channels. The early restitution of the action potential (e.g. see Boyett & Jewell, 1978, 1980) may also influence the Ca current due to its E_m-dependence. However, the primary effect of changes in action potential duration in this particular situation is probably that it alters the Ca load in the SR for the subsequent contraction (see below).

The rest of the early recovery phase is therefore likely to be attributable to either the recovery of the SR Ca release channel from inactivation or the movement of SR Ca from an "uptake compartment" to a "release compartment". The idea of an "uptake compartment" and a "release compartment" in mammalian cardiac muscle is a hypothetical construct incorporated into many models of the force-frequency relationship in part to

explain this delay between relaxation due to Ca sequestration by the SR and availability of Ca for release (e.g. Morad & Goldman, 1973; Edman & Jóhannsson, 1976; Wolfhart, 1979; Yue *et al.*, 1985). One of the possible sources of this delay was considered to be diffusion of Ca from the longitudinal SR (where Ca uptake occurs) to the terminal cisternae (specialized for Ca release). This would lend anatomical distinction to these two compartments. However, there are no apparent membrane boundaries within the SR and simple diffusion of Ca from longitudinal SR to junctional SR ($< 1\ \mu m$) should only take ~1 msec (and at most a very few msec). Thus while such anatomical movement of Ca may occur, it should occur more rapidly than I_{Ca} recovery and is unlikely to explain the 1 - 2 sec required for the early phase of mechanical restitution. Fabiato (1985b) has presented evidence for a slow recovery from inactivation of the SR Ca release channel (with a time constant of ~0.7 sec). Although this Ca release channel inactivation process has been difficult to characterize in intact tissue or single channels there is good reason to believe that this process exists (see pages 136-137). It also provides a logical explanation for the slower part of the early mechanical restitution. Bers *et al.* (1987) showed that SR Ca released by rapid cooling has recovered to its steady state value within ~200 msec, despite the fact that mechanical restitution of action potential-mediated contractions under these conditions requires more than 1 sec. The simplest interpretation of this result is that the SR Ca release channel is refractory to activation via Ca-induced Ca-release, but the Ca can still be released by rapid cooling (or caffeine, Fabiato, 1985b).

Thus, the limiting phase of early mechanical restitution is probably the recovery from inactivation of SR Ca release channels with recovery of sarcolemmal Ca channels being faster and diffusion of Ca within the SR being faster still (see Fig. 76). The hypothetical construct of Ca moving from the "uptake" to "release compartment" as described in some models could just as well be thought of as the SR Ca release channel recovering from inactivation. Indeed, as Yue *et al.* (1985) point out, these are usually functionally equivalent in the studies using "uptake" and "release compartments" and cannot be distinguished. It is also possible that contraction can increase due to a net gain in cellular and SR Ca during the pause. This possibility will be discussed more explicitly below with reference to longer rest periods.

The above explanation for early mechanical restitution also readily explains the well known functional response in cardiac muscle known as post extrasystolic potentiation (PESP, Hoffman *et al.*, 1956). This is where activation occurs prior to mechanical restitution such that only a weak contraction occurs (extrasystole), but the subsequent contraction is potentiated (PESP). If I_{Ca} recovers (even partially) before the extrasystole, some Ca will enter the cell at the extrasystole. If the SR Ca release channel is refractory, normal Ca release will not occur, resulting in a weak contraction. The Ca which entered during the extrasystole will however, contribute to a larger Ca accumulation by the SR. Then when the SR release channel has recovered by the next contraction, a greater SR Ca release and contraction results. This mechanism might also apply to mechanical alternans,

where contraction amplitude alternates from one beat to the next at a constant frequency (Wolfhart, 1982). If the normal refractory period for the SR Ca release channel is prolonged (e.g. due to elevated $[Ca]_i$) then Ca release would be weak and the Ca_i transient small (but Ca influx would still occur). By the next pulse the SR may no longer be refractory so a larger than normal Ca release may occur. Due to the larger Ca_i transient Ca current will inactivate faster (limiting Ca influx) and Ca extrusion from the cell (via Na/Ca exchange) will be enhanced. This would result in a lower Ca content for the next contraction. Thus, the smaller contraction might be smaller for two reasons: 1) because the SR has less Ca and 2) due to refractoriness of the SR release mechanism (see Fig. 84).

Rest Decay and SR Ca Depletion in Rabbit Ventricle

With increasing periods of rest in rabbit ventricular muscle the first post-rest contraction decreases in amplitude (see Fig. 77). This process, known as rest decay, occurs in ventricular muscle from most species and is thought to reflect a gradual decline in SR Ca content (Allen *et al.*, 1976). Figure 77 shows that the amplitude of RCCs also declines as a function of rest duration in a parallel fashion to stimulated twitches (Bridge, 1986; Bers, 1989). Since RCC amplitude depends only on Ca release from the SR (unlike twitches which also depend on Ca influx), this confirms the conclusion that rest decay really reflects gradual SR Ca loss. After longer rests (5-10 min) RCC amplitude continues to decline to zero, while twitch force plateaus at a level where the rested-state contraction is observed. The rested-state contraction probably results from the continued gradual emptying of the SR during rest, but with some finite level of residual contractile force which depends on Ca influx. The influence of rest on the I_{Ca} amplitude is also shown in Fig. 77. While decline in I_{Ca} could make some contribution to the early phase of rest decay (up to ~30 sec), the gradual decline in SR Ca content provides a more complete explanation.

Rest decay of twitches and RCCs can be dramatically slowed or even reversed in rabbit ventricular muscle by reduction of the transsarcolemmal [Na] gradient (Sutko *et al.*, 1986; Bridge, 1986; Bers & Bridge, 1988). Indeed, in the absence of external Na and Ca, rest decay is effectively abolished (see Figs. 44 & 49 and Bers *et al.*, 1989; Hryshko *et al.*, 1989c). Thus the SR Ca content appears to be critically linked to transsarcolemmal Ca movements via Na/Ca exchange. As discussed in Chapter 5, this probably results from competition between the SR Ca-pump and Na/Ca exchange for the Ca which leaks from the SR at some finite rate. If the SR Ca-pump were the only means of removing Ca from the cytoplasm, then whatever Ca leaked from the SR would be reaccumulated by the SR and the SR Ca load would stay constant. With a finite rate of net Ca extrusion via Na/Ca exchange (with the extracellular space acting as a sink), the SR will eventually be drained (even at constant $[Ca]_i$). The rate at which the SR is drained depends on the SR leak rate and the ratio of the SR Ca-pump rate to the Na/Ca exchange rate (assuming sarcolemmal Ca leak = 0). Thus, if Na/Ca exchange is prevented by decreasing the [Na] gradient or by depolarization, a larger fraction of the Ca from the SR will be resequestered and rest decay

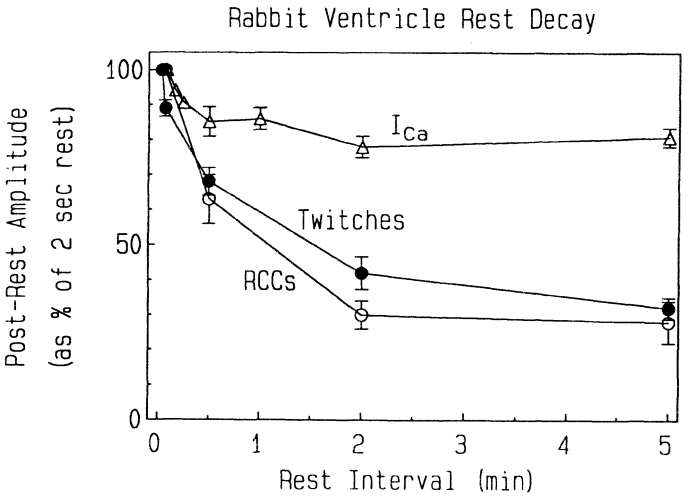


Figure 77. Rest decay of twitch force, SR Ca content (based on RCCs) and Ca current in rabbit ventricle. The amplitude of the twitch, RCC and I_{Ca} at the first pulse after the rest are shown as % of the value at the steady steady interpulse interval (2 sec). Contraction data are from multicellular preparations (Bers, 1989) and I_{Ca} data are from isolated ventricular myocytes (Hryshko & Bers, 1990). The rest decay of RCCs and twitches in isolated myocytes is very similar to that in muscle (Hryshko *et al.*, 1989c).

is slowed. If the SR Ca-pump rate is decreased (or the leak rate is increased, e.g. by ryanodine), then the competition between the SR Ca-pump and the Na/Ca exchange is biased toward Na/Ca exchange and rest decay is accelerated.

Clearly this is a simplified consideration of diastolic Ca fluxes since it neglects other possible Ca fluxes (e.g. mitochondrial Ca fluxes and sarcolemmal Ca leak and Ca-pump). However, the mechanisms considered above appear to be the most important ones and suffice to explain the main features of diastolic changes in SR Ca content. Thus, the process of rest decay in cardiac muscle appears to depend upon Ca extrusion via Na/Ca exchange and the balance of SR Ca-pump and leak. Rest can also result in increased SR Ca content and rest potentiation. This will be discussed specifically below.

Post-Rest Recovery and SR Refilling in Rabbit Ventricle

When stimulation is resumed in rabbit ventricular muscle after a long period of rest, the SR must become refilled to the steady state level. Figure 78 shows one way which we have used to assess the refilling of the SR. Figure 78A shows the post-rest recovery of twitches after a 30 sec rest interval (top panel) and also where RCCs were induced in place of indicated electrically evoked contractions. These RCCs provide an index of the amount of SR Ca available for release at each post-rest contraction. In general, the recovery of twitches and RCCs in Fig. 78B & C look similar, including the decrease in SR Ca at the

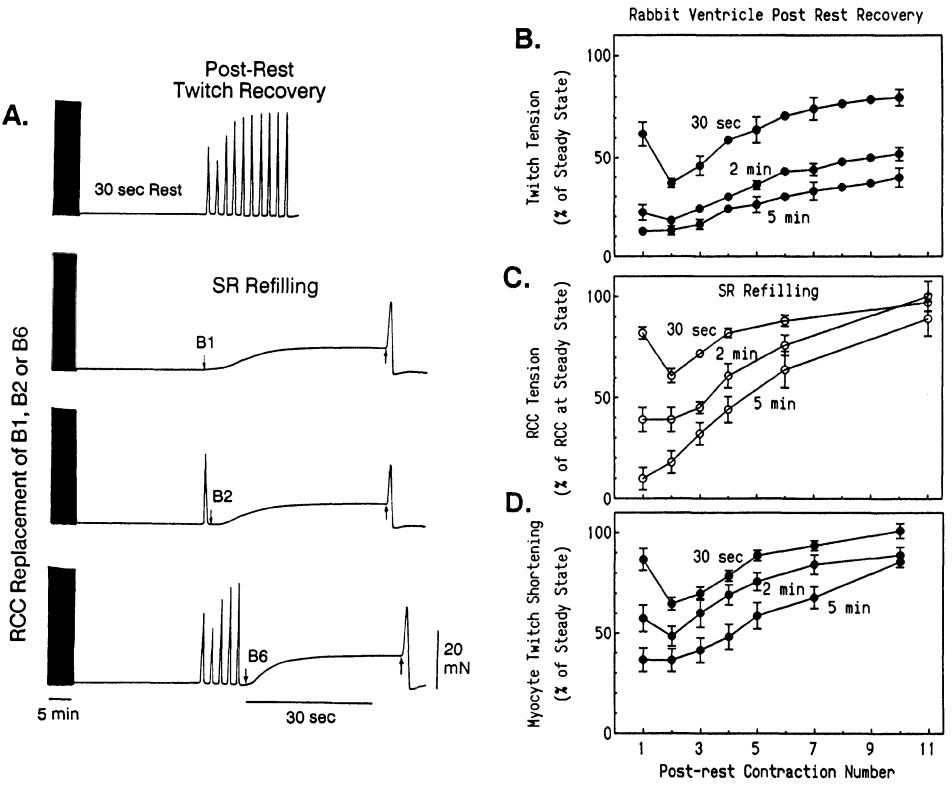


Figure 78. Refilling of the SR with Ca and post-rest recovery in rabbit ventricle (at 30°C and 0.5 Hz).. **A.** Recovery of twitch force after a 30 sec rest (top). The lower panels show repeated 30 sec rests where an RCC was induced in place of the first, second or sixth post-rest contraction (B1, B2, B6). **B.** Post-rest recovery of twitch force in rabbit ventricular muscle after three different rest periods. **C.** SR refilling with Ca during post-rest recovery assessed with RCCs as in A. **D.** Post-rest recovery of twitch contractions in isolated rabbit ventricular myocytes after the same three rest periods. (Panels A, B and C are redrawn from Bers, 1989).

second contraction after a 30 sec rest. However, it can also be seen that the RCCs recover back to steady state considerably faster than stimulated twitches (especially after long rest intervals). This indicates that something other than refilling of the SR is likely to contribute to the slow phase of recovery of twitch force after long rests (see below).

The half-time for refilling the SR appears to be about 4 beats. This agrees with results from extracellular Ca depletion studies in rabbit ventricle (Bers & MacLeod, 1986; MacLeod & Bers, 1987; Bers, 1987b). These Ca_o depletions, which reflect net Ca uptake by the cells, reach a maximum in 6-12 contractions after a rest with the half-maximum typically occurring at beat 4-5 (see Figs. 51 and 99). Ca current amplitude also increases to

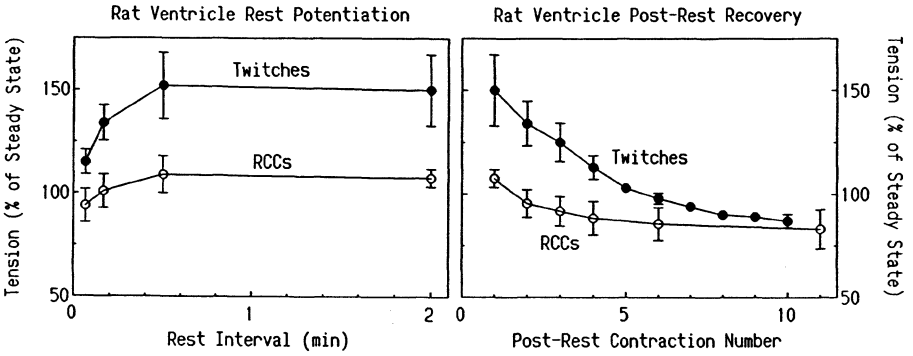


Figure 79. Rest potentiation and post-rest recovery of twitches and RCCs in rat ventricular muscle. Post-rest recovery of RCCs was performed as described in Fig. 78. Tension is expressed as percent of steady state twitch force (0.5 Hz, 30°C, redrawn from Bers, 1989).

its steady state level during the first 5 - 10 post-rest pulses (see Fig. 29). However, this I_{Ca} staircase is small and its real contribution to the post-rest recovery is not yet clear.

On the other hand, it is useful to consider from a quantitative perspective how Ca entry via I_{Ca} may contribute to refilling the SR. After a long rest (e.g. 5 min) the SR may be almost completely Ca depleted. During the first few pulses after such a rest the contractions are small and consequently there will be little Ca efflux. For these first few contractions the integrated I_{Ca} may fairly nearly reflect the net transsarcolemmal Ca flux. If we consider that the integrated I_{Ca} for each of the first 4-5 contractions brings in ~15 μmol/kg wet wt (see page 62), then the net Ca_i gain after 4-5 beats would be 60-75 μmol/kg wet wt. Since the SR is about half refilled by beat 4-5 (see Fig. 78C), this would correspond to an SR Ca capacity for these conditions (30°C, 0.5 Hz) of ~120-150 μmol/kg wet wt. This agrees quite well with various other estimates of SR Ca capacity discussed in chapter 6, where the amount of Ca lost from the SR during rest and regained during post-rest recovery was estimated to be ~100-300 μmol/kg wet wt in rabbit and guinea-pig ventricle (Pytkowski *et al.*, 1983; Lewartowski *et al.*, 1984; Bridge, 1986; Pierce *et al.*, 1987; Lewartowski & Pytkowski, 1987; Pytkowski, 1989; Bers *et al.*, 1989).

It is interesting that the post-rest recovery of twitches in isolated myocytes is faster than in multicellular preparations and more closely parallels the recovery of RCCs (see Fig. 78D). Diffusional restrictions are a major difference between these preparations. Thus it is possible that part of the slow recovery of twitches in Fig. 78B is due to interstitial depletion of Ca (or perhaps accumulation of K etc.). The interstitial $[Ca]_o$ does recover very slowly during twitches after long rest periods ($t_{1/2}$ ~ 1-3 min, Bers & MacLeod, 1986). On the other hand buffering $[Ca]_o$ with either citrate or NTA (nitrilotriacetic acid) can limit this Ca_o depletion, but the slow recovery phase of twitch contractions in multicellular preparations persists (unpublished observation). While it is possible that a slow recovery

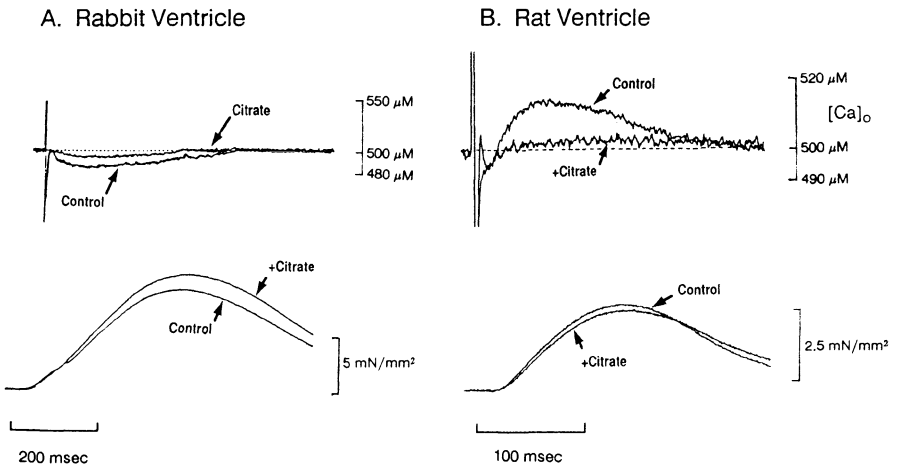


Figure 80. Changes in $[Ca]_o$ measured with double barreled Ca-selective microelectrodes during individual contractions in rabbit (A) and rat (B) ventricular muscle (0.5 Hz, 30 °C). The traces show $[Ca]_o$ (top) and tension (bottom) in the absence and presence of 10 mM citrate (which limits Ca_o depletion by buffering $[Ca]_o$, but now we know it also inhibits I_{Ca}, Bers *et al.*, 1991). The bath $[Ca]_o$ = 0.5 mM and is indicated by the dotted line. (A is from Shattock & Bers, 1989, with permission.)

of some intracellular process or rise in aNa_i could contribute to this slow recovery of twitches (Seibel, 1986), the difference between myocytes and multicellular preparation may provide a key.

