The Effect of Modulating Calcium-Induced Calcium Release on the Properties of Spontaneous and Systolic Calcium Release in Rat Ventricular Myocytes

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

Catherine Louise Overend

September 1999

ACKNOWLEDGEMENTS

I am grateful to my sponsors, the Wellcome Trust for providing the financial support necessary for me to undertake my Ph.D. studies. In particular for providing me with the opportunity to travel and present my work internationally, because this experience has been invaluable to me.

A big thank you to all my friends especially **Mary Díaz**, whose experience has been invaluable to me and who put up with my mess around the house for so long, and to **Becky Bynon** and **Louise Murray**. We have had loads of fun in Liverpool throughout my Ph.D. Thank you to **Mr and Mrs Bynon** and to the **Murray Family** for making me feel so "at home" in Liverpool and for supporting me in my frequent visits to Liverpool while writing my thesis.

To my fellow "Wellcome" students, **Colin, Kate, Maddy** and **Naomi,** and to everybody in the Physiology Department who made our first few months in Liverpool so exciting and enjoyable. Good luck for the future.

A big thank you to all my work colleagues; **Andy, Mary, William, Duncan, Ho Sook, Gillian, Lynda, Steve, Deb, Mike, Wendy** and **Joseph,** many of whom I consider to be my close friends and who have made my time in Liverpool (even working!) so enjoyable. I am especially grateful to Andy (**Dr Andrew Trafford**) for designing and allowing me to use his analysis program without which analysing results would have been horrendously laborious. Many thanks to **Professor David Eisner** for all his help and enthusiastic approach to discussing my work. Good luck in Manchester and your future careers everyone!

To my husband **David** for being so supportive and helpful during the most difficult stages of my Ph.D. studies. Thank you tonnes for proof-reading and putting the whole thing together and for putting up with my high stress levels over the months leading up to submission. I would also like to thank all my family and friends for their love and support throughout my University career. Thank you to **Mum, Dad, Helen** and **Jasper** especially, and to **Uncle Tim**: "Hopefully, now I can go and get a "proper job"!"

Finally a very hefty thank you to my supervisor **Dr Stephen O'Neill** for all his encouragement and support over the last four years. I am so very grateful for everything you have done for me; you are the best supervisor in the whole world!

LIST OF PUBLICATIONS

Overend, C. L., Mill, J. G., Cook, L. J., Cook, S. J., Eisner, D. A., & O'Neill, S. C. Tetracaine increases sarcoplasmic reticulum calcium content while inhibiting spontaneous release in rat ventricular myocytes. *Journal of Physiology* **496P**, 47P. 1996. Abstract

Overend, C. L., O'Neill, S. C., & Eisner, D. A. Tetracaine only transiently inhibits contractions of rat isolated ventricular myocytes. *Journal of Physiology* **504P**, 82P-82P. 1997. Abstract

Overend, C. L., Adams, W. A., O'Neill, S. C., & Eisner, D. A. The Ca^{2+} content of sarcoplasmic reticulum is limited by leak, not uptake, in rat cardiac isolated myocytes. *Journal of Physiology* **511P**, 85P-86P. 1998. Abstract

Overend, C. L., Eisner, D. A., & O'Neill, S. C. (1997). The effect of tetracaine on spontaneous Ca release and sarcoplasmic reticulum calcium content in rat ventricular myocytes. *Journal of Physiology* **502**, 471-479.*

Overend, C. L., O'Neill, S. C., & Eisner, D. A. (1998). The effect of tetracaine on stimulated contractions, sarcoplasmic reticulum Ca^{2+} content and membrane current in isolated rat ventricular myocytes. *Journal of Physiology* **507**, 759-769.*

Eisner, D. A., Trafford, A. W., Díaz, M. E., Overend, C. L., & O'Neill, S. C. (1998). The control of Ca release from the cardiac sarcoplasmic reticulum: regulation versus autoregulation. *Cardiovascular Research* **38**, 589-604.

Overend, C. L., Eisner, D. A., & O'Neill, S. C. Inhibition of sarcoplasmic reticulum Ca^{2+} release during metabolic inhibition in rat ventricular myocytes. *Journal of Physiology*. 1999. Abstract

* denotes papers attached in the Appendix

TABLE OF CONTENTS

TABLE OF FIGURES

ABSTRACT

The effects of modulation of CICR on spontaneous and systolic Ca^{2+} release were investigated in isolated rat ventricular myocytes. Spontaneous waves of Ca^{2+} release were initially abolished and then resumed at a lower frequency during exposure to 100 μM tetracaine. Both the duration of the initial quiescent period and oscillation frequency in tetracaine were dependent on the control oscillation frequency and the concentration of tetracaine applied. Electrophysiological quantification of the SR Ca^{2+} content of myocytes revealed a significant increase during exposure to tetracaine. The amplitude of spontaneous Ca^{2+} release was also increased such that despite decreased frequency, efflux per unit time activated by Ca^{2+} waves was not changed significantly. Using confocal microscopy, the spatial and temporal properties of Ca^{2+} waves were studied revealing that tetracaine inhibits the propagation of Ca^{2+} release. The increased SR Ca^{2+} content and the increased amplitude of Ca^{2+} release can reverse this effect.

Application of 100 μM tetracaine to electrically stimulated cells transiently depressed systolic Ca^{2+} release and contraction but had no effect in the steady state. Removal of tetracaine was associated with potentiation of systolic Ca^{2+} release followed by gradual recovery. Quantification of the SR Ca^{2+} content revealed that in tetracaine the SR Ca^{2+} content was significantly increased in the steady state. This increase was accounted for by inhibition of systolic Ca^{2+} release activating less Ca^{2+} efflux in the presence of the same or increased Ca^{2+} influx on the L-type Ca^{2+} current. As the SR Ca^{2+} content increases, more efflux is activated until eventually efflux and influx balance in the steady state. The transient potentiation of contraction on removal of tetracaine is due to the increased SR Ca^{2+} content, which increases the gain of CICR in the absence of inhibition of Ca^{2+} release.

The mechanism of post rest potentiation in rat cardiac tissue has not been conclusively elucidated by previous studies. This investigation provides evidence that changes in SR Ca^{2+} content and recovery of channels from inactivation could contribute to the potentiation of contraction observed in rat ventricle after a period of rest. Tetracaine enhances the degree of potentiation of contraction, which can only partially be attributed to its ability to enhance SR Ca^{2+} accumulation.

During myocardial ischaemia dramatic changes in the substrate and metabolite levels in cells occur and a number of these changes are known to affect the RyR. However, the overall effects of metabolic blockade on the sensitivity CICR in intact cells have been overlooked. Experiments were carried out to investigate the effect of metabolic inhibition on spontaneous Ca^{2+} release and SR Ca^{2+} content in isolated rat ventricular myocytes. The results show that CICR is inhibited during metabolic inhibition. This could contribute to the degree of damaging and potentially fatal Ca^{2+} overload experienced on reperfusion of ischaemic tissue.

ABBREVIATIONS

Å	Angstrom (unit of size)
4-AP	4-aminopyridine
AM	acetoxymethyl ester
ADP	adenosine diphosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
aNa_i	intracellular sodium activity
APD	action potential duration
β	Beta
Ba^{2+}	Barium ion
$BaCl_2$	Barium Chloride
BAPTA	1,2-bis(2-aminophenoxy)ethane
BDM	2,3-Butanedione 2-monoxime
BSA	Bovine Serum Albumin
cAMP	cyclic adenosine monophosphate
cADPR	cyclic adenosine diphosphate ribose
°C	Celsius, temperature
C	capacitance
$CaCl_2$	Calcium Chloride
CAM-kinase	Calmodulin-dependent protein kinase
CICR	Calcium-Induced Calcium Release
pCa	$Log_{10}[Ca^{2+}]$
Ca_i	Intracellular Ca^{2+}
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
$[Ca^{2+}]_o$	extracellular Ca^{2+} concentration
CCRM	Charge-coupled release mechanism
CN^-	Cyanide ion
$CsCH_3O_3S$	Caesium methanesulfonate
CsCl	Caesium Chloride

Cs_2EGTA	Caesium EGTA
CsOH	Caesium Hydroxide
DG	Diacylglycerol
DHP	Dihydropyridine
DHPR	Dihydropyridine receptor
DMSO	Dimethylsulfoxide
2-DOG	2-deoxyglucose
EC	Excitation-contraction
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N'N'-tetraacetic acid
F	Farad (unit of capacitance)
F_{400}	fluorescence emission at 400 nm
Fluo-4	Calcium indicator
FWHM	Full width at half maximum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
Hz	Hertz, frequency
I_{Ca}	Ca^{2+} current
I_{NaCa}	Na^{+}/Ca^{2+} exchange current
I_{ti}	Transient inward current
I-V relationship	Current-voltage relationship
Indo-1	Calcium indicator
$InsP_3$	Inositol (1,4,5)-Trisphosphate
JSR	Junctional SR
K^+	Potassium ion
K_{ATP}	ATP-sensitive potassium current
KCl	Potassium Chloride
l	Litre, volume
LSR	Longitudinal SR
M	Molar, concentration
mM	Millimolar
μM	Micromolar

Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
n	sample size
Na^+	sodium ion
NaCl	sodium chloride
$Na_2H_2PO_4$	sodium dihydrogen phosphate
NaOH	sodium hydroxide
N.A.	Numerical aperture
nA	nanoamp (unit of current)
Ω	ohm (unit of resistance)
P	probability
P_i	inorganic phosphate
P_o	channel open probability
pCa	$-\log[Ca^{2+}]$
PCr	phosphocreatine
pA	pico amp (unit of current)
pC	pico Coulomb (unit of charge)
pF	pico farad (unit of capacitance)
pH_i	intracellular pH
pH_o	extracellular pH
PKA	protein kinase A or cAMP-dependent protein kinase
PLC	Phospholipase C
PMT	Photomultiplier tube
Q	charge
R	Uncharged form of local anaesthetic
RCC	Rapid-cooling contracture
RH^+	Charged form of local anaesthetic
$R_{400:500}$	Ratio of 400 and 500 nm emission signals
RyR	Ryanodine Receptor / SR Ca^{2+} Release Channel
s	seconds (unit of time)
S	Siemans (unit of conductance)

S.E.M.	standard error of mean
Ser	Serine amino-acid residue
SERCA	Sarcoplasmic / Endoplasmic Reticulum Calcium ATPase
Sr^{2+}	Strontium ion
SR	Sarcoplasmic Reticulum
t-tubule	Transverse tubule
TD	Transient depolarisation
Thr	Threonine amino-acid residue
TTX	Tetrodotoxin
U	units of enzyme activity
UV	ultraviolet
µl	micro litre
µm	micro metre
µM	Micro molar
V	Volts (unit of potential)
VACR	Voltage-activated calcium release
V_{max}	Maximum rate
$[\ ]_i$	intracellular concentration
$[\ ]_o$	extracellular concentration
Xe	Xenon

Chapter 1

CHAPTER 1

INTRODUCTION

Calcium ions (Ca^{2+}) are implicated in the regulation of many different aspects of cell function including neuronal excitation, muscle contraction, secretion, cell division and apoptosis (see [36] for review). In many cases the initiating step in signal transduction is a transient increase in cytosolic free calcium ($[Ca^{2+}]_i$). However, prolonged exposure to elevated intracellular Ca^{2+} leads to cell death. Stringent regulation of intracellular Ca^{2+} is achieved through transport across the plasma membrane and buffering by intracellular Ca^{2+} stores and Ca^{2+}-binding proteins. *Excitation-contraction coupling* (EC-coupling) in cardiac muscle is a typical example of a Ca^{2+} signalling mechanism essential to normal cell function.

The contractile activity of the heart propels blood throughout the body. Its ability to function as a pump requires co-ordinated cycles of contraction, to expel blood into the circulation, and relaxation, to allow the chambers to refill with blood for the next cycle. At rest, the intracellular concentration of free Ca^{2+} ions ($[Ca^{2+}]_i \sim 200$ nM) is maintained at one ten thousandth that of the extracellular space ($[Ca^{2+}]_o \sim 1$-2 mM). This low intracellular concentration of Ca^{2+} is essential if the muscle is to remain relaxed, and this coupled with the electronegativity of the cell interior creates a large electrochemical gradient in favour of Ca^{2+} entry. This results in the continual Ca^{2+} leakage into the cell by passive diffusion, and the continual need for Ca^{2+} removal. Pumps and exchangers in the plasma

membrane maintain this large electrochemical gradient, while intracellular stores and Ca^{2+} buffers, such as the sarcoplasmic reticulum (SR) and calmodulin, also sequester Ca^{2+}, helping to maintain diastolic Ca^{2+} at very low levels.

In skeletal muscle the summation of contractions and recruitment of additional fibres can vary contractile force, and it can remain contracted for long periods of time. Cardiac muscle on the other hand, functions as a syncytium, with each cell contracting on every beat. The need for cardiac muscle to relax between beats to allow refilling of the chambers with blood means that tetanus would be a totally inappropriate response to meet increased demands on the muscle for the pumping of blood. Instead contractile force is varied by changes in the peak $[Ca^{2+}]_i$ reached during systole.

The purpose of the work described in this thesis was to investigate the effects of modulating SR Ca^{2+} release channel gating on spontaneous and stimulated Ca^{2+} release, contraction and sarcolemmal Ca^{2+} fluxes. This chapter provides an overview of Ca^{2+} regulation in cardiac muscle cells and reviews some of the mechanisms regulating the contraction and relaxation of cardiac muscle (See [40] for more detailed review).

1.1 Excitation-Contraction Coupling in Cardiac Muscle

The physiological stimulus for contraction is depolarisation of the surface membrane, caused by an action potential, which propagates throughout the cardiac syncytium to co-ordinate the contraction of individual cells. This electrical signal is transformed into contraction of the myofilaments, but the exact mechanism for this transduction remains a matter of considerable debate. The

role of Ca^{2+} in the control of cardiac contraction was first suggested in the last century by Ringer, who found that the heart would not contract in the absence of Ca^{2+} ions in the extracellular fluid [304]. It was later shown that simple Ca^{2+} diffusion into the cells of the frog skeletal muscle was not fast enough to account for EC coupling [175], implying the involvement of other mechanisms. An intracellular Ca^{2+} store, the sarcoplasmic reticulum (SR), was found to be essential to EC coupling in skeletal muscle [120, 139] and cardiac muscle [129, 130]. It is clear that depolarisation of the membrane somehow induces the release of a large amount of Ca^{2+} from this intracellular store. In skeletal muscle, conformational changes in the dihydropyridine receptor (DHPR) induced by depolarisation of the sarcolemma are communicated directly from the transverse tubule to the SR Ca^{2+} release channel to induce Ca^{2+} release [305, 306, 318], and there is no absolute requirement for Ca^{2+} entry [15]. The majority of evidence suggests that depolarisation of the sarcolemma does not play a direct role in cardiac muscle contraction [21, 62] (See [126] for review). Instead, the relatively small amount of Ca^{2+} that enters the cell during the action potential through voltage-gated Ca^{2+} channels is thought to trigger the release of Ca^{2+} from the SR. This hypothesis is known as ***"calcium-induced calcium release"*** or ***CICR***.

Muscle contraction is initiated by the sudden rise in cytosolic Ca^{2+}, caused by Ca^{2+} entry and its release from the SR. This sensitivity to calcium is mediated by *troponin*, which is a complex of three polypeptides, *troponin-T* (tropomyosin binding), *troponin-I* (inhibitory) and *troponin-C* (Ca^{2+}-binding). The complex of *tropomyosin* with *troponin-T* and *troponin-I* inhibits the interaction of actin and myosin, thereby preventing contraction. When *troponin-C* binds to Ca^{2+},

allosteric changes lead to the displacement of tropomyosin from its inhibitory position, thereby removing the steric hindrance produced by the other two troponin components allowing actin to interact with the myosin heads (See [413] for review). Hydrolysis of ATP then provides the chemical energy necessary for the myosin cross-bridges to pull the attached actin filaments towards the centre of the sarcomere, thereby producing contraction [150, 184, 185].

Relaxation of the heart muscle is essential if the ventricles are to fill with blood for the next cycle of contraction, and to achieve this $[Ca^{2+}]_i$ must be lowered to the diastolic level. This is achieved by sarcolemmal efflux mechanisms, primarily by sodium/calcium (Na^+/Ca^{2+}) exchange, and by resequestration of Ca^{2+} by the SR through the activity of the SR Ca^{2+}-ATPase, and will be discussed in detail later (Section 1.10).

1.2 Pathways for Sarcolemmal Ca^{2+} Entry during Contraction

Since the initial observation by Ringer [304] it has been known that cardiac contraction is highly dependent on extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). Furthermore, removal or replacement of Ca^{2+} in the solution bathing cells rapidly and reversibly abolishes Ca_i transients and contraction [271]. It is believed that Ca^{2+} can enter the cell during contraction through both voltage-activated Ca^{2+} channels and via reverse mode Na^+/Ca^{2+} exchange. These mechanisms of Ca^{2+} entry are discussed below.

1.2.1 Sarcolemmal Ca^{2+} Channels

Sarcolemmal voltage-dependent Ca^{2+} channels are integral membrane proteins that form gated-pores in the plasma membrane. The channels open in response to changes in membrane potential and allow Ca^{2+} ions to enter the cell passively down their steep concentration gradient. To explain the ability of changes in membrane potential to open and close voltage-sensitive ion channels, it is postulated that such channels contained a charged region in their polypeptide structure that could serve as a voltage sensor, changing conformation in response to depolarisation and repolarisation of the membrane [79, 192]. The existence of such voltage sensors in Ca^{2+} channels has been confirmed (see [252] for review).

The Ca^{2+} current serves a number of functions during the action potential of heart muscle. The inward movement of positive charge contributes to the electrical signal responsible for depolarisation of the heart cell membrane and contributes to pacemaker activity, impulse conduction and the plateau of the action potential. Perhaps most importantly for this thesis, the influx of Ca^{2+} causes a transient increase of intracellular $[Ca^{2+}]_i$, which can initiate further Ca^{2+} release from the SR in a graded manner, provide some activator Ca^{2+} for binding to troponin C and contributes to the maintenance of SR Ca^{2+} stores.

The co-existence of at least two types of Ca^{2+} channels has been demonstrated in canine atrial [31], rabbit sino-atrial node [163] and Guinea pig ventricular cells [266, 280]. These have been designated *L-type* and *T-type* Ca^{2+} channels because of their distinct kinetic properties.

The threshold for activation of ***L-type Ca^{2+} channels*** in cardiac myocytes is around –40 mV. Activation is followed by inactivation that reduces the current to

near zero within approximately 300 ms and seems to be governed by both voltage- and Ca^{2+}-dependent mechanisms [161, 202, 230]. The current-voltage (*I-V*) relationship of the L-type Ca^{2+} current is *bell-shaped*, because as the membrane is depolarised further, the driving force for Ca^{2+} entry is reduced and eventually reversed [32, 277].

Because the L-type Ca^{2+} channel binds to dihydropyridines (DHPs) with relatively high specificity it is frequently referred to as the dihydropyridine receptor or DHPR. The channel is inhibited by dihydropyridine (DHP) "Ca^{2+} antagonists" (e.g. nifedipine and nitrendipine) and activated by DHP "Ca^{2+} agonists" (e.g. BAY K8644). Isolation of the DHPR from skeletal muscle has revealed that it is composed of 5 subunits (α_1, α_2, β, γ and δ. See [64] for review). The α_1 subunit of both skeletal and cardiac channel possesses the main functional characteristics of the native channel, while the others are thought to play largely regulatory roles [181, 262]. A transmembrane spanning portion of this subunit bears a positively charged amino acid (arginine or lysine) at every third position with intervening non-polar residues [262] and has been proposed to act as the voltage sensor [79]. Its primary structure also includes potential cAMP-dependent phosphorylation sites (Ser residues 124, 1575, 1627, 1700, 1848 and 1928) [262]. The L-type Ca^{2+} current is believed to have central importance in loading and maintaining the stored Ca^{2+} content of the SR [123, 239], ensuring the continual availability of stored Ca^{2+} for subsequent contractions. During development, there is a progressive concentration of L-type Ca^{2+} channels in the junctional sarcolemma, in parallel with the formation of t-tubules, where they are situated in close apposition to the Ca^{2+} release channels of the SR [396].

The ***T-type Ca^{2+} channel*** has been described as **transient** and low threshold, activating at potentials of around –60 mV, and then rapidly inactivating (20 – 50 ms). The complete absence of T-type channels in many ventricular cells [54] suggests that they do not play an important role in normal working myocardium [31]. However, Ca^{2+} entry through T-type Ca^{2+} channels was recently demonstrated to be capable of triggering Ca^{2+} release from the SR, albeit with lower efficiency than the L-type Ca^{2+} current [330]. Their predominance in sino-atrial node tissue and the fact that their activation range overlaps with that of the pacemaker potential (between –65 mV and –40 mV) has lead to the suggestion that they play an important role in pace-making activity [163].

A novel third type of Ca^{2+} channel was identified in sarcolemmal vesicles from calf ventricle and termed ***B-type*** because they are thought to contribute to a steady background current at negative membrane potentials [308]. Similar background channel activity was also identified in rat ventricular myocytes [97]. Unlike L- and T-type channels, B-type channels do not require depolarisation for activation and display mean open times of greater than 100 ms. The existence of these channels could account for at least some of the resting Ca^{2+} *leak* conductance of the cardiac myocyte sarcolemma [224]. This may have implications for the frequency of spontaneous SR Ca^{2+} release and the effect of rest on contractility, as will be described in this thesis.

1.2.2 Reverse Mode Na^+/Ca^{2+} Exchange

The existence of a Na^+/Ca^{2+} exchanger in intact heart tissue has long been established [210, 303]. The stoichiometry of the exchanger is such that 3 Na^+ ions

are exchanged for a single Ca^{2+} ion [302], such that there is movement of positive charge in the direction of sodium translocation. A fundamental feature of the Na^+/Ca^{2+} exchanger is that elevation of $[Na^+]_i$ and depolarisation will promote the reverse mode, i.e. exchange of Na_i for Ca_o. Local increases in $[Na^+]_i$ at depolarised potentials, such as those during the action potential, will therefore allow Ca^{2+} entry on the exchanger, and this could trigger SR Ca^{2+} release. The ability of reverse-mode Na^+/Ca^{2+} exchange to trigger SR Ca^{2+} release was first reported by Berlin *et al* [33] in a Ca^{2+} overloaded preparation, and later in the presence of elevated $[Na^+]_i$ [20, 45]. In the absence of Ca^{2+} entry through L-type Ca^{2+} channels, due to pharmacological blockade with nisoldipine or using a voltage step insufficient to activate I_{Ca}, membrane depolarisation still elicited Ca^{2+} release from the SR in guinea-pig myocytes [225]. This release was dependent on Na^+ entry through TTX-sensitive Na^+ channels and on extracellular Ca^{2+}, and was attributed to Na^+ entry through TTX-sensitive Na^+ channels, followed by Na^+ efflux and Ca^{2+} influx on the Na^+/Ca^{2+} exchanger. Lederer *et al* proposed that to achieve increases in $[Na^+]_i$ sufficient to activate Ca^{2+} entry on the Na^+/Ca^{2+} exchanger, it must enter a restricted volume below the sarcolemma, with access to Na^+ channels, the Na^+/Ca^{2+} exchanger and some SR Ca^{2+} release channels, the so called sub-sarcolemmal *"fuzzy space"* [228]. Direct evidence for such a sub-sarcolemmal $[Na^+]_i$ gradient has been provided by electron microprobe microanalysis [394] (See [74] for review). Furthermore, in the absence of Na^+ entry, depolarisation alone may directly stimulate Ca^{2+} entry on the exchanger [214]. It has also been proposed that Na^+/Ca^{2+} exchange may supply a larger fraction of trigger Ca^{2+} than I_{Ca} in guinea-pig [232, 234].

Experimental conditions that favour Ca^{2+} entry on the exchanger, such as high $[Ca^{2+}]_i$ (reducing the amplitude of I_{Ca}) [121] and increased $[Na^+]_i$ [214, 231, 239] appear to favour the observation of this phenomenon, as might be expected. However, its role under normal physiological conditions remains the subject of considerable debate [54, 321, 329]. Ca^{2+} release in the absence of L-type Ca^{2+} may be an artefact of loss of voltage control [54, 321], as a consequence of the large I_{Na} current surge, which may allow the membrane to reach the voltages at which I_{Ca} is activated. Incomplete block of I_{Ca} may also explain such observations, and increased SR Ca^{2+} load, inevitable under conditions that favour Ca^{2+} entry on the exchanger, may lead to overestimation of the contribution of Na^+/Ca^{2+} exchange mediated Ca^{2+} influx in triggering Ca^{2+} release [54].

There is some evidence that Ca^{2+} entry on the Na^+/Ca^{2+} exchanger may trigger Ca^{2+} release at higher, more physiological temperature [388, 392], suggesting that the failure of some groups to observe Na^+/Ca^{2+} exchange-triggered Ca^{2+} release may be a consequence of using lower experimental temperatures. Further assessment of the quantitative importance of this mechanism is required. Some clarification of this situation was sought by studying the effect of over-expression of Na^+/Ca^{2+} exchanger in transgenic mice. Even when the Na^+/Ca^{2+} exchanger was over-expressed by up to nine-fold, Ca^{2+} entry on the exchanger produced a much smaller rise of $[Ca^{2+}]_i$ in comparison to I_{Ca} [3]. However, another group saw enhanced activation of CICR by reverse Na^+/Ca^{2+} exchange [408]. These differences were attributed to differences in $[Na^+]_i$, Na^+/Ca^{2+} exchange mediated Ca^{2+} release being observed at a higher, perhaps non-

physiological $[Na^+]_i$ and therefore not necessarily indicative of a physiological role for this mechanism of Ca^{2+} entry.

Recent work has suggested that for a given Ca^{2+} influx, the Na^+/Ca^{2+} exchanger may be less efficient than the I_{Ca} trigger at promoting Ca^{2+} release from the SR [121, 331]. This may be a consequence of tight functional coupling between the L-type Ca^{2+} channel and SR Ca^{2+} release channels [1, 322].

Perhaps the best description of the situation is that there may be a *"dynamic balance"* between the two trigger mechanisms [233]. The majority of evidence is in favour of the trigger for Ca^{2+} release from the SR being L-type Ca^{2+} current with a small, but significant, contribution from reverse Na^+/Ca^{2+} exchange [152].

1.2.3 Ca^{2+}-Induced Inactivation of L-type Ca^{2+} Channel

Experimental evidence suggests that the L-type Ca^{2+} channel is inactivated by Ca^{2+}, a potential negative feedback mechanism for the regulation of Ca^{2+} entry during EC-coupling [202, 230]. The rate of inactivation is decreased when cations other than Ca^{2+} (e.g. Ba^{2+} or Sr^{2+}) carry the current [230, 307, 411] and when $[Ca^{2+}]_i$ is heavily buffered [299]. Inactivation of I_{Ca} increases when I_{Ca} amplitude is increased by raising external Ca^{2+} or by enhancing Ca^{2+} entry by changing the voltage-clamp protocol, elevating $[Ca^{2+}]_o$ [162, 230, 307] or by increasing SR Ca^{2+} release [2, 320, 328]. During systole, SR Ca^{2+} release is strictly graded by the amount of trigger Ca^{2+} entry through L-type channels. It is interesting that Ca^{2+} released from the SR also has the ability to regulate I_{Ca}, representing a very important negative feedback mechanism in EC-coupling. With respect to this thesis, the balance of Ca^{2+} fluxes during systole is important

in regulating the Ca^{2+} content of the SR (See Chapter 5). Changes in the Ca^{2+} current must be matched in the steady state by changes in the extrusion of Ca^{2+}, largely via the Na^{+}/Ca^{2+} exchanger. Changes in I_{Ca} inactivation by altered SR Ca^{2+} release may facilitate recovery of SR Ca^{2+} load following inotropic intervention.

1.3 The Sarcoplasmic Reticulum

The sarcoplasmic reticulum (SR) represents a network of membrane bound tubular sacs with the ability to sequester Ca^{2+} from the cytosol. Its high luminal Ca^{2+} content in comparison to the cytosol means that during excitation, Ca^{2+} release is a passive process, while Ca^{2+} sequestration requires energy expenditure and is mediated through the activity of its intrinsic Ca^{2+}-adenosine triphosphatase (ATPase) pump. The importance of the SR as a source of Ca^{2+} in EC-coupling varies between species [131], tissue and with developmental stage [87]. For example, Bers [38] suggested that relative dependence on SR Ca^{2+} release follows the order adult rat ventricle > rabbit atrium > rabbit ventricle > frog ventricle.

The SR membrane can be divided both structurally and functionally into two regions [255]: the *sub-sarcolemmal cisternae* or *junctional SR*, which contain the SR Ca^{2+} release channels, and the more extensive *sarcotubular network* or *longitudinal* SR, containing densely packed arrays of Ca^{2+}-ATPase proteins. Ca^{2+} passively released from the junctional SR (JSR) activates contraction and is then actively pumped back into the longitudinal SR (LSR) during relaxation. Ca^{2+} ions move from the LSR by diffusion towards the JSR to complete the Ca^{2+} cycling of transport processes initiated by excitation.

1.3.1 The Sarcotubular Network or Longitudinal SR

This is the most extensive portion of the SR and is located at the centre of the sarcomere, surrounding the myofilaments. Its membrane is rich in the Ca^{2+}-ATPase proteins responsible for Ca^{2+} uptake by the SR and it has therefore been linked with Ca^{2+} sequestration and muscle relaxation.

1.3.2 The Sub-sarcolemmal Cisternae or Junctional SR

This portion of the SR is located at the junction between the SR and the invaginations of the sarcolemma, known as transverse- or t-tubules. At this junction, known as a dyad, the SR assumes a flattened shape and *"foot" proteins*, corresponding to the Ca^{2+} release channel of the SR [187, 259], project into the dyad space. In the lumen of the junctional SR high concentrations of the Ca^{2+} binding glycoprotein *calsequestrin* are found. The high capacity of this protein for binding Ca^{2+} allows the accumulation of large amounts of Ca^{2+} within this region of the SR, while its relatively low affinity allows bound Ca^{2+} to be released rapidly when required [407]. Over-expression of calsequestrin in transgenic mice causes reduced spark frequency and increased SR Ca^{2+} content in isolated ventricular myocytes [197]. This suggests that calsequestrin is important as a Ca^{2+} storage protein and that increasing store capacity may limit Ca^{2+} leak from the SR by regulating the Ca^{2+} release mechanism. The importance of SR luminal Ca^{2+} will be discussed later, but is of great importance to the regulation of CICR.

The capacity of the SR as a store for Ca^{2+} will be discussed in this thesis (See Chapter 3).

1.4 THE SR Ca^{2+} RELEASE CHANNEL

The Ca^{2+} release unit of the SR is a cation-selective channel that displays an unusually large conductance for monovalent and divalent cations (~75 pS in 50 mM Ca^{2+} [311]). This Ca^{2+} release channel is also frequently referred to as the *ryanodine receptor* (RyR), because of its sensitivity to the plant alkaloid ryanodine, to which it binds with high affinity and specificity. Taking advantage of the high specificity of ryanodine binding, [^{3}H]-ryanodine binding has been used as a marker for isolation and purification of the release channel [13, 335, 379], and as a measure of the open probability (P_o) of the channel, since ryanodine binds preferentially to open channels [257]. Since the RyR is not accessible for patch-clamping while maintaining the normal physiological environment of the channel, understanding of the gating and conductance characteristics of the channel has been achieved by studies using junctional SR vesicles enriched with RyRs [80, 212, 258, 387], or reconstitution of junctional SR membrane vesicles or isolated RyRs into planar lipid bilayers [317, 369, 404]. Comparison of the amino acid compositions of the skeletal and cardiac RyRs has revealed that they are similar, sharing 66% sequence homology [293], but different enough to suggest that they are encoded by different genes. These have been designated *ryr1* and *ryr2*, for skeletal and cardiac RyR genes respectively. The receptors from both tissues are arranged in a tetrameric structure [13], and electron microscopy has revealed that the large N-terminal part of each receptor protrudes into the

cytoplasm, forming the characteristic *"foot"* structure, which is synonymous with the RyR [187, 259]. An excellent review of the structure and function of RyRs is provided by Coronado *et al* [96].

1.4.1 Activation of the RyR by Cytosolic Ca^{2+}

The activation of RyRs by cytosolic Ca^{2+} is caused by increasing the channel open probability (P_o) [311], and the primary mechanism for this is an increase in the frequency of channel opening. The rate of Ca^{2+} release from cardiac SR is a biphasic function of [Ca^{2+}] with a maximum at 5–20 μM [258]. This has been hypothesised to result form the existence of two binding sites, a high affinity site that stimulates Ca^{2+} release and a low affinity site that inhibits release [269]. Such observations are in agreement with those of Fabiato in skinned myocytes [124]. However, the biphasic nature of this Ca^{2+} release may simply reflect the decrease in driving force for Ca^{2+} efflux, as extra-vesicular Ca^{2+} approaches the luminal concentration [96]. However, cytosolic Ca^{2+} alone cannot fully activate the channel [16] and other endogenous activators are required.

1.4.2 Endogenous and Pharmacological Modulators of the RyR

Like the skeletal RyR [14, 212, 310, 387, 404], the cardiac SR release channel is activated by endogenous Ca^{2+} and adenine nucleotide [258], and inhibited by Mg^{2+}, H^+ (low pH), and calmodulin [258]. Pharmacological activators include caffeine [309, 334], and inhibitors ruthenium red [80] and the local anaesthetics tetracaine [80], procaine [80, 369, 412] and cocaine [379].

Ryanodine at sub-micromolar concentrations increases the probability of channel opening, while at higher concentrations long lasting sub-conductance states appear, eventually blocking the channel at high (> 100 μM) concentrations [312].

1.4.2 Potential Modulation of the RyR by Other Endogenous Substances

The endogenous metabolite cyclic ADP-ribose (cADPR) was shown to cause Ca^{2+} release from a ryanodine-sensitive store in sea-urchin eggs [144, 261]. Its presence in cardiac muscle has raised the possibility that it acts as an endogenous regulator of CICR [143], but experimental results have lead to conflicting conclusions. Antagonists of cADPR have been shown to reduce contraction in isolated cardiac cells [301], suggesting that cADPR may potentiate CICR, by sensitising it to Ca^{2+}. However, other studies have demonstrated either no effect of cADPR or its antagonist 8-amino-cADPR [141, 154] or effects that are expected to be antagonised by physiological concentrations of ATP [332]. It has recently been suggested that the effects of cADPR are temperature dependent [186], possibly accounting for the lack of effect in previous experiments. More recently cADPR has been proposed to release Ca^{2+} from the SR independently of the RyR [221].

Closely associated with each skeletal RyR are four FK506-binding protein molecules (FKBP-12, 12 kDa each) [196]. Co-expression of these proteins with RyRs in insect cells showed that FKBP-12 increased the number of channels exhibiting full conductance, but decreased the channel P_o [60]. A slightly different form of FKBP co-purifies with the cardiac RyR [367]. This protein physically interacts stoichiometrically (FKBP: RyR 4:1) with the ryanodine

receptor, where it is thought to cause important inhibitory stabilising effects [199], and has also been implicated in the termination of elementary release events during CICR [403]. Binding of the immunosuppressant agents FK506 and rapamycin inhibit the activity of FKBP, thereby reversing its effects on the RyR [60, 199]. The cardiac ryanodine receptor binds to the FKBP-12 homologue FKBP-12.6. FKBP-12 deficient mice exhibit severe dilated cardiomyopathy, but have normal skeletal muscle function [326].

The SR Ca^{2+} release channel is ***activated*** by phosphorylation [107, 164, 360, 401], which may be of particular importance during β-adrenergic stimulation [360, 410].

Many of these endogenous and exogenous effectors could modulate EC-coupling through their ability to suppress or stimulate CICR. In this thesis the effects of such modulation of CICR on electrically stimulated and spontaneous contraction and the SR Ca^{2+} content will be discussed.

1.5 Calcium-Induced Calcium Release (CICR)

Solaro *et al* [341] attempted to define the total free $[Ca^{2+}]_i$ that must be bound to the myofilaments to produce a given level of tension. Based on these calculations it was suggested that the amount of Ca^{2+} transferred as I_{Ca} (up to 25 μmol/kg.w.w.) could be sufficient to activate contraction [188]. However, a fundamental error in these calculations was their failure to consider other intracellular buffers such as calmodulin and SR sequestration. Subsequent calculations demonstrated that even using data likely to over-estimate trans-

sarcolemmal influx, the change of free $[Ca^{2+}]_i$ caused by I_{Ca} is unlikely to activate the myofilaments appreciably [122] and release from the SR is a definite requirement in mammalian cardiac muscle.

In contrast to skeletal muscle, cardiac muscle has an absolute requirement for extracellular Ca^{2+} in order to contract. Rapid removal of extracellular Ca^{2+} from the solution bathing a cell abolishes Ca_i transients and therefore contraction [271]. In an elegant series of experiments, Fabiato & Fabiato used EGTA to buffer free $[Ca^{2+}]$ and to vary pCa ($-\log[Ca^{2+}]$) around skinned cardiac myocytes [127-129]. Cyclic contractions, attributed to cycles of release and resequestration of Ca^{2+} by the SR, were observed when $[Ca^{2+}]$ was raised slightly from pCa 7.6 to 7.4, while tonic contraction was not observed until pCa was increased to 6.0-6.5. This suggested that at higher pCa an additional large amount of Ca^{2+} must be released to elicit contraction [127, 128], corresponding to the regenerative release of CICR. These cyclic contractions were abolished when the SR was destroyed or functionally inhibited by high total [EGTA] [129]. It was concluded that these cyclic contractions corresponded to CICR from the SR.

To accompany these early investigations, the SR of canine ventricular tissue was demonstrated to be capable of binding more Ca^{2+} than is necessary to activate the myofilaments completely [340], suggesting that the SR is a more than adequate source of Ca^{2+} for contraction. Further evidence in support of a CICR mechanism was the demonstration that simulated Ca^{2+} current could trigger Ca^{2+} release in skinned myocytes [123] and SR Ca^{2+} release can be activated by rapid photolysis of caged Ca^{2+} in intact cells [272, 381]. The close relationship between the voltage dependencies of the I_{Ca}, Ca^{2+} transients and contraction provides

further evidence in favour of the CICR mechanism [32, 62, 92, 241, 277]. Ca^{2+} transients in crustacean muscle also closely follow the voltage dependence of peak Ca^{2+} current, leading to the suggestion that CICR is also responsible for contraction in crustacean muscle [158], providing a useful second model system for the study of CICR.

1.6 Grading of CICR

The hypothesis of CICR appears to suffer from its apparently inherent positive feedback. The trigger for Ca^{2+} release is also the product, which would be expected to stimulate further release, until depletion of the stores limits further release, the so-called ***all-or-none*** response. A number of observations suggest that this is not the case. Fabiato and Fabiato observed that the amplitude of cyclic contractions in skinned cardiac myocytes was increased when the free $[Ca^{2+}]$ that triggered them was increased [129], suggesting that the process is not *all-or-none* and must be graded with trigger Ca^{2+}. Under normal conditions local Ca^{2+} release does not propagate [286, 375]. The magnitude of the systolic Ca^{2+} transient and contraction is graded with the duration and amplitude of depolarisation and the resulting Ca^{2+} current [21, 32, 62, 92, 133, 241, 277], and if trigger I_{Ca} is terminated prematurely by further depolarisation to 100 mV, Ca^{2+} release is halted [92].

It is clear that to prevent the Ca^{2+} release process from going to completion some sort of negative control mechanism is required. A number of ways this could be achieved have been proposed and experimentally tested.

1.6.1 Ca^{2+}-Dependent Ryanodine Receptor Inactivation

Fabiato proposed that Ca^{2+} ions have two binding sites on the release channel receptor, one of high and the other low affinity [124]. The high affinity site is responsible for activation, while binding of Ca^{2+} to the low affinity site causes inactivation of the channel. A rapid increase in $[Ca^{2+}]_i$ causes binding of Ca^{2+} to the activating site, resulting in the release of Ca^{2+} from the SR. The inactivating site binds Ca^{2+} more slowly, and eventually terminates Ca^{2+} release. Stern proposed that inactivation of Ca^{2+} release could be modelled by rapid diffusion of Ca^{2+} away from the Ca^{2+} activation sites of the RyR [349]. An alternative suggestion is that as the SR becomes depleted of Ca^{2+}, the reduction of luminal Ca^{2+} will decrease the P_o and the conductance of Ca^{2+} through the channel of the channel [333].

The bell-shaped dependence of release channel opening on $[Ca^{2+}]$ [269] has been proposed to support the observations and hypothesis of Fabiato [124]. However, controversy remains over whether this could be a simple consequence of the reduced driving force for Ca^{2+} efflux [96].

Ca^{2+}-dependent inactivation of the RyR has been demonstrated at the level of the isolated canine cardiac RyR [317]. However, photo-release of caged Ca^{2+} at the onset of or during depolarisation-induced Ca^{2+} release failed to suppress contraction in isolated guinea pig and rat myocytes, as predicted from the Ca^{2+}-induced inactivation hypothesis [272]. A number of other groups have made observations inconsistent with Ca^{2+}-dependent inactivation of the RyR [92, 309]. It is apparent that free $[Ca^{2+}]$ must be very high (1 mM) to cause inactivation of the release channel [317]. Global increases in $[Ca^{2+}]_i$ are unlikely to be able to

achieve such levels, however in the microenvironment of the native RyR, the narrow space between the junctional SR and the t-tubular of the sarcolemma, Ca^{2+} may accumulate to much higher concentrations, thereby reaching the threshold for inactivation.

1.6.2 Ryanodine Receptor Adaptation

One particular observation inconsistent with the hypothesis of simple Ca^{2+}-dependent inactivation of RyR was that when Ca^{2+} is further increased channels appear to reopen and again eventually inactivate. This type of behaviour is referred to as *adaptation*. Isolated cardiac RyR channels were observed to adapt to maintained Ca^{2+} stimuli, thereby maintaining their ability to react to a further increment of $[Ca^{2+}]_i$ [156]. Following activation of a release channel its activity spontaneously decays until Ca^{2+} release stops. A model describing the mechanism of RyR adaptation was proposed by Cheng *et al* [83]. In the presence of physiological $[Mg^{2+}]_i$ RyR adaptation was observed to be a rapid (milliseconds) process, further accelerated by RyR phosphorylation by the cAMP-dependent protein kinase [382]. In intact myocytes this mechanism terminates CICR independent of trigger duration, which may also help to explain the ability of I_{Ca} to contribute towards SR loading at later times during Ca^{2+} entry [333, 409]. Adaptation cannot be explained by deactivation of the release channel by loss of Ca^{2+} from the activation site [386].

Adaptation may represent the negative feedback mechanism required to counter the inherent positive feedback of CICR, and could explain why fast Ca^{2+} stimuli are apparently more effective at eliciting Ca^{2+} release than are slow stimuli

[124]. Recently a role for FK506-binding protein in mediating this adaptation was proposed and supported experimentally [403].

1.6.3 "Common Pool" Model of CICR

A simplified schematic model of EC-coupling in an isolated myocyte is illustrated in Fig. 1.1. In the common pool model it is assumed that there is a single cytosolic pool of Ca^{2+}. The concentration of Ca^{2+} in this pool regulates Ca^{2+} release from the SR, and Ca^{2+} released from the SR enters the same cytosolic pool. This arrangement has obvious positive feedback and would be expected to operate with an all-or-none response to Ca^{2+} influx. The smooth grading of Ca^{2+} release as a function of trigger Ca^{2+} under normal conditions suggests that this is clearly not the case [32, 62, 92, 124, 241]. A mechanism such as those discussed previously must be provided to interrupt Ca^{2+} release, thereby breaking the positive feedback loop. Stern has proved rigorously that common pool models tend to give rise to all-or-none regenerative Ca^{2+} release, unless the system gain is very low [349, 352]. Under conditions of Ca^{2+} overload, spontaneous Ca^{2+} releases can propagate throughout the cell, suggestive of the potentially regenerative nature of the trigger signal.

1.6.4 "Local Control" model of CICR

To resolve the paradox of control imposed by CICR, local control models of EC-coupling have been proposed [349, 352], in which the trigger for Ca^{2+} release from the SR is Ca^{2+} in the micro-domain between the DHPR and the RyR,

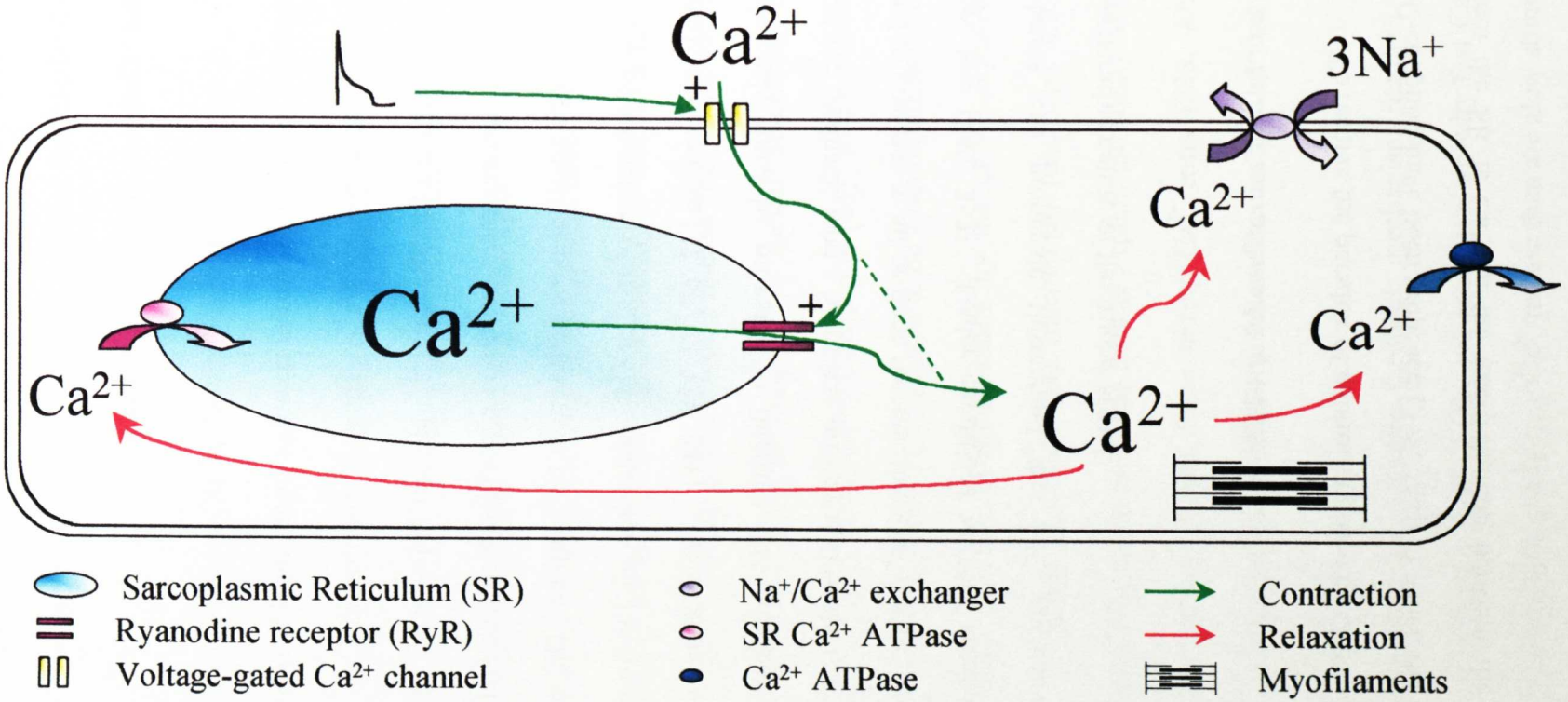

Figure 1.1. Simplified schematic representation of "common pool" Ca^{2+} cycling in a cardiac myocyte.

During the action potential Ca^{2+} enters the cell largely through voltage-dependent Ca^{2+} channels in the sarcolemma. This triggers further release from the SR through the RyR Ca^{2+} release channel, giving rise to a $[Ca^{2+}]_i$ transient, which activates the myofilaments causing contraction. During relaxation, Ca^{2+} is resequestered by the SR Ca^{2+}-ATPase, and extruded from the cell via Na^+/Ca^{2+} exchange and the sarcolemmal Ca^{2+}-ATPase.

generated by Ca^{2+} influx through the sarcolemmal DHPR. Two basic models were proposed by Stern; i) the *"Ca^{2+} synapse"* model, in which Ca^{2+} entry through an L-type Ca^{2+} channel directly activates Ca^{2+} release from a single adjacent RyR, and ii) the *"cluster bomb"* model, in which L-type channel Ca^{2+} entry activates a group of release channels clustered together in a single micro-domain. The Ca^{2+} synapse model requires unrealistically high conductance through RyRs in order to explain the high amplification of the trigger Ca^{2+} that is observed experimentally, and the cluster-bomb model is generally preferred. A great deal of experimental evidence supports the local control hypothesis.

The L-type Ca^{2+} current is much more efficient at gating the release of Ca^{2+} from the SR, and SR Ca^{2+} release more efficient at inactivating I_{Ca}, than global changes in $[Ca^{2+}]_i$ [1, 159, 322], suggesting privileged access between these two channels. In crustacean muscle local increases in $[Ca^{2+}]_i$ are also mediated by I_{Ca}, which is more effective in triggering CICR than global elevations of Ca^{2+}, for example mediated by photolysis of caged Ca^{2+} [160]. Furthermore, the voltage dependence of the gain of CICR is similar to that of unitary L-type Ca^{2+} current but not to that of the macroscopic current [398].

Co-localisation of L-type Ca^{2+} channels (DHPRs) and ryanodine receptors was demonstrated in chick myocardium [355], where the average spacing between a DHPR and RyR is less than the average spacing between RyRs. This confirms the arrangement necessary to avoid the positive feedback inherent to CICR, because Ca^{2+} influx has privileged access to coupled RyRs and Ca^{2+} release will not activate neighbouring release channels except at higher gain. The apparently

low Ca^{2+} sensitivity of release channels under normal conditions may help to protect against positive feedback of released Ca^{2+} on further release.

There are three mechanisms by which an active cluster can be extinguished. As reviewed by Stern [349], two of these are analogous to processes proposed to prevent regenerative Ca^{2+} release in the common pool models, Ca^{2+}-dependent inactivation (Section 1.6.1) and local depletion of releasable Ca^{2+}. As a third mechanism, it is known that there is a finite probability that enough channels in a cluster will close simultaneously to reduce the Ca^{2+} below the self-sustaining level. Channels remaining open at this time will close rapidly, and the cluster will remain inactive until stimulated again. This spontaneous "shut down" of active clusters of RyRs, in the absence of other mechanisms to extinguish Ca^{2+} release is referred to as **stochastic attrition**.

1.7 Ca^{2+} Sparks

Further evidence in support of local-control theories of EC-coupling has accumulated from observations of stereotypical, localised increases in $[Ca^{2+}]_i$, termed *Ca^{2+} sparks*, using the high temporal and spatial resolution of confocal fluorescence microscopy. These have been observed to occur spontaneously in resting myocytes [86, 157] and can also be evoked by membrane depolarisation [66, 242]. Their amplitude and spatio-temporal characteristic are identical and independent of species and the magnitude of L-type Ca^{2+} current activating them [85]. Consistent with their origin being stochastic Ca^{2+} release from the SR, their frequency is enhanced by nanomolar concentrations of ryanodine and they are inhibited by micromolar ryanodine [86]. It is believed that these events represent

the opening and closing of single SR Ca^{2+} release channels, or a small number acting in concert [86]. Sparks have been used to explain the spatial non-uniformity of global $[Ca^{2+}]_i$ transients activated by short pulse electrical stimulation [66]. The simplest explanation of this observation is that SR Ca^{2+} release takes place at discrete sites when a nearby Ca^{2+} channel is activated. The stochastic nature of channel gating and the finite latency in opening of the Ca^{2+} release channel results in not all sites being activated [67]. As this information accumulated the hypothesis that global Ca^{2+} transients are comprised of stochastic, independent local events similar to the Ca^{2+} sparks, originally described by Cheng *et al* [86], was formed. Gradation of Ca^{2+} transients in response to changes in L-type Ca^{2+} current can be explained by recruitment of variable numbers of Ca^{2+} sparks being activated [67, 85, 314], rather than by modulation of spark amplitude, as previously suggested [242].

In support of local control of EC-coupling, Ca^{2+} sparks are localised to the junction of the transverse tubule and the junctional SR [85, 319], where L-type Ca^{2+} channels and ryanodine receptors co-localise [73]. The probability of evoking a spark has voltage and time dependence similar to that of the single L-type Ca^{2+} channel [243, 398]. Ca^{2+} entry through L-type Ca^{2+} channels is also more efficacious in inducing Ca^{2+} sparks than global increases in $[Ca^{2+}]_i$ [243], and at high positive membrane potentials where Na^+/Ca^{2+} exchange mediated Ca^{2+} entry is activated but there is no Ca^{2+} entry through L-type Ca^{2+} channels, sparks were not observed, suggesting privileged access of L-type current to the RyR.

It has recently been proposed that normal Ca^{2+} release in the heart may involve as yet unresolved unitary Ca^{2+} release events much smaller than Ca^{2+}

sparks. These have been termed *Ca^{2+} quarks* [240]. This suggests that Ca^{2+} sparks are not indivisible and must involve the opening of more than a single RyR. That Ca^{2+} release involves more than one Ca^{2+} release site has also been suggested by Parker *et al* [296]. However, the indivisible single channel origin of a spark is preferred by Song *et al* [342], since depletion of SR Ca^{2+} does not prevent Ca^{2+} sparks in rat.

1.8 The Gain of CICR

Gain describes the relationship between Ca^{2+} entry on the Ca^{2+} current and Ca^{2+} released from the SR. At increased gain a given I_{Ca} will activate Ca^{2+} release more readily than at a lower gain. Gain is highly dependent on SR Ca^{2+} load [25, 166, 195, 235, 315, 343, 344], explaining why the process of CICR appears to be more readily triggered during Ca^{2+}-overload [86]. Most of these effects can be explained in terms of luminal effects of Ca^{2+} on the gating of RyRs and the increased driving force for Ca^{2+} efflux from the SR.

1.8.1 Regulation of SR Ca^{2+} Release by Luminal Ca^{2+}

The effect of SR luminal Ca^{2+} on the regulation of Ca^{2+} release was first suggested by Fabiato and Fabiato, who demonstrated that increased SR Ca^{2+} loading increased the magnitude of CICR in skinned cardiac myocytes [133]. It has become increasingly apparent that luminal Ca^{2+} may regulate Ca^{2+} release through RyR in both skeletal and cardiac muscle [174, 245, 335, 336, 376, 406] (see [337] for review). There are two possible explanations for this phenomenon.

The first possibility is that luminal Ca^{2+} could act by flowing through the RyR and regulate channel activity by gaining access to cytosolic activation and inactivation sites [173, 376, 406]. This is disputed by the finding that increased luminal Ca^{2+} failed to increase the open probability (P_o) of the cardiac release channel when cytosolic free Ca^{2+} was sub-optimal for release channel activation in sheep and dog [245, 336, 337]. Under these conditions, if Ca^{2+} flowing from the SR lumen had access to the cytosolic sites for activation, an increase in P_o would occur [336]. In the absence of other cytosolic activating ligands, increased luminal Ca^{2+} does not open the RyR [245, 335], so luminal Ca^{2+} cannot itself trigger channel opening. The second possibility is that there is a luminal receptor for Ca^{2+}, which somehow alters the cytosolic Ca^{2+} sensitivity of the RyR. The fact that in the presence of a second ligand (e.g. ATP or sulmazole) luminal Ca^{2+} can increase P_o [245, 336], could suggest that access to the luminal receptor for Ca^{2+} may be sterically limited and only available in the presence of other endogenous effectors. Under these conditions increased luminal Ca^{2+} shifts the activation of the RyR to much lower $[Ca^{2+}]_i$ [337]. Under conditions of Ca^{2+} overload, the marked increase in P_o that luminal Ca^{2+} exerts on the release channel in the presence of other endogenous activators, such as adenine nucleotides, may be enough to activate Ca^{2+} release. This could explain the ability of spontaneous Ca^{2+} release to propagate in Ca^{2+} overloaded cells [86, 263] and not under normal conditions [375]. These effects will also contribute to the potentiation of Ca^{2+} release at increased SR Ca^{2+} loads.

1.8.2 The Increased Driving Force for Ca^{2+} Efflux from the SR

Once Ca^{2+} release has been triggered, raised luminal [Ca^{2+}] is expected to result in larger than normal flux through the RyR due to the increased SR Ca^{2+} gradient and increased P_o [368]. This may be further amplified by Ca^{2+} released from the SR binding to the cytoplasmic activation site of the RyR. When strontium replaces Ca^{2+} in the lumen of the SR, this positive feedback effect is minimised [344].

1.9 Alternative Calcium Release Mechanisms

Difficulty in explaining the strictly graded nature of CICR and the fact that the voltage dependency of the Ca^{2+} current and Ca^{2+} transient are not exactly superimposible has been proposed to support the involvement of some other mechanism in regulating SR Ca^{2+} release [65, 226] (see [126] for review).

1.9.1 Voltage Activated or Charge-Coupled Ca^{2+} Release (VACR / CCCR)

In skeletal muscle, charge movements induced by depolarisation of the sarcolemma are communicated directly from the transverse tubule to the SR Ca^{2+} release channel to induce Ca^{2+} release [306, 318]. Intramembrane charge movement, Ca_i transient and tension development all show sigmoidal voltage dependence in this tissue [5]. In cardiac muscle on the other hand, the voltage dependence of Ca_i transients [21, 62], cell shortening [62, 92, 241] and tension [32] are ***bell-shaped*** and reflect the voltage dependence of the Ca^{2+} channel [277]. Furthermore, Ca^{2+} release can be induced by **repolarisation** from positive

potentials [21, 62]. This finding is totally inconsistent with intramembrane charge movement, by depolarisation of the membrane, as the trigger.

However, recent experimental evidence supports the existence of Voltage-activated Ca^{2+} release in cardiac myocytes. Ferrier and Howlett found that depolarisation could still produce SR Ca^{2+} release even in the absence of measurable Ca^{2+} entry [136] and it was recently found that this form of Ca^{2+} release is dependent on cAMP-dependent phosphorylation [138]. cAMP-dependent SR Ca^{2+} release activated directly by membrane depolarisation, without Ca^{2+} entry has also been observed by Hobai *et al* in rat, rabbit and guinea pig ventricular myocytes [177]. However, the inability of these groups to observe these effects in the absence of external Ca^{2+}, has lead to the suggestion that undetectable Ca^{2+} entry under conditions of high internal cAMP may be sufficient to trigger Ca^{2+} release by the classical CICR mechanism.

The ratio of ryanodine to dihydropyridine receptors is high (1:9), suggesting that direct interaction would be unable to achieve communication between these channels in cardiac myocytes [48, 396]. Niggli and Lederer found that Ca^{2+} release activated by flash photolysis of caged Ca^{2+} was unaffected by varying membrane potential, providing evidence for exclusion of even a modulatory role of voltage or charge movement in normal EC-coupling [279]. Finally, having established the importance of Ca^{2+} sparks as the fundamental event in EC-coupling, the observation that depolarisation alone is unable to elicit Ca^{2+} sparks, is further evidence to refute the importance of VACR in cardiac muscle [325].

1.9.2 Inositol (1,4,5)-Triphosphate ($InsP_3$)-Induced Calcium Release

When certain membrane receptors are stimulated there is an increase in the activity of phospholipase C (PLC), which breaks down phosphatidylinositol-4,5-bisphosphate into inositol (1,4,5)-triphosphate ($InsP_3$) and diacylglycerol (DG). $InsP_3$ is soluble in the cytoplasm, where it functions as an intercellular messenger, stimulating the release of Ca^{2+} from the ER of many cell types (See [35] for review). However, there is controversy over whether it is capable of triggering the release of Ca^{2+} from the SR of striated muscle [206, 282, 353] and $InsP_3$-induced release of Ca^{2+} from the SR appears to be too slow to be of importance in EC-coupling.

1.10 Relaxation of Cardiac Muscle

In order to perform efficiently as a pump the heart must relax between beats to allow refilling of the chambers with blood. To allow relaxation, $[Ca^{2+}]_i$ must be returned to the diastolic level, removing Ca^{2+} from troponin C and inactivating the myofilaments. Ca^{2+} is removed form the cytosol by a number of tightly regulated intracellular and sarcolemmal removal systems. The relative contributions of each of these mechanisms to cytosolic Ca^{2+} removal have been examined in several species and will be reviewed in some detail below.

1.10.1 The SR Ca^{2+}-ATPase (SERCA2)

Ca^{2+} accumulation by cardiac SR occurs through the activity of the SR Ca^{2+}-ATPase (SERCA2a), which transports Ca^{2+} from the cytosol into the lumen of the SR in a reaction that is also dependent on Mg^{2+}. The SR Ca^{2+}-ATPase is

selectively inhibited by nanomolar concentrations of thapsigargin [193, 208, 237, 365], micromolar 2,5-di-*tert*-butylhydroquinone (TBQ) [198] and cyclopiazoic acid [30]. It represents the major determinant of cardiac relaxation in mammalian species, as the time constant of the exponential decline of $[Ca^{2+}]_i$ is increased several fold by caffeine [43, 287] and by the specific SR Ca^{2+}-ATPase inhibitors thapsigargin [180, 193, 273, 285] and cyclopiazonic acid [30]. The affinity (K_m) and maximum rate (V_{max}) of rat ventricular SERCA2 were recently determined to be 0.28 μM and 0.21 $mM.s^{-1}$ respectively at room temperature [18]. The rate of Ca^{2+} transport is sensitive to small changes in free Ca^{2+} within the physiological range, such that when cytosolic Ca^{2+} rises to a value exceeding K_m the rate of sequestration rises to near maximal levels. As Ca^{2+} sequestration progresses, the reduction in cytosolic Ca^{2+} lowers the rate of accumulation until diastolic $[Ca^{2+}]_i$ is restored. Over-expression of SERCA2a in transgenic mice leads to abbreviated cardiac contraction and increased contractility [260].

The activity of the SR Ca^{2+}-ATPase is modulated by interaction with phospholamban. Phospholamban is an integral protein of the SR membrane and binds to SERCA2 in its unphosphorylated form, thereby inhibiting its activity, mainly by decreasing its Ca^{2+} sensitivity. Phosphorylation of phospholamban can occur at two different sites by a number of protein kinases – among them *cAMP-dependent protein kinase* (PKA), which phosphorylates at serine 16, and a membrane associated *calmodulin-dependent protein kinase* (CAM-kinase), which phosphorylates at threonine 17 [142]. This blocks the interaction of phospholamban with SERCA2 thereby relieving inhibition of the ATPase [209, 357], and enhancing Ca^{2+} uptake and relaxation. The inhibitory effect is regained

when phospholamban is dephosphorylated by *phosphoprotein phosphatases*. β-Adrenergic stimulation is believed to act by stimulating PKA mediated phosphorylation of phospholamban thereby increasing the amplitude of contraction and rate of relaxation by relieving the inhibition of the SR Ca^{2+}-ATPase [323, 357]. Genetic ablation of the gene encoding phospholamban in transgenic mice results in similar effects on contraction and relaxation, coupled with the loss of sensitivity to β-adrenergic stimulation [247]. The Ca^{2+}-ATPase is also sensitive to changes in pH [125] exhibiting slower rates of Ca^{2+} uptake as a consequence of decreased pH. This may be due to alteration in the phosphorylation of phospholamban [183].

1.10.2 Na^+/Ca^{2+} Exchange

The existence of a Na^+/Ca^{2+} exchanger in the sarcolemma of cardiac tissue has long been established [210, 303]. In the absence of SR Ca^{2+} sequestration inhibition of Na^+/Ca^{2+} exchange, by removing Na^+ from the bathing solution or by addition of Nickel (Ni^{2+}), dramatically reduces the rate of relaxation [274, 383]. This suggests that the Na^+/Ca^{2+} exchanger represents the main sarcolemmal route for Ca^{2+} extrusion from the cell [24, 47, 59, 98, 287]. The stoichiometry of the exchanger is such that 3 Na^+ ions enter the cell in exchange for the extrusion of a single Ca^{2+} ion [302], and the driving force for Ca^{2+} extrusion is the large electrochemical gradient favouring Na^+ movement into the cell. Ca^{2+} extrusion is therefore electrogenic resulting in the inward translocation of positive charge, an inward current, which depolarises the cell membrane. Currents associated with Na^+/Ca^{2+} exchange have been identified in guinea pig [20, 49, 135, 210, 211, 254]

and rat [55, 63]. The electrogenic nature of this extrusion mechanism means that it is also voltage dependent.

1.10.3 Sarcolemmal Ca^{2+}-ATPase

The Ca^{2+}-ATPase has higher affinity for Ca^{2+} ions (K_m in the range 0.1-0.5 μM) than the Na^+/Ca^{2+} exchanger, but lower maximal velocity of Ca^{2+} translocation. The density of the pump in the sarcolemma is also relatively low, representing less than 1% of the total protein content of the sarcolemma [77]. Studies of the contribution of the sarcolemmal ATPase to relaxation and the maintenance of diastolic $[Ca^{2+}]_i$ have been hindered by the lack of a specific inhibitor of the pump, and its role has been implicated from studies using inhibitors of the remaining Ca_i removal mechanisms. The Na^+/Ca^{2+} exchange independent decay of caffeine-induced Ca_i transient is attributed to the sarcolemmal Ca^{2+}-ATPase and mitochondrial sequestration. Separating these two components has been achieved by the use of mitochondrial uncouplers, such as FCCP [23, 26], or by elevating external Ca^{2+} to thermodynamically limit the sarcolemmal Ca^{2+}-ATPase [23]. Recently the use of carboxyeosin, a specific inhibitor of the pump [146, 147], has alleviated these problems and revealed the importance of Ca^{2+}-ATPase in regulating resting $[Ca^{2+}]_i$ [88], consistent with its high affinity but low pumping rate [77], and its appreciable contribution to Ca^{2+} efflux during relaxation of rabbit, ferret and rat ventricular myocytes [27, 89].

1.10.4 Mitochondria

Mitochondria are well known to have an enormous capacity for Ca^{2+} accumulation [267]. This has lead to the hypothesis that mitochondrial Ca^{2+} transport could play a role in EC-coupling. However, the Ca^{2+} affinity of the mitochondrial Ca^{2+} pump is low and the rate of transport slow at physiological $[Ca^{2+}]_i$, and unlikely to compete with the SR Ca^{2+} pump and Na^+/Ca^{2+} exchanger [43]. During caffeine contractures in Na^+ free solution relaxation was slowed by inhibition of the mitochondrial Ca^{2+} uptake using the oxidative phosphorylation uncoupler FCCP [26]. This series of experiments however lead to the conclusion that mitochondria are 50 fold slower than Na^+/Ca^{2+} exchange at cytoplasmic Ca^{2+} removal. Furthermore, Ca^{2+} sequestered by mitochondria also moves back into the cytoplasm within 40 seconds [23]. These observations suggest that mitochondria have limited ability to regulate intracellular Ca^{2+} during normal EC-coupling. Instead, it is possible that the Ca^{2+} transport mechanisms of the mitochondria could exist to regulate intra-mitochondrial processes, such as oxidative phosphorylation and therefore energy production, in the myocardium (See [40] for review).

1.11 Ca^{2+} Homeostasis in Cardiac Muscle

The intracellular concentration of calcium ions (100 nM) is actively maintained at one ten thousandth that of the extracellular space (1 mM). Low intracellular Ca^{2+} is essential to achieve relaxation of the muscle and to prevent cell injury as a consequence of Ca^{2+} overload, through spontaneous Ca^{2+} release or enzyme activation. The steep Ca^{2+} gradient between the cell and its bathing

solution is also essential to allow rapid Ca^{2+} entry during excitation. To maintain homeostasis Ca^{2+} entering the cell during the action potential must be extruded to ensure that no changes in Ca^{2+} content occur. The effect of modulators of CICR upon this equilibrium will be discussed in this thesis.

1.12 Spontaneous SR Ca^{2+} Release

Spontaneous oscillatory release of Ca^{2+} from the SR, not elicited by an action potential, is frequently observed under conditions of intracellular Ca^{2+} overload. Cyclic contractions were observed in skinned cardiac myocytes upon exposure to elevated $[Ca^{2+}]_o$ [127], and were attributed to spontaneous release of SR Ca^{2+}. Scattered light intensity fluctuations (SLIF) from intact multicellular preparations were also attributed to spontaneous Ca^{2+} release from the SR [217, 223, 351], and were increased by elevation of $[Ca^{2+}]_o$ or application of cardiac glycosides. Direct evidence that these mechanical fluctuations or oscillations were attributable to fluctuations in $[Ca^{2+}]_i$ has come from studies using Ca^{2+} indicators [116, 289, 399]. These phenomena are frequently attributable to conditions of high $[Ca^{2+}]_o$, low $[Na^+]_o$, high stimulation frequency, long depolarisations, Na^+ pump inhibition, or damage to the sarcolemma, affecting its ionic permeability, all of which are likely to increase $[Ca^{2+}]_i$ and probably lead to increased Ca^{2+} loading of the SR [42, 116, 265, 289]. Spontaneous oscillations are abolished by agents that interfere with SR Ca^{2+} loading or release, e.g. caffeine or ryanodine [116, 219, 380], and have thus been attributed to Ca^{2+} release from the SR.

In intact cells, spontaneous contractions are usually seen as waves of contraction that propagate along the cell, at a velocity of typically 100 $\mu m.s^{-1}$ at 22 °C [358]. Cheng *et al* [86] frequently observed an initiating "*macrospark*", believed to result from the summation of a number of closely spaced individual Ca^{2+} sparks, at the site of initiation of a spontaneous Ca^{2+} wave. The velocity, amplitude and width of Ca^{2+} waves appears to be fairly constant, and when two waves collide they extinguish themselves rather than summating, suggesting that there is a refractory zone behind the Ca^{2+} wave [190]. The frequency and propagation velocity is $[Ca^{2+}]_i$-dependent [218]. With increasing $[Ca^{2+}]_o$ there is an increase in the frequency of spontaneous Ca^{2+} release without an increase in the SR Ca^{2+} content [105]. This is thought to be because spontaneous Ca^{2+} release occurs when a critical threshold Ca^{2+} content is reached.

Two models have been proposed to explain the occurrence of these waves of Ca^{2+} and contraction. The first suggests that, at a point where the SR becomes Ca^{2+} overloaded, reaching its threshold Ca^{2+} content, Ca^{2+} may be dumped into the myoplasm, by RyR opening, and by the process of CICR this Ca^{2+} may initiate Ca^{2+} release from adjacent SR sites [17, 350]. This model is opposed by observation that CICR fails to propagate under normal conditions [286]. However, as discussed earlier, as the Ca^{2+} load of the SR increases, CICR among RyRs becomes progressively more dominant and less dependent on Ca^{2+} entering the cell through Ca^{2+} channels [343]. Locally activated contractions were also able to propagate under conditions of increased Ca^{2+} load due to Na^+ pump inhibition by ouabain [263]. The alternative hypothesis is that Ca^{2+} released spontaneously from a region of Ca^{2+} overloaded SR may be taken up by adjacent

SR, thereby allowing those regions to achieve the same critical level for Ca^{2+} release. This is refuted by the recent observation that thapsigargin, an inhibitor of SR Ca^{2+} uptake, does not inhibit the propagation of Ca^{2+} waves [246]. Instead thapsigargin actually increases the rate of Ca^{2+} wave propagation suggesting that Ca^{2+} uptake from the approaching wave front is not required for, and may actually inhibit, wave propagation.

1.12.1 Functional Implications of Spontaneous SR Ca^{2+} Release

Spontaneous Ca^{2+} release has a number of potentially damaging consequences. The occurrence of spontaneous oscillation before an electrically stimulated contraction results in a smaller than normal triggered contraction [361]. Failure of myofilaments to relax completely before the arrival of an action potential will compromise the heart's ability to pump blood [72, 361]. Cells that have just experienced spontaneous Ca^{2+} release may have a reduced SR Ca^{2+} content, and if a ventricular action potential arrives immediately after such an event, less Ca^{2+} will be available for release to activate the stimulated twitch. Cells experiencing these problems will be unable to contract to the same extent and will place constraints on the contraction of neighbouring cells because they do not behave independently. As a consequence of this, profound effects are likely to be experienced by the whole tissue, even when relatively few cells are suffering the direct effects of Ca^{2+} overload [219]. In this way, the extent to which twitches can be increased by mechanisms that enhance the intracellular Ca^{2+} load is limited by the occurrence of spontaneous Ca^{2+} release and contraction [71]. The fall in

tension during stimulated contractions observed under conditions of Ca^{2+} overload has also been attributed to the presence of Ca^{2+} oscillations [6].

Perhaps the most devastating consequence of spontaneous SR Ca^{2+} release is its association with the arrhythmogenic transient inward current (I_{ti}) [34, 134, 201, 203]. When Ca^{2+} is released from the SR, it is extruded from the cell largely through the Na^+/Ca^{2+} exchanger, which is electrogenic and depolarises the cell membrane. These oscillatory after-depolarisations, also known as transient depolarisations (TDs), can in turn lead to potentially fatal triggered arrhythmias in the heart when depolarisation is sufficient to reach the threshold for triggering an action potential [68, 137]. Such arrhythmias can be seriously detrimental when they occur at the "wrong" time in the cardiac cycle, resulting in ventricular fibrillation and a dramatic reduction in the heart's ability to pump blood effectively.

Digitalis intoxication and ischaemia represent two clinically relevant examples of pathological Ca^{2+} overload in cardiac tissue, which have potentially fatal consequences. Digitalis intoxication causes inhibition of the Na^+ pump leading to elevation of intracellular sodium activity (aNa_i) favouring Ca^{2+} accumulation of Ca^{2+} within the cell via the Na^+/Ca^{2+} exchanger. Ischaemia results in acidosis, favouring Na^+ entry on the Na^+/H^+ exchanger, while inhibition of the sodium pump, due to depletion of ATP compromises the cell's ability to extrude Na^+. This elevated Na^+ will once again favour accumulation of Ca^{2+} via the activity of Na^+/Ca^{2+} exchange.

1.12.2 Intracellular Sodium Activity (aNa_i) and Spontaneous SR Ca^{2+} Release

aNa_i and Na^+/Ca^{2+} exchange is extremely important in regulating contractile force. The relationship between aNa_i and tension is very steep [113, 170, 391]. It has been reported that aNa_i in rat ventricle is unusually high [324, 390] and unstimulated rat preparations exhibit spontaneous SR Ca^{2+} release when external Ca^{2+} is as low as 0.5 mM. This may be a consequence of reduced extrusion of Ca^{2+} or even Ca^{2+} influx via the Na^+/Ca^{2+} exchanger under the conditions of high aNa_i. Intracellular sodium alters the reversal potential of the Na^+/Ca^{2+} exchanger, such that at the high aNa_i of the rat, the reversal potential is more negative than the resting membrane potential, which means that at rest Ca^{2+} influx on the exchanger is favoured. In contrast, other species including rabbit ventricular myocytes do not show spontaneous release unless Ca_o is elevated to $\geq$ 10 mM. The variability in the frequency of spontaneous waves of Ca^{2+} release between different rat ventricular myocytes has recently been correlated with $[Na^+]_i$ [104]. Rat ventricular myocytes are therefore the tissue of choice for studying the properties of spontaneous Ca^{2+} release and were used throughout the experiments described in this thesis.

1.13 Local Anaesthetics

Local anaesthetics are a group of drugs that reversibly block the propagation of action potentials along nerve axons, by blocking the voltage-gated Na^+ channels in the membranes of these cells. Most of these drugs share similar physical properties and molecular structure, including a hydrophobic or lipophilic moiety imparting lipid solubility, and a hydrophilic moiety imparting water

solubility. Lipid solubility is a major determinant of intrinsic potency among these drugs. For example, comparing the ester-linked anaesthetics tetracaine and procaine, tetracaine is intrinsically more potent than procaine simply because of its enhanced lipid solubility. The main difference between these two drugs is the addition of a butyl group to the lipophilic aniline group of procaine, to produce tetracaine. Protein binding is thought to be the primary determinant of the anaesthetic duration. Tetracaine has a 13-fold higher affinity for proteins and its anaesthetic duration is 3 –7 times longer than that of procaine. The acid dissociation constant (pKa) is thought to determine the speed of action of local anaesthetics. They exist in the charged ionic form and the uncharged base form. The uncharged base is responsible for the onset of action because it is the form most likely to penetrate the membrane to reach its target sites.

The amide-linked anaesthetic lidocaine has been used for many years as a clinical anti-arrhythmogenic agent. Its therapeutic mechanism is likely to be due to its ability to reduce the excitability of the membrane by blocking Na^+ channels. This will also cause a decrease in intracellular [Na^+] [102, 112] and, via the activity of the Na^+/Ca^{2+} exchanger, will cause a reduction of intracellular [Ca^{2+}]. Such effects may contribute to its therapeutic ability and are applicable to the related drugs procaine and tetracaine.

Tetracaine has been shown to inhibit skeletal muscle contractions induced by caffeine or depolarisation of the membrane [12, 248], but does not affect myofilament sensitivity. Its effects are therefore thought to be due to inhibitions of Ca^{2+} release from the SR. Concentrations of tetracaine as low as 10 – 50 μM were found to affect SR Ca^{2+} release in some studies [99, 270], while much higher

concentrations of procaine (around 10 mM) are required to achieve similar effects, reflecting differences in their ability to reach their target site. The direct effect of tetracaine and procaine on the SR Ca^{2+} release channel was studied by Xu *et al* [404]. This study describes the effect of tetracaine on SR Ca^{2+} release in isolated cardiac myocytes, demonstrating its inhibitory effects in this muscle type also, despite the fundamental differences in the mechanism for triggering Ca^{2+} release between these muscle types. The effect of tetracaine on other membrane currents has also been studied. Tetracaine reduces the inwardly rectifying K^+ current at concentration of around 20 μM, while 10 fold higher concentrations suppress the delayed K^+ current in guinea pig ventricular myocytes [75]. In this species, in frog atrial myocytes and in sheep Purkinje fibres tetracaine has also been observed to inhibit the amplitude of the Ca^{2+} current with a K_i in the region of 100 μM [75, 81, 111]. The effects of tetracaine on these currents must obviously be taken into account when studying the effects of this drug on EC-coupling.

1.14 The Interval-Force Relationship

The interval force relationship describes the effect of changes in the rate of stimulation on the degree of contraction achieved by the myocardium. This aspect of EC coupling is important because changes in the rate and rhythm of cardiac contraction represent an important mechanism in the heart for responding to the changing metabolic demands of the body.

In most species increasing the frequency of stimulation results in an increase in the force of contraction (see [52] & [236] for review). This has been attributed to increasing aNa_i, which augments the Ca^{2+} load of the cell through via the

Na^+/Ca^{2+} exchanger [56, 95, 168]. The rat heart is exceptional in that it shows maximum force at low frequencies of stimulation and high frequencies result in decreased contractile force [178]. This is typical of tissues with short action potentials (rat myocytes and atrial cells) and is referred to as a negative force-frequency relationship. There are species differences in the handling of Ca^{2+} and the dependency on the SR as a source of Ca^{2+} during a single beat; for example the rat is more dependent on SR Ca^{2+} than rabbit [38]. Time-dependent repriming of the SR has been proposed to explain the effect of stimulation rate on the inotropic state of rat heart [292].

1.14.1 The Effect of Rest on Cardiac Contractility

The effect of a short period of rest (a period longer than the regular stimulation interval) is an extreme example of the force frequency relationship. During a short period of rest there is some finite leak of Ca^{2+} from the SR lumen into the cytoplasm, down its large concentration gradient. Once in the cytoplasm, this Ca^{2+} can be extruded from the cell (via Na^+/Ca^{2+} exchange or the sarcolemmal Ca^{2+}-ATPase) or resequestered by the SR (via the SR Ca^{2+}-ATPase). If Ca^{2+} is extruded, the SR Ca^{2+} content will decline at rest. This is demonstrated by the decline in the first post-rest contraction of most mammalian ventricular muscle, a phenomenon known as rest-decay. This depression of post rest contraction is paralleled by a reduction in SR Ca^{2+}, assessed by rapid-cooling contractures (RCCs) [39, 41, 44]. Loss of cellular Ca^{2+} has also been estimated from loss of ^{45}Ca during rest [194] or depletion / accumulation of extracellular Ca^{2+} assessed by extracellular microelectrodes on resumption of stimulation /

during a period of rest [250, 324]. It now seems that the gradual depletion of Ca^{2+} from these tissues depends on passive leak of Ca^{2+} from the SR and its extrusion from the cell via Na^+/Ca^{2+} exchange [39, 356].

The rat differs from other species in that there is no loss of Ca^{2+} from the resting myocardium, and post-rest potentiation is observed. To explain this, it is necessary to consider that leak of Ca^{2+} down its concentration gradient from outside the cell is also likely during a period of rest. Its ability to affect the SR Ca^{2+} load will depend on the ability of the SR Ca^{2+}-ATPase to compete with the Ca^{2+} extrusion mechanisms of the sarcolemma. In rat, the relatively high intracellular Na^+ could reduce Ca^{2+} extrusion by, or favour Ca^{2+} influx on, the Na^+/Ca^{2+} exchanger. Whether rest decay or rest potentiation occur is strongly dependent on $[Na^+]_i$ [324] and interventions that alter the electrochemical gradients for Ca^{2+} or Na^+ can lead to the observation of rest decay in the rat and rest potentiation in rabbit [44]. Despite the large amount of information available on this phenomenon, no conclusive evidence exists to explain rest potentiation in the rat. An increased SR Ca^{2+} content as might be expected in a species demonstrating resting influx of Ca^{2+}, could explain potentiation by increasing the gain of the Ca^{2+} release mechanism. Alternatively, increased recovery of channels from inactivation, or restoration of releasable SR Ca^{2+} between beats could contribute to post-rest potentiation. This study describes attempts to elucidate the mechanism of rest potentiation in isolated rat ventricular myocytes, in the hope that this will provide further insight into the regulation of cardiac contractility in the rat (Chapter 6).

1.15 Metabolic Inhibition and Ischaemia

The working heart rapidly consumes the ATP supplied to it by oxidative phosphorylation. When the blood flow to the heart is restricted, a condition referred to as myocardial ischemia, its supply of oxygen is removed and oxidative phosphorylation ceases. This is often the devastating result of coronary artery disease. Anaerobic glycolysis ensues in an attempt to maintain the supply of ATP, but in the absence of blood flow, removal of metabolites is prevented and glycolysis will eventually be prevented, resulting in total metabolic blockade. Under such conditions cardiac contractility is rapidly compromised and potentially fatal arrhythmias frequently commence. The accumulation of lactate (the end product of anaerobic glycolysis), inorganic phosphate (Pi), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and protons (H^+), and depletion of ATP and phosphocreatine (PCr) are the consequences of inhibition of oxidative ATP synthesis. The effects of these ions and metabolites on cardiac contractility has been the subject of intensive study, in the search for an explanation, and potential improvement of the poor prognosis for patients suffering coronary artery disease and cardiac ischaemia. The RyR is known to be affected by changes in $[H^+]$ [204, 310], $[Mg^{2+}]$ [130, 204, 405], [ATP] [258, 311], $[P_i]$ [140, 207], [ADP] [207] known to occur during metabolic inhibition and myocardial ischaemia [405], yet very little is known about their overall effects on the sensitivity of CICR in intact cells. This study includes a preliminary investigation of the effects of metabolic inhibition on CICR (Chapter 7). More detail concerning the effects of metabolic inhibition and myocardial ischaemia is given in Chapter 7.

1.16 SUMMARY OF THE AIMS OF THIS STUDY

It is clear that a number of potential control points exist to regulate systolic Ca^{2+} release and therefore the degree of contraction achieved during EC-coupling. These could involve changes in the amplitude of I_{Ca} and therefore the size of the trigger increase of $[Ca^{2+}]_i$, changes in the properties of the RyR, and its sensitivity to activation by $[Ca^{2+}]_i$, and changes in the Ca^{2+} content of the SR. It has been known for a number of years that the Ca^{2+} release from the SR can be activated by low concentrations of caffeine. Recent studies of the effects of caffeine on electrically stimulated contraction in rat ventricular myocytes have revealed that in the steady state there is no effect on contraction, because transient elevation of the SR Ca^{2+} release induced by the drug caused depletion of the SR Ca^{2+} content [284, 371]. This has lead to the suggestion that potentiation or inhibition of CICR alone will produce ***transient*** effects on systolic Ca^{2+} and contraction, because the effect on CICR will be compensated for by changes in the SR Ca^{2+} content.

The local anaesthetic tetracaine is known to inhibit Ca^{2+} release from the SR [12] and has been shown to reduce the frequency of spontaneous SR Ca^{2+} release [61, 278]. However, this could reflect their ability to reduce intracellular Na^+, by inhibition of sarcolemmal Na^+ channels (as for lidocaine and procaine [102, 112]) or Ca^{2+} channels [75], gradually reducing the Ca^{2+} load of the cell, rather than by direct inhibition of CICR. These two mechanisms predict opposite effects on the SR Ca^{2+} load of the cell and could be distinguished by quantitative assessment of the SR Ca^{2+} content of cells exposed to the drug. This study describes a comprehensive investigation of the effects of tetracaine on spontaneous Ca^{2+}

release, in the form of propagating Ca^{2+} waves and contraction, and SR Ca^{2+} content in rat ventricular mycoytes. Once the predominance of the CICR inhibitory effects of tetracaine was established the effects of tetracaine on stimulated contraction and associated Ca^{2+} fluxes were investigated.

