CALCIUM-DEPENDENT FACILITATION OF THE HIGH THRESHOLD CALCIUM CHANNEL CURRENT DURING REPETITIVE STIMULATION IN ISOLATED MYOCYTES OF THE GUINEA-PIG

by

Andrew Christopher Zygmunt

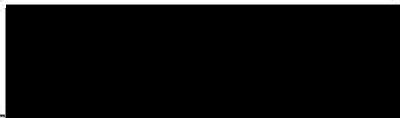
A DISSERTATION

Presented to the Department of Physiology
and the Oregon Health Sciences University
School of Medicine
in partial fulfillment of
the requirements for the degree of

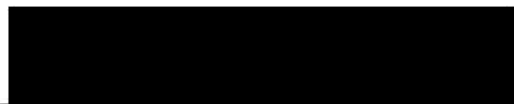
Doctor of Philosophy

April 1989

APPROVED:



(Dr. James Maylie, Assistant Professor in Charge of Thesis)



(Dr. John A. Resko, Chairman, Graduate Council)

TABLE OF CONTENTS

Page

LIST OF FIGURES vi

ACKNOWLEDGEMENTS viii

ABSTRACT ix

INTRODUCTION

Excitation-contraction Coupling in the Heart 1

Cardiac Contractility is Influenced by the Rate and Pattern of Stimulation 6

The Ca Current and Generation of Tension in the Heart 10

Rate-dependent Effects on the Cardiac Action Potential and the Ca Current 19

Modulation of the Ca current 26

Summary and Aims of this Study 31

References of the Introduction 34

MANUSCRIPT: Frequency-dependent Potentiation of High Threshold Calcium Channel Current in Guinea-pig Cardiocytes

SUMMARY 59

INTRODUCTION 62

METHODS

Cell Preparation 65

Solutions 65

Recording Conditions 66

Table of Contents (cont'd.) Page

RESULTS

Effects of Repetitive Depolarization on the Ca Current 69

Contribution of Currents Other Than Calcium 72

Kinetics of the Potentiation Process 76

Ca^{2+} Dependence of High Threshold Current Potentiation 79

The Effects of Decreased Loading and Release of Ca^{2+} from the Sarcoplasmic Reticulum on Potentiation 83

The Effects of Caffeine, Ryanodine and Isoproterenol on High Threshold Current Potentiation 89

DISCUSSION

Calcium Mediated Modulation of the High Threshold Ca Current 98

The Voltage Dependence of the Positive Current Staircase 100

Does the Release of Calcium from the Sarcoplasmic Reticulum Play a Role in Potentiation of the High Threshold Current? 101

Does Calcium Modulate Phosphorylation of the High Threshold Ca Channels During Repetitive Stimulation? 106

Table of Contents (cont'd.) Page

REFERENCES	109
DISCUSSION AND CONCLUSIONS	127
APPENDIX	132

LIST OF FIGURES

		Page
INTRODUCTION		
Figure 1:	Excitation-contraction coupling in the heart.	3
2:	Calcium fluxes in a cardiaocyte.	5
3:	Rate-dependent effects on contractility.	8
4:	Role of sarcoplasmic reticulum in rate inotropy.	18
5:	Ca-dependent reduction of Ca currents.	28
MANUSCRIPT		
Figure 1:	Frequency-dependent effects in low and high threshold Ca channel current.	70
2:	The effects of low threshold channel inactivation on potentiation of high threshold current.	73
3:	Application of 4-aminopyridine and potentiation of high threshold Ca current.	74
4:	The kinetics of activation and the relationship between the total flux of Ca^{2+} and Ca current potentiation.	77
5:	The restitution of the Ca current.	80
6:	The Ca2+ dependence of high threshold current potentiation.	82

List of Figures (cont'd.) Page

7: High threshold current potentiation under conditions designed to reduce Ca^{2+} loading and release from the sarcoplasmic reticulum. 85

8: The effects of ryanodine on Ca^{2+} dependent potentiation. 87

9: The effects of caffeine on the Ca current. 90

10: The effect of isoproterenol on caffeine induced enhancement of Ca current. 93

11: The effects of isoproterenol on high threshold current potentiation. 95

ACKNOWLEDGEMENTS

I wish to express my appreciation to my advisor, Dr. James Maylie, for sharing his insights and for serving as an example of someone who is both thoughtful and precise. Dr. J. Job Faber has contributed to my understanding of cardiac physiology and has been of immeasurable aid in the critical review of this research. Drs. Robert Hartshorne and Jon Abramson have made suggestions on many aspects of this work. I must also acknowledge the support of Dr. Stan Hillman, who participated in my early training as a comparative physiologist and suggested that I pursue a doctoral degree. There is one final person to thank, my wife, Andrea who has survived this experience with me and shares my joy.

ABSTRACT

Rate-dependent changes in cardiac contractility are thought to arise from accumulation of greater or lesser quantities of releasable stores of calcium within the sarcoplasmic reticulum (SR). This mechanism alone, however, may not account for the large increases in tension recorded in most mammalian species during the fast phase of the positive force staircase. Since the calcium influx may trigger release of calcium from the SR, rate-dependent increases in the Ca current may provide an additional mechanism by which tension is increased during the fast phase of the positive force staircase. This study was designed to test whether a beat dependent increase in the Ca current can be elicited by repetitive pulses in voltage clamp experiments of guinea-pig ventricular myocytes.

Whole cell currents were measured in acutely dissociated ventricular myocytes. Currents through sodium, potassium, and chloride channels were reduced by the substitution of ions that cannot traverse these channels in the internal and external solutions.

Stimulation from rest (-80 mV) with 5 pulses of 150 ms duration at a rate of 2 Hz resulted in depotentiation of low threshold Ca channel currents and potentiation of high

threshold Ca channel currents. Potentiation of the high threshold current was observed at potentials negative to the reversal potential. In 5 mM calcium the ratio of the inward peak of the high threshold Ca channel current measured during the 5th and the 1st pulses for steps to 20 mV was 1.37 ± 0.03 (SEM, n = 20).

Potentiation of the high threshold Ca channel current was beat dependent requiring 5-6 successive depolarizations to reach steady state. This potentiation was associated with a slowing in the rate of inactivation with each depolarization. The rate of rise of the Ca current and the steady state level at the end of a 150 ms pulse were unaffected by repetitive depolarization. Inactivation of low threshold Ca channels prior to activation of high threshold channels failed to significantly affect positive current staircases.

Dual pulse experiments revealed that a 10 ms first pulse was sufficient to maximally potentiate the Ca current measured during the second pulse. Restitution of the Ca current revealed a half time for recovery from the potentiated state of approximately 3 sec.

The positive current staircase persisted under conditions which reduced the loading and release of calcium from the SR.

Substitution of external Ca^{2+} with Ba^{2+}, Sr^{2+} or Na^{+}

reduced inactivation and abolished frequency-dependent potentiation. Positive current staircases were also abolished by a high concentration (1 μM) of isoproterenol, 0.5 to 1.0 mM caffeine, and following incubation of cells in solutions containing 0.2 or 0.4 μM ryanodine for 4.5 hrs.

Low concentrations of isoproterenol increased the positive current staircase by a fraction of approximately 0.3.

In conclusion, the potentiation of high threshold Ca channel current with repetitive depolarization is mediated by calcium. The data are consistent with a hypothesis in which calcium binds to a site near the inner pore of the Ca channel which results in the slowing of inactivation and an increase in peak Ca current. High concentrations of isoproterenol and low concentrations of caffeine which have previously been reported to block the fast phase of the force staircase also abolish the positive current staircase in isolated myocytes.

INTRODUCTION

Excitation-contraction coupling in the heart

The ventricular "working myocardium" is composed of individual cardiocytes measuring about 10-30 μm in diameter and 50-100 μm in length. The plasma membrane (sarcolemma), like its counterpart in skeletal muscle, is invaginated to form an extensive transverse-tubular system (t-tubules) (Rayns, Simpson & Bertaud, 1968; Fawcett & McNutt, 1969). When making contact with adjoining cells, the sarcolemma is highly specialized to form an intercalated disc. Within this region of the membrane, gap junctions form a low resistance pathway for the direct spread of depolarizing current from cell to cell. The ventricle must, therefore, be considered an electrical syncytium with a requirement that all ventricular cardiocytes contract during systole.

An internal membrane system, the sarcoplasmic reticulum (SR), consists of reticular tubules which lie close to myofibrils, the sarcolemma, and t-tubules. When lying in close opposition to the sarcolemma or t-tubules, the longitudinal SR is specialized to form bulbous swellings. These terminal cisternae occur in pairs when lying astride the t-tubules and this association of the terminal cisternae and t-tubules is termed the triad. The distance

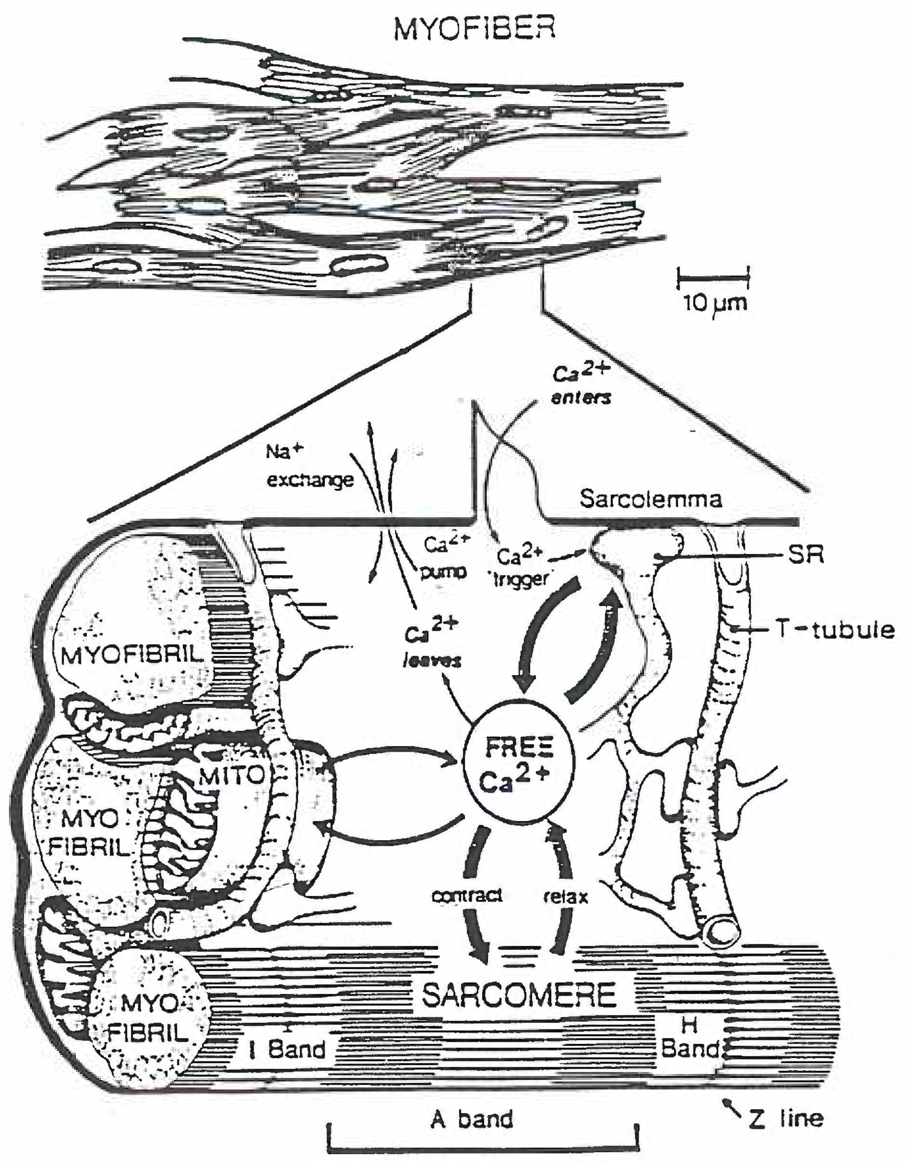
between the membranes of t-tubules and terminal cisternae is spanned by electron-dense feet which have recently been associated with the calcium release channel of the SR (Inui, Saito & Fleischer, 1987).

The initial event in the development of tension in the mammalian ventricle is the propogation of an action potential to the sarcolemma and the spread of depolarization to the T-tubules (Fig. 1). To cause contraction, this action potential must initiate a series of events which result in the binding of sufficient amounts of calcium to troponin to initiate contraction.

With depolarization of the sarcolemma, the influx of calcium through voltage-dependent Ca channels (Reuter, 1967, 1968 & 1979; McDonald, 1982) triggers the release of calcium from the SR (Fig. 1) (Fabiato, 1981 & 1982). Additional calcium enters the cell via a sarcolemmal Na - Ca exchange (Reuter & Seitz, 1968; Glitsch, Reuter & Scholz, 1970) and the net result is that systolic levels of intracellular free calcium approach $5x10^{-6}$ M (Winegrad, 1971; Solaro, Wise, Shiner & Briggs; 1974; Marban, Rink, Tsien & Tsien, 1980; Fabiato, 1981).

This elevation of intracellular calcium activates contractile proteins and also acts to close the Ca release channel of the SR, limiting further release of calcium from this source (Fabiato, 1985). Calcium has a somewhat

Fig. 1. Excitation-contraction in the heart. A sarcomere and its associated triad is illustrated. Contraction is initiated by the propagation of the action potential along the surface of the sarcolemma. With depolarization, calcium enters the cell via voltage gated Ca channels and electrogenic sodium-calcium exchange. Calcium which enters the cell through Ca channels serves to release calcium from the terminal cisternae of the sarcoplasmic reticulum (SR). Adapted from Opie, 1984.


MYOFIBER
10 μm
Ca2+
enters
Na+
exchange
Sarcolemma
Ca2+
pump
Ca2+
'trigger'
SR
T-tubule
Ca2+
leaves
MYOFIBRIL
FREE
Ca2+
MITO
MYO
FIBRIL
contract
relax
SARCOMERE
MYO
FIBRIL
I Band
H
Band
A band
Z line

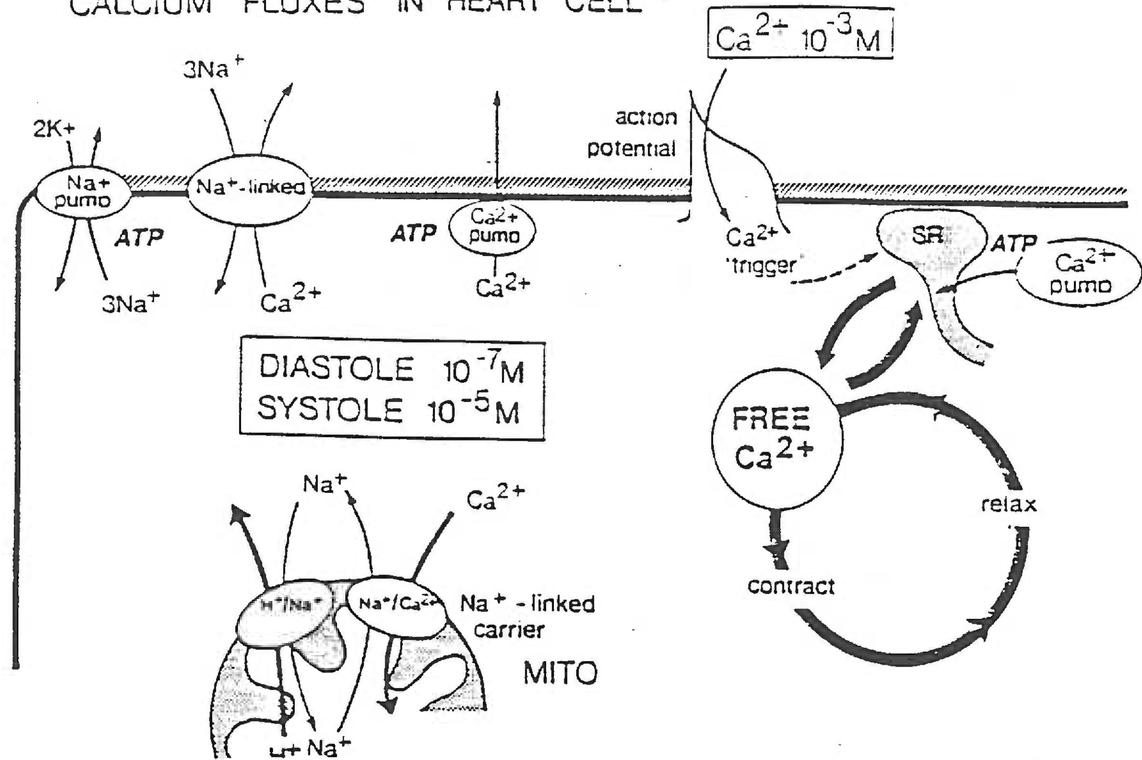
paradoxical role in the heart in that calcium opens SR release channels early in the course of the action potential and closes calcium release channels late in the action potential.

Relaxation precedes the repolarization of the membrane primarily as a result of inactivation of the Ca release channel and resequestration of calcium in the SR (Weber, Herz & Reiss, 1967; Harigaya & Schwartz, 1969; Solaro & Briggs, 1974).

With repolarization of the membrane, diastolic levels of intracellular free calcium (approximately 10^{-7} M) are maintained by the uptake of calcium into the SR and extrusion of calcium at the sarcolemma by a Na - Ca exchange (Mullins, 1979; Kimura, Miyamae & Noma, 1987)and an ATP-dependent calcium transport system (not shown in Fig. 1) (Caroni & Carafoli, 1980).

The nature of the calcium fluxes in a cardiocyte are summarized in figure 2. During diastole, calcium is taken up by the sarcoplasmic Ca^{2+}-dependent ATPase and extruded at the sarcolemma by an ATP-dependent transport system and by exchange of 3 sodium ions for 1 calcium ion. The slow rate of uptake of calcium by the mitochondria precludes a major role for this organelle in the beat to beat regulation of calcium in heart cells (Weber, Herz & Reiss, 1964b).

Fig. 2. Calcium fluxes in a cardiocyte. Calcium fluxes of the mitochondria (MITO), the sarcolemma and the sarcoplasmic reticulum (SR) are illustrated. See text for details. Adapted from Opie, 1984.


CALCIUM FLUXES IN HEART CELL
Ca2+ 10-3M
3Na+
2K+
Na+ pump
Na+-linked
ATP
3Na+
Ca2+
ATP
Ca2+ pump
Ca2+
action potential
Ca2+ 'trigger'
SR
ATP
Ca2+ pump
DIASTOLE 10-7M
SYSTOLE 10-5M
FREE Ca2+
relax
contract
Na+
Ca2+
H+/Na+
Na+/Ca2+
Na + - linked carrier
MITO
H+ Na+

With depolarization of the cell, there is calcium influx through the Ca channel and by exchange of extracellular calcium with intracellular sodium. The influx of calcium through Ca channels triggers the release of calcium from the SR to activate contraction. A change of intracellular free [Ca^{2+}] of approximately two orders of magnitude is required for maximal activation of cardiac contractile proteins (Weber, Herz & Reiss, 1964a; Fabiato, 1982).

Cardiac contractility is influenced by the rate and pattern of stimulation

The force of contraction of cardiac muscle is dependent upon both the rate and pattern of its stimulation (Bowditch, 1871; Koch-Weser & Blinks, 1963). The mechanisms which have been postulated for these changes in contractility will be considered after a brief description of the phenomena in mammalian ventricular muscle.

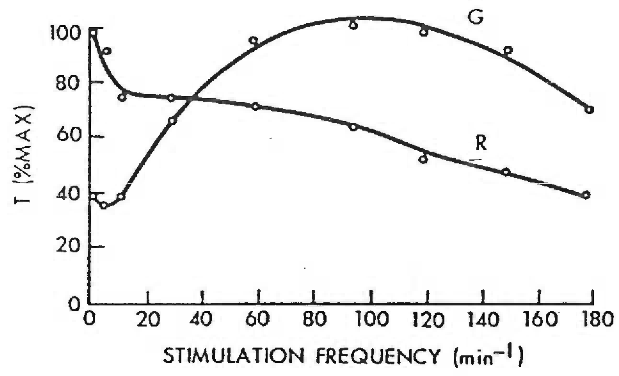
A weak force of contraction is generated at very high rates of stimulation in most mammalian species (Tuttle & Farah, 1962; Koch-Weser & Blinks, 1963). As the interval between stimulations is increased, steady-state tension increases to a maximal value and then declines with further increases in the interpulse interval (Fig. 3, top panel). When the interval between beats exceeds a certain duration, the strength of contraction is independent of

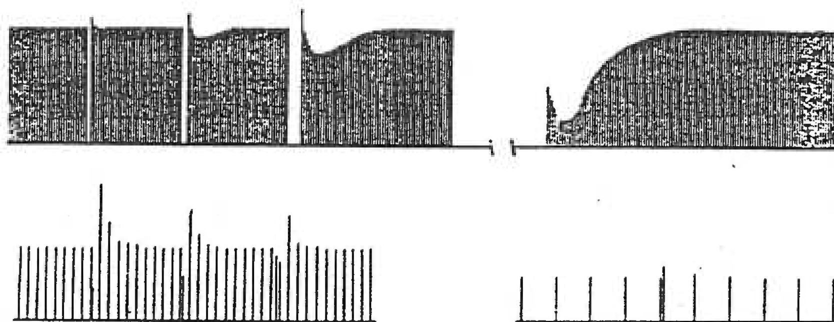
previous beats and such a contraction is said to be a rested-state contraction. The contraction in the rested-state is generally found to be quite low (Koch-Weser & Blinks, 1963; Allen, Jewell & Wood, 1976).

When the rate of stimulation is suddenly increased the strength of contraction does not immediately increase to the steady-state level determined by the new frequency. Instead, a positive force staircase is observed during which tension increases with each successive beat over the course of six to eight beats. This fast phase of the positive force staircase is followed by a further, but much slower increase in contractility to a new steady-state level over the course of several minutes (Koch-Weser & Blinks, 1963; Beresewicz & Reuter, 1977; Seibel, Karema, Takeya & Reiter, 1978; Seibel, 1986).

A positive force staircase can also be activated by a train of pulses following a rest period. The strength of contraction of the first pulse of this train is dependent on the rate of stimulation of the preceding train of pulses and the duration of the intervening rest interval (Allen, Jewell & Wood, 1976; Pidgeon, Lab, Seed, Elzinga, Papadoyannis & Noble, 1980). The restitution of contraction is described by the relationship between the rest interval and the tension of the first pulse which follows. As the rest interval is prolonged

Fig. 3. Rate-dependent effects on contractility. Top panel) Steady state frequency-tension curves in rat (R) and guinea-pig (G) papillary muscle. The rat is the one exception to the general mammalian pattern of the positive inotropic effect of an increase in frequency. Adapted from Forester & Mainwood, 1974. Middle panel) Records of force over time. The effect of rest in cat papillary muscle stimulated at 1.6 Hz: 2, 4, 10 beats omitted; 180 sec rest. C) Records of force over time. The effect of an extrasystolic beat: 0.32, 0.4, 0.63 sec after preceding beat. Adapted from Koch-Weser & Blinks, 1963.