Rest Potentiation and Post-Rest Recovery in Rat Ventricle

In contrast to the results above for rabbit ventricle, rat ventricle exhibits rest potentiation and a negative force-frequency relationship. Figure 79 shows that post-rest twitches and RCCs are potentiated in rat ventricular muscle, indicating that the SR gains Ca during rest (Bers, 1989). Although Bouchard & Bose (1989) did not see significant RCC changes with rest, the result in Fig 79 has recently been confirmed by Lewartowski & Zdanowski (1990) and Banijamali *et al.* (1990) using cafffeine-induced contractures and RCCs respectively. This supports the conclusion of Ragnarsdottir *et al.* (1982), based on twitch experiments, that rat ventricular cells gain Ca during rest. Figure 79 also shows that during post-rest recovery in rat ventricle the decline in SR Ca content roughly parallels the decline in twitch force. Thus, the negative force-frequency relationship or "staircase" in rat ventricle may be due in part to an actual decline in SR Ca content.

Ca Influx and Efflux in Rabbit and Rat Ventricle

These results suggest major fundamental differences in transsarcolemmal Ca fluxes in rat *vs* rabbit ventricle during both rest and contraction. Shattock & Bers (1989) compared Ca fluxes in rat *vs* rabbit ventricle during the cardiac cycle using extracellular Ca

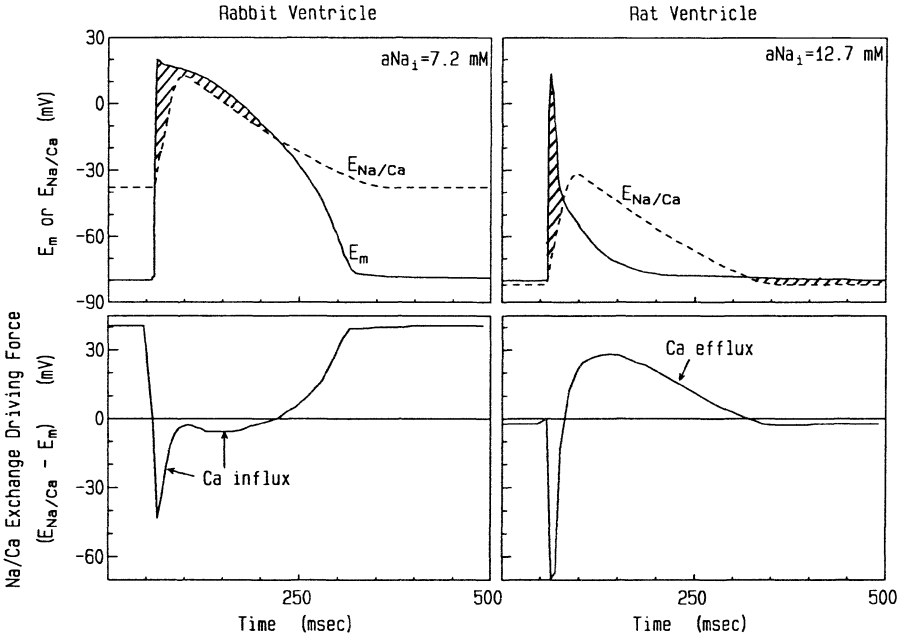


Figure 81. Schematic diagram of the estimated changes in the reversal potential of the Na/Ca exchange ($E_{Na/Ca}$) that accompany the action potential and Ca_i transient in rabbit and rat ventricle (top). The estimated changes in the net electrochemical driving force for Na/Ca exchange ($E_{Na/Ca}$-E_m) are shown in the bottom panel. We assumed a stoichiometry of 3Na:1Ca for the Na/Ca exchanger, aNa_i values measured in these preparations (Shattock & Bers, 1989) and, for simplicity, the Ca transient accompanying the contraction, has been assumed to be the same for both species. Resting $[Ca]_i$ was assumed to be 150 nM, rising to a peak of 1 μM, 40 msec after the upstroke of the action potential. The shape of the Ca_i transient was calculated as described by Bers (1987b). Note the similarity between the lower panels and the $[Ca]_o$ traces in Fig. 80. (Top panels were redrawn after Shattock & Bers, 1989, with permission).

microelectrodes. We demonstrated that during the twitch in rabbit ventricle net Ca influx occurs, but that during the twitch in rat ventricle net Ca efflux occurs (see Fig. 80). Since Na/Ca exchange is the main mechanism by which Ca is extruded from cardiac cells, we suggested that the large Ca efflux recorded in rat was due to Ca released by the SR and extruded by Na/Ca exchange. Indeed, the Ca efflux recorded in rat ventricle was caffeine sensitive and was larger at the first large post-rest contraction. We also compared resting intracellular Na activity (aNa_i) and found that aNa_i was significantly higher in rat (12.7 mM) than rabbit ventricle (7.2 mM). In rabbit ventricle Ca extrusion via Na/Ca exchange is thermodynamically favored at rest since the predicted reversal potential for Na/Ca exchange ($E_{Na/Ca}$) is positive to the membrane potential (E_m, see Fig. 81). During the action potential E_m exceeds $E_{Na/Ca}$ (even though $[Ca]_i$ is elevated) such that there is a modest driving force favoring Ca entry during the action potential. This is consistent with

the transient Ca_o depletions seen during contractions in rabbit ventricle (Fig. 80) and Ca loss during diastole. The rate of Ca extrusion during rest is small despite the large thermodynamic driving force because it is kinetically limited by the low resting $[Ca]_i$.

The resting aNa_i in rat ventricle is high enough that the $E_{Na/Ca}$ would be near the resting membrane potential (Fig. 81). In particular, after a train of stimuli aNa_i would be higher still, such that $E_{Na/Ca}$ *would* be negative to E_m and net Ca uptake would be favored. This can explain the rest potentiation and rest-dependent increase in SR Ca in rat ventricle (Fig. 79). DuBell & Houser (1987) also reported that resting $[Ca]_i$ was higher in rat than cat ventricular myocytes. In rat ventricle the action potential is also very short compared to rabbit ventricle and normally lacks an appreciable plateau phase (see Fig. 81). Thus, during the contraction when $[Ca]_i$ is high, $E_{Na/Ca}$ is positive to E_m so that there is a large driving force favoring Ca extrusion via Na/Ca exchange. In this way the competition between the SR Ca-pump and the Na/Ca exchanger is biased toward the latter and net Ca efflux occurs during the pulse (compare Fig. 80B and 81, lower right). During a potentiated post-rest contraction a larger Ca efflux will occur and the SR Ca content will be lower at the next contraction until a new steady state is achieved (where Ca influx and efflux must be the same over a complete cardiac cycle). This explains the decrease in contractions during post-rest recovery (Fig. 79) and also explains the well known negative "staircase" or force-frequency relationship in rat ventricle. If the depolarization in a rat ventricular myocyte is prolonged near 0 mV by a voltage clamp pulse (to resemble that in rabbit ventricle), the negative "staircase" can be converted to a positive "staircase" (Spurgeon *et al.*, 1988) as would be expected from Fig. 81. Figure 82 shows a similar result in guinea-pig ventricle which behaves much like rabbit ventricle. Increasing pulse duration leads to a positive "staircase", while reduction of pulse duration to 100 msec leads to a negative "staircase" (Isenberg & Wendt-Gallitelli, 1989). Thus, prolonging the duration of depolarization can increase intracellular (and SR) Ca loading by two means: 1) limiting the

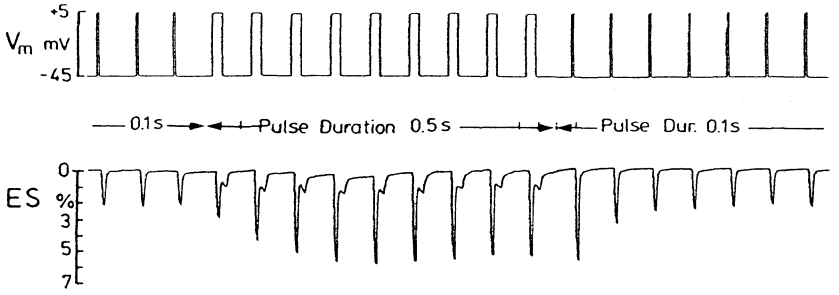


Figure 82. The duration of depolarization determines the direction of the contraction "staircase" in a guinea-pig ventricular myocyte. Membrane potential (V_m) and external shortening (ES) as a percent of resting cell length under voltage-clamp (37°C, $[Ca]_o$=1.8 mM). (From Isenberg & Wendt-Gallitelli, 1989, with permission).

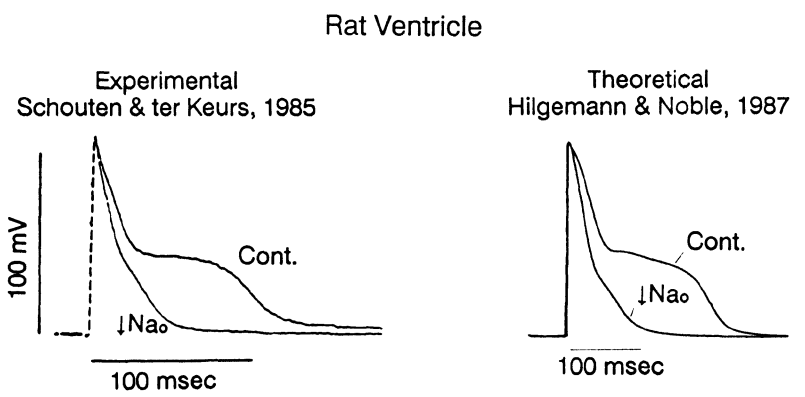


Figure 83. Action potentials recorded from rat ventricular muscle (left) stimulated at low frequency where contractions are large in a normal superfusate (Cont.) and after reduction of $[Na]_o$ from 150 to 30 mM ($\downarrow Na_o$). This behavior could be simulated in an action potential model simply by reducing the effective [Na] for Na/Ca exchange (right). (from Schouten & ter Keurs, 1985 and Hilgemann & Noble, 1987, with permission)

extrusion of Ca via Na/Ca exchange (or enhancing net influx) and 2) allowing continued Ca entry via sarcolemmal Ca channels.

Ryanodine greatly accelerates rest decay of RCCs in rabbit ventricle ($t_{1/2}$ ~ 1 sec), but does not abolish RCCs in rat ventricle under control conditions (Bers & Christensen, 1990). This is consistent with the forgoing discussion, since the low transsarcolemmal [Na] gradient may limit Ca extrusion via Na/Ca exchange (so that even a "leaky" SR may be able to retain Ca). Furthermore, by manipulating [Na] and [Ca] gradients rabbit ventricle could be made to behave more like rat ventricle (and *vice versa*, Bers & Christensen, 1990). For example, elevation of aNa_i in rabbit ventricle (by Na-pump inhibition) causes rest potentiation resembling that seen in rat ventricle. Reducing $[Ca]_o$ in rat ventricle can also convert rest potentiation into rest decay (more like rabbit ventricle). When rabbit ventricular contractions are potentiated by Na-pump inhibition or by high frequency prepulses, net Ca efflux is observed during the contraction as in Fig. 80 for rat ventricle (see Fig. 98B, Hilgemann & Langer, 1984; Bers & MacLeod, 1986; Bers, 1987b). Thus, the elevated aNa_i and rapid action potential repolarization in rat compared to rabbit ventricle can explain many of the functional differences observed between these two species.

While depolarization alters transsarcolemmal Ca fluxes, Ca extrusion via Na/Ca exchange can also modify the action potential. Schouten & ter Keurs (1985) demonstrated that removal of extracellular Na suppressed a slow component of the rat ventricular action potential which was most prominent at large contractions where $[Ca]_i$ is high (see Fig. 83). They attributed the slow depolarized phase of the action potential to inward current via Na/Ca exchange (i.e. 3 Na^+ influx for 1 Ca^{2+} extruded). Hilgemann & Noble (1987) were able to simulate this effect in their model of the rat ventricular action potential (Fig. 83).

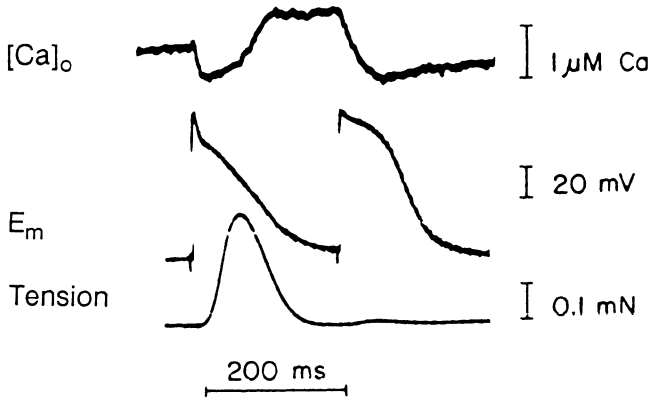


Figure 84. Paired-pulse stimulation of rabbit atrium in the presence of 2 mM 4-aminopyridine (to suppress transient outward K currents). The $[Ca]_o$ is assessed by the absorbance of the extracellular Ca indicator, tetramethylmurexide. Free $[Ca]_o$ is 150 μM, the basic frequency is 0.5 Hz and the paired-pulse is evoked 200 msec after the main pulse (from Hilgemann, 1986b, with permission).

Action potentials are also influenced by Ca fluxes during paired-pulse stimulation and mechanical alternans (Wolfhart, 1982; Hilgemann, 1986a,b). Figure 84 shows extracellular Ca depletion, action potentials and force in rabbit atrium during paired-pulse stimulation (in the presence of 4-aminopyridine to block transient outward K currents, Hilgemann, 1986b). During the large contraction the Ca_o depletion reaches a nadir and net Ca efflux is evident by the end of the contraction. This is analogous to the result with rat ventricle in Fig. 80 where high $[Ca]_i$ drives Ca efflux via Na/Ca exchange. The small paired-pulse produces a progressive net Ca_o depletion, a more prominent action potential plateau and only a tiny contraction. As described for the case of an extrasystole above, SR Ca release may be refractory at the paired-pulse so no SR Ca is released. Thus $[Ca]_i$ remains low during the action potential such that Ca_i-dependent inactivation of sarcolemmal Ca channels is minimal and Ca continues to enter (explaining the larger Ca_o depletion and the higher action potential plateau). The low $[Ca]_i$ also limits Ca extrusion via Na/Ca exchange during the paired-pulse. The net result is that the SR is more Ca loaded (and non-refractory) by the next regular pulse. In this case inward $I_{Na/Ca}$ may prevent even more rapid repolarization at the first pulse. However, the persistent inward I_{Ca} at the paired-pulse may more than compensate for the decreased amount of inward $I_{Na/Ca}$ at the small contraction. Since there is little Ca efflux during the small paired-pulse (where SR Ca release is refractory) one could almost consider that integrated Ca current is being "injected" into the SR! Of course the Ca which enters must be pumped into the SR, but then the SR has an extra aliquot of Ca available for the next contraction.

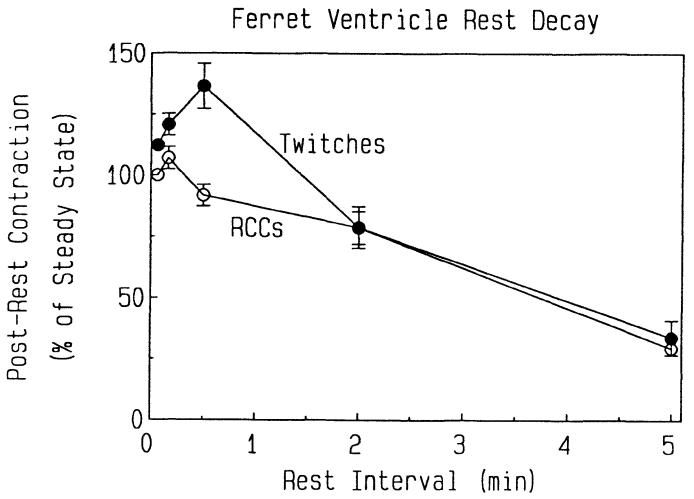


Figure 85. Rest decay of twitches and RCCs in ferret ventricular muscle (37°C, 0.5 Hz). Tension is shown normalized to the twitch or RCC after the interpulse interval (2 sec). Note that rest potentiation proceeds for at least 30 sec despite a decline in SR Ca assessed by RCC.

Potentiation of Contraction without Increasing SR Ca

So far the amplitude of post-rest contractions has been seen to parallel (at least qualitatively) the changes in SR Ca content assessed by RCCs (Figs. 77-79). Thus there was no reason to suppose any change in the fraction of SR Ca released (at least not after 1-2 sec of early mechanical restitution). However, this is not always the case. In several mammalian ventricular muscle preparations (notably dog and ferret) there is a prolonged period of rest potentiation (for up to ~2 min), despite a mainly monotonic decline in SR Ca content as assessed by RCCs (see Fig. 85, Hryshko *et al.*, 1989b; Bouchard & Bose, 1989). This result suggests that the fraction of SR Ca released during a twitch might increases over about the first 30-60 sec of rest. Since Ca current is decreased during this period in ferret ventricle (Hryshko *et al.*, 1990) as shown for rabbit in Fig. 77, it is possible that this reflects a very slow recovery phase from inactivation of the SR Ca release channel. Fabiato (1985b) found that this apparent recovery took several sec at very low [Ca] (pCa = 7.9) and it may take longer at the higher $[Ca]_i$ expected in cells.

FORCE-FREQUENCY RELATIONSHIPS

The relationship between stimulation pattern and contractile force has intrigued investigators since the early work of Bowditch (1871) and Woodworth (1902). This force-interval or force-frequency relationship has been the subject of numerous reviews (e.g.

