Factors affecting the calcium sensitivity of the contractile proteins of the heart.

A thesis submitted for the degree of

Doctor of Philosophy.

by

Christine Lamont.

Institute of Physiology,

Glasgow University

December, 1987.

Contents page.

Materials and Methods

Page no.

Chapter 1

Hysteresis in the pCa-tension relationship

Chapter 2

The influence of tonicity on isometric force production and calcium sensitivity

Chapter 3

The imidazoles and calcium sensitivity

Chapter 4

Cellular imidazoles as the natural enhancers of calcium sensitivity

Discussion

List of figures

TABLES

Abbreviations:

EGTA	Ethyleneglycol bis(B-aminoethylether)-N-N-tetraacetic acid
HDTA	Hexamethylene diamnine tetra-acetic acid
HEPES	N-2-hydroxylethylpiperaine-N'-2-ethansulphoic acid.
SR	Sarcoplasmic reticulum
C_{max}	Maximum calcium activated force
CICR	Calcium induced calcium release
Ca-	Calcium
c-b	Cross-bridge
pCa	$-\log[Ca^{2+}]$
$\overline{M}_n$	average molecular wt. definition on page 68
$\overline{M}_w$	weight average molecular wt. see page 68
COP	Colloid Osmotic Pressure
l.s.	Lattice spacing
s.l.	sarcomere length
$[Ca^{2+}]_i$	intracellular free calcium
'h'	Hill coefficient
pK_{app}	= pCa for half maximal activation
T	Relative tension
mM	millimolar
μm	micron or micrometer
pH_a	$-\log_{10}[H^+]_{activity}$

List of additional papers

CRICHTON, K.A., LAMONT, C., MILLER, D.J. & O'DOWD, J.J. (1987). Detection and estimation of carnosine, anserine and novel imidazoles in cardiac and skeletal muscle of several species by high-performance liquid chromatography. J.Physiol. (in press)

HARRISON, S.M., LAMONT, C. & MILLER, D.J. (1985). Carnosine and other natural imidazoles enhance muscle calcium sensitivity and are mimicked by caffeine and ARL-115 BS. J.Physiol. 371, P197.

HARRISON, S.M., LAMONT, C. & MILLER, D.J. (1985). Hysteresis in the Ca vs. tension relationship of chemically 'skinned' rat cardiac muscle. J.Physiol. 364, P76.

LAMONT, C. & MILLER, D.J. (1987). Oscillations in selectively skinned rat cardiac muscle. J.Physiol. 392, 14P.

LAMONT, C. & MILLER, D.J. (1987). Hypertonic shrinkage of the lattice spacing of chemically skinned cardiac muscle increases Ca-sensitivity and peak force. J.Physiol. 386, 57P.

MOSS, V.A., MILLER, D.J. & LAMONT, C. (1986). Real-time analysis of sarcomere length during activation of chemically skinned cardiac muscle. Proc. Roy. Soc (Edinburgh) 21, 581-582.

J. Physiol (1988) in the Press. (Mill Hill Meeting).

C. 68

Detection and estimation of carnosine, anserine and novel imidazoles in cardiac and skeletal muscle of several species by high-performance liquid chromatography

By CHRISTINE LAMONT, D. J. MILLER, J. J. O'DOWD and C. A. THOMSON. *Institute of Physiology, Glasgow University G12 8QQ*

We have reported that imidazole-containing compounds carnosine and *N*-acetyl histidine (endogenous to muscle), in common with imidazole drugs (such as caffeine and sulmazole), increase the calcium sensitivity of the contractile proteins (Harrison *et al.* 1986). To relate the occurrence of these substances to contractile characteristics, new separation and detection methods for high-performance liquid chromatography (HPLC) are under development. We have made a survey of skeletal and cardiac muscle, and tissues such as lung and kidney, from several species including frog, rat, and rabbit, body-wall muscle from the medicinal leech (*Hirudo medicinalis*) and, for heart muscle alone, guinea-pig and man.

Tissues samples (*c.* 100 mg wet wt.) are homogenized in 80% ethanol, 20% water at 20 °C and then extracted in the same mixture at 60 °C for 90 min. Extracts are centrifuged, the supernatant removed, evaporated to dryness and suspended in 0·1 M-phosphate buffer, pH 7·0, for examination with analytical HPLC (isocratic system; mobile phase, 0·1 M-phosphate, pH 2·0, stationary phase, Spherisorb ODS, 5 μm; detection at 210 nm). This combination of extraction and detection methods is selective for the compounds of interest. Absorbance peaks have been compared with commercial standards (carnosine, *N*-acetyl histidine 'NAH', anserine, histidine, methyl-histidine, alanine, tyrosine and others) or some synthesized specifically (*N*-acetyl forms of carnosine 'NAC', anserine 'NAA', 1-methyl histidine 'NAMH') using the method of Sobue *et al.* (1975). Selective differential staining techniques for substituted and non-substituted imidazoles (after Van Balgooy & Roberts, 1973) support the identifications by HPLC.

As in earlier reports (see Crush, 1970), we find high levels of carnosine and anserine in skeletal muscle, with fast fibres (extensor digitorum longus, EDL, from rat and rabbit) being higher than slow fibres (soleus from rat and rabbit). The mixed muscle (EDL, 40% fast oxidative, 45% fast glycolytic, FG, fibres) had higher levels than the 'pure' (90–95% FG) tensor fascia latae. Cardiac muscle, and human atrium in particular, has lower concentrations of these compounds. However, we have provisionally identifed NAH, previously only reported in frog heart (Kuroda & Ikoma, 1966), and NAC, NAA and NAMH (not previously reported) in the hearts and in several of the skeletal muscles. The latter compounds constitute a substantial fraction of the total muscle imidazoles, particularly in heart. For 13 human atrial samples, estimated total content of imidazoles was between 2 and 8 mmol kg^{-1} wet. wt. Confirmation of these identifications requires analysis with a second chromatographic system which is being developed. The effects of several of these novel compounds on the contractile machinery remain to be investigated.

We thank the MRC and the British Heart Foundation for financial support.

REFERENCES

CRUSH, K. G. (1970). *Comp. Biochem. Biophys.* **34**, 3–30.
HARRISON, S. M., LAMONT, C. & MILLER, D. J. (1986). *J. Physiol.* **371**, 197*P*.
KURODA, Y. & IKOMA, T. (1966). *Science, N.Y.* **152**, 1241–1242.
SOBUE, K., KONISHI, H. & NAKAJIMA, T. (1975). *J. Neurochem.* **24**, 1261–1262.
VAN BALGOOY, J. N. A. & ROBERTS, E. (1973). *Biochem. Pharmac.* **22**, 1405–1415.

J. Physiol. 386. (1987)

Hypertonic shrinkage of the lattice spacing of chemically skinned cardiac muscle increases Ca sensitivity and peak force

By CHRISTINE LAMONT and DAVID J. MILLER. *Institute of Physiology, Glasgow University, Glasgow G12 8QQ*

Changes in tonicity of the solutions bathing intact muscle fibres have a range of effects on force production. Godt & Maughan (1981) showed that Ca sensitivity of skinned skeletal fibres was increased when tonicity was increased by high molecular weight (MW 400000) Dextran, a 5% solution having maximum effect. We have observed a similar shift in Ca sensitivity and a substantial increase in maximum Ca-activated force in Triton-extracted trabeculae from rat cardiac muscle. Ca sensitivity is increased by about 0·2 pCa units (at half maximum activation) and maximum Ca-activated force (C'_{max}) by 15% with a 3% solution of 500000 MW Dextran. Fig. 1*A* shows an example of the reduction in C'_{max} when the high MW

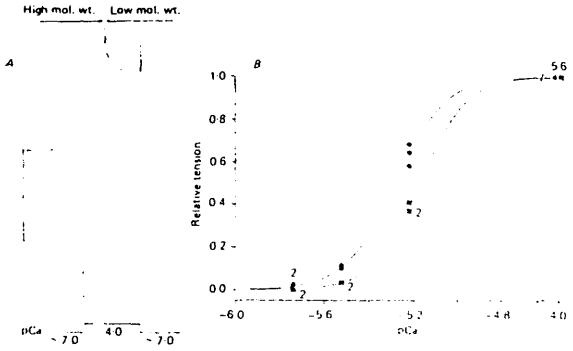


Fig. 1. Effects of altering osmolarity with high MW (500000) and low MW Dextran (9000, both 3% solution) on a Triton-treated rat ventricle trabecula. (For details of method and solution composition see Miller & Smith, 1985.) *A*, force developed at pCa 4·0 (10 mM total EGTA) first with high then low MW Dextran. Scale bars represent 1 min (horizontal) and 5 mg. wt (vertical). *B*, the normalized pCa-tension relationship obtained under the two conditions high (●) and low (■) MW. 20 °C. Sarcomere length 1·8 μm.

is exchanged for a low MW (9000) Dextran (3%). The shift in Ca sensitivity for the same preparation is illustrated in Fig. 1*B*. A 3% solution of low MW Dextran has no effect on either peak force or Ca sensitivity compared with control solutions. The major part of the effect on peak force (Fig. 1*A*) and Ca sensitivity develops within a minute or so, but approximately 20 min is required for equilibration (preparations 80 μm in diameter). Both effects occur at sarcomere lengths of 1·8–2·2 μm. These concentrations of high MW Dextran have been shown to shrink the myofilament lattice in chemically skinned skeletal fibres, whereas the low MW will not (Matsubara, Umazumi & Yagi, 1985). We conclude that the altered proximity of the thick and thin filaments alters Ca sensitivity in the heart as in skeletal muscle.

Financial support from the British Heart Foundation and M.R.C. is gratefully acknowledged.

REFERENCES

GODT, R. E. & MAUGHAN, D. W. (1981). *Pflügers Arch. (Eur. J. Physiol.)* 391, 334–337.
MATSUBARA, I., UMAZUMI, Y. & YAGI, N. (1985). *J. Physiol.* 360, 135–148.
MILLER, D. J. & SMITH, G. L. (1985). *J. Musc. Res. Cell Motil.* 6, 541–567.

[*From the Proceedings of the Physiological Society*, 19–20 *June* 1987
Journal of Physiology, 392, 14*P*, 1987]

Oscillations in selectively chemically skinned rat cardiac muscle

By Christine Lamont and D. J. Miller. *Institute of Physiology, University of Glasgow, Glasgow G12 8QQ*

Quiescent mammalian cardiac muscle has been widely reported to display 'chaotic' oscillatory behaviour (e.g. Lakatta & Lappé, 1981). We observe this activity using differential interference contrast microscopy on ventricle trabeculae (*ca.* 100 μm diameter, 2 mm length) from the rat (Miller, Sinclair, Smith & Smith, 1982). The experimental chamber permits solution exchange together with isometric force measurement and observation of the sarcomere patern continuously. Image display on a monochrome TV monitor enhances the otherwise low-contrast view. Preparations are selectively chemically 'skinned' with saponin, rendering the sarcolemma permeable to small ions and molecules but leaving the intracellular membrane systems (sarcoplasmic reticulum, SR and mitochondria) functional. In this state, more co-ordinated oscillatory behaviour can be evoked (Harrison & Miller, 1984; Fry & Miller, 1986). The demonstration illustrates that the force oscillations of about 0·2–1 Hz (20 °C) are associated with waves of localized shortening which propagate smoothly along the preparation. Preparations are initially exposed to a $[Ca^{2+}]$ below the threshold for steady-state force production (about 1 μM under present conditions of pH, pMg, etc). This $[Ca^{2+}]$ is only nominal since the level of Ca-EGTA buffer used is kept low (0·2 mM). If the $[Ca^{2+}]$ is raised slightly, to near or just above threshold, regular force oscillations occur: the cellular organelles have sufficient Ca-buffering capacity to control the $[Ca^{2+}]$ locally within the preparation. Waves generally start at one end of the muscle, but occasionally in two subsections of the preparation propagation occurs in opposite directions. Reduction of $[Ca^{2+}]$ or $[Na^{+}]$ terminates the oscillations abruptly; blocking respiration-dependent mitochondrial Ca-uptake slows them. These, and the effects of increasing $[Na^{+}]$ (Harrison & Miller, 1985) or of adding carnosine (Harrison, Lamont & Miller, 1986), will be demonstrated. Both interventions increase the frequency, and generally the amplitude, of the oscillations, indicating an involvement of both the mitochondria and SR in the processes controlling the oscillations.

Financial support from MRC and the British Heart Foundation is gratefully acknowledged.

REFERENCES

Fry, C. H. & Miller, D. J. (1986). In *Control and Manipulation of Calcium Movement*, ed. Parratt, J. New York: Raven Press.
Harrison, S. M., Lamont, C. & Miller, D. J. (1986). *J. Physiol.* **371**, 197*P*.
Harrison, S. M. & Miller, D. J. (1984). *J. Physiol.* **353**, 55*P*.
Harrison, S. M. & Miller, D. J. (1985). *J. Physiol.* **364**, 79*P*.
Lakatta, E. G. & Lappé, D. L. (1981). *J. Physiol.* **315**, 369–394.
Miller, D. J., Sinclair, J., Smith, A. & Smith, G. L. (1982). *J. Physiol.* **329**, 11*P*.

[*From the Proceedings of the Physiological Society*, 22–23 *February* 1985
Journal of Physiology, 364, 76*P*]

Hysteresis in the Ca *vs.* tension relationship of chemically 'skinned' rat cardiac muscle

By S. M. Harrison, Christine Lamont and D. J. Miller. *Institute of Physiology, University of Glasgow, Glasgow G12 8QQ*

Force is not a unique function of the sarcoplasmic free Ca concentration ($[Ca^{2+}]$; $-\log[Ca^{2+}] = pCa$) because of hysteresis in the pCa–tension relationship (Ridgway, Gordon & Martyn, 1983). We observe the same phenomenon in rat ventricular trabeculae after partial or complete chemical 'skinning' (with saponin or Triton X-100, respectively). Fig. 1 illustrates a typical response; steady-state tension at each pCa is higher as $[Ca^{2+}]$ is reduced than when increased; the pCa–tension relationship shifts 0·1–0·2 pCa units at half-maximum tension ($\frac{1}{2}C_{max}$; range for seven muscles, sarcomere length (SL) 2·2 μm determined by light microscopy). Hysteresis is reduced when $[Ca^{2+}]$ steps are reversed from submaximal activation levels, but enhanced at lower sarcomere lengths, when peak force is lower. This suggests that hysteresis (i) is not due to the level of force *per se* but rather to the $[Ca^{2+}]$ experienced by the Ca-binding proteins and (ii) it is not the consequence of the scatter of SLs varying during force production. Hysteresis is unaffected by 10 mM-caffeine which increases Ca sensitivity by 0·2 pCa units at $\frac{1}{2}C_{max}$. Preliminary observations suggest that hysteresis is virtually absent at SL above 2·3–2·4 μm; on the ascending limb of the length–tension curve, the effect of SL on Ca-sensitivity is restricted to the upgoing part of the hysteretic Ca–tension relationship. Relaxation of the heart thus requires a greater reduction of $[Ca^{2+}]$ than previously assumed.

Financial support by the M.R.C. and British Heart Foundation is gratefully acknowledged.

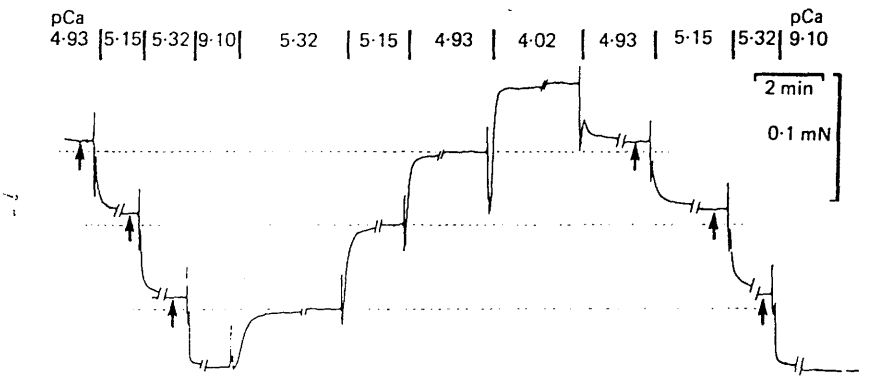


Fig. 1. Response of Triton-skinned rat ventricle trabecula (diam. 130 μm) to $[Ca^{2+}]$ steps (10 mM-EGTA, 2·2 mM-Mg^{2+}). A 2 min section is omitted at each break in the trace. Broken lines show the steady tension achieved when $[Ca^{2+}]$ is stepped upward. Arrows indicate that tension is higher when $[Ca^{2+}]$ is stepped downward. For pCa calculations and solution composition, see Miller & Smith (1984). Sarcomere length 2·2 μm.

REFERENCES

Miller, D. J. & Smith, G. L. (1984). *Am. J. Physiol.* **246**, C160–C166.
Ridgway, E. B., Gordon, A. M. & Martyn, D. A. (1983). *Science, N.Y.* **219**, 1075–1077.

MICRO 86
7-11 July, 1986 Microcomputers VI PM
14th March 1986

Real-time analysis of sarcomere length during activation of chemically-skinned cardiac muscle

V A Moss, D J Miller and C Lamont
Department of Physiology, The University, Glasgow G12 8QQ.

Experiments are being made to study the factors controlling the calcium-sensitivity of cardiac muscle. Sarcomere length (SL) has a major effect in two distinct ways; low SL results in lower Ca-sensitivity, at SL below about 2·2 μm hysteresis in the Ca-sensitivity develops which becomes greater at shorter SL. The SL of cardiac muscle is more varied in a given region of muscle than most skeletal fibres. For these reasons we needed to check that during cycles of activation and relaxation SL distribution did not alter significantly.

Sarcomere lengths are measured by monitoring the differential interference contrast image (optical magnification ~x1000) with a television camera on the microscope. The image analyser (Magiscan 2, Joyce-Loebl) has been programmed to digitise and analyse each frame from a standard 625 line, 25 frame/sec television camera. Frame capture occupies 37 msec, leaving just 3 msec to carry out the initial processing.

The sarcomeres in the muscle appear as a pattern of dark and light bands. A window (typically 512x12 pixels) is specified over the region of interest. For each frame the grey values (64 levels) of the pixels are averaged to a single line of 512 pixels by summing the 12 values at each position along the length of the window. This line is then saved before capturing the next frame. A sequence of up to 1024 frames can be collected (41 sec). To analyse fast movement at 50 frame/sec, each frame is split into its two interlaces by summing alternate pixels to give two lines of pixels from each frame.

The analysis is completed by scanning the stored values to find the positions on each frame of the minima or maxima (middle of the A or I bands respectively). With 35 sarcomeres being measured each sarcomere is only about 15 pixels long. In order to measure small length changes accurately it is therefore necessary to interpolate the positions using the pixels each side of the extreme values.

The pattern of calculated sarcomere positions is verified to correct for any missing or spurious bands. The distribution of sarcomere lengths can be displayed to show the time course of any change.

[*From the Proceedings of the Physiological Society*, 12–14 *September* 1985
Journal of Physiology, **371**, 197*P*, 1985]

Carnosine and other natural imidazoles enhance muscle Ca sensitivity and are mimicked by caffeine and AR-L 115BS

By S. M. Harrison, Christine Lamont and D. J. Miller. *Institute of Physiology, Glasgow University, Glasgow G12 8QQ, U.K.*

Reports of increased contractile protein Ca sensitivity produced by the imidazole-containing compounds caffeine (Wendt & Stephenson, 1983) and AR-L 115BS (sulmazole) (Herzig, Feile & Rüegg, 1981) led us to consider the possibility of a physiological correlate for this action. The chemical relationship of caffeine with other imidazoles in terms of contractile effect was noted by Chapman & Miller (1974). We have, therefore, investigated the effects of naturally occurring sarcoplasmic imidazoles, such as carnosine (Crush, 1970) and *N*-acetyl histidine (Kuroda & Ikoma, 1966) on the Ca sensitivity of chemically skinned cardiac and skeletal muscle (Fry & Miller, 1985). A reduction of 0·15 to 0·2 $\log_{10}$ units in the $[Ca^{2+}]$ required for half-maximal tension is observed with carnosine and *N*-acetyl histidine, equivalent to that reported for similar doses of caffeine. Maximum force is enhanced slightly (to 103·5 % (S.D. 4·5 %) mean of the mean effect in five muscles with 15 mM) by the natural imidazoles and strongly (to over 120 %) by sulmazole (AR-L 115BS, a benzimidazole, at 1 mM) but depressed by imidazole itself (to about 80 % with 15 mM). Ca loading of the sarcoplasmic reticulum (SR) and mitochondria in skinned cardiac fibres is also strongly facilitated by carnosine. Overall, this results in a shift upward and to lower $[Ca^{2+}]$ of the curve relating the $[Ca^{2+}]$ used to load the SR and amplitude of the caffeine contracture; significant loading of the SR occurs at pCa 7·2 with carnosine, whereas virtually none occurs without it. Reported differences in the Ca sensitivity of different skeletal muscle fibre types from studies on skinned fibres can be partially explained by the lack of carnosine from the experimental media: fast fibres have substantial levels *in vivo* (15–60 mM) and slow fibres very little (Crush, 1970). The present findings suggest, for example, that the low carnosine levels in dystrophic muscle (Stepanova & Grinio, 1968) will result in reduced Ca sensitivity, peak force and SR function, each contributing to diminished muscle performance. Since both myofilament regulatory proteins and membrane-bound Ca pumps are affected by cellular imidazoles other Ca-sensitive systems in muscle and non-muscle cells might be similarly influenced.

We thank the M.R.C. and the Smith–Kline Foundation for financial support, and Boehringer–Ingelheim for the gift of AR-L 115. S.H. holds an M.R.C. scholarship.

REFERENCES

Chapman, R. A. & Miller, D. J. (1974). *J. Physiol.* **242**, 615–634.
Crush, K. G. (1970). *Comp. Biochem. Biophys.* **34**, 3–30.
Fry, C. H. & Miller, D. J. (1985). In *Control and Manipulation of Calcium Movement*, ed. Parratt, J., Pp. 87–106. New York: Raven Press.
Herzig, J. W., Feile, K. & Rüegg, J. C. (1981). *Arzeneim.-Forsch.* **31**, 188–191.
Kuroda, Y. & Ikoma, T. (1966). *Science, N.Y.* **152**, 1241–1242.
Stepanova, N. G. & Grinio, L. P. (1968). *Vop. Med. Khim.* **14**, 210–215.
Wendt, I. R. & Stephenson, D. G. (1983). *Pflügers Arch.* **398**, 210–216.

Acknowledgments

I would like to thank my supervisor David J. Miller for his interest and encouragement during the period of this work.

I would also like to thank Professors O.F. Hutter and S. Jennett for the opportunity to study within the Institute and the Medical Faculty (Glasgow University) for the financial support.

Particular recognition must go to Simon Harrison for his invaluable help and patience in introducing me to the mysteries of the 'skinned' fibre and more importantly for his friendship. Dr V.A. Moss for his assistance with the computer-assissted image analysis, which made the assessments of s.l. distribution possible. Francis Burton for help with the computing both relevant and otherwise (special thanks for REF).

My warmest thanks go to all members of the Institute, for their friendship over the past three years, especially to Derek for encouraging me to take myself too seriously (Its worse than it really is!) and to Zaf for laughing at me when I did.

And last but not least thanks to Mum, Dad and Angus for their unerring love and support.

Summary

Force production by cardiac muscle can be altered by changing either the calcium available to the contractile proteins or their sensitivity to calcium. The work of this thesis principally concerns an investigation of factors affecting the calcium sensitivity of the contractile proteins of cardiac muscle.

The calcium sensitivity of the contractile proteins was examined using chemically-skinned muscle mainly from rat heart. Completely and partially skinned preparations were used. The completely chemically-skinned preparations had their cellular membranes disrupted by exposure to the non-ionic detergent Triton-X100. This should leave the isolated contractile proteins in their physiological configuration. The second type of preparation uses saponin-treatment. Saponin is an agent which precipitates cholesterol molecules from membranes. As the sarcolemma is richer in cholesterol than the subcellular membranes, brief exposure of preparations to saponin punctures the sarcolemma while leaving the subcellular membranes intact and functional. In both types of preparation the 'intracellular' conditions are under experimental control since the bathing solution is effectively an extension of the sarcoplasm. These preparations can be used to establish the relationship between 'intracellular' calcium and tension by measuring the tension

produced at a range of free calcium concentrations.

Four general aspects of the calcium sensitivity of the contractile proteins were investigated, (1) hysteresis in the calcium sensitivity of cardiac muscle, (2) the changes in calcium sensitivity with sarcomere length, (3) the effect of altering myofilamental lattice spacing by hypertonic shrinkage and (4) the effect of imidazole-containing compounds on calcium sensitivity

Chapter 1 and 2 report on an investigation of the hysteresis in, and the length dependence of, the pCa-tension relationship. Hysteresis means that a muscle can maintain a higher tension level than it can create de novo at any free calcium level. This phenomenon has only been reported in skeletal muscle to date: this thesis provides the first evidence for hysteresis in heart muscle. It is proposed that hysteresis is a special manifestation of the length-dependence of calcium sensitivity. The calcium sensitivity of the contractile protein's increases as the sarcomere length is increased. I propose that both phenomenon are the result of reduced myofilament separation (i) brought about in the case of hysteresis by force production and (ii) by the change in length in the case of length-dependence of Ca-sensitivity. Experiments designed to test this proposal are described in chapter 2. Lattice spacing was altered independently of force production or sarcomere length change by the use of hypertonic shrinkage techniques. This technique involves addition of long chain polymers (Dextran M.Wt.>40,000) to the bathing solutions

Discussion

General Introduction

Most of the work of this thesis involves the use of chemically skinned cardiac muscle to investigate the calcium sensitivity (Ca-sensitivity) of the contractile proteins, that is the relationship between the free calcium concentration ($[Ca^{2+}]$) and tension production.

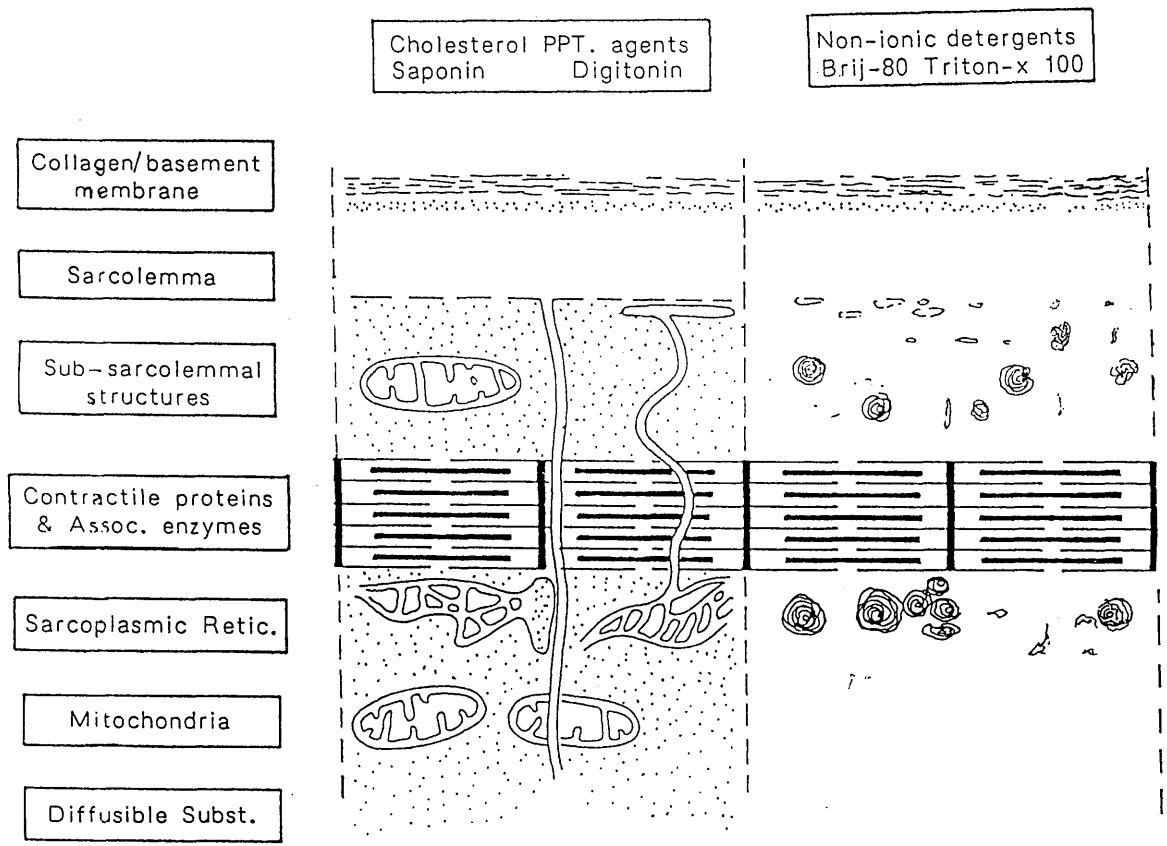
Three areas have been investigated: (1) Hysteresis in the pCa-tension relationship, (2) the length dependence of Ca-sensitivity and (3) the influence of imidazole-containing compounds.

The preparations used were cardiac trabeculae 'skinned' by chemical means. Two types of chemical skinning were employed, complete chemical skinning using the non-ionic detergent Triton-X100, and selective chemical skinning using the cholesterol-precipitating agent saponin. Figure 1 schematically illustrates the two types of preparation.

The right hand panel illustrates the Triton-treated state. In this condition all the cellular membranes are disrupted (Heleius & Simons, 1975) by the detergent leaving essentially the isolated contractile proteins, in what is presumably their physiological conformation (Miller, Elder & Smith, 1985)

Figure 1

A diagram illustrating the muscle in the saponin- and Triton-treated states. (See text for details).



The left hand panel illustrates the saponin-treated state. Saponin precipitates cholesterol molecules from membranes. The sarcolemma is richer in cholesterol than the intracelluar membrane systems, so brief exposures to saponin render the fibre's sarolemma permeable to small ions and molecules while leaving its sarcoplasmic membrane systems functionally intact (Endo & Kitazawa, 1978; Harrison, 1985). The saponin-treated preparation allows investigation of calcium uptake and release by the presumably functionally intact subcellular organelles. The calcium released by the fibre's organelles can be assessed indirectly as the force produced by the fibre.

The importance of the calcium sensitivity of the contractile proteins to the contractile force of the heart

It is generally assumed that all the myocardial cells contract during every cardiac cycle. Modulation of force must occur at the cellular level, unlike skeletal muscle where increased force is thought to be predominantly achieved by recruitment of motor units. At the cellular level, force production can be modified by altering either the availability of calcium to the contractile proteins or the relationship between calcium and tension, loosely termed Ca-sensitivity. The work of this thesis involves investigation of some of the factors which modify the muscle's apparent Ca-sensitivity, that is to say alter the relationship between intracellular calcium and tension

production.

Changes in Ca-sensitivity can be brought about by a multitude of factors including the organisation, the phosphorylation state and the ionic environment of the myofilaments.

In this thesis the organisation of the contractile filaments with respect to length and lattice spacing have been investigated in Section 1. The influence of imidazole containing compounds are investigated in Section 2.

The relationship between calcium and tension

The role of calcium in activation of muscle contraction is well established (Heilbrunn, 1940; Ebashi, Endo & Ohtsuki, 1969). In striated muscle, this occurs through calcium binding to troponin. This makes possible a series of steps leading to the interaction of the myosin cross-bridges with actin, producing force. The relationship between the intracellular free calcium concentration and the force produced has been widely investigated using skinned muscle preparations. With these preparations the 'intracellular' $[Ca^{2+}]$ can be varied, allowing investigation of the relationship between calcium and tension.

The Ca-tension relationship obtained from skinned fibres can be plotted in several ways, three of which are illustrated in figure 2. The data are frequently fitted to the Hill equation.

$$\frac{C}{C_{max}} = \frac{K_{Ca}[Ca^{2+}]^h}{1+K_{Ca}[Ca^{2+}]^h}$$

Where C is steady-state force; C_{max} is maximal calcium activated force, K_{Ca} (units M^{-h}) is an apparent affinity constant and 'h' is the Hill coefficient.

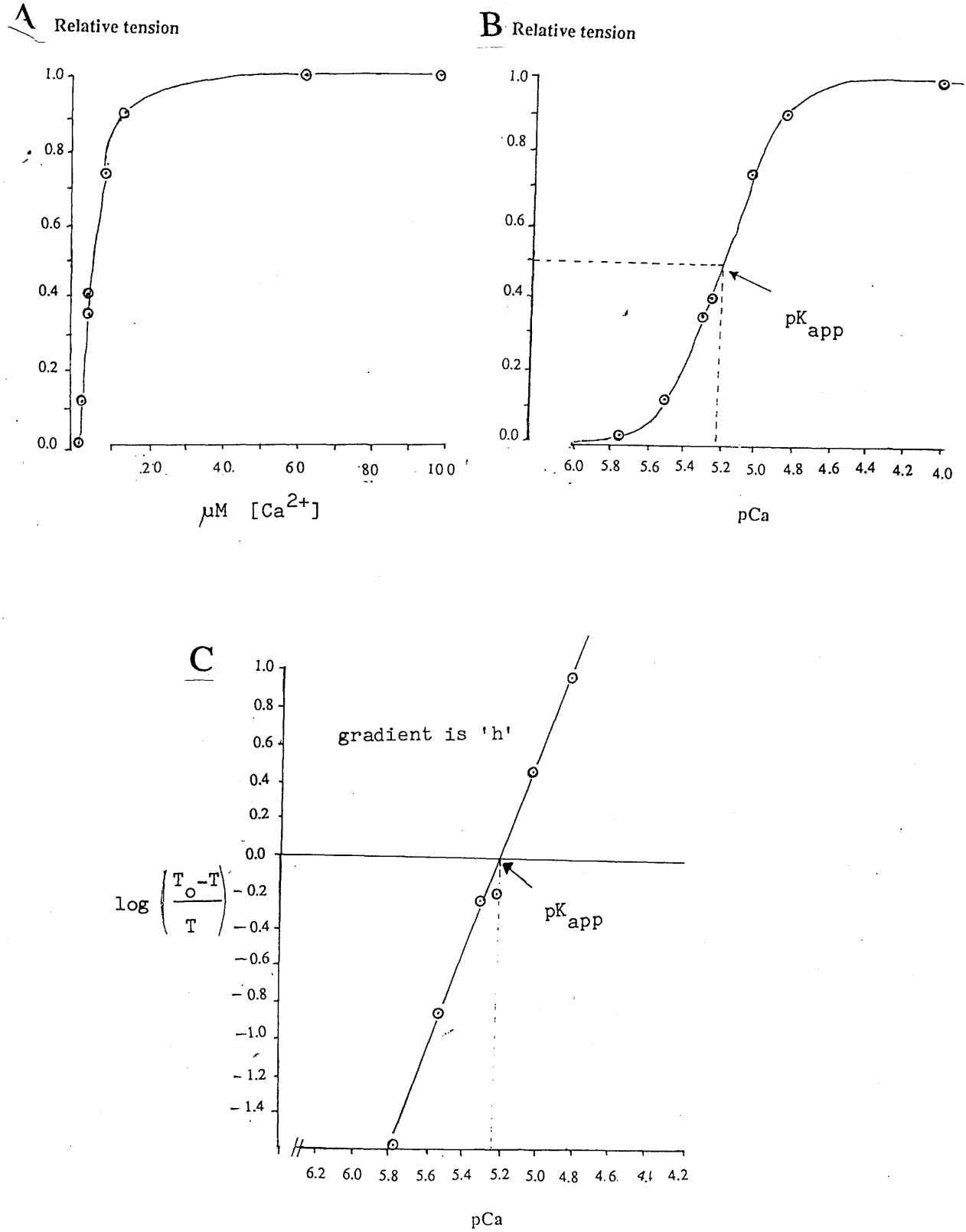


Figure 2

A typical set of data (steady-state tension at a selection of calcium levels) plotted in three ways. In (A) the data are plotted as relative tension against $[Ca^{2+}]$, in (B) as relative tension against pCa ($-\log[Ca^{2+}]$) and in (C) as a Hill plot, $\log (T_o - T/T)$ against pCa, where T is the relative tension and T_o is the maximum tension produced.

This equation provides a method of describing the Ca-tension relationship as two numbers; the K_{Ca} and the Hill coefficient ('h'), making quantification of the differences between curves simpler and objective. However, problems arise when attempts are made to correlate these parameters (K_{Ca} and 'h') with function. Statements such as.... "K_{Ca} represents the overall apparent association constant of the tension modulating site and is the product of the individual affinity constants for the calcium binding sites. 'h' the Hill coefficent of the curve gives an estimate of the minimum number of Ca^{2+} ions which combine with each modulating site." are found frequently in the literature. Unfortunately this may not be true as factors other than Ca-binding have been proposed to influence the position and nature of this pCa-tension relationship.

For example, Brandt, Cox, Kawai & Robinson (1982) propose the slope and position of the pCa-tension relationship relative to the underlying pCa-calcium binding relationship to be influenced by ratio of the c-b cycling time (the time a c-b takes to cycle back to the relaxed state once a cycle is initiated) to the mean lifetime of the troponin C-Ca^{2+} complex (TnC-Ca^{2+}). They propose that the pCa-tension relationship can be shifted to the left (i.e 'increased Ca-sensitivity') by increasing the time c-bs spend attached. If calcium remains bound to TnC with a mean lifetime that is shorter than the duration of a c-b cycle, e.g. the concentration of calcium required to produce half maximal

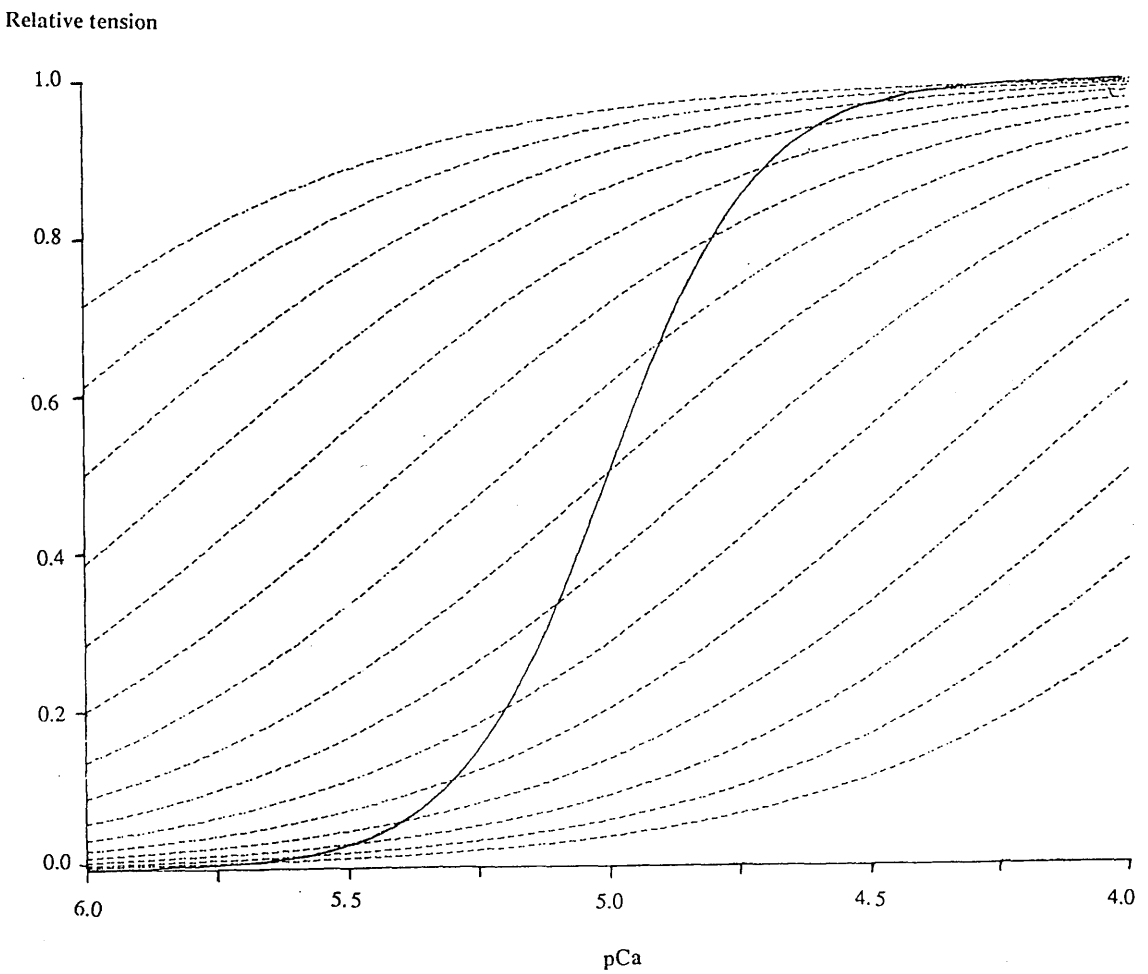


Figure 3

The dashed lines represent a family of hypothetical pCa-tension curves parallel to the Ca-binding curve assuming cardiac TnC binds one calcium (i.e. 'h' of 1). The appropriate curve would depend upon the c-b attached time (the average lifetime of the TnC-Ca^{2+} complex is assumed to be constant). The longer the c-b spends attached the further to the left would be the appropriate curve. If c-b attached time increased as tension increased the net result would be for the pCa-tension relationship to pass from one curve to the next on the left as tension increased, overall this would result in a curve with a steeper slope and a higher apparent calcium sensitivity i.e a pCa-tension relationship like the solid line.

force will be less than that to half-saturate the regulatory sites on TnC: the K_{Ca} of the pCa-tension relationship will be higher than that for the Ca-binding curve. This notion was based on the observation of Johnson, Charlton & Potter (1979) that calcium remains bound to TnC for a period shorter than the mean c-b cycle time. If c-b cycle time is constant the pCa-tension relationship will parallel the Ca-binding curve and the position relative to the binding curve will depend on the (constant) ratio between cycle time and calcium bound time. However, if some factor does change the c-b cycling rate, e.g. if the time it takes a c-b to complete a cycle increases progressively with increased tension, the experimentally observed pCa-tension relationship would be a composite curve produced by shifting through a family of curves. This family consists of curves all parallel to the Ca-binding curve but with midpoints shifted to the left depending on the c-b cycling time. A progressive decrease in the rate of c-b cycling would result in shift to the left in the curve relating calcium to tension, say as tension increased, the net result being an apparently greater Ca-sensitivity and steepening to the pCa-tension relationship i.e. a value for the Hill coefficient greater than that from the slope of the binding curve on which the relationship is based. This idea is illustrated schematically in figure 3. While it is logically correct one critisism of Brandt et al's analysis is that there is evidence for striated muscle and cardiac musle in particular that V_{max} increases with $[Ca^{2+}]$ (Herzig & Ruegg, 1980;

Podolin & Ford, 1983). Since V_{max} is usually taken as an index of c-b cycle rate, this suggests that the prolonging of attached time required in Brandt et als scheme may not occur. However, the relationship of V_{max} to attachment time is obscure.