G
R
100
80
60
40
20
0
T (%MAX)
0 20 40 60 80 100 120 140 160 180
STIMULATION FREQUENCY (min⁻¹)



contractility during the first rested beat increases up to a point (Fig. 3, middle panel) and then declines and eventually becomes independent of the previous contraction. Stimulation by a train of pulses of a muscle in the rested-state results in a rate staircase which is similar to the staircase elicited during continuous stimulation by a sudden increase in stimulation rate (Koch-Weser & Blinks, 1963).

An increase in contractility also follows an extrasystolic beat which is interposed between two regular pulses (Woodworth, 1902). The force developed during the extrasystole is generally less than that developed by the preceding contraction and is dependent on the interval between the extrasystole and the last regular beat (Fig. 3, bottom panel) (Koch-Weser & Blinks, 1963). Despite this depotentiation of extrasystolic tension, the contraction in response to the next pulse is enhanced ("post-extrasystolic potentiation"). Contractility returns to steady-state levels over the course of 6 to 8 beats at the basal rate of stimulation.

The concept that the force of contraction at any given moment is the result of two opposing factors which vary with the rate of stimulation was first expressed by Woodworth (1902). Blinks & Koch-Weser (1961) analyzed rate-dependent changes in cardiac contractility in terms

of a beat dependent accumulation or decay of a negative inotropic effect of activation (NIEA) which decreased contractility and a positive inotropic effect of activation (PIEA) which increased contractile strength. The search for the cellular basis of these transient changes in contractility has revealed much about the mechanisms which link electrical and contractile phenomena (excitation-contraction coupling) in the heart.

The Ca current and generation of tension in the heart

Control of cardiac contractility can not be achieved by recruitment of additional cells within the myocardium, since all myocardial cells contract during systole. The diversity of contractile states which arises from alterations in rate and rhythm of stimulation must, therefore, be expressed in each myocardial cell. The two interrelated processes of membrane depolarization and the release of calcium from the sarcoplasmic reticulum (SR) are thought to participate in control of myocardial contractility. Another modifier of force, sarcomere length, exists, but falls outside the scope of this work.

In the scheme described in figure 1, calcium influx through the Ca channel triggers the release of calcium from the SR. Fabiato (1982 & 1985) has hypothesized that the inotropic state of the muscle in response to the

release of calcium from the SR is a graded process which is dependent on 1) the level of SR stores of calcium, 2) the rate of change of myoplasmic free calcium, and 3) the level of free calcium used as a trigger (i.e. the Ca current).

An early report of voltage clamped Purkinje fibers revealed a slow inward current which was activated by depolarization and was dependent on the extracellular calcium concentration (Reuter, 1967). There is now general agreement that this current represents flux through voltage-dependent channels which are cation selective and preferentially permeable for divalent over monovalent ions (Lee & Tsien, 1984; Matsuda & Noma, 1984; Hess, Lansman & Tsien, 1986) and which are activated during the plateau phase of the cardiac action potential (Reuter, 1968; Reuter, 1979; McDonald, 1982). The relative permeability sequence for divalent cations is $Ca^{2+} > Sr^{2+} > Ba^{2+}$, with Mg^{2+} not measurably permeable (Hess, Lansman & Tsien, 1986).

Recent studies in canine atrial cells (Bean, 1985) and guinea-pig ventricular cells (Mitra & Morad, 1986) reveal two types of Ca channels. Low threshold, "T-type" Ca channels differ from high threshold, "L-type" Ca channels by having a smaller single channel conductance, a more negative threshold of activation, more rapid inactivation

and by being insensitive to dihydropyridines.

High and low threshold Ca channel currents may also be differentiated by whether inactivation is voltage- or calcium-dependent. Barium, which can substitute for calcium as a charge carrier in Ca channels, fails to activate a number of Ca^{2+} dependent cellular processes (Kolhardt, Haastert, & Krause, 1973; Siegelbaum & Tsien, 1980; Kass & Sanguinetti, 1984; Kimura, Miyamae & Noma, 1987). Substitution of barium for calcium in external solutions results in a slowing of inactivation and an increase in the amplitude of the high threshold current, but no change in inactivation or amplitude of the low threshold current (Bean, 1985). These findings are consistent with mechanisms of inactivation which are both voltage- and calcium-dependent in high threshold channels (Mentrard, Vassort & Fischmeister, 1984; Kass & Sanguinetti, 1984) and solely voltage-dependent in low threshold channels (Bean, 1985).

The potential dependence of the low and high threshold currents suggest that the flux of calcium during the plateau phase of the action potential flows, largely, through high threshold Ca channels (Bean, 1985; Mitra & Morad, 1986). There is considerable evidence that this flux of calcium during the action potential is essential for the development of tension in the heart.

Under voltage clamp conditions two types of contractile responses can be discerned in mammalian cardiac muscle. With depolarizations of less than 1 sec a phasic contraction is produced which relaxes completely before repolarization and has a time course similar to that of a normal cardiac contraction (Morad & Trautwein, 1968; Fozzard & Hellam, 1968, Beeler & Reuter, 1970a). Further prolongation of the duration of depolarization elicits a slow tonic contraction which is maintained until repolarization (Morad & Trautwein, 1968). The much faster phasic contraction predominates in the contractile response to a normal action potential (Morad & Goldman, 1973).

The phasic tension measured in cat ventricular trabeculae is dependent on potential and has a threshold near -50 mV and a maximum near 10 mV. Further depolarization results in a decrease in phasic tension (Morad & Goldman, 1973). The common voltage threshold and the decrease in both the Ca current and phasic tension with strong depolarizations has been confirmed in other preparations (Trautwein, McDonald & Tripathi, 1975; Maylie & Morad, 1984; Mitchell, Powell, Terrar & Twist, 1987). Support for the critical role of the Ca current in the development of tension in the heart is shown by the parallel decreases in the Ca current and contraction which

result from 1) a reduction of extracellular calcium (Beeler & Reuter, 1970a & 1970b; New & Trautwein, 1972a & 1972b), and 2) the partial block of the Ca current (McDonald, Pelzer & Trautwein, 1980; McDonald, Pelzer & Trautwein, 1981; Morgan, Wier, Hess & Blinks, 1983). The loss of contractility in calcium-free solutions is not due to depletion of SR stores of calcium, since caffeine causes release of calcium from the SR under both normal and calcium-free conditions (Smith, Valdeolmillos, Eisner & Allen, 1988). Calculation of the influx of calcium through Ca channels during systole suggests that this flux is insufficient to activate myofibrils directly, however, the findings suggest a close relationship between the Ca current and phasic contraction.

The basic characteristics of membrane control of tension in the mammalian heart are as follows: 1) only phasic contractions develop during depolarizations lasting less than 1 sec (see above), 2) for a muscle bathed in 5.4 mM calcium, an action potential of a few milliseconds duration produces significant tension (Morad & Trautwein, 1968), 3) the duration of the plateau of the cardiac action potential is an important determinant of subsequent contractility (Morad & Trautwein, 1968), and 4) tension requires from 6 to 8 beats to reach a new steady-state level following a change in action potential duration or

plateau (Wood et al., 1969; Beeler & Reuter, 1970b; Morad & Goldman, 1973).

A proposal that transmembrane Ca flux during the plateau of the action potential both triggers the release of calcium from the SR and serves to replenish SR stores of calcium is consistent with most aspects of the membrane control of cardiac contractility (Wood et al., 1969; Morad & Goldman, 1973; Fabiato, 1985). Morad and Trautwein (1968) report that tension is reduced in a beat dependent manner by repetitive stimulation with action potentials of short duration. They interpret the beat dependent decline in tension to mean that the Ca current which flows in the first 50 ms or so of the action potential serves to trigger SR release of calcium and that the flux of calcium during action potentials of longer duration serves to fill SR stores. Action potentials of short duration, therefore, deplete the SR of calcium. Morad & Cleeman (1987) have convincingly shown that the rapid release of the Ca current from a nifedipine block results in a recovery of the action potential within one beat, however, tension required 5 to 8 beats to fully recover. This beat dependent delay in response to alterations of action potential duration is hypothesized to result from delays in transport of calcium within the SR. In support of a dual role for the Ca current in both releasing and filling

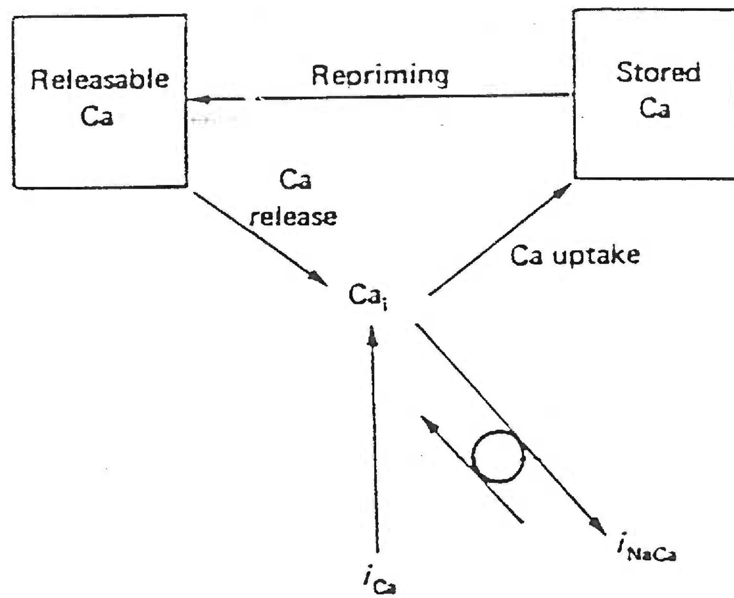
the SR stores of calcium, Fabiato (1985) reports that rapid pulses of calcium applied to mechanically skinned cardiac cells results in release of calcium from the SR, whereas calcium delivered at a slow rate results in inhibition of SR calcium release and accumulation of calcium in the SR. Additional evidence for the role of calcium in triggering the release of calcium from the SR comes from measurement of intracellular calcium transients with calcium indicator dyes. The similarities between the voltage relations of both the calcium current and the intracellular calcium signal as determined by FURA II (Barcenas-Ruiz & Wier, 1987) is consistent with the assumption that the flux of calcium through voltage dependent channels releases calcium from the SR.

An attempt can now be made to reconcile some of the effects of rate and pattern of stimulation on cardiac contractility in terms of a store of calcium which can be depleted and replenished by alterations in the duration of the cardiac action potential.

As previously described, the tension which is produced during an extrasystole is reduced when compared to the force developed by the last normal beat. The inotropic state of the muscle is, however, greater since the action potential which follows the extrasystole produces a larger than normal contraction. Contractility is then reduced to

steady state levels over the course of several beats. Of importance in considering a mechanism for this rhythm-dependent phenomena is the 1) time which has elapsed since the previous release of calcium from the SR, 2) and the history of depolarizations in terms of the action potential durations. Morad & Goldman (1973) hypothesized that contractility during the extra beat is reduced primarily as a result of a reduced amount of calcium available for release from the SR. Their assumption is that different regions of the SR are specialized for uptake and release of calcium (Fig. 4). The release of calcium is limited by the rate at which calcium is transported to the release site. The reduction in tension which results from the shortening of the interval between the extra beat and the last normal beat is consistent with this hypothesis. Following the extrasystolic beat, the SR takes up a fraction of the released calcium as well as the calcium which enters the cell during two action potentials (normal beat plus extrasystole). Tension is enhanced in the first normal beat following the extrasystole primarily as a result of the availability of an increased amount of releasable calcium within the SR. This amount of calcium is made available after some delay for transport to the release site. The uptake and recirculation of calcium results in the maintenance of the SR with each succeeding

Fig. 4. Diagram summarizing the role of the sarcoplasmic reticulum in cardiac rate inotropy. The Ca current triggers release of Ca from the releasable pool within the SR, and also replenishes SR calcium stores. The repriming process represents a delay in transporting calcium within the SR to release sites. The effect of an extrasystole is trigger release of calcium before the releasable pool has been replenished, and, therefore, the extrasystolic contraction is reduced below basal levels. The SR accummulates calcium during the extrasystolic beat and adds this to the store of releasable calcium. The postextrasystolic beat which follows releases replenished stores of calcium accumulated over the previous two beats. Adapted from Noble, 1984.


Releasable Ca
Repriming
Stored Ca
Ca release
Ca uptake
Ca_i
i_{NaCa}
i_{Ca}

beat. Contractility decays to steady state levels as a result of the conservation of a smaller fraction of calcium by the

To summarize, the Ca current plays a critical role in the development of tension in the heart, as demonstrated by the similar potential dependence of the Ca current and tension. The delays inherent in the uptake and recirculation of calcium within the SR may contribute to the changes in contractility associated with altered patterns of stimulation. However, the role of the Ca current in triggering the release and replenishing the stores of calcium in the SR suggests that intrinsic modulation of the Ca current during repetitive stimulation may also be of some importance when considering rate dependent changes in cardiac contractility.

Rate-dependent effects on the cardiac action potential and the Ca current

Rate and rhythm induced alterations of cardiac contractility are associated with changes in the duration of the action potential (reviewed by Boyett & Jewell, 1980). The duration of action potentials recorded from isolated Purkinje fibers and papillary muscles shortens when the rate of stimulation is increased, although the

total fraction of time spent in systole increases (Hiraoka & Hiraoka, 1975; Allen, 1977; Boyett, 1978). In sheep Purkinje fibers (Hiraoka & Hiraoka, 1975) and in rabbit ventricular muscle (Gibbs & Johnson, 1961) there is also shortening of the action potential duration at rates below 60 beats min^{-1}.

The type of action potential which is elicited in response to a premature stimulus is highly variable. A reduced plateau duration is observed in porcine ventricular muscles with premature action potentials (Gettes & Reuter, 1974). However, a "super-normal" premature action potential of long duration is observed in the guinea-pig (Anderson & Johnson, 1976) and the dog (Iinuma & Kato, 1979). Iinuma & Kato concluded that the mechanism involved was a rate-dependent enhancement of the Ca current, since an increase in extracellular calcium was found to prolong the plateau of the premature action potential. An alternative interpretation which Iinuma & Kato did not consider is that lengthening of the premature action potential plateau might have resulted from the rate dependent inhibition of an outward current carried by potassium (Gibbons & Fozzard, 1975; Aldrich, Getting & Thompson, 1979; Kenyon & Gibbons, 1979a & 1979b; Boyett, 1981). Recently, the mechanism of a similar increase in the amplitude and duration of premature action potentials

in rabbit ventricular cells was found to be the result of incomplete recovery from inactivation of an outward potassium current (Hiraoka & Kawano, 1987).

The value of these observations is that they suggest that rate and rhythm dependent changes in the duration of action potentials reflect underlying changes in ionic currents (Boyett & Jewell, 1980). Unfortunately, very little can be inferred about the amplitude of the Ca current from rate dependent changes in the action potential, unless parallel studies of ionic currents are undertaken.

Lux & Eckert (1974) reported that a delayed inward current in the monopolar ganglion cells of the snail was increased by a prior depolarization. They did not positively identify the ionic flux responsible for this current, however, its slow time course was more characteristic of a current carried by calcium ions rather than one carried by sodium. In a subsequent voltage clamp study, this current was shown to be blocked by the inorganic Ca channel blocker cobalt (Heyer & Lux, 1976); they corroborated the earlier study and reported that stimulation with a train of pulses to positive potentials resulted in a decrease in the calcium current with interpulse intervals of 20 ms and potentiation of the current at longer interpulse intervals.

Noble & Shimoni (1981a & 1981b) investigated the frequency dependence of Ca currents in frog atrial trabeculae using the double sucrose gap technique. They found that changes in the amplitude of the Ca current with repetitive stimulation were voltage-dependent. Small depolarizations resulted in decreases in Ca current and large depolarizations gave rise to positive current staircases. The use of sodium or strontium as a charge carrier in Ca channels did not reduce the facilitation of the Ca current, however, replacement of external calcium with barium reduced the positive current staircase. They concluded that voltage or calcium ions, in some manner, induced increases in the conductance of Ca channels and speculated that this facilitation of the Ca current may underlie positive tension staircases. Their findings concur with a report of rate-dependent enhancement of the Ca current in canine ventricular cells (Hiraoka & Sano, 1976), but must be contrasted with the results of experiments in mammalian heart where positive force staircases were associated with decreasing or non-changing Ca currents (Trautwein, 1973; Reuter, 1973; Gibbons & Fozzard, 1975; Simurda, Simudova, Braveny & Sumera, 1981).

Experiments reported in rat ventricular muscle have shown that an increase in the stimulation frequency results in either an increase (Payet, Schanne & Ruiz-

Ceretti, 1981), or a decrease in the Ca current (Mitchell, Powell, Terrar & Twist, 1985). These two studies differ in many respects, some of which may be important in considering their contrary findings. Payet et al. voltage clamped isolated trabeculae using the double sucrose gap technique. They reported that increases in the Ca current were associated with a marked reduction of the inactivation time constant. There is, however, no assurance that all cells within a multicellular preparation are clamped to the same potential when using the double sucrose gap technique (Morad & Goldman, 1973). It is possible that the voltage inhomogeneity of this preparation precludes an accurate assessment of frequency-dependent effects on the Ca current. Mitchell et al. (1985) used a single micro-electrode voltage clamp technique to investigate ventricular cells which were isolated by enzymatic dissociation. The time course of contraction was monitored by a photodiode in the microscope eyepiece. They reported that a 10 fold increase in the frequency of stimulation caused a gradual decline in the amplitude of contractions and the Ca current. In agreement with the earlier study by Payet et al. (1981), an early component of inactivation was reduced at the higher frequency. Mitchell et al. (1985) proposed that the rate-dependent decrease in the Ca current

resulted from an increase in SR calcium release and, as a result, an increase in calcium induced inactivation of the calcium current. This proposal is, however, inconsistent with their findings that both contractility and the rate of inactivation of the Ca current were reduced by an increase in stimulation frequency.

Mitra & Morad (1986) used the patch clamp technique in isolated guinea-pig myocytes to better separate the low and high threshold Ca channel currents from other ionic currents. They reported that a 5 fold increase in stimulation frequency resulted in a decrease in low threshold Ca current and an increase in high threshold current. Potentiation of Ca currents in isolated guinea-pig myocytes was also reported in cells which were stimulated by a train of pulses following a period of rest (Fedida, Noble, Shimoni & Spindler, 1985; Fedida, Noble, Shimoni & Spindler, 1987; Lee, 1987).

A variety of mechanisms have been proposed for the rate-dependent facilitation of Ca currents. Positive current staircases in the sino-atrial node of the rabbit have been modelled by assuming that the slow inward current consists of two components, a fraction due to flux through voltage gated Ca channels and a second fraction representing a sodium-calcium exchange current (Brown, Kimura, Noble, Noble & Taupignon, 1984). Brown et al. hypothesize that

it is a rate-dependent enhancement of sodium-calcium exchange which accounts for the entire positive current staircase observed in these cells. They did not, however, attempt to verify the validity of this proposal experimentally.

Rate-dependent facilitation of Ca current in chromaffin cells (Fenwick, Marty & Neher, 1982; Hoshi, Rothlein & Smith, 1984) and in guinea-pig ventricular cells (Lee, 1987) has been ascribed to an intrinsic voltage-dependent process which modulates Ca channels. Although a formal model of this process was not presented, Fenwick et al. suggests that depolarization of the membrane somehow results in a shift of the potential dependence of activation of Ca channels by approximately 1 mV. During a second depolarization this voltage shift is sensed and Ca channels are activated to a greater degree than during the first pulse.

Lee (1987) proposed a scheme for rate-dependent facilitation of the Ca current in which Ca channels have two distinct open states. The transition from a closed state to the first open state is made rapidly upon a single depolarization of the membrane. The transition to the second open state proceeds slowly and the channel is fully opened only after repeated depolarization. However, single channel studies in guinea-pig myocytes support a

three-state sequential scheme for activation of Ca channels with two closed states and only one open state (Cavalie, Ochi, Pelzer & Trautwein, 1983).

In summary, studies of rate-dependent alterations of cardiac action potentials are suggestive of underlying frequency-dependent changes in the Ca current. Modulation of the Ca current is confirmed by voltage clamp studies of cardiac muscle, sino-atrial nodal cells, and chromaffin cells. The contribution of this intrinsic modulation of the Ca current to the tension staircase of the mammalian myocardium is unclear, since rate-dependent changes in the Ca current do not always parallel changes in contractility. Even less is known of the mechanisms responsible for modulation of the Ca current, although the involvement of sodium-calcium exchange, as well as calcium-dependent and voltage-dependent processes has been proposed.

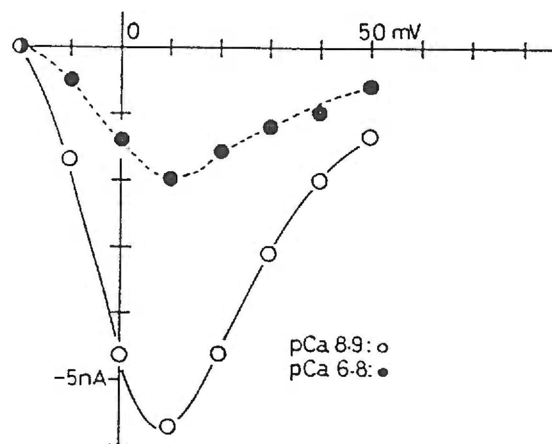
Modulation of the Ca current

The flux of ions through Ca channels is modulated by the level of Ca channel protein phosphorylation (Osterrieder, Brum, Hescheler, Trautwein, Flockerzi & Hofmann, 1982; Trautwein, Cavalie, Flockerzi, Hofmann & Pelzer, 1987), activation of guanine nucleotide binding protein (G protein) (Imoto, Yatani, Reeves, Codina, Birnbaumer &

Brown, 1988), and alteration of membrane surface potential by non-specific binding of ions (Ohmori & Yoshii, 1977). Hydrogen ions (Kaibara & Kameyama, 1988) and intracellular Ca^{2+} (Kokubun & Irisawa, 1984) have also been shown to alter the Ca current. Kokubun & Irisawa (1984) used the perfused patch pipette technique to investigate the effects of elevation of intracellular calcium on the Ca current (Fig. 5). An increase in intracellular calcium concentration from 1.3 nM to 150 nM decreased peak Ca currents by approximately 70 %.

The Ca current is increased by β-adrenergic stimulation (Reuter, 1983). The binding of agonists to β-adrenergic receptors results in the dissociation of the α-subunit from the G protein complex (Stryer & Bourne, 1986). The α-subunit activates adenylate cyclase and results in an increase in the amplitude of the Ca current. This increase in Ca current is mimicked by the elevation of intracellular c-AMP (Cachelin, de Peyer, Kokubun & Reuter, 1983) and is, presumably, due to phosphorylation of Ca channel proteins (Osterrieder, Brum, Hescheler, Trautwein, Flockerzi & Hofmann, 1982; Trautwein, Cavalie, Flockerzi, Hofmann & Pelzer, 1987). These effects of β-adrenergic stimulation are antagonized by the elevation of intracellular c-GMP, which may activate a phosphodiesterase (Hartzell & Fischmeister, 1986;

Fig. 5. The effect of elevation of $[Ca^{2+}]_i$ on the Ca current. A perfused patch pipette was used to raise calcium levels from pCa 8.9 to pCa 6.8 in an isolated guinea-pig ventricular myocyte. Adapted from Kokubun & Irisawa, 1984.