Changes in SR Ca^{2+} content have also been used to explain the phenomenon of rest decay in a number of species [39, 41, 44, 238, 364]. In the rat, rest potentiation predominates, yet there has been no conclusive study of the effect of rest on SR Ca^{2+} content in this species. This study describes an investigation of the effect of rest on Ca^{2+} content. The ability of caffeine and ryanodine to reduce post-rest potentiation has been attributed to their capacity to reduce Ca^{2+} accumulation by the SR. This study investigates the effect of tetracaine on rest potentiation and accumulation of Ca^{2+} by the SR.

Despite the abundance of information concerning the effects of manoeuvres likely to be experienced during ischaemia on the activity of the RyR, there is no clear understanding of the effects of metabolic blockade on CICR in intact myocytes. To resolve this a preliminary investigation of the effect of metabolic inhibition on spontaneous Ca^{2+} release and SR Ca^{2+} content has been made.

Chapter 2

CHAPTER 2

METHODS

2.1 CELL PREPARATION

Experiments were performed on single ventricular myocytes, isolated from rat hearts using a collagenase digestion protocol as previously described [114]. Wistar rats (either sex) weighing approximately 200 g were killed by stunning and cervical dislocation. The thoracic cavity was opened and the heart rapidly excised and washed with a nominally calcium-free HEPES-buffered solution (solution A) to remove residual blood and tissue debris. Next the heart was mounted onto the cannula of a Langendorff apparatus (primed with solution A) and perfused retrogradely through the aorta for 3 to 6 min (at a constant rate of 7 $ml.min^{-1}$, 37°C) with solution A. Solution A contained (in mM): NaCl, 134; Glucose, 11; HEPES, 10; KCl, 4; $MgSO_4$, 1.2; $Na_2H_2PO_4$, 1.2; titrated to pH 7.34 with NaOH and equilibrated with air at room temperature. The absence of calcium in the perfusate disrupts the integrity of cell-cell junctions [37] and reduces contractile activity, thereby preserving energy and reducing the potential for acidification of the tissue as a result of anaerobic respiratory products. Collagenase (Boehringer Mannheim GmbH., *type A*, activity 0.21 $U.mg^{-1}$) and protease (Sigma, *type XIV*, activity 5.1 $U.mg^{-1}$) were added to the perfusing solution to final concentrations of 0.14 $U.ml^{-1}$ and 0.13 $U.ml^{-1}$ respectively and the perfusion continued for a further 6 to 8 minutes. The perfusing solution was then switched to one containing taurine (Solution B) for a further 8 to 10 minutes

to wash away the enzymes. Solution B contained (in mM): NaCl, 115; Taurine, 50; Glucose, 11; HEPES, 10; KCl, 4; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; titrated to pH 7.34 with NaOH and equilibrated with air at room temperature. During periods of Ca^{2+}-free perfusion myocardial intracellular Na^+ increases. As a consequence of this, the reintroduction of Ca^{2+} to the perfusate causes in a massive influx of Ca^{2+}, via the sarcolemmal Na^+/Ca^{2+} exchanger, resulting in Ca^{2+} overload referred to as the "Ca^{2+} Paradox" [313]. Taurine effectively reduces the rise in intracellular Na^+ through the activity of a Na^+/Taurine symport [354]. This reduces the potential for Ca^{2+} overload on return to normal Ca^{2+}-containing Tyrode solution and increases the yield of "Ca^{2+} tolerant cells" [82, 189]. The softened ventricles were then removed and suspended in solution B before mincing and triturating (mechanical agitation) the tissue using a plastic pipette, to release single myocytes into solution. Cells suspended in solution B were then decanted into test tubes, before repeating the suspension and trituration steps a number of times, usually yielding 6 or 7 fractions. The cells were then viewed under a low power light-microscope and the fraction containing the highest proportion of apparently viable, quiescent myocytes was selected for use. Viable cells were assumed to be those that maintained a rod-like shape with clear cross-striations and sharp edges suggesting minimal membrane damage. Cells were maintained in solution B at room temperature until required. Millipore water (18.2 $M\Omega.cm^{-2}$) and analytical grade chemicals (Sigma Chemicals, Poole, UK and BDH, Poole, UK) were used in the preparation of all solutions. The glassware and tubing of the perfusion apparatus were cleaned daily using Millipore water and sterilised weekly by autoclaving.

Cells from the selected fraction were transferred to a tissue bath mounted on the stage of an inverted microscope. Once cells had settled onto the glass cover slip forming the bottom of the chamber, they were continually superfused with Tyrode solution (Composition detailed in Section 2.2) at a rate of 0.66 ± 0.03 $ml.min^{-1}$.

2.1.1 Optimisation of Myocyte Isolation

A number of different perfusion and digestion protocols were used while attempting to optimise the isolation of Ca^{2+} tolerant cardiac myocytes. These included changes in the type and concentration of collagenase used and the total omission of protease from the enzyme cocktail. Ca^{2+} was added to solution A to a final concentration of 100 μM during collagenase perfusion to enhance enzyme activity. 100 μM Ca^{2+} was also included in solution B in the hope that this would reduce the "Ca^{2+} Paradox" when cells were superfused with up to 5 mM Ca^{2+} containing solutions during experiments. BDM (10 mM) was used to reduce contractile activity, and therefore metabolic demand of isolated myocytes. The inclusion of 0.5 $mg.ml^{-1}$ Bovine Serum Albumin (BSA, essentially fatty acid free) in both perfusion solutions reduced over-digestion of cells by providing an alternative substrate for potentially damaging proteolytic enzymes that contaminate the collagenase.

2.2 Experimental Solutions

Control experimental Tyrode solution contained (mM): NaCl, 134; HEPES, 10; KCl, 4; $MgCl_2$, 1.2; Glucose, 11; $CaCl_2$, 1-5; titrated to pH 7.4 with NaOH. For voltage-clamp experiments, 5 mM 4-aminopyridine (4-AP) and 0.1 mM $BaCl_2$ were included, to avoid interference from outward currents. Some experimental solutions included tetracaine (0.1 – 1 mM), caffeine (up to 20 mM) and BDM (20 mM) alone or in combination, which were added to the appropriate control Tyrode solution without osmotic correction. Tetracaine was added from a 100 mM stock solution, prepared in water or Tyrode solution, and stored at room temperature.

For metabolic inhibition studies glucose was omitted from all experimental solutions and 10 mM 2-deoxyglucose (2-DOG) included to inhibit glycolysis. Cyanide was kept as concentrated stock solution (300 mM NaCN). Immediately before use, HEPES (2 M) was added to produce an HEPES-buffered stock solution of 200 mM NaCN. This solution was added to the Tyrode solution to a final concentration of 2 mM. To minimise CN^- loss from solution due to its volatility, vessels were sealed where possible. Glibenclamide (10 μM) was added to reduce K_{ATP} currents, which develop during metabolic blockade.

2.2.1 Carboxyeosin Loading

For metabolic inhibition studies, it was necessary to be confident that the changes observed were not due to inhibition of the energy requiring sarcolemmal Ca^{2+}-ATPase. To achieve this, this Ca^{2+}-ATPase was specifically inhibited under all conditions by pre-loading cells with carboxyeosin, a potent inhibitor of the

sarcolemmal Ca^{2+}-ATPase [146, 147]. We used 5-(and-6)-carboxyeosin diacetate succinimidyl ester (mixed isomers) from Molecular Probes, Eugene, OR USA. A 10 mM stock solution was prepared in dimethyl sulphoxide (DMSO). Cells were carboxyeosin-loaded by superfusion with a control solution containing 20 μM carboxyeosin for 10 minutes at room temperature. Cells were subsequently superfused with a control Tyrode solution for 10 minutes to allow carboxyeosin de-esterification.

2.2.2 Experimental Solution Changes

In the majority of experiments a custom-made 8-line solution changer was used. Miniature solenoid valves (Lee products, Gerrards Cross, Bucks.) controlling each line allowed remote switching between solutions bathing the cells. The fine common tip was placed as close to the objective as possible without interfering with the visual field. This ensured that it was close to any cell in view, allowing faster solution exchange of solutions bathing the experimental subject.

2.3 Intracellular Calcium Measurements

The fluorescent indicators Indo-1 and Fluo-4 were used to monitor changes in intracellular calcium. All these experiments were performed inside a Faraday cage in a darkened room at room temperature (23°C). The intensity of emission is dependent on such factors as illumination intensity, dye concentration in the cell, and effective cell thickness. Indo-1 exhibits a significant shift in the wavelength of emitted light as well as intensity when it binds Ca^{2+} [153]. Taking the ratio of

fluorescence at two different wavelengths effectively eliminates the effects of changes in cell thickness, non-uniform dye loading etc, because both wavelengths will be affected equally. Therefore the ratio can be used as an index of $[Ca^{2+}]_i$ and was the indicator of choice for global measurement of changes in $[Ca^{2+}]_i$. Fluo-4 exhibits a much larger shift in emission intensity when binding Ca^{2+} and is bleached more slowly in comparison with Indo-1. It was therefore the indicator of choice for confocal imaging.

2.3.1 Stimulation Protocol

In the majority of experiments using fluorescence microscopy, electrophysiological measurements were not performed. In this case, cells were stimulated at a frequency of 0.33 or 0.5 Hz, by field stimulation applied through two silver-wire electrodes positioned in the experimental chamber and connected to an isolated stimulator (model DS2, Digitimer Ltd, USA).

2.3.2 Indo-1 Fluorescence Measurements

Cells were loaded with the $[Ca^{2+}]_i$-sensitive fluorescent indicator Indo-1 (Molecular Probes. OR, USA) as the cell permeant acetoxymethyl (AM) ester form (2.5 μM for 5 min). A 1 mM stock-solution of Indo-1 AM was prepared in dimethyl sulphoxide (DMSO) plus 25 % (w/v) of the non-ionic detergent Pluronic F-127 (Molecular Probes. OR, USA). Once in the cytoplasm of the cells the AM-ester is cleaved by non-specific esterases to release the free-acid form of the dye from the parent molecule. The free-acid form remains trapped inside the cell

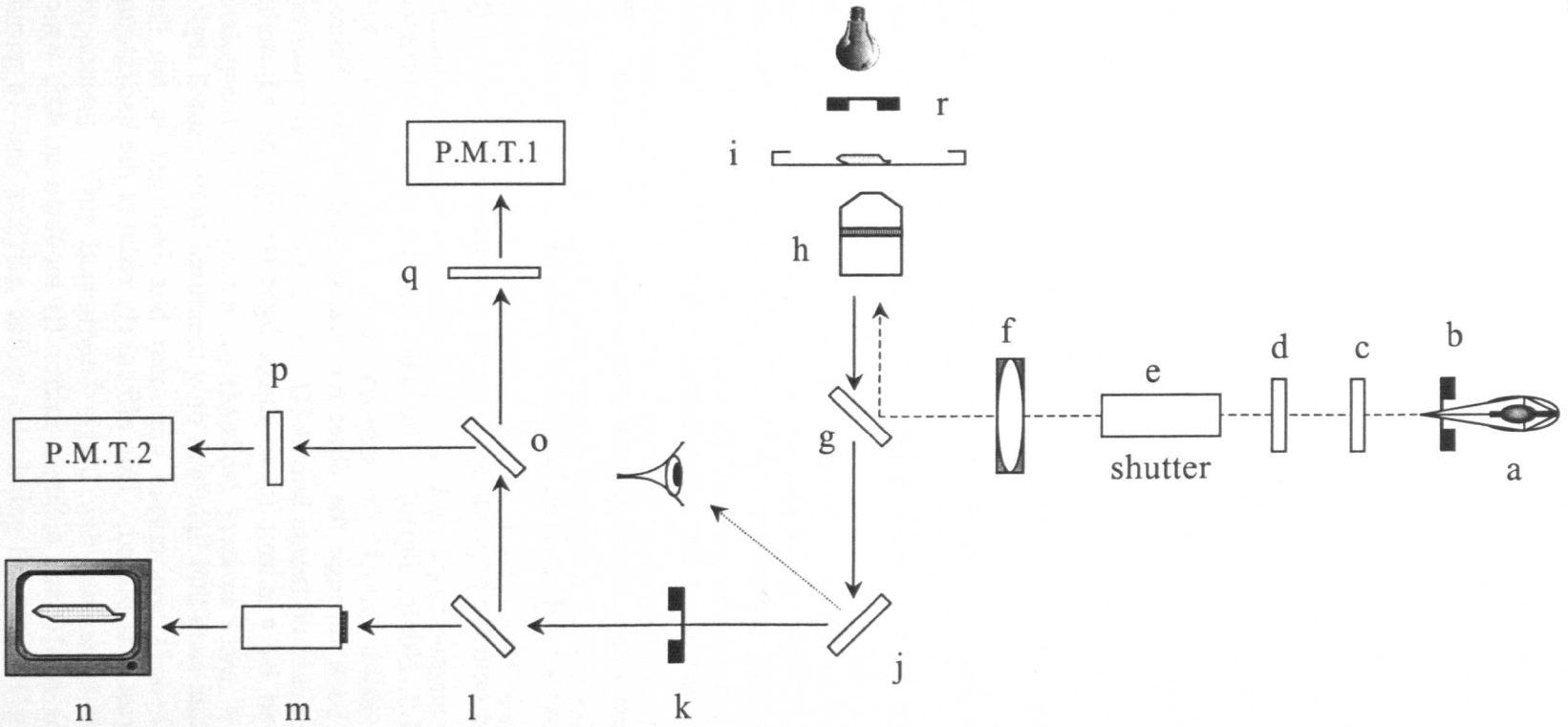

Figure 2.1. Diagram of the optical components of the set-up used for Indo-1 fluorescence measurement.

The lettering in the drawing was placed in the same order as each part is described in the text: a, xenon lamp; b, heat filter; c, neutral density filters; d, interference filter 340 nm; e, shutter; f, convex lens; g, dichroic mirror at 400 nm; h, oil immersion objective; i, preparation; j, sliding mirror; k, diaphragm; l, dichroic mirror 610 nm; m, video camera; n, monitor; o, dichroic mirror 450 nm; p, 400 nm filter; q, 500 nm filter; PMT, photomultiplier tubes 1 and 2 respectively; r, long pass filter 630 nm.

because of the limited permeability of the sarcolemma to charged molecules [378]. Cell loading with the dye was stopped by adding solution B to excess, and the cells were left for at least 30 minutes to allow de-esterification of the indicator. Cells were then allowed to settle in a bath mounted on the stage of an inverted microscope adapted for epifluorescence (Nikon Diaphot-TMD, Nikon, Japan), before superfusion with experimental solution.

A schematic diagram of the optical set-up used for $[Ca^{2+}]_i$ measurements using Indo-1, is illustrated in Fig. 2.1. Ultraviolet (UV) illumination was provided by a 150 W xenon lamp (XPS-100, Nikon, Japan) (a). Heat filter (b) and neutral density filters (c) were placed in the path of illumination to prevent excess heat and to reduce excitation light intensity respectively. The appropriate excitation wavelength was selected by the use of an interference filter (d) centred on 340 nm (10 nm full width at half maximum – FWHM). The illumination time was minimised through the use of an electromechanical shutter (e) in the excitation path, helping to prevent photo bleaching and photo-damage of the cells. The exciting light beam was then reflected and directed upward by a 400 nm dichroic mirror (g) and focussed onto the preparation (i) by an oil-immersion objective (h) (CF-Fluor 40×, N.A. 1.3, Nikon, Japan). The light emitted by the fluorescent indicator was passed back through the objective, transmitted by the dichroic mirror (g) and reflected by a mirror (j) to direct light through an adjustable diaphragm (k), positioned such that light was only measured from a small region where the cell of interest was placed. This effectively reduces the background signal. Light then hit a 610 nm dichroic mirror (l) mounted at 45°, which transmitted the longer wavelength emitted light (greater than 610 nm) to form an

image through a video camera (m) (Panasonic CCTV camera, Panasonic, Japan) and displayed on a monochrome monitor (n). Shorter wavelength light was reflected at (l) and was split by a 450 nm dichroic mirror (o), passing light to either the 400 nm or 500 nm PMTs (Thorn EMI Electron Tubes Ltd., England). In front of each PMT there were further ± 10 nm (FWHM) filters (p and q) centred at the appropriate wavelengths. To avoid interference from the microscope light with the fluorescence measurements, a 630 nm long pass filter (r) (Schott RG 630) was placed in front of the microscope lamp.

2.3.3 Recording the Indo-1 Signal

Fluorescence was excited at 340 nm and the ratio of emissions at 400 nm and 500 nm ($R_{400:500}$) was used as an index of $[Ca^{2+}]_i$ as described previously [283]. Figure 2.2 shows a schematic diagram of the signal recording equipment. The output of each PMT was connected to an amplifier (a) (Gould Instruments Division Inc. OH, USA) and passed through a custom-made circuit divider (b), which allowed for subtraction of the background signal and subsequently calculated the ratio signal ($R_{400:500}$). The analogue outputs from the circuit divider were displayed on a chart-recorder (c) (Gould 2400S ink recorder, Gould Instruments Division Inc. OH, USA) and stored on videotape (e) using a PCM-8 analogue/digital converter (d) (Medical Systems Corp. NY, USA). Taking the cell out of the field and recording background fluorescence completed each experiment. This background signal was subtracted from both wavelengths during off-line analysis, before calculating the ratio. For the purposes of these experiments Indo-1 was not calibrated with respect to $[Ca^{2+}]$ and results are

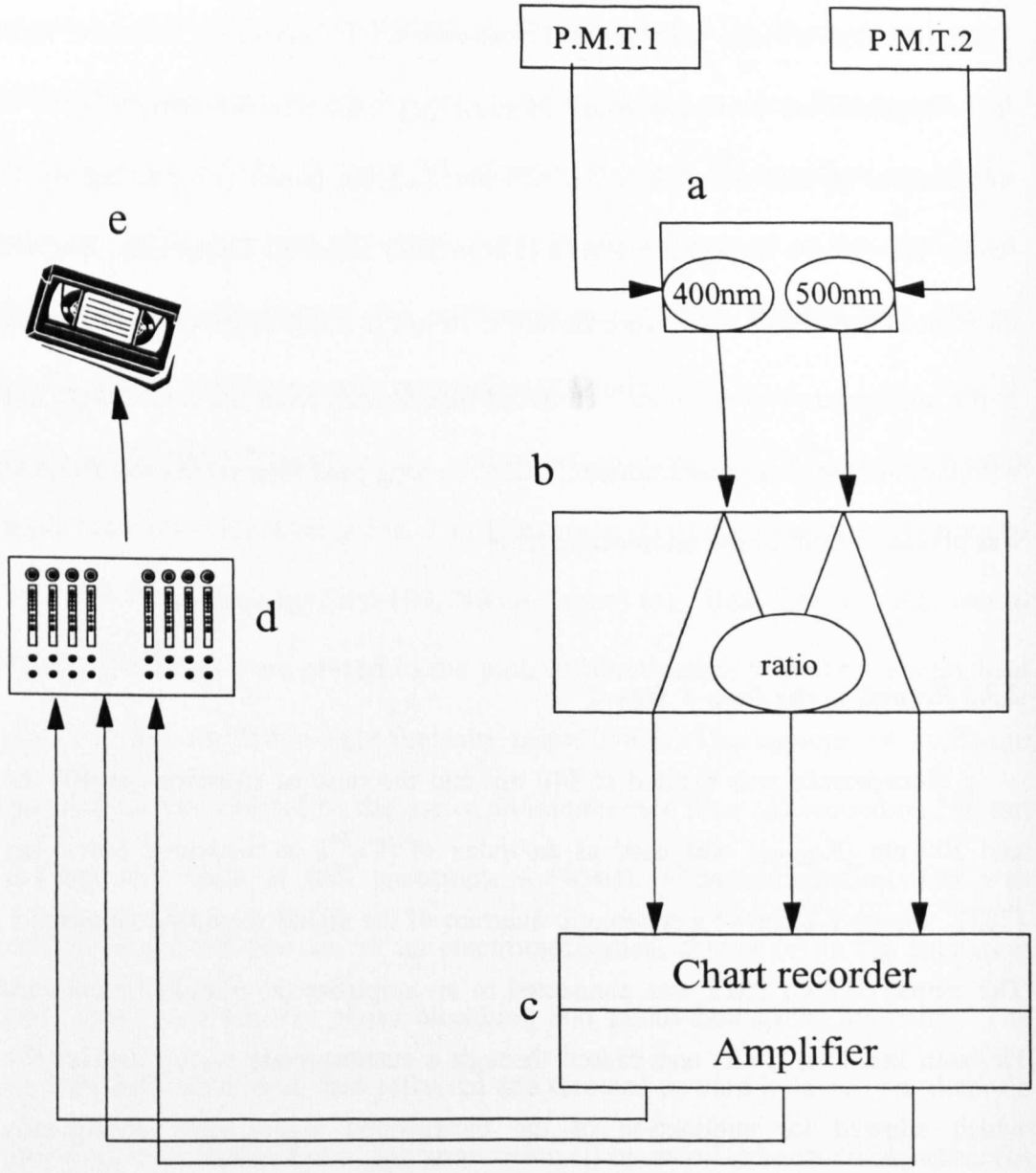

Figure 2.2. Schematic diagram of the equipment used to record the Indo-1 signal.

Each component is identified by letter: P.M.T.1 & 2, photomultiplier tubes 1 and 2 (for 400 and 500 nm signals respectively); **a**, amplifiers for both wavelengths; **b**, divider circuit; **c**, chart recorder; **d**, PCM-8 A/D-D/A converter; **e**, video-recorder.

expressed in terms of the ratio of light emitted at 400nm to that at 500nm. Signals where appropriate were played back from videotape and recorded using custom-designed software (Prof. D. A. Eisner) and analysed using Lotus 123 release 5 (Lotus Development Corp., Cambridge, MA, USA).

2.3.4 Tetracaine and Indo-1 Fluorescence

Application of tetracaine to Indo-1 loaded myocytes resulted in a maintained positive shift in the baseline of the Indo-1 ratio. This has been attributed to intrinsic fluorescence of tetracaine at 400 nm. Tetracaine also absorbs light at the excitation wavelength of 340 nm, and reduces the peak emission of fluorescence at 400 and 500 nm, possibly by reducing excitation intensity. The absorption spectrum of tetracaine was determined for a 200 μM solution of tetracaine in water between 300 and 550 nm. It absorbs strongly at 300 nm. Absorbance then falls rapidly towards zero at approximately 360 nm.

To investigate the effect of tetracaine on the emission spectrum of Indo-1, six separate solutions were prepared containing 1 μM Indo-1 free acid with and without 100 μM Ca^{2+} (replaced by 10 mM EGTA) and with and without 200 μM tetracaine. Using the excitation wavelength of 340 nM, the emission spectra were obtained over the range 350 – 520 nm. The spectra are illustrated in Figure 2.3. Since tetracaine is a base, it is accumulated in the more acidic cytoplasm. This means that it is not adequate to simply subtract the tetracaine fluorescence signal measured in the absence of the cell, to correct for this artefact. Subtracting the immediate increase of fluorescence emission at 400 nm (F_{400}) on application of tetracaine to the cell reduces the Indo-1 ratio to control levels.

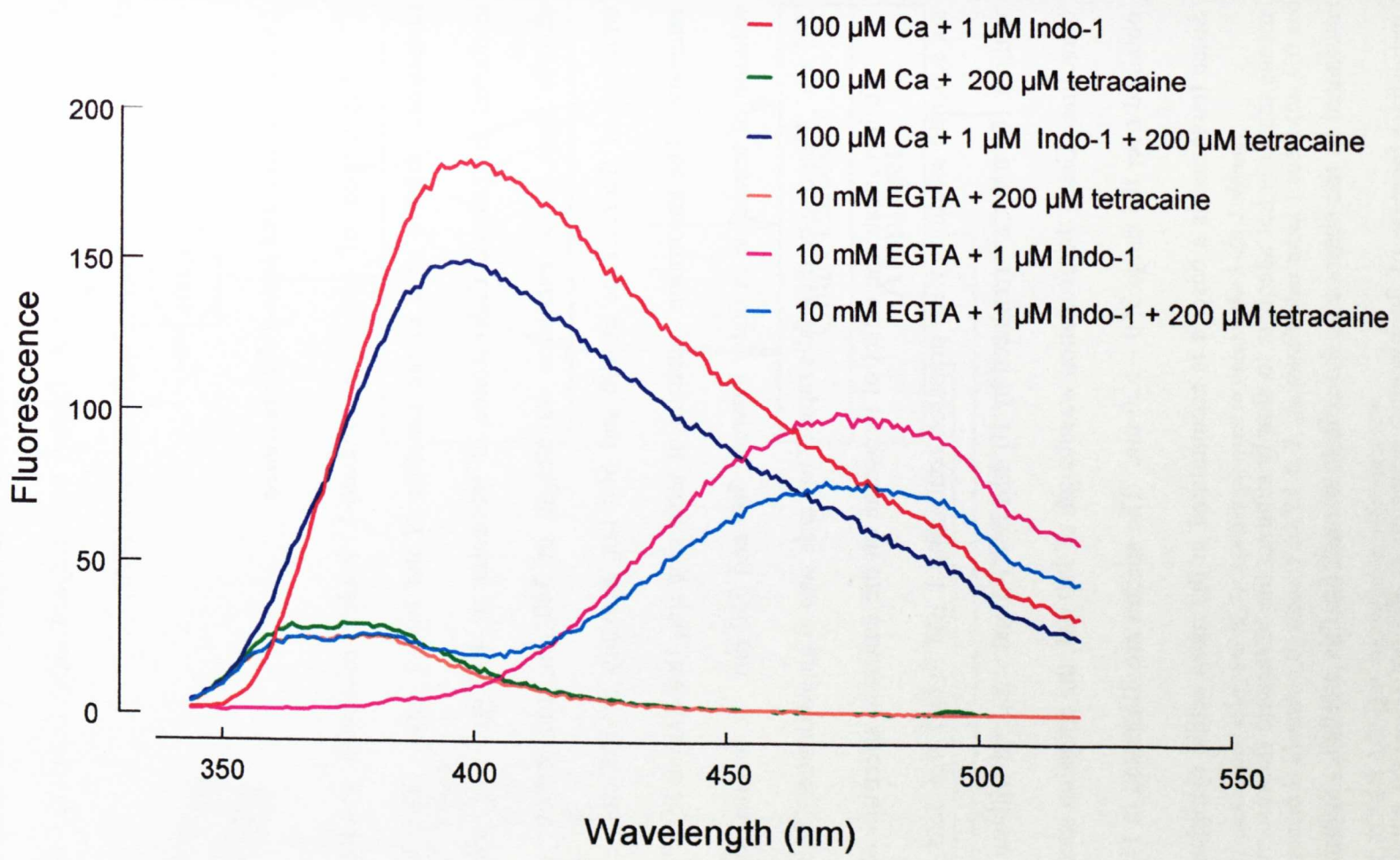

Figure 2.3. Emission spectra.

The emission spectra of solutions prepared as detailed above.

2.3.5 Measurement of Changes in [Caffeine]$_i$

Caffeine quenches the fluorescence of Indo-1 in a $[Ca^{2+}]_i$- and wavelength independent manner. By separating the caffeine- and $[Ca^{2+}]_i$-dependent components of Indo-1 fluorescence changes, it is possible to calculate the time course of changes in intracellular [caffeine] [283]. This was achieved by comparing the effects of stimulation with those of caffeine on individual cells, to determine the portion of signal at either wavelength that is due to changes in $[Ca^{2+}]_i$ alone. Indo-1 could therefore be used to measure $[Ca^{2+}]_i$ and $[caffeine]_i$ simultaneously during caffeine-induced contracture.

2.3.6 Confocal Microscopy

The principle of confocal microscopy is illustrated in Fig. 2.4. Source light passes through an aperture and is reflected by a beam splitter into the rear of the objective lens and is focussed onto the specimen. Light returning from the specimen passes back through the lens and beam splitter and is focussed through a second aperture, which allows only a portion of the light to reach the photomultipier (PMT). Placement of this adjustable aperture (detector aperture) before the PMT allows *out-of-focus* light to be rejected, thus facilitating the *optical sectioning* of the preparation. Events that occur outside the plane of focus will appear smaller in amplitude [298]. Cells were loaded with the cell permeable acetoxymethyl (AM) ester of the $[Ca^{2+}]_i$-sensitive fluorescent indicator Fluo-4 (Molecular Probes. Or, USA) at a concentration of 5 μM for 5 to 10 minutes. Loading was terminated by adding solution B to excess and cells left for at

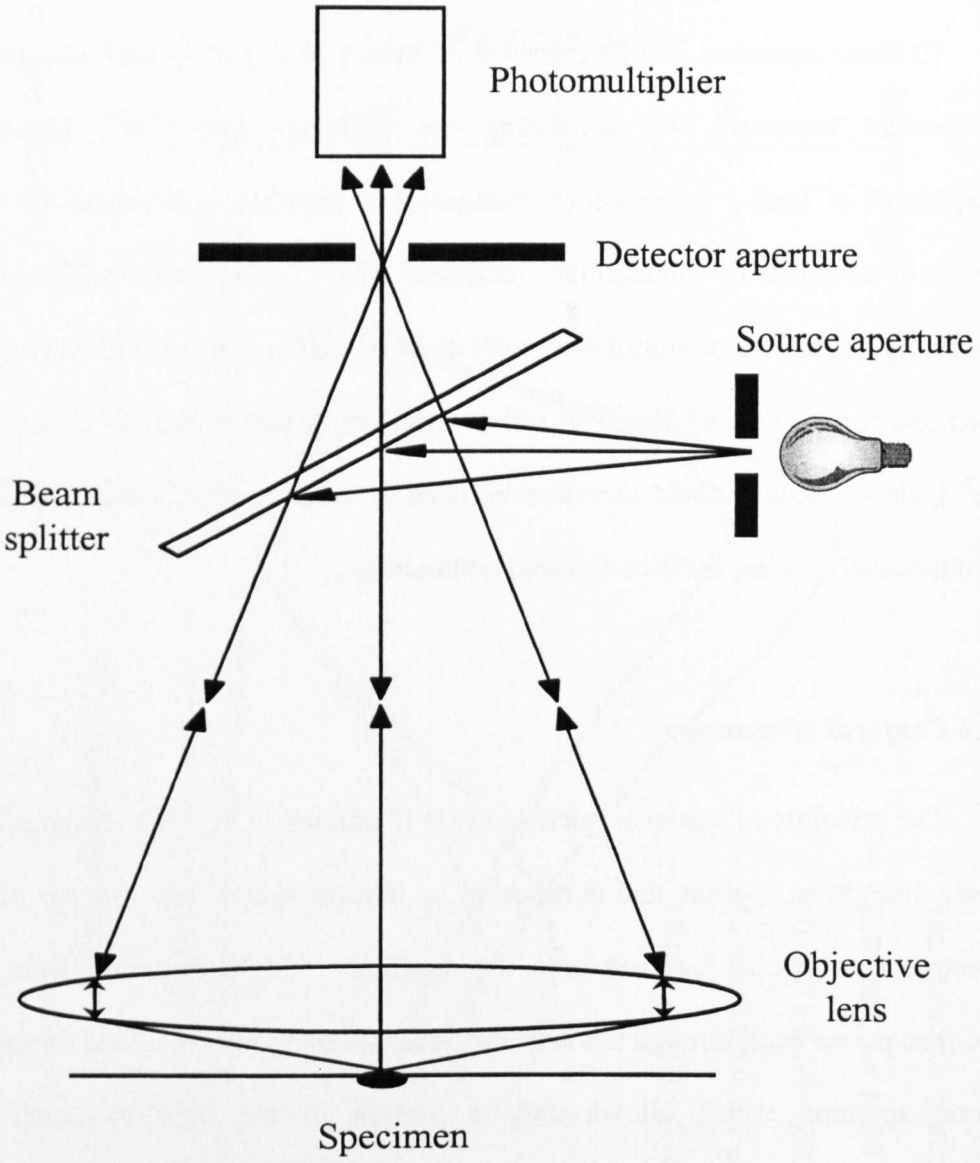

Figure 2.4. The principle of confocal microscopy.

See text for explanation.

least 30 minutes to allow de-esterification of Fluo-4 AM to the free-acid form of the dye. Cells were allowed to settle on the glass cover-slip forming the bottom of a bath mounted on the stage of a Nikon Eclipse TE300 inverted microscope (Nikon Inc. Kingston-upon Thames, UK) to which a Bio-Rad MRC-1024 confocal-imaging system (Bio-Rad, Hemel Hempstead, UK) was attached. Fluo-4 fluorescence was excited with light at 488 nm (100 mW argon-ion laser, attenuated intensity 3-10 %) and measured at wavelengths greater than 514 nm. The objective lens was an oil-immersion lens of ×60 and numerical aperture 1.4. The imaging system was operated in the "line-scan" mode with the confocal iris set to 3.5 mm. This setting is not optimal for optical sectioning of the preparation, but allows more light to reach the PMT, and allows visualisation of more *out-of-focus* Ca^{2+} sparks. In this mode a single line (chosen at random, but trying to avoid nuclei) across the cell is scanned repeatedly (2 ms per line), producing an image which illustrates spatial information horizontally and temporal information top to bottom. Lines were selected, where possible, that ran parallel to the longitudinal axis of the cell. This allowed monitoring of spark frequency over a larger area, and the possibility of calculating the propagation velocity of any spontaneous Ca^{2+} waves that occurred during the course of an experiment. To achieve this, in the majority of experiments a scan rotator (Scientific Design Systems Inc, UK) was used. Unfortunately rotation distorted the image such that cell appeared longer in the horizontal plane and narrower in the vertical plane of x-y images. It should be emphasised that the horizontal scale (given in microns in the figures and referred to in text to point out specific events) is not an accurate measure of distance along the cell, because of the distortion caused by the scan

rotator, and only relative values can be compared between cells. Off-line image processing and analysis was performed using Lasersharp software (Bio-Rad, Hemel Hempstead, UK).

2.4 ELECTROPHYSIOLOGY

Most of the experiments described in this work were performed under voltage-clamp on a set-up **not** adapted for epifluorescence, with the exception of the intracellular calcium measurements described above.

The perforated patch technique [179] was used in most experiments. A stock solution (60 $mg.ml^{-1}$ in DMSO) of amphotericin-B (Sigma Chemicals, UK) was prepared just before use and added to a K^+-free perforated patch pipette filling solution to a final concentration of 240 $\mu g.ml^{-1}$ [300]. The K^+-free perforated patch solution contained (mM); $CsCH_3O_3S$, 125; CsCl, 12; NaCl, 20; HEPES, 10; $MgCl_2$, 5; Cs_2EGTA, 0.1; titrated to pH 7.2 (room temperature) using CsOH. In some experiments, pipette Na^+ was reduced to 12 mM by equimolar substitution of NaCl with CsCl. The final solution was drawn into a 1 ml plastic syringe, modified for micro-electrode pipette filling, protected from light and stored on ice for up to three hours, after which time it was discarded.

For each experiment, after selecting a cell, a pipette was filled with the amphotericin-pipette solution, mounted on the headstage and gently lowered into the solution bath. A seal was achieved by touching the pipette onto the cell surface and applying gentle suction to increase seal resistance, until a giga-ohm seal was formed. The membrane potential was then set to –80 mV and cell access was assessed by following changes in the size and decreases in the time-course of

an inward current in response to a depolarising step. Due to the relatively high access resistance of the perforated-patch (about 20 MΩ), the switch-clamp facility of the Axoclamp-2B voltage clamp amplifier was used.

2.4.1 Micro-electrode Fabrication

Microelectrodes were fabricated from filamented-borosilicate glass (Clarke Electromedical Instruments. Pangbourne, UK) using a DMZ-Universal puller (Zeitz-Instrumente Vertriebs GmbH. Augsburg, Germany) and had final resistances of between 1.5 and 3 MΩ when filled with the perforated patch solution.

2.4.2 Recording electrophysiological signals

A stimulation frequency of 0.5 Hz was set. A Digitimer D4030 Event programmer (Digitimer Ltd., USA) controlled cycle length and command-step duration. An Axoclamp-2B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA) was used to select a command potential, controlling the amplitude of the voltage pulse, and for current recordings. Membrane voltage and current were monitored on a Tektonix 2211 storage oscilloscope (Tektronix Holland N.V., HEERENVEEN, The Netherlands) and displayed on computer. Raw and amplified current and voltage were digitised at 2 kHz using a DigiData 1200 Series Interface (Axon Instruments, Foster City, USA) and stored on computer using Axoscope software (version 1.1 or 7; Axon Instruments). A

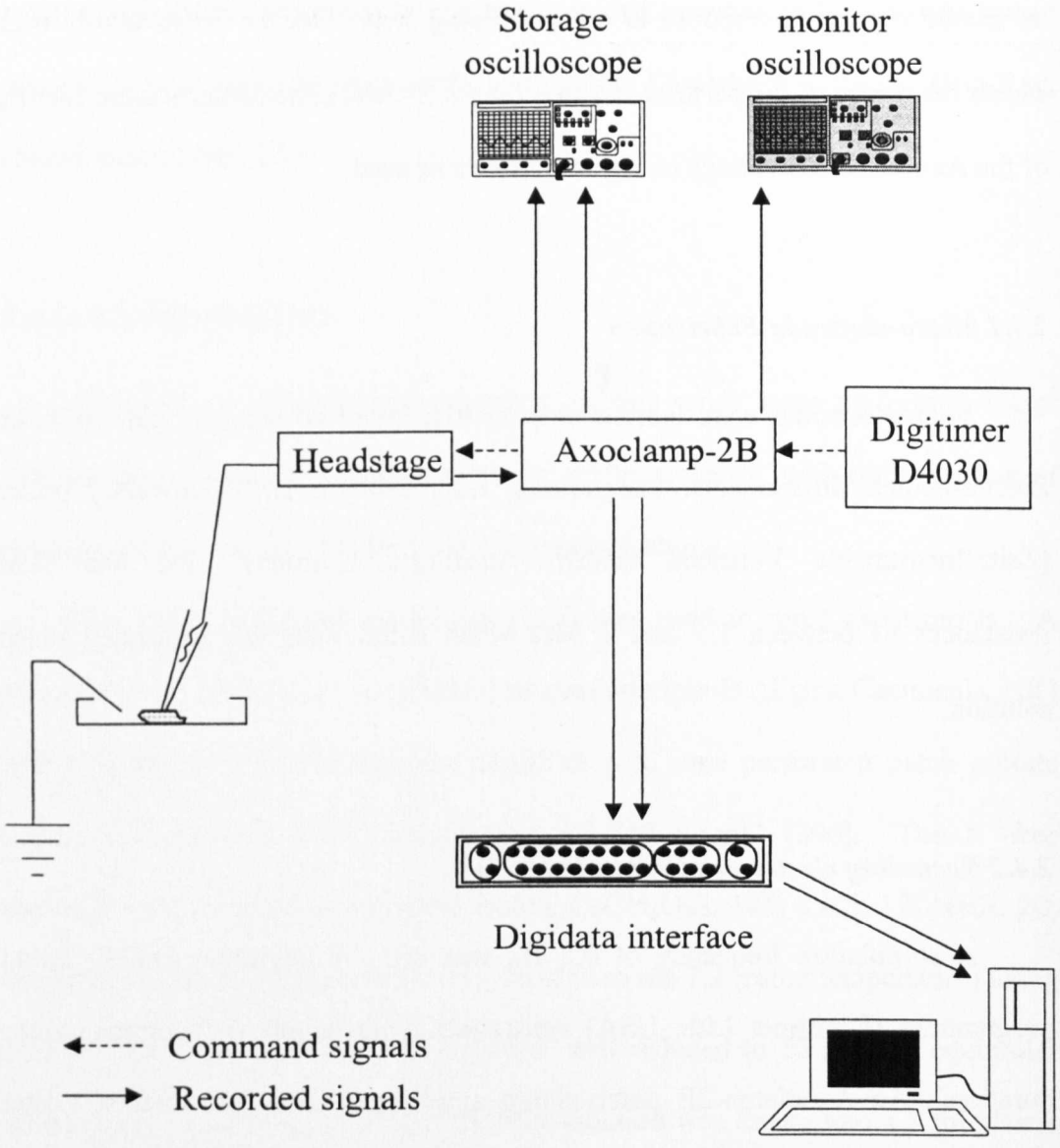

Figure 2.5. Schematic representation of the equipment used for recording electrophysiological events.

See text for full explanation.

schematic diagram of the set-up for measuring and recording electrophysiological signal is illustrated in Fig. 2.5.

2.5 Measurement of Cell Length

Cell length was measured using a custom-made video-based motion detector system with slight modifications from that published by Steadman *et al* [346]. Measurements were made either during experiments (on-line) or off-line from images stored on videotape. Figure 2.6 illustrates a schematic diagram of the set-up for monitoring and recording changes in cell length. A video-image of the myocyte was produced using a video camera (d) installed into the set-up, and passed through a video recorder (e) and the edge detector equipment (f) before being displayed on a monochrome monitor (g). The image was scanned with a series of parallel horizontal lines from top to bottom by the edge detection equipment. The voltage along each line varies in response to changes in light intensity, therefore detecting differences in light intensity between the cell and the surrounding field. The edge-detection system uses these voltage changes to electronically identify the end of the edges of the cell or specific sites of contrasting light intensity on the cell surface. The horizontal position of the detected edge is updated every 40 ms (dependent on the sweep rate of the camera).

The image was oriented by rotating the video camera such that the longitudinal axis of the cell was aligned parallel with the horizontal scan lines. The length and position of the edge detector on the monitor were adjusted to ensure that the movements of the cell occurred within the window boundaries. A

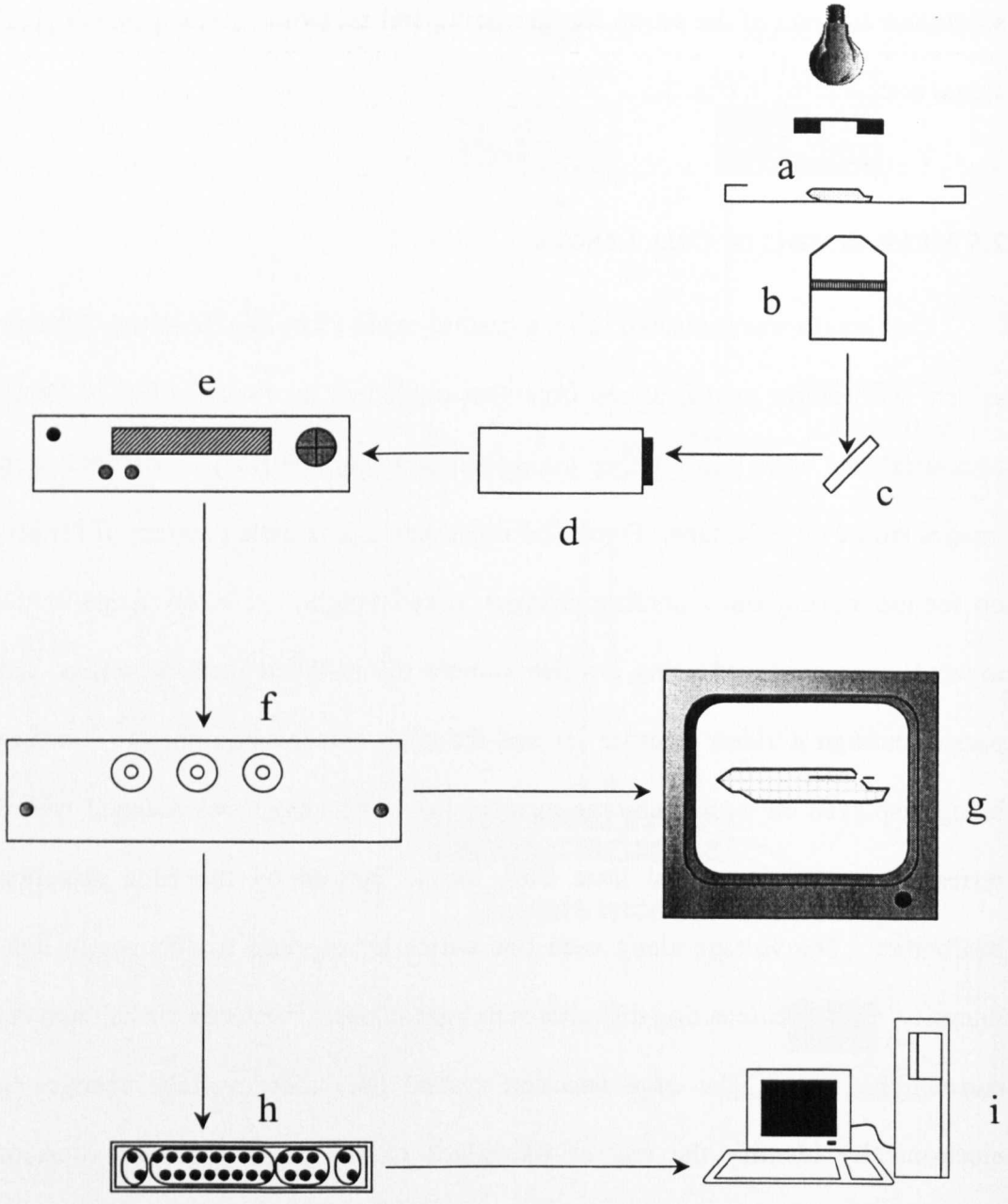

Figure 2.6. Schematic diagram of the cell length recording system.

Each component is identified by letter: **a**, preparation; **b**, ×40 objective; **c**, beam splitter; **d**, video camera; **e**, video recorder; **f**, video motion system; **g**, monitor; **h**, digidata interface; **i**, *Pentium* computer.

tracking dot followed the horizontal movements of a cell edge within the window, allowing continual assessment of the tracking quality. The threshold and edge transmission was adjusted manually to track the cell movements as closely as possible and to achieve a good signal. The motion signal was digitised (DigiData 1200 Series Interface) (h) and simultaneously displayed and stored on computer (i). The edge with most visual contrast was chosen for tracking.

Cell length measurement was not always successful. In some cases the cell rotated during the course of an experiment, and with patched cells it was not always possible to correct such movements. On other occasions, selecting an appropriate threshold was hindered because of the shape of the cell or poorly defined edges. This method often suffered from artefactual baseline changes.

2.6 Data Analysis and Statistics

Analysis of current and contraction records was performed using a custom-designed multi-channel analysis program (NEWABF), written by Dr. Andrew Trafford (Department of Veterinary Preclinical Sciences, University of Liverpool, UK), in combination with Lotus 123 release 5 (Lotus Development Corp., Cambridge, MA, USA).

Data is presented as mean ± S.E.M. of n experiments or samples. Statistical significance was tested using Student's t-tests where appropriate. Data was considered to be statistically significant when $P < 0.05$.

2.6.1 Calculation of Cell Volume

The total capacity of cells was estimated from the integral of the current surge elicited by applying a 10 mV hyperpolarising pulse current, using the relationship:

$$\mathbf{Q = C.V}$$

Therefore; $$\mathbf{C = Q/V}$$

Where **Q** represents the charge being transferred (measured from the integral in Coulombs); **C** represents the capacitance (measured in Farads) and; **V** represents voltage (in Volts). Cell surface area was calculated assuming a specific membrane capacitance of 1 $\mu F.cm^{-2}$. Total cell volume was calculated assuming a capacitance to volume ratio of 6.76 $pF.pl^{-1}$ [316]. This makes it possible to express values of SR Ca^{2+} content and other Ca^{2+} fluxes as μmoles per litre of total cell volume.

2.6.2 Calculation of SR Ca^{2+} Content and Sarcolemmal Ca^{2+} Fluxes

The inward Na^+/Ca^{2+} exchange currents produced by the application of 10 mM or 20 mM caffeine to voltage-clamped myocytes (holding potential –40 or –80 mV) were integrated and converted to total calcium fluxes as described previously [63, 383]. The influx of a single positive charge, carried by the entry of 3 Na^+ ions in exchange for each Ca^{2+} ion extruded means that entry of one positive charge corresponds to the extrusion of a single Ca^{2+} ion. Integrating the inward current provides a direct measure of the amount of charge entering the cell and therefore the amount of Ca^{2+} extruded on the Na^+/Ca^{2+} exchanger. Corrections were made for the fact that, during a caffeine response, some of the

calcium is removed from the cell by mechanisms other than Na^+/Ca^{2+} exchange, which do not generate a current. It has previously been shown that 68 % (2/3) of Ca^{2+} efflux is generated by Na^+/Ca^{2+} exchange [274, 383]. Therefore, to compensate for non-Na^+/Ca^{2+} exchange mediated Ca^{2+} efflux current integrals were multiplied by a factor of 1.5. This correction factor was used to convert Na^+/Ca^{2+} exchange fluxes associated with caffeine responses, spontaneous oscillations and repolarisation of a stimulatory pulse (tail current) into total cell Ca^{2+} efflux. The integrals of calcium currents, used to estimate Ca^{2+} influx, were divided by a factor of two to compensate for the fact that each calcium ion carries two positive charges into the cell. Charge (Q, measured from the integral in Coulombs) was converted to total Ca^{2+} fluxes using the Faraday constant 96500 $C.mol^{-1}$.

Chapter 3

CHAPTER 3

THE EFFECT OF TETRACAINE ON SPONTANEOUS RELEASE OF Ca^{2+} FROM THE SR

Under conditions of calcium overload, cardiac muscle exhibits spontaneous contractile activity resulting from the spontaneous release of Ca^{2+} from the sarcoplasmic reticulum (SR). This is thought to arise as a consequence of the stochastic opening of the SR Ca^{2+} release channels which, under non-overloaded conditions produce discrete localised increases of $[Ca^{2+}]_i$, referred to as Ca^{2+} sparks [86, 242], which are unable to initiate further SR Ca^{2+} release. Under Ca^{2+} overloaded conditions it appears that these Ca^{2+} sparks can initiate waves of elevated $[Ca^{2+}]_i$, which propagate along the length of the cell [84], presumably by the same CICR mechanism as is involved in normal EC-coupling. Increased SR luminal Ca^{2+} may shift the activation of the channel to a much lower cytosolic $[Ca^{2+}]_i$ [337], enhancing the ability of cytoplasmic Ca^{2+} to trigger further Ca^{2+} release. Furthermore, under conditions of Ca^{2+}-overload the increased SR Ca^{2+} content increases the amount of Ca^{2+} released for a given RyR opening [368], thereby increasing the gain of the Ca^{2+} release process. The local elevations of $[Ca^{2+}]_i$ activate sarcomere shortening and contractile waves also propagate along the cell, and are often referred to as mechanical oscillations. This spontaneous release is particularly prominent in rat myocytes, where it is frequently seen in cells under normal resting conditions [70, 217, 219, 222, 351]. This is thought to be a consequence of the relatively high $[Na^+]_i$ in rat myocytes [82, 324], which

will favour resting influx of Ca^{2+}, or reduce efflux, via the Na^+/Ca^{2+} exchanger, and their great dependence on the SR for normal contraction [87], in comparison to other species. The high cell-to-cell variability in the frequency of spontaneous Ca^{2+} waves has recently been attributed to variations in $[Na^+]_i$ [104].

This Ca^{2+} release activates Ca^{2+} extrusion from the cell, approximately two thirds via the electrogenic Na^+/Ca^{2+} exchanger, during each propagating wave [383]. This constitutes approximately one fifth of the Ca^{2+} content of the SR [105], and the resulting transient inward current (I_{ti}) can initiate potentially fatal arrhythmogenic action potentials [137, 229]. This spontaneous Ca^{2+} release activity therefore limits the usefulness of inotropic agents that elevate $[Ca^{2+}]_i$, which will favour Ca^{2+} overload of the SR.

Although these waves of SR Ca^{2+} release are largely of patho-physiological relevance in the heart, in other systems they are a normal aspect of cell function. Thus in a variety of secretory cells Ca^{2+} waves produced by Ca^{2+} release from the endoplasmic reticulum (ER) are the normal mechanism for coupling stimulus to response [200]. It is therefore important to understand the factors that influence the initiation and propagation of such Ca^{2+} signals.

3.1 Aims of This Chapter

Despite the volume of work in this area, there is little information about what determines the properties of spontaneous SR (or ER) Ca^{2+} release. For example, how do changes in the properties of SR Ca^{2+} release or re-uptake affect the magnitude, duration and frequency of the Ca^{2+} waves? In recent work it has been shown that changing the Ca^{2+} entry into the cell affects the frequency of

spontaneous Ca^{2+} waves but has no effect on the properties of the individual waves [105]. In this work the effects of tetracaine on spontaneous waves of Ca^{2+} release were investigated. Tetracaine is a local anaesthetic that has been shown to decrease the open probability of the SR Ca^{2+} release channel [404], an action that accounts for its inhibition of Ca^{2+} release from the SR [387] and of contraction in amphibian [12] and mammalian [100] skeletal muscle.

The results show that tetracaine decreases the frequency of spontaneous SR Ca^{2+} release. As a result of this there is an increase of SR Ca^{2+} content, which increases the magnitude of the Ca^{2+} waves thus demonstrating that, unlike many other manoeuvres [105], factors affecting the opening of the SR release channel have profound effects on the properties of individual spontaneous Ca^{2+} releases. Most striking is the demonstration that inhibiting Ca^{2+} release *increases* the magnitude of spontaneous Ca^{2+} release events.

3.2 Tetracaine Reduces the Frequency of Spontaneous SR Ca^{2+} Release

Figure 3.1 illustrates measurements of cell length made during an experiment to investigate the effects of various concentrations of tetracaine on contractions due to spontaneous SR Ca^{2+} release in a myocyte isolated from rat ventricle. Spontaneous Ca^{2+} releases are characterised by rapid changes in cell length followed using a video-edge detection system (Section 2.5). The application of tetracaine initially abolished this spontaneous Ca^{2+} release activity, before spontaneous contractions resumed at a frequency lower than control. Both the duration of the quiescent period and the degree of the steady-state decrease in

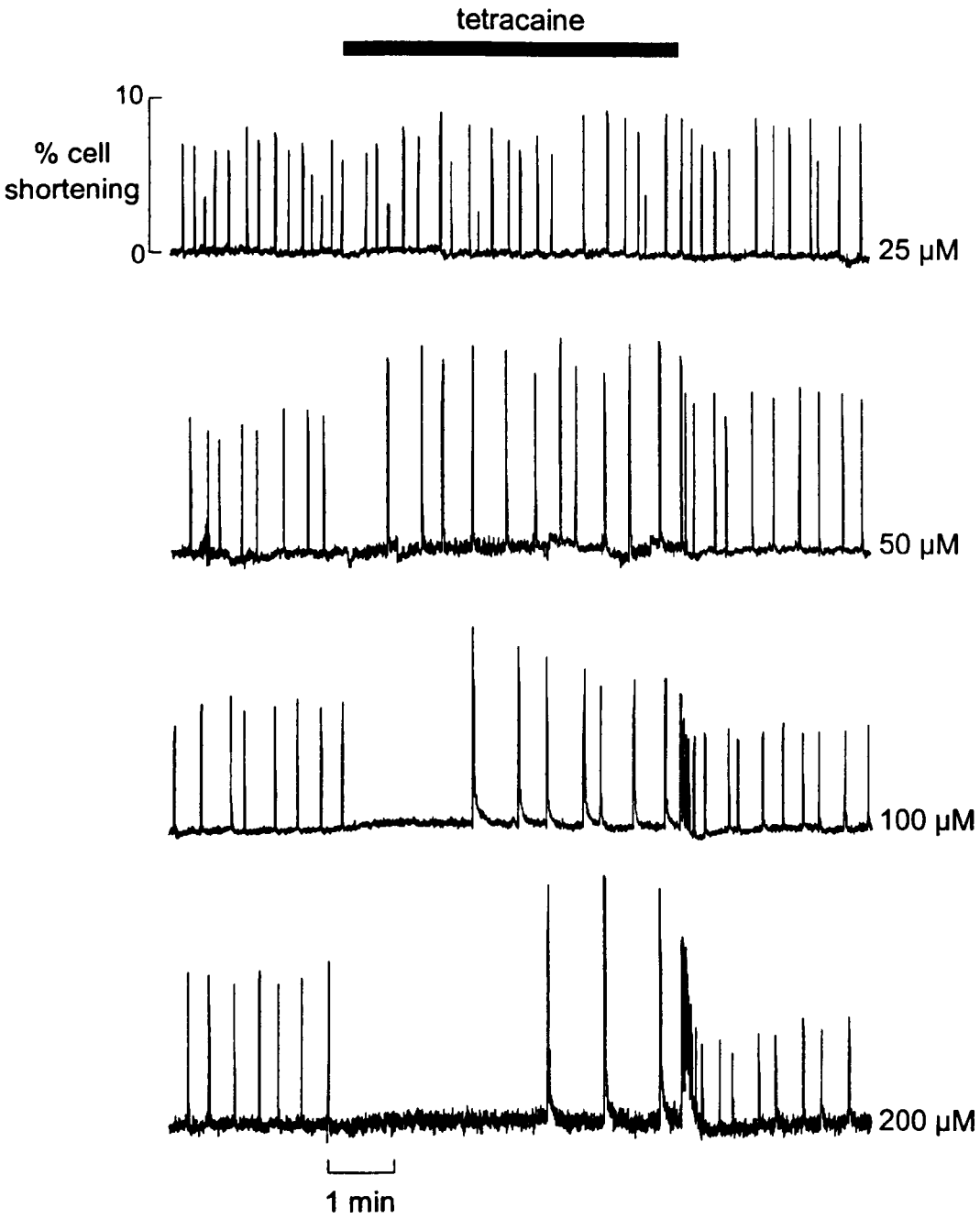

Figure 3.1. The effects of various concentrations of tetracaine on spontaneous SR Ca^{2+} release.

All traces illustrate cell shortening. Tetracaine was applied for the period illustrated by the bar, at concentrations of (from top to bottom): 25, 50, 100 and 200 µM.

frequency, increase with increasing tetracaine concentration. The dose-dependence of these effects is summarised by the mean data illustrated in Fig. 3.2. Another novel observation is that removal of tetracaine from the superfusing solution is associated with recovery of spontaneous release at a transiently elevated frequency (*"burst"*) in comparison to that observed in control. Spontaneous contraction frequency then falls towards the control level over the next 30 s to 1 min. The magnitude of this burst is also greater following exposure to higher tetracaine concentrations.

Previous work has shown that the frequency of spontaneous SR Ca^{2+} release is very variable between different cells [69, 104]. Fig. 3.3 shows that the effects of tetracaine depend on the initial rate of spontaneous Ca^{2+} release. Thus at a given concentration of tetracaine, the greater the interval between spontaneous contractions in the control condition, both the longer the initial quiescent period (lower panel) in tetracaine and the lower the steady-state frequency of spontaneous Ca^{2+} contractions (upper panel) in tetracaine. Identity is shown as a dashed line in each panel of Fig. 3.3, i.e. if tetracaine had no effect on the interval between spontaneous releases or produced no quiescent period, all the data points would lie along these dashed lines.

3.3 Tetracaine Increases the Amplitude of Spontaneous Ca^{2+} Release

The results described above show that, after an initial delay, in the presence of tetracaine, spontaneous Ca^{2+} release resumes at a lower frequency than in the control. The next series of experiments was designed to examine whether the properties of spontaneous Ca^{2+} release are also affected by tetracaine.

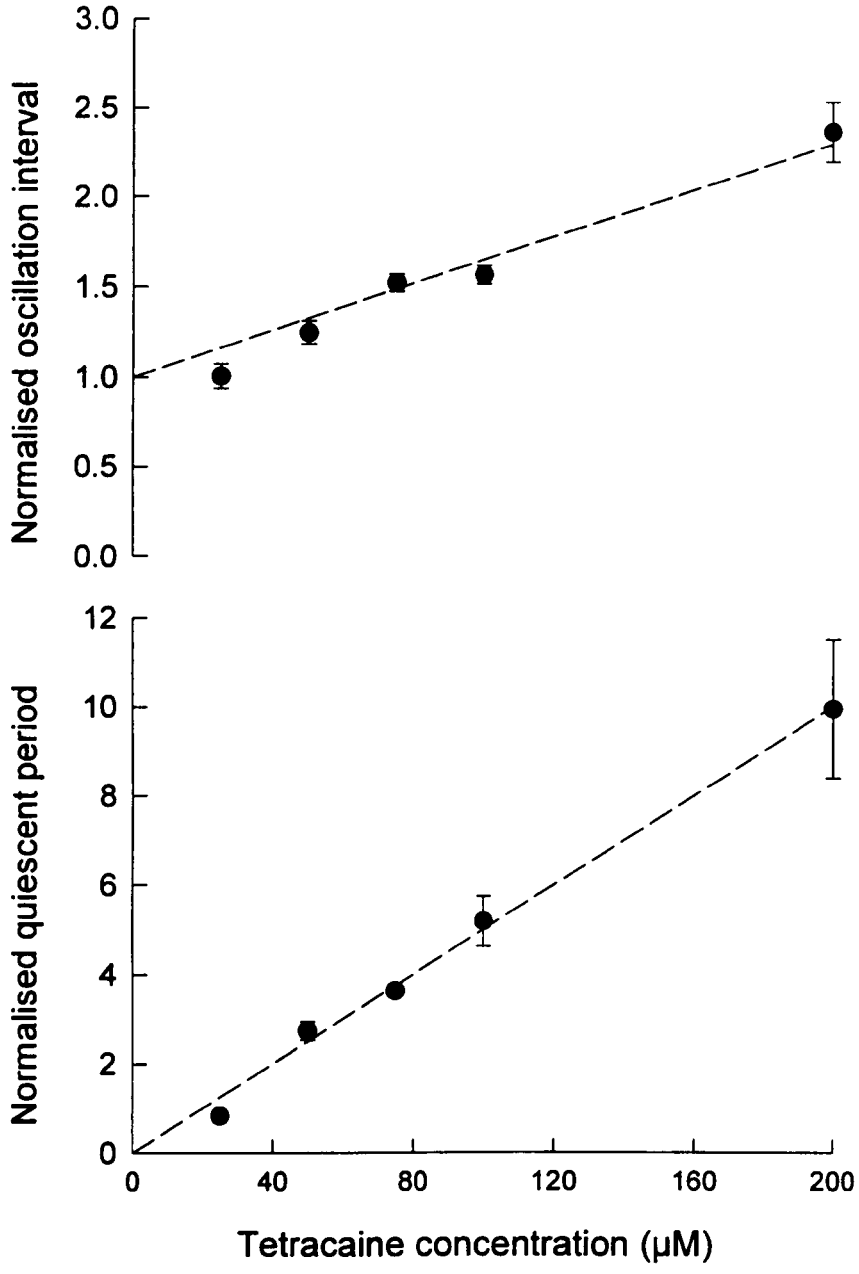

Figure 3.2. Concentration-dependence of the effects of tetracaine.

The abscissa shows the concentration of tetracaine. The ordinate shows: **top**, the interval between spontaneous releases in the presence of tetracaine and **bottom**, the duration of the quiescent period after adding tetracaine. The duration of the quiescent period and the oscillation interval were normalised by dividing by the control interval of spontaneous Ca^{2+} release before adding tetracaine. This had a mean value of 22 s. The quiescent period was calculated by subtracting the average control interval from the total period lacking spontaneous Ca^{2+} release. The lines are linear regressions that have been forced to go through values of 1.0 for the oscillation interval and 0 for the quiescent period in the absence of tetracaine. The symbols show mean ± S.E.M. of data from three cells. Error bars are not shown where they would be smaller than the symbols.

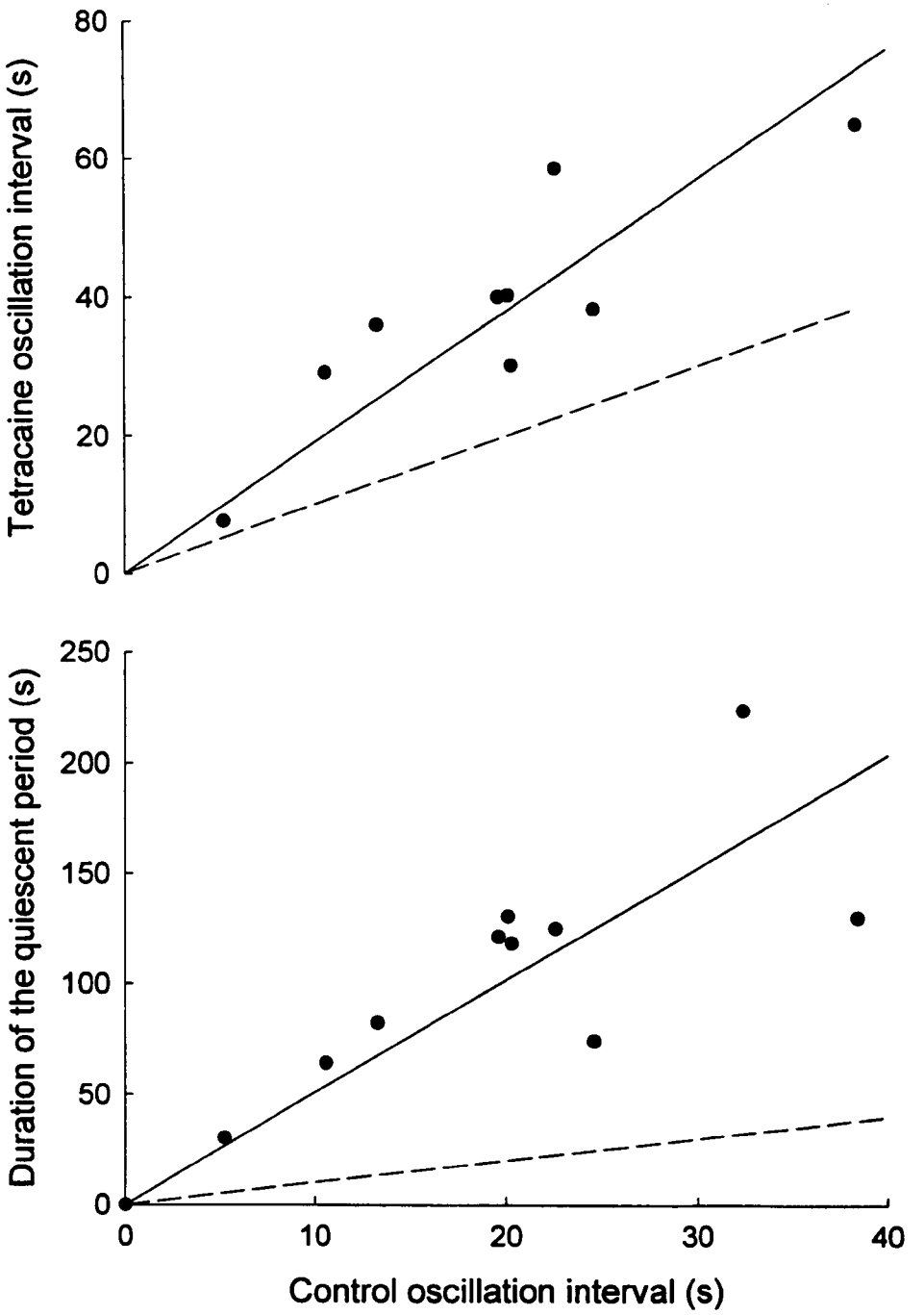

Figure 3.3. The relationship between the effects of tetracaine and the interval between spontaneous Ca^{2+} release events (contractile waves / oscillations) before adding tetracaine.

The **abscissa** shows the oscillation interval in control conditions before adding tetracaine (100 μM). The **ordinate** shows: top, the interval between spontaneous releases in the presence of tetracaine and bottom, the duration of the quiescent period after adding tetracaine. The dashed lines have unit slope. Each symbol represents a different cell.

In many experiments (e.g. Fig. 3.1) it is obvious that the magnitude of the spontaneous contractions is increased in the presence of tetracaine. It is difficult to quantify this effect from cell length measurements alone as the magnitude of the spontaneous contraction depends not only on that of the increase of $[Ca^{2+}]_i$ but also on how uniform it is throughout the cell and could also reflect changes in the Ca^{2+}-sensitivity of the myofilaments. The degree of contraction will depend on the fraction of the cell occupied by the Ca^{2+} wave at any one time (see Chapter 4 for a more detailed explanation). Figure 3.4 illustrates the effect of tetracaine on spontaneous Ca^{2+} release as assessed by measurement of global Ca_i changes using the Ca^{2+}-sensitive fluorescent indicator Indo-1. The upper panel demonstrates that there is an obvious problem with the use of this method in this study due to the intrinsic fluorescence of tetracaine at 400 nm, one of the emission wavelengths of Indo-1, causing a distinct step increase in fluorescence as soon as tetracaine was applied. To correct for this, the immediate increase in F_{400} (fluorescent emission at 400 nm) on application of tetracaine was subtracted (See Section 2.3.4), reducing the Indo-1 ratio to the control level, as illustrated in the lower panel of Fig. 3.4. This figure shows that there appears to be an increase in the magnitude of spontaneous Ca^{2+} releases during exposure to tetracaine, as suggested previously by the increased contraction amplitude in Fig. 3.1. This suggests that changes in the Ca^{2+}-sensitivity of the myofilaments cannot are not necessary to explain the increased amplitude of spontaneous contractions in the presence of this drug. However, measurement of global Ca^{2+} changes suffer from many of the limitations of measurement of changes in cell length, since they will also be affected by changes in the duration of the Ca^{2+} wave. An alternative estimate of

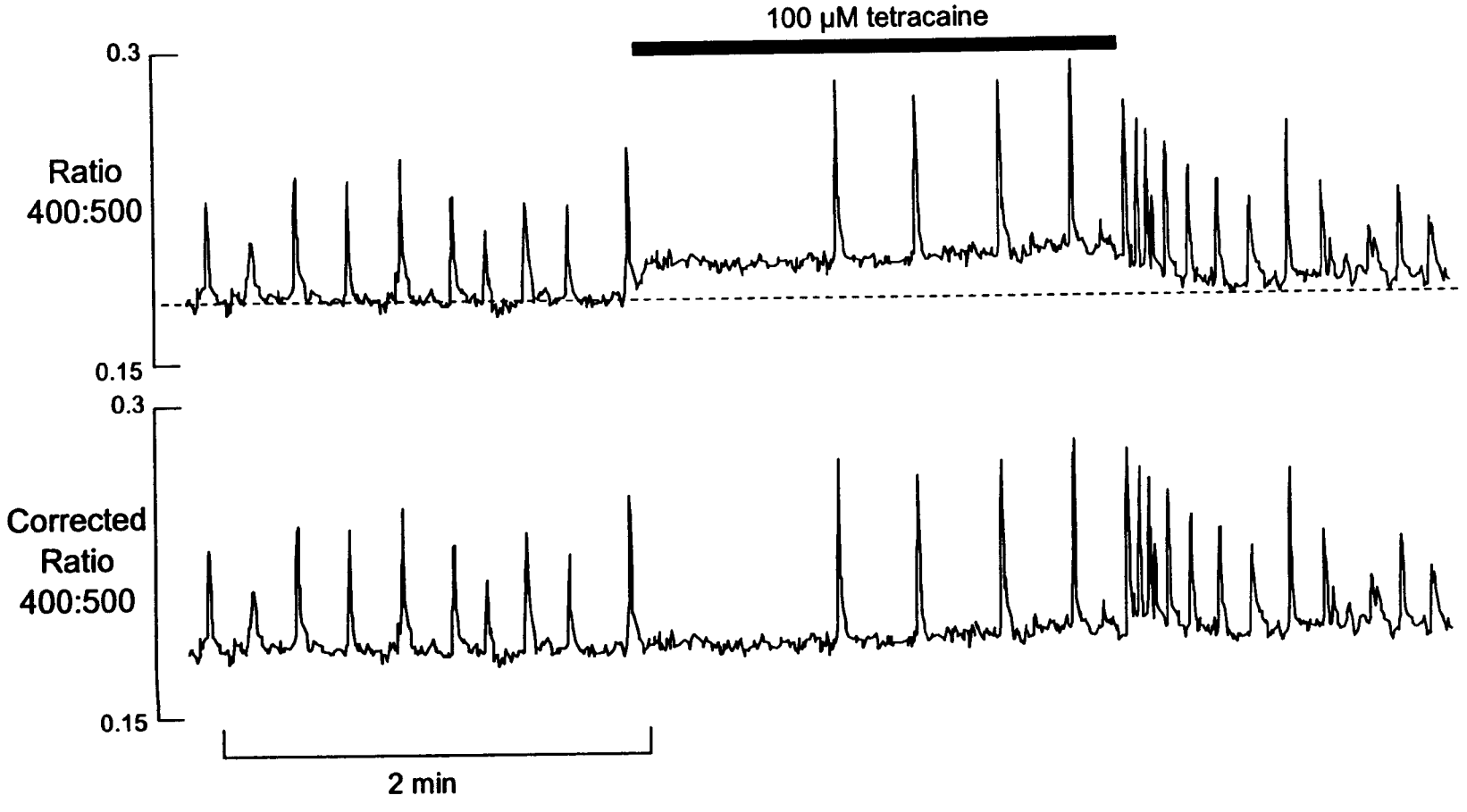

Figure 3.4. The effect of tetracaine on spontaneous Ca^{2+} release measured using Indo-1 ratio as an indication of global $[Ca^{2+}]_i$ changes.

The **upper** panel shows the raw ratio (400:500 nm). The **lower** panel illustrates the ratio after correcting for tetracaine's intrinsic fluorescence at 400 nm as detailed in the Methods section 2.3.4. Tetracaine was applied for the period indicated by the bar.

the magnitude of spontaneous SR Ca^{2+} release was achieved by measuring the integral of the transient inward Na^{+}/Ca^{2+} exchange current that is activated by the spontaneous Ca^{2+} release. Assuming that the properties of the Na^{+}/Ca^{2+} exchanger and the sarcolemmal Ca^{2+}-ATPase are not affected by tetracaine, then this integral, as well as giving a measure of the amount of Ca^{2+} pumped out of the cell, will also provide an indication of the amount of Ca^{2+} released from the SR.

Figure 3.5A shows a perforated patch voltage-clamp current record from an isolated myocyte under a similar protocol of solution changes to that of Fig. 3.1 & 3.4. This cell demonstrated an initial abolition of spontaneous Ca^{2+} releases (quiescent period) during exposure to 100 μM tetracaine, followed by their resumption, at reduced frequency. Figure 3.5B illustrates the transient inward currents associated with spontaneous Ca^{2+} releases (time points a – c in Panel A), and their integrals, on an expanded time scale. Spontaneous Ca^{2+} releases during exposure to tetracaine appear to be considerably larger with respect to both peak magnitude and integral, in comparison to those observed in control. However, these currents are also a measurement of global changes in $[Ca^{2+}]_i$ within the cell, and therefore suffer similar problems to those associated with cell length measurement and Indo-1 fluorescence ratio. These data are quantified in the histogram of Fig. 3.6A, which plots the mean effects of 100 μM tetracaine on the interval between spontaneous Ca^{2+} releases and their amplitude. It is clear that both parameters are increased by a factor of about two. The fractional changes relative to control are emphasised in Fig. 3.6B. From measurements of the magnitude and frequency of spontaneous Ca^{2+} releases, one can calculate the time-averaged calcium efflux from the cell produced by this spontaneous release

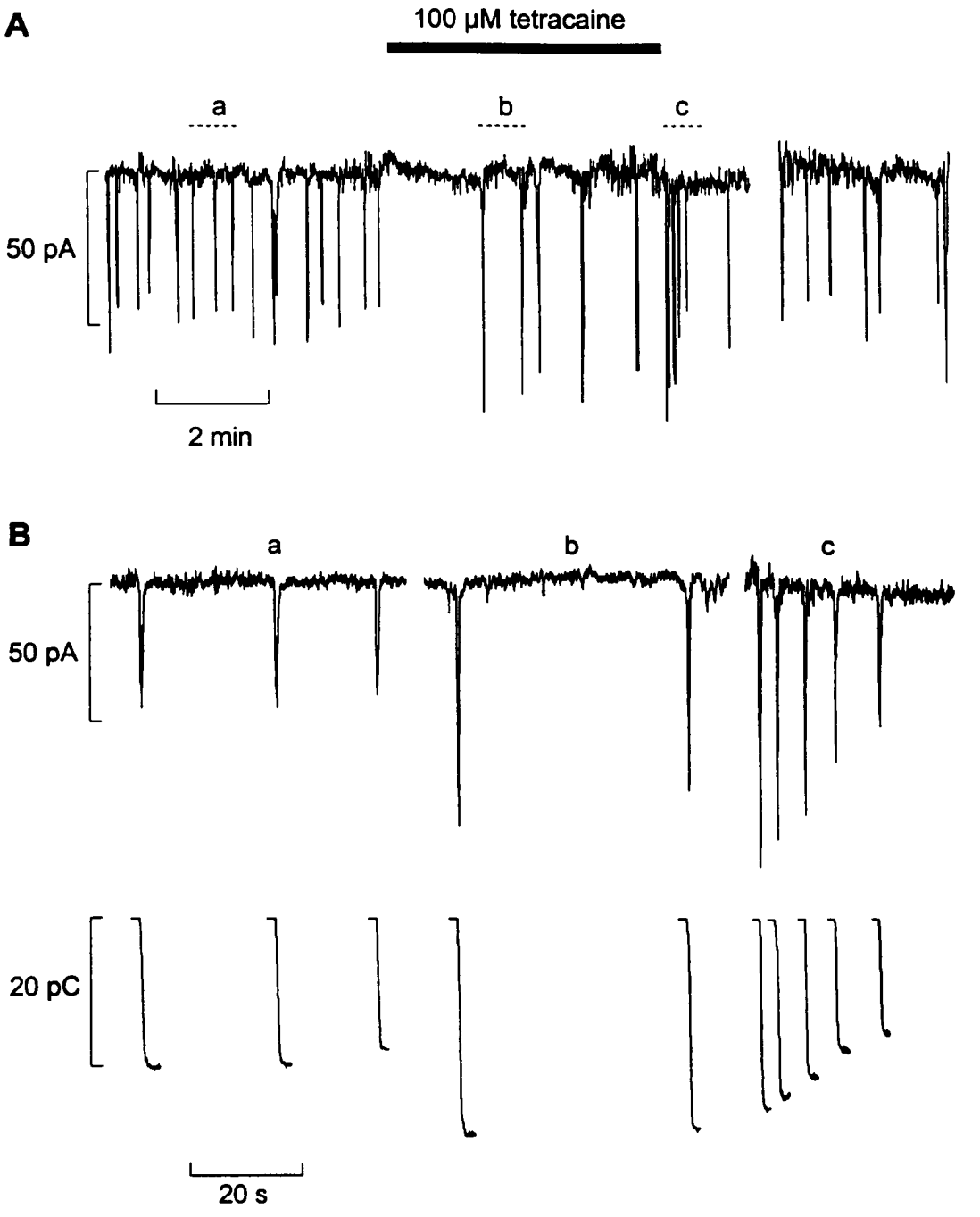

Figure 3.5. The effects of tetracaine on the membrane current accompanying spontaneous SR Ca^{2+} release.

A, time-course. The trace shows membrane current recorded from a cell held at -80 mV. Tetracaine (100 µM) was applied for the period illustrated by the bar. Tetracaine produced an outward shift of holding current of 4 pA. For clarity this was removed by subtracting a low-pass filtered version from the raw current trace. **B**, specimen records of: top, current and bottom, integrated current from the periods shown in A.

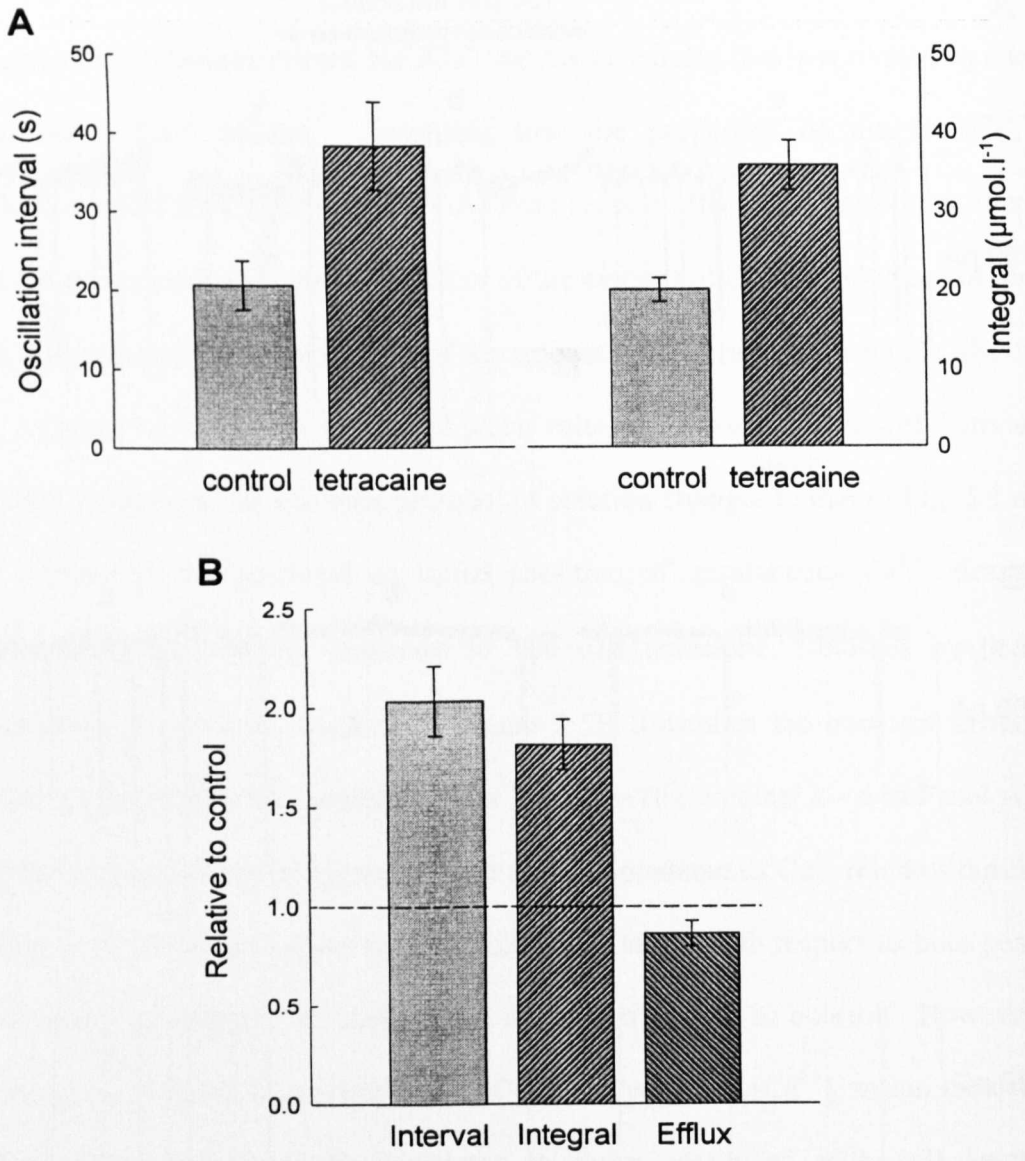

Figure 3.6. Summary of the effects of tetracaine on the magnitude and period of spontaneous SR Ca^{2+} releases.

A, histogram of the effects of tetracaine (100 μM) on: **left**, the interval between oscillations and **right**, the integral of the associated current (n = 10). The integral has been converted to the equivalent change of cell Ca^{2+} (See 2.6.2). Bars show ■ control and ▨ tetracaine. **B**, normalised data. The bars show mean data (n = 7) normalised to the value in the control solution of (from left to right): the interval between spontaneous releases; the integral of the transient inward current; the time-averaged efflux (calculated from the product of frequency and integral in each cell).

activity. On average, the time-averaged Ca^{2+} efflux via spontaneous release was 1.1 ± 0.1 $\mu mol.l^{-1}.s^{-1}$ in control and 0.9 ± 0.1 $\mu mol.l^{-1}.s^{-1}$ in 100 μM tetracaine (paired *t*-test $P > 0.15$, $n = 7$). The similarity of these values is emphasised by the right hand bar in Fig. 3.6B which shows the average efflux in tetracaine divided by that in control. Therefore the addition of tetracaine has little steady-state effect on Ca^{2+} loss from the cell activated via spontaneous Ca^{2+} release.

On removal of tetracaine there was consistently a progressive decrease in the magnitude of both the current oscillations and their integral (Fig. 3.5B, period c) toward the control levels, in parallel with the recovery of the control oscillation frequency.

3.4 Tetracaine Increases the SR Ca^{2+} Content

There are at least two explanations for the presence of the burst of spontaneous Ca^{2+} release on removal of tetracaine. (i) Tetracaine could have some complicated effect on the surface membrane such that its removal could produce a transient influx of calcium into the cell, which would then lead to an increase of SR Ca^{2+} loading and consequently SR Ca^{2+} release. (ii) The inhibitory effect of tetracaine on the SR Ca^{2+} release channel could allow an increase SR Ca^{2+} content. On removal of tetracaine, spontaneous release would ensue due to the Ca^{2+} overload of the SR. To discriminate between these hypotheses the SR Ca^{2+} content was measured using the integral of the Na^{+}/Ca^{2+} exchange current, activated by releasing SR Ca^{2+} by the application of 20 mM caffeine. In the maintained presence of caffeine, a known proportion (~67 %) of the calcium released from the SR leaves the cell on the Na^{+}/Ca^{2+} exchanger [274, 383]. The

integral of the transient inward current (due to Na^+/Ca^{2+} exchange) evoked by caffeine provides a quantitative measure of the SR Ca^{2+} content, after correcting for electro-neutral efflux mechanisms (See Section 2.6.2). Typical current records from a single ventricular myocyte under perforated patch voltage-clamp control are shown in the upper traces of Fig. 3.7A. The smooth curves in the lower panel represent the integrals of each current record and clearly show that tetracaine produces a reversible increase in the SR Ca^{2+} content, in this case from 118 $\mu mol.l^{-1}$ to 233 $\mu mol.l^{-1}$. On average (Fig. 3.7B) exposure to 100 μM tetracaine for 5 min increased the integral of the caffeine-evoked current by 80.3 ± 11.4 $\mu mol.l^{-1}$ from 108.0 ± 6.9 $\mu mol.l^{-1}$ (paired *t*-test, $P < 0.0001$, $n = 10$).

