THE ROLE OF TROPONIN C IN THE MODULATION OF MYOFIBRILLAR Ca^{2+}-SENSITIVITY

by

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A thesis submitted for the Degree of

Doctor of Philosophy

at the University of London

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DEDICATION

To Grandma May (muckafasha)

and Walti (someone special)

THE ROLE OF TROPONIN C IN THE MODULATION OF MYOFIBRILLAR Ca^{2+}-SENSITIVITY

It is desirable to treat the failing heart by increasing contractile force without raising level of intracellular Ca^{2+}. This could be achieved by increasing the sensitivity of myofilaments to Ca^{2+}, but "Ca^{2+}-sensitisers" have unwanted side-effects. In addition, the mechanism of the decrease in Ca^{2+}-sensitivity by low pH, or elevated Pi is unclear. I investigated the role of troponin-C (TnC) in these changes in Ca^{2+}-sensitivity by (i) comparing effects on cardiac and skeletal myofibrils (which have different forms of TnC) and (ii) measuring the Ca^{2+} affinity of TnC, both isolated and in the myofibrils.

The effects of pH, Pi and caffeine on skinned fibres were measured. Isolated skeletal and cardiac TnCs labelled with DANZ and IAANS were used to detect any direct effects of pH, Pi and caffeine on Ca^{2+}-binding to TnC. Neither Pi nor caffeine altered the Ca^{2+}-affinity of the TnC, but acidic pH decreased TnC Ca^{2+}-binding. Thus the negative inotropic effects of low pH in striated muscle are partially explained by a reduction in Ca^{2+}-affinity of the TnC. Pi and caffeine-induced modulation of Ca^{2+}-sensitivity may depend on other proteins, or may directly affect crossbridges.

I measured the Ca^{2+}-sensitising effects of novel compounds developed by Ciba-Geigy. The effects were species dependent and most pronounced in porcine muscle. CGP48506, like caffeine, had a greater Ca^{2+}-sensitising action in cardiac than skeletal fibres whereas CGP48508 unexpectedly worked the other way around. Neither compound affected Ca^{2+}-binding to isolated skeletal TnC so it appeared that their effects on skeletal muscle were not solely dependent on the moiety of TnC present.

To investigate the effects of CGP48506 on Ca^{2+}-binding to TnC in skinned psoas preparations, native TnC was exchanged for DANZ-labelled skeletal TnC. Results indicated that CGP48506 increased the Ca^{2+}-affinity of TnC. As binding of Ca^{2+} to isolated TnC was unaffected it appears that sensitisation depended on factor(s) in addition to TnC.

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ABBREVIATIONS

ADP	adenosine triphosphate
ATP	adenosine triphosphate
BDM	2,3-butanedione monoxime
BES	N,N-bis(2-hydroxyethyl)-2-aminoethane sulphonic acid
cAMP	adenosine 3':5' cyclic monophosphate
CaE	Ca^{2+}-EGTA buffer
C-G	Ciba-Geigy
CP	creatine phosphate
DANZ	dansylaziridine (5-dimethylaminonaphthalene-1-sulphonyl)
DMSO	dimethylsulphoxide
DTT	dithiothreitol
EDL	extensor digitorum longus
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N,N'-tetraacetic acid
F	force
HDTA	2,6-diaminohexane-N,N,N',N'-tetraacetic acid
H_n	Hill "n"
HR	high EGTA concentration relaxing solution
IAANS	2-(4-iodoacetamidoanalino)naphthalene 6-sulphonic acid
I_e	ionic equivalence
IS	ionic strength
LR	low EGTA concentration relaxing solution
MES	2-[N-morpholino]ethanesulphonic acid
MOPS	3-[N-morpholino]propanesulphonic acid
pCa	$-\log_{10}[Ca^{2+}]$
pCa_{50}	pCa required for 50% Ca^{2+}-activation
PDE	phosphodiesterase
PEP	pyroenolphosphate
Pi	inorganic phosphate
PMSF	phenylmethylsulphonylfluoride
Pr	propionate
pSr	$-\log_{10}[Sr^{2+}]$
SDS	sodium dodecyl sulphate
SEM	standard error of the mean, $\sigma/\sqrt{n}$
SL	sarcomere length
SR	sarcoplasmic reticulum
Tm	tropomyosin

Tn	troponin
TnC	troponin C
TnC-DANZ	troponin C labelled with the probe DANZ
TnC-IAANS	troponin C labelled with the probe IAANS
TnC-TnI	troponin C complexed with TnI
TnI	troponin I
TnT	troponin T
TRIS	(tris[hydroxy-methyl]amino methane)
UCL	University College London

ACKNOWLEDGEMENTS

I am very grateful to my supervisor, Dr. J. C. Kentish, for his invaluable guidance and patience throughout this study. I would also like to thank Prof. Dr. J. W. Herzig for his enthusiasm and encouragement during my visit to Ciba-Geigy in Basel, and Dr. G. Pay for his helpful advice.

Thanks are also due to Dr. P. Cummins for guiding me through the preparation of troponin C; to Prof. B. D. Gomperts for the loan of his fluorimeter; to Dr. W. Leijendekker for introducing me to the force & fluorescence apparatus; and to Heidi Depersin and Michelle Thonier for their cheerful assistance at Ciba-Geigy.

Finally I wish to thank members of the department of pharmacology at St. Thomas's for making space for me and providing much needed moral support during the final stages of this work.

CHAPTER 1: GENERAL INTRODUCTION TO THE REGULATION OF CONTRACTION IN STRIATED MUSCLE

1.1 INTRODUCTION

1.1.1 STRUCTURE OF STRIATED MUSCLE

Vertebrate striated muscle encompasses both skeletal and cardiac muscle which, although differing in action potential characteristics and source of Ca^{2+} for activation of contraction, have very similar microstructural organisation. Cardiac muscle consists of a syncytium with separate cellular units linked end to end by intercalated disks. The fibres thus formed bifurcate and interdigitate to form a complex network. Skeletal muscle consists of many fibres often parallel without crossbranches. Each fibre is usually composed of one cell extending the entire length of the muscle. Under the light microscope both skeletal and cardiac fibres appear striated with dark optical anisotropic "A bands" alternating with light isotropic "I bands". Each skeletal and cardiac fibre contains many myofibrils which are themselves composed of actin and myosin myofilaments. It is the arrangement of these myofilaments into sarcomeres (fig.1A) that causes the banding observed in striated muscle.

As shown in fig.1B, the thick filaments are composed of many myosin molecules lying in parallel with the heads protruding regularly from the backbone. Myosin consists of two α helix heavy chains twisted together to form a tail (LMM+S2) and two globular heads (S1), the latter each containing two light chains LC1 and LC2 (fig.1C). S2 is the flexible part of the tail near the heads and together these regions form the crossbridges which bind to actin. The myosin head also contains a catalytic site for the hydrolysis of ATP and is thus often referred to as myosin ATPase. The rate of hydrolysis is, however, very slow due to the slow rate of release of the products Pi and ADP.

The thin filaments are composed of two strands of actin monomers wound round each other (fig.1D) with a periodicity of 38 nm. α-helical tropomyosin (Tm) molecules are arranged along the grooves of the actin filaments with one Tm for every seven actin monomers. Associated with each Tm molecule is a troponin (Tn) complex composed of three subunits: troponin C, which binds Ca^{2+}; troponin I, which inhibits myosin ATPase; and troponin T, which binds Tm and TnI. The two dimensional arrangement of the actin and myosin filaments is shown in fig.1E. In three-dimensions each thick filament is surrounded by six thin filaments whereas each thin filament is surrounded by three thick filaments in a highly ordered lattice, where filament overlap occurs.

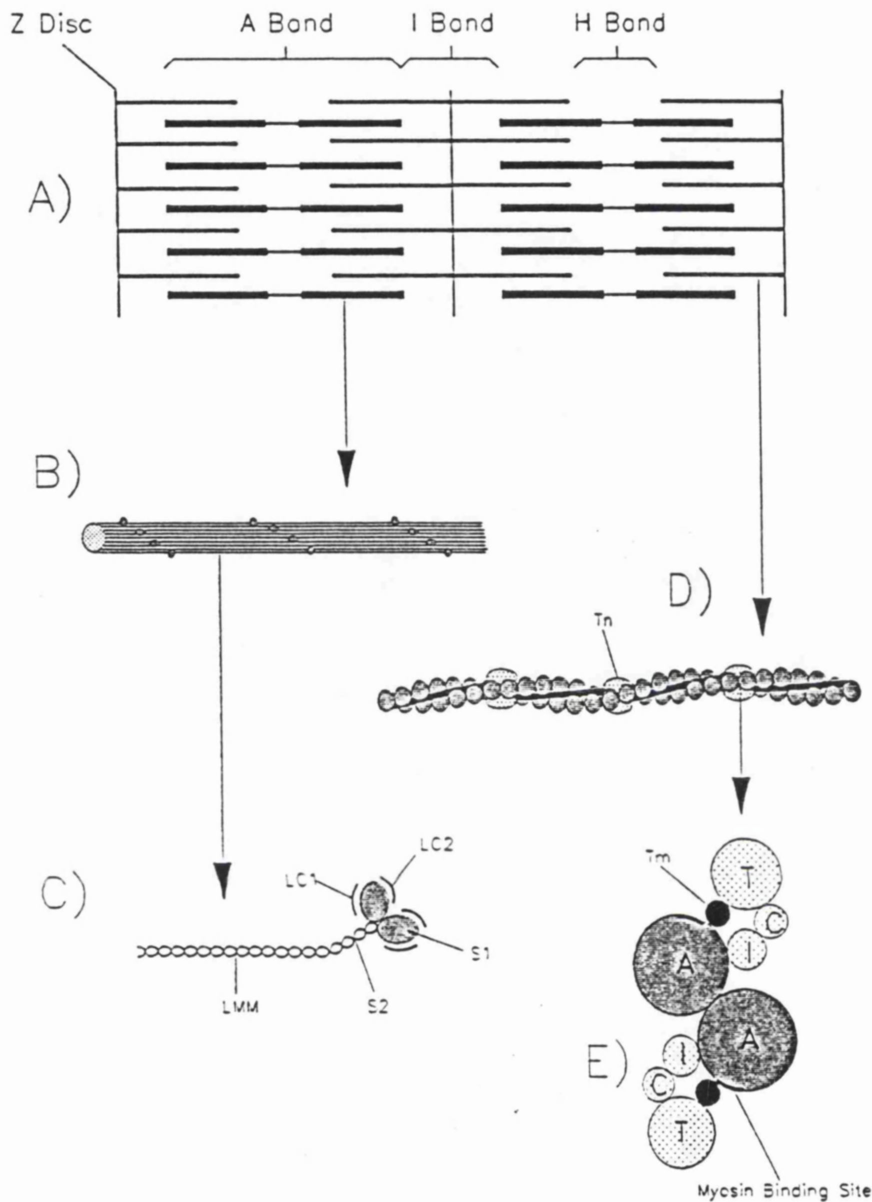

Fig.1 ORGANISATION OF CONTRACTILE PROTEINS. A) Thick myosin filaments and thin actin filaments are arranged in sarcomeres bounded by Z discs. The extent of filament overlap increases during contraction, when the sarcomeres shorten. Myosin filaments are orientated in opposite directions in each half of the sarcomere, leaving a central "tails only" region. B) Myosin filament consisting of myosin molecules arranged in a regular spiral with the heads protruding. C) Myosin molecule comprising a tail (LMM + S2) and two heads (S1) to which light chains (LC1 & LC2) bind. D) Actin filament consisting of two interwound strands of actin monomers with associated elongate tropomyosin molecules and troponin (Tn) complexes. E) Cross-section through a thin filament showing interactions of actin (A), tropomyosin (Tm) and troponins T, C and I.

1.1.2 SLIDING FILAMENT MECHANISM

Huxley (1957) proposed that force was generated by crossbridges cycling in the region of actin-myosin overlap such that the filaments slid past each other (Huxley & Hanson, 1954; Huxley & Niedergerke, 1954), thereby shortening the sarcomere (and muscle) without altering the length of the filaments themselves. Crossbridge binding was assumed asynchronous to prevent filaments slipping during contraction.

1.1.3 LENGTH-TENSION RELATIONSHIP

Gordon et al (1966) demonstrated that tension depended on SL and explained their results in terms of filament overlap. Using frog skeletal fibres they found that at SLs between 2.0 and 2.25 μm force was maximal, but increasing SL above 2.25 μm caused a linear decline in tension until no force was generated at a SL of 3.65 μm. Likewise shortening the SL from 2.0 to 1.67 μm also caused a gradual fall in tension which accelerated towards zero when SL was reduced from 1.67 to 1.27 μm. Gordon et al deduced that at SLs between 2.0 and 2.25 μm filament overlap (and hence crossbridge formation) was optimal. Increasing the SL reduced the extent of overlap, thereby decreasing actin-myosin interaction, whilst decreasing SL below 2.0 μm first caused the thin filaments from one side of the sarcomere to interfere with those on the other and then resulted in the thick filaments pressing against the Z disc, thereby exerting a restoring tension opposing sarcomere shortening. In cardiac muscle the presence of much connective tissue means that at SL above 2.3 μm preparations become damaged with a concurrent increase in resting tension. Thus there is no descending limb to the SL-tension relationship (Kentish, 1988). In addition, cardiac muscle usually operates at a SL on the ascending limb (2.1 μm) in contrast to skeletal muscle which, in vivo, is at a SL in the optimal range, for example 2.4 μm in rabbit psoas. In mammalian skeletal muscle the optimum SL range is 2.4-2.8 μm (Stephenson & Williams, 1982), due to longer filaments than in frog muscle.

a) Effects of Ca^{2+} on the Length-Tension Relationship

Changes in Ca^{2+} concentration have striking effects on length-tension relationships of both skeletal and cardiac muscle (Stephenson & Wendt, 1984; Allen & Kentish, 1985). In general, higher levels of Ca^{2+} increase the amount of force generated by fibres at a given SL, though the relationship between tension and SL also changes shape as $[Ca^{2+}]$ is raised (Kentish et al, 1986). Such effects are noted on both the ascending and descending limbs of graphs such as that in fig.12. As SL decreases, not only does the maximum Ca^{2+}-activated force decline (on the ascending limb) but the midpoint of the force-pCa curve moves to

the right indicating a decrease in myofilament Ca^{2+}-sensitivity. The Ca^{2+}-sensitivity of skeletal muscle was clearly less affected by SL changes than that of cardiac muscle (Babu et al, 1988). The mechanism of the length dependence of Ca^{2+}-sensitivity remains unclear although it is possible that the Ca^{2+} affinity of troponin C is increased as SL increases (see section on interactions between crossbridges and troponin). The role of crossbridges is, however, complicated by the fact that the Ca^{2+}-sensitivity of the fibres increases even at lengths in the descending limb of the length-tension relationship.

1.1.4 THE CROSSBRIDGE CYCLE

A simplified crossbridge cycle, by which force is generated, is illustrated and explained in fig.2.

1.1.5 EFFECTS OF [Ca^{2+}] ON FORCE AND ATPase ACTIVITY

In both cardiac and skeletal muscle the activation of the myofilaments is controlled by Ca^{2+}, which comes predominantly (in cardiac muscle) or entirely (in skeletal muscle) from the sarcoplasmic reticulum. Upon electrical stimulation of the muscle cell, levels of intracellular Ca^{2+} rise from 0.1 - 0.2 μM to 1 μM in cardiac muscle and 5 μM in skeletal fibres. Studies with skinned (permeabilised) fibres showed that although skeletal muscle is fully activated at 5 μM Ca^{2+}, the force generated by cardiac fibres could be doubled by raising intracellular [Ca^{2+}] from 1 μM to 5 μM (Fabiato, 1981). Thus living cardiac muscle is never more than half-maximally activated.

Skinned fibre experiments also demonstrated a sigmoidal relationship between force and pCa ($-\log_{10}[Ca^{2+}]$). Since one molecule of ATP is hydrolysed in each crossbridge cycle, myofibrillar ATPase activity provides a measure of crossbridge cycling rate. Studies on isolated myofibrils showed that ATPase activity is also sigmoidally related to pCa. The sigmoidal curves were fitted well by a modified form of the Hill equation (Hill, 1910):

$$\text{Relative Force or ATPase Activity} = \frac{F}{F_{max}} = \frac{[Ca]^n}{K^n + [Ca]^n}$$

where F = force or ATPase activity at Ca^{2+} concentration [Ca]

F_{max} = force at saturating Ca^{2+} concentration

n = Hill coefficient, a measure of cooperativity

K = Ca^{2+} concentration where F = 50% F_{max}

Each curve was characterised by its pCa_{50} (pCa for 50% F or -logK) and its

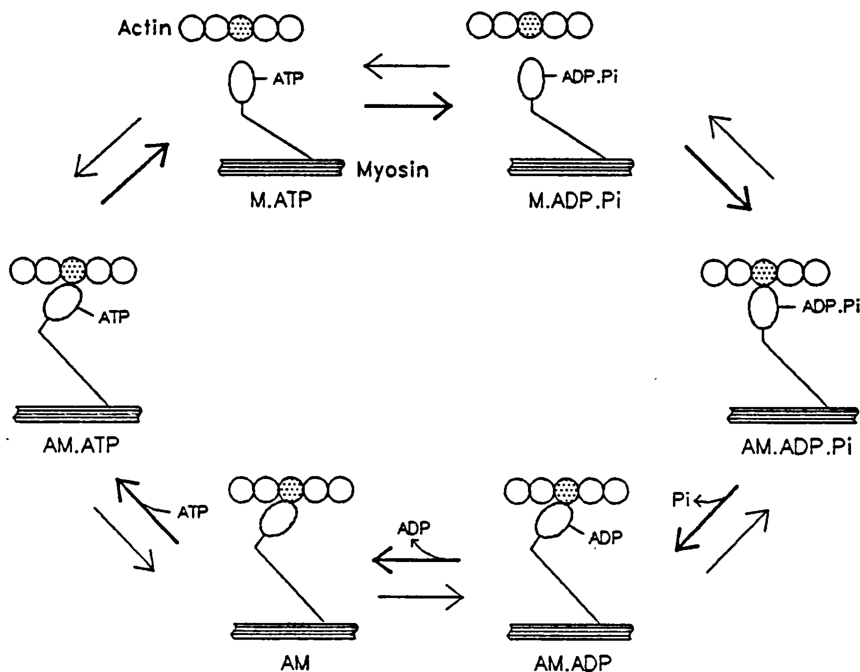

Fig.2 SIMPLIFIED CROSSBRIDGE CYCLE. In the absence of Ca^{2+} the crossbridges are assumed to be detached and generate no force. This corresponds to the M.ATP or M.ADP.Pi states. Although the hydrolysis of ATP by myosin ATPase is rapid, the release of the products is slow. Dissociation is accelerated by the binding of actin to myosin and the accompanying release of energy may be converted to mechanical work (crossbridge movement). Thus actin binds to the M.ADP.Pi state to form AM.ADP.Pi and stimulates the release of Pi (AM.ADP + Pi) then ADP (AM + ADP + Pi). Force is only produced after Pi release. Thus AM.ADP and AM correspond to strongly bound force generating crossbridge states whereas AM.ADP.Pi presumably corresponds to a weakly attached crossbridge state producing little or no force. Crossbridges only detach when actomyosin binds ATP and dissociates into actin and myosin (A + M.ATP) ready for a new cycle to begin. The sliding of actin and myosin filaments past each other during force generation is thought to be due to a rotation of the crossbridge such that the angle between the S1 head and the S2 region changes (fig.1), accompanying the release of Pi.

steepness as measured by H_n, the Hill coefficient. A H_n of one or less indicates that no cooperativity exists within the system. The Ca^{2+}-sensitivities of force and ATPase activity in skinned fibres were similar (Kuhn et al, 1990) thus it seems likely that Ca^{2+} regulates force and ATPase activity through a single mechanism.

1.1.6 REGULATION OF ACTIVATION

The Ca^{2+} activation of myofilaments is mediated through TnC, the Ca^{2+} binding subunit of Tn, the structure of which is discussed in section 1.1.7. It has been suggested (Huxley, 1973) that, at resting levels of Ca^{2+}, Tm sterically blocks the interaction of myosin with actin (fig.1) while TnI binds to actin thereby preventing movement of the Tm-Tn complex. During activation, levels of intracellular Ca^{2+} rise and Ca^{2+} binds to TnC (see sections 1.1.7 & 1.1.8) strengthening the bonds between TnC and TnI. This loosens the interaction between TnI and actin such that the Tm-Tn complex can move, with Tm slipping into the groove between the actin strands, freeing the myosin binding site on actin so crossbridge attachment can occur. Another possibility is that the regulatory proteins control crossbridge movement once myosin has bound to actin, as opposed to controlling crossbridge attachment (Chalovich et al, 1981). By inhibiting the dissociation of the enzyme-product complex, ATPase activity could be blocked.

It is also possible that the binding of myosin to actin might trigger changes in conformation within the Tm-Tn complex: it has been suggested (Bremel & Weber, 1972) that crossbridge attachment enhances the Ca^{2+} affinity of TnC. In addition there appears to be cooperativity within the system: either Ca^{2+} binding to one site on TnC facilitates Ca^{2+} binding to a second site, or there is cooperativity between Ca^{2+} binding to adjacent Tn complexes. Another possibility is that all Tm-Tn complexes on an actin strand form a cooperative system (Brandt et al, 1984). In any case, however, agents which alter the Ca^{2+} affinity of TnC would be expected to alter crossbridge binding and thus the Ca^{2+}-sensitivity of force.

1.1.7 STRUCTURE OF TROPONIN C

a) Primary Structure

Troponin C is a member of the EF hand family of proteins which are ideally suited to a role in the Ca^{2+} regulation of cellular processes since Ca^{2+} binds with dissociation constants in the range of $1\mu M$, compatible with the level of Ca^{2+} (0.1-10μM) in the cytoplasm. Four regions of internal homology are apparent in skeletal TnC (Collins et al 1973). Cardiac TnC also contains

homologous amino acid sequences although three regions have more in common than the fourth. Two aspartic acid residues in the fourth region of skeletal TnC are replaced by alanine and leucine in cardiac troponin C. This substitution greatly reduces the Ca^{2+} affinity of the corresponding binding site (site I) such that it is functionally redundant.

Rabbit skeletal TnC has 159 amino acids and a relative molecular mass of 17,846 (Collins et al, 1973). Bovine cardiac TnC has 160 amino acids and a relative molecular mass of 18,459 (van Eerd & Takahashi, 1976). Both proteins are highly acidic. The major polar donor groups from protein to Ca^{2+} are carbonyl and carboxylate centres such as aspartic and glutamic acids.

b) Gross Structure

The structure of TnC is illustrated in fig.3. The molecular conformation of avian skeletal TnC was resolved at 2.8 Å by Herzberg & James (1985) and 3 Å by Sundaralingam et al (1985) using x-ray crystallography. The protein is a 75 Å long dumbbell with two Ca^{2+} binding sites at each end. These sites and the two ends of the dumbbell are linked by regions of a-helix which have been labelled A-H from the N terminus by Potter and Johnson (1982). These authors also numbered the Ca^{2+} binding sites I-IV as shown in fig.3A.

c) Structural Changes on Ca^{2+} Binding

Ca^{2+} binding to any site on TnC causes a tightening of the corresponding loop with an increase in a-helix, thus reducing conformational flexibility. This is caused mainly by the formation of internal H bonds.

In addition, Ca^{2+} binding to skeletal TnC exposes (or forms) a hydrophobic cluster consisting of side chains from phenylalanines 19, 23, 26 & 75 which are close to binding sites I and II. This facilitates the reaction of Met-25 with the fluorescent probe DANZ (section 3.1.3). The binding of Ca^{2+} to skeletal TnC causes the burial of the -SH group of the sole cysteine residue Cys-98 thus protecting it from compounds which react with thiols. In contrast, the two cysteine residues of bovine cardiac TnC (Cys-35 & Cys-84) are partially buried in the absence of Ca^{2+} but increased levels of the cation cause exposure of both the thiol groups (Fuchs et al, 1989), facilitating their reaction with the probe IAANS (section 3.1.3).

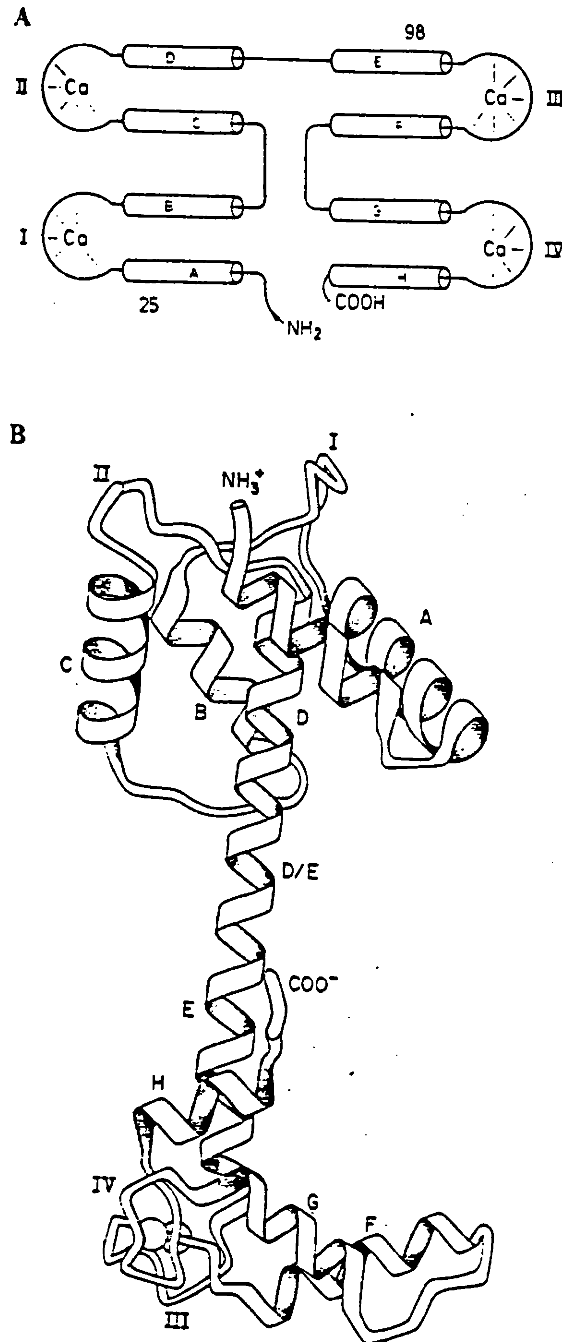

Fig.3 STRUCTURE OF TROPONIN C (from Ruegg, 1986). A) Simplified structure of skeletal TnC showing four Ca^{2+} binding domains (sites I-IV) and α helical regions (A-H). Notation taken from Potter & Johnson (1982). Skeletal TnC has two low affinity Ca^{2+} binding sites (I & II) and two high affinity sites (III & IV), whereas cardiac TnC has only one low affinity Ca^{2+} binding site (II). B) Structure of TnC as derived by Herzberg & James (1985) with domains labelled as above.

1.1.8 Ca^{2+} AND Mg^{2+} BINDING SITES ON TnC

a) Skeletal TnC

It was predicted from primary sequence determinations that skeletal TnC contained four possible Ca^{2+} binding sites (Collins et al, 1973). Potter & Gergely (1975), using equilibrium dialysis and Johnson et al (1978) using TnC-DANZ fluorescence, subsequently found that skeletal TnC has two high affinity Ca^{2+} binding sites, with a K_{Ca} of $2x10^7$ M^{-1}, and two low affinity sites with K_{Ca} $3x10^5$ M^{-1}. Studies of the kinetics of Ca^{2+} exchange with TnC-DANZ using stopped flow fluorimetry indicated that the Ca^{2+}-specific sites are most likely to regulate contraction (Johnson et al, 1979). Ca^{2+} binding to all four sites on skeletal TnC was complete within 2.4 ms (the mixing time of the instrument) and was thus diffusion limited. The half time of Ca^{2+} dissociation from the low affinity sites was 2-3 ms (a rate of 230-346 s^{-1}) whereas that from the high affinity sites was 700 ms (0.99 s^{-1}). The latter are therefore unsuitable for the regulation of contraction since the twitch is complete within 100 ms after excitation.

In the presence of 2 mM Mg^{2+}, competition with Ca^{2+} for the high affinity sites was observed, the K_{Ca} decreasing ten fold (Potter & Gergely, 1975). Thus skeletal TnC contained two Ca^{2+}-Mg^{2+} sites in addition to the two Ca^{2+}-specific sites. Mg^{2+} bound to the high affinity sites of TnC in the absence of Ca^{2+} with a half time of 6-7 ms (99-115 s^{-1}) and dissociated with a half time of 86 ms (8 s^{-1}; Johnson et al, 1979). Mg^{2+} bound to TnC at four sites all with a K_{Mg} of $2x10^3$ M^{-1} (Potter & Gergely, 1975).

On binding to TnC, TnI increased the K_{Ca} of both high and low affinity Ca^{2+} sites 10-fold, a result similar to that obtained on incorporation of TnC into troponin (Potter & Gergely, 1975). Johnson et al (1980) also labelled TnI with the fluorescent probe IAANS and incorporated it into skeletal troponin. Ca^{2+} binding to TnC produced conformational and hence fluorescence changes in TnI-IAANS which could be monitored by stopped flow fluorimetry. The rates of Ca^{2+} binding to the Ca^{2+}-specific and Ca^{2+}-Mg^{2+} sites of TnC, producing structural changes in TnI, were 100 s^{-1} and 9 s^{-1} respectively. Thus only the kinetics of Ca^{2+} exchange with the low affinity sites were fast enough to regulate contraction. The addition of 2 mM Mg^{2+} to TnC-TnI reduced the Ca^{2+} affinity of the high affinity sites 100 fold so that all four Ca^{2+} sites had a K_{Ca} of $4x10^6$ M^{-1} (Potter & Gergely, 1975). Since Mg^{2+} was found to have no effect on ATPase activity, Potter & Gergely (1975) suggested ATPase activity was regulated by Ca^{2+} binding to the Ca^{2+}-specific low affinity sites. Binding of Ca^{2+} or Mg^{2+} to the high affinity sites was proposed to cause a large change in TnC conformation which may then be fixed in a position that is a prerequisite for

activation (Potter & Gergely, 1975).

b) Cardiac TnC

Van Eerd & Takahashi (1976) predicted that replacement of two skeletal TnC aspartic acid residues with leucine and alanine in site I of cardiac TnC would result in Ca^{2+} binding only to sites II-IV. It was subsequently confirmed that cardiac TnC has two Ca^{2+}-Mg^{2+} sites with K_{Ca} 1-3x10^{7} M^{-1} and K_{Mg} 2x10^{3} M^{-1} (Leavis & Kraft, 1978, using tyrosine fluorescence; Holroyde et al, 1980, using equilibrium dialysis; and Johnson et al, 1980, using TnC-IAANS fluorescence), and one Ca^{2+}-specific site with K_{Ca} 2-5x10^{5} M^{-1} (Holroyde et al, 1980; Johnson et al, 1980). In addition, Holroyde et al (1980) and Johnson et al (1980) discovered several very low affinity Ca^{2+}-specific sites with K_{Ca} 9x10^{2} M^{-1} (too low to be of physiological relevance). Ca^{2+} binding to the single low affinity Ca^{2+}-specific site correlated with the activation of myofibrillar ATPase activity by Ca^{2+} and thus was most likely to be the regulatory site for contraction (Holroyde et al, 1980). This was supported by measurements of the kinetics of Ca^{2+} exchange with the low affinity site of TnC: the Ca^{2+} "on rate" was diffusion limited (occurring within 2.4 ms) whereas the "off rate" was 300-500 s^{-1} (Johnson et al, 1978). These rates were comparable to those of the Ca^{2+}-specific sites of skeletal TnC with the off rate being fast enough to regulate contraction.

When cardiac TnC was complexed with TnI or incorporated into troponin, the Ca^{2+} affinity of both high and low affinity sites (in the absence of Mg^{2+}) was increased 10-fold (Holroyde et al, 1980) similar to skeletal TnC. The addition of Mg^{2+} then decreased the Ca^{2+} affinity of the Ca^{2+}-Mg^{2+} sites 10-fold.

1.1.9 MODIFICATION OF THE FORCE-pCa RELATIONSHIP

Numerous factors can alter the relationship between intracellular Ca^{2+} concentration and force. Such alterations may include a shift along the x-axis of the sigmoidal pCa-force curve; potentiation or suppression of maximum force; or, more rarely, a change in slope of the sigmoidal curve. The first of these (a change in pCa_{50}) causes greatest changes in force at sub-maximal Ca^{2+} concentrations whereas alteration of maximum force causes force to change by the same proportion from zero to maximum $[Ca^{2+}]$.

Factors which desensitise the myofilaments to Ca^{2+} include: reductions in pH, increases in [Pi], increases in temperature (Stephenson & Williams, 1985), decreases in SL on the ascending limb of the length-tension relationship, and increases in $[Mg^{2+}]$ (Fabiato & Fabiato, 1975). In most cases the fall in force at a given sub-maximal $[Ca^{2+}]$ is caused by both depression of maximum force and decrease in pCa_{50}, though increasing the temperature from 5° C to 22° C causes a

rise in maximum force.

In contrast, increases in pH, decreases in [Pi] or [Mg^{2+}], increases in SL (at least in the range 2.2-3.6 μm; Stephenson & Williams, 1982), and falls in temperature all sensitise myofilaments to Ca^{2+}. Similar effects are achieved by compounds such as caffeine and theophylline, and by certain components of cardiac cytosol (taurine, Steele et al, 1990; N-acetyl histidine & carnosine, Harrison et al, 1986). Ca^{2+}-sensitisation is most commonly achieved by an increase in pCa_{50} though the pH, Pi, N-acetyl histidine and carnosine effects also involve potentiation of maximum force.

One purpose of the current study was to investigate the mechanism(s) by which acidosis, increases in [Pi], and caffeine alter myofibrillar Ca^{2+}-sensitivity. Such agents were selected in view of their relevance to hypoxia (reduced partial pressure of oxygen), ischaemia (lack of an adequate blood supply) and positive inotropy in heart failure, as discussed in section 1.1.13.

1.1.10 EFFECTS OF pH ON FORCE IN INTACT MUSCLE

a) Influence on Intracellular Ca^{2+} Levels

Lowering the pH reduces the slow inward (SI) current (mainly carried by Ca^{2+}) of the action potential in cardiac muscle (Kohlhardt et al, 1976; Sato et al, 1985). The inhibition of the SI current is mainly caused by a decrease in the probability of the Ca^{2+} channel being open but also by a reduction in the flow of Ca^{2+} through the open channels (Kaibara & Kameyama, 1988). Thus the amount of Ca^{2+} available in the sarcoplasm to load the SR or stimulate Ca^{2+}-induced Ca^{2+} release from cardiac SR (Fabiato, 1983) is decreased.

A decrease in pH also inhibits Na^{+}-H^{+} exchange: a rise in intracellular proton levels would stimulate H^{+} extrusion and hence Na^{+} influx. This would, in turn, promote Na^{+} efflux and thus Ca^{2+} influx via the Na^{+}-Ca^{2+} exchanger so sarcoplasmic Ca^{2+} concentration should rise. In fact cytosolic [Ca^{2+}] does rise during acidosis mainly because the affinity of TnC for Ca^{2+} is reduced so less Ca^{2+} binds to it.

In addition to its effects on the action potential and sarcolemmal ion-exchangers, acidosis alters the Ca^{2+} handling of the sarcoplasmic reticulum in both skeletal and cardiac muscle. A decrease in pH has been shown to inhibit the rate of Ca^{2+} uptake into the SR by the Ca^{2+}-ATPase of cardiac muscle (Fabiato & Fabiato,1978; Fabiato,1985) which would reduce the amount of Ca^{2+} available for the next release. It is also possible that the rate at which the SR loads and recycles Ca^{2+} between contractures is decreased by a fall in pH, as was suggested in a preliminary report by Boyett et al, 1989). The release of Ca^{2+}

from the SR appears to be inhibited by acidosis, with both the open probability and the unitary conductance of the SR Ca^{2+} release channel being decreased by low pH (Rousseau & Pinkos, 1990).

b) Interaction with the Contractile Apparatus

Acidosis reduces the force produced at a given Ca^{2+} concentration both by depressing maximum attainable force and by decreasing the Ca^{2+}-sensitivity of the contractile apparatus. A reduction in pH suppresses maximum force in both slow- and fast-twitch skeletal fibres and cardiac preparations (Donaldson & Hermansen, 1978; Fabiato & Fabiato, 1978), while simultaneously decreasing the Ca^{2+}-sensitivity of the myofilaments. Donaldson & Hermansen (1978) found a drop in pH from 7.0 to 6.5 caused 30% & 18% falls in maximum force of rabbit adductor magnus and cardiac trabeculae respectively, accompanied by 0.23 and 0.34 unit decreases in pCa_{50}. Similarly a pH change from 7.0 to 6.2 lowered maximum force of rat cardiac myocytes by 25% (Fabiato & Fabiato, 1978) with a fall in pCa_{50} of 0.4. Under the same conditions, maximum force of frog skeletal fibres fell by 28% and pCa_{50} by 0.3 units. Thus acidosis had a greater Ca^{2+}-desensitising action in cardiac than skeletal fibres though the pH-induced suppression of maximum force may be independent of fibre type.