The apparent Ca-sensitivity of the contractile proteins has been examined in this project in terms of changes in steady-state pCa-tension relationships. This section has been inserted to emphasise that although the Hill equation provides a method of describing the results, it does not necessarily provide information about the number of Ca-binding sites, their degree of cooperativity or their overall affinity for calcium as is sometimes suggested. Frequently in the literature a shift in the position of the pCa-tension relation is refered to as a change in the Ca-sensitivity of the preparation and by implication a change in the Ca-binding to troponin. However, if factors other than Ca-binding alter the position of the pCa-tension relation (e.g. the influence of c-b cycling time changes) changes in the relationship do not necessarily reflect changes in Ca-binding as the term Ca-sensitivity implies. This is why the term 'apparent Ca-sensitivity' has been employed in this study.

For the underlying mechanism of shifts in the position of the pCa-tension relation of skinned fibres to be understood corroborative evidence from other techniques is required, e.g. Ca-binding studies.

Materials and Methods

Male Wistar rats (145-155g.wt.) were killed by a blow to the head. The heart was rapidly excised and flushed through with Ringer's Solution at room temperature (20-24°C): comprising NaCl 150mM, KCl 5mM, $MgCl_2$ 1mM, $CaCl_2$ 2mM, and 5mM HEPES brought to a pH_a ($-\log_{10}[H^+]_{activity}$) of 7.00 with NaOH (approximately 1mM).

The atria were removed and the right ventricle opened, cutting as near as possible to the interventricular septum. Most experiments were carried out on small, free running trabeculae isolated from the right ventricle. The majority of preparations were taken from the base of the right ventricle near the valve. The trabeculae used were generally 1-3mm long with an average diameter of 70-120um when mounted at a s.l. of approximately 2.0μm.

In some experiments, frog (*Rana temporaria*), cod (*Gadus morhua*), shore crab (*Carcinus maenus*) and clawed toad (*Xenopus laevis*) skeletal fibres were used. With the batrachians, sections of single fibres, or small bundles of fibres were isolated from the sartorius or semitendinosus muscles. Single fast fibres or small bundles of two to six slow fibres were dissected from the myotomal muscle of the cod. All the skeletal fibres were dissected and mounted under light liquid paraffin.

All preparations were mounted for isometric force measurement in the assembly illustrated in figure 1A.

Mounting and force measurement

The preparations were attached to an Akers AE 875 transducer at one end and to the fixed point at the other by snares (see figure 1A).

The snares were made from stainless steel tubing (outside diameter 200μm, wall thickness 50μm, Goodfellows metals limited, no.FE227105/1, Cambridge). 3-4cm lengths of this tubing were threaded with a nylon monofilament (diameter 25μm) creating a snare which could be tightened from the top. One of these tubes was attached to the fixed point (a steel rod) and an other to the transducer. Three additional lengths of this tubing were glued to the side of the snare tube providing added rigidity in the plane in which the muscle exerts force. This method of stiffening the mounting kept it narrow in the plane of the long axis of the muscle permitting entry of the mounted muscle to the adapted microscope stage of the Differential Interference Contrast (DIC) microscope (see figure 1B). This mounting method means that only the snare tubes enter the experimental solutions minimising the solution change artefacts and meniscus effects.

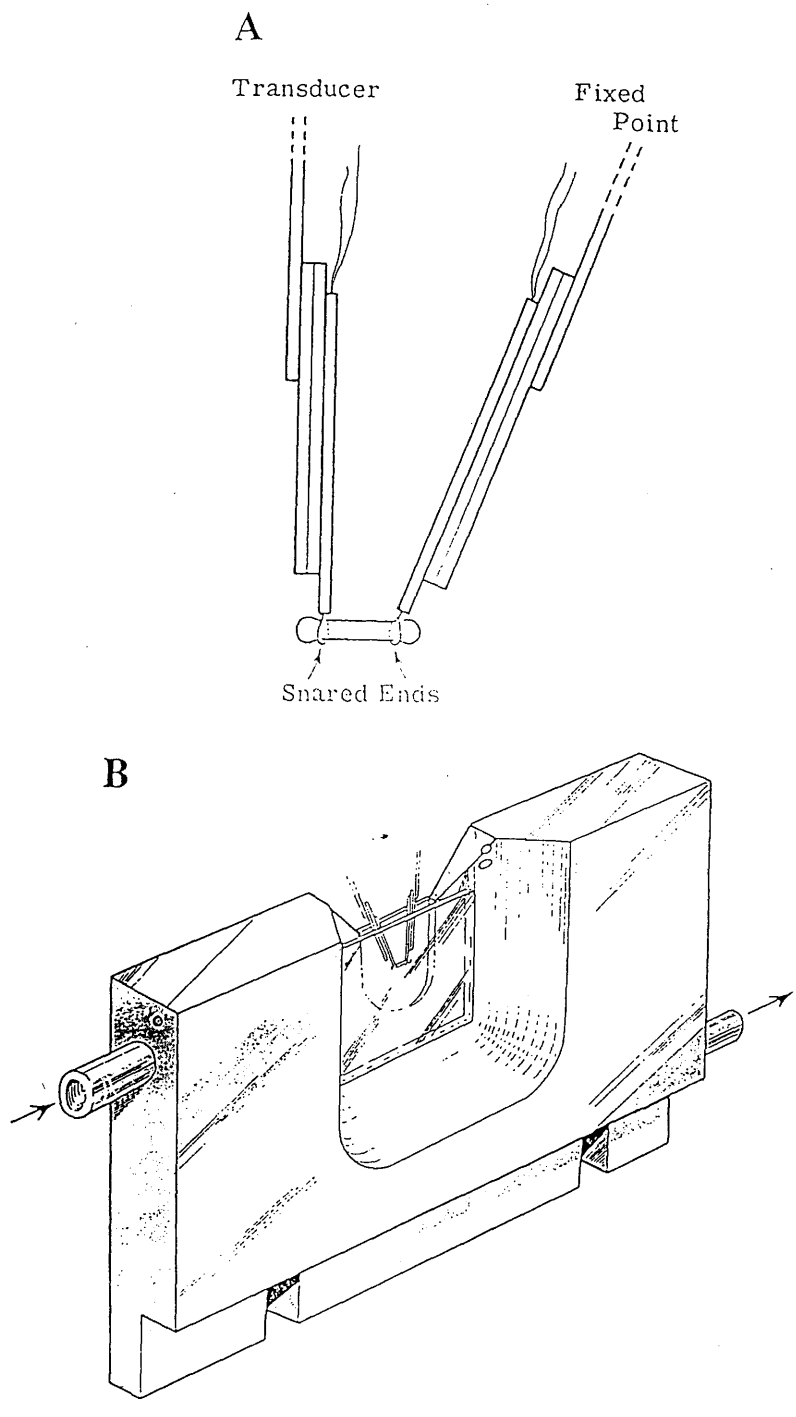


Figure 1

(A) Preparation mounting and transducer assembly. Schematic view of the mounting for preparations. Nylon mono-filament snares hold the muscle. The tube diameter (200µm) is infact generally twice that of the preparation. (B) Chamber for microscopic observation of a mounted trabecula. Schematic view of the chamber used in the DIC microscope for s.l. measurements. The gap of 1.5mm between the glass faces (two coverslips) allows the mounting to gain access to the bath while being narrow enough for the optical requirements of high magnification DIC. The stage can be temperature controlled by flowing water through the jacket (arrowed). The fluid contained in the observation chamber can be changed via tubes which open at its lip and base not shown.

The compliance of the mounting was assessed by measuring the displacement of the pins when known weights were applied. From these measurements a maximum of 0.3% change in overall muscle length would be expected with a preparation producing 50mg.wt. (5μm/50mg.wt.)

The transducer and the fixed point were both mounted on Narashige MM3 micromanipulator allowing movement in three planes. This permitted accurate control of the muscle length, which was crucial as sarcomere length (s.l.) was frequently one of the experimental variables.

Measurement of sarcomere length

Precise knowledge of the s.l. is critical in these experiments. It is well established that the contractile protein's calcium sensitivity is increased at longer s.l. (Endo, 1972; Endo, 1973; Hibberd & Jewell, 1982), and the extent of Ca-uptake and/or release by the SR in skinned preparations is length sensitive (Fabiato, 1980).

Sarcomere length was measured by Differential Interference Contrast (DIC) light microscopy. This microscope method allows a good check on local variations in sarcomere pattern, as well as enabling one to assess the dimensions of the muscle and any damage.

The dimensions of the preparation were routinely determined by measuring the diameter at the middle and towards each end of the preparation, in two planes at right angles. Values quoted in the text are averages of these values.

The microscope used was a modified Vickers M-17, using the DIC system after Smith (1969). The microscope lies supine to facilitate access to the space between the objective and the condenser. The stage has been replaced by the chamber illustrated in figure 1B.

The solutions in the chamber (volume 0.3ml) can be changed by syringes connected to small tubes which open at its base and lip (not shown). The relatively low contrast of the DIC image obtained from cardiac muscle was enhanced by viewing the image on a monochrome TV monitor. In all experiments, the s.l. was adjusted to the desired value while viewing the muscle in this chamber; the preparation was then transferred to the solution change system. From time to time during the experiment the s.l. was rechecked; usually no significant adjustments were required. The graticule in the microscope was calibrated with diffraction gratings (15,000lines/inch ~1.69μm spacing; 4,000lines/inch and 8,000lines/inch) viewed at the same magnification as the muscle. The grating was positioned where the mounted muscle would be viewed in the chamber shown in figure 1B.

In some experiments, s.l. was monitored continuously together with tension throughout a series of solution changes. The image was recorded on a standard domestic video recorder for subsequent study by computer-assisted image analysis (see Chapter 1, 'Analysis of sarcomere length changes by computer-assisted image analysis').

Solution exchange

A computer controlled bath system was used to change the bathing solution of the preparation. This system has been described in detail elsewhere (Miller, Sinclair, Smith & Smith, 1982; Smith, 1983).

The method is a development of that originally described by Ford & Podolsky (1972) and Ashley & Moisescu (1977). The experimental solutions were carried in a series of wells (5ml) in a perspex block. A solution change is effected by lowering the block and sliding it horizontally to bring the new solution under the muscle. The block is then raised to re-immerse the muscle. These movements are made by two stepper motors operating under microprocessor control and take 1-2s per solution change. The emergence of the muscle though the solution meniscus, as the bath is lowered, removes any significant adhering droplets. Checks confirm that the volume transferred from bath to bath are strictly negligible (Dr.G.L.Smith, personal communication). The solutions are stirred continuously by a small paddle. This system

permitted accurate timing of solution changes in pre-programmed runs and ensured that the muscle was immersed to exactly the same depth in each bath which is essential for reproducibility of tension levels.

Chemical supplies

Most of the chemicals were obtained from the Sigma Chemical Company Ltd, England, that is; carnosine, N-acetyl histidine, caffeine, creatine phosphate (CrP), adenosine triphosphate (ATP), saponin, creatine phosphokinase (CPK) and Ethylene glycol bis (B aminoehtyl ether)-N-N-tetra acetic acid (EGTA).

In most experiments disodium ATP and CrP were used, these were cheaper and more stable than the potassium or Tris salts commercially available. The disodium ATP was 'obtained from equine muscle prepared to be substantially vanadium free'. A few experiments were carried out with physiological sodium levels, in particular those involving the subcellular organelles which are highly sodium sensitive (Harrison 1985, Fry & Miller 1985). In these experiments $Tris_2CrP$ and K_2ATP were used.

The HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid) and Triton-X100 (spec. purified for membrane research) were obtained from Boehringer Mannheim GmbH - West Germany

The HDTA (1,6-Diaminohexane-N,N,N',N'-tetraacetic acid), imidazole, 1-methyl imidazole, 2-methyl imidazole and 4(5)-methyl imidazole were obtained from Fluka AG Switzerland

HCl, KOH, and NaOH 'Convol' concentrated volumetric solutions, 1.0 Molar $CaCl_2$ and $MgCl_2$ Stock solution, NaCl, KCl 'AnalaR'grade BDH Chemical, England.

Dextrans T9, T70 and T500 were obtained from Sigma and Pharmacia. For details see Chapter 2.

Solution composition

The solutions were made by mixing appropriate volumes of concentrated stock solutions, that is, 1M KCl, 100mM $MgCl_2$, K_2EGTA, $K_2CaEGTA$, K_2HDTA, 500mM HEPES and 50mM Caffeine.

The K_2EGTA and K_2HDTA were made by dissolving the required amount of EGTA or HDTA (with the appropriate compensations for the EGTA purity) in twice the amount of KOH. The solutions were made up to near final volume with distilled water. They were then heated (to 60°C) and thoroughly stirred for around one hour, until all the EGTA was dissolved. The solution was then made up to final volume with distilled water. Ca_2EGTA was made in a similar manner but equimolar $CaCO_3$ was added with the EGTA to the KOH. CO_2 is released leaving Ca_2EGTA. This required longer heating and stirring to ensure that all the CO_2 had been released. The ATP and CrP salts are added as powder. The final

composition of the solutions are listed in tables 1, 2 and 3 for rat cardiac, or frog, toad and crab skeletal muscle respectively.

The water was single distilled as Glasgow's water supply is very low in mineral and organic content making double distilling unnecessary.

TABLE 1 Solutions for rat cardiac muscle (concentrations in mM)

Solution	10Activating	10Relaxing	0.2Relaxing	Caffeine
KCl	100.0	100.0	100.0	100.0
$MgCl_2$	7.0	7.0	7.0	7.0
K_2EGTA	~	10.0	0.2	0.2
K_2HDTA	~	~	9.8	9.8
Ca_2EGTA	10.0	~	~	~
Na_2ATP	5.0	5.0	5.0	5.0
Na_2CrP	15.0	15.0	15.0	15.0
Hepes	25.0	25.0	25.0	25.0
Caffeine	~	~	~	10.0
KOH*	15.0	15.0	15.0	15.0

Table 2 Solutions for Frog skeletal muscle (concentrations in mM)

Solution	10Activating	10Relaxing	0.2Relaxing	Caffeine
KCl	60.0	60.0	60.0	60.0
$MgCl_2$	7.0	7.0	7.0	7.0
K_2EGTA	~	10.0	0.2	0.2
K_2HDTA	~	~	9.8	9.8
CaK_2EGTA	10.0	~	~	~
Na_2ATP	5.0	5.0	5.0	5.0
Na_2CrP	15.0	15.0	15.0	15.0
Hepes	40.0	40.0	40.0	40.0
Caffeine	~	~	~	10.0
KOH*	15.0	15.0	15.0	15.0

Table 3 Solutions for Crab skeletal muscle
(concentrations in mM)

Solution	10Activating	10Relaxing	0.2Relaxing
KCl	100.0	100.0	100.0
$MgCl_2$	7.0	7.0	7.0
K_2EGTA	~	20.0	0.2
K_2HDTA	~	~	19.8
CaK_2EGTA	20.0	~	~
Na_2ATP	5.0	5.0	5.0
Na_2CrP	15.0	15.0	15.0
Hepes	50.0	50.0	50.0
Caffeine	~	~	~
KOH*	14.0	14.0	14.0

*KOH required to bring the pH to 7.00 varied slightly (10-20mM)

All solutions contained 0.1mg/ml of CPK, which was added to each well in the final solution tray.

In those experiments where tonicity was altered, Dextran solid was added to the final solution listed above. This maintained constant volume molality for Ca^{2+} (and of the other ionic species), since the volume occupied by the aqueous phase remains constant (Moore, 1976; Godt & Maughan, 1981). Dextran was used as it is biologically inert and has no charge so does not produce ionic strength complications.

The calcium concentration range was achieved by mixing the 'activating' and 'relaxing' solutions in various ratios.

In some experiments 'oscillation solutions' were used. These were 0.2 Relaxing solutions with added calcium (added as $CaCl_2$). As the EGTA in 0.2 Relaxing solution is not saturated the addition of calcium resulted in hydrogen ion release. At this pH almost exactly $2H^+$ions are released per Ca-bound (Smith & Miller, 1985). KOH was, therefore, added at twice the concentraton of the calcium, so maintaining the pH of the final solution. These 'oscillation' solutions were used to provoke CICR, cycles of Ca-release and Ca-uptake occurring when the saponin-treated preparation was exposed to these weakly buffered solutions with calcium levels producing an intermediate level of activation.

pH

The activity of the contractile proteins and subcellular organelles (Fabiato & Fabiato, 1978c) and the metal ion binding constants of EGTA and ATP are strongly influenced by hydrogen ion concentration (Miller & Smith, 1984). This makes the pH, and consistency of pH, critical in the solutions used in this work.

HEPES, a Good's buffer (Good, Winget, Winter, Connolly, Izawa & Singh, 1966), was used in most experiments. The useful buffering range of HEPES 6.5-8.5 (pK_a=7.5 at 20°C I_e=0.1M), its low metal binding contants, and lack of biological side effects make it very suitable. Carnosine and N-acetyl histidine which are also pH buffers were also used in some of the experiments. For further discussion of these points see Chapters 3 and 4.

Measurement of pH

Illingworth (1981) pointed out that liquid-junction artefacts are a widespread source of substantial errors in the majority of general lab pH determinations. The ceramic plugs, a universal feature of combination electrodes, are the source of these errors.

The various combination electrodes in use in the laboratory were tested using a protocol suggested by Illingworth (1981). The electrode was immersed in standard pH (BDH) 4.0 solution with gentle stirring until the reading stabilised; the meter was set to read pH 4.00. It was then immersed in BDH pH 9.00 and the slope adjustment of the meter set to give a reading of 9.00. pH 7.00 solution was used to check the calibration. If the pH 7.0 solution did not give a reading of 7.00 +/- 0.01units the procedure was repeated. Some of the electrodes tested did not give stable readings and were abandoned at this stage.

With the newly 'calibrated' electrode, buffers 1,4,6 and 9 (see table 3) were tested in random order, with rinses in distilled water between measurements. Checks for zero shifts were made by frequently returning to buffer 5 (Table 4)

Table 4 (after Illingworth (1981))

Buffer no.	Composition	pH
1	50mM-Potassium hydrogen phthalate	4.040
4	250mM-KH_2PO_4/250mM-Na_2HPO_4	6.520
5	25mM-KH_2PO_4/25mM-Na_2HPO_4	6.865
6	2.5mM-KH_2PO_4/2.5mM-Na_2HPO_4	7.065
9	10mM-Disodium tetraborate	9.175

Using this test only two of the six electrodes tested gave results close to those expected with changes in the ionic strength. The unpredictable behaviour of most of the combination electrodes when the ionic strength was changed puts into serious doubt the calibration of the electrodes for 0.2M biological samples when the electrodes were calibrated (as is standard procedure in most laboratories) using the 0.05M commercial standards. The results improved when a separate reference cell (a calomel electrode) was employed (Corning, Calomel Reference Electrode 003 11 602h). Checks of this kind were repeated, and electrodes replaced or cleaned as necessary, at regular intervals throughout this study.

pH standard

To minimise the inconvenience associated with the accurate measurement of pH, the electrode (with separate reference) was routinely calibrated against a standard appropriate to the media used in these experiments. This comprised a solution of 25mM HEPES, 197mM KCl and 6.003mM KOH (from fresh 1M titration standard, BDH) which we calculate to have an ionic strength on 0.206M, pH_c of 7.00 and a pH_a of 7.134. The thermodynamic pK_2 of 7.629 at 20°C reported by Vega and Bates (1976), was used, giving a pK_{app} of 7.494 at I_e =0.2M. The use of this standard meant that the experimental media were subjected to a null detector method of pH measurement.

Ionic strength

Ionic strength (I) has been defined according to Equation 1 for the work reported here since Miller & Smith (1985) found that it gives a better description of solutions of the type used here than the more common definition of I (see also, Smith & Miller (1985) and Smith (1983) for a more detailed analysis of these points. This approach has been corroborated by Fink, Stephenson & Williams, 1986) Ionic strength is defined as the total of ionic equivalents, Ie:

$$I_e = 1/2 \sum C_j z_j \qquad \text{Equation (1)}$$

where C_j is the concentration of the jth ionic species, z_j is it valency (Miller & Smith 1985). In accordance with Bates, Roy & Robinson (1973) the zwitterion of HEPES (the predominant form at $pH<pK_2$) is assumed to have no effect on ionic strength. The basic solutions outlined in table 1 have an ionic strength of about 0.19M on the basis of Equ. 1.

Purity of the EGTA.

The question of the purity of commercial supplies of EGTA has been extensively investigated (Miller & Smith, 1984; Smith and Miller 1985; Bers, 1982) the solutions used in this project take full account of these findings.

It is standard practice in this laboratory to prepare calcium buffers by mixing two solutions, Ca-free EGTA and Ca-EGTA (i.e. the 'relaxing' and 'activating' solutions in tables 1 and 2). The latter is produced by mixing exactly equimolar $CaCO_3$ and Ca-free EGTA.

EGTA binds metal ions, other than calcium, to various degrees. The affinity constants for EGTA and the other ligands in the solutions (ATP, CrP, Carnosine) must be known in order to assess the amount of calcium bound to the EGTA and, therefore, to gauge the free calcium concentration of the EGTA containing solutions. The solution chemistry of EGTA for physiologically relevant metal ions has been described in detail (Smith 1983). A

computer program written by G.L.Smith and D.J.Miller was used to calculate the free calcium concentration of all the solutions used in this thesis (see figure 2).

In this program the affinities of EGTA and the other ligands for calcium and the other metal ions have been incorporated, giving a complete profile for free metal ions concentrations and ligand-metal concentrations in the solutions. The binding constants used in the REACT program are detailed in table 5 and corrected for ionic strength, pH and temperature. (see Miller and Smith 1984, Smith and Miller 1985).

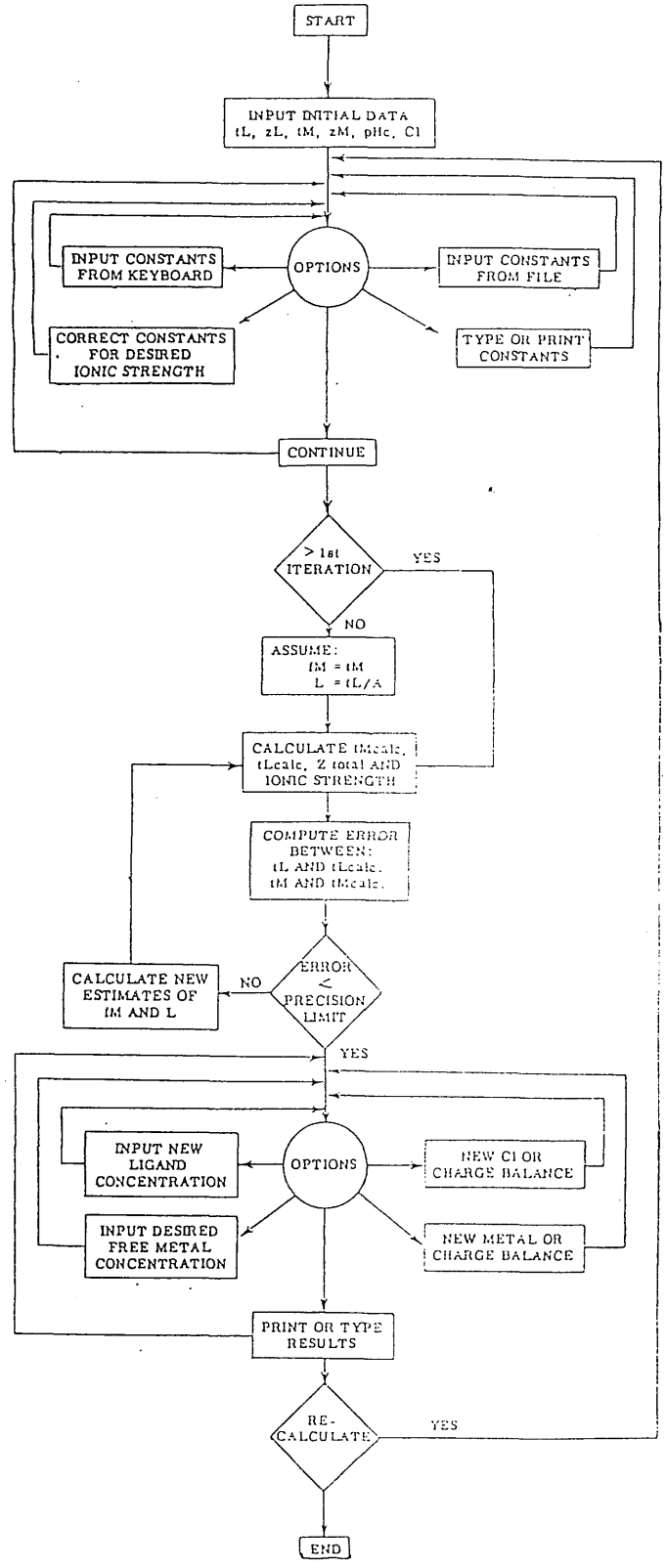


Figure 2 Flow diagram of the REACT progam.

The REACT program (Smith, 1983) was used to calculate the concentration of free metal ions in all the solutions used in this thesis.

TABLE 5 $\log_{10}$ of the stoichiometric affinity constant I=0.1, T=20°C.

		Hydrogen Constants	Ca^{2+}	Mg^{2+}
EGTA	K1	9.625	11.118	5.509
	K2	9.000	5.300	3.470
	K3	2.813		
	K4	2.117		
ATP	K1	6.950	3.982	1.800
	K2	4.050	4.324	2.740
	K3	1.000	0.903	
	K4	1.000	0.944	
CrP	K1	12.000	1.150	
	K2	4.508	1.300	
	K3	2.700		
Carnosine	K1	9.320	3.220	
	K2	6.750	3.100	
HEPES	K2	7.629		
HDTA	K1	10.810	4.600	3.700
	K2	9.790	4.800	3.660
	K3	2.700		
	K4	2.200		

From Smith and Martell (1974) except, EGTA (Smith and Miller 1985).

Calcium contamination

An estimate of the calcium contamination in the nominally calcium free solutions was derived by a pH-metric method. Typical results are plotted in figure 3.

In this example both solutions were initially titrated with KOH to a pH of about 9.0. At the start of the titration the pH falls, as H ions are released for calcium bound. Around 1.5 H^+ ions (Smith, 1983) are released for each calcium ion bound at this pH. An end point can be distinguished from the plot of volume of EGTA added against pH. At this point the contaminating calcium is completely bound. The subsequent increase in pH is simply due to the higher pH of the titrating solution. From this type of measurement values of 20μm contaminating calcium were obtained from relaxing solutions without their EGTA or full pH buffer capacity.

Carnosine, HDTA and Dextran were also checked for possiible calcium contamination. None of these compounds contained any detectable calcium contamination (i.e. <1μm for a 50mM solution).

Knowledge of the contamination calcium level is essential in calculations of the free calcium, especially the calcium 'free' relaxing solutions. There remains a degree of uncertainty about the free calcium levels, especially in those solutions in which the EGTA concentration is low. In these, even a small change in the contamination calcium level will alter the free calcium

substantially.

Free calcium levels calculated using React 3 under the conditions are detailed on the right of table 6.

TABLE 6

Free Calcium levels Calculated using REACT 3

Ratio Activating:Relaxing	pCa
1:1	6.246
3:1	5.746
5:1	5.524
8:1	5.319
10:1	5.222
15:1	5.048
25:1	4.836
Activating	4.199
Full	3.997
Relaxing	8.927
0.2 Relaxing	7.184

Assuming 20μm Calcium contamination

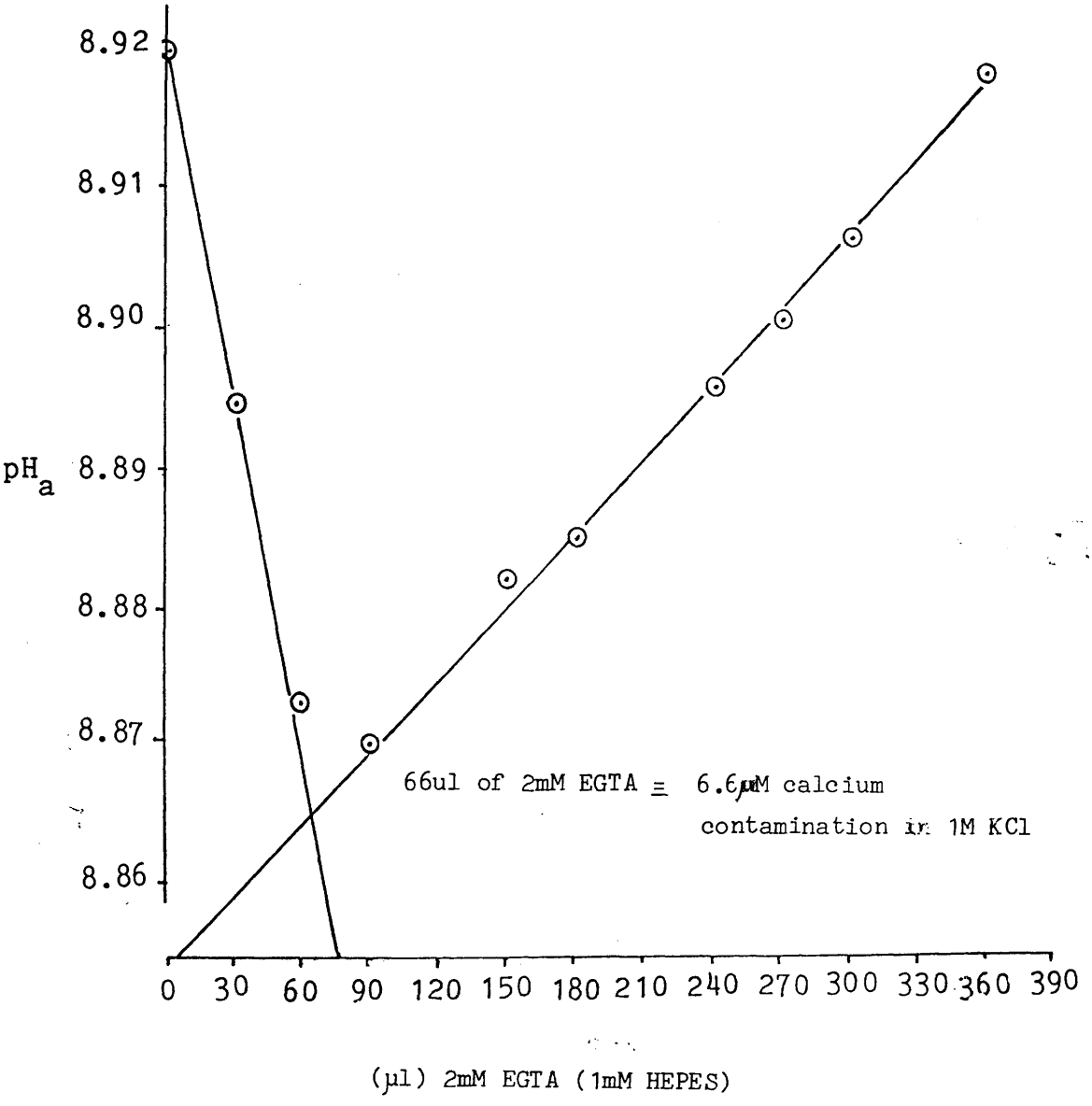
I=0.19M
K^+=0.14M
Na^+=0.04M
Mg^{2+}=0.007M
Cl^-=0.114M
pH_c=6.866
T=20°C

Full is an Activating solution with 0.1mM excess free calcium added to it.

Chemical skinning procedure

The mounted muscle was initially exposed to 'relaxing' solution (see table 1) including either the cholesterol precipitating agent saponin (50μg/ml) or Triton X-100 (1% v/v) for 20-30minutes. The chemical skinning agent was then removed by immersing the muscle in the relaxing solution before the first test calcium was applied.

Figure 3 Titration of 20ml of 1M KCl, 1mM HEPES with 2mM K_2EGTA, 1mM HEPES.



Data handling

The tension signal was routinely digitised (12bit) at appropriate rates (50-200ms per sample). The signal was prefiltered to avoid aliasing errors with an active filter (roll-off 12dB per octave, 3dB cut off at 15 or 25Hz) and amplified to use the full dynamic range of the A-D converter. Experimental data and calibration signals were stored on disc on a PDP-11 34 computer for subsequent analysis. A continuous chart recording (Linseis 1800, full scale deflection frequency response flat from DC to 1Hz, 3dB cut off at 4Hz) was also made. Tension responses are shown calibrated in either or both absolute force and relative force. For the latter, tension was normalised to maximum calcium activated force (C_{max}) which was determined at intervals throughout the experiment. This was done using either activating solution or activating solution with excess calcium added ('Full', table 6). Provided that relatively short time intervals elapsed between normalising maxima, a linear interpolation was found to describe adequately the decline of tension. The mean rate of decline of peak force for 10 preparations was 0.178%/min (range 0.024-0.045%); more rapid decline was taken as indicative of damage and the results were not used for quantitative work. This figure compares well with a mean of 1.2% per minute reported by Jewell and Kentish (1981) for Triton- treated rat ventricle preparations; the reduction in the rate of decline they report with dithiothreitol (to 0.12%) suggests that our preparations maintain contractile performance

well, despite the absence of SH protecting agents.

Hill equation

In these preparations, the pCa-tension relationship, is well described by the Hill equation (Miller and Smith, 1985; Fry & Miller, 1985).

$$\frac{C}{Cmax} = \frac{K_{Ca} \cdot [Ca^{2+}]^h}{1 + K_{Ca} \cdot [Ca^{2+}]^h} \qquad \text{Equation (2).}$$

Where C= steady-state tension, Cmax is maximum calcium activated force. K_{Ca} (units M^{-h}) is an apparent affinity constant and 'h' is the Hill coefficient. The curves were fitted by a least squares fitting procedure (after Levenberg & Marquat see Brown and Dennis 1972). This provides a two value description of the curve relating pCa-tension making quantification of differences in the relationships simpler.

In the text K_{app} (units M^{-1}) has generally been used as an alternative to K_{Ca}. K_{app} is numerically equal to the $\sqrt[h]{K_{Ca}}$. K_{app} is the reciprocal of the calcium concentration required to activate the preparations half maximally. See figure 2 of the introduction

pK_{app} is the pCa for half maximal activation.

Statistics

In table 1.1 a statistical method of paired comparison was applied to the pK_{app} up and down, (determined from the pCa-tension relationship as $[Ca^{2+}]$ was stepped, upwards and downwards respectively) and the Hill coefficients up and down. The null hypothesis was that 'up' and 'down' values are the same, i.e. $pK_{app}up=pK_{app}down$. To test this the difference between the pairs of data was compared to zero. The null hypotheses was rejected when the mean difference between the pairs of data was significantly different from zero at the 5% level. The statistic used was the t-test. This test is based on the assumption that the data are normally distributed. However, the t-test is robust i.e. not highly sensitive to departures from the assumption of normality. The robust nature of the t-test is important as with the small samples examined the normality can not be satisfactorily confirmed. Where n is the number of observations, μ.

$$t = \frac{\bar{x}-\mu}{s\sqrt{n}}$$

where the mean ($\bar{x}$)

$$\bar{x} = \frac{1}{n} \sum x$$

and standard deviation (s^2)

$$s^2 = \frac{1}{n-1}\left\{x^2 - \frac{1}{n}(\sum x)^2\right\}$$

With reference to the t-tables, to achieve a significance at the 5% level with 8 degrees of freedom (n-1) requires a t-value of 2.3 or more. The tested data marked * in Table 1.1 had a mean difference greater than 0, with a probability of greater than 0.05.

All the experiments reported here were carried out at room temperature (20-25°C).

Section 1

Hysteresis in, and the length dependence of calcium sensitivity

Chapter 1

Hysteresis in the pCa-tension relationship

Introduction

The relationship between intracellular free calcium concentration ($[Ca^{2+}]_i$) and tension has been extensively investigated both in intact muscle cells using Ca-indicators such as aequorin (Blinks, Weir, Hess & Prendergast, 1982) and in skinnned muscle were the sarcolemma is removed by mechanical (Natori, 1954; Fabiato & Fabiato, 1973) or chemical means (Julian, 1971; Solaro, Wise, Shiner & Briggs, 1974). The form of this relationship is central to the interpretation of contraction and relaxation, particularly when experimental interventions are likely to influence $[Ca^{2+}]_i$.

It has been tacitly assumed that the pCa-tension relationship of striated muscle is unique for any particular set of ionic conditions. However, it has recently been reported that a hysteresis exists in this relationship: that is, the contractile proteins are more sensitive to calcium as $[Ca^{2+}]_i$ is being reduced than as it is being raised (Ridgway, Gordon & Martyn, 1983).

Hysteresis, in the present context, means that a submaximal calcium level can maintain a higher tension level than it can create. This hysteresis was first reported by Ridgway, Gordon & Martyn (1983) in skinned barnacle skeletal muscle, and confirmed in vertebrate skeletal muscle (Gordon, Ridgway & Martyn, 1984). Ridgway et al discovered this phenomenon while attempting to find out if contraction affects Ca-binding to the regulatory proteins. The existence of an effect of contraction on Ca-sensitivity had been suggested on theoretical grounds (Adelstein & Eisenberg, 1980; Taylor, 1979) and there was evidence for it for myofibrils in the absence of ATP (Bremel & Weber, 1972; Fuchs, 1978). Ridgway et al's results were the first evidence for an effect of contraction on Ca-sensitivity in intact and skinned muscle fibres under physiological conditions. Their conclusion was that the relationship between $[Ca^{2+}]$ and force depends upon the history of the muscle, the muscle's Ca-sensitivity being increased by cross-bridge (c-b) formation.

The existence of hysteresis has not been generally accepted. There are negative reports for skeletal (Pagani, Shemin & Julian, 1986; Brandt, Gluck, Mini & Cerri, 1985; Williams & Stephenson, 1983,) and cardiac muscle (Fabiato, 1985).

In this chapter, I will report on the first observations of hysteresis in the pCa-tension relationship of cardiac muscle, and provide some explanation why its occurrence has not been more widely observed.

Results

The basic phenomenon

The results presented here provide the first positive evidence for hysteresis in the pCa-tension relationship of skinned cardiac muscle. Figure 1.1 shows a section of tension trace from a Triton-treated rat cardiac trabecula. In this sequence, which started from the activated state the $[Ca^{2+}]$ was first reduced, then increased and finally reduced again. At each calcium concentration, tension was allowed to equilibrate for several minutes to ensure that a steady tension level was achieved. The broken lines indicate the steady-state tension level as $[Ca^{2+}]$ was increased stepwise, which I shall term '<u>upgoing</u>'. Clearly, the tension level maintained as the $[Ca^{2+}]$ was reduced ('<u>downgoing</u>') was higher (indicated by the arrows) than as the calcium concentration was increased (upgoing). Results from sequences similar to those shown in Figure 1.1 are plotted as steady-state tension against $[Ca^{2+}]$ in Figure 1.2A. The abscissa is $-\log[Ca^{2+}]_c$, the ordinate is steady-state relative tension. The tension has been normalised to the maximal Ca-activated force (C_{max}) (see methods for details). C_{max} was about 60mg.wt.($\sim$0.6mN) in this sequence. The arrows indicate whether the data were obtained as $[Ca^{2+}]$ was being increased (filled circles) or decreased (open circles). In this and subsequent figures the pCa-tension relationships were fitted to the Hill Equation (Equation 2), using the best fit procedure

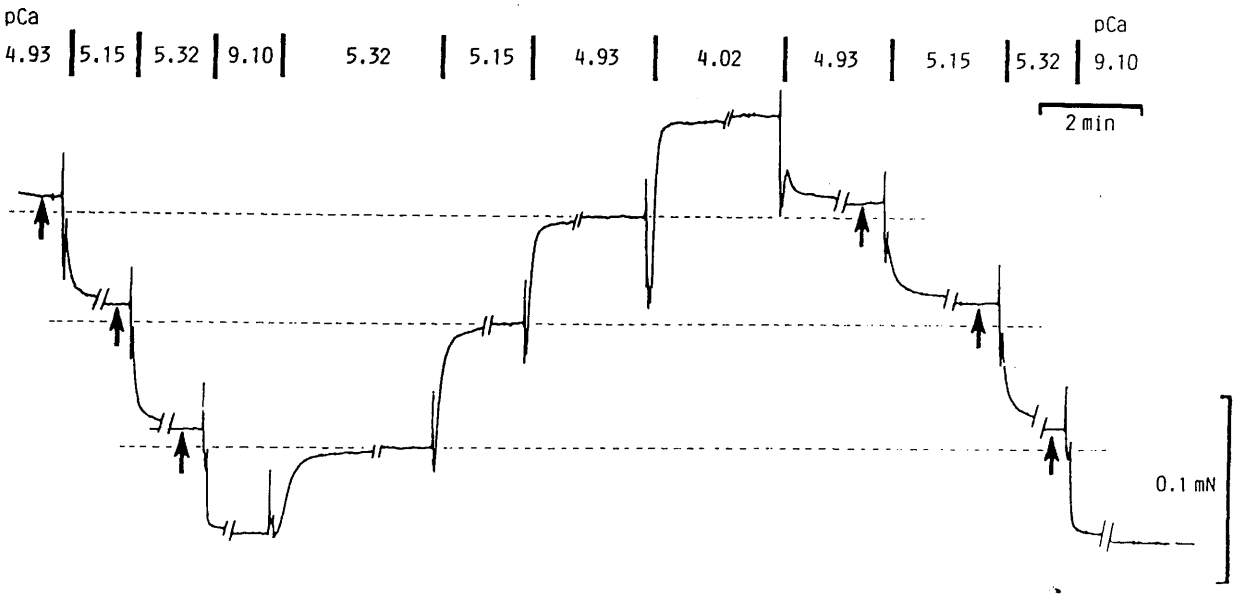


Figure 1.1

Tension recorded from a rat ventricle trabecula (Triton-treated) showing the basic phenomenon of hysteresis. The muscle was nearly fully activated at the start of this tension trace. The calcium level was then stepped downward to a subtension calcium level and then increased again to C_{max}, that is a downgoing and then an upgoing hysteresis sequence. The broken lines mark the steady-state tension level at each pCa on the 'upgoing' sequence. Decreasing the calcium again (downgoing) as in the first sequence yielded the same tension levels as the first (downgoing). Both of the downgoing sequencies tension levels are clearly above the 'upgoing' sequencies tension levels, as indicated by the arrows. The tension was allowed to stabilise at each of the test pCa levels. Breaks in the trace are of 2 min. The test $[Ca^{2+}]$ are expressed as pCa that is $-\log\{Ca^{2+}]$ s.l. 2.2μm, diameter 130μm.

described in the Methods section. In this example, pK_{app} increased from 5.38 to 5.50 M^{-1}, a 0.12 pCa unit decrease in the $[Ca^{2+}]$ required for half maximal activation on the downgoing limb. The Hill coefficient ('h') decreased from 2.4 on the upgoing limb to 1.8 on the downgoing limb.

The results shown in figure 1.1 and 1.2A are typical of 29 preparations. In all experiments the shift in the pCa-tension relationship as calcium was decreased was to a higher apparent Ca-sensitivity and the Hill coefficient 'h' remained constant or was reduced on the downgoing limb of the hysteresis relationship. Where the pCa-tension relationship was determined in detail (n=12).

the mean $pKapp_{up}$ 5.303 ± 0.116 (s.d),
the mean $pKapp_{down}$ 5.322 ± 0.117,
'h'up = 4.05 ± 1.44,
'h'down = 3.85 ± 1.48.

S.l. range 2.0-2.2μm.

Only 12 of the pCa-tension relationships were used in this average as in many experiments only a two or three submaximal pCas were used to evoke tension: while this allows a fair estimate of the pK_{app} to be made it does not provide a reliable definition of the slope of the Hill equation.