3.5 Where Does the Extra Ca^{2+} come from?

It is likely that the extra Ca^{2+} accumulated by the SR during exposure to tetracaine is gained during the initial quiescent period. In addition, one predicts that this extra calcium is lost during the *"burst"* of spontaneous Ca^{2+} release activity on removal of tetracaine. To test this hypothesis the Ca^{2+} gain during the period without spontaneous Ca^{2+} release was estimated from the Ca^{2+} efflux that would have occurred if spontaneous release activity had continued at its control frequency, using the average efflux per unit time. The average period between spontaneous releases in control was subtracted from the total interval between the last spontaneous release event in control and the first in tetracaine, revealing the *extra* interval due to application of tetracaine. This calculation gives the amount of Ca^{2+} efflux that ought to have taken place during the quiescent period. This Ca^{2+} is presumably accommodated within the SR. The *extra* Ca^{2+} lost from the

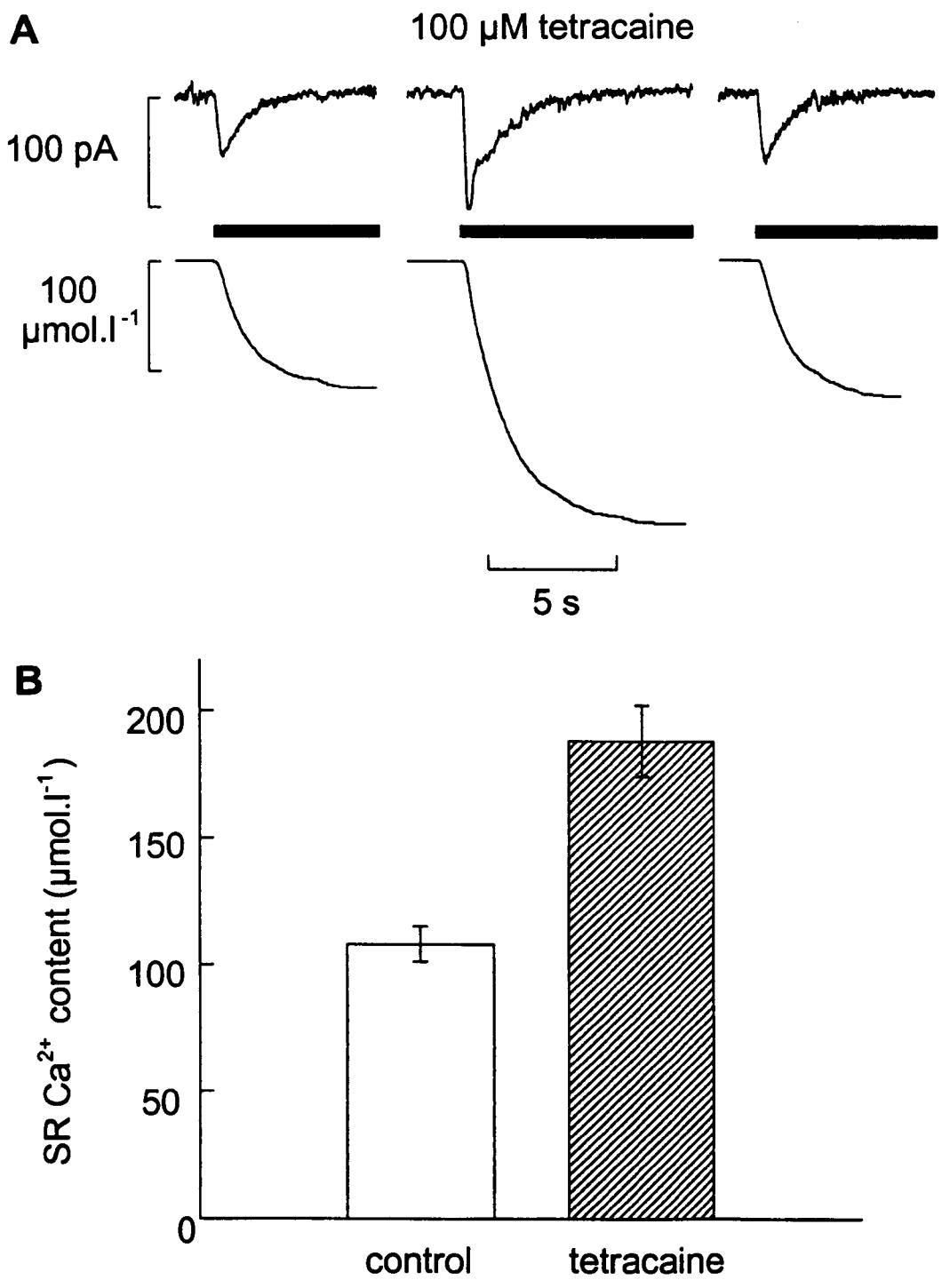

Figure 3.7. The effects of tetracaine on caffeine-induced Na^+/Ca^{2+} exchange current integral magnitude as an index of SR Ca^{2+} content.

A, original data. Traces show: top, current; bottom, integral. Panels show (from left to right): control; tetracaine (100 µM); recontrol. Caffeine (20 mM) was applied for the period indicated by the horizontal bars. **B**, histogram showing mean SR Ca^{2+} content (n = 10), as measured from the integral of the caffeine response in control (left) and during exposure to tetracaine (right).

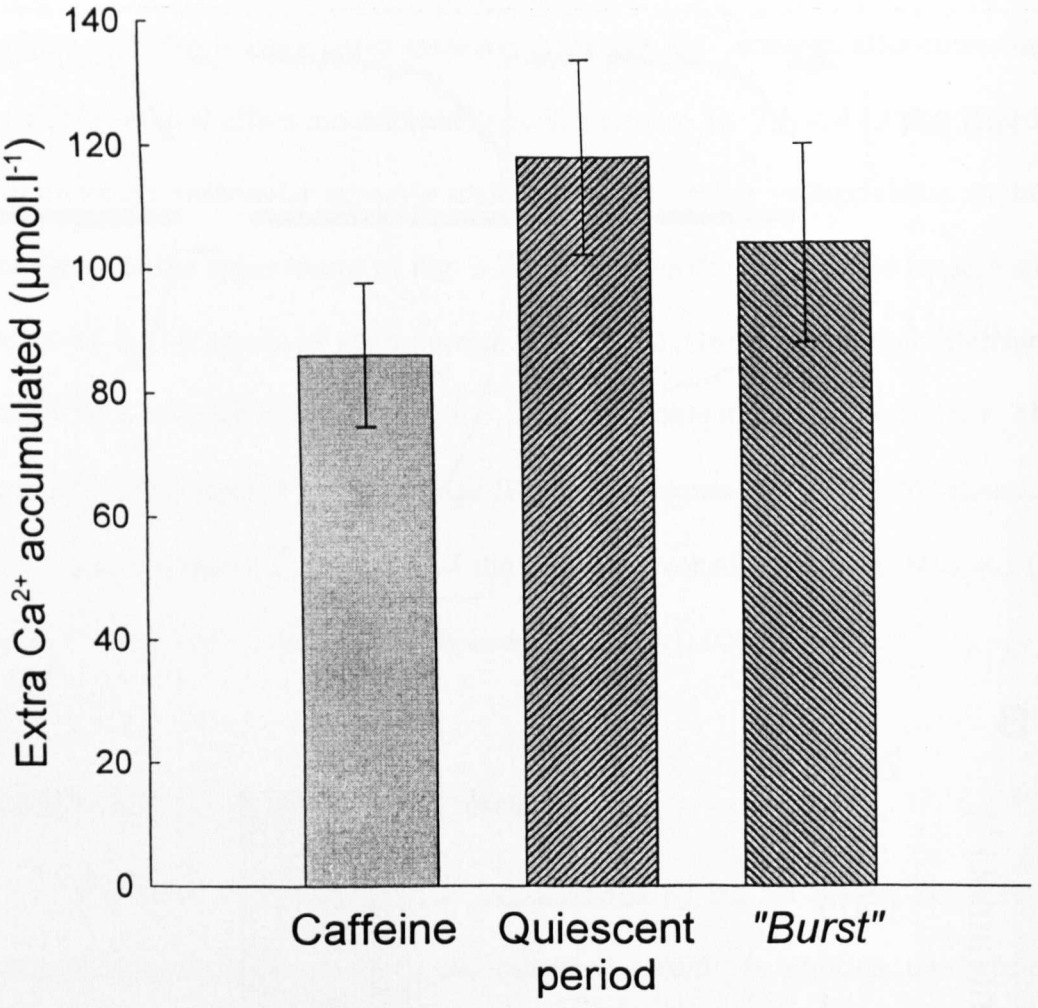

Figure 3.8. Comparison of three methods for estimating the Ca^{2+} gained by the cell during exposure to tetracaine (100 µM).

The bars show (from left to right): (**Caffeine**) the measured increase in the integral of the caffeine response; (**Quiescent period**) the calculated Ca^{2+} entry during the quiescent period on application of tetracaine (in this calculation the *extra* quiescent period produced by tetracaine *in addition* to the interval between spontaneous releases in control was used); (**"*Burst*"**) the calcium lost from the cell during the *burst* on tetracaine removal. This was obtained by integrating the current during the *burst* and subtracting from it the loss of calcium that would have occurred during an equivalent period of control oscillation frequency following tetracaine removal.

cell during the *burst* was calculated from the cumulative integral over a defined period commencing on removal of tetracaine. An estimate of Ca^{2+} loss, had spontaneous Ca^{2+} release occurred at the recontrol steady-state frequency, was subtracted from this value. On average these values compare well to the change of the caffeine response observed in tetracaine (Fig. 3.8). Average values for these estimation methods are 118.3 ± 15.7 $\mu mol.l^{-1}$ from the quiescent period, 104.5 ± 16.0 $\mu mol.l^{-1}$ for the burst and 86.3 ± 11.6 $\mu mol.l^{-1}$ from the difference in caffeine response. Each of these methods of estimation was compared with the other two using a paired *t*-test and they were found not to be significantly different (paired *t*-test, $P > 0.1$ for all, $n = 7$).

Figure 3.9 illustrates the relationship between spontaneous Ca^{2+} release and the resting influx of Ca^{2+} into a rat ventricular myocyte. The upper panel shows a raw current trace from which the integrals of the Na^{+}/Ca^{2+} exchange currents activated by Ca^{2+} spontaneously released from the SR were calculated and related to Ca^{2+} extruded from the cell (Ca^{2+} loss from the SR, represented as downward deflections in the lower panel). This is balanced by a resting influx of Ca^{2+} of 1.24 $\mu mol.l^{-1}.s^{-1}$.

3.6 The Effect of Tetracaine on the Threshold for Caffeine-Induced Ca^{2+} Release

Caffeine is thought to release Ca^{2+} from the SR by sensitising the CICR mechanism or RyR to Ca^{2+}, such that the resting $[Ca^{2+}]_i$ is sufficient to activate release [334]. Using Indo-1 it is possible to measure both $[Ca^{2+}]_i$ and $[caffeine]_i$ directly and simultaneously [283] (See Section 2.3.5). It is therefore possible to

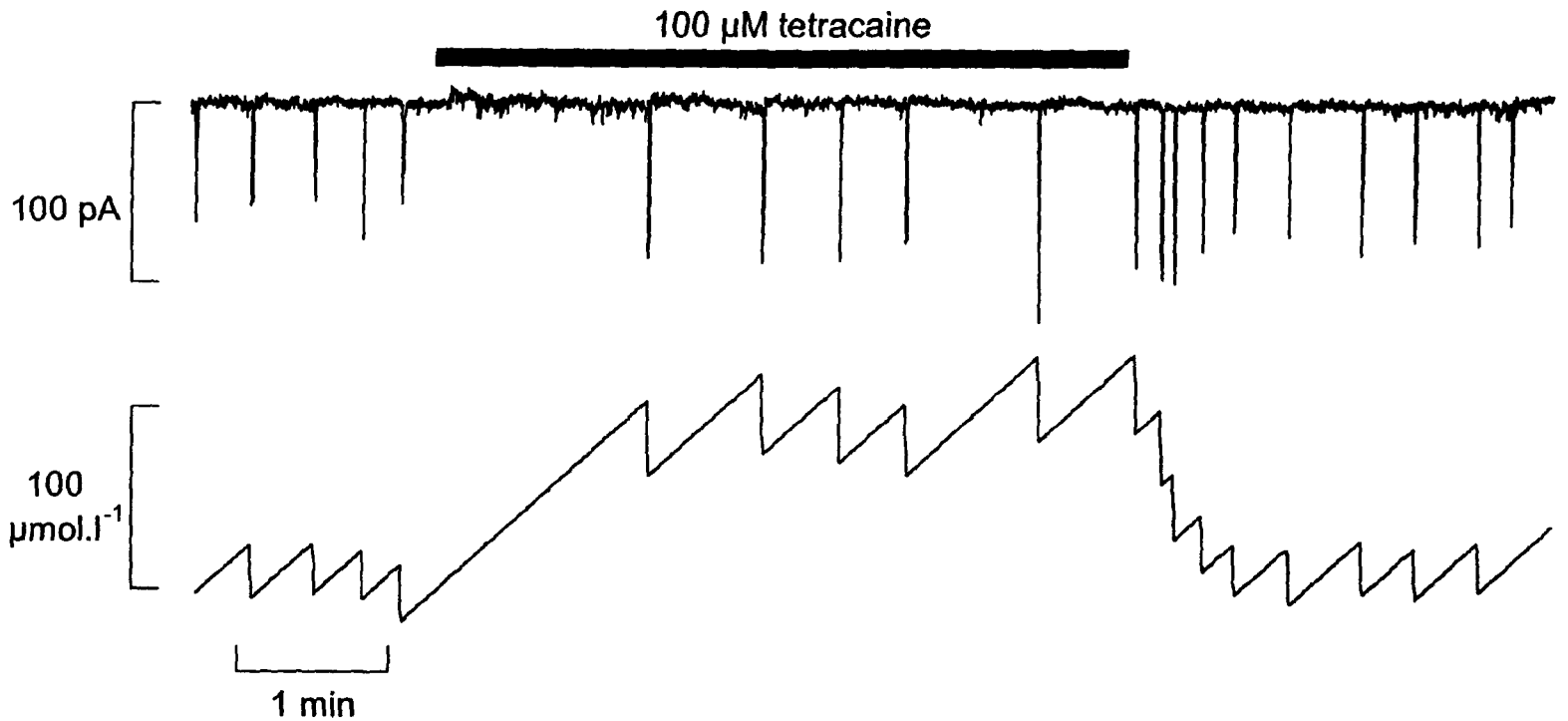

Figure 3.9. Calculation of the effects of tetracaine on SR Ca^{2+} content.

Top, membrane current; bottom, calculated Ca^{2+} content. Tetracaine (100 µM) was applied for the period indicated by the horizontal bar. Tetracaine produced an outward shift of holding current of 9 pA. For clarity this was removed by subtracting a low-pass filtered version from the raw current. The lower record was calculated by integrating the current records only for the period of each oscillation. To these integrals, a calcium influx of 1.24 $\mu mol.l^{-1}.s^{-1}$ was added. Note that the calculated Ca^{2+} content only considers changes due to sarcolemmal fluxes and ignores the faster components due to SR Ca^{2+} release and re-uptake during and immediately after spontaneous release.

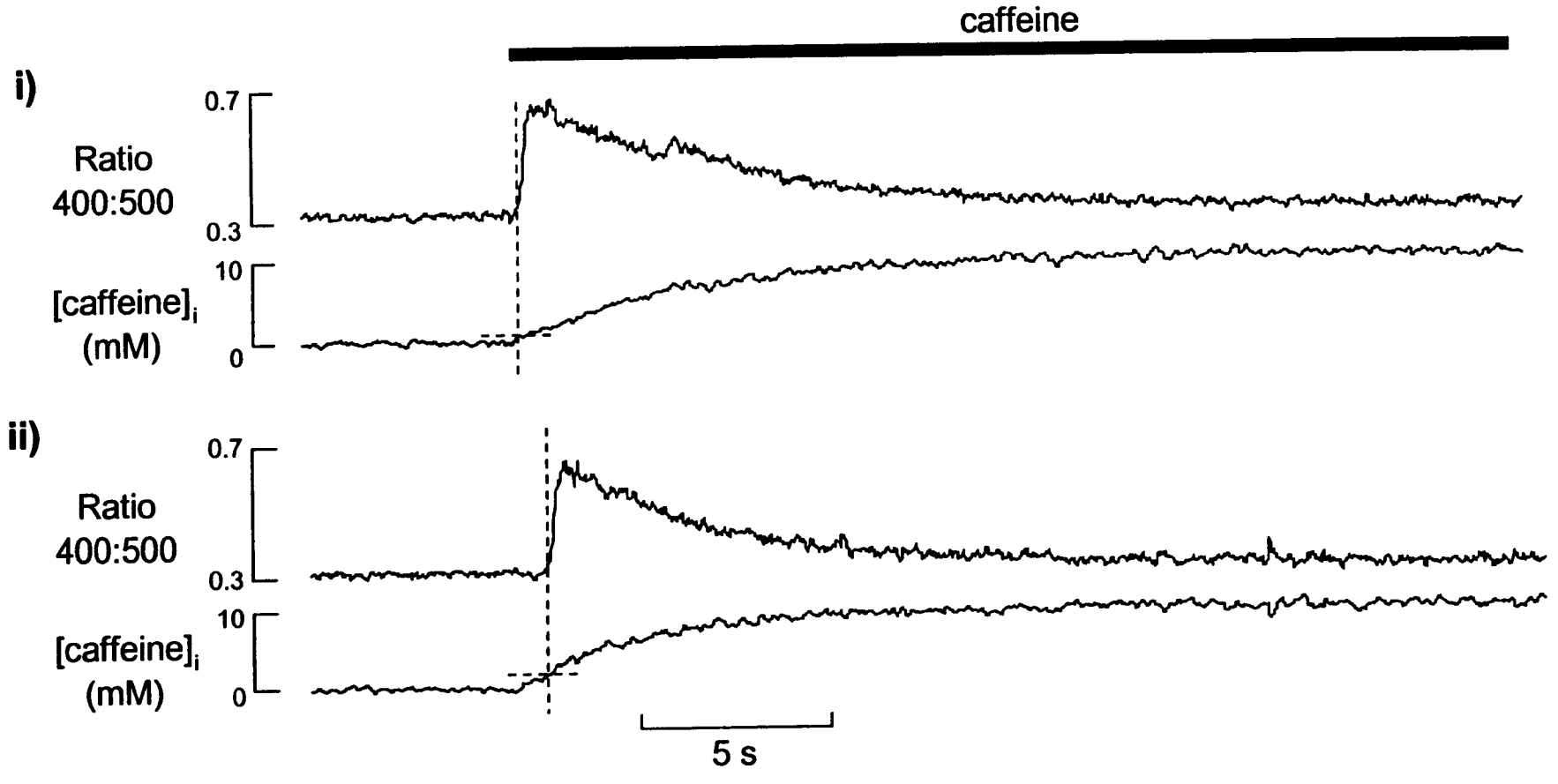

Figure 3.10. The effect of 100μM tetracaine on caffeine-induced Ca^{2+} release threshold.

Panels show i) Control conditions; Indo-1 ratio (top) and [caffeine]$_i$ (bottom) calculated from quench of Indo-1 signal. ii) During exposure to 100 μM tetracaine. Caffeine was applied for the period indicated by the bar. Increases in [caffeine]$_i$ have been synchronised.

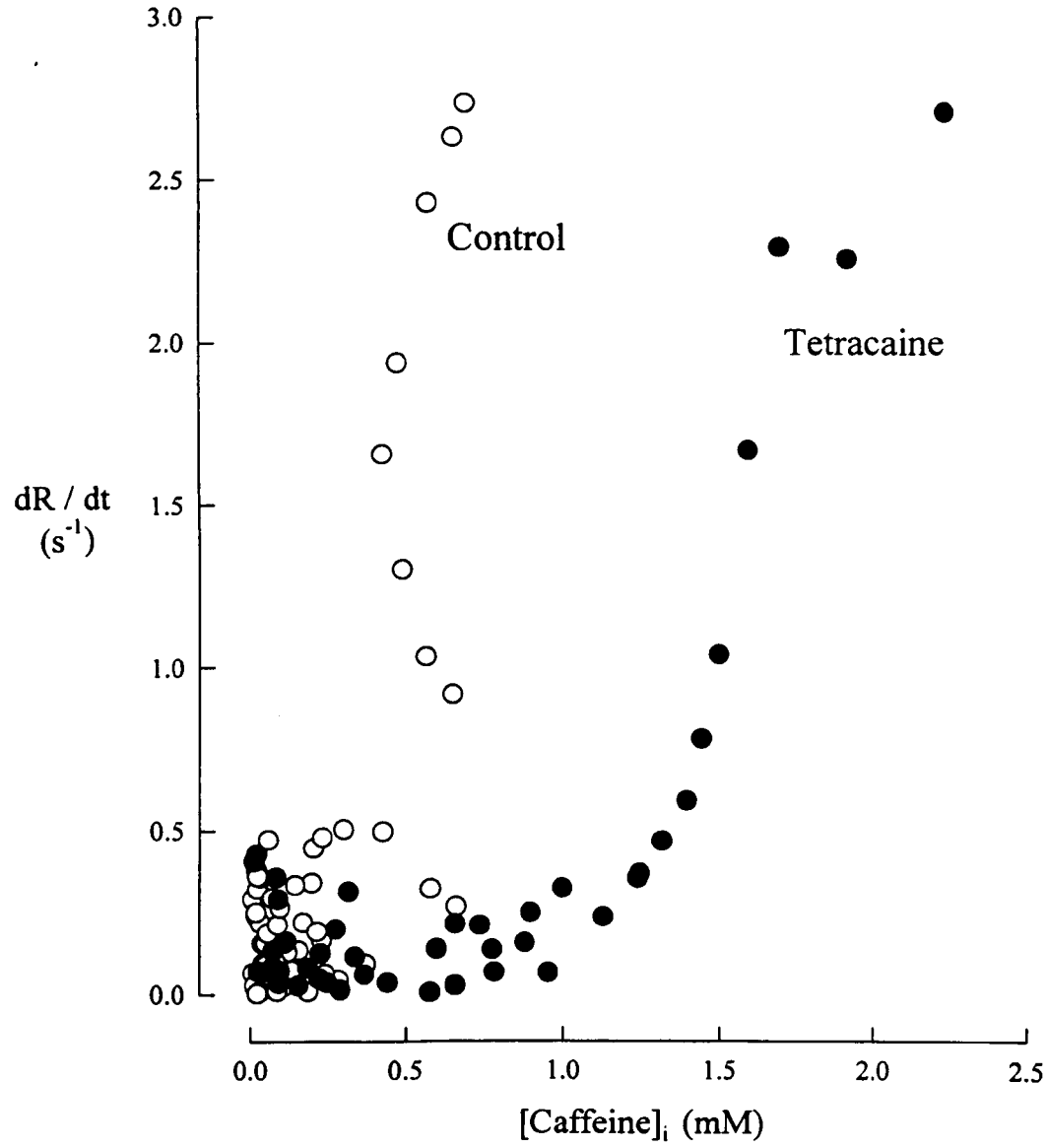

Figure 3.11. The instantaneous relationship between [caffeine]$_i$ and Ca^{2+} release.

The graph shows: ordinate, dR/dt (rate of change of ratio ($[Ca^{2+}]_i$); abscissa, $[caffeine]_i$. The data used was the same as that used to produce Fig. 3.10. Open circles represent control conditions. Filled circles represent results during exposure to 100 μM tetracaine.

determine the concentration of caffeine inside the cell that is required to release Ca^{2+} from the SR at resting $[Ca^{2+}]_i$. If tetracaine inhibits the SR Ca^{2+} release channel, one might predict that more caffeine would be required to release Ca^{2+}. Figure 3.10 shows the Indo-1 ratio and calculated $[caffeine]_i$ under control conditions and during exposure to 100 μM tetracaine. Tetracaine was added only a few seconds before caffeine application to avoid the potential complication of significant changes in SR Ca^{2+} content. The records have been arranged such that the increase in $[caffeine]_i$ are synchronised. The dashed line indicates the point of release. A higher concentration of caffeine inside the cell is required to release Ca^{2+} in the presence of 100 μM tetracaine. In four cells an average of 0.3 ± 0.02 mM caffeine was reached before release (defined as the ratio increasing by 2 standard deviations from the pre-caffeine mean) under control conditions. In 100 μM tetracaine this value was increased to 1.2 ± 0.05 mM caffeine. This method of estimating threshold $[caffeine]_i$ is somewhat subjective, depending on where the dashed line is placed. Figure 3.11 shows the derivative of the ratio (rate of change of $[Ca^{2+}]_i$) as a function of $[caffeine]_i$ from the same data as in Fig. 3.10. It is clear that the ratio begins to rise at a lower $[caffeine]_i$ under control conditions.

3.7 The Effect of High Concentrations of Tetracaine on SR Ca^{2+} Content

Quantification of the maximum possible SR Ca^{2+} load of rat ventricular myocytes was attempted under conditions of high external Ca^{2+} (5 mM Ca^{2+}

Tyrode solution) by applying high concentrations (up to 1 mM) of the Ca^{2+} release inhibitor tetracaine. The integral of the Na^{+}/Ca^{2+} exchange current, under voltage-clamp at a holding potential of –80 mV, activated by application of caffeine, which releases all Ca^{2+} from the SR, was used as a measure of the SR Ca^{2+} content.

Under normal conditions, following exposure to high concentrations of tetracaine the caffeine-induced contraction was often so large that the cell was torn from the patch-pipette or impaled itself and died, either way, terminating the experiment. To overcome this problem, the drug 2,3-butanedione 2-monoxime (BDM) was used, which affects the myofilaments, thereby reducing contraction [155, 253]. The effects of BDM are both dose-dependent and fully reversible upon washout. Shortly before caffeine application, the cell was exposed to 10 mM BDM to reduce the magnitude of the contraction resulting from caffeine-induced Ca^{2+} release. Like caffeine, BDM sensitises the ryanodine receptor to Ca^{2+}, and its application induces a small release of Ca^{2+} from the SR [297], producing an inward current, again attributed to Na^{+}/Ca^{2+} exchange mediated extrusion of Ca^{2+} from the cell [4]. This and the caffeine-induced Ca^{2+} release were quantified from the integrals of the Na^{+}/Ca^{2+} exchange currents, and summed to calculate the total SR content.

Figure 3.12 shows the effect of 15 minutes exposure to 1 mM tetracaine on the magnitude of this current, and its integral. In this case, the SR Ca^{2+} content was calculated to have increased approximately five-fold, from 90 to 499 $\mu mol.l^{-1}$ cell volume. In a further seven cells exposed to 1 mM tetracaine for between 10

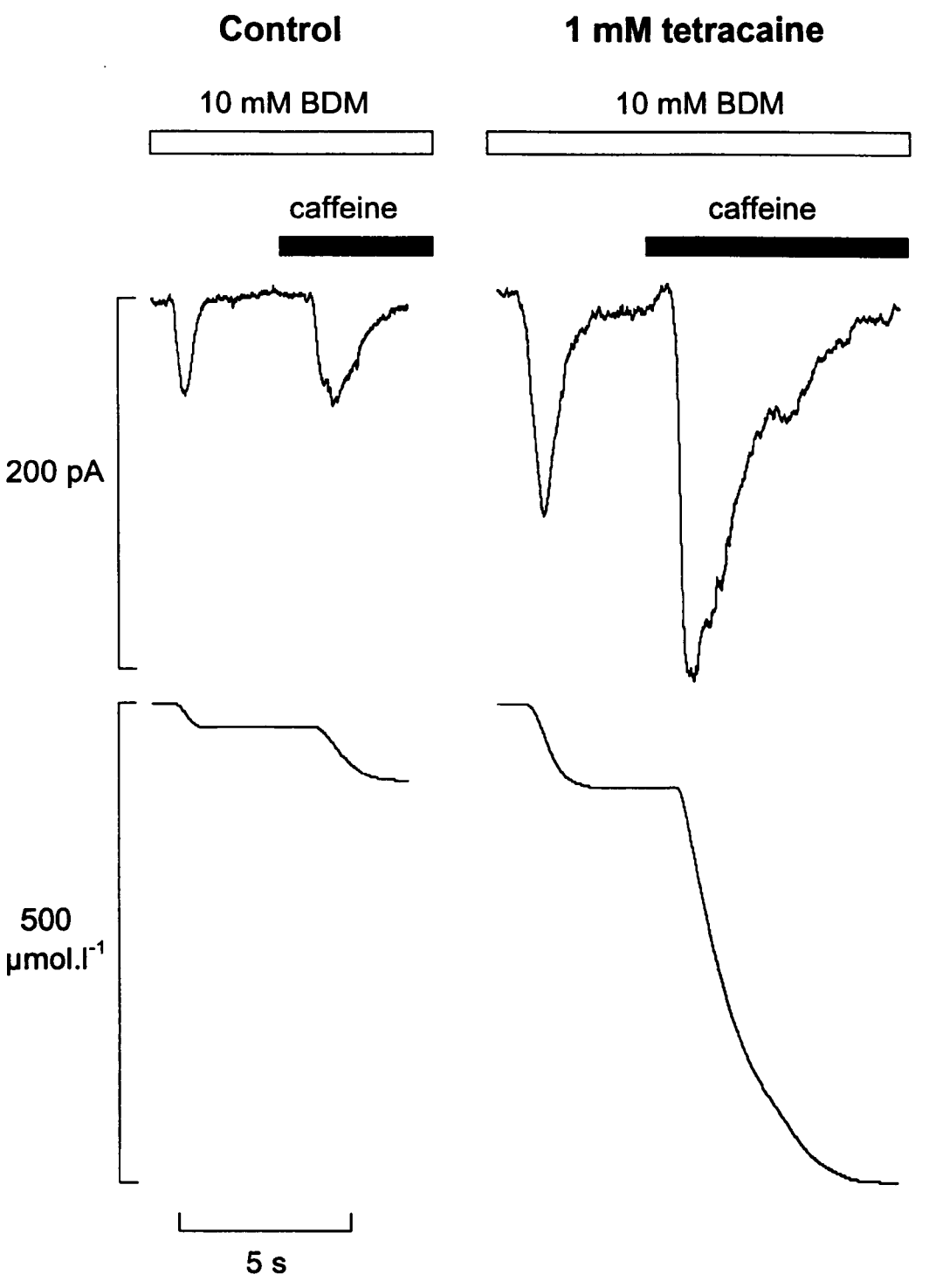

Figure 3.12. The effect of exposure to 1 mM tetracaine on SR Ca^{2+} content.

Upper panel shows Na^+/Ca^{2+} exchange currents activated by SR Ca^{2+} release induced by BDM and caffeine (applied for the periods indicated by the bars) under control conditions of 5 mM Ca^{2+} (left) and following exposure to 1 mM tetracaine for a period of 15 minutes. The lower panel shows their integrals converted into µmol per litre cell volume calculated as detailed in Section 2.6.2.

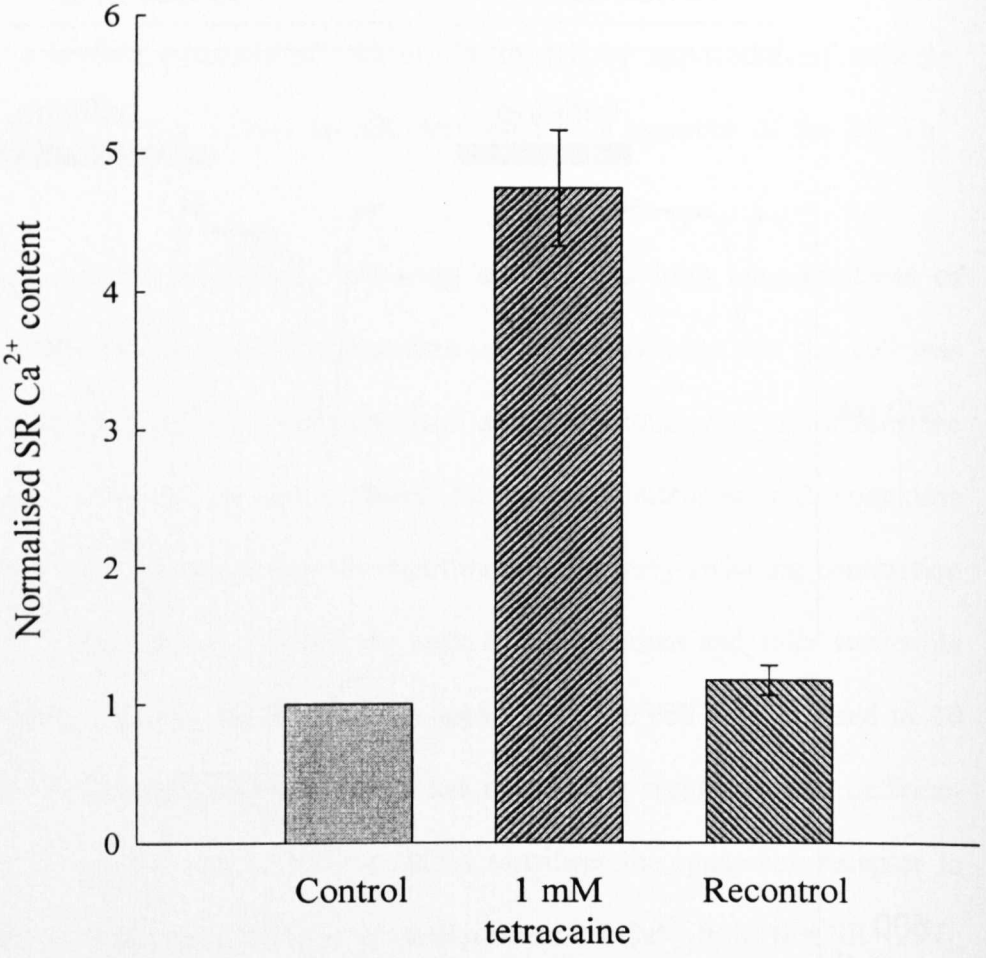

Figure 3.13. Summary of the effect of 1 mM tetracaine on SR Ca^{2+} content of rat ventricular myocytes exposed to 5 mM Ca^{2+}.

Histogram illustrating mean data ± S.E.M. of SR Ca^{2+} content (from left to right): Under control conditions (5 mM Ca^{2+}); during exposure to 1 mM tetracaine for periods of between 10 and 50 min; recontrol. All data was normalised with respect to the control SR Ca^{2+} content ($n = 7$).

and 50 min, the SR Ca^{2+} content increased from 90 to 450 $\mu mol.l^{-1}$ cell volume, as illustrated by the mean data in the bar chart of Fig. 3.13.

3.8 Discussion

The main finding of this work is that the reduction of spontaneous release frequency produced in rat ventricular cells by application of sub-millimolar concentrations of tetracaine is associated with an increase of sarcoplasmic reticulum (SR) Ca^{2+} content. This is associated with an increase in the size of the transient inward current and integral activated by propagating spontaneous Ca^{2+} release during exposure to tetracaine. These results are consistent with increased accumulation of Ca^{2+} in the SR and one might expect a greater flux of Ca^{2+} through the Ca^{2+} release channels (RyRs) when they are opened.

3.8.1 Tetracaine Inhibits SR Ca^{2+} Release and Increases the SR Ca^{2+} Content

The results of this work are consistent with tetracaine acting by inhibiting Ca^{2+} release from the SR. However, it is necessary to consider whether other actions of tetracaine could be involved. Tetracaine is a local anaesthetic and inhibits both sarcolemmal Na^+ & Ca^{2+} channels [75]. Inhibition of Na^+ channels will decrease $[Na^+]_i$ and, via Na^+/Ca^{2+} exchange, will decrease $[Ca^{2+}]_i$ and thence SR Ca^{2+} content. Inhibition of Ca^{2+} channels will directly decrease $[Ca^{2+}]_i$, by reducing the ability of Ca^{2+} to leak into the cell down its steep electrochemical gradient. These effects, however would produce (as is seen for lidocaine, [112]) a gradual decrease of the frequency of spontaneous Ca^{2+} release. If $[Ca^{2+}]_i$

recovered gradually on removal of tetracaine one would expect a *gradual* recovery of the frequency of spontaneous SR Ca^{2+} waves. This hypothesis is therefore unable to account for the immediate abolition of spontaneous SR Ca^{2+} release followed by its resumption or the *burst* of spontaneous activity observed on removal of tetracaine.

The appearance of spontaneous SR Ca^{2+} release in cardiac muscle is associated with increased SR Ca^{2+} content [42, 116, 265, 289]. One would expect, therefore, that abolition of spontaneous SR Ca^{2+} release would be associated with a decrease of SR Ca^{2+} content. The apparently paradoxical abolition of spontaneous Ca^{2+} release while increasing SR Ca^{2+} content caused by tetracaine can be explained if its main effect is to inhibit SR Ca^{2+} release. The structurally related local anaesthetic, procaine, has been shown to inhibit SR Ca^{2+} release in isolated cardiac SR vesicles [80], albeit at much higher concentrations (millimolar) than those used here for tetracaine. Procaine also inhibits calcium-activated channel activity of the cardiac ryanodine receptor in lipid bilayers [369, 412] and its application has been shown to increase the magnitude of the rapid-cooling contracture and therefore, presumably the SR Ca^{2+} content [215]. Although no studies have been published of the effect of tetracaine on the *cardiac* ryanodine receptor, a personal communication from L. Xu & G. Meissner shows that it has similar effects to those previously reported in skeletal muscle, where it inhibits spontaneous release of Ca^{2+} from skeletal muscle SR vesicles [387] and lowers the open probability of the calcium-activated skeletal muscle ryanodine receptor in lipid bilayer studies [404]. Work published recently [157] has shown that, in cardiac cells, tetracaine initially abolished spontaneous Ca^{2+} sparks before

a recovery associated with an increased SR Ca^{2+} load (as judged qualitatively by the caffeine-evoked increase of $[Ca^{2+}]_i$).

In previous work it has been shown that, in the absence of tetracaine, spontaneous SR Ca^{2+} release occurs when the SR Ca^{2+} content has reached a critical threshold level [105]. Increasing the magnitude of Ca^{2+} entry into the cell does not increase the Ca^{2+} content of the SR once spontaneous activity begins. Instead, spontaneous SR Ca^{2+} release occurs at a higher frequency because the SR refills to its threshold more rapidly. The apparent threshold may result from an effect of luminal Ca^{2+} on the gain of Ca^{2+}-induced Ca^{2+} release in either of two ways. (i) Directly through an intraluminal regulatory effect on the RyR, or (ii) indirectly because when a RyR opens, the greater the luminal Ca^{2+}, the greater the Ca^{2+} efflux from the SR and therefore the greater the gain of Ca^{2+}-induced Ca^{2+} release. In either case, the increased gain allows wave propagation, activating Ca^{2+} efflux from the cell and limiting the level to which the SR can fill.

The effects of tetracaine can be explained if one assumes that it increases the loading of the SR required for spontaneous release. This could arise because, by decreasing the opening probability of the SR Ca^{2+} release channel, tetracaine may decrease the frequency of Ca^{2+} "sparks" and thence inhibit the initiation of Ca^{2+} waves. The subsequent recovery of spontaneous release will arise because the increase of SR Ca^{2+} content (luminal Ca^{2+}) will increase the spark magnitude such that a larger fraction of the sparks are capable of initiating a propagating wave. This has recently received direct experimental support [157], although the possibility that the decrease of RyR opening probability will reduce the "gain" of CICR and interfere with the wave propagation, cannot be excluded and is

supported by the results presented in Chapter 4.

When a myocyte is exposed to tetracaine the SR will continue to fill with Ca^{2+} to a level beyond that reached in the control solution. This will produce the initial delay seen before spontaneous Ca^{2+} release resumes. The duration of this delay will depend on the rate at which Ca^{2+} is entering the cell. It is therefore noteworthy that the delay is proportional to the interval between spontaneous releases observed under control conditions (Fig. 3.3). Presumably, the higher the rate of Ca^{2+} entry into the cell, the greater the frequency of spontaneous release in control and the less time required to reach the *new* threshold level of SR luminal Ca^{2+}. The duration of the initial delay also increases with increasing tetracaine concentration probably because the concentration of luminal Ca^{2+} required to allow initiation of propagating Ca^{2+} release is increased.

In the presence of tetracaine, the amplitude of the spontaneous Ca^{2+} release, and the amount of Ca^{2+} pumped out of the cell per release increases. Presumably the increased SR Ca^{2+} content results in a larger release of Ca^{2+}. There is a corresponding decrease in the frequency of release such that the calculated time-averaged Ca^{2+} efflux, produced by spontaneous Ca^{2+} release in tetracaine, is similar to that in control. The unchanged spontaneous Ca^{2+} release-induced efflux suggests that the component of efflux activated by the resting calcium also remains unchanged, i.e. tetracaine probably does not change the resting $[Ca^{2+}]_i$. Attempts to measure this directly using the Ca^{2+}indicator Indo-1 were hindered by the intrinsic fluorescent properties of tetracaine. When tetracaine is applied to cells there is an immediate increase of fluorescence (Fig. 3.4), which is due, at least in part, to the fact that tetracaine is fluorescent. However there was no

subsequent change of resting fluorescence during prolonged exposure to tetracaine suggesting that $[Ca^{2+}]_i$ remains constant. Furthermore the constancy of the efflux suggests that tetracaine is not having a marked effect on Ca^{2+} entry as any decrease of Ca^{2+} entry would, in the steady-state, decrease efflux. It is known that tetracaine decreases Ca^{2+} fluxes through the L-type Ca^{2+} channel [75], however, it is likely that all these channels are closed at the normal resting membrane potential and, therefore, it is not surprising that tetracaine does not affect resting Ca^{2+} fluxes by this mechanism. The constancy of the time-averaged Ca^{2+} efflux is obviously required for the cell to maintain Ca^{2+} balance in the face of an unchanged Ca^{2+} entry. The initial delay before spontaneous Ca^{2+} release resumes in tetracaine is greater than the interval between spontaneous releases when they resume. This is consistent with the fact that the increase of SR Ca^{2+} content in tetracaine (i.e. the amount of Ca^{2+} gained during the initial delay) is greater than the extra Ca^{2+} pumped out of the cell per spontaneous release (i.e. the amount gained between waves in tetracaine).

This model can also account for the *burst* of spontaneous Ca^{2+} release seen after removing tetracaine. At this point the properties of CICR have been restored to normal but the SR Ca^{2+} content is elevated considerably above the control threshold for Ca^{2+} release. This will result immediately in Ca^{2+} release. It is noteworthy that the extra Ca^{2+} content of the SR is not removed from the cell on one very large spontaneous release and is presumably partly re-sequestered by the SR. This may be indicative of attenuation of Ca^{2+}-induced Ca^{2+} release because of the spontaneous elevation of $[Ca^{2+}]_i$ as suggested previously [124].

The data and calculations shown in Fig. 3.9 best illustrate the overall results

of this work. The upper trace shows the effects of tetracaine on membrane current. The inward currents associated with Ca^{2+} release were integrated to calculate the net loss of Ca^{2+} from the cell. In the steady state this must be balanced by a Ca^{2+} entry. The lower trace shows the calculated change of cell Ca^{2+} content assuming a constant Ca^{2+} influx into the cell of 1.24 $\mu mol.l^{-1}s^{-1}$, which was chosen to exactly balance the time-averaged Ca^{2+} efflux. Under control conditions this results in a gradual increase of cell Ca^{2+} punctuated by abrupt decreases during spontaneous SR Ca^{2+} release. It should be noted that the linear time course of the gain of Ca^{2+} between releases is a simplifying assumption: all that can be fixed are the initial and final points of the increase. When tetracaine is applied the spontaneous release stops for a period, while the Ca^{2+} influx continues and therefore the cell Ca^{2+} content increases. When spontaneous release resumes the integrals are larger and therefore the decrease of cell Ca^{2+} content on each release is larger and, in combination with the lower frequency, a new steady-state Ca^{2+} content is achieved. Finally, when tetracaine is removed, the burst reduces cell Ca^{2+} back down to the control level.

3.8.2 Tetracaine Reduces the Ca^{2+} Sensitivity of the Ryanodine Receptor

Tetracaine increases the concentration of caffeine required to trigger release of Ca^{2+} from the SR. Low concentrations of caffeine are believed to act by increasing the Ca^{2+} sensitivity of the RyR, such that lower $[Ca^{2+}]_i$ may still be capable of stimulating further Ca^{2+} release [284, 309, 334]. It acts at the cytosolic side of the SR membrane and increases the P_o of the RyR by increasing the frequency of channel opening. It is likely therefore that tetracaine opposes this

effect by desensitising the RyR and may act at the same site. This is consistent with the reversal of the effects of tetracaine on amphibian skeletal muscle by caffeine [248] and the ability of the related local anaesthetic procaine to inhibit caffeine-induced contracture [366] and to reverse the potentiating effects of caffeine [213] in skeletal muscle.

3.8.3 The Ca^{2+} Content of the SR is limited by Leak, not Uptake in Rat Ventricular Myocytes

In cardiac muscle, the Ca^{2+} content of the SR could be limited by the pumping ability of the SR Ca^{2+}-ATPase or by the tendency of Ca^{2+} to leak from the SR. It has previously been reported that the local anaesthetic tetracaine increases the amount of Ca^{2+} stored in the SR by blocking its release through the ryanodine receptor (Section 3.4 [294]). When the RyR was inhibited by 1 mM tetracaine in the presence of 5 mM external Ca^{2+}, the SR Ca^{2+} content was increased five-fold. This appears to suggest that even under the severe conditions of Ca^{2+} overload used in these investigations, the storage capacity of the SR cannot be limited by the ability of the Ca^{2+}-ATPase to pump Ca^{2+}. The thermodynamic limit of the [Ca^{2+}] gradient produced by the SR Ca^{2+} pump must be very much higher than that reached under normal conditions. This is at variance with the conclusions of Ginsburg *et al* [148], who suggest that in ferret ventricular myocytes the SR Ca^{2+} pump operates very close to its thermodynamic limit even under normal conditions. The limit for SR Ca^{2+} content in rat instead arises because as the SR Ca^{2+} content increases, spontaneous Ca^{2+} release occurs,

resulting in an efflux of Ca^{2+} from the cell, largely through the activity of the Na^{+}/Ca^{2+} exchanger.

3.8.4 Summary

In summary, the results of this work show that inhibition of the SR Ca^{2+} release channel causes an initial cessation of spontaneous SR Ca^{2+} release. However, the consequent increase of SR Ca^{2+} content overcomes the inhibition by 100 μM tetracaine, resulting in larger amplitude, but less frequent, Ca^{2+} releases such that cell Ca^{2+} homeostasis is maintained. In other words, given the need to maintain cell Ca^{2+} homeostasis, one obtains the initially unexpected result that inhibiting the Ca^{2+} release process can *increase* the magnitude of subsequent spontaneous Ca^{2+} releases. This result may well have implications for the different patterns of spontaneous Ca^{2+} release seen in other cell types (See [36] for review).

It has also been demonstrated that the Ca^{2+} content of the SR is limited by leak from the SR rather than by the ability of the SR Ca^{2+}-ATPase to pump Ca^{2+} against its concentration gradient. This suggests that the thermodynamic limit of the Ca^{2+} pump is very much higher than that normally reached even under the pathological conditions of Ca^{2+} overload.

Chapter 4

CHAPTER 4

THE EFFECT OF TETRACAINE ON THE SPATIAL AND TEMPORAL PROPERTIES OF SPONTANEOUS Ca^{2+} WAVES

Interventions that cause an increase in resting $[Ca^{2+}]_i$, such as increased $[Ca^{2+}]_o$, cardiac glycoside intoxication, can lead to the occurrence of propagating SR Ca^{2+} release (Ca^{2+} waves or oscillations) [289]. This form of Ca^{2+} release does not depend on an electrically stimulated Ca^{2+} current as a trigger. Spontaneous discharges of Ca^{2+} from the SR begin locally and then propagate as regenerative Ca^{2+} waves throughout the cell. These Ca^{2+} waves are particularly important because they can be responsible for contractile dysfunction and triggered arrhythmias [68, 137]. They are initiated at varying positions within a cell, but are usually fairly uniform in amplitude and propagation rate [358]. Ca^{2+} sparks have been implicated in the initiation of Ca^{2+} waves [84, 86] under conditions of Ca^{2+} overload. Typically Ca^{2+} waves propagate along the length of a myocyte at 100 $\mu m.s^{-1}$ at 22 °C [358] and have the spatial and temporal properties consistent with the hypothesis that Ca^{2+}-induced release of Ca^{2+} is mediated by the diffusion of Ca^{2+} from a site of initiation [17]. The frequency and propagation velocity of Ca^{2+} waves is highly dependent on bathing Ca^{2+}, which will in turn affect $[Ca^{2+}]_i$ [218].

The propagation of Ca^{2+} release along the cell could be caused by the following mechanisms: i) Ca^{2+} released spontaneously from one site may act on the cytoplasmic site of adjacent ryanodine receptors in a similar manner to that

involved during normal EC-coupling, thereby activating further Ca^{2+} release. ii) Ca^{2+} could be taken up into the SR surrounding the initial release site, where it becomes available to act at an intraluminal site. iii) a combination of these two effects where intraluminal Ca^{2+} increases the sensitivity of the SR release channel to cytosolic Ca^{2+}. Recent observations by Lukyanenko *et al* [246] that thapsigargin, an inhibitor of SR Ca^{2+} uptake, does not inhibit the propagation of Ca^{2+} waves, refute the second hypothesis. Instead thapsigargin actually increased the rate of Ca^{2+} wave propagation, so Ca^{2+} uptake from the approaching wave front is clearly not required for and may actually inhibit wave propagation. In support of the third hypothesis, increased intraluminal Ca^{2+} has been demonstrated to increase the sensitivity of Ca^{2+} release channels to cytosolic Ca^{2+} in lipid bilayers [174, 245, 335-337, 376, 406], and could explain the ability of Ca^{2+} release to propagate *only* under conditions of Ca^{2+} overload [86, 263, 286].

4.1 Aims of This Chapter

This chapter describes the use of line-scan images generated by laser-scanning confocal microscopy to examine the propagation of Ca^{2+} waves in Ca^{2+} overloaded rat ventricular myocytes. Tetracaine has been shown in the previous chapter to inhibit SR Ca^{2+} release and to increase the Ca^{2+} content of the SR [294] (See Chapter 5 [295]). It has been shown that, at least at low concentrations, tetracaine only temporarily suppresses spontaneous (sparks and waves) and electrically stimulated Ca^{2+} release [157, 294, 295]. The transient nature of this inhibition has been attributed to increases in the SR Ca^{2+} load, increasing the availability of Ca^{2+} for release and increasing the gain of CICR further through the

effects of luminal Ca^{2+} on the Ca^{2+} release channel. In this chapter the effect of different concentrations of tetracaine on the propagation of Ca^{2+} waves has been investigated. It is hoped that this will provide further insight into the effects of modulating CICR and SR Ca^{2+} load.

4.2 The Amount of Ca^{2+} Released during a Ca^{2+} Wave

The previous chapter describes estimates of the amplitude of Ca^{2+} waves during Ca^{2+} overload by measurement of global $[Ca^{2+}]_i$ using Indo-1 fluorescence emission ratio, changes in cell length and voltage-clamp measurement of the Na^+/Ca^{2+} exchange current activated by Ca^{2+} release. Each of these methods suffers from a number of problems, which could lead to false interpretation of a change in the signal amplitude as a change in the amplitude of Ca^{2+} release. Their main disadvantage stems from the fact that they represent global Ca^{2+} signals, i.e. signal averages over the whole cell and therefore suffer from the fact that they will not show local events, which Ca^{2+} waves and sparks have clearly been demonstrated to be [359, 397].

4.2.1 Global $[Ca^{2+}]_i$ Measurement

Global Ca^{2+} signals take no account of the area of the cell occupied by a Ca^{2+} wave. In addition, manoeuvres that affect the rate of propagation or SR Ca^{2+} uptake will affect the global Ca^{2+} signal even if the peak amplitude of the Ca^{2+} wave is unchanged. If the Ca^{2+} wave spreads out because Ca^{2+} uptake into the SR is slowed, this will increase the area of the cell occupied by the Ca^{2+} wave,

thereby increasing the global Ca^{2+} signal and contraction, without any change in the amount of Ca^{2+} released.

4.2.2 Cell Length Measurement

Cell length measurement suffers from even more limitations than global Ca_i measurement. A cell attached to the glass cover slip is particularly problematic. The Ca^{2+} wave activates the contraction of local sarcomeres as it travels along the myocyte. Areas that are actively contracting will pull relaxed areas along, and if a portion of the cell is attached to the base of the bath, it may cause stretching rather than movement and therefore less movement at the edges of the cell, than if it were free from its attachments. Furthermore, manoeuvres that affect the Ca^{2+} sensitivity of the myofilaments will affect the degree of contraction observed for a given increase in $[Ca^{2+}]_i$.

Figure 4.1 illustrates the effect of the site of initiation of a Ca^{2+} wave on global Ca_i measurement and contraction. When a Ca^{2+} wave is initiated near the end of a cell, it propagates as a single band of elevated $[Ca^{2+}]_i$ and contraction. If a Ca^{2+} wave is initiated toward the centre of the cell, it propagates in both directions resulting in two bands of elevated $[Ca^{2+}]_i$ and localised sarcomere shortening, and this will result in an increased global Ca_i signal and change in cell length. A Ca^{2+} wave initiated at the centre will generate a larger increase in global Ca^{2+} signal and an increased contraction amplitude, but will be of shorter duration in comparison to a Ca^{2+} wave initiated at the end of a cell.

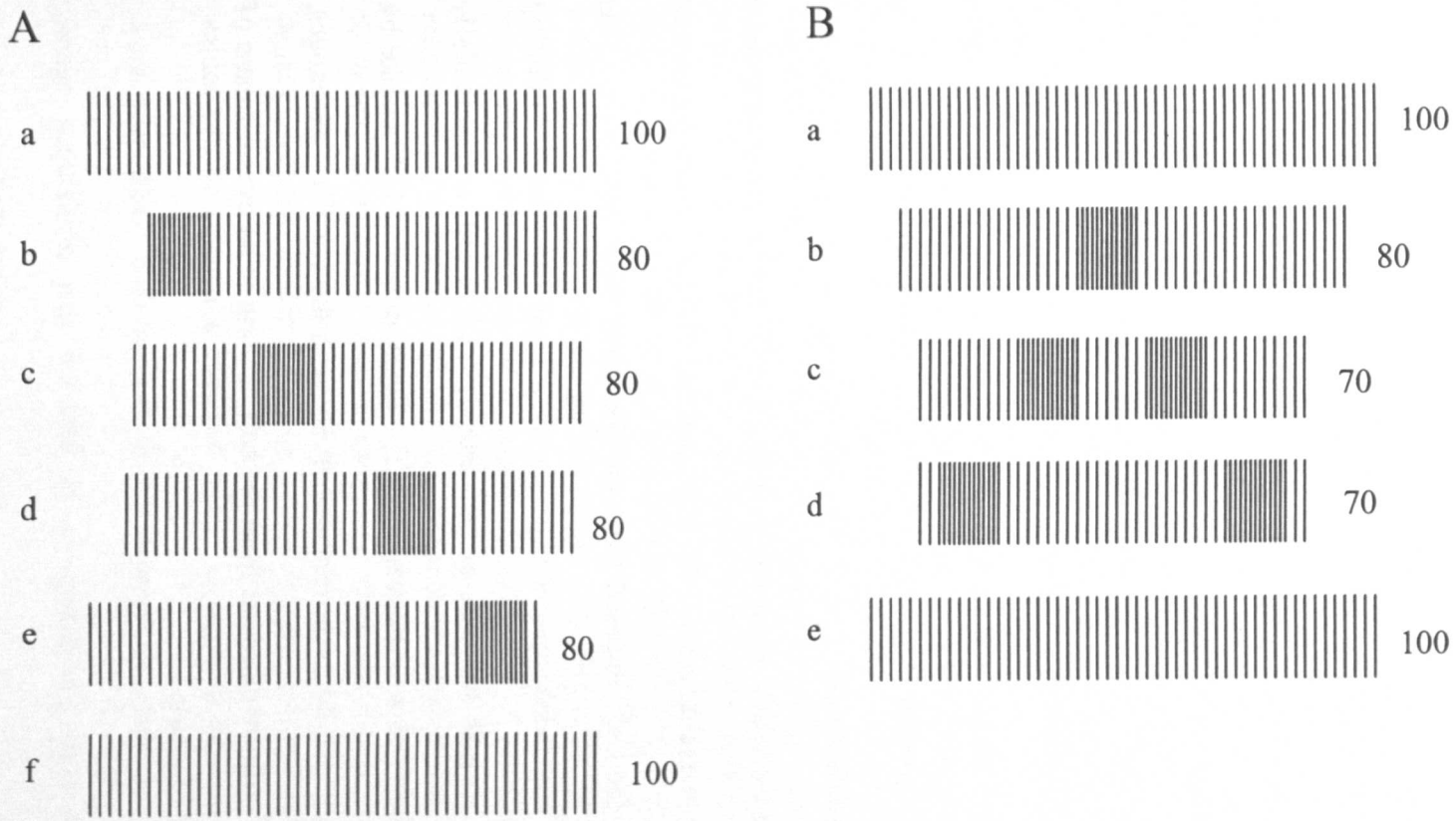

Figure 4.1. Schematic diagram of localised propagating myofilament activation initiated by spontaneous Ca^{2+} release.

A, a wave initiated near the edge of a cell propagates mono-directionally across the cell, giving rise to a single band of contraction. **B**, a wave initiated near the centre of the cell will propagate bi-directionally, giving rise to two discrete contractile bands. a – f represent the progression of time. The degree of cell shortening is represented as a percentage of the resting cell length indicated to the right of the figures. Adapted from [220].

4.2.3 The Magnitude of Na^+/Ca^{2+} Exchange Current

The integral of the Na^+/Ca^{2+} exchange current activated by Ca^{2+} release provides a direct measure of the amount of Ca^{2+} extruded from the cell during a spontaneous oscillation and is therefore an indication of the amount of Ca^{2+} released from the SR. However, comparing the amplitude of this integral under different experimental conditions relies upon the assumption that the same proportion of the Ca^{2+} released from the SR is extruded from the cell. Intracellular Na^+ will affect this: If $[Na^+]_i$ is increased, for example due to Na^+/K^+ pump inhibition, Ca^{2+} efflux on the exchanger becomes less favourable because of the reduced driving force for Na^+ entry. Under such conditions, more Ca^{2+} will be released due to the increased $[Ca^{2+}]_i$ but the Na^+/Ca^{2+} exchange current will under-estimate by how much. Conversely, if $[Na^+]_i$ is decreased, the driving force for Ca^{2+} efflux on the exchanger will be increased and Na^+/Ca^{2+} exchange current may over-estimate the Ca^{2+} released from the SR under these conditions. This could be the case with tetracaine, which acts as a local anaesthetic, inhibiting sarcolemmal Na^+ channels and leak of Na^+ into the cell at rest, and potentially reducing aNa_i as observed with lidocaine [112]. Furthermore, if Ca^{2+} uptake by the SR is inhibited, it will compete less effectively with the Na^+/Ca^{2+} exchanger, such that more Ca^{2+} will be extruded from the cell, leading to an over-estimate of the amplitude of Ca^{2+} release.

4.2.4 Line-scan Images of Propagating Ca^{2+} Release

Line-scan images of propagating Ca^{2+} release provide important spatial and temporal information by directly generating a plot of cell length and fluorescence intensity against time [400], allowing observation of Ca^{2+} release at all points along the scan line with respect to time. The horizontal dimension of the image represents distance along the scan line, while the vertical dimension represents increasing time (from top to bottom). Images were enhanced where appropriate using the "smoothing" facility of the Lasersharp software (Bio-Rad, UK) during off-line analysis.

Fluo-4 loaded rat ventricular myocytes were superfused with a control Tyrode solution containing 2 mM Ca^{2+}, to induce Ca^{2+} overload. Cells exhibiting frequent spontaneous Ca^{2+} waves were selected for study. Each cell was aligned horizontally and a scan line was selected parallel with the longitudinal axis of the cell. This was achieved using a scan rotator (Scientific Systems Design Inc, UK). Unfortunately rotation distorted the image such that cells appeared longer in the horizontal plane and narrower in the vertical plane of x-y images. It should be emphasised that the horizontal scale (given in microns in the figures and referred to in text to point out specific events) is therefore not an accurate measure of distance because of the distortion caused by the scan rotator, and only relative values can be compared between cells. To achieve this, propagation velocities were normalised with respect to the average propagation velocity in control. Image acquisition was initiated at an appropriate time under the different experimental conditions.

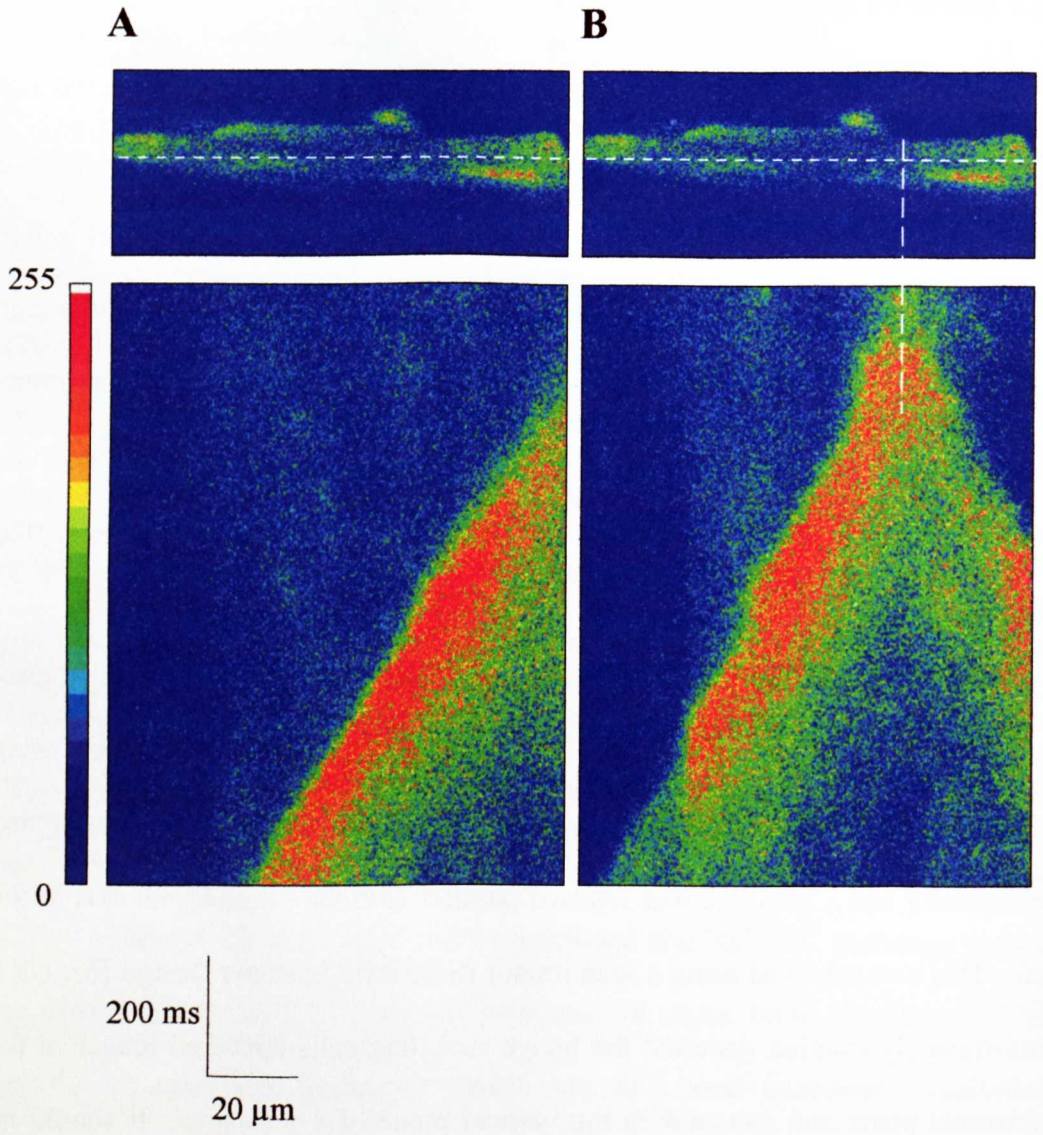

Figure 4.2. Typical confocal line-scan images of propagating Ca^{2+} waves.

Upper panels illustrate resting rat ventricular myocytes which were repeatedly scanned along a line running parallel with their longitudinal axis, for example as illustrated by the dashed line. **A**, typical line-scan image showing a spontaneous Ca^{2+} wave initiated at the edge of the cell and propagating mono-directionally towards the opposite end of the cell. **B**, typical line-scan image showing a centrally initiated spontaneous Ca^{2+} wave. In this case the Ca^{2+} wave propagates simultaneously towards both ends of the cell forming the typical inverted "V" pattern. The bar to the left of the images represents the fluorescence intensity scale.

Figure 4.2 illustrates typical line-scan images of a Ca^{2+} wave propagating along the cell. The upper panels show an x-y image of the cell being scanned. The dashed line represents an example of a selected scan line. Panel A illustrates a Ca^{2+} wave initiated at the right-hand edge of the cell, characterised by a single linear band of fluorescence travelling from right to left with time. This corresponds to the schematic example illustrated in Fig. 4.1A. Panel B shows a Ca^{2+} wave initiated more centrally, approximately one third of the total distance from the right-hand edge of the cell. The wave clearly propagates in both directions giving rise to the characteristic inverted "V" fluorescence pattern on the line-scan image as the wave of Ca^{2+} propagates simultaneously towards both ends of the cell (corresponding to Fig. 4.1B). The slope of the linear portions of these fluorescent bands gives an indication of the rate of propagation of the Ca^{2+} wave along the cell. In panel B the point of initiation of the wave in the resting myocyte is indicated by the intersection of the dashed scan line and the position in the line-scan image corresponding to the inflection of the inverted "V".

4.3 The Effect of Tetracaine on Ca^{2+} Wave Amplitude and Propagation Velocity

Figure 4.3 illustrates the effect of 200 μM tetracaine on the spatial and temporal properties of spontaneous waves of Ca^{2+} release in an isolated rat myocyte. During exposure to 200 μM tetracaine in this cell there was a clear increase in the peak fluorescence intensity associated with spontaneous Ca^{2+} release, suggestive of an increase in the amplitude of the Ca^{2+} waves. This is consistent with previous observations of the increased amplitude of global Ca^{2+}

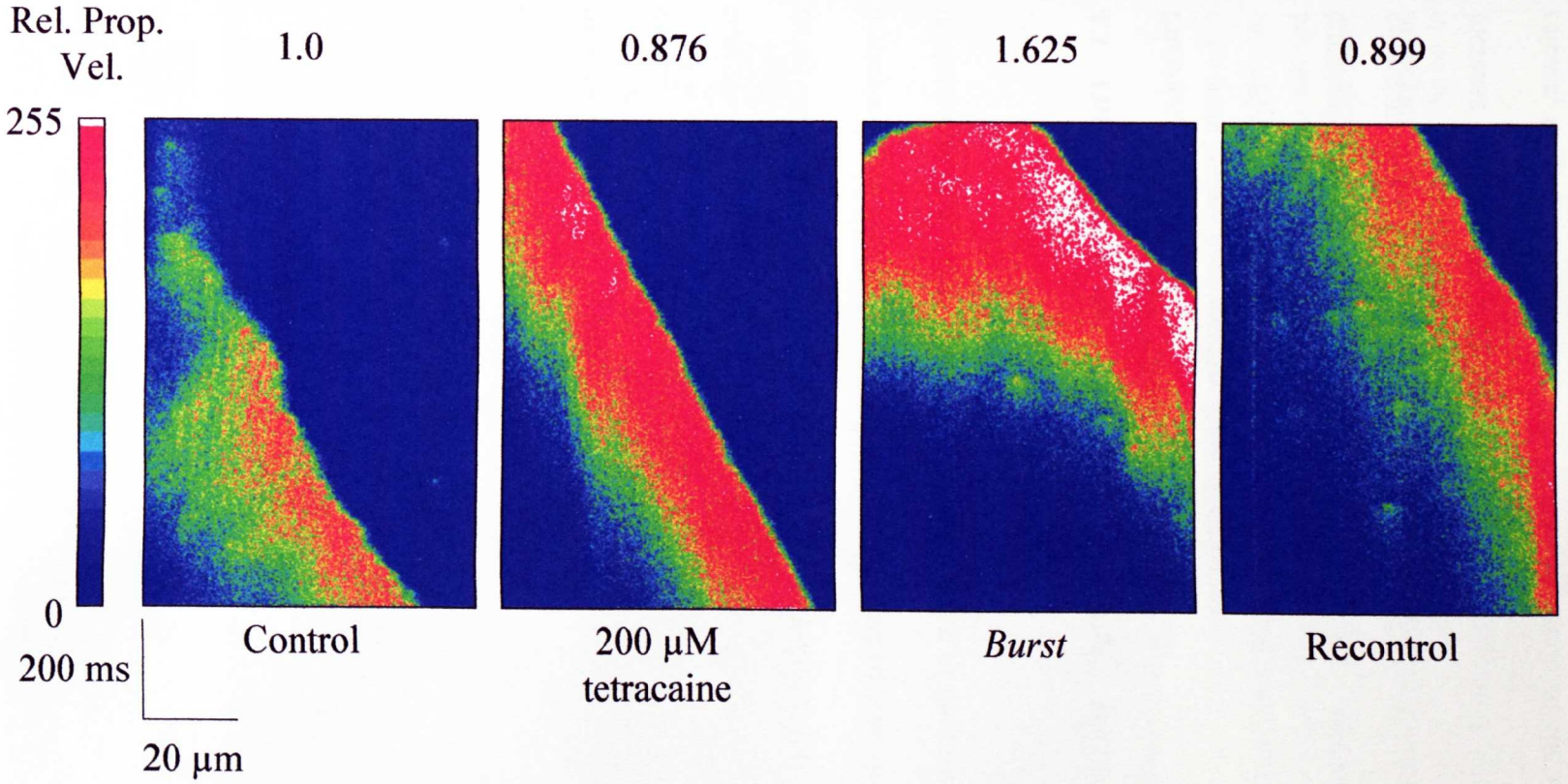

Figure 4.3. The effect of 200 µM tetracaine on the properties of Ca^{2+} waves in an isolated rat ventricular myocyte.

Panels illustrate line-scan images of typical Ca^{2+} waves during (from left to right); superfusion with a control 2 mM Ca^{2+} Tyrode solution; exposure to 200 µM tetracaine; the *burst* on removal of 200 µM tetracaine; and after the recovery of control oscillation frequency on return to control conditions ("recontrol"). The numbers above the images represent the values of propagation velocity for the Ca^{2+} waves shown, relative to that of the control waves shown. The bar to the left of the images represents the fluorescence intensity scale.

signals (See Chapter 3, Fig. 3.4), increased cell shortening (Fig. 3.1), and the increased Na^{+}/Ca^{2+} exchange current integral (Fig. 3.5) observed during exposure to 100 μM tetracaine. The rate of propagation of Ca^{2+} release along the cell in the example shown was ***slightly decreased*** during exposure to tetracaine, as indicated by a slight increase in the slope of the fluorescent band from left to right in the second panel compared to the control image. This is consistent with the hypothesis that tetracaine inhibits the propagation of Ca^{2+} release. The larger amplitude of Ca^{2+} release is probably due to the increased SR Ca^{2+} content likely to be experienced under conditions of tetracaine exposure (See Chapter 3). The compensatory effects of the increased SR Ca^{2+} content causing increased SR Ca^{2+} release could explain the fact that propagation velocity is only slightly affected by tetracaine in this cell. During the *burst* of spontaneous activity on removal of tetracaine (third panel) the amplitude of Ca^{2+} release was elevated further, at first, and propagation velocity increased to a maximum of 232.0 % of the average control velocity. The amplitude and propagation velocity of Ca^{2+} release then fell to the control levels eventually as illustrated by the recontrol line-scan image. This is consistent with the hypothesis that tetracaine inhibits the propagation of Ca^{2+} waves and when this inhibition is removed Ca^{2+} release can propagate more easily. Recontrol illustrates a Ca^{2+} wave after recovery of the control frequency and amplitude of Ca^{2+} release suggestive of recovery of the control SR Ca^{2+} content. In this cell, propagation velocity during exposure to tetracaine was approximately the same as the average under control conditions.

Figure 4.4 illustrates the effect of 200 μM tetracaine on Ca^{2+} wave properties in a different cell. In this example, 200 μM tetracaine caused an

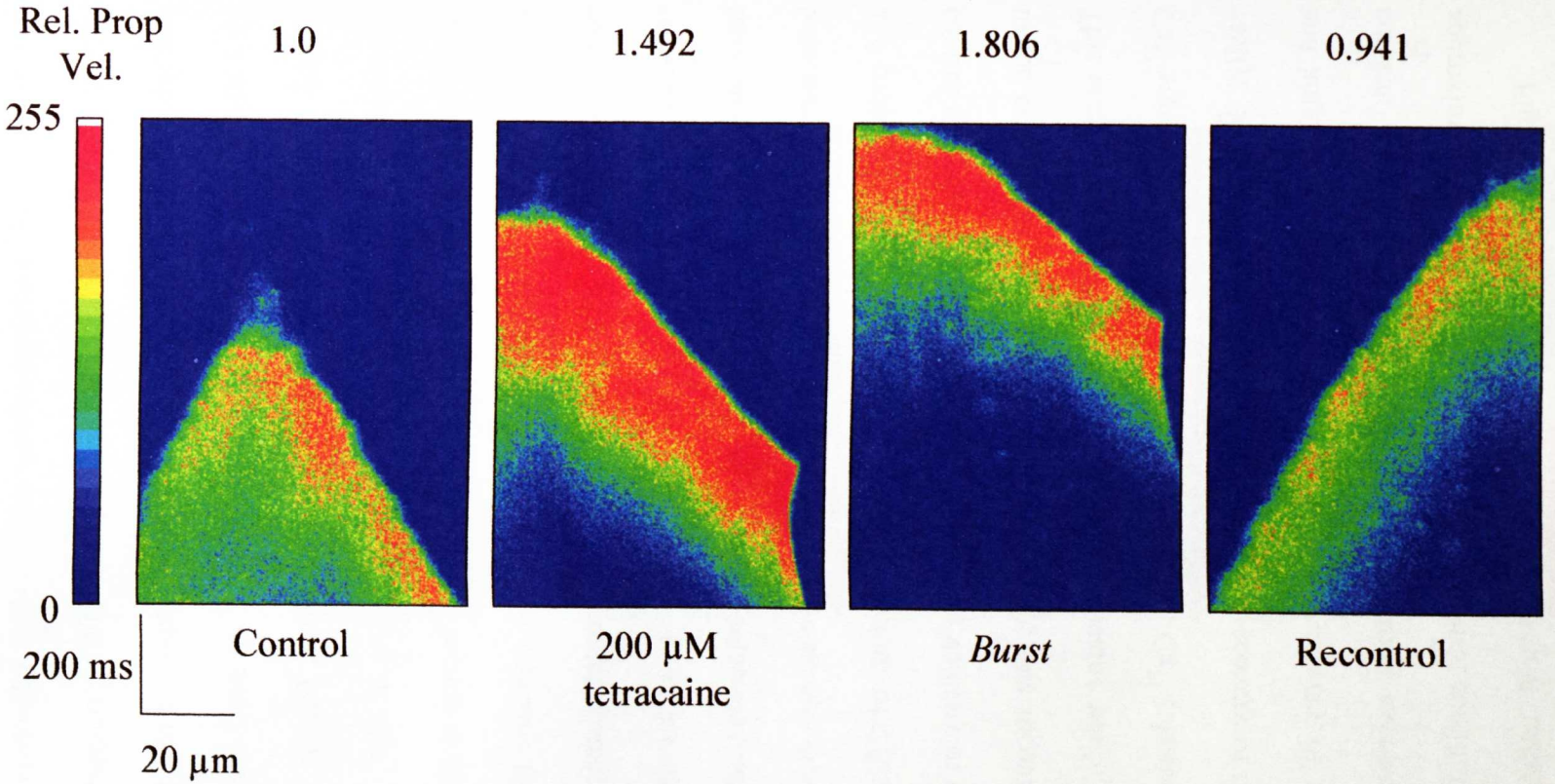

Figure 4.4. The effect of 200 µM tetracaine on the properties of Ca^{2+} waves in an isolated rat ventricular myocyte.

Panels illustrate line-scan images of typical Ca^{2+} waves during (from left to right); superfusion with a control 2 mM Ca^{2+} Tyrode solution; exposure to 200 µM tetracaine; the *burst* on removal of 200 µM tetracaine; and after the recovery of control oscillation frequency on return to control conditions ("recontrol"). The numbers above the images represent the values of propagation velocity for the Ca^{2+} waves shown, relative to that of the control waves shown. The bar to the left of the images represents the fluorescence intensity scale.

increase in wave amplitude and a more significant increase in propagation velocity. On removal of tetracaine a Ca^{2+} wave was elicited that was larger than those observed under control conditions, but smaller than those during exposure to tetracaine were. Its rate of propagation was increased beyond that of the wave observed in control and during exposure to 200 μM tetracaine. This observation suggests that in control conditions, waves of smaller amplitude are capable of propagating faster than waves of even higher amplitude in the presence of tetracaine. This supports the hypothesis that propagation of Ca^{2+} release is inhibited in the presence of tetracaine. When this inhibition is relieved propagation is potentiated above the control level because of the elevated SR Ca^{2+} content and the larger amplitude Ca^{2+} release. Ca^{2+} wave amplitude and propagation velocity again eventually fell towards the control level following removal of tetracaine (*Recontrol* panel).

4.4 The Concentration Dependence of the Effect of Tetracaine on the Properties of Spontaneous Ca^{2+} Release

Figure 4.5 compares the effect of 100 μM tetracaine and 500 μM tetracaine on the amplitude and propagation velocity of spontaneous Ca^{2+} waves in another rat ventricular myocyte. It is clear that 100 μM tetracaine increased the amplitude (fluorescence intensity) of the Ca^{2+} wave and its rate of propagation, as indicated by the decreased slope from right to left in the second image compared to control. Propagation velocity ***in this cell*** was increased by 63.9 ± 3.7 %, on average (*number of waves* = 12) during exposure to tetracaine, above the mean propagation velocity in control (*number of waves* = 11). With 500 μM tetracaine

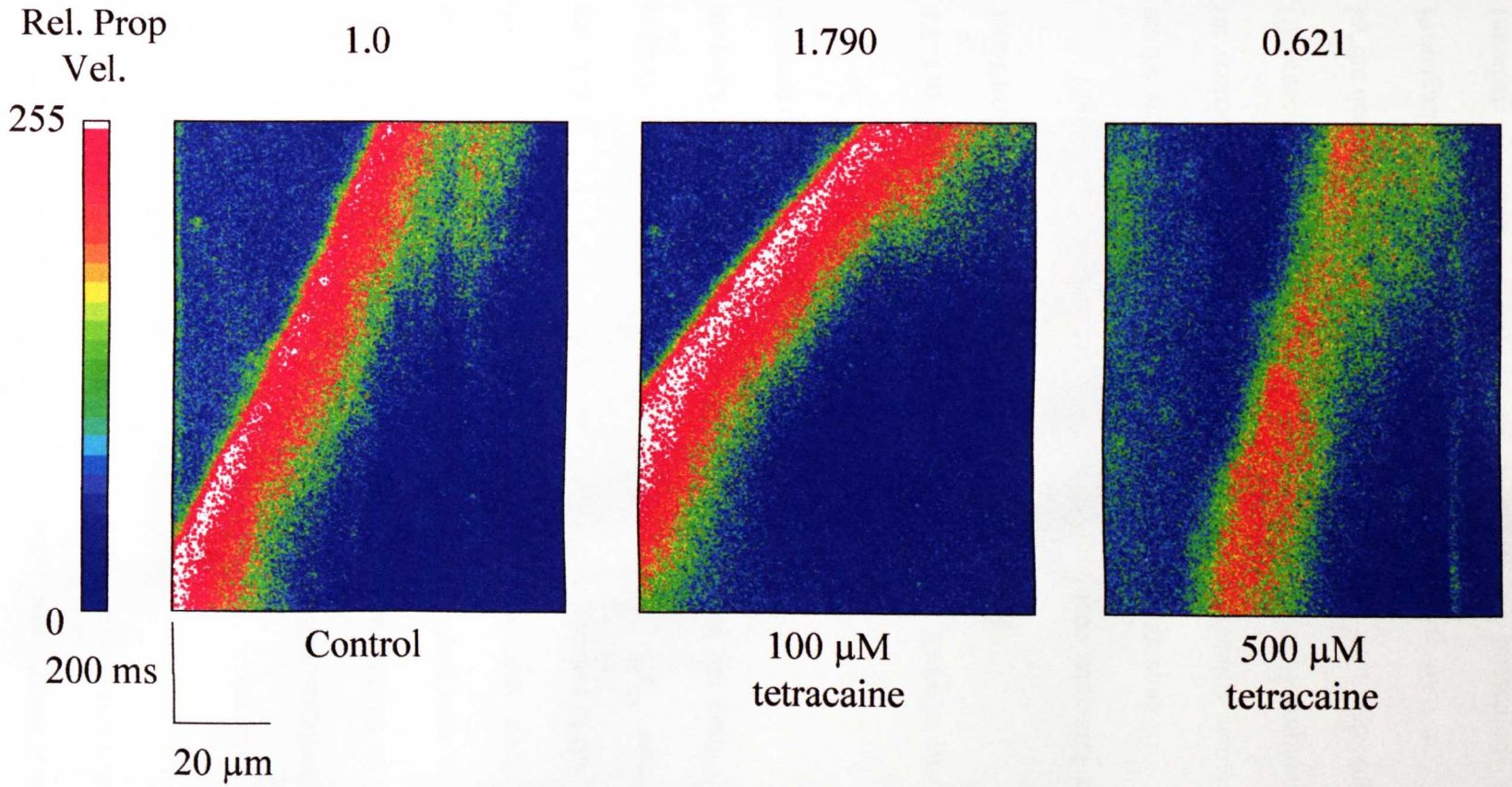

Figure 4.5. Comparison of the effects of 100 µM and 500 µM tetracaine on the properties of Ca^{2+} waves in an isolated rat ventricular myocyte.

Panels illustrate line-scan images of typical Ca^{2+} waves (from left to right); under control conditions (2 mM Ca^{2+}), during exposure to 100 µM tetracaine and 500 µM tetracaine. The numbers above the images represent the values of propagation velocity for the Ca^{2+} waves shown, relative to that of the control waves shown. The bar to the left of the images represents the fluorescence intensity scale.

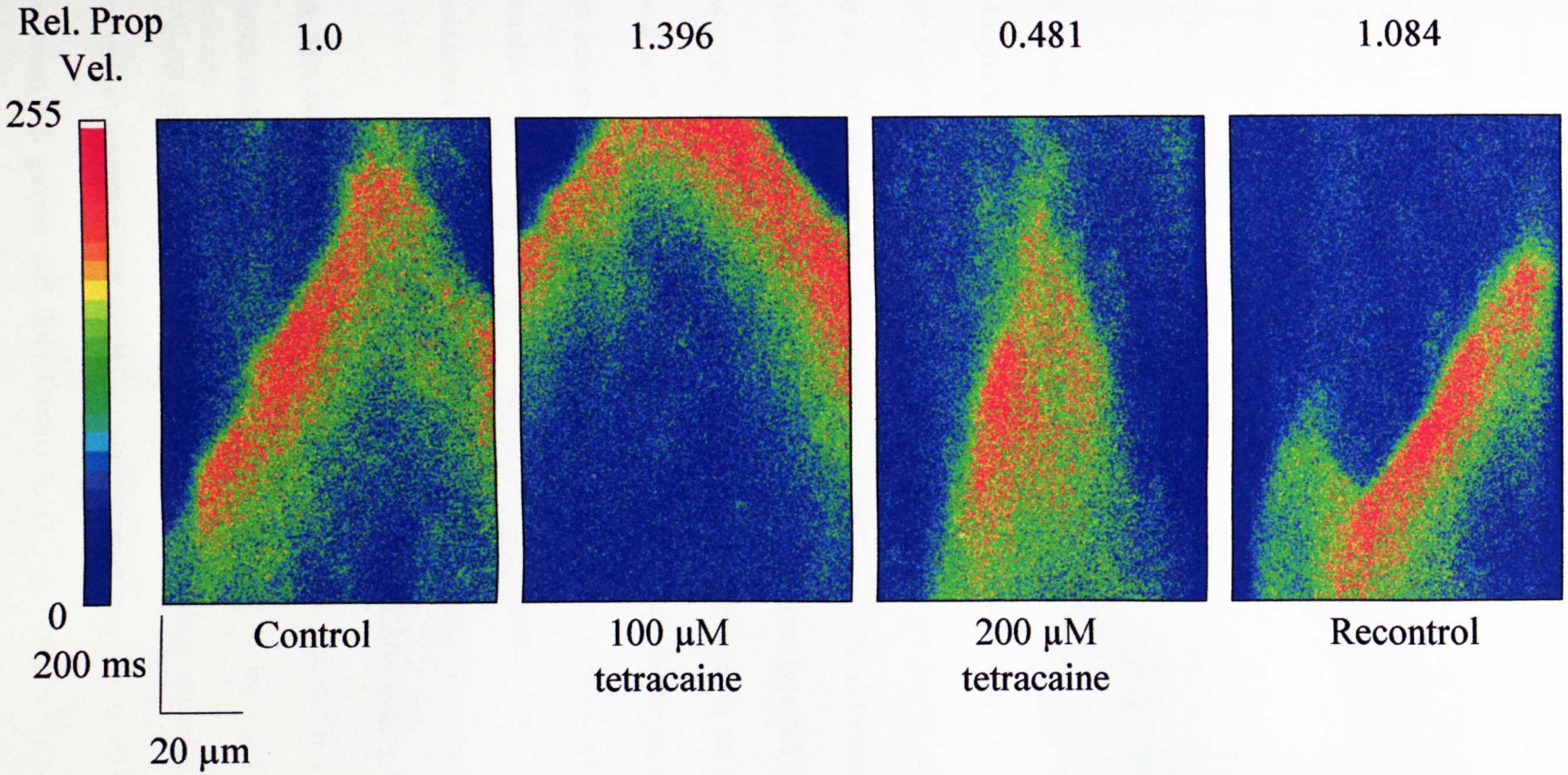

Figure 4.6. Comparison of the effect of 100 µM and 200 µM tetracaine on the properties of Ca^{2+} waves in an isolated rat ventricular myocyte.