A reduction in pH also decreases maximum myosin ATPase activity in both skeletal and cardiac skinned fibres, to a similar extent as its suppression of maximum force (Godt & Kentish, 1989). The Ca^{2+}-sensitivity of ATPase activity of skinned skeletal fibres is also reduced in acidosis (Blanchard et al, 1987), though to a lesser extent than force.

1.1.11 EFFECTS OF Pi ON FORCE IN INTACT MUSCLE

a) Influence on Intracellular Ca^{2+} Levels

Little information is available concerning direct effects of Pi on the Ca^{2+} handling of muscle cells. It is generally assumed to have no effect on intracellular Ca^{2+} concentration, though Smith et al (in press) indicated that 10 mM Pi decreased the Ca^{2+} content of the SR - probably by inhibiting Ca^{2+} uptake.

b) Interaction with the Contractile Apparatus

Similar to acidosis, Pi decreases both maximum force and the Ca^{2+}-sensitivity of the myofilaments. 15 mM Pi caused maximum force to fall by 31% and pCa_{50} by 0.25 units in skeletal fibres (Brandt et al, 1982), whereas 20 mM Pi suppressed peak tension of ventricle by 69% with a concomitant fall in pCa_{50} of 0.38 units (Kentish, 1986). More recently, Godt & Nosek (1989) showed that Pi had greater

effects on cardiac than on skeletal skinned fibres: 20 mM Pi suppressed maximum force of rabbit trabeculae and psoas by 55% and 30% respectively whilst decreasing the respective pCa_{50}s by 0.32 and 0.25 units.

The relationship between maximum force and log_{10}[Pi] is linear (Kentish, pers.comm.) thus a change in Pi level from say 1 mM to 5 mM will have a larger depressive effect on maximum force than a rise from 15 mM to 20 mM Pi. Maximum ATPase activity also decreased as Pi concentration increased (Kawai et al, 1987) though to a lesser extent than force. Schmidt-Ott et al (1990) demonstrated that 10 mM Pi reduced maximum ATPase activity and its pCa_{50} in human skinned atrial fibres, these effects being less than those of Pi on force.

1.1.12 EFFECTS OF CAFFEINE ON FORCE IN INTACT MUSCLE

a) Influence on Intracellular Ca^{2+} Levels

Caffeine prolongs the cardiac action potential (Blinks et al, 1972). Allen & Kurihara (1980) showed that caffeine increased twitch amplitude whilst slowing the Ca^{2+} transient. The ability of caffeine to produce transient contractures in striated muscle (Chapman & Miller, 1974) is generally attributed to its actions on the SR where it causes Ca^{2+} release (Weber & Herz, 1968), thereby causing a net outward movement of Ca^{2+} into the sarcoplasm. At least in cardiac muscle 5 mM caffeine promotes SR Ca^{2+} release by increasing the duration of the open state of the SR Ca^{2+} channels (Sitsapesan & Williams, 1990). At low concentrations caffeine increases force by increasing the Ca^{2+}-sensitivity of Ca^{2+}-induced Ca^{2+} release in cardiac cells (REF Eisners?). Thus less Ca^{2+} is required to trigger Ca^{2+} release from the SR. In addition, as discussed further in section 1.1.13, caffeine is a PDE inhibitor (Butcher & Sutherland, 1962) and therefore indirectly increases intracellular Ca^{2+} via a rise in cAMP levels.

b) Interaction with the Contractile Apparatus

Caffeine increases the force produced by skinned striated muscle at sub-maximal Ca^{2+} concentrations despite having a suppressive effect on maximum force at concentrations of 20 mM and above. Wendt & Stephenson (1983) showed that 20 mM caffeine sensitised the myofilaments to Ca^{2+} with increases in pCa_{50} of 0.32 and 0.15 units for cardiac and skeletal muscle respectively. 20 mM also depressed peak force of cardiac trabeculae by 16% and skeletal fibres by 19%.

The effects of caffeine were concentration-dependent: suppression of maximum force increased to 29% for both cardiac and skeletal muscle in the presence of 40 mM caffeine (Wendt & Stephenson, 1983). Likewise the increase

in pCa_{50} became larger as caffeine concentration rose.

Such effects of caffeine on the myofilaments were unrelated to its PDE inhibition as Wendt & Stephenson (1983) demonstrated that theophylline had similar effects to caffeine on skinned cardiac fibres although it is a more potent PDE inhibitor. The effects of caffeine on ATPase activity are unknown.

1.1.13 RELEVANCE OF pH, Pi AND CAFFEINE TO HEART FAILURE

a) Cardiac Failure, Ischaemia and Hypoxia

Cardiac failure is the inability of the heart to maintain an adequate cardiac output despite a satisfactory venous return. It usually results from either disease of the myocardium (e.g. myocardial infarction, cardiomyopathy) or disorders of the cardiac valves. Disturbance of blood supply e.g. by myocardial infarction or atherosclerosis may result in cardiac ischaemia with its restricted delivery of substrates and removal of waste products. Although hypoxia may result from cardiac defects where arterial and venous blood become mixed, it is usually caused by pulmonary disorders or an insufficient blood supply (e.g. during ischaemia). During cardiac hypoxia or ischaemia the continued utilisation of ATP in cellular processes results in a build up of ADP and Pi. Creatine phosphate (CP) is also broken down into creatine and Pi by the enzyme creatine kinase to regenerate ATP from ADP. In conjunction these reactions may increase sarcoplasmic levels of Pi from 4 mM to 20 mM in total hypoxia (Allen et al, 1985). In addition, anaerobic glycolysis increases with concomitant production of lactic acid, thereby causing an intracellular acidosis. Similar changes in pH and [Pi] may occur in fatigue of skeletal fibres (Dawson et al, 1978).

Cardiac output can be increased following cardiac failure by increasing the force of contraction of the heart:

b) Interactions which Increase Intracellular Ca^{2+} Concentration

Current treatment of the failing heart favours interventions which increase the strength of the heart by increasing the amount of Ca^{2+} available to the myofilaments. This can be achieved in a number of ways including inhibition of the sarcolemmal Na^{+}-K^{+}-ATPase; prolongation of cardiac action potential; inhibition of SR Ca^{2+} uptake and or stimulation of SR Ca^{2+} release; phosphodiesterase inhibition and adenylate cyclase stimulation. Cardiac glycosides are thought to inhibit Na^{+}-K^{+}-ATPase activity, thereby increasing intracellular [Na^{+}]. This decreases Na^{+}-Ca^{2+} exchange and thus increases intracellular [Ca^{2+}]. Ca^{2+} agonists such as Bay K 8644 and CGP28392 open Ca^{2+} channels in the sarcolemma, thereby increasing the Ca^{2+} transient.

Under normal circumstances, stimulation of β-receptors in the sarcolemma by catecholamines activates adenylate cyclase which in turn catalyses the formation of cyclic AMP from ATP. Cyclic AMP activates protein kinase which phosphorylates the Ca^{2+} channel, the SR and TnI thereby increasing the inward Ca^{2+} current (and hence force), stimulating Ca^{2+} uptake by the SR and decreasing the Ca^{2+}-sensitivity of the myofilaments (hence increasing relaxation rate). Cyclic AMP is in turn broken down by phosphodiesterase. Agents such as forskolin, noradrenalin, adrenalin, serotonin and dobutamine which stimulate adenylate cyclase raise intracellular levels of cAMP by triggering its production. Alternatively other compounds block the degradation of cAMP: the many phosphodiesterase inhibitors known include caffeine, piroximone, amrinone and milrinone. A rise in intracellular cAMP concentration causes an increase in sarcoplasmic Ca^{2+} levels which in turn leads to greater force production, despite the balancing effect of TnI phosphorylation.

For reasons given above, PDE inhibitors such as amrinone and milrinone have been used to treat heart failure. Improving the strength of the failing heart by raising the amount of Ca^{2+} available to the myofilaments does, however, have drawbacks. Firstly when resting sarcoplasmic Ca^{2+} increases, the SR becomes unstable and spontaneously releases Ca^{2+} causing arrhythmias (already a problem in acute heart failure). In addition, raised levels of cAMP may also cause arrhythmias. Secondly the oxygen consumption of the heart will be increased as more energy will be required to pump systolic Ca^{2+} back down to diastolic levels (in addition to the increase in ATP needed for production of greater force). Thirdly, if intracellular Ca^{2+} rises to approximately 1 μM, Ca^{2+}-dependent proteases are activated and digest the contractile proteins causing necroses. In severe chronic heart failure, levels of cAMP are often too low for phosphodiesterase inhibitors to be effective (Erdmann, 1989). Finally, it has also become apparent (Herzig, 1984) that cardiac insufficiency is not always related to a lack of Ca^{2+} available for activation, but is often at least partially caused by a reduction in Ca^{2+}-sensitivity of the myofilaments (as occurs in hypoxia). Such Ca^{2+}-desensitisation may reflect the fall in pH and or rise in Pi levels that often accompany heart failure.

c) Interventions which Increase Myofilament Ca^{2+}-Sensitivity

It seems appropriate to treat heart failure with drugs which directly sensitise the contractile apparatus to Ca^{2+}. In this case, maximum force would be achieved at lower Ca^{2+} concentrations which would reduce both the likelihood of arrhythmias and the energy required for Ca^{2+} cycling. One means by which Ca^{2+}-sensitivity of the myofilaments could be increased would be by increasing

could be sensitised to Ca^{2+} would be to alter the rate constants of crossbridge attachment and or detachment directly. Increasing the rate of attachment or decreasing detachment rate will raise the number of strongly bound crossbridges at any given moment, therefore increasing the force produced at a particular Ca^{2+} concentration.

COMPOUND	Ca^{2+} SENSITISING CONCENTRATION	PDE INHIBITION	REFERENCES
SULMAZOLE (AR-L 115 BS)	3-≥ 350 μM	YES	Herzig et al (1981)
PIMOBENDAN (UD-CG 115 BS)	10-≥ 400 μM	YES	Ruegg et al (1984)
PERHEXILINE	10-≥ 100 μM	?	Isac et al (1988)
BEPRIDIL	10-≥ 100 μM	YES?	Solaro et al (1986)
APP 201-533	≥ 100 μM	YES	Salzmann et al (1985)
ISOMAZOLE (LY-175326)	0.01-1.0 mM	YES	Lues et al (1988)
DPI 201-106	?≤ 1 μM≤ ?	NO	Scholtysik et al (1985)
MCI-154	0.1-100 μM	YES	Kitada et al (1987)
EMD 53998	3-30 μM	YES	Beier et al (1991)
ORG 30029	0.01-1.0 mM	YES	Miller & Steele (1990)

TABLE 1. BRIEF REVIEW OF COMPOUNDS WHICH SENSITISE THE MYOFILAMENTS TO Ca^{2+}.

One problem with myofilament Ca^{2+}-sensitisation as a means of treating cardiac insufficiency may be side effects produced in skeletal muscle which has a similar Ca^{2+}-regulation of actin-myosin interaction. Inotropic agents are therefore required which have an insignificant or no effect on Ca^{2+}-sensitivity of skeletal muscle whilst increasing force in the heart. In this case obvious targets for pharmaceutical agents would be TnC or TnI which have different cardiac and skeletal forms (Collins et al, 1973; van Eerd & Takahashi, 1976; Wilkinson & Grand, 1978).

To date the search for a suitable Ca^{2+}-sensitising agent has been hampered by the fact that most of the sensitisers discovered have also been PDE inhibitors (table 1). In addition, DPI 201-106 prolongs the action potential via an effect on Na^{+} channels (Buggisch et al, 1985). The first Ca^{2+}-sensitisers to be discovered

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1.2 AIMS OF THE CURRENT STUDY

As discussed above, pH, Pi and caffeine are all of importance in cardiac failure and its treatment, yet little is known about their method of interaction with the contractile apparatus. The aim of the current study was, therefore, to investigate the mechanism(s) by which pH, Pi, caffeine and novel cardiotonic agents developed at Ciba-Geigy alter the Ca^{2+}-sensitivity of myofilaments, with particular emphasis on the possible role of TnC. It was intended:

i) To compare directly the effects of the various agents on Ca^{2+} binding to isolated TnC and their effects on the myofilaments *in comparable physiological solutions*.

ii) To determine the effects of inotropic agents on the Ca^{2+} affinity of TnC in situ on the myofilaments and to relate this to force production.

CHAPTER 2: EFFECTS OF pH, Pi AND CAFFEINE ON FORCE PRODUCTION IN SKINNED FIBRES

2.1 INTRODUCTION

2.1.1 THE SKINNED FIBRE TECHNIQUE

The relationship between intracellular Ca^{2+} and force generation can be studied by measuring intracellular Ca^{2+} with indicators and comparing this to tension developed at the same time. Alternatively the selectively permeable membranes in muscle fibres can be removed or disrupted, by physical or chemical means, such that the Ca^{2+} in contact with the myofilaments can be directly controlled by suitable choice of bathing solutions. The latter method facilitates the study of factors which influence the Ca^{2+}-force relationship and is widely used.

Muscle preparations are most commonly permeabilised by detergents - notably glycerol and Triton X-100, but saponin, Brij, α-toxin and β-escin have also been employed. Glycerol skinning of skeletal fibres was first proposed by Szent-Gyorgyi (1949). Incubation of bundles of psoas fibres in 50% glycerol for at least 2 days produced permeabilised fibres which contracted in the presence of ATP. Although the mechanism of action of glycerol is unclear, it does appear to permeabilise both the sarcolemma and the SR since additional treatment of such skinned fibres with Triton X-100 does not alter the rate or magnitude of force development in ATP solution (section 2.2.2.c).

The most commonly used detergent for studies of cardiac myofilament activation is Triton X-100 since this disrupts both the sarcolemma and the SR. The efficacy of Triton X-100 as a skinning agent has been checked previously (Kentish, 1982; Miller & Smith 1985; Pan & Solaro, 1987). Incubation of cardiac trabeculae with 1% Triton X-100 (by volume) for 30 minutes at room temperature ensured that all the cells were permeable to Ca^{2+}, $MgATP^{2-}$, HCP^{2-}, and ferritin (RMM 750,000), and the selective permeability of the sarcolemma had been destroyed (Kentish, 1982). Miller & Smith (1985) demonstrated that the rate of contraction of Triton-skinned rat ventricular trabeculae followed a similar time course to that expected if the process were simply diffusion limited, with no ion selectivity present. Rate of contraction was accordingly related to the size of the preparations. Pan & Solaro (1987) treated canine cardiac fibres with 1% Triton X-100 at 4°C for 12 hours to ensure complete solubilisation or physical removal of the sarcolemma, SR and mitochondria. Following these (somewhat drastic) measures transmission electron microscopy revealed no evidence of any membranous structures left in

the preparations. Proteins, such as citrate synthase which is normally associated with mitochondria, were also shown to have been washed out of the fibres by the detergent extraction procedure; and by extrapolation it was assumed that proteins associated with the sarcolemma and SR had also been removed. It is however generally considered reasonable to assume that a 30 minute incubation of muscle fibres with Triton X-100 is sufficient to permeabilise all the cell membranes and render them non-functional even if fragments of them remain.

As glycerol and Triton X-100 are the most widely used skinning agents for skeletal and cardiac fibres respectively, these were the detergents chosen for the current study.

There are, however, drawbacks to the skinned fibre technique regardless of the skinning agent used. For a reasonable approximation of in vivo conditions, the permeabilised muscle should be bathed in a solution closely resembling the cytosol. The skinning procedure results in a loss of sarcoplasmic constituents (such as soluble enzymes and metabolites) which cannot practicably be included in the bathing solution. In addition the exact composition of the cytosol is unknown. Thus only a very simplified model of the latter can be used (see below). It is essential that only fibres of post-skinning diameter less than 200 μm are used since significant diffusion gradients may be set up within larger ones. Finally for studies of the Ca^{2+}-sensitivity of skinned fibres it is imperative to control accurately the level of free Ca^{2+}. This can be achieved using EGTA as a Ca^{2+} buffer though the Ca^{2+} affinity of EGTA is affected by pH, ionic strength and temperature so appropriate correction of the EGTA binding constants must be made to suit the experimental conditions (section 2.2.1.a). In addition the exact concentration of EGTA present must be known in order to calculate free Ca^{2+} concentration - EGTA from suppliers is rarely 100% pure (Miller & Smith, 1984) but it is possible to measure EGTA purity as described in section 2.2.1.b.

2.1.2 CHOICE OF BATHING SOLUTION FOR SKINNED FIBRES

The solutions chosen in the present study to mimic the cytosol were similar to those used previously by Kentish (1984, 1986) to enable direct comparison between the studies. Use of the same solutions for cardiac and skeletal muscle also enabled a direct comparison between muscle types. The pH of the solutions was set to 7.00 (Ruegg, 1986) and ionic strength to approximately 200 (Godt & Maughan, 1988). Solutions at pH 7.00 were buffered using 100 mM BES (pKa 7.1 at 25° C) to ensure tight control of pH, essential where EGTA was used to buffer Ca^{2+}. Ionic strength was adjusted with propionate (Pr) ions since it was not feasible to model the mixture of proteins and metabolites which perform the same role in the cytosol. The concentrations of K^+ (approximately 130 mM) and

Na^+ (30 mM) were based on intracellular estimates by Godt & Maughan (1988). Ca^{2+} concentration ranged between 1 nM and 0.3 mM to encompass the levels present in relaxed and contracting muscle (Ruegg, 1986). Free Mg^{2+} was set at approximately 1 mM (Fry, pers.comm.; Godt & Maughan, 1988) and chloride was limited to about 10 mM. 5 mM ATP and 10 mM CP were included in accordance with values estimated by Allen et al (1985). No creatine kinase was added as this is retained by detergent-skinned fibres (Miller & Smith, 1985). For experiments where the effects of pH were investigated, the solution was adjusted to pH 6.20 to match that observed in ischaemic intact muscle (Garlick et al, 1975). The pH buffer was also switched to MES which has a better buffering capacity (pKa 6.10 at 25 °C) than BES at reduced pH. For investigation of the action of Pi on skinned fibres, 20 mM Pi was included in the bathing solution in line with the level measured by Allen et al (1985) in ischaemic muscle. The inclusion of Pi was accompanied by the exclusion of sufficient Pr^- to maintain constant ionic strength. 20 mM caffeine was chosen as this has only a small effect on maximum force but a large Ca^{2+}-sensitising action (Wendt & Stephenson, 1983).

2.2 METHODS

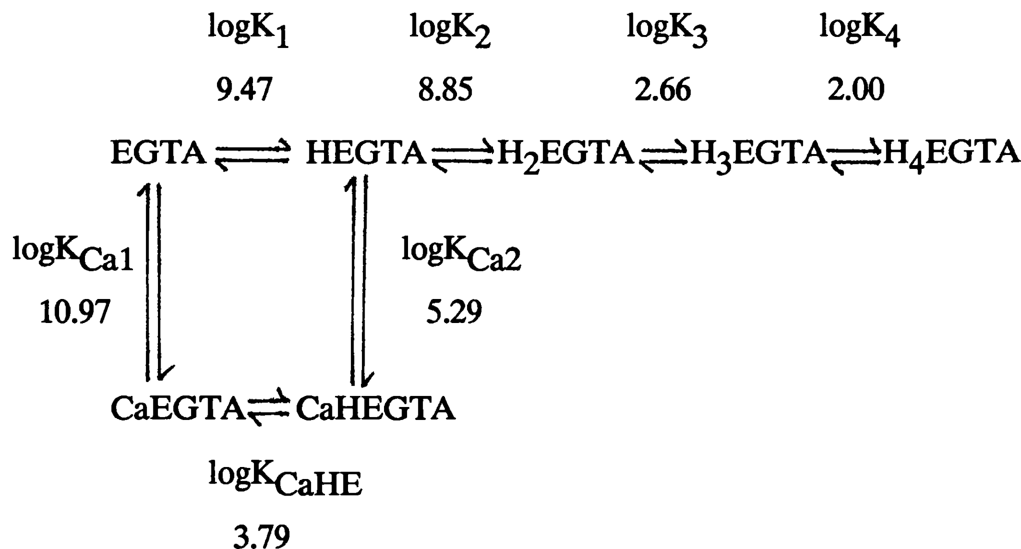
2.2.1 EGTA AS A Ca^{2+} BUFFER

In order to regulate Ca^{2+} levels at micromolar concentrations a high buffering capacity is required. The most commonly used buffer is EGTA as this has a high Ca^{2+} : Mg^{2+} selectivity (10^6) and functions well for Ca^{2+} concentrations in the micromolar range ($K'_D \approx$ 0.4 μM at pH 7.00). Two factors determine the Ca^{2+} concentration in a Ca^{2+}-EGTA buffer system: the affinity of EGTA for Ca^{2+} under the chosen conditions and the ratio of total Ca^{2+} : total EGTA. Both factors need to be accurately known if a reliable estimate of free Ca^{2+} is to be obtained. The determination of these factors is described below.

a) Calculation of the Affinity of EGTA for Ca^{2+}

i) *Effects of pH*

EGTA exists in a variety of states depending on the pH. At pH 7.0 the dominant form is H_2EGTA. However, Ca^{2+} binds to $EGTA^{4-}$ and so competes with H^+ for EGTA. This makes accurate control of the pH of experimental solutions containing the EGTA-Ca^{2+} buffering system absolutely essential. The equilibria involving EGTA, Ca^{2+} and H^+ are illustrated below. Affinity constants for each step are those of Martell and Smith (0.1M and 20° C):



The programme used to calculate free ion concentrations from the total concentrations took all these equilibria into account and so automatically allowed for the effect of pH. The affinity constants of Martell & Smith are concentration constants, i.e. were calculated from the concentrations of reactants and products. However, since the pH of my solutions was measured in terms of H^+ activity, it was more convenient to express K_1-K_4 as mixed constants (section 2.2.1.a.iv). K_{Ca1} and K_{Ca2} were left as concentration constants.

ii) *Correction of Association Constants for Ionic Strength*

Ionic strength affects affinity constants and needs to be taken into account if the chosen ionic strength differs from that used in the determination of the constants (which is usually the case).

IS = Ionic Strength = $0.5\Sigma |z_i^2| c_i$

where z = charge on ion i

c = molar concentration of ion i

The effect of ionic strength on the individual proton and Ca^{2+} EGTA association constants was corrected for using the following equations described by Miller & Smith (1984). It has been reported (Smith & Miller, 1985) that affinity constants are dependent on ionic equivalence (see below) rather than ionic strength thus ionic equivalence was used in the calculations:

$$\log K'_a = \log K_a + 2xy(\log f_j - \log f'_j)$$

where K'_a = association constant after correction for ionic strength

K_a = measured association constant (in concentration terms e.g. at 0.1 M;20°C) taken from Martell & Smith

x = absolute value of cation valency (e.g. 2 for Ca^{2+})

y = absolute value of anion valency (e.g. 4 for $EGTA^{4-}$)

f_j = activity coefficient of ion j at ionic equivalence at which the association constant was measured (see below)

f'_j = as f_j, but for the desired ionic equivalence and temperature

The activity constants were calculated from:

$$\log f_j = A((\sqrt{I_e}/(1+\sqrt{I_e}))-0.25 I_e)$$

where I_e = Ionic equivalence = $0.5\Sigma|z_i|c_i$
z = charge on ion i
c = molar concentration of ion i

and A = $1.8246\times10^6/(\epsilon T)^{1.5}$

where T = absolute temperature
ϵ = dielectric constant of water at temperature T

iii) *Correction of Association Constants for Temperature*

Since the term A (above) contains temperature-dependent terms, it was always calculated for the experimental temperature even if no adjustments for ionic strength were necessary. The individual association constants resulting from the above correction were then further modified using the Van't Hoff isochore, as described by Harrison & Bers (1989):

$$\log K''_a = \log K'_a + \Delta H(1/T - 1/T') / 2.303R$$

where $\log K''_a$ = association constant after correction for experimental temperature
$\log K'_a$ = association constant as above
ΔH = change in enthalpy (kcal/mol)
T = pre-correction absolute temperature
T' = experimental absolute temperature
R = Gas constant = 1.9872×10^{-3} kcal.K^{-1}mol^{-1}

Note ΔH for K_{Ca1} = -8.1 kcal.mol^{-1} (Boyd et al. (1965) J.Chem.Soc.Lond.V,7353-7358)

and ΔH for K_1 & K_2 = -5.8 kcal.mol^{-1} (Anderegg (1964) Helv.Chim.Acta 47,1801-1814)

The ΔH values for K_3, K_4 and K_{Ca2} are unavailable but correction of these terms for temperature is not usually necessary in physiological conditions anyway (Harrison & Bers, 1989).

As it was necessary to modify the association constants for both ionic strength and temperature, ionic strength was corrected first to give a more accurate result (Harrison & Bers, 1989).

iv) *Conversion from Concentration to Activity*

The association constants corrected for ionic strength and temperature were concentration constants. To convert K_1-K_4 from concentration to mixed constants (since pH is an activity measure) the following procedure was used:

Activity Constant = Concentration Constant + log f_H

where f_H = activity coefficient for protons under chosen conditions
f_H = 0.11 at ionic strength 0.1 M
f_H = 0.13 at ionic strength 0.2 M

Individual affinity constants corrected using the above procedures are shown in table 2. A similar procedure was used for the constants for Mg^{2+} binding to EGTA, and for Ca^{2+} and Mg^{2+} binding to ATP. These are also listed in table 2.

	MEASURED AFFINITY CONSTANTS IS = 0.1 M; I_e = 0.1 M	CALCULATED AFFINITY CONSTANTS IS = 0.2 M; I_e = 0.16 M		
	20° C	15° C	22° C	25° C
log K_1	9.58	9.547	9.413	9.364
log K_2	8.96	8.956	8.830	8.783
log K_3	2.77	2.720	2.707	2.705
log K_4	2.11	2.089	2.085	2.084
log K_{Ca1}	10.97	10.842	10.630	10.557
log K_{Ca2}	5.29	5.220	5.024	4.955
log K_{Mg1}	5.21	4.912	4.936	4.961
log K_{Mg2}	3.36	3.121	3.161	3.189
log $ATPK_1$	6.51	6.496	6.488	6.492
log $ATPK_2$	4.06	4.130	4.055	4.028
log $ATPK_{Ca1}$	3.77	3.550	3.466	3.446
log $ATPK_{Ca2}$	1.95	1.788	1.720	1.704
log $ATPK_{Mg1}$	4.06	3.769	3.783	3.804
log $ATPK_{Mg2}$	2.10	1.868	1.898	1.922

TABLE 2. MEASURED ASSOCIATION CONSTANTS AND THOSE CORRECTED FOR EXPERIMENTAL CONDITIONS. All proton constants are expressed in terms of activity constants and Ca^{2+} constants in concentration terms. Measured constants taken from Martell & Smith (1974). Affinity constants expressed as log M^{-1}. Temperature dependence of constants for MgEGTA and ATP were calculated using the following ΔH values (kcal.mol. $^{-1}$; Martell & Smith, 1974):

ΔH for KMg1	=	+5.0
ΔH for ATPK1	=	+1.2
ΔH for ATPK2	=	-3.7
ΔH for ATPKCa1	=	-1.0
ΔH for ATPKMg1	=	+4.5

v) *Steps Involved in Calculating Solutions*

Free ion concentrations were calculated from the total concentrations and the pH using a computer programme devised by Kentish. For solutions at pH 7.00, ionic strength approximately 200 and at 25°C the constants in table 3 were applied.

HBES	7.065	$HHDTA^{3-}$	10.920
$HEGTA^{3-}$	9.364	H_2HDTA^{2-}	9.900
H_2EGTA^{2-}	8.783	H_3HDTA^-	2.810
H_3EGTA^-	2.705	H_4HDTA	2.310
H_4EGTA	2.084	$CaHDTA^{2-}$	4.600
$KEGTA^{3-}$	0.960	$CaHHDTA^-$	3.790
$NaEGTA^{3-}$	1.380	Ca_2HDTA	0.001
$CaEGTA^{2-}$	10.557	$MgHDTA^{2-}$	4.800
$CaHEGTA^-$	4.955	$MgHHDTA^-$	3.590
$MgEGTA^{2-}$	4.961	HPO_4^{2-}	11.850
$MgHEGTA^-$	3.189	$H_2PO_4^-$	6.790
$HATP^{3-}$	6.492	$KHPO_4^-$	0.490
H_2ATP^{2-}	4.028	$NaHPO_4^-$	0.600
$KATP^{3-}$	0.900	$CaPO_4^-$	6.460
$NaATP^{3-}$	1.200	$CaHPO_4$	1.700
$CaATP^{2-}$	3.446	$MgPO_4^-$	3.400
$CaHATP^-$	1.704	$MgHPO_4$	1.880
$MgATP^{2-}$	3.804	HProp	4.722
$MgHATP^-$	1.922	HMES	6.070
CaHCP	1.150	$CaMES^+$	0.700
MgHCP	1.300	$MgMES^+$	0.800

TABLE 3. BINDING CONSTANTS USED IN THE CALCULATION OF FREE ION CONCENTRATIONS. All are in log form, taken from Martell & Smith, 1974 and Perrin & Dempsey, 1974). Constants involving protons were corrected to activity units. All were corrected for IS 200 and temperature 25°C, as described in section 2.2.1.a.

The proton association constants of the pH buffers were adjusted for ionic strength and temperature by first applying a suitable correction factor for departure of ionic strength from 100 mM to 200 mM (-0.025; Perrin & Dempsey, 1974), then applying a second factor (-0.016/ΔT BES; -0.011/ΔT MES) for deviation of temperature from 25°C (where necessary). Pi constants were uncorrected (for temperature and IS).

b) Determination of the Ratio Total Calcium : Total EGTA

Since the calcium : EGTA ratio was also a major factor determining free Ca^{2+} concentration in the solutions, it was essential to measure this ratio as accurately as possible. EGTA was added to the physiological solutions from nominally 50 mM stocks of K_2EGTA and CaK_2EGTA (section 2.2.2.a). The exact concentration of EGTA in the stock solutions was checked following the method of Smith & Miller (1985), a modified version of that of Moisescu & Pusch (1975) . A highly simplified solution based on the control one used in skinned fibre experiments (table 4) was made up containing 98 mM KPr, 2 mM BES and 2 mM K_2EGTA (from the 50 mM stock solution). Before making up to its final volume the pH of the solution was adjusted to approximately 9.40 with 1 M KOH (to ensure that the end point of the titration against Ca^{2+} occurred at a pH above that (approximately 6.0) where H^+ release from EGTA becomes less than 2 per Ca^{2+} bound). All stock solutions, except that for EGTA, were passed down a chelex column to remove contaminant Ca^{2+}. The pH of 20 ml of the solution was measured whilst stirring continuously. A Corning 250 pH electrode which had been calibrated with 25 mM K_2PO_4 + 25 mM NaH_2PO_4 (pH 6.865 at 25°C) and 50 mM phthalate (pH 4.008 at 25°C) was used for all pH measurements. Hamilton syringes (50 or 10μl as appropriate) were used for all $CaCl_2$ additions. 100 mM $CaCl_2$ (made up very accurately with a grade A volumetric flask from a supplied stock of 1 M $CaCl_2$) was added in 50μl or 5μl aliquots to the 2 mM EGTA solution and the pH was noted after each addition. As $CaCl_2$ is added, the pH drops as Ca^{2+} displaces H^+ from EGTA, until all the EGTA is saturated with Ca^{2+}. The procedure was carried out 3 times. Titration curves of volume of $CaCl_2$ added (μl) plotted against pH were drawn.

The graphs were all of the form of a gentle slope descending into a plateau. Extrapolation of straight lines through the plateau and points preceding the plateau for each graph gave an intersection which could be used to read off the amount of $CaCl_2$ required to react exactly with the EGTA present. Since the intersection occurred at approximately pH 7.6 it would be expected that Ca^{2+} and EGTA would react with a 1:1 ratio. The $CaCl_2$ solution was assumed to have a concentration of exactly 100 mM, thus the concentration of EGTA could be

deduced. The accuracy of the method was checked by comparing the results from the three titrations: these were always highly reproducible (EGTA purity was 96.3%). In practice, this measured purity was used to adjust the EGTA stock solutions so that they had a true EGTA concentration of 50 mM.

2.2.2 Ca^{2+}-SENSITIVITY MEASUREMENTS IN SKINNED FIBRES

a) Solutions

The solution composition for skinned fibre experiments is given in table 4.

	CONTROL	CAFFEINE	20 mM Pi	pH 6.2
HBES	100	100	100	-
EGTA	10	10	10	10
ATP	5	5	5	5
CP	10	10	10	10
$MgCl_2$	5.9-5.1	5.9-5.1	6.7-5.9	5.5-4.7
Mg^{2+}	1.4	1.4	1.3	1.4
KPr	55	55	20	50
KPi	-	-	20	-
HMES	-	-	-	100
CAFFEINE	-	20	-	-
pH	7.00	7.00	7.00	6.20
IS	193	193	197	200

TABLE 4. SOLUTIONS USED FOR MEASURING Ca^{2+}-SENSITIVITY IN SKINNED FIBRES. All concentrations expressed in mM terms. These concentrations were achieved by diluting 4 parts solution A with 1 part CaE solution to provide activating and relaxing solutions with a range of calcium concentrations from contaminant to 10.5 mM. $MgCl_2$ concentration was decreased in inverse proportion to the amount of Ca^{2+} added to maintain constant $[Mg^{2+}]$.

Stock solutions of 500 mM BES, 1 M KPr and 500 mM KPi were prepared, (adjusting the pH to 7.00 with KOH) and were passed down a chelex-100 (Biorad) column (in K^+ form) to remove contaminant Ca^{2+}. Na_2ATP, Na_2CP and caffeine were added as solids. pH was adjusted with either 1M KOH or 1M

HPr as appropriate, at 25°C. All chemicals were from BDH (AnalaR grade) unless stated otherwise. Among the exceptions are the following, which were obtained from SIGMA: caffeine; HBES; HMES; H_4EGTA; Na_2ATP & Na_2CP; and Triton X-100 from FLUKA. Solids were weighed on a OHAUS GALAXY 160D or GT400 balance to an accuracy of three decimal places. Volumes greater than 20 ml were measured in measuring cylinders whereas those less than 20 ml were added using appropriate Gilson pipetters. pH was set to either 7.00 or 6.20 with a calibrated Corning 250 pH meter at room temperature (25°C$\pm$1°C).

The final bathing solutions were made by combining four parts "physiological" solution A with one part CaE solution. Four different versions of solution A were prepared corresponding to control and test conditions outlined in table 4 - the only component of the solutions in this table missing from the solution A was EGTA. A range of CaE stock solutions was made up such that after dilution (1:4) of each with solution A the final Ca^{2+} concentrations ranged from zero (contaminant) to 0.3 mM, the final concentration of EGTA being 10 mM in each case. The same CaE solutions were used in combination with both control and test versions of solution A, though for the pH 6.20 bathing solution the concentrations of added Ca^{2+} required were lower since at this pH EGTA binds Ca^{2+} with lower affinity. The CaE solutions themselves were made by combining 50 mM K_2EGTA and 50 mM CaK_2EGTA, the proportion of the latter increasing from zero to 100% to give a range of total calcium concentration from zero to 50 mM, corresponding to a range of 0 - 10 mM in the final bathing solutions. In addition a solution of 52.5 mM total calcium (10.5 mM in the bathing solution) was prepared by adding 0.125 ml 1 M $CaCl_2$ to 50 ml 50 mM CaK_2EGTA. The range of CaE solutions made, together with final calcium concentrations and calculated pCa values in the bathing solutions are shown in table 5.

It was important to make the K_2EGTA and CaK_2EGTA stocks as accurately as possible so the final concentrations of EGTA and Ca^{2+} in the bathing solutions were known precisely. The purity of the EGTA was therefore measured (section 2.2.1.b) and used to adjust the stock solutions to exactly 50 mM EGTA. The CaK_2EGTA stock was made by heating to 80°C a solution containing dried H_4EGTA and $CaCO_3$ to give a final concentration of 50 mM of each. These combined to produce CaEGTA as CO_2 was released. The solution was neutralised with Ca^{2+}-free KOH and made up to final volume with double distilled water.