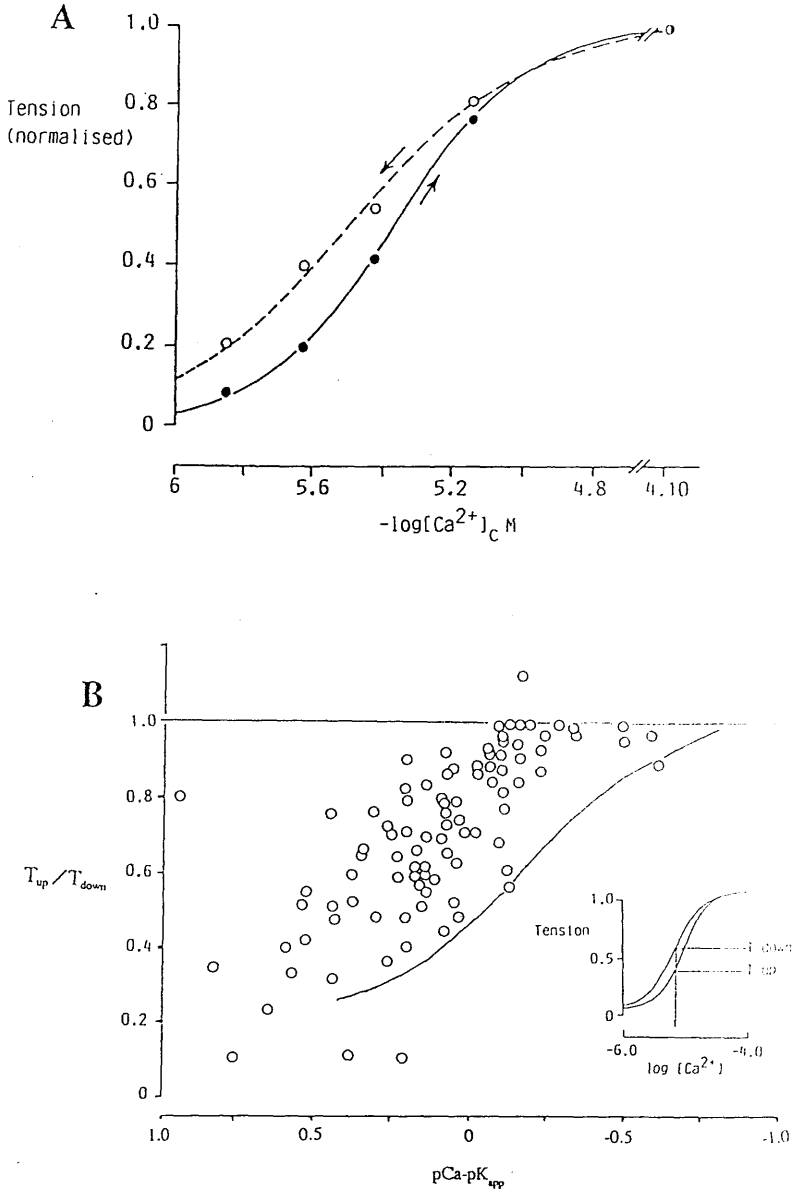


Figure 1.2

(A) pCa-tension relationship for a rat ventricle trabecula (Triton-treated). Results similar to those in figure 1.1 are plotted. The abscissa is pCa, the ordinate steady-state force, which has been normalised to C_{max}. Peak force is equivalent to ~60mg.wt. (=0.6mN approximately) in this sequence. The arrows on the curves indicate whether data were obtained as calcium was being stepped up or down. In this and subsequent figures curves were fitted according to the Hill Equation (2) using the best-fit procedure described in the methods section. pK_{app}=5.375 (M^{-1}) and 'h'=2.6 for the upward, and 5.5 and 1.8 for the downward limbs respectively, s.l. 1.9μm diameter 175μm.

(B) Accumulated data representing the extent of hysteresis of the type shown in 1.2A. The ratio of the tension achieved in the upward (T_{up}) and downward (T_{down}) direction is plotted on the ordinate. In order to standardise the plot for the differences in absolute Ca-sensitivity between individual preparations, the tension ratios are plotted against a calcium scale (abscissa) representing pCa-pK_{app} for the individual muscles (pK_{app} was established, by the best fit proceedure described in conjunction with Equation 2, for each experiment for both the upgoing and downgoing curves, the latter being used for obtaining pK_{app} since it is almost insensitive to s.l. (see figures 1.7). Data from 22 preparations are represented here.

The accumulated data from 22 preparations are presented in Figure 1.2B. The ratio of the tensions achieved in the upgoing (T_{up}) and the downgoing (T_{down}) directions at a given pCa are plotted on the ordinate. In order to standardise the plot for differences in the absolute Ca-sensitivity between preparations, the tension ratios were plotted against a calcium scale (abcissa) representing test $pCa-pK_{app}$ for each muscle. (pK_{app} was established by the best fit procedure in conjunction with the Hill equation, for each experiment for both upward and downward curves, the latter being used for obtaining pK_{app} as it is almost insensitive to s.l. as will be illustrated later in figure 1.7)

The cumulated data fall in a range between (i) little or no hysteresis, where the positions, and hence the slopes of the upgoing and downgoing curves are very similar, and (ii) greater hysteresis (see inset), where the positions and slopes differ appreciably. The theoretical lines on the main plot are derived from this example. The lower line corresponds to substantial hysteresis, and the upper line to the absence of hysteresis, respectively. The cumulated results fall satisfactorily within the general area indicated by these extremes. This plot also confirms that hysteresis is (with one exception) always in the direction T_{up} is less than or equal to T_{down} at a given level of $[Ca^{2+}]$.

Is hysteresis an experimental artefact?

(i) Carry over of calcium from the previous solution?

One possible explanation of hysteresis would be that it is an artefact due to calcium carry-over from the previous higher calcium level. However, the tension measurements are made in the same solution chamber on the upgoing and downgoing sequences at a given pCa. The up-down sequence is repeatable as seen in figure 1.1, so a carry-over of calcium from the preceeding higher $[Ca^{2+}]$ can be discounted as an explanation. The volume transferred from bath to bath is in fact very small, in the order of a few nanolitres. In combination with the well stirred, strongly calcium buffered (EGTA 10mM) solutions this precludes a calcium transfer large enough to increase the free calcium level.

(ii) Are sarcomere length changes associated with hysteresis?

A second possible explanation of hysteresis would be that an artefact results from a change in s.l. distribution when the muscle contracts. There are several reports in the literature that s.l. alters appreciably during force production under 'preparation isometric' conditions (Allen & Kentish, 1985; Kreuger & Pollack, 1975; Kentish, ter Keurs, Ricciardi, Bucx & Noble, 1986). However, if any redistribution of s.l. was to occur during contraction it would most likely result in a shorter s.l. in the active muscle, i.e. on the downgoing curve, as any damaged areas would be stretched by the functional areas contracting (this has in fact been shown by Kentish et al, 1986). This would not result in hysteresis in the direction observed, as the Ca-sensitivity would be lower at short s.l. (Hibberd & Jewell, 1982; Kentish, ter Keurs, Ricciardi, Bucx & Noble, 1986). One preparation was deliberately damaged centrally by crushing it with forceps. This preparation displayed negative hysteresis as predicted (that is less tension maintained at each calcium level as the muscle is relaxed).

However, it was important to determine if there was a redistribution of s.l. associated with force production, that is to check if the contractions were 'isometric'. Continuous video recordings of sarcomere pattern as observed with the DIC microscope during hysteretic sequences, were made. These data

were then subjected to computer-assisted image-analysis using the Magiscan system (Joyce-Loebl Ltd) with the assistance of Dr V A Moss (Moss, Miller & Lamont, 1986).

Analysis of sarcomere length changes by computer-assisted image analysis

An area of sarcomere pattern was defined for analysis with the run of sarcomeres perpendicular to the television raster lines. The intensity of the pixels (coded in 64 grey tones ≡ 6bit resolution) were averaged across the width of this area (typically about 5 to 10 raster lines) and expressed for each pixel along its length, in this case 65um, which amounted to 3% of the length of the muscle (see fig 1.3). Sarcomere length was equivalent to about 10-15 pixels which gives resolution better than that strictly available from the image at wavelengths in the visible spectrum. This information was displayed as a plot of intensity against distance along the muscle (figure 1.4A). The program then sought the intensity peaks (or troughs), found their mid points by an interpolating algorithm and gave the values for the separation of these midpoints, corresponding to the middles of the successive I (or A) bands. The mid-point positions are displayed in the figure as a series of points whose separation corresponds to the s.l. (vertical scale in figure 1.4B). With the discrimination criteria adopted, faint bands were not detected by the program so an integer multiple of the s.l. results (a gap in

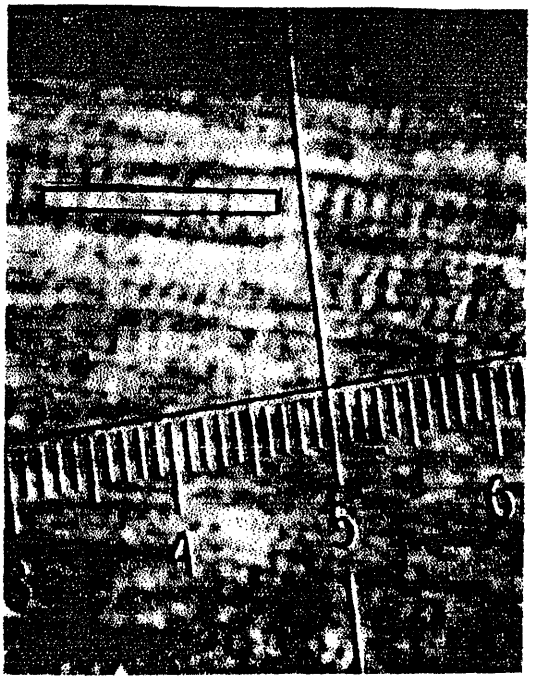


Figure 1.3

A screen dump of a section of rat cardiac muscle viewed through the DIC micsoscope. The pale bands correspond to the A-bands, the dark bands to the I-bands. One large graticule division corresponds to 16.9μm (s.l. approximately 2.2μm). The black block ids an area difined for analysis.

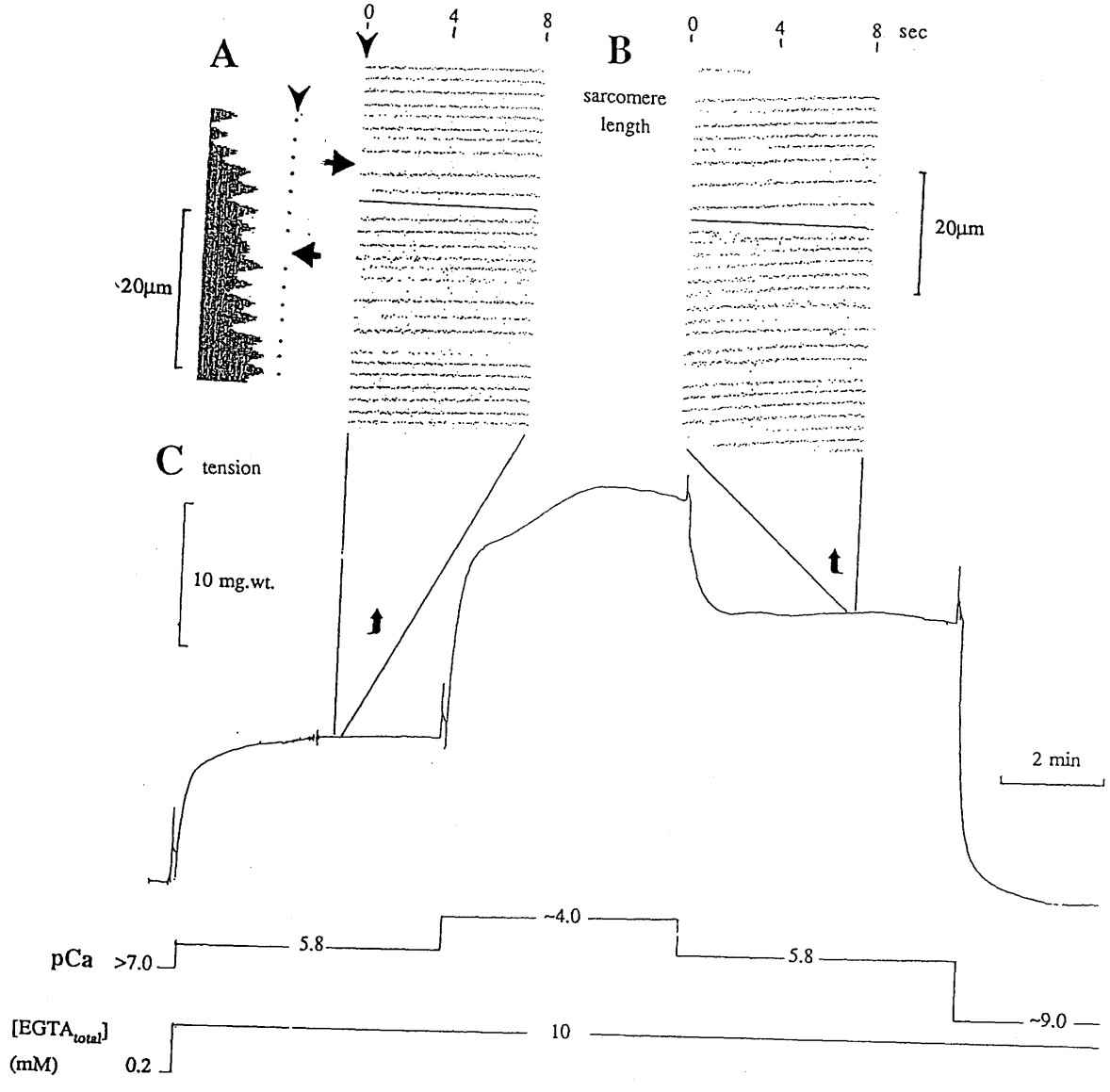


Figure 1.4

Computer-assisted image analysis as a check on s.l. during force production.

(A) Intensity analysis of a typical s.l. pattern. The 15 peaks represent the mid-points of the I-bands under analysis, the troughs represent the A-bands. (B) Two sequencies of analysis of the type shown in Panel A showing s.l. (ordinate) against time (abscissa). (C) The tension levels achieved by the muscle under analysis.

the array of dots). An example is indicated by the horizontal arrows in Figure 1.4A and 1.4B. Successive analyses over a period of time show any changes in s.l., s.l. distribution, or any displacement overall, with respect to time. Figure 1.4B shows two 8-second sections of analysis carried out on the same sarcomeres while tension was at steady-state on the upgoing and downgoing part of the hysteresis sequence.

The tension trace (1.4C) shows that, in this case, T_{down} was 1.8 times greater than T_{up} at pCa 5.75. The tension responses reached a steady-state less rapidly than those in the standard experimental chamber due to the relatively slow solution exchange.

Inspection of 1.4B shows that there is little obvious difference in s.l. between the 'upgoing' and 'downgoing' sections. A tiny lateral translation of the section under analysis occurred, amounting to a displacement of about 1um in 15min. This can be judged relative to the exactly horizontal line in the middle of each panel which corresponds to the position of a graticule line on the image, against which I-band centres drift upward (i.e. along the muscle's long axis. The mean s.l. ($\pm$ s.d.) for the whole of the sample period of 8-seconds (at 25 frames per second) was $2.10\pm0.25\mu m$ (upgoing) and $2.07\pm0.17\mu m$ (downgoing). This shows that there is no substantial s.l. redistribution or alteration associated with this substantial hysteresis.

Table 1.1

Image analysis of sarcomere length and distribution.

	Mean (μm)	s.d.(μm)	No. of regions sampled (*)
Relaxed state before contraction	2.06	0.21	6
Upgoing activation at pCa=5.75	2.07	0.19	5
Full activation at pCa=4.00	Sarcomere pattern not sufficiently distinct to permit analysis		
Downgoing activation at pCa=5.75	2.02	0.19	3
Relaxed state after contraction	2.08	0.20	5

(*) Regions were 40-80μm in length

The cumulated data from this analysis are presented in table 1.1. Sarcomere length and its distribution were assessed in at least 3 locations on the muscle. The results confirm, as the single analysis at one location on the muscle in figure 1.4 illustrates, that mean s.l. and s.l. distribution did not change significantly between the upgoing and downgoing limbs of the hysteresis loop. The table also includes estimates of the s.l. and distribution in the relaxed states before and after the hysteresis sequence, showing that the isometric conditions are well maintained, i.e. there is no major change in the s.l. or its distribution associated with force production. The significance of these observations, which is in marked contrast to others in the literature, will be considered in the Discussion of this

(iii) Is hysteresis due to loss of some diffusible cellular component?

A third possible explanation of hysteresis would be would be that it was due to loss of some cellular component when the sarcolemma is disrupted or removed. In several preparations hysteresis was observed both when the muscle was first saponin-treated and then subsequent to Triton-treatment. The degree to which the cellular membranes were maintained had no effect on the nature or magnitude of the hysteresis observed. This is good evidence that the phenomenon is not due to loss of larger cytoplasmic constituents since it has been shown that saponin-treated preparations retain most of their cytoplasmic enzymes (see Harrison and Miller 1984, Harrison 1985, Fry and Miller 1985, for details). Additional evidence against this explanation will be presented in the discussion.

Use of the term 'Hysteresis'

Hysteresis is derived from the Greek 'hystercin' which means 'to come late'. It is generally taken to mean a time lag in a physical effect. The 'hysteresis' described here is not a slow approach to the same steady level but a genuinely different steady-state. The hysteresis can be maintained for many minutes, as in figure 1.1. When the time in any one solution was further

increased, to 15min in some cases, no decrease in the magnitude of the hysteresis was observed (determined as the difference in upgoing and downgoing tension at a given pCa). This argues against hysteresis being a very slow approach to the same steady level, as the use of the term would suggest. The nature of the tension development also suggested a genuinely different steady-state. The tension appears asymptotically to approach the downgoing tension level. There is no suggestion of a continual downward drift to the upgoing tension level, nor, a continual upward drift in the upgoing tension level. I have continued to use the term hysteresis since, although it is not strictly appropriate, it is already established in the literature.

Partial hysteresis

Figure 1.5 is a section from a continuous tension trace, which shows that hysteresis is only partially reversed, or induced, when the muscle is not fully relaxed, or activated, respectively. The small numbers indicated the solution change number. In this sequence $[Ca^{2+}]$ was raised again after the initial upgoing and downgoing sequence without first returning the muscle to the fully relaxed state (solution change (8)). The submaximal tension achieved in this second upgoing exposure to pCa 5.05 is significantly higher (arrowed) than that achieved initially (3), or at the end of the sequence (11) which started again from the fully relaxed state. Similarly, as the last part of the trace shows (steps 11 to 14), when $[Ca^{2+}]$ is stepped

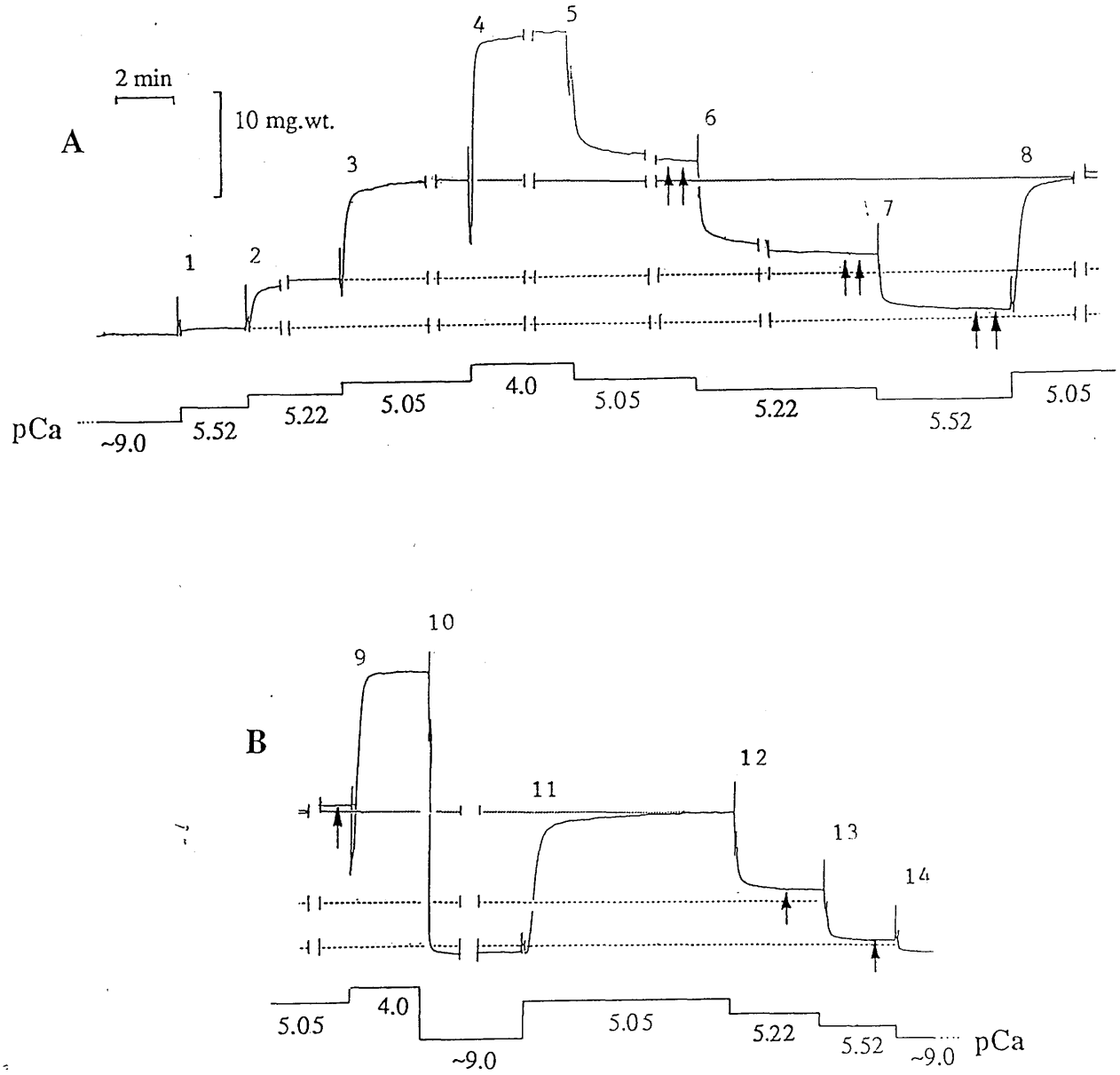


Figure 1.5

Partial hysteresis

A tension record from a rat ventricle trabecula (Triton-treated). Breaks in the trace are of 4 minutes duration. The broken lines indicate the steady-state tenson at each pCa on the initial upgoing curve. The small numbers indicate the solution exchange number. The double arrows indicate where a full hysteresis is observed. The single arrows indicate where a partial hysteresis is observed i.e. when the muscle has not been fully relaxed or fully activated (see text for details).

downwards after submaximal activation (11), the extent of the hysteresis is less (single arrows) than when the muscle was immediately previously fully activated (steps 5 to 8). Peak force is unaffected by the route with which it is approached. 'Partial' hysteresis of this type was observed in fourteen preparations.

Effect of increasing muscle calcium sensitivity

I was interested to test whether an alteration in the Ca-sensitivity of the contractile proteins would influence the magnitude of hysteresis. Caffeine at millimolar levels increases Ca-sensitivity (Wendt and Stephenson, 1983) and, as I shall report in chapters 3 and 4, imidazole and certain other compounds have similar effects. Figure 1.6 shows the results from three separate runs in one experiment with imidazole. The Ca-sensitivity was also increased by virtue of the higher pH_a employed; 7.2 in this case, as opposed to 7.0 employed in most of the experiments. As can be seen, hysteresis is still prominent and the shift in slope and pK_{app} are quantitatively similar to those shown earlier.

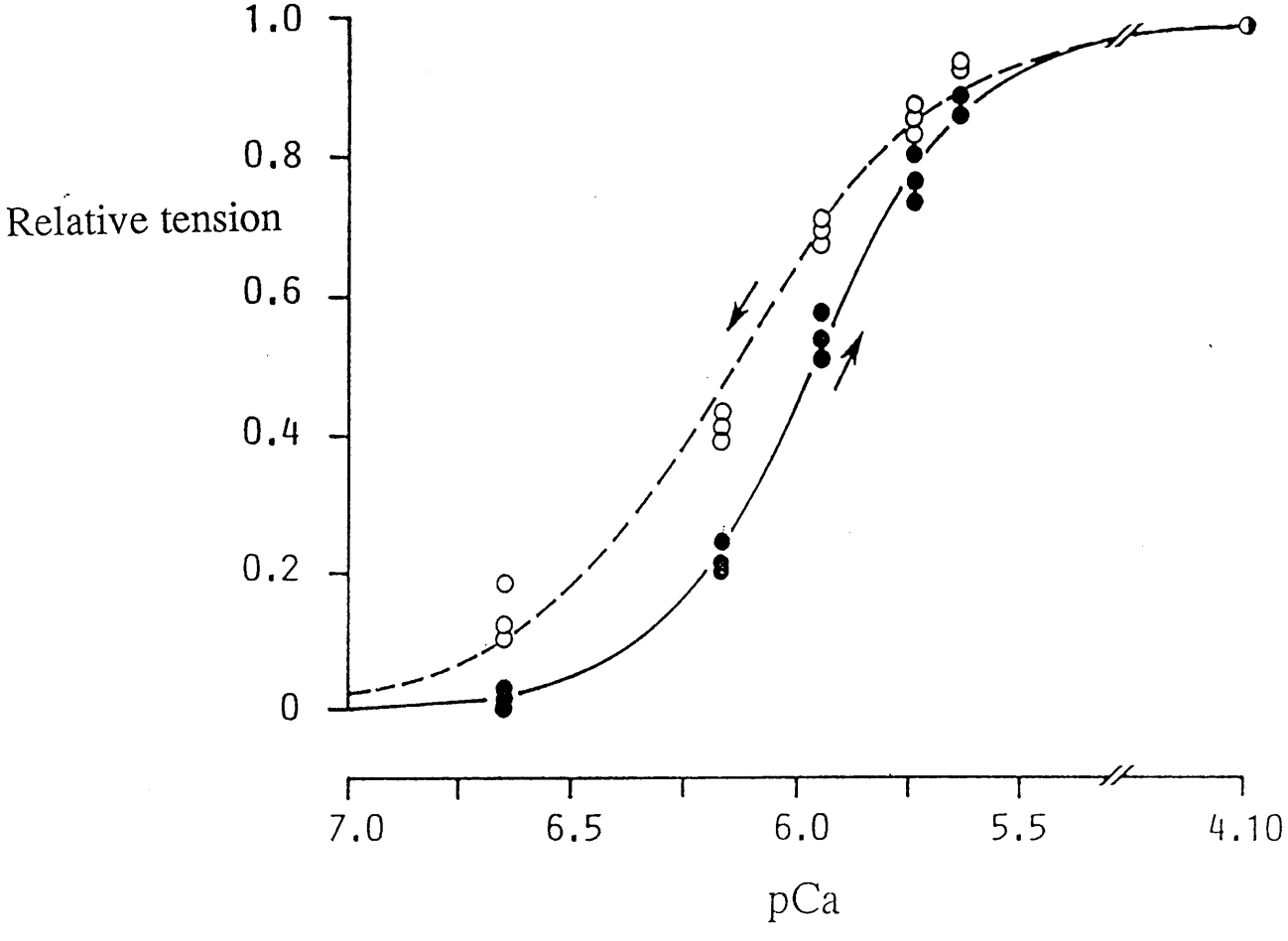


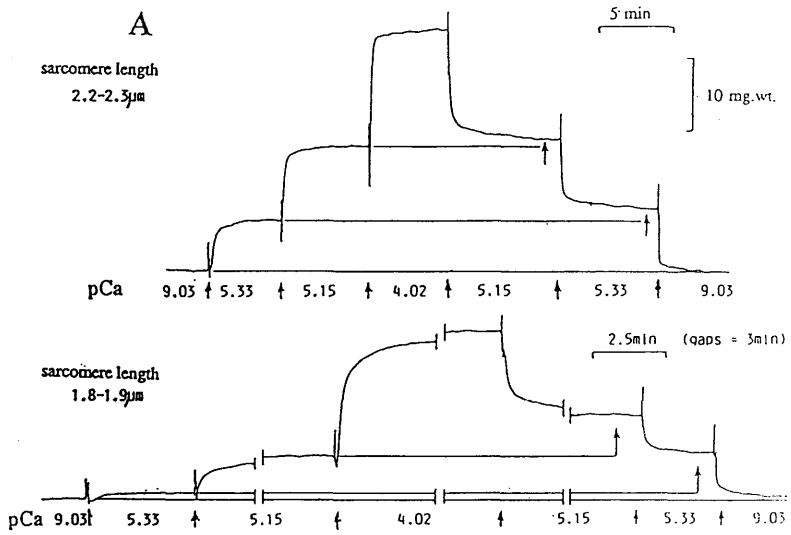
Figure 1.6

pCa-tension relationships for a rat ventricle trabecula (saponin-treated). Data from three separate runs on the same preparation are shown. The solutions all include 25mM imidazole, replacing HEPES. The pH_a was 7.20 instead of the 7.00 used elsewhere. The values for pK_{app} and 'h' were 5.98 and 2.5 for the upgoing curve (closed symbols) and 6.15 and 1.9 for the downgoing curve (open symbols). s.l. 2.1-2.2μm.

Is hysteresis force dependent?

Gordon, Ridgway and Martyn (1984) suggested from their experiments on skinned skeletal fibres from barnacle that tension production per se increases the Ca-sensitivity, i.e. hysteresis is force dependent. This conclusion might also be made on the basis of the partial hysteresis described above. A direct test of this hypothesis was to alter the s.l.. The prediction was that if hysteresis is force dependent, it should be diminished at low s.l. where absolute force production is reduced.

Figure 1.7 shows traces obtained from a preparation at two sarcomere lengths. The upper trace shows the response at a s.l. just at, or above the peak of the length tension curve. A relatively small hysteresis develops. The lower trace shows the responses to the same solutions at a shorter s.l. Note that the time base is faster than the upper trace. As the s.l. was reduced peak tension decreased by about 30%. This was expected from the length tension relationship (Gordon, Huxley & Julian, 1966; Jewell, 1974). The extent of the hysteresis was, however, substantially increased. Most strikingly, at pCa 5.33 very little tension develops as $[Ca^{2+}]$ is stepped up, but a higher steady level is maintained as $[Ca^{2+}]$ is stepped down. Results from this experiment are replotted as relative tensions normalised to the peak achieved at each s.l. Consider first the upgoing curves. The reduction in Ca-senstivity at the low s.l. can be seen. This reduction in Ca-sensitivity at lower s.l. has been reported by



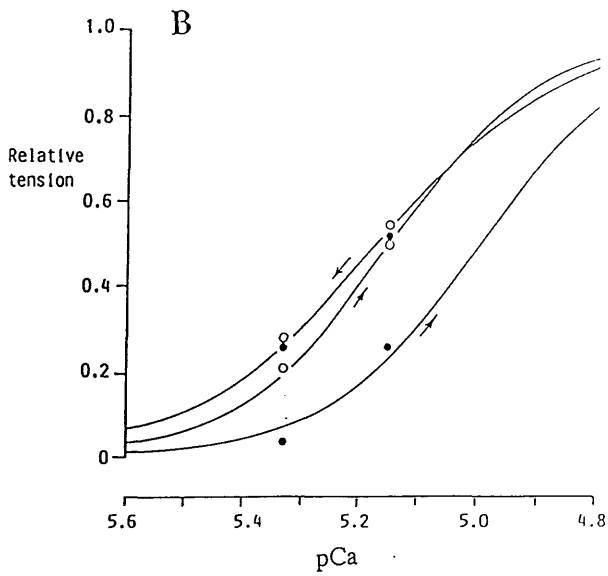


Figure 1.7

(A) Tension responses from a rat cardiac ventricle trabecula (saponin-treated). The upper trace was obtained at a s.l. of 2.2-2.3μm, the lower trace at s.l. of 1.8-1.9μm. The same solutions were used for each of the sequences. Gaps in the lower trace are of 3 minutes duration to confirm the full equilibration of tension. (B) The results from panel A are replotted as steady-state force, normalised with respect to maximum tension, at each s.l. Open symbols for s.l. 2.2μm, closed symbols for s.l. 1.8μm. The arrows indicate whether calcium was being stepped up or down.

other authors (for the ascending limb of the length-tension relationship in cardiac muscle by Hibberd & Jewell, (1982) and Kentish et al, 1986). However, it is striking, in this example, that the downgoing limb of the hysteretic relationship is almost the same at both s.l.s Thus, the major effects of s.l. on Ca-sensitivity (below 2.2μm) seems to be restricted to the upgoing limb of the hysteresis relationship. This point is reinforced later.

Table 1.2 presents the pK_{app} and Hill coefficients for 9 and 8 preparations at a s.l.s of less than 2.0μm and approximately 2.2μm respectively. The data support the point that the magnitude of hysteresis is greater at low s.l. The average shift in the pCa-tension relationship at a s.l. of less than 2.0μm was 0.12 and only 0.05 at a s.l. of 2.2μm.

Table 1.2

The influence of sarcomere length on hysteresis

Low sarcomere length, 2.0μm or less

	pK_{app}			Hill coefficient		
Expt no.	Up	Down	Difference	Up	Down	Diff.
93	5.00	5.18	+0.18	3.4	2.8	-
249	5.16	5.24	+0.08	3.4	3.3	-
250	5.57	5.65	+0.08	3.6	4.3	+
202	5.46	5.62	+0.16	2.2	1.3	-
206	5.42	5.70	+0.28	1.4	1.2	-
216	5.21	5.30	+0.09	3.0	2.5	-
276	5.21	5.25	+0.04	3.0	3.3	+
279	5.31	5.39	+0.08	4.3	4.5	+
252	5.22	5.28	+0.06	3.0	2.7	-
Mean	5.28	5.40	+0.12*	3.0!	2.8!	
s.d.	0.17	0.20	0.08	0.8	1.1	

Sarcomere length approximately 2.2μm

	pK_{app}			Hill coefficient		
Expt no.	Up	Down	Difference	Up	Down	Diff.
93	5.13	5.20	+0.07	2.4	2.4	0
215	5.48	5.54	+0.06	2.6	2.3	-
247	5.24	5.29	+0.05	2.8	2.3	-
252	5.24	5.29	+0.05	2.9	2.8	-
219	5.53	5.62	+0.09	2.6	2.6	0
216	5.27	5.32	+0.05	2.7	2.2	-
276	5.25	5.28	+0.03	3.6	3.7	+
279	5.38	5.39	+0.02	4.5	4.5	0
Mean	5.31	5.36	+0.05*	3.0!	2.8!	
s.d	0.14	0.14	0.02	0.7	0.8	

*= K_{up} and K_{down} are significantly different at P=0.005 level

!=not significantly different

Hysteresis becomes undetectably small when the muscle is stretched beyond a s.l. of 2.2μm though, as reported by others, Ca-sensitivity continues to increase (Fabiato & Fabiato, 1978, Moisescu & Thieleczek, 1979). This is illustrated in figure 1.8 Three s.l.s were investigated in this example; one below, one at and one above the peak of the length-tension relationship. The steady increase in Ca-sensitivity is exemplified by the increase in the absolute force level in response to the first exposure to a submaximally activating level of calcium (pCa 5.52) in traces A, B & C. Maximum calcium activated force rises between A and B and then falls again in C as s.l. is increased from 1.8 to 2.2 and then 2.3μm. The difference between the steady-state tension achieved during the first (upgoing) and second (downgoing) exposure to pCa 5.52 in traces A,B and C, i.e. the magnitude of the hysteresis, falls to zero. This figure reinforces the point that absolute force does not determine the amount of hysteresis: panels A and C represent similar absolute force levels with pronounced hysteresis in A but not in C. Trace B has the highest tension level but an intermediate level of hysteresis. Data for this experiment (Prep B) and two like it are presented in Table 1.2 as pK_{app} and Hill coefficients. Peak force is slower to develop at low s.l.s (see figures 1.7A and 1.8A). At the lower s.l.s the fibre's Ca-sensitivity is reduced, so the same calcium step does not produce such a sharp rise in tension as the intermediate calcium levels which the muscle experiences will produce less relative tension.

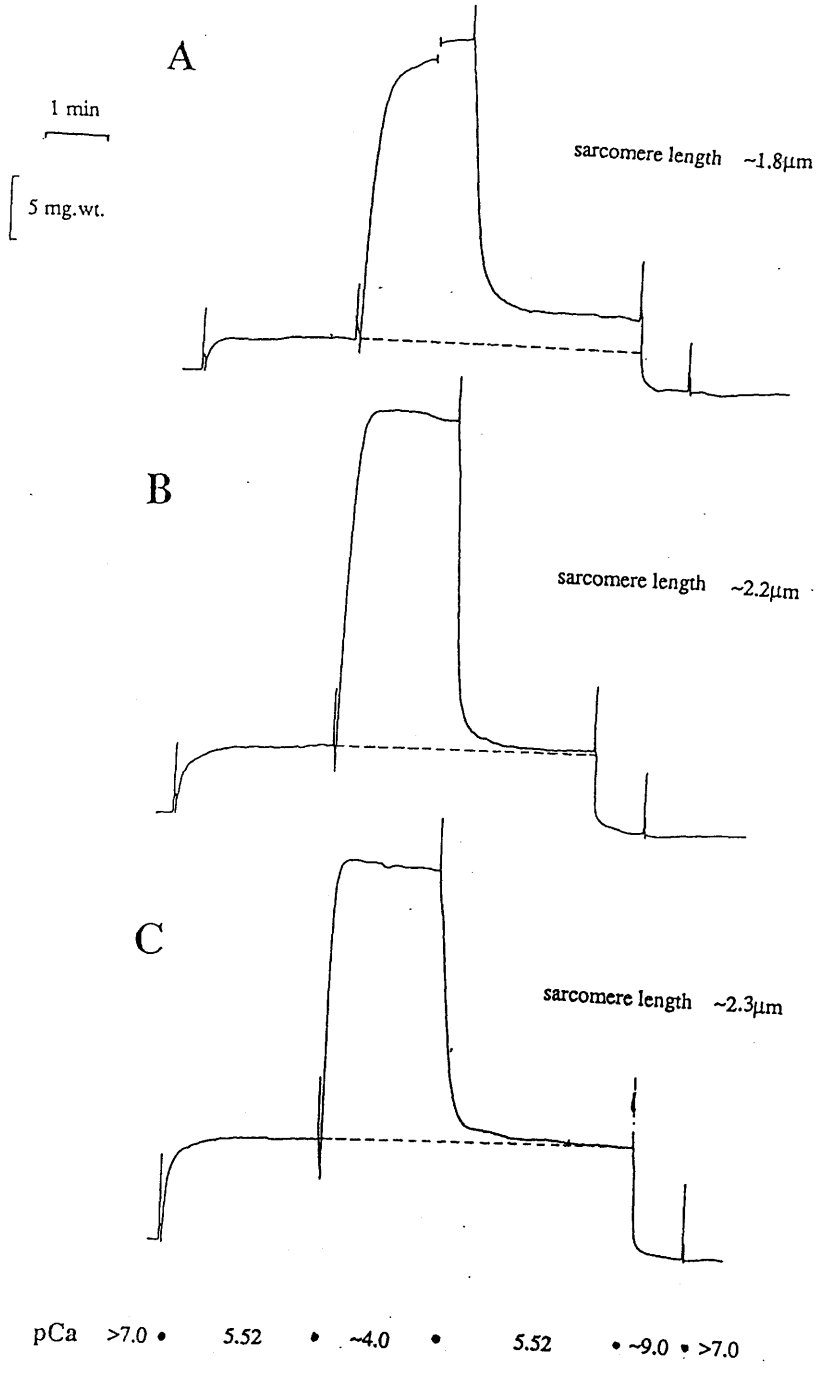


Figure 1.8

Reduction of hysteresis at long s.l.s. The same sequence of solution changes is repeated on the same preparation at 3 s.l.s; panel A s.l.~1.8μm, panel B s.l.~2.2μm and panel C s.l.~2.3μm. The pCa values applying to each sequence are indicated below the traces. The gap in panel A was 1 minute.

Table 1.3 Hysteresis at three s.l.s

Sarcomere length less than 2.2μm

Prep.	pK_{app}up	pK_{app}down	δpK	'h'up	'h'down	
A(216)	5.21	5.30	+0.09	2.96	2.48	-
B(276)	5.21	5.25	+0.04	3.06	3.28	+
C(279)	5.31	5.39	+0.08	4.26	4.46	+
Mean			0.07			

Sarcomere length approximately 2.2μm

Prep.	pK_{app}up	pK_{app}down	δpK	'h'up	'h'down	
A(216)	5.27	5.32	+0.05	2.69	2.19	-
B(276)	5.25	5.28	+0.03	3.57	3.70	+
C(279)	5.38	5.39	+0.01	4.49	4.46	-
Mean			0.03			

Sarcomere length greater than 2.2μm

Prep.	pK_{app}up	pK_{app}down	δpK	'h'up	'h'down	
A(216)	5.36	5.39	+0.03	2.37	1.67	-
B(276)	5.36	5.37	+0.01	4.55	4.30	-
C(279)	5.44	5.44	+0.00	4.56	4.40	-
Mean			0.01			

As in the example shown in figure 1.8 the magnitude of the hysteresis decreased (defined as $pKapp_{down}$-$pKapp_{up}$= δ pK) as the muscle was stretched. The mean shift in the pKapp was 0.07 pCa units at less than 2.2μm, 0.03 pCa units at 2.2μm, and only 0.01 at greater than 2.2μm.

The data all show the continuing increase in Ca-sensitivity with increasing s.l. For example preparation C's $pKapp_{up}$ increased from 5.31 to 5.38 as the preparation was stretched 2.2μm and from 5.38 to 5.44 as it was stretched above this level. This protocol was only successfully achieved with three preparations as stretching the preparations beyond a s.l. of 2.2μm frequently irreversibly damaged the preparation. Frequently once the preparation had been stretched beyond 2.2μm the tensions produced were unstable, the decline in peak tension being greatly accelerated. Other workers have found this and it has been attributed to a high internal connective tissue content in cardiac muscle.

Cumulated results for the effect of s.l. on hysteresis are plotted in figure 1.9. This figure is a plot of the ratio of relative tension (T) at the 2 s.l. (2.2μm or less: expressed as T_{short}/T_{long}) achieved on the upward (open symbols) and the downward (filled circles) parts of sequences of $[Ca^{2+}]$ changes from 6 preparations (Cumulated results from 6 preparations in which the tension was determined by at least 4 $[Ca^{2+}]$). The first three panels schematically illustrate, and the subscript describes, how the values plotted in 1.9D were calculated. A ratio of 1.0, near which most of the filled symbols are clustered, corresponds to no effect of s.l. on Ca-sensitivity. The collected results confirm that there is little or no significant effect of s.l. on the Ca-sensitivity of the downwgoing curve.