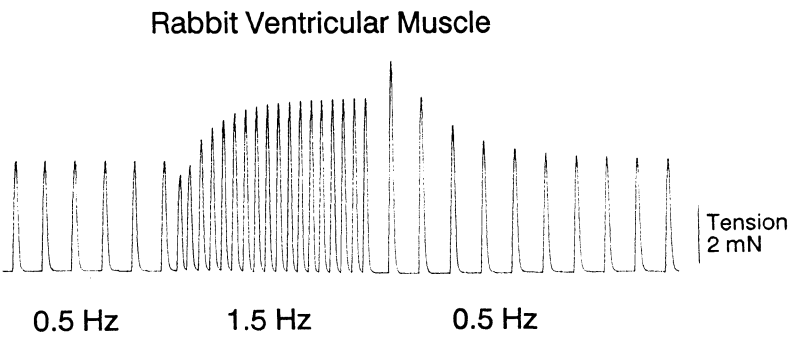


Figure 86. Effect of a transient increase in frequency (from 0.5 to 1.5 Hz) on twitch force in rabbit ventricular muscle (30°C).

Kruta, 1937; Braveny & Kruta, 1958; Blinks & Koch-Weser, 1961; Koch-Weser & Blinks, 1963; Wood *et al.*, 1969; Allen *et al.*, 1976; Edman & Jóhannsson, 1976; Johnson, 1979; Wolfhart & Noble, 1982; Lewartowski & Pytkowski, 1987; Schouten *et al.*, 1987). Most of the central fundamental mechanisms have been addressed above in discussion of mechanical restitution, rest decay (and potentiation), post-rest recovery, paired-pulses, alternans and post-extrasystolic potentiation. Indeed, it seems that most of the features of the force-frequency relationship can be fairly well understood within the context of the forgoing considerations. We can now be more specific about the cellular mechanisms responsible for changes in contractility than was possible when some of the classic reviews above were written.

For example, Figure 86 shows a classical response to a transient increase in stimulation frequency in rabbit ventricular muscle. The first pulse at higher frequency is decreased, probably reflecting insufficient time for the Ca release channel to recover from inactivation. Continued pacing at this higher frequency leads to a progressive positive "staircase" (which more than compensates for the infringement on restitution). At least three factors may be involved in this increase: 1) increased I_{Ca}, 2) higher diastolic $[Ca]_i$ (due to greater Ca influx from more pulses per second and also less time between contractions for Ca to be extruded from the cell) and 3) increased SR Ca available for release (as a consequence of the above and the higher average cytoplasmic [Ca]). Additionally the higher frequency increases aNa_i (Cohen *et al.*, 1982; January & Fozzard, 1984; Ellis, 1985; Boyett *et al.*, 1987). This will shift the delicate balance of the Na/Ca exchange system such that Ca extrusion is less favored and Ca influx is more favored. The result is that there is more Ca in the cell and in the SR. This is confirmed by extracellular Ca depletions and larger RCCs when stimulation frequency is increased in rabbit ventricle (Bers & MacLeod, 1986; Bers, 1989). Figure 87 shows that with increasing frequency, SR Ca content changes (assessed by RCCs, ○, △, □) roughly parallel changes in twitch force in rabbit, guinea-pig and rat ventricle. Bouchard & Bose (1989) did not see a significant

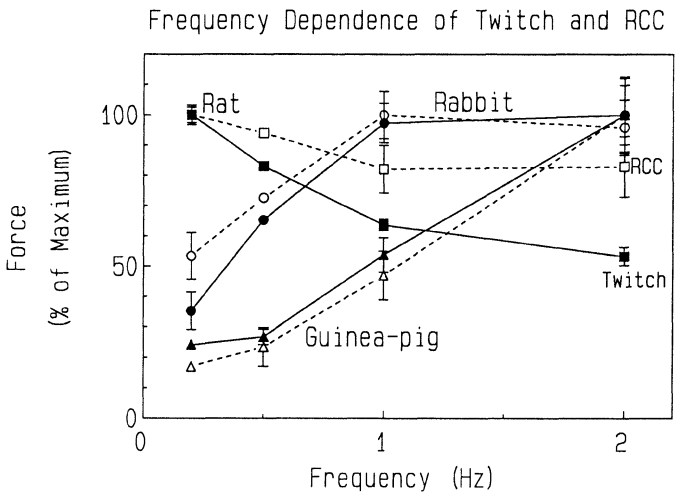


Figure 87. Effect of frequency (from 0.5 to 2 Hz) on twitch force in rabbit (circles), rat (squares) and guinea-pig ventricular muscle (triangles). Data for rabbit and rat are at 30°C (from Bers, 1989 and unpublished). Data for guinea-pig are at 36.5°C and were taken from Kurihara & Sakai (1985). RCCs were initiated within 5 sec of the last steady state stimulated contraction.

decline in RCC amplitude in rat ventricle with increasing frequency (and the decline in rat RCC in Fig. 87 is small). It is possible that the negative "staircase" in rat is due to a combination of a small decrease in SR Ca content and a decrease in fractional release at higher frequency (i.e. incomplete mechanical restitution).

When frequency is decreased in Fig. 86, the first pulse is large. This probably reflects a combination of the relatively high SR Ca content (described above) and also a greater fraction of SR Ca released (due to the greater time available for recovery from inactivation of the SR release channel). This large SR Ca release and longer diastolic interval stimulates substantial Ca extrusion via Na/Ca exchange. Consequently there is a progressive decline in the amount of Ca in the SR until steady state is reattained (e.g. where Ca efflux via Na/Ca exchange over one cycle matches the Ca influx via I_{Ca}). The stepwise decline from the potentiated contraction has also been used as an index of the fraction of released Ca that was resequestered (or recirculated) into the SR (Wolfhart & Noble, 1982; Schouten *et al.*, 1987). That is, if the second contraction is 70% of the potentiated one, then one might say that ~70% of the Ca released was recycled to the SR. Although this "recirculation fraction" assumes that Ca influx is negligible and that the relation between SR Ca released and peak force is linear, it may be of practical utility.

Thus, a largely positive steady state force-frequency relationship is expected for mammalian cardiac muscle, except for those tissues which have short action potentials (such as rat ventricle and many atrial preparations). In this case large Ca efflux may occur

during the twitch as in rat ventricle (Figs. 80-81). Even for these exceptions one can usually find conditions where a positive staircase is demonstrable (e.g. rat ventricle at low $[Ca]_o$ or high frequency, Forester & Mainwood, 1974; Henry, 1975). This might be expected based on the above discussion.

At the extreme of high frequency, encroachment on early mechanical restitution is likely to be responsible for limiting the positive staircase. Indeed, as diastolic $[Ca]_i$ rises, the problem of restitution may become more severe. At the lower extreme of frequency (e.g. rested state contraction) the force-frequency relationship may be dictated by the [Na] gradient and the pump/leak balance of the SR Ca-pump. Thus, for rabbit and guinea-pig ventricle which exhibit fairly monotonic rest decay, contractions continue to decline at very low frequencies. In some tissues (notably atrial muscle) there is often a large rested state contraction, suggesting that the SR does not become Ca-depleted even during long rests (Koch-Weser & Blinks, 1963). This would have to be attributed to either: 1) a low transsarcolemmal [Na] gradient (such that Na/Ca exchange cannot extrude Ca from the cell as described above for rat) 2) an SR with virtually no diastolic Ca leak or 3) a very slow phase of recovery from inactivation of the SR Ca release channel (as suggested above for the ferret ventricle).

In conclusion, there is great variability in the details of how $[Ca]_i$ appears to be regulated in different cardiac muscle preparations. However, this apparent complexity can be largely understood by considering variations in a small number of common systems which interact. Ca influx appears to be able to activate substantial contractions in mammalian as well as amphibian heart, but under normal conditions the SR is probably the major source of Ca for activation of mammalian cardiac muscle. Ca influx can serve to trigger SR Ca release and also contribute to the loading of the SR for the next contraction. Ca released from the SR can either be reaccumulated by the SR or extruded from the cell via Na/Ca exchange. Indeed, substantial Ca efflux can occur during contraction causing a net Ca loss from the cell and SR. In the steady state, however, the Ca extruded by Na/Ca exchange during one cardiac cycle is balanced with the Ca influx via I_{Ca} (and Na/Ca exchange). The Ca content of the SR can be gradually drained by the sarcolemmal Na/Ca exchange during rest and quickly refilled during post-rest recovery by the Ca entering via I_{Ca} in 5-10 contractions. Depending on the transsarcolemmal [Na] gradient, rest can either deplete the SR or fill the SR with Ca. Clearly a dynamic yet delicate balance exists in the control of intracellular Ca in the heart and changes in these systems can lead to inotropic and lusitropic changes.

CHAPTER 9

CARDIAC INOTROPY AND Ca OVERLOAD

My aim in this chapter is to discuss some of the general mechanisms involved in cardiac inotropy and their relationship with cellular Ca overload. I do not plan to provide a comprehensive review of either inotropic agents or cardiac pathophysiology. I will start by considering four fundamentally different examples of cardiac inotropy: 1) β-adrenergic activation, 2) α-adrenergic activation, 3) hypothermia and 3) cardioactive steroids (digitalis glycosides). Then I will discuss the ways in which intracellular Ca metabolism can go awry, with particular emphasis on Ca overload. Finally, I will address strategic sites for induction of cardiac inotropy. These discussions may help to bring some of the characteristics of specific cellular systems discussed in preceding chapters into a more integrative picture of cellular Ca regulation.

CARDIAC INOTROPY

β-Adrenergic Agents and Cardiac Inotropy

Isoproterenol, a β-adrenergic agonist, can produce large increases in cardiac contraction and the amplitude of the Ca_i transient (see Fig. 88). Three important effects of β-adrenergic agents have already been discussed: 1) decreased myofilament Ca sensitivity (Chapter 2), increased Ca current (Chapter 4) and enhanced SR Ca uptake (Chapter 6). All of the major β-adrenergic actions appear to be mediated by β_1-adrenergic receptors and I will focus on that pathway, although β_2-adrenergic receptors may also play some role (see Bode & Brunton, 1989). β-adrenergic agonists stimulate adenylate cyclase (see Fig. 33) with consequent elevation of cyclic AMP, activation of cyclic AMP-dependent protein kinase (PKA) and phosphorylation of a number of cellular proteins including: 1) troponin I, 2) sarcolemmal Ca channels and 3) phospholamban (associated with the SR Ca pump). As discussed in Chapter 4, β-adrenergic agonists can also increase Ca current by a more direct, G protein mediated pathway. The result of the stimulated I_{Ca} and SR Ca uptake is that the Ca_i transient is much larger and much faster after β-adrenergic agonists such as isoproterenol (see Fig. 88 and 90).

In the presence of β-adrenergic agonist, more Ca enters the cell at each excitation (due to increased I_{Ca}) and the SR accumulates a larger fraction of the cytosolic Ca pool

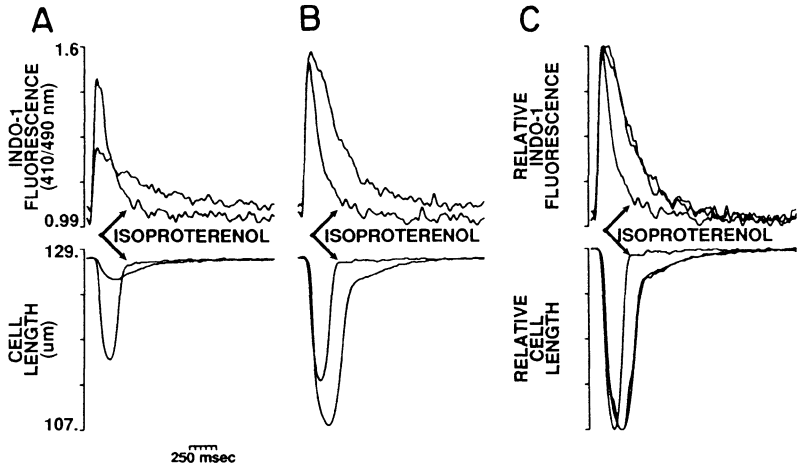


Figure 88. Effect of the β-adrenergic agonist, isoproterenol (0.5 μM) on Ca_i transients (top) and shortening of rat ventricular myocytes at 23°C. A. $[Ca]_o$=1 mM in the presence and absence of isoproterenol. B. a different cell with either 3 mM Ca_o or 1 mM Ca_o plus 0.5 μM isoproterenol, so that similar peak $[Ca]_i$ was reached in both cases (note smaller contraction with isoproterenol). C. Scaled traces from A and B (control traces) and the isoproterenol trace from B (note the faster time course with isoproterenol, from Spurgeon *et al.*, 1990 with permission).

during relaxation (due to SR Ca-pump stimulation). Thus, one expects a larger SR Ca load. The increased I_{Ca} might also increase the fraction of SR Ca which is released (via Ca-induced Ca-release, see Fig. 61). The result is a much larger peak $[Ca]_i$ during contraction with isoproterenol (see Figs. 88 & 90). Of course, the SR Ca-pump stimulation also accelerates relaxation and leads to an earlier peak and more rapid decline of the Ca_i transient. If Na/Ca exchange is unaltered, the SR Ca-pump stimulation will also bias the competition between these two mechanisms in favor of the SR Ca-pump (although the higher peak $[Ca]_i$ will also tend to stimulate Ca extrusion via Na/Ca exchange, see Fig. 81). This bias toward the SR Ca-pump can also explain why isoproterenol can abolish the negative "staircase" in rat ventricular myocytes (Fig. 89, Raffaeli *et al.*, 1987). That is, if Ca is not extruded via Na/Ca exchange at the large contractions (because the SR Ca-pump competes more effectively) the SR Ca load will not decrease progressively from beat to beat as shown in Fig. 79. It is also possible that isoproterenol shortens the time for the SR Ca release process to recover from inactivation. This could also contribute to the loss of the negative "staircase".

The dramatic increase in the Ca_i transient with β-adrenergic stimulation more than compensates for the decrease in myofilament Ca sensitivity, so contraction is also increased substantially. For some time it was thought that the decreased myofilament Ca sensitivity might contribute to the more rapid relaxation observed with β-adrenergic agonists (due to a faster Ca dissociation rate from troponin C). However, McIvor *et*

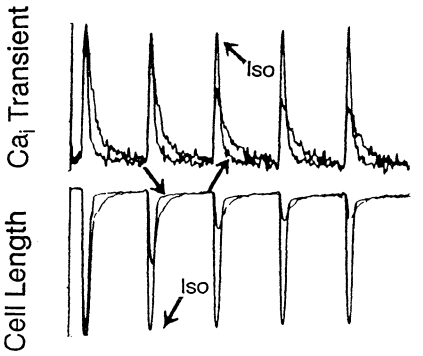


Figure 89. Isoproterenol abolishes the negative "staircase" in rat ventricular myocytes at 23°C. Upon resumption of stimulation after rest there is a prominent negative "staircase" of Ca_i transients (top, measured using indo-1 fluorescence) and contractions (bottom). Isoproterenol (0.5 μM) prevented the stepwise decline in contraction and peak $[Ca]_i$, possibly because the stimulated SR Ca-pump reacumulates most of the released Ca and less Ca is extruded by the Na/Ca exchange (from Raffaeli *et al.*, 1987 with permission).

al.(1988) and Endoh & Blinks (1988) showed that the acceleration of relaxation with isoproterenol could be attributed mainly to the SR Ca-pump stimulation rather than decreased myofilament Ca sensitivity. It is also possible that mechanical factors, such as the rate of cross-bridge detachment and geometric considerations are involved in the β-adrenergic induced acceleration of relaxation (Hoh *et al.*, 1988).

The enhanced I_{Ca} tends to elevate the plateau phase of the cardiac action potential, but β-adrenergic agonists also increase cardiac K conductance (Gadsby, 1983) which may contribute to a shortening of the action potential and hyperpolarization. β-adrenergic agonists also activate a Cl current in cardiac myocytes (Harvey & Hume, 1989), and the net result of effects on Ca, K and Cl currents is that action potential duration can be increased, decreased or unchanged (Tsien, 1977; Tsien *et al.*, 1986). The pacemaker current (I_f) is an inward current (carried by Na or K) which is activated by hyperpolarization (half-activated at ~-70 mV, DiFrancesco, 1981a,b; DiFrancesco & Tromba, 1989). β-adrenergic agonists also shift the E_m-dependence of I_f activation to more depolarized potentials, resulting in a more rapid diastolic depolarization (DiFrancesco, 1986; DiFrancesco *et al.*, 1986). β-adrenergic agonists also accelerate the decline in potassium conductance (g_K, Bennet *et al.*, 1986; Connors & Terrar, 1990) which may contribute to pacemaker activity (especially in cells with more positive diastolic potentials, such as sino-atrial nodal cells (Noble, 1985). All of these effects can contribute to the positive chronotropic effect of β-adrenergic agents. This increase in frequency, of course, can increase cardiac output in addition to the inotropic effect of β-adrenergic agonists. It is also worth noting that the increase in frequency itself would increase contractility, purely by virtue of the "staircase" effect discussed in Chapter 8 (i.e. independent of the inotropy due to β-adrenergic activation).

Thus, the increase in contractility *in vivo* with β-adrenergic agonists is a combination of the intrinsic inotropic effect of increased frequency and the other inotropic effects discussed above, which are directly attributable to β-adrenergic activation (e.g. increased I_{Ca} and SR Ca pumping). Thus, cardiac output can be greatly enhanced by β-adrenergic agonists.

β-adrenergic agonists have also been shown to stimulate the sarcolemmal (Na+K)ATPase and reduce aNa_i (Wasserstrom *et al.*, 1982; Lee & Vassalle, 1983: Désilets & Baumgarten, 1986). This will increase the transsarcolemmal [Na] gradient and increase the ability of the Na/Ca exchanger to extrude Ca. Since at steady state Ca influx and efflux must be matched, this may help Ca extrusion keep pace with the increased I_{Ca} (and frequency) associated with β-adrenergic activation. Nevertheless, the new steady state occurs with substantially larger Ca_i transients at each contraction.

Activation of β-adrenergic receptors also increases metabolism and glycogenolysis (via cyclic AMP-dependent protein kinase, which activates phosphorylase b kinase which in turn activates phosphorylase b, resulting in glycogen breakdown, Hayes & Mayer, 1981). The increased metabolic energy demand, in combination with the large demands due to the increased inotropic state and frequency can increase O_2 consumption dramatically (Rolett, 1974). This is a shortcoming of β-adrenergic agonists as an inotropic agent. That is, in energetic terms (and in terms of O_2 requirement) increasing contractility and heart rate via β-adrenergic agonists is expensive. β-adrenergic receptors are also "down-regulated" or removed from the membrane during chronic activation and in the failing human heart (Watanabe *et al.*, 1982; Stiles *et al.*, 1984; Bristow *et al.*, 1982, 1986). This makes β-adrenergic agonists more appropriate for acute rather than chronic inotropic therapy.