0
50 mV
-5nA
pCa 8·9 : o
pCa 6·8 : •

Fischmeister & Hartzell, 1987). The α-subunit of G protein may also increase the Ca current via a second pathway in which the α-subunit activates the Ca channel directly (Yatani, Codina, Imoto, Reeves, Birnbaumer & Brown, 1987; Imoto, Yatani, Reeves, Codina, Birnbaumer & Brown, 1988).

The activation of G proteins and the subsequent binding of a G protein subunit to adenylate cyclase has been shown to mediate the cellular response to β-adrenergic stimulation in the heart (Breitwieser & Szabo, 1985). Recent investigations have shown that the α-subunit of G proteins also directly regulates cardiac Ca channels (Yatani, Codina, Imoto, Reeves, Birnbaumer & Brown, 1987). The activated αG_s-subunit of G protein was reported to increase the peak opening probability and prolong the single channel open time of cardiac Ca channels incorporated in lipid bilayers (Imoto, Yatani, Reeves, Codina, Birnbaumer & Brown, 1988).

The preponderance of negatively charged proteins at the internal and external surfaces of the sarcolemmal membrane generates a surface potential which alters the electric field within the membrane (Hille, 1984). The non-specific binding of cations to neutralize this potential modifies the gating of voltage-dependent ionic channels within the membrane. A depletion of divalent cations at the outside

Sanguinetti, 1984) and the enhancement of the Ca current associated with digitalis inotropy in mammalian heart (Weingart, Kass & Tsien, 1978; Marban & Tsien, 1982).

It is clear from these studies in cardiac and skeletal muscle and neuronal tissues that there is considerable opportunity to alter the amplitude of the Ca current. It remains to be shown whether Ca currents and cardiac contractility show parallel changes with repetitive depolarization and whether voltage- or calcium-dependent mechanisms are involved in this process.

Summary and aims of this study

There is general agreement that the Ca current which contributes to the plateau of the cardiac action potential is critical for the generation of tension in the heart. The role of this current in rate- and rhythm-dependent changes in cardiac contractility is somewhat controversial. The limited investigations of rate-dependent effects on the Ca current in the heart have revealed either no change in the Ca current, or alterations of the calcium flux which are either parallel or opposite to contractile changes.

A scheme was reviewed for rate- and rhythm-dependent phenomena in the heart which is consistent with much of the available data (Morad & Goldman, 1973). However, the

force of contraction can change dramatically in the course of one or two beats (Koch-Weser & Blinks, 1963) and it is difficult to know whether sufficient calcium accumulates in the SR over that period to account for these increases in tension. If calcium serves to both fill the SR with calcium and trigger its release (Fabiato, 1985), then a rate-dependent increase in the Ca current may serve to release larger amounts of calcium from the SR during positive force staircases.

The objective of this project was to investigate the mechanisms of rate-dependent modulation of the Ca current in isolated ventricular myocytes of the guinea-pig. This preparation was chosen 1) since facilitation of the high threshold Ca channel current had previously been demonstrated in guinea-pig myocytes (Mitra & Morad, 1986), and 2) a similar process may play a role in digitalis inotropy (Weingart, Kass & Tsien, 1978; Marban & Tsien, 1982), protection of the myocardium from reexcitation during genesis of arrhythmia (Hiraoka & Sano, 1976), and generation of positive force staircases (Noble & Shimoni 1981a & 1981b).

The first aim of this study was to demonstrate that frequency-dependent potentiation of the inward current did not reflect rate-dependent alterations of contaminating ionic currents. The potentiation process was then

characterized in terms of its 1) potential dependence, 2) kinetics, 3) sensitivity to choice of Ca channel charge carrier, and 4) response to β-adrenergic stimulation. Pharmacologic interventions were used in an attempt to distinguish between a mechanism which involves modulation of Ca channel inactivation, and one in which increases in Ca currents are secondary to use-dependent inhibition of the release of calcium from the SR.

REFERENCES TO INTRODUCTION

Aldrich, R.W., Getting, P.A., & Thompson, S.H. (1979). Mechanism of frequency-dependent broadening of molluscan neurone soma spikes. *Journal of Physiology* 291, 531-544.

Allen, D.G. (1977). On the relationship between action potential duration and tension in cat papillary muscle. *Cardiovascular Research* 11, 210-218.

Allen, D.G., Jewell, B.R. & Wood, E.H. (1976). Studies of the contractility of mammalian myocardium at low rates of stimulation. *Journal of Physiology* 254, 1-17.

Anderson, T.W. & Johnson, E.A. (1976). The repolarization phase of the cardiac action potential: a comparative study of rate-induced changes in its waveform. *Journal of Molecular and Cellular Cardiology* 8, 103-121.

Bailey, L.E., Ong, S.D. & Queen, G.M. (1972). Calcium movement during contraction in the cat heart. *Journal of Molecular and Cellular Cardiology* 4, 121-138.

Barcenas-Ruiz, L. & Wier, W.G. (1987). Voltage dependence

Bowditch, H.P. (1871). Uber die Eigenthümlichkeiten der Reizbarkeitt, welche die Müskelfasern des Herzens zeigen. *Ber. sachs. ges. (Akad.) Wiss.* 652-689.

Boyett, M.R. (1978). An analysis of the effect of the rate of stimulation and adrenaline on the duration of the cardiac action potential. *Pflügers Archiv* 377, 155-166.

Boyett, M.R. (1981). A study of the effect of the rate of stimulation of the transient outward current in sheep cardiac purkinje fibres. *Journal of Physiology* 319, 1-22.

Boyett, M.R., & Jewel, B.R. (1980). Analysis of the effects of changes in rate and rhythm upon electrical activity in the heart. *Progress in Biophysics and Molecular Biology* 36, 1-52.

Breitwieser, G.E. & Szabo, G. (1985). Uncoupling of cardiac muscarinic and α-adrenergic receptors from ion channels by a guanine nucleotide analogue. *Nature* 317, 538-540.

Brown, H.F., Kimura, J., Noble, D., Noble, S.J. &

Taupignon, A. (1984). The slow inward current, i_{si}, in the rabbit sino-atrial node investigated by voltage clamp and computer simulation. *Proceedings of the Royal Society London B* 222, 305-328.

Cachelin, A.B., dePeyer, J.E., Kokubun, S. & Reuter, H. (1983). Ca^{2+} channel modulation by 8-bromocyclic AMP in cultured heart cells. *Nature* 304, 462-464.

Caroni, P. & Carafoli, E. (1980). An ATP-dependent Ca^{2+}-pumping system in dog heart sarcolemma. *Nature* 283, 765-767.

Cavalie, A., Ochi, R., Pelzer, D. & Trautwein (1983). Elementary currents through Ca^{2+} channels in guinea pig myocytes. *Pflügers Archiv* 398, 284-297.

DeRiemer, S.A., Strong, J.A., Albert, K.A., Greengard, P. & Kaczmarek, L.K. (1985). Enhancement of calcium current in *Aplysia* neurons by phorbol ester and protein kinase C. *Nature* 313, 313-316.

Fabiato, A. (1981). Myoplasmic free calcium concentration reached during the twitch of an intact isolated cardiac cell and during calcium-induced release of

calcium from the sarcoplasmic reticulum of a skinned cardiac cell from the adult rat or rabbit ventricle. *Journal of General Physiology* 78, 457-497.

Fabiato, A. (1982). Calcium release in skinned cardiac cells: variations with species, tissues, and development. *Federation Proceedings* 41, 2238-2244.

Fabiato, A. (1985). Simulated calcium current can both cause calcium loading in and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *Journal of General Physiology* 85, 291-320.

Fabiato, A. & Fabiato, F. (1975). Contractions induced by a calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. *Journal of Physiology* 249, 469-495.

Fawcett, D.W. & McNutt, N.S. (1969). The ultrastructure of the cat myocardium. *Journal of Cellular Biology* 42, 1-45.

Fedida, D., Noble, D., Shimoni, Y. & Spindler, A.J. (1985). Inward current and contraction in mammalian

cardiac cells: interventions that augment or reduce current staircases. *Journal of Physiology* 366, 84P.

Fedida, D., Noble, D. & Spindler, A.J. (1987). Inward current related to contraction in guinea-pig ventricular myocytes. *Journal of Physiology* 385, 565-589.

Fenwick, E.M., Marty, A. & Neher, E. (1982). Sodium and calcium channels in bovine chromaffin cells. *Journalof Physiology* 331, 599-635.

Fischmeister, R. & Hartzell, H.C. (1987). Cyclic guanosine 3',5'-monophosphate regulates the calcium current in single cells from frog ventricle. *Journal of Physiology* 387, 453-472.

Fozzard, H.A. & Hellam, D.C. (1968). Relationship between membrane voltage and tension in voltage clamped cardiac Purkinje fibres. *Nature* 218, 588-589.

Gettes, L.S. & Reuter, H. (1974). Slow recovery from inactivation of inward currents in mammalian myocardial fibres. *Journal of Physiology* 240, 703-724.

Gibbons, W.R. & Fozzard, H.A. (1975). Slow inward current

and contraction of sheep cardiac Purkinje fibres. *Journal of General Physiology* 65, 367-384.

Gibbs, C.L. & Johnson, E.A. (1961). Effect of changes in frequency of stimulation upon rabbit ventricular action potential. *Circulation Research* 9, 165-170.

Glitsch, H.G., Reuter, H. & Scholz, H. (1970). The effect of the internal sodium concentration on calcium fluxes in isolated guinea-pig auricles. *Journal of Physiology* 209, 25-43.

Grossman, A. & Furchgott, R.G. (1964). The effects of frequency of stimulation and calcium concentration on Ca^{45} exchange and contractility on the isolated guinea-pig auricle. *Journal of Pharmacology and Experimental Therapeutics* 143, 120-130.

Harigaya, S. & Schwartz, A. (1969). Rate of calcium binding and uptake in normal animal and failing human cardiac muscle. *Circulation Research* 25, 781-794.

Hartzell, H.C. & Fischmeister, R. (1986). Opposite effects of cyclic AMP on Ca^{2+} current in single heart cells. *Nature* 323, 273-275.

Hess, P, Lansman, J.B. & Tsien, R.W. (1986). Calcium channel selectivity for divalent and monovalent cations. *Journal of General Physiology* 88, 293-319.

Heyer, C.B. & Lux, H.D. (1976). Properties of a facilitating calcium current in pace-maker neurones of the snail, *Helix Pomatia*. *Journal of Physiology* 262, 319-348.

Hilgemann, D.W. (1986). Extracellular calcium transients at single excitations in rabbit atrium measured with tetramethylmurexide. *Journal of General Physiology* 87, 707-735.

Hille, B. (1984). Modifiers of gating. In *Ionic channels of excitable membranes*. pp 303-328 Sunderland: Sinauer Associates

Hiraoka, M. & Hiraoka, M. (1975). The role of the positive dynamic current on the action potential of cardiac Purkinje fibres. *Japanese Journal of Physiology* 25, 705-717.

Hiraoka, M. & Kawano, S. (1987). Mechanism of increased

amplitude and duration of the plateau with sudden shortening of diastolic intervals in rabbit ventricular cells. *Circulation Research* 60, 14-26.

Hiraoka, M. & Sano, T. (1976). Role of slow inward current in the genesis of ventricular arrhythmia. *Japanese Circulation Journal* 40, 1419-1427.

Horne, P., Triggle, D.J. & Venter, J.C. (1984). Nitrendipine and isoproterenol induce phosphorylation of a 42,000 dalton protein that co-migrates with the affinity labeled calcium channel regulatory subunit. *Biochemical and Biophysical Research Communications* 121, 890-898.

Hosey, M.M., Borsotto, M. & Lazdunski, M. (1986). Phosphorylation and dephosphorylation of dihydropyridine-sensitive voltage-dependent Ca^{2+} channel in skeletal muscle membranes by cAMP- and Ca^{2+}-dependent processes. *Proceedings of the National Academy of Sciences, USA* 83, 3733-3737.

Hoshi, T., Rothlein, J., & Smith, S.J. (1984). Facilitation of Ca^{2+} -channel currents in bovine adrenal chromaffin cells. *Proceedings of the National*

Academy of Sciences, USA 81, 5871-5875.

Iinuma, H., & Kato, K. (1979). Mechanism of augmented premature responses in canine ventricular muscle. *Circulation Research* 44, 624-629.

Imagawa, T., Leung, A.T., & Campbell, K.P. (1987). Phosphorylation of the 1,4-dihydropyridine receptor of the voltage-dependent Ca^{2+} channel by an intrinsic protein kinase in isolated triads from rabbit skeletal muscle. *Journal of Biological Chemistry* 262, 8333-8339.

Imoto, Y., Yatani, A., Reeves, J.P., Codina, J., Birnbaumer, L. & Brown, A.M. (1988). α-Subunit of G_s directly activates cardiac calcium channels in lipid bilayers. *American Journal of Physiology* 255, H722-H728.

Inui, M., Saito, A. & Fleischer, S. (1987). Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. *Journal of Biological Chemistry* 262, 1740-1747.

Kaibara, M. & Kameyama, M. (1988). Inhibition of the calcium channel by intracellular protons in single

ventricular myocytes of the guinea-pig. *Journal of Physiology* 403, 621-640.

Kavaler, F. (1959). Membrane depolarization as a cause of tension development in mammalian ventricular muscle. *American Journal of Physiology* 197, 968-970.

Kass, R.S. & Krafte, D.S. (1987). Negative surface charge density near heart calcium channels. *Journal of General Physiology* 89, 629-644.

Kass, R.S., & Sanguinetti, M.C. (1984). Calcium channel inactivation in the calf cardiac Purkinje fiber: Evidence for voltage- and calcium-mediated mechanism. *Journal of General Physiology* 84, 705-726.

Kenyon, J.L. & Gibbons, W.R. (1979a) Influence of chloride, potassium, and tetraethylammonium on the early outward current of sheep cardiac Purkinje fibers. *Journal of General Physiology* 73, 117-138.

Kenyon, J.L. & Gibbons, W.R. (1979b). 4-aminopyridine and the early outward current of sheep cardiac Purkinje fibers. *Journal of General Physiology* 73, 139-157.

Kimura, J., Miyamae, S. & Noma, A. (1987). Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig. *Journal of Physiology* 384, 199-222.

Koch-Weser, J. & Blinks, J.R. (1963). The influence of the interval between beats on myocardial contractility. *Pharmacological Reviews* 15, 601-665.

Kohlhardt, M., Haastert, H.P. & Krause, H. (1973). Evidence of non-specificity of the Ca channel in mammalian myocardial fiber membranes: substitution of Ca by Sr, Ba or Mg as charge carriers. *Pflügers Archiv* 342, 125-136.

Kokubun, S., & Irisawa, H. (1984). Effects of various intracellular Ca ion concentrations on the calcium current of guinea pig single ventricular cells. *Japan Journal of Physiology* 34, 599-611.

Langer, G.A. & Brady, A.J. (1963). Calcium flux in the mammalian ventricular myocardium. *Journal of General Physiology* 46, 703-720.

Lee, K.S. (1987). Potentiation of the calcium-channel

currents of internally perfused mammalian heart cells by repetitive depolarization. *Proceedings of the National Acadamy of Sciences, USA* 84, 3941-3945.

Lee, K.S. & Tsien, R.W. (1984). High selectivity of calcium channels in single dialysed heart cells of the guinea-pig. *Journal of Physiology* 354, 253-272.

Lewis, D.L. & Weight, F.F. (1988). The protein kinase C activator 1-oleoyl-2-acetylglycerol inhibits voltage-dependent Ca^{2+} current in the pituitary cell line AtT-20. *Neuroendocrinology* 47, 169-175.

Lux, H.D. & Eckert, R. (1974). Inferred slow inward current in snail neurones. *Nature* 250, 574-576.

Marban, E., Rink, T.J., Tsien, R.W. & Tsien, R.Y. (1980). Free calcium in heart muscle at rest and during contraction measured with Ca^{2+} - sensitive electrodes. *Nature* 286, 845-850.

Marban, E., & Tsien, R.W. (1982). Enhancement of calcium current during digitalis inotropy in mammalian heart: Positive feedback regulation by intracellular calcium? *Journal of Physiology* 329, 589-614.

Matsuda, H. & Noma, A. (1984). Isolation of calcium current and its sensitivity to monovalent cations in dialysed ventricular cells of guinea-pig. *Journal of Physiology* 357, 553-573.

Maylie, J. & Morad, M. (1984). A transient outward current related to calcium release and development of tension in elephant seal atrial fibres. *Journal of Physiology* 357, 267-292.

McDonald, T.F. (1982). The slow inward current in the heart. *Annual Review of Physiology* 44, 425-434.

McDonald, T.F., Pelzer, D. & Trautwein, W. (1980). On the mechanism of slow calcium block in heart. *Pflügers Archiv* 385, 175-179.

McDonald, T.F., Pelzer, D. & Trautwein, W. (1981). Does the calcium current modulate the contraction of the accompanying beat? *Circulation Research* 49, 576-583.

Mentrard, D., Vassort, G., & Fischmeister, R. (1984). Calcium-mediated inactivation of the calcium conductance in cesium-loaded frog heart cells. *Journal*

of General Physiology 83, 105-131.

Mines, G.R. (1913) On functional analysis by the actions of electrolytes. *Journal of Physiology* 46, 188-195.

Mitchell, M.R., Powell, T., Terrar, D.A. & Twist, V.W. (1985). Influence of a change in stimulation rate on action potentials, currents and contractions in rat ventricular cells. *Journal of Physiology* 364, 113-130.

Mitchell, M.R., Powell, T., Terrar, D.A. & Twist, V.W. (1987). Electrical activity and contraction in cells isolated from rat and guinea-pig ventricular muscle: a comparative study. *Journal of Physiology* 391, 527-544.

Mitra, R. & Morad, M. (1986). Two types of calcium channels in guinea pig ventricular myocytes. *Proceedings of the National Acadamy of Science, USA* 83, 5340-5344.

Morad, M. & Cleemann, L. (1987). Role of Ca^{2+} Channel in development of tension in heart muscle. *Journal of Molecular and Cellular Cardiology*. 19, 527-553.

Morad, M. & Goldman, Y. (1973). Excitation-contraction

coupling in heart muscle: membrane control of development of tension. *Progress in Biophysics and Molecular Biology* 27, 257-313.

Morad, M. & Trautwein, W. (1968). The effect of duration of the action potential on contraction in mammalian heart muscle. *Pflügers Archiv* 299, 66-82.

Morgan, J.P., Weir, W.G., Hess, P. & Blinks, J.R. (1983). Influence of calcium blocking agents on calcium transients and tension development in isolated mammalian heart muscle. *Circulation Research (Suppl. I)* 52, 47-52.

Mullins, L.J. (1979). The generation of electric current in cardiac fibers by Na/Ca exchange. *American Journal of Physiology* 236, C103-110.

New, W. & Trautwein, W. (1972a). Inward membrane currents in mammalian myocardium. *Pflügers Archiv* 334, 1-23.

New, W. & Trautwein, W. (1972b). The ionic nature of slow inward current and its relation to contraction. *Pflügers Archiv* 334, 24-38.

Noble, D. (1984). The surprising heart: a review of recent progress in cardiac electrophysiology. *Journal of Physiology* 353, 1-50.

Noble, S. & Shimoni, Y. (1981a). The calcium and frequency dependence of the slow inward current staircase in frog atrium. *Journal of Physiology* 310, 57-75.

Noble, S. & Shimoni, Y. (1981b). Voltage-dependent potentiation of the slow inward current in frog atrium. *Journal of Physiology* 310, 77-95.

Ohmori, H. & Yoshii, M. (1977). Surface potential reflected in both gating and permeation mechanisms of sodium and calcium channels of the tunicate egg cell membrane. *Journal of Physiology* 267, 429-463.

Opie, L. (1984). *The heart: physiology, metabolism, pharmacology and therapy*. New York:Grune & Stratton.

Osterrieder, W., Brum, G., Hescheler, J., Trautwein, W., Flockerzi, V. & Hofmann, F. (1982). Injection of subunits of cyclic AMP-dependent protein kinase into

cardiac myocytes modulates Ca^{2+} current. *Nature* 298, 576-578.

Payet, M.D., Schanne, O.F. & Ruiz-Ceretti, E. (1981). Frequency dependence of the ionic currents determining the action potential repolarization in rat ventricular muscle. *Journal of Molecular and Cellular Cardiology* 13, 207-215.

Pidgeon, J., Lab, M., Seed, A., Elzinga, G., Papadoyannis, D. & Noble, M.I.M. (1980). The contractile state of cat and dog heart in relation to the interval between beats. *Circulation Research* 47, 559-567.

Rayns, D.G., Simpson, F.O. & Bertaud, W.S. (1968). Surface features of striated muscle. *Journal of Cell Science* 3, 467-474.

Reuter, H. (1967). The dependence of slow inward current in Purkinje fibres on the extracellular calcium-concentration. *Journal of Physiology* 192, 479-492.

Reuter, H. (1968). Slow inactivation of currents in cardiac Purkinje fibres. *Journal of Physiology* 197, 233-253.

Reuter, H. (1973). Time- and voltage-dependent contractile responses in mammalian cardiac muscle. *European Journal of Cardiology* 1, 177-181.

Reuter, H. (1979). Properties of two inward membrane currents in the heart. *Annual Review of Physiology* 41, 413-424.

Reuter, H. (1983). Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature* 301, 569-574.

Reuter, H. & Seitz, N. (1968). The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *Journal of Physiology* 195, 451-470.

Rich, T.L. & Langer, G.A. (1975). A comparison of excitation-contraction coupling in heart and skeletal muscle: an examination of calcium-induced calcium-release. *Journal of Molecular and Cellular Cardiology* 7, 747-765.

Ringer, S. (1883). A further contribution regarding the influence of the different constituents of the blood

on the contraction of the heart. *Journal of Physiology* 4, 29-42.

Seibel, K. (1986). The slow phase of the staircase in guinea-pig papillary muscle, influence of agents acting on transmembrane sodium flux. *Naunyn-Schmiedeberg's Archiv of Pharmacology* 334, 92-99.

Seibel, K., Karema, E., Takeya, K. & Reiter, M. (1978). Effect of noradrenaline on an early and a late component of the myocardial contraction. *Naunyn-Schmiedeberg's Archiv of Pharmacology* 305, 65-74.

Siegelbaum, S.A. & Tsien, R.W. (1980). Calcium-activated transient outward current in cardiac Purkinje fibers. *Journal of Physiology* 299, 485-506.

Simurda, J., Simurdova, M., Braveny, P. & Sumbera, J. (1981). Activity-dependent changes of slow inward current in ventricular heart muscle. *Pflügers Archiv* 391, 277-283.

Smith, G.L., Valdeolmillos, M., Eisner, D.A. & Allen, D.G. (1988). Effects of rapid application of caffeine on intracellular calcium concentration in ferret

papillary muscles. *Journal of General Physiology* 92, 351-368.

Solaro, R.J. & Briggs, F.N. (1974). Estimating the functional capabilities of sarcoplasmic reticulum in cardiac muscle. *Circulation Research* 34, 531-540.