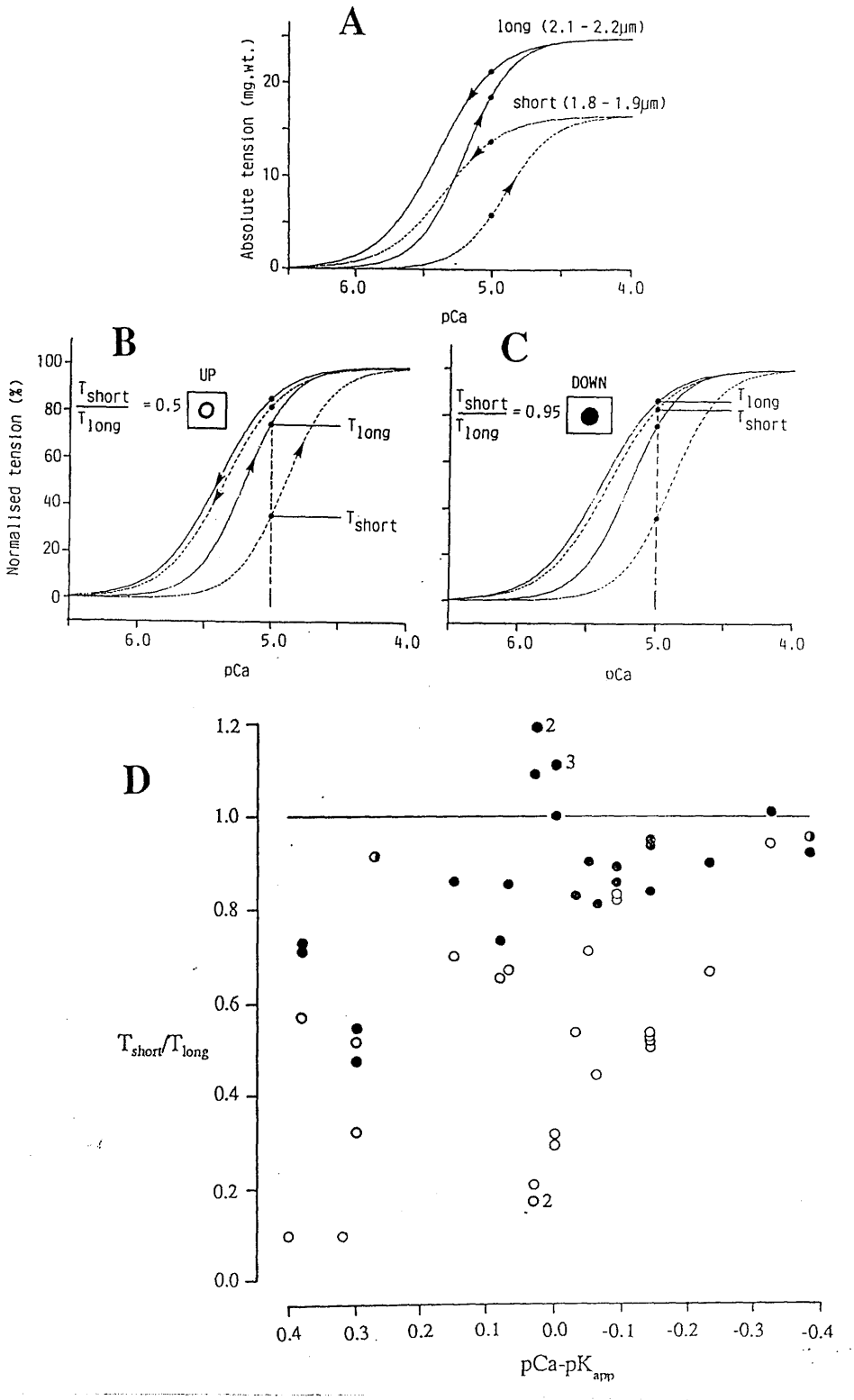


Figure 1.9

Differential effect of s.l. on the Ca-sensitivity of saponin- and Triton-treated rat cardiac trabeculae on the upgoing and downgoing limbs of hysteresis curves. Panels (A), (B), and (C) show hypothetical plots of the hysteresis pCa-tension relationships obtained at two s.l.s. (A) represents the raw data of tension against pCa; (B) and (C) show the effect of normalising to the maximum tension response. (B), shows how the relative tension at the longer (T_{long}) and shorter (T_{short}) s.l.s were compared to give values for the upward and (C) for the downward limbs. Actual data are plotted in panel (D). The ordinate represents the ratio of tension achieved at each given pCa at one shorter and one longer s.l. (as T_{short}/T_{long}). The abscissa is standardised for the differences in absolute calcium sensitivity between individual preparations (but not between s.l. for a given preparation) by plotting $pCa-pK_{app}$ for individual muscles (see figure 1.2B for details). Results from the upgoing (open symbols) and downgoing (closed symbols) parts of the curves can therefore be compared. S.l. long was typically 2.1-2.2μm, s.l. short was typically 1.7-2.0μm. Cumulated data for 6 preparations.

The data show quite a wide scatter which merits further comment. The reproducibility of the values between successive determinations of tension at a given pCa is generally good (an example is provided in figure 1.6). The comparison between values T_{short}/T_{long} clearly hinges upon such reproducibility. If it is postulated that the downward limb of the hysteresis curve is indeed independent of s.l. then the expected ratio for the closed circles will be unity and any real data should be scattered about 1.0. Inspection of 1.9C reveals that even a very small discrepancy between the downward curves at the two s.l.s will produce a T_{short}/T_{long} ratio that can differ significantly from 1.0, particularly at lower tension levels (higher pCa). The values obtained fall both above and below 1.0 but, given the sources of discrepancy noted here, seem satisfactorily close to 1.0. By contrast, for the upgoing curve, Ca-sensitivity is expected to fall with reducing s.l. so that T_{short}/T_{long} is always expected to be less that 1.0, if the reduction in s.l. is sufficient to reduce the Ca-sensitivity appreciably. This is the case for the collected data.

The Speed of development of hysteresis

The speed with which hysteresis develops is obviously important in establishing the physiological relevence of the phenomenon. The activation sequences described above are necessarily protracted; it often took several minutes to complete a sequence of force developments and to be convinced that a steady tension was achieved at each level. To check this point I tried to take advantage of the fact that hysteresis can be observed in the selectively skinned preparations. Caffeine contractures can be evoked under conditions of low calcium buffering capacity in saponin-treated muscle where the SR and mitochondria are functionally maintained (Endo & Kitazawa, 1978; Harrison, 1986). Tension peaks within about 1 second and depending upon the conditions, can reach C_{max}. It was intended to use the caffeine contraction to produce a large tension very quickly and during this caffeine contraction, to quickly impose the test pCa by the calcium jump technique (Ashley & Moisescu, 1977; Miller 1975): the buffer capacity is suddenly raised from e.g. 0.2mM to 10mM EGTA, as the calcium level is changed, causing the calcium level to be very quickly clamped throughout the preparation to the new level.

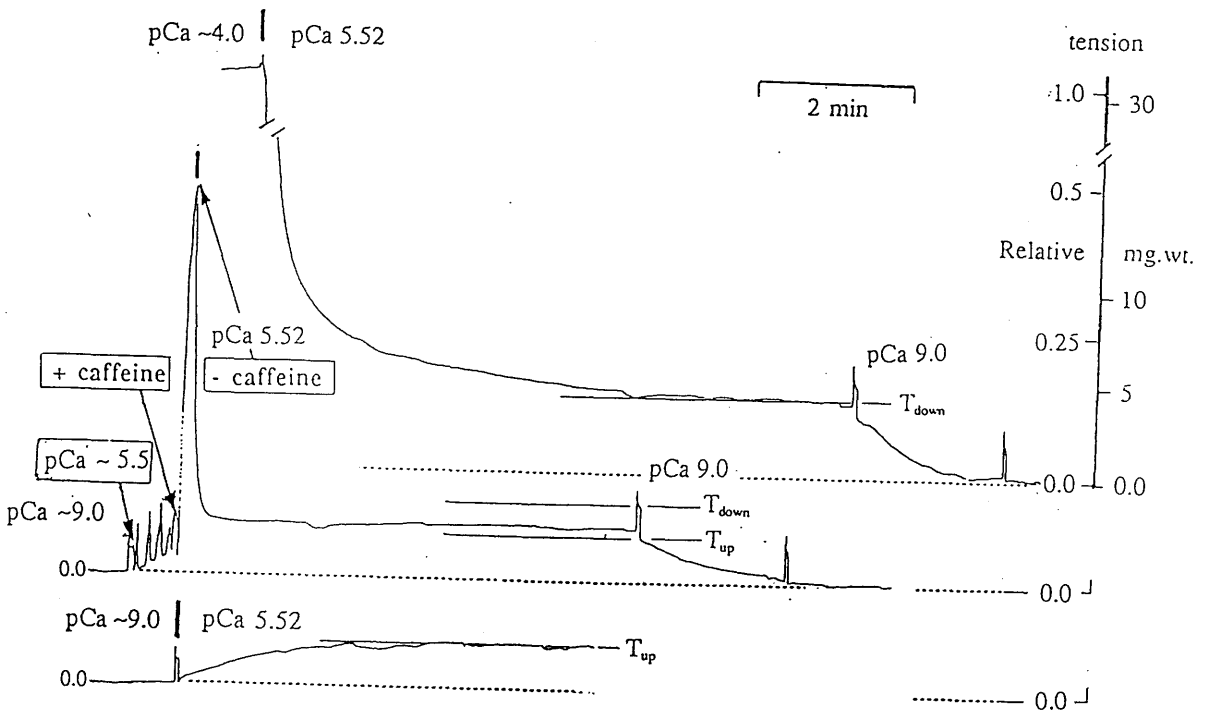


Figure 1.10

Rapid development of hysteresis after a caffeine contracture- middle trace.

A saponin-treated preparation was equilibrated at a low concentration of EGTA (0.2mM total) to minimise the buffering capacity. At the pCa chosen (~5.5) the muscle shows small tension oscillations. Addition of caffeine (10mM) induced a rapid transient contracture. At peak tension, the muscle was transferred to a high buffer-capacity solution (10mM EGTA). This 'clamps' the $[Ca^{2+}]$ throughout the preparation rapidly to the set level (pCa 5.52). The tension level at steady-state after the caffeine contracture can now be compared with that achieved in the same solution during standard upgoing (Tup level lowest trace pCa 9.00 to pCa 5.52) and downgoing (Tdown level from the uppermost trace pCa 4.00 to pCa 5.52) sequences. The caffeine contraction only reaches about half maximal tension and is very brief, but is sufficient to induce some hysteresis.

It seemed that if hysteresis developed within the very short time of the caffeine contracture, then it was likely that it could occur within the cell *in vivo*. This experiment however, turned out to be greatly complicated by the s.l. dependence of the caffeine contracture (Fabiato & Fabiato, 1975; Fabiato, 1980). To obtain a large hysteresis the s.l. must be low (as shown in figures, 1.7, 1.8, Tables 1.2 and 1.3). However, as the s.l. is reduced the magnitude of the caffeine contracture is greatly reduced. A great deal of time was invested in attempting to maximise the size of the caffeine contracture at low s.l. (14 experiments). The result presented here shows the maximum height of caffeine contracture attained at this low s.l. This 'maximum' contraction was achieved by applying the caffeine from an 'oscillating' solution (see methods section of details). This ment that only a partial activation of the muscle could be obtained by the caffeine contraction at low s.l.s. Within the experimental limitations, however, the result illustrated in figure 1.10, confirm that hysteresis will develop within about 20s at half maximal activation. The result provides another example of partial hysteresis; when the muscle is submaximally activated, (by the caffeine contraction) as here, the hysteresis resulting is less than that after full activation (C_{max}).

Discussion

Novelty of the phenomenon

Hysteresis is an apparent increase in the Ca-sensitivity of a muscle when it has immediately previously been exposed to a higher Ca-level; i.e a muscle can maintain a higher tension level than the given Ca-level can evoke de novo. The suggestion that the pCa-tension relationship might show hysteresis has not found universal acceptance. The present results provide the first positive evidence for the phenomenon in cardiac muscle. My finding that the magnitude of the effect is greatest at short s.l., and virtually absent above a s.l. of about 2.2um may help to explain why it has not been more widely observed. Some of the reports make no comment about the s.l. employed (e.g. Gordon et al, 1984) and negative reports have generally been obtained at s.l. greater than 2.2μm (Pagani, Shemin & Julian, 1986, Brandt, Gluck, Mini & Cerri, 1985, Williams & Stephenson, 1983; Fabiato, 1985).

Is hysteresis an experimental artefact?

(i) Sarcomere length change or redistribution?

The results in figure 1.4 confirm that significant changes in s.l. do not occur in these preparations during the activation sequences and hence can be dismissed as an explanation of hysteresis. Hysteresis is greatest at short s.l., and absent above about 2.3μm. This is difficult to reconcile with any model involving changes in the s.l. in the preparation as a consequence of force production. Any tendency of s.l. to alter during force production would be expected to result in shorter average s.l. (by stretching the damaged areas, as reported by Kentish et al 1986). On the ascending limb of the length tension curve (below 2.2um) where the greatest hysteresis is observed net s.l. reduction would result in negative hysteresis: After force production undamaged muscle would be at a lower s.l. and, therefore, less Ca-sensitive (Hibberd & Jewell, 1982). The hysteresis observed is diametrically opposite to this. It also follows that the estimate of hysteresis in the present experiments is an underestimate as any damage will tend to decrease the amount of hysteresis observed.

Our observation that s.l. and distribution are not appreciably altered during activation sequencies requires some further comment as many workers have reported alterations in s.l. under 'preparation isometric' conditions (Allen & Kentish 1985; Krueger & Pollack, 1975; Jewell, 1977; Winegrad, 1974; Kentish et al, 1986). I believe that there are two factors contributing to this difference. First, the method of mounting the preparation with the snares shown in figure 1B of the methods section. With this method the fraction of the muscle length which is damaged, even in the worst cases is less than 1%. Secondly, using light microscopy, rather than laser diffraction, to measure s.l. requires no rejection of preparations on optical grounds. Hibberd and Jewell (1982) have reported that, in their study, many preparations failed to give reliable laser diffraction patterns over the range of tensions and s.l. that they examined (1.9 to 2.5μm). These preparations were rejected from their study. Informal comment from other workers who use the laser diffraction method suggest that this approach is widespread. However, no preparations in this study had to be rejected on these grounds. Trabeculae almost always show a clear s.l. pattern, at least in a significant proportion of their length. However, there were often areas where the pattern was obscured by slight surface damage or endothelial cells. These preparations would presumably give unsatisfactory laser diffraction patterns.

It is also striking that the preparations used by Hibberd and Jewell had a slack length of 1.85-1.9μm (also those used by Dr.Kentish, personal communication). I commonly found slack lengths of 1.7μm with my preparations. This implies that the preparations selected by those using laser diffraction to measure s.l. may on average, have lower compliments of connective tissue than those used in this study. This suggestion is supported by the observation on single cardiac myocytes (rat) isolated by the use of collagenase: the resting s.l. is generally 1.9-2.2μm in such cells (Fabiato & Fabiato,1975).

(ii) Loss of some cellular component?

It could be suggested that hysteresis is due to the loss of some cellular component when the sarcolemma is disrupted or removed. As I have reported, there is no obvious difference in the magnitude or nature of the hysteresis exhibited by Triton- or saponin-treated preparations. This argues against the above but does not preclude it. However, there is evidence for hysteresis in intact muscle, which would argue strongly against hysteresis being due to loss of some cellular component (Gordon et al 1984). In barnacle single muscle fibres injected with aequorin to monitor $[Ca^{2+}]_i$ a long duration stimulus under voltage clamp conditions can produce a long duration calcium transient and force record which both approach steady levels. When the stimulus is briefly elevated to produce transiently a higher force level early in the contraction, the same steady-state calcium level can

eventually maintain a higher steady force i.e. hysteresis in the pCa-tension relationship of intact muscle.

Overall I conclude that hysteresis is a phenomenon without an obvious artefactual origin. Cardiac trabeculae prepared with either Triton or saponin show hysteresis in their Ca-sensitivity. Therefore, the phenomenon is not due to loss of some enzyme or other large diffusable element from the cytoplasm after comprehensive skinning with Triton, but retained in the saponin-treated state. There is no apparent change in s.l or distribution associated with it and it is not due to calcium contamination between solutions. Thus, it must be a property of the contractile machinery and the regulatory proteins.

Sarcomere length dependence of hysteresis

That the Ca-sensitivity of muscle is s.l. dependent is well established for both skeletal (Endo, 1972; Endo, 1973) and cardiac muscle (Hibberd & Jewell, 1982; Kentish, ter Keurs, Noble, Ricciardi & Schouten, 1983). Standard measurements of Ca-sensitivity are made by measuring the tension achieved in response either to single calcium steps or to a sequence of increasing calcium concentrations, starting from the fully relaxed state. These measurements are equivalent to those plotted on the upgoing curve of the hysteresis loops described here.

I observed an increase in the apparent Ca-sensitivity of preparations as their s.l. was increased, the magnitude of the hysteresis concomitantly decreasing (see Figure 1.8). This implied that both processes, hysteresis and the length dependence of Ca-sensitivity, might have a common mechanism. It was observed in all preparations examined that hysteresis was greatest at low s.l. and least at long s.l.

It thus seemed worth considering the proposed mechanisms for the length-dependence of Ca-sensitivity in an attempt to elucidate the mechanism of hysteresis. Unfortunately, although the length-dependence of Ca-sensitivity is now an established phenomenon, its mechanism remains obscure. Currently there is some debate as to whether the muscle's physical length or the tension it produces determines the myofibrillar Ca-sensitivity.

Does force modify Ca-sensitivity?

The generally held view is that in cardiac muscle Ca-sensitivity is determined primarily by the number of attached cross-bridges, and therefore by tension development, rather than by s.l. itself (Allen and Kentish 1985).

Allen and Kurihara (1979, 1982) working on intact cardiac muscle, showed the aequorin-signalled, calcium transient to decline more rapidly at longer s.l.s, while the duration of the tension transient increased. To explain this they proposed that the troponin binding constant increased as a function of tension

production. The first observations of hysteresis by Gordon et al (1984) and Ridgway et al (1983) was made to test this proposal. Allen and Kurihara's hypothesis would predict maximum hysteresis at 2.2μm where the number of effective c-bs is maximum. However the results presented in figures 1.7 and 1.8 show clearly that hysteresis is greatest at low s.l. when the absolute force is least. That hysteresis is not force-dependent is reinforced in figure 1.8 panels A and C. A is on the ascending limb of the length-tension relationship C on the descending, the two force levels are almost equal but the amount of hysteresis is quite different.

The idea that force modifies Ca-sensitivity is largely based on the biochemical work of Bremel and Weber (1972) who showed a substantial increase in troponin's affinity for calcium when myosin heads attach in rigor linkages. However, a more recent study by Fuchs and Planack (1983) using force producing (non-rigor) cross-bridges has shown that force, unlike rigor, does not influence troponin's Ca-binding affinity. The proposal that force production increases Ca-sensitivity cannot explain the increasing Ca-sensitivity on the descending limb of the length tension relationship. On the descending limb, tension is declining while the Ca-sensitivity is still increasing. An alternative or additional mechanism for the descending limb of the length tension relationship has to be proposed.

Overall, the current evidence is against force as the determinant of Ca-sensitivity, both in the case of the length dependence of Ca-senstivity and hysteresis.

Does the muscle's length per se modify the Ca-sensitivity?

In an extensive review of the length-dependence of Ca-sensitivity Stephenson and Wendt (1984) argue that the apparent increase in Ca-sensitivity is a direct consequence of the increase in muscle length rather than its tension production. This would also seem to be true of hysteresis as it appears to be length rather than tension which dictates its magnitude. Fuchs (1984,) tested Wendt and Stephenson's proposal by determining the Ca-binding in skeletal muscle at different s.l.s. The calcium saturation curves were found to be independent of s.l. over the s.l. range 1.6-3.9μm. However, more recent evidence for cardiac muscle (Hofmann & Fuchs, 1987) does show an increase in calcium binding as s.l. increases from 1.8 to 2.2μm. The attraction of Stephenson & Wendt's proposal is that it provides a single mechanism for the increase in Ca-sensitivity on both limbs of the length tension relationship and hysteresis. If the muscle's length affects its Ca-sensitivity this could be related either to changes in the overlap or internal spacing of the thick and thin filaments.

(i)Overlap

It could be proposed that the degree of overlap between the thick and thin filaments determines Ca-sensitivity. However, Ca-sensitivity increases on the ascending limb of the length-tension relationship (up to a s.l. of 2.2μm) where the overlap is complete, so a mechanism based simply on changes in overlap is not convincing. On the descending limb of the length tension relationship, Ca-binding sites move out of the region of overlap as the s.l. increase but even here the possible mechanisms are not simple. Only those binding sites which move out of the region of overlap experience a substantial change in their electrostatic enviroment. It would have to be proposed that the binding sites which have left the region of overlap are exerting long-range, allosteric effects on those sites controlling the force-producing cross-bridges. There is some support for this view as the regulatory proteins on the thin filament of vertebrate skeletal muscle are thought to act cooperatively as a unit with respect to calcium activation (Brandt, Diamond & Shachat, 1984). As with the force-dependent scheme, this mechanism does not account simply for the increase in Ca-sensitivity on the ascending and descending limbs of the length-tension relationship. In the case of hysteresis, the changes in Ca-sensitivity are clearly not associated with changes in filament overlap. The s.l. (the determinant of overlap) is constant as shown in figure 1.4 while the Ca-sensitivity is

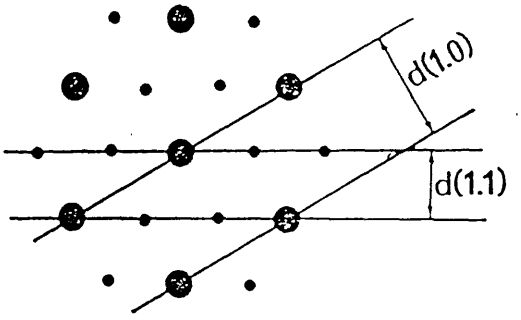
altering. Overall, it appears that overlap itself is unlikely to be the determinant of Ca-sensitivity.

(ii) Lattice spacing (l.s.)

It could be considered that the proximity of the thick and thin filaments determines the Ca-sensitivity. Interfilament distance (lateral spacing) varies as a function of length, as muscle has a constant volume (Huxley, 1953; Matsubara and Millman 1974, see figure 1.11): As the muscle is stretched the l.s. decreases over the full range of sarcomere lengths on the ascending and the descending limbs of the length-tension relationship. At present there is no direct evidence for l.s. changes altering Ca-binding to troponin. However, the data of Fuchs and Planack (1983) would include this condition since as they changed s.l. they simultaneously changed l.s. Ideally, information about calcium binding should be obtained when just the l.s. was changed with the methodology of Fuchs and Planack. The use of hypertonic shrinkage to decrease the l.s. with the muscle held at a constant s.l. would be the simplest possibility.

Figure 1.11

After figure 1 Matsubara and Millman (1974). Hexagonal lattice of thick and thin filaments, showing 1,0 all thick filaments and 1,1 planes two thin filaments to one thick filament.



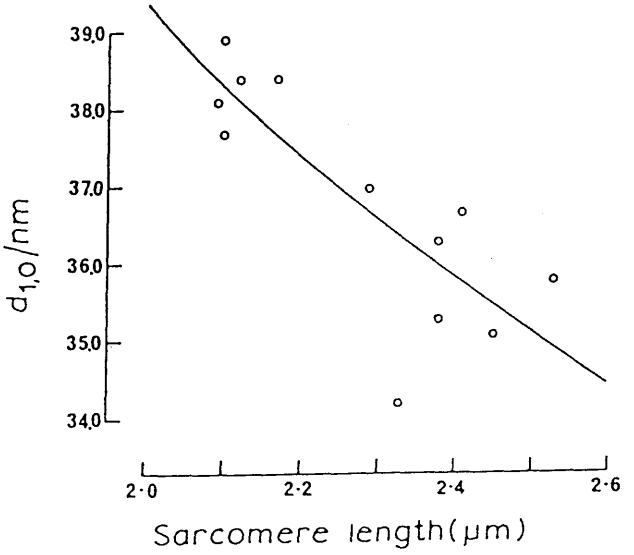


FIG. 3. The spacing of the 1,0 lattice planes ($d_{1,0}$) in resting papillary muscles plotted against sarcomere length. The curve represents a constant-volume relation: (sarcomere length) · $(d_{1,0})^2 = 3.0 \times 10^6$ nm^3.

Hysteresis and lattice spacing

If the length dependence of Ca-sensitivity and hysteresis are to have a common mechanism could a closer approximation of the myofilaments occur during hysteresis sequences?. Obviously, closer proximity of the filaments during hysteresis sequences cannot be achieved by increasing s.l. as the constancy of s.l. and distribution has already been determined. I proposed that it is achieved by force development. Closing down of the lattice has been shown to occur in skinned skeletal muscle when it is activated (Shapiro, Tawada & Podolsky, 1979; Matsubara, Umazume & Yagi, 1985). It follows from the currently accepted model of c-b action that as tension is generated by the c-bs there is a resultant which tends to draw the filaments together as well as the functionally more obvious one which produces shortening. Assuming that the lattice closes down during contraction in cardiac muscle, we can propose that the closing down produced by activation will result in an increase in the muscle's Ca-sensitivity. On the downgoing limb of the hysteresis sequences the muscle is in a more Ca-sensitive state as its lattice is compressed relative to the relaxed state. The application of a given calcium on the downgoing limb, therefore, resulting in a higher tension, as the myofibrils are more Ca-sensitive at the outset. This mechanism may appear on first inspection to be force dependent, the force dependence of hysteresis having already been discarded on experimental grounds. However, the amount of force produced will not be the sole factor

determining the amount of compression of the lattice, the initial lattice spacing will also contribute. From figure 1.11B we can see that at low s.l. the l.s. is at its greatest. In this state there is the greatest scope for l.s. compression.

Variablity of the phenomenon

The magnitude of the hysteresis was quite variable (see Table 1.2 & Figure 1.9). Some preparations displayed large amounts of hysteresis, others very little at the same s.l. If my proposal that hysteresis is due to an alteration in l.s. is true, an explanation of the variability of the phenomenon at a given s.l. may be sought in terms of the amount of swelling of the filament lattice in the individual preparations after chemical skinning. When the sarcolemma is removed or disrupted l.s. increases (Rome, 1967). The occurence of and reasons for, this swelling will be discussed in the next chapter. This raises the possibility that at least a part of the phenomenon is a direct consequence of the methodology i.e. what is generally termed an experimental artefact. The constriction of the lattice with force production and the effect of s.l. change are exaggerated in these swollen, skinned preparations. However, data from Matsubara & Millman (1974) as illustrated in figure 1.11 shows that l.s. does reduce with s.l. increase in intact cardiac muscle as constant volume behaviour would predict. Skinned fibres might therefore, tend to exaggerate the l.s. changes that I have argued, account for the length dependence of, and hysteresis in the

Ca-sensitivity of the cardiac contractile proteins.

In the next chapter I will examine the proposal that filament proximity alters Ca-sensitivity. I will test the hypothesis that the increased Ca-sensitivity with increased s.l. and hysteresis is due to reduced lattice spacing.

Chapter 2

The influence of tonicity on isometric force production and calcium sensitivity.

Introduction

In this chapter I have set out to investigate whether the proximity of the thick and thin filaments can alter the Ca-sensitivity of the contractile proteins. The proposal is that as the filaments are brought together, their apparent Ca-sensitivity increases. Lattice spacing changes would offer a simple common mechanism for the length dependence of, and hysteresis in, the Ca-sensitivity of the contractile proteins. The decreased l.s. associated with increased s.l. would explain the length dependence of Ca-sensitivity; the decreased l.s. with force production explains hysteresis (Shapiro, Tawada & Podolsky, 1979; Matsubara, Goldman & Simmons, 1984).

Results

Skinning and lattice spacing

The subject of the work in this chapter is the influence of l.s. on Ca-sensitivity. It therefore seemed pertinent to determine how the l.s. of cardiac muscle changes when the muscle is chemically skinned. It is well established that mechanically or chemically skinning skeletal muscle fibres leaves them in a swollen state, i.e. their l.s. is greater than *in vivo* (Rome, 1967; Godt & Maughan, 1977; Matsubara & Elliott, 1972). To investigate the extent of the swelling induced by chemically skinning cardiac muscle trabeculae, a few preparations were skinned in the microscope bath (Figure 1B methods section) and the changes in their overall diameters measured. The trabeculae were dissected and mounted under Ringer's solution (see Methods section for details of solution composition). The mounted muscle's bathing solution was changed from Ringer (I_e= 0.167M) to '10 relaxing' solution (I_e=0.19M), this produced a small (less than 5%) decrease in fibre diameter. The chemical skinning agent was then introduced to the '10 relaxing' solution. When skinned, the fibre's diameter was 5-10% greater than that of the intact fibre in the same solution (s.l. 2.2μm). The magnitude of the swelling did not appear to be dependent on the degree of skinning. A preparation initially skinned with saponin swelled no further when subsequently exposed to Triton-X100. The degree of swelling appeared to be preparation dependent.

What is Tonicity?

In skinned fibres the myofilament lattice allows free passage of H_2O, small ions and molecules, but excludes molecules with Mol.Wt.>40,000 daltons (Godt & Maughan, 1981). The bathing solution can, therefore, exhibit only that fraction of its total osmotic pressure which is due to molecules too large to enter the filament lattice. This fraction of the total osmotic pressure of a solution is termed tonicity. Evidently, therefore, a solution's tonicity cannot be predicted solely from its composition (as the osmotic pressure can) for the distinctive properties of the lattice are also involved. In fact, the tonicity of the solutions used here will even alter slightly as the lattice shrinks. With the shrunken lattice the size of the molecules excluded decreases, so more of the Dextran fraction will act tonically. In this study solution composition is defined only in terms of the amount and average molecular weight of the Dextrans employed (see Methods section for details of the solution chemistry).

Hypertonic shrinkage

Figure 2.1 is a diagrammatic representation of a chemically or mechanically skinned myofibril. In the standard solutions (see table 1 on the Methods section) the osmotic behaviour of the lattice is attributable to the fixed charges on the proteins. Ionic balance and overall charge balance is achieved by the uneven distribution of the mobile K^+ and Cl^- ions. The fixed charges on the myofilaments are net negative at physiological pH (Jennison & Elliott, 1981), resulting in a higher K^+ and a lower Cl^- concentration within the lattice than in the bulk bathing solution. (Donnan effect)

Compression of the fibre, and thus reduction of the interfilament space, can be brought about by the addition of long chain polymers to the bathing solution, which are too large to enter the filament lattice i.e. increasing the tonicity of the bathing fluid. Exclusion of these large polymers creates an osmotic gradient across the myofilament. To re-achieve osmotic balance water leaves the fibre. This reduction in interfilamental fluid volume results in radial compression if the length of the muscle is held constant (i.e. under isometric conditions). Charge balance is re-attained by the movement of the mobile K^+ and Cl^- ions. The fixed charges on the proteins remain constant so the same number of K^+ and Cl^- ions are required for charge balance. This means that the same amount of the mobile ions is now in a smaller volume, i.e. a higher concentration of both ions. Thus

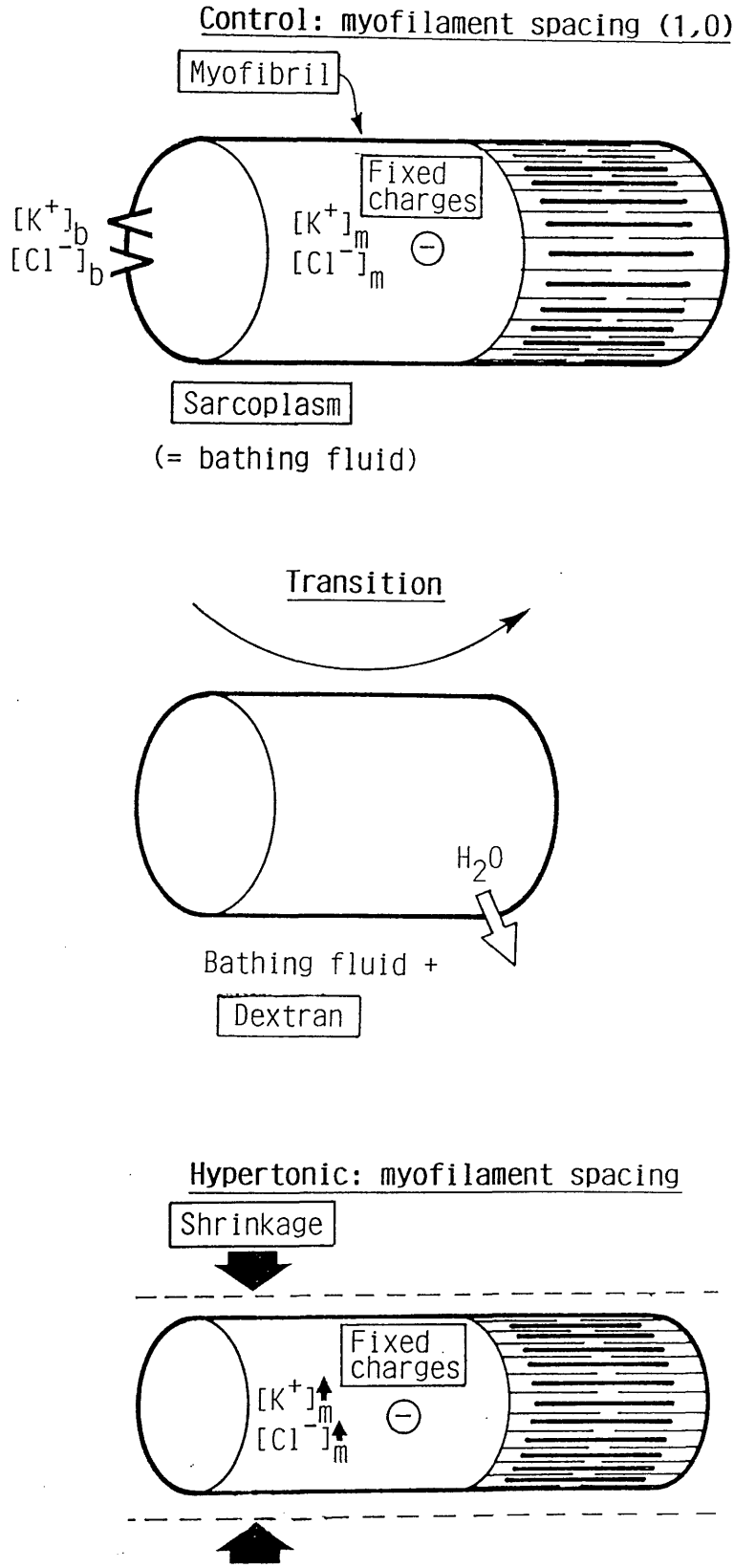


Figure 2.1 Hypertonic shrinkage of skinned muscle fibres.

hypertonic shrinkage technique allows alteration of the lattice spacing at constant sarcomere length.

We chose to use Dextran (a large polymer) to increase the tonicity of the bathing solutions as it is not charged (which would introduce ionic strength problems) and is biologically inert.

Hypertonic shrinkage and fibre diameter

Does increasing the tonicity of the solution bathing a skinned fibre preparation reduce the l.s. as predicted?. We had no direct method of establishing the l.s, so the muscle's diameter (as assessed using the DIC microscope) was used as a gauge of l.s. Decreases in fibre diameter were taken to indicate decreases in l.s. As predicted, the muscle's diameter decreased on the introduction of hypertonic solutions. A few preparations were mounted and skinned in the usual manner and then moved to the chamber illustrated in figure 1B of the Methods section. The optical characteristics of the hypertonic solutions (Dextran) did not alter the magnification of the preparation. This was determined by measuring the diameter of one of the mounting pins in the microscope with standard solutions and with hypertonic solutions. The tonicity of the bathing solution was altered by introducing Dextran T500 (M.Wt.~500,000) at various concentrations, to '10-relaxing' solution. The hypertonic solutions produced rapid shrinkage of the skinned fibre, most of

which occurred within the first minute, with up to 30 minutes being required before a stable fibre diameter was achieved. (This rapid initial shrinkage was on a comparable time scale to the onset of the effect on Ca-sensitivity and peak tension as we shall see later; figure 2.2) The shrinkage achieved with 3-5% T500 Dextran brought the fibre diameter back to its pre-skinning diameter, which we assume to be close to its *in vivo* diameter (This assumes that the intact fibre in Ringer's solution was close to *in vivo* diameter).

Larger concentrations of Dextran further decreased fibre diameter, to as little as 60% of the preskinning diameter with 20% T500. 10% T500 Dextran brought the fibre diameter back to slightly less than the preskinning diameter (within 5-10%). The smaller T70 Dextran, had a lesser tonic effect. Its tonicity was proportional to that predicted from our knowledge of its polydipersity as will be discussed in the section on Dextran purity.

Can lattice spacing alter calcium sensitivity?

Having shown that hypertonic shrinkage reduces the fibre's diameter and so by implication its l.s., I set out to investigate the effect of the fibre's l.s. in its apparent Ca-sensitivity.

Figure 2.2 illustrates the two main effects of hypertonic shrinkage on the skinned fibre. The figure shows a continuous section of tension trace from a Triton-treated rat cardiac trabecula with a s.l. of 2.0μm. The fibre was initially maximally activated at a pCa of 4.00 (i.e. 100μM free calcium). This solution was then switched to one with the same free calcium concentration but with 3% (i.e. 3g/100ml) T70 Dextran added. The hypertonicity produced shrinkage which increased C_{max} by 25%. $[Ca^{2+}]$ was then lowered allowing the muscle to relax. The second section of the trace shows the effect of hypertonic shrinkage at a submaximal level of activation. The muscle was partially activated at a pCa of 5.52 (i.e. 3μM free calcium), again the muscle was moved to a solution with the same calcium but with a higher tonicity. The tension produced by the fibre more than doubled. This figure shows the two main effects of shrinking the myofilament lattice: that is increasing Ca-sensitivity and C_{max}.

The effect of hypertonic shrinkage was determined at a range of calcium levels to determine the pCa-tension relationship. Figure 2.3 shows two pCa-tension curves, one in the presence of 3% T500 Dextran (filled circles) and the other in the presence of 3% T9 Dextran (shaded squares). Each of the test contractures was

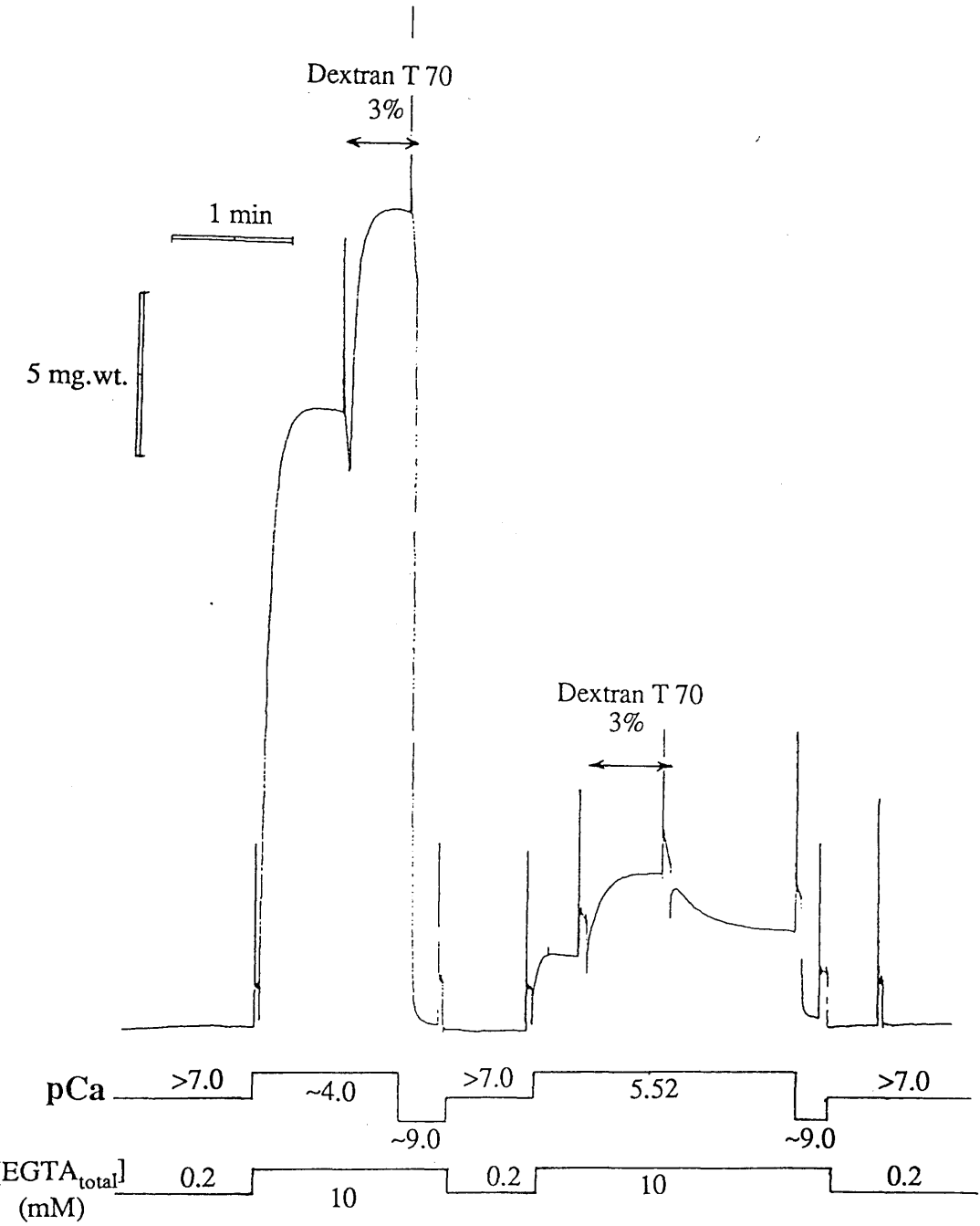


Figure 2.2

Effect of tonicity on maximal calcium-activated force and Ca-sensitivity.

A continuous section of tension trace from a rat cardiac trabecula (Triton-treated). Initially the calcium was raised to a level producing maximal calcium activation (pCa 4.00). The solution was then replaced with a solution of the same free calcium but with 3% T70 Dextran added. Tension rose. The muscle was then relaxed. Next the muscle was exposed to a pCa of 5.52 which produced approximately 20% of C_{max}, again 3% T70 Dextran was introduced, the tension level more than doubled. The Dextran was then removed again and the tension fell. Preparation diameter 90μm, s.l. 2.2μm.