As calcium concentration in the bathing solution rises, the amount of Ca^{2+} bound to the EGTA will increase, thereby displacing Mg^{2+} previously bound to the same sites. Free Mg^{2+} concentration will unfortunately therefore rise. To

maintain Mg^{2+} at a constant level, 4.15 mM $MgCl_2$ was added to the K_2EGTA used to make the CaE solutions. This boosted free Mg^{2+} levels in low Ca^{2+} concentration solutions in inverse proportion to the amount of Ca^{2+} present, thereby maintaining a constant free Mg^{2+} concentration in the bathing solutions.

TOTAL Ca IN CaE (mM)	TOTAL CALCIUM IN IN BATHING SOLN (mM)	CALCULATED pCa 25° C		
		CONTROL & CAFFEINE	Pi	pH 6.20
0.0	≈ 10 μM	9.39	9.39	7.81
2.5	0.5	-	-	6.09
5.0	1.0	-	-	5.76
10.0	2.0	-	-	5.41
12.5	2.5	-	-	5.29
15.0	3.0	-	-	5.18
20.0	4.0	6.56	6.56	4.99
25.0	5.0	-	-	4.82
30.0	6.0	-	-	4.64
35.0	7.0	6.02	6.02	4.46
37.5	7.5	-	-	4.36
40.0	8.0	5.79	5.79	-
42.5	8.5	5.64	5.64	4.12
45.0	9.0	5.44	5.44	3.98
46.25	9.25	-	5.31	-
47.5	9.5	5.13	5.13	3.81
48.75	9.75	-	4.87	-
50.0	10.0	4.40	4.46	3.63
52.5	10.5	3.68	3.80	3.45

TABLE 5. CALCULATED pCa VALUES FOR THE RELAXING AND ACTIVATING SOLUTIONS. Constants were as in table 3 at 25° C.

The bathing solutions containing Ca^{2+} were termed activating solutions since they could induce contraction of skinned fibres, whereas the bathing solution with no added CaK_2EGTA was known as high EGTA relaxing solution (HR). To speed up the rate of contraction, a "jump technique" (Moisescu, 1976) can be employed whereby a solution containing zero Ca^{2+} and a much reduced amount of EGTA is applied to the fibre immediately prior to activation. This has the

advantage of equilibrating the myofilament lattice with a very low concentration of EGTA (and Ca^{2+}) such that when the fibre is transferred to a Ca^{2+}-containing activating solution the Ca^{2+} will diffuse into the lattice and bind rapidly to TnC without competition from Ca^{2+}-free EGTA. Accordingly a solution containing HDTA, 0.65 mM $MgCl_2$, nominally zero Ca^{2+} and a low concentration of EGTA (some being necessary to buffer the contaminant Ca^{2+}) was prepared such that after dilution with solution A the concentration of EGTA was 0.6 mM and that of HDTA 19.7 mM. The latter was included as an inert replacement for EGTA since HDTA has a far lower affinity for Ca^{2+}. The final bathing solution was termed low EGTA relaxing solution or "LR".

10 ml of each bathing solution was made up and stored at -20° C for up to 2 months, together with any remaining solution A. The CaE stocks (including those for HR and LR) were stored indefinitely at 4° C.

b) Preparation of Skinned Fibres

i) *Glycerinated Rabbit Psoas*

Rabbit psoas fibres were prepared according to the method of Ferenczi et al (1984). A rabbit (New Zealand White, 2.5 kg) was killed by cervical dislocation and the psoas muscle exposed whilst still warm. The fascia was removed and a bundle of fibres approximately 2.5 cm long and 1.5-2.0 mm diameter was partially separated (ends not cut) from the main bulk of the muscle with small scissors. A piece of surgical thread was tied in a double knot around one end of the bundle, this being repeated at the opposite end. A wooden cocktail stick about 4 cm long was placed adjacent to the bundle, then each end of the bundle was tied to the stick with the remaining ends of the surgical thread. Finally the ends of the bundle were cut resulting in a piece of psoas held at its *in vivo* length along the stick. This was immediately immersed in pre-skinning solution (see below). The procedure was repeated until the bundles became difficult to separate from the rest of the muscle, at which point the second psoas muscle was exposed and used to obtain more preparations. A total of 5 or 6 bundles were generally prepared from each rabbit. The procedure was completed within 20 minutes.

The pre-skinning solution consisted of : 170 mM KPr; 5 mM EGTA; 2.5 mM Na_2ATP; 2.5 mM $MgCl_2$; 0.1 mM PMSF; 5 mM NaN_3 and 10 mM Imidazole, pH 6.76 at 21° C. PMSF was taken from a stock of 10 mM in propan-1-ol. Newly prepared psoas bundles were left in the preskinning solution for 24 hours at 4° C with one change of solution and frequent inversion of the tubes to ensure adequate mixing and to facilitate diffusion of the chemicals into the fibres. The bundles were next transferred to a skinning or "glycerination" solution consisting

of pre-skinning solution diluted 50% by volume with glycerol. They were stored in this at -20° C for up to two months but could be used within 2 days.

ii) *Triton-treated Rat Trabeculae*

Male Wistar rats (≈ 200g) were killed by cervical dislocation and the hearts rapidly excised. Each heart was rinsed three times in oxygenated (95%O_2/5%CO_2) tyrodes and was simultaneously massaged to remove excess blood. The tyrodes consisted of: 93 mM NaCl; 20 mM $NaHCO_3$; 1 mM Na_2HPO_4; 1 mM $MgSO_4.7H_2O$; 5 mM KCl; 10 mM glucose; 20 mM Na acetate and 5 u/l insulin. The solution was oxygenated for at least 30 minutes before the addition of 2 mM $CaCl_2$ just prior to use. Following rinsing, each heart was transferred to a deep Petri dish containing a sylgard base and was covered with fresh oxygenated tyrodes. The inside of the right ventricle was exposed and trabeculae which were mostly unattached along their lengths were dissected out together with a plug of ventricular wall at each end. Only trabeculae that were contracting strongly and had a diameter of 200 μm or less were used. These were generally positioned close to the valve tissue. The trabeculae were transferred to a shallow Petri dish containing relaxing solution (HR, see above) plus 1% (by volume) Triton X-100 and were left for 30 minutes to allow complete permeabilisation of the cell membranes (section 2.1.1). Fresh preparations of cardiac muscle were made for each days experiments. During each day "reserve" trabeculae were stored in skinning solution at 4° C.

c) Mounting the Preparations

Single glycerinated rabbit psoas fibres were dissected from a fibre bundle (under oil) and attached to the motor and transducer arms (section 2.2.2.e) with acetone/cellulose acetate glue. The remaining glycerol and oil prevented desiccation of the fibres during the mounting procedure. Following attachment the psoas fibres were slackened and immersed in relaxing solution (HR), in the first of a series of 12 wells, to equilibrate for approximately 10 minutes. The 0.5 ml wells were cut into a block of anodised aluminium and the sides constructed from glass coverslips to allow the passage of light through for SL measurement. Bath changes could be effected manually in 1-2 seconds. Occasionally the fibres were treated at this stage in a solution of 1% Triton X-100 in HR for 20-30 minutes but this had no effect on rate or magnitude of force development so it was concluded that the fibres had been adequately permeabilised by their treatment with glycerol.

Trabeculae were rapidly transferred, in a miniature spoon, from the petri dish to a shallow well containing relaxing solution (HR), prior to mounting. For

experiments with cardiac muscle the carbon arms on the motor and transducer were replaced with stainless steel tubes (250 μm diameter) through which nylon monofilament had been threaded to form loops (Harrison et al, 1988). The size of each loop could be altered by pulling or pushing the ends of the monofilament projecting from the top of the tubes. The ventricular plugs at the ends of the trabeculae were manoeuvred through the nylon loops which were then tightened to firmly grasp the tissue without cutting into it. The mounted trabecula was then transferred to relaxing solution in the first of a series of wells, was slackened, and left for approximately 10 minutes to equilibrate.

d) Measurement of Sarcomere Length

Sarcomere length (SL) of the fibres was monitored using a laser diffraction system (fig.4) similar to that developed by ter Keurs et al (1980). The beam from a He-Ne Spectraphysics laser (633 nm; 10 mW) was directed through the well and muscle fibre, the sarcomeres of the latter diffracting the light onto a Reticon 256G photodiode array via a series of lenses. The first of these, a x20 objective lens, focussed the zero order and diffracted light to different focal points since the former was an approximately parallel beam and the latter divergent. A camera lens then focussed the rediverging diffracted light onto the photodiode array and the zero order to one side. Finally a cylindrical lens focussed the vertical bands of diffracted light to points to increase the light intensity. Between the objective and camera lenses, a beam splitter directed 50% of the focussed zero order light onto a white screen, providing an image of the muscle fibre. This enabled the homogeneity of the preparation to be monitored and its depth to be measured.

The photodiode array was scanned continuously in 2 ms by a photodiode array driver (Reticon 100B) and the current output from the photodiodes converted to a video signal then sent to a Philips PM3206 oscilloscope to display the position and intensity of the diffraction pattern. This pattern was converted to SL using a SL calculator (devised by ter Keurs et al, 1980) which worked out the centre of gravity of the diffracted light in each peak. Thus the median SL could be computed after correction for the scattering of light from zero order.

The system was calibrated using a 300 lines / mm diffraction grating in the normal position of the centre of the well (representing a muscle fibre). This produced first and second order diffraction patterns at 1.67 and 3.33 μm which appeared as peaks on the oscilloscope. The digital readout of SL was adjusted to match these values before each experiment. The output from the SL calculator was also connected to a 4-channel Gould 2400S chart recorder so a record of SL was obtained throughout the experiment.

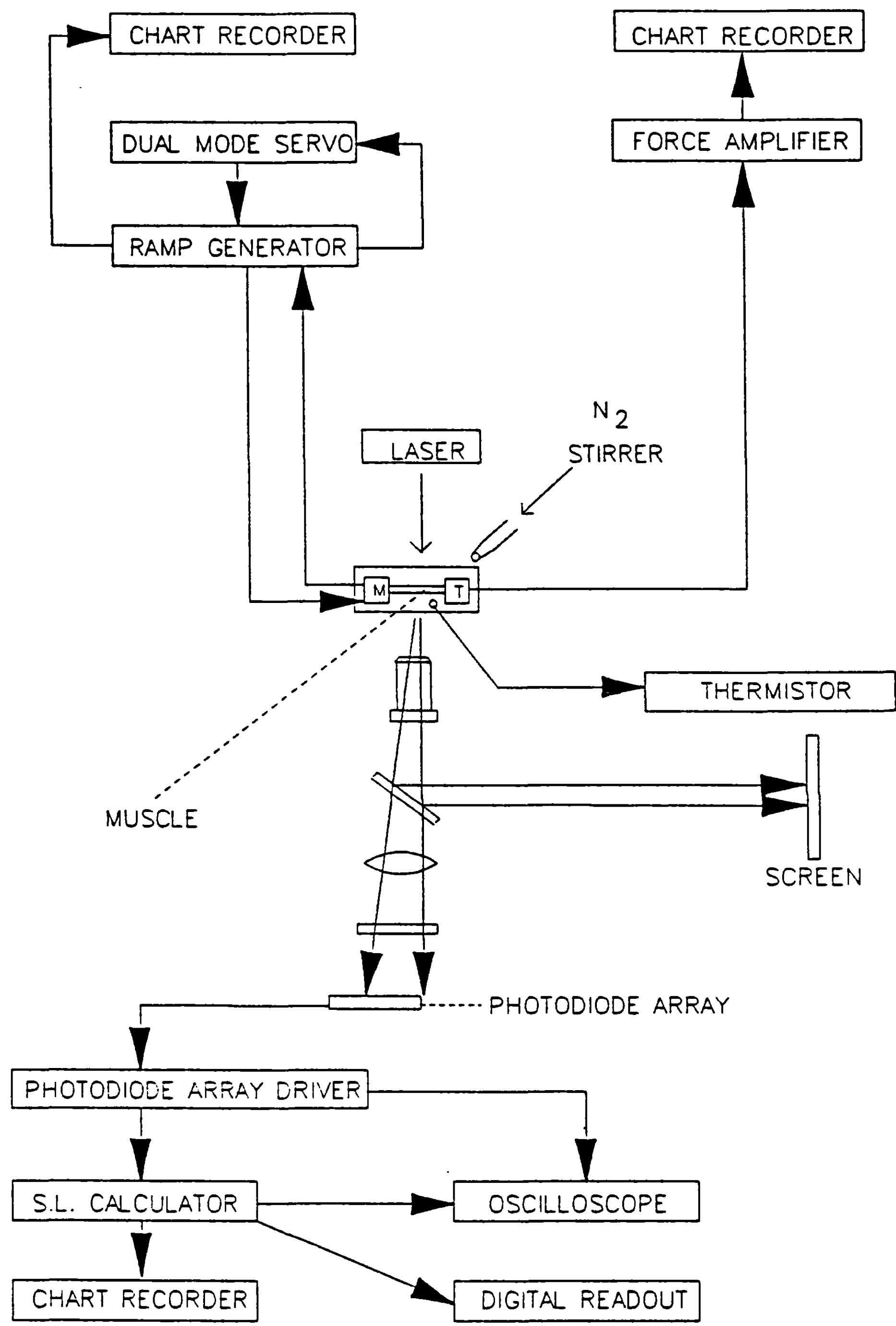


Fig.4 FLOW DIAGRAM OF FORCE AND SARCOMERE LENGTH MEASUREMENT. Further details of the equipment are given in sections 2.2.2.c-e.

e) Measurement of Force

Fibres were mounted between carbon arms on a motor (though this was used only as a fixed stop in these experiments) and a SensoNor AE801 force transducer (fig.4). The latter had a compliance of 5 μm/mN and a sensitivity of 2 V/mN. Changes in resistance of the transducer were measured by a wheatstone bridge circuit, and the accompanying changes in voltage, after amplification, were recorded on a Gould 2400S 4-channel chart recorder as a measure of force.

A ramp generator system was used to keep SL constant, particularly during experiments on the effect of SL on caffeine-induced Ca^{2+} sensitisation. The position settings from the ramp generator were fed into a Cambridge 300S dual mode servo which controlled the length of the muscle via a feedback system. The length output signal was recorded on the Gould 4-channel chart recorder.

The temperature of the baths was maintained at 25°C using a waterbath to pump water through channels bored in the metal base of the well system. A thermistor placed in the end well continually monitored the temperature which was displayed on a digital read-out. The solutions in the wells were continuously stirred by a jet of hydrated nitrogen directed onto the solution surface. Evaporation of solution from the wells was minimal during each run but where a fall in the level of the meniscus below the top of the bath was noticed the solution was removed and replaced with a fresh one.

i) *Protocol*

Psoas fibres and trabeculae were stretched, by moving the transducer arm with a micromanipulator, until their resting SLs were 2.4 and 2.1 μm respectively. A few maximal activations in 10 mM and 10.5 mM calcium solutions were carried out to determine the stability of the fibres. When three successive contractions gave near identical force levels with SL returning to its starting value in each relaxation the fibre was judged to be good enough to use. Preparations in which maximum force declined noticeably during contractions were rejected. Any fibres in which control maximum force declined during the experiment by more than 20% from the initial value were rejected. Those in which no stable resting sarcomere pattern could be obtained were also abandoned. Results were only analysed for fibres in which the resting sarcomere pattern remained clear throughout. Any drifting of SL away from that set in relaxing solution was compensated for by stretching or releasing the fibre to recover the initial length unless damage was visible in which case the fibre was rejected.

f) Effects of pH, Pi and Caffeine on Ca^{2+}-Sensitivity

To enable the application of three test conditions (pH 6.2, Pi and caffeine) to each fibre with minimum deterioration of the preparation, the following protocol was used. The first and third tests involved activation in a control solution followed by relaxation before activation in a test solution (protocol A). This protocol was carried out repeatedly, with solutions applied in order of increasing calcium levels from 4.0 to 10.5 mM (or 0.5 to 10.0 mM at pH 6.20). Maximum force was checked in 10 or 10.5 mM Ca solution at the start and end of each investigation of the test condition, plus after every four contractions (2 control and 2 test) during the run. Each time maximum force was measured in the absence and presence of the appropriate test substance or pH, as was resting tension. In between these runs the second test was carried out as above but omitting all control solutions except those giving maximal activations. The mean of the control pCa_{50} values obtained for the first and third tests could be used as the control pCa_{50} for the second test (assuming a linear change in pCa_{50} throughout the experiment). In practice the control Ca^{2+}-sensitivity of the skinned fibres remained constant from start to finish of the three tests: the change in pCa_{50} of the first and third test controls for rabbit psoas was $-0.03 \pm 0.03 (n=3)$ and that of rat trabecula was $-0.03 \pm 0.02 (n=5)$. Separate experiments established that although Ca^{2+}-sensitivity did not change with time, maximum force of rabbit psoas decreased by $17.5 \pm 0.90\%$ $(n=10)$ over 2.25 hours.

The order of the different test conditions was rotated such that in one experiment it was caffeine followed by Pi then pH 6.20, then in the next experiment caffeine was tested second and pH 6.20 first, etc. Although this general pattern was followed it was occasionally unavoidably disturbed when a preparation did not last well enough for all three factors to be tested.

i) *The Effects of Sarcomere Length on the Caffeine Response*

It was thought possible that the greater Ca^{2+} sensitising effect of caffeine observed in cardiac muscle compared to skeletal fibres (Wendt & Stephenson, 1983 & section 2.3.1.d) was related to SL. Cardiac muscle generally operates at a shorter (2.1 μm) SL in vivo than skeletal (2.4 μm). Experiments were therefore performed measuring the sensitising effects of caffeine in rabbit psoas fibres held at different SLs. Due to internal shortening of SL during activation, it was essential to control SL during contraction. It was not possible to repeat the work with rat trabeculae since in this case SL could not be measured during contraction using laser diffraction. This phenomenon has been noted in other studies (Kentish et al, 1986) and has been attributed to a change in the precise

alignment of the sarcomeres during activation, such that the myofilament lattice no longer acts as a diffraction grating.

Methods were as described above, except that the pH was set to 7.10 at 21°C to match that of the myoplasm in skeletal muscle (Roos & Boron, 1981). All experiments were carried out at 15°C as lowering the temperature seemed to stabilise the sarcomere pattern. The pH of the solutions therefore rose to 7.22 and binding constants were adjusted accordingly.

Each psoas fibre was repeatedly maximally activated in control solution until the force and SL during contraction were stable (fig.10). SL was then adjusted during activation to a value between 1.5 and 2.8 μm inclusive. When the SL was below 2.0 μm the fibre became slack during relaxation. Ca^{2+}-sensitivity of the fibre was then measured in the presence and absence of 20 mM caffeine according to protocol A described in section 2.2.2.f. SL during contraction was always checked, and if necessary adjusted to the original set value by moving the motor arm to stretch or release the fibre. Fibres in which the sarcomere pattern became unstable were rejected. At the end of this first run, the SL of the fibre was changed, during maximal activation, to a value at least 0.5 μm different from the previous one. Ca^{2+}-sensitivity with and without caffeine was again measured before returning the SL to the original value for a final check on Ca^{2+}-sensitivity in the absence and presence of caffeine. Thus each fibre was tested at two SLs with one sandwiched between two runs at the other. This protocol enabled any change in the Ca^{2+}-sensitivity of the fibre independent of SL and caffeine to be detected. The results from fibres which lasted only for the first two runs were not included in statistical analysis. Equal numbers of experiments were performed with the initial SL (2.1-2.8 μm) longer than the second (1.5-2.3 μm) and vice versa. Force-pCa relationships were fitted to the Hill equation and the shift in pCa_{50} induced by 20 mM caffeine was calculated for each of the three runs.

2.2.3 STATISTICAL ANALYSIS

Unless stated otherwise, values are given as mean $\pm$ SEM of n experiments.

Decline in maximum force during an experiment was calculated by expressing the maximum force at the end of the experiment as a percentage of the initial maximum force.

When calculating relative force, correction was always made for the decline (assumed to be linear) in maximum force during an experiment . % Relative Force was plotted against Ca^{2+} concentration and the resulting sigmoidal curves were fitted to the Hill equation by a computer programme. Mean pCa_{50} and H_n values were used to generate the curves shown in the results section. These curves were normalised to control maximum force.

"Student's" or "paired" t-tests were applied where appropriate to test the significance of differences. Significant results ($p<0.05$) are denoted by *. It was previously ascertained, using the Kolmogorov-Smirnov test (Sokal & Rohlf, 1969), that H_n and pCa_{50} were Normally distributed, since this is a prerequisite for using the t-test.

2.3 RESULTS

2.3.1 AGENTS INFLUENCING THE Ca^{2+}-SENSITIVITY OF SKINNED FIBRES

a) Controls

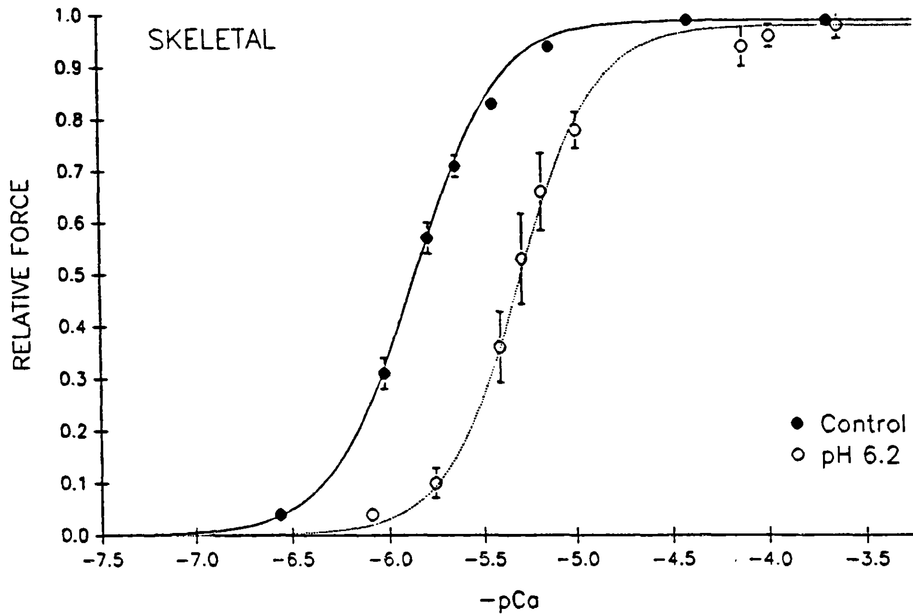
Under control conditions, the psoas fibres (mean diameter $65 \pm 7\,\mu m$, $n=5$) generated an average 84.4 ± 15.6 mN/mm^2 stress, whereas trabeculae (mean diameter $134 \pm 20\,\mu m$, $n=5$) produced only 25.0 ± 7.2 mN/mm^2. These values were of the same order of magnitude as those obtained in other studies (e.g. Brandt et al, 1984; Kentish, 1986).

The relationship between relative force and pCa was sigmoidal (fig.5) and could be fit to the Hill equation. As shown in table 6, the pCa_{50} for rabbit psoas fibres was approximately 5.85 whereas that of rat trabeculae was approximately 5.56. The corresponding H_n values were 1.93 and 2.62 respectively. Thus skeletal fibres were more sensitive to Ca^{2+} but force-pCa curves were less steep.

b) Effects of pH 6.20

The reduction of pH from 7.00 to 6.20 reversibly suppressed the maximum force (figs.6 & 7) by 42.3% ($n=4$) and 59.0% ($n=5$) in skeletal and cardiac fibres respectively. Acidosis had no consistent effect on resting tension of psoas muscle or rat trabeculae.

Reducing the pH to 6.20 desensitised skinned fibres to Ca^{2+} (figs.6 & 7), causing a rightwards shift of the force-pCa curve as shown in fig.5. The pCa_{50} of rabbit psoas was displaced by 0.55 ($n=4$) units and that of rat trabeculae by 1.09 ($n=5$) units. Thus the shift with cardiac muscle was twice that with skeletal fibres. Acidosis had no significant effect on the H_n of psoas fibres or of trabeculae (table 6).



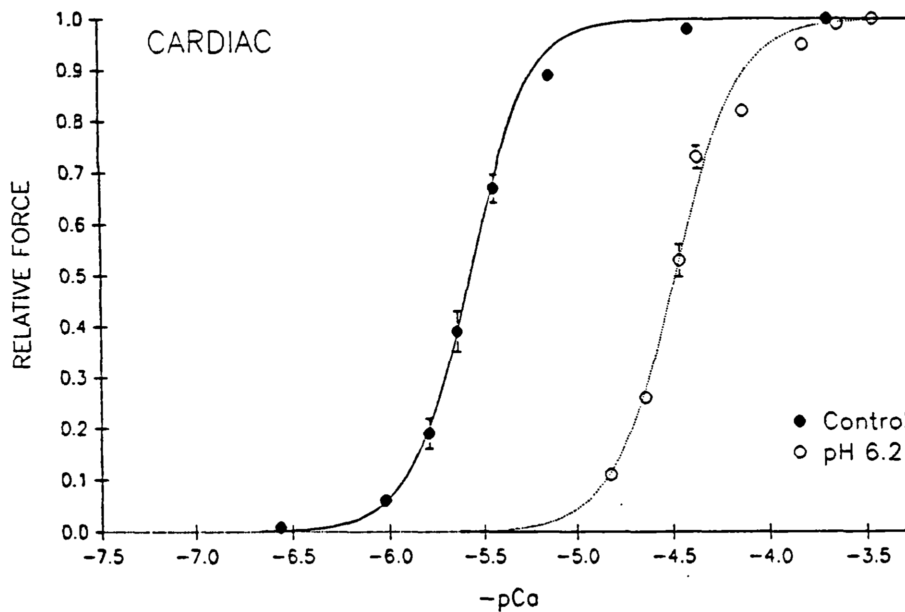


Fig.5 EFFECTS OF REDUCED pH ON Ca^{2+}-SENSITIVITY OF SKINNED FIBRES. Conditions were as described in section 2.2.2.f. Points represent mean data from 4 skeletal and 5 cardiac experiments and are normalised to control maximum force. Curves were generated from the appropriate mean pCa_{50} and Hill "n" values (table 6).

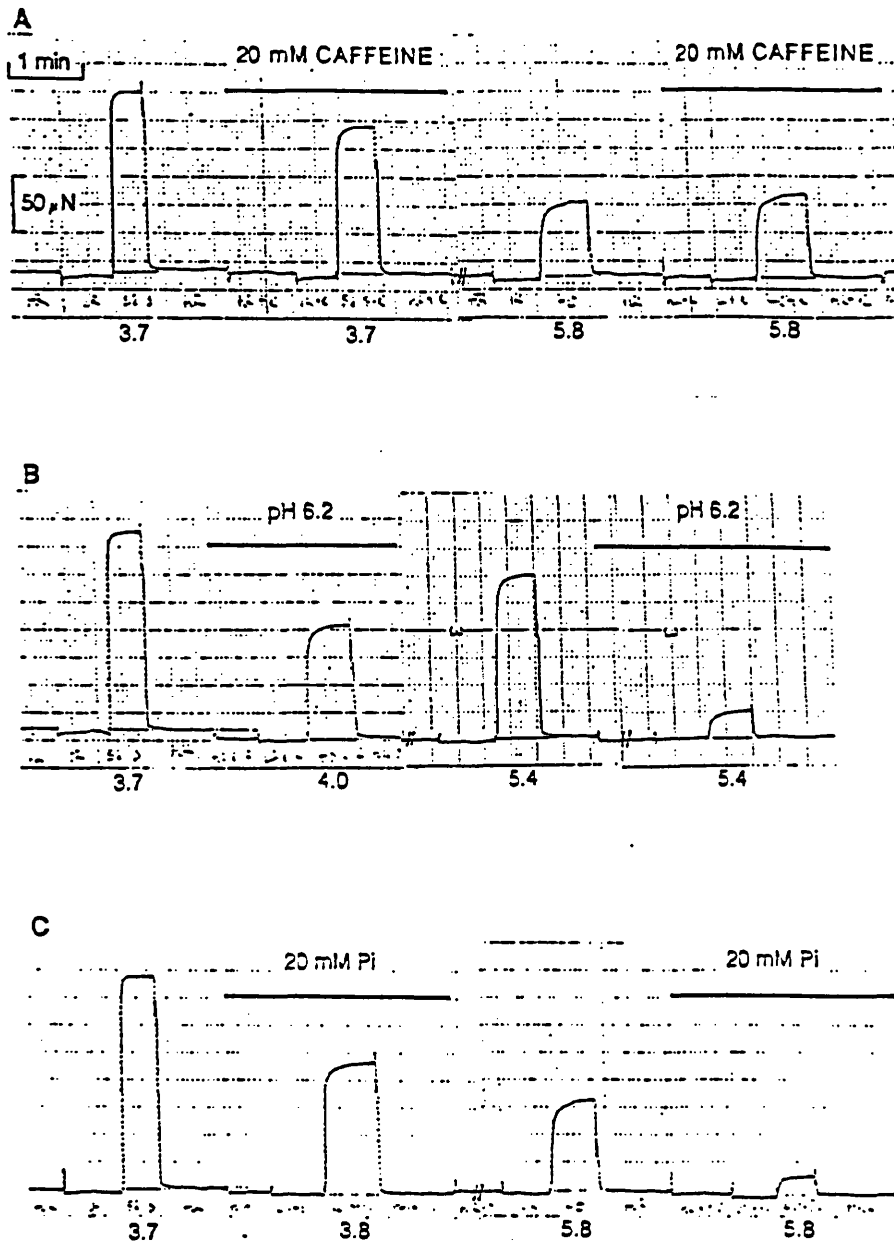


Fig.6 EFFECTS OF (A) CAFFEINE, (B) pH 6.20 AND (C) Pi ON FORCE OF RABBIT SKINNED PSOAS FIBRES. Maximal activations in the absence and presence of test conditions were followed by corresponding sub-maximal activations as described in section 2.2.2.f using solutions in section 2.2.2.a, table 4 at 25°C. Fibres were relaxed between contractions in 2 washes of HR and 1 of LR. Numbers below each trace represent pCa during activation. Black bars indicate duration of test condition. // indicates non-continuous record. Time and force scales in (B) and (C) are as in (A).

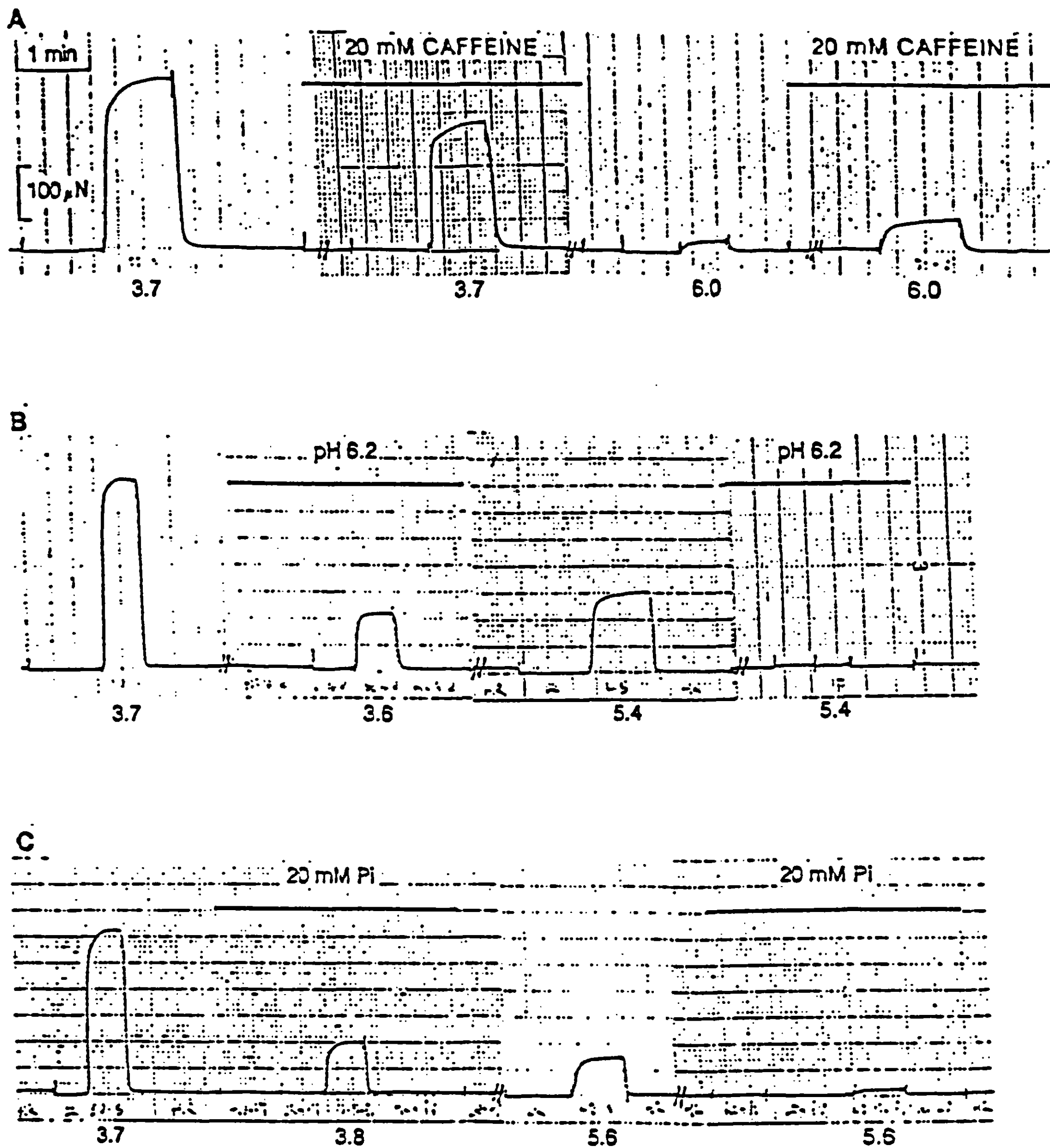


Fig.7 EFFECTS OF (A) CAFFEINE, (B) pH 6.20 AND (C) Pi ON FORCE OF RAT SKINNED TRABECULA. Maximal activations in the absence and presence of test conditions were followed by corresponding sub-maximal activations as described in section 2.2.2.f using solutions in section 2.2.2.a, table 4 at 25° C. Fibres were relaxed between contractions in 2 washes of HR and 1 of LR. Numbers below each trace represent pCa during activation. Black bars indicate duration of test condition. // indicates non-continuous record. Time and force scales in (B) and (C) are as in (A).

		pH 6.2	Pi	CAFFEINE
pCa_{50}				
Control	Sk	5.86±0.06(4)	5.87±0.04(5)	5.82±0.05(4)
	C	5.57±0.03(5)	5.55±0.04(5)	5.57±0.03(5)
Test	Sk	5.30±0.06(4)	5.57±0.04(5)	5.91±0.04(4)
	C	4.48±0.01(5)	5.36±0.03(5)	5.88±0.05(5)
Shift	Sk	-0.55±0.02(4)*	-0.30±0.02(5)*	0.09±0.01(4)*
	C	-1.09±0.02(5)*	-0.19±0.03(5)*	0.31±0.04(5)*
Hill "n"				
Control	Sk	1.90±0.10(4)	1.96±0.09(5)	1.94±0.13(5)
	C	2.72±0.17(5)	2.65±0.16(5)	2.50±0.19(5)
Test	Sk	2.13±0.14(4)	2.10±0.13(5)	1.83±0.10(4)
	C	2.55±0.14(5)	2.84±0.14(5)	2.64±0.15(5)
Shift	Sk	0.23±0.23(4)	0.14±0.09(5)	-0.11±0.05(4)
	C	-0.17±0.21(5)	0.19±0.26(5)	0.13±0.23(5)
Max. F.				
% Fall	Sk	42.3±1.8(4)*	42.4±2.1(5)*	18.6±2.4(4)*
	C	59.0±5.8(5)*	57.4±1.6(5)*	15.6±5.6(5)*

TABLE 6. EFFECTS OF LOWERING pH TO 6.20, 20 mM Pi AND 20 mM CAFFEINE ON SKINNED SKELETAL AND CARDIAC FIBRES. Solutions as described in section 2.2.2.a. All values expressed as mean±SEM (n). * denotes a significant difference from zero (Paired t-test). Suppression of maximum force expressed relative to force of first maximal activation.

c) Effects of Inorganic Phosphate

20 mM inorganic phosphate suppressed maximum force (figs.6 & 7) by 42.4% in rabbit psoas and 57.4% in rat trabecula. Phosphate had no consistent effect on resting tension of psoas or cardiac muscle.