Solaro, R.J., Wise, R.M., Shiner, J.S. & Briggs, F.N. (1974). Calcium requirements for cardiac myofibrillar activation. *Circulation Research* 34, 525-530.

Stryer, L. & Bourne, H.R. (1986). G proteins: a family of signal inducers. *Annual Review of Cellular Biology* 2, 391-419.

Trautwein, W. (1973). The slow inward current in mammalian myocardium. *European Journal of Cardiology* 1, 169-175.

Trautwein, W., Cavalie, A., Flockerzi, V., Hofmann, F. & Pelzer, D. (1987). Modulation of calcium channel function by phosphorylation in guinea pig ventricular cells and phospholipid bilayer membranes. *Circulation Research* 61, I-17-I-23.

Trautwein, W., McDonald, T.F. & Tripathi, O. (1975). Calcium conductance and tension in mammalian ventricular muscle. *Pflügers Archiv* 354, 55-74.

Tritthart, H., Kaufman, R., Volkmer, H.P., Bayer, R. & Crause, H. (1973). Ca movement controlling myocardial contractility. *Pflügers Archiv* 338, 207-231.

Tuttle, R.S. & Farah, A. (1962). The effect of ouabain on the frequency-force relation and on post-stimulation potentiation in isolated atrial and ventricular muscle. *Journal of Pharmacology* 135, 142-150.

Weingart, R., Kass, R.S., & Tsien, R.W. (1978). Is digitalis inotropy associated with enhanced slow inward current? *Nature* 273, 389-391.

Weber, A., Herz, R., & Reiss, I. (1964a). The regulation of myofibrillar activity by calcium. *Proceedings of the Royal Society B* 160, 489-501.

Weber, A., Herz, R., & Reiss, I. (1964b). Role of calcium in contraction and relaxation of muscle. *Federation Proceedings* 23, 896-900.

Weber, A., Herz, R., & Reiss, I. (1967). The nature of the cardiac relaxing factor. *Biochem Biophys Acta* 131, 188-194.

Wilson, D.L., Morimoto, K., Tsuda, Y. & Brown, A.M. (1983). Interaction between calcium ions and surface charge as it relates to calcium currents. *Journal of Membrane Biology* 72, 117-130.

Wingrad, S. (1971). Studies of cardiac muscle with high permaebility to calcium produced by treatment with ethylenediaminetetraacetic acid. *Journal of General Physiology* 58, 71-93.

Winegrad, S. & Shanes, A.M. (1962). Calcium flux and contractility in guinea pig atria. *Journal of General Physiology* 45, 371-394.

Wood, E.H., Heppner, R.L. & Weidmann, S. (1969). Inotropic effects of electric currents. *Circulation Research* 24, 409-445.

Woodworth, R.S. (1902). Maximal contraction, "staircase" contraction, refractory period, and compensatory pause of the heart. *American Journal of Physiology* 8, 213-249.

Yatani, A., Codina, J., Imoto, Y., Reeves, J.P., Birnbaumer, L., & Brown, A.M. (1987). A G protein directly regulates mammalian cardiac calcium channels. *Science* 238, 1288-1292.

SUMMARY

1. Frequency-dependent modulation of Ca channel currents was examined in single guinea-pig ventricular cells. Whole cell recordings of Ca channel currents were made in cells under conditions which reduce ionic flux through other channels.

2. Stimulation from rest with 5 pulses at a rate of 2 Hz resulted in depotentiation of low threshold Ca channel currents and potentiation of high threshold Ca channel currents. Frequency-dependent potentiation of high threshold current was associated with a reduction in the rate of inactivation.

3. In dual pulse experiments in which the duration of the first pulse was varied, a 10 ms first pulse was sufficient to maximally potentiate the Ca current measured during the second pulse. The Ca current in the second pulse increased with increasing flux of Ca^{2+} in the first pulse when its duration was less than 10 ms. It was independent of Ca^{2+} flux in the first pulse with pulses of longer duration.

4. Substitution of external Ca^{2+} with Ba^{2+}, Sr^{2+} or Na^{+} reduced inactivation and abolished frequency-dependent potentiation.

5. Low concentrations of isoproterenol (5-50 nM)

increased potentiation. However, 1 μM isoproterenol increased Ca current but abolished frequency-dependent potentiation.

6. Potentiation persisted under conditions designed to reduce the loading and release of Ca^{2+} from the sarcoplasmic reticulum i.e. i) the use of the Ca^{2+} chelators EGTA and BAPTA to reduce internal Ca^{2+} below 1 nM and ii) the addition of 40 μM ruthenium red to internal solutions adjusted to pH 6.9.

7. Interventions which modify Ca channel phosphorylation and maximize the high threshold Ca channel current abolished frequency-dependent potentiation. Caffeine (0.5 and 1 mM) increased Ca currents by more than 2 fold and abolished frequency-dependent potentiation. Similarly, Ca currents were large and the positive current staircase was abolished in cells which were incubated for more than 5 hrs in solutions containing 0.2 μM ryanodine. Addition of 1 μM isoproterenol to caffeine or ryanodine solutions failed to further increase the Ca current.

8. We conclude that potentiation of the high threshold Ca channel current with repetitive depolarization is mediated by calcium and hypothesize that calcium binds to an site near the Ca channel and modifies channel protein phosphorylation which results in a slowing of inactivation and an increase in peak Ca current.

INTRODUCTION

Cardiac contractility is strongly influenced by the rate and pattern of stimulation (Bowditch, 1871; Koch-Weser & Blinks, 1963). The voltage-dependence of cardiac contractility and the Ca current is similar, and reveals the importance of the Ca current in the initiation of tension in the heart (Beeler & Reuter, 1970; New & Trautwein, 1972; Morad & Goldman, 1973). The influx of calcium ions with depolarization may load the sarcoplasmic reticulum (SR) with Ca^{2+} (Bassingthwaighte & Reuter, 1972; Gibbons & Fozzard, 1975) and trigger SR release of Ca^{2+} (Fabiato, 1985a).

In a patch clamp study of ventricular myocytes in which contaminating currents were substantially reduced, a decrease in low threshold, T-type Ca channel current and a potentiation of high threshold, L-type Ca channel current has been observed with repetitive depolarization (Mitra & Morad, 1986). Recently, potentiation of the high threshold Ca current has been shown to be dependent on external Ca^{2+} in guinea-pig ventricular myocytes (Zygmunt & Maylie, 1988; Tseng, 1988; Fedida, Noble & Spindler, 1988a). A mechanism has been proposed in which the peak amplitude of the Ca current during repetitive stimulation is increased as a consequence of reduced levels of SR Ca^{2+}

release and Ca^{2+}-dependent inactivation of the Ca current (Tseng, 1988; Fedida et al. 1988a). The rate staircase in the guinea-pig ventricle, however, is associated with increased tension (Tuttle & Farah, 1962) and presumably an increase in SR Ca^{2+} release. Potentiation of the Ca current has also been observed in frog atrial and ventricular myocytes in which the SR is poorly developed (Noble & Shimoni, 1981a; Argibay, Fischmeister & Hartzell, 1988). Thus a mechanism of frequency-dependent potentiation of the Ca current may involve voltage (Lee, 1987) or Ca^{2+} enhanced activation of the high threshold channel (Zygmunt & Maylie, 1988; Fedida, Noble & Spindler, 1988b). Such modulation of the Ca channel might be accomplished by changes in the level of Ca channel phosphorylation by the actions of the cyclic-AMP dependent kinase (Osterrieder, Brum, Hescheler, Trautwein, Flockerzi & Hofmann, 1982; Trautwein, Cavalie, Flockerzi, Hofmann & Pelzer, 1987), or the activation of the Ca-calmodulin-dependent phosphatase (Chad & Eckert, 1986).

The experiments which follow were designed to separate the effects of voltage, time and calcium on the frequency-dependent potentiation of the high threshold Ca channel current in mammalian ventricular muscle. This potentiation persisted under experimental conditions designed to reduce the release of Ca^{2+} from the SR, and

was increased by low concentrations of isoproterenol (5-50 nM). We hypothesize that calcium binds to a site in close proximity to the inner pore of Ca channels, resulting in a decrease in the rate of calcium channel inactivation and a potentiation of current during subsequent depolarizations.

A preliminary report of this work has appeared (Zygmunt & Maylie, 1988).

METHODS

Cell preparation. Single ventricular myocytes were obtained from guinea-pig hearts by enzymatic dissociation (Mitra and Morad, 1985). Guinea-pigs (250-400 grams) were killed by either decapitation or cervical dislocation. A dissected heart was mounted on a Langendorff column. All solutions were warmed to 37°C and saturated with 100% oxygen. Retrograde perfusion of the aorta with nominally Ca-free Tyrode's solution for 5 min was followed by a 5 min perfusion with the same solution containing 130 units/ml collagenase (Worthington, U.S.A.; type CLS-2) and 0.2 mg/ml protease (Sigma, U.S.A.; type XIV). These enzymes were washed from the heart with Tyrode's solution containing 100 μM Ca^{2+}. Sections of the right and left ventricular free wall were dissected and single cells were dispersed by gentle agitation in 100 μM Ca^{2+} Tyrode's solution. Cells stored at room temperature (20-25°C) in 1 mM Ca^{2+} Tyrode's solution were used within 16 hours.

Solutions. The composition of the Ca-free Tyrode's solution (mM) was: NaCl, 145; KCl, 5; $MgCl_2$, 3; glucose, 10; N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), 10; and the pH was adjusted to 7.35 with NaOH. The composition of the external and internal solutions was such as to minimize currents through sodium, potassium,

and chloride channels. The external solution contained: $CaBr_2$, 2, 5, or 20; Tetraethylammonium (TEA) methanesulfonate, or TEA bromide 150; HEPES, 10; and the pH was adjusted to 7.35 with TEA hydroxide. In some of the experiments the external solution contained 1 mM $MgCl_2$, as indicated in the figure legends. The internal solution contained: Cs aspartate, 120; $MgCl_2$, 1; HEPES, 10; ATP (magnesium salt), 5; and its pH was adjusted to 7.2 with CsOH. The $[Ca^{2+}]_i$ was buffered to 4 nM with 20 mM ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA; apparent K_D = 0.16 μM, pH 7.2 Fabiato & Fabiato, 1979), or 10 nM with 5 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA, apparent K_D = 0.11 μM, Molecular Probes, USA). The free $[Mg^{2+}]_i$ was calculated to be 0.76 mM and 1.3 mM respectively. Stock solutions of $CdCl_2$, ruthenium red (Sigma, USA), isoproterenol, tetrodotoxin (TTX; Calbiochem, USA), 4-aminopyridine (4-AP, Sigma, USA) and ryanodine (Progressive Agri-Systems, USA) were diluted to the final concentrations indicated in the text.

Recording conditions. Acutely dissociated cells were placed in a 500 μl chamber on the stage of an inverted microscope (Zeiss model IM-35). The chamber was continuously superfused at a rate of 2 ml/min. Whole cell currents (Hamill, Marty, Neher, Sakmann & Sigworth, 1981)

were measured at room temperature (20-25°C) with an Axopatch 1B amplifier (Axon Instruments, USA). The patch pipettes filled with internal solution had tip resistances of 1-3 MΩ. Junction potentials were zeroed prior to formation of the membrane-pipette seal in 1 mM Ca^{2+} Tyrode's solution. The junction potentials between TEA solutions and Tyrode's solutions and between Cs aspartate solutions and Tyrode's solutions was measured to be approximately 4 mV and -11 mV, respectively. When presenting the data, voltage corrections were not made for this shift in junction potential with changes of external solution. Thus a correction factor of approximately -7 mV should be applied to our results to nullify the effects on voltage of changes in external solutions.

Whole cell currents were not electronically compensated for series resistance and capacitance. Linear leak and capacitance currents were subtracted with a scaled current from an average of 16 hyperpolarizing pulses from the holding potential of -80 mV before each test potential. Voltage steps were computer controlled and were applied with a time constant of 200 μs. Currents were amplified with an instrumentation gain amplifier with computer controlled automatic adjustment of gain which was built in this laboratory. Currents were filtered with a 4-pole Bessel filter at 10 kHz, digitized at 5 kHz and stored on

an LSI 11/73 computer. The current traces presented in the text were digitally filtered at 0.5 kHz (Gaussian filter; Colquhoun & Sigworth, 1983).

Input resistance immediately after establishment of whole cell recording in 1 mM Ca^{2+} Tyrode's solution was between 20 and 50 MΩ. Superfusion with the external solution increased the input resistance to between 1 and 3 GΩ within 4 min. Matsuda & Noma (1984) showed that the exchange of intracellular K^+ with Cs^+ at the tip of a patch pipette was essentially complete within 3 min after establishment of whole cell recording and no contractions were observed in our preparation within 1 min after whole cell recording. For currents recorded 7-13 min after establishment of whole cell recording in 5 mM Ca^{2+}, the time to peak of the Ca current for a single pulse from -80 to 20 mV was 10.0 ± 0.9 ms (mean ± S.E.M., n = 62 values). Peak Ca current (I_{Ca}) was -1509 ± 380 pA (n = 31), I_{Ca} density was -9.72 ± 0.95 pA/pF (n = 31) and cell capacitance was 156 ± 35 pF (n = 31).

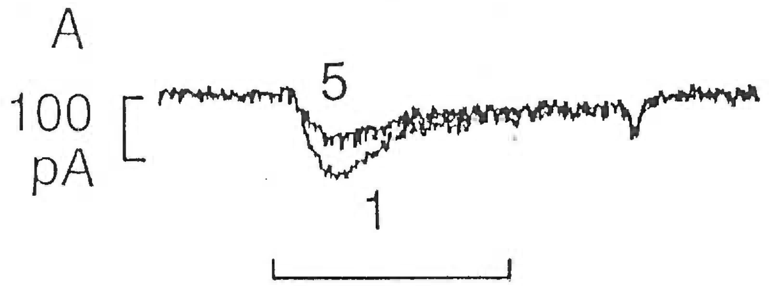
Average results presented throughout the text are given as mean ± S.E.M.

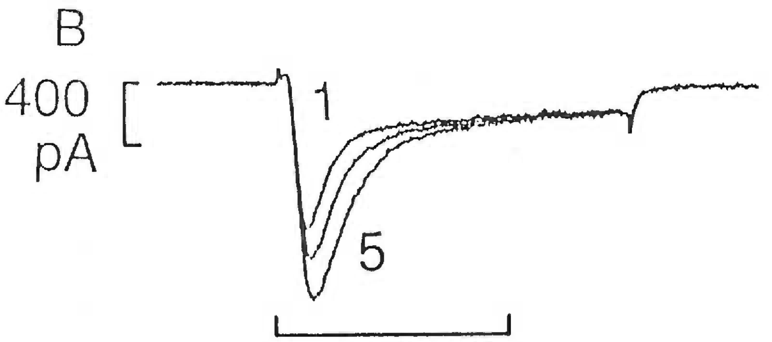
RESULTS

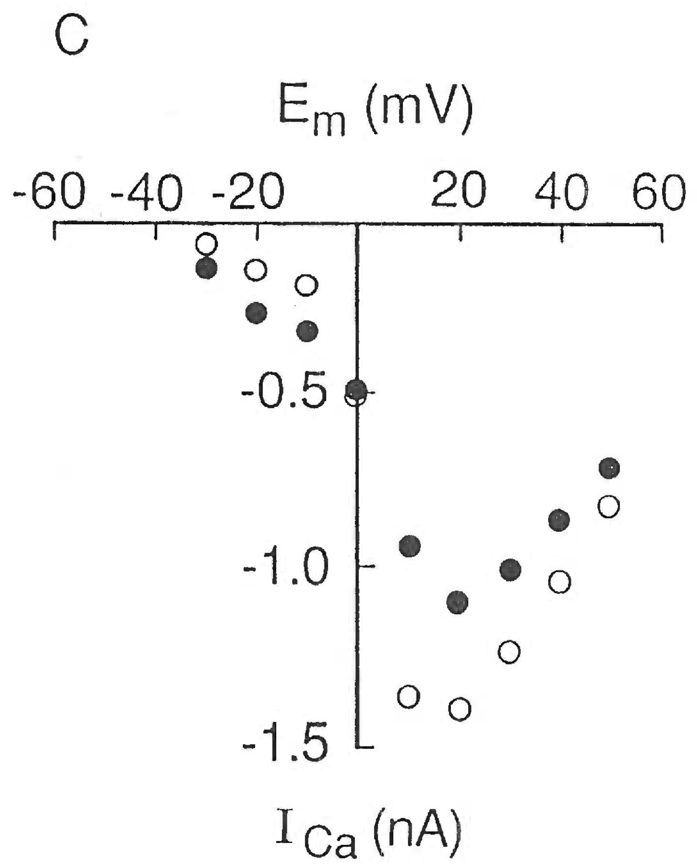
Effects of repetitive depolarization on the Ca current

Approximately every 30 sec the cells were depolarized from a holding potential of -80 mV to a given test potential with a train of 5 pulses of 150 ms duration and a frequency of 2 Hz. Depolarization to a -20 mV test potential, which predominantly activates low threshold Ca channels (Bean, 1985), resulted in a decrease in I_{Ca} (Fig. 1A). The ratio of the peak current measured during the 5th and 1st pulse (I_{P5}/I_{P1}) was 0.58 and I_{P2}/I_{P1} was 0.62. The current reduction was largely complete by the 2nd pulse which is consistent with a failure of the low threshold channels to recover from voltage-dependent inactivation at 2 Hz. Repetitive steps to 10 mV, which activate both low and high threshold Ca^{2+} channels, resulted in a beat dependent increase of current which reached a steady state within 5 beats (Fig. 1B). At a test potential of 10 mV, I_{P2}/I_{P1} was 1.10 and I_{P5}/I_{P1} was 1.30. Potentiation was observed at test potentials between 0 and 50 mV (Fig. 1C). The ratio, I_{P5}/I_{P1}, increased to a maximum at a test potential of 10 mV (Fig. 1C). In other experiments in which the potential dependence of potentiation was examined up to the reversal potential, this ratio decreased at test potentials more

Fig. 1. Effects of repetitive depolarization on Ca current and potential dependent enhancement of the Ca current. After a 30 sec rest period, the cell was depolarized from a holding potential of -80 mV to a test potential with a train of 5 pulses of 150 ms duration and a frequency of 2 Hz. Currents obtained during the 1st (1), 2nd, and 5th (5) pulses in 20 mM Ca^{2+} are shown. Time bar equals 100 ms, cell capacitance, 141 pF. A) Test potential of -20 mV. The 2nd and 5th current pulses are nearly identical and are depotentiated. B) Test potential of 10 mV. C) The peak current (I_{Ca}) during the 1st pulse (●) and the 5th pulse (O) is plotted against test potential (V_{test}). The ratios of I_{Ca} during the 5th and the 1st pulses are as follows: 0.52 at -30 mV, 0.54 at -20 mV, 0.58 at -10 mV, 1.03 at 0 mV, 1.45 at 10 mV, 1.27 at 20 mV, 1.22 at 30 mV, 1.19 at 40 mV, and 1.16 at 50 mV.


A
100
pA
5
1


B
400
pA
1
5


C
Em (mV)
-60
-40
-20
20
40
60
-0.5
-1.0
-1.5
ICa (nA)

positive than 10 mV, but remained greater than 1 up to the reversal potential of the current (n = 4). Potentiation was not observed at potentials more positive than the I_{Ca} reversal potential (n=3).

Potentiation at a test potential of 20 mV was observed in 20 mM Ca^{2+} and current ratios were $I_{P2}/I_{P1} = 1.17 \pm 0.04$ (n = 9) and $I_{P5}/I_{P1} = 1.39 \pm 0.06$. These ratios were $I_{P2}/I_{P1} = 1.20 \pm 0.02$ (n = 20) and $I_{P5}/I_{P1} = 1.37 \pm 0.03$ in 5 mM Ca^{2+} and were $I_{P2}/I_{P1} = 1.20 \pm 0.03$ (n = 14) and $I_{P5}/I_{P1} = 1.36 \pm 0.06$ in 2 mM Ca^{2+}.

Low threshold and high threshold Ca channel currents contribute to I_{Ca} at positive test potentials (Bean, 1985; Mitra & Morad, 1986). Since the effect of stimulation at 2 Hz is to reduce the low threshold current, the potentiation of the high threshold current at 10 mV may be underestimated. A prepulse to -40 mV which should inactivate the low threshold current (Bean, 1985) increased the positive current staircase of the high threshold current in 5 mM Ca^{2+} (Fig. 2). During the 100 ms prepulse to -40 mV the time to peak of the low threshold current was 10 ms and the current was completely inactivated 50 ms into the prepulse. With a step to 20 mV, I_{P5}/I_{P1} was 1.41 in the control (panel A) and 1.47 with a prepulse (panel B).

In general, the contribution of the low threshold

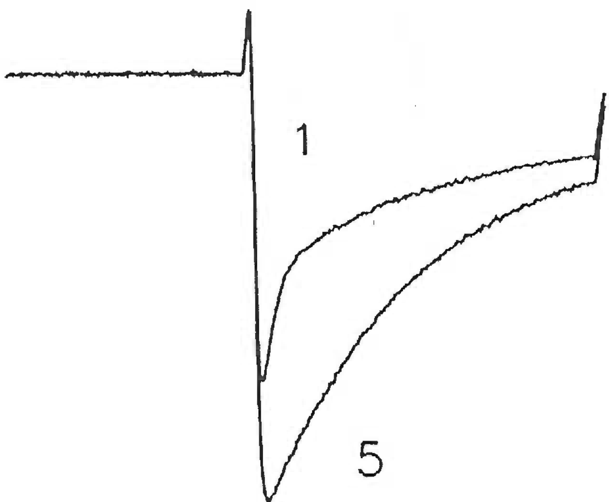
channel current at potentials greater then 10 mV was negligable. The calcium current during a single depolarization to -20 mV was -190 ± 56 pA (n=6) in 5 mM Ca^{2+}. The effect of a 70 ms prepulse to -30 mV was to reduce this current to -58 ± 18 pA (n=6). This prepulse effectively reduced the low threshold current, but had little effect on the positive current staircase. I_{P5}/I_{P1} at 20 mV was 1.30 ± 0.12 in the control and 1.30 ± 0.08 (n = 10) with a 70 ms prepulse.

Facilitation of I_{Ca} was associated with a reduction in the rate of inactivation. The initial rate of rise and the steady state current measured 150 ms after stepping to 20 mV were unchanged by repetitive depolarization (Fig. 2).