α-Adrenergic Agents and Cardiac Inotropy

In addition to β-adrenergic receptors, there are also α-adrenergic receptors in the heart and they are present at a similar density (Bode & Brunton, 1989; Benfey, 1990). Here I will limit discussion to the α_1 subtype which is the most prominent in cardiac muscle. Table 14 shows the density of several hormone receptors and ion transport proteins in whole tissue (fmol/mg homognate protein) and also the estimated surface density ($/\mu m^2$) on the sarcolemma (or the SR for the SR Ca-pump and junctional SR for the ryanodine receptor). Shümann *et al.* (1974, 1975) demonstrated inotropic effects attributable to α-adrenergic activation, but these receptors and their effects have only been appreciated fairly recently. In addition to the inotropic effects described below, α-adrenergic activation is also centrally involved in ventricular myocyte hypertrophy (Simpson, 1985). The hypertrophic response appears to be mediated via the α_{1a} receptor subtype (i.e. WB4101-sensitive), while the inotropic action may be mediated by the α_{1b} receptor subtype (i.e. Chloroethylclonidine-sensitive, Minneman *et al.*, 1988; Minneman, 1988; Simpson *et al.*, 1990; Michel *et al.*, 1990).

The magnitude of the inotropic effect of α-adrenergic activation varies in different ventricular preparations, being larger in rabbit and smaller in cat, guinea-pig and human

Table 14

Hormone Receptors and Ion Transporters in Cardiac Muscle

	Ligand	K_d (nM)	B_{max} (fmol/mg)	Density (/μm^2)†	Reference
β-Adrenergic					
Rat LV	DHA	2.3	71	12	Williams & Lefkowitz, 1978
Rabbit LV	DHA	5.2	62	11	Brodde *et al.*, 1982
G-P LV	IHYP	0.10	42	7	Hedberg *et al.*, 1980
Human LV	ICYP	0.18	79	14	Stiles *et al.*, 1983
Human RA	ICYP	0.26	84	14	Stiles *et al.*, 1983
α-Adrenergic					
Rat LV	DHE	2.7	26	5	Williams & Lefkowitz, 1978
Rabbit Hrt	DHE	11.5	80	14	Schümann & Brodde, 1979
G-P Heart	Prazosin	0.58	58	10	Karliner *et al.*, 1979
Muscarinic					
Rat Hrt	QNB	0.03	144	25	Fields *et al.*, 1978
Rat A	QNB	1.1	371	64	Wei & Sulakhe, 1979
Rabbit Hrt	QNB	0.03	57	10	Fields *et al.*, 1978
Na Channel					
Sheep V	Saxitoxin	0.22	20*	3*	Doyle *et al.*, 1985
SL Ca Channel					
Rabbit V	PN200-110	1.7	75	13	Lew *et al.*, 1991
Na/Ca exchanger					
Dog V	Rate	-	1087	187	Cheon & Reeves, 1988
SL Na-Pump					
Dog V	Ouabain	33	6000*	1032*	Colvin *et al.*, 1985
SR Ca Channel					
Rabbit V	Ryanodine	23	338	174	Bers, unpublished
SR Ca-Pump					
G-P V	E-PO_4	-	49,000‡	2600	Levitsky *et al.*, 1981

Abbreviations: DHA, dihydroalprenolol; IHYP, Iodohydroxybenzylpindolol; ICYP, iodocyanopindolol; DHE, dihydroergocryptine; QNB, quinuclidinyl benzilate; Hrt, heart; V, ventricle; A, atrium; L, left; R, right; SL, sarcolemma; E-PO_4, Phospho-enzyme. See Bode & Brunton (1989) for more hormone receptor values.

*The maximum, corrected, values in isolated SL for saxitoxin sites (678 fmol/mg) and ouabain sites (365 pmol/mg) were divided by the SL purification factor (35× for saxitoxin and ~60× for ouabain). In isolated SL vesicles, Colvin *et al.*, (1985) and Doyle *et al.* (1985) estimated ouabain and saxitoxin site density at 330 and 3.6-7.6/μm^2 respectively, using different assumptions than those below.

‡Assuming 120 mg homogenate protein/g wet weight

†B_{max} values can be extrapolated to surface density for SL receptors by assuming (for rabbit ventricle): 120 mg protein/g wet weight, 1.06 g wet weight/cm^3 heart, 25% extracellular space (0.75 cm^3 cell/cm^3 heart) and 0.6 μm^2 SL (including T-tubules)/μm^3 cell (Table 1, Page & Surdyk-Droske, 1979). For simplicity all SL density calculations used these assumptions, though others might be better in some cases. For the SR Ca-pump 1.96 μm^2 SR/μm^3 was used and for the ryanodine receptor 0.2 μm^2 junctional SR/μm^3 was used (Table 1).

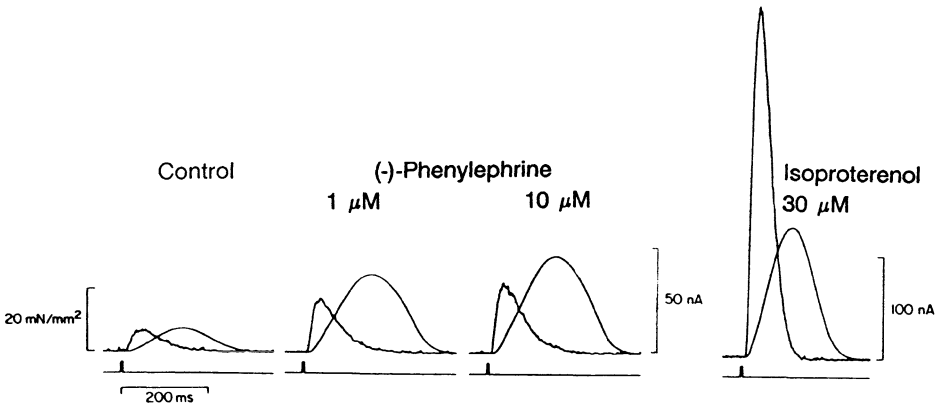


Figure 90. The influence of α-adrenergic activation and β-adrenergic activation on contractions and Ca_i transients (the earlier peaks, measured with aequorin) in rabbit ventricular muscle at 37.5°C, stimulated at 1 Hz and equilibrated continuously with 1 μM bupranolol (a β-adrenergic blocker). Phenylephrine was applied at several concentrations and then washed out. Then isoproterenol was added at a high enough concentration to overcome most of the β-adrenergic blockade by bupranolol. Note that the Ca_i transient in the presence of isoproterenol is recorded at reduced gain (modified from Endoh & Blinks, 1988, with permission).

(Hartmann *et al.*, 1988; Hescheler *et al.*, 1988; Hiramoto *et al.*, 1988; Jakob *et al.*, 1988). Figure 90 shows Ca_i transients and force during α-adrenergic and β-adrenergic activation in rabbit ventricle where the maximal α-adrenergic induced inotropy is about half that of the β-adrenergic response (Endoh & Blinks, 1988). With α-adrenergic activation the changes in the Ca_i transient and kinetics of contraction are much smaller than with β-adrenergic agonists, despite a similar extent of inotropy. Based on a shift in the relationship between peak $[Ca]_i$ and maximal tension development, Endoh & Blinks (1988) concluded that α-adrenergic activation increases the myofilament Ca sensitivity. This probably explains part of the inotropic effect, but an increase in Ca affinity of the myofilaments would be expected to decrease the amplitude of the Ca_i transient (since more of the activating Ca is bound to the myofilaments). Thus, the increase in the Ca_i transient implies that some other changes must occur such that either more Ca enters the cell or more is released from the SR. Despite early suggestive results (Brückner & Scholz, 1984) α-adrenergic activation does not appear to increase Ca current (Hescheler *et al.*, 1988; Hartmann *et al.*, 1988). In contrast to the increased cAMP with β-adrenergic agonists, α-adrenergic agonists stimulate cAMP phosphodiesterase activity and reduce cAMP (Buxton & Brunton, 1985). Thus, the increase of the Ca_i transient with α-adrenergic activation must have a different basis than that for β-adrenergic activation.

α-adrenergic activation also increases hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into $Ins(1,4,5)P_3$ and diacylglycerol (e.g. see Fig. 72, Brown & Jones,

1986; Poggioli *et al.*, 1986; Jones *et al.*, 1988; Otani *et al.*, 1988; Scholz *et al.*, 1988). Diacylglycerol can stimulate protein kinase C, but this pathway does not seem to be crucial since phorbol esters which also stimulate protein kinase C do not consistently mimic the effects of α-adrenergic activation (Nawrath, 1989). Ins(1,4,5)P_3 on the other hand can stimulate or at least modulate SR Ca release (see Chapters 6 & 7). While it has not been shown that intracellular injection of Ins(1,4,5)P_3 mimics all the effects of α-adrenergic activation, it is possible that the elevation of Ins(1,4,5)P_3 might modify Ca_i transients and contribute to the inotropic effects observed (see pages 143-144).

Activation of α-adrenergic receptors also increases action potential duration possibly due to inhibition of an outward K current (Toshe *et al.*, 1987). This action potential prolongation could shift the balance of sarcolemmal Ca fluxes during the cardiac cycle such that the SR Ca content is increased (e.g. by increasing the time where Ca influx occurs and decreasing the time where Ca extrusion occurs). Activation of α-adrenergic receptors can also lead to intracellular alkalinization which could contribute to the positive inotropic effect of these agents (Gambassi *et al.*, 1990).

Physiological catecholamine release (norepinephrine and epinephrine) activates both α and β-adrenergic receptors, but it is probable that the inotropic effect is mediated mainly by β-adrenergic activation. However, in species with a prominent α-adrenergic response, up to one-third of the inotropic response to norepinephrine can be attributed to α-adrenergic activation (Aass *et al.*, 1983; Nawrath *et al.*, 1988).

Hypothermic Inotropy

Reduction of temperature from 37°C in mammalian cardiac muscle results in an increase in developed force (Kruta, 1938; Sumbera et al., 1966; Blinks & Koch-Weser, 1963; Langer and Brady, 1968). This hypothermic inotropy results in a remarkable 400-500% increase in force at 25°C (see Fig. 91). Much of this large inotropic effect occurs immediately, when temperature is quickly changed between contractions (see Fig. 92). This rapid change suggests that alteration in SR Ca content or relatively slow biochemical changes are unlikely to be critical for most of this inotropic effect. Additionally, hypothermic inotropy of similar amplitude is still observed when normal SR function is depressed by ryanodine or caffeine (Fig. 91, Shattock & Bers, 1987). Myofilament Ca sensitivity and maximal force are both decreased by cooling (see page 26-27, Harrison & Bers, 1989a). This suggests that $[Ca]_i$ must either increase dramatically or come to more complete equilibration with the myofilaments at cooler temperature. Over this temperature range cooling decreases peak Ca current (Q_{10}~3, Cavalié et al., 1985; Briggs & Bers, 1990) and increases action potential duration (e.g. from ~220 to ~370 msec in rabbit ventricle, Shattock & Bers, 1987; Blinks & Koch-Weser, 1963). Despite a slowing of Ca channel inactivation with cooling, there is still less integrated Ca entry via I_{Ca} during depolarizations to 0 mV at lower temperature, even when longer pulses are used to simulate the longer action potentials at cooler temperatures (Briggs & Bers, 1990).

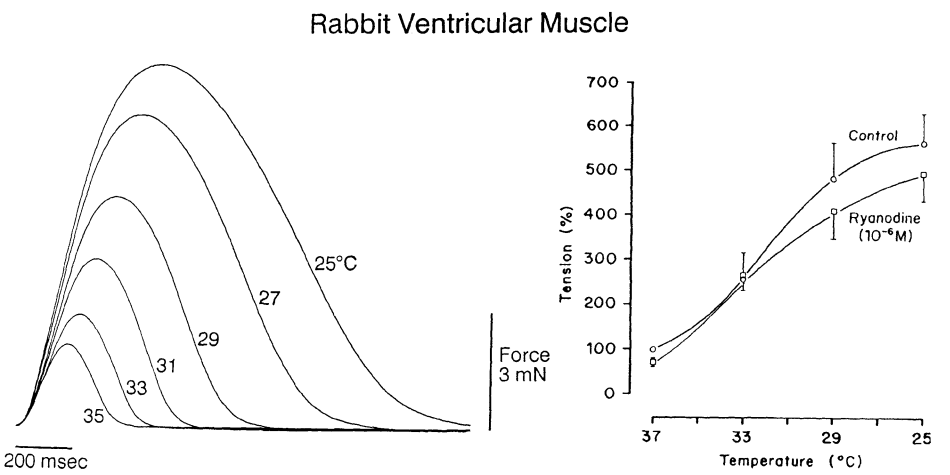


Figure 91. Hypothermia produces a marked inotropy in rabbit ventricular muscle (0.5 Hz). Steady state contractions are shown at the indicated temperatures (left). Pre-equilibration with 1 μM ryanodine does not prevent this hypothermic inotropy (right panel is from Shattock & Bers, 1987, with permission).

If Ca entry is reduced we would also expect a smaller Ca-induced release of Ca from the SR (particularly as SR Ca load is unlikely to change significantly between two consecutive twitches as in Fig. 92). On the other hand, since cooling increases the open probability of the cardiac SR Ca release channel (by increasing the duration of openings, Sitsapesan et al., 1991), a greater fraction of the SR Ca content might be released for a given Ca "trigger". Whether this effect would be sufficient to compensate for the lower peak Ca current is not known. Indeed, it is not known whether peak $[Ca]_i$ during a twitch is increased during hypothermic inotropy. The fact that the maximum rate of rise of force (+dF/dt) is not decreased at the first contraction at 25°C in Fig. 92 would seem to suggest that the SR Ca release is at least as large as at 35°C. Otherwise the slower development of contractile force expected from the myofilaments at low temperature (for a given [Ca]) should lead to a smaller +dF/dt. So perhaps peak $[Ca]_i$ *is* increased at lower temperature,

Na/Ca exchange activity is also decreased by cooling over this temperature range (Q_{10}~1.7, Debetto et al., 1990; Reuter & Seitz, 1968) which would reduce the potential contribution of this system to Ca influx during the early part of the action potential. On the other hand, reduced Na/Ca exchange would mean that Ca extrusion via this system during later stages of the action potential would be diminished (especially since the action potential is prolonged). Of course, the SR Ca-pump is also slowed by cooling and the overall result of slowing both main mechanisms by which Ca is removed from the cytoplasm is that relaxation is greatly delayed (see -dF/dt in Fig. 92). This prolongs the

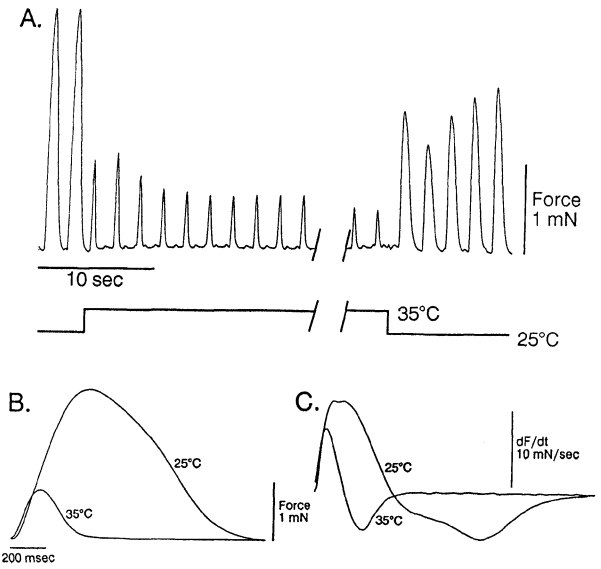


Figure 92. A quick change in temperature shows that most of the hypothermic inotropy take place rapidly. rabbit ventricular muscle was stimulated at 0.5 Hz with temperature switch >90% complete in ~300 msec (using the same set-up as for RCCs). In A, the break in the force record is ~2 min. Force (B) and dF/dt (C) are shown for the first contraction after cooling to 25°C and the last contraction at 35°C from panel A.

"active state" or time during which $[Ca]_i$ is high, and may allow the myofilaments to more completely equilibrate with the cytosolic [Ca] or at least prolong the period during which the myofilaments are activated. Because of the marked slowing in Na/Ca exchange and the SR Ca-pump, $[Ca]_i$ would be expected to reach a higher value for a given amount of SR Ca release. Thus, the $[Ca]_i$ at the time of peak contraction might be higher in the cold and the myofilaments may be more fully activated. In other words, at 35°C Ca removal from the cytoplasm (by the SR Ca-pump and Na/Ca exchange) may overlap temporally the myofilament activation, thereby limiting activation. At 25°C , the overlap of activation and relaxation may be much less (Fig. 92C).

In the steady state at lower temperature the Na-pump is inhibited (Q_{10} ~ 3, Eisner & Lederer, 1980) and intracellular Na activity rises (Shattock, 1984; Chapman, 1986). The rise of aNa_i can then lead to increases in diastolic $[Ca]_i$, SR Ca content and Ca influx due to the Na/Ca exchange system (see next section). Shattock and Bers (1987) showed that a lower steady state temperature (23 *vs* 37°C) resulted in an increase in SR Ca content (assessed by rapid cooling contractures). These Ca loading effects may be responsible for much of the slower phase of hypothermic inotropy which develops over a minute or two (in contrast to the more immediate changes discussed above).

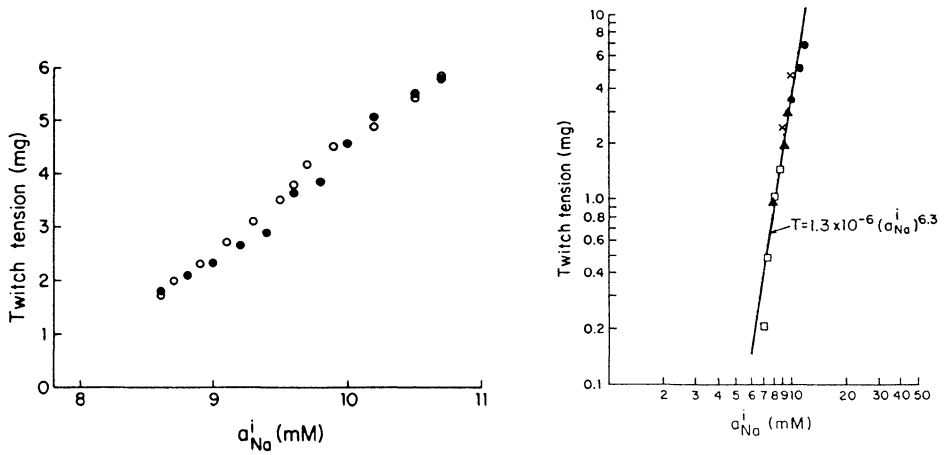


Figure 93. Twitch tension in dog Purkinje fiber depends on aNa_i, which was measured with Na-selective microelectrodes (36°C, 1 Hz). At left, measurements were made during the onset (•) and washout (□) of inotropy induced by 1 μM strophanthidin in one fiber. The right panel shows data from the same fiber (×) and from 3 other fibers on a log-log plot (from Lee & Dagostino, 1982 and Lee, 1985, with permission).