Both fibre types were desensitised to Ca^{2+} by phosphate (figs.6 & 7). The midpoint of the force-pCa relationship of skeletal fibres was shifted by 0.30 pCa units (n=5) as shown in fig.8. In cardiac preparations the shift was 0.19 (n=5) (fig.8). Cooperativity within the fibres, as measured by H_n, was unaffected by phosphate in psoas and trabeculae (table 6).

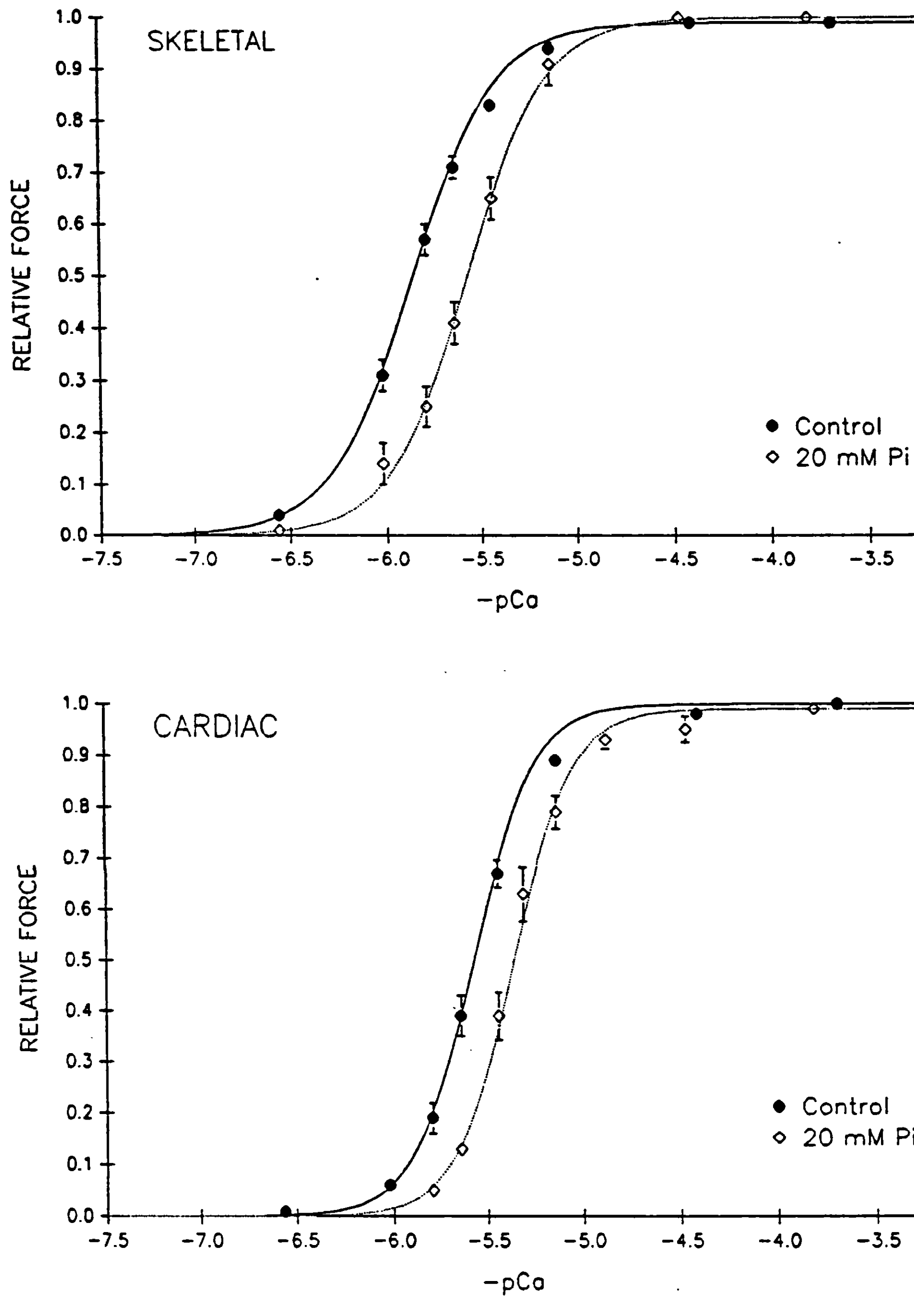
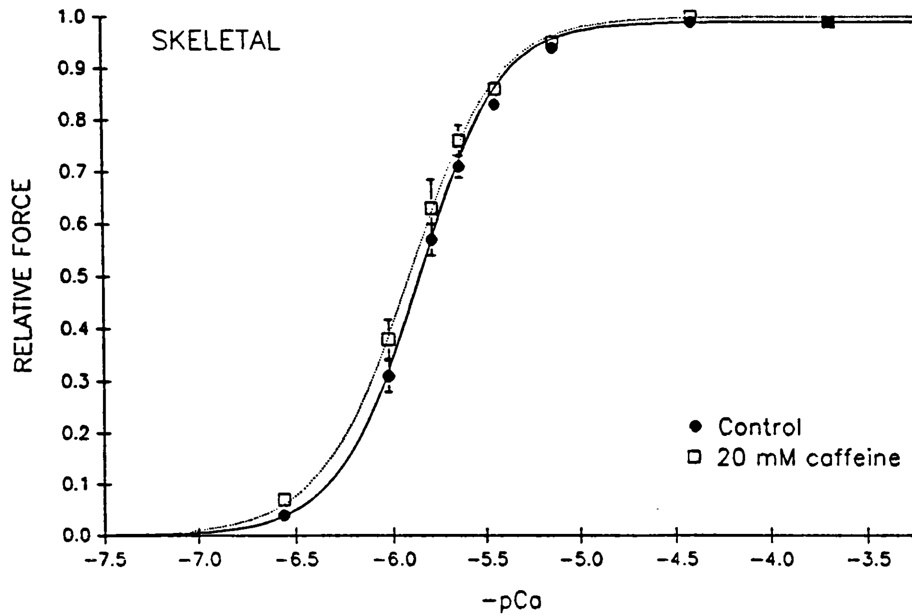


Fig.8 EFFECTS OF Pi ON Ca^{2+}-SENSITIVITY OF SKINNED FIBRES. Conditions were as described in section 2.2.2.f. Points represent mean data from 5 skeletal and 5 cardiac experiments and are normalised to control maximum force. Curves were generated from the appropriate mean pCa_{50} and Hill "n" values (table 6).



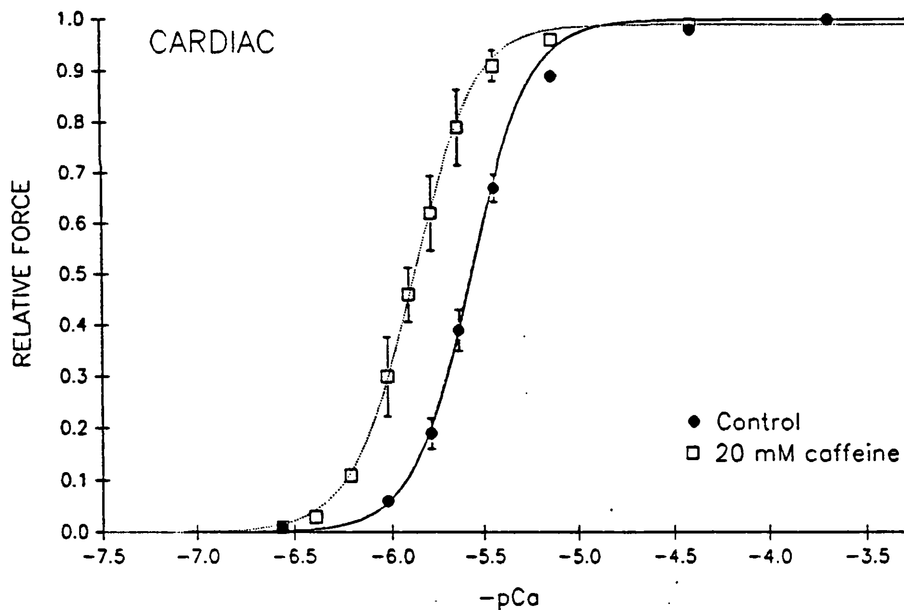


Fig.9 EFFECTS OF CAFFEINE ON Ca^{2+}-SENSITIVITY OF SKINNED FIBRES. Conditions were as described in section 2.2.2.f. Points represent mean data from 4 skeletal and 5 cardiac experiments and are normalised to control maximum force. Curves were generated from the appropriate mean pCa_{50} and Hill "n" values (table 6).

d) Effects of Caffeine

20 mM caffeine had no effect on resting tension but significantly reduced the maximum attainable force: by 18.6% (n=4) in skeletal muscle and 15.6% (n=5) in cardiac muscle (figs.6 & 7). The percentage suppression of maximum force by caffeine remained constant throughout the experiments with both psoas and trabeculae.

The addition of 20 mM caffeine increased the Ca^{2+}-sensitivity of skeletal (fig.6) and cardiac (fig.7) skinned fibres. Force-pCa curves were shifted to the left (fig.9) but caffeine had no effect on their steepnesses. The shift in pCa_{50} (table 6) for skeletal fibres was 0.09 (n=4) from the control value (5.82) and was 0.31 (n=5) for trabeculae (from 5.57). Thus trabeculae were more than three times as sensitive as psoas fibres to caffeine. Both the sensitising and suppressive effects of caffeine were fully reversible on return to control solutions.

i) *Role of Sarcomere Length in Caffeine-induced Ca^{2+}-Sensitisation of Psoas*

Results from many fibres had to be rejected since the SL changes applied between runs were often accompanied by deterioration of the sarcomere pattern. This was particularly noticeable during the third run of the experiments (i.e. the second control at the original SL), especially at high Ca^{2+} concentrations. Despite the persistence throughout the three runs of a good diffraction pattern during contraction of accepted fibres (fig.10), all fibres showed a decrease in Ca^{2+}-sensitivity between runs 1 and 3. The mean fall in pCa_{50} between runs 1 and 3 was 0.17 ± 0.04 (n=7) for controls and 0.15 ± 0.08 (n=7) for the corresponding caffeine curves. These values were not significantly different from each other so the presence of caffeine did not affect the fall in Ca^{2+}-sensitivity. Following from this, the caffeine-induced increase in Ca^{2+}-sensitivity compared to control was the same in runs 1 (0.14 ± 0.01, n=7) and 3 (0.16 ± 0.04, n=7), thus the sensitising effect of caffeine was not affected by the process which caused control pCa_{50} to fall during the experiments.

20 mM caffeine caused the midpoint of the force-pCa relationship to shift leftwards by 0.15 ± 0.01 (n=23) units (including data from all SLs studied). Fig.11 shows the caffeine-induced increases in pCa_{50} in each muscle at different SL. It appears that the ΔpCa_{50} in each muscle did not depend on SL. To check this, the difference between the mean caffeine-induced shift in pCa_{50} at the longer SL of a pair and that at the shorter SL was calculated and was divided by the magnitude of the SL change (to correct for different magnitudes of SL change in different experiments). The values of $\Delta pCa_{50}/\Delta SL$ obtained were not found to be significantly different from zero indicating that the effect of caffeine on pCa_{50} was independent of SL. It is apparent from fig.11 that although caffeine often

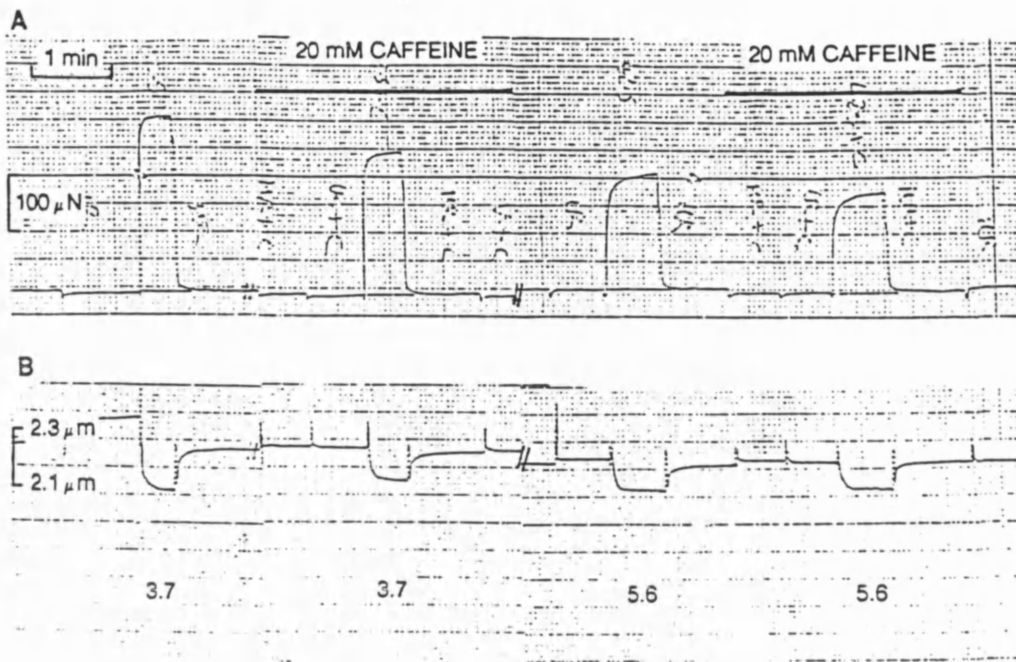


Fig.10 SIMULTANEOUS MEASUREMENT OF SARCOMERE LENGTH AND EFFECTS OF CAFFEINE ON FORCE OF RABBIT SKINNED PSOAS. Example of recordings of sarcomere length during relaxation and activation of skinned psoas in the absence and presence (indicated by black bars) of 20 mM caffeine. The effects of caffeine on force (A) were measured simultaneously with sarcomere length (B) at 15° C using conditions and solutions described in sections 2.2.2.a and 2.2.2.f. Fibres were relaxed between contractions in 2 washes of HR and 1 of LR. Numbers below trace show pCa during activation. Time scale in (A) also applies to (B). // indicates non-continuous record.

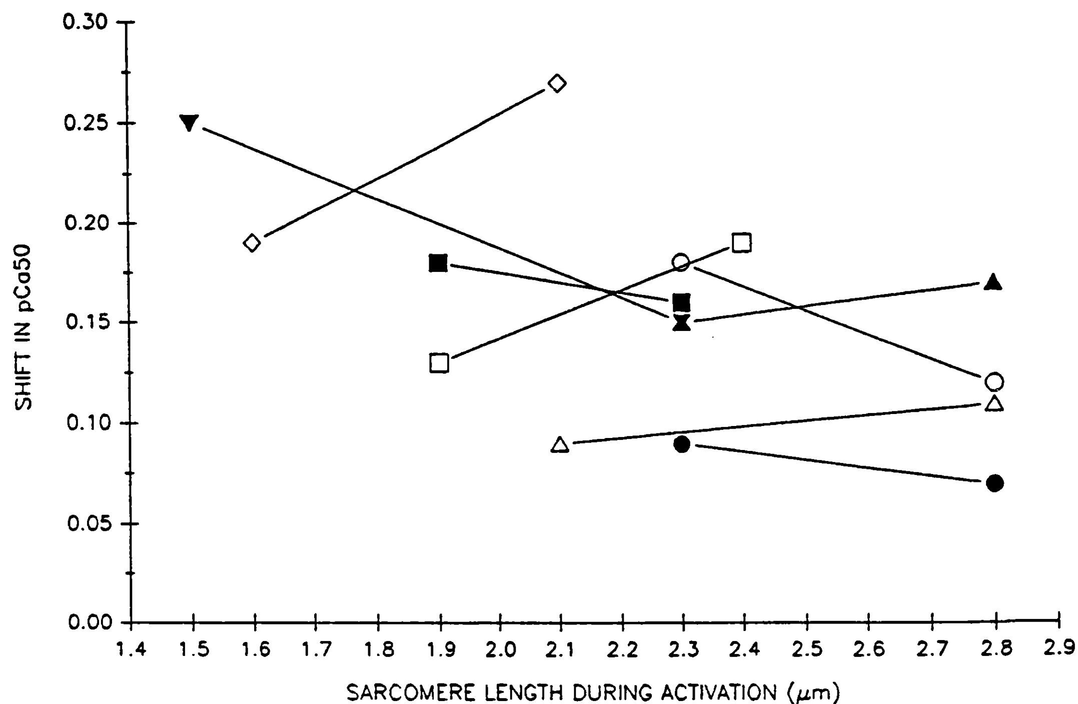
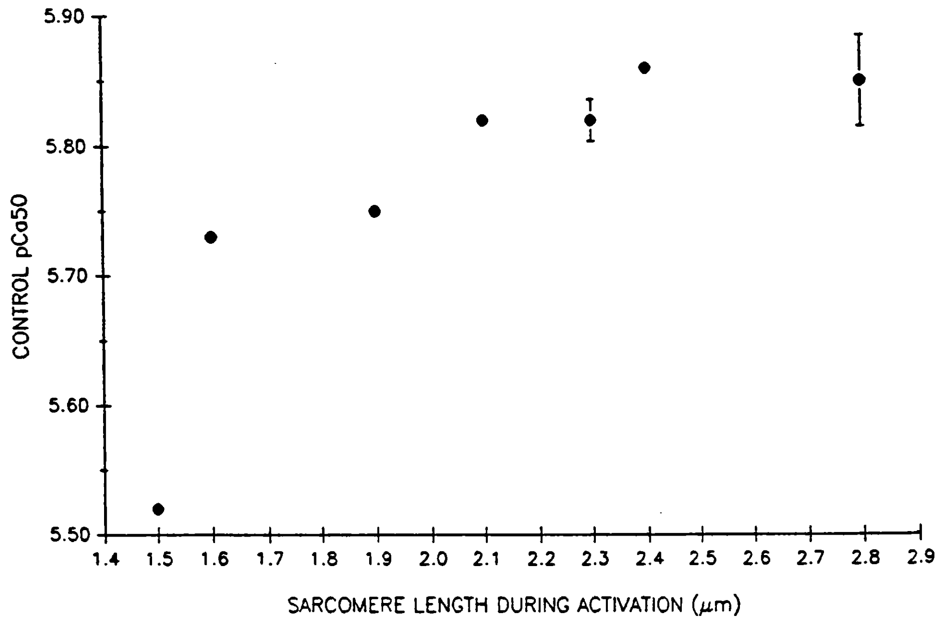


Fig.11 RELATIONSHIP BETWEEN ACTIVE SARCOMERE LENGTH AND CAFFEINE-INDUCED Ca^{2+}-SENSITISATION OF SKINNED RABBIT PSOAS. Conditions were as described in section 2.2.2.f.i. Different symbols represent data from different experiments: lines joining paired points indicate how sarcomere length affected the 20 mM caffeine-induced shift in pCa_{50} in an individual experiment. Lines with negative gradients occur where increasing SL decreases the effect of caffeine; lines with positive gradients indicate that increasing SL increases the Ca^{2+}-sensitisation by caffeine, whereas a horizontal line would equate to no effect of SL on caffeine-induced shifts in pCa_{50}.



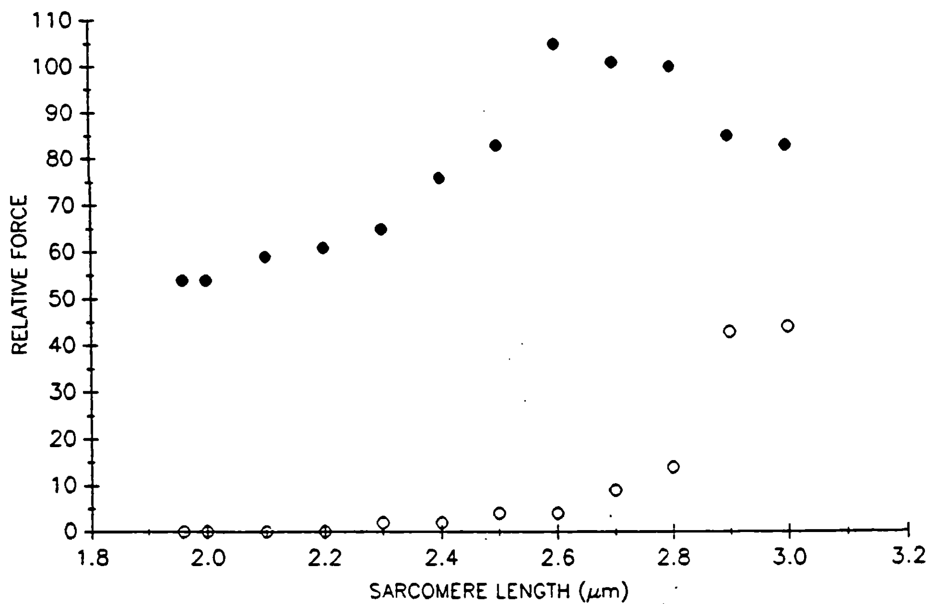


Fig.12 DEPENDENCE OF Ca^{2+}-SENSITIVITY AND MAXIMUM FORCE ON SARCOMERE LENGTH IN SKINNED PSOAS FIBRES. A) Data from experiments described in section 2.2.2.f.i (also fig.11). Points for sarcomere lengths 1.5, 1.6 and 2.4 μm are single determinations whereas those at SL 1.9, 2.1, 2.3 and 2.8 μm are mean values of data from 2, 2, 4 and 4 determinations respectively. Ca^{2+}-sensitivity increased as SL rose from 1.5 to 2.4 μm but further lengthening had no effect on pCa_{50}. B) Data from a single experiment where maximum and resting tension were measured at various SLs using solutions described in section 2.2.2.a but at 15°C and pH 7.22.

shifted pCa_{50} of individual fibres by different amounts at different SLs, the shifts were not consistently greater at longer SL or vice versa. Such inconsistencies are missed if mean caffeine-induced shift in pCa_{50} for each SL is calculated from the pooled results of all fibres.

In addition, 20 mM caffeine suppressed maximum force by 34$\pm$1.7% (n=23). Changes in active SL had no apparent effect on the extent of suppression but the depression of force increased throughout the experiments rising from 27.8$\pm$1.9% (n=8) at the start of run 1 to 42.6$\pm$1.6% (n=7) at the end of run 3. Decreases in SL below 2.6 μm were accompanied by a reduction in maximum force (fig.12) whereas peak tension remained unaffected by changes in SL between 2.6 μm and 2.8 μm (consistent with the results of Stephenson & Williams, 1982). The control pCa_{50}, but not H_n, of the fibres was also found to depend on SL, with Ca^{2+}-sensitivity increasing with lengthening of the sarcomeres up to 2.4 μm then remaining steady between SLs of 2.4 and 2.8 μm (fig.12).

2.4 DISCUSSION

2.4.1 AGENTS AFFECTING Ca^{2+}-SENSITIVITY OF SKINNED FIBRES

a) Controls

Both skeletal and cardiac skinned fibres showed decreases in maximum force during experiments. This decline was of similar magnitude in each case and was never more than 20% of the initial maximum force since at this point the preparations would have been rejected. Such a fall in force is commonly found in work with skinned fibres (Kentish, 1982 & 1984) although its mechanism is unclear. It appears to be related to time rather than contraction number, which suggests a possible diffusion out of the fibres of substances necessary for full activation, or degradation of the fibres e.g. by proteases. Fortunately, the detrimental process had no effect on Ca^{2+}-sensitivity since this remained stable with time (but see section 2.3.1.d.i). This indicates that the mechanism of Ca^{2+}-regulation of the myofibrils remained intact throughout the experiments. Thus the mode of action of the various agents on Ca^{2+}-sensitivity could be reliably studied.

Previous work with skinned trabeculae, using similar solutions (except $[Mg^{2+}]$ = 3 mM) to those in the present study, produced control pCa_{50}s of 5.56 (Kentish, 1984); 5.42 (Kentish et al, 1986) and 5.08 (Kentish, 1986). Adjustment of the current pCa_{50} (5.56) for the effect of an increase in $[Mg^{2+}]$ from 1 mM to 3 mM (ΔpCa_{50} = +0.13 / mM rise in $[Mg^{2+}]$, Fabiato & Fabiato, 1975) gives a pCa_{50} of 5.30 which is close to Kentish's earlier values. In his 1986 study, Kentish did

not monitor SL during the experiments so some shortening of the sarcomeres may have occurred, which might explain the surprisingly low pCa_{50} of 5.08.

It was found that the fast skeletal fibres were more sensitive to Ca^{2+} (pCa_{50} 5.85) than were trabeculae (pCa_{50} 5.56). Similar results were obtained by Fabiato & Fabiato (1978); Wendt & Stephenson (1983) and Godt & Nosek (1989). The latter found the pCa_{50} of rabbit psoas (pH 7.00) to be 5.84 and that of rabbit trabecula 5.63, very close to the present values. Donaldson and Hermansen (1978), however, proposed that fast twitch muscle (adductor magnus) was less Ca^{2+} sensitive than cardiac fibres. Comparison of the precise value of pCa_{50} from various studies has, however, only a limited value since Ca^{2+}-sensitivity is governed by other factors in addition to fibre type. Sarcomere length, temperature and free Mg^{2+} concentration all affect Ca^{2+}-sensitivity, as do pH and (indirectly) the size of the preparations. In addition different studies have used different Ca^{2+}-EGTA binding constants. Thus any differences between Ca^{2+}-sensitivities of fibres in different studies may simply reflect variation in experimental conditions. In the present case (and Godt & Nosek, 1989), the solutions were the same for skeletal and cardiac muscle, so the different Ca^{2+}-sensitivity was due to a true difference between muscle types or to a longer SL in skeletal muscle (as this may increase Ca^{2+}-sensitivity). Psoas fibres were set at SL 2.4 μm and trabeculae at 2.1 μm: had the skeletal fibres been at 2.1 μm the pCa_{50} would have been approximately 5.82 (fig.12). This is still close to the pCa_{50} of 5.85 observed at SL 2.4 μm so skeletal and cardiac muscles do differ in Ca^{2+}-sensitivity independent of SL.

In addition to the possible role of SL mentioned above, the increased Ca^{2+}-sensitivity of fast twitch fibres compared to cardiac fibres could be related to differences in Ca^{2+} affinity of the myofibrillar proteins. Experiments in which the native TnC is removed then replaced with TnC from another muscle type have demonstrated that Ca^{2+}-sensitivity is determined by the moiety of TnC present (Moss et al, 1986).

The H_n value reflects the minimum number of sites between which there is cooperativity. Previous studies (Brandt et al, 1980; Brandt et al, 1982) have shown that the Ca^{2+}-binding sites of TnC cannot account for all the cooperative effects in skinned psoas fibres, since H_n values up to 5.8 were recorded. The steepness of the force-pCa relationship of fast twitch skeletal fibres is usually greater than that of cardiac muscle. Godt & Nosek (1989), for example, measured a H_n of 7.71 for psoas and 4.34 for trabeculae. Donaldson & Hermansen (1978), however, reported a lower H_n for adductor magnus (1.90) than cardiac fibres (2.14). Kentish et al (1986), reported a H_n value of 5.37 (n=6) for rat trabeculae maintained at SL 2.15 μm during contraction. In another study

Kentish (1986) found H_n was 1.96 ± 0.12 (n=7) for rat trabeculae where SL was not fixed.

The current finding that the steepness of the force-pCa curve for rabbit psoas was less (H_n 1.9) than that for rat trabeculae (H_n 2.6) was somewhat surprising. Reasons for the differences between the current results and those of Donaldson and Hermansen compared to those detailed above are unclear. It appeared that the cooperativity of skeletal fibres had been affected by something that did not affect cardiac muscle (the cooperativity of the latter was reasonably close to that obtained in previous studies). Since the same solutions were used to measure Ca^{2+}-sensitivity of both fibre types and comparable protocols were employed from the time the preparations were mounted, it is possible that the storage of the psoas fibres in glycerol solution (section 2.2.2.b) had somehow reduced the extent of cooperativity. The magnitude of H_n, however, was not dependent on the length of time the fibres had been stored. Another possibility is that there was greater internal shortening in the psoas fibres compared to both psoas in other studies and trabeculae. SL was not controlled during activation of fibres in the current study (apart from experiments investigating the role of SL in caffeine-induced Ca^{2+}-sensitisation of fibres). Thus it is possible that sarcomeres in the centre of fibres shortened whilst those at the ends lengthened causing a fall in H_n similar to that observed in cardiac preparations (Kentish et al, 1986; Kentish, 1986). The mean H_n for fibres at SL 2.3 or 2.4 μm in the "SL & caffeine" experiments, however, was 2.08 ± 0.22 (n=5) which is similar to that obtained above without control of active SL.

b) Effects of pH 6.20

i) *Maximum Force*

The pH reduction from 7.00 to 6.20 depressed maximum force by 42.3%, and in trabeculae by 59.0%. Although many studies have demonstrated that acidosis lowers maximum tension, the extents of the observed effects have varied considerably. Donaldson & Hermansen (1978) showed that reducing the pH from 7.0 to 6.5 caused decreases in maximum force of 18%, 30% and 12% for rabbit cardiac, adductor magnus and soleus fibres respectively. In the same year Fabiato & Fabiato found reductions of 25% for cardiac myocytes and 28% for skeletal fibres with a drop in pH from 7.0 to 6.2. More recently, Blanchard et al (1987) measured the depression of maximum force (again with a pH change from 7.0 to 6.5) in rabbit psoas and soleus Triton-skinned fibres, obtaining values of >30% and zero respectively. The largest known suppression in cardiac muscle was the 34% recorded for adult rat by Solaro et al, 1988, once more with pH falling from

7.0 to 6.5. This was almost identical to the 32% depression in maximum force obtained for neonatal rat heart in the same study.

Depression of maximum force appears to be elicited via a different mechanism to that which alters myofibrillar Ca^{2+}-sensitivity: the addition of Ca^{2+} does not reverse the pH-induced depression of maximum force (Fabiato & Fabiato, 1978); and although fibres from adults and neonates differ in the effects of pH on their Ca^{2+}-sensitivity, the effect of pH on maximum force is the same for both fibre types (Solaro et al, 1988). Maximum myosin ATPase activity of cardiac myofibrils may be affected more than skeletal ones by a reduction in pH. Kentish & Nayler (1979) obtained a suppression of maximum ATPase activity of 20% for rabbit ventricular isolated myofibrils as pH changed from 7.20 to 6.40 whereas there was no observable effect in rabbit gastrocnemius under the same conditions. Blanchard & Solaro (1984) found no significant differences in maximum ATPase activity for canine cardiac myofibrils at pH 7.0, 6.5 and 6.2, and in a preliminary study Blanchard et al (1987) also found no effect of a decrease in pH from 7 to 6.5 on maximal Ca^{2+}-activated ATPase of rabbit psoas or soleus myofilaments. In contrast, Godt & Kentish (1989), found relative decreases in maximum force and MgATPase activity of both skinned rabbit psoas and rat trabeculae were similar when pH fell from 7.0 to 6.5. In skeletal fibres maximum force fell by 24% and MgATPase activity by 17% whereas corresponding values for trabeculae were 38% and 30% respectively. Thus acidosis apparently reduces maximum ATPase activity in skinned fibre preparations.

The mechanism of the pH-induced effects on maximum force is unknown. Nosek et al (1987) suggested that a reduction in the pH of skinned skeletal fibres would cause inorganic phosphate to shift its equilibrium from the HPO_4^{2-} to the $H_2PO_4^-$ form and, since the latter is thought to be responsible for the Pi-induced suppression of maximum force, this would enhance the direct depression caused by pH. No evidence has so far been found for this effect in cardiac fibres (Kentish, 1991). Since pH may have a smaller effect on maximum ATPase activity than on force it seems possible that both crossbridge cycling rate and force generated by each crossbridge are decreased in acidosis. This view was supported by the results of Fabiato & Fabiato (1978) who found that Ca^{2+}-independent rigor tension was reduced when pH fell from 7.0 to 6.2. This indicated that the force per crossbridge was lowered by acidosis at least under conditions when crossbridge detachment was prevented. More recently, preliminary work by Kentish & Palmer (1989) demonstrated, using instantaneous stiffness measurements at maximal activation, that the number of attached crossbridges was decreased by a reduction in pH from 7.4 to 6.2. In addition, as

pH fell the number of bound crossbridges fell by a smaller proportion than force, indicating that the remaining attached crossbridges were each generating less force than at the control pH, i.e. there was a decrease in mean force per attached crossbridge. Finally, Blanchard et al. (1987), suggested that the ATPase activity/force ratio could be used as an indicator of the energy cost of force maintenance. At maximum activation the energy cost of contraction in psoas fibres was increased by a reduction in pH since force fell while ATPase activity remained stable (though the latter was only measured in myofibrils where sarcomere arrangement is variable). Thus it appears that in this case crossbridge cycling was unaffected by pH whereas the mean force per crossbridge presumably decreased. This provides evidence for the existence of at least two states of strongly bound crossbridges: one state in which normal force is generated and a second in which cycling continues but force production is reduced (for example by decreases in pH). Godt & Kentish (1989) noted that maximum ATPase activity and force of skinned striated muscle fibres decreased in acidosis to similar extents. It therefore appears that in skinned muscle the ATPase activity/force ratio is unchanged by a fall in pH. In this case it would be expected that only crossbridge cycling not mean force per crossbridge would have fallen.

ii) *Ca^{2+}-Sensitivity*

Acidosis had a far greater effect on Ca^{2+}-sensitivity of cardiac than of skeletal muscle with respective shifts in pCa_{50} of 1.09 and 0.55 units towards higher Ca^{2+} concentrations. Cooperativity (as indicated by H_n) was unaffected by pH changes. Many studies have demonstrated a decrease in the pCa_{50} of force development as pH is lowered in cardiac skinned fibres. Donaldson & Hermansen (1978) found the effects of pH varied in extent according to muscle type: a fall in pH from 7.0 to 6.5 decreased the pCa_{50} of the force-pCa relationship by 0.15 units in soleus, 0.23 units in adductor magnus and 0.34 units in cardiac fibres from rabbit. Similarly, Fabiato & Fabiato (1978) measured a greater desensitising effect of acidosis in rat permeabilised cardiac cells than in frog skeletal fibres (a change in pH from 7.00 to 6.20 resulted in pCa_{50} shifts of 0.5 and 0.3 units respectively). No significant changes in the steepness of the force-pCa relationship were detected in any of the above studies.

The change in pCa_{50} per unit change in pH in Solaro et al's (1988) work was 1.22 for adult rat trabeculae (2 mM Mg^{2+}) whereas it was 0.68 for adult rabbits (1 mM Mg^{2+}) (Donaldson & Hermansen, 1978). Fabiato & Fabiato (1978) obtained a corresponding value of 0.63 for rat cardiac cells (0.3 mM Mg^{2+}) compared to 1.36 in the present study (1 mM Mg^{2+}). The discrepancy between Donaldson & Hermansens' results and those of Solaro et al and the present study

may reflect a species difference. Fabiato & Fabiatos' small pH effect could relate to their low [Mg^{2+}] although Donaldson & Hermansen (1978) showed that increasing [Mg^{2+}] from 1 mM to 10 mM *reduced* the pH-induced shift in pCa_{50} to 0.32 units. In both the current study and that of Fabiato & Fabiato (1978) SL was controlled at 2.1-2.3 μm and the same pH change was studied (thereby eliminating differences caused by possible non-linearity of the relationship between ΔpCa_{50} and ΔpH). One possibility is that Fabiato & Fabiato used younger rats (acidosis-induced decreases in Ca^{2+}-sensitivity increase with age; Solaro et al, 1988) though their rats' weights were similar to those in the current study. Alternatively use of imidazole as a pH buffer by Fabiato & Fabiato (and Donaldson & Hermansen) may reduce the effects of pH.

Canine and rat neonatal heart tissues are less sensitive to acidosis than the corresponding adult forms (Solaro et al, 1986; Solaro et al, 1988). In skinned rat myofibrils, for example, reducing the pH from 7.0 to 6.5 caused pCa_{50} to fall by 0.61 units in adult tissue but only by 0.27 units in neonatal muscle. It appeared that the different pH-sensitivities of adult and neonate tissues, and possibly cardiac and skeletal myofilaments, were due to differences in the thin filament proteins or troponin complex. Solaro et al (1986, 1988) suggested the responsibility may lie with TnI since it has different isoforms in neonates and adults and in cardiac and skeletal muscle. TnC was also considered a candidate for the differential action on cardiac and skeletal muscle since the cardiac and skeletal forms are different and it is directly involved in Ca^{2+} regulation of the myofilaments. The role of TnC in the effect of pH on Ca^{2+}-sensitivity was therefore tested in the present study using the isolated cardiac and skeletal proteins (chapter 3).

c) Effects of Pi

i) *Maximum Force*

20 mM Pi reduced the maximum forces of skeletal and cardiac skinned fibres by 42.4% and 57.4% respectively. These values were broadly similar to those obtained in previous studies. Brandt et al (1982) showed that 15 mM Pi caused maximum force to fall by 31% in rabbit psoas. Kentish (1986) found that 20 mM Pi reduced maximum force of rat ventricle by 69% on average though there was considerable scatter (see below). In 1989 Godt & Nosek showed that 20 mM Pi suppressed maximum force of skinned rabbit psoas and trabeculae by 30 and 55% respectively.