Contribution of currents other than calcium

The observed facilitation of I_{Ca} could result from a use-dependent inhibition of a transient outward current. Such a use-dependent inhibition of a TEA and 4-AP sensitive transient outward current has been described in cardiac Purkinje fibres (Gibbons & Fozzard, 1975; Kenyon & Gibbons, 1979a & 1979b; Boyett, 1981), and in rabbit ventricular cells, the interval-dependent increase in the amplitude and duration of the action potential plateau was shown to result from inactivation of a 4-AP sensitive current (Hiraoka & Kawano, 1987). Figure 3 shows the

current traces of the 1st and 5th pulses in the control (panel A) and approximately 14 min after addition of 4-AP (panel B). The ratio of I_{P5}/I_{P1} was 1.51 in control and 1.57 after 14 min of exposure to 4-AP. Shown in Fig. 3C is the ratio, I_{P5}/I_{P1}, and the decrease over time of I_{Ca} during the 1st and 5th pulse. I_{P5}/I_{P1} and the rundown of Ca


A
1
5

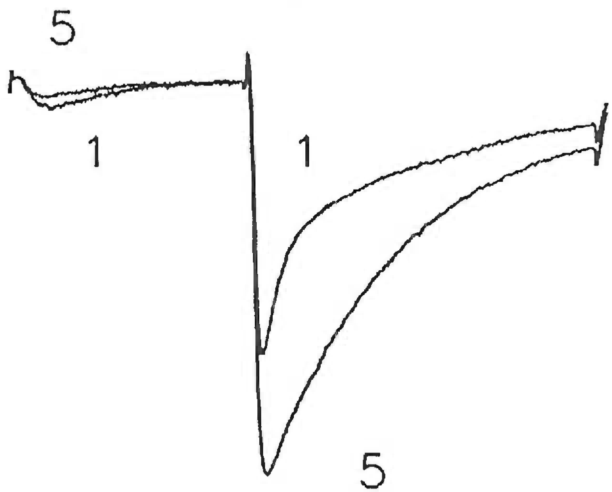
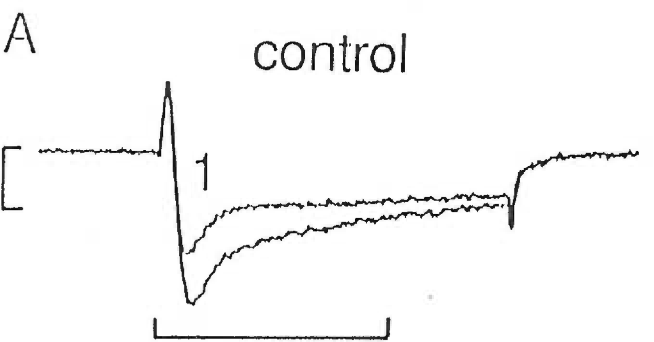
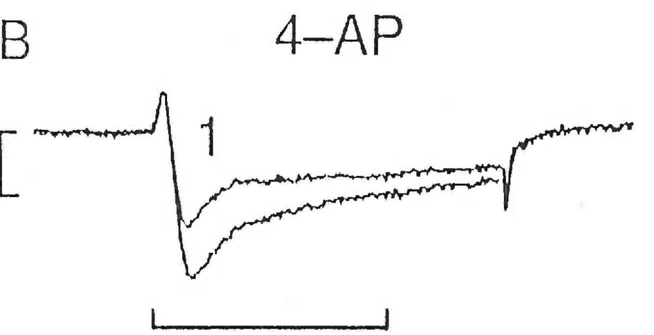
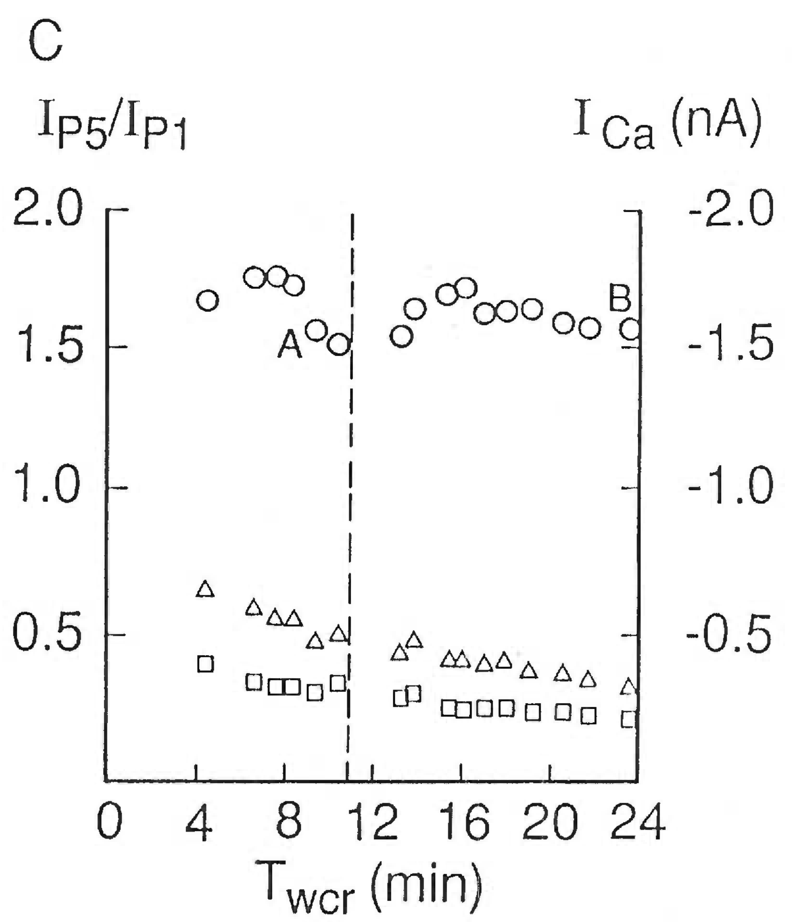

B
5
1
1
5

Fig. 3. Application of 4-aminopyridine and potentiation of high threshold Ca current. The cell was depolarized from a holding potential of -80 mV to a test potential of 30 mV at a rate of 2 Hz. Currents during the 1st (1) and 5th pulse in 20 mM Ca^{2+}. Current bar, 200 pA; time bar, 100 ms; cell capacitance, 100 pF. A) Current traces from cell 9.3 min after whole cell formation and prior to 4-AP application. These traces correspond to the data point marked A in the right panel. B) Current traces from cell 23.6 min after whole cell formation, following a 14 min exposure to 1 mM 4-AP. These traces correspond to the data point marked B in the right panel. C) The ratio of currents during the 5th and 1st pulse (I_{P5}/I_{P1}) (O); and peak current (I_{Ca}) during the 1st ( ) and 5th ( ) pulse is plotted against time since establishment of whole cell recording (T_{wcr}). 4-AP (1 mM) was applied 10.4 min after establishment of whole cell recording indicated by the dashed line.


A
control
1


B
4–AP
1


C
I_{P5}/I_{P1}
I_{Ca} (nA)
2.0
1.5
1.0
0.5
-2.0
-1.5
-1.0
-0.5
A
B
0
4
8
12
16
20
24
T_{wcr} (min)

current were not appreciably affected by the application of 1 mM 4-AP. In 7 other myocytes which were repetitively depolarized in 20 mM Ca^{2+}, 1 mM 4-AP did not reduce potentiation at a test potential of 30 mV after an exposure of 9-20 min. These data demonstrate that 4-AP does not affect I_{Ca} or the positive current staircase in guinea-pig ventricular myocytes, and suggests that transient outward currents do not contaminate current records in this preparation.

Matsuda & Noma (1984) have observed a voltage-dependent leakage current carried by Cs^+ at potentials more positive than 30 mV in guinea-pig myocytes. In order to assess the effect of an outward Cs^+ current on high threshold current potentiation, Cs^+ in the standard pipette solution was replaced with either TEA or n-methylglucamine. With repetitive depolarization to 20 mV in 20 mM Ca^{2+}, I_{P5}/I_{P1} was 1.36 ± 0.06 ($n = 2$) in TEA internal solution and 1.22 ± 0.07 ($n = 3$) in n-methylglucamine internal solution in 2 mM Ca^{2+}. There was, therefore, no evidence of a Cs^+ current interfering with potentiation.

Ionic flux through Na^+ channels was reduced by the substitution of impermeant ions for Na^+ in external and internal solutions and with a 70 ms prepulse to -30 mV prior to each test pulse. In 4 cells, currents measured during successive steps to a test potential of 20 mV were

insensitive to tetrodotoxin (10 μM TTX). Finally, time dependent currents were not observed following the blockade of Ca channels with 100 μM $CdCl_2$ (n = 6).

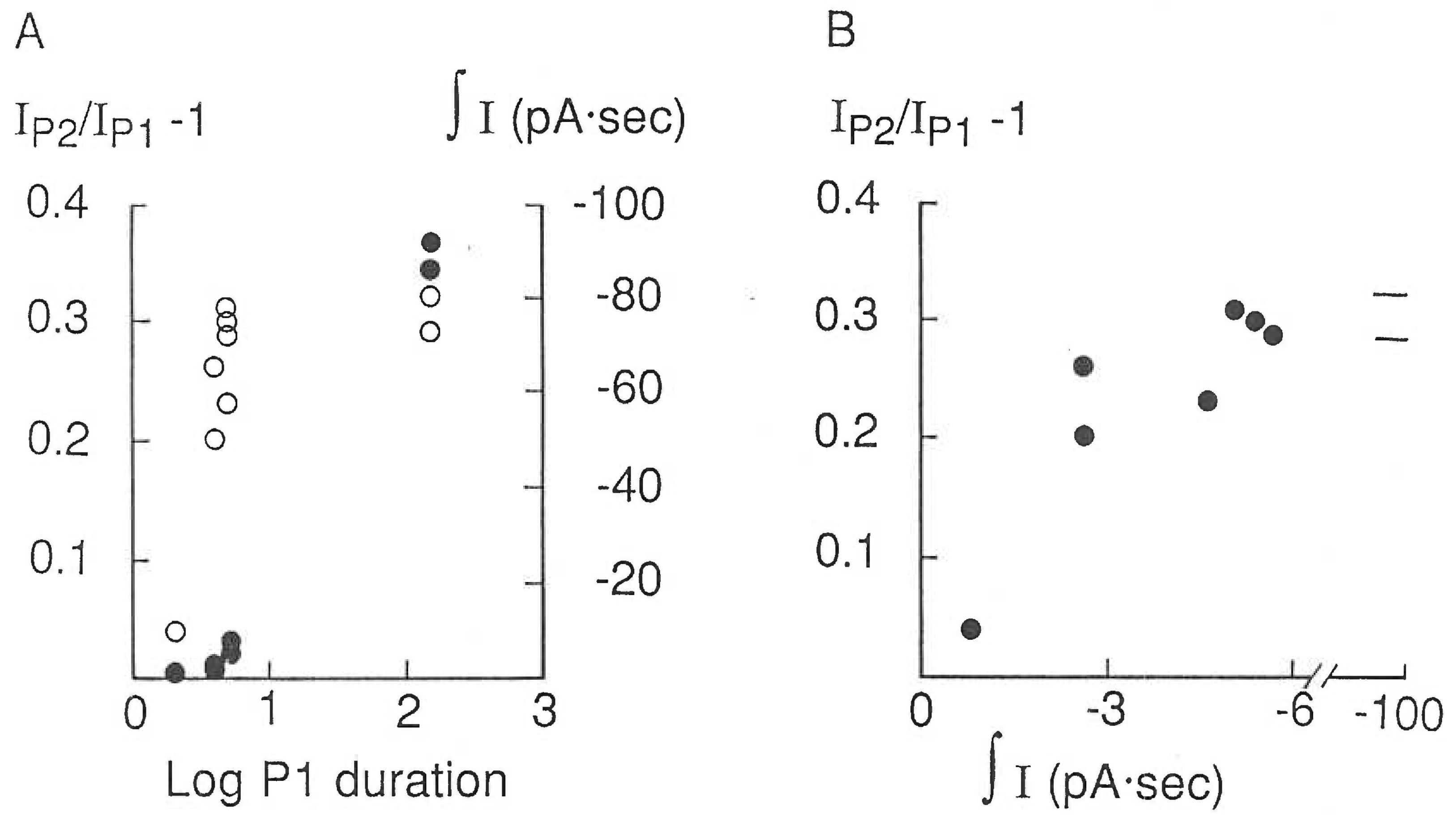
The above data suggests the reasonably complete isolation of I_{Ca} in these dialyzed myocytes.

Kinetics of the potentiation process

The minimum time needed for activation of potentiation was determined with a dual pulse protocol in which the duration of the first pulse was varied. Figure 4 shows the fractional increase in currents during the 2nd pulse from an experiment in which the duration of the 1st pulse was varied between 2 and 150 ms. The peak inward current of the 2nd pulse, plotted as the fractional increase of the ratio I_{P2}/I_{P1} versus the log of duration of the 1st pulse (open circles, panel A), increased to its maximal value with a 5 ms first pulse. The fractional increase in current was 0.29 (average of 4 trials) with a 5 ms first pulse duration and was not further increased by a 150 ms pulse. In 6 other cells a 1st pulse duration of 10 ms was sufficient for maximal potentiation of the 2nd pulse. The longest pulse duration tested was 700 ms, which resulted in the same degree of potentiation as a 10 ms pulse. Activation of current potentiation was rapid and independent of pulse duration between 10 and 700 ms.

Calcium is required for the enhancement of Ca currents

Fig. 4. The kinetics of activation and the relationship between the total flux of Ca^{2+} and Ca current potentiation in 5 mM Ca^{2+}. After a 30 sec rest period, the cell was depolarized with a dual pulse protocol in which the duration of the 1st pulse (P1) was varied. The cell was depolarized from a holding potential of -80 mV to a test potential of 30 mV and returned to the holding potential for 350 ms before a 2nd step (P2) was taken to 30 mV for 150 ms. Cell capacitance, 143 pF. The standard pipette solution was modified to reduce the release of Ca^{2+} by the sarcoplasmic reticulum and contained (mM): cesium aspartate, 120; $MgCl_2$, 1; HEPES, 10; ATP (magnesium salt), 5; EGTA, 20; ruthenium red, 40 uM; and pH was adjusted to 6.9 with CsOH. A) 1st pulse durations of 2,4,5, and 150 ms are plotted against the fractional increase in current during the 2nd 150 ms pulse ($I_{P2}/I_{P1}-1$) (O); and the total flux of Ca^{2+} ($\int I$) during the 1st pulse (●). In calculating the fractional increase in current there was a need to extrapolate the magnitude of I_{P1} from control pulses of 150 ms duration recorded throughout the experiment, since: i) peak current did not fully develop with a pulse of less than 15 ms duration, and, ii) Ca current was observed to decrease over time. B) $(I_{P2}/I_{P1})-1$ is plotted against the total flux of Ca^{2+} during the 1st pulse. Off scale, the horizontal dashes indicate 2 150 ms pulses (plotted in A).


A
IP2/IP1 -1
∫ I (pA·sec)
0.4
0.3
0.2
0.1
-100
-80
-60
-40
-20
0
1
2
3
Log P1 duration
B
IP2/IP1 -1
0.4
0.3
0.2
0.1
0
-3
-6
-100
∫ I (pA·sec)

by strophanthidin (Marban & Tsien, 1982). To see whether the activation of potentiation of the Ca current was related to the net influx of calcium, the current traces from the 1st pulse were integrated to estimate the total influx of calcium. The Ca currents, corrected for linear leak and capacitance were filtered at 0.2 Hz and integrated from the beginning of the test pulse to the point at which tail currents were deactivated. The limits of integration included those currents which are thought to result from movement of charge with channel gating (Bean & Rios, 1989) and any Ca tail currents. The on and off gating charges are of equal magnitude at a holding potential of -110 mV but of opposite sign and would not contribute to the calculated ionic flux. The relationship between pulse duration and integral of the Ca current is plotted in Fig. 4A (closed circles). A 2 ms pulse resulted in a net influx of 0.8 pA•sec and a fractional increase in current of 0.03. The tail current accounted for the entire flux of Ca^{2+} with a 2 ms pulse. A 4 ms depolarization resulted in significant potentiation of the current and a net influx of 2.6 pA•sec. Ca^{2+} influx continued to increase between 5 and 150 ms pulses. In figure 4B the relationship between net Ca^{2+} flux and enhancement of I_{Ca} is initially steep and then saturates at larger influxes. In an additional two cells we also

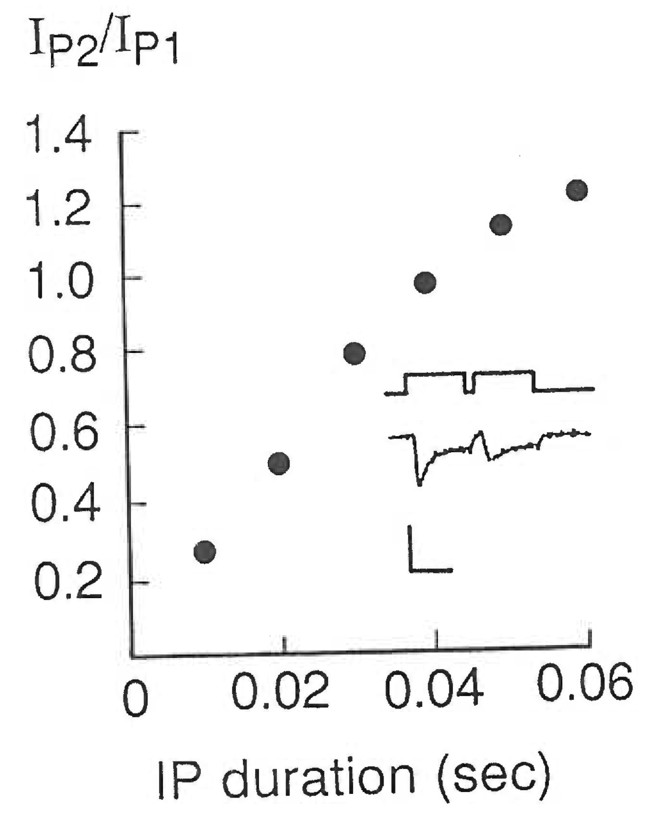
observed this increase of Ca current with increasing net ionic influx of calcium in the previous pulse. I_{Ca} of the second pulse was independent of Ca^{2+} flux with first pulse durations of 10 ms and longer (n = 7). The relationship between ionic flux and potentiation of I_{Ca} is consistent with a calcium mediated response which quickly saturates.

The interval between pulses determines the relative contributions of potentiation and recovery from inactivation, and therefore, the amplitude of the current during succeeding pulses. The effect of changing the interval between two pulses to 10 mV on the amplitude of the 2nd pulse current is shown in Fig. 5. A short interpulse duration of 10 ms resulted in a fractional recovery of I_{Ca} during the second pulse of 0.28 of the first pulse current (Fig 5A). Increasing the interval to 60 ms resulted in recovery from inactivation and a 1.3 fold increase of the current during the 2nd pulse. Reversal of potentiation in a second cell is shown at a slower time course (Fig. 5B). The half time for reversal of potentiation in this cell was 3.7 sec. In 4 cells, the mean half time was 4.4 ± 0.6 sec. Decay of potentiation was less rapid than recovery from inactivation.

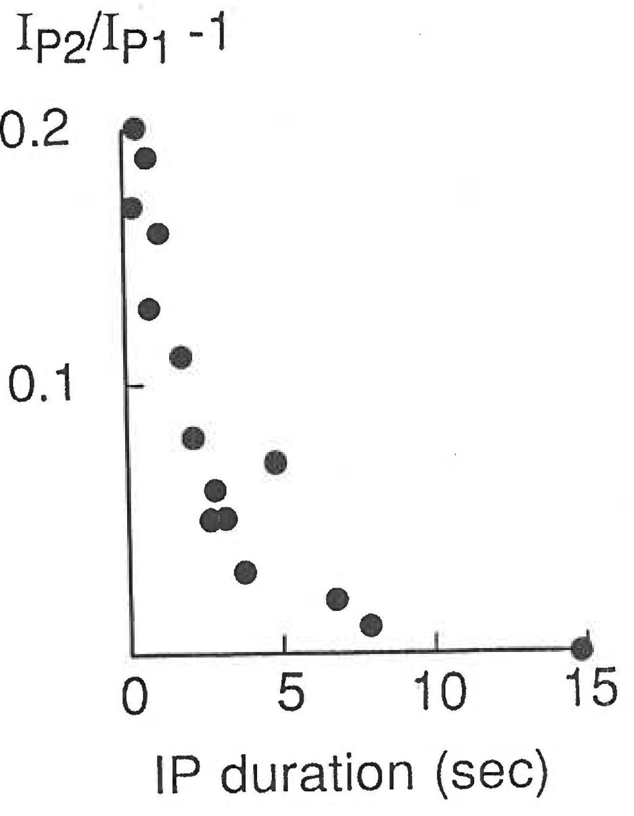
Ca^{2+} dependence of high threshold current potentiation

If facilitation of the high threshold Ca current is mediated by calcium as the previous data suggest,

Fig. 5. Recovery from inactivation and potentiation determined using a dual pulse protocol in which the interval between two 150 ms steps to 10 mV was changed. After waiting at least 30 sec, cells were depolarized from a holding potential of -80 mV to a test potential of 10 mV for 150 ms and returned to the holding potential before a 2nd 150 ms pulse was taken to 10 mV. Current and time calibrations in inset are 600 pA and 50 ms. Left panel) The ratio of peak current during the 2nd and 1st pulses (I_{P2}/I_{P1}) is plotted against interpulse (IP) duration. The inset shows a current trace and voltage template for an IP of 20 ms. Cell capacitance 106 pF, in 20 mM Ca^{2+}. Right panel) The fractional increase in current ($I_{P2}/I_{P1}-1$) is plotted against IP duration in a second cell. Cell capacitance 160 pF, in 5 mM Ca^{2+}.