Cardioactive Steroids: Glycoside Inotropy

Digitalis is undoubtedly the oldest cardiac inotropic agent. Withering (1785) described its use in heart failure, known then as dropsy, and cardioactive steroids remain among the most efficacious inotropic agents. Since the recognition of ouabain (and digitalis glycosides) as specific inhibitors of the plasma membrane Na-pump (Glynn, 1964; Skou, 1965), it has become increasingly clear that this action is primarily responsible for positive and negative inotropic effects as well as arrhythmogenic effects of these agents. There have been some indications that very low concentrations of glycoside can stimulate the Na-pump, thereby reducing aNa_i and still produce inotropy (Blood, 1975; Cohen *et al.*, 1976; Godfraind & Ghysel-Burton, 1977; Noble, 1980). However, this action may be due to autonomic neural secretion of catecholamines and consequent stimulation of β-adrenergic receptors (Hougen *et al.*, 1981). The powerful cardiovascular effect of these Na-pump inhibitors and some additional experimental results have fueled a search for a "physiological" digitalis or endogenous natriuretic factor which inhibits the Na-pump (e.g. see Hamlyn, 1989). However, in this section I will focus on the mechanisms by which glycoside-induced Na-pump inhibition leads to cardiac inotropy and Ca overload.

Inhibition of the Na-pump by strophanthidin (a rapidly acting and reversible cardioactive steroids) leads to increased aNa_i and contractility (see Fig. 93). Indeed, the relationship between aNa_i and developed tension can be very steep, particularly in cardiac Purkinje fibers where force can be doubled with only a 1 mM increase of aNa_i (and Hill coefficients of 3-6 have been reported, Lee & Dagostino, 1982; Wasserstrom et al. 1983; Im & Lee, 1984; Eisner et al., 1984). In ventricular muscle the relationship may be less steep,

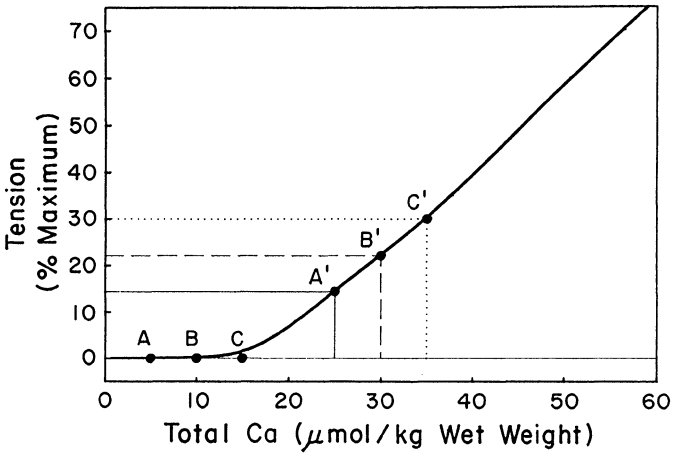


Figure 94. Increased diastolic $[Ca]_i$ can increase developed tension, even with the same amount of activating Ca. The relationship between total myoplasmic Ca and tension is from Fabiato (1983), but in units of μmol/kg wet wt (i.e. 0.4 × μM/liter non-mitochondrial cell water) and fit by the expression $T=127/\{1+(70420/[Ca\text{-}total]^{2.8})\}$ (see Fig. 20). A, B and C correspond to three diastolic $[Ca]_i$ levels and A′, B′ and C′ correspond to the tension and total Ca_i reached when 20 μmol/kg wet wt is added to A, B and C respectively (from Bers, 1987b, with permission).

but this may be due to a relative "ceiling effect" (e.g. in rabbit ventricular muscle at ~29°C and 0.5 Hz) where control twitches are already ~40% of the maximum force the myofilaments can generate (Harrison & Bers, 1989a).

Glynn (1964), Repke (1964) and Langer (1965) first suggested that reciprocal Na and Ca movements might be involved in the inotropic effect. This was several years before Na/Ca exchange was demonstrated and this hypothesis could be more clearly stated (Reuter & Seitz, 1968; Baker et al., 1969; Langer & Serena, 1970). Now it is quite clear that relatively small increases in aNa_i can have a large impact on the balance of Ca fluxes mediated by Na/Ca exchange. For example, an increase of aNa_i from 7 mM to 10 mM would shift the reversal potential for Na/Ca exchange ($E_{Na/Ca}$, by ~30 mV in the negative direction; see Fig. 38). This will tend to increase Ca influx via Na/Ca exchange during the action potential and also limit Ca extrusion via Na/Ca exchange during relaxation and diastole. The result is that resting $[Ca]_i$ can increase (Lee et al., 1980; Marban et al., 1980; Bers & Ellis, 1982; Sheu & Fozzard, 1982; Allen et al., 1984a; Weingart & Hess, 1984; Wier & Hess, 1984). It is worth considering what impact the elevated diastolic $[Ca]_i$ may have by itself on contraction if all other things are equal.

Figure 94 shows the relationship between total cytoplasmic Ca and force, calculated from Fabiato's (1983) data (see Fig. 20). In this case, three different diastolic $[Ca]_i$ values are indicated: A (125 nM), B (300 nM) and C (480 nM) corresponding to 5, 10 and 15

μmol Ca/kg wet weight. These values of $[Ca]_i$ are typical of control resting $[Ca]_i$ (A) and values reported during inotropic effects of Na-pump inhibition (B, C). These values are also below or just at the threshold for myofilament activation. If we assume that activation adds a "bolus" of 20 μmol/kg wet weight of Ca to the cytoplasm A goes to A', B to B' and C to C' (peak $[Ca]_i$ ~ 1-2 μM). Thus, increasing resting $[Ca]_i$ from A to B or C would increase developed force (57 or 114%), even though the phasic supply of activating Ca is constant. Therefore increased diastolic $[Ca]_i$ may contribute to the inotropic effect of cardiac glycosides.

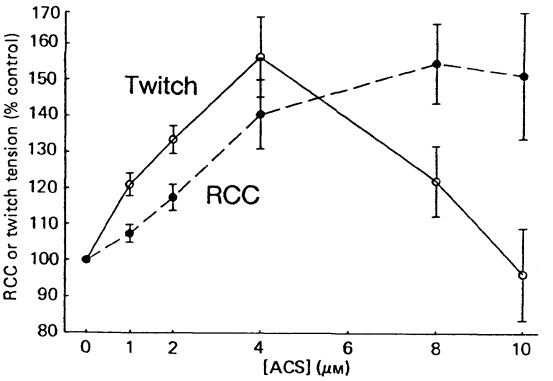


Figure 95. Effect of Na-pump inhibition by acetylstrophanthidin (ACS) on twitch tension and RCC amplitude in rabbit ventricular muscle at 30°C, paced at 0.5 Hz (from Bers & Bridge, 1988, with permission).

The rise in aNa_i and the shift in $E_{Na/Ca}$ will shift the competition between SR Ca-pump and Na/Ca exchange more in favor of the SR Ca-pump during relaxation. Along with the higher diastolic and mean $[Ca]_i$ under these conditions this may be expected to increase the SR Ca load. Indeed, SR Ca content assessed by rapid cooling contractures is increased with Na-pump inhibition (see Fig. 95). Interestingly, the RCC amplitude remains high with higher ACS concentration, despite a progressive decline in the amplitude of twitch contractions. The negative inotropic effect of high concentrations of cardioactive steroids will be addressed below. The increase in SR Ca content with glycosides probably results in greater SR Ca release (Wier & Hess, 1984; Allen et al., 1985b). This increased SR Ca release is likely to contribute to the inotropic effect of cardioactive steroids. Indeed, some investigators have implied that the entire inotropic response to cardiac glycosides can be attributed to an increase in systolic Ca release from the SR (Morgan, 1985; Akera, 1990). While this may be a dominant contributor to the inotropic effect, it may not be that simple.

For example, if the SR is inhibited by caffeine or ryanodine, the inotropic effect of ACS can be just as large as under control conditions (see Fig. 96). While this result does not mean that the increase in SR Ca does not normally play an important role, it does

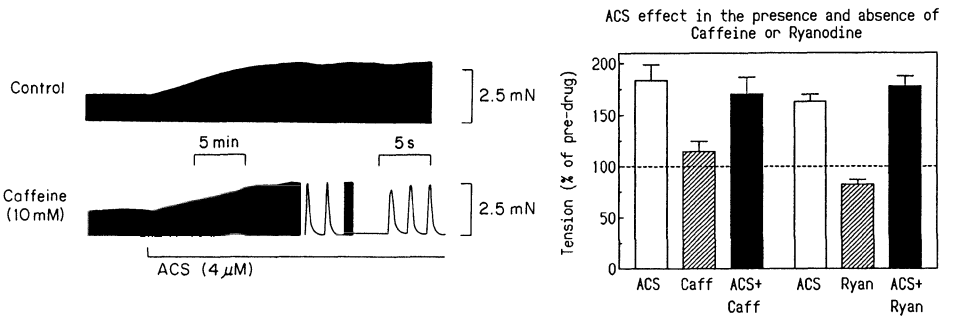


Figure 96. Acetylstrophanthidin (ACS) increases twitch tension in the absence or presence of caffeine or ryanodine. Rabbit ventricular muscle (at 30°C, 0.5 Hz) was exposed to 4 μM ACS. After washout, the muscle was equilibrated with 10 mM caffeine and then ACS was applied again. ACS increased force by a similar amount in control and after equilibration with 10 mM caffeine or 500 nM ryanodine (right, adapted from Bers, 1987b, with permission).

demonstrate that the inotropy still occurs without a normally functioning SR. Thus, changes in diastolic $[Ca]_i$ and transsarcolemmal Ca fluxes may also contribute importantly to the glycoside inotropy.

Na-pump inhibition and elevation of aNa_i is expected to favor Ca influx via Na/Ca exchange and make Ca extrusion less favorable. Under normal conditions the amount of Ca influx via Na/Ca exchange is probably insufficient to activate appreciable contraction. This can be appreciated by the ability of the Ca channel antagonist nifedipine to virtually abolish contraction under control conditions (and in the presence of caffeine or ryanodine, see Fig. 97A). However, when aNa_i is increased by the Na-pump inhibitor ACS (with the SR suppressed by caffeine or ryanodine) contraction is not very strongly inhibited by nifedipine, despite a large decrease in action potential duration (Fig. 97). Increasing action potential duration back to control under these conditions can also return twitches to roughly control levels (Bers *et al.*, 1988). This indicates that when aNa_i is elevated, enough Ca can enter the cell via Na/Ca exchange to activate contraction directly. It has also been suggested that Ca influx via Na/Ca exchange can "trigger" SR Ca release (Bers et al., 1988; Leblanc & Hume, 1990). While this "triggering" action may still be controversial, it is clear that with high aNa_i large amounts of Ca can enter the cell via Na/Ca exchange (see also pages 80-81). But *does* Ca influx via Na/Ca exchange increase measurably with Na-pump inhibition?

I studied the influence of ACS on transsarcolemmal Ca fluxes in rabbit ventricle using extracellular Ca microelectrodes (Bers, 1987b). ACS sometimes increased Ca_o depletion (or net Ca uptake) in a simple manner (Fig. 98A). Sometimes ACS increased the initial rate of Ca_o depletion, which then gave way to a net efflux of Ca during the contraction (Fig. 98B, second trace). This is reminiscent of the Ca efflux observed during

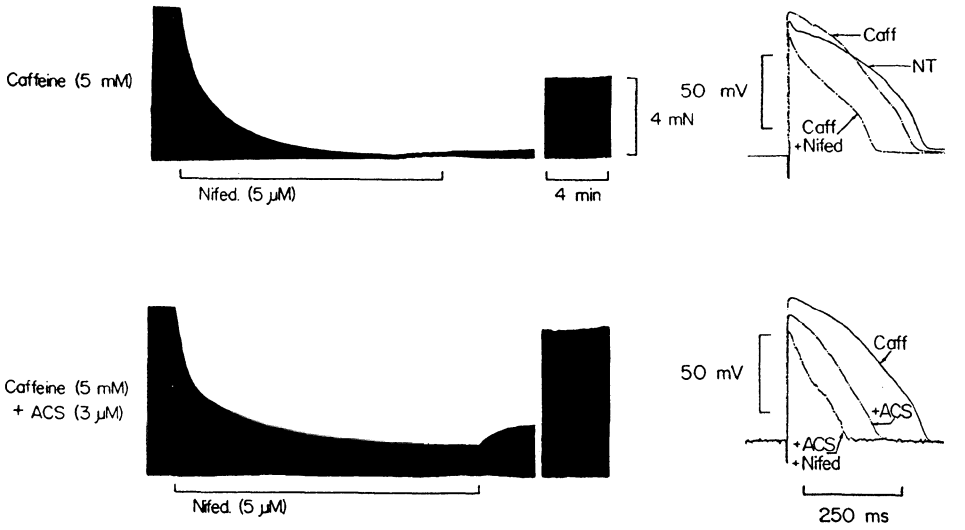


Figure 97. The Ca channel blocker, nifedipine, nearly abolishes twitch tension under control conditions (not shown) or in the presence of 5 mM caffeine (top). However, after Na-pump inhibition (by ACS), nifedipine does not abolish tension, even though the action potential duration is greatly reduced (right). Experiments were with rabbit ventricular muscle at 30°C, stimulated at 0.5 Hz (adapted from Bers *et al.*, 1988, with permission).

contraction in rat ventricle (Fig. 80) and may have the same cellular basis. That is, the large SR Ca release (twitch force was 225% of control in Fig. 98B) and the shorter action potential in ACS, may stimulate Ca extrusion via Na/Ca exchange (much as in the rat ventricle in Fig. 81).

The fact that Ca efflux occurs during contraction and probably overlaps temporally with Ca influx makes it hard to assess changes in unidirectional Ca influx. To limit this problem, I studied the effect of ACS on Ca influx under conditions where Ca efflux is minimized (Fig. 99). This occurs at the first few post-rest contractions in the presence of ryanodine. As discussed on pages 105-107, ryanodine causes rapid SR Ca depletion at rest, which is reflected by the rapid increase in $[Ca]_o$ when stimulation is stopped in Fig. 99A *vs* C. Thus, at the first post-rest contraction, the SR is empty and may accumulate much of the Ca which enters the cell at the first contraction. The post-rest contraction is small and the presumed low $[Ca]_i$ will not stimulate much Ca efflux via Na/Ca exchange. Therefore only Ca influx is appreciable during the first two post-rest contractions and Ca_o depletion occurs without recovery between beats (Fig. 99C or E). During the first few pulses, the amount of Ca_o depletion increases with each beat, consistent with the "staircase" of Ca current (Fig. 29) and possibly more Ca entry via Na/Ca exchange. Addition of ACS in control (Fig. 99B) prevents the loss of SR Ca during rest (i.e. slowed rest decay) and no slow rise in $[Ca]_o$ is seen during rest. Since the SR and cell did not lose Ca during rest, the

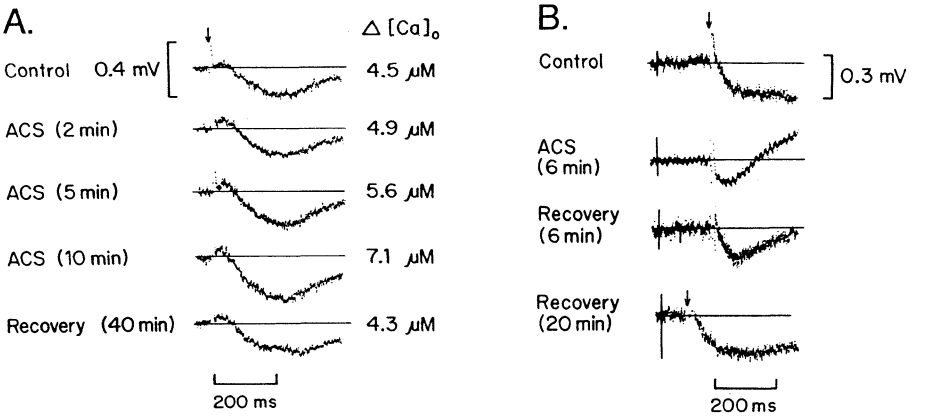


Figure 98. Extracellular Ca depletions in rabbit ventricular muscle measured with Ca-selective microelectrodes (30°C, 0.5Hz, $[Ca]_o$=0.3 mM). A. Na-pump inhibition (by ACS) increased the amplitude of Ca_o depletions (see numbers at right), reflecting more Ca entry into the cell. B. ACS increased the initial rate of Ca_o depletion, but then Ca started to come out of the cells, as evidenced by the increase in $[Ca]_o$ (from Bers, 1987b, with permission).

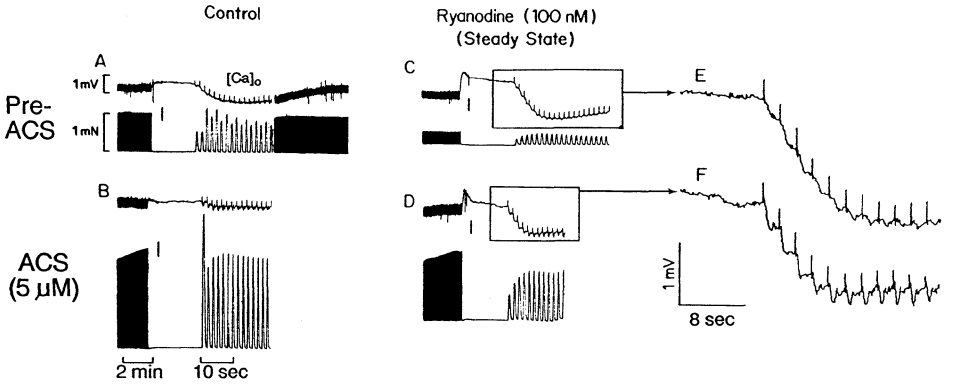


Figure 99. Effects of Na-pump inhibition (by ACS) on Ca fluxes and contractions in rabbit ventricular muscle in the absence (A,B) and presence of 100 nM ryanodine (C-F). Extracellular [Ca] was measured with a Ca-selective microelectrode and downward deflections reflect Ca_o depletion and net Ca uptake by the cells. The vertical bars indicate a change in recording rate. ACS was applied several min before B and washed out prior to equilibration with ryanodine (C & E). Then ACS was again added in the continued presence of ryanodine (D & F). The conditions were 30°C, 0.5Hz, $[Ca]_o$=0.3 mM (modified from Bers, 1987b, with permission).

post-rest contraction is large and no cumulative Ca_o depletion occurs (i.e. the SR is already Ca loaded). When ACS is applied in the presence of ryanodine (Fig. 99D), the SR is still

emptied during rest, although more slowly than with ryanodine alone (Fig. 99C). ACS also slows the ryanodine-induced loss of SR Ca in rabbit ventricle assessed by RCCs (Bers & Christensen, 1990).