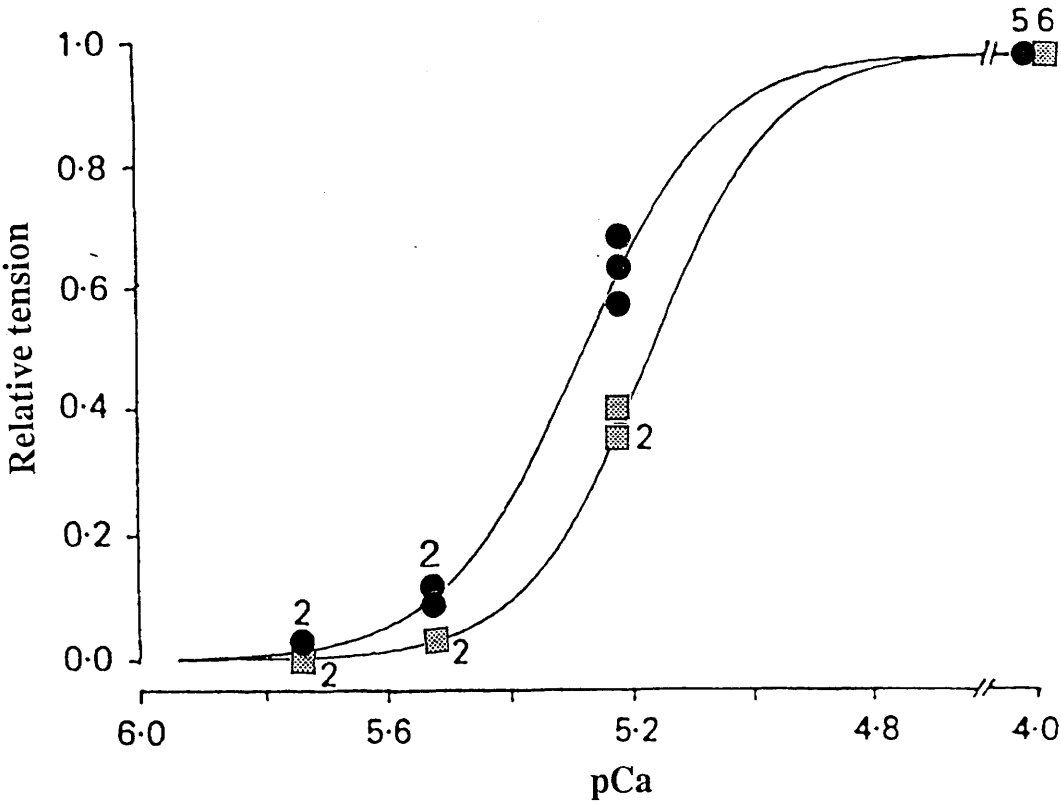


Figure 2.3

The pCa-tension relationship from a Triton-treated rat cardiac trabecula. The squares in the presence of 3% T9 Dextran, the filled circles in the presence of 3% T500 Dextran. The pK_{app} and 'h' were 5.17 and 4.2 in the presence of 3% T9 Dextran and 5.28 and 3.8 in the presence of 3% T500 Dextran. s.l. 2.2μm.

evoked directly from the relaxed state. The tension is expressed relative to C_{max} under each condition. In this example T500 Dextran increased peak force by 15%. The curves were determined with the appropriate Dextran continuously present as introduction and removal of the large Dextran led to fluctuations in the fibre diameter. The pCa for half maximal activation (pK_{app}) decreased by 0.11 pCa units and the Hill coefficient decreased by 0.4 units in this example. The small Dextran (T9 $\bar{M}_n$~9,000) was used as a check that Dextran itself did not affect Ca-sensitivity, and so to confirm that the action of the large molecular weight Dextran was through its tonicity effect. The T9 Dextran will readily penetrate the lattice and, hence, is without tonicity effect.

Dextran Purity

Dextrans are high molecular weight polymers of D-glucopyranose sythesised by bacteria belonging to the family Lactobacilleae. Native Dextran isolated from the culture filtrate is extremely polydisperse, containing molecules of all sizes from oligomers of a few hundred molecular weight to Dextrans with molecular weights of several hundred million. Commercially the native Dextran is repeatedly fractionated to produce narrow and well defined molecular weight samples.

The average molecular weight of polmers such as Dextran is expressible in several ways. The number average molecular weight, $\bar{M}_n$, given by:

$$M_n = \frac{\sum n_i M_i}{\sum n_i}$$

i.e. the ratio of the total weight of the sample to the number of moles of molecules. This gives no information about the spread of molecular weights in the sample.

The weight average molecular weight ($\bar{M}_w$)is given by:

$$\bar{M}_w = \frac{\sum n_i M_i^2}{\sum n_i M_i}$$

In this parameter each molecular size is weighted according to its weight fraction in the sample.

$\bar{M}_w$ is usually greater than $\bar{M}_n$ for heterogenous samples. The ratio $\bar{M}_w/\bar{M}_n$ gives an indication of the heterogeneity of the sample; i.e. $\bar{M}_w/\bar{M}_n=1$ for samples where all the molecules have the same molecular weight.

The initial supply of Dextran was obtained from The Sigma Chemical Company, who provide only the average molecular weights of their samples ($\bar{M}_n$). On the advice of Dr.J.A.Riegel (see Riegel, 1986) I switched to Pharmacia Dextrans. T70 had a $\bar{M}_w$ = 66,300 and a $\bar{M}_n$ = 36,400, the ratio = 1.82. T500 had a $\bar{M}_w$

=539,000 and a $\bar{M}_n$ = 197,000, the ratio = 2.73.

From the plots provided by Pharmacia, of molecular weight against weight percentage we could estimate that 30% by weight of the sample of T70 Dextran would have a molecular weight under 40,000 and would, therefore, not contribute to tonicity. With the T500 Dextran, less than 1 percentage of the sample would have a molecular weight less than 40,000 daltons. Therefore only 70% of the Pharmacia T70 Dextran could have a tonicity effect, even less in the case of the Sigma T70 Dextran.

These observations provide an explanation for the results in figure 2.4. The figure shows the effect of nominally osmotically equivalent percentage concentrations (weight/volume) amounts of T70 and T500 Sigma Dextrans. The T500 Dextran has a more pronounced effect moving the pCa-tension relationship by 0.30 pCa units while the T70 moved it by only 0.23 pCa units. We would predict that c. 1.5% T70 would be about as effective as c. 4% T500 if the myofilament lattice does filter at 40,000 M.Wt.

As a consequence of this observation the later experiments were carried out using T500 Pharmacia Dextran to avoid complications with small molecular weight Dextrans.

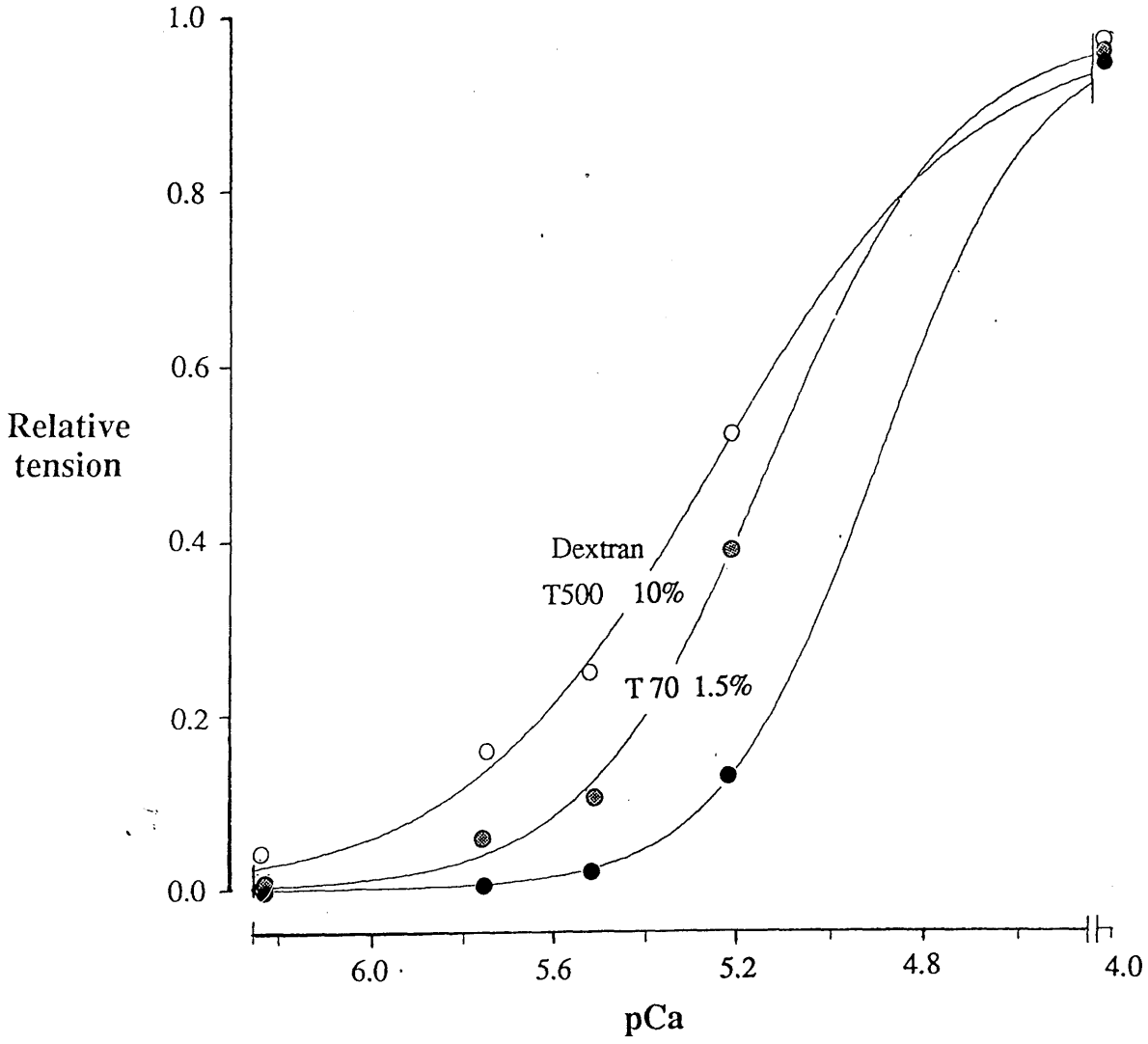


Figure 2.4

Effects of Dextrans of nominal osmotic equivalence.

The effect of nominally osmotically equivalent amounts of Sigma T500 and T70 Dextran on the pCa-tension relationship of a Triton-treated rat cardiac trabecula. The pK_{app} and 'h' were 4.90 and 2.6 (filled circles) in the absence of Dextran, 5.13 and 2.2 in the presence of 1.5% T70 Dextran (filled squares) and 5.24 and 1.6 in the presence of 10% T500 Dextran (open circles). s.l. 1.8μm.

The degree of hypertonic shrinkage and the change in calcium sensitivity.

When fibre dimeter was reduced from that of the skinned swollen state to that probably appropriate *in vivo*, with 3-5% T500 Dextran, the maximum increase in Ca-sensitivity was achieved, mean=0.08±0.06 (s.d) n=5. Cumulated data are presented in table 2.1. If fibre diameter was further decreased by 10% T500 Dextran (at a s.l. of 2.2μm), almost no effect on the apparent Ca-sensitivity was observed (see figure 2.5). This level of hypertonicity decreases the fibre's diameter to 5-10% below that *in vivo* at 2.2μm. At lower s.l. 10% T500 Dextran increases the Ca-sensitivity see figure 2.4. The 3% T70 which was naively predicted to be 7 times as effective as 3% of the T500 on the grounds of the amount of Dextran, (assuming all of the T70 M.Wt.>40,00 daltons) was actually equipotent to between 3 and 5% T500 Dextran as predicted above. 3% T70 Dextran produced close to *in vivo* dimensions in the fibre and again increased the Ca-sensitivity by the greatest amount.

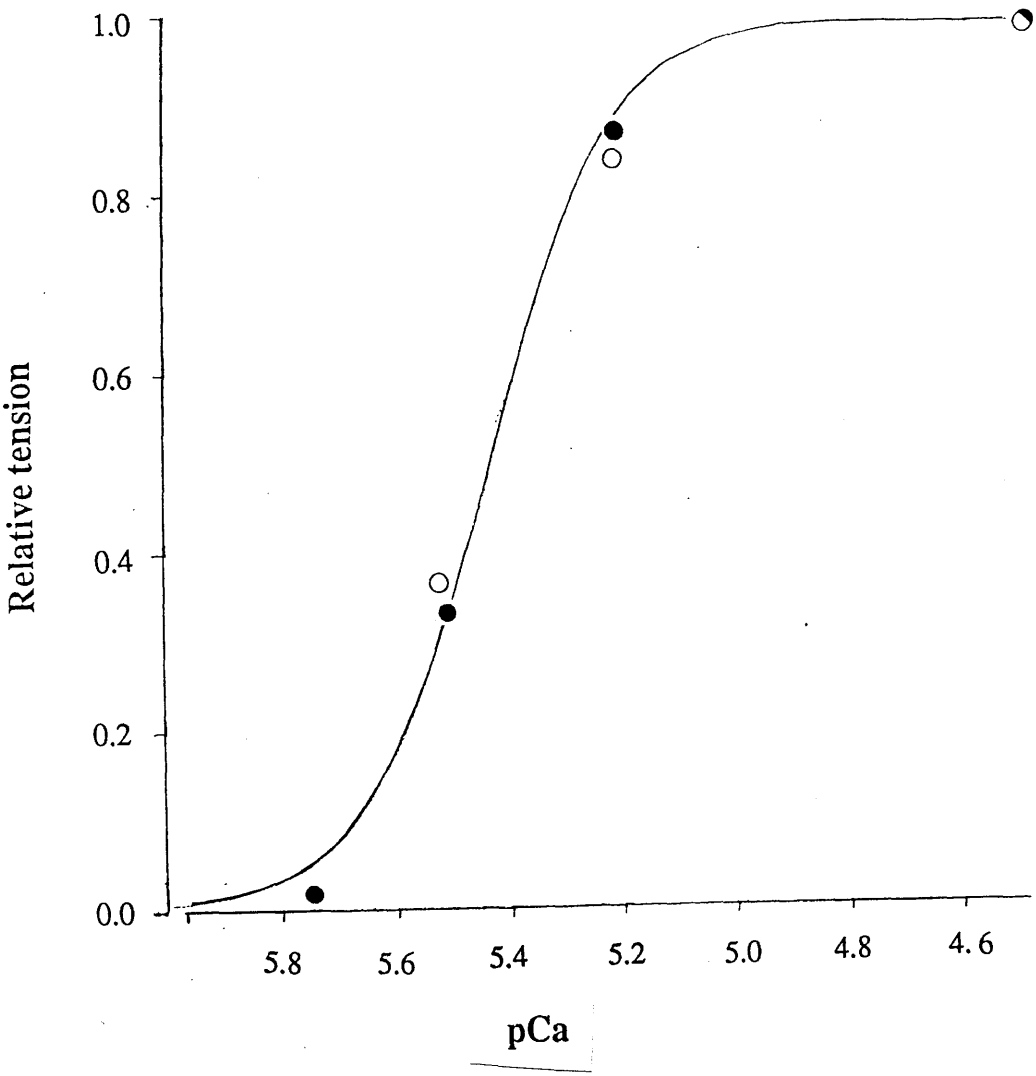


Figure 2.5

The effect of a large degree of hypertonic shrinkage on the Ca-sensitivity of a rat ventricle trabecula (Triton-treated). The filled circles are in the absence of Dextran, the open circles in the presence of 10% T500 Dextran. S.l. 2.2μm,

Table 2.1

Cumulated data the effect of 3% T500 Dextran

	Control		3% T500 Dextran		
Expt.	logKapp	'h'	logKapp	'h'	Delta
287	5.100	3.9	5.137	3.9	0.037
288	5.248	7.4	5.284	5.0	0.036
292	5.129	3.6	5.290	1.8	0.161*
297	5.169	4.2	5.289	3.1	0.120*
302	5.455	5.0	5.485	4.7	0.030
mean±s.d.	5.220±0.14		5.30±0.20		0.080±0.06

Where those marked * had a s.l. of 2.0μm or less.

The length dependence of calcium sensitivity and lattice spacing.

It was indicated in the discussion of chapter 1 that the working hypothesis for this series of experiments is that l.s. provides a mechanism for the length dependence of Ca-sensitivity i.e. that a decrease in l.s. increases the Ca-sensitivity. The hypothesis is that the increase in Ca-sensitivity produced by hypertonic shrinkage should be greatest when muscle s.l. is low, i.e. when the l.s is greatest. To test this the influence of hypertonic shrinkage on Ca-sensitivity was determined at two s.ls. With the muscle initially at a relatively low s.l.(~2.0μm), hypertonic shrinkage increased the Ca-sensitivity of the myofilaments by 0.15 pCa units (Figure 2.6 panel A). The muscle was then stretched to a s.l. of 2.2μm. This increased the

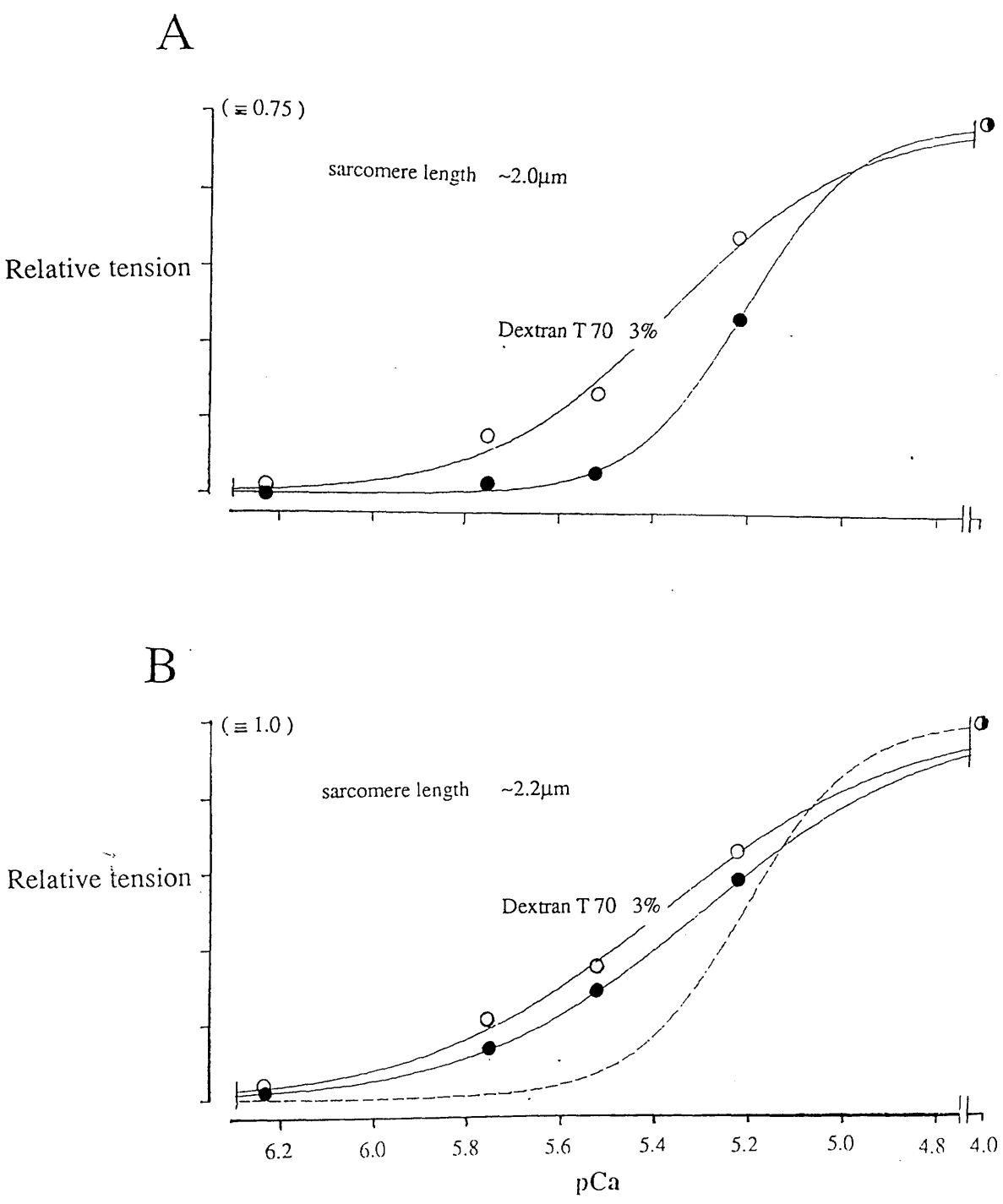


Figure 2.6

The influence of hypertonic shrinkage on the calcium sensitivity and length dependence of calcium sensitivity in skinned cardaic muscle. Panels A and B show pCa-tension relationships from a rat cardiac trabecula. In panel A the s.l. was 2.0 μm. Addition of 3% T70 Dextran (open symbols) increased the Ca-sensitivity (pK_{app} from 5.21 to 5.36, 'h' from 3.76 to 2.27) in a manner similar to that produced by the increase in s.l. In panel B the s.l. was increased to 2.2μm. Addition of Dextran produced a much shift in the pCa-tension relationship (pK_{app} from 5.30 to 5.38, 'h' from 1.81 to 1.72)

Ca-sensitivity of the preparation by 0.09 pCa units and increased the peak tension by 25% in the absence of Dextran. The shift in the pCa for half maximal activation of the muscle, is the length dependence of Ca-sensitivity (Endo, 1972, 1973; Hibberd & Jewell, 1982), As predicted, at this s.l. the hypertonic shrinkage increased the Ca-sensitivity less (by only 0.08 pCa units) than at the shorter s.l. The reduced effect of hypertonic shrinkage on the Ca-sensitivity at a s.l. of 2.2μm is illustrated in figure 2.5 panel B. The pCa-tension relationship at 2.0μm in the absence of Dextran is indicated by the dashed line in panel B.

The length dependence of lattice shrinkage

If, as proposed, l.s. is a determinant of muscle Ca-sensitivity, then lattice shrinkage at a low s.ls would be greater than at the longer s.l.(2.2μm) as the change in Ca-sensitivity is greatest there (see Figure 2.6).

I examined the changes in the diameter produced by hypertonic shrinkage at two s.l in one muscle. Using the DIC microscope. At a s.l. of 2.2μm the muscle examined had an average diameter of 190μm. Addition of 2.5% T500 Dextran to the '10-relaxing' solution caused the muscle's average diameter to decrease to 180μm (that is by 5.3%). The muscle was then released (at this lower length the sarcomere pattern was indistinct) to a s.l. of ~2.0μm. The fibre diameter increased to 210μm. In this state, addition of 2.5% T500 Dextran reduced the average diameter

to 180μm, i.e. by 14.3%. As proposed, the diameter of the muscle decreased more at the short s.l. (~2.0μm). The hypertonic shrinkage at both s.l. brought the fibres diameter to approximately the same value. This observation is worthy of further comment. This observation might have been predicted on the basis of results like those illustrated in figure 2.6, where the Ca-sensitivity in the presence of the hypertonic solutions are nearly identical at two s.l.s. It seems from figures 2.6 and 1.7 that lattice compression by moderate hypertonic shrinkage, by hysteresis or by change in s.l. to approximately 2.2μm may result in the fibre being compressed to nearly the same l.s, and so, brought to the same Ca-sensitivity. The greater influence of hypertonic shrinkage at low s.l. is also suggested in table 2.1. The effect of 3% T500 Dextran on Ca-sensitivity being greatest in the two preparations at low s.l. (marked with a *).

The effect of hypertonic shrinkage on peak tension (C_{max})

As indicated the effect of the Dextran is not purely on the Ca-sensitivity but also on the peak tension, see figure 2.2 for an example of this. This effect is also sarcomere length dependent as indicated in figure 2.6. The effect of hypertonic shrinkage of the lattice being greatest at low s.l. e.g. in one preparation 125% at 2.0μm and 110% at 2.2μm. The mean effect of the 3% T500 Dextran from the five preparations tabulated in Table 2.2 was 109.7±8.9% (s.d.) of C_{max} in the absence of the Dextran. The two at low s.l. effects was 102.3% and 114.3%. However, in

any experiment where the effect on peak tension at two s.l. was measured, the effect on peak force was always greatest at the lower s.l.

Table 2.2

Cumulated data on the effect of 3% T500 Dextran on C_{max}.

Expt. no.	$\%C_{max}$ in the absence of Dextran
287	121.8
288	109.5
292	102.3*
297	114.3*
302	100.0

n=5, x=109.7% and s.d.=8.9%

Where those marked * had a s.l. of 2.0μm or less

Discussion

Skinning and lattice spacing

The swelling of the myofilament lattice upon skinning is well documented (Rome, 1967; Matsubara & Elliott, 1972; Godt & Maughan, 1977; Brandt & Maughan, 1981; Metznger & Moss, 1987). In this study the average fibre diameter increased by 5-10%, (I_e=0.19M 20^{o}C). Why should the removal or disruption of the sarcolemma produce swelling of the myofibril?. It seems unlikely that the membrane exerts a transverse mechanical force on the filament lattice, as the sarcolemma is concave in places and does not look taut (Blinks, 1965). It is more likely that the restraining influence of the sarcolemma on the filament lattice is by restriction of ion and colloid movement between the fibre fluid and the bathing fluid. Two mechanisms can be proposed for this: (i) loss by diffusion of some high molecular weight substance present within the fibre which is excluded from the myofibril, this substance exerting a tonic compressive force on the myofibril, (ii) change in the ionic enviroment, within the lattice, for example a change in the ionic strength of the sarcoplasm. It has been proposed that reducing the ionic strength increases the electrostatic repulsive force between the filaments by reducing the counter ions in the interfilamental space (Elliott, 1968), leading to expansion of the lattice. It seems unlikely that our solutions are lower than physiological ionic strength. It would, however, be interesting to investigate the

extent of the swelling with skinning at a range of ionic strengths.

Lattice spacing and calcium sensitivity

In the last chapter, I proposed that altering the l.s. would alter the Ca-sensitivity. This proposal seems to have been borne out by the results presented in this chapter. When the l.s. was reduced by hypertonic shrinkage the muscle exhibited a higher apparent Ca-sensitivity (see figures 2.2, 2.3, 2.4, 2.6 and Table 2.1). The length-dependence of the change in Ca-sensitivity with hypertonic shrinkage (Figure 2.6) in addition to the length-dependence of the hysteresis described in chapter 1 (see figures 1.7, 1.8, Tables 1.2 and 1.3) provides strength to the initial proposal that lattice spacing changes might provide a common mechanism for the length-dependence of, and hysteresis in, the Ca-sensitivity of the myofilaments.

The length-dependence of calcium sensitivity

As indicated in the discussion of chapter 1, there are two opposing views about the mechanism underlying the length-dependence of Ca-sensitivity. One view is that the force produced by the fibre increases the Ca-sensitivity, the other is that the length of the muscle *per se* alters the Ca-sensitivity. In chapter 1, I proposed a unifying hypothesis that both factors alter the Ca-sensitivity by changing lattice spacing. Force production and increasing the s.l. reduce the fibre's l.s. The evidence suggests that the changes in Ca-sensitivity with s.l. and force production (hysteresis) observed in these preparation are due to changes in l.s. The length-dependence of Ca-sensitivity is very similar in nature to the shift in the pCa-tension relationship produced by hypertonic shrinkage. In fact, hypertonic shrinkage abolishes the effect of length change on Ca-sensitivity (see figure 2.6). However, I feel that the evidence reported and reviewed here suggests that while the explanation based on l.s. is correct, the l.s. change may be a phenomenon of the skinned fibre rather than of the intact cell.

In these experiments two factors brought the lattice spacing back near to that in life (i) tonicity and (ii) force. That tonicity does this can be inferred from my obervations of fibre diameter with hypertonic solutions. The suggestion that force brings the lattice back near to *in vivo* dimensions is based on the observation of Matsubara, Goldman & Simmons (1984) that

the d(1,0) (see figure 1.11A which illustrates d(1,0)) spacing of skinned skeletal fibres is returned to physiological dimensions during maximal activation (i.e. full activation, 'downgoing' hysteresis curves).

When the l.s. was returned to physiological size either by the force produced by the fibre (as in hysteresis) or by increasing the tonicity of the solutions bathing the muscle, the length-dependence of Ca-sensitivity was greatly diminished if not totally abolished (see figures 1.7, 1.8 and 1.9 and tables 1.2 and 1.3, e.g. common downgoing hysteresis curves at different s.l. and the small difference in Ca-sensitivity at 1.8 and 2.2μm s.l. when the lattice is compressed by dextran figure 2.6). This suggests that the swollen state of the skinned fibre myofilament lattice is a contributary factor to the occurrence of length-dependence of Ca-sensitivity and hysteresis. The ability of hypertonic shrinkage to increase the apparent Ca-sensitivity appears to be due to its ability to bring the lattice back to pre-skinning values. Greater degrees of hypertonic shrinkage (which bring the lattice below physiological size) do not increase the Ca-sensitivity (see figure 2.5, and the lack of effect of 100mg/ml polyvinylpyrrolidone M.Wt, 40,000, Fabiato & Fabiato, 1978)

On the other hand, there are several observations made by others on intact skeletal and cardiac muscle which suggest that hysteresis, and the length-dependence of Ca-sensitivity may not occur only in skinned fibres. For example, the first observations of hysteresis were made on intact barnacle fibres using aequorin to assess the calcium level, the muscle producing a higher tension level if the muscle had immediately previously been exposed to a higher calcium level (Ridgway et al, 1983). There are observations on intact cardiac muscle showing the aequorin-signalled calcium transient to decline more rapidly at longer s.l.s, while the duration of the tension transient increased (Allen & Kurihara, 1979, 1982) an observation which was explained an increase in Ca-sensitivity with increased s.l.

Overall, it appears that l.s. changes do provide the common mechanism for hysteresis in, and the length dependence of Ca-sensitivity in skinned fibres. However, it must be borne in mind that that these phenomenon are at the very least exaggerated in the skinned fibre.

Possible Mechanisms for the change in apparent Ca-sensitivity with lattice spacing.

The simplest interpretation of the increase in apparent Ca-sensitivity of the contractile proteins is that the affinity of troponin for calcium increases. It could, however, be that myofilamental separation alters the way in which the steps after calcium binding are transmitted to the contractile proteins. Unfortunately, there are no reports of changes of Ca-binding associated with hysteresis or hypertonic shrinkage; and no concensus as to the effect of s.l. change (see discussion of chapter 1). Alternatively filament spacing could affect the force at any given Ca-level by affecting the distribution of c-bs (by changing rate constants for transitions between states) or the force produced by each c-b, so changing the apparent Ca-sensitivity. These points are considered next.

(A) Force per cross-bridge at different angles of attachment

It would seem likely that changes in cross-bridge working distance could influence their performance. The l.s. decreases by 10% as the s.l. is increased from 2.0 to 2.2μm. The current view, however, is that c-bs accommodate these changes in working distance and maintain constant force per bridge over a wide range of muscle lengths by dint of their doubly-hinged configuration. (Huxley, 1969; Julian, Moss & Sollins, 1978)

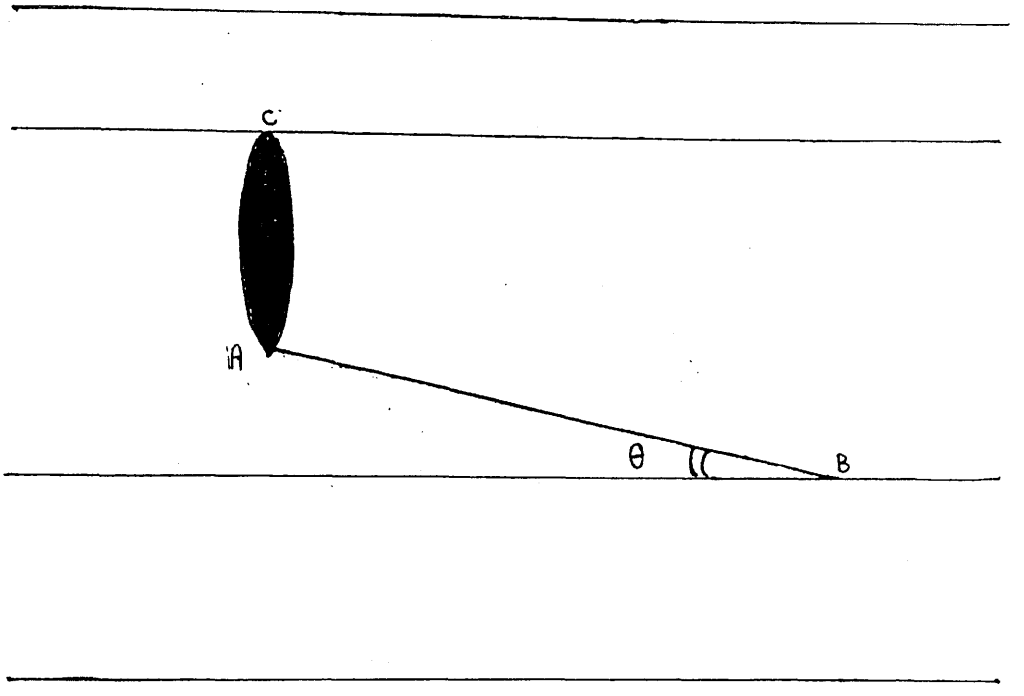


Figure 2.7

A possible arrangement of a muscle cross-bridge in two dimensions, drawn approximately to scale at a sarcomere length of 2.1μm (23nm filament separation). The thin filament (upper) has a diameter of 8nm, the thick filament (lower) has a diameter of 14nm. Note that point A need not lie in the same plane as the filaments.

A simplistic two dimensional model of the influence of c-b angle on its force production is shown in figure 2.7. This is a two dimensional model assuming the c-b to be perpendicular to the plane of the axis of the filaments (this a simplifying assumption which is known to be untrue (Squire, 1972: Schoenberg, 1980)). The angle subtended by the c-b to the (myosin) thick filament θ is critical to the longitudinal force exerted by the filament. Because of the assumption omitting the azimuthal angle this model provides an overestimate of the change in the angle of c-b attachment. The force produced by the c-b is cosine θ X longitudinal force exerted at the thin filament which will reduce as the filament spacing gets larger.

If the range of filament separations (thick: thin) is 15nm to 22nm, using the model with the dimensions shown in figure 2.7 θ will range from 4.89^{o} to 12.77^{o}. Cos θ from 0.996 to 0.975. Thus, even at the widest separation, 97.5% of the force generated by the c-b will act axially. This model predicts very little change in force output per c-b with l.s.. Schoenberg (1980) mathematically modelled the changes in force at different l.s. using the current 3 dimensional models of c-b attachment and concluded that the simplest models produce total axial forces which are proportional to the number of c-bs and independent of l.s. Overall it appears unlikely that the angle of attachment of the c-b with changes l.s. alters the force output of the muscle. Since change in force with l.s. would have provided an explanation of the increases in peak force with l.s. (see figure

2.2 and table 2.2) an observation which otherwise remains unexplained.

(B) Number of c-bs attached at different lattice spacings

(i) Changes in the number of sites of attachment with lattice spacing

A second possible influence of geometry on force production would be that the filament separation altered the number of c-bs formed. Squire (1972) pointed out that the cross-bridges attach to actin sites located at a variety of azimuthal angles with respect to the plane of the axes of the filaments as the axial and azimuthal periodicity of the actin helix is different from that of myosin. If there was not some flexibility in the cross-bridges to attach to sites at different axial and azimuthal positions, very few cross-bridges would be able to attach. Squire (1972), suggested that about 2/3rds of the cross-bridges can attach to actin sites if the range of azimuthal location of sites allowing attachment is 90°. Potentially the force output of a muscle, could be affected by the l.s. by changing the angular distribution of site locations allowing c-bs to attach. It might also change the overall force or even the force per cross-bridge if all the azimuthal locations do not behave similarly.

(ii) Change in the number of c-bs attached at different lattice spacings

As mentioned in the section on the relationship between calcium and tension, Brandt et al (1982) have proposed that the relationship between calcium and tension can be shifted to the left by increasing the time the c-bs spend attached. Thus if the c-bs were to 'hang-on' longer at narrower l.s (an idea suggested by Hibberd & Jewell, 1982) this would result in a greater average number of c-bs at intermediate $[Ca^{2+}]$ and so a higher apparent Ca-sensitivity. Osmotic compression has been reported to increase fibre stiffness suggesting that c-b attachment to the thin filaments has increased (Berman & Maughan, 1982; Kawai, Cornacchia & Schulman, 1984). These studies have demonstrated that variations in the lateral spacing of the filament lattice could affect the likelyhood of c-b attachment. Metzner & Moss (1987) have shown V_{max} to be decreased with small degrees of compression (5% T500 Dextran) an observation consistent with the idea that the reduced c-b cycling rate at narrower lattice spacings might underly the apparent increase in Ca-sensitivity observed in this study.

Overall conclusions

Overall the conclusion is that the increase in peak force and the augmented Ca-sensitivity of the myofilaments produced by both hypertonic shrinkage and stretching the muscle are a consequence of the geometry of the filaments in particular of their greater proximity. The mechanism for this remains obscure. It could be produced by changes in Ca-binding with l.s. or by changes in the number of c-bs attached. The skinned fibre results presented do not allow separation of the two types of increase. Until independent evidence is obtained using other experimental techniques no overall conclusion can be drawn about the mechanism underlying changes in Ca-sensitivity with changes in lattice spacing. The swollen state of the skinned fibres lattice should be borne in mind when extrapolating results from the skinned fibre to that in the intact cell.

Section 2

The Imidazoles and calcium sensitivity

Chapter 3

The Imidazoles and calcium sensitivity

Introduction

Caffeine is a widely used tool in muscle physiology. Until recently the site of its action in muscle was associated, either directly or indirectly, with the cell's membrane systems, e.g. acting directly on the sarcoplasmic reticulum (SR) (Weber & Herz, 1968; Endo, Tanaka & Ogawa, 1970), the sarcolemma or indirectly via inhibition of phosphodiesterase on the surface membrane adenylate cyclase, which in turn alters the phosphorylation of troponin I and so the sensitivity of the contractile proteins.

Experiments utilising the calcium-sensitive photoprotein aequorin to monitor intracellular calcium transients in mammalian cardiac muscle have shown caffeine to potentiate the twitch force while concomittantly decreasing the size of the calcium transient (Allen & Kurihara, 1980; Morgan & Blinks, 1982). This suggested that caffeine might be increasing the Ca-sensitivity of the contractile proteins by increasing the calcium bound at a given free $[Ca^{2+}]$.

In 1983, Wendt and Stephenson presented the first clear evidence that caffeine could act directly on the myofibrils. Caffeine shifted the pCa-tension relationship towards higher pCa, that is it increased the apparent Ca-sensitivity.

The results presented in this chapter are an investigation of the chemical structural requirements for caffeine's dual action on the myofilaments and the SR. The findings with some of caffeine's chemical relatives will be put into a wider context in the subsequent chapter.

Results

Chapman and Miller (1974) examined the features of chemical str[illegible]e which explain caffeine's abilityto provoke contractions in intact frog heart muscle. Later Chapman & Leoty (1976) made similar observation on mammalian cardiac muscle. Figure 3.1 shows a section of an experimental trace. The first contracture in each panel was evoked by the withdrawal of sodium from the bathing solution. In panel A the sodium-withdrawal contracture was followed by a transient contracture evoked by 5mM caffeine. This contracture was slightly larger than that produced by sodium withdrawal. In panel B application of 20mM imidazole evoked a much smaller contracture (~10% of the size of the caffeine contraction in panel A). The imidazole contracture differed in time course as well as magnitude to the caffeine contracture, being slower to rise and maintained. At the time Chapman and Miller concluded that the imidazole ring was sufficient to evoke contractions provided its 3 nitrogen was unsubstituted. They proposed that the contractions were solely due to calcium release from the SR. Figure 3.2 shows the chemical structures of caffeine, imidazole and Sulmazol (an imidazole-containing cardi[illegible]otonic agent Herzig, Feile & Ruegg, 1981; Scholz, 1984 Review) with the active (3) nitrogen circled.

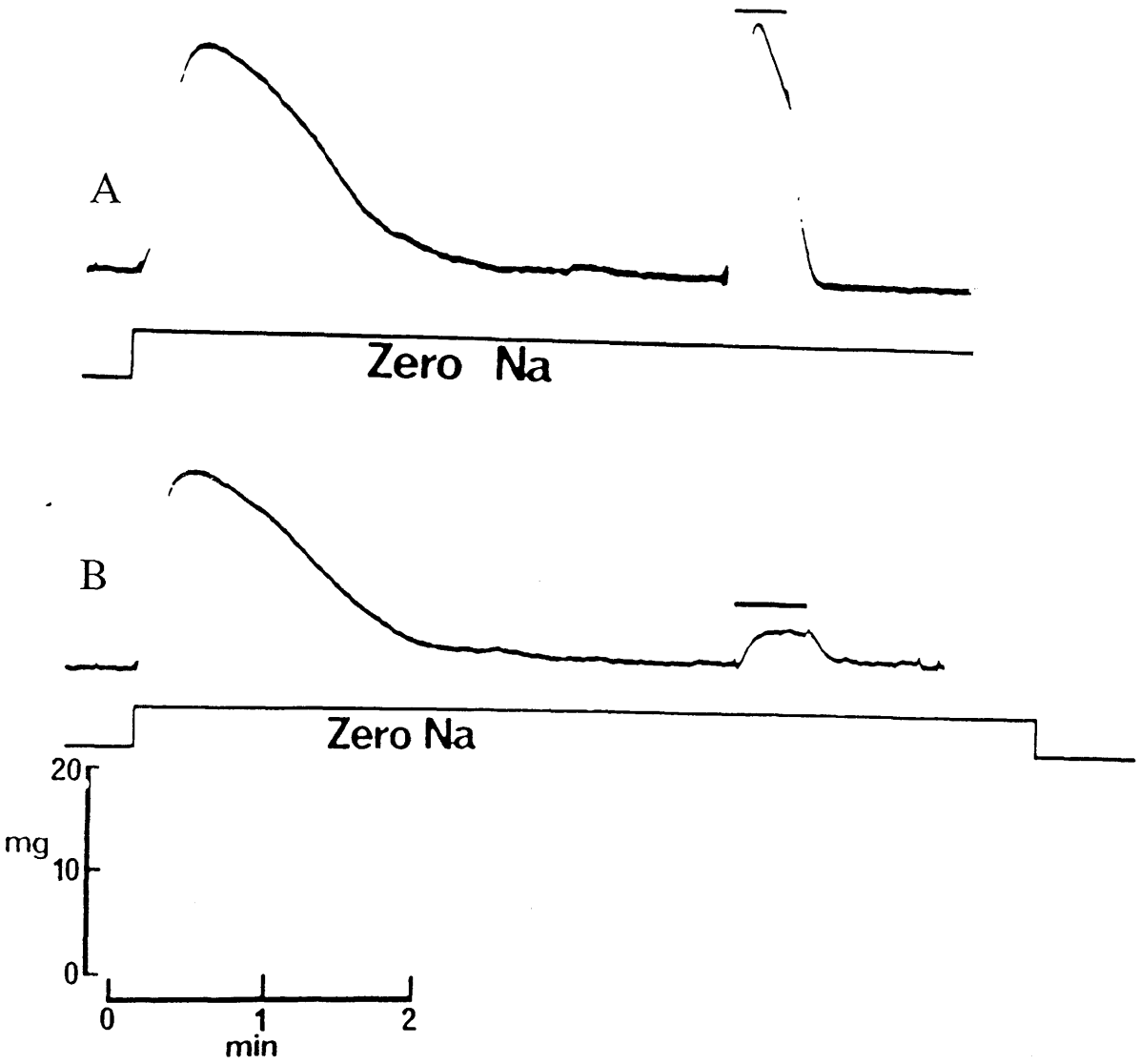
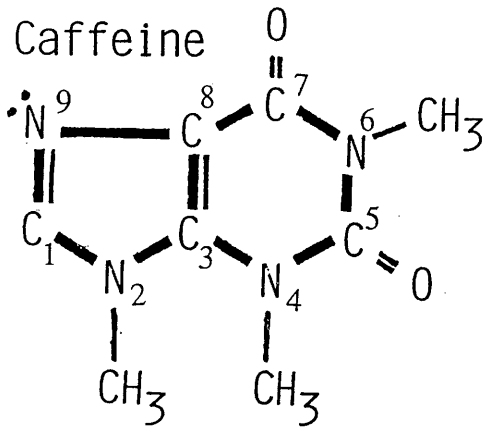


Figure 3.1

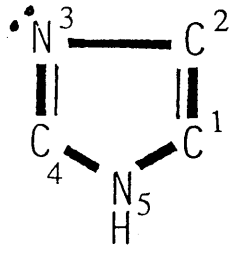
Caffeine and imidazole were tested for their ability to evoke contractures in intact frog cardiac muscle after a sodium withdrawal contraction had fully relaxed. 5mM caffeine and 20mM imidazole were appplied for the duration of the bars above the tension traces, A and B respectively. The experiment was carried out at 19^{o}C and the Ringer contained 2mM calcium.