More recently, Stienen et al (1990) found that 15 mM Pi reduced the maximum force of frog anterior tibialis fibres by 36%. They also discovered that

the size of the force decrease depended not only on the concentration of Pi in the solutions but also on the size of the fibres used. Depression of force in thick fibres was smaller than in bundles of myofibrils dissected out of the fibres. This confirmed the observations made by Kentish (1986) that Pi accumulation (0.1 - 0.3 mM in trabeculae, twice as much in skeletal fibres) resulting from ATP hydrolysis and CP breakdown would be greatest in larger fibres, thus a change in Pi concentration in the bathing solution would have a lesser effect in these fibres than in thinner ones. This was reflected in the variation in the sizes of the force depression caused by 20 mM Pi (40-80%) which was fairly well correlated with fibre diameter (Kentish 1986). The accumulation of Pi would also be expected to be greater in skeletal than in cardiac fibres since the former have a higher ATPase activity - this may explain the smaller effects of Pi on maximum force of skeletal fibres.

It has also been suggested that Pi may suppress maximum force by reducing the free energy available for work (A) when ATP is hydrolysed (Dawson et al, 1978):

$$A = -(\Delta G^{\circ} + RT\ln([ADP][Pi]/[ATP]))$$

where ΔG° = apparent standard free energy change under stated conditions,
R = gas constant
T = temperature in Kelvins

Since A will tend to decrease as [ADP] or [Pi] increase, Kentish (1986) was able to test the hypothesis by seeing if increasing amounts of ADP elicited the same effects on maximum force as did raising Pi levels. He showed that 5 mM ADP actually potentiated the force by 7% in contrast to a 39% suppression by 5 mM Pi. In addition experiments where either 1 mM Pi and 5 mM ADP or 5 mM Pi and 1 mM ADP were applied simultaneously (thereby keeping [ADP][Pi] constant) resulted in a greater suppression of force when the Pi concentration was 5 mM.

It appears that Pi acts on the force-generating step of the crossbridge cycle. By reversing the transition of crossbridges from the weak-binding to the strong-binding (force producing) state, Pi could reduce the maximum attainable force (Webb et al, 1986). Hibberd et al (1985) used caged ATP to trigger relaxation of psoas fibres from rigor. The rate of relaxation was higher in the presence of Pi indicating that the rate of change of the crossbridges from the strongly bound to the weakly bound state had been increased. If more crossbridges are in the non-force generating form then ATPase activity would be expected to decrease. Kawai et al (1987) presented evidence that ATPase activity was reduced in the

presence of Pi. Thus it is likely that Pi does induce transition of crossbridges from the strongly bound to the loosely attached state.

The fact that Pi seems to have a greater suppressive action in cardiac than skeletal muscle may be due to a greater susceptibility of the cardiac MgATPase to Pi. Alternatively it may be due to an artifact caused by more Pi accumulation in skeletal fibres.

ii) *Ca^{2+}-Sensitivity*

When these experiments were done, the current study was, as far as it is known, the first in which the Ca^{2+} desensitisation by Pi was directly compared for skeletal and cardiac skinned fibres. Subsequently Godt & Nosek (1989) reported that 20 mM Pi shifted the pCa_{50} of psoas by 0.25 and trabecula by 0.32 units in rabbit. Their results contrasted with those of the present study where 20 mM Pi shifted the midpoint of the force-pCa relationship by 0.30 units in rabbit psoas and 0.19 units in rat trabeculae. Thus the Ca^{2+}-activation of skeletal muscle was more sensitive to Pi than cardiac preparations, but in neither case did Pi affect the steepness of the curve (H_n). Brandt et al (1982) found that raising the level of Pi from zero to 15 mM in rabbit skinned psoas fibres caused a fall in pCa_{50} of 0.25 units. Kentish (1986) demonstrated similar effects in skinned rat trabeculae: increasing amounts of Pi in the millimolar range decreased the pCa_{50} with the greatest effects occurring between zero and 10 mM Pi. 20 mM Pi caused a rightwards shift in pCa_{50} of 0.38 units. Only in Brandt et al's (1982) study was an effect of Pi on the steepness of the force-pCa relationship observed: a rise in Pi from zero to 7.5mM was accompanied by an increase in Hill 'n' from 3.7 to 5.1. However, it would probably be unwise to attempt quantitative comparisons between the results obtained by different laboratories where the solutions used were different. Kentish (1986) used solutions very similar to those in the current study, the main differences being his inclusion of DTT, creatine kinase and 3 mM Mg^{2+} instead of 1 mM. The reason why he observed a greater effect of Pi on pCa_{50} of rat trabecula is unclear but may be related to his use of a higher free magnesium concentration.

In addition, the current results contrast with those of Godt & Nosek (1989) who found that Pi had a greater Ca^{2+}-desensitising effect in cardiac than skeletal fibres. Both studies used 1 mM Mg^{2+}. The possibility that the trabeculae in the present study had a reduced ΔpCa_{50} in the presence of Pi because of Pi accumulation in the preparation was discounted as the size of the preparations was no different from that used by Godt & Nosek. Interestingly Godt & Nosek used rabbit trabeculae as opposed to those from rat so the difference between their results and those in the current study may relate to species differences: rat

cardiac muscle may be more resistant to Pi.

It seems increasingly likely that Pi somehow affects the state of the crossbridges, forcing a high proportion into the weakly bound configuration. This is discussed further in chapter 6.

d) Effects of Caffeine

i) *Maximum Force*

20 mM caffeine was found to reversibly depress maximum force by 18.6% in skeletal and 15.6% in cardiac muscle. These effects were greater than those observed by Wendt & Stephenson (1983), using rat EDL (suppression = 8%), soleus (9%) and trabeculae (6%) with 20 mM caffeine and SL 2.4 μm. These authors noted that the effect of caffeine on maximum force was concentration dependent with no depression occurring with less than 20 mM caffeine, and approximately 30% suppression induced by 40 mM caffeine. De Beer et al (1988), suggested that 10 mM caffeine reduced maximum force of rabbit gracilis or papillary muscle (it was not stated which!). Furthermore the effect was dependent on sarcomere length: at SL 1.4 μm the depression was 2% of maximum force, at SL 2.0 μm it was 4% and at 2.8 μm 20%. Since the current study employed a SL of 2.1 μm for trabeculae and 2.4 μm for rabbit psoas it might be expected that the latter would show greater depression by caffeine than trabeculae, simply because of the different SLs. A slightly greater suppression was indeed observed in psoas fibres. However, later experiments indicated that the reduction of maximum force by caffeine was independent of SL (see section 2.3.1.d.i).

The mechanism of suppression of maximum force by caffeine may involve an alteration in myofilament lattice spacing similar to that induced by other apparently inert substances such as some sugars (a suggestion first published by Wendt & Stephenson, 1983). De Beer et al (1988) observed that caffeine caused myofibrils to assume a swollen barrel-like appearance (increasing myofilament spacing) in transmission electron micrographs. Godt & Maughan (1981), however, showed that increasing fibre width by 20% did not change peak tension, thus changes in lattice spacing do not necessarily alter maximum force.

A reduction in maximum force could result from a fall in the tension generated within each crossbridge or from a drop in the proportion of crossbridges in the strongly bound (force producing) state. The depression of force is not related to changes in Ca^{2+}-affinity of the contractile apparatus since increasing the levels of Ca^{2+} does not reverse the effects of the suppression, and in any case caffeine has a concomitant Ca^{2+}-sensitising action on the

myofilaments.

The effect of caffeine on maximum Ca^{2+}-activated force does not appear to be related to the double bonded 9-position nitrogen thought to be involved in Ca^{2+}-sensitisation (section 6.2) since similar imidazole-containing compounds with N also at the 9-position elicit different effects on peak force. Sulmazole (1 mM) enhanced maximum tension by more than 20% (Harrison et al, 1986; Herzig et al, 1981) compared to 3.5% by 15 mM carnosine or N-acetyl histidine (Harrison et al, 1986). On the other hand 15 mM imidazole suppressed maximum force by 20% (Harrison et al, 1985).

ii) *Ca^{2+}-Sensitivity*

Caffeine also had a Ca^{2+} sensitising action on the skinned fibres but the increase in pCa_{50} was three times greater in cardiac muscle (0.31) than fast skeletal muscle (0.09). This supported the findings of Wendt & Stephenson (1983), in which 20 mM caffeine increased the pCa_{50} of rat trabecula by 0.32 units, of soleus by 0.30 units and EDL by 0.15 units. Thus caffeine has a greater Ca^{2+} sensitising effect in cardiac and slow twitch muscles than in skeletal preparations. The mechanism of the Ca^{2+} sensitisation of striated muscle by caffeine remains subject to debate. Wendt & Stephenson (1983) ruled out the possibility that cAMP-dependent protein kinase was involved since theophylline (a more potent phosphodiesterase inhibitor than caffeine) had similar effects to caffeine on Ca^{2+}-sensitivity of skinned fibres. Likewise the effects of caffeine were not modified by the addition of up to 0.5 mM cyclic AMP in the presence or absence of cAMP dependent protein kinase. In addition the same study with cardiac fibres showed no effects on the caffeine-induced pCa_{50} shift of a decrease in temperature from 20 - 5°C; a lowering of free Mg^{2+} from 1 mM to 0.1 mM; omission of the ATP regenerating system; or the presence of 1 mM azide or 5 mM procaine. The force-pSr relationship was affected by caffeine in the same way as the force-pCa one. Increasing the ionic strength (using KCl) from 225 mM to 385 mM reduced the effect of 10 mM caffeine on the change in pCa_{50} from 0.20 to 0.13 units. Wendt & Stephenson therefore suggested that caffeine could act by inhibiting the binding of ions which would normally compete with Ca^{2+} at the force controlling site. This would not only increase the apparent affinity of Ca^{2+} for this site but would also be consistent with the finding that an increase in the concentration of ions present (at ionic strength 385) decreased the effect of caffeine.

It is also possible that caffeine could alter the environment of the regulatory site of TnC, thereby affecting its Ca^{2+} affinity more directly. This might also explain why caffeine sensitised cardiac fibres to Ca^{2+} to a greater extent than

skeletal preparations since the two muscle types have different forms of TnC Wilkinson (1980). This possibility was investigated in the current study by testing the effects of 20mM caffeine on Ca^{2+} binding to isolated cardiac and skeletal TnC (chapter 3).

iii) *Role of SL in Caffeine Response*

During the experiments there was a steady decline in the maximum force generated by fibres. This was independent of the presence of caffeine and the SL, and presumably occurred by a similar means to that described in the previous control section. In contrast, however, to the results obtained in section 2.2.2.f, if SL was altered between the two runs (1 & 3) at the original SL the Ca^{2+}-sensitivity of the fibres decreased. The pCa_{50} for run 2 was obviously different because of the change in SL as well as any fall in Ca^{2+}-sensitivity such as that observed above. The mechanism of the desensitisation of psoas to Ca^{2+} was probably not related to time since previous experiments (section 2.2.2.f) have shown Ca^{2+}-sensitivity of psoas to remain constant over several hours. The change in pCa_{50} between runs 1 and 3 was also independent of SL since similar changes in Ca^{2+}-sensitivity were observed in fibres set at low and high SLs. The fall in Ca^{2+}-sensitivity between runs 1 and 3 was the same in the presence and absence of caffeine. Two possible explanations remain: the first is that the desensitisation of the fibres was caused by the change in experimental conditions (pH 7.22, 15°C) from those used in earlier experiments (section 2.2.2.a). This is unlikely because no drop in Ca^{2+}-sensitivity was observed for fibres tested in control solution after testing at pH 6.20, and a reduction in temperature would be expected to stabilise fibres if anything. The second explanation is that changing SL irreversibly alters the Ca^{2+}-sensitivity of psoas fibres such that a return to the same SL still results in a decreased sensitivity of the fibre to Ca^{2+}. It is not clear why this should be the case, nor has this been observed in other studies. It was assumed that the decline was linear, so that the test pCa_{50} could be compared to the pCa_{50} of the two controls.

Fortunately the degree of sensitisation of the fibres to Ca^{2+} by 20 mM caffeine remains unaffected by the loss of sensitivity to Ca^{2+} of the controls in runs 1 and 3. Thus the dependence of the Ca^{2+} sensitising effect of caffeine on SL could still be investigated. It was found that the shift in pCa_{50} induced by caffeine in a fibre was similar whatever the SL although there was some variation between fibres in the extent of Ca^{2+} sensitisation (fig.11). No significant difference was found (using the paired t-test) between caffeine-induced shifts in pCa_{50} at different SLs for each fibre. Thus the effect of caffeine was independent of SL in the range 1.5 - 2.8 μm. The difference in caffeine-sensitivity of cardiac

and skeletal muscles is therefore not due to the longer SL of the latter.

These results were in apparent contrast to those of De Beer et al (1988) who concluded that the caffeine effect decreased as SL increased, and that there was no effect of caffeine on pCa_{50} at SL 2.8 μm. Unfortunately these authors failed to correct the relative forces for the variations in depression of maximum force at the different SLs. In fact correction of the results of De Beer et al (1988) for the depression of maximum force by 10 mM caffeine yields shifts in pCa_{50} of 0.30 at SL 1.4 μm, 0.16 at 2.0 μm and 0.14 at 2.8 μm. These results were probably obtained from skeletal (gracilis) preparations since it is unlikely that cardiac muscles could be stretched to a SL of 2.8 μm without causing extensive damage. Thus changes in SL between 2.0 and 2.8 μm may not affect the Ca^{2+}-sensitisation by caffeine. De Beer et al probably measured resting SL i.e. before and after activation so their results could be inaccurate given that inconsistencies in SL may develop during contraction. Thus the results of the current study are far more convincing since SL was kept constant during activation. In addition the use of 20 mM caffeine, instead of the 10 mM used by de Beer et al, increased the size of the caffeine-induced shift in pCa_{50} so that significant differences were more likely to be detected.

Interestingly the suppression of maximum force by caffeine increased steadily throughout each experiment. The extent of suppression (34% on average) was greater than that observed previously with skinned psoas fibres (section 2.3.1.d), and was independent of SL. Although maximum force declined steadily during the experiments the increase in suppression by caffeine was not related to the size of the control maximum force since changing SL did not alter the effect of caffeine. The fact that, at least in skeletal muscle, SL had no effect on the Ca^{2+}-sensitising action of caffeine indicated that the differences in this action observed between cardiac and skeletal fibres were unlikely to be the result of the different *in vivo* SLs of these muscle types. The possibility remained, however, that differences between skeletal and cardiac TnC moieties may be responsible for the greater effect of caffeine in cardiac muscle.

2.5 SUB-CONCLUSIONS

a) Acidosis desensitised both cardiac and skeletal skinned fibres to Ca^{2+}, the fall in pCa_{50} being greater in the former than the latter. Maximum force was also suppressed more in cardiac fibres, the drop in tension probably resulting from decreases in both crossbridge cycling rate and force generated per crossbridge.

b) Pi had a larger Ca^{2+}-desensitising effect on skeletal than cardiac muscle, but depressed maximum force more in cardiac than skeletal fibres. The suppression of maximum force by Pi may be caused by a shift in equilibrium of crossbridge state from the strongly attached to the weakly bound form.

c) Caffeine increased the Ca^{2+}-sensitivity of cardiac preparations and, to a lesser extent, skeletal fibres but this difference was not related to the different active SLs of the muscles. Caffeine suppressed maximum force similarly in both muscle types.

d) Acidosis, Pi and caffeine may alter Ca^{2+}-sensitivity by directly influencing Ca^{2+} binding to TnC, by affecting other components of the Tn complex, or by direct actions on the crossbridges. The suppression of maximum force is governed by a different mechanism to that changing Ca^{2+}-sensitivity.

CHAPTER 3: INFLUENCE OF pH, Pi AND CAFFEINE ON CALCIUM BINDING TO ISOLATED TROPONIN C

3.1 INTRODUCTION

3.1.1 BACKGROUND

One explanation for the Ca^{2+}-sensitising effects of caffeine and desensitising effects of Pi and low pH in skinned fibres (discussed in chapter 2) could be that such agents act directly on the troponin C to alter its Ca^{2+} affinity. To investigate this, I measured Ca^{2+} binding to isolated TnC, labelled covalently with the fluorescent probes DANZ or IAANS for skeletal or cardiac TnC respectively.

3.1.2 FLUORESCENCE

It is generally better to use fluorescence rather than absorbance to follow binding of ligands or conformational changes in molecules. Many molecules will absorb light but only those labelled appropriately will emit visible radiation (fluorescence), thus the latter is a more specific parameter.

a) Factors affecting Fluorescence Measurement

a)Since some of the absorbed energy is dissipated by internal conversions, the fluorescence emitted by a molecule will have a lower energy and hence a greater wavelength (Planck's law) than the absorbed radiation. It is advantageous to maximise this difference (Stoke's shift) between excitation and emission wavelengths so the exciting light will not interfere with fluorescence measurement. Both DANZ and IAANS (see below) have much shorter optimum excitation wavelengths (340 and 328 nm respectively) than emission wavelengths (520 and 450 nm). Use of narrow band width filters to restrict the emitted light collected also reduces interference from exciting light.

b)Scattering of exciting and emitted light by particles in the light path will cause a reduction in intensity of emitted light reaching the exit slit. Tyndall scattering varies according to the inverse square of the wavelength. Use of as long an excitation wavelength as possible is therefore advisable to minimise scattering as well as absorbance of light by the solvent. This has to be balanced against the need for a large Stoke's shift (see above). To minimise the effects of scattering, and to avoid "contamination" by non-absorbed exciting light, emitted fluorescence was collected perpendicular to the exciting light.

c)The concentration of fluorescent molecule also governs the intensity of fluorescence measured. Fluorescence increases linearly with concentration of

absorbing molecules when the latter is low, then saturates and finally decreases s concentration increases further. The fall in fluorescence is attributed to increased amounts of the exciting light being absorbed by molecules furthest from the exit slit - the resulting fluorescence may then be too far from the slit to be collected efficiently. Such "self-quenching" can be avoided by using low concentrations of labelled protein: 1-3 μM are commonly employed with labelled TnC.

d)Agents which alter the environment of a fluorescent probe on a molecule can also affect fluorescence intensity. Quenching agents e.g. heavy metal ions donate electrons to fill the ground state of an excited probe thus blocking the return of the original electron. This obviously reduces fluorescence as do rises in temperature (by increasing internal conversion); drastic changes in pH (by altering the ionic state and or conformation of the labelled molecule) and chemical modifications of fluorescently labelled proteins (where attachment of the probe may be destabilised or its local environment altered). To avoid such problems standard conditions of 25°C and pH 7.00 were used (except where the effects of pH on Ca^{2+} binding to TnC were investigated).

3.1.3 TnC LABELLED WITH FLUORESCENT PROBES

a) Skeletal TnC-DANZ

The fluorescent probe N-dansylaziridine (DANZ) reacts at pH 6.5-8.6 with cysteine and other sulphydryl containing compounds whilst showing little, if any, reactivity towards weaker nucleophiles such as amides or alcohols (Scouten et al 1974). When removed from an aqueous medium and transferred to a hydrophobic environment, the fluorescence intensity of such probes as DANZ increases with an accompanying decrease in wavelength of the emission maximum. Although the probe was usually specific for thiol groups, Scouten et al suggested that binding of DANZ to weaker nucleophiles would be possible if the surrounding microenvironment was such that this nucleophility was increased.

In their 1978 study of skeletal TnC labelled with DANZ, Johnson et al proposed that the fluorescent probe was bound to the Met-25 residue. Skeletal TnC contains only one cysteine residue and this becomes buried on the binding of Ca^{2+} thus rendering it inaccessible to reaction with DANZ. The presence of at least four mol. Ca^{2+} per mol. TnC during incubation with DANZ doubled the affinity of the protein for the probe. This suggested that Ca^{2+} binding to the Ca^{2+}-specific sites of TnC exposed a hydrophobic pocket (phenylalanines 19, 23, 26 & 75) to which DANZ was attracted. This in turn positioned the reactive sulphonimide part of the probe close to the side chain of Met-25 thus promoting binding.

Confirmation that the DANZ label was binding to Met-25 was obtained by subsequent fractionation of the TnC-DANZ and analysis of the fluorescence containing portions. Most of the DANZ was bound to Met-25 although at DANZ : TnC incubation ratios of more than 0.5 mol./mol. the probe also seemed to bind to other sites. This extra labelling would have the unwanted effect of increasing background fluorescence without responding to Ca^{2+} binding to the Ca^{2+}-specific sites (Johnson et al, 1978).

Removal of Ca^{2+} from TnC-DANZ does not result in the dissociation of probe from the protein. The labelling of TnC is thus irreversible in these conditions and changes in TnC-DANZ fluorescence may be used to measure Ca^{2+} (and Mg^{2+}) binding to skeletal TnC. Such information is only of value if dansylation of the protein does not seriously disrupt its physical and biological properties. The consecutive increases in α-helix of TnC occurring on the binding of Ca^{2+} to the Ca^{2+}-Mg^{2+} and Ca^{2+}-specific sites can be measured by circular dichroism and were shown to be unaffected by the presence of the DANZ label (Johnson et al, 1978). The affinity constants for Ca^{2+} and Mg^{2+} binding to the low and high affinity sites of TnC-DANZ were the same as those measured by equilibrium dialysis techniques (Potter & Gergely, 1975). Thus Ca^{2+} and Mg^{2+} binding also remain unaltered by the labelling of TnC with DANZ. Rotational activity of the protein can be followed using fluorescence depolarisation spectroscopy which determines rates of rotation of proteins labelled with fluorescent dyes. Using this method TnC-DANZ was shown to rotate with the same characteristics as the unlabelled protein - dansylation therefore does not introduce new areas of flexibility or asymmetry into the protein. Nor does it significantly alter the 37 Å diameter of hydrated TnC (Johnson et al, 1978). The Ca^{2+} (or Mg^{2+}) induced changes in TnC fluorescence are therefore a true reflection of the situation in unlabelled TnC.

The binding of Ca^{2+} to the low affinity sites of skeletal TnC-DANZ caused fluorescence intensity to increase 2.1 fold with a concomitant blue shift of optimal emission wavelength from 524 to 514 nm (excitation was at 340 nm) - Johnson et al (1978). Ca^{2+} affinity for these sites was calculated to be $4x10^5$ M^{-1} which was almost identical to that measured by Potter and Gergely (1975). By comparing the observed Ca^{2+}-dependence of relative fluorescence with computer models assuming Ca^{2+} binding to one, either or both of the low affinity sites, Johnson et al predicted that occupancy of either Ca^{2+}-specific site could induce the observed changes in fluorescence. No effect of Mg^{2+} was apparent on TnC-DANZ fluorescence induced by Ca^{2+} binding to these low affinity sites. Ca^{2+} binding to the high affinity sites of TnC-DANZ caused a small ($\approx 20\%$) decrease in fluorescence.

Incorporation of TnC-DANZ into troponin altered the fluorescence responses to Ca^{2+} binding such that the addition of sufficient Ca^{2+} to saturate the Ca^{2+}-specific sites resulted in only a 10% increase in fluorescence. Despite the difficulties involved in working with such a small response, Johnson et al (1978) were able to estimate a Ca^{2+} affinity of $3.3 x 10^6 M^{-1}$ for the Ca^{2+}-specific sites. Interaction of TnC with TnI and TnT thus increased the affinity of the Ca^{2+}-specific sites ten fold. These results correlated perfectly with those of Potter and Gergely (1975), confirming that the low affinity sites on TnC are most likely to regulate contraction.

b) Cardiac TnC-IAANS

Bovine cardiac TnC lacks the Met-25 residue of skeletal TnC and does not react significantly with DANZ (Johnson et al, 1978). Iodoacetic acid and its derivatives, e.g. IAANS, react more rapidly with -SH groups at pH 8.0 than with other nucleophilic side chains (Hirs, 1967). Care must, however, be taken to add only sufficient IAANS to react with the thiols available since at higher concentrations modification of other amino acids such as methionine, lysine, histidine and tyrosine is likely to occur.

Cardiac TnC contains two partially buried cysteine residues: Cys-35 in site I and Cys-84 in helix D (Fuchs et al, 1989). The binding of Ca^{2+} results in a change in conformation of TnC such that both cysteines become exposed to solvent. This allows the reaction of these cysteines with IAANS. Cys-35 and Cys-84 were equally labelled with IAANS, as demonstrated by fractionation of the resultant TnC-IAANS and analysis of the fluorescence-bearing peptides (Johnson et al, 1980).

As with skeletal TnC-DANZ a small decrease in wavelength of the emission maximum (from 454 to 452 nm) plus a large increase in fluorescence intensity occurred on the binding of Ca^{2+} to the Ca^{2+}-specific site (site II) of cardiac TnC-IAANS (Johnson et al, 1980). The excitation maximum occurred at a wavelength of 328 nm.

Labelling of cardiac TnC with IAANS did not affect the physical properties of the protein. The two increases in α-helix noted on Ca^{2+} binding to the low affinity site and Ca^{2+} or Mg^{2+} binding to the high affinity sites (as measured by circular dichroism) were the same in native and labelled TnC (Johnson et al, 1980). The affinity constants for Ca^{2+} and Mg^{2+} binding to the Ca^{2+} specific and Ca^{2+}-Mg^{2+} sites of TnC-IAANS were the same as those measured by equilibrium dialysis techniques (see below; Holroyde et al,1980). Dialysis of labelled and unlabelled TnC against $^{45}CaCl_2$ revealed an equal amount of Ca^{2+} binding in each case, thus the stoichiometry of Ca^{2+} to protein was also

unaffected by the IAANS probe (Johnson et al,1980). Any changes in fluorescence of TnC-IAANS induced by Ca^{2+} or Mg^{2+} binding are therefore a true indication of the behaviour of unlabelled cardiac TnC.

Ca^{2+} bound to the single low affinity site of bovine cardiac TnC-IAANS with a K_{Ca} of 4.5×10^5 M^{-1} and to the very low affinity sites with K_{Ca} 5×10^2 M^{-1} (Johnson et al,1980). A concomitant 2.1-fold increase in fluorescence was noted as pCa was decreased from 7 to 2, with 62% of the fluorescence caused by Ca^{2+} binding to the single low affinity site and 38% caused by Ca^{2+} interacting with the very low affinity sites. No effect of Mg^{2+} was apparent on TnC-IAANS fluorescence induced by binding at either of these.

In the absence of Mg^{2+}, Ca^{2+} binding to the two high affinity sites caused a decrease in fluorescence of at least 5% and probably in the region of 15% if the superimposed fluorescence increase as Ca^{2+} binds to low affinity sites is deducted (Johnson et al,1980). Mg^{2+} binds to the high affinity sites with a K_{Mg} of 3.5×10^3 M^{-1} (as compared to 1×10^3 M^{-1} measured by Holroyde et al,1980) and a 20% decrease in fluorescence.

As in the case of unlabelled cardiac TnC (Holroyde et al,1980) the binding of TnC-IAANS to TnI or its incorporation into the troponin complex resulted in a ten-fold increase in affinity of the low affinity Ca^{2+}-specific site, with a K_{Ca} of 3×10^6 M^{-1} (Johnson et al,1980). Ca^{2+} binding to this site was accompanied by a 20% decrease in fluorescence although no change in fluorescence occurred with Ca^{2+} binding to the very low affinity (K_{Ca} 5×10^2 M^{-1}) sites. Interaction of Mg^{2+} in the absence of Ca^{2+} (and probably Ca^{2+} in the absence of Mg^{2+}) with the high affinity sites caused fluorescence to fall by 7%.

3.1.4 EFFECTS OF pH ON TnC

Although the effects of acidosis on the Ca^{2+}-sensitivity of myofilaments had been fairly well established by 1987, when the current study was begun, (section 1.1.10), the possible role of TnC in the pH-sensitive step had not been confirmed. It seemed plausible that the Ca^{2+}-sensitivity of skinned fibres could be reduced by a lowering of the Ca^{2+} affinity of Tn at decreased pH (Katz & Hecht, 1969). The existence of a direct involvement of TnC was first implied in preliminary experiments by Robertson et al. (1978). They showed a fall in pH from 7.5 to 6.5 shifted the pCa-fluorescence curve of TnC-IAANS rightwards by 0.8 units. This implied that the Ca^{2+}-specific site of cardiac TnC was pH sensitive. pH had no effect on the Ca^{2+}-induced tyrosine fluorescence of isolated bovine cardiac TnC (and hence no effect on Ca^{2+} binding to the Ca^{2+}-Mg^{2+} sites). Stull & Buss (1978) measured Ca^{2+} binding to isolated cardiac troponin and, although they found a reduction in Ca^{2+} affinity with a pH change from 7.2 to 6.5, this

appeared to be dependent on the presence of EGTA. In 1980 the situation was not made much clearer by the finding of Kohama that pH had no effect on Ca^{2+} binding to cardiac Tn when EGTA was used as the Ca^{2+} buffer, unless Mg^{2+} was absent.

The first study of the pH dependence of Ca^{2+} binding to TnC *in situ* in cardiac myofibrils was carried out in 1984 by Blanchard & Solaro. They measured the amounts of Ca^{2+} bound to canine skinned ventricular myofilaments and myosin using centrifugation techniques with $^{45}Ca^{2+}$ in both the presence and absence of EGTA. Independently of the presence of EGTA, a reduction in pH from 7.0 to 6.2 shifted the midpoint of the Ca^{2+} binding curve by 0.83 units towards lower pCas. Thus in cardiac myofibrils Ca^{2+} binding to TnC was reduced by decreasing the pH . However, there was a large potential error in the curve-fitting used to determine affinity constants from the binding data. A similar conclusion was reached for skeletal TnC: in a corresponding study using the same methods, reducing the pH from 7.0 to 6.2 decreased the pCa_{50} by 0.5 units (Blanchard et al, 1984).

In apparent contrast to the results of Blanchard et al, Ogawa (1985) showed that Ca^{2+} binding to the low affinity sites of isolated skeletal TnC was unaffected by a fall in pH from 7.2 to 6.5. The confusion in the literature over the effects of pH on the Ca^{2+} affinity of TnC made it important to study such effects further.

3.1.5 EFFECTS OF INORGANIC PHOSPHATE AND CAFFEINE ON TnC

Prior to the start of this study, there were no reports on the effects of Pi or caffeine on Ca^{2+} binding to TnC.

3.2 METHODS

3.2.1 PREPARATION OF LABELLED SKELETAL TnC

TnC-DANZ samples were kindly provided by R. J. Solaro in the form of freeze-dried (lyophilised) solid. TnC had been isolated from rabbit back skeletal muscle according to the method described in El-Saleh & Solaro (1988). Labelling with DANZ had been carried out following the method of Johnson et al (1978).

It was later found that the TnC was best stored in solution so the solid was dissolved in 100 mM BES, 67 mM KCl at pH 7.00 (4°C), frozen rapidly at -80°C then kept at -20°C.

3.2.2 PREPARATION OF LABELLED CARDIAC TnC

Isolation and fractionation of cardiac troponin was carried out in the lab. of Dr. P. Cummins (Birmingham).

a) Isolation of Troponin

Troponin was isolated from bovine heart (fig.13) according to the method of Tsukui & Ebashi (1973) with the following modifications. LiCl was only added once to the regulated actin with a single centrifugation at 7,000xg. The centrifugation at 100,000xg following solubilisation of the Tn pellet in 1 mM $NaHCO_3$ was also omitted (which would have resulted in a poorer yield). 80.9 mg Tn per 100 g muscle were obtained (concentration measured using an extinction coefficient for Tn of 3.8 at 280 nm (Cummins, pers.comm.).

b) Fractionation of the Tn Complex

Troponin was split into its subunits according to the method of Cummins (pers comm. fig.14) which was based on that of Greaser & Gergely (1971). The main differences from the latter were that urea was equilibrated with the chromatography resin both before and after loading onto the column, air bubbles being removed by vacuum suction, and NaCl was used instead of KCl for the salt gradient (KCl and SDS precipitate together). DEAE-cellulose was used instead of sephadex as the latter does not give complete separation of subunits. The procedure resulted in the separation of TnI, TnC, TnT and contaminant Tm with a yield of 8.9 mg TnC per 100 g muscle (11 mg TnC/100 mg Tn), as measured using an extinction coefficient of 3.0 for cardiac TnC at 276 nm (Szynkiewicz et al, 1985).

c) Labelling of Cardiac TnC with IAANS

TnC was labelled with IAANS as shown in fig.15, following the method of Johnson et al (1980). The ratio of TnC : IAANS after labelling was calculated from the absorbance of TnC-IAANS at 276 nm and 325 nm ($E_{276}^{1\%}$ TnC = 3.0, Szynkiewicz et al, 1985; E_{325} IAANS = 24,900 M^{-1}, Johnson et al, 1980)), and was never less than 1, indicating that the IAANS did not bind to sites additional to Cys-35 or Cys-84.

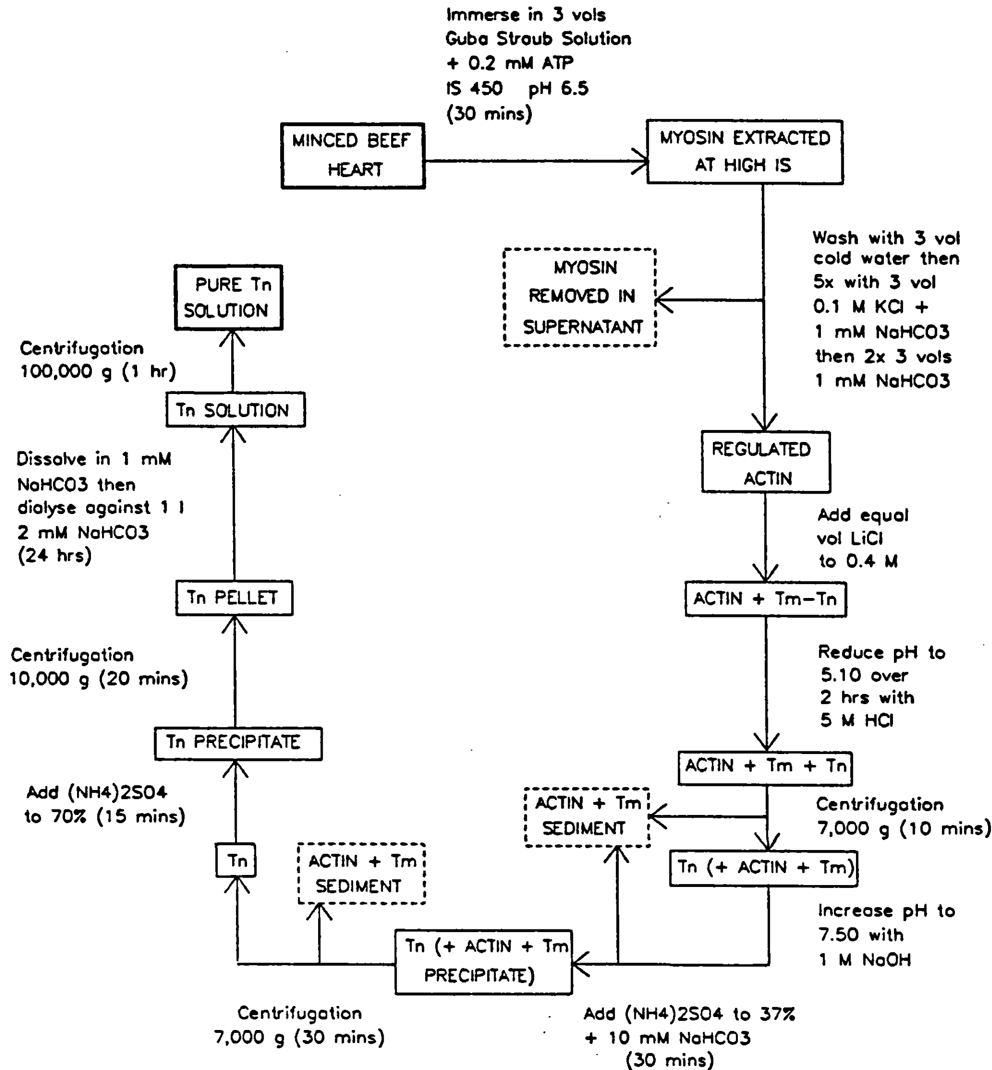


Fig.13 PREPARATION OF ISOLATED CARDIAC Tn. All steps were carried out at 4°C. Density of the muscle was assumed to be 1 g/ml for calculation of volumes. Guba-Straub solution contained 0.3 M KCl; 0.1 M KH_2PO_4; 0.05 M K_2HPO_4, pH 6.5 at 4°C. The duration of each step is indicated in brackets where necessary. The muscle was filtered through 2 pieces of sterile absorbent gauze, to remove myosin, after immersion in Guba-Straub solution and after each wash with cold water; 0.1 M KCl + 1 mM $NaHCO_3$; and 1 mM $NaHCO_3$. The supernatant from the first centrifugation was filtered through glass wool to remove traces of fat. The Tn pellet from the third centrifugation was dissolved in 1.5 ml 1 mM $NaHCO_3$ per 100 ml original muscle. Fractions enclosed in dashed-line boxes were discarded. The final pure Tn solution was stored at 4°C prior to its fractionation into subunits. IS = ionic strength (mM).