With the background above we can now compare the effect of ACS on Ca influx in Fig. 99E *vs* F, under conditions where Ca efflux has been minimized. At the first post-rest contraction, ACS substantially increases the initial rate of Ca_o depletion (~6 fold) and the extent (~2 fold). As steady state is approached (where Ca influx = Ca efflux over each cycle) both Ca influx and Ca efflux appear to be increased by ACS. The former is presumably due to greater Ca influx via Na/Ca exchange favored by the elevated aNa_i. The latter probably results from the increased peak $[Ca]_i$ during the twitch which "drives" Ca efflux via Na/Ca exchange and overcomes the shift in $E_{Na/Ca}$ favoring Ca influx. Ryanodine helps to temporally separate Ca influx and efflux phases since peak $[Ca]_i$ occurs later. This allows us to conclude that Na-pump inhibition can lead to increased Ca influx and efflux during the action potential (whereas Pizarró *et al.* 1985 did not discern any effect of 1 μM strophanthidin on Ca fluxes in frog ventricle). Le Grand *et al.* (1990) recently showed that ouabain increases both L- and T-type Ca currents. It is possible that the higher average $[Ca]_i$ in the presence of Na-pump inhibition contributes to this increase of I_{Ca} as suggested by Marban & Tsien (1982). This I_{Ca} facilitation may occur in a manner similar to the I_{Ca} staircase phenomenon shown in Fig. 29.

In conclusion, the inotropy induced by cardioactive steroids can be attributed primarily to Na-pump inhibition and consequent shifts in Na/Ca exchange, making Ca influx more favorable and Ca efflux less favorable. During an individual contraction these shifts can increase contraction by increasing diastolic $[Ca]_i$, increasing SR Ca content (and release) and increasing Ca influx early in the contraction. These are of course all interrelated and it is difficult to determine unequivocally the fractional contribution of each effect. When the cell gains too much Ca due to the shift in Na/Ca exchange, negative inotropic and arrhythmogenic effects occur. These will be discussed in the next section.

Ca MISMANAGEMENT AND NEGATIVE INOTROPY

Ca Overload and Spontaneous SR Ca Release

At high concentrations of cardioactive steroids the positive inotropic action gives way to a negative inotropic effect (e.g. see Fig. 95). Other toxic effects of glycosides also become apparent: 1) higher increased resting force, 2) oscillatory after-contractions and 3) oscillatory afterdepolarizations. It now seems clear that all of these effects are secondary to cellular Ca overload and spontaneous Ca release from the SR during diastole.

Fabiato & Fabiato (1972) observed that cyclical contractions occured in skinned rat ventricular myocytes when the bathing [Ca] was $\geq$100 nM. They attributed these to "spontaneous release" of Ca from the SR. Lakatta & Lappé (1981; Lappé & Lakatta,

1980) reported changes in scattered light intensity fluctuations (SLIF) from intact multicellular preparations. These changes in light scattering were also attributed to spontaneous SR Ca release and SLIF was increased by elevation of $[Ca]_o$ or application of ouabain. Interestingly, rat ventricle often shows spontaneous SR Ca release and SLIF under normal resting conditions, whereas rabbit ventricle only shows them at high $[Ca]_o$ or with Na-pump inhibition (Kort & Lakatta, 1984, 1988b; Capogrossi *et al.*, 1986a). This is consistent with the notion raised in Chapter 8 that resting rat ventricular muscle is "living on the edge" in terms of Ca balance (i.e. that it gains Ca during rest because resting aNa_i is high, Shattock & Bers, 1989). Furthermore, spontaneous oscillations in rat ventricle were suppressed for several sec after a stimulated (synchronous) contraction (Capogrossi & Lakatta, 1985; Kort & Lakatta, 1988a). If that stimulated contraction "drives" net Ca efflux via Na/Ca exchange (as in Fig. 80) and decreases the cellular and SR Ca content, it may take several sec for cellular Ca to increase back to the point where spontaneous Ca release occurs again. With higher Ca overload this delay can be abolished and SLIF can then be elevated immediately after the twitch (Kort & Lakatta, 1988a). This is what one would expect as the Ca overload (and Ca influx during the twitch) increase to the point where they exceed the rate at which the cell can extrude Ca (i.e. at sufficient aNa_i and Ca overload, the SR in rat ventricle SR may not "unload" during contraction).

Spontaneous fluctuations of $[Ca]_i$ are also associated with this spontaneous contractile activity and $[Ca]_i$ may reach very high levels ($\geq$10 μM) (Orchard *et al.*, 1983; Wier *et al.*, 1983; Allen *et al.*, 1984a, 1985b; Eisner & Valdeolmillos, 1986). The frequency and amplitude of these oscillations increase with increasing $[Ca]_o$ or Na-pump inhibition (up to a maximal fundamental frequency of ~4 Hz based on Fourier analysis). Both the $[Ca]_i$ and tension fluctuations were abolished by caffeine or ryanodine.

The functional consequence of these spontaneous SR Ca releases can be appreciated from Fig. 100. When a stimulated twitch occurs soon after a spontaneous release, the stimulated Ca_i transient and contraction are depressed (Allen *et al.*, 1985b; Capogrossi *et al.*, 1986b; Capogrossi & Lakatta, 1989). The weaker Ca release may result from either incomplete mechanical restitution (e.g. refractoriness of the SR Ca release channel, Ishide *et al.*, 1990) or a net loss of Ca from the cell (e.g. due to the large SR Ca release at negative E_m where Ca extrusion by Na/Ca exchange is favored). In a multicellular preparation such spontaneous Ca release can occur in random cells, but as the number of Ca overloaded cells increases a progressive decline in contractile force is expected. However, the negative inotropic effect in multicellular preparations is much more serious than this simple additive expectation. This is because the cells are not independent. A weakly activated cell will have high compliance and be stretched by more fully activated cells. Such a fully activated cell will also produce less force and shortening velocity as a consequence of its shorter sarcomere length. Additionally, the shortening that was required to stretch the more compliant cell will not contribute to external work. Thus, the negative consequences of these inhomogeneous spontaneous Ca releases are greatly

amplified. These spontaneous contractions may thus limit the extent to which twitches can be potentiated by mechanisms that increase Ca_i load (e.g. cardioactive steroids, reduced $[Na]_o$, elevated $[Ca]_o$ etc., Capogrossi *et al.*, 1988).

In intact single myocytes, these spontaneous contractions are typically manifest as propagating waves (~100 μm/sec) of contraction starting from an initiating focus or multiple foci (Stern *et al.*, 1984; Kort *et al.*, 1985; Capogrossi & Lakatta, 1985; Capogrossi *et al.*, 1987; Wier *et al.*, 1987; Berlin *et al.*, 1989; Takamatsu & Wier, 1990). The Ca_i transients and contraction amplitude can be comparable to a twitch contraction (see Fig. 100). However, these contractions are typically damped by their slow propagation in comparison to the twitch where $[Ca]_i$ changes almost uniformly within the cell (Wier *et al.*, 1987). Such contractile waves have also been reported to propagate from a damaged site in thin multicellular preparations (Mulder *et al.*, 1989; Daniels & ter Keurs, 1990). Thus, it seems probable that the propagation can procede from one cell to the next.

As discussed in Chapter 7 and by Takamatsu & Wier (1990), the mechanism for these spontaneous SR Ca releases and waves in cardiac myocytes is not clearly defined. While some investigators attribute them to propagated Ca-induced Ca-release (Mulder *et al.*, 1989; Backx *et al.*, 1989), Fabiato (1983) has pointed out that SR Ca release in these Ca overload conditions is fundamentally different. In particular, the process described by Fabiato would be inactivated by the elevated Ca. O'Neill *et al.* (1990b) also showed directly that Ca-induced Ca-release in non-overloaded cells would not propagate. It is possible that the intra-SR [Ca] (or trans-SR [Ca] gradient) is important in the spontaneous SR Ca release associated with Ca overload. A simple model is to assume that when the SR Ca content reaches some critical level, the release channel opens. This may still propagate as the Ca released from one SR zone may cause the Ca content of a neighboring zone to reach the critical level for spontaneous Ca release. At this point it is still not entirely clear whether the "physiological" Ca-induced Ca-release mechanism is involved.

RCCs are not appreciably reduced at high concentrations of ACS, where twitch force is depressed (see Fig 95). This may be due to the synchronization of SR Ca release at an RCC, and the fact that the RCC reflects the sum of the SR Ca released and the cytosolic Ca. The plateau of RCC amplitude at high [ACS] in Fig. 95 may also indicate that the mean cellular Ca (SR + cytosolic) does not decrease. On the other hand, a progressive increase of Ca uptake might be expected, but could be partially limited by Ca extrusion during contractions when $[Ca]_i$ is high.

These spontaneous Ca releases also appear to be synchronized by electrical stimulation and may lead to the aftercontractions and afterdepolarizations commonly associated with Ca overload and digitalis toxicity (e.g. see Fig. 101). The same mechanism is probably responsible for the spontaneous SR Ca release and the aftercontraction (Stern *et al.*, 1983; Allen *et al.*, 1984a). The mechanism of synchronization is not obvious, because if the stimulated contraction simply overrides the local spontaneous cycle, one would not

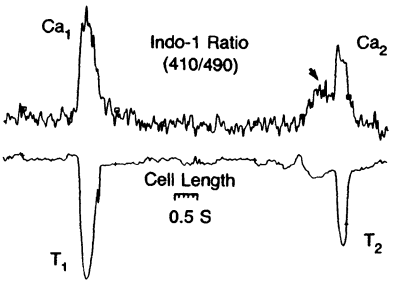


Figure 100. The appearance of a spontaneous Ca_i transient and contraction during the diastolic interval in a rat ventricular myocyte stimulated at 0.2 Hz and with 3 mM $[Ca]_o$ at 23°C. $[Ca]_i$ was measured by indo-1 fluorescence (from Lakatta *et al.*, 1989, with permission).

expect a depression of the contraction following a spontaneous release. It is possible that the stimulated contraction leads to inactivation of Ca release throughout the fiber, so that as all the SR regions recover from inactivation they reach the point of spontaneous release more contemporaneously. After a stimulated contraction, the level of Ca overload may also be increased because of Ca entry (via I_{Ca}) during the action potential. Higher Ca overload also increases the frequency of spontaneous SR Ca release and may thereby increase the synchrony of aftercontractions.

The progressive decline in amplitude of oscillatory aftercontractions (see Fig. 101) may be partly due to desynchronization. It might also be partly due to a real decline in the amplitude of Ca release in each cell as the cell recovers from the acute Ca load associated with the action potential and Ca current. Part of this recovery may, in fact be due to Ca extrusion via Na/Ca exchange which will be favored at diastolic E_m, especially during a Ca release. In support of this, the amplitude of cyclical contractions can remain constant in skinned cells where Ca efflux cannot occur (Fabiato & Fabiato, 1978b). Additionally inward current which is at least partly attributable to Ca extrusion via Na/Ca exchange is associated with the aftercontraction (see below). It seems probable that both desynchronization and net Ca loss contribute to the progressive decline in the amplitude of aftercontractions.

Afterdepolarizations and Triggered Arrhythmias

Aftercontractions are associated with afterdepolarizations, but it is now clear that the release of intracellular Ca is responsible for both. That is, release of SR Ca activates the myofilaments (aftercontraction), but also activates a transient inward current (I_{ti}) which is responsible for the afterdepolarization (Lederer & Tsien, 1976; Kass *et al.*, 1978). Ca_i can activate two currents which may contribute to I_{ti}: the Na/Ca exchange current ($I_{Na/Ca}$) and a nonspecific cation current (Colquhoun *et al.*, 1981). Cannell & Lederer

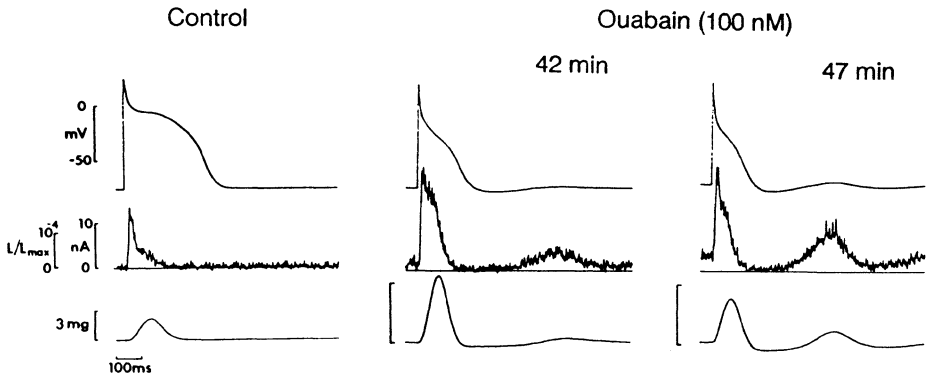


Figure 101. Ouabain-induced afterdepolarizations and aftercontractions in a canine cardiac Purkinje fiber at 35°C, 1 Hz. Addition of the Na-pump inhibitor decreased action potential duration, increased the Ca_i transient (assessed by aequorin luminescence) and increased contractile force. However, eventually afterdepolarizations, "afterglimmers" and aftercontractions become apparent (modified from Wier & Hess, 1984, with permission).

(1986) found substantial I_{ti} in sheep cardiac Purkinje fibers in the absence of Na_o (i.e. where $I_{Na/Ca}$ could not be responsible). Their experiments indicated that the Ca_i-activated nonspecific cation current can contribute to I_{ti} and that Na or Ca could carry the inward current. There is also strong evidence that $I_{Na/Ca}$ contributes to I_{ti} (Arlock & Katzung, 1985; Karaguzian & Katzung, 1982; Noble, 1984; Mechman & Pott, 1986; Lipp & Pott, 1988). Indeed, Fedida *et al.* (1987b) concluded that most of the I_{ti} observed in their guinea-pig ventricular myocytes was attributable to $I_{Na/Ca}$ (based on removal of Na_o). Kimura (1988) also concluded that ~85% of I_{ti} in guinea-pig ventricular myocytes was attributable to $I_{Na/Ca}$ (at E_m=-80 mV and $[Ca]_i$=0.5 μM). The Ca_i-activated nonspecific cation current was responsible for the remaining 15% of I_{ti}. This conclusion is perhaps not surprising since, as discussed on pages 78-86, Na/Ca exchange can extrude Ca rapidly and generate a substantial inward current in doing so.

These I_{ti} are responsible for the oscillatory afterdepolarizations observed with Ca overload. The development of the oscillatory afterdepolarizations can, in turn lead to triggered arrhythmias in the heart (Ferrier & Moe, 1973; Ferrier *et al.*, 1973; Rosen *et al.*, 1973a,b; Ferrier, 1977; Wit & Rosen, 1986). Figure 102 shows that in an isolated myocyte spontaneous waves can lead to sufficient depolarization to reach the threshold for triggering an action potential (Capogrossi *et al.*, 1987). These authors also showed that Ca release induced by caffeine application could induce an action potential. Thus, the process of Ca overload can lead to triggered arrhythmias via a fairly well defined sequence of events. While the Ca overload secondary to Na-pump inhibition is perhaps the most extensively studied, this same sequelae occur with other causes of cellular Ca overload.

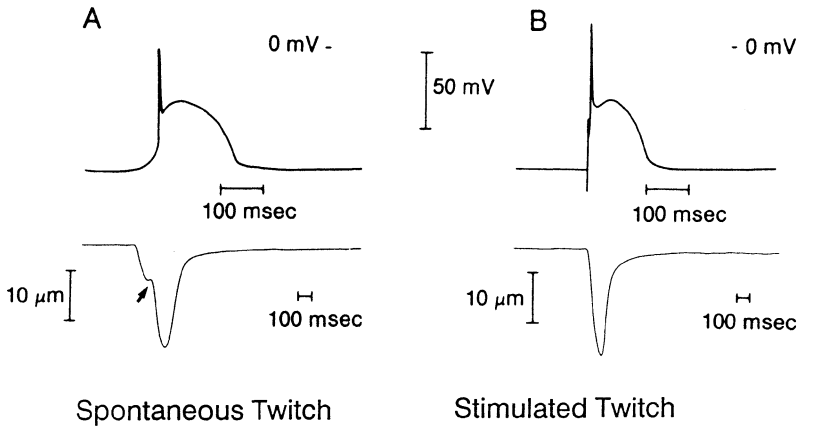


Figure 102. Membrane potential and cell length during a twitch resulting from a multifocal spontaneous Ca release (A) and an electrically driven twitch (B) in a rat ventricular myocyte (at 37°C). These contractions are considered to be multifocal whenever two waves are moving in one cell, even if they both originated from a single site near the middle of the cell. Multifocal contractions more often depolarize the cell sufficiently to trigger an action potential (from Capogrossi *et al.*, 1987, with permission).

Other causes of Ca overload may include reduction of $[Na]_o$, increased Na permeability (e.g. with monensin), elevated $[Ca]_o$, Ca channel agonists, large depolarizations, high frequency stimulation, decreased membrane Ca or Na permeability barrier or decrease in energy supply required to maintain normal ionic gradients.

In conclusion, cellular Ca overload can lead to spontaneous releases of SR Ca which can contribute to:

1) Increased mean resting $[Ca]_i$ (basal and average from spontaneous SR Ca releases),
2) Increased diastolic force,
3) Greater inactivation of Ca-induced Ca-release at a normal pulse,
4) Asynchrony of Ca release (due to refractoriness in areas of recent Ca release),
5) Reduced twitch force
6) Partial synchronization of spontaneous release after systole
   (due to the bolus of Ca influx and maybe synchrony of SR Ca release inactivation),
7) An added compliance that contracting cells must stretch,
8) Ca_i-dependent increase of inward I_{ti} ($I_{Na/Ca}$ and Ca-activated nonselective I),
9) Depolarization (afterdepolarization),
10) Triggered arrhythmias

Acidosis

Acidosis has been known to depress myocardial contractility for more than 100 years (Gaskell, 1880), and it is important to consider because acidosis is a major consequence of myocardial ischemia and may contribute to the ischemic decline in force.