(section of figure 44. D.J.Miller's PhD thesis 1973)

Caffeine



Imidazole



AR-L 115 BS (Sulmazol)

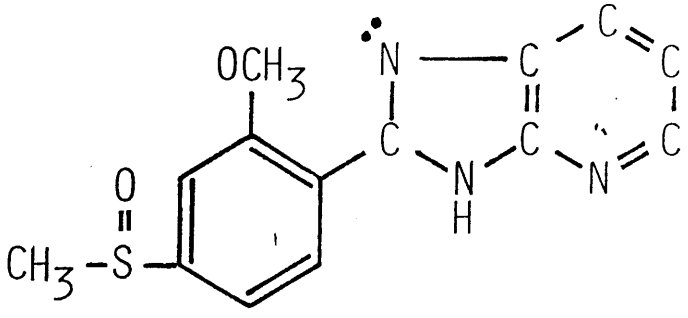


Figure 3.2

The chemical structures of caffeine, imidazole ans Sulmazol (AR-L 115BS).

In the light of Wendt and Stephenson's (1983) results the contractures reported by Chapman and Miller can now be viewed as having at least two possible components; that is, (i) calcium release from the SR and (ii) augmented Ca-sensitivity of the myofilaments. I propose that transient caffeine contractions are predominantly due to calcium release from intracellular stores, and the smaller maintained imidazole contractions, due to enhancement of the myofilament's Ca-sensitivity.

Caffeine and calcium sensitivity

The results to be presented confirm Wendt and Stephenson's (1983) findings. Chemically-skinned cardiac trabeculae of the rat were activated in strongly calcium-buffered solutions (10mM EGTA) in the absence and presence of 10-20mM caffeine. In all preparations caffeine was found to shift reversibly the curve relating steady-state force and free $[Ca^{2+}]$ towards lower free $[Ca^{2+}]$. The increase in apparent Ca-sensitivity was the same irrespective of the manner in which the fibre was skinned and consequently of the degree to which the cellular membrane systems were retained. The effect of caffeine was completely reversible with a time course compatible with diffusion.

Imidazole and calcium sensitivity

From the results of Chapman and Miller (1974) it could be predicted that imidazole itself might increase myofilamental Ca-sensitivity.

The effect of imidazole on the Ca-sensitivity of skinned fibres was indistinguishable from that produced by caffeine. An example of a pCa-tension relationship for a cardiac muscle trabecula in the absence and presence of 10mM imidazole is shown in figure 3.3. Imidazole was applied, replacing the standard pH buffer (HEPES) on a mole for mole basis, as it buffers pH significantly in the physiological range. HEPES, the standard pH buffer for these experiments is a zwitterion and makes a diminished contribution to ionic strength ($\sim$70% of its concentration at pH 7.0, Vega and Bates, 1976) while the dissociated form of imidazole contributes to the ionic strength. The imidazole-containing solutions will, therefore, have a slightly higher ionic strength than the standard HEPES containing solutions. The increased ionic strength would slightly decrease the muscle's apparent Ca-sensitivity (Ashley & Moisescu, 1977) leading to an underestimate of the effect of imidazoles in increasing the Ca-sensitivity.

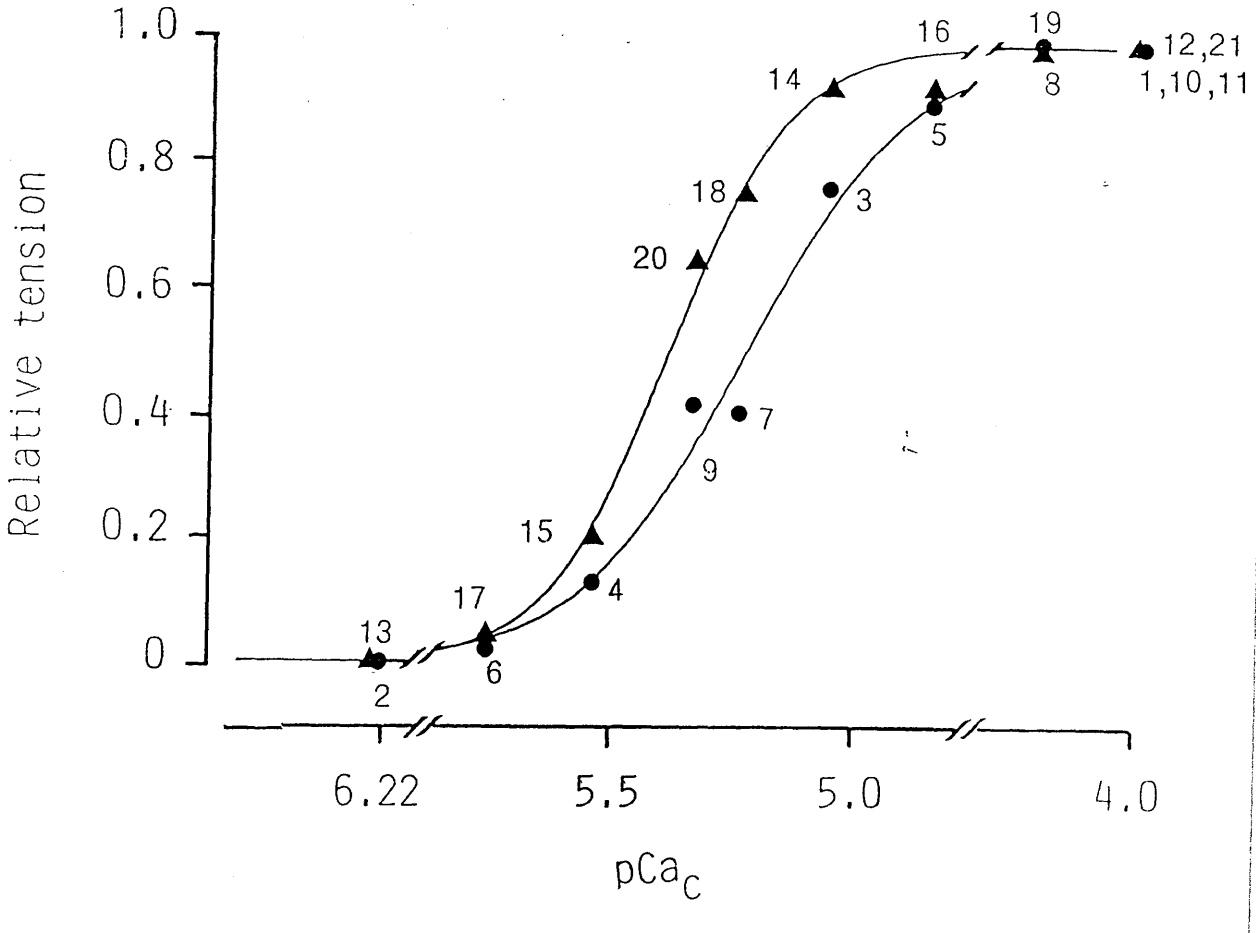


Figure 3.3

The increase in Ca-sensitivity produced by 10mM imidazole

Steady-state force at a given pCa is plotted for a rat ventricle trabecula chemically skinned with saponin. The small numbers indicate the sequence in which the determinations were made. The force is normalised to maximum calcium activated force under each condition, imidazole depressed C_{max} to 87%. The pCa for half maximal activation was increased from 5.21 to 5.37 and the Hill exponent from 2.62 to 3.58.

Caffeine, imidazole and peak tension

Caffeine had little or no effect on peak tension at concentrations from 10-20mM. Average 101.5%±5% (s.d) (n=4). This is contrary to the results of Wendt and Stephenson (1983) who showed 10-20mM caffeine to depress peak tension appreciably.

Imidazole had a much more pronounced effect on peak force. At concentrations of 10-20mM a slight depression of peak force was observed (peak force ranged from 80-94%, n=3). Peak tension was decreased to approximately 80% in the example shown in Figure 3.3. Part of this depression of peak force produced by imidazole can be explained by its ionic strength effect. Solutions with a higher ionic strength have been shown to depress peak tension (Ashley & Moisescu, 1977). However, the depression of peak force was greater than could be explained by this ionic strength effect alone. The effect of higher doses of imidazole on peak force was difficult to quantify as the ionic strength effect is difficult to compensate for. 60mM imidazole depressed peak tension to 80 and 77% in two preparations when no compensations were made for ionic strength.

Imidazole and calcium release from the SR

I propose that the action of caffeine on the intact muscle is predominatly due to calcium release (Chapman & Miller, 1974), the imidazole contraction is due to increased Ca-sensitivity. To investigate this proposal, the ability of these compounds to evoke calcium release from the SR was ascertained.

Caffeine's ability to release calcium from the SR is well known (Bianchi, 1961; Nayler, 1963; Weber & Herz, 1968; Endo, Tanaka & Ogawa, 1972). The saponin-treated preparation provides an ideal model to study calcium release from the SR. In this state the intracellular membrane systems are thought to be functionally intact (Endo & Kitazawa, 1978; Harrison, 1985) and in their physiological configuration. The contractile protein's tension production can act as a meter of Ca-release by the SR.

Protocol

Fibres were initially exposed to a free [Ca^{2+}] which allowed the SR to take up calcium, termed '<u>loading</u>'. The [Ca^{2+}] was then lowered. In the examples shown (see Figure 3.4A, 4.7, 4.8 and 4.9) the loading calcium level was one which produced tension. It should be pointed out, however, that calcium loading can be achieved in these preparations at calcium levels below the threshold for tension production (Harrison, 1985). Once the muscle was 'loaded' and 'relaxed' the calcium buffering capacity was reduced ([EGTA] lowered from 10mM to 0.2mM) to prevent the

released calcium being completely buffered by the EGTA before reaching the contractile proteins. The test compound was then applied in 0.2 Relaxing solution. The application of caffeine (1mM or more) could provoke a fast transient contracture (the magnitude of which depended critically on the calcium loading conditions, the composition of the bathing fluid and the sarcomere length). These contractures are due to release of calcium from the SR. This can be demonstrated by their dependence on prior Ca-loading conditions (Fabiato & Fabiato, 1975; Harrison, 1985) and their abolition with Triton-X100 which disrupts the membranes (Heleius & Simmons, 1979).

After Ca-loading and relaxing the muscle in the manner described above, application of imidazole provokes no contracture, whereas immediately subsequent application of caffeine provokes a contracture, confirming that the SR contains calcium. Application of imidazole at concentrations up to 60mM fails to provoke contractures. This suggests that imidazole's chemical structure is insufficient to provoke Ca-release from the subcellular stores.

A selection of other compounds were tested for their ability to release calcium, using the protocol described above. Of the compounds tested only those with more than one ring in their structure provoked contractions, e.g. the Ca-sensitivity enhancing drug Sulmazol (structure illustrated in figure 3.2) was able to provoke calcium release (see figure 3.4). However,

1,9,dimethyl xanthine, 1 methyl, 2 methyl and 4(5)methyl imidazole did not produce contractures, all of which produced shifts in Ca-sensitivity, except the 1,9,dimethyl xanthine. A selection of examples of the contractures or the lack of contractures produced by these compounds are illustrated in figure 3.4.

Overall it appeared that both a double-ringed structure and an unsubstituted 9 nitrogen position of the xanthine ring (that is the 3 nitrogen of the imidazole ring) is required for a compound to evoke calcium release. However, the imidazole ring alone is sufficient to produce the increase in the apparent Ca-sensitivity of the contractile proteins.

Discussion

Possible mechanisms for the apparent increase in Ca-sensitivity of the contractile proteins

(i) Calcium release from the subcellular organelles

Despite the variation in the degree to which cellular membranes were retained in the Triton- and saponin-treated preparations, the effect of caffeine or imidazole on the apparent Ca-sensitivity was the same. This implies that the cellular membranes were neither necessary for, nor involved in, the apparent increase in Ca-sensitivity produced by caffeine or imidazole. It is clear that the shift in the pCa-tension relationship produced by caffeine could not result from calcium release within the preparation (e.g. from the SR) as the effect is undiminished by distruction of the subcellular organelles (i.e. by Triton treatment). In addition the steady-state force was measured in solutions which were buffered for calcium at a level (10mM) well above that necessary to prevent caffeine contractions. Any release of calcium from a cellular store, if it did occur, would be immediately controlled by the calcium buffering capacity of the solution and could not affect the force even transiently.

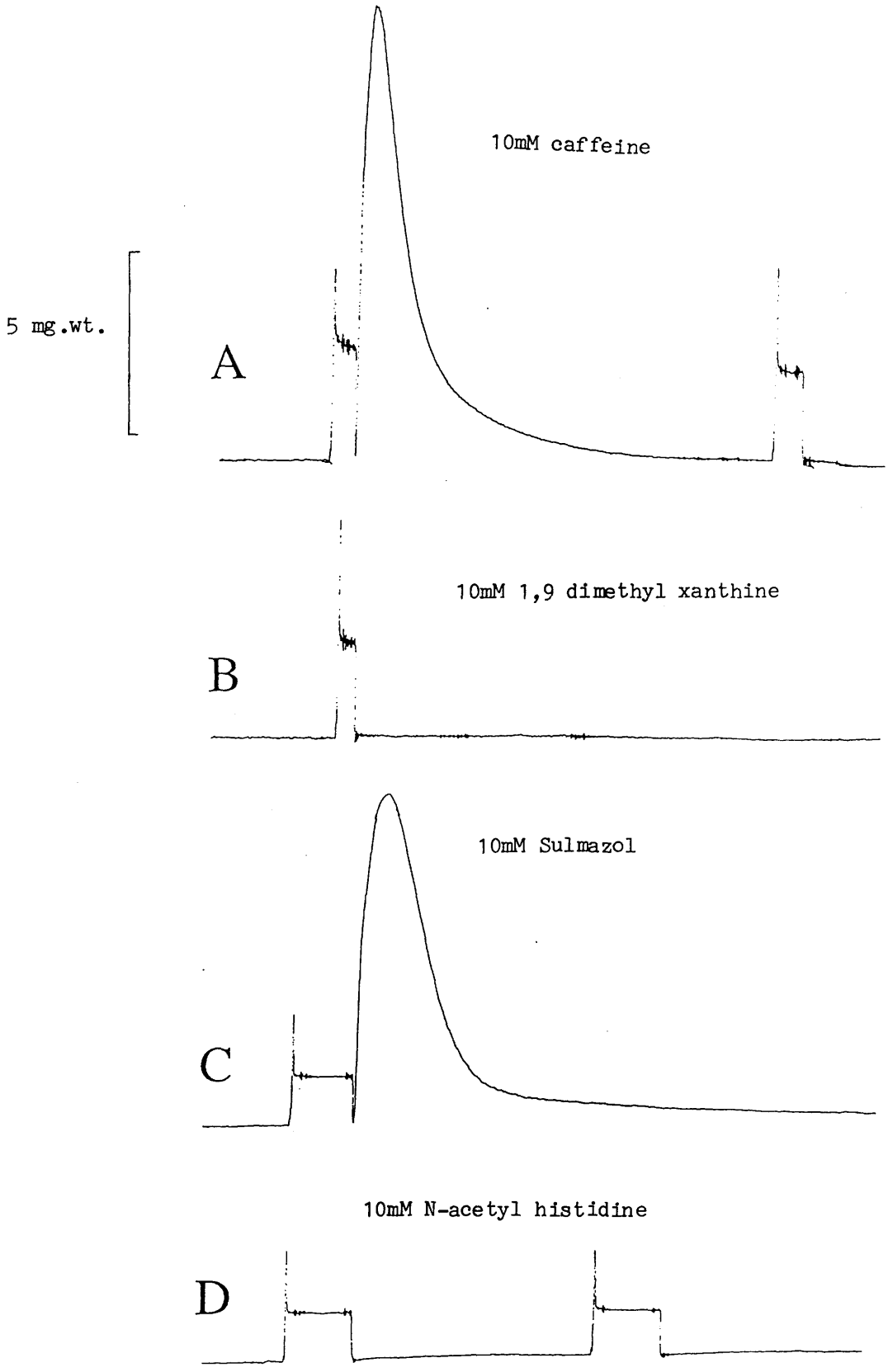


Figure 3.4

The effect of application of (A) 10mM caffeine, (B) 10mM N-acetlyl histidine, (C) 10mM Sulmazol and (D) 10mM 1,9 dimethyl xanthine to a well calcium loaded saponin-treated rat cardiac trabecula in 0.2 Relaxing solution.

Finally, imidazole is equipotent to caffeine in increasing the apparent Ca-sensitivity of the contractile proteins, but has no potency in evoking calcium release from subcellular stores, as shown in this chapter. Overall, the possibility that the increase in apparent Ca-sensitivity is a result of calcium released from the cellular stores can be discounted.

(ii) cAMP-dependent phosphorylation

The ability of caffeine to inhibit phosphodiesterase raises the possibility that it might alter cylic nucleotide levels which could in turn modulate Ca-sensitivity. Indeed it is likely that the regulation of cardiac myofibrils Ca-sensitivity is influenced by phosphorylation of troponin-I by the cAMP-dependent protein kinase. However, several factors argue against caffeine's ability to increase the apparent Ca-sensitivitity of the myofilaments, being due to phosphorylation.

First, caffeine and imidazole have quantitatively similar effects on Ca-sensitivity at the same concentrations even though caffeine is a phosphodiesterase inhibitor (Butcher & Sutherland, 1962) while imidazole is a facilitator of phosphodiesterase (Butcher & Sutherland, 1962).

Secondly, caffeine produces the same effect in cardiac and skeletal muscle (Wendt & Stephenson, 1983). cAMP-dependent protein kinase is found in both cardiac and skeletal muscles. However, activation of this enzyme results in phosphorlyation of cardiac troponin but not normally skeletal muscle troponin (Stull, 1980).

Finally, Wendt and Stephenson (1983) were unable to produce an effect such as that obtained with caffeine, or to modify the effect produced by caffeine, by addition of exogenous cAMP. In fact the effect of cAMP on the steady-state force measured was in the opposite direction to that of caffeine (Wendt & Stephenson, 1983; Fabiato & Fabiato, 1978; Herzig & Ruegg, 1979),

Overall, it appears that the effect of caffeine or imidazole on Ca-sensitivity is not mediated by c-AMP dependent phosphorylation.

(iii) pH effect

The possibility that the effect was due to a pH change or improved pH buffering can also be discounted. The constancy of pH was determined as described in the Methoods section. Increasing the pH buffer capacity of the solutions made no detectable difference to the steady-state tension levels. Imidazole, and its close relatives, are pH buffers while caffeine which produces an indistinguishable effect cannot enolize or ionize. Additionally, the Ca-sensitivity enhancing drugs such as Sulmazol act strongly

even at concentrations too low to have a direct pH effect (less than 0.1mM).

(iv) Calcium binding

The simplest mechanism for the increased Ca-sensitivity would be that imidazole somehow augments Ca-binding. This could be a direct effect on troponin C or an indirect effect on the competitive binding of cations other that calcium to the regulatory sites (Stephenson & Wendt, 1983). The lesser shift in the force-pCa curve produced by caffeine at higher ionic strengths (Wendt & Stephenson, 1983; Steele, personal communication) is consistent with the effect being on Ca-binding.

Currently, there are no data to indicate whether imidazole or caffeine can alter the amount of calcium bound to the myofilaments. However, Solaro & Ruegg (1982) have shown Sulmazol, (one of a group of compounds currently under development as inotropes, which act in the same way as caffeine and imidazole to increase myofilamental Ca-sensitivity, Herzig et al, 1981), an imidazole-containing compound, to increase the calcium bound to cardiac myofilaments. The effect of Sulmazol on Ca-binding suggests that the action of the other imidazole-containing compounds on Ca-sensitivity may be mediated via increased Ca-binding. This explanation is also favoured by Allen and Kurihara's (1980) observation that caffeine potentiates the twitch while concommitantly decreasing the size of the calcium

transient. This observation would be consistent with higher calcium binding in the presence of caffeine.

Overall the most likely explanation of the increase in apparent Ca-sensitivity produced by the imidazole-containing compounds is that they in some way augment Ca-binding to the myofilaments.

Possible mechanism for caffeine's ability to release calcium from the SR and imidazole's failure

(i) Direct action of the molecules on the SR membranes

Caffeine's ability to release calcium from the SR is thought to mimic calcium-induced calcium-release (CICR), which is the mechanism by which contraction is thought to be elicited physiologically (Fabiato, review 1983) calcium enters the cell during the action potential elicits a positive feedback by which calcium induces calcium release from the intracelluar stores. It is proposed that caffeine binds to some site on the SR membrane which provokes the calcium release through the same channel as CICR. Imidazole's inability to induce calcium release may be a result of its inability to bind to the SR membrane and so to trigger calcium release. However, there is no direct evidence for caffeine or imidazole binding to the SR membrane.

A second mechanism that has been proposed (Endo, Tanaka & Ogawa, 1970) for the ability of caffeine to provoke calcium release from the SR is that caffeine lowers the threshold for calcium-induced calcium-release. A suggestion which I will return to in the next chapter.

(ii) Phosphorylation

Caffeine and imidazole have opposite effects on phosphodiesterase, as they do on calcium release. Phosphodiesterase inhibition leads to elevated levels of cAMP, which in turn facilitates phosphorylation. This mechanism for calcium release is unlikely as the effect of the caffeine is fast, the contractures peaking within 1s of caffeine application, the time for the onset of the contraction being sufficient for caffeine's diffusion into the cell, but inadequate for build up of cAMP. There is no evidence for cAMP directly causing Ca-release, though there is some evidence from skeletal muscle that it can augment Ca-uptake by the SR (Fabiato & Fabiato, 1978).

The mechanism by which caffeine evokes calcium release from the subcellular organelles is not clearly understood. These results provide no clarification of this situation, other than to establish that an unsubstituted 3 Nitrogen of the imidazole ring and some feature in addition to a simple imidazole ring is required for caffeine-like provoked Ca-release from intracellular stores.

Irrespective of the mechanism by which caffeine enhances the apparent Ca-sensitivity of the contractile proteins, this effect must be borne in mind when interpreting the force response of a muscle in the presence of this drug. Care must be taken for example, when interpreting the caffeine contractures of skinned muscle in terms of calcium release from the SR.

The effect of imidazole itself on Ca-sensitivity must also be borne in mind. It is frequently used as a pH buffer in muscle physiology and biochemistry, often in concentrations as high as 60mM which would greatly increase the Ca-sensitivity and decrease C_{max} of the fibres.

Knowing that the imidazole ring can enhance the apparent Ca-sensitivity of the myofilaments led me to consider what the physiological basis for this phenomenon might be. It is common to approach such questions by considering whether the compounds of interest are mimicking the action of some endogenous substance. Are there natural cellular imidazoles which act to increase the Ca-sensitivity of the contractile system?.

Chapter 4

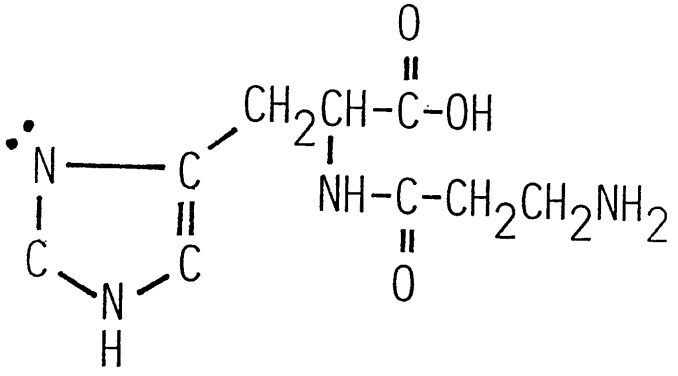
Cellular imidazoles as the natural enhancers of calcium sensitivity

Introduction

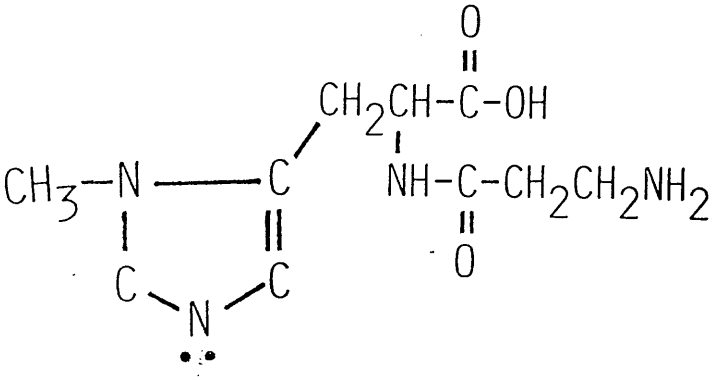
In the last chapter I established the imidazole ring to be sufficient to augment myofilamental Ca-sensitivity directly in the same manner as caffeine. This raised the possibility that there may be endogenous sarcoplasmic imidazole-containing compounds which increase the Ca-sensitivity of the contractile proteins.

Cellular imidazoles

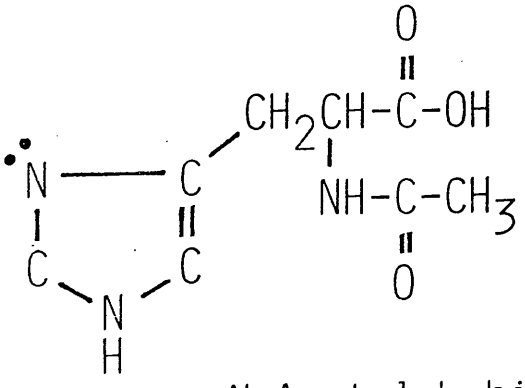
The most abundant cellular imidazoles are the histidine dipeptides which are found predominantly in muscle tissues. These compounds are found in concentrations from 1 to 60mM in vertebrate skeletal muscle (Crush, 1970). The most abundant of these are carnosine (β-alanyl-L-histidine), anserine (β-alanyl-L-1-methyl histidine), ophidine (β-alanyl-L-3-methyl histidine), and homocarnosine (γ-aminobutyryl histidine). The chemical structures of three of these compounds; carnosine, anserine and N-acetyl histidine are shown in figure 4.1.



Carnosine



Anserine



N-Acetyl-L-histidine

Figure 4.1

The Chemical structures of carnosine, anserine and N-acetyl histidine.

The distribution of the histidine dipeptides

The histidine dipeptides are found predominantly in skeletal muscle, though they have been found in other tissues for example the brain (Perry, Berry, Hansen, Diamond & Mok, 1971) and the nasal olfactory bulb (Margolis, 1974). Most vertebrate skeletal muscle contains histidine dipeptides, the type and amount of which varies greatly. Terrestrial mammalian and amphibian muscle contains predominantly carnosine (generally at levels between 2-20mmol/kg of muscle). Bird and some fish muscle, in contrast, has higher levels of anserine than of carnosine. Invertebrate muscle generally has little or none of the histidine dipeptides though some Mollusca and Crustacea (Lukton & Olcott, 1958; Crush, 1970) have been reported to contain carnosine.

Histidine dipeptide content of skeletal muscle

Carnosine was first discovered in bovine muscle at the turn of the century (Gulewitsch & Amiradzibi, 1900) and anserine as its name suggests in the goose (Anser sp.), a few years later (Tolkachevskaya, 1929; Ackerman, Timpe & Poller, 1929). Since shortly after their discovery it has been recognised that these compounds are not uniformly distributed, but vary quite dramatically with muscle type. The concentrations of carnosine and anserine are generally greater in white muscle than in red (Zapp & Wilson, 1938, Davey, 1960; Castellini & Somero, 1981; Christman, 1968) e.g. in the white muscle of skipjack tuna these

compounds contributed 147±3.5mmol/kg of muscle (n=5) but in the red muscle the concentration was only 21.3±2.1mmol/kg of muscle (Abe, Brill, Hochachka, 1986). These compounds are absent in crab muscle (Crush, 1970) while levels as high as 150mM have been found in the white muscle of the tuna (Abe et al, 1986). Examples of the levels of histidine dipeptides found in a selection of muscles are given in table 4.1.

Table 4.1

Compound	Concentration mmol/kg wet tissue	Species	Muscle	Reference
Carnosine	15	Rana temporaria	Sartorius	Burton 1983a
Carnosine Anserine	5.5 2.5	Rat	Gastrocnemius	Tamaki, et al, 1977
Carnosine	1-8	Homo sapiens	Various	Christman 1971
Carnosine	2.72 0.62	Rabbit	Skeletal Heart	Sobue et al 1975
Anserine	18.0	Atlantic salmon		Crush, 1970

The histidine dipeptide content of cardiac muscle

While much information is available concerning the concentrations of carnosine and anserine in skeletal muscle, there are only a few divergent reports of these compounds in heart muscle. The first positive identification of carnosine and anserine in heart muscle (frog and rabbit) was made by Yudaev (1949) using paper chromatography. The following year Parshin & Gorunkhina made the first quantification of these compounds in cardiac muscle. They arrived at figures of 4-6mmol/kg. of fresh heart tissue. As the methods for quantification of these compounds were refined the levels estimated decreased dramatically, Schmidt and Cubiles (1955) using paper electrophoresis detected no carnosine or anserine in the ventricles of several species of vertebrates, Abraham et al, (1961) detected no carnosine or homocarnosine in rat heart and Sobue, Konishi & Nakajima (1974) found only 0.6mM carnosine in rabbit heart. However, Kuroda & Ikoma (1966) identified a novel histidine-containing compound, N-acetyl histidine, in extracts of frog heart muscle. Preliminary work from this laboratory (Crichton, Lamont, Miller & O'Dowd, 1987) has suggested that the bulk of the histidine dipeptides found in cardiac muscle are acetylated and so have not been detected by the methods used to measure carnosine and anserine. Preliminary observations suggest there may by 1-8mM imidazole-containing compounds in heart muscle sarcoplasm.

Despite the considerable interest in the histidine dipeptides, no concensus has been reached on their function. They have been implicated in a wide variety of processes including: pH buffering (Davey, 1960) myosin ATPase regulation (Avena & Bowen, 1969), divalent cation binding (Brown & Antholine, 1979; Brown, 1981), neurotransmission (Rochel & Margolis, 1982) modulation of muscle phosphorylases (Fedyna. Mertiz-Smith, Kavinsky & Johnson, 1982; Johnson & Aldstadt, 1984) and SR Ca-uptake (Lopina & Boldyrev, 1975).

In this chapter I report an examination of the influence of the histidine dipeptides on skinned muscle function. I have investigated the action of the endogenous imidazole-containing compounds on the Ca-sensitivity of the contractile proteins and the mobilisation of calcium by the subcellular organelles.

Results

The effect of the histidine dipeptides on calcium sensitivity

The chemical structures of the imidazole containing compounds; carnosine, anserine and N-acetyl histidine are illustrated in figure 4.1. They all contain an imidazole ring with a free 3 nitrogen , which was shown in the last chapter to be chemically sufficient to increase the contractile protein's apparent Ca-sensitivity i.e. shift the pCa-tension relationship to the left.

The effect of carnosine on the calcium sensitivity of skeletal muscle

The occurence of the histidine dipeptides in skeletal muscle sarcoplasm suggests they might physiologically enhance the Ca-sensitivity of the contractile protein's. The basic observation of an increase in the calcium sensitivity produced by these compounds is shown in figure 4.2. In this preparation a small bundle of muscle fibres (*frog sartorious*) were chemically skinned with Triton-X100. The preparation was activated approximately half-maximally from the relaxed state by increasing the bathing $[Ca^{2+}]$ to a pCa of 5.85. Once a steady tension level had been achieved the muscle's bathing solution was changed to one with the same free $[Ca^{2+}]$ but without carnosine, tension

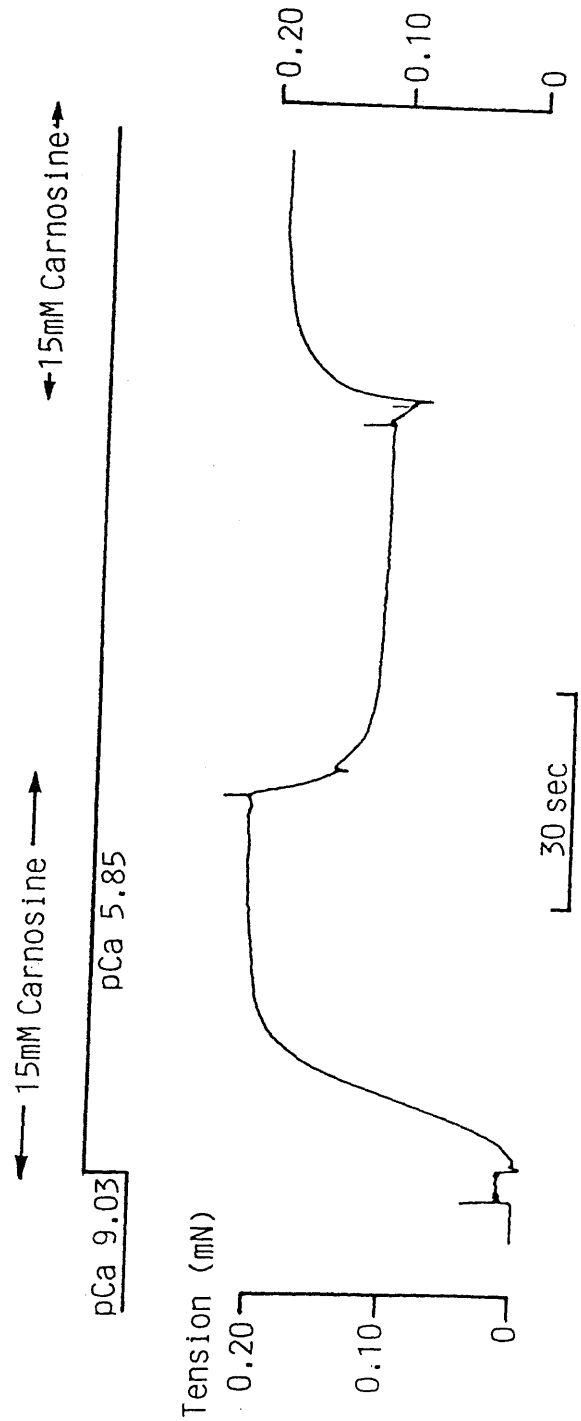


Figure 4.2 Effect of carnosine on Triton-treated frog sartorius fibre.

Force produced by Triton-treated frog sartorius fibres. The effect of removing and reapplying 15mM carnosine are shown. pCa 5.58 produced about half-maximal tension in this muscle. The speed of response indicates that the effects of carnosine develop with a time-course limited by diffusion. S.l. 2.2μm, I_e 0.14M, three fibres overall diameter 90μm.

fell. When the muscle was returned to the original carnosine-containing solution, tension redeveloped. The increase in Ca-sensitivity produced by carnosine resulted in a rapid increase in tension which reversed at a similar rate. Figure 4.3 shows the data from a similar experiment plotted as relative tension against pCa. In this example the presence of carnosine shifted the pCa-tension relationship to the left and possibly steepened it (there are too few data to be confident about the effect on slope). Carnosine was replaced by HEPES in the solutions on a mole for mole basis to maintain the buffer capacity of the solutions (Carnosine pK_2=6.83, Bates-Smith, 1938) as outlined for imidazole in the last chapter.

Many skeletal fibres were mounted (n=19) but few proved to be functionally viable long enough for their pCa-tension relationship to be determined accurately. It appeared that the mounting system used (see figure 1.A methods section) was not suitable for skeletal fibre preparations. The fibres were generally too small to mount singly (i.e. with diameters less than 50μm) and the mounting of small bundles of fibres was unsatisfactory as frequently during activation sequencies (despite the use of the Ca-jump technique, Ashley & Moisescu, 1977; Miller, 1975) one or more of the fibres broke. In those preparation where a range of pCas with and without carnosine was sucessfully tested, the tension measurements were made cumulatively as outlined in the subscript to figure 4.3 (a methodology not used with the cardiac preparations). The

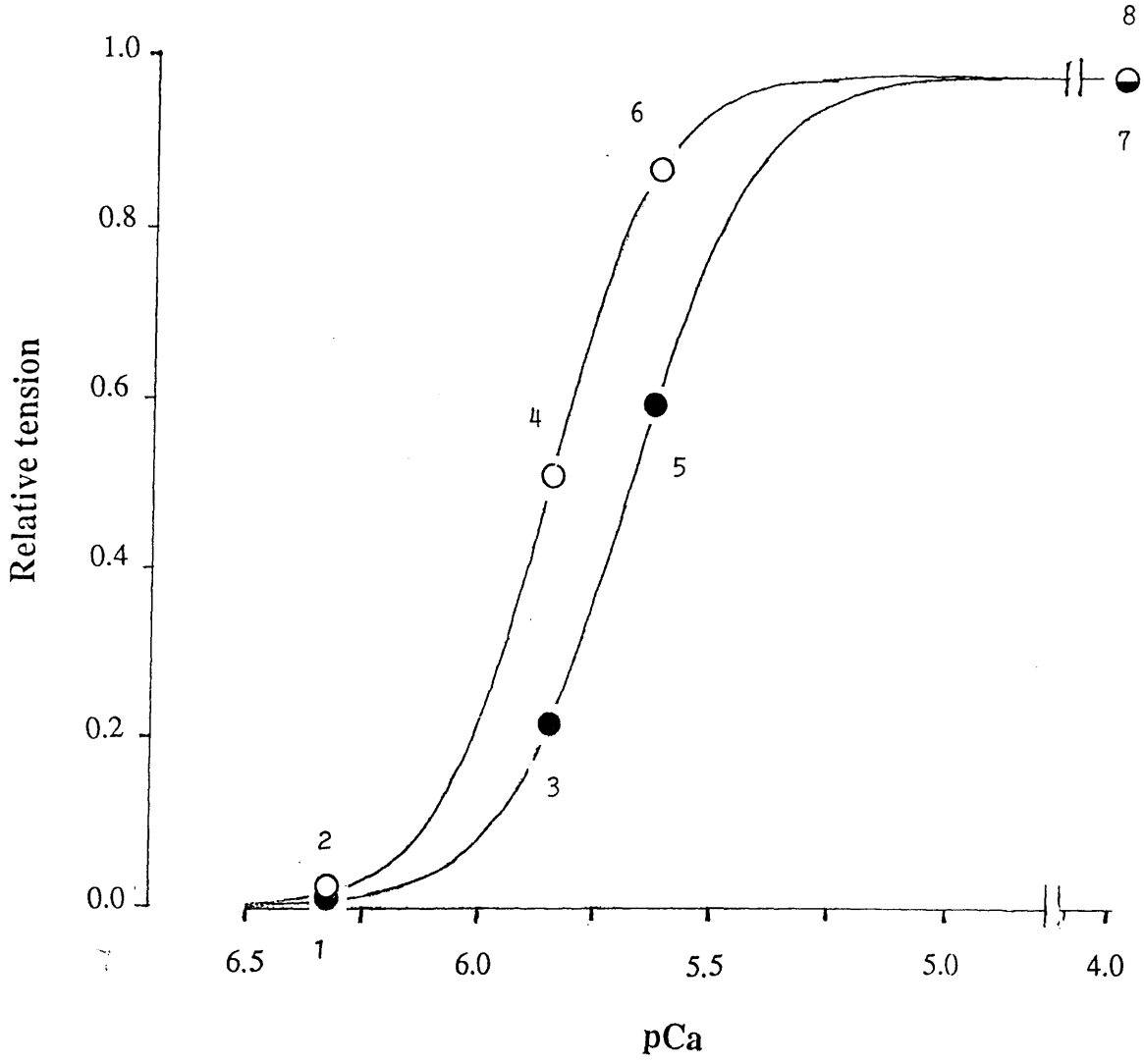


Figure 4.3

The pCa-tension relationship for a single Triton-treated sartorius fibre from the frog. The open circles indicate the presence of 15mM carnosine, the filled circles indicate its absence. The pCa was calculated using REACT with the appropriate alterations for the frog solutions used in this experiment (see table 2 methods section). The determinations were made cumulatively in the order indicated by the small numbers. The determinations were made in this manner as the fibres became damaged very quickly making single step determinations of several pCas with and without carnosine impossible in the time available. The pK_{app} increased from 5.68 to 5.85 and 'h' from 3.3. to 3.8 with the application of 15mM carnosine.

cumulative determinations were made with increasing Ca-levels to avoid the problems associated with hysteresis in the pCa-tension relationship, described in chapter 1. Most of the preparations lasted long enough for an intermediate Ca-level with and without carnosine to be determined, though few survived long enough for the pCa-tension relationship with and without carnosine to be described. My subjective impression was that the shifts in the pCa-tension relationship quantified in the four preparations detailed in Table 4.2 were typical.

Sulmazol also increased the Ca-sensitivity of skinned skeletal muscle fibres. However, none of the fibres in which Sulmazol was applied survived long enough for an estimate of the magnitude of the effect in terms of K_{app} shift to be made.

The effect of carnosine on the calcium sensitivity of crab skeletal muscle fibres

As mentioned in the introduction, the levels of the histidine dipeptides in invertebrate muscles are generally low. Parker and Ring (1970) have shown the ATPases of muscles from invertebrates to be unaffected by the imidazole dipeptides. This led me to ask if the Ca-sensitivity of crab skeletal muscle would be affected by carnosine. This muscle had no detectable imidazole content (Crush, 1970). A single muscle fibre from one of walking legs of a crab (Carcinus maenus) was dissected free, split into two longitudinally and mounted in the assembly illustrated in

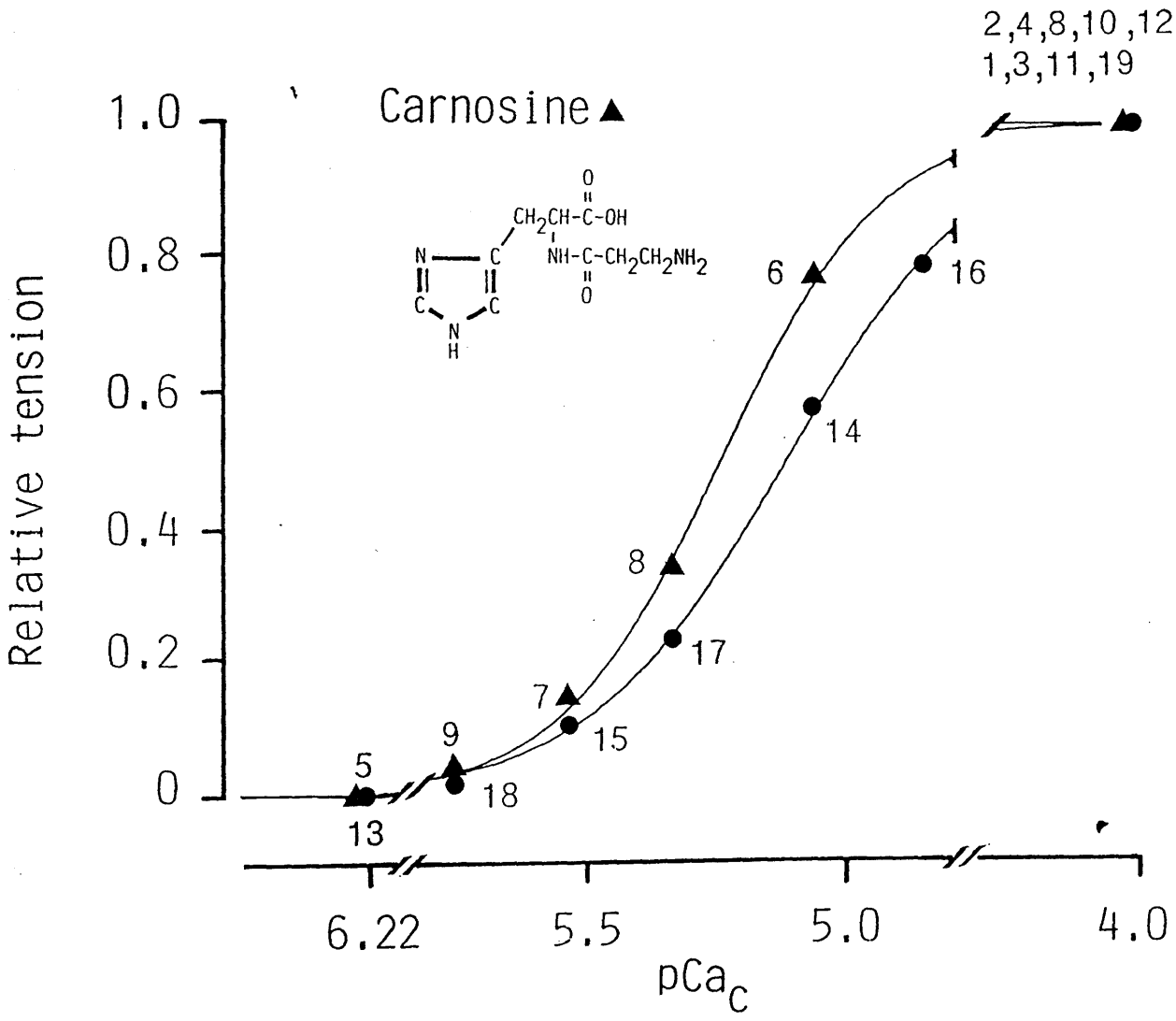


Figure 4.4

The increase in calcium sensitivity produced by carnosine in cardiac muscle.