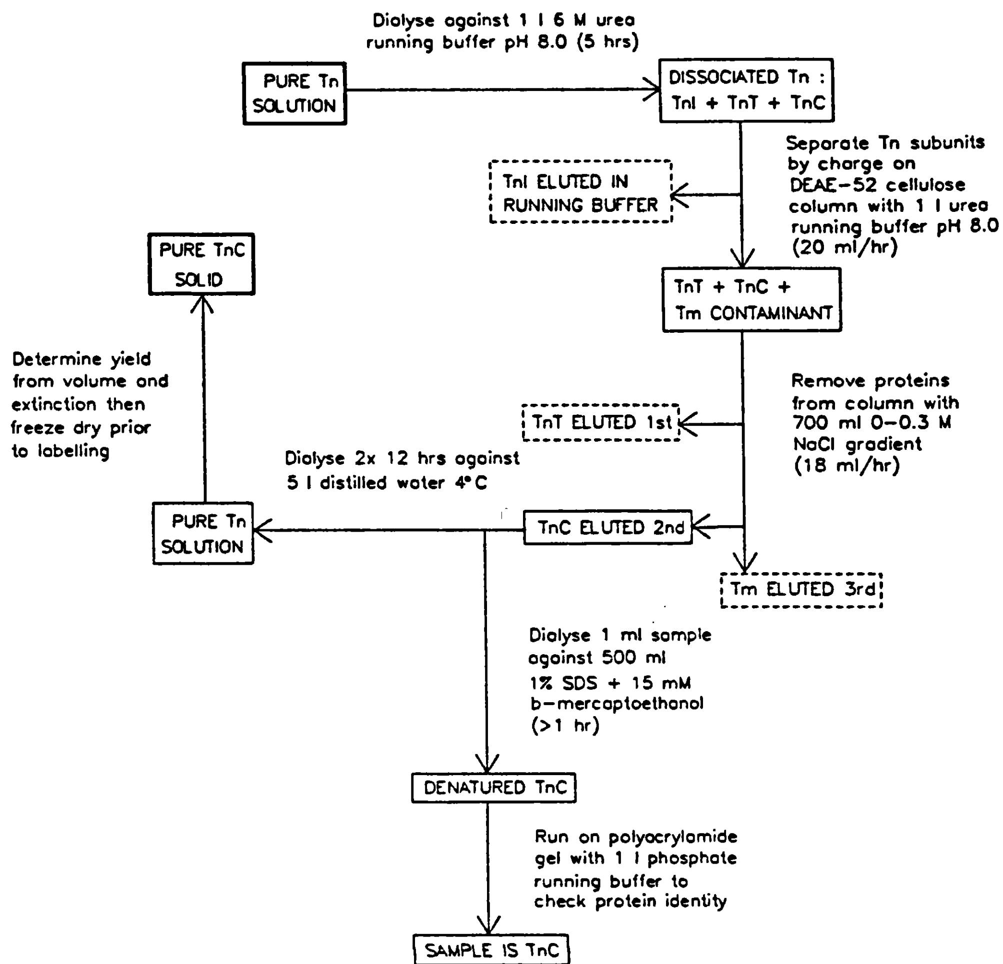


Fig.14 FRACTIONATION OF THE Tn COMPLEX. All steps were carried out at room temperature unless indicated. Urea running buffer consisted of: 6 M urea; 50 mM TRIS-HCl; 5 mM EGTA & 15 mMβmercaptoethanol. 8 M urea stock was deionised using Duolite MB6113 mixed exchange resin. DEAE-52 cellulose resin (Whatman, pre-swollen) was adjusted to pH 8.0 with NaOH, batch washed with urea running buffer then loaded onto a 25.0x2.4 cm LKB 2137 chromatography column and equilibrated overnight with 1 litre urea running buffer (20 ml/hr). The sample was loaded with 5 ml buffer wash-in. Proteins eluted by the NaCl gradient were collected in test tubes by an automatic sampler and identified through extinction readings. Gels consisted of 0.1% SDS; 0.1 M phosphate buffer, pH 7.0; 11.85% acrylamide; 0.16% bisacrylamide; 0.075% ammonium persulphate & 1.25×10^{-3}% TEMED. The running buffer was 0.1 M phosphate buffer + 0.1% SDS, pH 7.1. 40 μl samples boiled for 2 mins with sucrose & Bromophenol Blue (BPB) stain were loaded with a TnC marker. The gel was run at 50 mA for 100 mins then 150 mA until BPB reached the bottom of the gel. Protein bands were stained with Anderson's brilliant blue R. Fractions in dashed-line boxes were discarded.

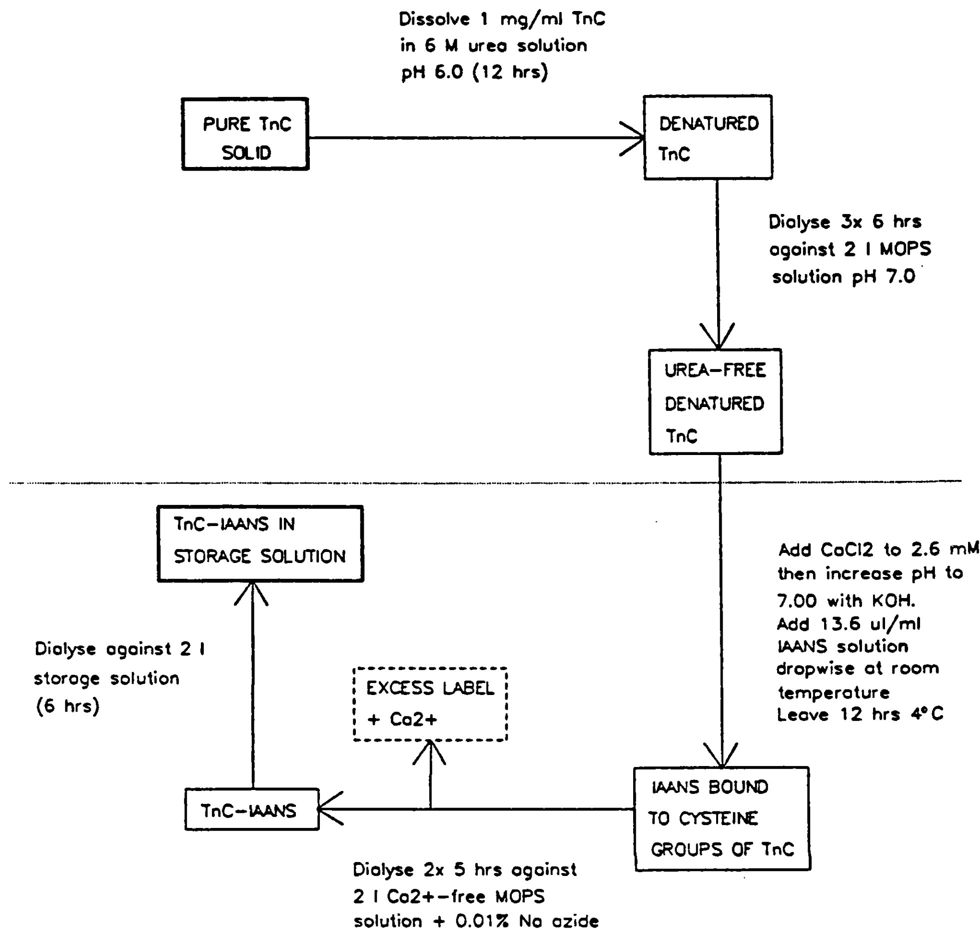


Fig.15 LABELLING OF CARDIAC TnC WITH IAANS. All stages were carried out at 4°C unless indicated. Steps below the dotted line were performed in the dark. 8 M urea stock was deionised using Duolite MB6113 mixed exchange resin. Urea solution contained: 6 M urea; 20 mM imidazole; 1 mM EGTA & 0.1 mM DTT, pH 6.00 at 4°C. MOPS solution contained: 10 mM MOPS; 90 mM KCl; 2 mM EGTA & 0.1 mM DTT, pH 7.00 at 4°C. IAANS solution consisted of 1 mg/ml IAANS (Molecular Probes) in 10 mM phosphate buffer, pH 7.00 at 4°C. Following addition of IAANS to the TnC the solution was left overnight at 4°C on a gentle rotator. Ca^{2+} was removed from MOPS solution by passing MOPS and KCl stocks down a Chelex column. Storage solutions were 10 mM MOPS; 40 mM KCl & 0.01% Na azide, pH 7.00 at 4°C (El-Saleh, pers.comm.) or 100 mM BES; 2 mM EGTA & 67 mM KCl, pH 7.00 at 4°C. TnC-IAANS solutions were divided into 100 or 500 μl aliquots, frozen at -80°C and stored at -20°C in foil-covered eppendorf tubes.

Skeletal	LOW IS	CONTROL	CAFFEINE	Pi	pH 6.2
BES	100.00	100.00	100.00	100.00	87.00
MES	-	-	-	-	13.00
EGTA	1.74	1.74	1.74	1.74	1.74
ATP	-	4.35	4.35	4.35	4.35
CP	-	8.70	8.70	8.70	8.70
KPr	-	85.26	85.26	55.68	69.60
$MgCl_2$	-	4.44	4.44	5.05	4.44
Mg^{2+}	-	1.15	1.15	1.10	1.12
KCl	67.00	8.71	8.71	8.71	8.71
CAFFEINE	-	-	20.00	-	-
KPi	-	-	-	20.00	-
TnC-DANZ	3.0 μM	3.0 μM	3.0 μM	3.0 μM	3.0 μM
pH	7.00	7.00	7.00	7.00	6.20
IS	114	202	202	209	194

Cardiac	LOW IS	CONTROL	CAFFEINE	Pi	pH 6.2
BES	87.00	87.00	87.00	87.00	-
MES	-	-	-	-	87.00
EGTA	1.74	1.74	1.74	1.74	1.74
ATP	-	4.35	4.35	4.35	4.35
CP	-	8.70	8.70	8.70	8.70
KPr	-	85.26	85.26	55.68	69.60
$MgCl_2$	-	4.44	4.44	5.05	4.44
Mg^{2+}	-	1.15	1.15	1.10	1.15
KCl	63.49	5.20	5.20	5.20	5.20
CAFFEINE	-	-	20.00	-	-
KPi	-	-	-	20.00	-
MOPS	1.30	1.30	1.30	1.30	1.30
Na AZIDE	0.20	0.20	0.20	0.20	0.20
TnC-IAANS	3.0 μM	3.0 μM	3.0 μM	3.0 μM	3.0 μM
pH	7.00	7.00	7.00	7.00	6.20
IS	105	186	186	199	184

TABLE 7. SOLUTIONS USED FOR Ca^{2+}-SENSITIVITY MEASUREMENTS OF ISOLATED SKELETAL TnC-DANZ AND CARDIAC TnC-IAANS. Amounts given are total mM concentrations. Ca^{2+} was added as aliquots of 100 mM $CaCl_2$ as described in section 3.2.3.b.

3.2.3 MEASUREMENT OF Ca^{2+} BINDING TO ISOLATED TnC: EFFECTS OF pH, Pi AND CAFFEINE

Ca^{2+} binding was assessed from the fluorescence increase as $CaCl_2$ was added sequentially to a solution containing EGTA and labelled TnC.

a) Solutions

Since previous studies of isolated TnC had used non-physiological low ionic strength solutions, similar solutions were prepared (table 7) to enable comparison between the current work and previous results. Johnson et al (1978) used a control solution of 10 mM phosphate buffer; 90 mM KCl & 2 mM EGTA, pH 7.00. A similar solution was therefore prepared but with the following changes: to reduce pH changes on the addition of $CaCl_2$ and to eliminate possible effects of Pi on TnC, the phosphate buffer was replaced by 100 mM BES. The concentration of K^+ was kept constant by reducing KCl concentration to 67 mM. Since the TnC-DANZ had been stored in 100 mM BES, 67 mM KCl solution, addition of TnC-DANZ to 3 μM caused dilution of the 2 mM EGTA to 1.74 mM. The calculation of free calcium took the EGTA dilution into account. Similar logic applied to TnC-IAANS control solution: the protein had been stored in 10 mM MOPS, 40 mM KCl, 0.01% Na azide therefore the addition of TnC-IAANS to 3 μM to the low ionic strength solution diluted the BES, EGTA and KCL accordingly.

In order to compare directly results obtained using isolated TnC with those obtained using skinned fibres, it was necessary to use very similar solutions in each case. Thus control, caffeine, Pi and pH 6.2 solutions (table 7) were prepared for fluorescence work from the same stocks and with similar accuracy to those for skinned fibre work (section 2.2.2.a). The addition of labelled TnC with its solvent caused a slight dilution of the solution constituents. The variations in concentrations of ATP, creatine phosphate and KPr between the solutions used for fibres and isolated protein experiments were too small to significantly affect Ca^{2+}-sensitivity. The only significant change from the skinned fibre solutions was the presence of 1.74 mM EGTA (instead of 10 mM in the skinned fibre solutions). This was necessary to reduce the change in pH on the addition of $CaCl_2$. The computer calculation of free calcium took all dilutions into account as well as the introduction of new ions with the TnC. The titration of each solution against $CaCl_2$ caused a gradual but small (maximum 0.08 units in control solution) fall in pH. This was measured by "scaling up" the addition of $CaCl_2$ to each of the experimental solutions. Aliquots (30 or 15 μl) of 100 mM $CaCl_2$ were added successively to 18 ml (15.66 ml experimental solution plus 2.34 ml TnC storage solution) each of the control, caffeine, pH 6.20 and Pi solutions, and the

pH was measured after every addition of $CaCl_2$. The measured decreases in pH were taken into account in calculations of free calcium, but were too small to affect the Ca^{2+}-sensitivity of the TnC itself. Free magnesium concentrations did not increase by more than 0.1 mM over the entire Ca^{2+} titration and this would not be sufficient to significantly affect the TnC. The effects on the EGTA concentration of dilution by the $CaCl_2$ added were calculated to have minimal effects on free calcium levels.

All solutions were stored at -20° C in 10 ml aliquots and were kept for a maximum of 2 months.

b) Protocol

Fluorescence was measured using a Perkin Elmer LS-5 Fluorimeter. For work with TnC-DANZ the settings were as follows: excitation wavelength 340 nm; emission wavelength 520 nm; excitation slit 10 nm; emission slit 5 nm; scan speed 240 nm/min; recording speed 10 nm/cm (response 2; Full Scale 1). The equivalent settings for TnC-IAANS were identical except that the excitation wavelength was changed to 328 nm and that of emission to 450 nm. Settings of scan speed, recording speed and response were specifically chosen to optimise the definition of the spectra (and later the point readings) whilst minimising the amount of noise. This was of especial importance in work with TnC-IAANS since the fluorescence was rather subject to fluctuations for no apparent reason. A solution of 100 mM BES; 67 mM KCl; 2 mM EGTA; 1 μM Fura-2 & 50 μM $CaCl_2$ employed as a "spot-check" standard between the TnC runs confirmed that the fluctuations in fluorescence of TnC-IAANS were not due to a drifting of some component in the fluorimeter. Fluorescence readings were time averaged over 4 seconds (sampled every 0.4 secs.) to increase the accuracy of the fluorescence readings obtained during titration of $CaCl_2$ against the labelled TnC.

The quartz cuvette held 0.6 ml solution and this was stirred continuously with a magnetic flea. To ensure complete mixing of solutions the cuvette contents were also twice sucked into a plastic pipette and vigorously expelled back into the cuvette after each addition of new solution. All experiments were performed at 25 ° C. Samples of labelled TnC stock solution were added to the experimental solutions in the cuvette to give a final concentration of 3 μM TnC. The remaining TnC stock was stored at 4° C in the dark prior to use.

Initial scans of the excitation and emission wavelengths were carried out at the start of each experiment to confirm that the optimum settings for the measurement of fluorescence had been chosen. Each experiment consisted of an initial control followed by the three test runs (in rotational sequence in different

experiments), always ending with a control to check reproducibility. Occasionally a control run was also included midway through the experiment. Temperature within the cuvette was monitored throughout at random intervals.

An initial average was taken of the fluorescence produced by the labelled TnC in the control solution. Aliquots (1.0 μl) of 100 mM $CaCl_2$ were added to the cuvette with a 1 μl Hamilton syringe and after each addition the solution was thoroughly mixed before an average fluorescence reading was again taken. The aliquots were reduced to 0.5 μl when the fluorescence began to change noticeably to improve the definition of the results. No more than 18 μl total $CaCl_2$ was added to the cuvette to avoid problems associated with excessive dilution. When the addition of $CaCl_2$ no longer resulted in a change in fluorescence, the procedure was halted and occasionally a further pair of complete scans of emission and excitation wavelengths was carried out. This enabled any shifts in the spectra induced by the presence of excess Ca^{2+} to be detected.

At the end of each run the cuvette and Hamilton syringe were thoroughly cleaned and dried. The whole titration procedure could then be repeated with fresh solutions (control or otherwise). The fluorescence of each of the control and test solutions before the addition of the TnC was also determined. Excitation and emission scans were performed on each solution using the same wavelengths and settings as for the TnC scans.

3.2.4 STATISTICAL ANALYSIS

Absolute fluorescence readings (or means) were expressed in terms of relative fluorescence using the formula:

$$\text{RELATIVE FLUORESCENCE} = \frac{\text{ABSOLUTE FLUORESCENCE - MINIMUM FLUORESCENCE}}{\text{FLUORESCENCE WITH } 12.5\,\mu\text{l } CaCl_2 \text{ - MINIMUM FLUORESCENCE}}$$

The scale of relative fluorescence thus ranged between zero and one. This normalisation enabled easy comparison between results from control and test runs. Correction for the proportional decreases in fluorescence produced on dilution of the labelled TnC by successive additions of $CaCl_2$ to 2.083 mM had no significant effect on relative fluorescence values and was therefore unnecessary. Such correction was only required where pCa was less than 2.5 ($>3\%$ dilution of TnC) and even at pCa 1.89 increased relative fluorescence by only 0.04.

pCa-fluorescence curves were fitted to the Hill equation. Changes in fluorescence of TnC-IAANS were triphasic (fig.18): relative fluorescence

decreased between pCas 9.0 and 6.2, increased sigmoidally between pCas 6.2 and 3.8, then increased further between pCa 3.8 and 1.5. For curfitting to the middle pCa range it was assumed that minimum fluorescence occurred where relative fluorescence was zero and maximum fluorescence after the addition of $CaCl_2$ to 2.083 mM (12.5 μl). The influences on these points of the fluorescence changes in the other pCa ranges was taken into account during curfitting by making minimum and maximum fluorescence variable.

3.3 RESULTS

3.3.1 LABELLING OF CARDIAC TnC WITH IAANS

Early attempts to label cardiac TnC with IAANS were beset with problems. Although the labelled protein was fluorescent, it initially showed no increase in fluorescence on the addition of Ca^{2+}. Thus although the TnC presumably had IAANS bound at the correct sites (the excitation and emission spectra were as expected for zero added Ca^{2+}), the binding of Ca^{2+} either failed to induce the expected change in conformation of the TnC or it failed to be correctly transmitted to the IAANS. The presence of excess Ca^{2+} during the incubation of TnC with the IAANS would have induced the optimum conformation of the protein for the binding of the label (by exposing hydrophobic groups - section 3.1.3). The IAANS should, therefore, have been firmly bound to the TnC and it is difficult to envisage a condition whereby all the fluorescent properties of the label are as expected except for the absence of a fluorescence enhancement when the environment of the probe becomes more hydrophobic (as occurs on Ca^{2+} binding to TnC). This strongly suggests that it is the structure of the TnC that is at fault rather than the IAANS itself.

A lack of fluorescence response to Ca^{2+} could be caused by the TnC failing to undergo the expected structural changes in the presence of Ca^{2+}. In a second attempt at labelling it was found that the inclusion of DTT in the dialysis solutions used after the labelling stage greatly improved the fluorescence enhancement by Ca^{2+}. This implies that the oxidation of sulphydryl residues was at least partly responsible for the lack of fluorescence increase and it may have "locked" the TnC molecule in a non-responsive conformation e.g. by the formation of disulphide bridges.

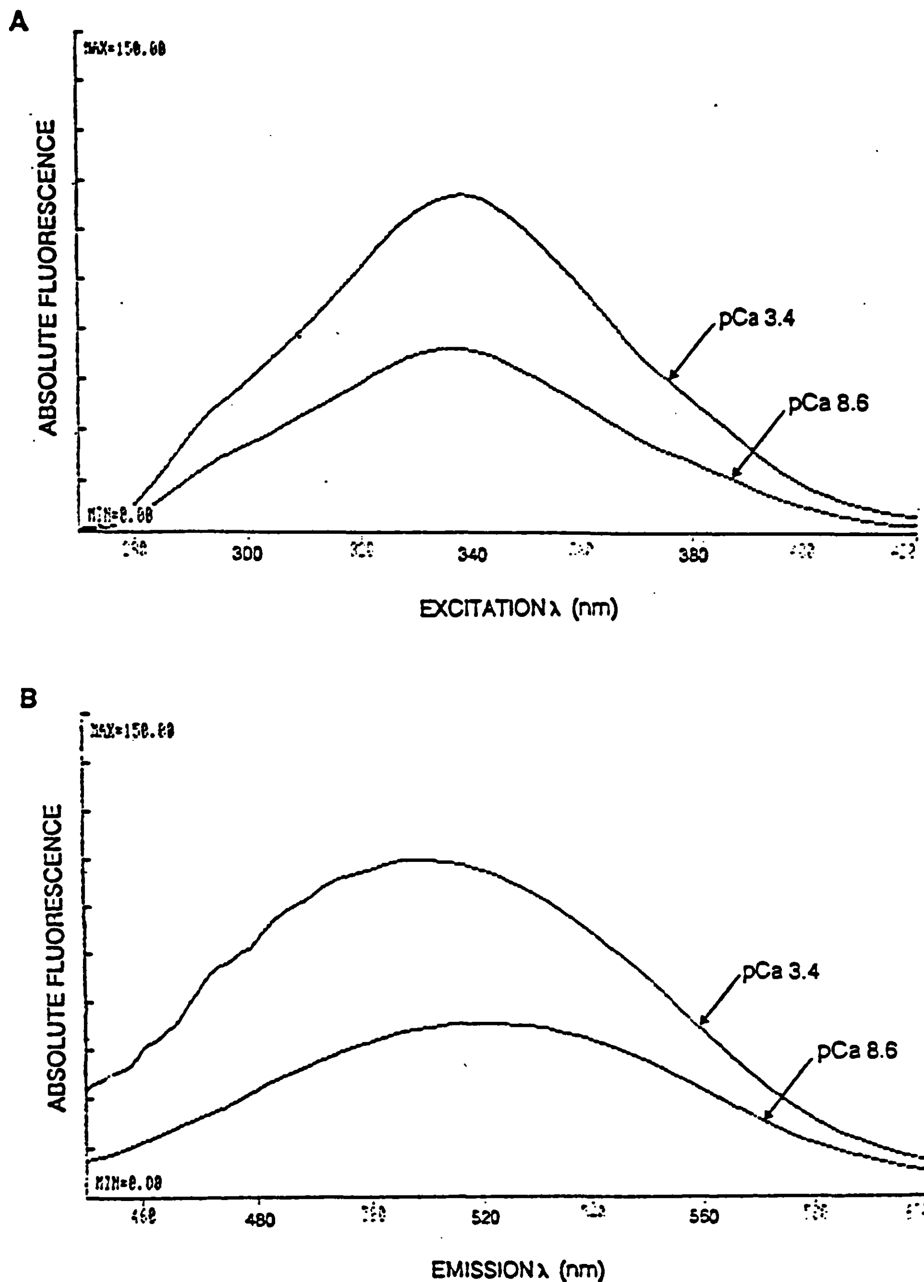


Fig.16 EFFECTS OF Ca^{2+} ON (A) EXCITATION AND (B) EMISSION SPECTRA OF ISOLATED TnC-DANZ. Fluorescence spectra were measured for 3 μM TnC-DANZ using the high ionic strength control solution described in section 3. 2.3.a, before and after the addition of sufficient Ca^{2+} to saturate all Ca^{2+}-binding sites of TnC. Fluorimeter settings were as in section 3.2.3.b except that in A) excitation wavelength was scanned over 270 to 420 nm with emission collected at 520 nm and in B) emission wavelength was scanned over 450 to 600 nm with excitation wavelength 340 nm.

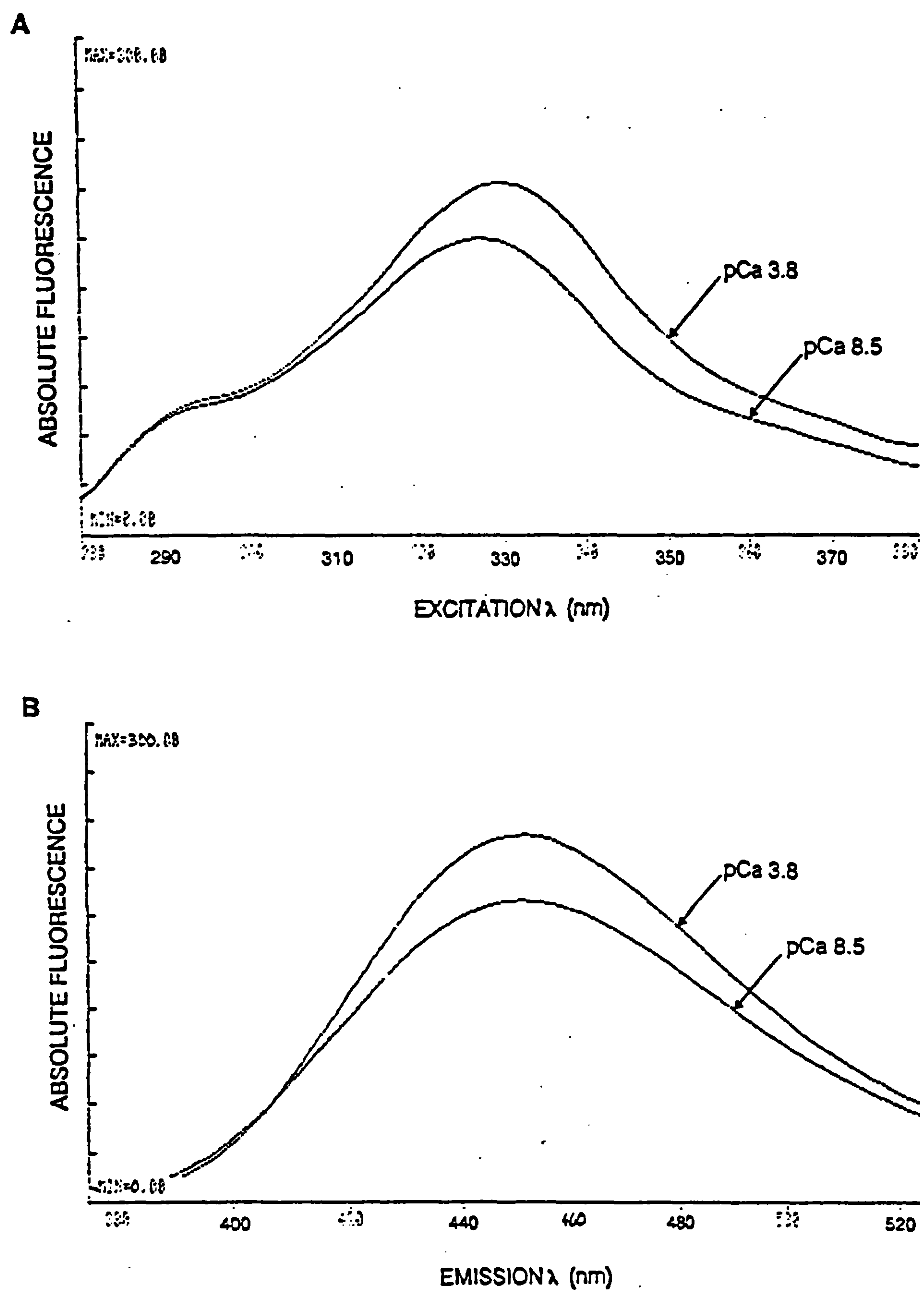


Fig.17 EFFECTS OF Ca^{2+} ON (A) EXCITATION AND (B) EMISSION SPECTRA OF ISOLATED TnC-IAANS. Fluorescence spectra were measured for 3 μM TnC-DANZ using the high ionic strength control solution described in section 3.2.3.a, before and after the addition of sufficient Ca^{2+} to saturate all Ca^{2+}-binding sites of TnC. Fluorimeter settings were as in section 3.2.3.b except that in A) excitation wavelength was scanned over 280 to 380 nm with emission collected at 450 nm and in B) emission wavelength was scanned over 375 to 525 nm with excitation wavelength 328 nm.

3.3.2 EMISSION AND EXCITATION SPECTRA

a) Experimental Solutions

Both DANZ and IAANS labels were non-fluorescent in high IS control solution. None of the control or test solutions produced noticeable fluorescence, with the exception of those containing 20 mM caffeine excited at 328 nm with emission collected at 450 nm. The fluorescence accounted for 5% of the minimum fluorescence measured in the presence of TnC-IAANS at high ionic strength. To enable the comparison of absolute fluorescence produced by TnC under test conditions with that emitted in the control circumstances, the fluorescence attributed to the caffeine solution was subtracted prior to further calculations.

b) TnC-DANZ

The optimal excitation and emission wavelengths for TnC-DANZ were found to be 338 and 520 nm respectively (fig.16). As pCa fell from 8.58 to 3.44, fluorescence increased (section 3.3.3) with a concomitant shift in peak emission wavelength to 508 nm. These results were similar to those of Johnson et al (1978). Excitation and emission wavelengths of 340 and 520 nm respectively were used for the experiments since both excitation and emission spectra were broad (fig.16) and peak fluorescence was unaffected by small deviations in wavelength.

c) TnC-IAANS

Maximum fluorescence occurred at excitation and emission wavelengths of 327 and 452 nm respectively with TnC-IAANS (fig.17), close to the values of 328 and 454 nm reported by Johnson et al (1980). TnC-IAANS experiments were therefore performed with excitation 328 nm and emission 450 nm (small deviations in these wavelengths had negligible effects on peak fluorescence). Decreasing pCa from 8.71 to 4.02 caused an enhancement of fluorescence (section 3.3.3) with no change in optimal emission wavelength. Interestingly lowering the pCa from 4.02 to 1.89 resulted in a further increase in fluorescence. This is discussed further in section 3.3.4.a.

3.3.3 CHANGES IN ABSOLUTE FLUORESCENCE

a) TnC-DANZ

At low IS (100) a fall in pCa from 8.58 to 3.44 resulted in a $89 \pm 2\%$ (n=7) increase in peak fluorescence. In control high IS (200) solution the same change in Ca^{2+} caused a $110 \pm 1\%$ (n=4) rise in fluorescence (fig.16) and a similar

enhancement ($104 \pm 2\%$, $n=6$) was obtained in the control used for Pi experiments (with another batch of TnC). Minimum and maximum fluorescence refer to absolute fluorescence values over a range of total $CaCl_2$ concentrations from zero to 2.083 mM total.

Pi affected neither minimum nor maximum fluorescence of TnC-DANZ, the enhancement of fluorescence as pCa rose from 8.50 to 3.95 being $98 \pm 2\%$ ($n=9$). In contrast, lowering pH to 6.20 increased both minimum fluorescence (by $23 \pm 3\%$, $n=4$) and maximum fluorescence (by $15 \pm 3\%$, $n=4$) with a $97 \pm 1\%$ ($n=4$) enhancement of fluorescence on the addition of Ca^{2+} to pCa 3.73. Caffeine suppressed maximum absolute fluorescence very slightly (by $8 \pm 2\%$, $n=4$) while TnC-DANZ fluorescence was enhanced by $89 \pm 1\%$ ($n=4$) as the pCa rose to 3.82.

b) TnC-IAANS

At low IS, peak fluorescence increased by $31 \pm 1\%$ ($n=6$) as pCa fell from 6.2 to 3.8. Fluorescence at pCa 8.5 was approximately 40% of that at pCa 3.8 (fig.18) therefore a direct change from pCa 8.5 to 3.8 resulted in a 19% increase in fluorescence (fig.17).

With the TnC-IAANS used for caffeine and pH studies, decreasing pCa from 6.40 to 3.82 in high IS control solution caused an increase in fluorescence of $18 \pm 1\%$ ($n=4$), a far smaller response than was observed with TnC-DANZ. The fresh batch of TnC-IAANS used for the phosphate studies had a larger increase ($31 \pm 1\%$, $n=9$) on the addition of $CaCl_2$.

The fluorescence enhancements on changing from pCa 6.40 (or 6.49) to 3.82 (or 3.95) were $17 \pm 1\%$ ($n=4$) and $29 \pm 1\%$ ($n=9$) for caffeine and Pi solutions respectively. Increasing the pCa of the pH 6.20 solution from 7.08 to 3.68 caused TnC-IAANS fluorescence to increase by $31 \pm 1\%$ ($n=4$). The reduction in pH increased minimum and maximum fluorescence levels by $25 \pm 10\%$ and $39 \pm 10\%$ ($n=4$) respectively. 20 mM caffeine decreased both minimum fluorescence (by $13 \pm 2\%$, $n=4$) and maximum fluorescence (by $14 \pm 2\%$, $n=4$). Pi had no effect on TnC-IAANS absolute fluorescence levels.

3.3.4 Ca^{2+}-SENSITIVITY OF LABELLED SKELETAL AND CARDIAC TnC

a) Controls

At pCa values greater than 6.2, the fluorescence of TnC-IAANS decreased sigmoidally as the Ca^{2+} concentration rose (fig.18). At pCa 8.5 the fluorescence was approximately 40% of that at pCa $\approx$ 4.0). The pCa_{50} of this fluorescence change was estimated by eye to be 6.95, corresponding to a K_{Ca} of 8.9×10^6 M^{-1}

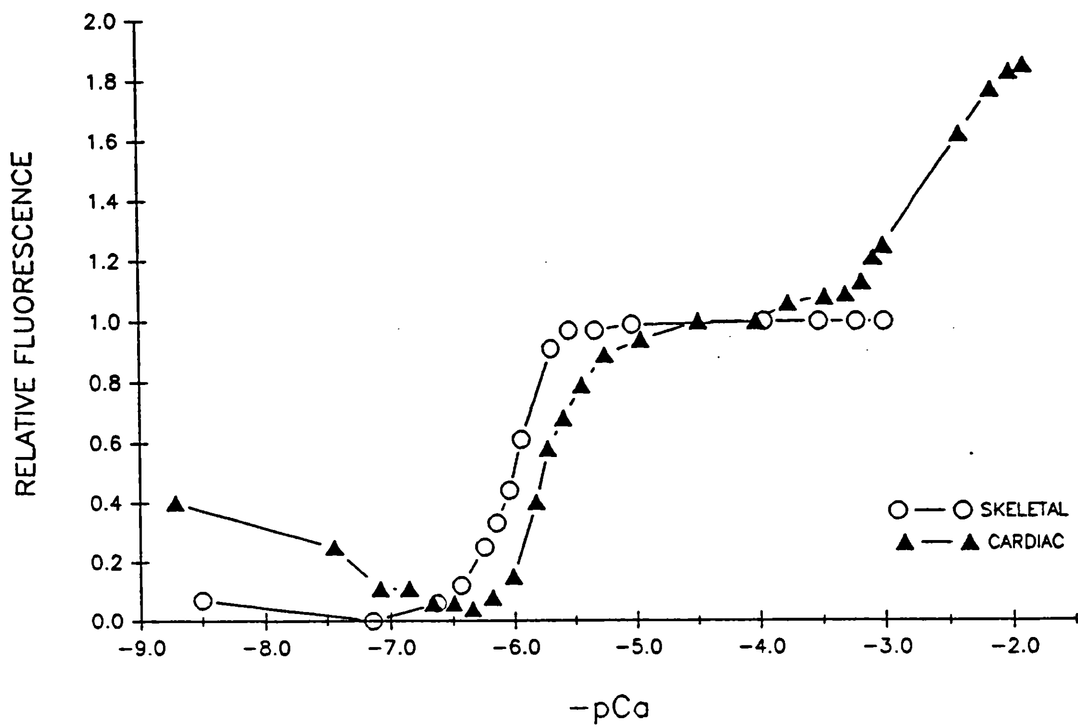


Fig.18 Ca^{2+}-SENSITIVITY OF TnC-DANZ AND TnC-IAANS FLUORESCENCE. Each point represents data from a single TnC-DANZ experiment or mean data from 6 TnC-IAANS experiments, using 3 μM labelled TnC in low ionic strength solution (table 7). Conditions as described in section 3.2.3.b.

assuming no significant increase in the fluorescence occurred above pCa 8.5. In fact the fluorescence change had not reached a plateau by pCa 8.5 so the estimated Ca^{2+} affinity would be rather too low. No such fluorescence increase, however, was noted for TnC-DANZ. Between pCas 6.2 and 3.8, the fluorescence of both TnC-DANZ and TnC-IAANS increased sigmoidally (fig.18). As this reflected Ca^{2+} binding to the Ca^{2+}-specific sites, most experiments concentrated on this pCa range. Labelled cardiac TnC (but not TnC-DANZ, fig.18) showed an additional rise in fluorescence as total calcium concentration rose above 0.3 mM (pCa less than 3.8). The relationship between pCa and relative fluorescence in this range was again sigmoidal (pCa_{50} approximately 2.7), although such results cannot have any physiological relevance since intracellular free calcium never rises so high.