The situation is potentially complicated by the fact that changing pH can modify virtually every cellular system involved in Ca regulation and force development. Nevertheless, some important conclusions can be drawn from experimental work aimed at evaluating this complex problem (see review by Orchard & Kentish, 1990).

Respiratory acidosis (created by increasing extracellular CO_2) produces much more rapid decline in contraction than does metabolic acidosis (e.g. induced by decreasing $[HCO_3]_o$, Fry & Poole-Wilson, 1981). Since respiratory acidosis changes intracellular pH much faster than does metabolic acidosis, this has been taken as evidence that the major negative inotropic effect is due to intracellular (rather than extracellular) acidosis. Thus, even though low pH_o inhibits Ca current (Irisawa & Sato, 1986; Krafte & Kass, 1988) and would thereby decrease contraction, that effect must not be major. On the other hand intracellular acidosis can also decrease I_{Ca} (Sato *et al.*, 1985; Irisawa & Sato, 1986; Kaibara & Kameyama, 1988), which would also be expected to decrease the Ca_i transient in the cell.

Indeed, many of the individual effects of decreased pH_i may be expected to diminish the Ca_i transient. It was therefore somewhat surprising when it was demonstrated that acidosis increases the amplitude of the Ca_i transients measured with aequorin, but decreases contractile force (see Fig. 103, Allen & Orchard, 1983; Orchard, 1987; Allen *et al.*, 1989). This suggests that the major negative inotropic effect of acidosis may be at the level of the myofilament responsiveness to $[Ca]_i$.

Acidosis does indeed decrease the myofilament Ca sensitivity and decreases the

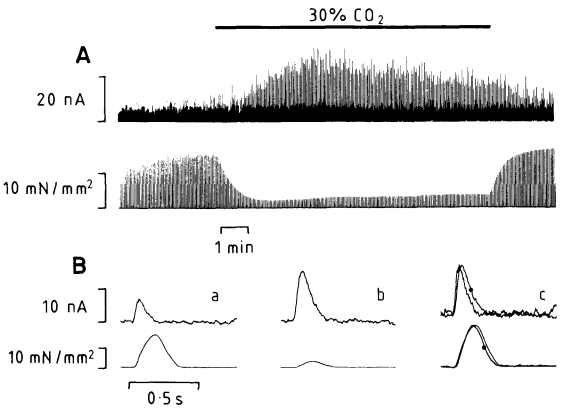


Figure 103. Changes in the Ca_i transient (top) and contraction (bottom) during respiratory acidosis in ferret papillary muscle (pH_o was reduced from 7.4 to 6.7 by increasing $[CO_2]$ at 30°C and 0.33 Hz). The Ca_i transient was monitored using aequorin. Note that the peak $[Ca]_i$ is increased during acidosis and although tension falls rapidly there is a partial recovery. B. averaged aequorin and tension traces for control (a), after 1.5 min of acidosis (b) and with those traces scaled and superimposed (where • denotes the trace during acidosis, from Orchard and Kentish, 1990, with permission).

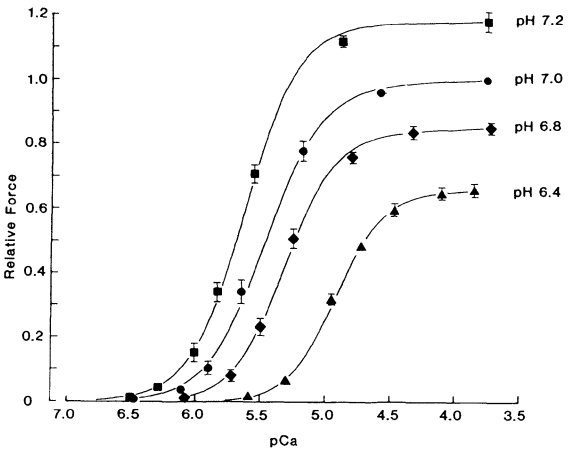


Figure 104. The effect of pH on the myofilament Ca sensitivity in triton-"skinned" guinea-pig ventricular muscle at 30°C. Force is normalized to the maximum at pH 7.0 (from Orchard and Kentish, 1990, with permission).

maximum force production (Fig. 104, Donaldson & Hermansen, 1978; Fabiato & Fabiato, 1978a; Kentish & Nayler, 1979; Blanchard & Solaro, 1984). Blanchard & Solaro (1984) concluded that much of the shift in myofilament Ca sensitivity could be attributed to a decrease in the affinity of ^{45}Ca binding to cardiac troponin C. The effect of pH on Ca binding to troponin C is also amplified by a pH-sensitive change in the affinity of troponin I for troponin C (El-Saleh & Solaro, 1988; Solaro *et al.* 1989). The reduction in maximum force appears to be a separate effect and also amplifies the depressant effect of acidosis on force development (Orchard & Kentish, 1990). Acidosis decreases maximum force much more than it does myofibrillar ATPase activity (Kentish & Nayler, 1979; Blanchard & Solaro, 1984). This may mean that acidosis decreases the maximum force (and efficiency) of each crossbridge as well as the turnover rate of the ATPase. These effects of pH on myofilament Ca sensitivity can thus be expected to be of major importance in the negative inotropic effect of acidosis.

The surprising increase of the Ca_i transient during acidosis merits some further discussion. As presented in Fig. 94, an increase of diastolic $[Ca]_i$ could contribute to an increase in peak $[Ca]_i$. Aequorin is very sensitive to systolic changes in $[Ca]_i$, but is not particularly sensitive to diastolic $[Ca]_i$ changes. Studies using Ca-selective microelectrodes and newer fluorescent Ca indicators have demonstrated that intracellular acidosis also increases diastolic $[Ca]_i$ (Bers & Ellis, 1982; Khomoto *et al.*, 1990, Nakanishi *et al.*, 1990). This might then, by itself contribute to the larger Ca_i transient during acidosis in Fig. 103. The increased intracellular $[H^+]$ could increase diastolic $[Ca]_i$ by competing with Ca ions for intracellular Ca binding sites (Bers & Ellis, 1982; Vaughan-Jones *et al.*, 1983).

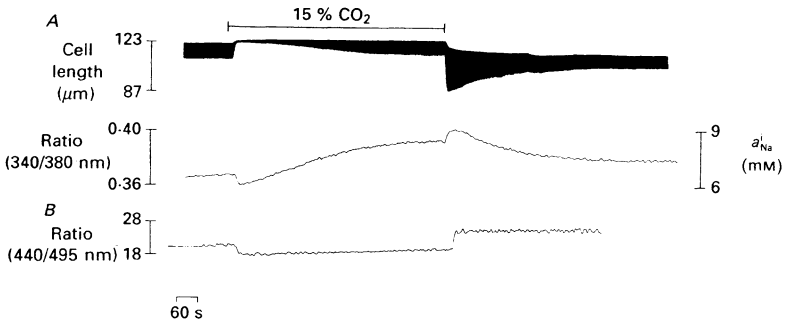


Figure 105. Respiratory acidosis decreases contraction (top), and pH_i (bottom, assessed by BCECF fluorescence) in a rat ventricular myocyte at 26°C, stimulated at 1 Hz. Intracellular Na activity (assessed by SBFI fluorescence) gradually increases during acidosis. This increase aNa_i may contribute to the contractile recovery (via a shift in Na/Ca exchange). The initial decrease in the SBFI fluorescence trace was attributed to a pH effect on SBFI (from McCall *et al.*, 1990, with permission).

However, this should only be a transient effect, since the $[Ca]_i$ should eventually return to control level as transport systems remove the Ca displaced from binding sites. The transient nature of this effect might contribute to the gradual decline in the Ca_i transients during sustained acidosis (Fig. 103).

Low intracellular pH also stimulates proton extrusion via Na/H exchange, especially when pH_o is relatively normal. Indeed, for acid loads, cardiac cells appear to rely on Na/H exchange for pH_i regulation, while for alkali loads the Cl/HCO_3 exchange appears to be more important (Vaughan-Jones, 1982; Piwnica-Worms *et al.*, 1985; Ellis & MacLeod, 1985). Thus, extrusion of protons via Na/H exchange increases aNa_i, particularly at normal pH_o (Deitmer & Ellis, 1980; Bountra & Vaughan-Jones, 1989). Normally, it might be expected that the sarcolemmal Na-pump would reduce the aNa_i back to the control level, but decreasing pH below 7.5 also inhibits the Na-pump (e.g. Sperelakis & Lee, 1971). The result is that aNa_i increases during respiratory acidosis with a time course similar to the slow recovery phase of contraction (Fig. 105, McCall *et al.*, 1990). Thus, the increase in aNa_i may be responsible for the slow recovery of contractile force via a shift in Na/Ca exchange. Under conditions where extracellular pH was held constant Bountra & Vaughan-Jones (1989) showed that a decrease in pH_i could lead to an increase in aNa_i and twitch tension in guinea-pig papillary muscle. They attribute this positive inotropic effect to shifts of Na/Ca exchange which were sufficient to overcome the largely depressant effects of intracellular acidosis. It seems possible then, that the gain in cellular Na (and consequently Ca) might contribute to the increased Ca_i transient observed with acidosis.

The Na/Ca exchange system is also inhibited by low pH (Philipson *et al.*, 1982). Together with the increased aNa_i discussed above the ability of the cell to extrude Ca via Na/Ca exchange may be severely compromised. As with cardiac glycosides, this will contribute to the larger Ca_i transients seen during acidosis, but could also lead to Ca overload and consequently to arrhythmias (Coraboeuf *et al.*, 1976; Kurachi, 1982). If spontaneous SR Ca release occurs during acidosis, the negative inotropic effects would be further amplified (as discussed on pages 186-188).

Acidosis also decreases cardiac SR Ca uptake in both isolated SR vesicles and "skinned" myocytes (see Chapter 6, Shigekawa, 1976; Mandel *et al.*, 1982; Fabiato & Fabiato, 1978a; Fabiato, 1985e). However, it is possible that the increased Ca load described above may more than compensate for the inhibitory effect of pH on SR Ca uptake. SR Ca content could then be higher than normal during acidosis. The release of Ca from the cardiac SR also appears to be decreased by acidosis. Fabiato (1985e) showed that the optimal free [Ca] trigger for Ca-induced Ca-release was higher at low pH (i.e. a larger "trigger" Ca is required). The opening of isolated SR Ca release channel is also inhibited by acidosis (Ma *et al.*, 1988; Rousseau & Pinkos, 1990). The result is that we might expect a lower fractional release of Ca from the SR at low pH, but again this might be partially offset by a larger SR Ca content.

So far the increased Ca_i transient has been assumed to reflect a greater amount of Ca added to the cytoplasm (i.e. SR Ca release plus Ca influx). However, acidosis also decreases the Ca binding to the myofilaments (Fig. 104, Blanchard & Solaro, 1984). Thus, even an unchanged SR Ca release will be expected to increase the amplitude of the Ca_i transient. This can be appreciated in quantitative terms using Fabiato's (1983) estimations of intracellular Ca buffering constituents (see Figs. 19, 20 & 94 and page 47). For a Ca_i transient from 0.1 μM to 1 μM $[Ca]_i$, requires the release (or influx) of ~50 times that amount into the sarcoplasm (i.e. 50 μmol/liter non-mitochondrial cell volume or 20 μmol/kg wet wt). Since troponin C is responsible for about 40% of the Ca buffering at this $[Ca]_i$, reduction of the affinity of troponin C for Ca will increase peak $[Ca]_i$ and Ca binding to all of the other sites (assuming their affinity is not similarly reduced). Using a decrease in Ca affinity based on the $K_{1/2}$ values in Fig. 104 (for pH 7.2 and 6.4) the amplitude of the Ca_i transient would be increased about 50%. Therefore, part of the increased Ca_i transient in Fig. 103 might simply be attributable to reduced sarcoplasmic Ca buffering at low pH.

In conclusion, the effects of acidosis on cellular Ca and force production are complex, but some important aspects seem clear. The decrease in myofilament Ca sensitivity and maximum force are probably the main factors responsible for the negative inotropic effect of acidosis. The decrease in myofilament Ca binding may contribute to the increased Ca_i transient. Proton extrusion via Na/H exchange, the inhibition of Na-pump and of Na/Ca exchange may contribute to the increased cellular (and SR) Ca load. This increased cellular Ca load can contribute to the larger Ca_i transient, but can also lead to cellular Ca overload.

Hypoxia and Ischemia

I will not discuss the effects of hypoxia or ischemia on the regulation of intracellular Ca or contractile force in any detail (see reviews by Reimer & Jennings, 1986; Allen & Orchard, 1987; Chien & Engler, 1990; Downey, 1990; Jennings *et al.*, 1990; Tani, 1990). In a very simplistic sense acidosis can be thought of as a more controlled stepping stone toward understanding pathological changes associated with the more clinically relevant situations of hypoxia and ischemia. Indeed, acidosis is an early consequence of hypoxia (due to shifting of metabolism to glycolysis and lactic acid production) and ischemia (where lactic acid and other metabolites are also not washed away). In this context it can be expected that some of the discussion about acidosis above may be extended to hypoxia and ischemia, but the situation becomes much more complex (e.g. with energetic considerations which have not been developed in this book). I will briefly discuss three issues in this arena which are more or less extensions of the foregoing discussions.

As discussed above, acidosis decreases myofilament Ca sensitivity and maximum force. During ischemia (or hypoxia with inhibition of glycolysis) high energy phosphates are gradually depleted and inorganic phosphate (P_i) can increase from ~1 mM to 20 mM (Allen & Orchard, 1987). This high [P_i] by itself depresses the Ca sensitivity of the myofilaments (Herzig & Rüegg, 1977; Kentish, 1986). In combination with the concomitant intracellular acidosis, myofilament Ca sensitivity can be profoundly depressed. The combination may work synergistically if, as in skeletal muscle, the inhibitory form of P_i is diprotonated (H_2PO_4, Nosek *et al.*, 1987). This form would make up a higher fraction of the total P_i pool at lower pH and could exert a more powerful depressant effect. Thus, the decline in force generation in ischemia, as in acidosis is probably largely attributable to decreased myofilament activation. Again, as with acidosis, force can be severely depressed while Ca_i transients remain large (Lee *et al.*, 1988; Allen *et al.*, 1989).

As high energy phosphates are further depleted intracellular [ATP] declines and ATP-sensitive K channels become activated (Noma, 1983). These K channels are inhibited by μM ATP (K_i(ATP) = 20 μM, Nichols & Lederer, 1990) and appear to be preferentially inhibited by ATP produced by glycolysis (Weiss & Lamp, 1987, 1989). There is also evidence that these channels can be activated and can cause shortening of the action potential during the early contractile failure in rat ventricular myocytes under complete metabolic blockade (Lederer *et al.*, 1989). Nichols & Lederer (1990) showed that despite the low K_i(ATP), there are so many of these channels that even a decline in [ATP] to 1 mM (from 5 mM) is sufficient to shorten the action potential duration appreciably as is observed during ischemia. At lower [ATP] the action potential may be effectively prevented. During early ischemia there is also an increase in ^{42}K efflux which can be prevented by glyburide, a selective blocker of the ATP-sensitive K channel (Weiss & Lamp, 1989). Thus, these ATP-sensitive K channels may serve as a protective mechanism, whereby energetically compromised cells can be prevented from depolarizing. Without

depolarization $[Ca]_i$ should not rise phasically and the cell can better conserve energy, since the myofilaments and Ca pumps should have minimal energy consumption in this state.

Ischemia or metabolic blockade produces acidosis as well as a progressive gain in cellular Na and Ca (as discussed above for acidosis, but now with possible energetic limitations). The increase in aNa_i via Na/H exchange is limited by the fact that extracellular pH is also low in ischemia (Bountra & Vaughan-Jones, 1989). Upon reperfusion, the extracellular pH can be quickly returned to normal without recovery of the intracellular acidosis, such that there is a large outwardly directed $[H^+]$ gradient. This is precisely the condition where Na/H exchange can extrude protons rapidly, but at the expense of increasing aNa_i (Bountra & Vaughan-Jones, 1989). The elevation of aNa_i would be expected to increase Ca content via Na/Ca exchange. Lazdunski *et al.* (1985) hypothesized that this mechanism could be responsible for the large cellular Ca accumulation associated with reperfusion after ischemia. While the quantitative contribution of this pathway has not been assessed, it seems likely that it would at least contribute to the Ca overload observed on reperfusion.

In conclusion, I should reiterate that the three topics related to hypoxia and ischemia discussed briefly above were selected for discussion, because they involve the same sort of Ca regulatory systems which I have focused on. For comprehensive discussion of hypoxia or ischemia one of the aforementioned reviews should be consulted.

SITES FOR INDUCTION OF CARDIAC INOTROPY

There are two main means by which cardiac contractility can be increased. First, the amplitude or timecourse of the Ca_i transient can be altered such that more Ca is supplied to the myofilaments. This additional Ca could be due to modification of any of the Ca transport systems previously discussed. Second, the Ca sensitivity of the myofilaments can be increased. This second possibility really includes both increased Ca affinity of troponin C and increased force for a given degree of Ca occupancy of troponin C. Below I will discuss the sites for inotropic intervention with reference to some of the newer inotropic agents which have been more extensively studied. I will focus on some of the advantages and disadvantages of specific strategies, rather than review results with many individual agents. Most of the newer inotropic agents affect several sites, so I will try to discuss the individual sites as well as the integrated effects.