I_{P2}/I_{P1}
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.02
0.04
0.06
IP duration (sec)

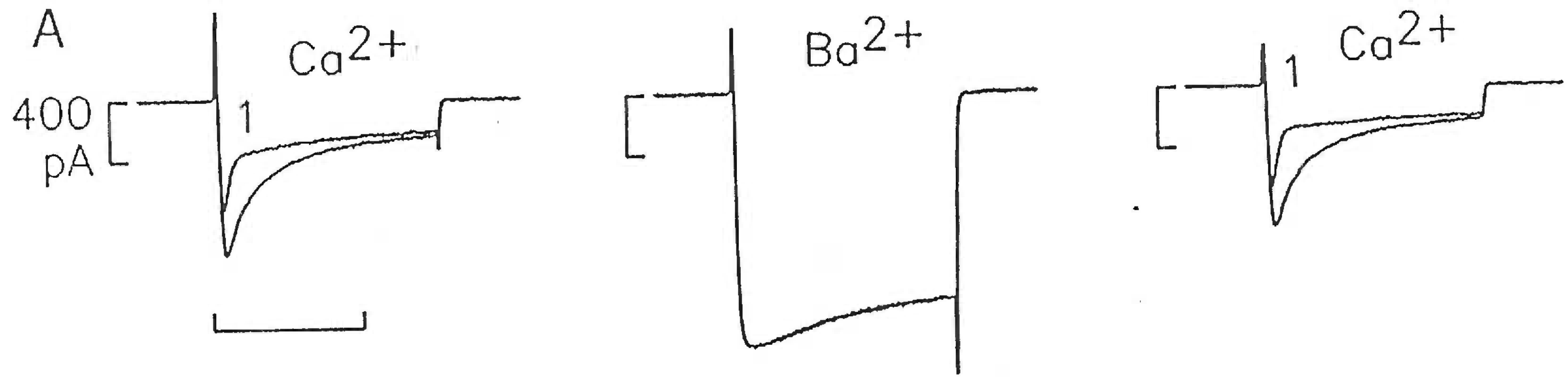


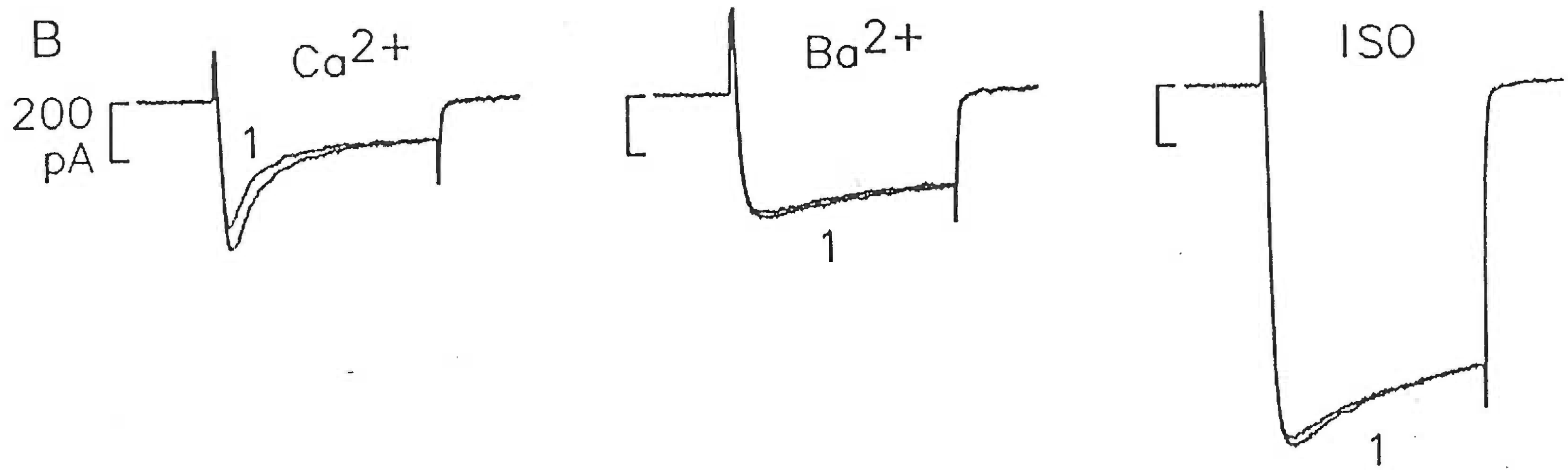
I_{P2}/I_{P1} -1
0.2
0.1
0
5
10
15
IP duration (sec)

frequency-dependent potentiation should be sensitive to the choice of Ca channel charge carrier. Ba^{2+} and Sr^{2+}, which can substitute for Ca^{2+} as a charge carrier in Ca channels, fail to activate a number of Ca^{2+} dependent cellular processes (Kolhardt, Haastert, & Krause, 1973; Siegelbaum & Tsien, 1980; Kass & Sanguinetti, 1984). Equimolar substitution of Ba^{2+} for external Ca^{2+} increased the high threshold current, reduced its rate of inactivation and abolished use-dependent potentiation (Fig. 6A). These effects required 2 min to fully develop. (The frequency-dependent decrease of the low threshold current was not affected by Ba^{2+}.) Subsequent superfusion with Ca^{2+} solution restored potentiation back to control levels within 2 min (Fig. 6A right traces). The loss of frequency-dependent potentiation in barium was not due to an inability to further increase the current, since the β-adrenergic agonist isoproterenol was effective in increasing the Ba^{2+} current approximately 3-fold (Fig. 6B). The loss of frequency-dependent potentiation was obtained in 29 other cells in barium and 5 cells in strontium. The loss of potentiation was not specific to Ba^{2+} and Sr^{2+}, since potentiation was also abolished when sodium was used as the charge carrier (144 mM $[Na^+]_o$; n = 2).

Frequency-dependent potentiation of Ca current does not

Fig. 6. The Ca^{2+} dependence of high threshold current potentiation. Repetitive steps to 0 mV from a holding potential of -80 mV at a rate of 2 Hz. Currents during the 1st (1) and 5th pulse. Time bar, 100 ms. A) Currents at a test potential of 0 mV: in 2 mM external Ca^{2+} (left), following exchange of external Ca^{2+} with 2 mM Ba^{2+} (middle), and after restoration of external Ca^{2+} (right). Cell capacitance, 66 pF. B) A 70 ms prepulse to -40 mV precedes each step to the test potential of 0 mV in a 2nd cell: in 2 mM external Ca^{2+} (left), following exchange of external Ca^{2+} with 1 mM Ba^{2+} (middle), and after exposure to 1 μM isoproterenol (ISO) in Ba^{2+} solution (right). The current staircase was positive in Ca^{2+}, and negative in Ba^{2+} solutions. Cell capacitance, 76 pF.


A
400
pA
Ca2+
1
B
200
pA
Ca2+
1


Ba2+
Ca2+
1
Ba2+
1
ISO
1

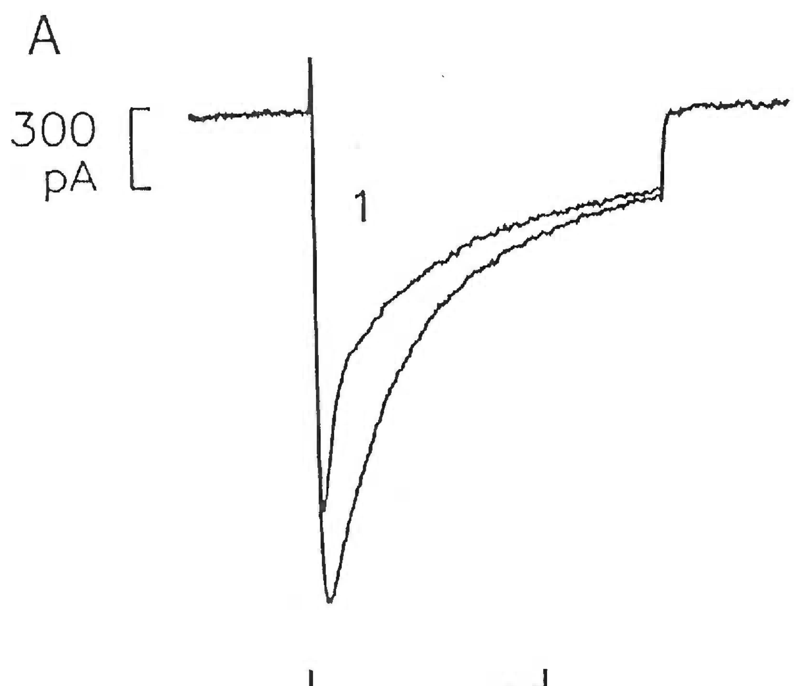
appear to be a response to the displacement of protons from EGTA for two reasons; i) Kaibara and Kameyama (1988) found an inhibitory effect of intracellular protons on the high threshold Ca channel in guinea-pig ventricular myocytes, and ii) potentiation was observed when 5 mM BAPTA was used as the Ca chelator in the internal solution. At pH 7.2, BAPTA is in its ionized form and the binding of Ca^{2+} does not displace protons (Tsien, 1980). In 5 mM Ca^{2+}, I_{P5}/I_{P1} was equal to 1.24 ± 0.09 ($n = 4$) in cells dialyzed with BAPTA solutions.

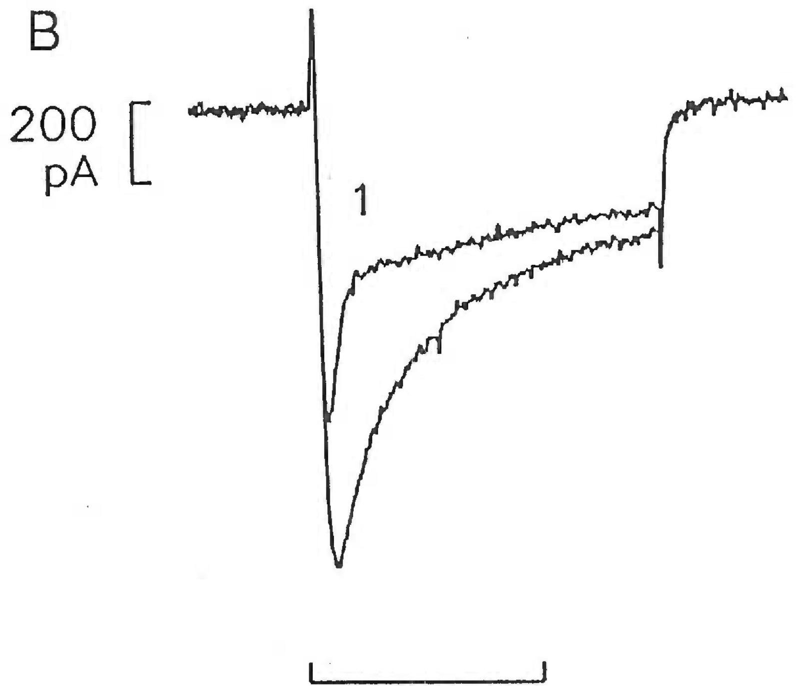
These results are consistent with the hypothesis that use-dependent enhancement of the high threshold Ca channel current is Ca^{2+} dependent.

The effects of decreased loading and release of Ca^{2+} from the sarcoplasmic reticulum on potentiation

Two very different mechanisms offer some explanation for the Ca^{2+} dependence of the positive current staircase in dialyzed myocytes. Common to both mechanisms is that repetitive depolarization from a rested state results in a reduction in Ca channel inactivation. Inactivation of cardiac Ca channels is both voltage- and calcium-dependent (Kass, & Sanguinetti, 1984; Lee, Marban & Tsien, 1985). The modulation of Ca channel gating by calcium involves the binding of calcium to a specific site, since neither

Fig. 7. High threshold current potentiation under conditions designed to reduce Ca^{2+} loading and release from the sarcoplasmic reticulum. Cells were repetitively depolarized at a rate of 2 Hz from a holding potential of -80 mV to a prepulse potential of -30 mV for 70 ms and then to the test potential for 150 ms. External Ca^{2+}, 5 mM; test potential, 20 mV; time bar, 100 ms. A) Internal Ca^{2+} was buffered below 1 nM in the pipette solution containing (mM): cesium aspartate, 120; $MgCl_2$, 1; HEPES, 10; BAPTA, 5; ATP, 5; and pH was adjusted to 6.9 with CsOH. Cell capacitance, 108 pF. B) Currents in a second cell 10 min after establishment of whole cell recording. Internal Ca^{2+} was buffered below 1 nM in the pipette solution containing (mM): cesium aspartate, 120; $MgCl_2$, 1; HEPES, 10; EGTA, 20; ATP, 5; and pH was adjusted to 6.9 with CsOH. Ruthenium red (40 μM) was added to the pipette solution to block release of Ca^{2+} from the sarcoplasmic reticulum. Cell capacitance 122 pF.

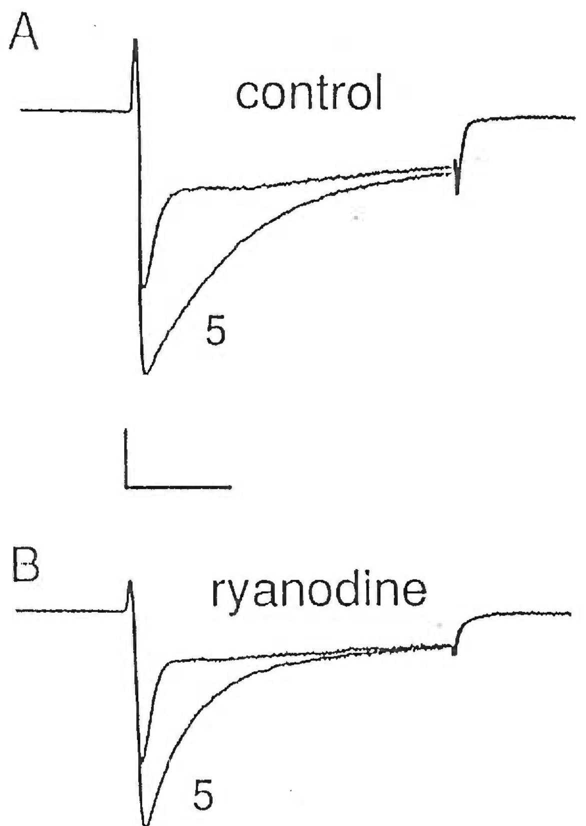

A
300 pA
1

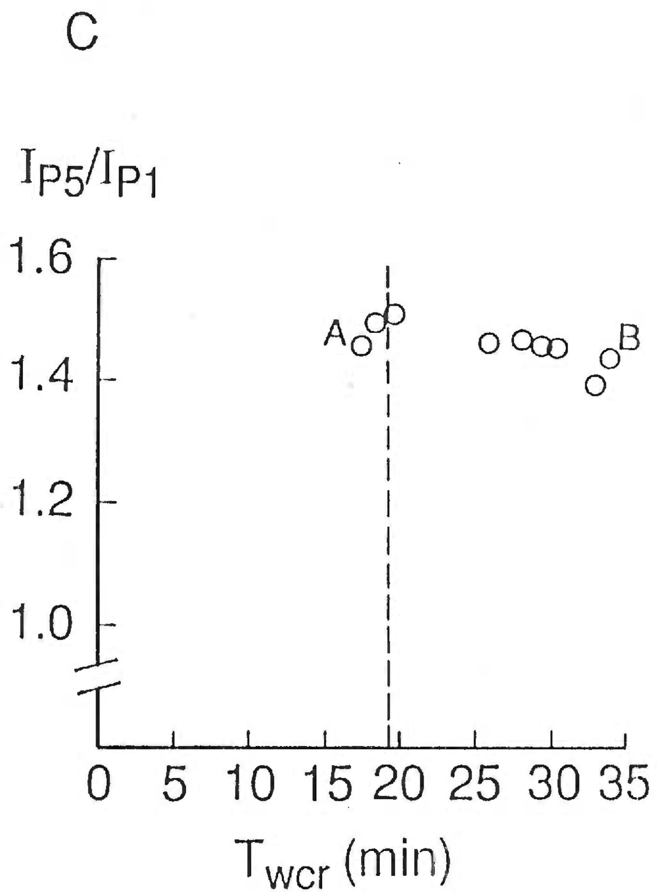

B
200 pA
1

1.16 ± 0.02, n = 4). In 6 other experiments, internal Ca^{2+} was buffered below 1 nM with EGTA. Release from the SR was reduced by the addition of 40 μM ruthenium red to internal solutions (Fabiato & Fabiato, 1973; Baylor, Hollingworth & Marshal, 1988). The release of calcium from the SR was also reduced by the adjustment of internal pH to 6.9 (Ma, Fill, Knudson, Campbell & Coronado, 1988). Figure 7B shows current traces during the 1st and 5th pulse to a test potential of 30 mV. In 5 mM Ca^{2+}, depolarization to 30 mV at 2 Hz resulted in an average I_{P5}/I_{P1} ratio equal to 1.38 ± 0.08 (n = 6) in currents recorded 11-24 min after establishment of whole cell recording.

The effect on potentiation of the addition of ryanodine (2 μM) to the external solution is shown in Fig. 8. The switch to the ryanodine solution was made 19 min after establishment of whole cell recording, and stimulation was continued (Fig. 8C). Prior to ryanodine exposure and 17.4 min after establishment of whole cell recording, I_{P5}/I_{P1} was equal to 1.45 (Fig. 8A). Following a 14 min exposure to ryanodine, the magnitude of I_{P1} was reduced from -1723 pA in the control to -1309 pA, however, potentiation was not significantly reduced (I_{P5}/I_{P1} = 1.43, Fig. 8B). Potentiation persisted at ryanodine concentrations of 0.2 (n = 2), 0.4 (n = 1), and 2 μM (n = 4). Potentiation was

Fig. 8. The effect of 2 μM ryanodine on Ca^{2+} dependent potentiation. The cell was repetitively stimulated from a holding potential of -80 mV to 20 mV at 2 Hz in 5 mM Ca^{2+}. Current and time calibrations; 500 pA, 50 ms. Cell capacitance, 108 pF. Current traces during the 1st and 5th (5) pulse. A) Current traces in control solution, 17.4 min after establishment of whole cell recording (T_{wcf}). This trace corresponds to the data point labeled A in the right panel. B) Current traces after 14 min exposure to ryanodine, corresponding to data point B in right panel. C) Plot of potentiation (I_{P5}/I_{P1}) against T_{wcf} over the course of the experiment. The switch to ryanodine solution was made at the time indicated by the dashed line.


A
control
5
B
ryanodine
5


C
I_{P5}/I_{P1}
1.6
1.4
1.2
1.0
A
B
0 5 10 15 20 25 30 35
T_{wcr} (min)

abolished in 1 cell in 15 μM ryanodine (20 min exposure), and this effect was reversed with washout of the drug. It is possible that ryanodine abolishes potentiation in lower concentrations if sufficient time is allowed for receptor binding.

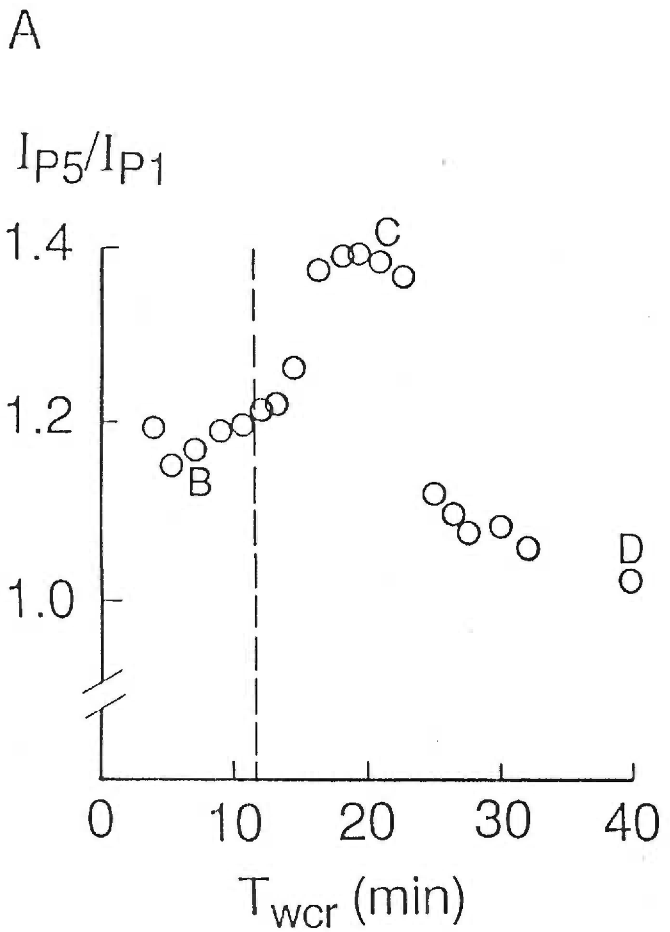
With a concentration of 5 nM ryanodine, the time constant of association for ryanodine and its receptor is approximately 40 min (Pessah, I.N., Francini, A.O., Scales, D.J., Waterhouse, A.L. & Casida, J.E., 1986). We tested the effects of 0.2 μM ryanodine on potentiation in cells which were incubated in ryanodine for 3 to 9 hrs at room temperature.

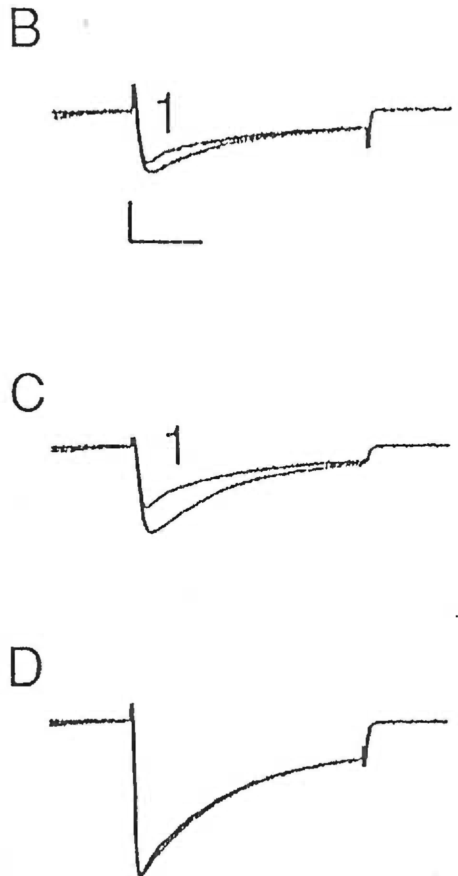
A positive current staircase was observed in two cells incubated in 0.2 μM ryanodine for 3 to 4 hrs (I_{P5}/I_{P1} = 1.15 ± 0.05, n = 2). I_{P1} in these two cells was 2173 ± 978 pA and current density was 21.1 ± 5.8 pA/pF. Further incubation of 4 cells exposed to 0.2 μM ryanodine for over 5 hrs increased the Ca current and abolished the positive current staircases. I_{P1} in these 4 cells was 4447 ± 1530 pA (42.0 ± 9.9 pA/pF) for cells in 0.2 μM ryanodine. This loss of potentiation was not the result of "rundown" of potentiation over time, since positive current staircases were observed in two cells which were stored in ryanodine-free solutions for over 8 hrs (I_{P5}/I_{P1} = 1.34 ± 0.04, n = 2).

The effects of caffeine, ryanodine and isoproterenol on high threshold current potentiation

Caffeine (10 mM) has been reported to abolish use-dependent enhancement of the Ca current in ventricular myocytes (Tseng, 1988). We tested the effects of lower concentrations of caffeine on potentiation. Caffeine at a concentration of 0.1 mM had no effect on Ca currents or frequency-dependent potentiation (n = 3). Increasing concentrations to 0.5 mM (n = 6) and 1.0 mM (n = 11) increased I_{Ca}, but abolished potentiation. Potentiation was not immediately abolished; rather, the effect of caffeine required several minutes during which the cells were repetitively stimulated. Figure 9 shows the effect on potentiation of 0.5 mM caffeine over the course of one experiment. The switch to caffeine solution (Fig 9A, indicated by vertical bar) resulted initially in an increase in both I_{Ca} and potentiation. I_{P5}/I_{P1} increased from approximately 1.2 in control, to 1.4 after an 8 min exposure to caffeine. Continued superfusion of caffeine solution resulted in further increases in I_{Ca} and a loss of potentiation. The amplitude of I_{P1} increased from -478 pA to -618 pA near the time of peak potentiation, and was

Fig. 9. The effects of 0.5 mM caffeine on high threshold Ca current. Stimulation protocol described in legend of Fig. 7. Test potential, 20 mV in 5 mM Ca^{2+}. Current and time calibrations, 400 pA and 50 ms; cell capacitance, 73 pF. A) A plot of potentiation (I_{P5}/I_{P1}) against time since establishment of whole cell recording (T_{wcf}). The switch to caffeine solution was made at the time indicated by the dashed line. B) Currents during the 1st (1) and 5th pulse in control; C) after 11 min exposure to 0.5 mM caffeine; D) and 28 min exposure to caffeine. Traces correspond to those points marked B, C, and D in left panel.