Panels illustrate line-scan images of typical Ca^{2+} waves (from left to right); under control conditions (2 mM Ca^{2+}), during exposure to 100 µM tetracaine, 200 µM tetracaine and after recovery of control oscillation frequency on return to control conditions ("recontrol"). The numbers above the images represent the values of propagation velocity for the Ca^{2+} waves shown, relative to that of the control waves shown. The bar to the left of the images represents the fluorescence intensity scale.

the amplitude of Ca^{2+} release was decreased in comparison with control and the rate of propagation of the Ca^{2+} wave was also decreased to 58.5 % (*number of waves* = 1) of the average control velocity. This observation is consistent with the hypothesis that propagation velocity is dependent on the amplitude of Ca^{2+} release.

In the example illustrated in Figure 4.6, comparing the effect of 100 μM and 200 μM tetracaine in a ***different cell***, 100 μM tetracaine increased Ca^{2+} wave amplitude slightly. Average propagation velocity was increased by only 14.6 ± 15.2 % (*number of waves* = 9) above the average control velocity (*number of waves* = 8). During exposure to 200 μM tetracaine Ca^{2+} waves were of slightly smaller amplitude and propagation velocity was decreased to 51.7 ± 8.2 % of the average under control conditions. This is in contrast with the examples shown in Figs. 4.4, where 200 μM tetracaine produced an increase in the amplitude of the Ca^{2+} wave and its propagation velocity. In this cell the effects of 200 μM tetracaine are more similar to those of 500 μM tetracaine in Fig. 4.5. It is also different from the observations of Fig. 4.3, where 200 μM tetracaine caused an increase of Ca^{2+} wave amplitude and marginal depression of Ca^{2+} release propagation velocity. It appears that intermediate concentrations of tetracaine show variability in their effects on the properties of Ca^{2+} release.

Figure 4.7 illustrates a histogram showing average data of the rate of propagation of Ca^{2+} waves in isolated rat ventricular myocytes. Normalised propagation velocity was increased during exposure to 100 μM tetracaine by 29.6 ± 11.0 % ($P < 0.05$, $n = 9$) above control (Fig. 4.7A). It was increased further, to 168.9 ± 28.5 % ($P < 0.05$, $n = 8$) of control (100 %), during the burst of

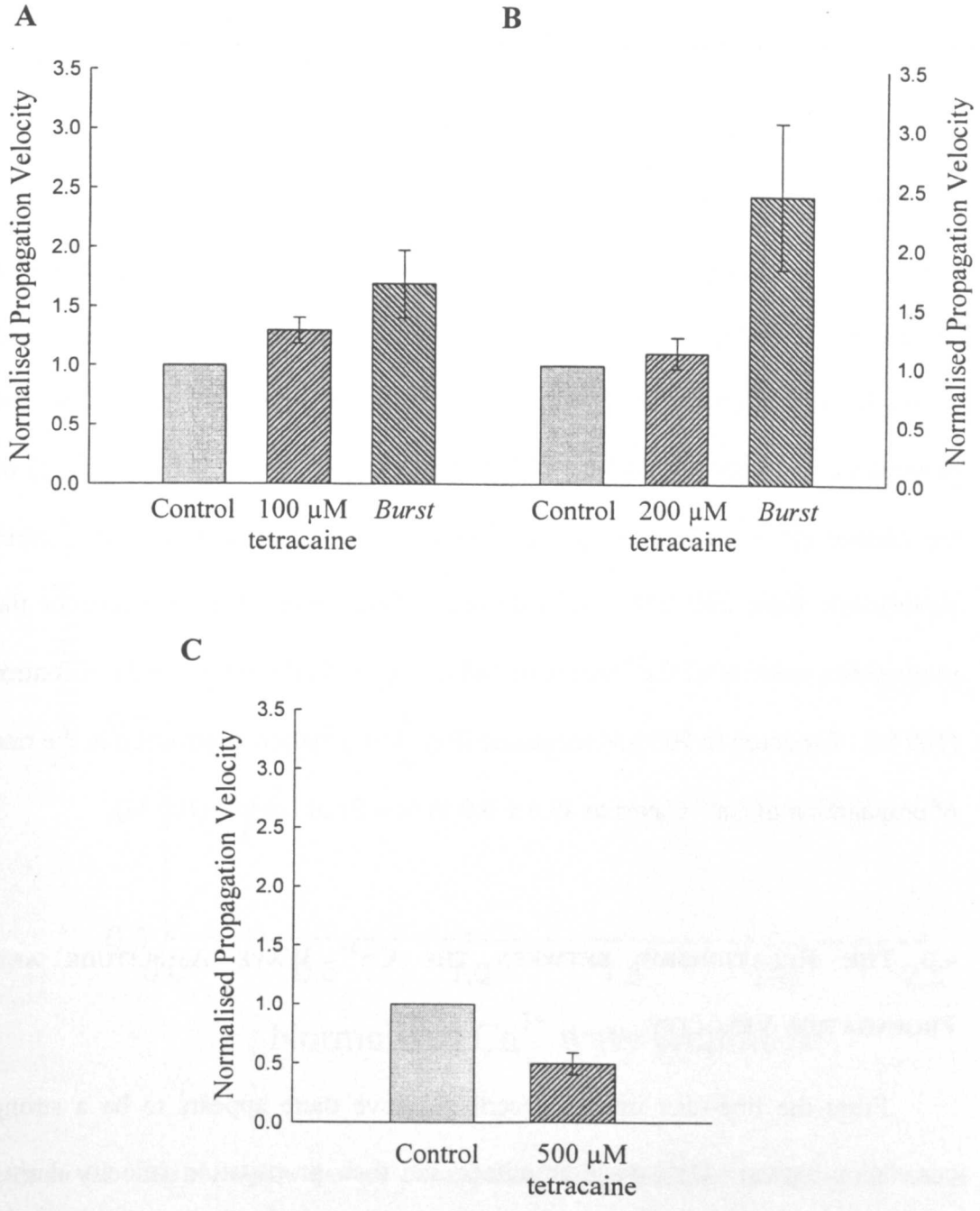

Figure 4.7. The effect of tetracaine on the propagation velocity of Ca^{2+} waves.

Histograms showing mean data of the effect on normalised propagation velocity of: **A**, 100 µM tetracaine ($P < 0.05$, $n = 9$) and its removal ($P < 0.05$, $n = 8$); **B**, 200 µM tetracaine ($P > 0.05$, $n = 7$) and its removal ($P < 0.05$, $n = 3$); **C**, 500 µM tetracaine ($P > 0.05$, $n = 2$).

spontaneous activity following removal of 100 μM tetracaine. This is consistent with the increased SR Ca^{2+} load of the cell overcoming the inhibition imposed by tetracaine, by luminal effects on the SR release channel or by the increased flux from the SR having increased capacity to activate Ca^{2+} release from adjacent SR sites. During exposure to 200 μM tetracaine 2 out of 7 cells demonstrated a reduction in propagation velocity. The remainder showed an increase in propagation velocity. On average there was no significant change in the propagation velocity (Fig. 4.7B), which was calculated to be 111.1 ± 13.3 % of the control ($P > 0.05$, $n = 7$). In 3 cells the burst on returning to control superfusion from 200 μM was analysed. This revealed an increase in the propagation velocity of Ca^{2+} waves to 244.0 ± 61.5 % ($P < 0.05$, $n = 3$) of control (100 %). Exposure to 500 μM tetracaine (Fig. 4.9C) caused a reduction in the rate of propagation of Ca^{2+} waves to 49.6 ± 9.0 % ($n = 2$) of control (100 %).

4.5 The Relationship between the Ca^{2+} Wave Amplitude and Propagation Velocity

From the line-scan images described above there appears to be a strong correlation between Ca^{2+} wave amplitude and their propagation velocity during exposure to tetracaine. This was investigated more directly by producing a plot of propagation velocity against Ca^{2+} wave amplitude (both normalised with respect to mean control values) during exposure to tetracaine. The different symbols represent the different concentrations of tetracaine applied to the cells. There appears to be a linear relationship between the rate of propagation of a Ca^{2+} wave and the amplitude of Ca^{2+} release.

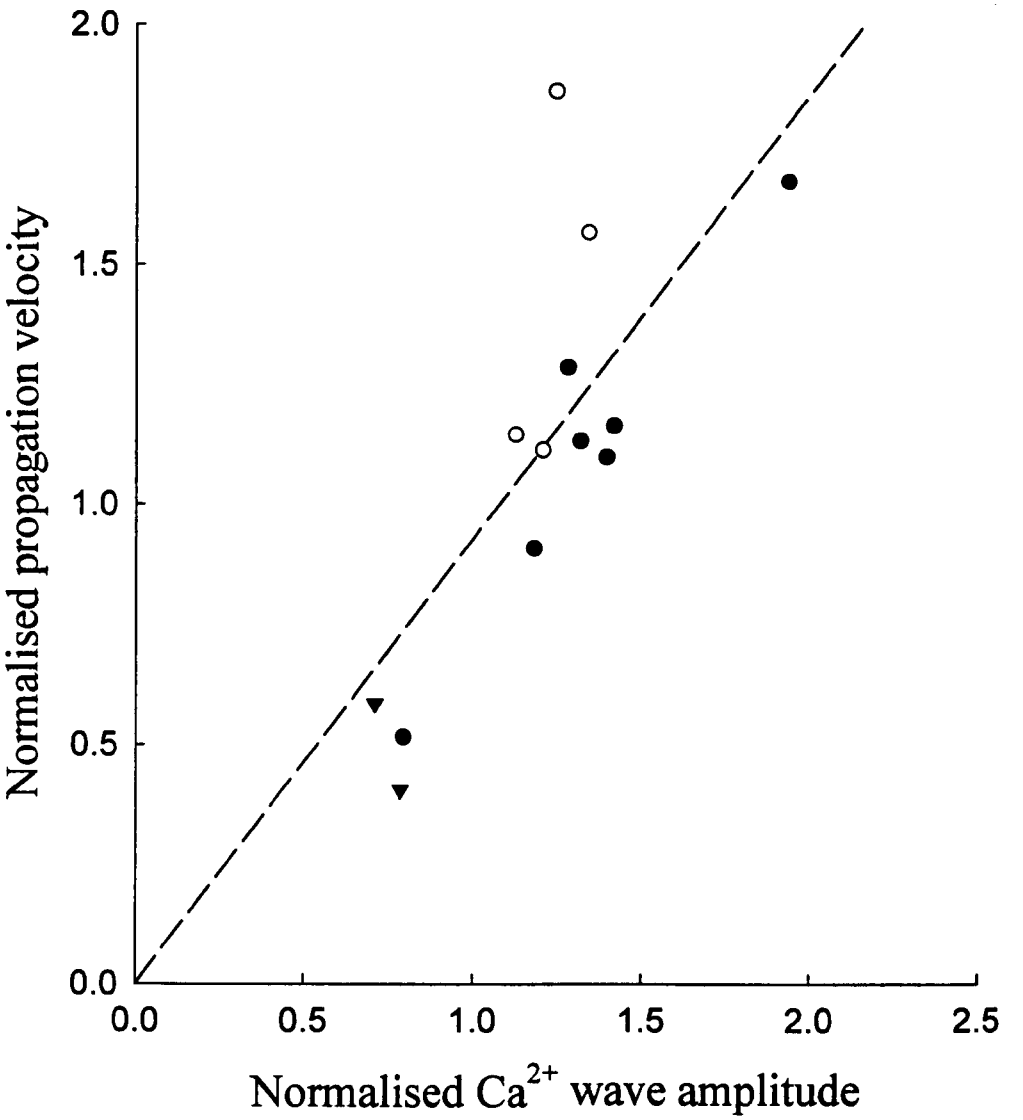

Figure 4.8. The correlation between the amplitude and propagation velocity of Ca^{2+} waves during exposure to tetracaine.

The different symbols represent the different concentrations of tetracaine applied, and each symbol represents a different cell exposed to that concentration: ○ 100 μM, ● 200 μM, ▼ 500 μM tetracaine. Average wave propagation velocity was normalised with respect to the control for each cell and plotted as the ordinate. The abscissa shows the corresponding average amplitude of the Ca^{2+} waves normalised with respect to control. The line is a best-fit linear regression of all the data points ($r^2 = 0.608$, $n = 13$).

4.6 DISCUSSION

One might expect that manoeuvres that inhibit CICR would decrease the ability of Ca^{2+} diffusing from an area of initiation to trigger Ca^{2+} release from adjacent SR. However, the results presented here show that in the presence of low concentrations of tetracaine ($\leq$ 200 μM) the propagation of SR Ca^{2+} release can be facilitated thereby potentiating the velocity of Ca^{2+} waves.

4.6.1 Tetracaine Inhibits the Propagation of Spontaneous SR Ca^{2+} Release

During the *burst* of spontaneous activity on removal of tetracaine, even waves of smaller amplitude in comparison to those during exposure to tetracaine are capable of propagating faster (Fig. 4.4), providing clear evidence that propagation velocity is inhibited during exposure to tetracaine. 500 μM tetracaine was capable of inhibiting Ca^{2+} release despite the predicted increase of SR Ca^{2+} content (See Chapter 3). This was demonstrated by its ability to reduce the amplitude of Ca^{2+} release and its ability to propagate along the cell. Ca^{2+} waves were very infrequent under these conditions and were often observed to terminate prematurely. Tetracaine is believed to act by desensitising the RyR to cytosolic Ca^{2+} [284, 309] (See Section 3.6), often producing exactly opposite effects to, and antagonising, those of the RyR sensitiser caffeine [115, 248]. The ability of tetracaine to inhibit the propagation of Ca^{2+} release is consistent with the ability of increasing the sensitivity of release sites to cytosolic Ca^{2+} by the focal application of caffeine to allow the propagation of Ca^{2+} release under conditions of normal SR

Ca^{2+} load [246, 374]. Interestingly the propagation velocity of Ca^{2+} waves initiated by focal application of caffeine was significantly lower during continued exposure to 500 μM caffeine [246]. This was also correlated with and attributed to the decreased amplitude of Ca^{2+} waves as a consequence of reduced SR Ca^{2+} load.

4.6.2 The Amplitude of SR Ca^{2+} Release is correlated with its Ability to Propagate

The inhibitory effects of tetracaine on CICR and the ability of SR Ca^{2+} release to propagate is compensated for in a number of cells (See Figs. 4.4, 4.5 & 4.6 during exposure to 100 μM tetracaine) by the increased amplitude of the Ca^{2+} release. Figure 4.8 clearly illustrates the strong correlation between Ca^{2+} wave amplitude and propagation velocity during exposure to tetracaine. Propagation is favoured by an elevated increase of $[Ca^{2+}]_i$ during spontaneous Ca^{2+} release, probably because of increased Ca^{2+} diffusion from the wave front due to its increased amplitude. This correlation between the Ca^{2+} wave amplitude and propagation velocity is consistent with the results of Trafford *et al* [374], using digital imaging fluorescence microscopy. The effect of low concentration of caffeine on Ca^{2+} wave properties has also been investigated [246, 327], revealing its ability to depress Ca^{2+} wave amplitude, probably as a result of decreased SR Ca^{2+} load, and the propagation velocity of Ca^{2+} release along the cell. Preliminary investigation of the properties of Ca^{2+} release during the *burst* of spontaneous activity observed on initial application of caffeine (c.f. the *burst* on removal of inhibition by tetracaine) suggests that caffeine can enhance Ca^{2+} wave

propagation in the absence of significant changes in SR Ca^{2+} load [Personal communication with Miss Gillian Sibbring and Dr. Andrew Trafford].

4.6.3 The Variable Sensitivity of Myocytes to Inhibition of Ca^{2+} Release by Tetracaine

There was considerable variability in the effects of 200 μM tetracaine on the amplitude and propagation characteristics of Ca^{2+} waves between different cells (Compare Figs. 4.3, 4.4 & 4.6). This is masked when looking at the mean data of Fig. 4.7, but highlighted by Fig. 4.8. Cells that were exposed to 500 μM tetracaine demonstrated lower amplitude Ca^{2+} waves of correspondingly lower propagation velocity. Cells exposed to 100 μM tetracaine occupied the upper right-hand area of the graph. This suggests that the greater the inhibition by tetracaine the smaller the amplitude of the Ca^{2+} wave, reducing its ability to propagate. The variability of the effect of a single concentration of tetracaine on Ca^{2+} wave properties appears to represent differences in the sensitivity of myocytes to tetracaine. However, it may represent changes in the availability of tetracaine to its intracellular sites of action (e.g. the RyR).

Local anaesthetics cross the cell membrane in their uncharged form. The ratio of the uncharged to charged form of an anaesthetic will therefore affect the amount of agent available to its sub-cellular sites of action. Lipid solubility is another major determinant of anaesthetic potency. Removal of the butyl group from tetracaine yields the local anaesthetic procaine (Fig. 4.9A); an anaesthetic with significantly lower potency because of its 25-fold reduced lipid solubility (See hydrophobic partition coefficients [387, 404]). The ratio of the charged

($[RH^+]$) to uncharged ($[R]$) form of an anaesthetic at different pH values can be calculated from the pKa using the Henderson-Hasselbach equation (given below). Assuming the pKa of tetracaine to be 8.24 [50];

$$\log \frac{[RH^+]}{[R]} = pKa - pH$$

At pH 7.4, 12.6 % of tetracaine applied extracellularly exists in the uncharged, membrane permeable form (R). Procaine, on the other hand, with a pKa of 8.95 [50] is more than 97 % charged at pH 7.4 and even smaller quantities will partition into and cross lipid bilayers. The uncharged form of the anaesthetic will distribute itself equally across the cell membrane such that $[R]_e = [R]_i$. Applying a concentration of 100 μM tetracaine at pH 7.4 yields an external $[R]_e$ of 12.6 μM. The internal concentration will be equal ($[R]_i = 12.6$). From this it is possible to calculate the total concentration of tetracaine accumulated inside the cell ($[R]_i + [RH^+]_i$) and the concentration of the charged form ($[RH^+]_i$) for given intracellular pH values. Figure 4.9B shows a graph illustrating the effect of intracellular pH (pH_i) on the intracellular accumulation of tetracaine ($[tetracaine]_i = [R]_i + [RH^+]_i$) when applied at external concentrations of 100 μM and 200 μM. It is clear that small changes in pH_i around 7.2 can cause a significant change in intracellular tetracaine concentration and therefore the availability of tetracaine to intracellular site of action, i.e. the RyR. Decreasing intracellular pH by 0.2 units from 7.2 to 7.0 causes an increase of $[tetracaine]_i$ from 151 μM to 232 μM, an increase of more than 50 %.

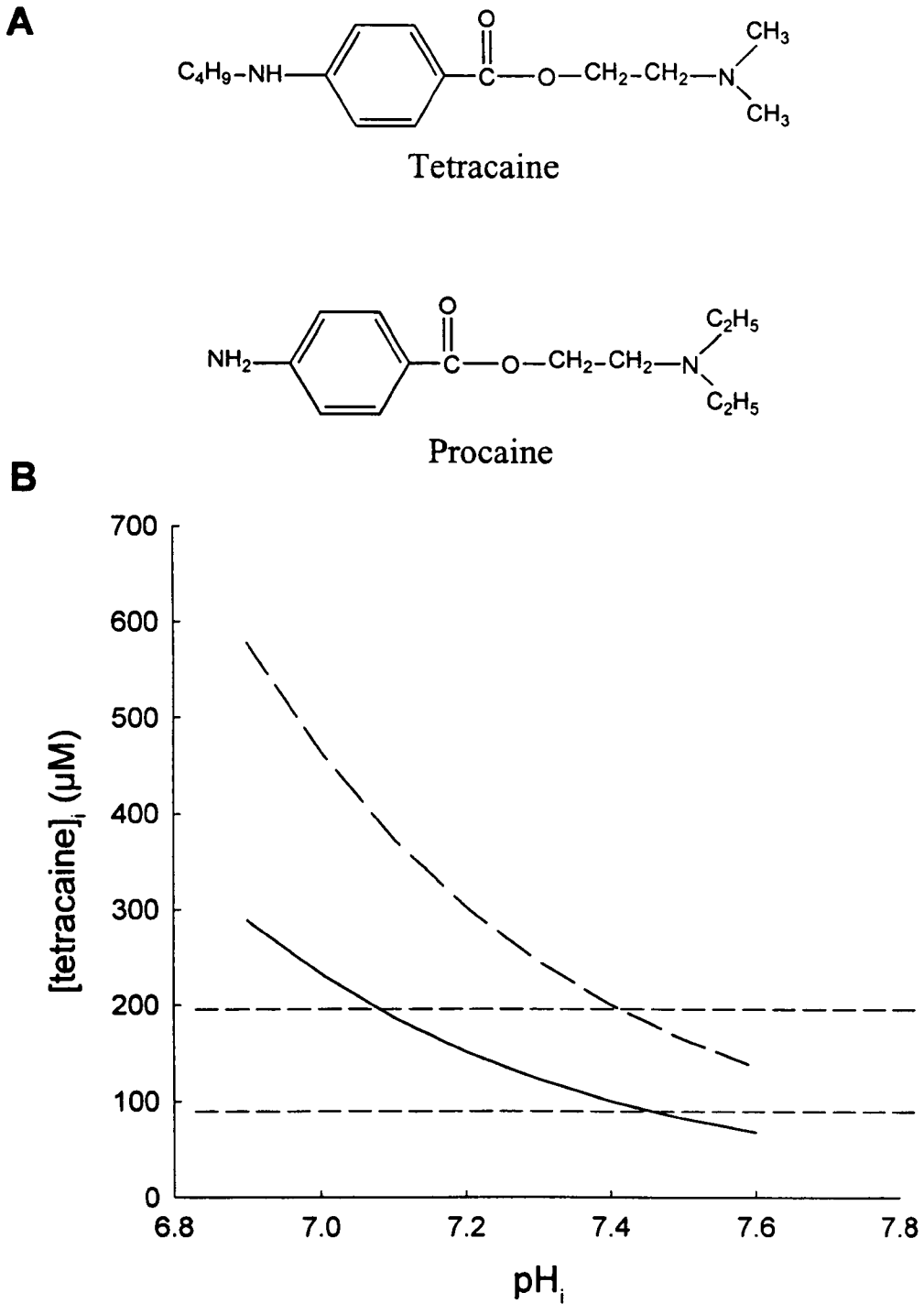

Figure 4.9. The effect of intracellular pH on the ability of cells to accumulate tetracaine.

A, the structure of tetracaine and procaine. **B**, the relationship between $[tetracaine]_i$ and pH_i. Graph shows $[tetracaine]_i$ calculated from the Henderson-Hasselbach equation (assuming the pKa of tetracaine = 8.24 [50], extracellular pH = 7.4, tetracaine applied at 100 μM and 200 μM) plotted as the ordinate. The abscissa shows corresponding intracellular pH values.

It is possible that differences in intracellular pH between cells could be a consequence of the variability in intracellular aNa_i recently found in rat ventricular myocytes [104]. This could affect intracellular pH through the activity of the Na^+/H^+ exchanger [119, 385].

Chapter 5

CHAPTER 5

THE EFFECT OF TETRACAINE ON STIMULATED CONTRACTIONS, SR Ca^{2+} CONTENT AND SARCOLEMMAL Ca^{2+} FLUXES IN RAT ISOLATED VENTRICULAR MYOCYTES

Ca^{2+}-induced Ca^{2+} release (CICR) underlies excitation-contraction coupling in cardiac muscle. Ca^{2+} is released from the sarcoplasmic reticulum (SR) through a specialised release channel termed the ryanodine receptor (RyR). This receptor is activated during an action potential by Ca^{2+}, which enters the cell through voltage-activated L-type Ca^{2+} channels in the sarcolemma.

Certain agents modify the sensitivity of the RyR to Ca^{2+} and as such are considered to be potential regulators of contraction. For example both the compound cyclic ADP-ribose [301] and phosphorylation of the ryanodine receptor [107] have been suggested to enhance the sensitivity of the RyR to triggering by local elevation of $[Ca^{2+}]_i$, thereby increasing the gain of CICR and thence the magnitude of the systolic Ca^{2+} transient. Local anaesthetics such as procaine and tetracaine inhibit Ca^{2+} fluxes through the ryanodine receptor [157, 412] and suppress SR Ca^{2+} release and contraction in cardiac and skeletal muscle [12, 81, 213, 215, 348]. Tetracaine may therefore be a useful model compound for studying the effects of regulators of CICR. It has previously been shown that tetracaine produces a transient suppression of *spontaneous* Ca^{2+} release from the SR (Chapter 3, [294] & [157]). This effect was attributed to the combination of

(i) an inhibitory effect of tetracaine on CICR, which is then gradually overcome by (ii) a subsequent increase of SR Ca^{2+} content. These results were shown to be consistent with inhibition of CICR. There was, however, no direct measurement of the gain of CICR. Furthermore there was no information about the effects of tetracaine on the normal, stimulated release of Ca^{2+} from the SR.

5.1 Aims of This Chapter

The structurally related local anaesthetic, procaine, has been shown to inhibit SR Ca^{2+} release directly [412], and to increase SR Ca^{2+} content (as assessed by rapid cooling contracture), an effect which has been attributed to a reduction in trans-sarcolemmal Ca^{2+} efflux [215]. The initial aim of this work was to obtain *quantitative* measurements of changes in the gain of CICR (the relationship between trigger Ca^{2+} and SR Ca^{2+} release, See Section 1.8) and SR Ca^{2+} content during exposure to low concentrations of tetracaine (100 or 200 μM). However, it is important to consider other possible actions of tetracaine, which acts as a local anaesthetic by blocking Na^+, Ca^{2+} and K^+ ion channels in nerve and muscle preparations at concentrations similar to those affecting the SR Ca^{2+} release channel [75, 81, 176]. Some of the inotropic effects of tetracaine could be attributable to effects on these sarcolemmal ionic fluxes. The work presented here provides evidence that such contributions are small, and that the transient nature of the effects of tetracaine on contraction can be attributed to depression of SR Ca^{2+} release and consequent changes of SR Ca^{2+} content. In conclusion agents that affect CICR alone will only have transient effects on contraction.

5.2 The Effect of Tetracaine on the Amplitude of Stimulated Contraction

Figure 5.1 shows the effect of tetracaine on the amplitude of contraction and associated Ca^{2+} transients (Indo-1 ratio) in a field-stimulated rat ventricular myocyte. The Indo-1 ratio was corrected for the intrinsic fluorescence of tetracaine as described in Section 2.3.4. Application of 100 μM tetracaine transiently reduced the amplitude of contraction. Contraction then gradually recovered towards the control level over a period of 1 - 2 min, despite the maintained presence of the drug. The magnitude of peak contraction was also more variable in the presence of tetracaine in this cell, although this was not a consistent observation. Removal of tetracaine was associated with a transient elevation of contraction amplitude above the control level, which subsequently recovered to the control level. Similar effects of tetracaine addition and removal were seen on the systolic Ca^{2+} transient record (Fig. 5.1A, lower panel). This suggests that the observed changes in contraction amplitude are a consequence of changes in the Ca^{2+} transient magnitude, and do not reflect effects of the drug on the contractile apparatus of the cell. The amplitude of the Ca^{2+} transient does not recover to the control level, while the contraction appears to demonstrate recovery to the control level. The possibility that the lack of complete recovery of the Ca^{2+} transient is due to errors in correction for the intrinsic fluorescence of tetracaine (See Section 2.3.4) cannot be excluded. However, the changes in the amplitude of the Ca^{2+} transient which occur in the maintained presence of tetracaine and the overshoot on removal of tetracaine must be due to changes of $[Ca^{2+}]_i$ rather than artefactual changes of fluorescence.

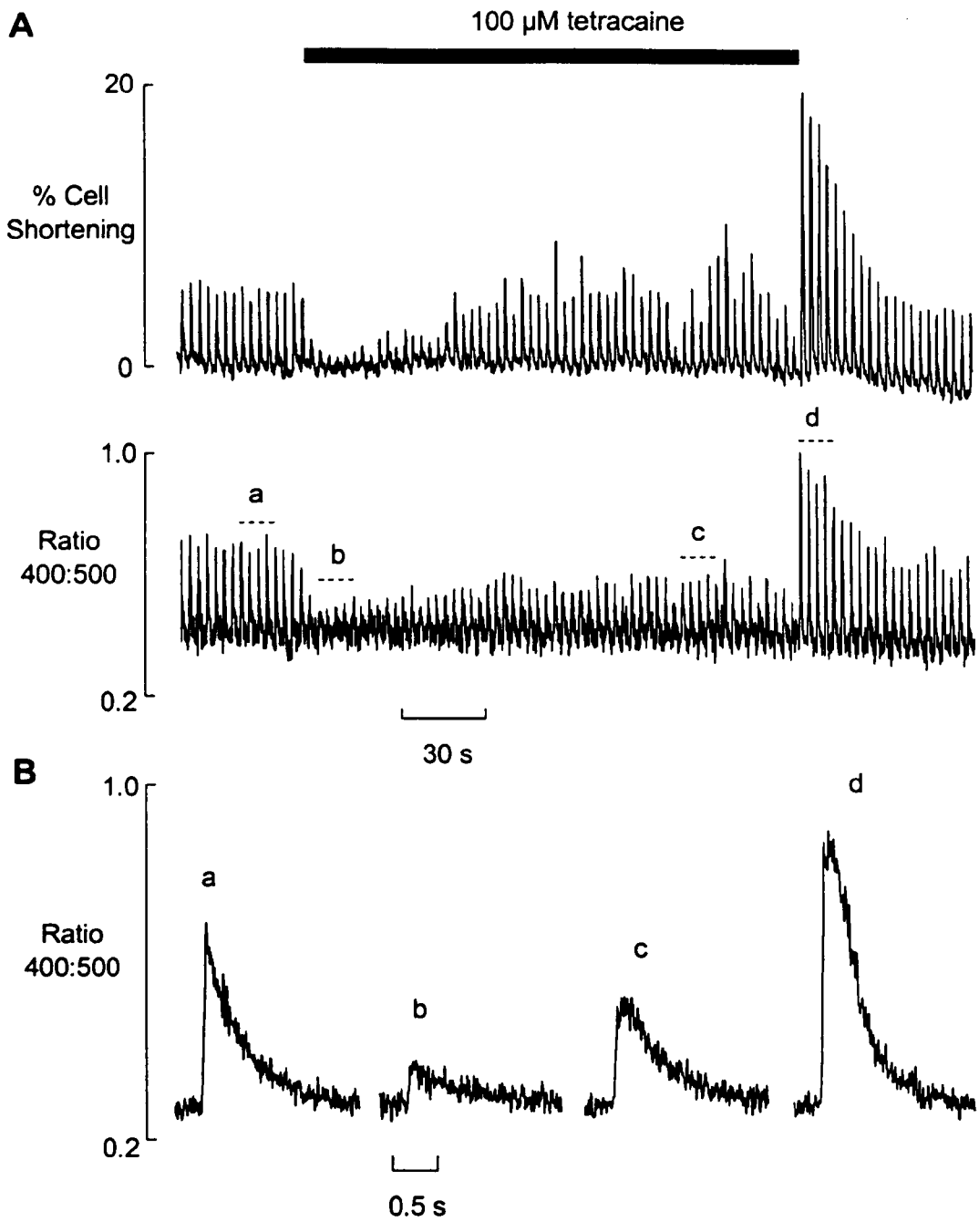

Figure 5.1. The effects of tetracaine on contraction amplitude and systolic $[Ca^{2+}]_i$ transients.

A, time course of cell shortening (top) and indo-1 ratio ($R = F_{400}:F_{500}$) (bottom). The cell was field stimulated at a frequency of 0.3 Hz. Tetracaine (100 μM) was applied for the period indicated by the bar. The indo-1 ratio was corrected for the intrinsic fluorescence of tetracaine (see Methods 2.3.4). **B**, specimen mean (n = 5) Ca^{2+} transients taken from the periods (a – d) indicated in the lower panel of A.

5.3 Could Changes in I_{Na} and I_{Ca} Explain the Inotropic Effects of Tetracaine?

It is likely that the effects of tetracaine are a result of reduced Ca^{2+}-induced Ca^{2+} release (CICR). However, tetracaine has well documented effects on Na^+ and Ca^{2+} currents across the sarcolemma (I_{Na} and I_{Ca} respectively) [75, 176]. Inhibition of I_{Na} will decrease excitability and any effect this could have on the results of this investigation were eliminated by using voltage-clamp to hold the membrane at a potential of –40 mV (under such conditions I_{Na} should be inactivated). Inhibition of I_{Ca} will directly decrease Ca^{2+} entry. The experiment illustrated in Fig. 5.2 shows a typical result in a voltage-clamped cell. The effects of tetracaine on contraction (Fig. 5.2A) are qualitatively similar to those seen in field-stimulated cells: tetracaine produces an initial decrease of contraction followed by recovery and then an overshoot on removal of tetracaine. The transient overshoot of contraction amplitude on removal of the drug, in this case, was accompanied by spontaneous Ca^{2+} release and propagating waves of contraction, indicating some degree of Ca^{2+} overload at this point in the experiment. The presence of spontaneous Ca^{2+} release also limits the size of the overshoot. Figure 5.2B shows specimen records of current and contraction. The immediate major depression of contraction (b) is accompanied by a modest decrease (to 83%) of the peak magnitude of the Ca^{2+} current. This is associated paradoxically with a slight *increase* in the integral of the Ca^{2+} current, representing increased Ca^{2+} influx (See Fig. 5.5B and section 5.5). However, while contraction recovers towards control levels, there is no significant increase in the amplitude of the Ca^{2+} current (c). Similarly the overshoot in contraction

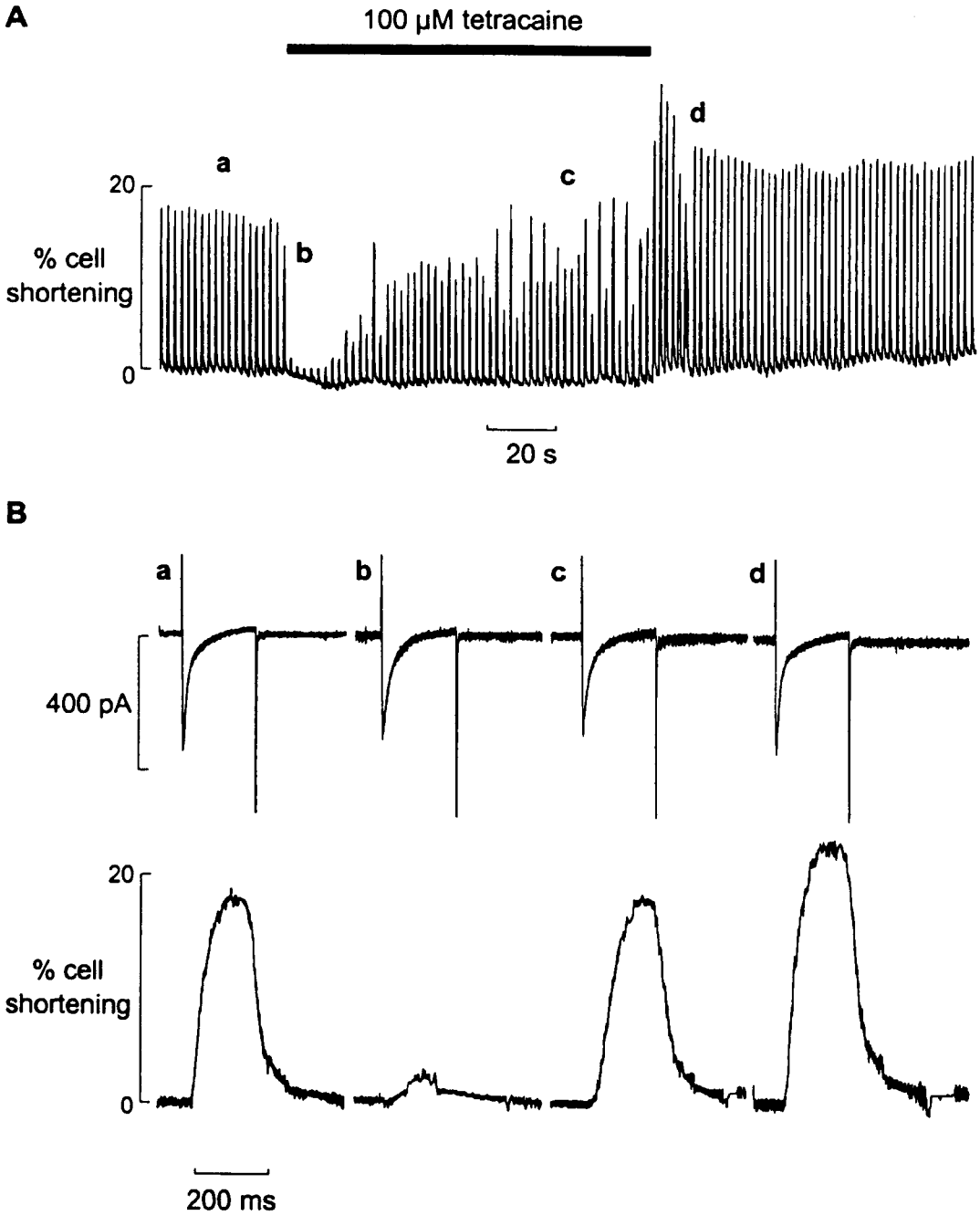

Figure 5.2. The effects of tetracaine on contraction and membrane current in a voltage clamped rat ventricular myocyte.

A, time course of changes of contraction. Tetracaine (100 µM) was applied for the period indicated by the bar. The membrane potential was held at -40 mV, and 200 ms duration depolarising pulses to 0 mV were applied at a frequency of 0.5 Hz. **B**, specimen records of membrane current (top) and contraction (bottom) obtained at the times (a – d) indicated in A.

amplitude on removal of tetracaine (d) is not accompanied by an increase in peak Ca^{2+} current above the control level. Changes in the peak amplitude of the Ca^{2+} current, therefore, cannot explain the recovery of contraction amplitude during continued superfusion with tetracaine, nor the transient overshoot of contraction observed on its removal. On average, in eleven cells, the minimum level of contraction reached in 100 μM tetracaine was 40.0 ± 4.5 % of the control level and this recovered to 96.2 ± 2.6 % in the steady-state. This level of contraction during steady-state exposure to tetracaine was not significantly different from that in control ($P > 0.1$, $n = 11$). On removal of tetracaine the mean peak level of contraction reached was 170.8 ± 16 % of the control level ($P < 0.01$, $n = 11$).

In Figure 5.3 the effect of tetracaine was compared with that produced by deliberately reducing the Ca^{2+} current, by reducing the size of the depolarising step (from +40 to +30 mV). This provides some idea of the extent to which inhibition of the L-type Ca^{2+} current may account for changes in contraction. Reducing the size of the depolarisation reduced the peak Ca^{2+} current considerably more than did exposure to 100 μM tetracaine (here to 54 % of the control peak I_{Ca} c.f. 83 % in tetracaine). However, the contraction elicited by the smaller pulse was only marginally reduced in magnitude, in contrast to the dramatic reduction of contraction amplitude observed during early exposure to tetracaine. This confirms that only a small proportion of the effects of tetracaine on contraction amplitude can be due to reduction of the L-type Ca^{2+} current.

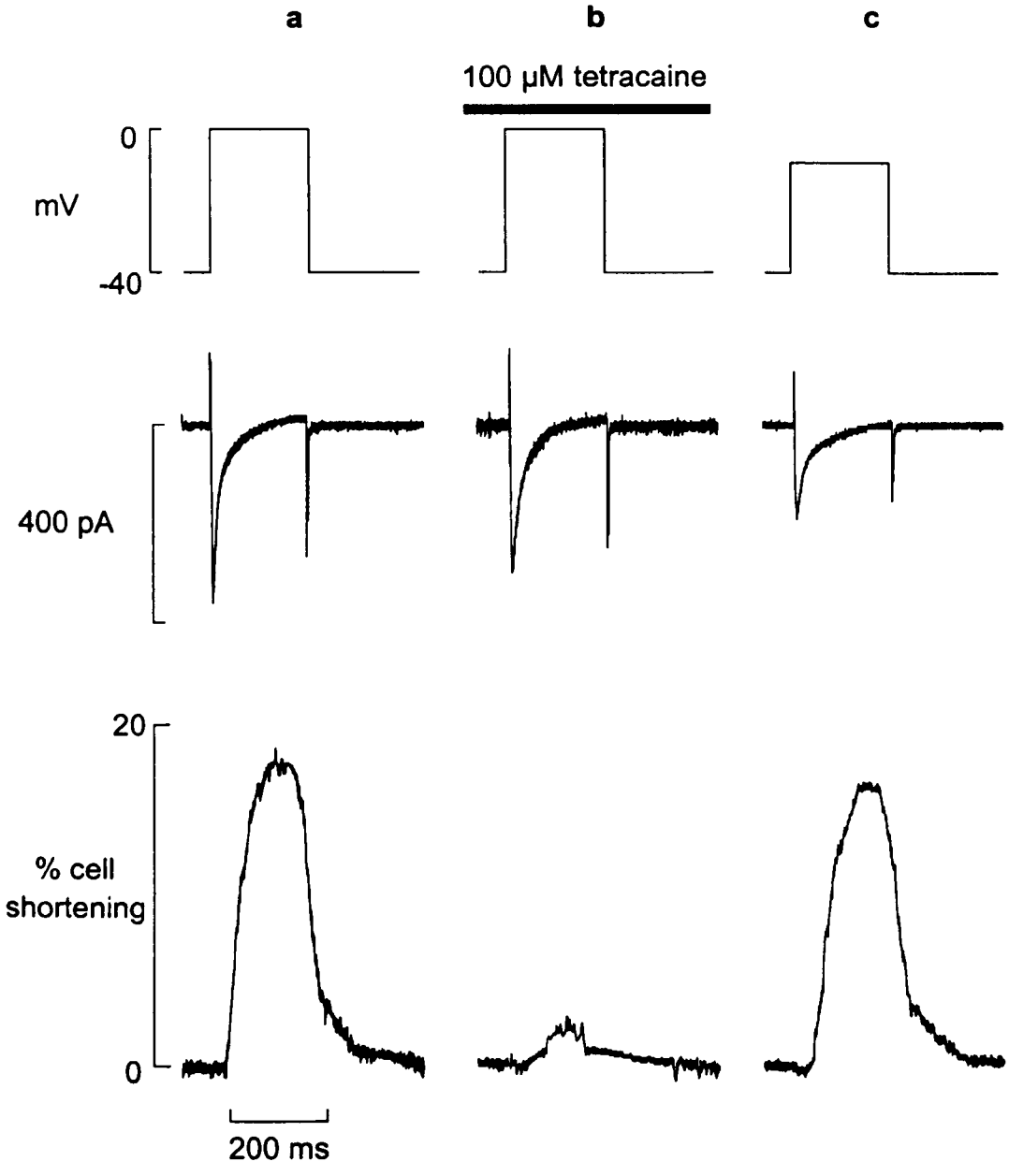

Figure 5.3. Comparison of the effects of tetracaine with those of decreasing the size of depolarisation.

In all panels the traces show (from top to bottom): membrane potential, current, and cell length. In panels a and b the depolarising pulse was to 0 mV. Records show: panel **a**, control; **b**, after 4 s exposure to tetracaine (100 µM); **c**, in the absence of tetracaine (depolarisation to -10 mV). Membrane potential was held at -40 mV and depolarising pulses of 200 ms duration were applied to elicit contraction.

5.4 Direct Measurements of SR Ca^{2+} Content

The transient elevation of contraction amplitude observed on removal of tetracaine may be indicative of an increase in the SR Ca^{2+} load of the cells following exposure to tetracaine. This is further suggested by the presence of spontaneous oscillations in some cells, immediately following removal of the drug (Fig. 5.2A). It is likely that any extra Ca^{2+} gained by the cell, during exposure to tetracaine, is accommodated in the SR. The effect of tetracaine on the SR Ca^{2+} content was quantified more directly by measuring the caffeine-induced Na^{+}/Ca^{2+} exchange currents in stimulated myocytes under control conditions and during tetracaine superfusion. Figure 5.4A illustrates typical caffeine traces and their associated integrals as a measure of SR Ca^{2+} content (See Section 2.6.2). It is clear that exposure to tetracaine increases the size of the integral and therefore of the SR Ca^{2+} content. The histogram (Fig. 5.4B) shows mean data ± S.E.M. of the steady state SR Ca^{2+} content during exposure to tetracaine in comparison with control. The steady state SR Ca^{2+} content during exposure to tetracaine was 44.9 ± 6.4 % higher than that under control conditions ($n = 7$, $P < 0.0001$).

5.5 Measurement of Ca^{2+} Flux Balance in Tetracaine

The results presented in the previous section provide direct quantitative measurements of an increase of SR Ca^{2+} content during stimulation in the presence of 100 μM tetracaine. The aim of the next series of experiments was to investigate the origin of this increase. In order to achieve this, measurement of the sarcolemmal fluxes of Ca^{2+} elicited by voltage-clamp pulses from a holding

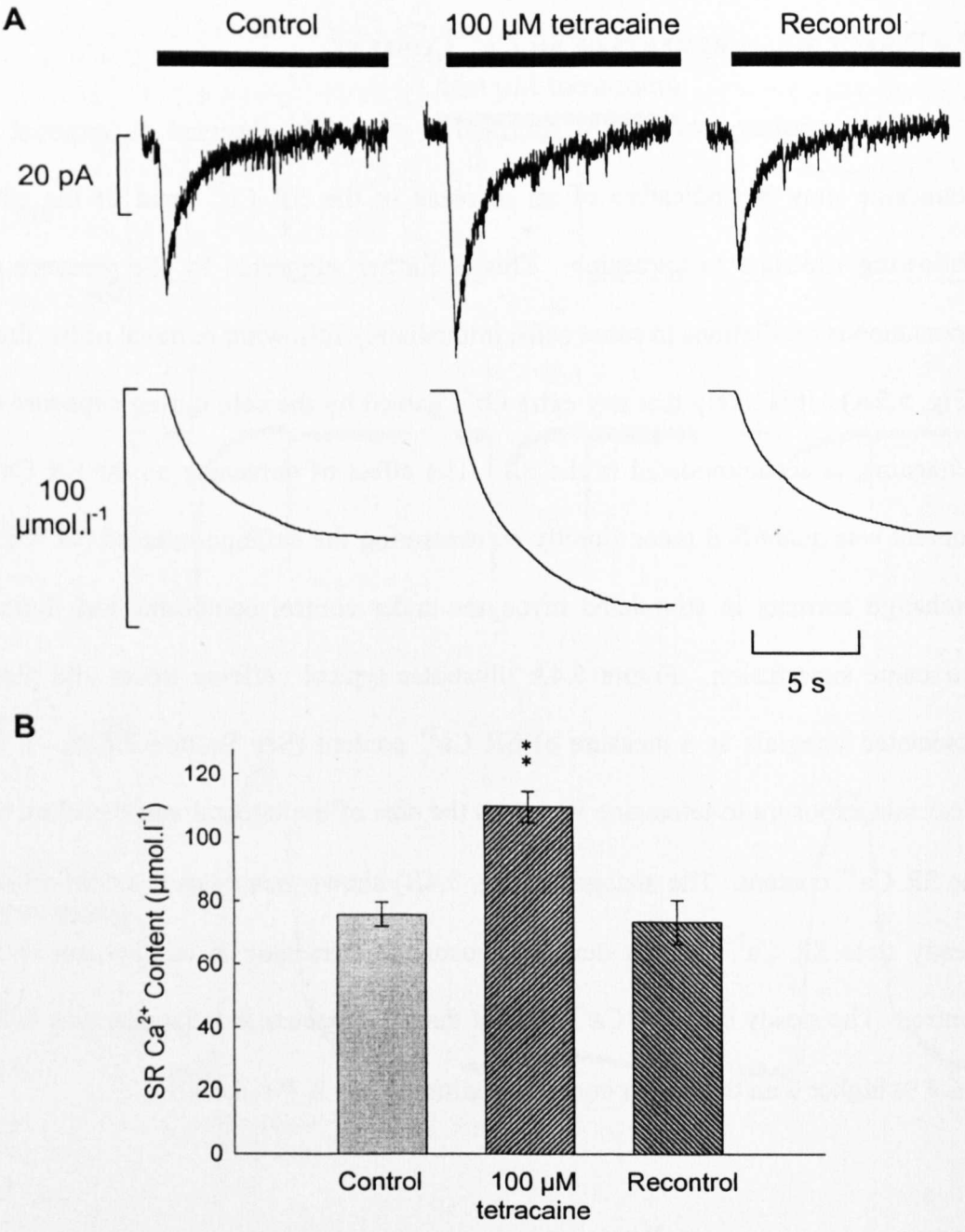

Figure 5.4. The effect of tetracaine on the SR Ca^{2+} content of electrically stimulated rat ventricular myocytes.

A, measurement of SR Ca^{2+} content. Caffeine (20 mM) was applied for the period indicated by the bars. Traces show current (top) and integrated current (bottom) converted into SR Ca^{2+} content as described in Methods 2.6.2. Records show (from left to right): control; after 1.5 min exposure to tetracaine (100 µM); recontrol (1.5 min after removing tetracaine). **B**, histogram showing mean data ± S.E.M. ($P < 0.0001$, $n = 7$) of SR Ca^{2+} under control (left), during exposure to 100 µM tetracaine (centre) and following recovery of control steady state after removal of tetracaine (right).

potential of –40 mV to 0 mV was performed. I_{Na} should be inactivated at these voltages. As described previously [276, 372] (See Methods 2.6.2), Ca^{2+} entry was calculated by integrating the L-type Ca^{2+} current and Ca^{2+} efflux from the Na^+/Ca^{2+} exchange current tail activated on repolarisation [57, 135]. To facilitate the measurements, short (100 ms duration) pulses were used in order to minimise Ca^{2+} efflux during depolarisation, when the L-type Ca^{2+} current will obscure any Na^+/Ca^{2+} exchange flux. The contraction record in Fig. 5.5A again shows a transient reduction of amplitude on application of tetracaine, followed by a recovery, and overshoot on removal of the drug. Figure 5.5B illustrates sample current records. The integrated currents for this cell, show that under control conditions (a) depolarisation produces a gain of about 4 $\mu mol.l^{-1}$ (cell volume) via the L-type Ca^{2+} current per cycle. On repolarisation there is a loss of Ca^{2+} from the cell, which is of the same magnitude as the initial gain. In other words influx equals efflux. On average in 12 cells the Ca^{2+} entry during the Ca^{2+} current was 4.19 ± 0.43 $\mu mol.l^{-1}$ in comparison to average efflux of 4.55 ± 0.37 $\mu mol.l^{-1}$ (all fluxes expressed with respect to total cell volume). These are not significantly different ($P > 0.10$). The records illustrated in Fig. 5.5B(b) show the early effects of tetracaine. Despite the reduction in the peak magnitude of the Ca^{2+} current, the integrated Ca^{2+} entry is greater (presumably due to reduced Ca^{2+}-induced inactivation of the current, because of the smaller Ca^{2+} transient [2, 328]). The main effect of tetracaine on membrane current is an initial decrease of the Na^+/Ca^{2+} exchange current tail on repolarisation due, presumably, to the decreased magnitude of the systolic Ca^{2+} transient providing less activation of the Na^+/Ca^{2+} exchanger. The net effect is that the cell has gained about 3 μmoles

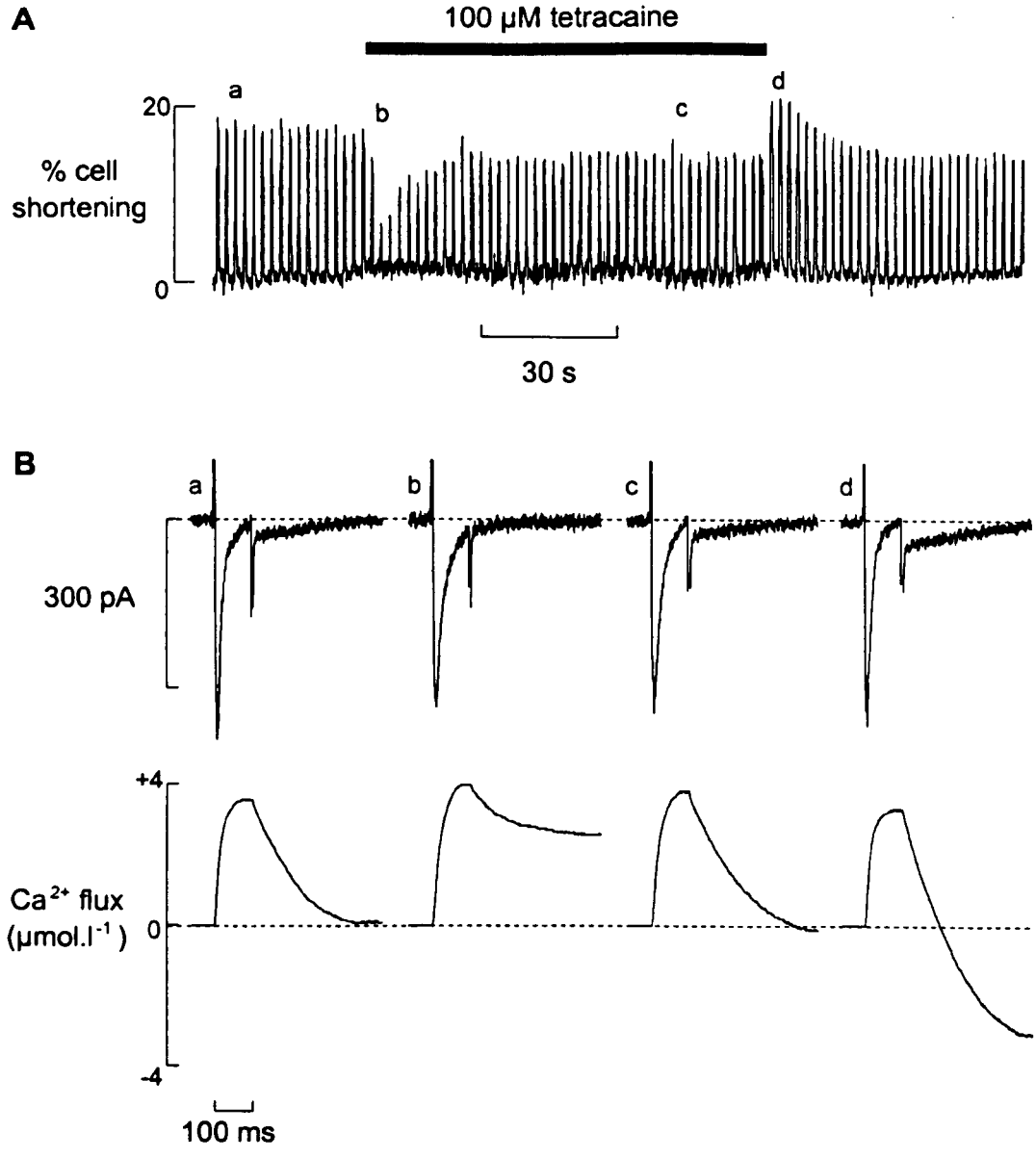

Figure 5.5. Transient loss of Ca^{2+} flux balance during application and removal of tetracaine.

A, time course of the effects on contraction of applying tetracaine for the period indicated by the bar. **B**, specimen records of membrane current (top) and cumulative integral (bottom). Membrane potential was held at -40 mV and depolarising pulses of 100 ms duration were applied at 0.5 Hz. The Ca^{2+} flux traces show the cumulative integral of the Ca^{2+} current (initial upward deflection) followed by a downward deflection due to Ca^{2+} efflux. The records were obtained at times (a – d) shown in A. (See Methods 2.6.2 for calculation of sarcolemmal Ca^{2+} movements). The vertical positions of the current traces have been aligned to facilitate comparison. Tetracaine produced an outward shift of holding current of 7 pA, and this has been removed to facilitate comparison between records.

$Ca^{2+}.l^{-1}$ at the end of the record shown. During the period of exposure to tetracaine, as the size of the systolic Ca^{2+} transient and contraction increases, so does that of the inward current tail on repolarisation. Therefore, in the steady state in tetracaine (c) Ca^{2+} entry on depolarisation exactly balances the loss on repolarisation such that there is no net change of cell Ca^{2+} per cycle. The average data confirm that influx (4.00 ± 0.42 $\mu mol.l^{-1}$) and efflux (4.64 ± 0.38 $\mu mol.l^{-1}$) are not significantly different and therefore balance in tetracaine ($P > 0.05$), once a steady-state level of contraction is achieved. When tetracaine is removed there is a small decrease of the integrated Ca^{2+} current. This is accompanied by a much larger increase of Ca^{2+} loss on repolarisation due to the larger Ca^{2+} transient. On this pulse there is a net loss of cell Ca^{2+} of almost 4 $\mu mol.l^{-1}$. Although not shown, as the contraction and systolic Ca^{2+} transient decrease towards control levels, the Ca^{2+} loss on repolarisation decreases to control levels and Ca^{2+} flux balance is once again achieved. This post-tetracaine depletion of Ca^{2+} presumably accounts for the gradual reduction of contraction towards the control steady-state level.

The net changes of Ca^{2+} illustrated in Figure 5.5 take place over each cycle of contraction and relaxation. It appears that exposure to tetracaine causes a net gain of Ca^{2+} by the cell, and this is balanced by a loss of Ca^{2+} from the cell on its removal. The total amount of Ca^{2+} gained and lost in this way can therefore be calculated by summing the net Ca^{2+} flux for each cycle. An example of this type of calculation is illustrated in Fig. 5.6. This calculation was performed using the same data as Fig. 5.5. The solid bar indicates the period of tetracaine superfusion. The upper panels represent the values of Ca^{2+} influx and efflux respectively,

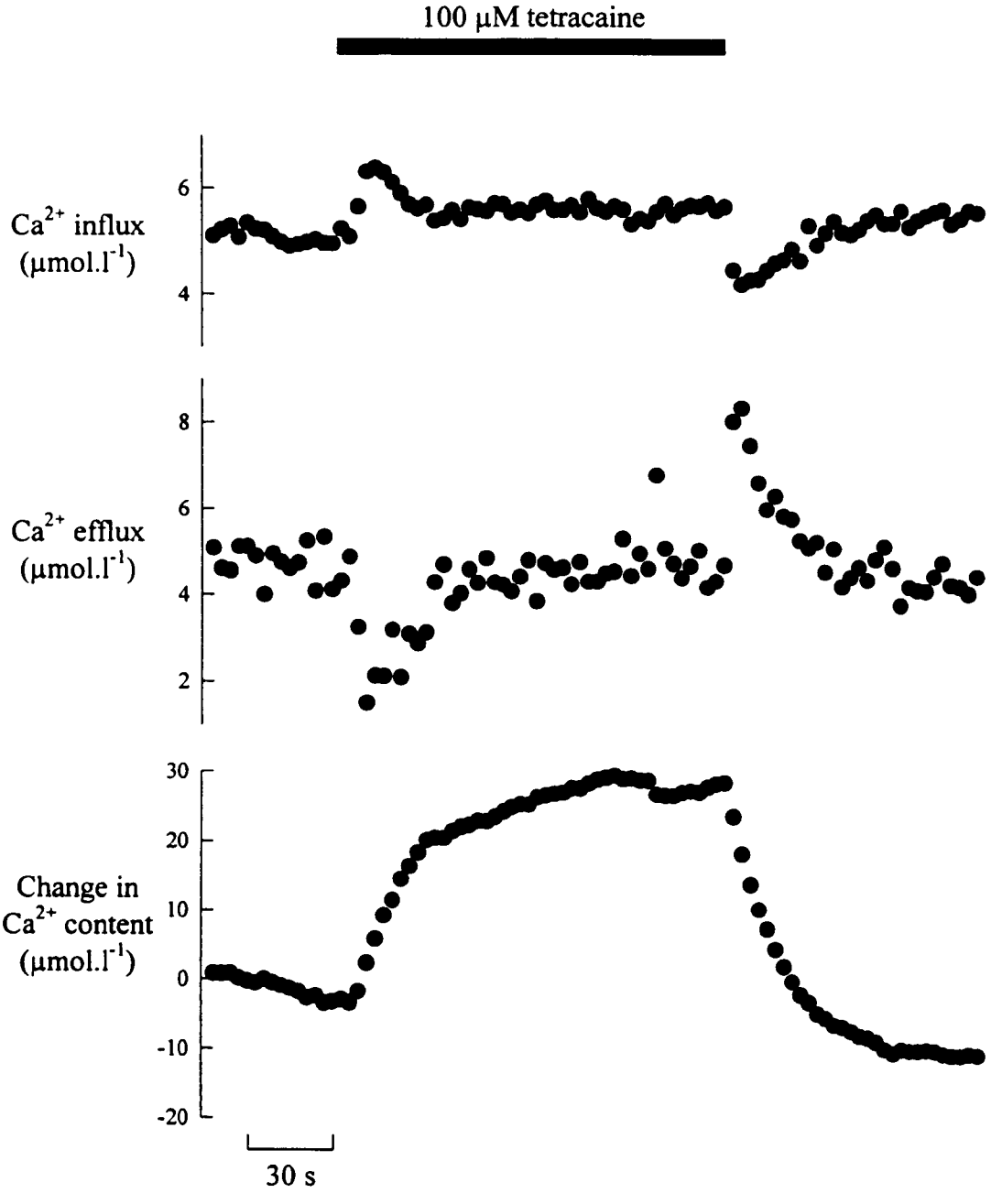

Figure 5.6. Net accumulation of Ca^{2+} during exposure to and removal of tetracaine.

The traces show (from top to bottom) the traces show: calculated Ca^{2+} entry via the L-type Ca^{2+} current; Ca^{2+} efflux on repolarisation (calculated as shown in Fig. 5.5); cumulative change of cell Ca^{2+} content. Ca^{2+} content (presumably SR) is expressed per unit total cell volume.

calculated from the integrals of Ca^{2+} current and Na^{+}/Ca^{2+} current, associated with each cycle of contraction. The bottom panel illustrates the cumulative difference between influx and efflux, i.e. the total amount of Ca^{2+} gained or lost by the cell since the start of the record. The data presented earlier show that, on average, in either control or tetracaine in the steady-state Ca^{2+} influx and efflux are equal. However any small difference will add up with this cumulative method. The average steady-state values of Ca^{2+} influx and efflux for control, tetracaine and recontrol were calculated. These steady-state values were then subtracted from the integral of each pulse in the appropriate solution. As shown in Fig. 5.5, application of tetracaine is associated with a reduction in Ca^{2+} efflux from the cell, and a transient elevation of Ca^{2+} entry above the control level, probably as a consequence of reduced Ca^{2+}-induced inactivation of I_{Ca}. Both these effects will contribute to increasing the Ca^{2+} content of the cell. As the cell gains Ca^{2+}, contraction recovers towards the control level, and with it efflux from the cell, activated by the increasing magnitude of associated Ca^{2+} transients. On removal of tetracaine there is a transient elevation of the efflux integral, producing a net loss of Ca^{2+} from the cell. There is also a significant undershoot of Ca^{2+} entry into the cell, which will further contribute to reducing cell Ca^{2+} content. This is because the larger Ca_i transient will cause greater inhibition of the L-type Ca^{2+} current [328]. The net Ca^{2+} loss, on removal of tetracaine, calculated in this way (35.5 ± 3.3 $\mu mol.l^{-1}$, $n = 11$) is similar to ($P > 0.05$) the calculated amount of Ca^{2+} gained in the presence of tetracaine (33.7 ± 3.1 $\mu mol.l^{-1}$, $n = 11$).

The histogram of Fig. 5.7 shows that, in six cells, the increase of SR Ca^{2+} content measured directly from the integral of the caffeine-evoked Na^{+}/Ca^{2+}

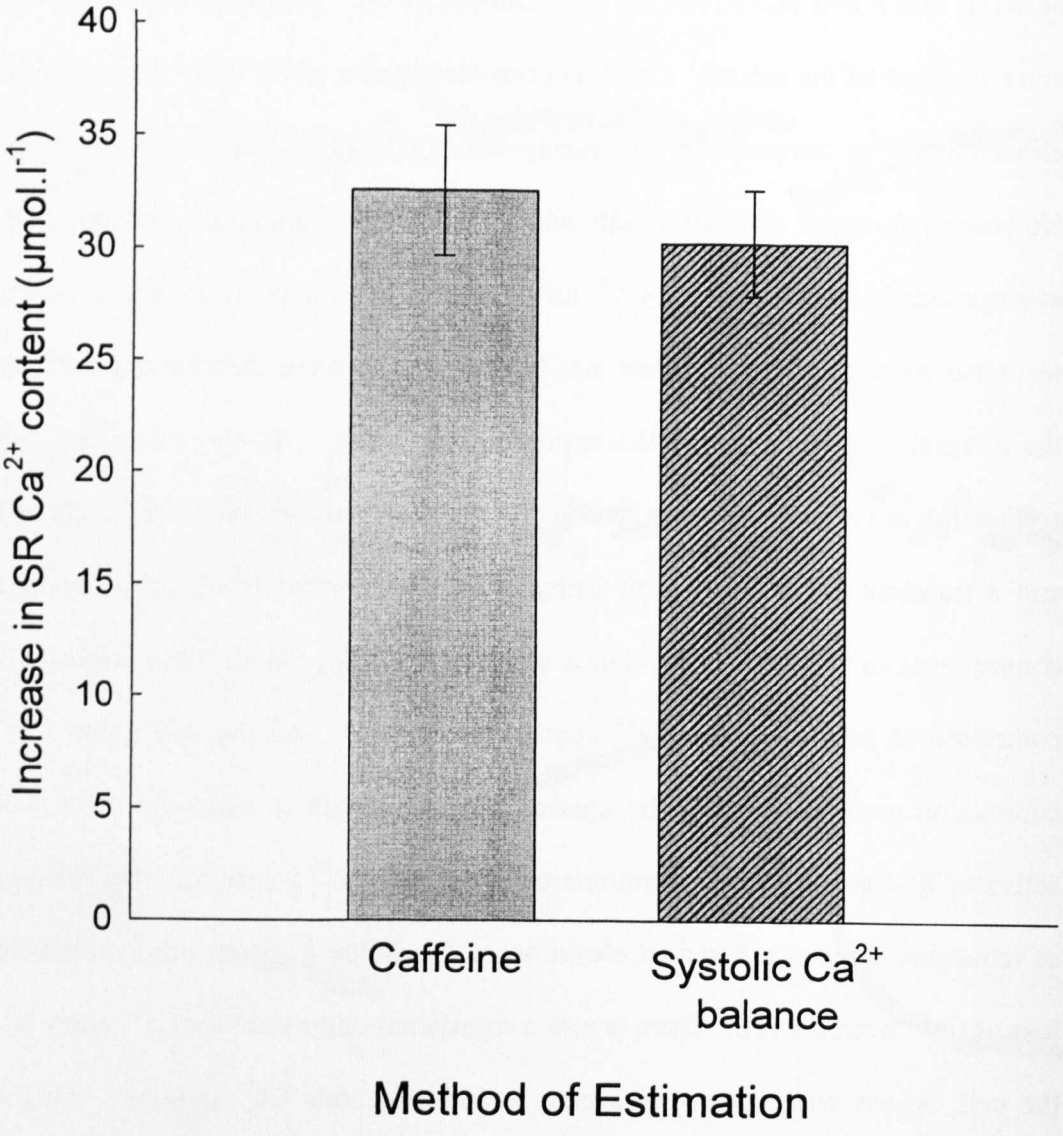

Figure 5.7. The gain of measured SR Ca^{2+} content matches that calculated from the sarcolemmal fluxes.

Histogram comparing the measured changes of SR Ca^{2+} (left) with those calculated as in Fig. 5.6 (see Methods 2.6.2) from the cumulative integrals (right).

exchange current (32.6 ± 2.9 μmol.l^{-1}) is very similar to (although statistically different from, $P < 0.05$) that estimated from the balance of Ca^{2+} fluxes (30.3 ± 2.4 μmol.l^{-1}).

5.5.1 The Effect of a Higher Concentration of Tetracaine on Ca^{2+} Fluxes

In the steady state, the flux of Ca^{2+} into the cell in Fig. 5.5 is minimally affected by 100 μM tetracaine. This is reflected by the fact that contraction amplitude recovers towards the control level in the steady state (where influx and efflux are balanced). Inhibition of the Ca^{2+} current may however contribute to any reduction in the steady state amplitude of contraction below that observed under control conditions, during prolonged exposure to tetracaine. This effect is more obvious in Fig. 5.8, which illustrates the effect of 200 μM tetracaine on contraction and associated sarcolemmal currents and Ca^{2+} fluxes. Changes in contraction amplitude during exposure to 200 μM tetracaine follow a similar, although somewhat exaggerated, pattern as observed with lower concentrations of the drug. Exposure to 200 μM tetracaine (indicated by the bar) eventually produces a new steady state level of contraction, which is below the control level. In this case, recovery time is also considerably prolonged. The associated I_{Ca} (Fig. 5.8B, top panel) is inhibited to a greater extent by tetracaine, thereby reducing Ca^{2+} entry into the cell to a greater extent. In this case the Ca^{2+} current integral was reduced to 62.1 % of the average control value by exposure to 200 μM tetracaine. In the same cell a reduction in the magnitude of I_{Ca} to 82.6 % of the average control value was observed during exposure to 100 μM tetracaine. The decrease in Ca^{2+} current integral during exposure to 200 μM tetracaine was

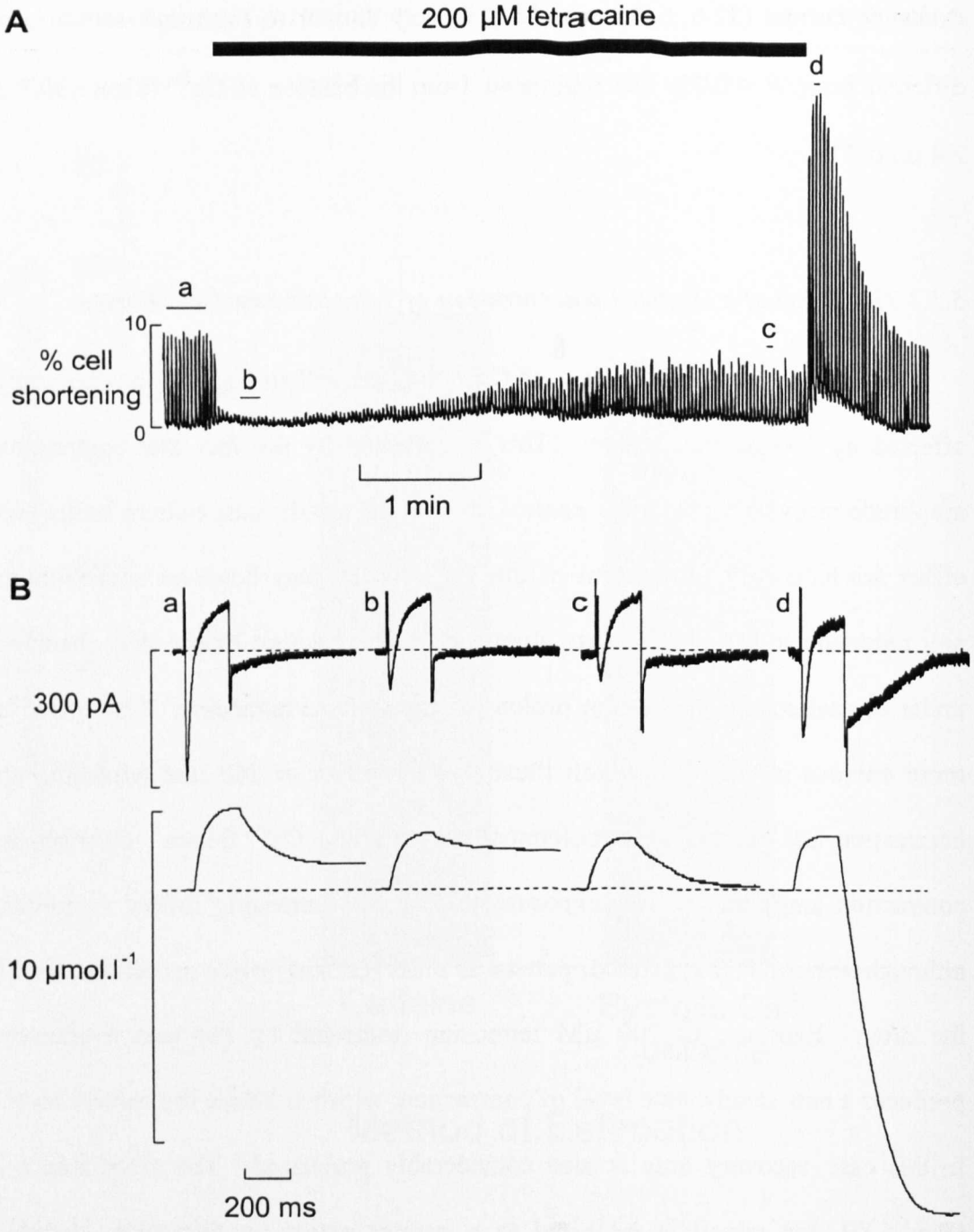

Figure 5.8. The effect of 200 µM tetracaine on contraction and membrane current.

A, time course of cell shortening in response to electrical stimulation elicited by 200 ms depolarising steps to 0 mV from a holding potential of -40 mV. Tetracaine (200 µM) was applied as indicated by the bar. **B**, specimen records of membrane current (top) and cumulative integral (bottom). The records were obtained at the times (a – d) shown in A. The records in a and b are the averages of 10 and 5 pulses respectively. Single pulses are shown in c and d.

reflected by a reduction in the steady state amplitude of contraction to 54.8 % of the control level, in comparison to 85.5 % during exposure to 100 μM tetracaine.

5.6 DISCUSSION

The most striking feature of the results obtained in this study is the transient nature of the inotropic effects of tetracaine. Tetracaine transiently suppresses electrically stimulated contraction and systolic $[Ca^{2+}]_i$ transients, and its removal is associated with a transient elevation of contraction and $[Ca^{2+}]_i$ transient amplitude above the control level. These experiments were designed to investigate the mechanism by which tetracaine achieves these inotropic effects in rat ventricular myocytes. It has been shown that the transient effects of tetracaine addition and removal cannot be accounted for by changes of I_{Ca}, but are likely to be due to a combination of inhibition of CICR and increasing SR Ca^{2+} content.

During normal excitation-contraction coupling, Ca^{2+} ions are released from the SR by Ca^{2+}-induced release, triggered by Ca^{2+} entry through voltage-gated sarcolemmal Ca^{2+} channels. The concentrations of tetracaine used in this study decrease Ca^{2+} entry slightly, by direct inhibition of the L-type Ca^{2+} current [75, 81]. However, it has been shown that this effect cannot account for the transient nature of the depression of contraction, and the effects of tetracaine are therefore likely to reflect predominantly a decrease in the ability of I_{Ca} to stimulate Ca^{2+} release from the SR. Qualitatively, the biphasic nature of the effects of tetracaine are similar (but in the opposite direction) to those found for low concentrations of caffeine [158, 284]. Caffeine produces a transient increase of systolic contraction and $[Ca^{2+}]_i$ transient followed by a decay back to control levels. On removal of

caffeine, there is an undershoot of the contraction and $[Ca^{2+}]_i$ transient amplitude before recovery of the steady state control level. The transient increase on application of caffeine was explained as resulting from a stimulation of CICR [284]. Its effects are only transient because the increased release decreases the SR Ca^{2+} content. This hypothesis has recently received quantitative support [371].

5.6.1 Measurement of Sarcolemmal Fluxes and SR Ca^{2+} Content during Tetracaine Exposure.

This work has examined the balance of Ca^{2+} fluxes across the membrane during the application of tetracaine. This was achieved by comparing the magnitude of Ca^{2+} entry during the depolarising pulse, via the L-type Ca^{2+} current, with Ca^{2+} efflux on repolarisation. The initial effect of applying tetracaine on sarcolemmal fluxes is to decrease the Ca^{2+} efflux (as a result of the decreased $[Ca^{2+}]_i$ transient), and to increase slightly Ca^{2+} entry into the cell. Peak I_{Ca} is actually decreased, however the depressed Ca_i transient reduces the effect of Ca^{2+}-induced inactivation of the L-type Ca^{2+} current with the result that the integral of this current is transiently increased, overcoming the small direct inhibitory effect of tetracaine on I_{Ca}. This causes a net predicted accumulation of cell Ca^{2+} on each stimulus, in contrast with the flux balance observed under control conditions. The increase of SR Ca^{2+} content can be calculated from the net integral. The results (Fig. 5.6) show that there is a gradual increase of cell Ca^{2+} content on each twitch in tetracaine, until a new balance is achieved when efflux again equals influx. This results from the increase of Ca^{2+} efflux due to the gradual increase of the systolic Ca^{2+} transient. On removal of tetracaine the

inhibitory effect on CICR is removed and the larger SR Ca^{2+} content produces a larger systolic Ca^{2+} release than in the control steady state. As a consequence of this Ca^{2+} efflux from the cell is also increased above control levels. This results in the gradual decrease of SR Ca^{2+} content until the final control steady state is re-established.

The changes of SR Ca^{2+} content referred to above were calculated from the integrated current records. This method has the advantage that an estimate of change of SR content can be obtained after each pulse. The method is, however, rather indirect and, in particular makes no allowance for changes of fluxes between pulses. Proof that this is a valid method is given by the fact that (i) under control conditions and in the steady-state in tetracaine, influx and efflux balance, and (ii) the increase of SR Ca^{2+} content estimated by this method agrees quantitatively with that measured directly by applying 20 mM caffeine to release all the SR Ca^{2+} content (Fig. 5.7). The measurement of increased SR Ca^{2+} content is also in agreement with the results of Komai *et al* who demonstrated that the cooling contracture was increased by the related local anaesthetic procaine [215].

5.6.2 Changes of the Gain of CICR.

The present data show that tetracaine decreases the amount of Ca^{2+} release from the SR in response to a given size of Ca^{2+} current. In other words tetracaine decreases the gain of CICR (See Introduction 1.8). This effect is then overcome by an increase of SR Ca^{2+} content. The increase of SR Ca^{2+} release by an increase of SR Ca^{2+} content is in agreement with previous work [25, 157, 245]. It is interesting to note that the fractional recovery of contraction is greater than that of

the SR Ca^{2+} content. This presumably reflects the combination of (a) the steep relationship between $[Ca^{2+}]_i$ and contraction and (b) the fact that increasing SR Ca^{2+} content produces a fractionally larger increase of Ca^{2+} release [25, 372]. Increased Ca^{2+} flux has been demonstrated in isolated RyR reconstituted in lipid bilayers when luminal Ca^{2+} is increased [368].

5.6.3 Implications for Other Work.

The results of this paper have shown that tetracaine, despite having a *maintained* depressant effect on CICR, has only a *transient* effect on the magnitude of the systolic Ca^{2+} transient. This is consistent with the *transient* effects of caffeine on systolic Ca^{2+} [284, 370]. The argument put forward to explain the effects of tetracaine should also apply to other compounds that inhibit Ca^{2+} release. As long as inhibition of Ca^{2+} release is not complete, the mechanism by which it occurs (i.e. direct blockade of the pore vs. effects on gating) should not influence the result. These observations have two consequences. (i) If one is looking to see whether a compound affects CICR then the magnitude of the systolic Ca^{2+} transient or contraction is an inappropriate measure (at least in the steady-state). Indeed if the compound acts slowly then the changing SR Ca^{2+} content will ensure that no effect will be seen. To overcome this, it is necessary to measure SR Ca^{2+} content at the same time. Alternatively, one can make use of the fact that compounds which affect CICR (like tetracaine), although having no steady-state effect on the magnitude of the stimulated contraction, produce a steady-state effect on the frequency of spontaneous SR release (See Chapter 3) [157, 294]. (ii) These results and those of Trafford et al [371] suggest that the

maintained inotropic effects of substances such as cyclic ADP ribose [301] or phosphorylation of the ryanodine receptor [107] cannot simply be attributed to effects on CICR and it is likely that these agents affect other processes as well.

Tetracaine has well known effects on the L-type Ca^{2+} current [81], and in this study this effect has been shown to result in a maintained depression of steady-state contraction amplitude following recovery from the initial transient depression of contraction below the steady state amplitude (200 μM tetracaine Fig. 5.8). These effects are more pronounced with increasing tetracaine concentration, being less severe during exposure to 100 μM in comparison with 200 μM tetracaine. There are two possible mechanisms whereby changing the magnitude of the L-type Ca^{2+} current can cause maintained effects on the steady state contraction amplitude. i) Increasing the Ca^{2+} current will increase the trigger for CICR, thereby stimulating Ca^{2+} release and the amplitude of contraction, and decreasing I_{Ca} will decreased the trigger. ii) An increase of Ca^{2+} entry during each pulse will limit any decrease of SR Ca^{2+} content, or if decreased will exacerbate it. Is it possible that agents that activate SR Ca^{2+} release and apparently cause a maintained increase in systolic contraction also stimulate the Ca^{2+} current, thereby increasing the trigger for Ca^{2+} release from the SR further and limiting SR Ca^{2+} loss, as a consequence of the increased Ca^{2+} release.

Anything that affects the balance between SR Ca^{2+} uptake and Ca^{2+} extrusion from the cell will also alter the effect of CICR modulation on SR Ca^{2+} content. The maintained effect of FK506 on systolic $[Ca^{2+}]_i$ and contraction has been attributed to inhibition of Na^+/Ca^{2+} exchange [251] and changes in the duration of the action potential [108].

Chapter 6

CHAPTER 6

POST-REST POTENTIATION, SR Ca^{2+} CONTENT AND THE EFFECT OF TETRACAINE

Contractile force is graded predominantly by Ca^{2+} entry through the sarcolemma and its release from the sarcoplasmic reticulum (SR). Alterations in the contributions of these two sources to the stimulation of contraction can explain the interval-force relationship in the mammalian heart. Studies of frequency and rest-dependent changes in contraction amplitude have been a valuable tool in understanding Ca^{2+} handling by the SR. When test intervals are longer than the regular stimulation interval and force is increased above the steady state value, it is referred to as post-rest potentiation. Post-rest potentiation is an interesting aspect of Ca^{2+} handling, which is demonstrated very clearly in the rat. In rat ventricular myocytes, the first few contractions elicited after a period of rest are increased in magnitude. The contraction amplitude then decays in a "negative staircase", with an exponential time course, eventually resulting in the recovery of the pre-rest steady state contraction amplitude (Fig. 6.1). The degree of rest potentiation is also strongly dependent upon the rate of stimulation (Fig. 6.1b), although the apparent increase of potentiation observed at higher stimulation frequencies may reflect predominantly increased mechanical restitution and be less dependent on changes in Ca^{2+} handling by the SR.

It has long been established that rest potentiation can be attributed largely to effects on SR Ca^{2+} handling, but exactly which aspect of this system and its

regulators produces the potentiation remains disputed. It could represent a change in the availability of Ca^{2+} that can be released (a longer interval allowing transfer of Ca^{2+} from uptake to release sites in the SR) [244], a change in the fraction of SR Ca^{2+} released [41], as a consequence of recovery of channels (I_{Ca} or RyR) from inactivation, or a change in the SR Ca^{2+} content [276], and therefore the gain of the Ca^{2+} release mechanism. The role of these mechanisms remains unresolved.

Studies have investigated the effect of rest periods on the cardiac cellular Ca^{2+} content using extracellular Ca^{2+} sensitive micro-electrodes to monitor Ca^{2+} movement across the sarcolemma [38, 250, 324]. These studies have revealed that rest is associated with a depletion of extracellular Ca^{2+} in rat myocytes [324], consistent with increased intracellular Ca^{2+} being responsible for the characteristic potentiation of contraction following a period of rest. Conversely, rabbit demonstrates a depletion of extracellular Ca^{2+} during stimulation and extrusion of Ca^{2+} during rest [250, 324], probably as a consequence of Ca^{2+} efflux on the Na^{+}/Ca^{2+} exchanger [356]. $^{45}Ca^{2+}$ exchange measured in rat atrial strips revealed no apparent change in $^{45}Ca^{2+}$ flux during rest, despite observing potentiation of contraction [244]. Rapid cooling contracture and caffeine-induced contracture have been used to estimate SR Ca^{2+} content in a number of studies. Both these techniques have their limitations and have produced inconsistent results, some investigators observing an apparent increase in SR Ca^{2+} content in parallel with potentiation of contraction in rat [19, 39, 44], and others reporting no apparent change in SR Ca^{2+} content [28, 41, 244] using these techniques. The effect of rest interval on the release of Ca^{2+} from the SR has been investigated in guinea-pig,

using the caffeine-induced Na^+/Ca^{2+} exchange current, measured under voltage-clamp, to quantify changes in SR Ca^{2+} content with rest [364]. The results of this investigation showed that there is a decay of SR Ca^{2+} content associated with the characteristic rest-induced negative inotropy in this species. Negretti *et al* [275] demonstrated that rest-potentiation in rat ventricular myocytes was correlated with changes in the amount of Ca^{2+} stored in the SR, by measuring changes in trans-sarcolemmal fluxes of Ca^{2+}. They compared the changes in SR Ca^{2+} content, measured directly from caffeine-induced Na^+/Ca^{2+} exchange currents, caused by switching from short to long pulses, and correlated these with changes in sarcolemmal Ca^{2+} fluxes. However, they did not provide direct evidence of an increase in the SR Ca^{2+} content during rest, using the caffeine-evoked current technique. There are no reports of using this well-established technique to directly examine changes in SR Ca^{2+} content associated with the rest-induced potentiation of contraction observed in the rat.