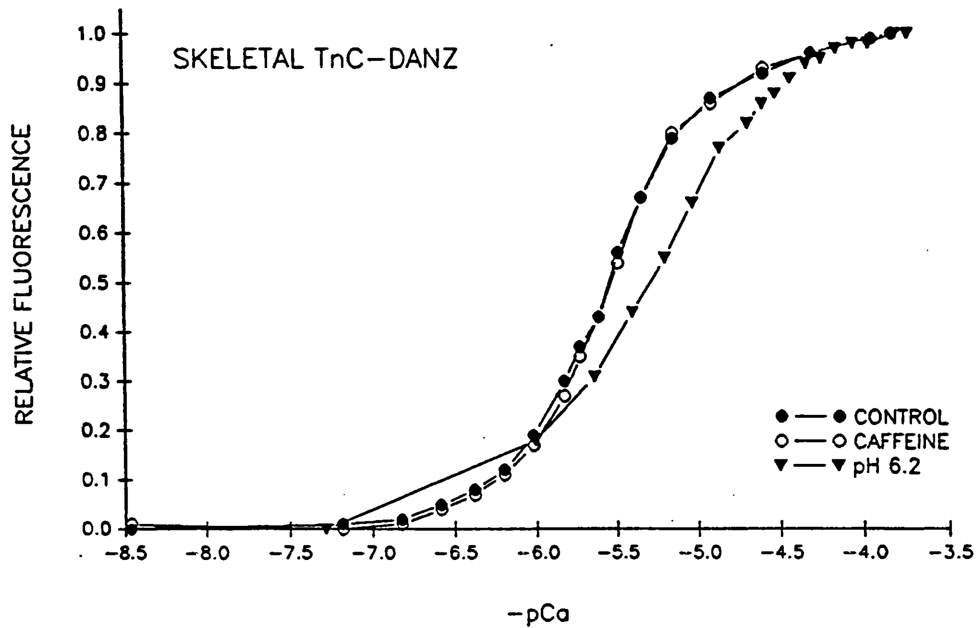
Initial experiments were done at 0.1 M IS to compare results with previous work. At this low IS the pCa_{50} of TnC-DANZ was 5.83 ± 0.05 (n=4) and H_n 1.68 ± 0.18 (n=4). Corresponding values for TnC-IAANS were 5.36 ± 0.16 (n=4) and 1.28 ± 0.25 (n=4) respectively. Thus the Ca^{2+}-sensitivity of TnC-DANZ was greater than that of TnC-IAANS, though the H_n values were not significantly different.

At physiological IS (0.2 M) the overall pCa-fluorescence curves were similar to those at low IS. As shown in fig.19 and table 8, labelled skeletal TnC was still more sensitive to Ca^{2+} than cardiac TnC in control solutions (pCa_{50}s were 5.56 ± 0.02 and 5.34 ± 0.06 respectively) and H_n values were once again similar for skeletal and cardiac TnC.

b) <u>Effects of pH 6.2</u>

Reducing pH from 7.00 to 6.20 caused rightwards displacements of the fluorescence-pCa curves of both TnC-DANZ (ΔpCa_{50} = 0.28) and TnC-IAANS (ΔpCa_{50} = 0.64 units), as shown in fig.19, table 8. The shift for cardiac TnC was twice that with skeletal TnC (as was found for skinned fibres) although the pH-induced changes in pCa_{50} of isolated TnC were less than those found in skinned fibre experiments (ΔpCa_{50} was 0.55 for rabbit psoas and 1.09 for rat trabecula, section 2.3.1).

Acidosis also reduced the H_n values for skeletal isolated TnC (fig.19, table 8) in contrast to the results from skinned fibres. Finally, the decrease in TnC-IAANS fluorescence observed at pCas above 6.5 in control solution was abolished by the fall in pH (fig.19).



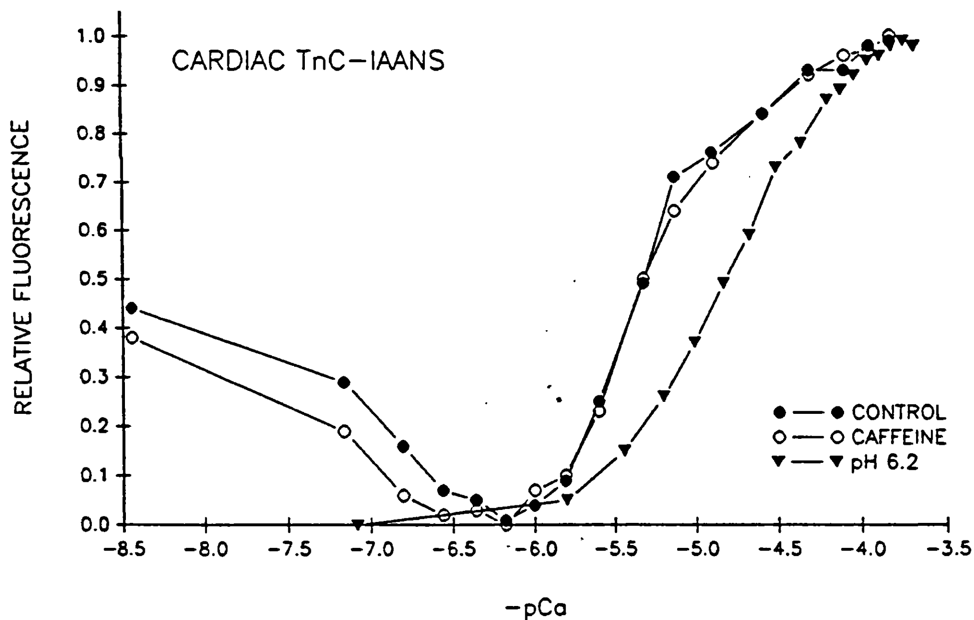


Fig.19 EFFECTS OF CAFFEINE AND REDUCTION OF pH ON Ca^{2+}-BINDING TO LABELLED ISOLATED SKELETAL AND CARDIAC TnC. Conditions were as described in section 3.2.3 using high ionic strength solutions and 3 μM labelled TnC. Points represent mean data from 4 experiments. Lowering the pH from 7.00 to 6.20 reduced the Ca^{2+}-sensitivity of both skeletal and cardiac TnC whereas the addition of 20 mM caffeine at 7.00 had no effect.

			CAFFEINE	pH 6.2	Pi
pCa_{50}					
	CONTROL	SKELETAL	5.56±0.02	5.56±0.02	5.52±0.05
		CARDIAC	5.34±0.06	5.34±0.06	5.10±0.12
	TEST	SKELETAL	5.54±0.01	5.28±0.01	5.48±0.03
		CARDIAC	5.35±0.06	4.78±0.04	5.27±0.05
	SHIFT	SKELETAL	-0.02±0.03	-0.28±0.01*	-0.03±0.02
		CARDIAC	-0.01±0.07	-0.64±0.05*	0.05±0.04
HILL n					
	CONTROL	SKELETAL	1.36±0.06	1.36±0.06	1.43±0.10
		CARDIAC	1.48±0.20	1.48±0.20	1.12±0.15
	TEST	SKELETAL	1.43±0.05	1.01±0.02	1.28±0.06
		CARDIAC	1.19±0.27	1.25±0.12	0.90±0.07
	SHIFT	SKELETAL	0.07±0.05	-0.35±0.04*	-0.15±0.03*
		CARDIAC	-0.29±0.08*	-0.32±0.20	-0.34±0.16

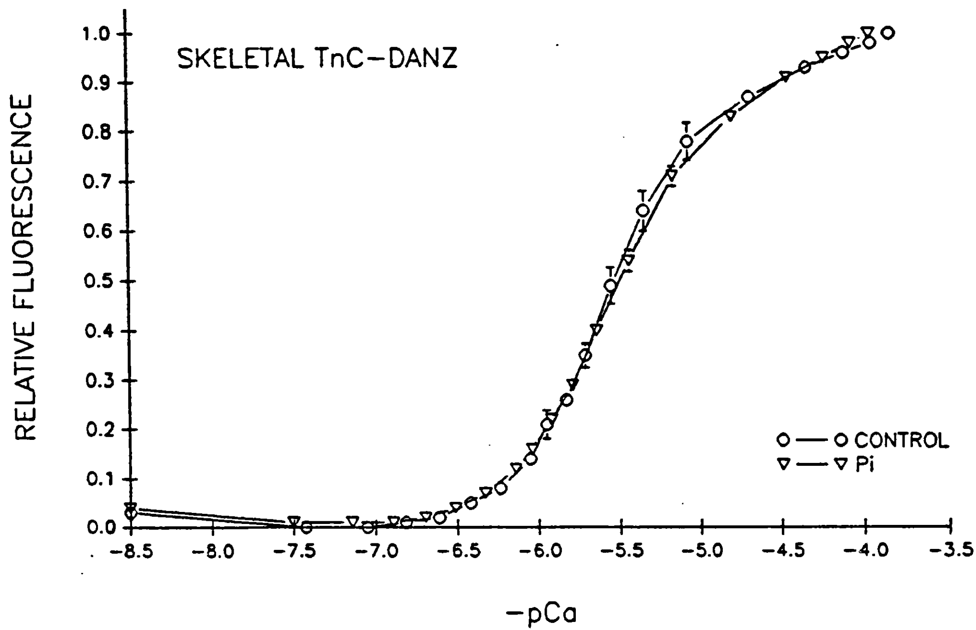
TABLE 8. EFFECTS OF 20 mM CAFFEINE, REDUCTION OF pH TO 6.20 AND 20 mM Pi ON Ca^{2+}-SENSITIVITY OF SKELETAL TnC-DANZ AND CARDIAC TnC-IAANS. All values expressed as mean±SEM. n=4 in each case. Solutions as described in table 7. Experiments with Pi used a different batch of labelled TnC (skeletal and cardiac) than those with caffeine and pH.

c) Effects of Pi

20 mM Pi had no effect on the midpoint of the pCa-fluorescence curves of TnC-DANZ or TnC-IAANS (fig.20, table 8) though it did reduce the H_n of TnC-DANZ. Thus the Pi-induced rightwards shifts in pCa_{50} (0.30 and 0.19 units, section 2.3.1) observed in skinned skeletal and cardiac fibres respectively were not matched by similar effects of Pi on isolated TnC.

d) Effects of Caffeine

As shown in fig.19 and table 8, 20 mM caffeine had no effect on the fluorescence-pCa curves of TnC-DANZ or TnC-IAANS, apart from a slight suppression of TnC-IAANS fluorescence at pCa values above 6.5 and a reduction in H_n for Ca^{2+} binding to labelled cardiac TnC. This was in contrast to the Ca^{2+}-sensitising action of caffeine on skinned skeletal and cardiac fibres where pCa_{50} was shifted by 0.09 and 0.31 units respectively (section 2.3.1).



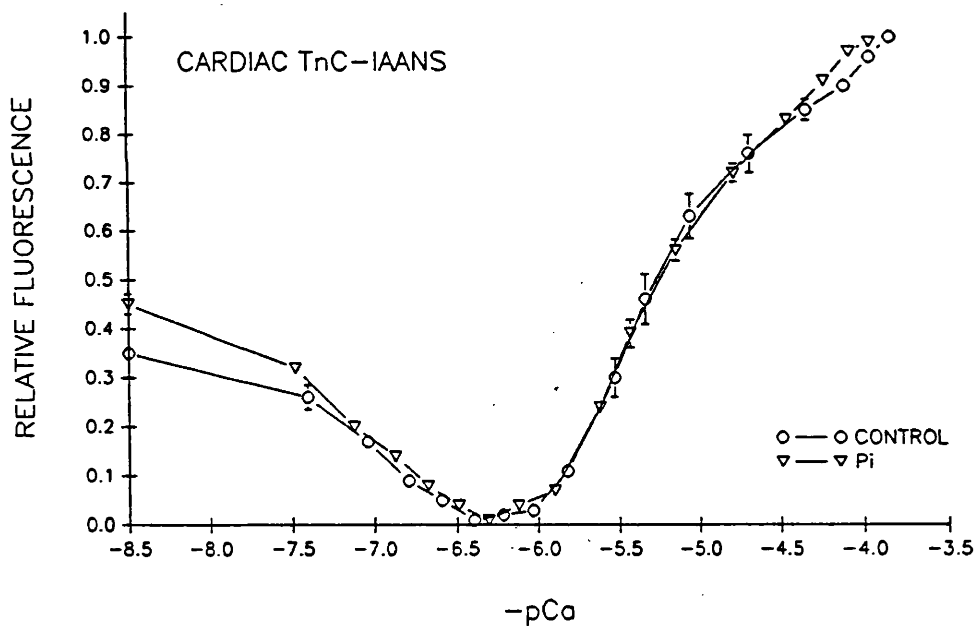


Fig.20 EFFECTS OF Pi ON Ca^{2+}-BINDING TO LABELLED ISOLATED SKELETAL AND CARDIAC TnC. Conditions as described in section 3.2.3 using high ionic strength solutions and 3 μM labelled TnC. Points represent mean data from 4 experiments with TnC-DANZ and from 8 control runs or 9 Pi runs with TnC-IAANS. 20 mM Pi had no effect on the Ca^{2+}-sensitivity of either skeletal or cardiac TnC.

3.4 DISCUSSION

3.4.1 Ca^{2+}-BINDING TO TnC: COMPARISON WITH OTHER STUDIES

Previous studies all used solutions of low ionic strength (0.1 M). The pCa-fluorescence relationship of TnC-DANZ in low ionic strength solution had a mean pCa_{50} of 5.83 and H_n 1.68. Corresponding values for TnC-IAANS were 5.36 and 1.28 respectively. Thus, assuming no interaction between sites is necessary for fluorescence, skeletal TnC had a Ca^{2+} affinity (K_{Ca}) of $6.8x10^5$ M^{-1} and cardiac TnC a K_{Ca} of $2.3x10^5$ M^{-1}. The TnC-DANZ result was close to the $4x10^5$ M^{-1} K_{Ca} obtained by Johnson et al (1978) and that of $3x10^5$ M^{-1} obtained by Potter & Gergely (1975), the latter using unlabelled skeletal TnC Such a comparison enables two immediate conclusions to be drawn: firstly since Johnson et al (1978) and Potter & Gergely (1975) attributed their association constants to Ca^{2+} binding to the low affinity "Ca^{2+} specific" sites of skeletal TnC, it is more than likely that the $CaCl_2$ was being titrated against the same sites in this current study. Secondly, the fact that the three values for the same constant are so similar indicates that the Ca^{2+} binding properties of the TnC used here have not been irreversibly altered by the TnC isolation, purification or labelling steps.

Ca^{2+} binding to the high affinity Ca^{2+}-Mg^{2+} sites of TnC-DANZ causes a 17% decrease in fluorescence (Johnson et al, 1978). The K_{Ca} of these sites was found to be $2x10^7$ M^{-1} in unlabelled TnC (Potter & Gergely, 1975), which corresponds to a pCa_{50} of 7.30. In the current study no effect of Ca^{2+} on TnC-DANZ fluorescence was noted at such high pCa values (fig.18) but the reasons for this are unclear.

The K_{Ca} of $2.3x10^5$ M^{-1} for TnC-IAANS was similar to those obtained for the Ca^{2+} specific site of TnC by Johnson et al ($4.5x10^5$ M^{-1}, 1978) using TnC-IAANS, and Holroyde et al ($2.5x10^5$ M^{-1}, 1980) using unlabelled cardiac TnC and equilibrium dialysis. Not only does this indicate that the binding site being titrated against Ca^{2+} in the current study was the low affinity Ca^{2+} specific site of TnC, it also demonstrates that the Ca^{2+} binding properties of this site were unaffected by the addition of IAANS to the protein, or by the isolation and purification techniques employed.

The H_n values of 1.68 and 1.28 for skeletal and cardiac TnC respectively indicate that only skeletal TnC shows cooperativity in Ca^{2+} binding at low ionic strength. This could reflect facilitation of Ca^{2+} binding to the second Ca^{2+}-specific site once the first had been occupied. Such cooperativity would not be expected in cardiac TnC which has only one low affinity site.

3.4.2 Ca^{2+}-BINDING TO TnC IN PHYSIOLOGICAL SOLUTION

a) Controls

The addition of sufficient Ca^{2+} to saturate the low affinity sites of TnC-DANZ in physiological buffer caused fluorescence to increase by 110%. This was identical to the 2.1 fold increase reported by Johnson et al (1978) at low IS. Thus TnC-DANZ was able to respond to the binding of Ca^{2+} despite a change in ionic strength. The Ca^{2+}-saturation of the low affinity site of TnC-IAANS in physiological solution was accompanied by a 18% (1.2 fold) increase in fluorescence as compared with the 68% rise observed by Johnson et al (1980) at low IS. This may reflect the problems encountered in obtaining Ca^{2+}-sensitive TnC-IAANS (section 3.3.1). Some TnC-IAANS, for example, may still have been insensitive to Ca^{2+} as a result of structural changes during purification or labelling. Alternatively IAANS may have bound to sites other than Cys-35 & Cys-84 giving a high level of Ca^{2+}-insensitive background fluorescence.

Direct comparison of TnC Ca^{2+} binding at low and high IS would be unwise as K^+ and Na^+ concentrations varied between the corresponding solutions in addition to IS per se. These cations are known to affect Ca^{2+}-sensitivity of skinned fibres regardless of any effects of ionic strength (Kentish, 1984) and thus may alter Ca^{2+} binding to TnC.

The pCa_{50} of the pCa-fluorescence curve for TnC-DANZ was 5.56 (K_{Ca} $3.6x10^5$ M^{-1}) and that of TnC-IAANS 5.34 (K_{Ca} $2.2x10^5$ M^{-1}). Corresponding pCa_{50} values for skinned psoas and trabeculae were 5.85 and 5.56 under control conditions (section 2.3.1). Thus skeletal TnC and skinned fibres were more sensitive to Ca^{2+} than their cardiac counterparts. It was also clear, however, that although the Ca^{2+}-sensitivity of the skinned fibres may be partly governed by the nature of TnC present, it does not depend entirely on TnC. There was a difference in pCa_{50} of 0.22 between the two TnC moieties but 0.33 between the corresponding fibre types. The "extra" difference between pCa_{50} of skeletal and cardiac fibres may be explained by factors which reduce the Ca^{2+}-sensitivity of cardiac myofilaments but play no role in skeletal fibres e.g. phosphorylation of TnI (Robertson et al, 1982).

In agreement with Johnson et al (1980), the current results showed a decrease in TnC-IAANS fluorescence as pCa fell from 6.2 to 8.5. Since Ca^{2+} binds to the Ca^{2+}-Mg^{2+} sites in cardiac TnC with a K_{Ca} of $2x10^7$ M^{-1} (Holroyde et al, 1980) it is possible that the observed fluorescence change is related to Ca^{2+} binding at these sites. Johnson et al further observed a decrease in fluorescence of TnC-IAANS by 5-15% as Ca^{2+} bound to the Ca^{2+}-Mg^{2+} sites in the absence of Mg^{2+}. However, a 20% drop in fluorescence also occurs when Mg^{2+} binds,

with a K_d of 0.3 mM (Johnson et al, 1980). Since the solutions used in the present study contained 1 mM Mg^{2+} it would be expected that almost all the possible fluorescence change on Mg^{2+} binding to the Ca^{2+}-Mg^{2+} sites would have occurred. Subsequent addition of $CaCl_2$ would cause little further change in fluorescence since the binding sites would already have Mg^{2+} associated with them.

This fluorescence response at pCas above 6.2 was decreased by 20 mM caffeine, abolished by decreasing pH from 7.00 to 6.20 but was unaffected by 20 mM Pi. It was completely absent in skeletal TnC-DANZ. The cause of the observed fluorescence change of TnC-IAANS at high pCas, however, remains unclear.

Labelled cardiac TnC also differed from TnC-DANZ in that a further increase in fluorescence was observed at pCa values below 3.8. The pCa_{50} of this additional fluorescence change was approximately 2.7 (K_{Ca} $5.0x10^2$ M^{-1}) which may reflect Ca^{2+} binding to the very low affinity mutated site I. Similar results were obtained by Johnson et al (1980).

The control H_n values for skeletal and cardiac TnC were 1.36 and 1.48 respectively for caffeine and pH 6.20 experiments at physiological ionic strength (or 1.43 and 1.12 respectively for Pi experiments). The values for skeletal (but not cardiac) TnC were significantly greater than one, suggesting cooperativity in Ca^{2+} binding. It is reasonable to assume that Ca^{2+} binding to the first of the two Ca^{2+}-specific sites of skeletal TnC could facilitate Ca^{2+} binding to the second of these sites, as occurred at low ionic strength. Alternatively, skeletal TnC may have a H_n greater than one even in the absence of cooperativity (Grabarek & Gergely, 1983). These authors modelled the response of a fluorescent probe to Ca^{2+} binding to two low affinity sites on TnC, assuming varying degrees of cooperativity between sites and different fluorescence responses to Ca^{2+} binding. With no cooperativity the Hill coefficient was between 1.0 and 1.2 (depending on the type of fluorescence response). For H_n <1.4, however, various pairs of cooperativity parameter and binding constants gave results consistent with experimental data, depending on the choice of fluorescence response. Thus it was possible to have a Hill coefficient > 1 without cooperativity or a H_n of 1 even with strong negative cooperativity. Hill coefficients of 1.4-2.0 were only achieved if positive cooperativity was present and were less dependent on the type of fluorescence response.

Since the H_n values obtained for skeletal TnC in the present study were 1.36 and 1.43 (close to Grabarek & Gergelys lower limit for certain cooperativity) it is not clear whether this reflects true cooperativity (with the full fluorescence change occurring on Ca^{2+} binding to either site) or no cooperativity but only

partial change in fluorescence occurring on Ca^{2+} binding to either site.

In theory values of H_n should not be greater than one for cardiac TnC since only one Ca^{2+}-specific site is present. This was confirmed by the current results where the H_n of TnC-IAANS was not significantly different from unity. Accurate curfitting of cardiac data was difficult because of the additional fluorescence changes occurring at pCas above 6.2 and below 3.8 (fig.18). Making minimum and maximum fluorescence both variable and extending the range of pCa values over which the points were fitted all affected the H_n values considerably (whereas pCa_{50} remained unaffected), but these steps improved the accuracy of the estimates.

The H_n values for skeletal and cardiac TnC (1.36 and 1.48 respectively) were less than the corresponding values (1.9 and 2.6) for skinned psoas and trabecula (section 2.3.1). Thus there was little or no cooperativity in Ca^{2+} binding to the low affinity sites of isolated TnC but cooperativity was present in the force-pCa relationship of skinned fibres. This implied that interactions between the Ca^{2+} binding sites on TnC do not facilitate force development alone but may do so in conjunction with other proteins present in skinned fibres. TnI, Tm or actin, which are also involved in the activation process may have a role in cooperativity. It has been suggested (Bremel & Weber, 1972; Guth & Potter, 1987) that crossbridge binding per se may influence Ca^{2+} binding to TnC, as is discussed further in chapter 5.

b) Effects of pH 6.2

Reducing pH from 7.00 to 6.20 caused increases of 15 and 37% in minimum and maximum absolute fluorescence of skeletal and cardiac labelled TnC. This may reflect an enhancement of the hydrophobic nature of the fluorescent probes' surroundings in each case since quenching is reduced in non-polar environments. Protonation results in the adoption of a more compact structure by TnC (Ingraham & Swenson, 1983), increasing the stability of both low and high affinity Ca^{2+} binding sites. Thus it is perhaps not surprising that pH should indirectly affect probes used to detect environmental changes in these regions (neither DANZ nor IAANS are themselves pH-sensitive). Such results will not interfere with investigations of Ca^{2+} affinity if the effects of acidosis on labelled TnC fluorescence in the absence of Ca^{2+} are independent from any pH effects on Ca^{2+} binding-induced fluorescence. The pH-induced decreases in minimum and maximum absolute fluorescence were not significantly different from each other. Thus the addition of Ca^{2+} did not affect the responses of the probes to pH-induced changes in TnC conformation.

A fall in pH from 7.00 to 6.20 caused the pCa_{50} of the pCa-fluorescence curve

of TnC-DANZ to drop by 0.28 units and that of TnC-IAANS by 0.64 (table 8). El-Saleh & Solaro (1988) showed that a fall in pH from 7.0 to 6.5 (at IS 85) was accompanied by a 0.21 unit shift in pCa_{50} of TnC-DANZ fluorescence. Assuming a linear relationship between change in pH and change in pCa_{50} (true at least for TnC-TnI and reconstituted Tn; El-Saleh & Solaro, 1988), a decrease in pH from 7.00 to 6.20 would be expected to produce a 0.34 unit fall in pCa_{50}. This is slightly greater than the observed 0.28 units but this may reflect the different experimental conditions. In a preliminary report, a change in pH from 7.5 to 6.5 caused a 0.8 unit decrease in pCa_{50} in TnC-IAANS (Robertson et al, 1978) which corresponds to a drop of 0.64 units for a pH fall from 7.00 to 6.20. This closely parallels the results of the current study. Previous experiments assessing the effects of pH on Ca^{2+} binding produced inconsistent results (section 3.1.4) ranging from no observed effects on Tn (Ogawa, 1985; Stull & Buss, 1978) to significant changes in Ca^{2+} affinity of TnC (Robertson et al, 1978; El-Saleh & Solaro, 1988). The present results indicate that acidosis does reduce the Ca^{2+}-sensitivity of both skeletal and cardiac TnC though the effect on cardiac TnC was approximately twice that on the skeletal form.

The mechanism by which acidosis decreases Ca^{2+} binding probably reflects pH-induced changes in TnC structure. Leavis & Lehrer (1974) measured conformational changes in skeletal TnC using tyrosyl fluorescence and ellipticity changes which were the same whether induced by protons or divalent metals. Protons appeared to interact with carboxyl groups with abnormal pKa values (6.0) to which Ca^{2+} might also bind. Similarly Ingraham & Swenson (1983) showed that protonation of TnC made the Ca^{2+}-Mg^{2+} and Ca^{2+}-specific sites more compact, increasing their thermal stability. Thus protons compete directly with Ca^{2+} for binding at the low affinity sites of TnC.

The two-fold difference between pH-sensitivity of Ca^{2+} binding to skeletal and cardiac TnC could reflect the structural differences between the two forms of TnC. If acidosis only affected Ca^{2+} binding to one of the two Ca^{2+}-specific sites of skeletal TnC, the overall pH-induced change in pCa_{50} would be half that expected were both sites affected. Since cardiac TnC has only one low affinity site its change in pCa_{50} during acidosis would be twice that observed for skeletal TnC. Such effects could also explain the reduction in H_n observed for skeletal TnC at low pH since cooperativity between Ca^{2+} binding to the two Ca^{2+}-specific sites would also be reduced by competition between protons and Ca^{2+} at one site. As no effect of pH on H_n is found in cardiac TnC, which has only one functional regulatory site (site II), it seems possible that only site II is rendered less Ca^{2+} sensitive by a reduction in pH. This may reflect the susceptibility of the residues intrinsic to, or surrounding, site II to protonation as opposed to a more

resistant environment around site I. The increases in cooperativity when TnC is incorporated into skinned fibres (with H_n values of 1.9 and 2.6 for psoas and trabecula respectively (section 2.3.1)) probably reflect interactions between, for example, crossbridges and TnC or adjacent Tn units.

This study represents the first time that the properties of skinned muscles and isolated TnC were examined under similar conditions. This makes it possible to say how much of the changes in pCa_{50} in skinned fibres can be accounted for by the properties of TnC. Skinned skeletal and cardiac fibres were desensitised to Ca^{2+} by a reduction in pH from 7.00 to 6.20, with respective falls in pCa_{50} of 0.55 and 1.09 (table 6). Using similar solutions, the corresponding falls in pCa_{50} of isolated TnC-DANZ and TnC-IAANS were 0.28 and 0.64 respectively. Thus the two-fold greater effect of pH on cardiac compared to skeletal fibres can be attributed partly to the two-fold greater sensitivity of cardiac TnC to pH changes. It is clear, however, that although direct competition between Ca^{2+} and protons binding to the low affinity sites of TnC causes part of the desensitisation to Ca^{2+} of skinned fibres, another mechanism is also involved.

These results are consistent with those of Blanchard & Solaro (1984) who demonstrated that Ca^{2+} binding to TnC in skinned cardiac fibres was reduced more than the Ca^{2+} affinity of isolated TnC by a decrease in pH. However, their Ca^{2+} measurement in skinned fibres could not easily distinguish between high and low affinity sites, unlike the present study. These conclusions are different from those of Gulati & Babu (1989), who found that replacement of TnC in hamster trabeculae gave a similar pH sensitivity whether the TnC added was cardiac or fast skeletal TnC.

The pH-induced shifts in pCa_{50} of isolated TnC were approximately half those observed in skinned fibres (see above). Incorporation of TnC into the Tn complex causes a 10-fold increase in Ca^{2+}-affinity of the Ca^{2+}-specific sites of TnC (Potter & Gergely, 1975). El-Saleh & Solaro (1988) demonstrated that acidosis halves the affinity of TnI for TnC, thereby diminishing the TnI-induced enhancement of Ca^{2+} binding to TnC. Thus as pH decreased, the fall in pCa_{50} of TnC-DANZ complexed with TnI was twice that of TnC-DANZ alone. Similar results were found with TnC-IAANS (Solaro et al, 1989). The mechanism by which protons reduce the affinity of TnI for TnC is unknown but two pH-dependent sites of interaction between the proteins have been found (El-Saleh & Solaro, 1988). It seems likely that protons react with histidine and carboxyl groups associated with these sites, causing structural changes in TnC.

Additional evidence that regulatory subunit interactions determine pH-sensitivity came from comparative studies on systems containing different isoforms of the regulatory proteins. Neonatal rat trabeculae were less sensitive to

pH changes than were adult trabeculae which have different TnI and TnT isoforms (Solaro et al, 1988). Fast and slow twitch skeletal fibres contain different forms of TnC but similar TnI isoforms: pH changes affected slow twitch more than fast twitch fibres (Donaldson & Hermansen, 1978) - although Metzger & Moss (1987) found both fibre types had similar falls in pCa_{50} during acidosis.

Alternatively, acidosis might decrease reciprocal coupling (Guth & Potter, 1987) between crossbridge formation and Ca^{2+} binding to TnC (discussed further in chapter 6).

In conclusion, the desensitisation of myofilaments to Ca^{2+} by acidosis is caused by direct competition of protons and Ca^{2+} for the low affinity sites of TnC, plus pH-induced decreases in the affinity of TnI for TnC or reciprocal coupling. Differences in pH-sensitivity of skeletal and cardiac skinned fibres are related to differences in their constituent TnC molecules.

c) Effects of Inorganic Phosphate

20 mM Pi reduced the H_n of TnC-DANZ fluorescence from 1.43 to 1.28 (table 8), thus the cooperativity of Ca^{2+} binding to the low affinity sites of skeletal TnC was decreased. No such effect was observed with TnC-IAANS, however, nor in either skinned skeletal or cardiac fibres (table 6) so such a change in H_n is unlikely to be of physiological significance.

Pi had no effects on the Ca^{2+} affinity of the regulatory sites of isolated TnC-DANZ or TnC-IAANS (table 8 & fig.20). These results contrasted with those from a preliminary study by El-Saleh (1989) where 28.5 mM Pi caused a rightward shift of the pCa-fluorescence relationship of TnC-DANZ by 0.105 and TnC-IAANS by 0.438 units. In addition El-Saleh reported that complexing TnC with TnI enhanced the desensitising effect of Pi on the Ca^{2+}-specific sites, though to what extent was unclear. Since no details were given of the experimental conditions, it is difficult to explain the differences between El-Saleh's results and those in the current study. Contrasting results have also been obtained from studies measuring the effects of Pi on TnC in skinned fibres. Preliminary work by Takayasu & Solaro (1990) using $^{45}Ca^{2+}$ indicated that 20 mM Pi did not affect the Ca^{2+} affinity of the regulatory sites of TnC in skinned cardiac fibres at pH 7.0 or 6.5. Guth & Potter (1987) and Morano & Ruegg (1991) replaced native TnC in skinned rabbit psoas fibres with TnC-DANZ and demonstrated that Pi decreased TnC-DANZ fluorescence less than force in skinned rabbit psoas at sub-maximal Ca^{2+}. Interpretation of these results was complicated by the finding that weakly and strongly bound crossbridges altered the fluorescence of TnC-DANZ, attachment of weak crossbridges enhancing fluorescence by less than strongly attached ones (section 5.4.3.a). Morano &

Ruegg concluded that Pi caused the accumulation of loosely bound crossbridges with no contribution to force and a reduced contribution to fluorescence. It was assumed that 5 mM Pi had no direct effect on TnC-DANZ. It may be expected that a Pi-induced transition from strongly to weakly attached crossbridges would be accompanied by an indirect reduction in Ca^{2+}-affinity of TnC since the latter has been shown to increase in relation to the number of force-generating crossbridges (Guth & Potter, 1987). This, however, is not supported by Takayasu & Solaro's (1990) findings using non-fluorimetric techniques.

The current results and those of Guth & Potter (1987), Takayasu & Solaro (1990) and Morano & Ruegg (1991) suggest that direct reduction of the Ca^{2+}-affinity of TnC by Pi does not play a major role, if any, in the Pi-induced desensitisation of skinned fibres to Ca^{2+}. Thus differences in TnC moieties in skeletal and cardiac muscle are unlikely to explain the different Pi-sensitivities of these muscles (table 6). Possible mechanisms by which Pi may reduce the Ca^{2+}-sensitivity of skinned fibres are discussed further in chapter 6.

d) Effects of Caffeine

As discussed in section 2.4.1.d, it was considered possible that the different degrees of Ca^{2+} sensitisation by caffeine in skeletal and cardiac skinned fibres may reflect differences in TnC of the muscle types. 20 mM caffeine induced a rise in pCa_{50} of 0.09 in psoas fibres and 0.31 in trabeculae. However, caffeine had no effect on pCa_{50} of isolated skeletal TnC-DANZ or cardiac TnC-IAANS. Thus it did not increase the Ca^{2+} affinity of the low affinity sites of TnC directly. In the myofilaments, TnC is closely linked to other proteins, such as TnI, TnT and Tm, which could alter its response to caffeine. The different effects of caffeine in skeletal and cardiac muscle might then be attributed to the different skeletal and cardiac versions of TnI, for example. If caffeine altered the interaction of TnC with TnI then the usual 10-fold enhancement by TnI of TnC Ca^{2+} affinity may be increased. The extent of this effect may depend on the isoform of TnI or on the phosphorylation state of the cardiac form. It is also possible that caffeine reduces the 10-fold decrease in the K_{Ca} of TnC when Tn is incorporated into thin filaments (Zot et al, 1983). Alternatively, caffeine may act more indirectly to increase the Ca^{2+} affinity of TnC, perhaps via the promotion of crossbridge formation linked to reciprocal coupling. This is discussed further in Ch6.