A
IP5/IP1
1.4
1.2
1.0
0
10
20
30
40
Twcr (min)
B
C
D


B
1
C
1
D

-1475, pA after a 28 min exposure to caffeine (Fig 9; right panel).

In these experiments, exposure to concentrations of caffeine as high as 10 mM did not result in contracture of the myocytes.

In elephant seal atrial trabeculae stimulated at 0.4 Hz, 1 mM caffeine increased phasic tension, but abolished the fast phase of the force staircase (Maylie & Morad, 1984). In the one preparation tested, a guinea-pig papillary muscle bathed in 5 mM Ca^{2+} Tyrode's solution was stimulated continuously at 0.2 Hz. This stimulation was interrupted by a train of 10 pulses at 0.4 Hz. The tension recorded at 0.4 Hz increased with each beat to a new steady state level within six beats. This beat dependent fast phase of the positive force staircase (Koch-Weser & Blinks, 1963) was abolished with 0.5 and 1.0 mM caffeine, however, phasic tension increased with caffeine. Since tension increased with 0.5 and 1 mM caffeine, it is unlikely that SR stores of Ca^{2+} were depleted at these concentrations of caffeine.

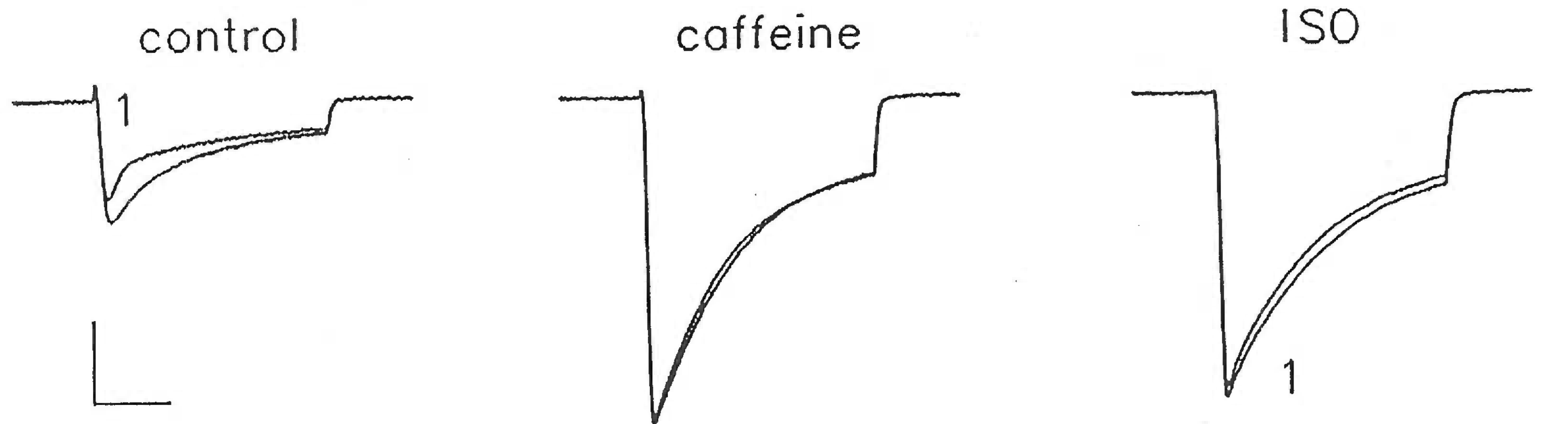
In the present study, the loss of potentiation in 1 mM caffeine was accompanied by a greater than 2 fold increase in I_{Ca} (n = 11). We, therefore, tested whether loss of potentiation might represent inhibition of phosphodiesterase activity and maximum phosphorylation of

Ca^{2+} channels, rather than depletion of SR stores of Ca^{2+}. Cells in 5 mM Ca^{2+} were periodically stimulated with a train of 5 pulses at 2 Hz in control solutions and solutions containing 1.0 mM caffeine. After allowing sufficient time for the loss of potentiation in caffeine, the external solution was exchanged for a solution containing 1 mM caffeine plus 1 μM isoproterenol. The results from a representative experiment are shown in Fig. 10. The magnitude of I_{P1} was increased from -1096 pA in 5 mM Ca^{2+} to -3678 pA in the presence of 1 mM caffeine. Following a 5 min exposure to a combination of caffeine and 1 μM isoproterenol, I_{P1} was equal to -3500 pA. A slight reduction in I_{Ca} following superfusion of isoproterenol was observed in a number of these experiments and can probably be attributed to rundown of the Ca current. I_{P1} was increased by greater than 2 fold in 0.5 (n = 2) and 1 mM caffeine (n = 4) and was not additionally enhanced by isoproterenol in these six cells.

Similarily, 1 μM isoproterenol did not further enhance Ca currents in 3 cells exposed to 0.2 μM ryanodine for over 5 hrs.

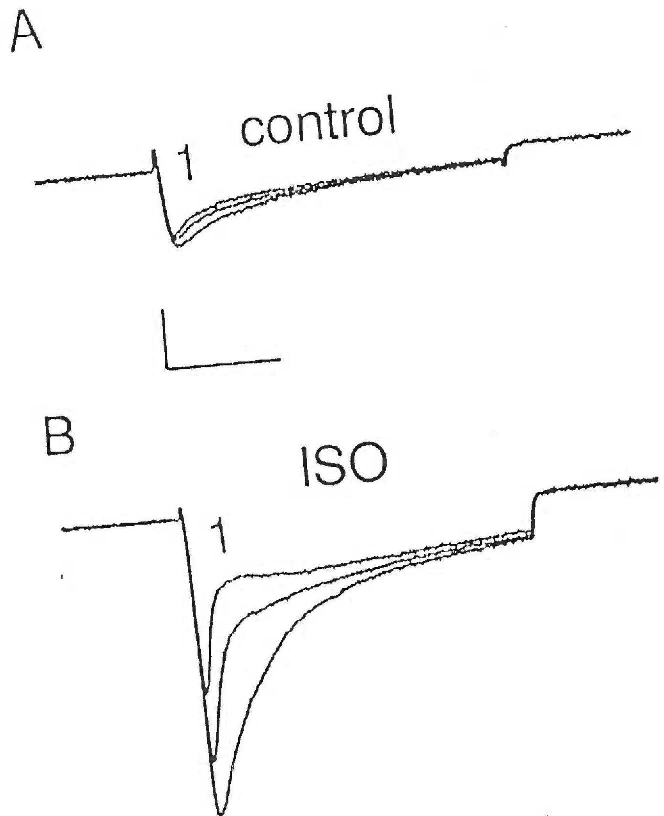
If the effects of 0.5 and 1 mM caffeine on use-dependent enhancement of high threshold current are the result of elevation of 3'-5' cyclic adenosine monophosphate (cAMP)

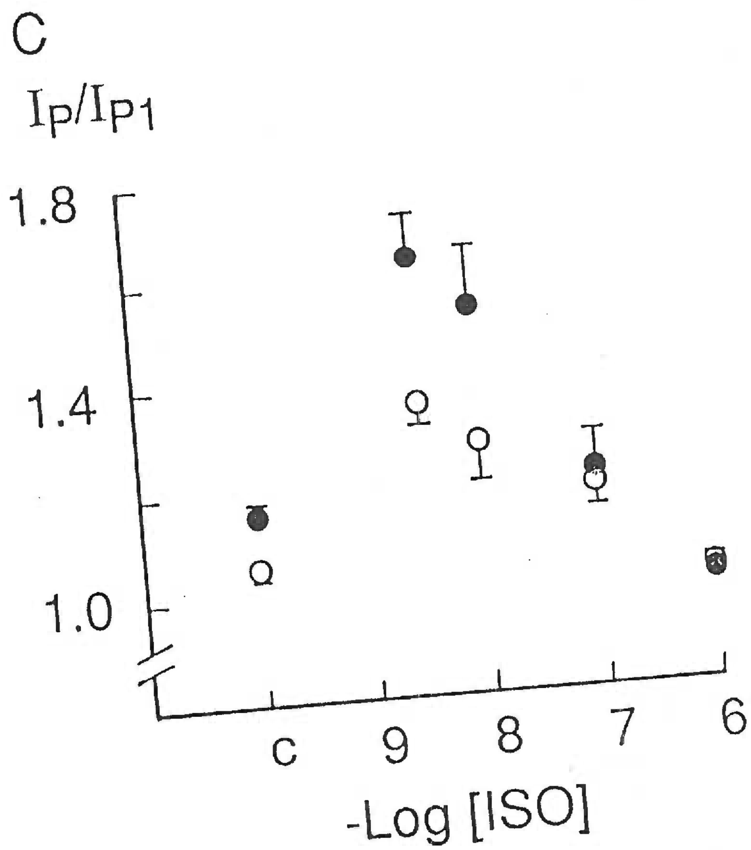
Fig. 10. The effect of isoproterenol (ISO) on caffeine induced enhancement of Ca current. Stimulation protocol described in legend of Fig. 7. Test potential, 20 mV in 2 mM Ca^{2+}. Current and time calibrations, 800 pA and 50 ms; cell capacitance, 143 pF. Currents during the 1st (1) and 5th pulse: prior to exposing the cell to 1 mM caffeine (left), after 8 min in caffeine solution (middle), and after an additional 5 min superfusion of external solution containing 1 mM caffeine plus 1 μM ISO (right).


control
1
caffeine
ISO
1

levels, similar effects might be induced by isoproterenol. The effect of isoproterenol on currents during the 1st, 2nd, and 5th pulse is shown for one cell in Fig. 11A. Following superfusion of 10 nM isoproterenol, the magnitude of I_{P1} increased from -545 pA in the control to -1398 pA. I_{P5}/I_{P1} increased dramatically from a control value of 1.19 to 1.71 with isoproterenol. Isoproterenol did not appear to activate ionic flux through Na^+, K^+, or Cl^- channels, since time dependent currents were not observed in the presence of isoproterenol and 100 μM $CdCl_2$. The effects of low concentrations of isoproterenol on the positive current staircase most likely involves the modulation of the Ca channel, rather than changes in the release of Ca^{2+} from the sarcoplasmic reticulum. Potentiation of the positive current staircase with 10 nM isoproterenol was also observed in two cells dialyzed by internal solutions containing 40 μM ruthenium red to reduce SR release. In cells which were stimulated at 2 Hz in 5 mM Ca^{2+}, superfusion of 5 nM isoproterenol (Fig. 11C) resulted in an increase in I_{P2}/I_{P1} from 1.05 ± 0.01 (n = 14) to 1.34 ± 0.03 (n = 7). The magnitude of I_{P2}/I_{P1} was 1.26 ± 0.06 (n = 9) in 10 nM isoproterenol, and this ratio decreased with higher concentrations of isoproterenol. Isoproterenol at a concentration of 1 μM abolished frequency-dependent potentiation of Ca current

Fig. 11. The effects of isoproterenol (ISO) on high threshold current potentiation. Stimulation protocol described in legend of Fig. 7. Test potential, 20 mV in 5 mM Ca^{2+}. Current and time calibrations, 500 pA and 50 ms; cell capacitance, 136 pF. A) Currents during the 1st (1), 2nd, and 5th pulses in control. B) Same cell as in (A) after a 7 min exposure to 10 nM isoproterenol (ISO). C) A plot of I_{P2}/I_{P1} (O) and I_{P5}/I_{P1} (O) against [ISO]. The data are presented as the mean ± S.E.M. for controls (C; n = 17), 5 nM ISO (n = 7), 10 nM ISO (n = 9), 100 nM ISO (n = 3), and 1 μM ISO (n = 3).


A
control
1
B
ISO
1


C
Ip/Ip1
1.8
1.4
1.0
c
9
8
7
6
-Log [ISO]

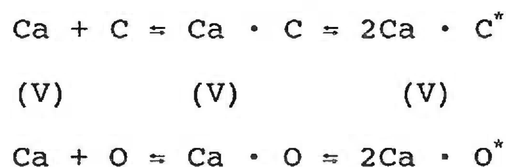
(n = 3). The use of I_{P5}/I_{P1} as a measure of potentiation (Fig. 11C) resulted in a similar pattern in which 5 nM isoproterenol increased potentiation, and higher concentrations progressively reduced potentiation. These results suggest that caffeine, ryanodine and isoproterenol abolish potentiation by a mechanism which maximizes peak Ca current, rather than by depletion of the SR.

DISCUSSION

The major finding of this paper is that the positive current staircase of high threshold channels is dependent on having calcium in the external solution and is potentiated by low concentrations of isoproterenol. Potentiation of the high threshold Ca current was observed at potentials negative to the reversal potential under conditions which limit the contribution of ionic fluxes through other voltage-dependent channels (Matsuda & Noma, 1984) and abolish Na^{+} - Ca^{2+} exchange (Kimura, Noma & Irisawa, 1986). Ryanodine, caffeine, and high concentrations of isoproterenol maximally potentiated I_{Ca} and abolished the positive current staircase. We conclude that potentiation of the high threshold Ca current is activated by calcium via a mechanism which involves modification of Ca channel inactivation.

The mechanism by which calcium both initiates inactivation and slows its rate may be similar to that proposed for the interactions of calcium with the calcium release channel of the sarcoplasmic reticulum (Fabiato, 1985; Rios & Pizarro, 1988). Rios & Pizarro (1988) hypothesize that a fraction of the sarcoplasmic Ca release channels in skeletal muscle are both opened and closed by calcium. We propose a modification of their scheme to

explain the dual actions of calcium in the heart.



$$Ca + C \rightleftharpoons Ca \cdot C \rightleftharpoons 2Ca \cdot C^{*}$$
$$(V) \qquad (V) \qquad (V)$$
$$Ca + O \rightleftharpoons Ca \cdot O \rightleftharpoons 2Ca \cdot O^{*}$$

A Ca channel with two calcium binding sites is hypothesized. The open state is obtained when voltage (V) opens Ca channels. With the influx of calcium, the activator site is rapidly occupied by calcium (Ca · O). More slowly, an inactivator site equilibrates with calcium and the channel inactivates (2Ca · O). Calcium bound to the activator site initiates a molecular rearrangement of the inactivation site such that the rate of inactivation is slowed (transformation between states Ca · O and 2Ca · O). As myoplasmic levels of free calcium are reduced, the channel returns to the closed state. Potentiation of the Ca current in succeeding depolarizations results from the reduced rate of inactivation initiated in the previous beat. Since the Ca channel might possibly inactivate without opening, a parallel scheme is shown for the closed state of the channel.

Calcium mediated modulation of the high threshold Ca current. The best evidence that the positive current staircase of high threshold Ca channels is activated by calcium is that

facilitation during a train of pulses is abolished with replacement of external Ca^{2+} with Ba^{2+}, Sr^{2+} or Na^{+}.

Calcium does not appear to act via an alteration of the surface potential during repetitive stimulation. Depletion of Ca^{2+} surrounding the external opening of the Ca channel and accumulation in the region of the inner pore of the channel should have resulted in a decrease in Ca channel flux (Wilson, Morimoto, Tsuda & Brown, 1983 and negative current staircases for voltages up to the calcium reversal potential.

If the alteration of surface charge was to shift the voltage dependence of activation to more negative potentials (Ohmori & Yoshii, 1977; Wilson, Morimoto, Tsuda & Brown, 1983) the result should have been potentiation at all potentials.

If both processes were operative small depolarizations should have resulted in positive current staircases whereas large depolarizations should have elicited negative current staircases. These surface potential effects should be independent of whether Ba^{2+} or Ca^{2+} is chosen as the Ca channel charge carrier (Wilson, Morimoto, Tsuda & Brown, 1983).

Thus, a mechanism based on surface potential effects is inconsistent with the following findings: 1) the potential dependence of I_{Ca} during the first and the fifth pulses

was not significantly shifted along the voltage axis (Fig. 1) 2) the initial rate of rise of the Ca current was unaffected by repetitive depolarization (Fig 2B) 3) I_{Ca} was potentiated at positive potentials up to the reversal potential 4) and potentiation was abolished in barium solutions. Kass and Krafte (1987) report that the voltage dependence of inactivation in guinea-pig myocytes is shifted to more negative potentials by the depletion of divalent cations surrounding the external pore of the Ca channel. Again, we can not reconcile our results with this effect of altering the surface charge, since repetitive stimulation slowed the rate of inactivation and failed to reduce the steady-state current.

The voltage dependence of the positive current staircase. The facilitation of I_{Ca} in bovine chromaffin cells has been shown to be voltage dependent (Hoshi, Rothlein & Smith, 1984). In the present study, the increase in Ca current with repetitive stimulation is greater at 10 mV than at 30 mV, even though the amplitude of the Ca current during the first pulse is larger at 30 mV (Fig. 1). This apparent voltage dependence of the potentiation process may reflect the voltage dependence of calcium entry. The driving force for calcium entry is greater with steps to 10 mV than with steps to 30 mV. Inactivation of I_{Ca} is largely calcium dependent (Kass, & Sanguinetti, 1984; Lee, Marban

& Tsien, 1985) and is greater at 10 mV than at 30 mV (Eckert & Chad, 1984). Therefore, the peak current at 10 mV is reduced by inactivation to a greater degree. A calcium mediated process which decreases the rate of inactivation of the Ca current would have a maximal effect at this potential, which is consistent with our findings (Fig 1C).

Does the release of calcium from the sarcoplasmic reticulum play a role in potentiation of the high threshold current? Facilitation of Ca currents in guinea-pig ventricular cells is thought to involve the depletion of Ca^{2+} near the binding sites for inactivation (Tseng, 1988; Fedida et al. 1988a). In this scheme, the increased calcium buffering capacity of cells loaded with EGTA slows the uptake of Ca^{2+} by the SR. Repetitive stimulation would, therefore, result in a beat dependent decrease of SR calcium release and is consistent with the finding that facilitation is Ca^{2+} dependent and potentiated in the presence of internal EGTA (Fedida et al. 1988a). However, the first contraction after rest in intact mammalian ventricular muscle is generally smaller than contractions during the preceding period of stimulation, presumably due to the progressive leakage of calcium from the SR during the rest interval (Koch-Weser & Blinks, 1963; Allen, Jewell & Wood, 1976). This interval-dependent decrease in tension in intact muscle suggests

that the SR may also be depleted of calcium in single cells loaded with EGTA. We have attempted to differentiate between a mechanism for the positive current staircase which involves the SR and one in which calcium binds to a site close to the pore of the Ca channel and slows inactivation.

For a mechanism which involves the SR, potentiation is decreased by interventions which 1) reduce the filling of the SR, 2) block the release of Ca^{2+}, 3) or increase the rate of filling of SR stores. In the present study, the uptake of Ca^{2+} into the SR was reduced by an increase in the intracellular calcium buffering capacity. Internal calcium was buffered to 6 nM in the standard EGTA solution and 10 nM in the standard BAPTA solution. Following establishment of whole cell recording, internal calcium transients were below the threshold for the activation of myofibrils since contraction was not elicited by depolarization or application of 10 mM caffeine. Although this is equivocal evidence that the SR is depleted of calcium, a positive current staircase was also observed in cells in which no calcium was added to internal solutions ($[Ca^{2+}]_i$ < 1 nM; Fig. 7). It is more difficult to argue for loading of the SR under conditions in which the free calcium of the internal solutions was well below the apparent dissociation constant (4.3×10^{-7} M) for the

binding of Ca^{2+} to the sarcoplasmic reticulum ATPase (Inesi, Kurzmack, Coan & Lewis, 1980). Tseng (1988) has reported that the facilitation of the Ca current was abolished in 10 mM BAPTA and argued that this loss of potentiation is a consequence of a reduction in the release of Ca^{2+} from the SR. We have shown that potentiation is reduced with 5 mM BAPTA, however, this reduction could equally well result from the fast kinetics of BAPTA (Tsien, 1980) and a reduction in calcium near the inner pore of the channel.

In order to differentiate between these two effects of BAPTA, the release of calcium from the SR was reduced with ruthenium red (Fabiato & Fabiato, 1973; Baylor, Hollingworth & Marshal, 1988) or with ryanodine (Sutko & Willerson, 1980; Fabiato, 1985b). Ruthenium red (40 μM) failed to abolish the facilitation of the Ca current (Figures 4 & 7). Ryanodine at concentrations between 10 nM and 1 μM is reported to deplete the SR of calcium in isolated rat myocytes (Hansford & Lakatta, 1986). These authors showed that this depletion was nearly complete within 10 min of applying 100 nM ryanodine. Higher concentrations of ryanodine are reported to block the release of calcium from the SR (Meissner, 1986). We have shown that 0.2 to 2 μM ryanodine failed to abolish the positive current staircase (Fig. 8). Ryanodine has been

potential plateau in cardiac muscle (Wier, Yue & Marban, 1985). Since the Ca current is known to contribute to the duration of the action potential plateau (Reuter, 1979) this is further evidence that ryanodine may increase Ca current or slow its inactivation. Although the period of exposure to ryanodine was not reported, ryanodine was shown to decrease the amplitude of I_{Ca} and slow its rate of inactivation (Tseng, 1988; Fedida et al. 1988a). It is possible the decrease in amplitude of I_{Ca} amounted to rundown of the Ca current in these studies. We have shown that the normal increase in I_{Ca} in response to 1 μM isoproterenol is abolished in cells incubated in ryanodine for long periods of time. After showing that much higher concentrations of ryanodine were needed to affect SR release in skinned preparations, Fabiato (1985b) argued that low concentrations of ryanodine act in intact cardiac cells through processes or structures which are destroyed in the skinning procedure. Recently, binding of ryanodine has been localized to the region of the feet structures of junctional terminal cisternae of the SR (Inui, Saito & Fleischer, 1987).

The facilitation of the Ca current was enhanced with low concentrations of isoproterenol and abolished with high concentrations of isoproterenol (Fig. 11). A mechanism which involves the SR is inconsistent with these findings,

since catecholamines are also reported to increase the uptake of calcium into the SR and enhance tension repriming in guinea pig atria (Kirchberger, Tada, Repke & Katz, 1972; Shimoni, 1987).

We have also shown that low concentrations of caffeine over time mimic the concentration-dependent effects of isoproterenol (Fig. 9). It has been reported that caffeine (10 mM) abolished facilitation of the Ca current in guinea-pig myocytes (Tseng, 1988; Fedida et al. 1988a). We hypothesize that facilitation is abolished not by reducing SR calcium release, but by maximizing the Ca current. To support this contention: 1) the positive current staircase was abolished with 1 μM isoproterenol, and 2) caffeine and ryanodine abolished facilitation of I_{Ca} only under conditions which also abolished the increase in current normally observed with 1 μM isoproterenol.

Does calcium modulate phosphorylation of the high threshold Ca channels during repetitive stimulation? The best evidence that calcium may modulate Ca channel phosphorylation is that low concentrations of isoproterenol potentiate the positive current staircase (Fig. 11). A diverse group of calcium dependent kinases are reported to phosphorylate a spectrum of proteins, and some of these proteins are also substrates for cAMP-dependent kinases (Reichardt & Kelly,

1983). In addition, membrane bound calcium dependent kinases are reported to modulate phosphorylation levels of the dihydropyridine-sensitive Ca channel in skeletal muscle (Hosey, Borstto & Lazdunski, 1986; Imagawa, Leung & Campbell, 1987) and in cardiac microsomal membranes (Horne, Triggle & Venter, 1984).