6.1 Aims of This Chapter

This work examines the phenomenon of post-rest potentiation in voltage-clamped rat ventricular myocytes. The effects of rest on the peak amplitude and integral of the L-type Ca^{2+} current (I_{Ca}), responsible for triggering the first post-rest beat were investigated, along with the effect of rest interval on SR Ca^{2+} content, using caffeine-evoked Na^+/Ca^{2+} exchange current integrals. To further investigate the role of the SR in rest potentiation the effect of tetracaine exposure on this phenomenon was also examined. If changes in SR Ca^{2+} content are responsible for rest potentiation, inotropic interventions that affect SR Ca^{2+}

handling are likely to affect the degree of potentiation. The basis of the changes induced by this inotropic intervention is also discussed.

6.2 The Frequency Dependence of Rest Potentiation

Figure 6.1 illustrates the effect of stimulation frequency on the degree of rest-potentiation. Contraction was elicited, under voltage-clamp, by 100 ms pulses to 0 mV from a holding potential of –40 mV. It is clear that a period of rest interrupting a train of stimulation transiently increases the amplitude of the first contraction when stimulation is resumed at both 0.5 Hz and 1 Hz stimulation frequency. The degree of potentiation appears to be greater at the higher stimulation frequency. Post-rest contraction was 323.8 % of the pre-rest steady state at 1 Hz stimulation frequency, in comparison to a post-rest twitch potentiation of only 54.7 % at 0.5 Hz stimulation. However, it is important to note that the steady state contraction amplitude at the higher stimulation frequency is depressed to 50.1% of the pre-rest steady state amplitude at the lower stimulation frequency. This was accompanied by a decrease in the magnitude of peak I_{Ca} and its integral to 57.9% and 67.4% respectively, of the pre-rest steady state values at 0.5 Hz stimulation. This suggests that the limited time between stimuli at the higher frequency does not allow full recovery from inactivation of Ca^{2+} channels. The potentiation of contraction was accompanied by an increase in peak Ca^{2+} current of 30.5 % during 0.5 Hz stimulation and 80.3 % at 1 Hz. This suggests that recovery of Ca^{2+} channels from inactivation, thereby enabling them to pass increased peak Ca^{2+} current during depolarisation, could account for at least some of the rest potentiation observed.

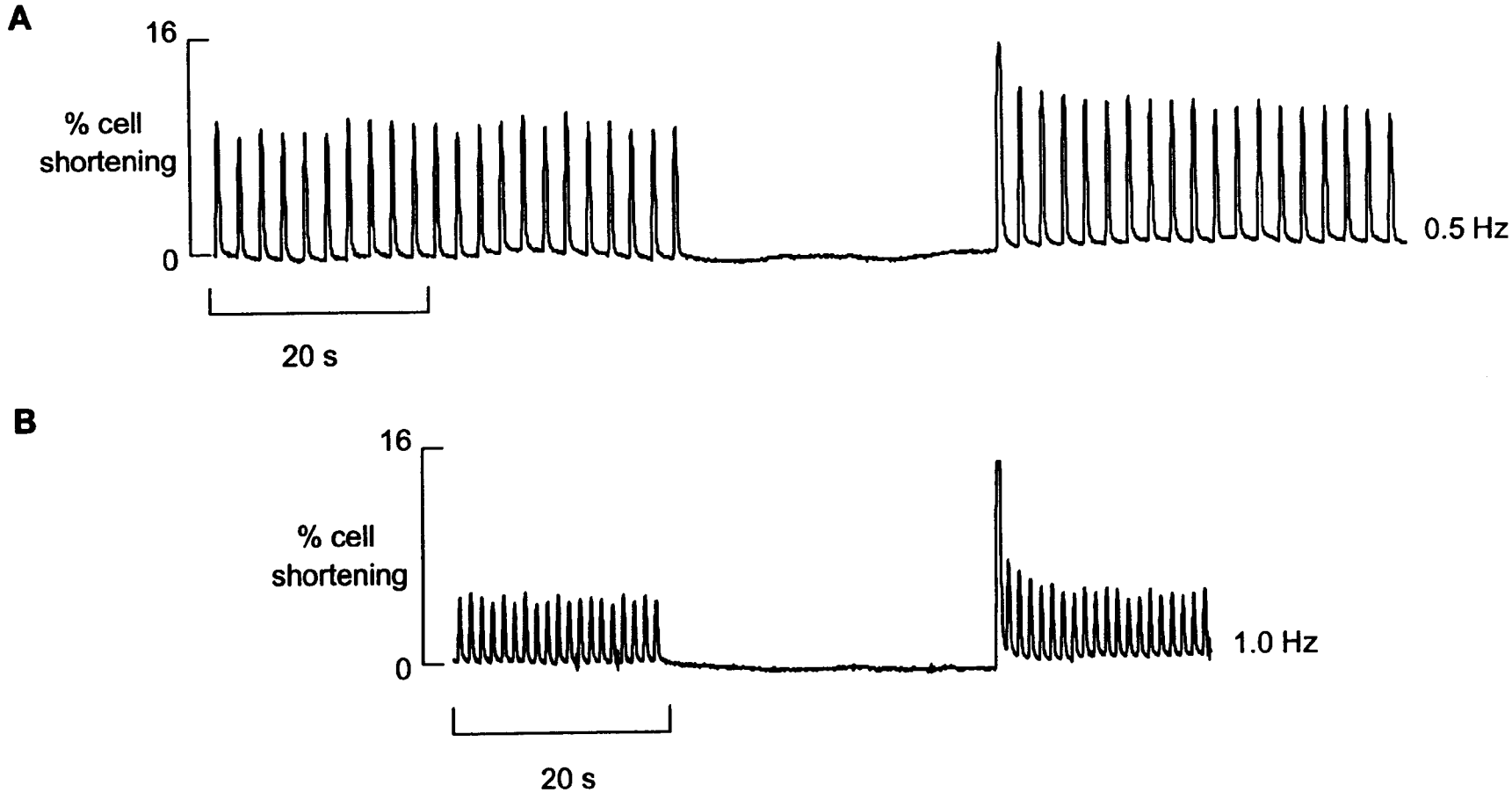

Figure 6.1. The effect of stimulation frequency on contraction amplitude in the steady state and following rest.

Panels show changes in cell length (contraction) during stimulation at 0.5 Hz (upper panel **A**) and 1 Hz (lower panel **B**). The myocyte was voltage-clamped at a holding potential of –40 mV and depolarising pulses of 100 ms duration to 0 mV were applied to elicit contraction.

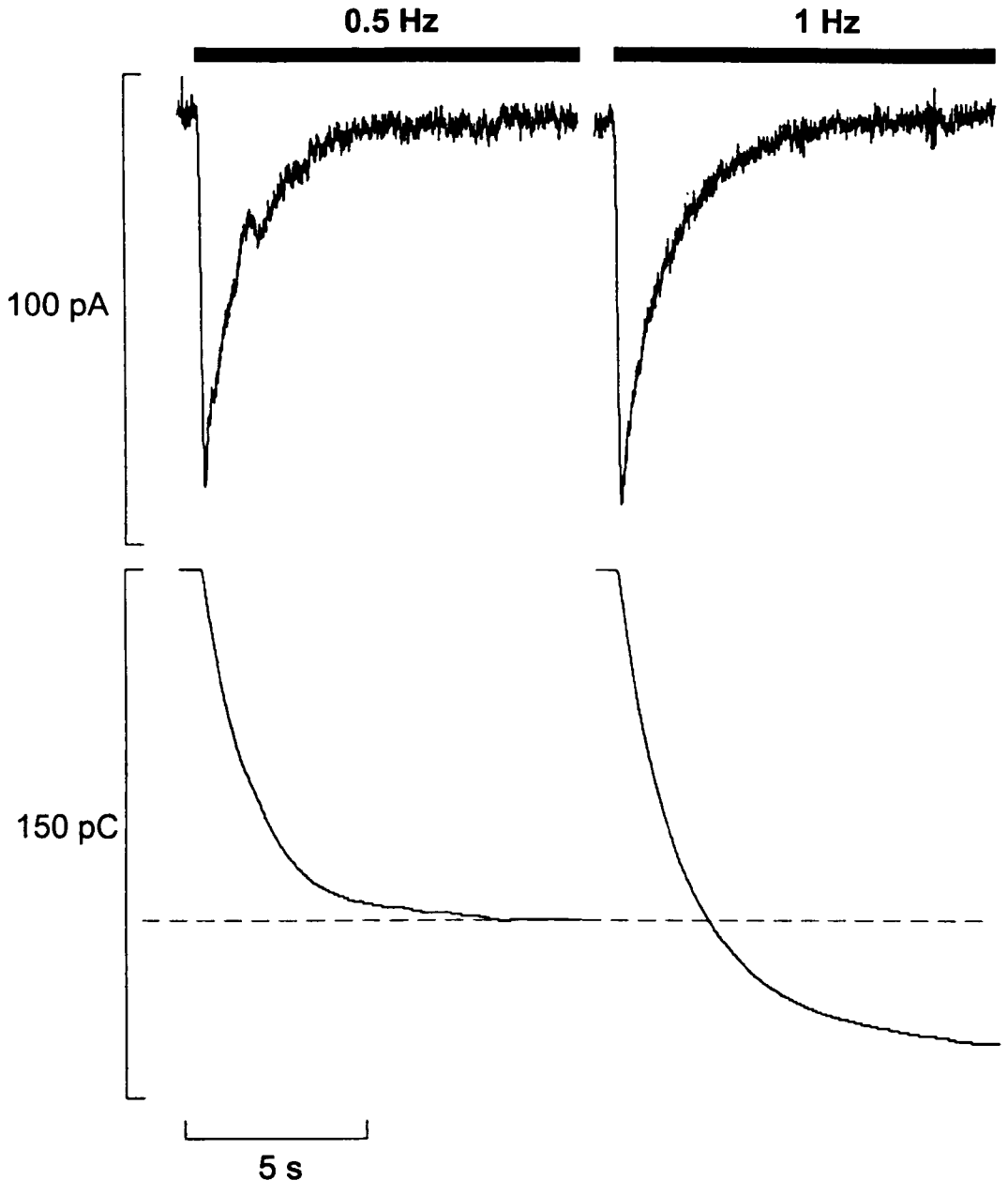

Figure 6.2. The effect of stimulation frequency on SR Ca^{2+} content.

20 mM Caffeine was applied for the periods indicated by the bar, immediately after stimulation was stopped. Traces show current (top) and integrated current (bottom).

Figure 6.2 shows the effect of stimulation frequency on caffeine-evoked Na^+/Ca^{2+} exchange currents. These are related to SR Ca^{2+} content as described previously (Section 2.6.2 [63, 383]). It is clear that the SR Ca^{2+} content is increased during higher frequency stimulation. This may be a consequence of an increase of time-averaged Ca^{2+} influx across the sarcolemma, activated by the more frequent depolarising pulses, providing more Ca^{2+} to the SR. This is in contrast to the results of Bouchard and Bose [53] who found no significant change in the SR Ca^{2+} content with increasing stimulation frequency. This may simply reflect differences in the sensitivity of the techniques used to assess the SR Ca^{2+} content, RCCs versus I_{NaCa} integral in this study. The gain of CICR should be increased with increased SR Ca^{2+} content [25, 166, 195, 235, 315, 343, 344], yet the increased stimulation frequency has a negative inotropic effect in rat ventricle. This supports the hypothesis that the reduced Ca^{2+} current and ability of the SR to refill between depolarising pulses may limit twitch amplitude at high stimulation frequencies. The negative force frequency relationship in the rat is clearly not a result of reduced global SR Ca^{2+} loading. Instead it may reflect rate dependent changes in the coupling of excitation and contraction or in the amount of Ca^{2+} available to release sites in the SR. The steady state twitch amplitude is clearly not a reliable indicator of SR Ca^{2+} content in the rat.

6.3 The Effect of Rest on the Magnitude of Contraction and I_{Ca} Peak and Integral

Isolated rat ventricular myocytes were superfused with a control 2 mM Ca^{2+} Tyrode solution and voltage-clamped at a holding potential of –40 mV. 100 ms or

200 ms duration pulses to 0 mV were used to elicit contraction. Periods of rest of between 15 and 30 s interrupted stimulation, and the effect of this rest period on contraction amplitude, peak Ca^{2+} current and its integral were determined. Figure 6.3 illustrates mean data showing the effect of a 15 s rest interval on the normalised Peak I_{Ca} (A), I_{Ca} integral (B) and contraction amplitude (C) of the first post-rest beat. It is clear that rest transiently increases the amplitude of contraction by 35.2 ± 4.8 % of the control amplitude ($P < 0.001$, $n = 9$), which eventually recovers to the pre-rest steady state level. The peak amplitude of I_{Ca} was increased slightly from 0.457 ± 0.093 to 0.490 ± 0.101 nA following a 15 s rest interval ($P < 0.05$, $n = 9$). The integral of I_{Ca} was reduced very slightly on average from 4.319 ± 0.231 to 4.178 ± 0.250 $\mu mol.l^{-1}$ with a 15 s rest ($P > 0.05$, $n = 9$). Although the integral of I_{Ca} was not significantly changed following a 15 s rest interval, the peak of the Ca^{2+} current was significantly increased, and may contribute to the potentiation of contraction observed.

Figure 6.4 illustrates the effect of a 30 s rest on the peak I_{Ca} (A), I_{Ca} integral (B) and contraction amplitude (C) of the first post-rest beat normalised with respect to the pre-rest values. Contraction amplitude was increased by 40.4 ± 3.3 % following a 30 s rest interval ($P < 10^{-6}$, $n = 10$). Peak I_{Ca} was increased from 0.453 ± 0.086 to 0.489 ± 0.092 nA after a 30 s rest ($P < 0.01$, $n = 10$), while the integral of I_{Ca} was decreased slightly from 4.254 ± 0.230 to 4.138 ± 0.270 $\mu mol.l^{-1}$ ($P > 0.05$, $n = 10$). Once again the peak of Ca^{2+} current was significantly increased. Since this is thought to represent the trigger for CICR, its increased magnitude, probably a result of increased recovery of Ca^{2+} channels from

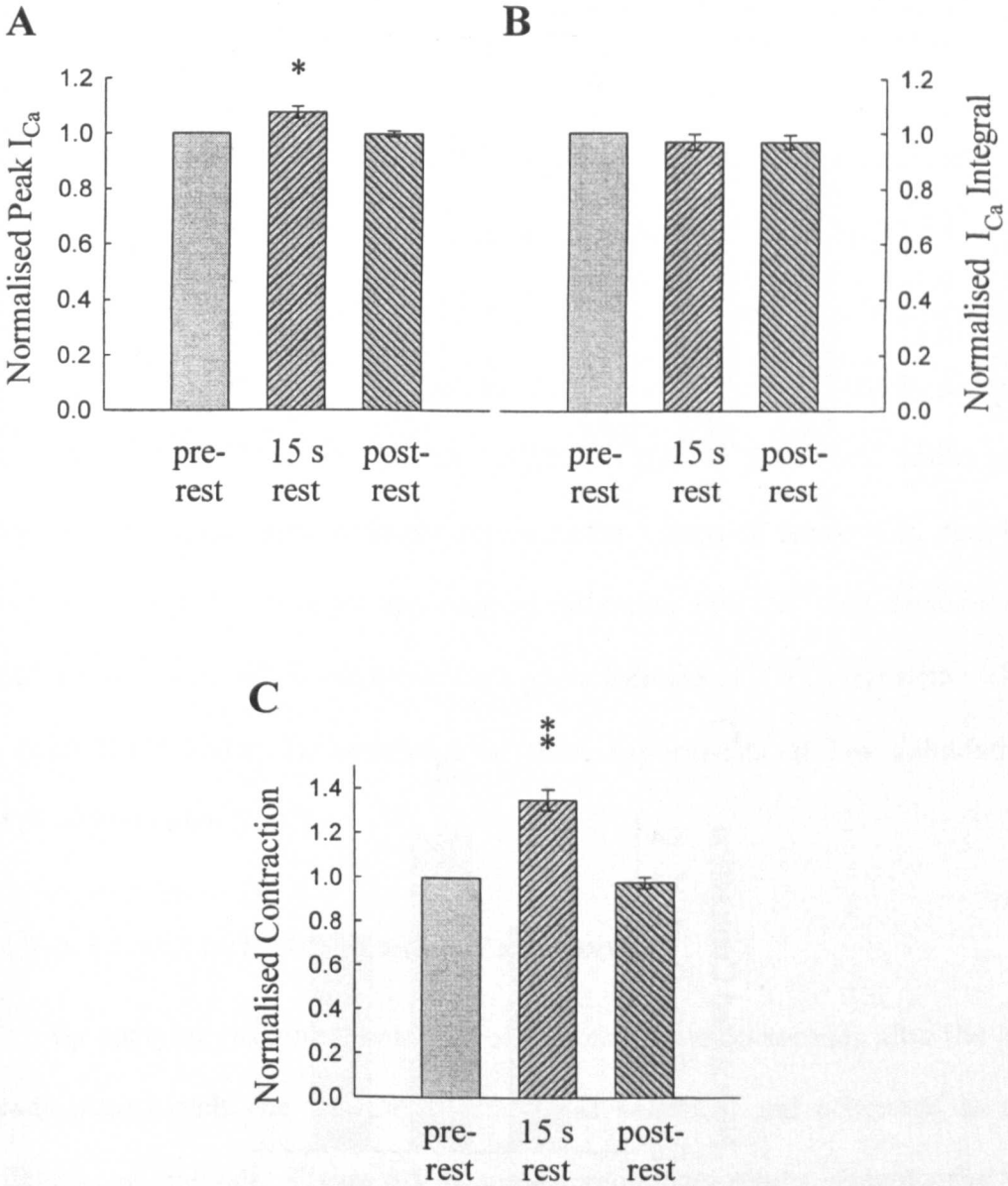

Figure 6.3. The effect of a 15 s rest interval on I_{Ca} and contraction.

Mean data ± S.E.M. showing the effect of rest on; **A**, the peak of I_{Ca}; **B**, the integral of I_{Ca}; and **C**, contraction amplitude. All results were normalised with respect to the pre-rest values ($n = 9$ for all). * and ** indicate significance at the 5 % and 1 % level respectively.

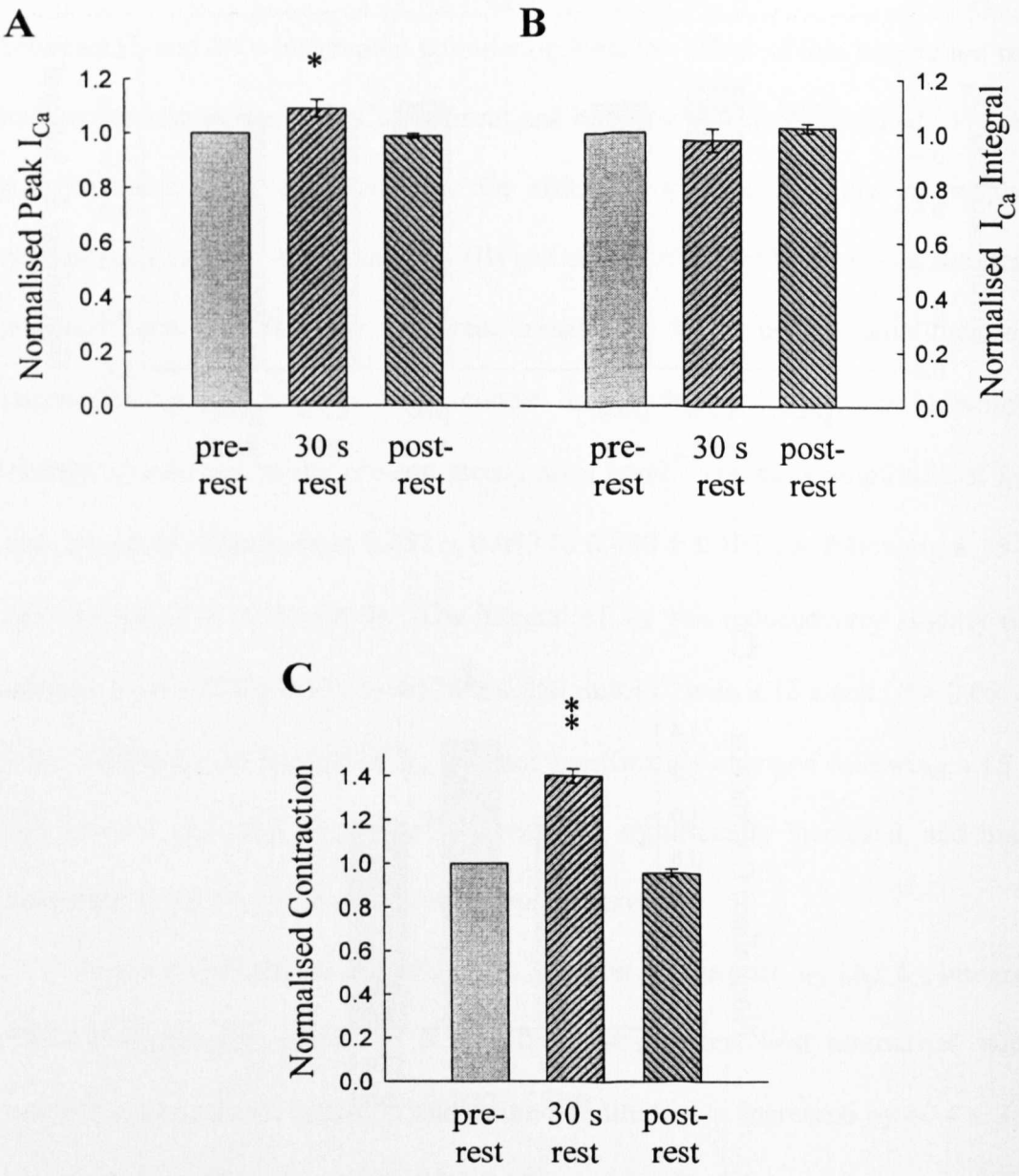

Figure 6.4. The effect of a 30 s rest interval on I_{Ca} and contraction.

Mean data ± S.E.M. showing the effect of rest on; **A**, the peak of I_{Ca}; **B**, the integral of I_{Ca}; and **C**, contraction amplitude. All results were normalised with respect to the pre-rest values (n = 10 for all). * and ** indicate significance at the 5 % and 1 % level respectively.

inactivation, will contribute to the potentiation of contraction, even at this low stimulation frequency.

The degree of potentiation appears to be only slightly affected by increasing the duration of the rest period. Twitch amplitude was increased by 35.2 ± 4.8 % after 15s and by 40.4 ± 3.3 % following 30 s rest. This could be because some cells exhibited spontaneous Ca^{2+} release during the longer rest periods, limiting the accumulation of Ca^{2+} by the SR. Alternatively, the potentiated contraction observed after a period of rest may represent the maximum contraction possible because the myofilaments are approaching saturation with Ca^{2+} and demonstrate smaller changes in twitch amplitude for a given increase in $[Ca^{2+}]_i$ transient. This is quite likely under the conditions of these experiments of low stimulation frequency and high $[Ca^{2+}]_o$.

6.4 The Effect of Rest on the SR Ca^{2+} Content

By applying caffeine at intervals of between 2 and 30 seconds after the last steady state twitch, the SR Ca^{2+} content was estimated and compared at the different rest intervals. Figure 6.5 illustrates preliminary results, showing the SR Ca^{2+} content of a cell rested for the periods indicated above the figure. The caffeine-evoked inward I_{NaCa} currents (upper panel) and their integrals (lower panel) are shown as an index of SR Ca^{2+} content immediately following steady state stimulation pre-rest, following a 15 s rest interval and post-rest in the steady state. Figure 6.6 shows mean data of the increase in SR Ca^{2+} content experienced following 15 s and 30 s rest intervals. The SR Ca^{2+} content increased significantly during a rest period (12.5 ± 1.3 % following 15 s rest and 18.6 ± 2.6 % following

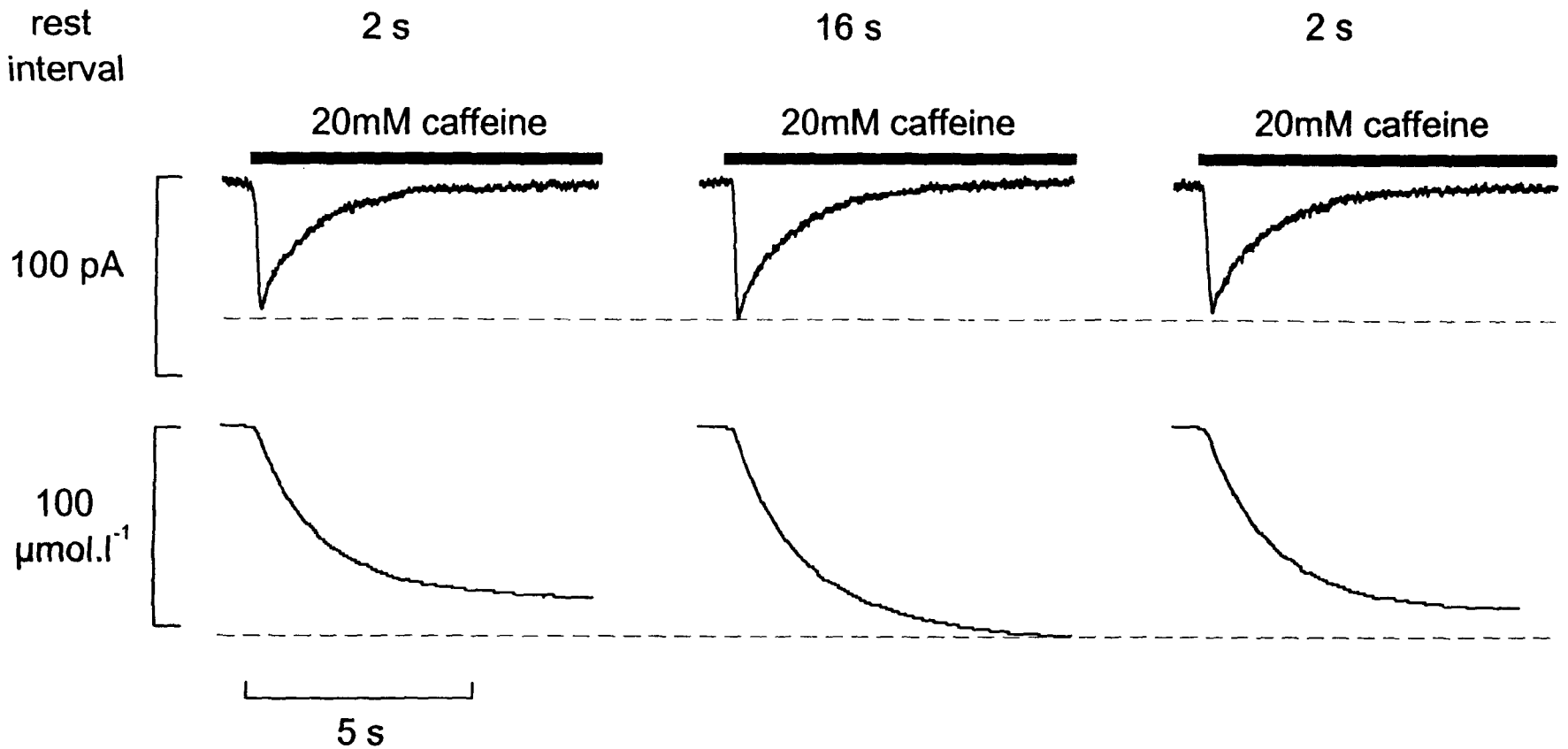

Figure 6.5. The effect of 15 s rest on caffeine-evoked Na^+/Ca^{2+} exchange current and SR Ca^{2+} content.

Upper panels show Na^+/Ca^{2+} exchange currents evoked by 20 mM caffeine (from left to right); ~2 s after the last triggered contraction (pre-rest); ~16 s after the last triggered contraction; 2 s after the last triggered contraction following a period of rest and recovery of the steady state (post-rest). Lower panel illustrates the integrals of the currents expressed in terms of SR Ca^{2+} content.

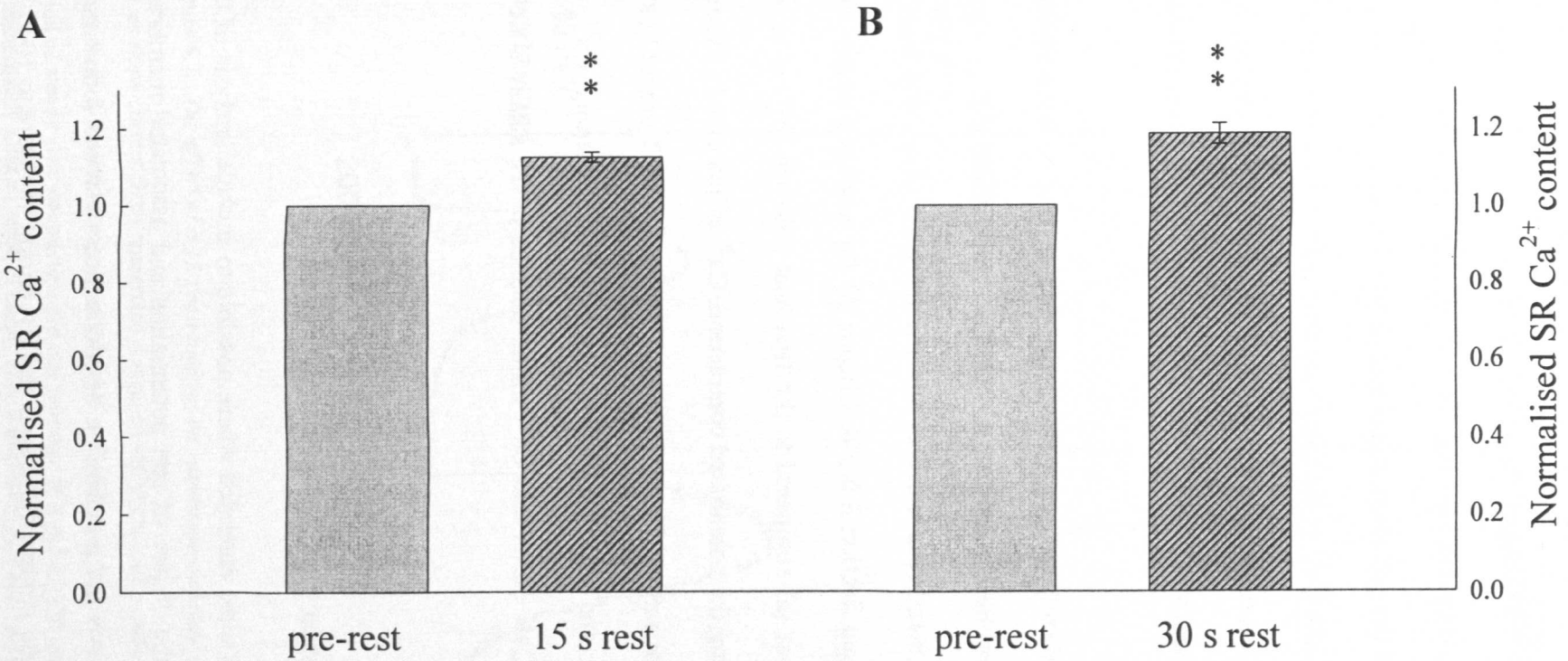

Figure 6.6. The effect of rest on SR Ca^{2+} content.

Histograms showing mean data ± S.E.M. of the increase in SR Ca^{2+} content observed following; **A**, 15 s rest ($P < 0.01$, $n = 9$) and **B**, 30 s rest intervals ($P < 0.01$, $n = 6$). The data was normalised with respect to the pre-rest control SR Ca^{2+} content.

30 s rest, $P < 0.01$, $n = 9$ and 6 respectively). This is in agreement with a resting influx of Ca^{2+} in rat ventricular myocytes [276, 324], and the results of Lewartowski et al, who demonstrated that SR Ca^{2+} depleted rat myocytes can re-accumulate Ca^{2+} even if the cell is not stimulated [238]. The fact that the SR does not continue to accumulate Ca^{2+} at the same rate after a 15 s rest (or the 30 s SR Ca^{2+} content would be increased by twice that of a 15 s rest) can be explained by the presence of spontaneous SR Ca^{2+} release during the extended rest period in some cells.

The extra Ca^{2+} accumulated during this period of rest must be lost before the post-rest steady state can be re-established. This is clearly illustrated in Figure 6.7, which shows raw membrane currents and the integrals of I_{Ca} and I_{NaCa} in terms of Ca^{2+} fluxes (See Section 2.6.2 & Chapter 5) for averaged steady state pulses during the pre-rest (a) compared to the first beat post-rest (b) and steady state post-rest (c). During the potentiated contraction, Ca^{2+} efflux clearly exceeds Ca^{2+} entry during depolarisation. The cell extrudes approximately 2 $\mu mol.l^{-1}$ extra Ca^{2+} on the first beat and will continue to extrude the remaining Ca^{2+} on subsequent beats until Ca^{2+} efflux and Ca^{2+} influx balance in the steady state as described in Chapter 5.

6.5 The Effect of Tetracaine on Rest Potentiation

The investigations were extended where possible to include analysis of the effect of tetracaine on the degree of rest potentiation and associated membrane currents. Figure 6.8 shows the potentiation of contraction observed following a 15 s rest interval (A) under control conditions and (B) during exposure to 100 μM

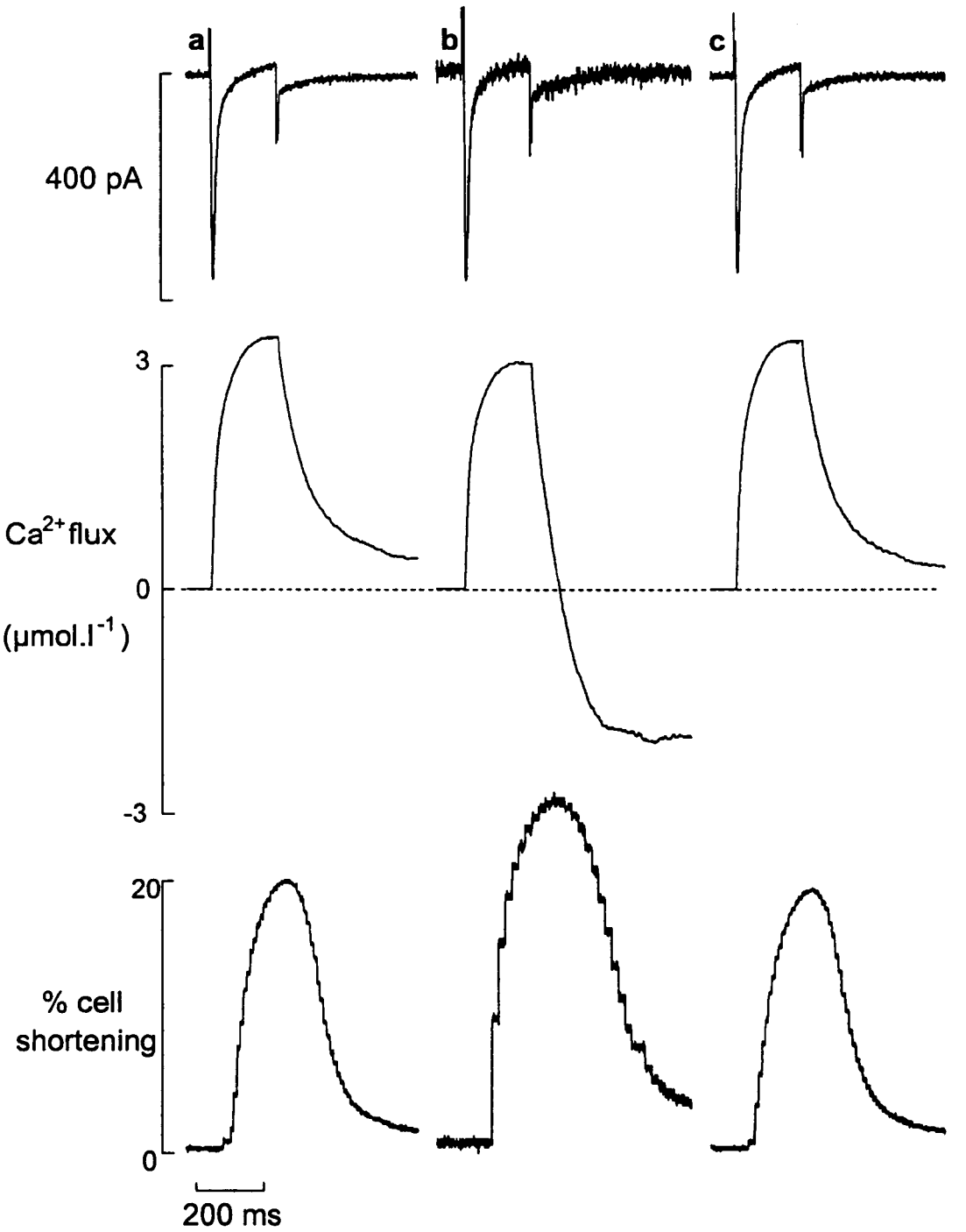

Figure 6.7. The effect of a 15 s rest interval on membrane currents and Ca^{2+} fluxes.

Traces show current (top), integrated current (middle) and contraction (bottom) elicited by 200 ms pulses form a holding potential of –40 mV to 0 mV at a frequency of 0.5 Hz: **A**, following a period of steady state contraction pre-rest (average of 5 pulses); **B**, after a 15 s rest (single pulse); **C**, immediately after a period of steady state contraction post-rest (average of 5 pulses).

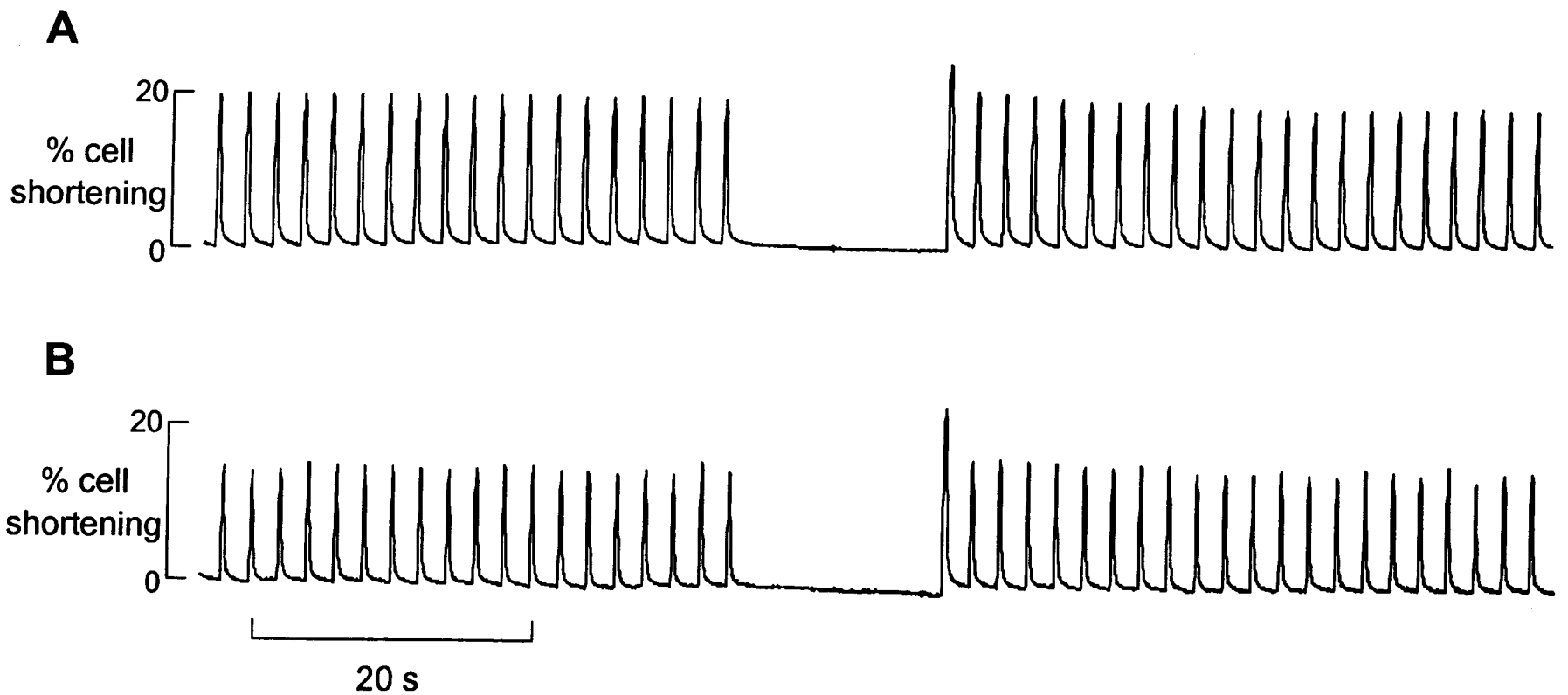

Figure 6.8. The effect of tetracaine on post-rest potentiation of contraction.

Traces show stimulated contractions (changes in cell length) elicited by 200 ms voltage-clamp pulses from a holding potential of –40 mV to 0 mV at 0.5 Hz, interrupted by a 15 s rest interval: **A**, under control conditions and; **B**, during exposure to 100 μM tetracaine.

tetracaine. It is clear from Fig. 6.8 that contraction amplitude appears to be slightly suppressed during exposure to tetracaine, but despite this, the first post-rest contraction is increased beyond the level of the control steady state contraction. The amplitude of steady state contractions in the presence of 100 μM tetracaine was reduced to an average of 70.9 ± 6.0 % (n = 5) of the control amplitude. This was accompanied by a reduction of I_{Ca} integral to 85.9 ± 3.6 % of the control value (n = 5, $P < 0.05$) and peak I_{Ca} was reduced to 60.5 ± 3.6 % of the control (n = 5, $P < 0.05$). The reduction in the amplitude of contraction is clearly correlated with a reduction of both peak I_{Ca} and its integral suggesting that both have a significant role in grading the amount of Ca^{2+} released from the SR and the degree of contraction activated.

Despite the negative inotropic effects of tetracaine the degree of rest potentiation observed was increased. Figure 6.9 illustrates mean data showing the effect of a 15 s rest interval on (from left to right) peak I_{Ca} (A), I_{Ca} integral (B) and contraction amplitude (C). Figure 6.10 shows the mean effect of a 30 s rest interval on the same parameters. After a 15 s rest the first contraction was increased by 86.7 ± 21.1 % compared with the pre-rest steady state contraction amplitude in tetracaine. Similarly, following a 30 s rest, the first post-rest contraction was increased by 94.4 ± 8.1 % above the pre-rest steady state value. There appears to be no significant increase in the degree of rest potentiation with increasing duration of the rest interval beyond 15 s, as is also observed under control conditions. As under control conditions, the large increase in contraction amplitude following 15 s and 30 s rest intervals is accompanied by a small but

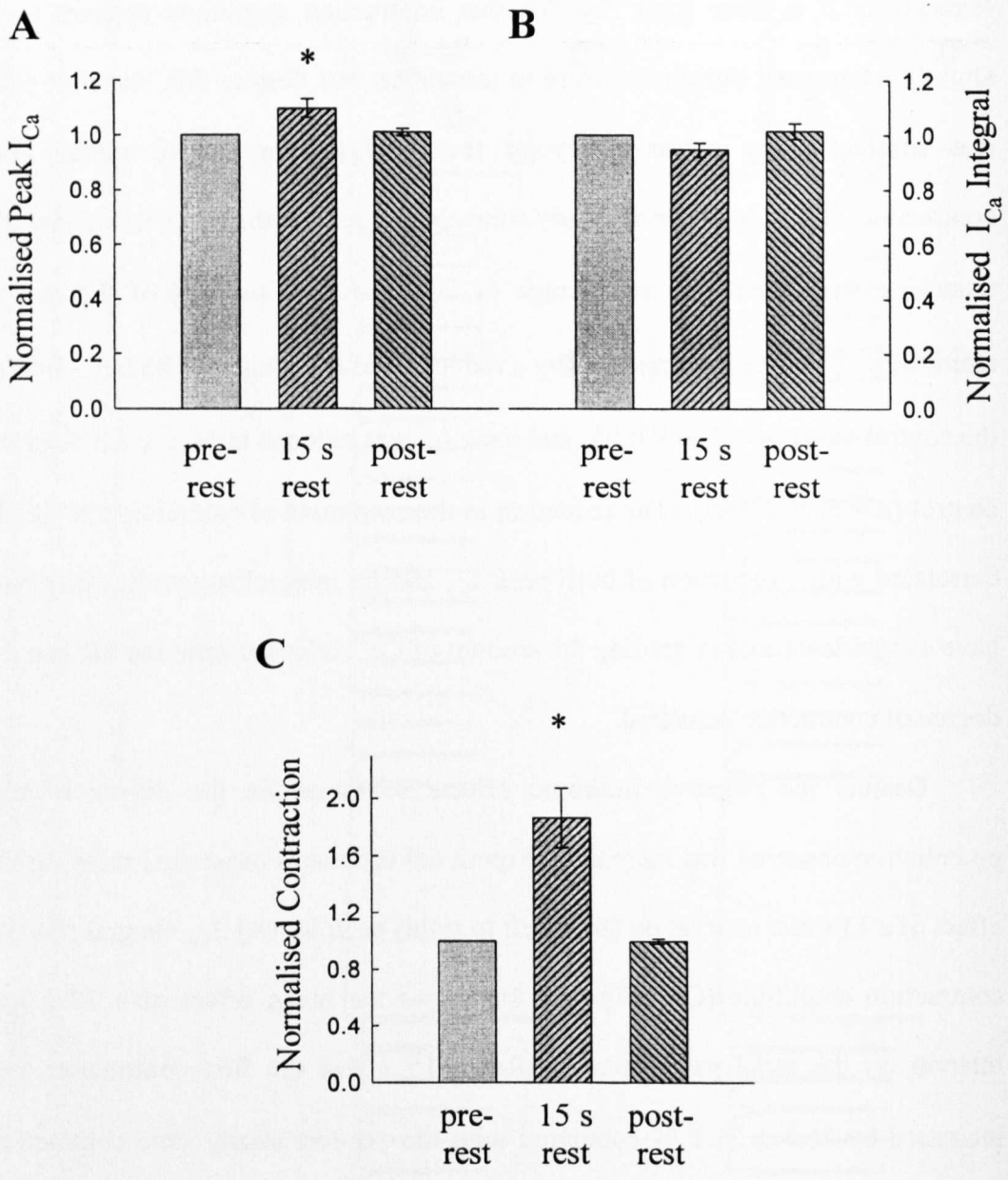

Figure 6.9. The effect of a 15 s rest interval on I_{Ca} and contraction during exposure to 100 μM tetracaine.

Mean data ± S.E.M. showing the effect of rest on; **A**, the peak of I_{Ca}; **B**, the integral of I_{Ca}; and **C**, contraction amplitude. All results were normalised with respect to the pre-rest values (n = 5 for all). * indicates significance at the 5 % level.

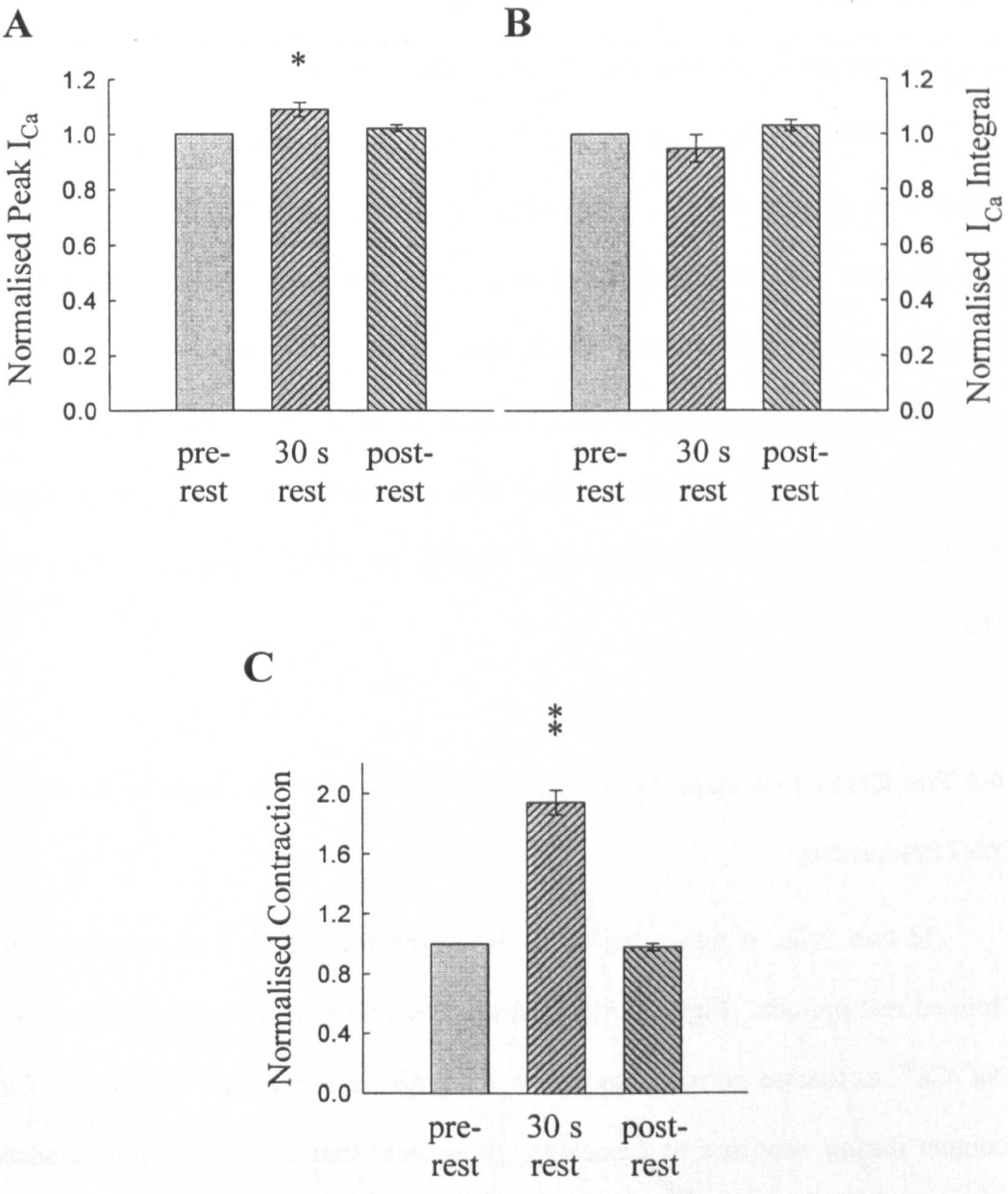

Figure 6.10. The effect of a 30 s rest interval on I_{Ca} and contraction during exposure to 100 μM tetracaine.

Mean data ± S.E.M. showing the effect of rest on; **A**, the peak of I_{Ca}; **B**, the integral of I_{Ca}; and **C**, contraction amplitude. All results were normalised with respect to the pre-rest values (n = 5 for all). * and ** indicate significance at the 5 % and 1 % level respectively.

statistically significant increase in peak I_{Ca} ($P < 0.05$, $n = 5$ for both) and an insignificant decrease of I_{Ca} integral ($P > 0.05$, $n = 5$ for both).

Tetracaine also appears to speed up recovery of the steady state contraction amplitude. This is clearly illustrated by the fact that only the first beat post-rest appears to be potentiated in Fig. 6.8. Figure 6.11 shows the I_{Ca} and I_{NaCa}, and their integrals, of averaged pre-rest steady state pulses compared with the first beat post-rest and steady state post-rest. This can be attributed to activation of a very large Ca^{2+} transient and contraction, which activates a large Ca^{2+} efflux on the Na^{+}/Ca^{2+} exchanger, thereby rapidly reducing the SR Ca^{2+} content to the steady state level.

6.6 The Effect of Rest Interval on SR Ca^{2+} Content during Exposure to Tetracaine

In two cells, it was possible to determine the SR Ca^{2+} content following defined rest periods. Figure 6.12 illustrates the effect of rest on caffeine-evoked Na^{+}/Ca^{2+} exchange currents and their integrals as a measure of the SR Ca^{2+} content during exposure to tetracaine. It is clear that a 15 s rest increased the magnitude of the inward current associated with Ca^{2+} extrusion from the cell (its integral) during exposure to 20 mM caffeine, indicating that the SR Ca^{2+} content is increased during a rest interval in the presence of tetracaine.

Agents that suppress Ca^{2+} accumulation by the SR, e.g. caffeine and ryanodine, are often referred to as inhibitors of the SR. This is a misleading term, as it does not describe their actions fully, failing to specify whether they affect Ca^{2+} release, uptake or accumulation. In fact both caffeine and ryanodine can

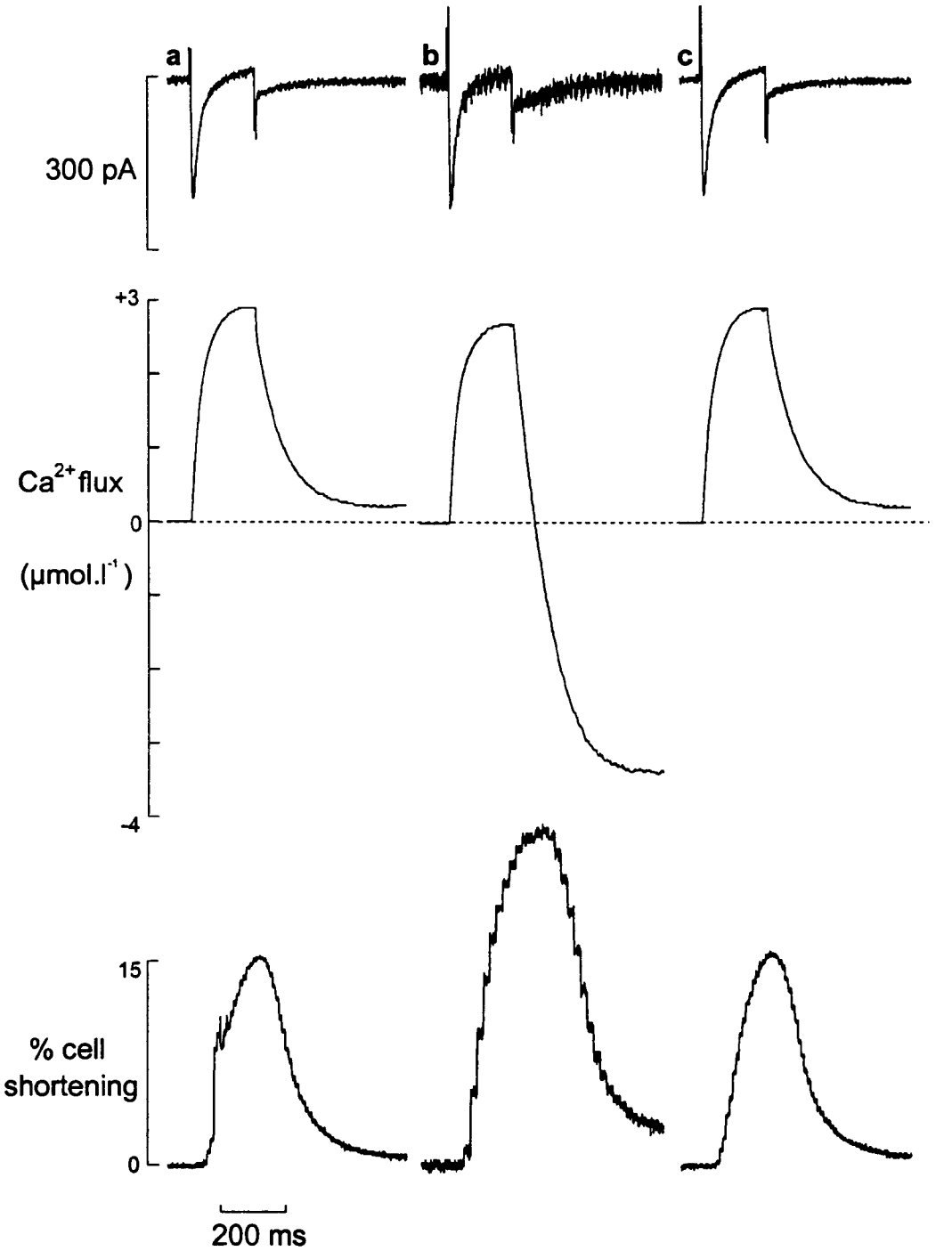

Figure 6.11. The effect of rest on membrane currents and Ca^{2+} fluxes during exposure to 100 μM tetracaine.

Traces show current (top), integrated current (middle) and contraction (bottom) elicited by 200 ms pulses from a holding potential of –40 mV to 0 mV at a frequency of 0.5 Hz: **A**, following a period of steady state contraction pre-rest (average of 5 pulses); **B**, after a 15 s rest (single pulse); **C**, immediately after a period of steady state contraction post-rest (average of 5 pulses). 100 μM tetracaine was continuously present during this period.

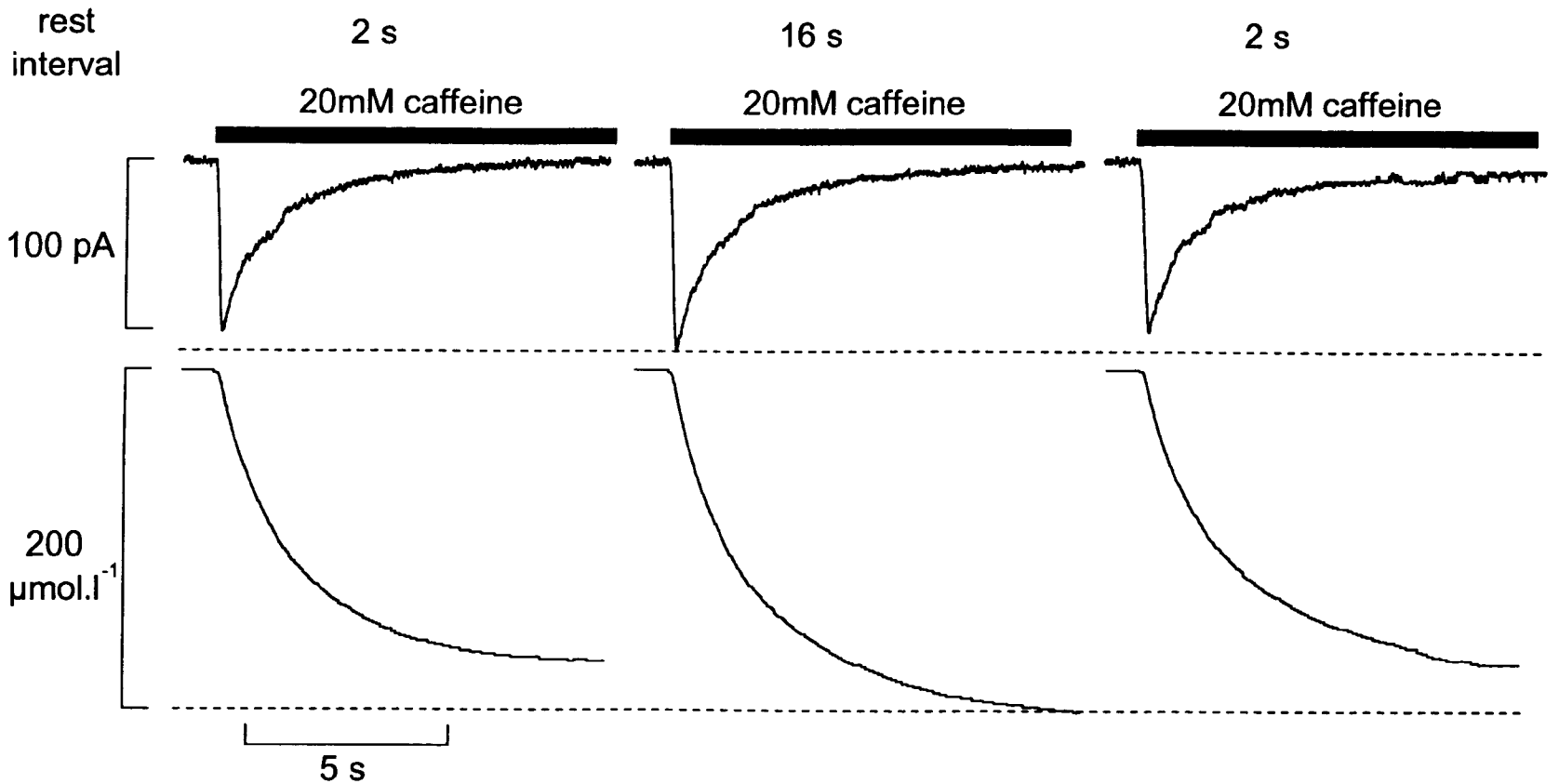

Figure 6.12. The effect of 15 s rest on caffeine-evoked Na^+/Ca^{2+} exchange current and SR Ca^{2+} content during exposure to 100 µM tetracaine.

Upper panels show Na^+/Ca^{2+} exchange currents evoked by 20 mM caffeine (from left to right); ~2 s after the last triggered contraction (pre-rest); ~16 s after the last triggered contraction; 2 s after the last triggered contraction following a period of rest and recovery of the steady state (post-rest). Lower panels illustrate the integrals of the currents expressed in terms of SR Ca^{2+} content.

stimulate Ca^{2+} release from the SR thereby affecting accumulation of Ca^{2+}. It is through this activity that this type of SR inhibitor can reduce post-rest potentiation. Inhibitors of SR Ca^{2+} release, such as tetracaine, on the other hand, could enhance potentiation, by increasing SR Ca^{2+} accumulation. The SR Ca^{2+} content was increased from the steady state pre-rest Ca^{2+} content of 174.5 $\mu mol.l^{-1}$ to 193.2 $\mu mol.l^{-1}$ following a 15 s rest during exposure to tetracaine. This increase of 18.7 $\mu mol.l^{-1}$ is slightly higher than that observed during a 15 s rest under control conditions of 16.4 $\mu mol.l^{-1}$ in the same cell.

In these experiments basic observation suggests that low concentrations of tetracaine may enhance post-rest potentiation, despite its well-characterised transient negative inotropic effects on contraction under these conditions [295]. At least some of the potentiation of contraction following a period of rest under these conditions could be attributed to an increase in SR Ca^{2+} content. The ability of tetracaine to enhance post-rest potentiation may be a consequence of increased Ca^{2+} accumulation by the SR in the presence of this release inhibitor. This could be a consequence of reduced SR Ca^{2+} leak in the absence of any effect on SR Ca^{2+} uptake or an increase of SR Ca^{2+} uptake by stimulating the SR Ca^{2+}-ATPase. The following results describe a more direct approach to estimating the ability of tetracaine to enhance SR Ca^{2+} accumulation.

6.7 The Effect of Tetracaine on SR Ca^{2+} Accumulation

The effect of tetracaine on SR Ca^{2+} accumulation in un-stimulated rat ventricular myocytes was achieved by applying 20 mM caffeine to myocytes to empty the SR of Ca^{2+}, and varying the loading time between successive caffeine

exposures. The Na^+/Ca^{2+} exchange current activated by caffeine was used to calculate SR Ca^{2+} content at the intervals tested, revealing the pattern of Ca^{2+} accumulation. This protocol was used under control conditions and during exposure to 100 μM tetracaine in the same cell to allow a statistical comparison of the rates of Ca^{2+} accumulation. In all five cells analysed increasing the loading time between successive caffeine exposures increased the SR Ca^{2+} content, so long as the cell remained quiescent. This is again consistent with the observation by Lewartowski *et al*, who demonstrated that SR Ca^{2+} depleted rat myocytes can re-accumulate Ca^{2+} even if the cell is not stimulated [238]. Figure 6.13A shows a typical record of the increase in SR Ca^{2+} content with time under control conditions and during exposure to 100 μM tetracaine. The increase in Ca^{2+} content was found to be linear over the period for which the cells remained quiescent under control conditions and during exposure to 100 μM tetracaine. In this cell spontaneous oscillations occurred between 90 and 120 s under control conditions and between 150 and 180 s during exposure to tetracaine. The linear range of SR Ca^{2+} accumulation was subjected to regression analysis to provide a value for the rate of Ca^{2+} accumulation in each cell. Figure 6.13B illustrates mean data from the regression analysis showing the effect of 100 μM tetracaine on the rate of Ca^{2+} accumulation in the SR. SR Ca^{2+} accumulation was increased from 0.776 ± 0.182 $\mu mol.l^{-1}.s^{-1}$ under control conditions to 0.920 ± 0.226 $\mu mol.l^{-1}.s^{-1}$ during exposure to 100 μM tetracaine ($n = 5$, $P < 0.05$). So it appears that the ability of tetracaine to enhance Ca^{2+} accumulation by the SR, possibly as a consequence of reducing Ca^{2+} leak through RyRs, could contribute to its ability to enhance post-rest potentiation. However, the difference in the rate of

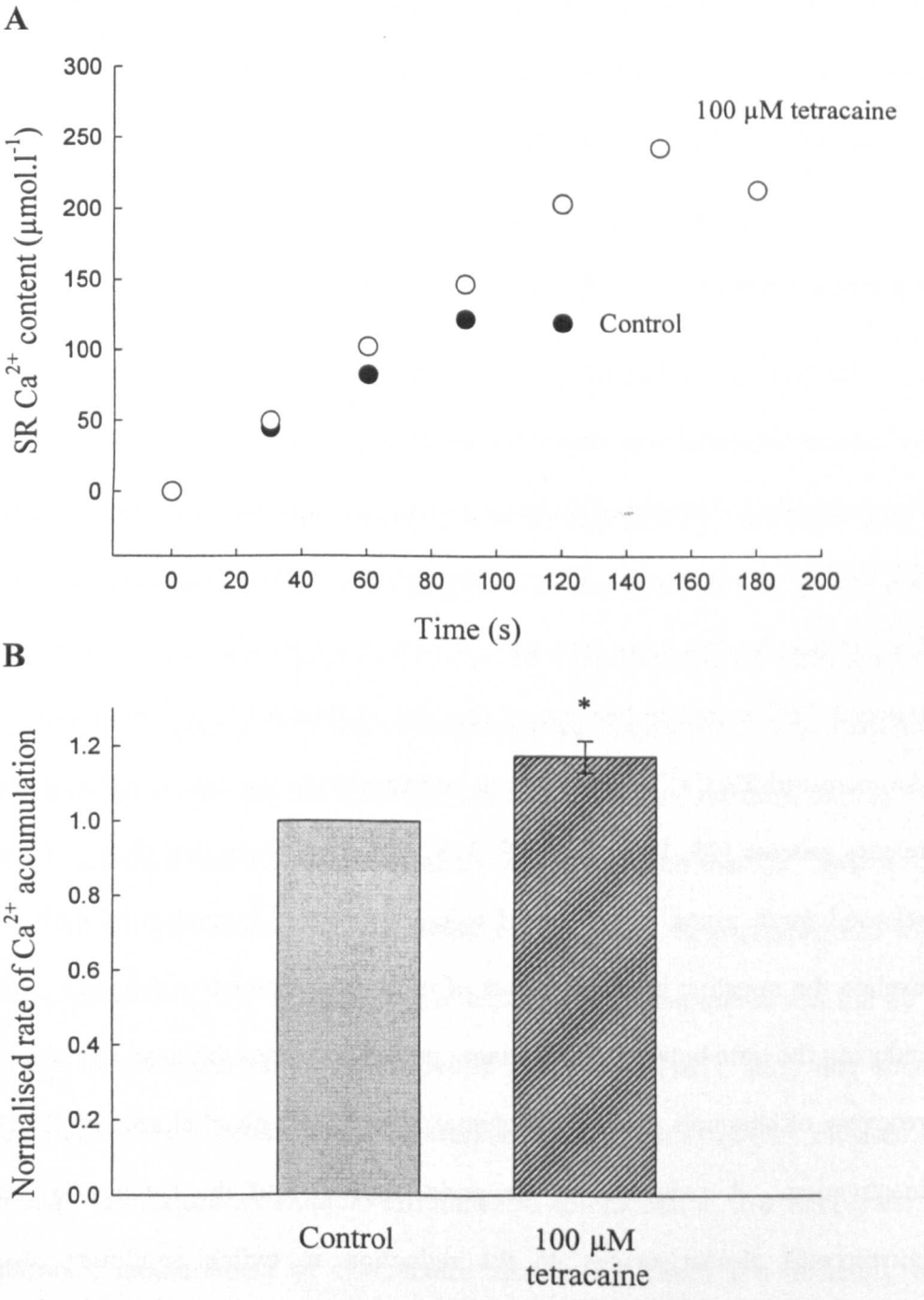

Figure 6.13. The effect of tetracaine on the rate of Ca^{2+} accumulation by the SR in un-stimulated voltage-clamped rat ventricular myocytes.

A, representative graph showing: ordinate, SR Ca^{2+} content from caffeine-evoked inward current at holding potential of –80 mV; abscissa, time (s). **B**, bar chart showing the mean data ± S.E.M. of the effect of tetracaine on the linear rate of SR Ca^{2+} accumulation by the SR in un-stimulated myocytes ($P < 0.05$, $n = 5$). Data was normalised with respect to control to eliminate the effect of variations in the control rate of Ca^{2+} accumulation.

accumulation observed here can only account for an extra SR Ca^{2+} accumulation of 2.2 $\mu mol.l^{-1}$ over a 15 s rest interval, and such a small difference seems unlikely explain the extent of potentiation seen.

6.8 Discussion

Increasing the frequency of stimulation had a negative inotropic effect in isolated rat ventricular myocytes under the experimental conditions of this study. Paradoxically the increased frequency of stimulation also produced an increase in SR Ca^{2+} load of the cell. This is probably the result of increase time averaged Ca^{2+} influx through more frequently activated L-type Ca^{2+} channels and perhaps reduced Ca^{2+} extrusion because of the limited time between depolarising pulses. An increased SR Ca^{2+} content would be expected to increase the gain of the Ca^{2+} release process [25, 166, 195, 235, 315, 343, 344], such that more Ca^{2+} will be released for a given amplitude of trigger $[Ca^{2+}]_i$. A number of factors could explain the negative inotropic effect of increased stimulation frequency. Firstly, reducing the time between depolarising pulses decreases the time available for the recovery of channels (both sarcolemmal L-type Ca^{2+} channel and SR RyR) from inactivation. A reduction in the peak amplitude of the L-type Ca^{2+} current corresponds almost exactly to the reduction in twitch amplitude observed. However, it is necessary also to consider the increased SR Ca^{2+} content under this condition, which would be expected to result in increased Ca^{2+} release for the same trigger I_{Ca}. There may be some contribution of time-dependent refilling of the release sites of the SR Ca^{2+} store. Perhaps this is consistent with the results of Orchard and Lakatta [292], who proposed that the depression of twitch with

increasing stimulation frequency in rat myocardium might be due to the fact that replenishment of the SR with Ca^{2+} is inversely related to stimulation frequency. After a period of rest, the enhancement of post-rest potentiation observed at the higher stimulation frequency could therefore be accounted for by increased recovery of Ca^{2+} current and full replenishment of SR Ca^{2+} release sites.

6.8.1 Rest Potentiation is Associated with an Increase of SR Ca^{2+} Content

Despite that fact that rest decay has been successfully correlated with a depletion of SR Ca^{2+} with rest, investigations of the effect of rest on SR Ca^{2+} content of rat ventricle have produced inconsistent results using the techniques of measurement of rapid-cooling and caffeine-induced contracture. Rapid-cooling inhibits Ca^{2+} uptake by the SR Ca^{2+}-ATPase and Ca^{2+} removal from the cell by Na^{+}/Ca^{2+} exchange and the sarcolemmal Ca^{2+}-ATPase, such that Ca^{2+} that leaks from the SR will not be removed rapidly as it is at normal temperatures, and will activate contraction [58]. A possible limitation of this technique is that the Ca^{2+} sensitivity of myofilaments is also reduced in the cold [167], such that small changes in the SR Ca^{2+} content may be masked, because the extra Ca^{2+} released is not enough to produce measurable difference in contraction at low temperature. Furthermore, measurement of contracture also suffers from the difficulty of measuring cell length accurately and relies on the assumption that the myofilaments are not approaching saturation by the amount of Ca^{2+} released from the SR. The use of caffeine-induced contracture as an index of SR Ca^{2+} content relies strongly on the ability to prevent removal of Ca^{2+} from the cytosol. Caffeine also enhances the Ca^{2+} sensitivity of the myofilaments [395] such that

small changes in released Ca^{2+} may be exaggerated. In the experiments described here a period of rest caused potentiation of contraction, and this was associated with an increased SR Ca^{2+} content, as assessed by the caffeine-evoked Na^{+}/Ca^{2+} exchange current. These results provide direct evidence that an increase in SR Ca^{2+} content during intervals of rest could explain the phenomenon of rest potentiation in rat ventricle.

6.8.2 Increasing the Rest Interval does not change the Apparent Degree of Potentiation

Increasing the period of rest from 15 s to 30 s did not cause further enhancement of the rest-potentiation. This may be in agreement with previous reports of saturation of the SR with Ca^{2+} [53]. This was also suggested by the observation that a number of cells exhibited spontaneous release of Ca^{2+} during the longer rest periods, preventing the further increase of SR Ca^{2+} content.

However, the SR Ca^{2+} content was increased more on average during a 30 s rest interval in comparison with the 15 s rest (See Fig. 6.6). An alternative explanation as to why potentiation of contraction is not enhanced further during a 30 s rest interval is needed. During the potentiated twitch the myofilaments may be approaching saturation with Ca^{2+} in these cells, such that increments in $[Ca^{2+}]_i$ transient are not detectable in the amplitude of contraction. This would explain why the potentiated twitch was always of similar amplitude at different stimulation frequencies and during exposure to tetracaine. This is serious limitation to the interpretation of these results, since it is now clear that the degree

of twitch potentiation observed from a contraction record may not be an accurate indication of systolic Ca^{2+} release from the SR.

The decay of potentiation was enhanced in the presence of tetracaine because the potentiated twitch under these conditions activated more Ca^{2+} extrusion, speeding up the recovery of the steady state SR Ca^{2+} content. This suggests that during exposure to tetracaine more Ca^{2+} was released from the SR during the first post-rest beat than under control conditions, thereby activating more Ca^{2+} extrusion on the Na^{+}/Ca^{2+} exchanger. However, the potentiated twitch during exposure to tetracaine was of similar amplitude to that observed under control conditions, lending support to the hypothesis that the myofilaments may be approaching saturation during the potentiated twitch.

6.8.3 Enhancement of Rest Potentiation by Tetracaine

Tetracaine appears to enhance post-rest potentiation. This is consistent with the ability of the related drug cocaine to enhance post-rest potentiation despite having a negative inotropic action on steady state stimulated contraction in this muscle preparation [78]. Agents that suppress Ca^{2+} accumulation by the SR, e.g. caffeine and ryanodine, have been shown to reduce or abolish post-rest potentiation [264]. Inhibitors of SR Ca^{2+} release, such as tetracaine, on the other hand, could increase SR Ca^{2+} accumulation and this could explain their ability to enhance rest potentiation. This study has provided evidence that tetracaine can enhance the accumulation of Ca^{2+} by the SR. However, the increased accumulation of Ca^{2+} could only account for a small increase of SR Ca^{2+} content

above that observed under control conditions, and is therefore unlikely to explain the ability of tetracaine to enhance post-rest potentiation fully.

During exposure to tetracaine there is an increase in the steady state SR Ca^{2+} content (See Chapter 5). At increased SR Ca^{2+} content the Ca^{2+} current could be more efficient at eliciting Ca^{2+} release due to the increased gain of the CICR mechanism, so the effect of any recovery of peak Ca^{2+} current may be exaggerated by increased gain. However, since the increase in SR Ca^{2+} content is itself the result of the reduced gain of CICR due to inhibition of the RyR by tetracaine, this is unlikely to be the case.

The enhanced ability to accumulate Ca^{2+} in the SR during exposure to tetracaine may be a consequence of reduced leak of Ca^{2+} through RyRs. The extra accumulation of Ca^{2+} was found to be an average of 0.144 $\mu mol.l^{-1}.s^{-1}$ in the presence of 100 μM tetracaine. This compares quite well with the calculated unidirectional efflux of Ca^{2+} from rat SR of 0.32 $\mu M.s^{-1}$ reported by Bassani and Bers [29]. This was assumed to be primarily due to occasional opening of SR Ca^{2+} release channels. The extra accumulation of Ca^{2+} during exposure to tetracaine could therefore be accounted for by partial inhibition of RyR openings and a reduction in the amount of time averaged Ca^{2+} efflux from the SR. This is consistent with the observation that this concentration of tetracaine does not produce complete inhibition of Ca^{2+} release, as evidenced by the recovery of spontaneous [157, 294] (See Chapter 3 & 4) and stimulated contraction [295] (See Chapter 5) during continued exposure to the drug.

The source of the Ca^{2+} that accumulates in the SR at rest is likely to be extracellular Ca^{2+}, which constantly leaks into the cell down its large

electrochemical gradient. Most other species demonstrate rest decay and depletion of the SR at rest, because the Na^+/Ca^{2+} exchanger or the sarcolemmal Ca^{2+}-ATPase rapidly extrudes any Ca^{2+} that leaks into the cytoplasm across the sarcolemma or from the SR. The rat is known to possess a much higher intracellular Na^+ than most other species [324, 390], which imposes a bias on the Na^+/Ca^{2+} exchanger in favour of Ca^{2+} entry and reduced Ca^{2+} efflux at resting membrane potentials. This will be enhanced under the conditions of these experiments, where the holding potential of –40 mV in most experiments will further enhance this bias. Recent work by Ho Sook Choi has lent support to the hypothesis that the resting influx of Ca^{2+} in the rat can be attributed largely to a Ni^{2+} sensitive Na^+/Ca^{2+} exchange mechanism [90].

Chapter 7

CHAPTER 7

THE EFFECT OF METABOLIC INHIBITION ON Ca^{2+} HANDLING BY THE SR

The working heart rapidly consumes the energy supplied to it, and is therefore strongly dependent on an uninterrupted supply of substrates, especially oxygen, which cannot be stored. Myocardial Ischemia (reduced blood-flow to the heart muscle) is one of the most common causes of serious illness and premature death in developed countries. Its main cause is the obstruction of the coronary arteries that supply the myocardium, and is synonymous with coronary heart disease. Reduced blood flow deprives the heart muscle of oxygen and other essential substrates, reducing mechanical performance and making the heart more susceptible to lethal arrhythmias. Although the most important effect of ischaemia is the lack of oxygen, ischaemia and hypoxia have different effects on the myocardium [9]. This is probably due to the inability to remove metabolites from the myocardium in the absence of adequate blood flow and consequent inhibition of glycolysis. Exactly how the changes in ions and metabolites that occur during ischaemia cause the changes in contractility is still uncertain, but it may involve modulation of both the contractile proteins, generation of the action potential and CICR.

In the absence of oxygen for oxidative phosphorylation anaerobic glycolysis ensues. The end product of anaerobic glycolysis is lactate, which in absence of an adequate blood supply will accumulate and eventually cause inhibition of

anaerobic glycolysis and total metabolic blockade. Phosphate (P_i), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) are the products of ATP hydrolysis and accumulate when oxidative ATP production ceases. When the heart's ability to produce ATP is compromised, $[ATP]_i$ is expected to fall, however, large changes in $[ATP]_i$ have not been observed in cells exposed to metabolic inhibition [117]. Instead, there is a large fall in phosphocreatine (PCr) and a large increase in inorganic phosphate (P_i) [8, 118]. Free $[Mg^{2+}]_i$ is also increased in the absence of ATP.

Intracellular acidosis has been attributed to the accumulation of lactate as a result of anaerobic glycolysis, which is utilised under conditions of hypoxia [7, 8]. During ischaemia even anaerobic glycolysis may be limited due to the accumulation of metabolites. Acidosis can still occur in the absence of glycolysis and has been attributed to the release of protons during ATP hydrolysis [114]. The accumulation of protons in the ischaemic heart is largely brought about by the hydrolysis of high-energy phosphate compounds, producing inorganic phosphate, which acts as a weak acid releasing protons. However, during the early stages of ischaemia this is likely to be opposed by the proton consuming reaction of phosphocreatine hydrolysis, which will cause a short-lived alkalosis [8, 172] and limit acidosis. In the absence of glycolysis, this alkalosis may be more significant, because of the reduced contribution of lactate accumulation.

$$PCr + H^+ \longrightarrow Cr + P_i$$

Although the degree, source and timing of acidosis remain the subject of debate, there is no question that the ischaemic heart rapidly becomes acidotic [191]. Proton accumulation is likely to have a number of consequences. i) It will

inhibit glycolysis. ii) Protons can displace Ca^{2+} ions from anionic binding sites, and reduce contractility by decreasing the Ca^{2+}-sensitivity of the myofilaments and maximal Ca^{2+} activated contraction [51, 132, 165, 216, 290]. iii) Protons also depress Ca^{2+} uptake [125, 204] and release from the SR [288, 291].

The hydrolysis of ATP and phosphocreatine releases large amounts of phosphate (increased from 4 mM to 20 mM [8]), which also reduces the Ca^{2+} sensitivity of the cardiac contractile proteins and maximum Ca^{2+} activated force [109, 205, 220]. In the steady state therefore, both acidosis and increased $[P_i]_i$ will reduce the contractility of the myocardium. A number of studies have separated the contribution of these two effects on contractility revealing that increasing $[P_i]_i$ is the dominant effect [110, 220]. Increasing free $[Mg^{2+}]_i$ also decreases myofilament sensitivity [124].

Ischaemia is also associated with a massive loss of potassium ions (K^+) from the cell. This is believed to be due to the opening of ATP-sensitive K^+ channels (K_{ATP}) in the sarcolemma when $[ATP]_i$ falls below a critical level [151, 281]. The ATP produced by glycolysis preferentially blocks these channels [393] and this has lent support to the hypothesis that glycolysis is essential to the integrity of membrane function, while the ATP derived from oxidative phosphorylation is used to maintain contractile activity [171]. Activation of K_{ATP} channels causes an outward current and shortening of the action potential [76, 151, 227], such that at sufficiently low $[ATP]_i$ levels, the action potential may be completely prevented. Action potential shortening has been observed in the absence of changes in bulk $[ATP]_i$ necessary to activate K_{ATP} [117]. However, changes in $[ATP]_i$, in as yet unresolved discrete subcellular compartments, may be adequate to explain such

observations [172]. This efflux of positive ions from the cell would be expected to hyperpolarise the membrane, thereby reducing excitability and has therefore been proposed to serve as a protective mechanism, whereby energetically compromised cells are prevented from depolarising, thereby conserving the energy that would otherwise be consumed by further contractile activity. However, extracellular accumulation of K^+ is a typical phenomenon during ischaemia and will depolarise the resting membrane potential, such that any spontaneous activity is more likely to cause triggered arrhythmias.

During ischaemia there is also a progressive accumulation of Na^+ [106, 249] and Ca^{2+} [338] in the cytoplasm. Na^+ entry is favoured under conditions of acidosis, which stimulates the extrusion of protons and influx of Na^+ on the Na^+/H^+ exchanger [101, 169], although this mechanism is slowed by inhibition of glycolysis [402]. Regulation of aNa_i by the Na^+/K^+-ATPase (sodium pump) is also compromised at low pH and $[ATP]_i$ [268, 345]. The elevation of aNa_i favours Ca^{2+} entry on the Na^+/Ca^{2+} exchanger and may lead to increased Ca^{2+} loading of the SR. Acidosis alone has been shown to increase the SR Ca^{2+} content of rat ventricular myocytes through effects on aNa_i and $[Ca^{2+}]_i$ [169]. Increased intracellular $[H^+]$ may also directly increase diastolic $[Ca^{2+}]_i$ by competing with Ca^{2+} ions for intracellular Ca^{2+} binding sites [46, 384]. A further source of Ca^{2+} entry has recently been identified to be a Ca^{2+} leak channel activated by metabolic inhibition [389]. The reduction in ATP availability may also inhibit Ca^{2+}-ATPase pumps of the sarcolemma and SR, thereby reducing Ca^{2+} removal from the cytosol [338]. Although the intracellular [ATP] is unlikely to fall to levels that

limit Ca^{2+} uptake into the SR, intracellular phosphate and ADP also affect the free energy of ATP hydrolysis according to the equation:

$$\Delta G_{ATP} = \Delta G^{0}_{ATP} + RT \ln \frac{[ATP]}{[ADP][P_i]}$$

Such that as $[P_i]$ and [ADP] increase the free energy of hydrolysis of ATP will fall, and less energy is available to fuel the translocation of Ca^{2+} from the cytosol. The mechanisms of Ca^{2+} overload have been reviewed by Tani [362].

Low pH reduces SR Ca^{2+} release [204] by reducing the open probability of the RyR [310]. However, low pH has also been demonstrated to reduce Ca^{2+} uptake into the SR of saponin-treated ferret ventricular muscle, such that these two effects counter-balance and there is no effect on the steady state SR Ca^{2+} content [204]. Similar observations were also made with increasing $[Mg^{2+}]_i$.

ATP increases the opening probability of the RyR [258, 311], such that the reduction in intracellular $[ATP]_i$ that occurs during ischaemia may therefore be expected to suppress SR Ca^{2+} release. Increased free $[Mg^{2+}]_i$ has also been shown to depress RyR opening [130, 204, 405]. The acidification that occurs during hypoxia and ischaemia will also depress SR release channel activity [405]. P_i also has as yet unresolved effects on the SR Ca^{2+} release channel. P_i can release Ca^{2+} from the SR, thereby decreasing the SR Ca^{2+} content of saponin-treated rat cardiac trabeculae [339, 347]. However, there was an increase of spontaneous activity on removal of P_i [339], which could be consistent with the removal of P_i mediated inhibition of Ca^{2+} release. Fruen *et al* [140] suggested that, in cardiac muscle, P_i induced release of SR Ca^{2+} could not be attributed to an activation of the RyR,

while in contrast, sheep cardiac RyR appears to be activated by P_i [207]. ADP was also shown to activate the RyR, albeit to a lesser degree than ATP [207]. Under conditions of ischaemia, the fall of ATP is likely to reduce RyR activity more than the increase of ADP can stimulate it, such that the combined effect of these changes alone is likely to be a decrease of channel open probability. The regulation of isolated cardiac SR release channels at the intracellular concentrations of Ca^{2+}, H^+, Mg^{2+} and adenine nucleotide thought to be present under normal and ischaemic myocardium has been investigated [405]. This study suggested that during exposure to sustained ischaemic conditions RyR activity may be suppressed, but the effects of ischaemia on SR Ca^{2+} handling in intact cells remains unclear.