Modulation of Myofilament Ca Sensitivity

Several agents are known to increase the myofilament Ca sensitivity (e.g. caffeine, theophylline, pimobendan, sulmazole, isomazole, adibendan, perhexiline and bepridil, see references on page 30 and Rüegg & Morano, 1989). This strategy is a rather direct means to increase contractility. That is, increasing myofilament Ca sensitivity will lead to greater force for the same amount of activating Ca (SR Ca release + Ca influx). If the

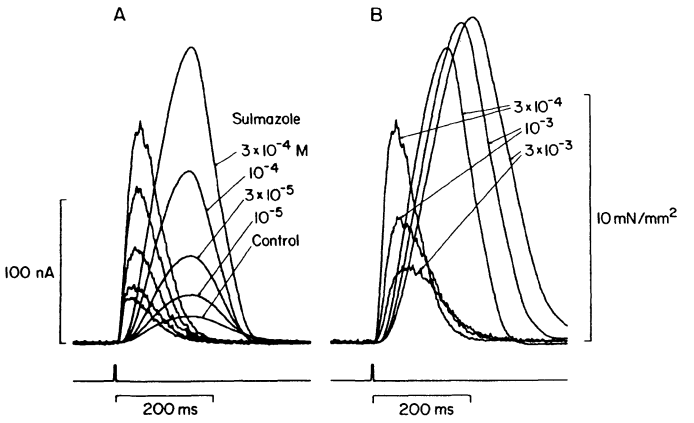


Figure 106. The effect of sulmazole on contractions and Ca_i transients (assessed by aequorin luminescence) in dog ventricle at 37.5°C, 0.5 Hz in the presence of 1 μM bupranolol (a β-adrenergic blocker). A. the peak $[Ca]_i$ and tension both increase with increasing [sulmazole] up to 300 μM. B. as [sulmazole] was further increased, tension continued to increase, but peak $[Ca]_i$ decreased, indicating an increase in myofilament Ca sensitivity (from Blinks & Endoh, 1984, with permission).

sensitization works by increasing Ca affinity of troponin C, the peak $[Ca]_i$ during the contraction is expected to be lower. This is a consequence of the fact that troponin C is a major buffer of Ca_i and >90% of the activating Ca is buffered during the Ca_i transient. Thus the peak $[Ca]_i$ is determined by both the amount of activating Ca and the amount of intracellular Ca buffering. Figure 106B shows that this effect can be seen with sulmazole (AR-L 115 BS), which increases myofilament Ca binding and sensitivity (Solaro & Rüegg, 1982). That is, at higher sulmazole concentration the force increases, but the peak $[Ca]_i$ decreases (Blinks & Endoh, 1986). Unfortunately this interpretation is complicated because sulmazole is not purely a Ca sensitizer. Sulmazole also inhibits a cyclic nucleotide phosphodiesterase (PDE, Endoh *et al.*, 1985) and has some caffeine-like action to open the SR Ca release channel (Williams and Holmberg, 1990). The PDE inhibition may increase cAMP and thereby activate the same cascade of effects as β-adrenergic agonists and cAMP (see above). The net results expected are: 1) an increase in I_{Ca}, 2) an increase or a decrease in SR Ca release (depending on the relative predominance of β-adrenergic *vs* SR Ca release activating effects) and 3) an increase or a decrease in myofilament Ca sensitivity (depending on the relative predominance of direct myofilament sensitization *vs* β-adrenergic induced decrease in Ca sensitivity).

Because of the multiple effects, we do not know whether the amount of activating Ca supplied to the myofilaments is increased or decreased by sulmazole. We may however speculate that the β-adrenergic type effects lead to the effects seen in Fig. 106A at lower sulmazole concentrations. In Fig 106A both the Ca_i transient and contraction are

increased with increasing sulmazole concentrations. The caffeine-like action opening the SR Ca release channel may also contribute to the slowing of the decline in $[Ca]_i$ and prolongation of contraction in Fig. 106B. The myofilament Ca sensitizing effect would also contribute to the prolonged contraction (since the off-rate of Ca from troponin C may be reduced). While these other aspects complicate the interpretation, the increase in force with a decrease in $[Ca]_i$ is strong evidence that the Ca sensitizing effect is important in the inotropic action of sulmazole. A compound which appears at this time to have mainly a Ca sensitizing effect, rather than multiple effects is EMD 53998 (Allen & Lee, 1989; Ferroni *et al.*, 1989).

It is generally accepted that the on-rate of Ca binding to troponin C is so fast that it is effectively diffusion-limited ($k_{on} \sim 4 \times 10^7$ $M^{-1}sec^{-1}$, Robertson *et al.*, 1981). If this is correct, then changes in Ca affinity would depend primarily on changes in the rate of dissociation of Ca from troponin C. Smith & England (1990) found that the rate of Ca dissociation from the bovine cardiac troponin-tropomyosin complex was unaffected by sulmazole or pimobendan, but reduced by isomazole, perhexiline and bepridil. These results are consistent with an increased Ca binding with isomazole, perhexiline and bepridil (Solaro *et al.*, 1986). However, this does not agree with previous results which showed an increase in Ca binding to dog myofilaments with sulmazole or pimobendan (Solaro & Rüegg, 1982; Jaquet & Heilmeyer, 1987). This also raises the point that myofilament Ca sensitivity can be increased without a change in Ca binding. For example, alterations in cross-bridge cycling or the coupling between contractile proteins "downstream" from Ca binding could increase the "gain" of activation by Ca. Along these lines, Rüegg *et al.* (1989) found that peptides derived from the myosin head can alter the interaction between troponin I and actin and thereby increase myofilament Ca sensitivity.

A pure increase in myofilament Ca sensitivity can increase the amount of force for a given amount of activating Ca. A significant fundamental advantage in this strategy is that the balance of transsarcolemmal Ca fluxes need not be altered. Thus, the cells are not subject to Ca overload (and its negative inotropic and arrhythmogenic consequences). A potential disadvantage with Ca sensitizers can be appreciated by considering the parallel shift of the pCa-force relationship in Fig. 107 (dashed curve). This is typical of observations with many Ca sensitizers. The shift of the curve toward lower [Ca] means that incomplete relaxation may become a problem at the diastolic [Ca]. This might be of particular concern in pathological conditions where diastolic $[Ca]_i$ can be elevated. Thus, it becomes crucial to know what the diastolic $[Ca]_i$ is with respect to the threshold for contractile activation (and these values may change with conditions). The dotted curve in Fig. 107 shows an increase in the steepness of the pCa-force relationship (with a smaller shift in $pCa_{1/2}$). This change could be more beneficial, since the threshold for contractile activation is not shifted to lower $[Ca]_i$ and the myofilament Ca sensitivity is greatly increased. However, as a practical matter it should be kept in mind that >90% of the Ca which is released from the SR (or enters the cell) is bound (1 μM free *vs* an added total of

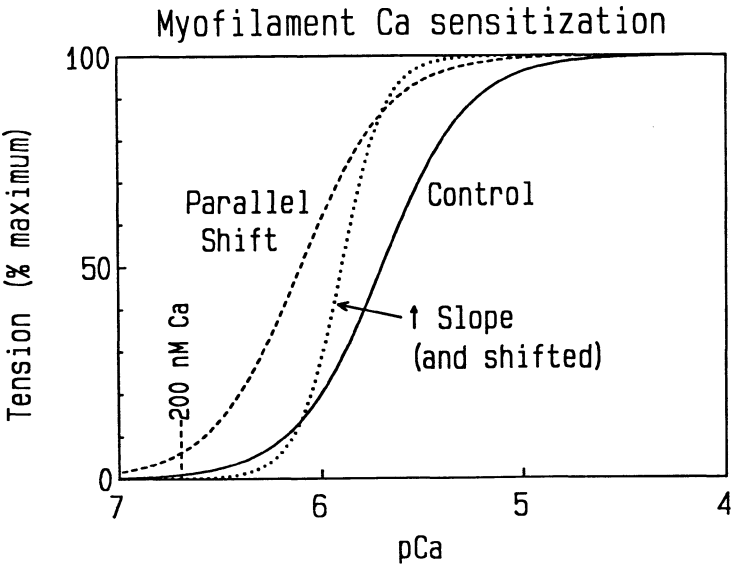


Figure 107. Increasing myofilament Ca sensitivity can limit relaxation. For control, a Hill curve was used with $pCa_{1/2}$=5.7 and n=2. A parallel shift of myofilament Ca sensitivity is assumed to change $pCa_{1/2}$ to 6.1 (with n=2). The myofilament Ca sensitivity can also be increased by a combination of a smaller shift of $pCa_{1/2}$ to 5.9 and an increase in slope (to n=4).

~50 μmol/liter non-mitochondrial cell volume). The steeper pCa-force relationship may allow a greater fraction of the activating Ca to bind to troponin C (rather than other intracellular Ca buffers), but the absolute amount of activating Ca may provide some limitation. That is, if most of the activating Ca is already binding to troponin C, there may be little Ca available for further saturation of troponin C (unless the amount of activating Ca is increased). The other inotropic mechanisms discussed below all deal with changing the amount of activating Ca.

Na/Ca Exchange Modulation

Cardioactive steroids are really the prototypical inotropic agents which function via Na/Ca exchange, even though the Ca movements are secondary to elevation of aNa_i. This has been addressed in detail above (see pages 180-186). I will simply point out that any agent which elevates intracellular Na (or reduces the transsarcolemmal [Na] gradient) will have the same consequences. This includes agents which serve as Na-ionophores (e.g. monensin), or agents which promote Na influx by prolonging the open times of Na channels during the action potential (e.g. veratridine, batrachotoxin, grayanotoxin, DPI 201-106, SDZ 210-921, see Scholtysik *et al.*, 1989). Direct inhibitors of Na/Ca exchange may also be expected to produce the same type of effects (e.g. benzamil, dichlorobenzamil).

Unfortunately, all of the Na/Ca exchange inhibitors identified so far are not selective enough to be useful (see Chapter 5). DPI 201-106 is a Na channel activator which has been extensively studied as an inotropic agent. It does increase contractility and prolongs action potential duration (Buggisch *et al.*, 1985; Scholtysik *et al.*, 1985; Kihara *et al.*, 1989). The increase in action potential duration will also limit Ca extrusion via Na/Ca exchange. Like many other newer inotropes, DPI 201-106 has multiple effects. It now seems clear that it also increases myofilament Ca sensitivity and may also have Ca channel blocking effects (Scholtysik *et al.*, 1985; Siegl *et al.*, 1988; Kihara *et al.*, 1989).

Na channel activation might have some functional advantage over Na-pump inhibition. Leblanc & Hume (1990) suggested that Na entry via Na channels may activate Ca entry via Na/Ca exchange due to the higher $[Na]_i$ near the sarcolemma. They also concluded that this Ca entry could trigger SR Ca release. In this context, the Na channel activators might generate more Ca influx (and SR Ca release) during the action potential, but with less of the global Na and Ca loading which leads to Ca overload. That is, Ca influx via Na/Ca exchange may increase phasically during contraction, but the cell may be able to extrude much of the extra Na and Ca gain during diastole. Though clearly speculative, it is even possible that this sort of enhanced Ca entry via Na/Ca exchange contributes to the positive inotropic effect of certain agents which stimulate Na/Ca exchange (e.g. lauryl sulphate or phospholidase D, Philipson & Nishimoto, 1984; Philipson , 1984; Philipson *et al.*, 1985).

As discussed earlier, Ca overload and the negative inotropic and arrhythmogenic consequences are a general disadvantage for digitalis or any inotropic strategy which shifts the Na/Ca exchange system so that it is less effective at extruding Ca from the cell. Since Na/Ca exchange seems to be the main means by which Ca is extruded from the cell (see Chapter 5), prevention of Ca extrusion can increase cellular Ca load and force. Indeed, from a historical perspective, this has clearly been the most successful type of inotropic agent. However, at higher concentrations (or more effective prevention of Ca extrusion) Ca overload can be expected (see pages 186-191).

Ca Current Modulation

Dihydropyridine Ca channel agonists such as Bay K 8644 can increase I_{Ca} and produce a dramatic positive inotropic effect (Schramm *et al.*, 1983). The potentially great advantage with Ca channel activators is that Ca influx is increased precisely when it can best contribute to inotropy. That is, Ca influx is increased during the action potential, when it can increase: 1) the fraction of SR Ca release (via Ca-induced Ca-release), 2) the amount of Ca supplied directly to the myofilaments and 3) SR Ca loading. During diastole, Ca extrusion via Na/Ca exchange should not be compromised (as it is with Na-pump inhibition). Thus, during diastole the cell may be able to extrude the larger amount of Ca which enters during a steady state action potential in the presence of Bay K 8644. This interpretation is consistent with measurements of net Ca fluxes in rabbit ventricle using

extracellular Ca selective microelectrodes (Bers & MacLeod, 1986). We found that Bay K 8644 does not increase the net Ca uptake nearly as much as an increase in frequency (where Ca efflux may be compromised). Thus, Ca channel activators may produce a large inotropic effect, with less of the Ca overload problem which limits cardiac glycoside action.

The main disadvantage with Ca channel activators is the major effects of these agents on other tissues (e.g. causing smooth muscle vasoconstriction and central nervous system effects). If a Ca channel activator which was highly selective for cardiac muscle Ca channels could be found, it would be an excellent candidate for an inotropic agent. This goal has so far been elusive. Compared to Bay K 8644, Sandoz 202-791 has less vasoconstricting action for a given cardiac inotropic effect (Bechem *et al.*, 1988; Hof *et al.*, 1985) and the Lilly compound (LY249933) produced modest cardiac inotropy and vasodilation (Holland *et al.*, 1989), but these drugs are not nearly selective enough. Given the molecular and functional diversity of Ca channels (see Chapter 4), there is certainly reason to hope that a highly cardiac muscle selective compound can be found. Most of the current work has been with dihydropyridines, but the search need not be limited to this class of compounds. As we learn more about the molecular structure of Ca channels and how they differ, progress in this area could accelerate.

Phosphodiesterase Inhibition

Many inotropic agents being studied are PDE inhibitors. These include caffeine, theophylline, amrinone, milrinone, enoximone, piroximone, saterinone, pimobendan, adibendan and sulmazole (Butcher & Sutherland, 1962; Alousi *et al.*, 1983; Endoh *et al.*, 1985; Weishaar *et al.*, 1988; von der Leyen *et al.*, 1988). There are at least four classes of PDE in the heart (I-IV, see Table 15, Beavo, 1988; Beavo & Reifsnyder, 1990; Schmitz *et al.*, 1989). Caffeine, theophylline, isobutylmethylxanthine (IBMX) and other methylxanthines are relatively non-selective inhibitors of PDE I-III, but most of the more promising new inotropes appear to selectively inhibit PDE III. Agents which inhibit PDE I, II & IV do not appear to be particularly good inotropes. Bode *et al.* (1989) showed that the most abundant PDE in heart (PDE I) was absent in isolated cardiac myocytes, suggesting that PDE I is restricted to other cells in the heart. The four classes of PDE were named, based on their order of elution from DEAE-cellulose columns, but as indicated in Table 15, they can also be characterized by the substrates and regulatory factors that influence their activity.

These PDE III inhibitors prevent the breakdown of cAMP to 5'-AMP and thereby can increase the level of cAMP in the cell. This, of course, can activate the same series of effects activated by β-adrenergic agonists (see above pages 171-174). An advantage over β-adrenergic agonists is that these agents can still inhibit PDE III, even when β-adrenergic receptors are down-regulated and cells become unresponsive to catecholamines. However, if the rate of production of cAMP by adenylate cyclase is very low, PDE inhibition cannot be expected to induce very high levels of cAMP. This limitation does appear to occur in

TABLE 15

Classification of Cardiac Phosphodiesterase Inhibitors (after Schmitz *et al.*, 1989)

Class	Substrates	Key Regulator	Inhibitors
I	cAMP cGMP	Ca-Calmodulin-stimulated	Zaprinast
II	cAMP cGMP	cGMP-stimulated	-
III	cAMP	cGMP-inhibited	Milrinone, amrinone, pimobendan, sulmazole...
IV	cAMP	cAMP-specific cGMP-insensitive	Rolipram, RO 201724

the failing human heart where β-adrenergic down-regulation may also limit cAMP formation (Schmitz *et al.*, 1989; Bristow, 1982, 1986). It is possible to circumvent this limitation by direct activation of adenylate cyclase and cAMP production with forskolin. However, the side effects of forskolin (including excessive vasodilation) are prohibitive.

When cAMP is increased by PDE III inhibition, we expect an increase in I_{Ca}, an increase of SR Ca pumping and a decrease in myofilament Ca sensitivity. While in some cases these effects have been reported, they are again confounded by multiple effects of most of these agents. For example, sulmazole, pimobendan, and adibendan are also direct myofilament sensitizers. This myofilament Ca sensitizing effect might more than offset the Ca desensitizing effect expected from cAMP-dependent phosphorylation of troponin I (see Fig. 106). Sulmazole and milrinone also exhibit caffeine-like actions on the SR Ca release channel, which may supercede the cAMP-depedent phosphorylation of phospholamban and stimulation of the SR Ca pump (Rapundalo *et al.*, 1986; Holmberg *et al.*, 1990; Williams & Holmberg, 1990). Thus the net results on Ca fluxes and contractile force can be rather complicated.

A major advantage with PDE III inhibitors compared to Ca channel activators (and catecholamines) is that the cardiac inotropy is accompanied by vasodilation rather than vasoconstriction. This effect is a consequence of the relaxant effect of cAMP in vascular smooth muscle (Somlyo & Himpens, 1989). This combination of effects is desirable in a cardiac inotrope and the current emphasis on development of these PDE III inhibitors as inotropic agents seems justified. Disadvantages with PDE III inhibitors are side effects and that they are not effective if the cAMP pool is low.

SR Ca Uptake and Release

A final possibility which might be considered is specific modulation of the SR Ca uptake or release process. While some of the above agents stimulate SR Ca uptake (e.g. β-adrenergic type effects) and some promote opening of the SR release channel, perhaps

agents which selectively alter these processes would be beneficial. For example, an agent which interacts specifically with the SR Ca pump or phospholamban to stimulate SR Ca uptake, could increase the amount of SR Ca available for release in cardiac muscle (without increasing Ca influx or desensitizing the myofilaments as with β-adrenergic agonists). Another potentially beneficial example would be an agent which interacts specifically with the SR Ca release channel and stabililizes the channel with respect to the spontaneous Ca release seen with Ca overload. Such an agent would decrease spontaneous SR Ca releases and allow the cell to tolerate a higher Ca load and greater positive inotropic state before it gives way to negative inotropy and arrhythmogenesis. The action would need to be selective for the Ca overload induced SR Ca release, so that normal E-C coupling can still occur. Obviously, this makes it important to answer the question of whether the spontaneous SR Ca release is controlled in a manner which is different than the normal physiological mechanism.

CONCLUSION

As we know more about the molecular nature of the processes regulating Ca movements and contractile force it should be possible to design inotropic agents to act specifically at strategic locations in the heart. A clear understanding of how these processes work and interact is an important prerequisite. I hope that this chapter and this book help the reader develop a greater understanding of the dynamic yet delicate balance of Ca in cardiac muscle cells, particularly as it relates to the control of contractile force.

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