It is tantalizing to speculate that with depolarization, a transient rise in calcium near the inner pore of the Ca channel may activate kinases which phosphorylate proteins associated with the sites for inactivation. Alternatively, the transient rise in current with depolarization may phosphorylate substrates for cAMP-dependent kinases such that the concentration dependence of the relation between intracellular cAMP and Ca current is shifted. The relatively slow recovery from the potentiated state (Fig. 5) is consistent with the need to return to basal levels of phosphorylated substrate.

Potentiation of the high threshold calcium channel current is beat dependent requiring 5-6 successive depolarizations to reach steady state. This beat dependence is similar to the beat dependence of the fast phase of the force staircase (Maylie, 1982) and the decay of the altered inotropic state following postextrasystolic potentiation, or a change in rate of stimulation, which require 5-8 beats to reach control levels (Morad &

Goldman, 1973). Calcium dependent modulation of the high threshold calcium channel current represents an intrinsic process that may have a role in the force staircase.

REFERENCES

Aldrich, R.W., Getting, P.A., & Thompson, S.H. (1979). Mechanism of frequency-dependent broadening of molluscan neurone soma spikes. *Journal of Physiology* 291, 531-544.

Allen, D.G., Jewell, B.R. & Wood, E.H. (1976). Studies of the contractility of mammalian myocardium at low rates of stimulation. *Journal of Physiology* 254, 1-17.

Argibay, J.A., Fischmeister, R. & Hartzell, H.C. (1988). Inactivation, reactivation and pacing dependence of calcium current in frog cardiocytes: correlation with current density. *Journal of Physiology* 401, 201-226.

Barcenas-Ruiz, L. & Wier, W.G. (1987). Voltage dependence of intracellular $[Ca^{2+}]_i$ transients in guinea pig ventricular myocytes. *Circulation Research* 61, 148-154.

Bassingthwaighte, J.B. & Reuter, H. (1972). Calcium movements and excitation-contraction coupling in cardiac cells. In *Electrical Phenomena in the Heart*, ed. DeMello, W.C. pp. 353-362. New York: Academic Press.

Baylor, S.M., Hollingworth, S. & Marshall, M.W. (1989). Effects of intracellular ruthenium red on excitation-contraction coupling in intact frog skeletal muscle fibres. *Journal of Physiology* 408, 617-635.

Bean, B.P. (1985). Two kinds of calcium channels in canine atrial cells. *Journal of General Physiology* 86, 1-30.

Bean, B.P. & Rios, E. (1989). Non-linear charge movement in mammalian cardiac ventricular cells. *Journal of General Physiology*, in press.

Beeler, G.W. & Reuter, H. (1970). The relation between membrane potential, membrane currents and activation of contraction in ventricular myocardial fibres. *Journal of Physiology* 207, 211-229.

Bowditch, H.P. (1871). Uber die Eigenthumlichkeiten der Reizbarkeitt, welche die Muskelfasern des Herzens zeigen. *Ber. sachs. ges. (Akad.) Wiss.* 652-689.

Boyett, M.R. (1981). A study of the effect of the rate of stimulation of the transient outward current in sheep cardiac purkinje fibres. *Journal of Physiology* 319, 1-22.

Boyett, M.R., & Jewel, B.R. (1980). Analysis of the effects of changes in rate and rhythm upon electrical activity in the heart. *Progress in Biophysics and Molecular Biology* 36, 1-52.

Chad, J.E., & Eckert, R. (1986). An enzymatic mechanism for calcium current inactivation in dialysed Helix neurones. *Journal of Physiology* 378, 31-51.

Colquhoun, D. & Sigworth, F.J. (1983). Fitting and statistical analysis of single-channel records. In *Single-Channel Recording*, ed. Sakmann, B. & Neher, E. pp. 191-263. New York: Plenum Press.

Eckert, R. & Chad, J.E. (1984). Inactivation of Ca channels. *Progress in Biophysics and Molecular Biology* 44, 215-267.

Fabiato, A. (1985a). Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *Journal of General Physiology* 85, 247-289.

Fabiato, A. (1985b). Simulated calcium current can both cause calcium loading in and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *Journal of General Physiology* 85, 291-320.

Fabiato, A. (1985c). Effects of ryanodine in skinned cardiac cells. *Federation Proceedings* 44, 2970-2976.

Fabiato, A. & Fabiato, F. (1973). Activation of skinned cardiac cells. *European Journal of Cardiology* 1/2, 143-155.

Fabiato, A. & Fabiato, F. (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *Journal of Physiology (Paris)* 75, 463-505.

Fedida, D., Noble, D. & Spindler, A.J. (1987). Inward current related to contraction in guinea-pig ventricular myocytes. *Journal of Physiology* 385, 565-589.

Fedida, D., Noble, D. & Spindler, A.J. (1988a). Use-dependent reduction and facilitation of Ca^{2+} current in guinea-pig myocytes. *Journal of Physiology* 405, 439-

460.

Fedida, D., Noble, D. & Spindler, A.J. (1988b). Mechanism of the use dependence of Ca^{2+} current in guinea-pig myocytes. *Journal of Physiology* 405, 461-475.

Gettes, L.S., & Reuter, H. (1974). Slow recovery from inactivation of inward currents in mammalian myocardial fibres. *Journal of Physiology* 240, 702-724.

Gibbons, W.R. & Fozzard, H.A. (1975). Slow inward current and contraction of sheep cardiac Purkinje fibres. *Journal of General Physiology* 65, 367-384.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* 391, 85-100.

Hansford, R.G. & Lakatta, E.G. (1986). Ryanodine releases calcium from sarcoplasmic reticulum in calcium-tolerant rat cardiac myocytes. *Journal of Physiology* 390, 453-467.

Hess, P. Lansman, J.B., & Tsien, R.W. (1984). Different modes of Ca channel gating behavior by dihydropyridine Ca agonists and antagonists. *Nature* 311, 538-544.

Hilgemann, D.W., Delay, M.J. & Langer, G.A. (1983). Activation-dependent cumulative depletions of extracellular free calcium in guinea pig atrium measured with antipyrylazo III and tetramethlymurexide. *Circulation Research* 53, 779-793.

Hiraoka, M. & Kawano, S. (1987). Mechanism of increased amplitude and duration of the plateau with sudden shortening of diastolic intervals in rabbit ventricular cells. *Circulation Research* 60, 14-26.

Horne, P., Triggle, D.J. & Venter, J.C. (1984). Nitrendipine and isoproterenol induce phosphorylation of a 42,000 dalton protein that co-migrates with the affinity labeled calcium channel regulatory subunit. *Biochemical and Biophysical Research Communications* 121, 890-898.

Hosey, M.M., Borsotto, M. & Lazdunski, M. (1986). Phosphorylation and dephosphorylation of dihydropyridine-sensitive voltage-dependent Ca^{2+}

of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. *Journal of Biological Chemistry* 262, 1740-1747.

Isenberg, G. (1977). Cardiac purkinje fibres: the slow inward current under the influence of modified $[Ca^{2+}]_i$. *Pflügers Archiv* 371, 61-69.

Josephson, I.R., Sanchez-Chapula, J. & Brown, A.R. (1984). A comparison of calcium currents in rat and guinea pig single ventricular cells. *Circulation Research* 54, 144-156.

Kaibara, M. & Kameyama, M. (1988). Inhibition of the calcium channel by intracellular protons in single ventricular myocytes of the guinea-pig. *Journal of Physiology* 403, 621-640.

Kass, R.S. & Krafte, D.S. (1987). Negative surface charge density near heart calcium channels. *Journal of General Physiology* 89, 629-644.

Kass, R.S., & Sanguinetti, M.C. (1984). Calcium channel inactivation in the calf cardiac Purkinje fiber:

Evidence for voltage- and calcium-mediated mechanism. *Journal of General Physiology* 84, 705-726.

Kenyon, J.L. & Gibbons, W.R. (1979a) Influence of chloride, potassium, and tetraethylammonium on the early outward current of sheep cardiac Purkinje fibers. *Journal of General Physiology* 73, 117-138.

Kenyon, J.L. & Gibbons, W.R. (1979b). 4-aminopyridine and the early outward current of sheep cardiac Purkinje fibers. *Journal of General Physiology* 73, 139-157.

Kimura, J., Noma, A. & Irisawa, H. (1986). Na - Ca exchange current in mammalian heart cells. *Nature* 319, 596-597.

Kirchberger, M.A., Tada, M., Repke, D.I. & Katz, A.M. (1972). Cyclic nucleo-side 3', 5'-monophosphate-dependent protein kinase stimulation of calcium uptake by canine cardiac microsomes. *Journal of Molecular and Cellular Cardiology* 4, 673-680.

Koch-Weser, J. & Blinks, J.R. (1963). The influence of the interval between beats on myocardial contractility. *Pharmacological Reviews* 15, 601-665.

Kohlhardt, M., Haastert, H.P. & Krause, H. (1973). Evidence of non-specificity of the Ca channel in mammalian myocardial fiber membranes: substitution of Ca by Sr, Ba or Mg as charge carriers. *Pflugers Archiv* 342, 125-136.

Kohlhardt, M., Herdey, A. & Kubler, M. (1973). Interchangeability of Ca ions and Sr ions as charge carriers of the slow inward current in mammalian myocardial fibers. *Pflügers Archiv* 344, 149-158.

Kohlhardt, M., Krause, H., Kubler, M., & Herdey, A. (1973). Kinetics of inactivation and recovery of the slow inward current in the mammalian ventricular myocardium. *Pflügers Archiv* 355, 1-17.

Kokubun, S., & Irisawa, H. (1984). Effects of various intracellular Ca ion concentrations on the calcium current of guinea pig single ventricular cells. *Japan Journal of Physiology* 34, 599-611.

Lee, K.S. (1987). Potentiation of the calcium-channel currents of internally perfused mammalian heart cells by repetitive depolarization. *Proceedings of the National*

Acadamy of Sciences, USA 84, 3941-3945.

Lee, K.S., Marban, E., & Tsien, R.W. (1985). Inactivation of calcium channels in mammalian heart cells: Joint dependence on membrane potential and intracellular calcium. *Journal of Physiology* 364, 395-411.

Ma, J., Fill, M., Knudson, C.M., Campbell, K.P. & Coronado, R. (1988). Ryanodine receptor of skeletal muscle is a gap junction-type channel. *Science* 242, 99-102.

Marban, E., & Tsien, R.W. (1982). Enhancement of calcium current during digitalis inotropy in mammalian heart: Positive feedback regulation by intracellular calcium? *Journal of Physiology* 329, 589-614.

Matsuda, H. & Noma, A. (1984). Isolation of calcium current and its sensitivity to monovalent cations in dialysed ventricular cells of guinea-pig. *Journal of Physiology* 357, 553-573.

Maylie, J.G. (1982). Excitation-contraction coupling in neonatal and adult myocardium of cat. *American Journal of Physiology* 242, H834-H843.

Maylie, J. & Morad, M. (1984). A transient outward current related to calcium release and development of tension in elephant seal atrial fibres. *Journal of Physiology* 357, 267-292.

Meirow, D. & Shimoni, Y. (1988). Tension reactivation and potentiation in guinea pig atrium: the effects of isoprenaline, calcium and rate changes. *Proceedings of the Royal Society of London B* 233, 33-43.

Meissner, G. (1986). Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *Journal of Biological Chemistry* 261, 6300-6306.

Mentrard, D., Vassort, G., & Fischmeister, R. (1984). Calcium-mediated inactivation of the calcium conductance in cesium-loaded frog heart cells. *Journal of General Physiology* 83, 105-131.

Mitchell, M.R., Powell, T., Terrar, D.A. & Twist, V.W. (1984). Ryanodine prolongs Ca-currents while suppressing contraction in rat ventricular muscle cells. *British Journal of Pharmacology* 81, 013-015.

Mitra, R., & Morad, M. (1985). A uniform enzymatic method for dissociation of myocyte from hearts and stomachs of vertebrates. *American Journal of Physiology* 249, H1056-H1060.

Mitra, R. & Morad, M. (1986). Two types of calcium channels in guinea pig ventricular myocytes. *Proceedings of the National Acadamy of Science, USA* 83, 5340-5344.

Morad, M. & Cleemann, L. (1987). Role of Ca^{2+} Channel in development of tension in heart muscle. *Journal of Molecular and Cellular Cardiology.* 19,

Morad, M. & Goldman, Y. (1973). Excitation-contraction coupling in heart muscle: membrane control of development of tension. *Progress in Biophysics and Molecular Biology* 27, 257-313.

New, W. & Trautwein, W. (1972). The ionic nature of slow inward current and its relation to contraction. *Pflügers Archiv* 334, 24-38.

Noble, S. & Shimoni, Y. (1981a). The calcium and frequency dependence of the slow inward current

staircase in frog atrium. *Journal of Physiology* 310, 57-75.

Noble, S. & Shimoni, Y. (1981b). Voltage-dependent potentiation of the slow inward current in frog atrium. *Journal of Physiology* 310, 77-95.

Ohmori, H. & Yoshii, M. (1977). Surface potential reflected in both gating and permeation mechanisms of sodium and calcium channels of the tunicate egg cell membrane. *Journal of Physiology* 267, 429-463.

Osterrieder, W., Brum, G., Hescheler, J., Trautwein, W., Flockerzi, V. & Hofmann, F. (1982). Injection of subunits of cyclic AMP-dependent protein kinase into cardiac myocytes modulates Ca^{2+} current. *Nature* 298, 576-578.

Pessah, I.N., Francini, A.O., Scales, D.J., Waterhouse, A.L. & Casida, J.E. (1986). The calcium-ryanodine receptor complex
solubilization and partial characterization from skeletal muscle junctional sarcoplasmic reticulum vesicles. *Journal of Biological Chemistry* 261, 8643-8648.

Reichardt, L.F. & Kelly, R.B. (1983). A molecular description of nerve terminal function. *Annual Review of Physiology* 52, 871-926.

Reuter, H. (1979). Properties of two inward membrane currents in the heart. *Annual Review of Physiology* 41, 413-424.

Rios, E. & Pizarro, G. (1988). Voltage sensors and calcium channels of excitation-contraction coupling. *News in Physiological Sciences* 3, 223-227.

Seibel, K. (1979). The two phases of the staircase of mammalian ventricular muscle in relation to inotropic mechanisms. *Naunyn-Schmiedeberg's Archiv of Pharmacology* 308, R6.

Seibel, K. (1986). The slow phase of the staircase in guinea-pig papillary muscle, influence of agents acting on transmembrane sodium flux. *Naunyn-Schmiedeberg's Archiv of Pharmacology* 334, 92-99.

Shattock, M.J., Warner, K.C., Tidball, J.G. & Bers, D.M. (1987). Two different electrophysiological responses to ryanodine: evidence for two populations of muscles

isolated from the rabbit right ventricle. *Journal of Molecular and Cellular Cardiology* 19, 751-762.

Shimoni, Y (1981). Parameters affecting the slow inward channel repriming process in frog atrium. *Journal of Physiology* 320, 269-291.

Shimoni, Y. (1987). The effects of catecholamines on tension reactivation in cardiac muscle. *Proceedings of the Royal Society of London B* 231, 231-249.

Siegelbaum, S.A. & Tsien, R.W. (1980). Calcium-activated transient outward current in cardiac Purkinje fibers. *Journal of Physiology* 299, 485-506.

Smith, J.S., Imagawa, T., Ma, J., Fill, M., Campbell, K.P. & Coronado, R. (1988). Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *Journal of General Physiology* 92, 1-26.

Sutko, J.L. & Willerson, J.T. (1980). Ryanodine alteration of the contractile state of rat ventricular myocardium: comparison with dog, cat, and rabbit ventricular tissues. *Circulation Research* 46, 332-343.

Tsien, R.Y. (1980). New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* 19, 2396-2404.

Trautwein, W., Cavalie, A., Flockerzi, V., Hofmann, F. & Pelzer, D. (1987). Modulation of calcium channel function by phosphorylation in guinea pig ventricular cells and phospholipid bilayer membranes. *Circulation Research* 61, I-17-I-23.

Tseng, G. (1988). Calcium current restitution in mammalian ventricular myocytes is modulated by intracellular calcium. *Circulation Research* 63, 468-482.

Tuttle, R.S. & Farah, A. (1962). The effect of ouabain on the frequency-force relation and on post-stimulation potentiation in isolated atrial and ventricular muscle. *Journal of Pharmacology* 135, 142-150.

Weingart, R., Kass, R.S., & Tsien, R.W. (1978). Is digitalis inotropy associated with enhanced slow inward current? *Nature* 273, 389-391.

Wilson, D.L., Morimoto, K., Tsuda, Y. & Brown, A.M. (1983). Interaction between calcium ions and surface charge as it relates to calcium currents. *Journal of Membrane Biology* 72, 117-130.

Wier, W.G., Yue, D.T. & Marban, E. (1985). Effects of ryanodine on intracellular Ca^{2+} transients in mammalian cardiac muscle. *Federation Proceedings* 44, 2989-2993.

Zygmunt, A.C. & Maylie, J. (1988). Calcium-dependent potentiation of high threshold calcium channels during repetitive depolarization in guinea pig ventricular myocytes. *Biophysical Journal* 53, 18a.

DISCUSSION AND CONCLUSIONS

Comparison with other studies

We have shown that high threshold Ca channel currents exhibit positive current staircases with repetitive stimulation. The loss of this potentiation when barium, strontium, or sodium is used as a charge carrier for Ca channels suggests that this process is primarily mediated by calcium, rather than by voltage.

These findings must be contrasted with those for potentiation of Ca currents in chromaffin cells (Fenwick, Marty & Neher, 1982; Hoshi, Rothlein & Smith, 1984). Fenwick et al. (1982) found that depolarization of the cell with two pulses to positive potentials resulted in an increase in Ca current measured during the second pulse. This potentiation of the Ca current was independent of the voltage of the first pulse. In a few of these trials, facilitation of the Ca current measured in the second pulse was not inhibited, even when the potential of the first pulse was positive to the reversal potential for the calcium current. Under these conditions, the current during the first pulse is outward. However, their conclusion that facilitation of the Ca current was not mediated by calcium must be reevaluated given the rapid activation of the potentiation process by calcium in our

study (Fig. 4). It is entirely possible that during dual pulse stimulation in chromaffin cells, influx of calcium upon repolarization of the cell between pulses is sufficient to activate the potentiation process.

However, Hoshi et al. (1984) have convincingly shown that facilitation of the Ca current in chromaffin cells is a voltage-dependent phenomena, since facilitation was unaffected by charge carrier species or internal free Ca^{2+} concentration.

It appears that the mechanisms of potentiation of Ca currents in guinea-pig ventricular cells and bovine chromaffin cells are fundementally different. The reasons for the discrepancy between the present study and a recent report of voltage-dependent facilitation in isolated guinea-pig ventricular myocytes is not clear (Lee, 1987).

We have corroborated the report by Mitra & Morad (1986) that there is a differential response to increases in frequency in low and high threshold Ca channel currents. We have gone on to show that the activation of low threshold channels is not neccessary for potentiation of high threshold channels (Fig. 2). Potentiation of high threshold channels is, therefore, not the result of a calcium induced transformation of low threshold channels to a higher conductance state. It is less clear whether influx of calcium through low threshold channels

participates in the potentiation phenomena.

Potentiation is also not the result of a "pumping down" of the intracellular free calcium in the vicinity of the Ca channel inactivation sites by a sodium-calcium exchange at the sarcolemma as proposed by Argibay, Fischmeister & Hartzell (1988), since sodium free internal and external solutions were used in this study. The ATP-dependent extrusion of calcium at the sarcolemma which may be operative in this preparation is thought to be half maximally activated at an internal calcium level of 500 nM, well above the levels in this preparation (Caroni & Carafoli, 1980). It is not clear that this pump could operate in a manner that would transiently lower calcium below basal levels. Some Ca channels may be inhibited by diastolic levels of intracellular calcium (Plant, Standen & Ward, 1983), however, potentiation was not abolished at calcium levels below 1 nM in this study (Fig. 7). One would have to assume that Ca channels could still be recruited at these low levels of calcium.

The positive current staircase is abolished by uncoupling of control over phosphorylation and maximizing the Ca current.

Tseng (1988) has hypothesized that frequency-dependent potentiation of the Ca current is secondary to the use-dependent inhibition of SR calcium release in EGTA loaded

cells. However, we have shown that lowering intracellular Ca levels below 1 nM with either EGTA or BAPTA does not abolish potentiation of the Ca current. Instead, the positive current staircase is abolished by those mechanisms which also abolish the normal increase in current with exposure to 1 μM isoproterenol.

Isoproterenol is thought to activate G_s proteins and the α-subunit can directly activate the Ca channel (Stryer & Bourne, 1986; Yatani, Codina, Imoto, Reeves, Birnbaumer & Brown, 1987). However, the mechanism of the positive current staircase in cardiac cells does not appear to involve such direct interactions, since the stable analog of GDP failed to abolish potentiation of the Ca current (Appendix).

We hypothesize that caffeine and ryanodine, which are thought to empty the SR stores of calcium acts also at some additional site to alter the Ca current. In support of this hypothesis, ryanodine has been reported to separate t-tubules from underlying structures (Penefsky, 1974). Although certainly speculative, these ultrastructural changes may underlie increases in Ca current evoked by ryanodine and caffeine. The SR calcium release channel which is associated with feet structures in the region of cardiac triads has been reported to have binding domains for both ryanodine and caffeine (Inui,

Saito & Fleischer, 1987). If these structures are also associated with Ca channels, as has been proposed in skeletal muscle (Rios & Pizarro, 1988) a mechanism may be possible by which ryanodine and caffeine can alter cardiac Ca channels.

Calcium dependent calmodulin pathways probably do not play a role since the chlorinated napthalenesulfonamide derivative W-12 had no appreciable effect on potentiation of the Ca current. A very high concentration was necessary of the calmodulin blocker W-7 in order to abolish the current staircase (Appendix).

REFERENCES

Penefsky, Z.J. (1974). Ultrastructural studies of the site of action of ryanodine on heart muscle. *Pflügers Archiv* 347, 185-198.

Plant, T.D., Standen, N.B. & Ward, T.A. (1983). The effects of injection of calcium ions and calcium chelators on calcium channnel inactivation in *Helix* neurones. *Journal of Physiology*, 334, 189-212.

APPENDIX

In order to test the effect of blocking the G protein pathway, the availability of GTP was reduced by the inclusion in the pipette solution of 200 μM GDPγS, the stable analog of GDP. Since GTP is necessary for activation of G proteins (Stryer & Bourne, 1986), this should inhibit the G protein pathway. In 2 mM Ca^{2+}, the ratio of currents during the fifth and first pulses was 1.49 ± 0.06 (n = 3) following repetitive stimulation to 20 mV.

Calmodulin dependent pathways were also blocked with the calmodulin blockers W-7 and W-12. The effect of a very high concentration of W-7 (100-200 μM) was to block the positive current staircase in 7 cells. It was not possible to test the reversibility of this effect. However, the blocker W-12 had little effect in 4 other cells at a concentration of 10 μM. In 2 mM Ca^{2+}, the ratio of currents during the fifth and first pulses was 1.23 ± 0.03 (n = 4) following repetitive stimulation to 20 mV.