7.1 Aims of This Chapter

The main aim of this study was to examine the effect of metabolic inhibition on SR Ca^{2+} release in isolated rat ventricular myocytes. Since metabolic inhibition mimics some of the changes that occur during late ischaemia, the changes in Ca^{2+} handling that occur during and after metabolic blockade may provide some insight into the changes that occur during ischaemia and reperfusion. Modulators of CICR have previously been shown to affect the frequency of spontaneous SR Ca^{2+} release and the SR Ca^{2+} content in isolated myocytes (See Chapters 3, 4 & 5 and references [157, 284, 294, 295, 371]). The effects of metabolic inhibition on SR Ca^{2+} handling were investigated by examining the effect of superfusion with a solution containing cyanide and 2-deoxglucose (replacing glucose) on the frequency of spontaneous SR Ca^{2+} release

in the form of Ca^{2+} sparks and Ca^{2+} waves in resting myocytes. The effect of these conditions on the SR Ca^{2+} content of electrically stimulated mycoytes was also investigated.

7.2 The Effect of Metabolic Inhibition on the Occurrence of Ca^{2+} Sparks

The effect of metabolic inhibition on Ca^{2+} sparks was examined using confocal microscopy in the line-scan mode as described in Methods 2.3.6. Fluo-4 loaded rat ventricular myocytes were superfused with a control 2 mM Ca^{2+} Tyrode solution containing 2-deoxyglucose to inhibit glycolysis. Cells were stimulated to contract using an external stimulator and field electrodes. A series of line-scan images was then acquired under control conditions, immediately after stimulation was stopped. Stimulation was then continued and metabolic inhibition was induced by exposure to 2 mM NaCN in the superfusing solution. When the twitch was abolished and there was evidence that the cell was going into sustained contracture a second series of line-scan images was taken and compared to those under control conditions. Images were enhanced using the "*smoothing*" facility of the Lasersharp software (Bio-Rad, UK). Figure 7.1 compares typical line-scan images taken under control conditions, during exposure to cyanide and on removal of cyanide. Ca^{2+} sparks were evident under control conditions as localised elevations of fluorescence intensity. During exposure to cyanide the cell demonstrated a sustained contracture, as illustrated by the fact that the line-scan image is narrower in the horizontal plane. It is also clear that the resting fluorescence intensity of the cell is increased above that of control,

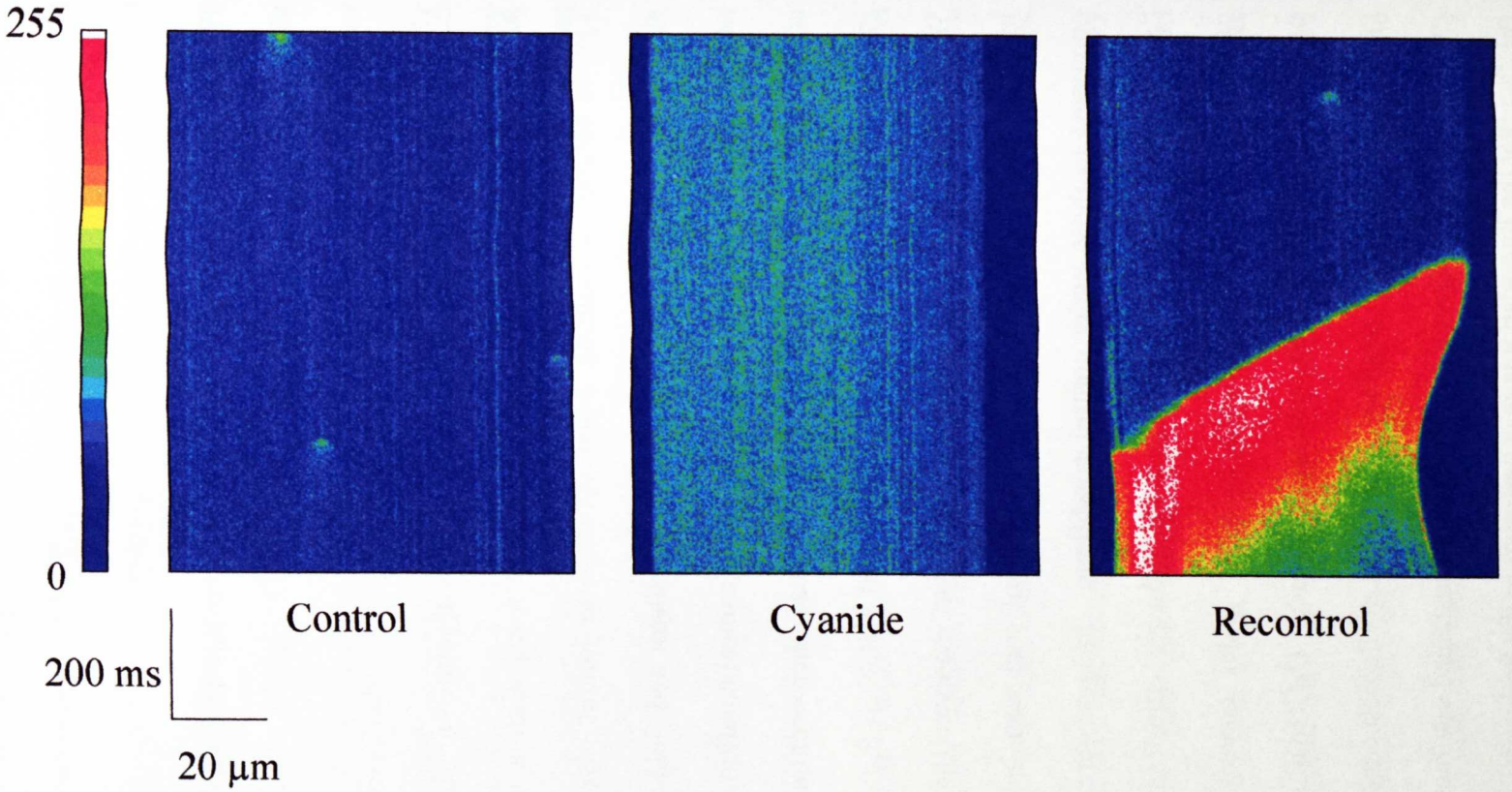

Figure 7.1. The effect of metabolic inhibition and its removal on spontaneous Ca^{2+} sparks and waves in an isolated rat ventricular myocyte.

Panels illustrate typical line-scan images from a Fluo-4 loaded myocyte under control conditions (left), during exposure to cyanide (centre) and following removal of cyanide (right). 2-deoxyglucose was present throughout the experiment. Image acquisition was started immediately after stopping stimulation under control conditions and when twitches ceased and contracture began to develop during exposure to cyanide. Electrically stimulated twitches could not be activated after removal of cyanide in this cell. The bar to the left of the images represents the fluorescence intensity scale.

but no sparks are visible in the image shown. However, this may be due to the difficulty of distinguishing Ca^{2+} sparks on such high background fluorescence. In ***this cell***, the average frequency of Ca^{2+} sparks was calculated from a series of 21 line-scans to be 3.4 ± 0.5 s^{-1} under control conditions (n = 21), and this was reduced to an average of 0.2 ± 0.1 s^{-1} (from 20 line-scan images). On removal of cyanide, the cell initially contracted further before relaxing slightly, although not to the control cell length. Ca^{2+} sparks were evident and Ca^{2+} waves were frequently observed despite their absence during the pre-cyanide (normal) control period. This is indicative of Ca^{2+} overload of the cell and is typical of the damage experienced on reperfusion of ischaemic tissue.

An important limitation to interpretation of these results is that the elevated background fluorescence intensity during exposure to cyanide could reduce the ability to detect Ca^{2+} sparks under these conditions. Figure 7.2 shows a similar series of line-scan images from another cell. In this cell, resting fluorescence was increased even more dramatically during exposure to cyanide, but a Ca^{2+} spark is still visible at the top of the image, approximately one third of the total distance from the right-hand edge, despite the elevated background. In this cell Ca^{2+} spark frequency in line-scan images was reduced from 0.9 ± 0.2 s^{-1} to 0.3 ± 0.1 s^{-1} during exposure to cyanide.

There is still an element of doubt as to whether Ca^{2+} sparks would be visible against the increased resting Ca^{2+}-sensitive fluorescence during contracture in cyanide. Attempts to negotiate this problem involved switching to a low Ca^{2+} (100 μM) bathing solution just before line-scan acquisition was initiated. However, this posed the limitation that Ca^{2+} spark frequency was significantly

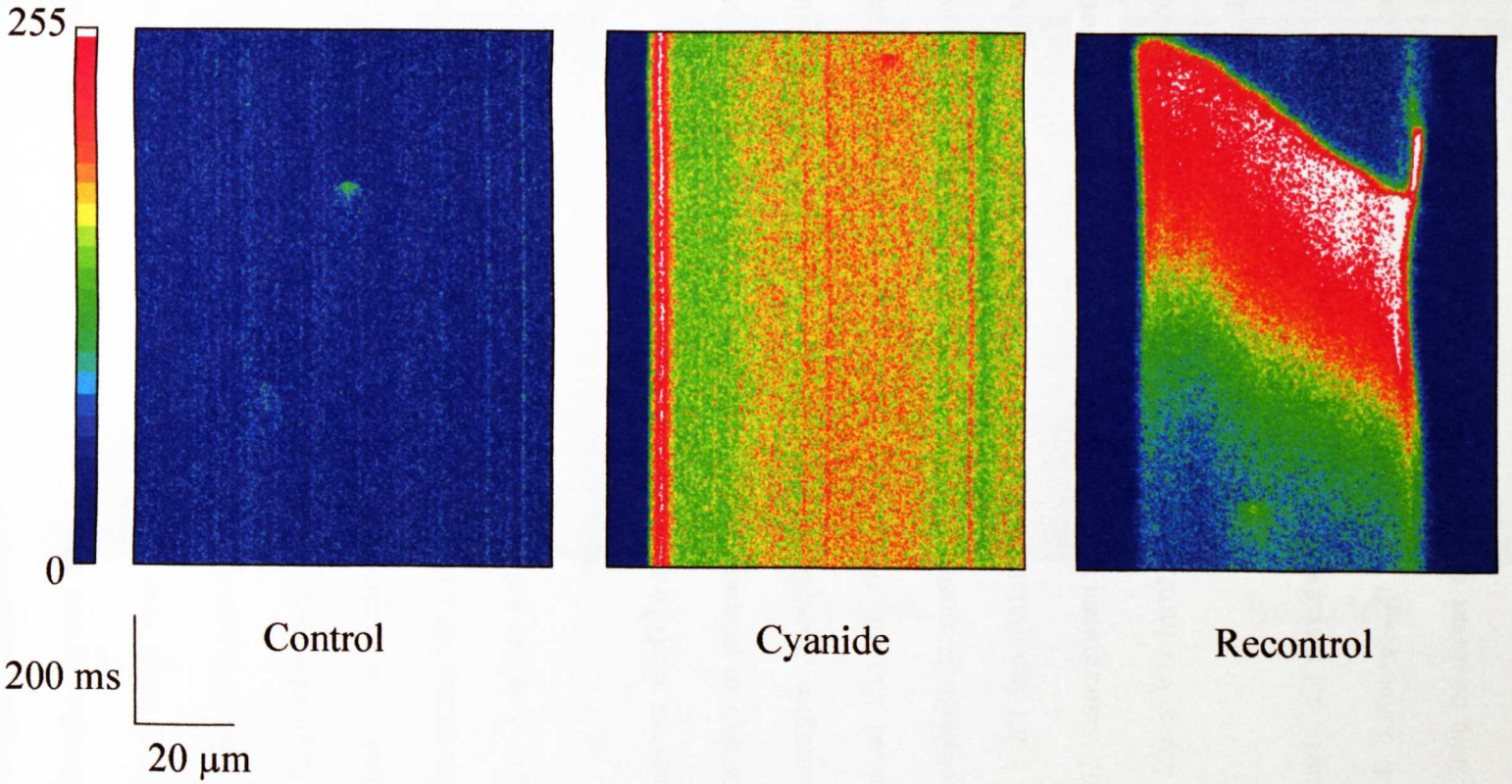

Figure 7.2. The effect of metabolic inhibition and its removal on spontaneous Ca^{2+} sparks and waves in an isolated rat ventricular myocyte.

Panels illustrate typical line-scan images from a Fluo-4 loaded myocyte under control conditions (left), during exposure to cyanide (centre) and following removal of cyanide (right). 2-deoxyglucose was present throughout the experiment. Image acquisition was started immediately after stopping stimulation under control conditions and when twitches ceased and contracture began to develop during exposure to cyanide. Electrically stimulated twitches could not be activated after removal of cyanide in this cell. The bar to the left of the images represents the fluorescence intensity scale.

reduced in most cells under these conditions. Figure 7.3 illustrates typical line-scan images from a cell during superfusion with 2 mM Ca^{2+} (A), 100 μM Ca^{2+} (B) and subsequently during exposure to cyanide (C) and on after removal of cyanide (D). The superfusing solution was switched to the lower $[Ca^{2+}]_o$ just before line-scan acquisition in examples B, C and D. It is clear that Ca^{2+} spark frequency is dramatically depressed during superfusion with a solution of lower $[Ca^{2+}]$. The frequency of sparks in the line-scan images was reduced from 9.9 ± 1.3 s^{-1} (*number of line-scans* = 12), during superfusion with 2 mM Ca^{2+}, to 1.0 ± 0.2 s^{-1} during exposure to 100 μM external Ca^{2+} (*number of line scans* = 27). This is consistent with the hypothesis that Ca^{2+} flux through sarcolemmal L-type Ca^{2+} channels is responsible for triggering Ca^{2+} sparks, or that reducing $[Ca^{2+}]_o$ may reduce the SR Ca^{2+} content and therefore spark frequency by reducing the gain of CICR or the driving force for Ca^{2+} efflux from the SR. Reducing external Ca^{2+} will reduce the driving force for Ca^{2+} entry, thereby reducing the amount of Ca^{2+} entering the cell through stochastically opened sarcolemmal channels and the frequency of successful RyR triggering. In this cell, the resting fluorescence was still increased slightly during exposure to cyanide, but no sparks were evident during the period of line-scan acquisition. On removal of cyanide there was an increase of Ca^{2+} spark frequency 2.0 ± 0.4 s^{-1} (n = 20), but Ca^{2+} waves were not observed in comparison with cells exposed to 2 mM Ca^{2+} control Tyrode.

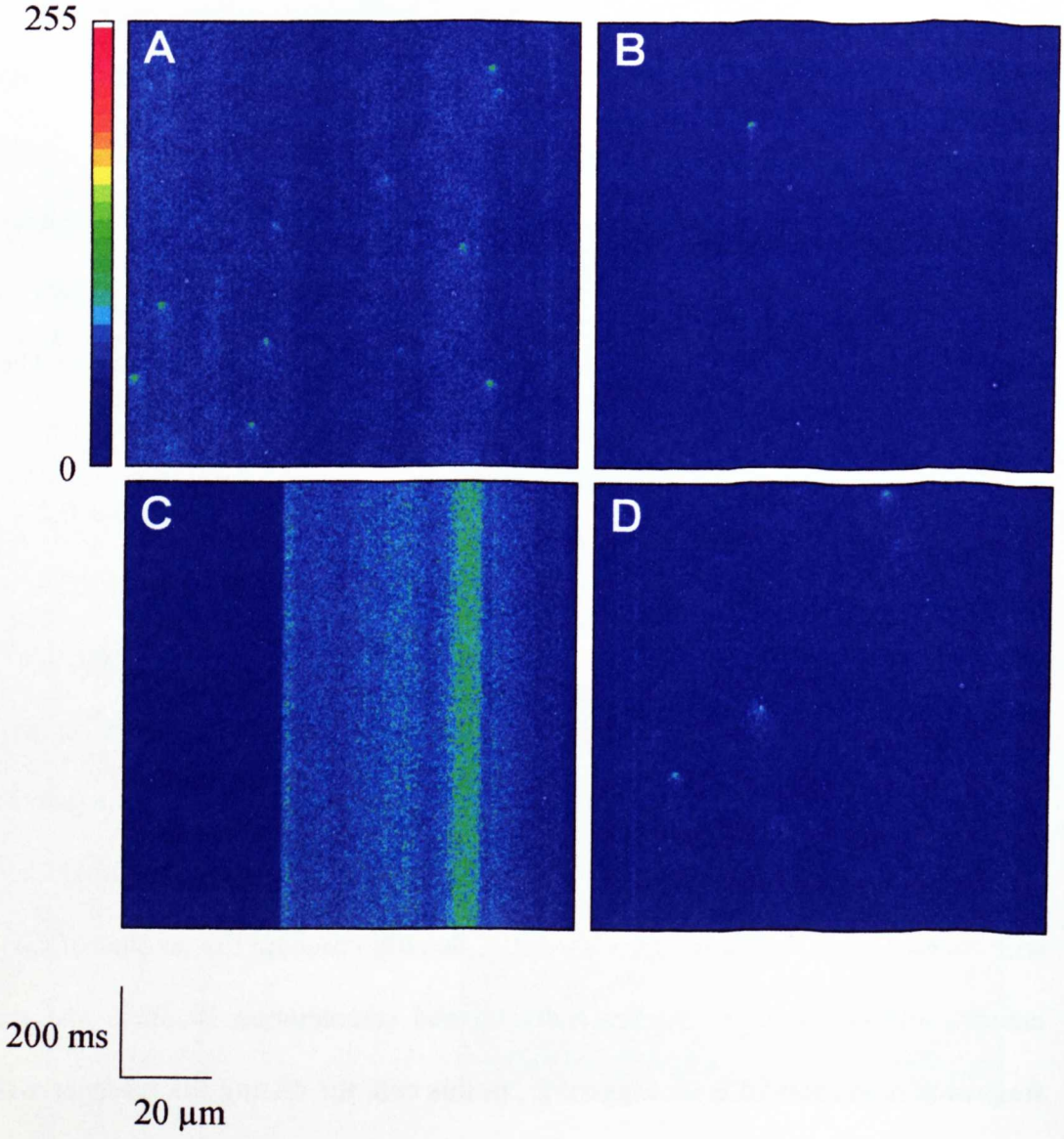

Figure 7.3. The effect of lowering $[Ca^{2+}]_o$ on Ca^{2+} sparks and background fluorescence intensity.

Panels illustrate typical line-scan images from a Fluo-4 loaded myocyte during exposure to: **A**, 2 mM $[Ca^{2+}]_o$; **B**, 100 µM $[Ca^{2+}]_o$; **C**, cyanide in the presence of 100 µM $[Ca^{2+}]_o$; **D**, on removal of cyanide in the presence of $[Ca^{2+}]_o$. The cell was electrically stimulated in the presence of 2 mM Ca^{2+}. Line-scan acquisition was initiated immediately after stopping stimulation and in B-D after switching to a solution containing 100 µM Ca^{2+}.

7.3 The Effect of Metabolic Inhibition on the Frequency of Mechanical Spontaneous Oscillations

The effect of cyanide exposure on the frequency of propagating waves of spontaneous SR Ca^{2+} release was investigated by monitoring changes in cell length. Figure 7.4 illustrates a typical record of changes in cell length activated by spontaneous Ca^{2+} release in a resting rat myocyte. Cyanide was applied for the period indicated by the solid bar, and caused a gradual reduction in the frequency of spontaneous Ca^{2+} release, which was eventually abolished before the cell began to develop contracture. On removal of cyanide the cell immediately relaxed from its contracture and spontaneous Ca^{2+} release was soon resumed at around the control frequency. This is consistent with the hypothesis that metabolic inhibition depresses SR Ca^{2+} release. Similar results were obtained in a further three cells, although some developed contracture before spontaneous Ca^{2+} release was observed during exposure to cyanide.

There appears to be a lengthening of the cell during exposure to cyanide, indicated by the change in baseline in Fig. 7.4. It is thought that this could be explained by the decreased sensitivity of the myofilaments to $[Ca^{2+}]_i$ during exposure to metabolic inhibition, due to elevated $[H^+]_i$ and $[P_i]_i$. The development of contracture later in the record provides evidence that there has been a significant fall in $[ATP]_i$, which is believed to be responsible for the decrease of cross bridge recycling and rigor contracture during metabolic blockade [22].

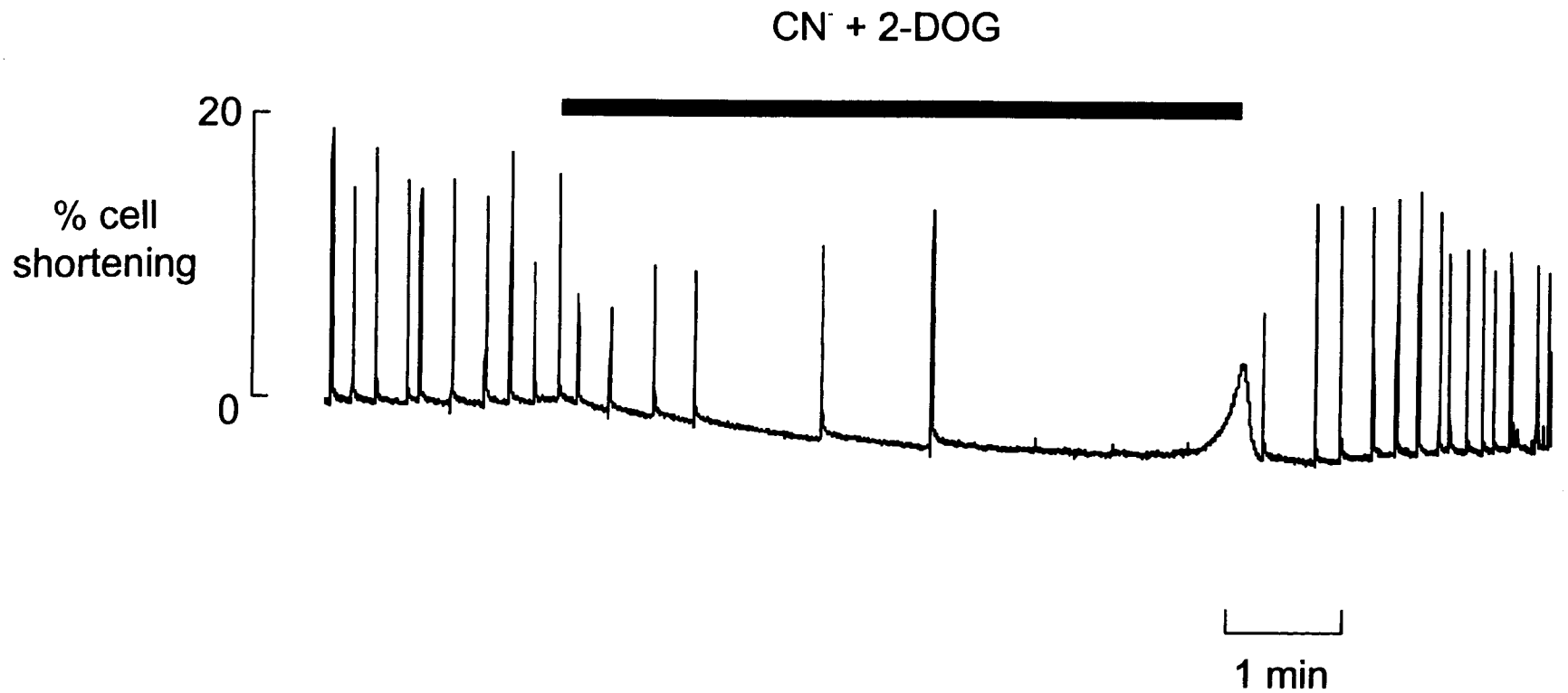

Figure 7.4. The effect of metabolic inhibition on the frequency of spontaneous Ca^{2+} waves in a rat ventricular myocyte.

Typical record of cell length from a spontaneously contracting myocyte. The cell was superfused with control Tyrode solution (containing 2 mM Ca^{2+} and 2-deoxyglucose). 2 mM Cyanide was present for the period indicated by the bar. Deflections in the length record represent contractions activated by propagating waves of SR Ca^{2+} release.

7.4 The Effect of Metabolic Blockade on the SR Ca^{2+} Content of Electrically Stimulated Myocytes

The reduced frequency of spontaneous Ca^{2+} release described so far could be attributed to inhibition of Ca^{2+} uptake by the SR, which would reduce the SR Ca^{2+} load and the frequency of spontaneous Ca^{2+} release. Alternatively, these results could be explained by the direct inhibition of RyRs reducing the gain of the CICR process. These two mechanisms predict opposite changes in the SR Ca^{2+} content of cells, the latter causing an increase of SR Ca^{2+} content [294, 295]. To discriminate between these two effects the SR Ca^{2+} contents of isolated rat ventricular myocytes were quantified and compared under control conditions and during exposure to metabolic blockade.

Caffeine-evoked inward currents were used to measure the SR Ca^{2+} content of voltage-clamped, electrically stimulated rat ventricular myocytes. Under conditions of metabolic inhibition the activity of the sarcolemmal Ca^{2+}-ATPase may be compromised, reducing its ability to compete with the Na^+/Ca^{2+} exchanger for extrusion of Ca^{2+}. This will affect the contribution of these two mechanisms to Ca^{2+} extrusion during exposure to caffeine, such that an increase of the inward current could reflect the increased contribution of Na^+/Ca^{2+} exchange to Ca^{2+} extrusion rather than an increase in Ca^{2+} release and SR Ca^{2+} content. To avoid such complications, the sarcolemmal Ca^{2+}-ATPase was specifically inhibited by loading cells with carboxyeosin prior to each experiment, as detailed in Methods 2.2.1. Ca^{2+} extrusion was then assumed only to be mediated by Na^+/Ca^{2+} exchange and it was not necessary to compensate for non-Na^+/Ca^{2+} exchange mediated Ca^{2+} efflux by multiplying integrals by a factor of 1.5 as described in

previous chapters. 10 μM glibenclamide was also included in the bathing solution to inhibit K_{ATP} sensitive currents, which may otherwise result in loss of efficient voltage-clamp control. Figure 7.5 illustrates typical Na^+/Ca^{2+} exchange currents activated by application of 20 mM caffeine to an electrically stimulated rat myocyte under control conditions and following exposure to 2 mM cyanide to block oxidative phosphorylation for two minutes. Voltage-clamp command pulses from a holding potential of –40 mV to 0 mV activated stimulation. Caffeine was applied immediately after stimulation was stopped under the two conditions and cyanide was added after the recovery of the steady state level of contraction under control conditions. It is clear that the caffeine-evoked current and its integral are increased following two minutes exposure to cyanide. This particular cell showed only slight depression of contraction during exposure to cyanide for this period. Other cells demonstrated similar moderate, if any, depression of contraction and moderately increased SR Ca^{2+} content over periods of 1 – 4 minutes stimulation in the presence of cyanide. Figure 7.6 illustrates a histogram of average data comparing the SR Ca^{2+} content of seven cells under control conditions and after exposure to cyanide for between 1 and 4 minutes. SR Ca^{2+} content was increased by 47.4 ± 8.2 % from 79.4 ± 5.7 $\mu mol.l^{-1}$ to 115.2 ± 6.6 $\mu mol.l^{-1}$ ($P < 0.001$, $n = 7$). Because metabolic inhibition appears to cause a certain degree of permanent damage to the cell, measurement of SR Ca^{2+} content following removal of cyanide was not achieved in this study.

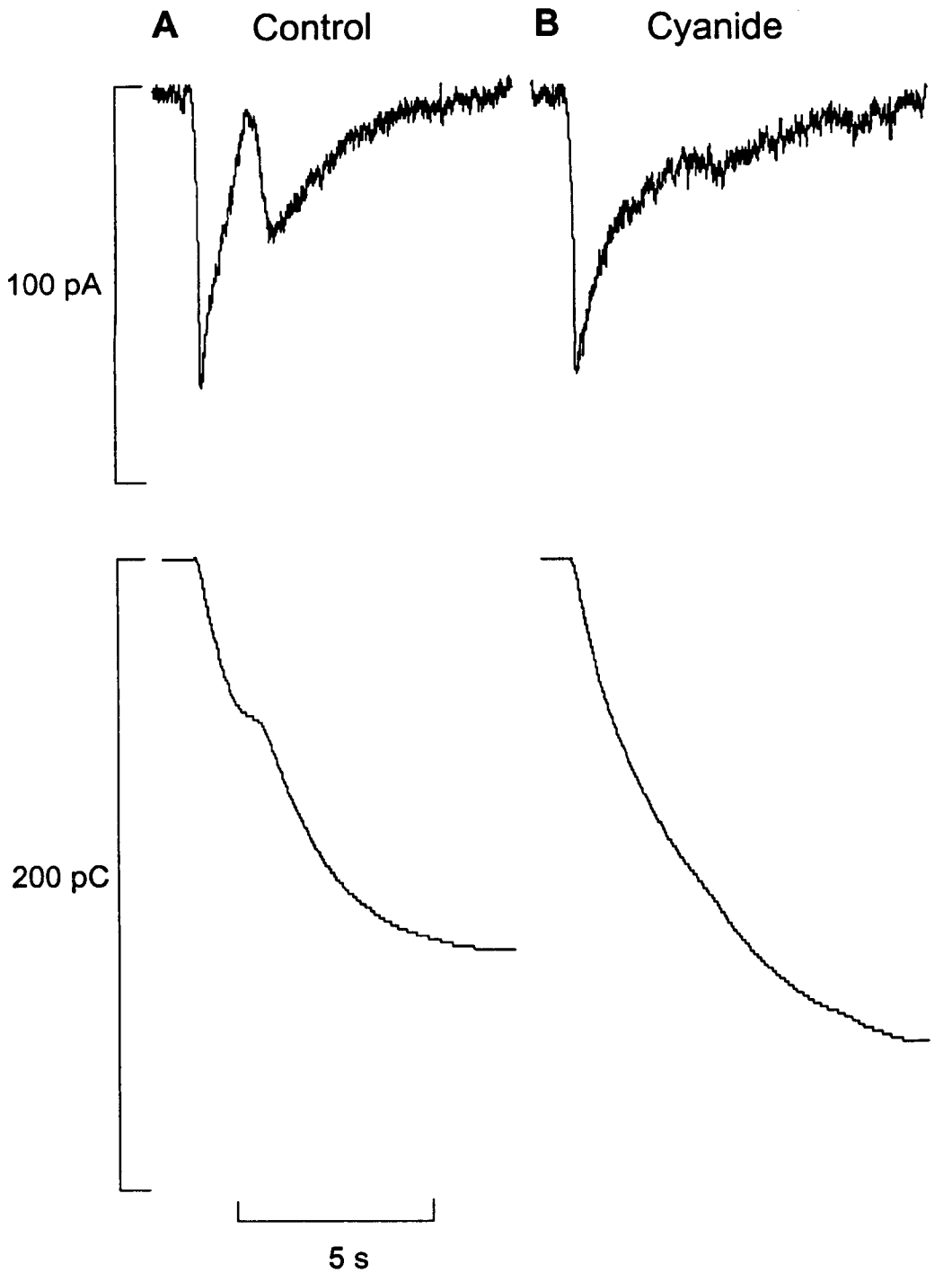

Figure 7.5. The effect of metabolic inhibition on caffeine-evoked Na^+/Ca^{2+} exchange currents and their integrals.

The **upper** panel illustrates Na^+/Ca^{2+} exchange currents activated by the application of 20 mM caffeine: **A**, under control conditions and **B**, following exposure to cyanide for a period of 2 minutes. Caffeine was applied immediately after stopping stimulation. The **lower** panel shows the integrals calculated from the inward currents of the upper panel. 2-deoxyglucse was present throughout the experiment.

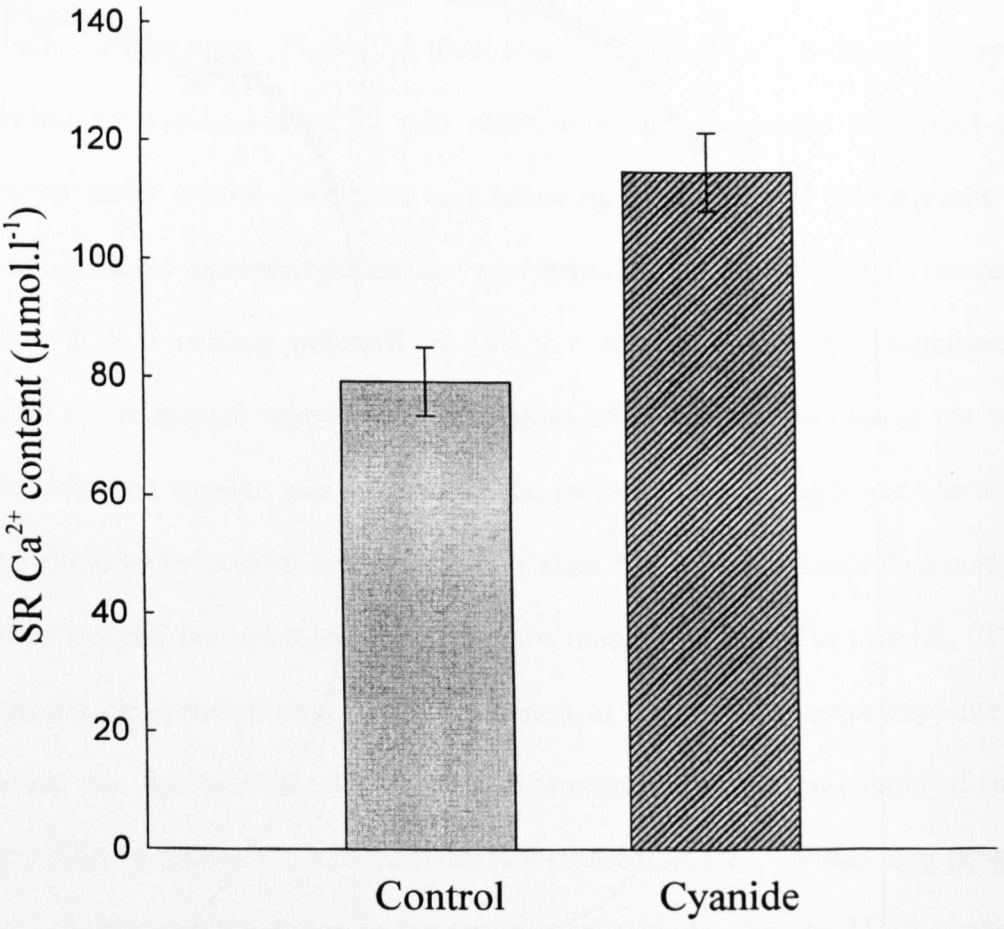

Figure 7.6. The effect of metabolic inhibition on the SR Ca^{2+} content of electrically paced rat ventricular myocytes.

Histogram showing mean data ± S.E.M. of SR Ca^{2+} content under control conditions and after exposure to cyanide for between 1 and 4 minutes ($P < 0.001$, $n = 7$). 2-deoxyglucose was present under both conditions in all experiments.

7.5 Discussion

Despite the knowledge that many of the changes that occur during metabolic inhibition and therefore myocardial ischaemia have been shown to affect the RyR [256, 310, 405], their effects on CICR in intact myocytes has been previously overlooked. This is probably a consequence of the assumption that SR Ca^{2+} uptake is compromised under conditions of limited ATP and energy supply. However, changes in CICR could be critical to the heart's ability to tolerate ischaemia.

7.5.1 SR Ca^{2+} Release is inhibited During Metabolic Blockade

The results presented here demonstrate three separate techniques of assessing the effect of metabolic inhibition on the process of CICR. It has been shown that metabolic blockade causes the inhibition of spontaneous Ca^{2+} release from the SR in the form of Ca^{2+} sparks and propagating Ca^{2+} waves, and an increase the SR Ca^{2+} content of electrically paced rat myocytes. Together, the results provide clear evidence of inhibition of SR Ca^{2+} release and a reduction in the sensitivity of the process of CICR, essential to normal cardiac function.

Despite the apparent suppression of SR Ca^{2+} release, no suppression of electrically evoked contraction was observed during exposure to cyanide. This can be explained by the knowledge that changes in RyR sensitivity can be rapidly compensated for by changes in SR Ca^{2+} content (See Chapter 5 and references [295, 371]), and changes in the sensitivity of the CICR mechanism will not cause changes in twitch amplitude in the steady state. Twitch is normally abolished

relatively early during metabolic inhibition, due to activation of ATP-sensitive K^+ channels and consequent shortening of the action potential. This will not affect twitch stimulated electrically through the voltage-clamp protocol, which was designed specifically to activate L-type Ca^{2+} channels and Ca^{2+} influx to trigger SR Ca^{2+} release and contraction. The fact that twitch was minimally affected during metabolic inhibition under the conditions of these experiments is consistent with the hypothesis that early contractile failure is usually a consequence of action potential shortening due to activation of K_{ATP} channels [227].

There are a number of reasons to suspect that the SR Ca^{2+}-ATPase may be inhibited under conditions of metabolic blockade and that this may contribute to the elevation of intracellular free $[Ca^{2+}]_i$. Elevated $[Mg^{2+}]_i$ and $[H^+]_i$, which are likely to be experienced during metabolic inhibition, have been shown to decrease SR Ca^{2+} uptake in saponin-treated ferret ventricular muscle [204]. The free energy of ATP hydrolysis will be reduced due to elevated $[ADP]_i$ and $[P_i]_i$, and may fall below that necessary to pump Ca^{2+} into the SR against its concentration gradient [9]. However, a number of studies have failed to observe changes in $[ATP]_i$ sufficient to cause significant inhibition of ATP-dependent Ca^{2+} pumps. Unfortunately these studies suffer from the limitation that ATP appears to be functionally compartmentalised [103, 171, 393] and significant changes in sub-cellular '*pockets*' of ATP sufficient to compromise such processes may be missed when examining the cell as a whole. The inhibition of Ca^{2+} uptake by the SR has also been proposed to explain observations of reduced Ca^{2+} release from the SR under conditions of acidosis [288]. The hypothesis of reduced Ca^{2+} uptake by the SR is at variance with the observation in this study of an increased SR Ca^{2+}

content during metabolic inhibition. The results of this study **are** consistent with the observations of Goldhaber *et al* [149] in guinea pig ventricular myocytes that CICR is inhibited during rigor contracture. The observation in this study of increased SR Ca^{2+} content (Fig. 7.5 & 7.6) and reduced frequency of spontaneous Ca^{2+} release (Fig. 7.4) before the onset of contracture also suggests that inhibition of CICR may be a relatively early consequence of inhibiting oxidative phosphorylation.

The increase in fluorescence observed during early contracture in these cells supports the hypothesis of increased $[Ca^{2+}]_i$ associated with the early development of contracture [22, 114] and are at variance with the failure to note increases in aequorin luminescence during metabolic inhibition [9-11, 94, 338]. Clusin [93] has suggested that aequorin may be more sensitive to Ca^{2+} released from the SR than to uniformly distributed Ca^{2+}. Furthermore, the sensitivity of aequorin to diastolic levels of $[Ca^{2+}]_i$ is relatively low and may also be further reduced by $[Mg^{2+}]_i$, which is likely to be increased during metabolic inhibition [22]. Although the development of contracture was coincident with the increase of $[Ca^{2+}]_i$, the results described here cannot ascertain whether the increase of $[Ca^{2+}]_i$ precedes the development of contracture. The source of such an increase in $[Ca^{2+}]_i$ is unclear, but could be a consequence of influx on the Na^+/Ca^{2+} exchanger as a consequence of the increased aNa_i expected under these conditions [101, 169], activation of B-type Ca^{2+} channels [389] or decreased Ca^{2+} extrusion via the sarcolemmal ATPase [338].

7.5.2 The Functional Implications of Inhibition of CICR during Metabolic Blockade

Ca^{2+} oscillations and associated arrhythmias on reperfusion of tissue (when oxidative phosphorylation restarts) were previously thought to be a consequence of rapid uptake of Ca^{2+} from the myoplasm by the SR Ca^{2+}-ATPase, which is rapidly reactivated by the availability of ATP. The results presented here suggest that the SR Ca^{2+} content is already elevated during exposure to metabolic blockade, due to direct inhibition of SR Ca^{2+} release. When oxidative phosphorylation is resumed, the inhibition of the SR Ca^{2+} release channel will be removed as the cell recovers its control levels of metabolites. Spontaneous Ca^{2+} release activity then resumes and the SR Ca^{2+} content should eventually fall to the control level. Na^{+}/Ca^{2+} exchange has also been shown to contribute to the rise of $[Ca^{2+}]_i$ on resumption of oxidative phosphorylation [338].

The opening of ATP-sensitive K^{+} channels will hyperpolarise the membrane and reduce the excitability of the heart, possibly contributing to maintenance of the precious energy reserves by reducing contractility. However, the accumulation of extracellular K^{+} in the absence of its removal by an adequate blood supply and inhibition of the Na^{+}/K^{+}-ATPase during ischaemia will depolarise the cell membrane, thereby increasing the risk of arrhythmia. Inhibition of CICR may represent a protective mechanism to avoid the generation of such arrhythmias. Reducing the frequency of spontaneous Ca^{2+} release means that oscillations of Ca^{2+} and therefore membrane potential are less likely to summate and drive membrane potential to threshold. However, the increased SR Ca^{2+} content as a consequence of inhibition of CICR may be ultimately

deleterious since it will contribute to the degree of Ca^{2+} overload and therefore enhancement of spontaneous SR Ca^{2+} release observed on resumption of oxidative phosphorylation, typical of reperfusion damage. At this time the SR Ca^{2+} release properties will be returned to normal in the presence of an elevated SR Ca^{2+} content. It is clear that inhibition of CICR could represent an important aspect of modulation of EC-coupling during ischaemia and reperfusion, which may contribute to the heart's ability to survive ischaemia and the damage experienced on subsequent recovery of normal metabolism.

Chapter 8

CHAPTER 8

GENERAL DISCUSSION

Many aspects of the mechanism of CICR, which is widely accepted to underlie EC-coupling in cardiac muscle, remain poorly understood. Previously very little was known about the effect of modulators of CICR on spontaneous and stimulated Ca^{2+} release from the SR. The work described here has made a significant contribution to the understanding of this aspect of Ca^{2+} handling in the heart. This has relevance to the effects of certain endogenous substances and pharmacological agents, many of which are believed to modulate the activity of the SR Ca^{2+} release channel (the RyR) and, as detailed in this study, may be of importance during myocardial ischaemia (Chapter 7). Detailed discussion already accompanies each chapter, therefore this section aims to discuss the more general implications of this work and proposals for possible future investigations to further advance our knowledge of CICR.

8.1 Spontaneous SR Ca^{2+} Release as an Index of CICR Modulation

Tetracaine has been used for many years as a tool for investigating the effects of inhibition of SR Ca^{2+} release in striated muscle [12, 145, 182, 248]. This study has provided evidence that its effects on spontaneous Ca^{2+} release are largely due to the inhibition of Ca^{2+} release from the SR, probably by reducing the sensitivity of the RyR to cytosolic [Ca^{2+}]. From this study it is clear that reducing the sensitivity of CICR initially abolishes spontaneous Ca^{2+} release in the form of

propagating Ca^{2+} waves, in agreement with its effect on spontaneous Ca^{2+} sparks in work published by Györke *et al* [157]. As a consequence of inhibiting spontaneous Ca^{2+} release, Ca^{2+} accumulates in the SR, resulting in an increased SR Ca^{2+} content. This increases the gain of the CICR process and, as result it can overcome the inhibition imposed and spontaneous Ca^{2+} release resumes. The amplitude of spontaneous SR Ca^{2+} release was also increased during exposure to 100 μM tetracaine, probably as a consequence of the increased SR Ca^{2+} content increasing the driving force for Ca^{2+} efflux from the SR lumen into the cytoplasm [368]. This activates more Ca^{2+} extrusion, such that more time is required for the SR to reach the threshold Ca^{2+} content to allow initiation and propagation of a spontaneous wave of Ca^{2+} release. This is the basis of the maintained reduction of oscillation frequency observed in this study. In contrast to these effects are those of applying a low concentration of caffeine (100 – 500 μM), which causes a *burst* of spontaneous activity on initial application of the drug, due to sensitisation of CICR, and resultant rapid reduction of SR Ca^{2+} content. Spontaneous Ca^{2+} release events in the steady state are smaller in amplitude, because of the reduced SR Ca^{2+} content and reduced driving force for Ca^{2+} to leave the SR. The reduced $[Ca^{2+}]_i$ transient activates less Ca^{2+} efflux from the cell, and the frequency of oscillations is thus increased [373]. These results have lead to the proposal that changes in the magnitude and frequency of spontaneous Ca^{2+} release represent a useful index of changes in RyR function [115, 294, 373].

It is not clear from this study how increasing the inhibition of CICR further (using higher concentrations of tetracaine) will affect the steady state frequency of spontaneous Ca^{2+} release. The effects of tetracaine on the frequency of

spontaneous oscillations are concentration dependent up to 200 μM (See Fig. 3.2). However, in Chapter 4, the effect of tetracaine on the spatial and temporal properties of Ca^{2+} release revealed that 500 μM tetracaine caused a reduction of Ca^{2+} wave amplitude and propagation of Ca^{2+} release was often terminated prematurely. This suggests that at higher concentrations the increase of SR Ca^{2+} content is no longer able to fully compensate for the inhibition imposed by tetracaine. Fewer channels will be sensitive to cytosolic Ca^{2+} resulting in a smaller Ca^{2+} release. With these observations in mind, the amplitude of spontaneous SR Ca^{2+} release is clearly not an adequate index of changes of RyR function as suggested previously [373]. In this situation, if Ca^{2+}-uptake by the SR and leak into the cell is unaffected by tetracaine one would expect that smaller Ca^{2+} waves will occur more frequently than comparatively larger Ca^{2+} waves during exposure to lower concentrations of tetracaine. However, tetracaine may affect SR Ca^{2+} uptake and resting Ca^{2+} influx at these concentrations. It will be necessary to investigate the effects of higher concentrations of tetracaine on the frequency and amplitude of spontaneous Ca^{2+} release and its affects on SR Ca^{2+} accumulation. Changes in SR Ca^{2+} accumulation and/or flux of Ca^{2+} into the cell will also affect the frequency of spontaneous SR Ca^{2+} release and limit the usefulness of steady state oscillation frequency as an index of modulation of CICR, since relatively few agents are expected to affect only the RyR.

8.2 Effects of Modulating CICR on SR Ca^{2+} Content and Stimulated Contraction

Stimulation of CICR might be expected to increase the amount of Ca^{2+} released on stimulation and therefore the force of contraction. This has previously been used to explain the inotropic effects of a number of agents, e.g. cADP-ribose [301], rapamycin [199] and FK-506 [251]. The results presented in Chapter 5 of this study demonstrate that reducing the sensitivity of CICR alone, by using low concentrations of tetracaine, has ***no maintained*** effect on cardiac contractility [295]. Similarly the effects of low concentrations of caffeine have been shown to be transient [284, 371]. The transient nature of these effects has again been attributed to alteration of the gain of the CICR mechanism and consequent changes in Ca^{2+} fluxes across the sarcolemma. This results in a change in the SR Ca^{2+} content of myocytes, which itself affects the gain of the CICR process having the reverse effect. Together these findings strongly suggest that modulation of CICR alone ***cannot produce maintained effects*** on the magnitude of systolic Ca^{2+} release.

8.3 The Physiological Significance of the Transient Nature of CICR Modulation

In answer to the question "why have a mechanism for transient modulation of systolic Ca^{2+} release" the effects of β-adrenergic stimulation represent a neat example. β-adrenergic stimulation increases the rate of force development, the maximal force of contraction and the rate of relaxation. A biochemical cascade, ultimately causing an increase in intracellular [cAMP] initiates these effects. This

increase of cAMP activates the cAMP-dependent protein kinase (PKA), which phosphorylates voltage-sensitive Ca^{2+} channels [377], phospholamban [357] and the RyR [360, 410]. These events result respectively in enhancement of Ca^{2+} influx, stimulation of Ca^{2+} uptake by the SR and stimulation of the RyR, activating SR Ca^{2+} release and potentiating the systolic Ca^{2+} transient [107]. Removing the inhibition imposed by phospholamban will allow the SR Ca^{2+}–ATPase to compete more effectively with Na^{+}/Ca^{2+} exchange, such that the duration of the Ca^{2+} transient is shortened, and Ca^{2+} efflux from the cell will be limited despite elevated Ca^{2+} release as a consequence of RyR stimulation. This will facilitate the maintenance of the SR Ca^{2+} content and will contribute to the maintained increase of systolic Ca^{2+} transient. The ***immediate transient*** response to stimulation of CICR compensates for the delay in the response to phosphorylation of phospholamban to increase the SR Ca^{2+} content, allowing the two mechanisms to work co-operatively producing an almost immediate response to β-adrenergic stimulation. These effects are modelled very well in the review article by Eisner *et al* [115]. Augmentation of the Ca^{2+} current will further limit any decrease of SR Ca^{2+} content due to stimulation of CICR and the increased trigger will further enhance systolic Ca^{2+} release.

8.3.1 The Possible Frequency and Temperature Dependence of these Effects

The effects of modulating CICR on stimulated Ca^{2+} release may be dependent on the frequency of stimulation. In the rat there is believed to be significant Ca^{2+} uptake during diastole, confirmed in this study by the observation of spontaneous waves of Ca^{2+} release (Chapters 3, 4 & 7) and rest potentiation

(Chapter 6). Although diastolic Ca^{2+} uptake appeared to be minimal in the study described in Chapter 4, because the change in sarcolemmal Ca^{2+} influx was almost completely balanced by the efflux activated on repolarisation. At very low stimulation frequencies, the diastolic gain of Ca^{2+} by the SR may mask transient effects on systolic Ca^{2+} release of CICR modulation. Reducing the length of diastole may reduce Ca^{2+} uptake during this period thereby limiting the rate at which the SR Ca^{2+} content is increased when RyR is inhibited. This would slow the recovery from transient depression of contraction observed when CICR is inhibited. It would also increase the rate at which the SR Ca^{2+} load is decreased during stimulation of CICR. The investigations described so far have been carried out using stimulation frequencies far below the normal rates of cardiac contraction for rat. It is important that in the future some investigation of the effects of modulating CICR be made at normal heart rates, the normal heart rate of rat ventricle being up to 400 min^{-1}.

The temperature dependence of the effects of cADP-ribose on contractility [186], highlight the inability to extrapolate the results presented in this study to the effects of such agents under normal physiological temperatures. This could be attributed to temperature dependent changes in the availability of endogenous effectors or changes in the contribution of cytosolic Ca^{2+} removal mechanisms to relaxation, thereby altering the ability of the SR to compete with other extrusion mechanisms. It is therefore important to assess the effect of modulators of CICR under more physiological temperatures. Preliminary experiments performed by Dr Andrew Trafford (personal communication) have revealed that the qualitative effects of caffeine application on stimulated contraction are similar at 37 °C. At

higher temperatures a new equilibrium between the different Ca^{2+} uptake and efflux pathways will be established, but the effects of tetracaine are likely to be the same qualitatively if not quantitatively. For example if the SR Ca^{2+}-ATPase is activated more at higher temperature than the Na^{+}/Ca^{2+} exchanger, one might observe a faster increase of SR Ca^{2+} content and therefore faster recovery from the transient depression of contraction.

8.3.2 The Effect of more gradual Modulation of CICR

The effect of tetracaine on CICR can be compensated for by an increase in SR Ca^{2+} content. Exactly the reverse occurs during stimulation of CICR with low concentrations of caffeine [284, 371]. Both these agents reach their targets rapidly, causing inhibition / activation of CICR almost as soon as they are applied to cells. If agents were to reach their target sites more slowly, causing more gradual changes in the sensitivity of CICR, then it is possible that the change in SR Ca^{2+} content could compensate simultaneously for any manipulation of RyR sensitivity, such that transient effects on systolic Ca^{2+} release are not observed. This means that the lack of effect of any agent or change of experimental conditions on systolic $[Ca^{2+}]_i$ or contraction ***does not*** mean that it has no effect on CICR. This knowledge has already proven useful in achieving clear evidence that CICR is inhibited during metabolic blockade and the SR Ca^{2+} content is elevated, while no transient depression of contraction was observed in this study. This previously overlooked phenomenon could have an important role in limiting the occurrence of potentially fatal arrhythmias during ischaemia. The effects of a manoeuvre like metabolic inhibition could be attributed to the gradual changes in

the concentrations of ions and metabolites with potential effects on CICR. The transient nature of CICR modulation on stimulated contraction means that its protective effect can be achieved without compromising the heart's ability to pump blood. The failure of contraction under these conditions must be due to the failure of the action potential caused by opening of K_{ATP} channels, a mechanism to reduce the energy demand of the heart and to preserve at least low levels of ATP.

8.4 Resting Influx of Ca^{2+} and its Accumulation by the SR

A constant Ca^{2+} influx into rat myocytes occurs at rest, as suggested by the occurrence of spontaneous Ca^{2+} release (Chapters 3 & 4), the phenomenon of rest potentiation and the ability of resting myocytes to accumulate Ca^{2+} in their SR (Chapter 6). This influx could represent flux through L-type Ca^{2+} channels, B-type Ca^{2+} channels [97, 224] or reverse mode Na^+/Ca^{2+} exchange. In Guinea pig ventricular myocytes resting Ca^{2+} accumulation was shown not to be inhibited by Ni^{2+} or nifedipene, suggesting that Na^+/Ca^{2+} exchange and Ca^{2+} flux through L-type channels contribute little to Ca^{2+} influx across the membrane in this species [363]. The route of Ca^{2+} entry into rat myocytes has recently been investigated by Ho Sook Choi, and is largely attributed to Na^+/Ca^{2+} exchange under normal resting conditions [90], as expected for a species with such a high aNa_i.

Ca^{2+} flux through B-type channels is augmented under conditions of metabolic inhibition [389]. Coupled with the increased ability of the SR to accumulate Ca^{2+} due to inhibition of CICR under these conditions this could contribute further to the degree of Ca^{2+} overload experienced during ischaemia

and responsible for the damage on reperfusion. It would be interesting to see how Ca^{2+} accumulation by the SR is changed under conditions of metabolic inhibition (as achieved for tetracaine in Chapter 6). The inhibition of SR Ca^{2+} release and increased Ca^{2+} influx predict that it will be enhanced, and this could compensate for decreased Ca^{2+} uptake due to reduced ATP availability or thermodynamic limitations on the SR Ca^{2+}-ATPase. Specific inhibition of the B-type Ca^{2+} channel under these conditions by intracellular protamine [91] could reduce the increase of SR Ca^{2+} content and the degree of Ca^{2+} overload. Such an effect should be investigated because of its tremendous potential as a therapeutic agent in the treatment of ischaemic heart disease.

The rate of Ca^{2+} accumulation by the SR appears to be enhanced by tetracaine. It was proposed that this is due to some inhibition of Ca^{2+} release through random openings of the RyR and the occurrence of Ca^{2+} sparks. It appears therefore that the occurrence of spontaneous Ca^{2+} sparks could limit the ability of the SR to accumulate Ca^{2+}. The frequency of such openings will be increased during exposure to low concentrations of caffeine and it would be interesting to see whether such a manoeuvre could reduce the rate of SR Ca^{2+} accumulation.

Despite the well known relationship between Ca^{2+} spark amplitude and SR Ca^{2+} load [86], there has been no quantitative investigation of the effects of SR Ca^{2+} content on the spatial and temporal properties of spontaneous Ca^{2+} sparks, particularly in non-Ca^{2+} overloaded myocytes. Song *et al* observed that the frequency of spark generation was independent of Ca^{2+} load, although spark amplitude depends on the SR Ca^{2+} load [342], after correcting for the difficulties

in detecting smaller amplitude sparks against the background fluorescence. It would also be interesting to monitor the properties of Ca^{2+} sparks during Ca^{2+} loading of the SR, showing their dependence on the SR Ca^{2+} load. It might be expected that at a low Ca^{2+} load the frequency and amplitude of Ca^{2+} release would be reduced. There are a number of problems with assessing the frequency of Ca^{2+} sparks under different conditions. Any manoeuvre that affects spark amplitude will increase the apparent frequency because larger amplitude Ca^{2+} release events that occur below the plane of focus may diffuse into the plane of focus and be detected. At lower amplitude these sparks would be missed. This could explain the apparent increase in Ca^{2+} spark frequency observed on resumption of spontaneous Ca^{2+} release in the presence of high concentrations of tetracaine by Györke *et al* [157]. Such problems should be taken into consideration.

8.5 Some of the Limitations of This Study

An important limitation to the interpretation of the results presented in this study is that the conclusions drawn from changes in SR Ca^{2+} content rely on the assumption that the contribution of Na^+/Ca^{2+} exchange to Ca^{2+} extrusion during caffeine-induced Ca^{2+} release is unaffected by the presence of tetracaine. This appears to a reasonable assumption since the presence of tetracaine had no effect on the steady state efflux of Ca^{2+} per unit time in resting rat ventricular myocytes (See Chapter 3).

Since the rat shows many characteristics that are a consequence of its high resting $[Na^+]_i$, manoeuvres that potentially alter this could affect Ca^{2+} influx and

therefore the SR Ca^{2+} load of the cell. The local anaesthetics lidocaine and procaine have been shown to reduce the effects of manoeuvres designed to increase aNa_i, by inhibiting Na^+ entry through TTX sensitive Na^+ channels. It would be interesting to see how intracellular Na^+ is affected by superfusion with tetracaine. The results presented in this study suggest that any changes in aNa_i that occur with low concentrations of tetracaine cannot compensate for its effects on CICR. At higher concentrations however, we have observed smaller amplitude Ca^{2+} waves, which in the absence of change in Ca^{2+} influx would be expected to occur at a higher frequency than larger amplitude oscillations in the presence of lower concentrations. Instead their frequency was very low, suggesting that Ca^{2+} influx must be affected by the presence of higher concentrations of tetracaine. This could be caused by effects on the L-type Ca^{2+} channels [75, 81] or by lowering aNa_i and the driving force for Ca^{2+} entry on the Na^+/Ca^{2+} exchanger. Further investigation of the effects of higher concentrations of tetracaine on the amplitude of spontaneous Ca^{2+} waves and their frequency needs to be made.

Bibliography

BIBLIOGRAPHY

[1] **Adachi-Akahane S, Cleemann L, Morad M.** Calcium cross-signalling between DHP and ryanodine receptors in cardiomyocytes. *Heart Vessels* 1995; **Supplement 9**: 163-166.

[2] **Adachi-Akahane S, Cleemann L, Morad M.** Cross-signalling between L-type Ca^{2+} channels and ryanodine receptors in rat ventricular myocytes. *J.Gen.Physiol.* 1996; **108**: 435-454.

[3] **Adachi-Akahane S, Lu L, Li Z, Frank JS, Philipson KD, Morad M.** Calcium signalling in transgenic mice over-expressing cardiac Na^{+}-Ca^{2+} exchanger. *J.Gen.Physiol.* 1997; **109**: 717-729.

[4] **Adams WA, Trafford AW, Eisner DA.** 2,3-butanedione monoxime (BDM) decreases sarcoplasmic reticulum Ca^{2+} content by stimulating Ca^{2+} release in isolated rat ventricular myocytes. *Pflügers Archiv.* 1998; **436**: 776-781.

[5] **Adrian RH, Almers W.** Charge movement in the membrane of striated muscle. *J.Physiol.(Lond.)* 1976; **254**: 339-360.

[6] **Allen DG, Eisner DA, Pirolo JS, Smith GL.** The relationship between intracellular calcium and contraction in calcium-overloaded ferret papillary muscles. *J.Physiol.(Lond.)* 1985; **364**: 169-182.

[7] **Allen DG, Lee JA, Smith GL.** The consequences of simulated ischaemia on intracellular Ca^{2+} and tension in isolated ferret ventricular muscle. *J.Physiol.(Lond.)* 1989; **410**: 297-323.

[8] **Allen DG, Morris PG, Orchard CH, Pirolo JS.** A nuclear magnetic resonance study of metabolism in the ferret heart during hypoxia and inhibition of glycolysis. *J.Physiol.(Lond.)* 1985; **361**: 185-204.

[9] **Allen DG, Orchard CH.** Intracellular calcium concentration during hypoxia and metabolic inhibition in mammalian ventricular muscle. *J.Physiol.(Lond.)* 1983; **339**: 107-122.

[10] **Allen DG, Orchard CH.** Measurements of intracellular calcium concentration in heart muscle: The effects of inotropic interventions and hypoxia. *J.Mol.Cell.Cardiol.* 1984; **16**: 117-128.

[11] **Allshire A, Piper HM, Cuthbertson KS, Cobbold PH.** Cytosolic free Ca^{2+} in single rat heart cells during anoxia and reoxygenation. *Biochem J* 1987; **244**: 381-385.

[12] **Almers W, Best PM.** Effects of tetracaine on displacement currents and contraction of frog skeletal muscle. *J.Physiol.(Lond.)* 1976; **262**: 583-611.

[13] **Anderson K, Lai FA, Liu Q-Y, Rousseau E, Erickson HP, Meissner G.** Structural and functional characterization of the purified cardiac ryanodine receptor-Ca^{2+} release channel complex. *J.Biol.Chem.* 1989; **264**: 1329-1335.

[14] **Antoniu B, Kim DH, Morii M, Ikemoto N.** Inhibitors of Ca^{2+} release from the isolated sarcoplasmic reticulum. I. Ca^{2+} channel blockers. *Biochim.Biophys.Acta* 1985; **816**: 9-17.

[15] **Armstrong CM, Benzanilla FM, Horowicz P.** Twitches in the presence of ethylene glycol (*beta*-aminoethylether)-N,N'-tetraacetic acid. *Biochim.Biophys.Acta* 1972; **267**: 605-608.

[16] **Ashley RH, Williams AJ.** Divalent cation activation and inhibition of single calcium release channels from cardiac sarcoplasmic reticulum. *J.Gen.Physiol.* 1990; **95**: 981-1005.

[17] **Backx PH, de Tombe PP, van Deen JHK, Mulder BJM, ter Keurs HE.** A model for propagating calcium-induced calcium release mediated by calcium diffusion. *J.Gen.Physiol.* 1989; **93**: 963-977.

[18] **Balke CW, Egan TM, Wier WG.** Processes that remove calcium from the cytoplasm during excitation-contraction coupling in intact rat heart cells. *J.Physiol.(Lond.)* 1994; **474**: 447-462.

[19] **Banijamali HS, Gao W-D, MacIntosh BR, ter Keurs HEDJ.** Force-interval relations of twitches and cold contractures in rat cardiac trabeculae: Effect of Ryanodine. *Circ.Res.* 1991; **69**: 937-948.

[20] **Barcenas-Ruiz L, Beuckelmann DJ, Wier WG.** Sodium-calcium exchange in heart: Membrane currents and changes in $[Ca^{2+}]_i$. *Science* 1987; **238**: 1720-1722.

[21] **Barcenas-Ruiz L, Wier WG.** Voltage dependence of intracellular $(Ca^{2+})_i$ transients in guinea pig ventricular myocytes. *Circ.Res.* 1987; **61**: 148-154.

[22] **Barry WH, Peeters GA, Rasmussen CAJr, Cunningham MJ.** Role of changes in $[Ca^{2+}]_i$ in energy deprivation contracture. *Circ.Res.* 1987; **61**: 726-734.

[23] **Bassani JWM, Bassani RA, Bers DM.** Ca^{2+} cycling between sarcoplasmic reticulum and mitochondria in rabbit cardiac myocytes. *J.Physiol.(Lond.)* 1993; **460**: 603-621.

[24] **Bassani JWM, Bassani RA, Bers DM.** Relaxation in rabbit and rat cardiac cells: species-dependant differences in cellular mechanisms. *J.Physiol.(Lond.)* 1994; **476**: 279-293.

[25] **Bassani JWM, Yuan W, Bers DM.** Fractional SR Ca^{2+} release is regulated by trigger Ca^{2+} and SR Ca^{2+} content in cardiac myocytes. *Am.J.Physiol.* 1995; **268**: C1313-C1329.

[26] **Bassani RA, Bassani JWM, Bers DM.** Mitochondrial and sarcolemmal Ca^{2+} transport reduce $[Ca^{2+}]_i$ during caffeine contractures in rabbit cardiac myocytes. *J.Physiol.(Lond.)* 1992; **453**: 591-608.

[27] **Bassani RA, Bassani JWM, Bers DM.** Relaxation in ferret ventricular myocytes: role of the sarcolemmal Ca^{2+} ATPase. *Pflügers Archiv.* 1995; **430**: 573-578.

[28] **Bassani RA, Bers DM.** Na^{+}-Ca^{2+} exchange is required for rest-decay but not for rest-potentiation of twitches in rabbit and rat ventricular myocytes. *J.Mol.Cell.Cardiol.* 1994; **26**: 1335-1347.

[29] **Bassani RA, Bers DM.** Rate of diastolic Ca^{2+} release from the sarcoplasmic reticulum of intact rabbit and rat ventricular myocytes. *Biophys.J.* 1995; **68**: 2015-2022.

[30] **Baudet S, Shaoulian R, Bers DM.** Effects of thapsigargin and cyclopiazonic acid on twitch force and sarcoplasmic reticulum Ca^{2+} content of rabbit ventricular muscle. *Circ.Res.* 1993; **73**: 813-819.

[31] **Bean BP.** Two kinds of calcium channels in canine atrial cells: differences in kinetics, selectivity, and pharmacology. *J.Gen.Physiol.* 1985; **86**: 1-30.

[32] **Beeler GW, Reuter H.** The relation between membrane potential, membrane currents and activation of contraction in ventricular myocardial fibres. *J.Physiol.(Lond.)* 1970; **207**: 211-229.

[33] **Berlin JR, Cannell MB, Lederer WJ.** Regulation of twitch tension in sheep cardiac Purkinje fibers during calcium overload. *Am.J.Physiol.* 1987; **253**: H1540-H1547.

[34] **Berlin JR, Cannell MB, Lederer WJ.** Cellular origins of the transient inward current in cardiac myocytes: role of fluctuations and waves of elevated intracellular calcium. *Circ.Res.* 1989; **65**: 115-126.

[35] **Berridge MJ.** Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu.Rev.Biochem.* 1987; **56**: 159-193.

[36] **Berridge MJ.** Elementary and global aspects of calcium signalling. *J.Physiol.(Lond.)* 1997; **499**: 291-306.

[37] **Berry MN, Friend DS, Scheuer J.** Morphology and metabolism of intact muscle cells isolated from adult rat heart. *Circ.Res.* 1970; **26**: 679-687.

[38] **Bers DM.** Ca^{2+} influx and sarcoplasmic reticulum Ca^{2+} release in cardiac muscle activation during post-rest recovery. *Am.J.Physiol.* 1985; **248** : H366-H381.

[39] **Bers DM.** SR Ca^{2+} loading in cardiac muscle preparations based on rapid-cooling contractures. *Am.J.Physiol.* 1989; **256**: C109-C120.

[40] Bers DM: *Excitation-contraction coupling and cardiac contractile force*, Dordrecht/Boston/London, Kluwer Academic Publishers, 1993,

[41] **Bers DM, Bassani RA, Bassani JWM, Baudet S, Hryshko LV.** Paradoxical twitch potentiation after rest in cardiac muscle: increased fractional release of SR calcium. *J.Mol.Cell.Cardiol.* 1993; **25**: 1047-1057.

[42] **Bers DM, Bridge JHB.** Effect of acetylstrophanthidin on twitches, microscopic tension fluctuations and cooling contractures in rabbit ventricle. *J.Physiol.(Lond.)* 1988; **404**: 53-69.

[43] **Bers DM, Bridge JHB.** Relaxation of rabbit ventricular muscle by Na^+-Ca^{2+} exchange and sarcoplasmic reticulum calcium pump: ryanodine and voltage sensitivity. *Circ.Res.* 1989; **65**: 334-342.

[44] **Bers DM, Christensen DM.** Functional inter-conversion of rest decay and ryanodine effects in rabbit and rat ventricle depends on Na^+/Ca^{2+} exchange. *J.Mol.Cell.Cardiol.* 1990; **22**: 715-723.

[45] **Bers DM, Christensen DM, Nguyen TX.** Can Ca^{2+} entry via Na^+-Ca^{2+} exchange directly activate cardiac muscle contraction. *J.Mol.Cell.Cardiol.* 1988; **20**: 405-414.

[46] **Bers DM, Ellis D.** Intracellular calcium and sodium activity in sheep heart Purkinje fibres. Effect of changes of external sodium and intracellular pH. *Pflügers Archiv.* 1982; **393**: 171-178.

[47] **Bers DM, Lederer WJ, Berlin JR.** Intracellular Ca^{2+} transients in rat cardiac myocytes: role of Na^+-Ca^{2+} exchange in excitation-contraction coupling. *Am.J.Physiol.* 1990; **258**: C944-C954.

[48] **Bers DM, Stiffel VM.** Ratio of ryanodine to dihydropyridine receptors in cardiac and skeletal muscle and implications for E-C coupling. *Am.J.Physiol.* 1993; **264**: C1587-C1593.

[49] **Beuckelmann DJ, Wier WG.** Sodium-calcium exchange in guinea-pig cardiac cells: exchange current and changes in intracellular Ca^{2+}. *J.Physiol.(Lond.)* 1989; **414**: 499-520.

[50] **Bianchi CP, Bolton TC.** Action of local anaesthetics on coupling systems in muscle. *J.Pharmocol.Exp.Ther.* 1967; **157**: 388-405.

[51] **Blanchard EM, Solaro RJ.** Inhibition of the activation and troponin calcium binding of dog cardiac myofibrils by acidic pH. *Circ.Res.* 1984; **55**: 382-391.

[52] **Blinks JR, Koch-Weser J.** Analysis of the effects of changes in rate and rhythm upon myocardial contractility. *Journal of Pharmacology* 1961; **134**: 373-389.

[53] **Bouchard RA, Bose D.** Analysis of the interval-force relationship in rat and canine ventricular myocardium. *Am.J.Physiol.* 1989; **257**: H2036-H2047.

[54] **Bouchard RA, Clark RB, Giles WR.** Role of sodium-calcium exchange in activation of contraction in rat ventricle. *J.Physiol.(Lond.)* 1993; **472**: 391-413.

[55] **Bouchard RA, Clark RB, Giles WR.** Effects of action potential duration on excitation-contraction coupling in rat ventricular myocytes. *Circ.Res.* 1995; **76**: 790-801.

[56] **Boyett MR, Hart G, Levi AJ, Roberts A.** Effects of repetitive activity on developed force and intracellular sodium in isolated sheep and dog Purkinje fibres. *J.Physiol.(Lond.)* 1987; **388**: 295-322.

[57] **Bridge JH, Smolley JR, Spitzer KW.** The relationship between charge movements associated with I_{Ca} and $I_{Na\text{-}Ca}$ in cardiac myocytes. *Science* 1990; **248**: 376-378.

[58] **Bridge JHB.** Relationships between the sarcoplasmic reticulum and sarcolemmal calcium transport revealed by rapidly cooling rabbit ventricular muscle. *J.Gen.Physiol.* 1986; **88**: 437-473.

[59] **Bridge JHB, Smolley JR, Spitzer KW.** The relationship between charge movements associated with I_{Ca} and $I_{Na\text{-}Ca}$ in cardiac myocytes. *Science* 1990; **248**: 376-378.

[60] **Brillantes A-MB, Ondrias K, Scott A, Kobrinsky E, Ondriasova E, Moschella MC, Jayaraman T, Landers M, Ehrlich BE, Marks AR.** Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* 1994; **77**: 513-523.

[61] **Buxton N, Overend CL, Mill JG, Cook LJ, Cook SJ, O'Neill SC, Eisner DA.** The effects of tetracaine on spontaneous Ca^{2+} oscillations in rat isolated ventricular myocytes. *J.Physiol.(Lond.)* 1996; **495P**: 86P (abstract)

[62] **Callewaert G, Cleemann L, Morad M.** Epinephrine enhances Ca^{2+} current-regulated Ca^{2+} release and Ca^{2+} reuptake in rat ventricular myocytes. *Proc.Natl.Acad.Sci,USA.* 1988; **85**: 2009-2013.

[63] **Callewaert G, Cleemann L, Morad M.** Caffeine-induced Ca^{2+} release activates Ca^{2+} extrusion via Na^{+}-Ca^{2+} exchanger in cardiac myocytes. *Am.J.Physiol.* 1989; **257**: C147-C152.

[64] **Campbell KP, Leung AT, Sharp AH.** The biochemistry and molecular biology of the dihydropyridine-sensitive calcium channel. *Trends in Neuroscience* 1988; **11**: 425-430.

[65] **Cannell MB, Berlin JR, Lederer WJ.** Effect of membrane potential changes on the calcium transient in single rat cardiac muscle cells. *Science* 1987; **238**: 1419-1423.

[66] **Cannell MB, Cheng H, Lederer WJ.** Spatial Non-Uniformities of $[Ca^{2+}]_i$ during Excitation-Contraction Coupling in Cardiac Myocytes. *Biophys.J.* 1994; **67**: 1942-1956.

[67] **Cannell MB, Cheng H, Lederer WJ.** The control of calcium release in heart muscle. *Science* 1995; **268**: 1045-1049.

[68] **Capogrossi MC, Houser SR, Bahinski A, Lakatta EG.** Synchronous occurrence of spontaneous localised calcium release from the sarcoplasmic reticulum generates action potentials in rat cardiac ventricular myocytes at normal resting membrane potential. *Circ.Res.* 1987; **61**: 498-503.

[69] **Capogrossi MC, Kort AA, Spurgeon HA, Lakatta EG.** Single adult rabbit and rat cardiac myocytes retain the Ca^{2+}- and species-dependent systolic and diastolic contractile properties of intact muscle. *J.Gen.Physiol.* 1986; **88**: 589-613.

[70] **Capogrossi MC, Lakatta EG.** Frequency modulation and synchronization of spontaneous oscillations in cardiac cells. *Am.J.Physiol.* 1985; **248**: H412-H418.

[71] **Capogrossi MC, Stern MD, Spurgeon HA, Lakatta EG.** Spontaneous Ca^{2+} release from the sarcoplasmic reticulum limits Ca^{2+}- dependent twitch potentiation in individual cardiac myocytes; A mechanism for maximum inotropy in the myocardium. *J.Gen.Physiol.* 1988; **91**: 133-155.

[72] **Capogrossi MC, Suárez-Isla BA, Lakatta EG.** The interaction of electrically stimulated twitches and spontaneous contractile waves in single cardiac myocytes. *J.Gen.Physiol.* 1986; **88**: 615-633.

[73] **Carl SL, Felix K, Caswell AH, Brandt NR, Ball Jr WJ, Vaghy PL, Meissner G, Ferguson DG.** Immunolocalization of sarcolemmal dihydropyridine receptor and sarcoplasmic reticulum triadin and ryanodine receptor in rabbit ventricle. *J.Cell.Biol.* 1995; **129**: 672-682.

[74] **Carmeliet E.** A fuzzy sub-sarcolemmal space for intracellular Na^+ in cardiac cells? *Cardiovasc.Res.* 1992; **26**: 433-442.

[75] **Carmeliet E, Morad M, Van der Heyden G, Vereecke J.** Electrophysiological effects of tetracaine in single guinea-pig ventricular myocytes. *J.Physiol.(Lond.)* 1986; **376**: 143-161.

[76] **Carmeliet E, Vereecke J, Vleugels A.** Ionic currents during hypoxia voltage-clamped cat ventricular muscle. *Circ.Res.* 1980; **47**: 501-508.

[77] **Caroni P, Carafoli E.** The Ca^{2+}-pumping ATPase of heart sarcolemma. *J.Biol.Chem.* 1981; **256**: 3263-3270.

[78] **Carpentier RG, Coleman BR, Patel DJ.** Cocaine enhances post-rest and paired-stimulation potentiation in rat papillary muscle. *J.Pharmocol.Exp.Ther.* 1993; **266**: 41-46.

[79] **Catterall WA.** Structure and function of voltage-sensitive ion channels. *Science* 1988; **242**: 50-61.

[80] **Chamberlain BK, Volpe P, Fleischer S.** Inhibition of calcium-induced calcium release from purified cardiac sarcoplasmic reticulum vesicles. *J.Biol.Chem.* 1984; **259**: 7547-7553.

[81] **Chapman RA, Leoty C.** The effects of tetracaine on the membrane currents and contraction of frog atrial muscle. *J.Physiol.(Lond.)* 1981; **317**: 475-486.

[82] **Chapman RA, Rodrigo GC.** Intracellular amino acids and the regulation of $[Na]_i$: an effect upon calcium tolerance in isolated guinea-pig ventricular myocytes. *J.Physiol.(Lond.)* 1990; **426**: 16P (abstract)

[83] **Cheng H, Fill M, Valdivia H, Lederer WJ.** Models of Ca^{2+} release channel adaptation. *Science* 1995; **267**: 2009-2010.

[84] **Cheng H, Lederer MR, Lederer WJ, Cannell MB.** Calcium sparks and $[Ca^{2+}]_i$ waves in cardiac myocytes. *Am.J.Physiol.* 1996; **270**: C148-C159.

[85] **Cheng H, Lederer MR, Xiao R-P, Gómez AM, Zhou Y-Y, Ziman B, Spurgeon H, Lakatta EG, Lederer WJ.** Excitation-contraction coupling in heart: new insights from Ca^{2+} sparks. *Cell Calcium* 1996; **20**: 129-140.

[86] **Cheng H, Lederer WJ, Cannell MB.** Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science* 1993; **262**: 740-744.

[87] **Chiesi M, Wrzosek A, Grueninger S.** The role of the sarcoplasmic reticulum in various types of cardiomyocytes. *Mol.Cell.Biochem.* 1994; **130**: 159-171.

[88] **Choi HS, Eisner DA.** The role of the sarcolemmal Ca^{2+}-ATPase in the regulation of resting calcium concentration in rat ventricular myocytes. *J.Physiol.(Lond.)* 1998; **515**: 109-118.

[89] **Choi HS, Eisner DA**: The effects of inhibition of the sarcolemmal Ca^{2+}-ATPase on systolic calcium fluxes and intracellular calcium concentration in rat ventricular myocytes. *J.Physiol.(Lond.)* 1999;(in press)

[90] **Choi HS, Trafford AW, Eisner DA**: Measurement of Ca^{2+} entry and exit in quiescent rat ventricular myocytes. *J.Physiol.(Lond.)* 1999;(in press)

[91] **Clague JR, Harvey R, Langer GA.** Protamine and other polycationic drugs inhibit calcium leak in cardiac cells during metabolic inhibition and free radical exposure. *J.Pharmocol.Exp.Ther.* 1993; **267**: 1349-1354.

[92] **Cleemann L, Morad M.** Role of Ca^{2+} channel in cardiac excitation-contraction coupling in rat: evidence from Ca^{2+} transient and contraction. *J.Physiol.(Lond.)* 1991; **432**: 283-312.

[93] **Clusin WT.** Do caffeine and metabolic inhibitors increase free calcium in the heart? Interpretation of conflicting intracellular calcium measurements. *J.Mol.Cell.Cardiol.* 1985; **17**: 213-220.

[94] **Cobbold PH, Bourne PK.** Aequorin measurements of free calcium in single heart cells. *Nature* 1984; **312**: 444-446.

[95] **Cohen CJ, Fozzard HA, Sheu S-S.** Increase in intracellular sodium ion activity during stimulation in mammalian cardiac muscle. *Circ.Res.* 1982; **50**: 651-662.

[96] **Coronado R, Morrissette J, Sukhareva M, Vaughan DM.** Structure and function of ryanodine receptors. *Am.J.Physiol.* 1994; **266**: C1485-C1504.

[97] **Coulombe A, Lefèvre IA, Baro I, Coraboeuf E.** Barium- and calcium-permeable channels open at negative membrane potentials in rat ventricular myocytes. *J.Membrane Biol.* 1989; **111**: 57-67.

[98] **Crespo LM, Grantham C, Cannell MB.** Kinetics, stoichiometry and role of the Na^{+}-Ca^{2+} exchange mechanism in isolated cardiac myocytes. *Nature* 1990; **345**: 618-621.

[99] **Csernoch L, Huang CL-H, Szucs G, Kovács L.** Differential effects of tetracaine on charge movements and Ca^{2+} signals in frog skeletal muscle. *J.Gen.Physiol.* 1988; **92**: 601-612.

[100] **Csernoch L, Szentesi P, Sárközi S, Szegedi C, Jona I, Kovács L.** Effects of tetracaine on sarcoplasmic calcium release in mammalian skeletal muscle fibres. *J.Physiol.(Lond.)* 1999; **515**: 843-857.

[101] **Deitmer JW, Ellis D.** Interactions between the regulation of the intracellular pH and sodium activity of sheep cardiac Purkinje fibres. *J.Physiol.(Lond.)* 1980a; **304**: 471-488.

[102] **Deitmer JW, Ellis D.** The intracellular sodium activity of sheep heart Purkinje fibres: Effects of local anaesthetics and tetrodotoxin. *J.Physiol.(Lond.)* 1980b; **300**: 269-282.

[103] **Dizon J, Burkhoff D, Tauskela J, Whang J, Cannon P, Katz J.** Metabolic inhibition in the perfused rat heart: evidence for glycolytic requirement for normal sodium homeostasis. *Am.J.Physiol.* 1998; **274**: H1082-H1089.

[104] **Díaz ME, Cook SJ, Chamunorwa JP, Trafford AW, Lancaster MK, O'Neill SC, Eisner DA.** Variability of Spontaneous Ca^{2+} Release Between Different Rat Ventricular Myocytes Is Correlated with Na^{+}-Ca^{2+} Exchange and $[Na^{+}]_i$. *Circ.Res.* 1996; **78**: 857-862.

[105] **Díaz ME, Trafford AW, O'Neill SC, Eisner DA.** Measurement of sarcoplasmic reticulum Ca^{2+} content and sarcolemmal Ca^{2+} fluxes in isolated rat ventricular myocytes during spontaneous Ca^{2+} release. *J.Physiol.(Lond.)* 1997; **501**: 3-16.

[106] **Donoso P, Mill JG, O'Neill SC, Eisner DA.** Fluorescence measurements of cytoplasmic and mitochondrial sodium concentration in rat ventricular myocytes. *J.Physiol.(Lond.)* 1992; **448**: 493-509.

[107] **duBell WH, Lederer WJ, Rogers TB.** Dynamic modulation of excitation-contraction coupling by protein phosphatases in rat ventricular myocytes. *J.Physiol.(Lond.)* 1996; **493**: 793-800.

[108] **duBell WH, Wright PA, Lederer WJ, Rogers TB.** Effect of the immunosupressant FK506 on excitation-contraction coupling and outward K^{+} currents in rat ventricular myocytes. *J.Physiol.(Lond.)* 1997; **501**: 509-516.

[109] **Ebus PJ, Stienen GJM, Elzinga G.** Influence of phosphate and pH on myofibrillar ATPase activity and force in skinned cardiac trabeculae from rat. *J.Physiol.(Lond.)* 1994; **476.3**: 501-516.

[110] **Eisner DA, Elliott AC, Smith GL.** The contribution of intracellular acidosis to the decline of developed pressure in ferret hearts exposed to cyanide. *J.Physiol.(Lond.)* 1987; **391**: 99-108.

[111] **Eisner DA, Lederer WJ, Noble D.** Caffeine and tetracaine abolish the slow inward calcium current in sheep cardiac Purkinje fibres. *J.Physiol.(Lond.)* 1979; 76P-77P.

[112] **Eisner DA, Lederer WJ, Sheu SS.** The role of intracellular sodium activity in the anti-arrhythmic action of local anaesthetics in sheep Purkinje fibres. *J.Physiol.(Lond.)* 1983; **340**: 239-257.

[113] **Eisner DA, Lederer WJ, Vaughan Jones RD.** The quantitative relationship between twitch tension and intracellular sodium activity in sheep cardiac Purkinje fibres. *J.Physiol.(Lond.)* 1984; **355**: 251-266.

[114] **Eisner DA, Nichols CG, O'Neill SC, Smith GL, Valdeolmillos M.** The effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. *J.Physiol.(Lond.)* 1989; **411**: 393-418.

[115] **Eisner DA, Trafford AW, Díaz ME, Overend CL, O'Neill SC.** The control of Ca^{2+} release from the cardiac sarcoplasmic reticulum: regulation versus autoregulation. *Cardiovasc.Res.* 1998; **38**: 589-604.

[116] **Eisner DA, Valdeolmillos M.** A study of intracellular calcium oscillations in sheep cardiac Purkinje fibres measured at the single cell level. *J.Physiol.(Lond.)* 1986; **372**: 539-556.

[117] **Elliott AC, Smith GL, Allen DG.** Simultaneous measurements of action potential duration and intracellular ATP in isolated ferret hearts exposed to cyanide. *Circ.Res.* 1989; **64**: 583-591.

[118] **Elliott AC, Smith GL, Eisner DA, Allen DG.** Metabolic changes during ischaemia and their role in contractile failure in isolated ferret hearts. *J.Physiol.(Lond.)* 1992; **454**: 467-490.

[119] **Ellis D, MacLeod KT.** Sodium dependant control of intracellular pH in Purkinje fibres from sheep heart. *J.Physiol.(Lond.)* 1985; **359**: 81-105.

[120] **Endo M, Tanaka M, Ogawa Y.** Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. *Nature* 1970; **228**: 34-36.

[121] **Evans AM, Cannell MB.** The role of L-type Ca^{2+} current and Na^{+} current-stimulated Na^{+}/Ca^{2+} exchange in triggering SR calcium release in guinea-pig cardiac ventricular myocyte. *Cardiovasc.Res.* 1997; **35**: 294-302.