The pCa-tension relation for a Triton-treated rat cardiac trabecula. The determinations were made as single steps from the relaxed state. The small numbers indicate the sequence in which the determinations were made. Force is normalised to C_{max} under each condition. Carnosine potentiate C_{max} by 8%. The pCa for half maximal activation (pK_{app}) increased form 5.10 to 5.23 and the Hill exponent form 2.29 to 2.81. The filled triangles indicate the presence of 10mM carnosine, the filled circles its absence.

figure 1A of the Methods section. No increase in Ca-sensitivity was produced by 25mM carnosine. The calcium-sensitising drug Sulmazol (10mM), a more potent augmentor of apparent Ca-sensitivity also failed to increase crab muscle's apparent Ca-sensitivity or C_{max}. The composition of the solutions used are detailed in table 3 of the Methods section.

The effect of carnosine and the calcium sensitivity of cardiac muscle.

From the literature it would appear that the imidazole dipeptide content of cardiac muscle may be low. However, as figure 4.4 shows, carnosine enhances the Ca-sensitivity of cardiac muscle. In this example the presence of 10mM carnosine decreased the calcium required for half-maximal activation and steepened the pCa-tension relationship.

I tested the effect of several of the endogenous imidazole compounds on saponin- and Triton-treated rat cardiac trabeculae. The pCa-tension relationships, were determined in the presence and absence of the imidazole-containing compounds. The results of these experiments are collected in table 4.2.

From table 4.2 it can be seen that carnosine increased Ca-sensitivity in a dose-dependent manner. For example in experiment 269 where 10, 20, 30, 40 and 80mM carnosine were applied, the shift in the pCa-tension relationship to lower free Ca-levels increased from 0.031 pCa units to 0.274 pCa units in a

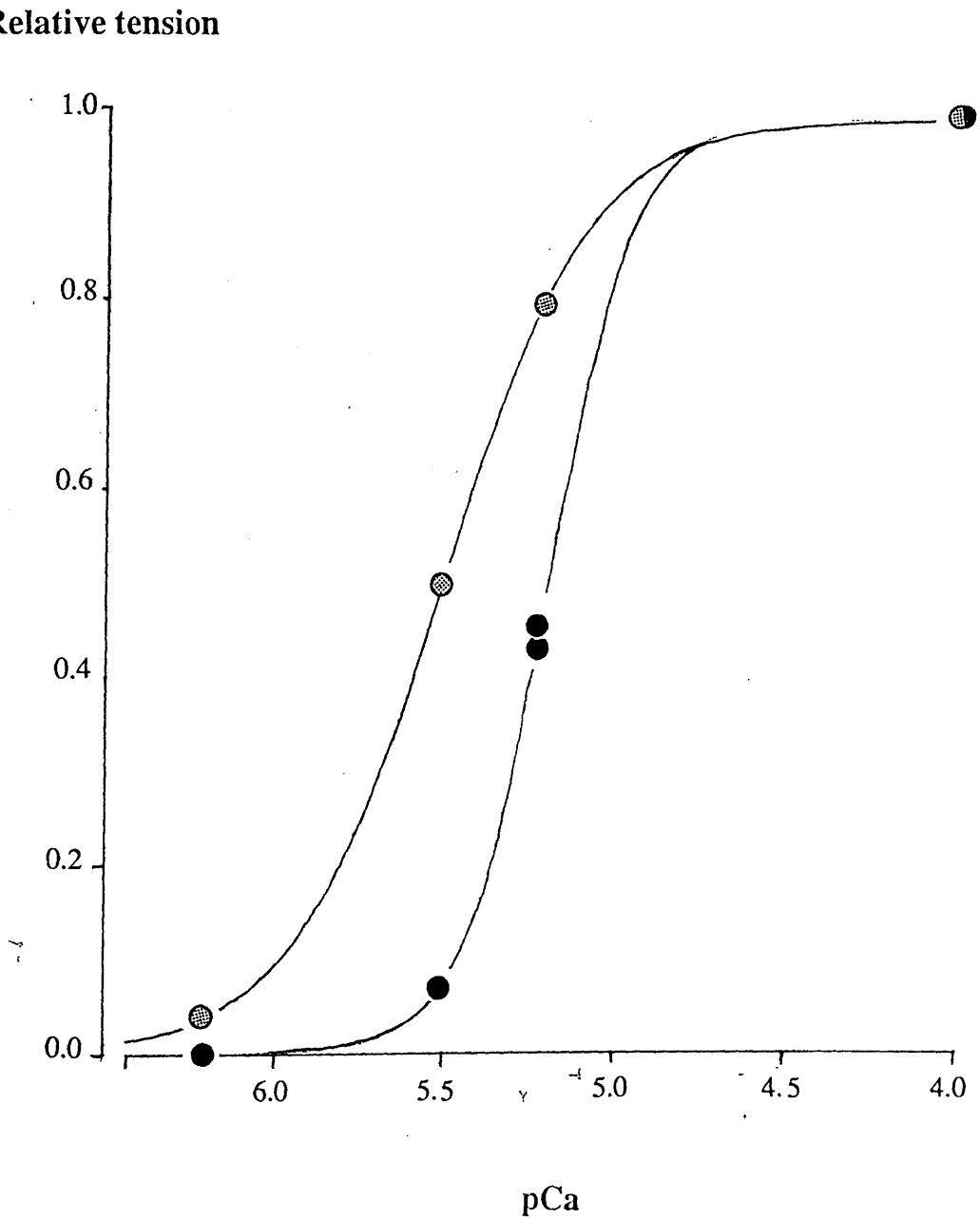


Figure 4.5

The pCa-tension relationship for a Triton-treated rat cardiac trabecula. The determinations were made as single steps from the relaxed state. Sulmazol potentiated C_{max} by 52%. The pK_{app} increased from 5.197 to 5.513 and 'h' decreased from 3.5 to 2.0. The shaded circles indicate the prescence of 10mM Sulmazol, the filled circles its absence .

dose dependent manner. The shift in pK_{app} against the dose of carnosine for the experiments tabulated in Table 4.2 are plotted in figure 4.5. From this plot it is clear that carnosine increases Ca-sensitivity in a dose-dependent manner. It appears that the effect of carnosine on pK_{app} is not saturated even at 80mM carnosine.

Sulmazol was a much more potent augmentor of cardiac muscle Ca-sensitivity. 1mM Sulmazol produced as large a shift as 10-20mM carnosine. 10mM Sulmazol produced a shift as large as 60-80mM carnosine. The effect of 10mM Sulmazol on the pCa-tension relationship is shown in figure 4.6.

β-alanine and Ca-sensitivity

Carnosine consists of two amino acids L-histidine and β-alanine. The evidence presented in chapter 3 suggests that the Ca-sensitising ability of carnosine is dependent upon the imidazole ring of the L-histidine, β-alanine having no effect on Ca-sensitivity. To test this hypothesis β-alanine (20mM) was introduced to the standard solutions in the same manner as carnosine (though not replacing the pH buffer, as β-alanine does not buffer H^+). No effect on intermediate tension levels or peak tension was produced by β-alanine i.e. it did not alter the apparent Ca-sensitivity or C_{max}.

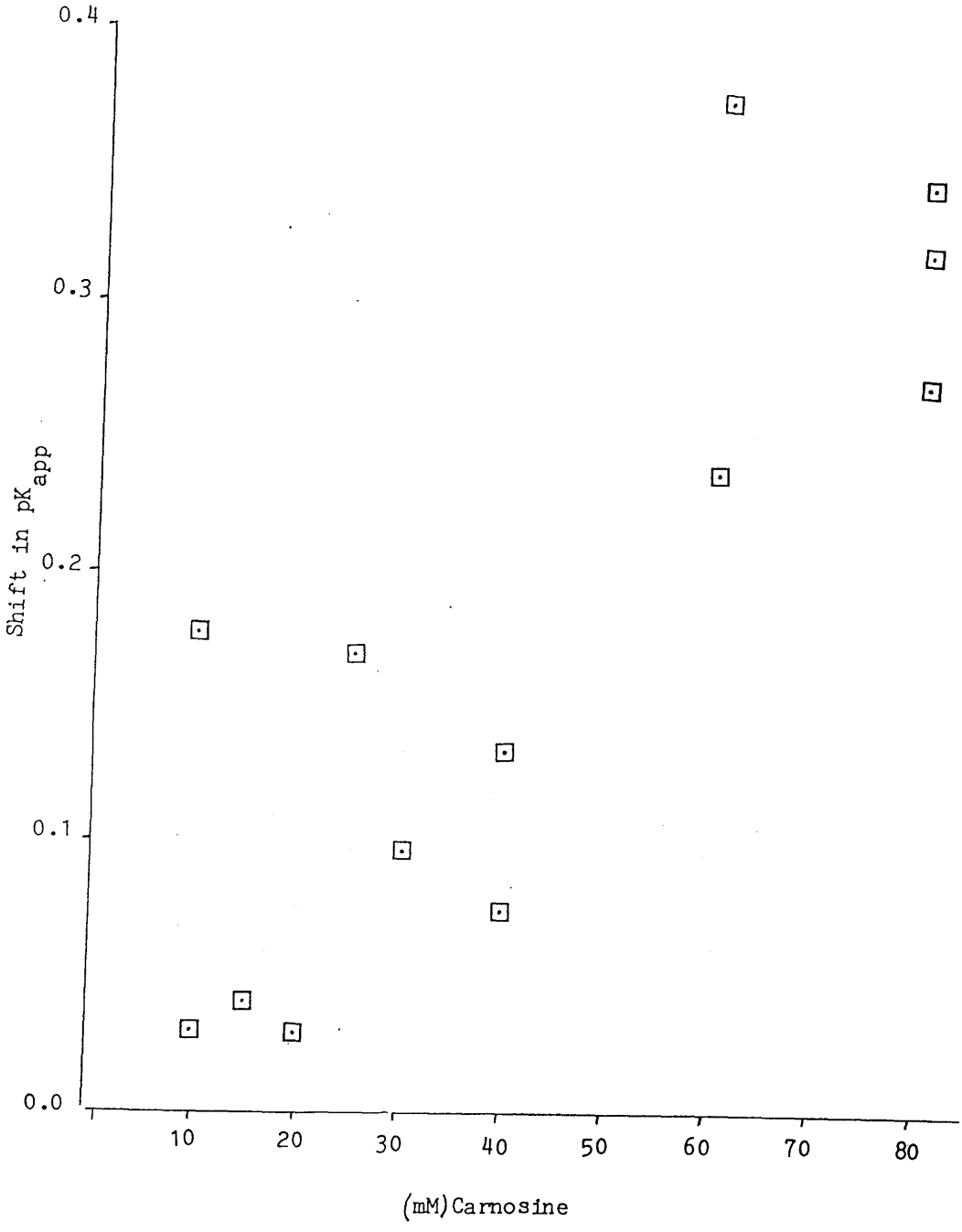


Figure 4.6

The dose dependency of the shifts in the pCa-tension relationship produced by carnosine. A plot of the shift in the pCa-tension relationship ($pKapp_{carnsine}$ - $pKapp_{control}$) (ordinate) against the dose of carnosine applied (abscissa).

Table 4.2

Effect of various imidazole containing compounds on the pCa-tension relationship

Cardiac (rat ventricle)

			Control		Experimental		
Compound	Conc (mM).		pKapp	'h'	pKapp	'h'	Delta
Carnosine	10	(204)T	5.097	2.3	5.277	2.8	0.180
	10	(269)T	5.267	4.8	5.298	4.8	0.031
	15	(231)S	5.504	5.2	5.544	4.8	0.040
	20	(269)T	5.267	4.8	5.297	4.6	0.030
	25	(240)S	5.542	3.8	5.709	2.8	0.167
	30	(269)T	5.267	4.8	5.360	4.3	0.093
	40	(268)T	5.401	4.4	5.535	5.3	0.134
	40	(269)T	5.267	4.8	5.342	4.4	0.075
	60	(224)S	5.274	4.4	5.515	2.9	0.241
	60	(225)T	5.095	3.8	5.474	5.5	0.379
	80	(268)T	5.401	4.4	5.728	3.3	0.327
	80	(270)T	5.300	3.1	5.657	3.7	0.357
	80	(269)T	5.267	4.8	5.541	4.7	0.274
N-acetyl histidine	15	(219)T	5.097	2.3	5.277	2.8	0.180
Sulmazol	1	(256)S	5.029	2.5	5.159	5.5	0.130
	1	(257)S	5.150	3.5	5.197	3.5	0.047
	10	(273)S	5.197	3.5	5.513	2.0	0.316

Skeletal

Carnosine	15	(223)T	5.604	1.9	5.728	1.5	0.124
Rana temporaria		T	5.605	2.0	5.750	2.2	0.100
sartorius fibre		T	5.680	3.3	5.850	3.6	0.170
Carnosine	25	(283)T	5.420	3.5	5.797	7.2	0.377
Xenopus leavis semimembranosus fibre.							

T=Triton-treated
S=Saponin-treated
The no. in the brackets indicate Expt no.
Delta = $pKapp_{Expt} - pKapp_{Control}$

The effect of the natural imidazoles on peak tension

Force was normalised to maximum Ca-activated tension (C_{max}) under each condition. While this normalisation is necessary to apply the Hill equation and so quantify the pCa-tension curves, it obscures an additional effect of these compounds; their effects on C_{max}.

In cardiac muscle, C_{max} is enhanced slightly by 10-15mM carnosine (101.3% (range 97-107%) mean of the mean effect in five preparations). An example of the effect of 15mM carnosine on peak tension is shown in figure 4.8B. By contrast, higher doses of carnosine generally depressed peak tension (possibly because of ionic strength effects), although it did appear that 80mM carnosine augmented C_{max} in expt 268.

The effects of imidazole-containing compounds on C_{max} are tabulated in table 4.3. Problems were encountered when determining the effect of the C_{max} of skeletal muscle. Repeated determinations of C_{max} with and without carnosine were achieved with only one skeletal preparation (223). In this experiment no effect of carnosine on C_{max} was observed. However, frequently when the carnosine was introduced at C_{max} the fibres broke, implying that carnosine was increasing C_{max} in skeletal fibres.

Table 4.3

Imidazoles and C_{max}

Compound	Conc. (mM)	Expt no.	Average C_{max}
Cardiac			
Carnosine	10	204	107.0
	15	231	97.0
	15	232	102.5
	15	220	98.5
	15	222	102.0
	25	240	87.0
	40	268	105.0
	60	224	93.3
	60	225	98.0
	80	268	110.0
N-acetyl histidine	15	219	93.0
	60	227	94.0
Sulmazol	1	227	123.0
	1	291	111.4
	10	273	152.0
Frog skeletal			
Carnosine	15	223	100.0

Can carnosine provoke calcium release?.

On the basis of the work in chapter 3 it would be predicted that carnosine could not initiate calcium release from the SR. This proposal was examined using the protocol descibed in Chapter 3. As predicted carnosine could not evoke contracures in saponin-treated rat cardiac trabeculae under conditions where caffeine was effective.

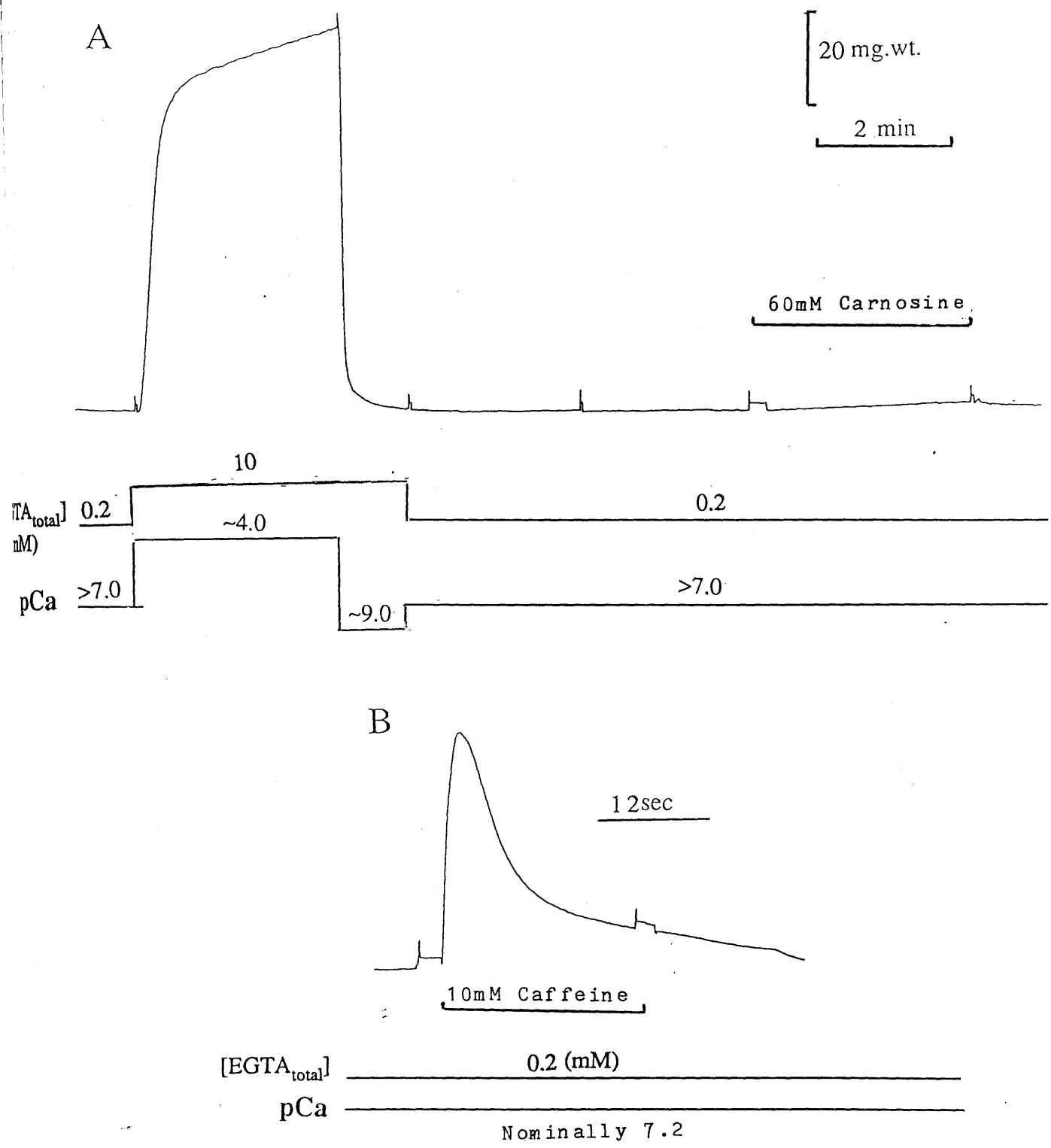


Figure 4.7

A continuous section of tension trace from a saponin-skinned rat ventricle trabecula. The preparation was initially exposed to a calcium level which allow the SR to load with calcium (pCa 4.00). The muscle was then relaxed and the calcium buffer capacity reduced from 10 to 0.2mM EGTA. In this weakly buffered state 60mM carnosine was applied. No contracture was produced. To confirm that the SR did contain calcium the muscle was immediately subsequently exposed to 10mM caffeine, this produced a contracture 60% of the size of C_{max}.

An example of this is provided in figure 4.7. A saponin-treated rat cardiac trebecula was initially loaded at a pCa of 4.02 for 3 minutes. Placing the Ca-loaded fibre in a weakly calcium buffered solution (0.2mM EGTA) containing 6 mM carnosine did not provoke a contraction, immediately subsequent exposure to 10mM caffeine produced a contraction, 60% of the size of C_{max}.

N-acetyl histidine also failed to evoke contractures at concentrations up to 60mM (The lack of effect of 10mM N-acetyl histidine is illustrated in figure 3.4B)

Carnosine and calcium uptake by the sarcoplasmic reticulum

Although carnosine could not provoke Ca-release from the SR, it seemed likely that carnosine would influence SR function (Boldyrev, Lebedev & Ritov, 1969). This set of experiments were designed to investigate carnosine's influence on Ca-uptake by the SR.

The influence of carnosine on the Ca-loading of the SR in saponin-treated muscles was examined using the size of the caffeine contracture as an index of the Ca-content of the SR.

Protocol The fibre's calcium stores were depleted to standardise its initial calcium content (see below). The preparation was then exposed to a Ca-level which allows the muscle to Ca-load. After the muscle was relaxed, its calcium-buffering capacity reduced (from 10mM to 0.2mM) and in this weakly-buffered state 10mM caffeine was applied. The size of this caffeine contracture provoked was taken as an index of the calcium content of the SR.

Depletion Depletion of the calcium stores was achieved by placing the fibre in a '10 Relaxing' solution with 10mM caffeine ('emptying' solution). The caffeine provokes calcium release and the released calcium will be bound by the EGTA (10mM). It was found that if the 'emptying' solution was applied directly from a 10 Relaxing solution the SR was not emptied. This is shown by the ability of caffeine to provoke a subsequent contracture when the buffer capacity was reduced to 0.2mM EGTA. If the 'emptying solution' was applied from a '0.2 Relaxing' solution or an 'oscillating solution' (see methods section table 1 for details of the solution composition) most of the stored calcium was lost to the emptying solution, i.e. little or no contracture was produced if the Ca-buffer capacity was subsequently reduced (0.2mM EGTA) and 10mM caffeine applied. The explanation for this observation is that caffeine lowers the threshold for CICR, but not so far as to be effective at pCa>9.0 (as when equilibrated in 10mM EGTA). This is good evidence against a simple 'Ca-release' model for caffeine's action which would be

effective in both test conditions.

Loading Loading was achieved by raising the calcium to a level at which the SR could take up calcium. This level was very variable. Some fibres loaded from the '0.2 relaxing' solutions (pCa~7.00), others did not load at levels less than pCa 6.6 (10mM EGTA). In both of the figures used here for illustrative purposes (4.8 and 4.9) calcium-loading was carried out at a pCa of 4.00. This was done for two reasons. First at this pCa the fibres do not load optimally (Harrison, 1985), so there is room for carnosine to increase calcium-loading. Second, the contraction produced by a pCa of 4.00 during the loading period gives a measure of C_{max} against which to compare the contracture size. Optimal Ca-loading was generally found to occur at ~ pCa 6.3 which is close to the threshold for tension production. None of the experiments where the fibre was loaded at this pCa were used for illustrative purposes as the loading of the SR was near optimal and so any change in calcium loading with carnosine was minimal.

Release At the end of the loading period [Ca^{2+}] was reduced to a pCa of ~9.00 (10 Relaxing solution) for 1 minute. The buffer capacity was then reduced (0.2mM EGTA). The muscle was allowed to equilibrate in this solution for approximately 6 minutes. Finally, the muscle was transferred to 0.2 Relaxing solution including 10mM caffeine. The introduction of caffeine provoked Ca-release from the intracellular stores. Provided the

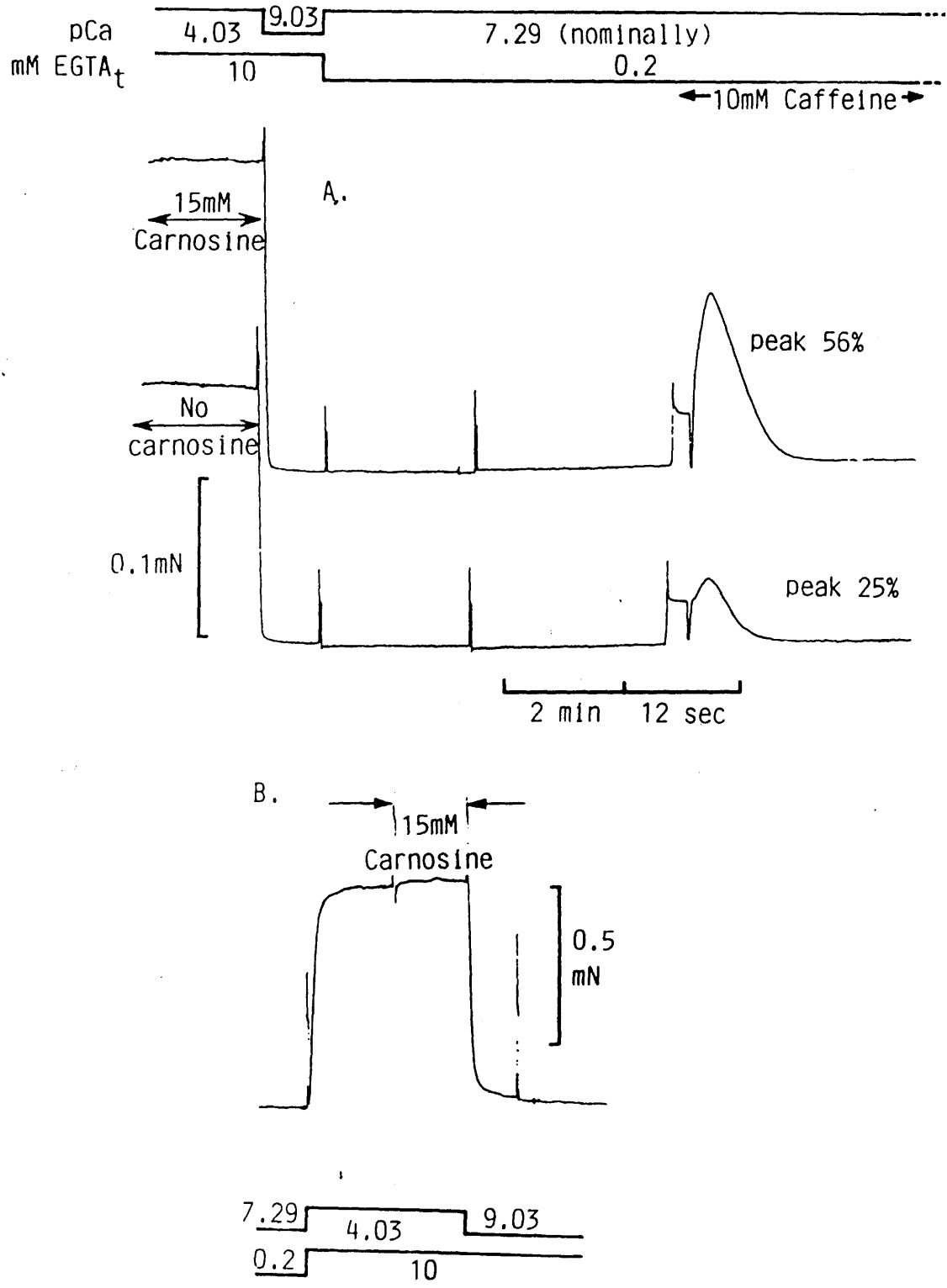
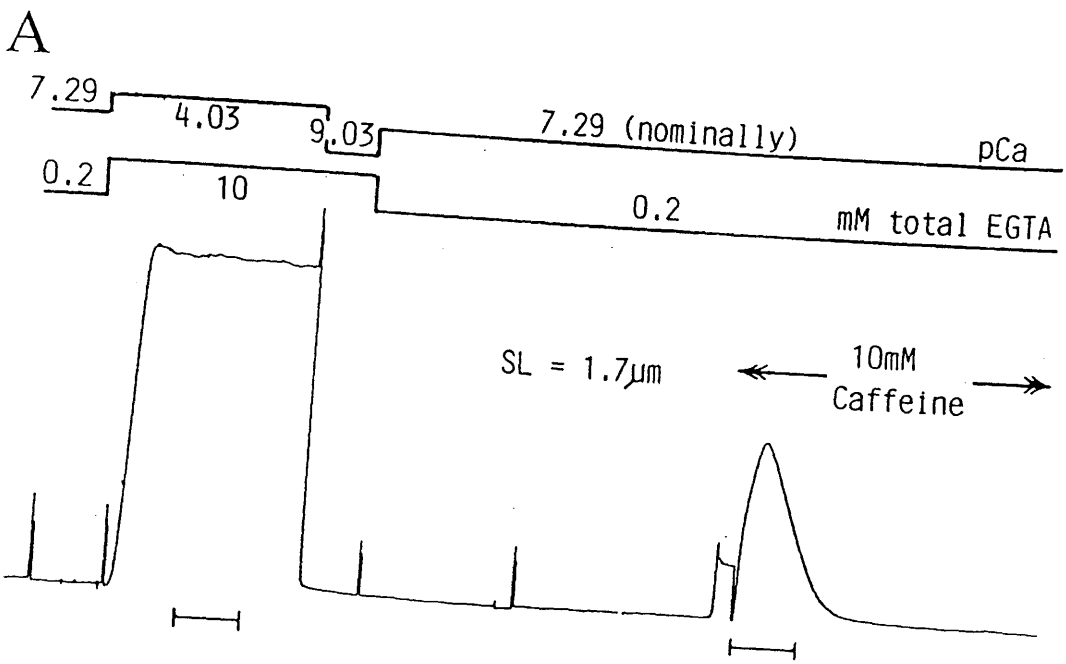


Figure 4.8

Tension response of a saponin-skinned rat ventricle trabecula. (A) The contracture produced by 10mM caffeine (note the faster time base) after loading the SR by maximally activating the muscle as shown. The upper trace shows the response with 15mM carnosine in the loading solution, the lower trace without carnosine. The percentage figures express peak caffeine contracture tension relative to the corresponding C_{max}. (B) Potentiation of C_{max} (by 3%) by carnosine in the same preparation.

muscle was calcium 'depleted' between runs, and the loading conditions were consistent the size of the caffeine contraction was reproducible (±5%). The size of the caffeine contraction is taken as an indicator of calcium release, assuming Ca-release provoked by caffeine to be an indicator of calcium content of the SR (e.g. Fabiato & Fabiato, 1978; Endo, Tamaka & Ogawa, 1972).

The effect of introducing carnosine during the loading period is illustrated in figure 4.8. This shows the last section of the protocol described above for two runs, the upper with 15mM carnosine present during the loading period, the lower with the same free $[Ca^{2+}]$ during the loading period but in the absence of carnosine. The figure comprises of the end of the loading period, the relaxing of the muscle, equilibration in 0.2 Relaxing solution and the contraction evoked by application of 10mM caffeine. In this example the muscle was loaded at a pCa of 4.02 which produces maximal calcium-activated tension. The caffeine contraction was diminished by setting the s.l. below 2.2μm which reduces the Ca-sensitivity of the contractile proteins (length dependence of Ca-sensitivity) as well as reducing the Ca-loading by the SR. This set of conditions were chosen to leave 'room' for augmentation of Ca-uptake. In the upper trace with carnosine present during the loading period, the caffeine contraction was 56% of peak tension. Without carnosine (lower trace) the caffeine contraction was only 25% of peak tension. Clearly the presence of carnosine during the load period, greatly increased the magnitude of the resulting caffeine contraction and therefore by



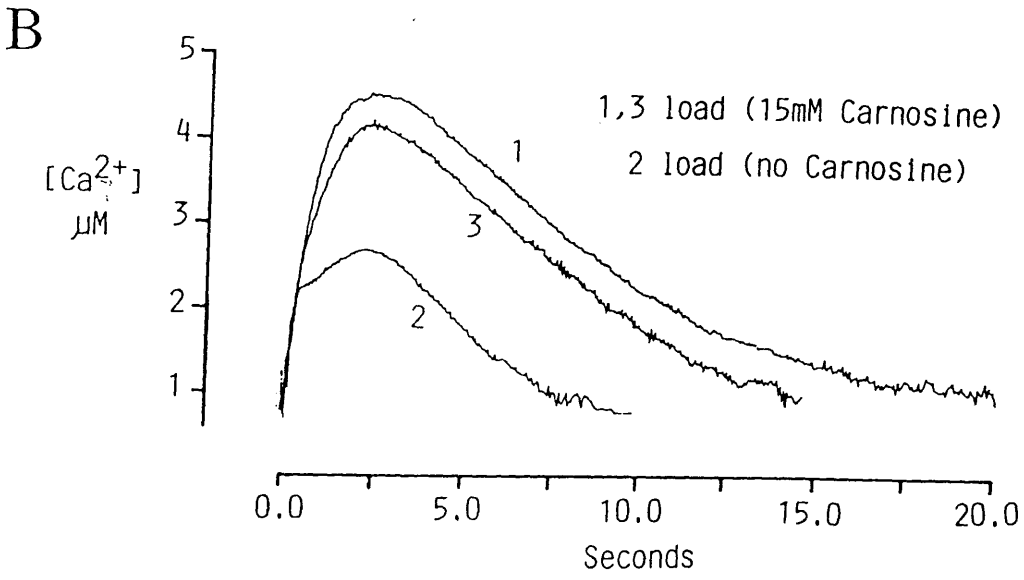


Figure 4.9

Panel (A) outlines the experimental protocol. The muscle was loaded at pCa of 4.00, (this produced maximum activated tension) relaxed and the Ca-buffer capacity reduced from 10mM to 0.2mM EGTA. In this weakly buffered state 10mM caffeine was applied provoking a contracture. In panel (B) three contractures produced by 10mM caffeine on three separate runs on the same muscle after loading the SR as shown in panel are superimposed (A). The traces in panel (B) are shown as calcium, the tension responses have been tranformed to calcium using the appropriate pCa-tension relation. Trace 2 was obtained without carnosine in the load solution, traces 1 and 3 were obtained with 15mM carnosine present in the load solution.

implication the amount of calcium accumulated by the SR, as the Ca-sensitivity of the contractile proteins and stimulus to Ca-release by the SR were unchanged.

Overall, the presence of carnosine in the loading solution results in a shift to lower $[Ca^{2+}]$ in the curve relating $[Ca^{2+}]$ to caffeine contracture amplitude (this was observed in 3 preparations).

Figure 4.9 provides a second example of this, the upper panel (A) outlines the experimental protocol, the muscle was loaded at a pCa of 4.02 at a s.l. of 1.7µm. The three superimposed traces in panel B were obtained using the protocol in Panel A. Traces 1 and 3 were produced with 15mM carnosine present, during the load period, trace 2 in the absence of carnosine. The three tension traces were transformed to $[Ca^{2+}]$, using the appropriate pCa-tension relationship (ie. allowing for the increase in Ca-sensitivity produced by caffeine). The increased free Ca^{2+} achieved by Ca-release from the SR when carnosine was present during the load period can be seen when comparing traces 1 and 3, to trace 2. Clearly the amount of calcium released when carnosine was present during loading is greater than in trace 2 since the extra Ca-binding by the EGTA and diffusional losses are greater during the larger contracture. This suggests that carnosine reversibly increases the capability of the SR to take up calcium ions.

Carnosine and it influence on CICR

Another more physiological type of calcium release is that produced by calcium itself (CICR). Saponin-treated preparations produced oscillations in tension when placed in weakly-buffered solution with an intermediate calcium level. An example of this is shown in figure 4.10. These oscillations in tension are thought to be due to cycles of calcium release and reuptake. The calcium release is thought to be provoked by a local increase in calcium around the SR which provokes calcium release by positive feedback. The large synchronised tension oscillations are thought to be due to synchronised waves of CICR, a suggestion which is supported by the apppearance of the muscle when viewed in the DIC microscope, waves of contraction can been seen to pass along the muscle in synchrony with the large oscillations in tension (Lamont & Miller, 1987). The upper panel of figure 4.10 shows the oscillations in a 0.2mM EGTA solution with a pCa nominally of 5.32. During the first half of the trace, 15mM carnosine was present. Approximately half way along the trace the muscle's bathing solution was replaced by one of the same [EGTA] and $[Ca^{2+}]$ but without the carnosine. The removal of carnosine dramatically reduced the height and amplitude of the tension oscillation. A portion of this reduction in tension level is due to the decreased Ca-sensitivity of the contractile protein's as the carnosine is removed (see figure 4.4). To eliminate this factor, the digitised tension trace was transformed from tension to calcium applying the steady-state pCa-tension relationship for

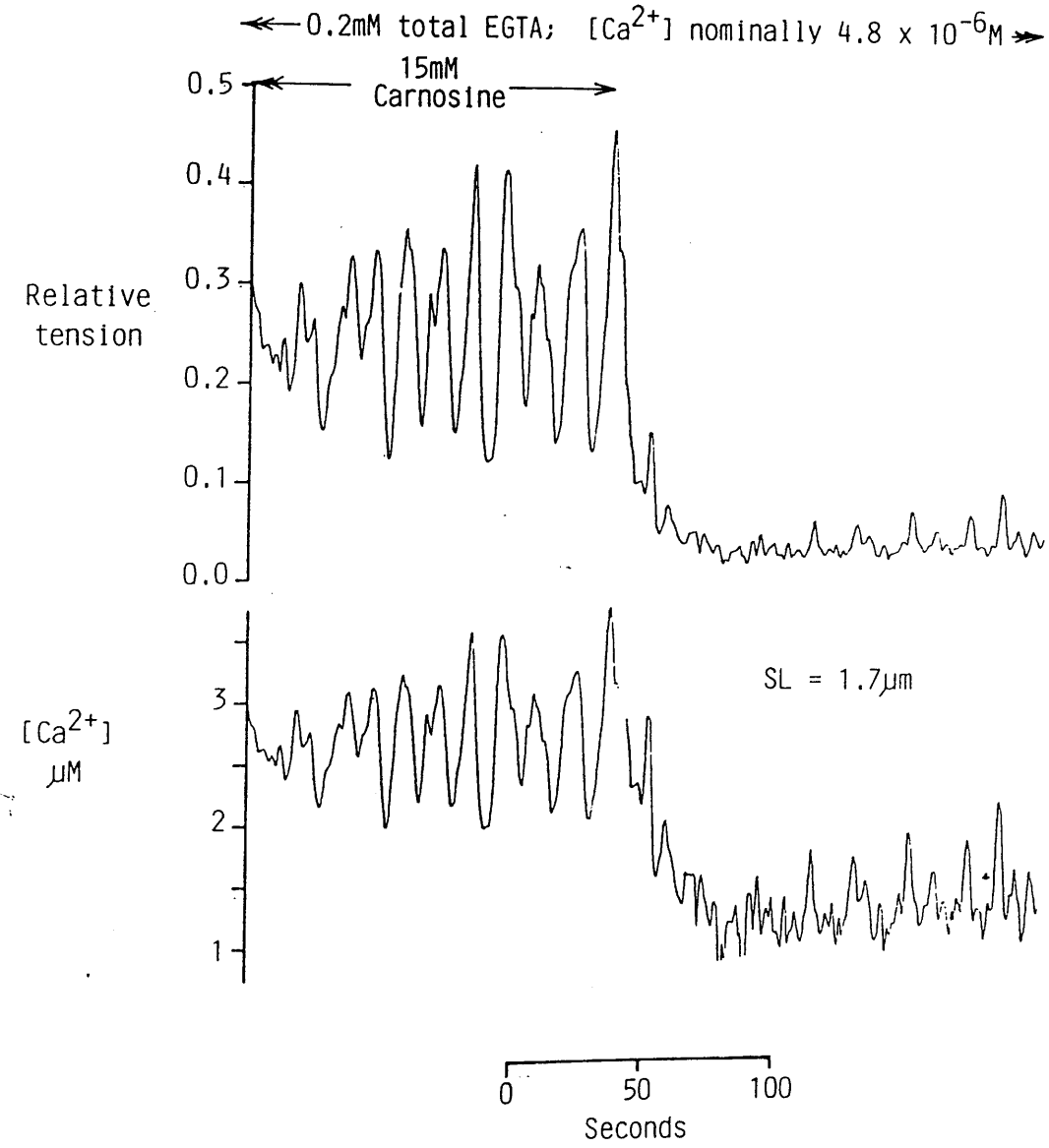


Figure 4.10

Spontaneous tension oscillations initiated by a weakly Ca-buffered solution. At the same Ca-level carnosine greatly enhances oscillation amplitude. s.l. 1.7μm

the preparation in the absence and presence of 15mM carnosine (illustrated in figure 4.4). The Ca-sensitivity of the muscle in this experiment was established in the presence and absence of 15mM carnosine in the standard manner (see methods section). There are some doubts about the applicability of this pCa-tension relationship to the CICR oscillations. The standard pCa-tension relationship is obtained under steady-state conditions and the muscle is activated uniformly, whereas the tension in the CICR oscillations is neither steady-state nor uniform. Additionally, it can be seen when observing the oscillating muscle using the DIC microscope that in sections of the preparation local shortening occurs, while in others it lengthens. Even with these reservations, the figure makes clear that changes in Ca-sensitivity alone cannot explain the effect of carnosine. The trace, thus suggests that there are greater oscillations in free calcium in the presence of carnosine and that these oscillations are centred around a higher absolute Ca-level. After complete skinning, the muscle was re-exposed to the 'oscillation' solutions and the tension levels in the solution re-established. This showed the oscillations maxima and minima to be respectively above and below the tension level produced by the preparation without the organelles operative. See figure 4.11

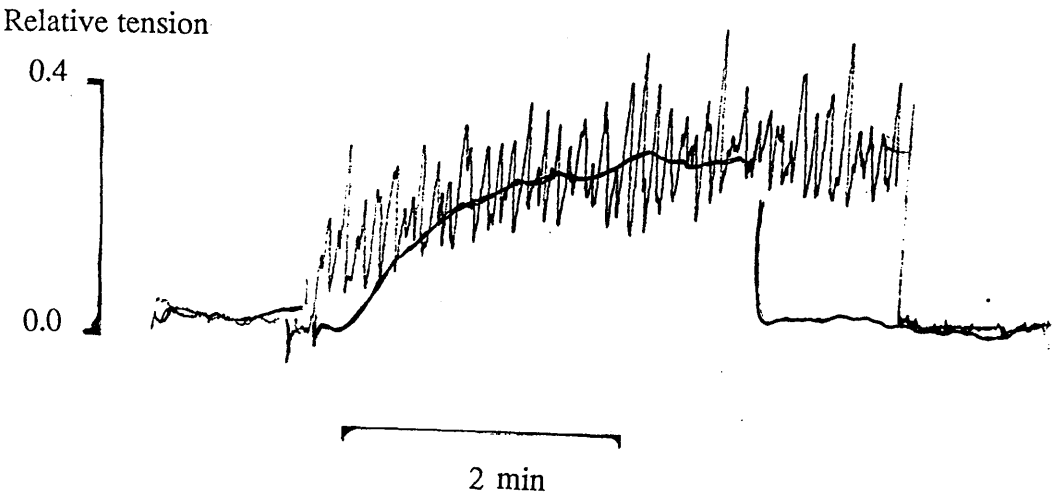


Figure 4.11

Spontaneous tension oscillations produced by a saponin-treated rat cardiac trabecula in a weakly Ca-buffered solution. Superimposed on this trace is the tension response to the same solution when the muscle has subsequently been Triton-treated. From this figure it can be seen that the oscillations in tension produced by the saponin-treated preparation were above and below the tension level produced by the muscle when its cellular membrane systems were disrupted by Triton.