[122] **Fabiato A.** Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *Am.J.Physiol.* 1983; **245**: C1-C14.

[123] **Fabiato A.** Simulated calcium current can both cause calcium loading in and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J.Gen.Physiol.* 1985a; **85**: 291-320.

[124] **Fabiato A.** 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. *J.Gen.Physiol.* 1985b; **85**: 247-289.

[125] **Fabiato A.** Use of aequorin for the appraisal of the hypothesis of the release of calcium from the sarcoplasmic reticulum induced by a change of pH in skinned cardiac cells. *Cell Calcium* 1985c; **6**: 95-108.

[126] **Fabiato A.** Appraisal of the physiological relevance of two hypothesis for the mechanism of calcium release from the mammalian cardiac sarcoplasmic reticulum: calcium-induced release versus charge-coupled release. *Mol.Cell.Biochem.* 1989; **89**: 135-140.

[127] **Fabiato A, Fabiato F.** Excitation-contraction coupling of isolated cardiac fibers with disrupted or closed sarcolemmas. Calcium-dependent cyclic and tonic contractions. *Circ.Res.* 1972; **31**: 293-307.

[128] **Fabiato A, Fabiato F.** Activation of skinned cardiac cells. Subcellular effects of cardioactive drugs. *European Journal of Cardiology* 1973; **1**: 143-155.

[129] **Fabiato A, Fabiato F.** Contractions induced by a calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. *J.Physiol.(Lond.)* 1975a; **249**: 469-495.

[130] **Fabiato A, Fabiato F.** Effects of magnesium on contractile activation of skinned cardiac cells. *J.Physiol.(Lond.)* 1975b; **249**: 497-517.

[131] **Fabiato A, Fabiato F.** Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned cells from adult human, dog, cat, rabbit, rat, and frog hearts and from fetal and new-born rat ventricles. *Annals New York Academy of Sciences* 1978a; **307**: 491-522.

[132] **Fabiato A, Fabiato F.** Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. *J.Physiol.(Lond.)* 1978b; **276**: 233-255.

[133] **Fabiato A, Fabiato F.** Use of chlorotetracycline fluorescence to demonstrate Ca^{2+}-induced release of Ca^{2+} from the sarcoplasmic reticulum of skinned cardiac cells. *Nature* 1979; **281**: 146-148.

[134] **Fedida D, Noble D, Rankin AC, Spindler AJ.** The arrhythmogenic transient inward current I_{TI} and related contraction in isolated guinea-pig ventricular myocytes. *J.Physiol.(Lond.)* 1987; **392** : 523-542.

[135] **Fedida D, Noble D, Shimoni Y, Spindler AJ.** Inward current related to contraction in guinea-pig ventricular myocytes. *J.Physiol.(Lond.)* 1987; **385**: 565-589.

[136] **Ferrier GR, Howlett SE.** Contractions in guinea-pig ventricular myocytes triggered by a calcium-release mechanism separate from Na^+ and L-currents. *J.Physiol.(Lond.)* 1995; **484**: 107-122.

[137] **Ferrier GR, Saunders JH, Mendez C.** A cellular mechanism for the generation of ventricular arrhythmias by acetylstrophanthidin. *Circ.Res.* 1973; **32**: 600-609.

[138] **Ferrier GR, Zhu J, Redondo IM, Howlett SE.** Role of cAMP-dependent protein kinase A in activation of a voltage-sensitive release mechanism for cardiac contraction in guinea-pig myocytes. *J.Physiol.(Lond.)* 1998; **513**: 185-201.

[139] **Ford LE, Podolsky RJ.** Regenerative calcium release within muscle cells. *Science* 1970; **167**: 58-59.

[140] **Fruen BR, Mickelson JR, Shomer NH, Roghair TJ, Louis CF.** Regulation of the sarcoplasmic reticulum ryanodine receptor by inorganic phosphate. *J.Biol.Chem.* 1994; **269**: 192-198.

[141] **Fruen BR, Mickelson JR, Shomer NH, Velez P, Louis CF.** Cyclic ADP-ribose does not affect cardiac or skeletal muscle ryanodine receptors. *FEBS Lett.* 1994; **352**: 123-126.

[142] **Fujii J, Maruyama K, Tada M, MacLennan DH.** Expression and site-specific mutagenesis of phospholamban. *J.Biol.Chem.* 1989; **264**: 12950-12955.

[143] **Galione A.** Cyclic ADP-ribose: a new way to control calcium. *Science* 1993; **259**: 325-326.

[144] **Galione A, Lee HC, Busa WB.** Ca^{2+}-induced Ca^{2+} release in sea urchin egg homegenates: modulation by cyclic ADP-ribose. *Science* 1991; **253**: 1143-1146.

[145] **García J, Avila-Sakar AJ, Stefani E.** Differential effects of ryanodine and tetracaine on charge movement and calcium transients in frog skeletal muscle. *J.Physiol.(Lond.)* 1991; **440**: 403-417.

[146] **Gatto C, Hale CC, Xu W, Milanick MA.** Eosin, a potent inhibitor of the plasma membrane Ca^{2+} pump, does not inhibit the cardiac Na^{+}-Ca^{2+} exchanger. *Biochemistry* 1995; **34**: 965-972.

[147] **Gatto C, Milanik MA.** Inhibition of red blood cell calcium pump by eosin and other fluorescein analogues. *Am.J.Physiol.* 1993; **264**: C1577-C1586.

[148] **Ginsburg KS, Weber CR, Bers DM.** Control of maximum sarcoplasmic reticulum Ca^{2+} load in intact ferret ventricular myocytes. *J.Gen.Physiol.* 1998; **111**: 491-504.

[149] **Goldhaber JI, Parker JM, Weiss JN.** Mechanisms of excitation-contraction coupling failure during metabolic inhibition in guinea-pig ventricular myocytes. *J.Physiol.(Lond.)* 1991; **443**: 371-386.

[150] **Goldman YE, Brenner B.** Special topic: Molecular mechanism of muscle contraction. *Annu.Rev.Physiol.* 1987; **49**: 629-709.

[151] **Gramolini A, Renaud J-M.** Blocking ATP-sensitive K^+ channel during metabolic inhibition impairs muscle contractility. *Am.J.Physiol.* 1997; **272**: C1936-C1946.

[152] **Grantham CJ, Cannell MB.** Ca^{2+} influx during the cardiac action potential in guinea pig ventricular myocytes. *Circ.Res.* 1996; **79**: 194-200.

[153] **Grynkiewicz G, Poenie M, Tsien RY.** A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J.Biol.Chem.* 1985; **260**: 3440-3450.

[154] **Guo X, Laflamme MA, Becker PL.** Cyclic ADP-Ribose does not regulate sarcoplasmic reticulum Ca^{2+} release in intact cardiac myocytes. *Circ.Res.* 1996; **79**: 147-151.

[155] **Gwathmey JK, Hajjar JJ, Solaro RJ.** Contractile deactivation and uncoupling of crossbridges. Effects of 2,3-Butanedione monoxime on mammalian myocardium. *Circ.Res.* 1991; **69**: 1280-1292.

[156] **Györke S, Fill M.** Ryanodine receptor adaptation: control mechanism of Ca^{2+} -induced Ca^{2+} release in heart. *Science* 1993; **260**: 807-809.

[157] **Györke S, Lukyanenko V, Györke I.** Dual effects of tetracaine on spontaneous calcium release in rat ventricular myocytes. *J.Physiol.(Lond.)* 1997; **500**: 297-309.

[158] **Györke S, Palade P.** Calcium induced calcium release in crayfish skeletal muscle. *J.Physiol.(Lond.)* 1992; **457**: 195-210.

[159] **Györke S, Palade P.** Role of local Ca^{2+} domains in activation of Ca^{2+}-induced Ca^{2+} release in crayfish muscle fibres. *Am.J.Physiol.* 1993; **264**: C1505-C1512.

[160] **Györke S, Palade P.** Ca^{2+}-dependent negative control mechanism for Ca^{2+} induced Ca^{2+} release in crayfish muscle. *J.Physiol.(Lond.)* 1994; **476**: 315-322.

[161] **Hadley RW, Lederer WJ.** Intramembrane charge movement in guinea-pig and rat ventricular myocytes. *J.Physiol.(Lond.)* 1989; **415**: 601-624.

[162] **Hadley RW, Lederer WJ.** Ca^{2+} and voltage inactivate Ca^{2+} channels in guinea pig ventricular myocytes through independent mechanisms. *J.Physiol.(Lond.)* 1991; **444**: 257-268.

[163] **Hagiwara N, Irisawa H, Kameyama M.** Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. *J.Physiol.(Lond.)* 1988; **395**: 233-253.

[164] **Hain J, Onoue H, Mayrleitner M, Fleischer S, Schindler H.** Phosphorylation modulates the function of the calcium release channel of sarcoplasmic reticulum from cardiac muscle. *J.Biol.Chem.* 1995; **270**: 2074-2081.

[165] **Hamilton DL, Orchard CH.** The effect of acidosis on the relationship between Ca^{2+} and force in isolated ferret cardiac muscle. *J.Physiol.(Lond.)* 1987; **391**: 48P (abstract)

[166] **Han S, Schiefer A, Isenberg G.** Ca^{2+} load of guinea-pig ventricular myocytes determines efficacy of brief Ca^{2+} currents as trigger for Ca^{2+} release. *J.Physiol.(Lond.)* 1994; **480**: 411-421.

[167] **Harrison SM, Bers DM.** Temperature dependence of myofilament Ca^{2+} sensitivity of rat, guinea-pig, and frog ventricular muscle. *Am.J.Physiol.* 1990; **258**: C274-C281.

[168] **Harrison SM, Boyett MR.** The role the Na^{+}-Ca^{2+} exchanger in the rate dependent increase in contraction in guinea-pig ventricular myocytes. *J.Physiol.(Lond.)* 1995; **482**: 555-566.

[169] **Harrison SM, Frampton JE, McCall E, Boyett MR, Orchard CH.** Contraction and intracellular Ca^{2+}, Na^{+}, and H^{+} during acidosis in rat ventricular myocytes. *Am.J.Physiol.* 1992; **262**: C348-C357.

[170] **Harrison SM, McCall E, Boyett MR.** The relationship between contraction and intracellular sodium in rat and guinea-pig ventricular myocytes. *J.Physiol.(Lond.)* 1992; **449**: 517-550.

[171] **Hasin Y, Barry WH.** Myocardial metabolic inhibition and membrane potential, contraction and potassium uptake. *Am.J.Physiol.* 1984; **247**: H322-H329.

[172] **Hearse DJ.** Oxygen deprivation and early myocardial contracile failure: A reassessment of the possible role of adenosine triphosphate. *The.American.Journal.of.Cardiology.* 1979; **44**: 1115-1121.

[173] **Herrmann-Frank A, Lehmann-Horn F.** Regulation of the purified Ca^{2+} release channel / ryanodine receptor complex of skeletal muscle sarcoplasmic reticulum by luminal calcium. *Pflügers Archiv.* 1996; **432**: 155-157.

[174] **Hidalgo C, Donoso P.** Luminal calcium regulation of calcium release from sarcoplasmic reticulum. *Biosci.Rep.* 1995; **15**: 387-397.

[175] **Hill AV.** The abrupt transition from rest to activity in muscle. *Proc.R.Soc.Lond.(Biol.)* 1949; **136**: 399-420.

[176] **Hille B.** Local anesthetics: Hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J.Gen.Physiol.* 1977; **69**: 497-515.

[177] **Hobai IA, Howarth FC, Pabbathi VK, Dalton GR, Hancox JC, Zhu J-Q, Howlett SE, Ferrier GR, Levi AJ.** "Voltage-activated Ca^{2+} release" in rabbit, rat and guinea-pig cardiac myocytes, and modulation by internal cAMP. *Pflügers Archiv.* 1997; **435**: 164-173.

[178] **Hoffman BF, Kelly JJJr.** Effects of rate and rhythm on contraction of rat papillary muscle. *Am.J.Physiol.* 1959; **197**: 1199-1204.

[179] **Horn R, Marty A.** Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J.Gen.Physiol.* 1988; **92**: 145-159.

[180] **Hove-Madsen L, Bers DM.** Sarcoplasmic reticulum Ca^{2+} uptake and thapsigargin sensitivity in permeabilized rabbit and rat ventricular myocytes. *Circ.Res.* 1993; **73**: 820-828.

[181] **Hryshko LV, Bers DM.** Ca^{2+} current facilitation during postrest recovery depends on Ca^{2+} entry. *Am.J.Physiol.* 1990; **259**: H951-H961.

[182] **Huang CL-H.** Effects of local anaesthetics on the relationship between charge movements and contractile thresholds in frog skeletal muscle. *J.Physiol.(Lond.)* 1981; **320**: 381-391.

[183] **Hulme JT, Colyer J, Orchard CH.** Acidosis alters the phosphorylation of Ser^{16} and Thr^{17} of phospholamban in rat cardiac muscle. *Pflügers Archiv.* 1997; **434**: 475-483.

[184] **Huxley AF, Simmons RM.** Proposed Mechanism of Force Generation in Striated Muscle. *Nature* 1971; **233**: 533-538.

[185] **Huxley HE.** The Mechanism of Muscular Contraction. *Science* 1969; **164**: 1356-1366.

[186] **Iino S, Cui Y, Galione A, Terrar DA.** Actions of cADP-Ribose and its antagonists on contraction in guinea pig isolated ventricular myocytes: Influence of temperature. *Circ.Res.* 1997; **81**: 879-884.

[187] **Inui M, Saito A, Fleischer S.** Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures. *J.Biol.Chem.* 1987; **262**: 15637-15642.

[188] **Isenberg G.** Ca^{2+} entry and contraction as studied in isolated bovine ventricular myocytes. *Zeitschrift.Fur.Naturforschung.* 1982; **37**: 502-512.

[189] **Isenberg G, Klockner U.** Calcium tolerant ventricular myocytes prepared by preincubation in a "KB medium". *Pflügers Archiv.* 1982; **395** : 6-18.

[190] **Ishide N, Urayama T, Inoue K-I, Komaru T, Takishima T.** Propagation and collision characteristics of calcium waves in rat myocytes. *Am.J.Physiol.* 1990; **259**: H940-H950.

[191] **Jacobus WE, Pores IH, Lucas SK, Weisfeldt ML, Flaherty JT.** Intracellular acidosis and contractility in the normal and ischemic heart as examined by ^{31}P NMR. *J.Mol.Cell.Cardiol.* 1982; **14 Suppl. 3**: 13-20.

[192] **Jan L, Jan Y.** Voltage-sensitive ion channels. *Cell* 1989; **56**: 13-25.

[193] **Janczewski AM, Lakatta EG.** Thapsigargin inhibits Ca^{2+} uptake, and Ca^{2+} depletes sarcoplasmic reticulum in intact cardiac myocytes. *Am.J.Physiol.* 1993; **265**: H517-H522.

[194] **Janczewski AM, Lewartowski B.** The effect of prolonged rest on calcium exchange and contractions in rat and guinea-pig ventricular myocardium. *J.Mol.Cell.Cardiol.* 1986; **18**: 1233-1242.

[195] **Janczewski AM, Spurgeon HA, Stern MD, Lakatta EG.** Effects of sarcoplasmic reticulum Ca^{2+} load on the gain function of Ca^{2+} release by Ca^{2+} current in cardiac cells. *Am.J.Physiol.* 1995; **268**: H916-H920.

[196] **Jayaraman T, Brillantes A-M, Timerman AP, Fleischer S, Erdjument-Bromage H, Tempst P, Marks AR.** FK506 binding protein associated with the calcium release channel (ryanodine receptor). *J.Biol.Chem.* 1992; **267**: 9474-9477.

[197] **Jones LR, Suzuki YJ, Wang W, Kobayashi YM, Ranesh V, Franzini-Armstrong C, Cleemann L, Morad M.** Regulation of Ca^{2+} signalling in transgenic mouse cardiac myocytes overexpressing calsequestrin. *J.Clin.Invest.* 1998; **101**: 1385-1393.

[198] **Kabbara AA, Stephenson DG.** Effects of 2,5-di-*tert*-butylhydroquinone on rat cardiac muscle contractility. *Am.J.Physiol.* 1997; **272**: H1001-H1010.

[199] **Kaftan E, Marks AR, Ehrlich BE.** Effects of rapamycin on ryanodine receptor/Ca^{2+}-release channels from cardiac muscle. *Circ.Res.* 1996; **78**: 990-997.

[200] **Kasai H, Petersen OH.** Spatial dynamics of second messengers: IP_3 and cAMP as long-range and associative messengers. *Trends in Neuroscience* 1994; **17**: 95-100.

[201] **Kass RS, Lederer WJ, Tsien RW, Weingart R.** Role of calcium ions in transient inward currents and aftercontractions induced by strophanthidin in cardiac Purkinje fibres. *J.Physiol.(Lond.)* 1978; **281**: 187-208.

[202] **Kass RS, Sanguinetti MC.** Inactivation of calcium channel current in the calf cardiac Purkinje fiber: Evidence of voltage- and calcium-mediated mechanisms. *J.Gen.Physiol.* 1984; **84**: 705-726.

[203] **Kass RS, Tsien RW, Weingart R.** Ionic basis of transient inward current induced by strophanthidin in cardiac Purkinje fibres. *J.Physiol.(Lond.)* 1978; **281**: 209-226.

[204] **Kawai M, Konishi M, Kurihara S.** Magnesium and hydrogen ions inhibit sarcoplasmic reticulum function in cardiac muscle. *J.Mol.Cell.Cardiol.* 1996; **28**: 1401-1413.

[205] **Kentish JC.** The effects of inorganic phosphate and creatine phosphate on force production in skinned muscles from rat ventricle. *J.Physiol.(Lond.)* 1986; **370**: 585-604.

[206] **Kentish JC, Barsotti RJ, Lea TJ, Mulligan IP, Patel JR, Ferenczi MA.** Calcium release from cardiac sarcoplasmic reticulum induced by photorelease of calcium or Ins(1,4,5)P_3. *Am.J.Physiol.* 1990; **258**: H610-H615.

[207] **Kermode H, Sitsapesan R, Williams AJ.** ADP and inorganic phosphate activate the sheep cardiac sarcoplasmic reticulum Ca^{2+}-release channel. *J.Physiol.(Lond.)* 1995; **487P**: 144P-145P (abstract)

[208] **Kijima Y, Ogunbunmi E, Fleischer S.** Drug action of thapsigargin on the Ca^{2+} pump protein of sarcoplasmic reticulum. *J.Biol.Chem.* 1991; **266**: 22912-22918.

[209] **Kim HW, Steenaart NAE, Ferguson DG, Kranias EG.** Functional reconstitution of the cardiac sarcoplasmic reticulum Ca^{2+}-ATPase with phospholamban in phospholipid vesicles. *J.Biol.Chem.* 1990; **265**: 1702-1709.

[210] **Kimura J, Miyamae S, Noma A.** Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig. *J.Physiol.(Lond.)* 1987; **384**: 199-222.

[211] **Kimura J, Noma A, Irisawa H.** Na^{+}-Ca^{2+} exchange current in mammalian heart cells. *Nature* 1986; **319**: 596-597.

[212] **Kirino Y, Osakabe M, Shimizu H.** Ca^{2+}-induced Ca^{2+} release from fragmented sarcoplasmic reticulum: Ca^{2+}-dependent passive Ca^{2+} efflux. *J.Biochem.(Tokyo.)* 1983; **94**: 1111-1118.

[213] **Klein MG, Simon BJ, Schneider MF.** Effects of procaine and caffeine on calcium release from the sarcoplasmic reticulum in frog skeletal muscle. *J.Physiol.(Lond.)* 1992; **453**: 341-366.

[214] **Kohmoto O, Levi AJ, Bridge JHB.** Relation between reverse sodium-calcium exchange and sarcoplasmic reticulum calcium release in guinea pig ventricular cells. *Circ.Res.* 1994; **74**: 550-554.

[215] **Komai H, Redon D, Rusy BF.** Procaine enhancement of the rapid cooling contracture and inhibition of the decay of potentiated state in rabbit papillary muscle. *J.Mol.Cell.Cardiol.* 1995; **27**: 2543-2550.

[216] **Komukai K, Ishikawa T, Kurihara S.** Effects of acidosis on Ca^{2+} sensitivity of contractile elements in intact ferret myocardium. *Am.J.Physiol.* 1998; **274**: H147-H154.

[217] **Kort AA, Lakatta EG.** Calcium-dependent mechanical oscillations occur spontaneously in unstimulated mammalian cardiac tissues. *Circ.Res.* 1984a; **54**: 396-404.

[218] **Kort AA, Lakatta EG.** Propagation velocity and frequency of spontaneous microscopic waves in intact rat papillary muscles are Ca^{2+} dependent. *Biophys.J.* 1984b; **45**: 94a (abstract)

[219] **Kort AA, Lakatta EG.** Spontaneous sarcoplasmic reticulum calcium release in rat and rabbit cardiac muscle: relation to transient and rested-state twitch tension. *Circ.Res.* 1988; **63**: 969-979.

[220] **Kusuoka H, Weisfeldt ML, Zweier JL, Jacobus WE, Marban E.** Mechanism of early contractile failure during hypoxia in intact ferret heart: evidence for modulation of maximal Ca^{2+}-activated force by inorganic phosphate. *Circ.Res.* 1986; **59**: 270-282.

[221] **Lahouratate P, Guibert J, Faivre J-F.** cADP-ribose releases Ca^{2+} from cardiac sarcoplasmic reticulum independently of ryanodine receptor. *Am.J.Physiol.* 1997; **273**: H1082-H1089.

[222] **Lakatta EG.** Functional implications of spontaneous sarcoplasmic reticulum Ca^{2+} release in the heart. *Cardiovasc.Res.* 1992; **26**: 193-214.

[223] **Lakatta EG, Lappé DL.** Diastolic scattered light fluctuation, resting force and twitch force in mammalian cardiac muscle. *J.Physiol.(Lond.)* 1981; **315**: 369-394.

[224] **Lamont C, Eisner DA.** The sarcolemmal mechanisms involved in the control of diastolic intracellular calcium in isolated rat cardiac trabeculae. *Pflügers Archiv.* 1996; **432**: 961-969.

[225] **Leblanc N, Hume JR.** Sodium current-induced release of calcium from cardiac sarcoplasmic reticulum. *Science* 1990; **248**: 372-376.

[226] **Lederer WJ, Berlin JR, Cohen NM, Hadley RW, Bers DM, Cannell MB.** Excitation-contraction coupling in heart cells. *Annals New York Academy of Sciences* 1990; **588**: 190-206.

[227] **Lederer WJ, Nichols CG, Smith GL.** The mechanism of early contractile failure of isolated rat ventricular myocytes subjected to complete metabolic inhibition. *J.Physiol.(Lond.)* 1989; **413**: 329-349.

[228] **Lederer WJ, Niggli E, Hadley RJ.** Sodium-calcium exchange in excitable cells: Fuzzy Space. *Science* 1992; **248**: 283.

[229] **Lederer WJ, Tsien RW.** Transient inward current underlying arrhythmogenic effects of cardiotonic steroids in Purkinje fibres. *J.Physiol.(Lond.)* 1976; **263**: 873-100.

[230] **Lee KS, Marban E, Tsien RW.** Inactivation of calcium channels in mammalian heart cells: Joint dependence on membrane potential and intracellular calcium. *J.Physiol.(Lond.)* 1985; **364**: 395-411.

[231] **Levesque PC, Leblanc N, Hume JR.** Role of reverse-mode Na^+-Ca^{2+} exchange in excitation contraction coupling in the heart. *Annals New York Academy of Sciences* 1991; **639**: 386-397.

[232] **Levi AJ, Brooksby P, Hancox JC.** One hump or two? The triggering of calcium release from the sarcoplasmic reticulum and the voltage dependence of contraction in mammalian caridac muscle. *Cardiovasc.Res.* 1993; **27**: 1743-1757.

[233] **Levi AJ, Li J, Spitzer KW, Bridge JHB.** Effect on the Indo-1 transient of applying Ca^{2+} channel blocker for a single beat in voltage-clamped guinea-pig cardiac myocytes. *J.Physiol.(Lond.)* 1996; **494**: 653-673.

[234] **Levi AJ, Spitzer KW, Kohmoto O, Bridge JHB.** Depolarization-induced Ca^{2+} entry via Na^+-Ca^{2+} exchange triggers SR release in guinea pig cardiac myocytes. *Am.J.Physiol.* 1994; **266**: H1422-H1433.

[235] **Lewartowski B, Hansford RG, Langer GA, Lakatta EG.** Contraction and sarcoplasmic reticulum Ca^{2+} content in single myocytes of guinea pig heart: effect of ryanodine. *Am.J.Physiol.* 1990; **259**: H1222-H1229.

[236] **Lewartowski B, Pytkowski B.** Cellular mechanism of the relationship between myocardial force and frequency of contractions. *Prog.Biophys.molec.Biol.* 1987; **50**: 97-120.

[237] **Lewartowski B, Wolska BM.** The effect of thapsigargin on sarcoplasmic reticulum Ca^{2+} content and contractions in single myocytes of guinea-pig heart. *J.Mol.Cell.Cardiol.* 1993; **25**: 23-29.

[238] **Lewartowski B, Zdanowski K.** Net Ca^{2+} influx and sarcoplasmic reticulum Ca^{2+} uptake in resting single myocytes of the rat heart: comparison with guinea-pig. *J.Mol.Cell.Cardiol.* 1990; **22**: 1221-1229.

[239] **Lipp P, Niggli E.** Sodium current-induced calcium signals in isolated guinea-pig ventricular myocytes. *J.Physiol.(Lond.)* 1994; **474**: 439-446.

[240] **Lipp P, Niggli E.** Submicroscopic calcium signals as fundamental events of excitation-contraction coupling in guinea-pig cardiac myocytes. *J.Physiol.(Lond.)* 1996; **492**: 31-38.

[241] **London B, Krueger JW.** Contraction in voltage-clamped, internally perfused single heart cells. *J.Gen.Physiol.* 1986; **88**: 475-505.

[242] **López-López JR, Shacklock PS, Balke CW, Wier WG.** Local, stochastic release of Ca^{2+} in voltage-clamped rat heart cells: visualization with confocal microscopy. *J.Physiol.(Lond.)* 1994; **480**: 21-29.

[243] **López-López JR, Shacklock PS, Balke CW, Wier WG.** Local calcium transients triggered by single L-type calcium channel currents in cardiac cells. *Science* 1995; **268**: 1042-1045.

[244] **Lukas A, Bose R.** Mechanisms of frequency-induced potentiation of contractions in isolated rat atria. *Naunyn-Schmiedeberg's Arch.Pharmacol.* 1986; **334**: 480-487.

[245] **Lukyanenko V, Györke I, Györke S.** Regulation of calcium release by calcium inside the sarcoplasmic reticulum in ventricular myocytes. *Pflügers Archiv.* 1996; **432**: 1047-1054.

[246] **Lukyanenko V, Subramanian S, Györke I, Wiesner TF, Györke S.** The role of luminal Ca^{2+} in the generation of Ca^{2+} waves in rat ventricular myocytes. *J.Physiol.(Lond.)* 1999; **518**: 173-186.

[247] **Luo W, Grupp IL, Harrer J, Ponniah S, Grupp G, Duffy JJ, Doetschman T, Kranias EG.** Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of β-agonist stimulation. *Circ.Res.* 1994; **75**: 401-409.

[248] **Luttgau HC, Oetliker H.** The action of caffeine on the activation of the contractile mechanism in striated muscle fibres. *J.Physiol.(Lond.)* 1968; **194**: 51-74.

[249] **MacLeod KT.** Effects of hypoxia and metabolic inhibition on the intracellular sodium activity of mammalian ventricular muscle. *J.Physiol.(Lond.)* 1989; **416**: 455-468.

[250] **MacLeod KT, Bers DM.** Effects of rest duration and ryanodine on changes of extracellular [Ca^{2+}] in cardiac muscle from rabbits. *Am.J.Physiol.* 1987; **253**: C398-C407.

[251] **McCall E, Li L, Satoh H, Shannon TR, Blatter LA, Bers DM.** Effects of FK-506 on contraction and Ca^{2+} transients in rat cardiac myocytes. *Circ.Res.* 1996; **79**: 1110-1121.

[252] **McCleskey EW, Womack MD, Fieber LA.** Structural properties of voltage-dependent calcium channels. *Int.Rev.Cytol.* 1993; **137C**: 39-54.

[253] **McKillop DFA, Fortune NS, Ranatunga KW, Geeves MA.** The influence of 2,3-butanedione 2-monoxime (BDM) on the interaction

between actin and myosin in solution and in skinned muscle fibres. *J.Muscle.Res.Cell Mot.* 1994; **15**: 309-318.

[254] **Mechmann S, Pott L.** Identification of Na^+-Ca^{2+} exchange current in single cardiac myocytes. *Nature* 1986; **319**: 597-599.

[255] **Meissner G.** Isolation and characterization of two types of sarcoplasmic reticulum vesicles. *Biochim.Biophys.Acta* 1975; **389**: 51-68.

[256] **Meissner G.** Ryanodine receptor/Ca^{2+} release channels and their regulation by endogenous effectors. *Annu.Rev.Physiol.* 1994; **56**: 485-508.

[257] **Meissner G, el-Hashem A.** Ryanodine as a functional probe of the skeletal muscle sarcoplasmic reticulum Ca^{2+} release channel. *Mol.Cell.Biochem.* 1992; **114**: 119-123.

[258] **Meissner G, Henderson JS.** Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotide, and calmodulin. *J.Biol.Chem.* 1987; **262**: 3065-3073.

[259] **Meissner G, Rousseau E, Lai FA, Liu QY, Anderson KA.** Biochemical characterization of the Ca^{2+} release channel of skeletal and cardiac sarcoplasmic reticulum. *Mol.Cell.Biochem.* 1988; **82**: 59-65.

[260] **Meyer M, Dillman WH.** Sarcoplasmic reticulum Ca^{2+}-ATPase overexpression by adenovirus mediated gene transfer and in transgenic mice. *Cardiovasc.Res.* 1998; **37**: 360-366.

[261] **Mészáros LG, Bak J, Chu A.** Cyclic ADP-ribose as an endogenous regulator of the non-skeletal type ryanodine receptor Ca^{2+} channel. *Nature* 1993; **364**: 76-79.

[262] **Mikami A, Imoto K, Tanabe T, Niidome T, Mori Y, Takeshima H, Narumiya S, Numa S.** Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. *Nature* 1989; **340**: 230-233.

[263] **Mill JG, O'Neill SC, Lamont C, Trafford AW, Eisner DA.** Propagation of locally activated contractions in isolated rat ventricular myocytes. *J.Physiol.(Lond.)* 1993; **459**: 81P (abstract)

[264] **Mill JG, Vassallo DV, Leite CM, Campagnaro P.** Influence of the sarcoplasmic reticulum on the inotropic responses of the rat myocardium resulting from changes in rate and rhythm. *Brazilian J.Med.Biol.Res.* 1994; **27**: 1455-1465.

[265] **Minamikawa T, Cody SH, Williams DA.** In situ visualization of spontaneous calcium waves within perfused whole rat heart by confocal imaging. *Am.J.Physiol.* 1997; **272**: H236-H243.

[266] **Mitra R, Morad M.** Two types of calcium channels in guinea pig ventricular myocytes. *Proc.Natl.Acad.Sci,USA.* 1986; **83**: 5340-5344.

[267] **Miyata H, Silverman HS, Sollott SJ, Lakatta EG, Stern MD, Hansford RG.** Measurement of mitochondrial free Ca^{2+} concentration in living single rat cardiac myocytes. *Am.J.Physiol.* 1991; **261**: H1123-H1134.

[268] **Morris AC, Hagler HK, Willerson JT, Buja LM.** Relationship between calcium loading and impaired energy metabolism during Na^{+}, K^{+} pump inhibition and metabolic inhibition in cultured neonatal rat cardiac myocytes. *J.Clin.Invest.* 1989; **83** : 1876-1887.

[269] **Nagasaki K, Kasai M.** Fast release of calcium from sarcoplasmic reticulum vesicles monitored by chlortetracycline fluorescence. *J.Biochem.(Tokyo.)* 1983; **94**: 1101-1109.

[270] **Nasri-Sebdani M, Traore F, Cognard C, Potreau D, Poindessault JP, Raymond G.** The depressing effect of tetracaine and ryanodine on the slow outward current correlated with that of contraction in voltage-clamped frog muscle fibres. *Pflügers Archive* 1990; **416**: 106-112.

[271] **Näbauer M, Callewaert G, Cleemann L, Morad M.** Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science* 1989; **244**: 800-803.

[272] **Näbauer M, Morad M.** Ca^{2+}-induced Ca^{2+} release as examined by photolysis of caged Ca^{2+} in single ventricular myocytes. *Am.J.Physiol.* 1990; **258**: C189-C193.

[273] **Negretti N, O'Neill SC, Eisner DA.** The effects of inhibitors of sarcoplasmic reticulum function on the systolic Ca^{2+} transient in rat ventricular myocytes. *J.Physiol.(Lond.)* 1993a; **468**: 35-52.

[274] **Negretti N, O'Neill SC, Eisner DA.** The relative contributions of different intracellular and sarcolemmal systems to relaxation in rat ventricular myocytes. *Cardiovasc.Res.* 1993b; **27**: 1826-1830.

[275] **Negretti N, Varro A, Eisner DA.** An estimate of net Ca^{2+} loss from the cell during a single beat in rat ventricular myocytes. *J.Physiol.(Lond.)* 1994; **475**: 78P (abstract)

[276] **Negretti N, Varro A, Eisner DA.** Estimate of net calcium fluxes and sarcoplasmic reticulum calcium content during systole in rat ventricular myocytes. *J.Physiol.(Lond.)* 1995; **486**: 581-591.

[277] **New W, Trautwein W.** The ionic nature of slow inward current and its relation to contraction. *Pflügers Archiv.* 1972; **334**: 24-38.

[278] **Nieman CJ, Eisner DA.** Effects of caffeine, tetracaine, and ryanodine on calcium-dependent oscillations in sheep cardiac Purkinje fibers. *J.Gen.Physiol.* 1985; **86**: 877-889.

[279] **Niggli E, Lederer WJ.** Voltage-independent calcium release in heart muscle. *Science* 1990; **250**: 565-568.

[280] **Nilius B, Hess P, Lansman JB, Tsien RW.** A novel type of cardiac calcium channel in ventricular cells. *Nature* 1985; **316**: 443-446.

[281] **Noma A.** ATP-regulated K^+ channels in cardiac muscle. *Nature* 1983; **305**: 147-148.

[282] **Nosek TM, Williams MF, Zeigler ST, Godt RE.** Inositol trisphosphate enhances calcium release in skinned cardiac and skeletal muscle. *Am.J.Physiol.* 1986; **250**: C807-C811.

[283] **O'Neill SC, Donoso P, Eisner DA.** The role of $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$-sensitization in the caffeine contracture of rat myocytes: measurement of $[Ca^{2+}]_i$ and $[caffeine]_i$. *J.Physiol.(Lond.)* 1990; **425**: 55-70.

[284] **O'Neill SC, Eisner DA.** A mechanism for the effects of caffeine on Ca^{2+} release during diastole and systole in isolated rat ventricular myocytes. *J.Physiol.(Lond.)* 1990; **430**: 519-536.

[285] **O'Neill SC, Lamont C, Negretti N, Eisner DA.** The effect of thapsigargin on the calcium transient and twitch of single myocytes isolated from the heart. *J.Physiol.(Lond.)* 1992; **446**: 148P-148P (abstract)

[286] **O'Neill SC, Mill JG, Eisner DA.** Local activation of contraction in isolated rat ventricular myocytes. *Am.J.Physiol.* 1990; **258**: C1165-C1168.

[287] **O'Neill SC, Valdeolmillos M, Lamont C, Donoso P, Eisner DA.** The contribution of Na^+-Ca^{2+} exchange to relaxation in mammalian cardiac muscle. *Ann.N.Y.Acad.Sci.* 1991; **639**: 444-452.

[288] **Orchard CH.** The role of the sarcoplasmic reticulum in the response of ferret and rat heart muscle to acidosis. *J.Physiol.(Lond.)* 1987; **384**: 431-449.

[289] **Orchard CH, Eisner DA, Allen DG.** Oscillations of intracellular Ca^{2+} in mammalian cardiac muscle. *Nature* 1983; **304**: 735-738.

[290] **Orchard CH, Hamilton DL, Astles P, McCall E, Jewell BR.** The effect of acidosis on the relationship between Ca^{2+} and force in isolated ferret cardiac muscle. *J.Physiol.(Lond.)* 1991; **436**: 559-578.

[291] **Orchard CH, Houser SR, Kort AA, Bahinski A, Capogrossi MC, Lakatta EG.** Acidosis facilitates spontaneous sarcoplasmic reticulum Ca^{2+} release in rat myocardium. *J.Gen.Physiol.* 1987; **90**: 145-165.

[292] **Orchard CH, Lakatta EG.** Intracellular calcium transients and developed tension in rat heart muscle. A mechanism for the negative interval-strength relationship. *J.Gen.Physiol.* 1985; **86**: 637-651.

[293] **Otsu K, Willard HF, Khanna VK, Zorzato F, Green NM, MacLennan DH.** Molecular cloning of cDNA encoding the Ca^{2+} retease channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. *J.Biol.Chem.* 1990; **265**: 13472-13483.

[294] **Overend CL, Eisner DA, O'Neill SC.** The effect of tetracaine on spontaneous Ca^{2+} release and sarcoplasmic reticulum calcium content in rat ventricular myocytes. *J.Physiol.(Lond.)* 1997; **502**: 471-479.

[295] **Overend CL, O'Neill SC, Eisner DA.** The effect of tetracaine on stimulated contractions, sarcoplasmic reticulum Ca^{2+} content and membrane current in isolated rat ventricular myocytes. *J.Physiol.(Lond.)* 1998; **507**: 759-769.

[296] **Parker I, Zang W-J, Wier WG.** Ca^{2+} sparks involving multiple Ca^{2+} sites along Z-lines in rat heart cells. *J.Physiol.(Lond.)* 1996; **497**: 31-38.

[297] **Phillips RM, Altschuld RA.** 2,3-Butanedione 2-monoxime (BDM) induces calcium release from canine cardiac sarcoplasmic reticulum. *Biochem.Biophys.Res.Com.* 1996; **229**: 154-157.

[298] **Pratusevich VR, Balke CW.** Factors shaping the confocal image of the calcium spark in cardiac muscle cells. *Biophys.J.* 1996; **71**: 2942-2957.

[299] **Qu Y, Campbell DL.** Modulation of L-type calcium current kinetics by sarcoplasmic reticulum calcium release in ferret isolated right ventricular myocytes. *Can.J.Cardiol.* 1998; **14**: 263-272.

[300] **Rae J, Cooper K, Gates P, Watsky M.** Low access resistance perforated patch recordings using amphotericin B. *J.Neurosci.Meth.* 1991; **37**: 15-26.

[301] **Rakovic S, Galione A, Ashamu GA, Potter BVL, Terrar DA.** A specific cyclic ADP-ribose antagonist inhibits cardiac excitation-contraction coupling. *Current Biology* 1996; **6**: 989-996.

[302] **Reeves JP, Hale CC.** The stoichiometry of the cardiac sodium-calcium exchange system. *J.Biol.Chem.* 1984; **259**: 7733-7739.

[303] **Reuter H, Seitz N.** The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *J.Physiol.(Lond.)* 1968; **195**: 451-470.

[304] **Ringer S.** A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. *J.Physiol.(Lond.)* 1883; **4**: 29-42.

[305] **Rios E, Brum G.** Involvement of dihydropyridine recepotrs in excitation-contraction coupling in skeletal muscle. *Nature* 1987; **325**: 717-720.

[306] **Rios E, Gonzales A.** The mechanical hypothesis of excitation-contraction coupling. *J.Muscle.Res.Cell Mot.* 1991; **12**: 127-135.

[307] **Romanin C, Karlsson J-O, Schindler H.** Activity of cardiac L-type Ca^{2+} channels is sensitive to cytoplasmic calcium. *Pflügers Archiv.* 1992; **421**: 516-518.

[308] **Rosenberg RL, Hess P, Tsien RW.** Cardiac calcium channels in planar lipid bilayers. *J.Gen.Physiol.* 1988; **92**: 27-54.

[309] **Rousseau E, Meissner G.** Single cardiac sarcoplasmic reticulum Ca^{2+}-release channel: activation by caffeine. *Am.J.Physiol.* 1989; **256**: H328-H333.

[310] **Rousseau E, Pinkos J.** pH modulates conducting and gating behaviour of single calcium release channels. *Pflügers Archiv.* 1990; **415**: 645-647.

[311] **Rousseau E, Smith JS, Henderson JS, Meissner G.** Single channel and $^{45}Ca^{2+}$ flux measurements of cardiac sarcoplasmic reticulum calcium channel. *Biophys.J.* 1986; **50**: 1009-1014.

[312] **Rousseau E, Smith JS, Meissner G.** Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channel. *Am.J.Physiol.* 1987; **253**: C364-C368.

[313] **Ruaño-Arroyo G, Gerstenblith G, Lakatta EG.** 'Calcium paradox' in the heart is modulated by cell sodium during the calcium-free period. *J.Mol.Cell.Cardiol.* 1984; **16**: 783-793.

[314] **Santana LF, Cheng H, Gómez AM, Cannell MB, Lederer WJ.** Relation between the sarcolemmal Ca^{2+} current and Ca^{2+} sparks and local control theories for cardiac excitation-contraction coupling. *Circ.Res.* 1996; **78**: 166-171.

[315] **Santana LF, Gomez AM, Kranias EG, Lederer WJ.** Amount of calcium in sarcoplasmic reticulum: influence on excitation-contraction coupling in heart muscle. *Heart Vessels* 1997; **Suppl. 12**: 44-49.

[316] **Satoh H, Delbridge LMD, Blatter LA, Bers DM.** Surface:Volume relationship in cardiac myocytes studied with confocal microscopy and membrane capacitance measurements: Species dependence and developmental effects. *Biophys.J.* 1996; **70**: 1494-1504.

[317] **Schiefer A, Meissner G, Isenberg G.** Ca^{2+} activation and Ca^{2+} inactivation of canine reconstituted cardiac sarcoplasmic reticulim Ca^{2+} release channels. *J.Physiol.(Lond.)* 1995; **489**: 337-348.

[318] **Schneider MF, Chandler WK.** Voltage-dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature* 1973; **242**: 244-247.

[319] **Shacklock PS, Wier WG, Balke CW.** Local Ca^{2+} transients (Ca^{2+} sparks) originate at transverse tubules in rat heart cells. *J.Physiol.(Lond.)* 1995; **487**: 601-608.

[320] **Sham JSK.** Ca^{2+} release-induced inactivation of Ca^{2+} current in rat ventricular myocytes: evidence for local Ca^{2+} signalling. *J.Physiol.(Lond.)* 1997; **500**: 285-295.

[321] **Sham JSK, Cleemann L, Morad M.** Gating of the cardiac Ca^{2+} release channel: the role of Na^{+} current and Na^{+}-Ca^{2+} exchange. *Science* 1992; **255**: 850-853.

[322] **Sham JSK, Cleemann L, Morad M.** Functional coupling of Ca^{2+} channels and ryanodine receptors in cardiac myocytes. *Proc.Natl.Acad.Sci,USA.* 1995; **92**: 121-125.

[323] **Sham JSK, Jones LR, Morad M.** Phospholamban mediates the β-adrenergic-enhanced Ca^{2+} uptake in mammalian ventricular myocytes. *Am.J.Physiol.* 1991; **261**: H1344-H1349.

[324] **Shattock MJ, Bers DM.** Rat vs. rabbit ventricle: Ca^{2+} flux and intracellular Na assessed by ion-selective microelectrodes. *Am.J.Physiol.* 1989; **256**: C813-C822.

[325] **Shorofsky SR, Izu L, Wier WG, Balke CW.** Ca^{2+} sparks triggered by patch depolarization in rat heart cells. *Circ.Res.* 1998; **82**: 424-429.

[326] **Shou W, Aghdasi B, Armstrong DL, Guo Q, Bao S, Charng M-J, Mathews LM, chneider MD, amilton SL, atzuk MM.** Cardiac defects and altered ryanodine receptor function in mice lacking FKB12. *Nature* 1998; **391**: 489-492.

[327] **Sibbring GC, Trafford AW, Eisner DA.** Changes in the properties of Ca^{2+} waves when Ca^{2+}-induced Ca^{2+} release is potentiated in cardiac muscle. *Biophys.J.* 1999; **76**: A308.

[328] **Sipido KR, Callewaert G, Carmeliet E.** Inhibition and rapid recovery of Ca^{2+} current during Ca^{2+} release from sarcoplasmic reticulum in guinea pig ventricular myocytes. *Circ.Res.* 1995; **76**: 102-109.

[329] **Sipido KR, Carmeliet E, Pappano A.** Na^{+} current and Ca^{2+} release from the sarcoplasmic reticulum during action potentials in guinea-pig ventricular myocytes. *J.Physiol.(Lond.)* 1995; **489**: 1-17.

[330] **Sipido KR, Carmeliet E, Van de Werf F.** T-type Ca^{2+} current as a trigger for Ca^{2+} release from the sarcoplamsic reticulum in guinea-pig ventricular myocytes. *J.Physiol.(Lond.)* 1998; **508**: 439-451.

[331] **Sipido KR, Maes M, Van de Werf F.** Low efficiency of Ca^{2+} entry through the Na^{+}-Ca^{2+} exchanger as trigger for Ca^{2+} release from the sarcoplasmic reticulum. *Circ.Res.* 1997; **81**: 1034-1044.

[332] **Sitsapesan R, McGarry SJ, Williams AJ.** Cyclic ADP-ribose competes with ATP for the adenine nucleotide sites on the cardiac ryanodine receptor Ca^{2+} release channel. *Circ.Res.* 1994; **75**: 596-600.

[333] **Sitsapesan R, Montgomery RAP, Williams AJ.** New insights into the gating mechanisms of cardiac Ryanodine receptors revealed by rapid changes in ligand concentration. *Circ.Res.* 1995; **77**: 765-772.

[334] **Sitsapesan R, Williams AJ.** Mechanisms of caffeine activation of single calcium-release channels of sheep cardiac sarcoplasmic reticulum. *J.Physiol.(Lond.)* 1990; **423**: 425-439.

[335] **Sitsapesan R, Williams AJ.** Regulation of the gating of the sheep cardiac sarcoplasmic reticulum Ca^{2+}- release channel by luminal Ca^{2+}. *J.Membrane Biol.* 1994; **137**: 215-226.

[336] **Sitsapesan R, Williams AJ.** The gating of the sheep skeletal sarcoplasmic reiculum Ca^{2+}-release channel is regulated by luminal Ca^{2+}. *J.Membrane Biol.* 1995; **146**: 133-144.

[337] **Sitsapesan R, Williams AJ.** Regulation of current flow through ryanodine receptors by luminal Ca^{2+}. *J.Membrane Biol.* 1997; **159**: 179-185.

[338] **Smith GL, Allen DG.** Effects of metabolic blockade on intracellular calcium concentration in isolated ferret ventricular muscle. *Circ.Res.* 1988; **62**: 1223-1236.

[339] **Smith GL, Steele DS.** Inorganic phosphate decreases the Ca^{2+} content of the sarcoplasmic reticulum in saponin-treated rat cardiac trabeculae. *J.Physiol.(Lond.)* 1992; **458**: 457-473.

[340] **Solaro RJ, Briggs FN.** Estimating the functional capabilities of sarcoplasmic reticulum in cardiac muscle. *Circ.Res.* 1974; **34**: 531-540.

[341] **Solaro RJ, Wise RM, Shiner JS, Briggs FN.** Calcium requirements for myofibrillar activation. *Circ.Res.* 1974; **34**: 525-530.

[342] **Song L-S, Stern MD, Lakatta EG, Cheng H.** Partial depletion of sarcoplasmic reticulum calcium does not prevent calcium sparks in rat ventricular myocytes. *J.Physiol.(Lond.)* 1997; **505**: 665-675.

[343] **Spencer CI, Berlin JR.** Control of sarcoplasmic reticulum calcium release during calcium loading in isolated rat ventricular myocytes. *J.Physiol.(Lond.)* 1995; **488**: 267-279.

[344] **Spencer CI, Berlin JR.** Calcium-induced release of strontium from the sarcoplasmic reticulum of rat cardiac ventricular myocytes. *J.Physiol.(Lond.)* 1997; **504**: 565-578.

[345] **Sperelakis N, Lee EC.** Characterization of (Na^+/ K^+)-ATPase isolated from embryonic chick hearts and cultured chick heart cells. *Biochim.Biophys.Acta* 1971; **233**: 562-579.

[346] **Steadman BW, Moore KB, Spitzer KW, Bridge JH.** A video system for measuring motion in contracting heart cells. *IEEE Transactions on Biomedical Engineering* 1988; **35**: 264-272.

[347] **Steele DS, McAinsh AM, Smith GL.** Effects of creatine phosphate and inorganic phosphate on the sarcoplasmic reticulum of saponin-treated rat heart. *J.Physiol.(Lond.)* 1995; **483**: 155-166.

[348] **Stephenson DG, Wendt IR.** Effects of procaine on calcium accumulation by the sarcoplasmic reticulum of mechanically disrupted rat cardiac muscle. *J.Physiol.(Lond.)* 1986; **373**: 195-207.

[349] **Stern MD.** Theory of excitation-contraction coupling in cardiac muscle. *Biophys.J.* 1992; **63**: 497-517.

[350] **Stern MD, Capogrossi MC, Lakatta EG.** Propagated contractile waves in single cardiac myocytes modeled as regenerative calcium induced calcium release from the sarcoplasmic reticulum. *Biophys.J.* 1984; 94a (abstract)

[351] **Stern MD, Kort AA, Bhatnagar GM, Lakatta EG.** Scattered-light intensity fluctuations in diastolic rat cardiac muscle caused by spontaneous Ca^{2+}-dependent cellular mechanical oscillations. *J.Gen.Physiol.* 1983; **82**: 119-153.

[352] **Stern MD, Lakatta EG.** Excitation-contraction coupling in the heart: the state of the question. *FASEB J.* 1992; **6**: 3092-3100.

[353] **Suárez-Isla BA, Irribarra V, Oberhauser A, Larralde L, Bull R, Hidalgo C, Jaimovich E.** Inositol (1,4,5)-trisphosphate activates a calcium channel in isolated sarcoplasmic reticulum membranes. *Biophys.J.* 1988; **54**: 737-741.

[354] **Suleiman M-S, Rodrigo GC, Chapman RA.** Interdependence of intracellular taurine and sodium in guinea pig heart. *Cardiovasc.Res.* 1992; **26** : 897-905.

[355] **Sun X, Protasi F, Takahashi M, Takeshima H, Ferguson DG, Franzini-Armstrong C.** Molecular Architecture of Membranes Involved in Excitation-Contraction coupling of Cardiac Muscle. *J.Cell.Biol.* 1995; **129**: 659-671.

[356] **Sutko JL, Bers DM, Reeves JP.** Post-rest inotropy in rabbit ventricle: Na^{+}-Ca^{2+} exchange determines sarcoplasmic reticulum Ca^{2+} content. *Am.J.Physiol.* 1986; **250**: H654-H661.

[357] **Tada M, Kadoma M.** Regulation of the Ca^{2+} pump ATPase by cAMP-dependent phosphorylation of phospholamban. *BioEssays.* 1989; **10**: 157-163.

[358] **Takamatsu T, Wier WG.** Calcium waves in mammalian heart: quantification of origin, magnitude, waveform, and velocity. *FASEB J.* 1990a; **4**: 1519-1525.

[359] **Takamatsu T, Wier WG.** High temporal resolution video imaging of intracellular calcium. *Cell Calcium* 1990b; **11**: 111-120.

[360] **Takasago T, Imagawa T, Shigekawa M.** Phosphorylation of the cardiac ryanodine receptor by cAMP-dependent protein kinase. *J.Biochem.* 1989; **106**: 872-877.

[361] **Tameyasu T, Kasugai H, Tanaka M, Harada H.** Sarcomere dynamics in a spontaneous contraction wave and its effect on the following, electrically triggered twitch in rat myocyte. *J.Gen.Physiol.* 1994; **103**: 625-645.

[362] **Tani M.** Mechanisms of Ca^{2+} overload in reperfused ischemic myocardium. *Annu.Rev.Physiol.* 1990; **52**: 543-559.

[363] **Terracciano CMN, MacLeod KT.** Reloading of Ca^{2+}-depleted sarcoplasmic reticulum during rest in guinea pig ventricular myocytes. *Am.J.Physiol.* 1996; **271**: H1814-H1822.

[364] **Terracciano CMN, Naqvi RU, MacLeod KT.** Effects of rest interval on the release of calcium from the sarcoplasmic reticulum in isolated guinea pig ventricular myocytes. *Circ.Res.* 1995; **77**: 354-360.

[365] **Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP.** Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc.Natl.Acad.Sci,USA.* 1990; **87**: 2466-2470.

[366] **Thorpe WR, Seeman P.** The site of action of caffeine and procaine in skeletal muscle. *J.Pharmocol.Exp.Ther.* 1971; **179**: 324-330.

[367] **Timerman AP, Jayaraman T, Wiederrecht G, Onoue H, Marks AR, Fleischer S.** The ryanodine receptor from canine heart sarcoplasmic

reticulum is associated with a novel FK-506 binding protein. *Biochem.Biophys.Res.Com.* 1994; **198**: 701-706.

[368] **Tinker A, Lindsay ARG, Williams AJ.** Cation conduction in the calcium release channel of the cardiac sarcoplasmic reticulum under physiological and pathophysiological conditions. *Cardiovasc.Res.* 1993; **27**: 1820-1825.

[369] **Tinker A, Williams AJ.** Charged local anesthetics block ionic conduction in the sheep cardiac sarcoplasmic reticulum calcium release channel. *Biophys.J.* 1993; **65**: 852-864.

[370] **Trafford AW, Díaz ME, Eisner DA.** Alteration of sarcolemmal and sarcoplasmic reticulum calcium fluxes in isolated rat ventricular myocytes produced by modulation of calcium induced calcium release. *J.Physiol.(Lond.)* 1997; **501P**: 133-133 (abstract)

[371] **Trafford AW, Díaz ME, Eisner DA.** Stimulation of Ca^{2+}-induced Ca^{2+} release only transiently increases the systolic Ca^{2+} transient: measurements of Ca^{2+} fluxes and SR Ca^{2+}. *Cardiovasc.Res.* 1998; **37**: 710-717.

[372] **Trafford AW, Díaz ME, Negretti N, Eisner DA.** Enhanced calcium current and decreased calcium efflux restore sarcoplasmic reticulum Ca^{2+} content following depletion. *Circ.Res.* 1997; **81**: 477-484.

[373] **Trafford AW, Díaz ME, Sibbring GC, Eisner DA.** The use of spontaneous rather than stimulated sarcoplasmic reticulum Ca^{2+} release to identify agents that modulate Ca^{2+}-induced Ca^{2+} release in cardiac muscle. *J.Physiol.(Lond.)* 1998; **509P**: 146P-147P (abstract)

[374] **Trafford AW, Lipp P, O'Neill SC, Niggli E, Eisner DA.** Propagating calcium waves initiated by local caffeine application in rat ventricular myocytes. *J.Physiol.(Lond.)* 1995; **489**: 319-326.

[375] **Trafford AW, O'Neill SC, Eisner DA.** Factors affecting the propagation of locally activated systolic Ca^{2+} transients in rat ventricular myocytes. *Pflügers Archiv.* 1993; **425**: 181-183.

[376] **Tripathy A, Meissner G.** Sarcoplasmic reticulum lumenal Ca^{2+} has access to cytosolic activation and inactivation sites of skeletal muscle Ca^{2+} release channel. *Biophys.J.* 1996; **70**: 2600-2615.

[377] **Tsien RW.** Calcium channels in excitable membranes. *Annu.Rev.Physiol.* 1983; **45**: 341-358.

[378] **Tsien RY.** A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* 1981; **290**: 527-528.

[379] **Tsushima RG, Kelly JE, Wasserstrom JA.** Characteristics of cocaine block of purified cardiac sarcoplasmic reticulum calcium release channels. *Biophys.J.* 1996; **70**: 1263-1274.

[380] **Valdeolmillos M, Eisner DA.** The effects of ryanodine on calcium-overloaded sheep cardiac Purkinje fibers. *Circ.Res.* 1985; **56**: 452-456.

[381] **Valdeolmillos M, O'Neill SC, Smith GL, Eisner DA.** Calcium-induced calcium release activates contraction in intact cardiac cells. *Pflügers Archiv.* 1989; **413**: 676-678.

[382] **Valdivia HH, Kaplan JH, Ellis-Davies GCR, Lederer WJ.** Rapid adaptation of cardiac ryanodine receptors: modulation by Mg^{2+} and phosphorylation. *Science* 1995; **267**: 1997-2000.

[383] **Varro A, Negretti N, Hester SB, Eisner DA.** An estimate of the calcium content of the sarcoplasmic reticulum in rat ventricular myocytes. *Pflügers Archiv.* 1993; **423**: 158-160.

[384] **Vaughan-Jones RD, Lederer WJ, Eisner DA.** Ca^{2+} ions can affect intracellular pH in mammalian cardiac muscle. *Nature* 1983; **301**: 522-524.

[385] **Vaughan Jones RD, Eisner DA, Lederer WJ.** The effects of intracellular Na^{+} on contraction and intracellular pH in mammalian cardiac muscle. *Adv.Myocardiol.* 1985; **5**: 313-330.

[386] **Vélez P, Györke S, Escobar AL, Vergara J, Fill M.** Adaptation of single cardiac ryanodine receptor channels. *Biophys.J.* 1997; **72**: 691-697.

[387] **Volpe P, Palade P, Costello B, Mitchell RD, Fleischer S.** Spontaneous calcium release from sarcoplasmic reticulum - effect of local anaesthetics. *J.Biol.Chem.* 1983; **258**: 12434-12442.

[388] **Vornanen M, Shepherd N, Isenberg G.** Tension-voltage relations of single myocytes reflect Ca^{2+} release triggered by Na^{+}/Ca^{2+} exchange at 35°C but not at 23°C. *Am.J.Physiol.* 1994; **267**: C623-C632.

[389] **Wang S-Y, Clague JR, Langer GA.** Increase in calcium leak channel activity by metabolic inhibition or hydrogen peroxide in rat ventricular myocytes and its inhibition by polycation. *J.Mol.Cell.Cardiol.* 1995; **27**: 211-222.

[390] **Wasserstrom JA.** Intracellular sodium ion activity in rat ventricular myocardium. *Fed.Proc.* 1983; **42**: 1113 (abstract)

[391] **Wasserstrom JA, Schwartz DJ, Fozzard HA.** Relation between intracellular sodium and twitch tension in sheep cardiac Purkinje strands exposed to cardiac glycosides. *Circ.Res.* 1983; **52**: 697-705.

[392] **Wasserstrom JA, Vites AM.** The role of Na^+-Ca^{2+} exchange in activation of excitation-contraction coupling in rat ventricular myocytes. *J.Physiol.(Lond.)* 1997; **493**: 529-542.

[393] **Weiss JN, Lamp ST.** Glycolysis preferentially inhibits ATP-sensitive K^+ channels in isolated guinea pig cardiac myocytes. *Science* 1987; **238**: 67-69.

[394] **Wendt-Gallitelli MF, Voigt T, Isenberg G.** Microheterogeneity of subsarcolemmal sodium gradients, electron probe microanalysis in guinea-pig ventricular myocytes. *J.Physiol.(Lond.)* 1993; **472**: 33-44.

[395] **Wendt IR, Stephenson DG.** Effects of caffeine on Ca^{2+}-activated force production in skinned cardiac and skeletal muscle fibres of the rat. *Pflügers Archiv.* 1983; **398**: 210-216.

[396] **Wibo M, Bravo G, Godfraind T.** Postnatal maturation of excitation contraction coupling in rat ventricle in relation to the subcellular localization and surface density of 1,4-dihydropyridine and ryanodine receptors. *Circ.Res.* 1991; **68**: 662-673.

[397] **Wier WG, Cannell MB, Berlin JR, Marban E, Lederer WJ.** Cellular and subcellular heterogeneity of $[Ca^{2+}]_i$ in single heart cells revealed by fura-2. *Science* 1987; **235**: 325-328.

[398] **Wier WG, Egan TM, López-López JR, Balke CW.** Local control of excitation-contraction coupling in rat heart cells. *J.Physiol.(Lond.)* 1994; **474**: 463-471.

[399] **Wier WG, Kort AA, Stern MD, Lakatta EG, Marban E.** Cellular calcium fluctuations in mammalian heart: direct evidence from noise analysis of aequorin signals in Purkinje fibers. *Proc.Natl.Acad.Sci,USA.* 1983; **80**: 7367-7371.

[400] **Williams DA.** Mechanisms of calcium release and propagation in cardiac cells. Do studies with confocal microscopy add to our understanding? *Cell Calcium* 1993; **14**: 724-735.

[401] **Witcher DR, Kovacs RJ, Schulman H, Cefali DC, Jones LR.** Unique phosphorylation site on the cardiac ryanodine receptor regulates calcium channel activity. *J.Biol.Chem.* 1991; **266**: 11144-11152.

[402] **Wu M-L, Vaughan-Jones RD.** Effect of metabolic inhibitors and second messengers upon Na^+-H^+ exchange in the sheep cardiac Purkinje fibre. *J.Physiol.(Lond.)* 1994; **478**: 301-313.

[403] **Xiao R-P, Valdivia HH, Bogdanov K, Valdivia C, Lakatta EG, Cheng H.** The immunophilin FK506-binding protein modulates Ca^{2+} release channel closure in rat heart. *J.Physiol.(Lond.)* 1997; **500**: 343-354.

[404] **Xu L, Jones R, Meissner G.** Effects of local anesthetics on single channel behavior of skeletal muscle release channel. *J.Gen.Physiol.* 1993; **101**: 207-233.

[405] **Xu L, Mann G, Meissner G.** Regulation of cardiac Ca^{2+} release channel (Ryanodine receptor) by Ca^{2+}, H^{+}, Mg^{2+}, and adenine nucleotides under normal and simulated ischemic conditions. *Circ.Res.* 1996; **79**: 1100-1109.

[406] **Xu L, Meissner G.** Regulation of cardiac muscle Ca^{2+} release channel by sarcoplasmic reticulum lumenal Ca^{2+}. *Biophys.J.* 1998; **75**: 2302-2312.

[407] **Yano K, Zarain-Herzberg A.** Sarcoplasmic reticulum calsequestrins: structural and functional properties. *Mol.Cell.Biochem.* 1994; **135**: 61-70.

[408] **Yao A, Su Z, Nonaka A, Zubair I, Lu L, Philipson KD, Bridge JHB, Barry WH.** Effects of over-expression of the Na^{+}-Ca^{2+} exchanger on $[Ca^{2+}]_i$ transients in murine ventricular myocytes. *Circ.Res.* 1998; **82**: 657-665.

[409] **Yasui K, Palade P, Györke S.** Negative control mechanism with features of adaptation controls Ca^{2+} release in cardiac myocytes. *Biophys.J.* 1994; **67**: 457-460.

[410] **Yoshida A, Takahashi M, Imagawa T, Shigekawa M, Takisawa H, Nakamura T.** Phosphorylation of ryanodine receptors in rat myocytes during β-adrenergic stimulation. *J.Biochem.* 1992; **111**: 186-190.

[411] **Yue DT, Backx PH, Imredy JP.** Calcium-sensitive inactivation in the gating of single calcium channels. *Science* 1990; **250**: 1735-1738.

[412] **Zahradníková A, Palade P.** Procaine effects on single sarcoplasmic reticulum Ca^{2+} release channels. *Biophys.J.* 1993; **64**: 991-1003.

[413] **Zot AS, Potter JD.** Structural aspects of troponin-tropomyosin regulation of skeletal muscle contraction. *Ann.Rev.Biophys.Chem.* 1987; **16**: 535-559.

Appendix

The effect of tetracaine on spontaneous Ca^{2+} release and sarcoplasmic reticulum calcium content in rat ventricular myocytes

C. L. Overend, D. A. Eisner* and S. C. O'Neill

Department of Veterinary Preclinical Sciences, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK

1. The effects of tetracaine were studied on voltage-clamped rat ventricular myocytes, which exhibited Ca^{2+} overload as identified by spontaneous Ca^{2+} release from the sarcoplasmic reticulum (SR) as shown by the associated contractions. This Ca^{2+} release was initially abolished by tetracaine before returning at a lower frequency, but greater amplitude, than the control. On removal of tetracaine, there was a burst of spontaneous Ca^{2+} release activity. All these effects were dose dependent, from 25 to 200 μM tetracaine.

2. The spontaneous Ca^{2+} release activated an inward Na^{+}–Ca^{2+} exchange current as Ca^{2+} was pumped out of the cell. The integral of this current (i.e. the Ca^{2+} efflux) was increased in the presence of tetracaine. The calcium efflux per unit time was unaffected by tetracaine.

3. The SR Ca^{2+} content was increased by tetracaine, as shown by the integral of the caffeine-evoked Na^{+}–Ca^{2+} exchange current. The increase of SR Ca^{2+} content was equal to the extra Ca^{2+} lost from the cell during the burst on removal of tetracaine, and to estimates of the extra calcium gained over the quiescent period following addition of tetracaine.

4. It is concluded that partial inhibition of calcium-induced calcium release increases SR Ca^{2+} content. In the steady state, cell Ca^{2+} balance is maintained as the lower frequency of spontaneous release (that activates efflux) is compensated for by their greater size.

Under conditions of calcium overload, cardiac muscle exhibits spontaneous contractile activity resulting from the spontaneous release of Ca^{2+} ions from the sarcoplasmic reticulum (SR). This is thought to arise as a consequence of the stochastic opening of the SR Ca^{2+} release channels, which, under non-overloaded conditions, produce discrete localized increases of $[Ca^{2+}]_i$ or Ca^{2+} sparks (Cheng, Lederer & Cannell, 1993; López-López, Shacklock, Balke & Wier, 1994). Under Ca^{2+}-overloaded conditions, it appears that these sparks can initiate waves of $[Ca^{2+}]_i$, which propagate along the cell (Cheng, Lederer, Lederer & Cannell, 1996). This is possibly because the higher the SR Ca^{2+} content, the greater the amount of Ca^{2+} released for each SR Ca^{2+} channel opening and therefore the greater the gain of the release process.

About one-fifth of the Ca^{2+} content of the SR is pumped out of the cell by the electrogenic Na^{+}–Ca^{2+} exchange during each propagating wave (Díaz, Trafford, O'Neill & Eisner, 1997) and the resulting inward current can initiate potentially arrhythmogenic action potentials (Lederer & Tsien, 1976). This spontaneous calcium release activity therefore limits the usefulness of inotropic agents that elevate $[Ca^{2+}]_i$. Although these waves of SR Ca^{2+} release are largely of pathophysiological relevance in the heart, in other systems they are a normal aspect of cell function. Thus, in a variety of secretory cells, Ca^{2+} waves produced by Ca^{2+} release from the endoplasmic reticulum (ER) are the normal mechanism for coupling stimulus to response (Kasai & Petersen, 1994).

Despite the volume of work in this area, there is little information about what determines the properties of the spontaneous SR (or ER) Ca^{2+} release. For example, how do changes in the properties of SR Ca^{2+} release or re-uptake affect the magnitude, duration and frequency of the Ca^{2+} waves? In recent work, we have shown that changing the Ca^{2+} entry into the cell affects the frequency of the waves but has no effect on the properties of the individual waves (Díaz *et al.* 1997). Here we investigate the effects of tetracaine, a local anaesthetic that has been shown to decrease the open probability of the SR Ca^{2+} release channel (Xu, Jones & Meissner, 1993), an action that accounts for its inhibition of Ca^{2+} release from the SR (Volpe, Padade, Costello, Mitchell & Fleischer, 1983) and of contraction in striated muscle (Almers & Best, 1976).

The results show that tetracaine decreases the frequency of spontaneous SR Ca^{2+} release. As a result of this there is an

* To whom correspondence should be addressed.

increase of SR Ca^{2+} content, which increases the magnitude of the Ca^{2+} waves, thus demonstrating that, unlike many other manoeuvres, factors affecting the opening of the SR release channel have profound effects on the properties of individual Ca^{2+} releases. Most striking is the demonstration that inhibiting Ca^{2+} release increases the magnitude of the release.

METHODS

Experiments were carried out on cardiac myocytes isolated from rat ventricles using a collagenase and protease digestion protocol as previously described (Eisner, Nichols, O'Neill, Smith & Valdeolmillos, 1989). Rats were killed by stunning and cervical dislocation. Voltage-clamp control was achieved with the perforated patch technique (Horn & Marty, 1988) using amphotericin B (240 $\mu g\ ml^{-1}$). Due to the relatively high access resistance of the perforated patch (about 20 MΩ), the switch-clamp facility of the Axoclamp-2A voltage-clamp amplifier (Axon Instruments) was used. Data were digitized using a Digidata board and Axoscope software (Axon Instruments) and analysed using programs kindly written for us by Dr A. W. Trafford. Pipettes (1–3 MΩ in resistance) were filled with the following solution (mM): 125 $CsCH_3O_3S$, 12 CsCl, 20 NaCl, 10 Hepes, 5 $MgCl_2$ and 0·1 EGTA; titrated to pH 7·2 with CsOH. To avoid interference from outward currents, all experiments were carried out in the presence of 5 mM 4-aminopyridine and 0·1 mM $BaCl_2$. Cells were bathed in a control solution of the following composition: 135 NaCl, 4 KCl, 10 Hepes, 11 glucose, 2 $CaCl_2$ and 1 $MgCl_2$; titrated to pH 7·4 with NaOH. In some experiments (e.g. Figs 1, 2 and 3), the voltage clamp was not used. In these experiments, 4-aminopyridine and $BaCl_2$ were omitted. All experiments were carried out at 22 °C. In some experiments caffeine was used to release Ca^{2+} from the SR. In initial experiments, we found that in the presence of tetracaine, 10 mM caffeine often produced a series of oscillations of inward current rather than a single release. This is similar to the effect of a lower concentration of caffeine in the absence of tetracaine and presumably reflects antagonism between tetracaine and caffeine (Almers & Best, 1976). For this reason 20 mM caffeine was used in subsequent experiments.

Measurement of SR Ca^{2+} content and Ca^{2+} efflux

The assignment of the $[Ca^{2+}]_i$-dependent membrane currents as Na^+–Ca^{2+} exchange and the conversion of integrated Na^+–Ca^{2+} exchange current records to total calcium fluxes have been described previously (Varro, Negretti, Hester & Eisner, 1993; Negretti, Varro & Eisner, 1995). Briefly, it is necessary to first correct for the fraction of the efflux that is not produced by Na^+–Ca^{2+} exchange, and then relate the fluxes to cell volume. The volume was calculated from the cell membrane capacitance using the capacitance-to-volume ratio of 6·76 $pF\ pl^{-1}$ (Satoh, Delbridge, Blatter & Bers, 1996). It should be noted that, as in our previous work, the SR Ca^{2+} content is expressed in relation to cell (and not SR) volume.

RESULTS

Figure 1 illustrates measurements of cell length made during an experiment to investigate the effects of various concentrations of tetracaine on contractions due to spontaneous SR Ca^{2+} release in a rat isolated ventricular myocyte. The application of tetracaine initially abolished this spontaneous activity before spontaneous contractions resumed at a frequency lower than the control. Both the

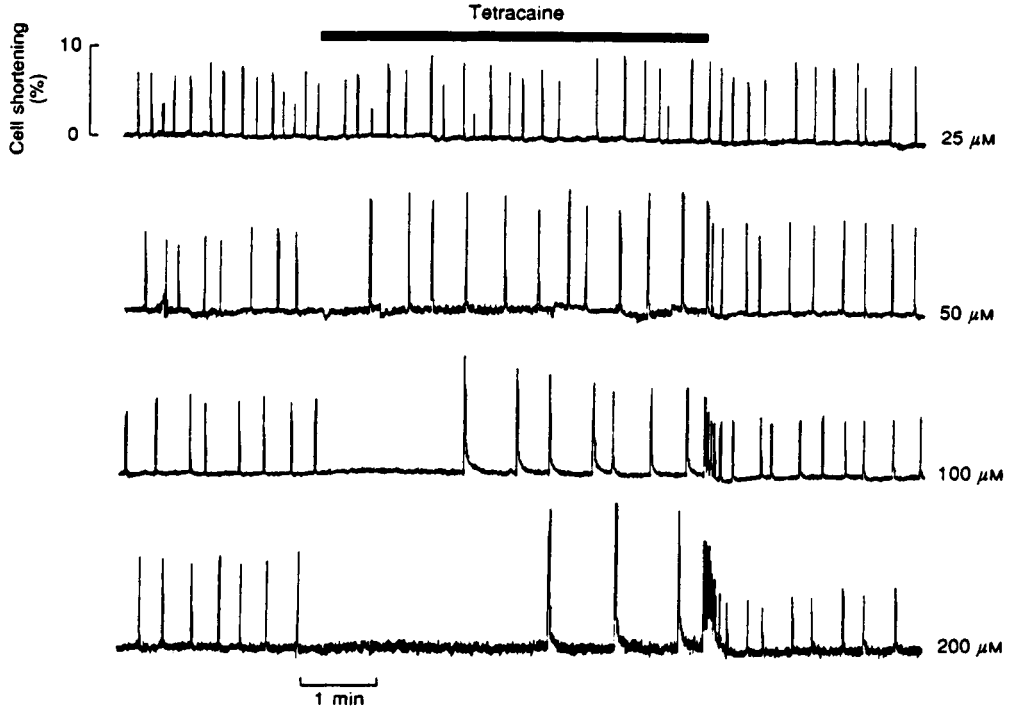

Figure 1. The effects of various concentrations of tetracaine on spontaneous SR Ca^{2+} release
All traces show cell shortening. Tetracaine was applied for the period shown above at concentrations of (from top to bottom): 25, 50, 100 and 200 μM.

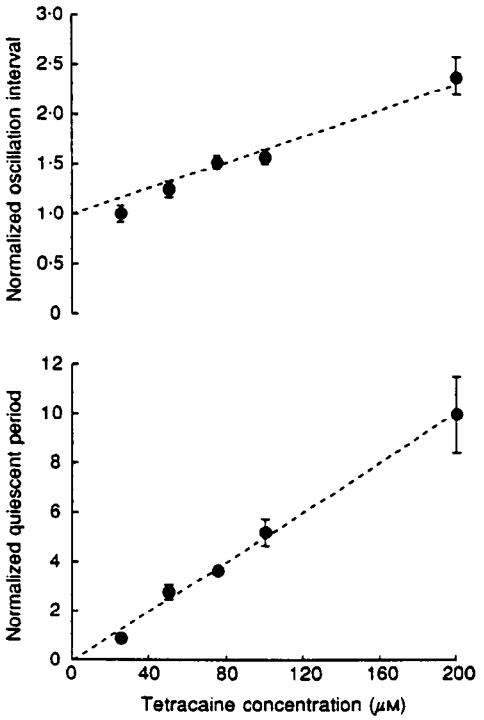

Figure 2. Concentration dependence of the effects of tetracaine
The abscissa shows the concentration of tetracaine. The ordinate shows: top, the interval between spontaneous releases in the presence of tetracaine; bottom, the duration of the quiescent period after adding tetracaine. Both the duration of the quiescent period and the oscillation interval have been normalized by dividing by the control interval of spontaneous release before adding tetracaine. This had a mean value of 22 s. The quiescent period was calculated by subtracting the average control interval from the total period lacking spontaneous release. The lines are linear regressions that have been forced to go through values of 1·0 for the oscillation interval and 0 for the quiescent period in the absence of tetracaine. The symbols show means ± S.E.M. of data from three cells. Error bars are not shown where they would be smaller than the symbols.

duration of the quiescent period and the steady-state decrease of frequency increase with increasing concentration of tetracaine. The dose dependence of these effects are summarized by the mean data illustrated in Fig. 2. Another novel observation is that removal of tetracaine from the superfusing solution is associated with recovery of spontaneous release at a transiently elevated frequency ('burst') in comparison with that observed in control. Spontaneous contraction frequency then falls towards the control level over the next 30 s to 1 min. The magnitude of this burst is also greater following exposure to higher tetracaine concentrations.

Previous work has shown that the frequency of spontaneous SR Ca^{2+} release is very variable between different cells (Capogrossi, Kort, Spurgeon & Lakatta, 1986; Díaz *et al.* 1996). Figure 3 shows that the effects of tetracaine depend on the initial rate of spontaneous release. Thus at a given concentration of tetracaine, the greater the interval between

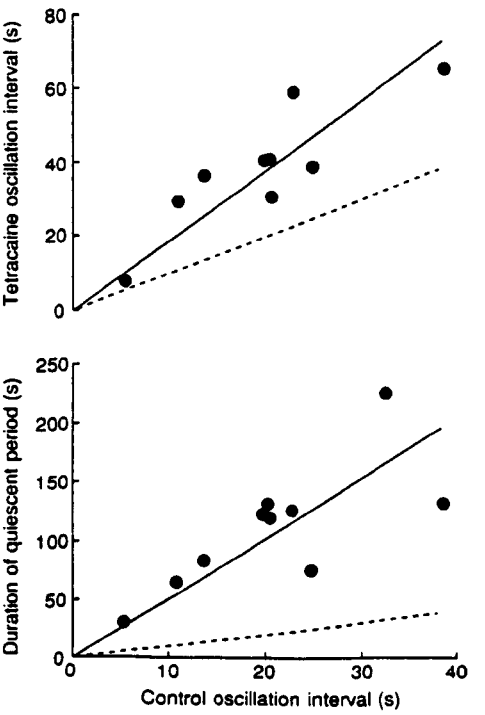

Figure 3. The relationship between the effects of tetracaine and the interval between spontaneous release events before adding tetracaine
The abscissa shows the interval in control conditions before adding tetracaine (100 μM). The ordinate shows: top, the interval between spontaneous releases in the presence of tetracaine; bottom, duration of the quiescent period after adding tetracaine. The interrupted lines have unit slope. Each symbol represents a different cell.

spontaneous releases in the control condition, both the longer the initial quiescent period in tetracaine and the lower the steady-state frequency of spontaneous Ca^{2+} release in tetracaine. The line of identity is shown as the dashed line in each panel of Fig. 3, i.e. if tetracaine had no effect on the interval between spontaneous releases or produced no quiescent period, all the data points would lie along these dashed lines.

The data above show that, after an initial delay, in the presence of tetracaine, spontaneous release resumes at a lower rate than in the control. We have also examined whether the properties of the spontaneous release are affected by tetracaine. In many experiments (e.g. Fig. 1), it is obvious that the magnitude of the spontaneous contractions is increased in the presence of tetracaine. It is difficult to quantify this effect from cell length measurements alone, as the magnitude of the spontaneous contraction depends not only on that of the increase of $[Ca^{2+}]_i$ but also on how uniform it is throughout the cell. Instead, we have measured the integral of the transient inward Na^+–Ca^{2+} exchange current that is activated by the spontaneous Ca^{2+} release. If we assume that the properties of Na^+–Ca^{2+} exchange are not affected by tetracaine, then this integral, as well as giving a measure of the amount of Ca^{2+} pumped out of the cell, also gives an indication of the amount of calcium released from the SR.

Figure 4 shows perforated patch voltage-clamp current records from an isolated myocyte under a similar protocol of solution changes as that in Fig. 1. This cell demonstrated an initial abolition of spontaneous Ca^{2+} releases (quiescent period) during exposure to 100 μM tetracaine, followed by their resumption, at reduced frequency. The lower trace in Fig. 4 illustrates the transient inward currents associated with spontaneous Ca^{2+} releases, and their integrals, on an expanded time scale. Spontaneous Ca^{2+} releases during exposure to tetracaine are considerably larger with respect to both peak magnitude and integral, in comparison with those observed in control. These data are quantified in the histogram of Fig. 5*A*, which plots the mean effects of 100 μM tetracaine on the interval between spontaneous releases and their amplitude. It is clear that both parameters are increased by a factor of about two. The fractional changes relative to control are emphasized in Fig. 5*B*. From the measurements of magnitude and frequency of spontaneous releases, one can calculate the time-averaged calcium efflux from the cell produced by this spontaneous Ca^{2+} release activity. On average, the time-averaged Ca^{2+} efflux via spontaneous release was $1{\cdot}1 \pm 0{\cdot}1$ μmol l^{-1} s^{-1} in control and $0{\cdot}9 \pm 0{\cdot}1$ μmol l^{-1} s^{-1} in 100 μM tetracaine (paired t test, $P > 0{\cdot}15$, $n = 7$). The similarity of these values is emphasized by the right-hand bar in Fig. 5*B*, which shows the average efflux in tetracaine divided by that

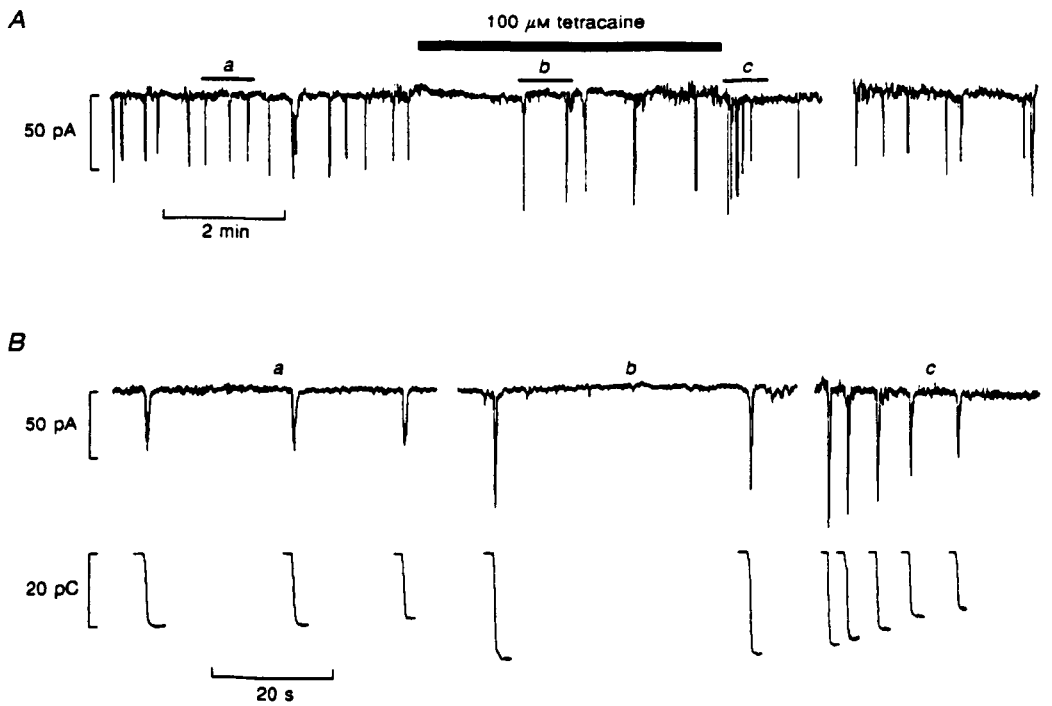

Figure 4. The effects of tetracaine on the membrane current accompanying spontaneous SR Ca^{2+} release

A, time course; the trace shows the membrane current recorded from a cell held at −80 mV. Tetracaine (100 μM) was applied for the period shown. Tetracaine produced an outward shift of holding current of 4 pA. For clarity this was removed by subtracting a low-pass-filtered version from the raw current trace. *B*, specimen records of: top, current; bottom, integrated current from the periods shown in *A*.

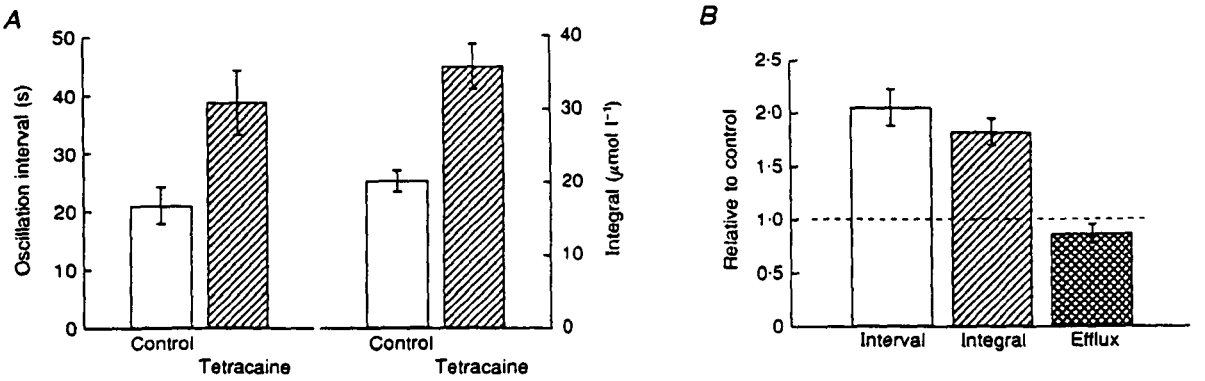

Figure 5. Summary of the effects of tetracaine on the magnitude and period of spontaneous SR Ca^{2+} release

A, histogram of the effects of tetracaine (100 μM) on: left, interval between oscillations ($n = 10$); right, integral of the associated current. The integral has been converted to the equivalent change of cell calcium (see Methods). □, control; ▨, tetracaine. *B*, normalized data. The bars show mean data ($n = 7$) normalized to the value in the control solution of (from left to right): the interval between spontaneous releases, the integral of the transient inward current and the time-averaged efflux (calculated from the product of frequency and integral in each cell).

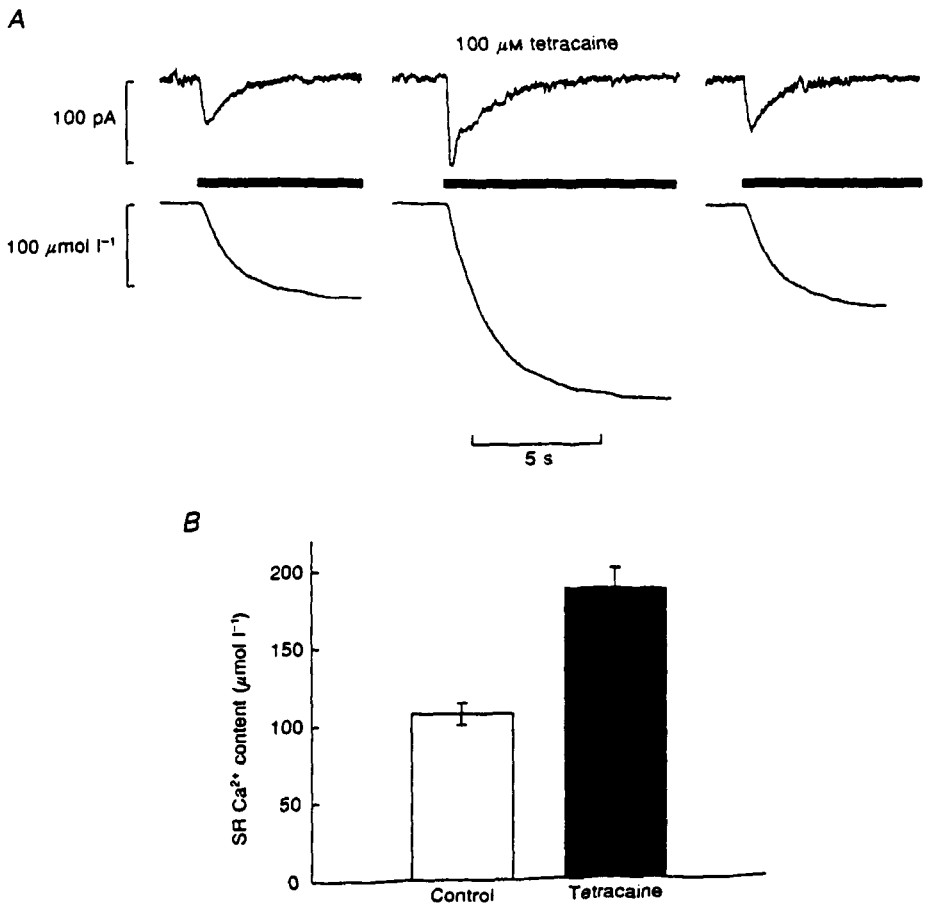

Figure 6. The effects of tetracaine on the response to caffeine

A, original data. The traces show: top, current; bottom, integral. The panels show (from left to right): control, tetracaine (100 μM) and recontrol. Caffeine (20 mM) was applied for the periods indicated by the horizontal bars. *B*, histogram showing mean SR Ca^{2+} content ($n = 10$), as measured from the integral of the caffeine response in control (left) and tetracaine (right).

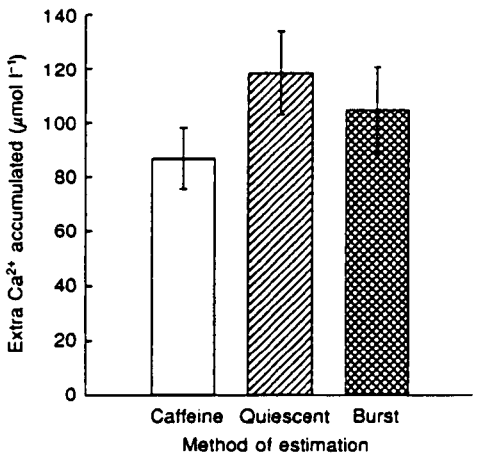

Figure 7. Comparison of three methods for estimating the Ca^{2+} gained by the cell during exposure to tetracaine (100 μM)

The bars show (from left to right): (1) the measured increase in the integral of the caffeine response; (2) the calculated Ca^{2+} entry during the quiescent period on application of tetracaine (in this calculation we used the extra quiescent period produced by tetracaine *in addition* to the interval between spontaneous releases in control); (3) the calcium lost from the cell during the burst on tetracaine removal. This was obtained by integrating the current during the burst and subtracting from it the loss of calcium which would have occurred during an equivalent period of control oscillation frequency following tetracaine removal.

in control. Therefore the addition of tetracaine has little steady-state effect on Ca^{2+} loss from the cell via spontaneous Ca^{2+} release.

On removal of tetracaine there was consistently a progressive decrease in the magnitude of both the current oscillations and their integral (Fig. 4) towards the control levels in parallel with the recovery of frequency.

There are at least two explanations for the presence of the burst of spontaneous Ca^{2+} release on removal of tetracaine. (i) Tetracaine could have some complicated effect on the surface membrane such that its removal could produce a transient influx of calcium into the cell, which would then lead to an increase of SR Ca^{2+} loading and consequently to SR Ca^{2+} release. (ii) The inhibitory effect of tetracaine on the SR Ca^{2+} release channel could increase SR Ca^{2+} content. On removal of tetracaine, spontaneous release would ensue due to the Ca^{2+} overload of the SR. We have discriminated between these hypotheses by measuring the SR Ca^{2+} content using the integral of the Na^+–Ca^{2+} exchange current, activated by releasing the SR Ca^{2+} by application of 20 mM caffeine (this concentration ensures a single, complete caffeine-induced calcium release). In the maintained presence of caffeine, a known proportion (~67%) of the calcium released from the SR leaves the cell by the Na^+–Ca^{2+} exchanger. The integral of the transient inward current (due to Na^+–Ca^{2+} exchange) evoked by caffeine provides a quantitative measure of the SR calcium content. Typical current records from a single ventricular myocyte under perforated patch voltage-clamp control are shown in the upper traces of Fig. 6A. The smooth curves in the lower panel represent the integrals of each current record and clearly show that tetracaine produces a reversible increase in the SR content, in this case from 118 to 233 μmol l^{-1}. On average (Fig. 6*B*), exposure to 100 μM tetracaine for 5 min increased the integral of the caffeine-evoked current by

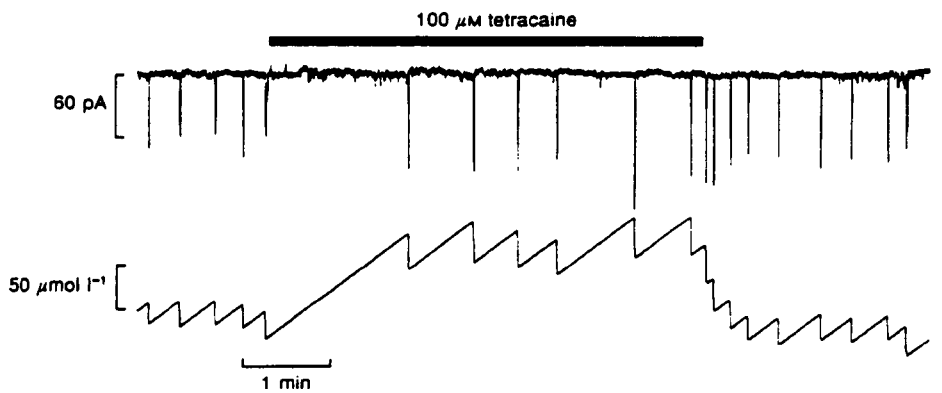

Figure 8. Calculation of the effects of tetracaine on SR Ca^{2+} content

Top, membrane current; bottom, calculated Ca^{2+} content. Tetracaine (100 μM) was applied for the period shown. Tetracaine produced an outward shift of holding current of 9 pA. For clarity this was removed by subtracting a low-pass-filtered version from the raw current. The lower record was calculated by integrating the current records only for the period of each oscillation. To these integrals, a calcium influx of 2·1 μmol l^{-1} s^{-1} was added. Note that the calculated Ca^{2+} content only considers changes due to sarcolemmal fluxes and ignores the faster components due to SR Ca^{2+} release and re-uptake during and immediately after spontaneous release.

80·3 ± 11·4 μmol l^{-1} from 108·0 ± 6·9 μmol l^{-1} (paired t test, $P < 0·0001$, $n = 10$).

It is likely that the extra Ca^{2+} accumulated by the SR in tetracaine is gained during the initial quiescent period. In addition, one predicts that this extra calcium is lost during the burst of spontaneous release activity on removal of tetracaine. We have tested this as follows. The calculated calcium gain during the period without spontaneous Ca^{2+} release was estimated from that which would have occurred if spontaneous release activity had continued at its control frequency, using the average efflux per unit time. We have taken the total interval between the last spontaneous release event in control and the first in tetracaine, and subtracted from this the average period between spontaneous releases in control, thereby leaving the extra interval due to application of tetracaine. This calculation gives the amount of calcium efflux that ought to have taken place during the quiescent period. This calcium, we assume, is accommodated within the SR. We estimated the extra Ca^{2+} lost from the cell during the burst by calculating the cumulative integral over a defined period commencing on removal of tetracaine. From this we subtracted an estimate of the Ca^{2+} loss had spontaneous Ca^{2+} release occurred at the steady-state frequency re-established following the burst. On average, these values compare well with the change of the caffeine response observed in tetracaine (Fig. 7). Average values for these estimation methods are 118·3 ± 15·7 μmol l^{-1} from the quiescent period, 104·5 ± 16·0 μmol l^{-1} for the burst and 86·3 ± 11·6 μmol l^{-1} from the difference in caffeine response. Each of these methods of estimation was compared with the other two using a paired t test, and they were found not to be significantly different (paired t test, $P > 0·1$ for all, $n = 7$).

DISCUSSION

The main finding of this work is that the reduction of spontaneous release frequency produced in rat ventricular cells by application of submillimolar concentrations of tetracaine is associated with an increase of SR calcium content. This is associated with an increase in the size of the transient inward current and integral activated by spontaneous releases in tetracaine. With more calcium in the SR, one might expect a greater flux of calcium through the calcium release channels when they are opened.

The results in this paper are consistent with tetracaine acting by inhibiting Ca^{2+} release from the SR. We must, however, consider whether other actions of tetracaine could be involved. Tetracaine is a local anaesthetic and inhibits both sarcolemmal Na^+ and Ca^{2+} channels (Carmeliet, Morad, Van der Heyden & Vereecke, 1986). Inhibition of Na^+ channels will decrease $[Na^+]_i$ and, via Na^+–Ca^{2+} exchange, will decrease $[Ca^{2+}]_i$ and thence SR Ca^{2+} content. Inhibition of Ca^{2+} channels will directly decrease $[Ca^{2+}]_i$. These effects, however, will produce (as is seen for lidocaine; Eisner, Lederer & Sheu, 1983) a gradual decrease of the frequency of spontaneous Ca^{2+} release. When tetracaine is removed, one would expect a *gradual* recovery of the rate of spontaneous SR Ca^{2+} release. This hypothesis is therefore unable to account for the immediate abolition of spontaneous SR release followed by redevelopment or the burst on removal of tetracaine.

The appearance of spontaneous SR Ca^{2+} release in cardiac muscle is associated with increased SR calcium content (Bers & Bridge, 1988). One would expect, therefore, that abolition of spontaneous SR Ca^{2+} release would be associated with a decrease in SR calcium content. The apparently paradoxical effect of tetracaine to abolish spontaneous release while increasing SR calcium content can be explained if its main effect is to inhibit SR calcium release. Such an inhibition has been demonstrated in isolated cardiac SR vesicles for the structurally related local anaesthetic procaine (Chamberlain, Volpe & Fleischer, 1984). Procaine also inhibits calcium-activated channel activity of the cardiac ryanodine receptor in lipid bilayers (Tinker & Williams, 1993; Zahradnikova & Palade, 1993) and its application has been shown to increase the magnitude of the rapid cooling contracture and therefore, presumably the SR Ca^{2+} content (Komai, Redon & Rusy, 1995). Although no studies have been published on the effect of tetracaine on the *cardiac* ryanodine receptor, a personal communication from L. Xu & G. Meissner shows that it has similar effects to those previously reported in skeletal muscle where it inhibits spontaneous release of calcium from skeletal muscle SR vesicles (Volpe *et al.* 1983) and lowers the open probability of the calcium-activated skeletal muscle ryanodine receptor in lipid bilayer studies (Xu *et al.* 1993). Work published recently in abstract form (Gyorke, Lukyanenko & Gyorke, 1997) has shown that, in cardiac cells, tetracaine initially abolished Ca^{2+} sparks before a recovery associated with an increased SR Ca^{2+} load (as judged qualitatively by the caffeine-evoked increase of $[Ca^{2+}]_i$).

In previous work we have shown that, in the absence of tetracaine, spontaneous SR Ca^{2+} release occurs when the SR Ca^{2+} content has reached a threshold level (Díaz *et al.* 1997). Increasing the magnitude of Ca^{2+} entry into the cell does not increase the Ca^{2+} content of the SR once spontaneous activity begins. Rather, spontaneous SR Ca^{2+} release occurs at a higher frequency because the SR refills to threshold more rapidly. The apparent threshold may result from an effect of luminal Ca^{2+} on the gain of calcium-induced Ca^{2+} release either (i) directly through an intraluminal regulatory effect on the ryanodine receptor or (ii) indirectly because when an SR Ca^{2+} channel opens, the greater the luminal Ca^{2+}, the greater the Ca^{2+} efflux and therefore the greater the gain of calcium-induced Ca^{2+} release. In either case, the greater gain allows wave propagation, activating calcium efflux, thus limiting the level to which the SR can fill.

On the above analysis the effects of tetracaine can be explained if we assume that it increases the loading of the SR required for spontaneous release. This could arise because, by decreasing the opening probability of the SR Ca^{2+} release

channel, tetracaine may decrease the frequency of Ca^{2+} 'sparks' and thence inhibit the initiation of Ca^{2+} waves. The subsequent recovery of spontaneous release will arise because the increase of SR Ca^{2+} content will increase the spark magnitude such that a larger fraction of the sparks result in a propagating wave. This has recently received direct experimental support (Gyorke *et al.* 1997), although we cannot exclude the idea that the decrease of opening probability will decrease the 'gain' of calcium-induced calcium release and may interfere with the propagation of a wave.

When tetracaine is applied, the SR will continue to fill with calcium to beyond the level reached in the control solution. This will produce the initial delay seen before spontaneous release resumes. The duration of this delay will depend on the rate at which Ca^{2+} is entering the cell. It is therefore noteworthy that the delay is proportional to the interval between spontaneous releases observed under control conditions (Fig. 3). Presumably, the higher the rate of Ca^{2+} entry into the cell, the greater the frequency of spontaneous release in control and the less time required to reach the new threshold level of luminal SR. The duration of the initial delay also increases with increasing tetracaine concentration, probably because the concentration of luminal Ca^{2+} required to initiate release increases. In the presence of tetracaine, the amplitude of the spontaneous releases and the amount of Ca^{2+} pumped out of the cell per release increases. Presumably the larger SR Ca^{2+} content results in a larger release of calcium. There is a corresponding decrease in the frequency of release such that the calculated time-averaged Ca^{2+} efflux produced by spontaneous Ca^{2+} release is similar to that in control. The unchanged spontaneous calcium release-induced efflux suggests that the component of efflux activated by the resting calcium is also unchanged, i.e. tetracaine probably does not change the resting calcium. We attempted to measure this directly. However, when tetracaine is applied, there is an immediate increase of fluorescence which is due, at least in part, to the fact that tetracaine is fluorescent. This therefore hampered measurement of resting $[Ca^{2+}]_i$. However, there was no subsequent change of resting $[Ca^{2+}]_i$ during prolonged exposure to tetracaine, suggesting that $[Ca^{2+}]_i$ is constant. Furthermore, the constancy of the efflux suggests that tetracaine is not having a marked effect on Ca^{2+} entry as any decrease of Ca^{2+} entry would, in the steady state, decrease the efflux. It is known that tetracaine decreases Ca^{2+} fluxes through the L-type Ca^{2+} channel (Carmeliet *et al.* 1986). It is, however, likely that all these channels are closed at the normal resting potential and, therefore, not surprising that tetracaine does not affect resting Ca^{2+} fluxes by this mechanism. The constancy of the time-averaged Ca^{2+} efflux is obviously required for the cell to maintain Ca^{2+} balance in the face of an unchanged Ca^{2+} entry. The fact that the initial delay before spontaneous release resumes is greater than the interval between spontaneous releases subsequently in tetracaine is consistent with the fact that the increase of SR Ca^{2+} content in tetracaine (i.e. the amount of calcium gained during the initial delay) is greater than the extra amount pumped out of the cell per spontaneous release (i.e. the amount gained between waves in tetracaine).

This model can also account for the burst of spontaneous Ca^{2+} release seen after removing tetracaine. At this point the properties of calcium-induced Ca^{2+} release have been restored to normal but the SR Ca^{2+} content is elevated considerably above the control threshold for Ca^{2+} release. This will result immediately in Ca^{2+} release. It is noteworthy that the extra Ca^{2+} content of the SR is not removed from the cell on one spontaneous release and is presumably partly re-sequestered by the SR.

The overall results of this paper are best illustrated by the data and calculations shown in Fig. 8. The upper trace shows the effects of tetracaine on membrane current. The inward currents associated with calcium release were integrated to calculate the net loss of calcium from the cell. In the steady state this must be balanced by a calcium entry. The lower trace in Fig. 8 shows the calculated change of cell Ca^{2+} content assuming a constant Ca^{2+} influx into the cell of 2·1 μmol l^{-1} s^{-1}, which was chosen to exactly balance the time-averaged Ca^{2+} efflux. Under control conditions, this results in a gradual increase of cell Ca^{2+} punctuated by abrupt decreases during spontaneous SR Ca^{2+} release. It should be noted that the linear time course of the gain of Ca^{2+} between releases is a simplifying assumption: all we can fix are the initial and final points of the increase. When tetracaine is applied, the spontaneous release stops for a period, the Ca^{2+} influx continues and therefore the cell Ca^{2+} content increases. When spontaneous release resumes, the integrals are larger and therefore the decrease of cell Ca^{2+} content on each release is larger and, in combination with the lower frequency, a new mean steady-state Ca^{2+} content is achieved. Finally, when tetracaine is removed, the burst reduces cell Ca^{2+} back down to the control level.

In summary, the results of this paper show that the inhibition of the SR Ca^{2+} release channel produces an initial cessation of SR Ca^{2+} release. However, the consequent increase of SR Ca^{2+} content overcomes this, resulting in larger amplitude but less frequent Ca^{2+} releases such that cell Ca^{2+} homeostasis is maintained. In other words, given the need to maintain cell Ca^{2+} homeostasis, one obtains the initially unexpected result that inhibiting the Ca^{2+} release process increases the magnitude of the spontaneous Ca^{2+} release. This result may well have implications for the different patterns of spontaneous Ca^{2+} release seen in other cell types (Petersen, Toescu & Petersen, 1991).

Note added in proof

Since this paper was submitted for publication, a full version of the work of S. Györke, V. Lukyanenko & I. Györke has been published (*Journal of Physiology* **500**, 297–309 (1997)).

ALMERS, W. & BEST, P. M. (1976). Effects of tetracaine on displacement currents and contraction of frog skeletal muscle. *Journal of Physiology* **262**, 583–611.

BERS, D. M. & BRIDGE, J. H. (1988). Effect of acetylstrophanthidin on twitches, microscopic tension fluctuations and cooling contractures in rabbit ventricle. *Journal of Physiology* **404**, 53–69.

CAPOGROSSI, M. C., KORT, A. A., SPURGEON, H. A. & LAKATTA, E. G. (1986). Single adult rabbit and rat cardiac myocytes retain the Ca^{2+}- and species-dependent systolic and diastolic contractile properties of intact muscle. *Journal of General Physiology* **88**, 589–613.

CARMELIET, E., MORAD, M., VAN DER HEYDEN, G. & VEREECKE, J. (1986). Electrophysiological effects of tetracaine in single guinea-pig ventricular myocytes. *Journal of Physiology* **376**, 143–161.

CHAMBERLAIN, B. K., VOLPE, P. & FLEISCHER, S. (1984). Inhibition of calcium-induced calcium release from purified cardiac sarcoplasmic reticulum vesicles. *Journal of Biological Chemistry* **259**, 7547–7553.

CHENG, H., LEDERER, W. J. & CANNELL, M. B. (1993). Calcium sparks: elementary events underlying excitation–contraction coupling in heart muscle. *Science* **262**, 740–744.

CHENG, H., LEDERER, M. R., LEDERER, W. J. & CANNELL, M. B. (1996). Calcium sparks and $[Ca^{2+}]_i$ waves in cardiac myocytes. *American Journal of Physiology* **270**, C148–159.

DÍAZ, M. E., COOK, S. J., CHAMUNORWA, J. P., TRAFFORD, A. W., LANCASTER, M. K., O'NEILL, S. C. & EISNER, D. A. (1996). Variability of spontaneous Ca^{2+} release between different rat ventricular myocytes is correlated with Na^+–Ca^{2+} exchange and $[Na^+]_i$. *Circulation Research* **78**, 857–862.

DÍAZ, M. E., TRAFFORD, A. W., O'NEILL, S. C. & EISNER, D. A. (1997). Measurement of sarcoplasmic reticulum Ca^{2+} content and sarcolemmal Ca^{2+} fluxes in isolated rat ventricular myocytes during spontaneous Ca^{2+} release. *Journal of Physiology* **501**, 3–16.

EISNER, D. A., LEDERER, W. J. & SHEU, S. S. (1983). The role of intracellular sodium activity in the anti-arrhythmic action of local anaesthetics in sheep Purkinje fibres. *Journal of Physiology* **340**, 239–257.

EISNER, D. A., NICHOLS, C. G., O'NEILL, S. C., SMITH, G. L. & VALDEOLMILLOS, M. (1989). The effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. *Journal of Physiology* **411**, 393–418.

GYORKE, S., LUKYANENKO, V. & GYORKE, I. (1997). Dual effects of tetracaine on spontaneous calcium release in ventricular myocytes. *Biophysical Journal* **72**, A163.

HORN, R. & MARTY, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *Journal of General Physiology* **92**, 145–159.

KASAI, H. & PETERSEN, O. H. (1994). Spatial dynamics of second messengers: IP_3 and cAMP as long-range and associative messengers. *Trends in Neurosciences* **17**, 95–100.

KOMAI, H., REDON, D. & RUSY, B. F. (1995). Procaine enhancement of the rapid cooling contracture and inhibition of the decay of the potentiated state in rabbit papillary muscle. *Journal of Molecular and Cellular Cardiology* **27**, 2543–2550.

LEDERER, W. J. & TSIEN, R. W. (1976). Transient inward current underlying arrhythmogenic effects of cardiotonic steroids in Purkinje fibres. *Journal of Physiology* **263**, 73–100.

LÓPEZ-LÓPEZ, J. R., SHACKLOCK, P. S., BALKE, C. W. & WIER, W. G. (1994). Local, stochastic release of Ca^{2+} in voltage-clamped rat heart cells: visualization with confocal microscopy. *Journal of Physiology* **480**, 21–29.

NEGRETTI, N., VARRO, A. & EISNER, D. A. (1995). Estimate of net calcium fluxes and sarcoplasmic reticulum calcium content during systole in rat ventricular myocytes. *Journal of Physiology* **486**, 581–591.

PETERSEN, C. C., TOESCU, E. C. & PETERSEN, O. H. (1991). Different patterns of receptor-activated cytoplasmic Ca^{2+} oscillations in single pancreatic acinar cells: dependence on receptor type, agonist concentration and intracellular Ca^{2+} buffering. *EMBO Journal* **10**, 527–533.

SATOH, H., DELBRIDGE, L. M. D., BLATTER, L. A. & BERS, D. M. (1996). Surface : volume relationship in cardiac myocytes studied with confocal microscopy and membrane capacitance measurements: Species dependence and developmental effects. *Biophysical Journal* **70**, 1494–1504.

TINKER, A. & WILLIAMS, A. J. (1993). Charged local anaesthetics block ionic conduction in the sheep cardiac sarcoplasmic reticulum calcium release channel. *Biophysical Journal* **65**, 852–864.

VARRO, A., NEGRETTI, N., HESTER, S. B. & EISNER, D. A. (1993). An estimate of the calcium content of the sarcoplasmic reticulum in rat ventricular myocytes. *Pflügers Archiv* **423**, 158–160.

VOLPE, P., PALADE, P., COSTELLO, B., MITCHELL, R. D. & FLEISCHER, S. (1983). Spontaneous calcium release from sarcoplasmic reticulum – effect of local anaesthetics. *Journal of Biological Chemistry* **258**, 12434–12442.

XU, L., JONES, R. & MEISSNER, G. (1993). Effects of local anaesthetics on single channel behavior of skeletal muscle release channel. *Journal of General Physiology* **101**, 207–233.

ZAHRADNIKOVA, A. & PALADE, P. (1993). Procaine effects on single sarcoplasmic reticulum Ca release channels. *Biophysical Journal* **64**, 991–1003.

Acknowledgements

We are indebted to Dr L. Xu and Dr G. Meissner for helping us by examining the effects of tetracaine on the cardiac SR release channel and providing us with their unpublished data. This work was supported by grants from The Wellcome Trust and the European Community. C.L.O. was in receipt of a Wellcome Prize Studentship.

Author's email address

D. A. Eisner: eisner@liverpool.ac.uk

Received 27 February 1997; accepted 8 May 1997.

The effect of tetracaine on stimulated contractions, sarcoplasmic reticulum Ca^{2+} content and membrane current in isolated rat ventricular myocytes

C. L. Overend, S. C. O'Neill and D. A. Eisner

Department of Veterinary Preclinical Sciences, University of Liverpool, Liverpool L69 3BX, UK

(Received 20 August 1997; accepted after revision 12 November 1997)

1. The effects of tetracaine were examined on rat ventricular myocytes. In both field-stimulated and voltage-clamped cells tetracaine (100–200 μM) produced an initial decrease of contraction before a recovery towards the control level. Removal of tetracaine produced a transient overshoot of contraction to levels greater than the control.

2. The transient decrease of contraction produced by tetracaine was accompanied by a small transient increase in the integral of the L-type Ca^{2+} current and a larger transient decrease of the Na^+–Ca^{2+} exchange current on repolarization. These are attributed to decreased systolic release of Ca^{2+}. On removal of tetracaine there was an increase of the Na^+–Ca^{2+} exchange current. Before the addition of tetracaine, calculated Ca^{2+} influx and efflux across the sarcolemma were approximately equal. On adding tetracaine, efflux was transiently less than influx and, on removal of tetracaine, efflux was greater than influx.

3. These changes in Ca^{2+} fluxes result in an increase of cell Ca^{2+} during exposure to tetracaine. The calculated magnitude of this increase was equal to that measured directly by applying caffeine (20 mM) to release sarcoplasmic reticulum (SR) Ca^{2+} and integrating the resulting Na^+–Ca^{2+} exchange current.

4. It is concluded that the effects of tetracaine can be accounted for by depression of calcium-induced Ca^{2+} release (CICR). The response is transient because the inhibition is compensated for by an increase of SR Ca^{2+} content such that there is no *steady-state* effect on the magnitude of the systolic Ca^{2+} transient. The consequences of this result for the effects of other modulators of CICR are discussed.

Calcium-induced calcium release (CICR) underlies excitation–contraction coupling in cardiac muscle. Calcium is released from the sarcoplasmic reticulum (SR) through a specialized release channel termed the ryanodine receptor (RyR). This receptor is activated during an action potential by calcium, which enters the cell through voltage-activated L-type calcium channels in the sarcolemma. Certain agents modify the sensitivity of the ryanodine receptor to calcium and as such are considered to be potential regulators of contraction. For example, both the compound cyclic ADP-ribose (Rakovic, Galione, Ashamu, Potter & Terrar, 1996) and phosphorylation of the ryanodine receptor (duBell, Lederer & Rogers, 1996) have been suggested to increase the gain of CICR and thence the magnitude of the systolic Ca^{2+} transient. Local anaesthetics such as procaine and tetracaine inhibit Ca^{2+} fluxes through the ryanodine receptor (Zahradníková & Palade, 1993; Györke, Lukyanenko & Györke, 1997) and suppress Ca^{2+} release and contraction in both cardiac and skeletal muscle (Almers & Best, 1976; Chapman & Leoty, 1981; Stephenson & Wendt, 1986; Klein, Simon & Schneider, 1992; Komai, Redon & Rusy, 1995). Tetracaine may therefore be a useful model compound for studying the effects of regulators of CICR. We have previously shown that tetracaine produces a transient suppression of *spontaneous* Ca^{2+} release from the SR (Overend, Eisner & O'Neill, 1997) (cf. Gyorke *et al.* 1997). This effect was attributed to the combination of (i) an inhibitory effect of tetracaine on CICR which is then gradually overcome by (ii) a subsequent increase of SR Ca^{2+} content. These results were shown to be consistent with inhibition of CICR. There was, however, no direct measurement of the gain of CICR. Furthermore there was no information about the effects of tetracaine on the normal, stimulated release of Ca^{2+} from the SR.

The related local anaesthetic, procaine, has been shown to directly inhibit SR calcium release (Zahradníková & Palade, 1993), and to increase SR calcium content (as assessed by rapid cooling contracture), an effect which has been attributed to a reduction in trans-sarcolemmal Ca^{2+} efflux (Komai *et al.* 1995). The initial aim of this paper was to

obtain *quantitative* measurements of changes of CICR gain and SR Ca^{2+} content. Before this can be done, however, it is important to consider other possible actions of tetracaine which, for example, acts as a local anaesthetic by blocking sodium, calcium and potassium ion channels in nerve and muscle preparations at concentrations similar to those affecting the SR release channel (Hille, 1977; Chapman & Leoty, 1981; Carmeliet, Morad, Van der Heyden & Vereecke, 1986). Therefore, some of the inotropic effects of tetracaine could be attributable to effects on these sarcolemmal ionic fluxes. We present evidence suggesting that such a contribution is small, and that the transient nature of the effects of tetracaine on contraction can be attributed to depression of SR Ca^{2+} release and consequent changes of SR Ca^{2+} content. We conclude that agents which only affect CICR will only have transient effects on contraction.

METHODS

Experiments were carried out on cardiac myocytes isolated from rat ventricles using a collagenase and protease digestion protocol as previously described (Eisner, Nichols, O'Neill, Smith & Valdeolmillos, 1989). Rats were killed by stunning and cervical dislocation.

Electrophysiology

Voltage clamp control was achieved using the perforated patch technique (Horn & Marty, 1988) using amphotericin B. Due to the relatively high access resistance of the perforated patch (about 20 MΩ) we used the switch clamp facility of the Axoclamp-2A voltage clamp amplifier (Axon Instruments). Pipettes (1–3 MΩ in resistance) were filled with the following solution (mM): $CsCH_3O_3S$, 125; CsCl, 12; NaCl, 20; Hepes, 10; $MgCl_2$, 5; Cs-EGTA, 0·1; titrated to pH 7·2 with CsOH. Amphotericin B was dissolved in DMSO and added to the filling solution to a final concentration of 240 $\mu g\ ml^{-1}$, before use. Cells were bathed in a control solution of the following composition (mM): NaCl, 135; KCl, 4; Hepes, 10; glucose, 11; $CaCl_2$, 2; $MgCl_2$, 1; titrated to pH 7·4 with NaOH. To avoid interference from outward currents, all voltage clamp experiments were performed in the presence of 5 mM 4-aminopyridine and 0·1 mM $BaCl_2$. Tetracaine was added where appropriate from a stock solution of 100 mM (in H_2O), without osmotic correction. In some experiments (e.g. Fig. 1) voltage clamp was not used, and in this case 4-aminopyridine and $BaCl_2$ were omitted. All experiments were carried out at 22 °C.

Fluorescence measurements

In experiments designed to measure $[Ca^{2+}]_i$, cells were loaded with the acetoxymethyl ester form of indo-1 (Molecular Probes; 2·5 μM for 5 min, followed by at least 30 min for de-esterification) and placed in a bath on the stage of an inverted epifluorescence microscope (Nikon Diaphot TMD, Nikon, UK). Fluorescence was excited at 340 nm and measured at 400 and 500 nm. The ratio of the emitted fluorescence (400 nm/500 nm) was used as an index of $[Ca^{2+}]_i$. In other experiments cells were not loaded with the indicator. Fluorescence measurements in the presence of tetracaine were hampered by the fact that the drug itself fluoresces with an intensity which is considerably greater at 400 than at 500 nm. The intensity of this fluorescence is greater in the presence of a cell presumably because tetracaine (a weak base) is accumulated within the cytoplasm (which is acidic with respect to the extracellular fluid). The time course of the rise of this artifactual increase of fluorescence is complete within 2–3 s. We have therefore adjusted the indo-1 fluorescence traces (Fig. 1) by subtracting off a step increase of fluorescence at both 400 and 500 nm. This correction is fairly crude and the results should be interpreted accordingly. In particular, it is not possible to compare accurately the magnitude of a Ca^{2+} transient in tetracaine with one in control solution, although changes of fluorescence once tetracaine has been applied will be recorded faithfully.

In some experiments caffeine was used to release Ca^{2+} from the SR. As reported previously (Overend *et al.* 1997), in the presence of tetracaine, 10 mM caffeine often produces a series of oscillations of inward current, rather than a single release. This is similar to the effect of a lower concentration of caffeine in the absence of tetracaine and presumably reflects antagonism between tetracaine and caffeine (Almers & Best, 1976). For this reason 20 mM caffeine was used to measure SR calcium content. Under these conditions a single release of calcium resulted, and the accompanying Na^+–Ca^{2+} exchange current could be measured easily.

Data were digitized using a Digidata 1200 series interface board (Axon Instruments) and analysed using software (ABFAN2) written by Dr A. W. Trafford (Department of Veterinary Preclinical Sciences, University of Liverpool).

Quantification of SR Ca^{2+} content and sarcolemmal Ca^{2+} fluxes

Inward Na^+–Ca^{2+} exchange currents produced by the application of caffeine (20 mM) to voltage-clamped cells were integrated and converted to total cell calcium fluxes as described previously (Varro, Negretti, Hester & Eisner, 1993; Negretti, Varro & Eisner, 1995). Briefly, it is necessary to first correct for that fraction of the efflux which is *not* produced by Na^+–Ca^{2+} exchange, and then relate the fluxes to cell volume. The volume was calculated from the cell membrane capacitance using the capacitance-to-volume ratio 6·76 $pF\ pl^{-1}$ (Satoh, Delbridge, Blatter & Bers, 1996). It should be noted that, as in our previous work, the SR Ca^{2+} content is expressed with relation to cell (and not SR) volume. We have previously shown that 67 % of Ca^{2+} efflux is generated by Na^+–Ca^{2+} exchange (Negretti, O'Neill & Eisner, 1993). The remainder occurs via the sarcolemmal Ca^{2+}-ATPase and mitochondrial sequestration. To take into account non-Na^+–Ca^{2+} exchange efflux of Ca^{2+}, current integrals were multiplied by a factor of 1·5. This correction was used for both caffeine- and repolarization (tail current)-activated current. The integrals of calcium currents were simply divided by a factor of 2 to compensate for the fact that each calcium ion carries two positive charges into the cell.

Statistics

All values are presented as means ± S.E.M. for n experiments. Significance was assessed using a paired t test.

RESULTS

Figure 1 shows the effect of tetracaine on the amplitude of contraction and associated calcium transients in field-stimulated rat ventricular myocytes. Application of 100 μM tetracaine transiently reduced the amplitude of contraction. Contraction then gradually recovered towards the control level over a period of 1–2 min, despite the maintained presence of the drug. The magnitude of peak contraction was also more variable in the presence of tetracaine. Removal of tetracaine was associated with a transient elevation of contraction amplitude above the control level, which

subsequently recovered to the control level. Similar effects of tetracaine addition and removal were seen on the systolic Ca^{2+} transient record (Fig. 1*A*, lower trace). This suggests that changes in contraction amplitude are a consequence of changes in the Ca^{2+} transient magnitude, and do not reflect effects of the drug on the contractile apparatus. The amplitude of the Ca^{2+} transient does not recover to the control level, while the contraction appears to demonstrate recovery to the control level. We cannot exclude the possibility that the lack of complete recovery of the Ca^{2+} transient is due to errors in correction for the intrinsic fluorescence of tetracaine (see Methods). However, the changes in the amplitude of the Ca^{2+} transient which occur during the maintained presence of tetracaine, and the overshoot on removal of tetracaine must be due to changes of $[Ca^{2+}]_i$ rather than artifactual changes of fluorescence.

It is likely that the effects of tetracaine are a result of reduced calcium-induced calcium release (CICR). However, tetracaine has well-documented effects on sodium and calcium currents across the sarcolemma (I_{Na} and I_{Ca}, respectively) (Hille, 1977; Carmeliet *et al.* 1986). The former will decrease excitability, and this effect can be eliminated by using voltage-clamped cells. The latter will directly decrease Ca^{2+} entry. The experiment illustrated in Fig. 2 shows a typical result in a voltage-clamped cell. The effects of tetracaine on contraction (Fig. 2*A*) are qualitatively similar to those seen in field-stimulated cells: tetracaine produces an initial decrease of contraction followed by recovery and then an overshoot on removal of tetracaine. The transient overshoot of contraction amplitude on removal of the drug, in this case, was accompanied by spontaneous oscillations, indicating some degree of calcium overload at this point in the experiment. Figure 2*B* shows specimen records of current and contraction. The immediate major depression of contraction (*b*) is accompanied by a modest decrease (to 83%) of the peak magnitude of the calcium current. This is associated with an increase in the integral of the Ca^{2+} current (see later). However, while contraction recovers towards control levels, there is no recovery of the calcium current (*c*). Similarly, the overshoot in contraction amplitude on removal of tetracaine (*d*) is not accompanied by an increase in peak calcium current above the control level. Changes in the peak amplitude of the

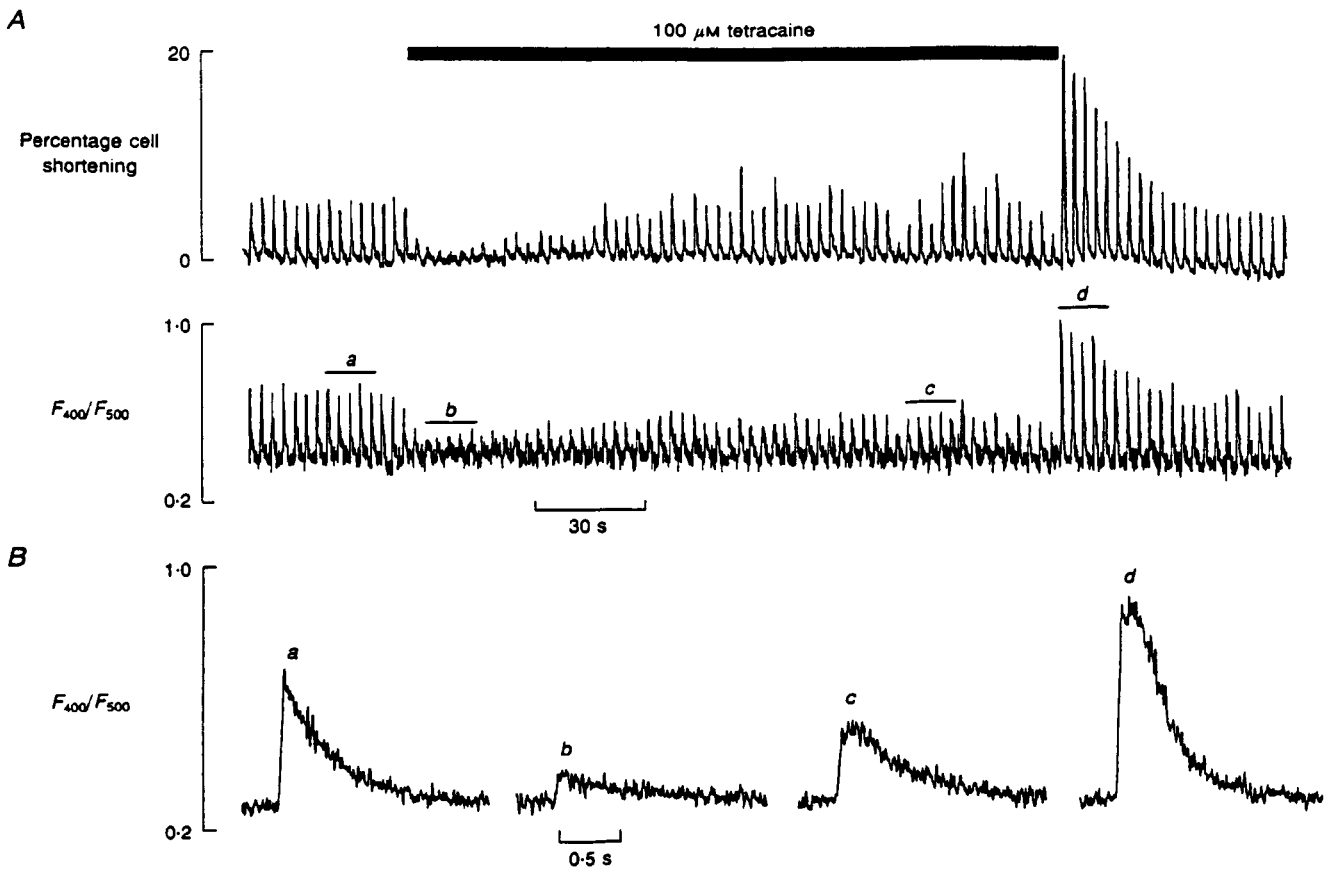

Figure 1. The effects of tetracaine on contraction amplitude and systolic $[Ca^{2+}]_i$

A, time course of cell shortening (top) and indo-1 ratio ($R = F_{400}/F_{500}$) (bottom). The cell was field stimulated at a frequency of 0·3 Hz. Tetracaine (100 μM) was applied for the period indicated by the bar. The indo-1 ratio was corrected for the intrinsic fluorescence of tetracaine (see Methods). *B*, averaged specimen ($n = 5$) Ca^{2+} transients taken from the periods (*a*–*d*) indicated in *A*.

calcium current, therefore, cannot explain the recovery of contraction amplitude during continued superfusion with tetracaine, nor the transient overshoot of contraction observed on its removal. On average, in twelve cells, the minimum level of contraction reached in 100 μM tetracaine was 40·0 ± 4·5% of the control level and this recovered to 96·2 ± 2·6% in the steady state. This level of contraction during steady-state exposure to tetracaine was not significantly different from that in control ($P > 0·1$). On removal of tetracaine the mean peak level of contraction reached was 170·8 ± 16% of the control level.

In an attempt to gauge to what extent inhibition of the L-type calcium current may account for changes in contraction, in Fig. 3 we compared the effect of tetracaine with that produced by deliberately reducing the calcium current, by reducing the size of the depolarizing step (from 40 to 30 mV). Reducing the size of the depolarization reduced the peak calcium current considerably more than did exposure to 100 μM tetracaine (here, to 54% of the control peak I_{Ca}, cf. 83% in tetracaine). However, the contraction elicited by the smaller pulse was only marginally reduced in magnitude, in contrast to the dramatic reduction of contraction amplitude observed during early exposure to tetracaine. This confirms that only a small proportion of the effects of tetracaine on contraction amplitude can be due to reduction of the L-type calcium current.

Measurement of Ca^{2+} flux balance in tetracaine

The transient elevation of contraction amplitude observed on removal of tetracaine may be indicative of an increase in the SR calcium load of the cells following exposure to tetracaine. This is further suggested by the presence of spontaneous oscillations in some cells immediately following removal of the drug (Fig. 2). Results presented later in this paper provide quantitative measurements of this increase of SR Ca^{2+} content. The aim of the series of experiments described below was to investigate the origin of this increase. In order to do this we have measured the sarcolemmal fluxes of calcium. As in our previous work (Negretti *et al.* 1995; Trafford, Díaz, Negretti & Eisner, 1997*b*), Ca^{2+} entry was measured by integrating the L-type Ca^{2+} current and Ca^{2+} efflux from the Na^+–Ca^{2+} exchange current tail activated on repolarization (Fedida, Noble, Shimoni & Spindler, 1987;

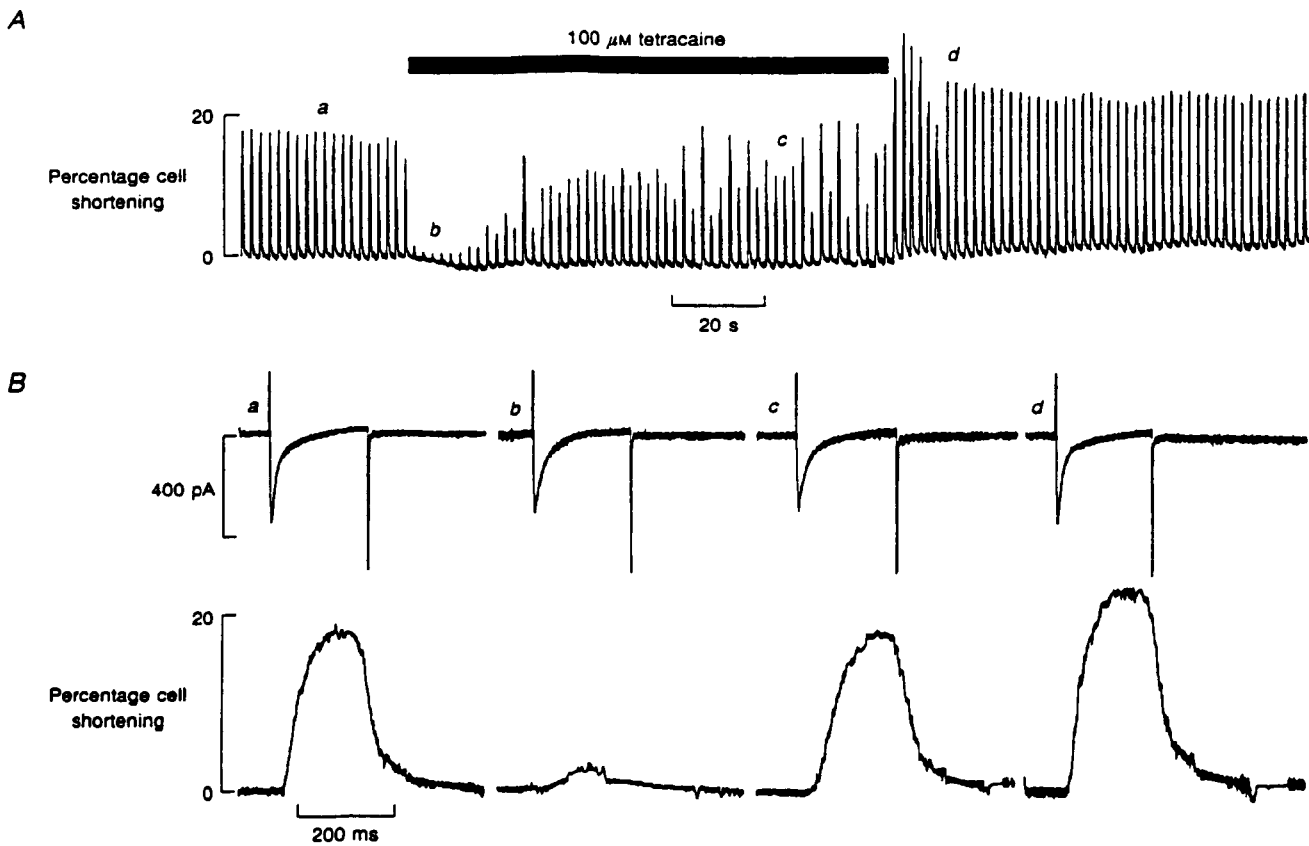

Figure 2. The effects of tetracaine on contraction and membrane current in a voltage-clamped cell

A, time course of changes of contraction. Tetracaine (100 μM) was applied for the period indicated by the bar. The membrane potential was held at −40 mV, and 200 ms duration depolarizing pulses to 0 mV were applied at a frequency of 0·5 Hz. *B*, specimen records of membrane current (top) and contraction (bottom) obtained at the times (*a*–*d*) indicated in *A*.

Bridge, Smolley & Spitzer, 1990). To facilitate the measurements, short (100 ms duration) pulses were used in order to minimize Ca^{2+} efflux during depolarization, when any Na^+–Ca^{2+} exchange flux will be obscured by the L-type Ca^{2+} current. The contraction record in Fig. 4 again shows a transient reduction of amplitude followed by a recovery on application of tetracaine and overshoot on removal of tetracaine. Figure 4*B* illustrates sample current records. The integrated currents for this cell show that in control conditions (Fig. 4*Ba*) depolarization produces a gain of about 4 μmol (l cell volume)$^{-1}$ Ca^{2+} via the L-type Ca^{2+} current. On repolarization there is a loss of calcium from the cell which is of the same magnitude as the initial gain. In other words, influx equals efflux. On average, in twelve cells, the Ca^{2+} entry during the Ca^{2+} current was 4·19 ± 0·43 μmol l^{-1} in comparison with average efflux of 4·55 ± 0·37 μmol l^{-1} (all fluxes expressed with respect to total cell volume). These are not significantly different ($P > 0·10$). The records illustrated in Fig. 4*Bb* show the early effects of tetracaine. Despite the reduction in the peak magnitude of the Ca^{2+} current, the integrated Ca^{2+} entry is greater (presumably due to reduced Ca^{2+}-induced inactivation of the current, because of the smaller Ca^{2+} transient: Sipido, Callewaert & Carmeliet, 1995; Adachi-Akahane, Cleemann & Morad, 1996). The main effect of tetracaine on membrane current is, however, a decrease of the Na^+–Ca^{2+} exchange current tail on repolarization due, presumably, to the decreased magnitude of the systolic Ca^{2+} transient. The net effect is that the cell has gained about 3 μmol Ca^{2+} l^{-1} at the end of the record shown. During the period of exposure to tetracaine, as the sizes of the systolic Ca^{2+} transient and contraction increase, so does that of the current tail on repolarization. Therefore, in the steady state in tetracaine (Fig. 4*Bc*) Ca^{2+} entry on depolarization exactly balances the loss on repolarization, such that there is no net change of cell Ca^{2+}. The mean data confirm that influx (4·00 ± 0·42 μmol l^{-1}) and efflux (4·64 ± 0·38 μmol l^{-1}) are not significantly different and, therefore, balance in tetracaine ($P > 0·05$) once a steady-state level of contraction is achieved. When tetracaine is removed there is a small decrease of the integrated calcium current. This is accompanied by a much larger increase of Ca^{2+} loss on repolarization due to the larger Ca^{2+} transient. On this pulse there is a net loss of cell calcium of almost 4 μmol l^{-1}. Although not shown, as the contraction and systolic Ca^{2+} transient decrease towards control levels, the Ca^{2+} loss on repolarization decreases to control levels and Ca^{2+} flux balance is once again achieved. This post-tetracaine depletion of calcium presumably

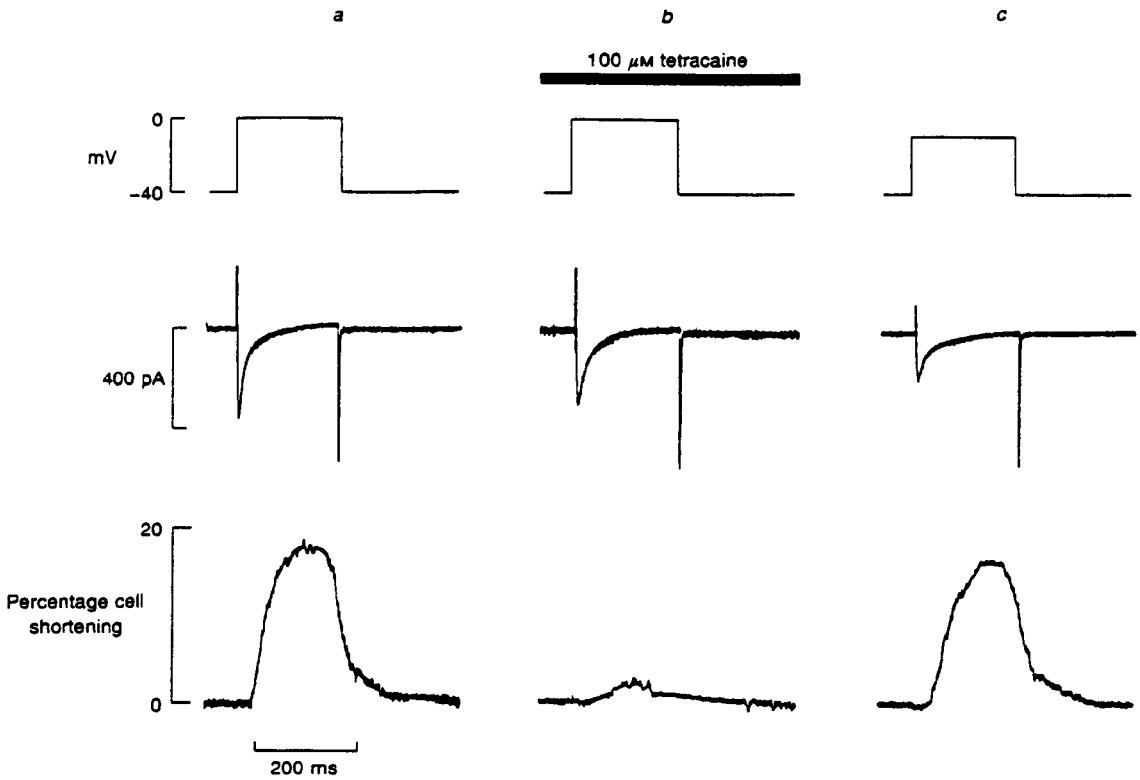

Figure 3. Comparison of the effects of tetracaine with those of decreasing the size of depolarization

In all panels the traces show (from top to bottom): membrane potential, current, cell length. In *a* and *b*, the depolarizing pulse was to 0 mV. Panel *a*, control; *b*, after 4 s exposure to tetracaine (100 μM); *c*, in the absence of tetracaine (depolarization to −10 mV). Membrane potential was held at −40 mV and depolarizing pulses of 200 ms duration were applied to elicit contraction.

accounts for the gradual reduction of contraction towards the control steady-state level.

The net changes of calcium illustrated in Fig. 4 take place over each cycle of contraction and relaxation. It appears that exposure to tetracaine causes a net increase of calcium by the cell, and this is balanced by a loss of calcium from the cell on its removal. The total amount of calcium gained and lost in this way can therefore be calculated by summing the net calcium flux for each cycle. An example of this type of calculation is illustrated in Fig. 5. This calculation was performed using the same data as in Fig. 4. The bar illustrates the period of tetracaine superfusion. The upper panels represent the values of Ca^{2+} influx and efflux, respectively, calculated from the integrals of Ca^{2+} current and tail current, associated with each cycle of contraction. The bottom panel illustrates the cumulative difference between influx and efflux, i.e. the total amount of calcium gained or lost by the cell since the start of the record. The data presented earlier show that, on average, in either control or tetracaine, in the steady state Ca^{2+} influx and efflux are equal. However, any small differences will add up with this cumulative method. We have therefore calculated the mean steady-state values of calcium influx and efflux for control, tetracaine and recontrol. These steady-state values were subtracted from the integral of each pulse in the appropriate solution. As shown in Fig. 4, application of tetracaine is associated with a reduction in calcium efflux from the cell, and a transient elevation of calcium entry above the control level, probably as a consequence of reduced calcium-induced inactivation of I_{Ca}. Both these effects will contribute to increasing the calcium content of the cell. As the cell gains calcium, contraction recovers towards the control level, and with it efflux from the cell, activated by the increasing magnitude of associated Ca^{2+} transients. On removal of tetracaine there is a transient elevation of the efflux integral, producing a net loss of

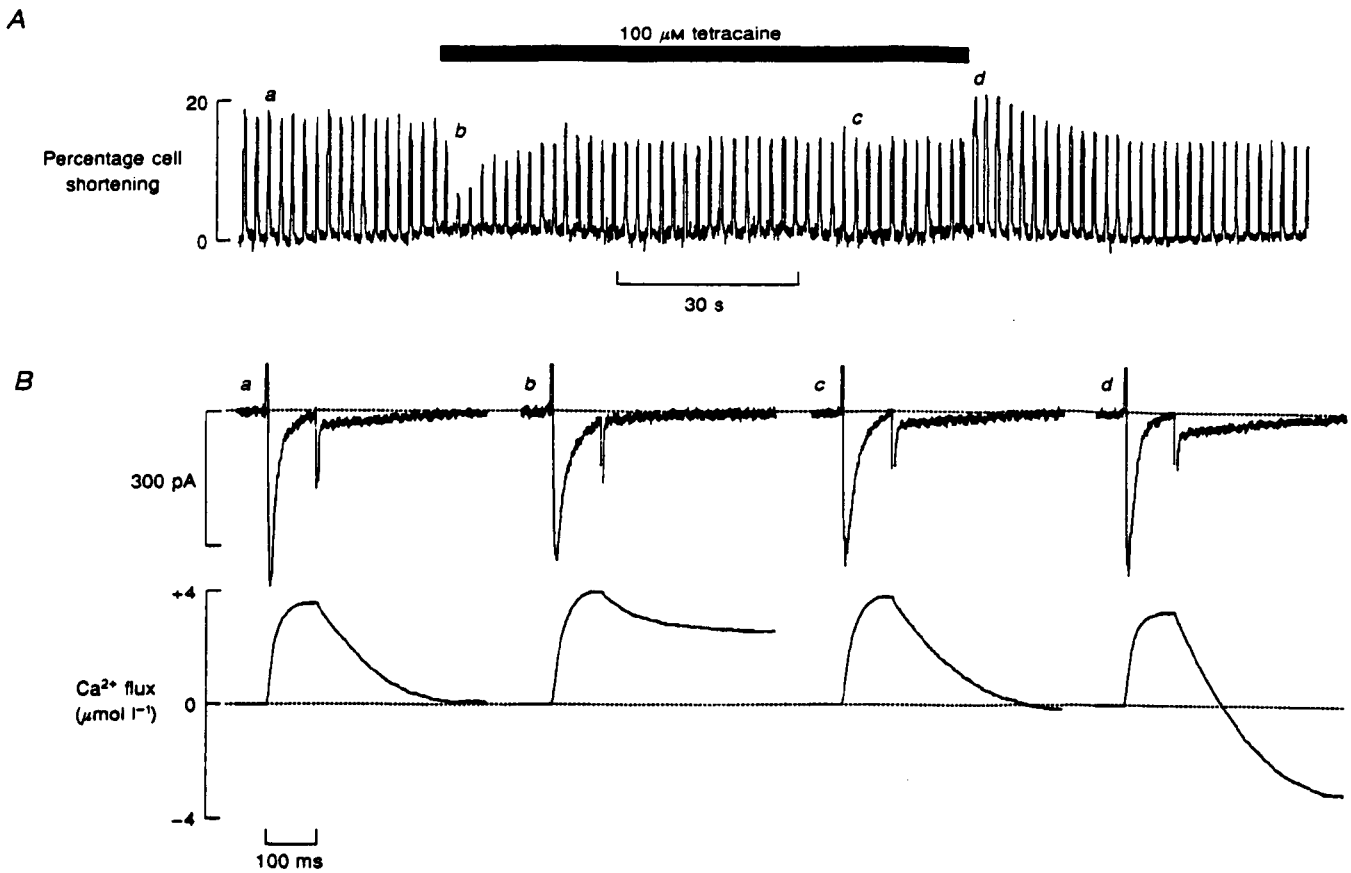

Figure 4. Transient loss of Ca^{2+} flux balance during application and removal of tetracaine

A, time course of the effects on contraction of applying tetracaine for the period shown by the bar. *B*, specimen records of membrane current (top) and cumulative integral (bottom). Membrane potential was held at −40 mV and depolarizing pulses of 100 ms duration were applied at 0·5 Hz. The Ca^{2+} flux traces show the cumulative integral of the calcium current (initial upward deflection) followed by a downward deflection due to the Ca^{2+} efflux. The records were obtained at the times (*a–d*) shown in *A*. (See Methods for calculation of sacrolemmal Ca^{2+} movements.) The vertical positions of the current traces have been aligned to facilitate comparison. Tetracaine produced an outward shift of holding current of 7 pA and this has been removed to facilitate comparison between records.

calcium from the cell. There is also a significant undershoot of calcium entry into the cell, which will further contribute to reducing cell calcium content. The net calcium loss on removal of tetracaine calculated in this way ($35{\cdot}5 \pm 3{\cdot}3$ μmol l^{-1}, $n = 11$) is similar to ($P > 0{\cdot}05$) the calculated amount of calcium gained in the presence of tetracaine ($33{\cdot}7 \pm 3{\cdot}1$ μmol l^{-1}, $n = 11$).

In the steady state, the flux of calcium into the cell in Fig. 4 is minimally affected by 100 μM tetracaine. This is reflected in the fact that contraction amplitude recovers towards the control level in the steady state (where influx and efflux are balanced). Inhibition of the calcium current may, however, contribute to any observed reduction in the steady-state amplitude of contraction below that observed under control conditions, during prolonged exposure to tetracaine. This effect is more obvious in Fig. 6, which illustrates the effect of 200 μM tetracaine on contraction and associated sarcolemmal currents and calcium fluxes. Changes in contraction amplitude during exposure to 200 μM tetracaine follow a similar, although somewhat exaggerated, pattern as observed with lower concentrations of the drug. Exposure to 200 μM tetracaine (indicated by the bar) eventually produces a new steady-state level of contraction, which is below the control level. In this case, recovery time is also considerably prolonged. The associated I_{Ca} (Fig. 6*B*, top panel) is inhibited to a greater extent by tetracaine, thereby reducing calcium entry into the cell more significantly. In this case, the calcium current integral was reduced to 62·1% of the mean control value by exposure to 200 μM tetracaine. In the same cell a reduction in the magnitude of I_{Ca} to 82·6% of the mean control value was observed during exposure to 100 μM tetracaine. The decrease in the Ca^{2+} current integral during exposure to 200 μM tetracaine was reflected by a reduction in the steady-state amplitude of contraction to 54·8% of the control level, in comparison with 85·5% during exposure to 100 μM tetracaine.

Direct measurements of SR Ca^{2+} content

It is likely that the extra calcium gained by the cell during exposure to tetracaine is accommodated in the SR. We took this type of analysis one step further by comparing the effect of tetracaine on the SR calcium content more directly, by measuring the caffeine-induced Na^+–Ca^{2+} exchange currents in stimulated myocytes under control conditions and during tetracaine superfusion. Figure 7*A* illustrates typical caffeine traces and their associated integrals. It is clear that exposure to tetracaine increases the size of the integral and therefore of the SR Ca^{2+} content. The histogram of Fig. 7*B* shows that, in six cells, the increase of SR Ca^{2+} content measured directly with this technique ($32{\cdot}6 \pm 2{\cdot}9$ μmol l^{-1}) is very similar to (although statistically different from, $P < 0{\cdot}05$) that estimated from the integrated current records ($30{\cdot}3 \pm 2{\cdot}4$ μmol l^{-1}).

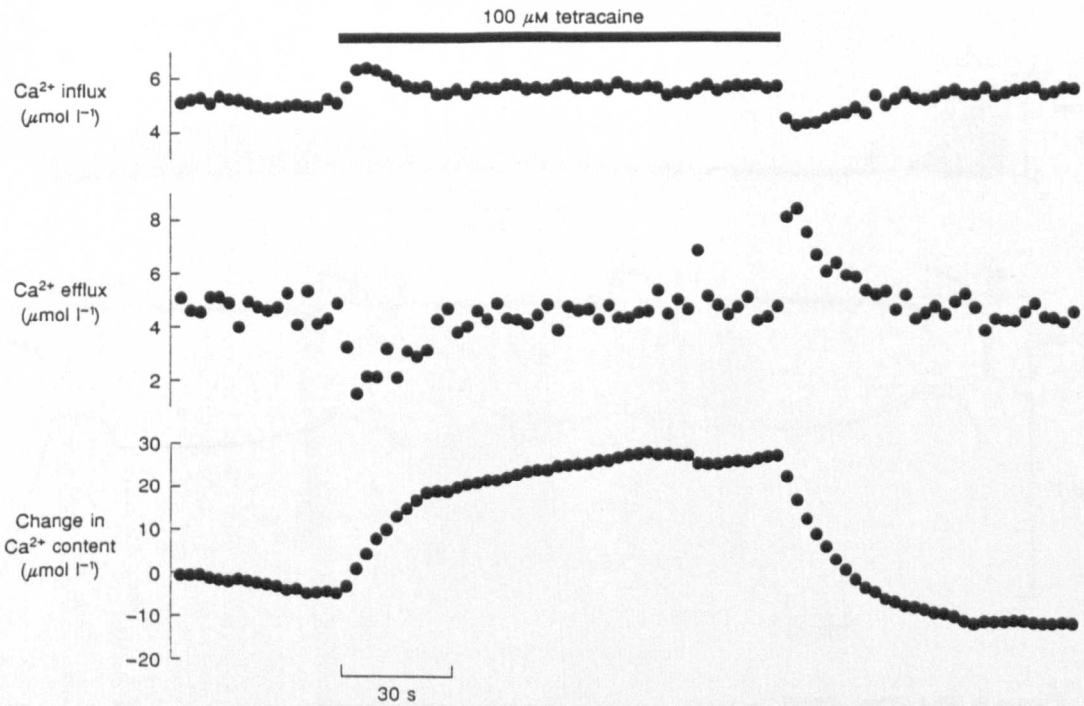

Figure 5. Net accumulation of calcium during exposure to and removal of tetracaine

The traces show (from top to bottom): calculated Ca^{2+} entry via the L-type Ca^{2+} current, Ca^{2+} efflux on repolarization (calculated as shown in Fig. 4), cumulative change of cell Ca^{2+} content. Calcium content (presumably SR) is expressed per unit total cell volume.

DISCUSSION

The most striking feature of the results obtained in this study is the transient nature of the inotropic effects of tetracaine. Tetracaine transiently suppresses electrically stimulated contraction and systolic $[Ca^{2+}]_i$, and its removal is associated with a transient elevation of contraction and $[Ca^{2+}]_i$ amplitude above the control level. These experiments were designed to investigate the mechanism by which tetracaine achieves these inotropic effects in rat ventricular myocytes. We have shown that the transient effects of tetracaine addition and removal cannot be accounted for by changes of I_{Ca}, but are likely to be due to a combination of inhibition of CICR and increased SR Ca^{2+} content.

During normal excitation–contraction coupling, calcium ions are released from the SR by calcium-induced release, triggered by calcium entry through voltage-gated sarcolemmal Ca^{2+} channels. The concentrations of tetracaine used here decrease calcium entry slightly, by direct inhibition of the L-type calcium current (Chapman & Leoty, 1981; Carmeliet *et al.* 1986). However, we have shown that this effect cannot account for the transient nature of the depression of contraction, and the effects of tetracaine are therefore likely to reflect, predominantly, a decrease in the ability of I_{Ca} to stimulate calcium release from the SR. Qualitatively, the biphasic nature of the effects of tetracaine is similar (but in the opposite direction) to that found for low concentrations of caffeine (O'Neill & Eisner, 1990; Györke & Palade, 1992). Caffeine produces a transient increase of systolic contraction and $[Ca^{2+}]_i$ followed by a decay back to control levels. On removal of caffeine, there is an undershoot of the magnitude, before recovery back to control levels. The transient increase on application of caffeine was explained as resulting from a stimulation of CICR (O'Neill & Eisner, 1990). It is only transient because the increased release decreases the SR Ca^{2+} content. This hypothesis has recently received quantitative support (Trafford *et al.* 1998).

Measurement of sarcolemmal fluxes and SR Ca^{2+} content during tetracaine application

In this paper we have examined the balance of fluxes across the membrane during the application of tetracaine. This was done by comparing the magnitude of Ca^{2+} entry during the depolarizing pulse via the L-type Ca^{2+} current with the Ca^{2+}

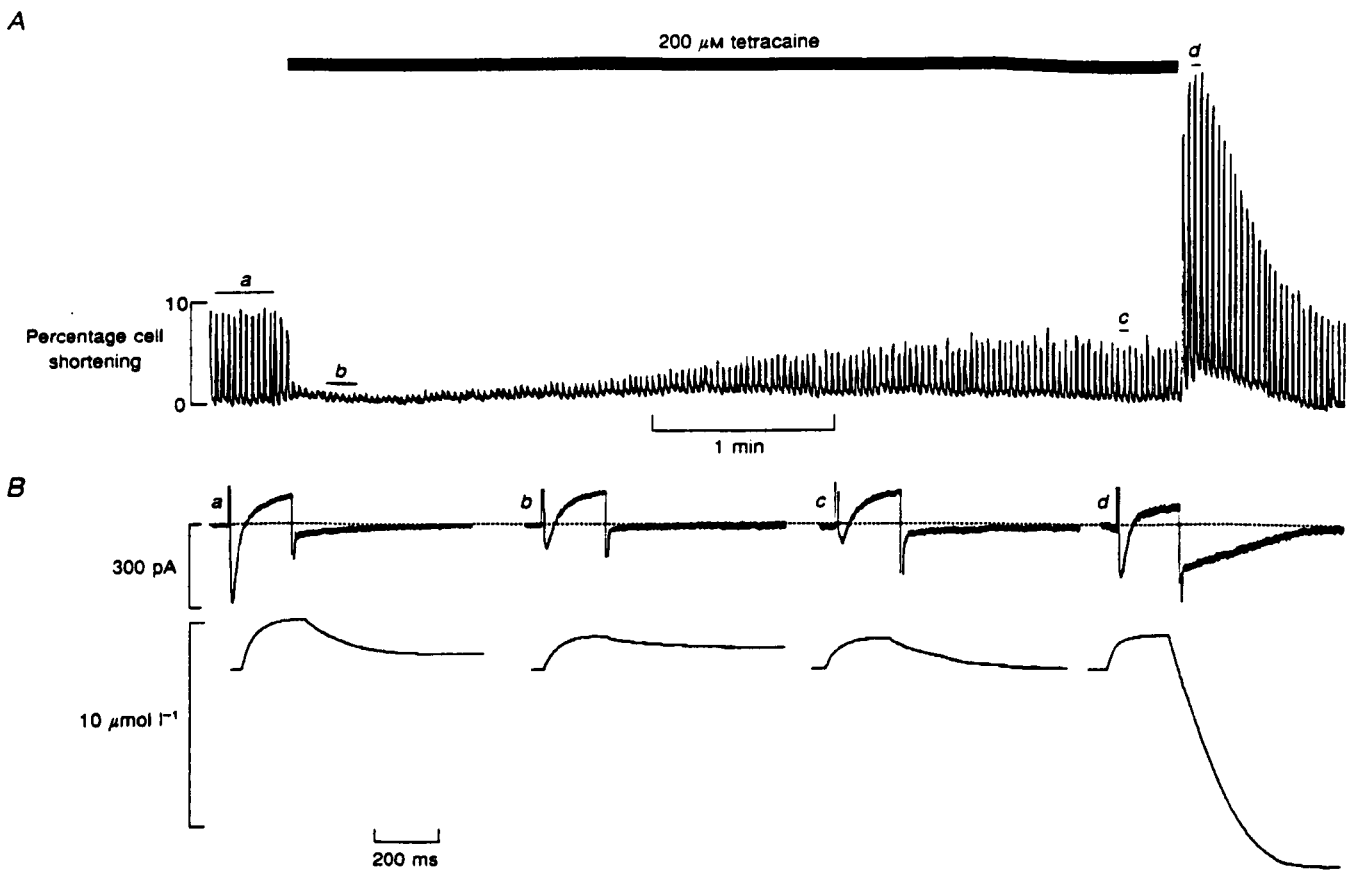

Figure 6. The effect of 200 μM tetracaine on contraction and current

A, time course of cell shortening in response to electrical stimulation elicited by 200 ms depolarizing steps to 0 mV from a holding potential of −40 mV. Tetracaine (200 μM) was applied as indicated by the bar. *B*, specimen records of membrane current (top) and cumulative integral (bottom). The records were obtained at the times (*a*–*d*) shown in *A*. The records in *a* and *b* are the means of 10 and 5 pulses, respectively. Single pulses are shown in *c* and *d*.

efflux on repolarization. The initial effect of applying tetracaine on sarcolemmal fluxes is to decrease the Ca^{2+} efflux (as a result of the decreased Ca^{2+} transient) and to increase the Ca^{2+} entry into the cell. The latter effect is due to decreased inactivation of the Ca^{2+} current (presumably due to decreased calcium-induced inactivation) overcoming the small direct inhibitory effect of tetracaine on the Ca^{2+} current. This results in a net predicted increase of cell calcium on each stimulus in contrast to the balance under control conditions. The increase of SR Ca^{2+} content can be calculated from the net integral. The results (Fig. 5) show that there is a gradual increase of cell Ca^{2+} content on each beat in tetracaine until a new balance is achieved, when efflux again equals influx. This results from the increase of Ca^{2+} efflux due to the gradual increase of the systolic Ca^{2+} transient. On removal of tetracaine, the inhibitory effect on CICR is removed and the larger SR Ca^{2+} content produces a larger systolic Ca^{2+} release than in the control. Therefore the Ca^{2+} efflux from the cell is increased above control levels. This results in a decrease of SR Ca^{2+} content until the final steady state is reached.

The changes of SR Ca^{2+} content referred to above were calculated from the integrated current records. This method has the advantage that an estimate of change of SR content can be obtained after each pulse. The method is, however, rather indirect and, in particular, makes no allowance for changes of fluxes between pulses. That this is a valid method is shown by the fact that (i) under control conditions and in the steady state in tetracaine, influx and efflux balance and (ii) the estimate of SR Ca^{2+} gain from this

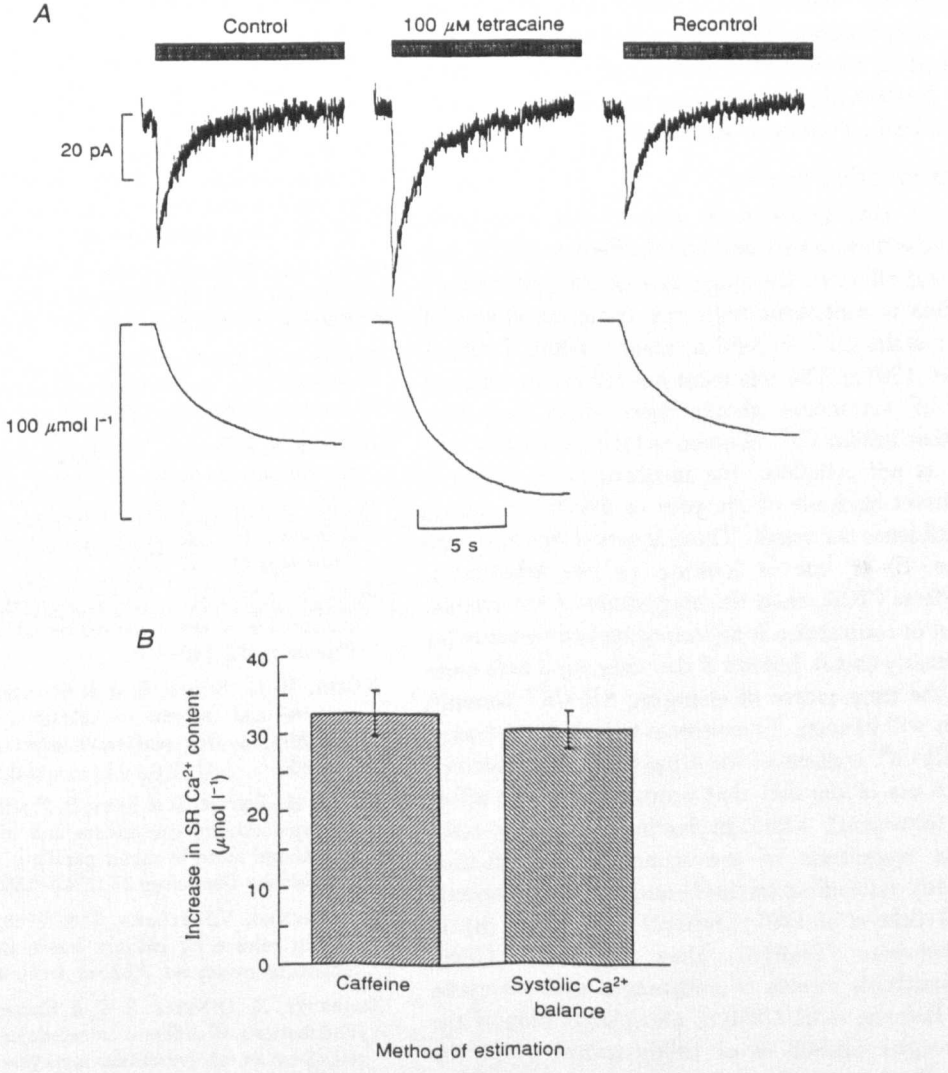

Figure 7. The gain of measured SR Ca^{2+} content matches that calculated from the sarcolemmal fluxes

A, measurement of SR Ca^{2+} content. Caffeine (20 mM) was applied for the period indicated by the bars. Traces show current (top) and integrated current (bottom). From left to right: control; after 1·5 min exposure to tetracaine (100 μM); recontrol (1·5 min after removing tetracaine). *B*, histogram comparing the measured changes of SR Ca^{2+} (left) with those calculated as in Fig. 5 from the cumulative integrals (right).

method agrees quantitatively with that measured directly by applying 20 mM caffeine to release all the SR Ca^{2+} content. The measurement of increased SR Ca^{2+} content is also in agreement with qualitative results showing that the cooling contracture was increased by the related local anaesthetic procaine (Komai *et al.* 1995).

Changes of the gain of CICR

The present data show that tetracaine decreases the amount of Ca^{2+} release from the SR in response to a given size of Ca^{2+} current. In other words tetracaine decreases the gain of CICR. This effect is then overcome by an increase of SR Ca^{2+} content. The increase of SR Ca^{2+} release by an increase of SR Ca^{2+} content is in agreement with previous work (Bassani, Yuan & Bers, 1995; Lukyanenko, Györke & Györke, 1996; Györke *et al.* 1997). It is interesting to note that the fractional recovery of contraction is greater than that of the SR Ca^{2+} content. This presumably reflects the combination of (i) the steep relationship between $[Ca^{2+}]_i$ and contraction and (ii) the fact that increasing SR Ca^{2+} content produces a fractionally larger increase of Ca^{2+} release (Bassani *et al.* 1995; Trafford *et al.* 1997*b*).

Implications for other work

The results of this paper have shown that tetracaine, despite having a maintained depressant effect on CICR, has only a transient effect on the magnitude of the systolic Ca^{2+} transient. This is consistent with the transient effects of caffeine on systolic Ca^{2+} (O'Neill & Eisner, 1990; Trafford, Díaz & Eisner, 1997*a*). The argument put forward to explain the effects of tetracaine should also apply to other compounds that inhibit Ca^{2+} release; as long as inhibition of Ca^{2+} release is not complete, the mechanism by which it occurs (i.e. direct blockade of the pore *vs.* effects on gating) should not influence the result. These observations have two consequences. (i) If one is looking to see whether a compound affects CICR, then the magnitude of the systolic Ca^{2+} transient or contraction is an inappropriate measure (at least in the steady state). Indeed if the compound acts more slowly than the time course of changing SR Ca^{2+} content, then no effect will be seen. To overcome this, it is necessary to measure SR Ca^{2+} content at the same time. Alternatively, one can make use of the fact that compounds which affect CICR (like tetracaine), although having no steady-state effect on the magnitude of the stimulated contraction, produce a steady-state effect on the frequency of spontaneous SR release (Györke *et al.* 1997; Overend *et al.* 1997). (ii) As discussed elsewhere (Trafford, Díaz & Eisner, 1998), maintained inotropic effects of substances such as cyclic ADP-ribose (Rakovic *et al.* 1996) or phosphorylation of the ryanodine receptor (duBell *et al.* 1996) cannot simply be attributed to effects on CICR.

Adachi-Akahane, S., Cleemann, L. & Morad, M. (1996). Cross-signaling between L-type Ca^{2+} channels and ryanodine receptors in rat ventricular myocytes. *Journal of General Physiology* **108**, 435–454.

Almers, W. & Best, P. M. (1976). Effects of tetracaine on displacement currents and contraction of frog skeletal muscle. *Journal of Physiology* **262**, 583–611.

Bassani, J. W. M., Yuan, W. & Bers, D. M. (1995). Fractional SR Ca release is regulated by trigger Ca and SR Ca content in cardiac myocytes. *American Journal of Physiology* **268**, C1313–1319.

Bridge, J. H., Smolley, J. R. & Spitzer, K. W. (1990). The relationship between charge movements associated with I_{Ca} and $I_{Na\text{-}Ca}$ in cardiac myocytes. *Science* **248**, 376–378.

Carmeliet, E., Morad, M., Van der Heyden, G. & Vereecke, J. (1986). Electrophysiological effects of tetracaine in single guinea-pig ventricular myocytes. *Journal of Physiology* **376**, 143–161.

Chapman, R. A. & Leoty, C. (1981). The effects of tetracaine on the membrane currents and contraction of frog atrial muscle. *Journal of Physiology* **317**, 475–486.

duBell, W. H., Lederer, W. J. & Rogers, T. B. (1996). Dynamic modulation of excitation–contraction coupling by protein phosphatases in rat ventricular myocytes. *Journal of Physiology* **493**, 793–800.

Eisner, D. A., Nichols, C. G., O'Neill, S. C., Smith, G. L. & Valdeolmillos, M. (1989). The effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. *Journal of Physiology* **411**, 393–418.

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

Györke, S., Lukyanenko, V. & Györke, I. (1997). Dual effects of tetracaine on spontaneous calcium release in rat ventricular cells. *Journal of Physiology* **500**, 297–310.

Györke, S. & Palade, P. (1992). Calcium-induced calcium release in crayfish skeletal muscle. *Journal of Physiology* **457**, 195–210.

Hille, B. (1977). Local anesthetics: Hydrophilic and hydrophobic pathways for the drug-receptor reaction. *Journal of General Physiology* **69**, 497–515.

Horn, R. & Marty, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *Journal of General Physiology* **92**, 145–159.

Klein, M. G., Simon, B. J. & Schneider, M. F. (1992). Effects of procaine and caffeine on calcium release from the sarcoplasmic reticulum in frog skeletal muscle. *Journal of Physiology* **453**, 341–366.

Komai, H., Redon, D. & Rusy, B. F. (1995). Procaine enhancement of the rapid cooling contracture and inhibition of the decay of the potentiated state in rabbit papillary muscle. *Journal of Molecular and Cellular Cardiology* **27**, 2543–2550.

Lukyanenko, V., Györke, I. & Györke, S. (1996). Regulation of calcium release by calcium inside the sarcoplasmic reticulum in ventricular myocytes. *Pflügers Archiv* **432**, 1047–1054.

Negretti, N., O'Neill, S. C. & Eisner, D. A. (1993). The relative contributions of different intracellular and sarcolemmal systems to relaxation in rat ventricular myocytes. *Cardiovascular Research* **27**, 1826–1830.

Negretti, N., Varro, A. & Eisner, D. A. (1995). Estimate of net calcium fluxes and sarcoplasmic reticulum calcium content during systole in rat ventricular myocytes. *Journal of Physiology* **486**, 581–591.

O'Neill, S. C. & Eisner, D. A. (1990). A mechanism for the effects of caffeine on Ca^{2+} release during diastole and systole in isolated rat ventricular myocytes. *Journal of Physiology* **430**, 519–536.

Overend, C. L., Eisner, D. A. & O'Neill, S. C. (1997). The effect of tetracaine on spontaneous Ca^{2+} release and sarcoplasmic reticulum calcium content in rat ventricular myocytes. *Journal of Physiology* **502**, 471–479.

Rakovic, S., Galione, A., Ashamu, G. A., Potter, B. V. L. & Terrar, D. A. (1996). A specific cyclic ADP-ribose antagonist inhibits cardiac excitation-contraction coupling. *Current Biology* **6**, 989–996.

Satoh, H., Delbridge, L. M. D., Blatter, L. A. & Bers, D. M. (1996). Surface:volume relationship in cardiac myocytes studied with confocal microscopy and membrane capacitance measurements: Species dependence and developmental effects. *Biophysical Journal* **70**, 1494–1504.

Sipido, K. R., Callewaert, G. & Carmeliet, E. (1995). Inhibition and rapid recovery of Ca^{2+} current during Ca^{2+} release from sarcoplasmic reticulum in guinea pig ventricular myocytes. *Circulation Research* **76**, 102–109.

Stephenson, D. G. & Wendt, I. R. (1986). Effects of procaine on calcium accumulation by the sarcoplasmic reticulum of mechanically disrupted rat cardiac muscle. *Journal of Physiology* **373**, 195–207.

Trafford, A. W., Díaz, M. E. & Eisner, D. A. (1997*a*). Alteration of sarcolemmal and sarcoplasmic reticulum calcium fluxes in isolated rat ventricular myocytes produced by modulation of calcium-induced calcium release. *Journal of Physiology* **501.P**, 133*P*.

Trafford, A. W., Díaz, M. E. & Eisner, D. A. (1998). Stimulation of Ca-induced Ca release only transiently increases the systolic Ca transient: measurements of Ca fluxes and s.r. Ca. *Cardiovascular Research* **37** (in the Press).

Trafford, A. W., Díaz, M. E., Negretti, N. & Eisner, D. A. (1997*b*). Enhanced calcium current and decreased calcium efflux restore sarcoplasmic reticulum Ca content following depletion. *Circulation Research* **81**, 477–484.

Varro, A., Negretti, N., Hester, S. B. & Eisner, D. A. (1993). An estimate of the calcium content of the sarcoplasmic reticulum in rat ventricular myocytes. *Pflügers Archiv* **423**, 158–160.

Zahradníková, A. & Palade, P. (1993). Procaine effects on single sarcoplasmic reticulum Ca release channels. *Biophysical Journal* **64**, 991–1003.

Corresponding author

D. A. Eisner: Department of Veterinary Preclinical Sciences, University of Liverpool, Liverpool L69 3BX, UK.

Email: eisner@liv.ac.uk