The CICR oscillations were augmented in a similar manner by N-acetyl histidine (15mM).

Discussion

I suggested in the last chapter that the ability of caffeine and imidazole to augment the Ca-sensitivity of the contractile proteins might be mimicking the action of some endogenous substance. It was asked whether natural cellular imidazoles fill this role. I have examined the effect of these compounds on two calcium regulated processes; the Ca-sensitivity of the contractile proteins and calcium uptake by the sarcoplasmic reticulum. The results reported in this chapter show that the cellular imidazoles (e.g. the histidine dipeptides) do augment Ca-sensitivity. In addition, these compounds enhance Ca-uptake by the SR.

The histidine dipeptides and the Ca-sensitivity of skeletal muscle

Histidine dipeptides are present in the sarcoplasm of vertebrate skeletal muscle at concentrations of 1-60mM. They have the structural characteristics predicted in chapter 3 to make them Ca-sensitisers, i.e. an imidazole ring with an unsubstituted nitrogen. As predicted, these compounds increased the contractile protein's apparent Ca-sensitivity, apparently in the same manner as caffeine and imidazole.

15mM carnosine is found in the sartorius muscle of the frog (Rana temporaria) (Burton, 1983). Application of this concentration of carnosine to a Triton-treated fibre from this muscle, increased its Ca-sensitivity as is illustrated in figures 4.2 and 4.3 (see also table 4.2). This shows that the presence of carnosine at its physiological concentration increases the Ca-sensitivity of the contractile proteins. It can be deduced that the apparent Ca-sensitivity in the intact state will be higher than that observed using skinned fibres in the absence of these compounds. Thus, previous estimates of Ca-sensitivity from skinned skeletal fibres are lower than physiological. In the example shown in figure 4.3 the calcium level required to activate half-maximally the fibre would be estimated as 2.1uM, but with the appropriate cellular concentration of carnosine present this would be reduced to 1.4uM.

It was mentioned in the introduction to this chapter that the level of the histidine dipeptides varies dramatically with muscle type. The concentrations of carnosine and anserine are generally much higher in white muscle than in red (Zapp & Wilson, 1938; Davey, 1960; Castellini & Somero, 1981; Christman, 1968). It would be interesting to determine the potency of these compounds on the calcium sensitivity of various muscle types. From the preliminary observations reported in this chapter it appeared that the shift in the pCa-tension relationship in the frog sartorius (Rana temporaria) muscle was smaller than that in clawed toad semimembranosus muscle (Xenopus leavis) (see table

4.2). This implies that there may be differences in the potency of carnosine's effect on calcium sensitivity as well as the already established differences in the distribution of the compound with muscle type and species. The effect of caffeine on Ca-sensitivity has already been shown to vary with muscle type (Wendt & Stephenson, 1983). The shift in the pCa-tension relationship of rat cardiac and soleus muscle was greater than that in rat extensor digitorum longus muscle.

Effect of carnosine on crab skeletal muscle

Invertebrate muscles generally contains little or no carnosine or anserine (Crush, 1970). I found that Carnosine (20mM) and Sulmazol (10mM) had no effect on the tension produced by chemically skinned crab skeletal muscle: Ashley and Griffiths (1984) have previously reported Sulmazol (AR-L 115 BS) to have no effect on the Ca-sensitivity of skinned barnacle fibres (Balanus nubilus). These observations suggest that the Ca-sensitising effects of the imidazoles may be limited to vertebrate muscle. Comparative studies of the effect of carnosine on myofibrillar-ATPase activity of vertebrate and invertebrate muscles (Parker and Ring, 1970) has shown carnosine activation of myofibrillar-ATPase activity to be limited to myofibrils from muscles that normally contain carnosine and/or anserine as major cellular constituents. The lack of effect of carnosine on the Ca-sensitivity of invertebrate skeletal muscle may indicate minor

structural dissimilarities in the Ca-binding sites of the regulatory proteins from vertebrate and invertebrate muscle. Decapod muscle is unusual amongst the invertebrates in having only actin regulation and is therefore vertebrate-like except with regard to the action of the Ca-sensitisers.

The effect of carnosine on the Ca-sensitivity of cardiac muscle

Carnosine augments Ca-sensitivity of cardiac muscle in the same manner as caffeine and imidazole described in the last chapter. An example of the increase in rat cardiac muscle's Ca-sensitivity is presented in figure 4.4. (see table 4.2 for cumulated data). Carnosine increased the Ca-sensitivity in a dose-dependent manner. From the plot of shift in pK_{app} against the concentracion of carnosine in figure 4.6 it can be suggested that the effect of carnosine is not saturated even at 80mM.

The physiological levels of imidazole-containing compounds in cardiac muscle have not been clearly established. The levels of histidine dipeptides in cardiac muscle are generally thought to be low or neglible . The highest value reported was 0.6mM carnosine in rabbit heart (Sobue et al, 1975). However, preliminary measurements of the imidazole-containing compound content of heart muscle extracts, made in this laboratory using HPLC (high performance liquid chromatography), suggest total imidazole levels as high as 8mmole per Kg wet weight (further corrections to sarcoplasmic volume are required, Crichton et al,

1987). On the basis of this observation it can be proposed that the effect observed with 10-15mM carnosine (see figures 4.4 and table 4.2) may be close to physiological levels.

Effects on carnosine on SR function

The second effect of the imidazole dipetides reported is their influence on SR function. Since the late 1960s it has been thought that the sarcoplasmic imidazole-containing compounds influence SR function. This idea might relate to the effect of caffeine, whose structural similarities were mentioned earlier, which provokes Ca-release from the SR. Boldyrev, Lebedev & Ritov, (1969) showed carnosine to reverse fatigue in frog nerve-muscle preparations under myoneural blockade. They suggested that this action was intracellular and perhaps associated with the SR. This suggestion was borne out when Lopina and Boldyrev (1975) showed carnosine to activate strongly the overall ATPase activity of isolated skeletal muscle SR. They showed this to be specific stimulation of the Mg-K-dependent component with concomitant suppression of the Mg-dependent activity. Simultantaneously with the stimulation of the Mg-K-ATPase, carnosine accelerated the absorption of calcium by the reticulum by a factor of 1.5-2.0 compared with Tris buffer. The results reported here confirm Lopina and Boldyrev's (975) observation that the presence of carnosine during the calcium 'loading' period increases the calcium uptake by the SR. I have shown the same phenomenon on

cardiac muscle SR in what is presumed to be its physiological configuration (see figures 4.8 and 4.9). The ability of the SR to take up calcium, as viewed in either isolated SR or in skinned muscle preparations will be underestimated when the cellular imidazoles have been omitted from the solutions employed.

Physiological consequencies of the histidine dipeptides

What are the physiological consequencies of the effects of carnosine?. It is generally thought that the intracellular free calcium level in fast twitch fibres (e.g. the frog sartorius fibres) is supramaximal during the all-or-none twitch response, and that force cannot normally be altered in these muscles by changes in intracellular free calcium or Ca-sensitivity of the contractile proteins. It is generally thought that greater force in the intact twitch muscles within the body is graded by the recruitment of additional fibres rather than changes in the force per fibre. However, it should be noted that there is contrary evidence based on the sub-tetanic rate of driving of motor control under voluntary contraction (Milner-Brown, Stein & Ymen, 1973). Therefore, it seems that changes in Ca-sensitivity or Ca-mobilisation may make little difference to the performance of fast twitch skeletal fibres unless a large effect of C_{max} were present. However, the situation in slow tonic fibres and cardiac muscle is certainly different. In slow tonic fibres where the response to stimulation is graded, this gradation is proposed to be due to an increase of the intracellular Ca^{2+} concentration

resulting from varied depolarisation (Miledi, Parker & Schalow, 1981) so an increase in the Ca-sensitivity of the contractile proteins or an increased availabilty of calcium would augment the muscle's force output.

In cardiac muscle all the cells are thought to be activated during every cardiac cycle. The gradation in force production can be produced by altering either the calcium available to the regulatory proteins or their Ca-sensitivity. Clearly, the evidence presented here shows that carnosine can affect both of these factors by increasing the Ca-sensitivity of the contractile proteins and the ability of the SR to take up calcium. The increased ability of the SR to take up calcium will have several effects. The intracellular calcium level will be reduced. This will result in a greater calcium entry during the action potential because the driving force is greater. A greater and faster change in the calcium concentration surrounding the SR will produce a larger calcium release (Fabiato, 1983). The total calcium content of the SR will be greater. So both the trigger for calcium release and the amount available to be released will be augmented.

Effects of imidazole containing compound on muscle

From the literature it is evident that the role of the histidine dipeptides is not understood despite extensive investigation. The histidine dipeptides have been implicated in a wide variety of process likely to affect muscle performance. They influence actomyosin ATPases (Avena & Bowen, 1969), calcium accumulation by the SR (Lopina & Boldyrev, 1975), the buffering of pH (Davey, 1960), divalent ion chelation (Brown & Antholine 1984) and the regulating glycolysis (Johnson & Aldstadt, 1984). However, despite the considerable interest in these compounds no concensus has been reached as to their function though several roles have been suggested for them.

One of the first proposals to explain the distribution and function of the histidine dipeptides was that they are cellular pH buffers. This action undoubtably occurs. The histidine dipeptides are pH buffers in the physiological range e.g. carnosine (pK_2 6.83) and anserine (pK_2 7.04 Bates-Smith, 1938, Deutsch & Eggleton, 1938) and have been shown to constitute 20-30% of the total buffer capacity of skeletal muscle (Davey, 1960). The compounds would exhibit their maximum buffering capacity if pH should fall below pH 7.0-7.3 (Davey, 1960). They will tend to neutralise the lactic acid produced by anaerobic glycolysis in skeletal muscle (Davey, 1960a). As would, therefore be expected the highest levels of these dipeptides are found in muscles which can metabolise anaerobically (Bates-Smith,1938;

Castellini & Somero, 1981; Hochachka & Somero, 1984), e.g. the skipjack tuna (Katusuwonus pelamis) which can engage in burst swimming, sustained by elevated capacities for anaerobic glycolysis in its white muscle, (Guppy & Hochachka 1978) has 150mM total histidine dipeptide. Many of the effects of these compounds can be attributed to their ability to buffer H^+ ions. However, changes in pH, or pH buffer capacity, cannot explain all of the reported effects of these compounds.

A second established area of histidine dipeptide function is as divalent cation chelators. Brown (1981) has shown that carnosine and the other histidine dipeptides chelate Cu^{2+} and Zn^{2+}. This role brings together several effects, for example their involvement in intracellular transport of copper to the mitochondria for activation of cytochrome oxidase at the end of the electron transport chain and in the regulation of glycolysis. In the regulation of enzymes such as fructose 1,6,bisphosphatase (isolated from white skeletal muscle) activity is increased by carnosine and anserine and inhibited by zinc(II) and copper(II) ions (Ikeda, Kimura, Hama & Tamaki, 1980).

Both these phenomena are likely to influence muscle performance substantially but neither can explain the effect on Ca-uptake or calcium sensitivity reported here. The solutions used for these experiments are well buffered for pH and buffer capacity was frequently kept constant so that changes in buffer capacity cannot explain the results. The effect of chelation of

Cu^{2+} or Zn^{2+} is irrelevant given the low mineral content of our distilled water and the EGTA buffering of the solutions (zinc and copper binding constants for EGTA are respectively 50 and 10^4 times greater than for calcium) alterations in levels of these cations cannot occur.

Factors affecting carnosine levels

Several factors have been shown to alter the cellular histidine dipeptide levels for example denervation and trauma result in reduced levels (Crush, 1970). All types of muscle atrophy are characterised by a decreased carnosine level, for example, patients with progressive muscular dystrophy have decreased carnosine content (Stepanova and Grinio, 1960). All these are conditions which are associated with impaired muscular performance. These observations are now not surprising in view of the important functions attributable to the histidine dipeptides from the current work.

Overall conclusions

Overall it appears that carnosine increases the Ca-sensitivity, peak force production and SR calcium loading and, therefore, must be an important determinant of force production *in vivo*. The reduction of carnosine in dystrophic muscle, denervated muscle and skeletal muscle after trauma may explain, at least in part, the muscular weakness observed. Skinned fibre

and isolated organelle work may yield misleading estimates of Ca-sensitivity and Ca-transport. Perhaps unwittingly, some investigators will have partly compensated for this by using imidazole as a pH buffer.

I have shown the sarcoplasmic imidazoles to sensitise two calcium regulated processes; the contractile proteins and calcium mobilisation by intracellular organelles. The effects of caffeine and Sulmazol on the former can now be viewed as mimicking the action of the natural imidazoles. The opposing effects on Ca-release/uptake by caffeine and others on one hand and the cellular imidazoles on the other might reflect common features in action on Ca-affinity of the Ca-binding sites presumably associated with uptake or release. The actions of the natural imidazoles may prove to be a general feature of calcium regulated systems. Other calcium sites would include Ca-dependent K channels, the Na-Ca exchanger in the sarcolemma and the Ca-Na exchanger on the inner mitochondrial membrane.

ABE, H., BRILL, R.W. & HOCHACHKA, P.W. (1986). Metabolism of L-Histidine, Carnosine and Anserine in Skipjack Tuna. Physiol. Zool. 59, 439-450.

ACKERMANN, D., TIMPE, O. & POLLER, K. (1929). Uber das Anserin, einen neuen Bestandteil der Vogelmuskalatur. Hoppe-Seyler's Z. Physiol. Chem. 183, 1-10.

ADELSTEIN, R.S. & EISENBERG, E. (1980). Regulation of kinetics of the actin-myosin-ATP interaction. Ann. Rev. Biochem. 49, 921-956.

ALLEN, D.G. & KENTISH, J.C. (1985). The cellular basis of the length-tension relation in cardiac muscle. J. Mol. Cell. Cardiol. 17, 821-846.

ALLEN, D.G. & KURIHARA, S. (1979). Calcium transients at different muscle lengths in rat ventricular muscle. J.Physiol. 292, 68-69P.

ALLEN, D.G. & KURIHARA, S. (1980). Calcium transients in mammalian ventricular muscle. Eur. Heart J. 1, 5-15.

ALLEN, D.G. & KURIHARA, S. (1982). The effect of muscle length on intracellular calcium transients in mammalian cardiac muscle. J.Physiol. 327, 79-94.

ASHLEY, C.C. & GRIFFITHS, P.J. (1984). Potentiation of contraction by AR-L 115BS in striated muscle fibers from the barnacle Balanus nubilus. J.Physiol. 360, 58P.

ASHLEY, C.C. & MOISESCU, D.G. (1977). Effect of changing the composition of the bathing solutions upon the isometric tension-pCa relation in bundles of crustacean myofibrils. J.Physiol. 270, 627-652.

AVENA, R.M. & BOWEN, W.J. (1969). Effect of Carnosine and Anserine on Muscle Adenosine Triphosphates. J.Biol.Chem. 244, 1600-1604.

BATES, R.G., ROY, R.N. & ROBINSON, R.A. (1973). Buffer Standards of Tris [Hydroxmethyl]methylglycine "Tricine" for the Physiological Range pH 7.2 to 8.5. Anal.Chem. 45, 1663-1666.

BERMAN, M. & MAUGHAN, D.W. (1982). Axial stiffness modulii of compressed-lattice and sucrose-loaded skinned skeletal muscle fibres. Biophys.J. 37, 363A.

BERS, D.M. (1982). A simple method for the accurate determination of free $[Ca^{2+}]$ in Ca-EGTA solutions. Am.J.Physiol. 242, C404-408.

BIANCHI, C.P. (1961). The effect of caffeine on radiocalcium movements in frog satorius. J Gen Physiol 44, 845-858.

BLINKS, J.R. (1965). Influence of osmotic strngth on ccross-section and volume of isolated isolated single cells. J.Physiol. 177, 42-57.

BLINKS, J.R. & ENDOH, M. (1984). Sulmazol (ARL-115 BS) alters the relation between [Ca^{2+}] and tension in living canine ventricle muscle. J.Physiol. 253, 63P.

BLINKS, J.R., WEIR, W.C., HESS, P. & PRENDERGAST, F.G. (1982). Measurement of Ca^{2+} concentration in living cells. Prog. Biophys. 40, 1-114.

BOLDYREV, A.A., LEBEDEV, A.V. & RITOV, V.B. (1969). Adenosine Triphosphatase activity and the absorption of Ca^{2+} ions by Sarcoplasmic Reticulum fragments. Biochemistry Biokhimiya 34, 96-100.

BRANDT, P.W., COX, R.N. & KAWAI, M. (1980). Can the binding of calcium to two regulatory sites on troponin C determine the steep pCa-tension relationship of skeletal muscle. Proc. Natl. Acad. Sci. USA 77, 4717-4720.

BRANDT, P.W., COX, R.N. & KAWAI, M. (1982). Shifts in the position of the pCa/tension curve of skinned rabbit psoas fibers correlated with the kinetics of the cross-bridge cycle. Biophys.J. 37, 121A.

BRANDT, P.W., DIAMOND, M.S. & SCHACHAT, F.H. (1984). The thin Filament of Vertebrate Skeletal muscle Co-operatively activates as a unit. J. Mol. Biol. 180, 379-384.

BRANDT, P.W., GLUCK, B., MINI, M. & CERRI, C. (1985). Hysteresis of the mammalian pCa/Tension relationship is small and muscle specific. J.Musc.Res.Cell.Motil. 6, 197-205.

BREMEL, R.D. & WEBER, A. (1972). Cooperation within Actin Filament in Vertebrate Skeletal Muscle. Nature 238, 97-101.

BROWN, C.E. (1981). Interactions among Carnosine, Anserine, Ophidine and Copper in Biological Adaption. J.Theor.Biol. 88, 245-256.

BROWN, C.E. & ANTHOLINE, W.E. (1979). Chelation Chemistry of Carnosine. Evidence that Mixed Complexes May occur in Vivo. J.Physical Chem. 83, 3314-3319.

BROWN, K.M. & DENNIS, J.S. (1972). Derivative free analogs of the Levenberg- Marquadt and Gauss algorithms for non linear least squares approximation. Numersche Mathematik 18, 289-297.

BURTON, R.F. (1983). The composition of animal cells: solutes contributing to osmotic pressure and charge balance. Comp.Biochem.Physiol. 76, B663-671.

BUTCHER, R.W. & SUTHERLAND, E.W. (1962). Adenosine 3',5'-Phosphate in Biological matterials. J.Biol.Chem. 237, 1244-1250.

CASTELLINI, M.A. & SOMERO, G.N. (1981). Buffering Capacity of Vertebrate Muscle: Correlations with Potentials for Anaerobic Function. J.Comp.P.Physiol. 143, 191-198.

CHANG, L.C. (1972). Content of free amino acids and natural dipeptides in the myocardium of vertebrates. (Russian) Dolk.Akad.Nauk SSSR 205, 1479-1482.

CHAPMAN, R.A. & LEOTY, C. (1976). Which of Caffeine's Chemical Relatives are able to evoke contractions in Mammalian Heart?. In Recent Advances in Studies on Cardiac Structure and Metabolism, pp425-430.

CHAPMAN, R.A. & MILLER, D.J. (1974). Structure-activity relations for caffeine: a comparative study of the inotropic effects of the methylxanthines, imidazoles and related compounds on the frog heart. J.Physiol. 242, 615-634.

CHAPMAN, R.A. & MILLER, D.J. (1974). The effects of caffeine on the contraction of the frog heart. J.Physiol. 242, 589-613.

CHRISTMAN, A.A. (1968). Anserine and Carnosine Content of Muscle Tissue. Fed.Proc. 27, 280.

CHRISTMAN, A.A. (1971). Determination of anserine, carnosine and other histidine compounds in muscle extractives. Anal. Chem. 39, 181.

CRICHTON, K.A., LAMONT, C., MILLER, D.J. & O'DOWD, J.J. (1987). Detection and estimation of carnosine, anserine and novel imidazoles in cardiac and skeletal muscle of several species by high-performance liquid chromatography. J.Physiol. (in press).

CRUSH, K.G. (1970). Carnosine and Related Substances in Animal Tissues. Comp.Biochem.Physiol. 34, 3-30.

DAVEY, C.L. (1960). Tha significance of Carnosine and Anserine in Striated Skeletal Muscle. Archs.Biochem.Biophys. 89, 303-308.

EBASHI, S., ENDO, M. & OHTSUKI, I. (1969). Control of muscle contraction. Quat. Rev. Biophys. 2, 351-384.

ELLIOTT, G.F. (1968). Force-Balances and Stability in Hexagonally-packed Polyelectolyte Systems. J.Theor.Biol. 21, 71-87.

ENDO, M. (1972). Stretch-induced Increase in Activation of Skinned Muscle fibres by Calcium. Nature 237, 211-212.

ENDO, M. (1973). Length dependence of activation of skinned muscle fibres by calcium. Cold. Spring Harb. Symp quant. Biol.37, 505-510.

ENDO, M. & KITAZAWA, T. (1978). E-C coupling studies on skinned cardiac fibres. In Biophysical aspects of cardiac muscle., pp307-327.

FABIATO, A. (1980). S.l. dependence of Ca-release from the SR of skinned cardiac cells demonstrated by differential microspectrophotometry with Arsenazo III . J Gen Physiol 76, 15A.

FABIATO, A. (1983). Calcium-induced release of alcium from the rat sarcoplasmic reticulum. Am.J.Physiol. 245, C1-14.

FABIATO, A. (1985). 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 6, 95-108.

FABIATO, A. (1985). Apprasial of the hypothesis of "Depolarisation-induced" release of calciumfrom the sarcolasmic reticulum in skinned cardiac cells from the rat or pigeon ventricle. In Structure and Function of the Sarcoplasmic Reticulum, pp112-113.

FABIATO, A. & FABIATO, F. (1973). Activation of skinned cardiac cells. Subcellular effects of cardioacctive drugs. Eur. J. Cardiol. 1, 143-155.

FABIATO, A. & FABIATO, F. (1975). Contractions induced by a calcium triggered release of calcium from the SR of single cardiac cells. J.Physiol. 249, 469-495.

FABIATO, A. & FABIATO, F. (1975). Dependence of the contractile activation of skinned cardaic cells on sarcomere length. Nature 256, 54-56.

FABIATO, A. & FABIATO, F. (1978). Cyclic AMP-induced enhancent of calcium accumulation by the sarcoplasmic reticulum with no modification of the sensitivity of the myofilaments to calcium in skinned fibres from a fast skeletal muscle. Biochim Biophys Acta 539, 253-260.

FABIATO, A. & FABIATO, F. (1978). Myofilament-Generated Tension Oscillations during Partial Calcium Activation and Activation Dependence of the Sarcomere Length-Tension Relation of Skinned Cardiac Cells. J Gen Physiol 72, 667-699.

FABIATO, A. & FABIATO, F. (1978). Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. J.Physiol. 276, 233-255.

FEDYNA, J.S., MORITZ-SMITH, C., KASVINSKY, P.J. & JOHNSON, P. (1982). Carnosine and Anserine are Effectors of Muscle Phosphorylase. J.Cell.Biol. 95, A358.

FINK, R.H.A., STEPHENSON, D.G. & WILLIAMA, D.A. (1986). Potassium and ionic strength effects on isometric force of skinned twitch muslce fibres of the rat and toad. J.Physiol. 370, 317-337.

FORD, L.E. & PODOLSKY, R.J. (1972). Calcium uptake and force development by skinned muscle in EGTA buffered solutions. J.Physiol. 223, 1-19.

FRY, C.H., HARDING, D.P. & MILLER, D.J. (1985). Separation of different Ca^{2+} exchange process in isolated rat ventricular myocytes. J.Physiol. 372, P6.

FRY, C.H. & MILLER, D.J. (1985). The contribution of mitochondria to calcium metabolism and tension generation in cardiac muscle. In Control and Manipulation of Calcium Movement, pp111-222.

FUCHS, F. (1978). On the relation between filament overlap and the number of calcium binding sites on glycerated muscle fibers. Biophys.J. 21, 273-277.

FUCHS, F. (1984). Calcium-troponin affinity in relation to sarcomere length in relaxed skeletal muscle fibres. Fed. Proc. 43, P531.

FUCHS, F. & FOX, C. (1982). Parallel measurements of bound calcium and force in glycerinated rabbit psoas muscle fibers. Biochim Biophys Acta 679, 110-115.

FUCHS, F. & PLANCHAK, R. (1983). Is Ca^{2+}-troponin affinity force-dependent?. Biophys.J. 41, 262A.

GERCKEN, V.G., BISCHOFF, H. & TROTZ, M. (1980). Myocardial protection by a carnosine-buffered cardioplegic solution. (German) Arzneimittelforsch 30, 2140-2143.

GODT, R.E. & MAUGHAN, D.W. (1977). Swelling of skinned muscle fibres of the frog. Experimental observations. Biochem.J. 19, 103-116.

GODT, R.E. & MAUGHAN, D.W. (1981). Influence of Osmotic Compression on Calcium Activation and Tension in Skinned Muscle Fibers of the Rabbit. Pfluegers Arch. 391, 334-337.

GODT, R.E. & MORGAN, J.L. (1981). Effect of osmotic compression on calcium activation and force production of skinned rabbit muscle fibres. Biophys.J. 33, 30A.

GOLDMAN, Y.E., MATSUBARA, I. & SIMMONS, R.M. (1979). Lateral filament spacing in frog skinned muscle fibres in the relaxed and rigor states. J.Physiol. 295, P80-81.

GOOD, N.E., WINGET, G.D., WINTER, W., CONNOLLY, T.N., IZAWA, S. & SINGH, M.M. (1966). Hydrogen Ion Buffers for Biological Research. Biochemistry Biokhimiya 5, 467-477.

GORDON, A.M., HUXLEY, A.F. & JULIAN, F.J. (1966). The variation in isometric tension with sarcomere length in vertebrate muscle fibres. J.Physiol. 184, 170-192.

GORDON, A.M., HUXLEY, A.F. & JULIAN, F.J. (1966). Tension development in highly stretched vertebrate muscle fibres. J.Physiol. 184, 143-169.

GORDON, A.M., RIDGWAY, E.B. & MARTYN, D.A. (1984). Calcium Sensitivity is Modified by Contraction. Adv.Exp.Med.Biol. 170, 553-563.

GULEWITSCH, W. & AMIRADZIBI, S. (1900). Der Dtsch. Chem. Ges. 33, 1902.

HARRISON, S.M. (1985). The involvement of mitochondria in the intracellular control of contraction in cardiac muscle. PhD Thesis, Glasgow University.

HARRISON, S.M. & MILLER, D.J. (1984). Mitochondrial contribution to relaxation demonstrated in skinned cardiac muscle of the rat. J.Physiol. 353, 55P.

HARRISON, S.M. & MILLER, D.J. (1984). Sodium-induced calcium release in chemically skinned cardiac fibres. Proc.Int.U.Physiol.Sci. (Jer) 84, 388.

HARTLAGE, P.L., MOBLEY, E., BLANKENSHIP, P.R. & AUGUST ROESEL, R. (1983). Carnosine Excretion in Muscular Dystrophy. Fed.Proc. 42, 791.

HEILBRUNN, I.V. (1940). The action of calcium on muscle protoplasm. Physiol. Zool. 13, 88-95.

HELEIUS, A. & SIMONS, K. (1975). Solubilisation of membranes by detergents. Biochim Biophys Acta 415, 29-79.

HERZIG, J.W., FEILE, K. & RUEGG, J.C. (1981). Activating effects of AR-l 115BS on the Ca^{2+} sensitivitity, force, stiffness and unloaded shortening velocity (Vmax) in isolated contractile structures from mammalian heart muscle. Arzneimittelforsch 31, 177-182.

HERZIG, J.W. & RUEGG, J.C. (1979). Investigation of gylcerinated cardiac muscle fibres in relation to the problem of regulation of cardiac contractility. Basic Res. Cardiol. 75, 26-33.

HIBBERD, M.G. & JEWELL, B.R. (1982). Calcium and length-dependent force production in rat ventricular muscle. J.Physiol. 329, 527-540.

HOFMANN, P.A. & FUCHS, F. (1987). Bound calcium and force development in detergent extracted cardiac muscle bundles: effect of sarcomere length. Biophys.J. 51, 463A.

HUXLEY, H.E. (1953). X-ray analysis and problems of muscle. Proc.R.Soc.Lond.B 141, 59-62.

HUXLEY, H.E. (1969). The mechanism of muscular contraction. Science 164, 1356-1366.

ILLINGWORTH, J.A. (1981). A common source of error in pH measurements. Biochem.J. 195, 259-262.

JENNISON, K. & ELLIOTT, G.F. (1981). Charge measurements of muscle proteins. Biophys.J. 33, 26A.

JEWELL, B.R. (1974). Introduction: the changing face of the length-tension relation. In The Physiological Basis of Starling's Law of the Heart, pp7-12.

JEWELL, B.R. (1977). A re-examination of the influence of muscle length on myocardial performance. Circ.Res. 40, 221-230.

JEWELL, B.R. & KENTISH, J.C. (1981). Dithiothreitol reduces the deterioration of 'skinned' preparations of cardiac muscle. J.Physiol. 319, 83-84P.

JOHNSON, J.D., CHARLTON, S.C. & POTTER, P.D. (1979). A fluorescence stopped flow analysis of Ca-exchange with troponin C. J.Biol.Chem. 254, 3497-3502.

JOHNSON, P. & ALDSTADT, J. (1984). Effects of Carnosine and Anserine on Muscle and Non -Muscle Phosphorylases. Comp.Biochem.Physiol. 78, B331-333.

JULIAN, F.J. (1971). The effect of calcium on the force velocity relation for briefly glycerinated frog muscle fibres. J.Physiol. 218, 117-145.

JULIAN, F.J., SOLLINS, M.R. & MOSS, R.L. (1978). Sarcomere length non-uniformity in relation to tetanic responses of stretched skeletal muscle fibers. Proc.R.Soc.Lond.B 200, 109-116.

KAWAI, M., SCHULMAN, M.I. & CORNACCHIA, T. (1984). Cross-bridge kinetics of rabbit skeletal muscle fibres when thelattice space is altered by dextran T500 and ionic strength changes. Biophys.J. 45, 7A.

KENTISH, J.C.,ter KEURS, H.E.D.T., RICCIARDI, L., BUCX, J.J. & NOBLE, M.I.M. (1986). Comparison between the sarcomere length force relations of intact and skinned trabeculae from the rat right ventricle (Influence of calcium concentrations on these relations). Circ.Res. 58, 755-768.

KERRICK, W.G.L. & BEST, P.M. (1974). Calcium ion release in mechanically disrupted heart cells. Science 183, 435-437.

KRASNER, B. & MAUGHAN, D. (1981). Dextran T500 decreases skinned fiber width , tension and ATP ase. Biophys.J. 33, 27A.

KREUGER, J.W. & POLLACK, G.H. (1975). Myocardial sarcomere dynamics during isometric contracions. J.Physiol. 251, 627-643.

KURODA, Y. & IKOMA, T. (1966). N-acetyl histidine Isolated from Frog Heart. Science 152, 1241-1242.

LAMONT, C. & MILLER, D.J. (1987). Oscillations in selectively skinned rat cardaic muscle. J.Physiol. 392, 14P.

LOPINA, O.D. & BOLDYREV, A.A. (1975). Effects of the Dipeptides Carnosine and Anserine on the Accumulation of Calcium by S.R. fragments (in Russian) Dolk.Akad.Nauk SSSR 220, 1218-1221.

LUKTON, A. & OLCOTT, H.S. (1958). Content of free imidazole compounds in the muscle tissue of aquatic animals. Food Research 23, 611-618.

MARGOLIS, F.L. (1974). Carnosine in the primary olfactory pathway. Science 184, 909-911.

MARTELL, A.E. & SMITH, R.M. (1974). Critical Stability constants. Amino Acids.

MATSUBARA, I. & ELLIOTT, G.F. (1972). X-ray Diffraction Studies on Skinned Single Fibres of Frog Skeletal Muscle. J. Mol. Biol. 72, 657-669.

MATSUBARA, I., GOLDMAN, Y.E. & SIMMONS, R.M. (1984). Changes in the Lateral Filament Spacing of Skinned Muscle Fibres when Cross-bridges Attach. J. Mol. Biol. 173, 15-33.

MATSUBARA, I. & MILLMAN, B.M. (1974). X-ray diffraction studies on cardiac muscle. In The Physiological Basis of Starling's Law of the Heart, pp31-41.

MATSUBARA, I., UMAZUME, Y. & YAGI, N. (1984). Lateral Shrinkage of the myofilament lattice in chemically skinned muscle during contraction. In Contractile Mechanisms in Muscle, pp711-723.

MATSUBARA, I., UMAZUME, Y. & YAGI, N. (1985). Lateral filament spacing in chemically skinned murine muscles during contraction. J.Physiol. 360, 135-148.

MAUGHAN, D. & BERMAN, M. (1982). Radial compression of functionally skinned cardaic bundles abolishes Ca-activated force. Biophys.J. 37, 363A.

MESHKOVA, N.P. & KAYAVKINA, O.E. (1965). Participation of Carnosine and Anserine in the Glycolytic and Oxidative Processes in Muscle Tissue. Biochemistry Biokhimiya 30, 88-94.

METZGER, J.M. & MOSS, R.L. (1987). Shortening velocity in skinned single muscle fibres: Influence of sarcomere length. Biophys.J. 52, 127-131.

MILLER, D.J. (1975). Diffusion delays and the rate of contracture development in frog heart muscle. Pfluegers Arch. 359, R23.

MILLER, D.J. & CHAPMAN, R.A. (1972). A common Molecular Feature that Distinguishes which of the Methylxanthines, Imidazoles and related compounds are able to induce contractures in frog heart. Pharmac.Res.Comm. 4, 321-325.

MILLER, D.J., ELDER, H.Y. & SMITH, G.L. (1985). Ultrastructural and X-ray microanalytical studies of EGTA- and detergent treatd heart muscle. J.Musc.Res.Cell.Motil. 6, 525-540.

MILLER, D.J., SINCLAIR, J., SMITH, A.D. & SMITH, G.L. (1982). Measurement of sarcomere length and automated exchange of bathing solutions applied to the study of intact and chemically 'skinned' cardiac muscle. J.Physiol. 329, 11P.

MILLER, D.J. & SMITH, G.L. (1984). EGTA purity and the buffering of calcium ions in physiological solutions. Am.J.Physiol. 246, C160-166.

MILLER, D.J. & SMITH, G.L. (1985). The contractile behaviuor of EGTA- and detergent treated heart muscle. J.Musc.Res.Cell.Motil. 6, 541-567.

MILNER-BROWN, H.S., STEIN, R.B. & YEMM, R. (1973). Changes in firing rate of volumtarymotor units during linearly changing voluntary contractions. J.Physiol. 230, 317-390.

MOISESCU, D.G. (1976). The kinetics of reation in calcium activated skinned muscle fibres. Nature 262, 610-613.

MOISESCU, D.G. & THIELECZEK, R. (1978). Calcium and Strontium concentration changes within skinned muscle preparations following a change in the external bathing solution. J.Physiol. 275, 241-262.

MOORE, W.J. (1976). Physical Chemistry. Longman, 1976.

MORGAN, J.P. & BLINKS, J.R. (1982). Intracellular Ca^{2+} transients in cat papillary muscle. Can. J. Pharmacol. 60, 524-528.

MOSS, V.A., MILLER, D.J. & LAMONT, C. (1986). Real-time analysis of sarcomere length during activation of chemically skinned cardiac muscle. Proc. Roy. Microsc. Soc. (Edinburgh) 21, 581-582.

NATORI, I. (1954). The property of contraction process of isolated myofibrils. Jikeikai. Med. J. 1, 119-126.

NAYLER, W.G. (1963). Effects of Caffeine on cardiac contractile activity and radiocalcium movement. Am.J.Physiol. 204, 969-974.

PAGANI, E.D., SHEMIN, R. & JULIAN, F.J. (1986). Tension-pCa Relations of Saponin-skinned Rabbit and Human Heart Muscle. J.Mol. Cell. Cardiol. 18, 55-66.

PARKER, C.J. & RING, E. (1970). A Comparative study of the effect of carnosine on Myofibrillar-ATPase Activity of Vertebrate and Invertebrate Muscles. Comp.Biochem.Physiol. 37, 413-419.

PERRY, T.L., HANSEN, S., DIAMOND, S. & MOK, C. (1971). Regional distribution of amino acids in human brain obtained at autopsy. J.Neurochem 18, 513.

RIDGWAY, E.B., GORDON, A.M. & MARTYN, D.A. (1983). Hysteresis in the Force-Calcium relation in Muscle. Science 219, 1075-1077.

RIEGEL, J.A. (1986). Measurement of COP of microlitre and sunbmicrolitre volumes. J. Exp. Biol. 122, 433-437.

ROCHEL, S. & MARGOLIS, F.L. (1982). Carnosine release from the olfactory bulb synaptosomes is calcium dependent and deploarisation stimulated. J.Neurochem 38, 1505-1514.

ROME, E. (1967). Light and X-ray diffraction studies of the filament lattice of glyceral-extracted rabbit psoas muscle. J. Mol. Biol. 37, 331-344.

SCHMIDT, G. & CUBILES, R. (1955). Comparative Studies on the Occurrence on the Carnosine-Anserine Fraction in Skeletal Muscle and Heart. Archs.Biochem.Biophys. 58, 227-231.

SCHOENBERG, M. (1980). Geometrical factors influencing muscle force development. Biophys.J. 30, 69-78.

SCHOENBERG, M. (1980). Geometric Factors influencing muscle force development 1. The effect of filament spacing upon axial forces. Biophys.J. 30, 51-68.

SCHOLZ, H. (1984). Inotropic Drugs and their Mechanisms of Action. Jacc 4, 389-397.

SEVERIN, S.E. (1976). Physiological activity of natural derivatives of imidazole. (Russian) Vestn.Akad.Med.Nauk 9, 31-37.

SEVERIN, S.E., VAL FSON, P.L., GRIGOROVICH, Y.A. & SOLOV EVA, G.A. (1963). The biological role of carnosine. Biochemistry Biokhimiya 28, 415-420.

SEVERIN, S.E. & YU, Y.S. (1958). The effect of the dipeptide anserine and carnosine on oxidative phosphorylation in isolated mitochondria of the pectoral muscle of the pigeon. Biochemistry Biokhimiya 23, 816-822.

SHAPIRO, P.J., TAWADA, K. & PODOLSKY, R.J. (1979). Xray diffraction in skinned muscle fibres. Biochem.J. 25, A18.

SMITH, F.H. (1969). Double refracting interference microscope. Patent Specification No.1248771.

SMITH, G.L. (1983). A functional an structural study of cardiac muscle subjected to membrane disruption techniques. PhD Thesis, Glasgow University.

SMITH, G.L. & MILLER, D.J. (1985). Potentiometric measurements of stoichiometric and apparent affinity constants of EGTA for protons and divalent ions including calcium. Biochim Biophys Acta 839, 287-299.

SOBUE, K., KONISHI, H. & NAKAJIMA, T. (1975). Isolation and identification of N-acetylhomocarnosine and N-acetylcarnsine from brain and muscle. J.Neurochem 24, 1261-1262.

SOLARO, J.R. & RUEGG, J.C. (1982). Stimulation of Ca^{2+} binding and ATPase activity of dog cardiac myofibrils by ARL115 BS. Circ.Res. 51, 290-294.

SOLARO, R.J., WISE, R.M., SHINER, J.S. & BRIGGS, F.N. (1974). Calcium reqirements for cardiac myofibrillar activation. Circ.Res. 34, 525-530.

SQUIRE, J.M. (1975). General model of myosin filament structure II Myosin filament and cross-bridge interaction in vertebrate striated and insect flight muscles. J. Mol. Biol. 72, 125-138.

STEPHENSON, D.G. & WENDT, I.R. (1984). Length dependence of changes in sarcoplasmic calcium concentration and myofibrillar calcium sensitivity in striated muscle fibres. J.Musc.Res.Cell.Motil. 5, 243-273.

STEPHENSON, D.G. & WILLIAMS, D.A. (1983). Slow Aphibian Muscle Fibres Become Less Sensitive to Ca^{2+} with Increasing Sarcomere Length. Pfluegers Arch. 397, 248-250.

STEVANOVA, N.G. & GRINIO, L.P. (1968). [The content of carnosine and anserine in muscles in the course of progressive muscular dystrophy.] Russian Vop Med Khim 14, 210-215.

STULL, J.T. (1980). Phosphorylation of Contractile Proteins in Relation to Muscle Function. In Advances in Cyclic Nucleotide Research, , pp40-85.

TAMAKI, N., HARADA, M., KAWANO, H.A.M. & HAMA, T. (1977). Anserine and Carnosine contents on Muscular Tisssue of Rat and Rabbit. J. Nutr. Sci. Vitaminol. 23, 319-329.

TAYLOR, E.W. (1979). Mechanisms of Actomyosin ATPase and the problems of muscle contraction. Critical. Rev. Biochem. 6, 103-164.

TOLKATSCHEWSKIA, N. (1929). Z. Physiol Chem 223, 57.

VEGA, C.A. & BATES, R.G. (1976). Buffers for the Physiological Range: Thermodynamic Constants of Four Substituted Aminoethanesulfonic Acids from 5 to 50 degrees C. Anal.Chem. 48, 1293-1296.

WEBER, A. & HERZ, R. (1968). The relationship between caffeine contracture of intact muscle and the effect of caffeine on reticulum. J Gen Physiol 52, 750-759.

WENDT, I.R. & STEPHENSON, D.G. (1983). Effects of caffeine on Ca-activated force production in skinned cardiac and skeletal muscle fibers of the rat. Pfluegers Arch. 398, 210-216.

WILLIAMS, D.A. & STEPHENSON, D.G. (1983). A study of 'hysteresis' in the force-pCa relation in muscle. Biophys.J. 45, A99.

WINEGRAD, S. (1974). Resting sarcomere length-tension relation in living frog heart. J Gen Physiol 64, 343-355.

YUDAEV, N.A. (1949). Detection of carnosine and anserine in heart muscle by partition paper chromatography. Dolk.Akad.Nauk SSSR 68, 119-121.

ZAPP, J.A. & WILSON, D.W. (1938). The distribution of carnosine and anserine in various muscles of different species. J.Biol.Chem. 126, 19-27.