20 mM caffeine had no effect on the H_n of isolated TnC-DANZ but decreased that of TnC-IAANS. Since no cooperativity is possible with Ca^{2+} binding to the single low affinity site of cardiac TnC, a reduction in H_n cannot reflect a decrease in cooperativity and is therefore unlikely to be of physiological importance.

The decrease in fluorescence of TnC-IAANS at pCas below 6.2 was reduced by caffeine (fig.19), though it is unclear whether the pCa_{50} was also changed because fluorescence had not reached a plateau at pCa 8.5.

Caffeine suppressed equally minimum and maximum absolute fluorescence of TnC-IAANS and depressed maximum fluorescence of TnC-DANZ slightly. This presumably indicates that caffeine either directly or indirectly increased quenching of the fluorescent probes, though not to an extent sufficient to invalidate their use as detectors of Ca^{2+} binding.

3.5 SUB-CONCLUSIONS

a) The effects of acidosis, Pi and caffeine on the Ca^{2+}-sensitivity of isolated TnC were compared with their effects on skinned fibres *using the same physiological solutions*.

b) Acidosis reduced the Ca^{2+}-affinity of cardiac, and to a lesser extent, skeletal TnC. Approximately 50% of the acidosis-induced desensitisation of skinned fibres can be attributed to competition between protons and Ca^{2+} possibly at the Ca^{2+}-specific site II of TnC. The remaining 50% is probably due to a pH-induced decrease in affinity of TnI for TnC or to a reduction in the degree of reciprocal coupling between TnC Ca^{2+}-binding and crossbridge formation. Differences in pH-sensitivity of cardiac and skeletal fibres are related to the different TnC moieties.

c) Neither Pi nor caffeine altered the Ca^{2+}-sensitivity of isolated TnC. The different Pi- and caffeine-sensitivities of cardiac and skeletal fibres are therefore not governed solely by TnC moiety. Pi and caffeine presumably affect the Ca^{2+}-sensitivity of muscle by altering interactions between Tn components or by altering crossbridge state.

CHAPTER 4: Ca^{2+}-SENSITISATION OF MYOFILAMENTS BY NOVEL INOTROPIC AGENTS

4.1 INTRODUCTION

4.1.1 BACKGROUND

Six months of my PhD were spent working for Ciba-Geigy in Basel. The company had recently developed a novel inotropic agent (BA41899), which had no effect on PDE activity. It was of interest to investigate whether the compound had a direct effect on the myofilaments and, following subsequent separation of its enantiomers (CGP48506 and CGP48508), which have different optical activities and are non-superimposable mirror images, whether this effect of BA41899 was stereoselective. Although caffeine did not directly affect TnC, it was possible that BA41899 would increase the Ca^{2+} affinity of TnC and this was investigated using its enantiomers. Two types of experiments were performed: those with skinned fibres (part 1) and some with isolated TnC (part 2).

PART 1: Ca^{2+}-SENSITIVITY OF FORCE IN SKINNED FIBRES

4.2 METHODS

4.2.1 PREPARATION OF FIBRES

a) Porcine Septomarginalis

Porcine hearts were obtained from a slaughterhouse and large (2.5 x 0.5cm) trabeculae rapidly excised from the right ventricle. These were transferred to the laboratory on ice in a solution of 20 mM histidine; 5 mM K_2EGTA; 10 mM NaN_3; 1 mM DTT and 50% Glycerol, pH 7.0 at 4°C. The outer layer of connective tissue was peeled back to expose the subendocardium from which small (1 mm x 1 cm) bundles of septomarginalis fibres were removed. These bundles were then immersed in a skinning solution similar to that above, but also including 0.1% Triton X-100 and at pH 7.30, for 30 minutes with continuous gentle agitation at room temperature (23°C). The bundles were then transferred to the same solution without Triton-X and with a pH of 7.00 at 4°C and were stored at -20°C for up to 2 months.

Bundles of porcine septomarginalis fibres of approximately 5 mm length and 200 μm diameter were dissected from the glycerinated tissue for use in experiments.

b) <u>Rat Psoas</u>

Bundles of fibres approximately 1.5 cm in length and with diameters of 1.0 - 1.5 mm were dissected from the psoas muscles of male Sprague Dawley (300-400 g) rats (killed by cervical dislocation) and skinned as described above for pig septomarginalis.

Prior to mounting rat psoas in the experimental set-up further dissection was necessary to obtain bundles of 2-4 fibres of $\approx$ 5 mm length and 200 μm diameter, as measured with an eye-piece graticule. It proved impossible to dissect out single fibres without incurring damage during the separation from neighbouring fibres and connective tissue, although this may have improved with practice. Any fibres that were visibly bent or stretched during dissection were abandoned in case the sarcomere arrangement had been disrupted (especially important since SL was not monitored during the experiments).

c) <u>Rabbit Psoas</u>

Rabbit psoas muscle was prepared according to the method described in section 2.2.2.b.

d) <u>Porcine Sacrospinalis</u>

Deep fibres of pig sacrospinalis (skeletal muscle lying dorsal to the spine) were obtained from a slaughterhouse; psoas was not available since it is sold as pork fillet for human consumption. The sacrospinalis muscle was removed from a freshly killed (still warm) pig by a technician with a large carving knife - outermost parts of the muscle were therefore assumed to be too damaged for use and were discarded. It was also likely that the carcass had been rinsed with water which may have caused intracellular damage to outer regions of the muscle by upsetting the osmotic balance. The large (10 cm x 2.5 cm x 1 cm) strip of muscle obtained consisted of bundles of fibres orientated perpendicularly to the long axis of the strip, and with lengths of approximately 2.5 cm. Chunks comprising many bundles of fibres were rapidly dissected from the inner regions of the muscle and were placed in cold pre-skinning solution (as used for rabbit psoas). Each chunk had dimensions of approximately 1.5 cm x 0.5 cm x 0.5 cm with fibres aligned with the longest axis.

Back in the laboratory, approximately 30 minutes later, the chunks of muscle were further dissected under the microscope to yield fibre bundles about 1cm long with diameters 1.0-2.0 mm. These were then treated the same as rabbit psoas, being transferred to fresh pre-skinning solution and left for 24 hours at 4° C with frequent inversion. The porcine muscle was not, however, kept at its in vivo length so some rearrangement or shortening of sarcomeres may have occurred.

This sacrospinalis muscle was finally stored at -20°C in skinning solution diluted 50% with glycerol. Single fibres could be dissected out for experimentation.

4.2.2 SOLUTIONS

Ca^{2+}-sensitivity measurements were carried out using either solutions in routine use at Ciba-Geigy or solutions used at UCL. Both solutions were used in experiments with rabbit psoas.

Ciba-Geigy solutions consisted of a relaxing solution ("M"-solution) and a maximally-activating solution ("T"-solution). "M-solution" contained : 5 mM K_2EGTA; 10 mM Na_2ATP; 12 mM $MgCl_2$ (3 mM Mg^{2+}); 20 mM Imidazole; 5 mM NaN_3; 5 mM Pyroenolphosphate (PEP) and 10 μl/ml pyruvate kinase; pH 6.70, IS 104. Pyruvate kinase was not present in the stock solution of M and T but 10 μl were added to each ml of relaxing or activating solution using a multivolume dispenser just prior to the experiment (Pyruvate kinase activity is lost on freezing). The PEP/Pyruvate kinase system was necessary to provide a means of regenerating ATP from ADP:

"T-solution" was the same as M-solution except 5 mM CaEGTA was included instead of the K_2EGTA. These solutions were stored at -20°C for up to one month before use and pH was always checked and if necessary adjusted to 6.70 after defrosting. pH 6.7 was chosen to represent the cytosolic pH in the failing heart. Combination of M- and T-solutions in various ratios enabled a range of free Ca^{2+} concentrations from zero (contaminant, pCa 8.69) to 47.7 μM (pCa 4.32) to be achieved. Free Ca^{2+} was calculated using a computer program written by Herzig using the following binding constants: $logK_1$ 9.58; $logK_2$ 8.97; $logK_3$ 2.80; $logK_4$ 2.12; $logK_{Ca1}$ 11.0; $logK_{Ca2}$ 4.72; $logK_{Mg1}$ 5.21; $logK_{Mg2}$ 2.76; $logATPK_1$ 6.62; $logATPK_2$ 4.17; $logATPK_{Ca1}$ 3.60; $logATPK_{Ca2}$ 1.96; $logATPK_{Mg1}$ 4.00 and $logATPK_{Mg2}$ 0.12 (notation as in section 2.2.1). pCa values calculated with these constants were not significantly different from those calculated using the constants in table 2 at 22°C.

These solutions were used to determine the Ca^{2+}-sensitivity of rat psoas, rabbit psoas and pig sacrospinalis fibres, in the absence and presence of BA41899. BA41899 was relatively insoluble in water and was therefore prepared as a stock solution of 20 mM dissolved in 100% DMSO (Fluka). Aliquots of this stock were added to the relaxing and activating solutions to achieve the desired concentrations of drug. Equivalent volumes of 100% DMSO without BA41899

were added to control solutions in case DMSO had any effect on the fibres (Kurebayashi & Ogawa, 1985). In experiments where the effects of caffeine were investigated, caffeine was added in solid form to stock M- and T-solutions to give a concentration of 20 mM. The caffeine stock solutions were then combined in the same way as the control ones with the addition of pyruvate kinase and DMSO. All experiments were carried out at room temperature (23° C).

Evaporation of the solutions during the experiments was minimised by storing the solutions in baths (Wannli) covered by an inverted petri dish on paper towel soaked in distilled water.

Ca^{2+}-sensitivity measurements of rabbit psoas and pig septomarginalis were made in the absence and presence of 20 mM caffeine with solutions in routine use at UCL (table 4). The effects of BA41899 and its enantiomers CGP48506 and CGP48508 on Ca^{2+}-sensitivity were also tested on rabbit psoas and porcine septomarginalis. The compounds were prepared as 20 mM stock solutions in 100% DMSO. Aliquots of these were then added to the UCL relaxing and activating solutions to achieve the desired concentration of drug. Equivalent volumes of 100% DMSO were also added to control solutions. All experiments were carried out at room temperature (23° C).

4.2.3 MEASUREMENT OF FORCE

Isometric force was measured by gluing one end of each fibre, or of a group of 2-4 fibres, to a fixed glass rod (motor arm) and the opposite end to a carbon arm linked to a SensoNor AE801 force transducer. Force responses were recorded on a chart recorder (W+W Recorder 600 TARKAN). Special 1.0 ml capacity baths (Wannli) had been made by cutting the bases off tablet vials to create glass cylinders (diameter 1.2 cm, depth 5 mm). The baths were filled with solution prior to the start of the experiment and were placed in turn on a lowered pedestal below the mounted fibres. To incubate the fibres the pedestal was raised to a fixed stop where the solution covered the preparation. Bath changing was accomplished in 1-2 seconds. The pedestal and hence the Wannli were rotated throughout each experiment to improve diffusion of the solutions into the fibres.

Rat psoas fibre bundles (stiff as a result of rigor) were also glued onto a dual fibre set-up, in which two bundles of fibres could be immersed simultaneously in the same solution-filled Wannli. This apparatus was similar in principle to that described above but had two transducer elements per motor arm and a two channel chart recorder (electronics made by Ciba-Geigy).

4.2.4 PROTOCOLS

a) Use of M and T Solutions

The fibres were immersed in M-solution to equilibrate for ten minutes. Before the start of the experiment proper a test maximal activation was carried out and resting force was adjusted by stretching or relaxing the fibres to give a just measurable resting tension.

In experiments to determine the effects of BA41899 on Ca^{2+}-sensitivity of the skinned fibres either of two protocols was followed:

i) *Ciba-Geigy Method*

This protocol was based on that previously used at Ciba-Geigy for similar tests done on porcine cardiac tissue. After two washes in M-solution (each 6 minutes), fibres were incubated in two washes of T-solution (12 & 6 minutes) followed by 6 minutes in T-solution containing BA41899. The fibres were then relaxed (in M-solution) for 30 minutes or until resting tension was level. The double incubations in a given solution ensured that the true representative force had been reached. BA41899 was only applied once to minimise the duration of maximal activation. In some experiments the fibres were returned to T-solution between the drug and M-solutions to make sure that maximum force could still be attained. This however was usually omitted again to minimise duration of maximal contraction.

Following a 12 minute immersion in fresh M-solution increasing levels of Ca^{2+} were applied to the fibres. At each Ca^{2+} concentration two 12 minute incubations in control solution were followed by one in test solution (+ BA41899) then a return to control. Fibres were then transferred to a slightly higher Ca^{2+} concentration solution (culminating in T-solution) without an intermediate relaxation stage, and this pattern of incubations was repeated. Incubation times were shortened to 6 minutes (apart from the BA41899 washout) in work with rabbit and porcine fibres. The effects of 20 μM, 50 μM and 100 μM BA41899 were tested on rat and rabbit tissue, and 100μM BA41899 on porcine skeletal tissue.

ii) *UCL Method*

A second protocol, based on procedures followed at University College London, was also used in some cases to test the effects of BA41899 on Ca^{2+}-sensitivity of rabbit psoas and porcine sacrospinalis. This method used contractions of only 1.0-1.5 minutes duration coupled with complete relaxation of fibres between applications of Ca^{2+}-activation solutions. The fibres were initially maximally activated in T-solution for 1 minute followed by incubation in M-

solution until resting tension became stable. This was repeated until a steady force in T-solution was obtained : early contractions often showed a marked decline in force probably due to pulling of fibres out of the glue.

After an initial test with T-solution (± BA41899), Ca^{2+} solutions were applied in order of increasing Ca^{2+} concentration. At each Ca^{2+} concentration, two 1-1.5 minute incubations in M-solution were followed by one in activating solution, one in activating solution plus BA41899 then a return to M-solution. In early experiments double incubations were used to check that true force had been reached for a particular Ca^{2+} concentration before immersion of the fibres in BA41899 solution. This was reduced to a single incubation in later experiments as no change in force was ever noted during a second immersion in the same solution.

After testing of BA41899 on each porcine fibre, the effects of 20 mM caffeine were determined in the same fibre, the protocol being the same as that described for BA41899 using the UCL method. A 30 minute relaxation in M-solution was allowed between the final BA41899 application and the first Ca^{2+} activation in the caffeine experiment.

b) Use of UCL Solutions

The effects of caffeine, BA41899, CGP48506 and CGP48508 on Ca^{2+} sensitivity of rabbit psoas and pig septomarginalis were monitored using the UCL method described in the previous section. This set of experiments was performed during a second visit to Ciba-Geigy, thus the repetition of work demonstrating the effects of BA41899 on rabbit psoas served as a control to ensure continuity of results from one visit to the next (6 months later). The only difference from the UCL method described above was the composition of the solutions, which were the same as those in table 4, with 10 mM (after dilution with solution A) total Ca solution used as the maximal activation solution (c.f. T-solution) and zero (contaminant) Ca solution (HR) used as the relaxing solution (c.f. M-solution).

4.2.5 STATISTICAL ANALYSIS

Analysis of the results was carried out as described in section 2.2.3.

4.3 RESULTS

4.3.1 C-G AND UCL METHODS OF Ca^{2+}-SENSITIVITY MEASUREMENT

Rabbit psoas was used to compare Ca^{2+}-sensitivity measurements obtained according to the Ciba-Geigy and UCL methods (table 9). The results obtained using the C-G technique were not significantly different from those obtained for

rat psoas (table 9). Thus the general stability and Ca^{2+}-sensitivity of rat and rabbit fibres were similar. In addition the Ciba-Geigy and UCL methods gave the same Ca^{2+}-sensitivity for the rabbit psoas although resting tension did appear to remain lower in the UCL method (increasing by 3.3% as compared to 8.0% during the experiment). In later work, therefore, pCa_{50} and H_n values obtained using the two protocols were combined for statistical analysis.

	C-G *Rat*	C-G *Rabbit*	UCL *Rabbit*
pCa_{50}	5.79±0.04 (11)	5.76±0.04(7)	5.74±0.03(6)
Hill "n"	2.08±0.10 (11)	1.94±0.13(7)	2.18±0.11(6)
Decline in Maximum Force	24.2±3.3 (11)	21.4±3.6(7)	15.7±2.2(6)
Increase in Resting Force	5.7±1.3 (11)	8.0±1.6(7)	3.3±1.3(6)*

TABLE 9. COMPARISON OF CIBA-GEIGY AND UCL METHODS FOR DETERMINING Ca^{2+}-SENSITIVITY OF SKINNED PSOAS FIBRES. Measurements made using C-G solutions (section 4.2.2). All forces expressed as % force of 1st maximal activation. All values expressed as mean±SEM (n). * denotes significant difference from corresponding C-G result.

4.3.2 EFFECTS OF INOTROPIC AGENTS ON THE Ca^{2+}-SENSITIVITY OF SKINNED FIBRES

a) Effects of BA41899

The effects of the novel compound BA41899 were tested, using a range of concentrations, on rat and rabbit psoas muscle (tables 10 & 11; fig.21). 20μM BA41899 slightly sensitised both rat and rabbit fibres to Ca^{2+} with increases in pCa_{50} of 0.04 and 0.07 units respectively. The shifts in pCa_{50} increased to 0.12 units in both species with 50μM BA41899, and to 0.21 with 100μM BA41899. In contrast, no effect of the test compound was observed on H_n at any of the concentrations used.

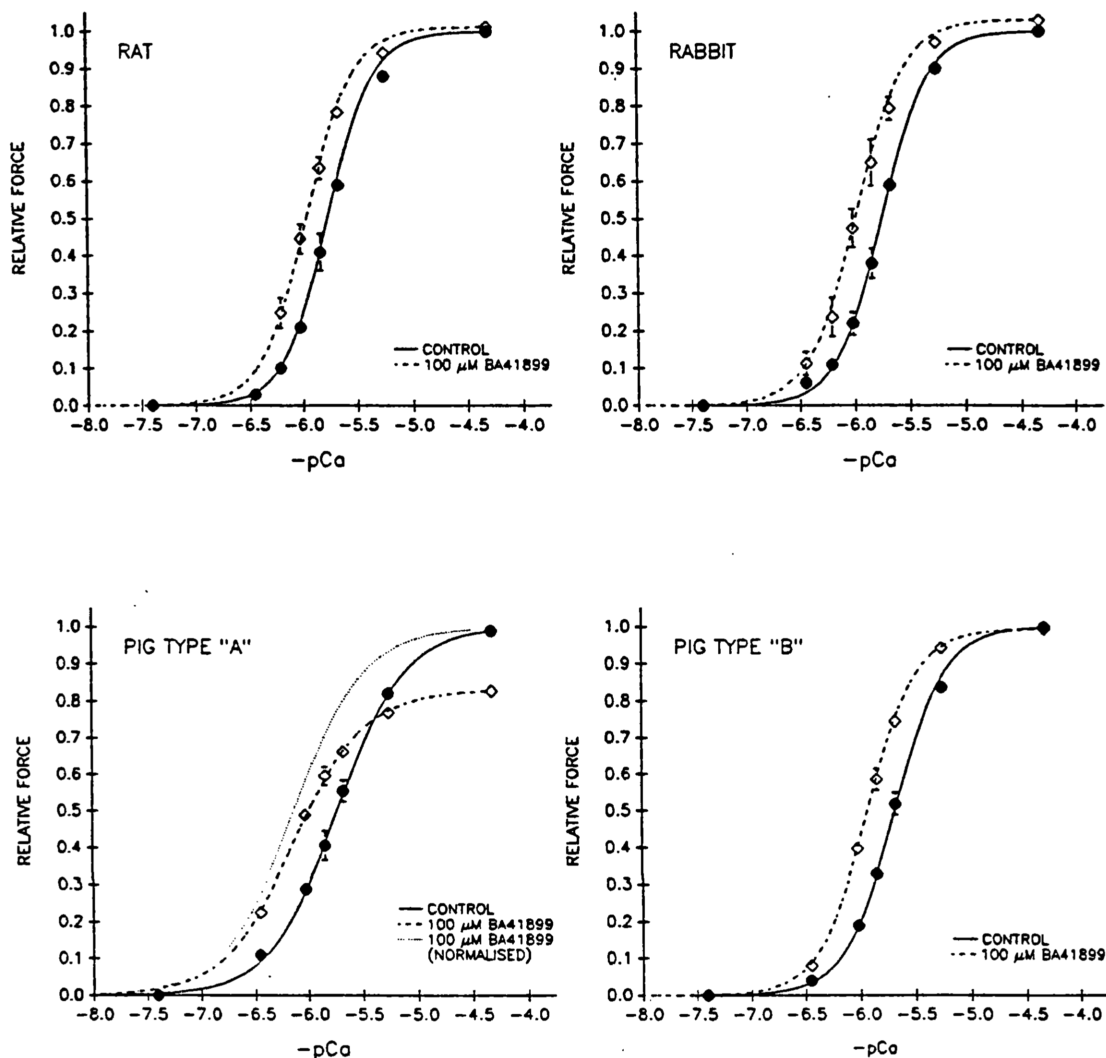


Fig.21 EFFECTS OF BA41899 ON MAXIMUM FORCE AND Ca^{2+}-SENSITIVITY OF SKINNED SKELETAL FIBRES. Experiments performed with Ciba-Geigy solutions according to the Ciba-Geigy protocol, described in section 4.2.4.a.i. Points represent mean data ± SEM from 4 experiments (rabbit psoas) or 7 experiments (rat psoas; types A and B porcine sacrospinalis). Curves were generated from mean pCa_{50} and Hill "n" values (table 11) and, where stated, normalised to control maximum force.

			20 μM BA41899	50 μM BA41899
pCa_{50}	Control	Rat	5.77±0.03(6)	5.72±0.02(5)
		Rabbit	5.75±0.05(4)	5.73±0.06(4)
	Test	Rat	5.81±0.03(6)	5.84±0.02(5)
		Rabbit	5.82±0.04(4)	5.85±0.05(4)
	Shift	Rat	0.04±0.01(6)*	0.12±0.01(5)*
		Rabbit	0.07±0.01(4)*	0.12±0.02(4)*
Hill n	Control	Rat	2.11±0.07(6)	2.21±0.06(5)
		Rabbit	2.09±0.14(4)	2.01±0.26(4)
	Test	Rat	2.06±0.09(6)	2.31±0.08(5)
		Rabbit	2.08±0.11(4)	1.81±0.11(4)
	Shift	Rat	-0.05±0.06(6)	0.11±0.11(5)
		Rabbit	-0.01±0.03(4)	-0.20±0.16(4)
Max F	% Fall	Rat	0.0±0.7(7)	-1.2±1.0(5)
		Rabbit	-1.8±0.9(4)	-1.8±1.3(4)

TABLE 10. EFFECTS OF BA41899 ON SKINNED PSOAS FIBRES FROM RAT AND RABBIT. Experiments performed using C-G solutions and the C-G or UCL protocol. All values expressed as mean±SEM(n). * denotes a significant difference from zero (Paired t-test). Suppression of maximum force by the test compounds was measured during the first maximal activation (expressed relative to control).

The Ca^{2+} sensitising action of 100 μM BA41899 was also measured in pig sacrospinalis fibres (table 11, fig.21) and, in experiments performed six months later, using pig septomarginalis and rabbit psoas again to check reproducibility (fig.22). It became apparent during work with BA41899 (and caffeine, discussed below) that two groups of fibres (nominally called types "A" and "B") were present in pig sacrospinalis muscle (table 11). These were indistinguishable in terms of dimensions (mean diameter was 140±8.0 μm (n=7) for type A and 121±8.5 μm (n=7) for type B).

			BA41899	CAFFEINE
pCa_{50}	Control	Rat	5.75 ± 0.04(7)	-
		Rabbit	5.76 ± 0.04(4)	-
		Pig A	5.73 ± 0.04(7)	5.67 ± 0.07(4)
		Pig B	5.68 ± 0.03(7)	5.57 ± 0.04(5)
	Test	Rat	5.96 ± 0.03(7)	-
		Rabbit	5.98 ± 0.05(4)	-
		Pig A	6.13 ± 0.03(7)	5.93 ± 0.05(4)
		Pig B	5.93 ± 0.02(7)	5.72 ± 0.02(5)
	Shift	Rat	0.21 ± 0.02(7)*	-
		Rabbit	0.21 ± 0.00(4)*	-
		Pig A	0.40 ± 0.02(7)*	0.26 ± 0.03(4)*
		Pig B	0.25 ± 0.01(7)*	0.15 ± 0.03(5)*
Hill n	Control	Rat	2.03 ± 0.11(7)	-
		Rabbit	2.05 ± 0.13(4)	-
		Pig A	1.38 ± 0.06(7)	1.31 ± 0.11(4)
		Pig B	1.88 ± 0.17(7)	1.58 ± 0.07(5)
	Test	Rat	2.03 ± 0.15(7)	-
		Rabbit	1.95 ± 0.11(4)	-
		Pig A	1.32 ± 0.04(7)	1.28 ± 0.09(4)
		Pig B	1.94 ± 0.12(7)	1.34 ± 0.04(5)
	Shift	Rat	0.00 ± 0.13(7)	-
		Rabbit	-0.10 ± 0.07(4)	-
		Pig A	-0.05 ± 0.06(7)	-0.03 ± 0.07(4)
		Pig B	0.09 ± 0.09(7)	-0.24 ± 0.06(5)
Max F	% Fall	Rat	-1.1 ± 0.7(7)	-
		Rabbit	-2.8 ± 1.7(4)	-
		Pig A	17.7 ± 2.1(7)*	6.5 ± 1.0(4)*
		Pig B	0.7 ± 1.2(7)	3.4 ± 1.3(5)

TABLE 11. EFFECTS OF 100 μM BA41899 AND 20 mM CAFFEINE ON SKINNED SKELETAL FIBRES. Experiments performed using C-G solutions and either the C-G protocol (BA41899 + rat, rabbit & pig) or the UCL protocol (BA41899 & caffeine + rabbit & pig). Pig A and pig B represent 2 types of fibres found in porcine sacrospinalis. All values expressed as mean $\pm$ SEM(n). * denotes a significant difference from zero (Paired t-test). Suppression of maximum force by the test compounds was measured during the first maximal activation (expressed relative to control).

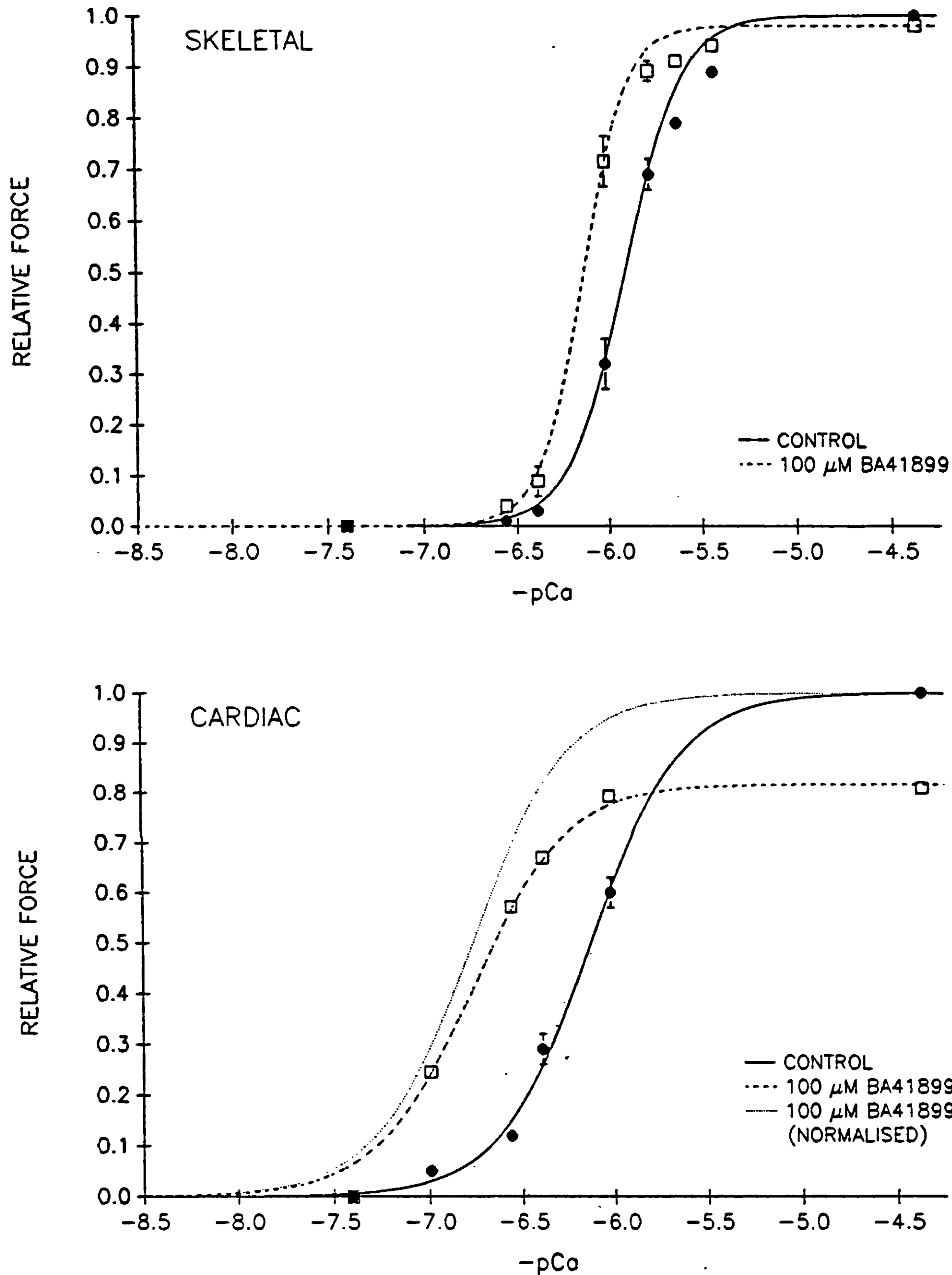


Fig.22 EFFECTS OF BA41899 ON SKINNED SKELETAL AND CARDIAC MUSCLE FIBRES. Experiments performed with UCL solutions (table 4) according to the UCL protocol described in section 4.2.4.a.ii. Points represent mean data $\pm$ SEM from 6 experiments (rabbit psoas) or 4 experiments (porcine septomarginalis). Curves were generated from mean pCa_{50} and Hill "n" values (table 12) and, where stated, normalised to control maximum force.

			CAFFEINE	BA41899
pCa_{50}	Control	Sk	5.98±0.02(4)	5.91±0.02(6)
		C	6.10±0.02(4)	6.13±0.02(4)
	Test	Sk	6.09±0.01(4)	6.14±0.01(6)
		C	6.37±0.01(4)	6.77±0.00(4)
	Shift	Sk	0.11±0.01(4)*	0.23±0.01(6)*
		C	0.26±0.02(4)*	0.64±0.02(4)*
Hill n	Control	Sk	3.75±0.22(4)	2.81±0.10(6)
		C	1.73±0.04(4)	1.74±0.03(4)
	Test	Sk	2.19±0.09(4)	3.62±0.49(6)
		C	1.90±0.14(4)	1.69±0.03(4)
	Shift	Sk	-1.56±0.16(4)*	0.81±0.11(6)*
		C	0.17±0.17(4)	-0.05±0.02(4)
Max F	% Fall	Sk	16.3±0.1(4)*	2.0±0.5(6)
		C	16.0±3.7(4)*	18.3±0.6(4)*

TABLE 12. EFFECTS OF CAFFEINE AND BA41899 ON SKINNED RABBIT PSOAS AND PORCINE SEPTOMARGINALIS FIBRES. Experiments performed with UCL solutions and according to the UCL protocol. BA41899 applied at 100 μM and caffeine at 20 mM. All values expressed as mean±SEM(n). * denotes a significant difference from zero (Paired t-test). Suppression of maximum force by the test compound was measured during the 1st maximal activation (expressed relative to control). Sk = skeletal (rabbit psoas); C = cardiac (porcine septomarginalis).

Type A sacrospinalis fibres were more affected by 100 μM BA41899 than were type B fibres (fig.21), the respective increases in pCa_{50} being 0.40 and 0.25 (table 11). The Ca^{2+} sensitising actions of the compound were much greater in porcine cardiac than in skeletal muscle, with pCa_{50} shifting by 0.64 units (fig.22). In no case was the steepness of the force - pCa relationship altered by BA41899. The change in pCa_{50} induced by 100 μM BA41899 (table 12) in repeat experiments with rabbit psoas was no different to that observed in earlier work (0.23 units, table 11) despite the fact that the control pCa_{50} of 5.91 was greater than the 5.76 observed in the former experiments.

BA41899 had varying effects on maximum force. At concentrations of 20 μM

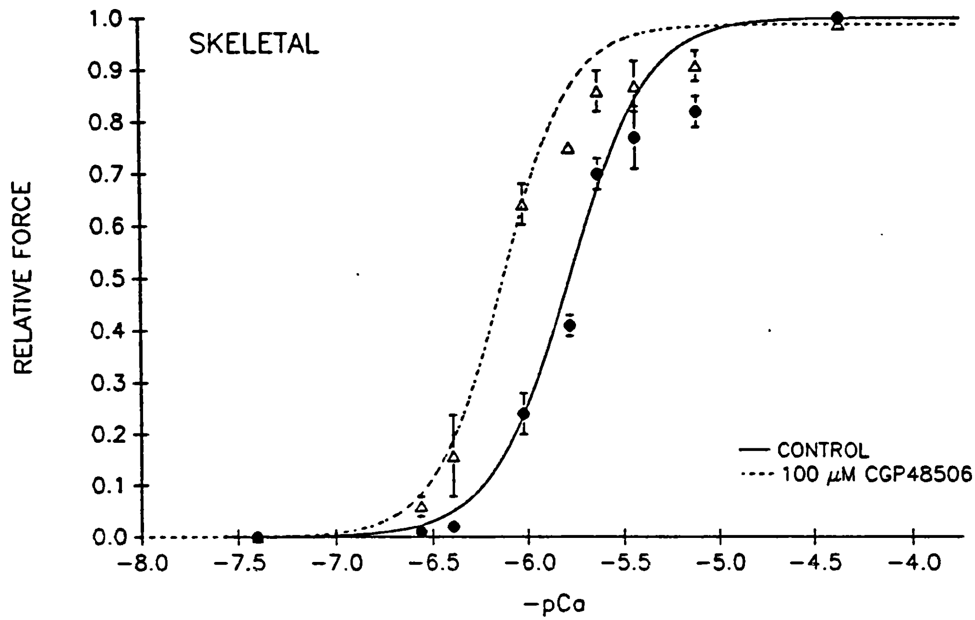
or 50 μM no effect was found in rat or rabbit psoas (table 10). 100 μM BA41899 reduced maximum force of porcine type A sacrospinalis and septomarginalis by 17.7% and 18.3% respectively but had no effect on rat or rabbit psoas and pig type B sacrospinalis fibres (fig.21, tables 11 & 12).

There were therefore considerable differences in the degrees of Ca^{2+}-sensitisation induced by 100 μM BA41899 in different fibre types. Fast twitch (psoas) muscle responded with the smallest increase in pCa_{50} and cardiac muscle the greatest. A similar pattern was observed in the suppression of maximum force by BA41899. The responses of type B fibres resembled those of psoas and were therefore presumed fast twitch. Type A fibres, however, were more sensitive to BA41899 than type B fibres and were probably slow-twitch. Interestingly the type B fibre results fell between those of fast twitch and cardiac muscle - though comparison with the latter can only be tentative as different solutions were used in each case.

b) Effects of Enantiomers of BA41899

The Ca^{2+} sensitising actions of the two enantiomers of BA41899 (CGP48506 and CGP48508) were investigated using rabbit psoas (skeletal) and pig septomarginalis (cardiac). 100 μM CGP48506 increased pCa_{50} by 0.35 units in the skeletal muscle and by 0.88 in the cardiac preparation (table 13, fig.23). In both cases CGP48506 had a greater Ca^{2+} sensitising effect than did the same concentration of BA41899. In comparison, 100 μM CGP48508 produced a leftwards displacement of the pCa_{50} of rabbit psoas by 0.14 units but had no effect on the Ca^{2+}-sensitivity of pig septomarginalis (table 13, fig.24). It did, however, suppress maximum force generated by the cardiac muscle by 11.5% compared to a mere 2.5% in rabbit psoas. The depression of maximum force of septomarginalis caused by CGP48506 (20.5%) was greater than that of its enantiomer (11.5%), but CGP48506 had no effect on the maximum tension of rabbit psoas (table 13, fig.23).

The enantiomers thus showed marked differences in their effects on cardiac and skeletal fibres, with CGP48506 increasing the Ca^{2+}-sensitivity of septomarginalis more than psoas (similar to caffeine - table 6) whereas CGP48508 very unusually increased the pCa_{50} of skeletal fibres whilst having no effect on that of cardiac muscle.



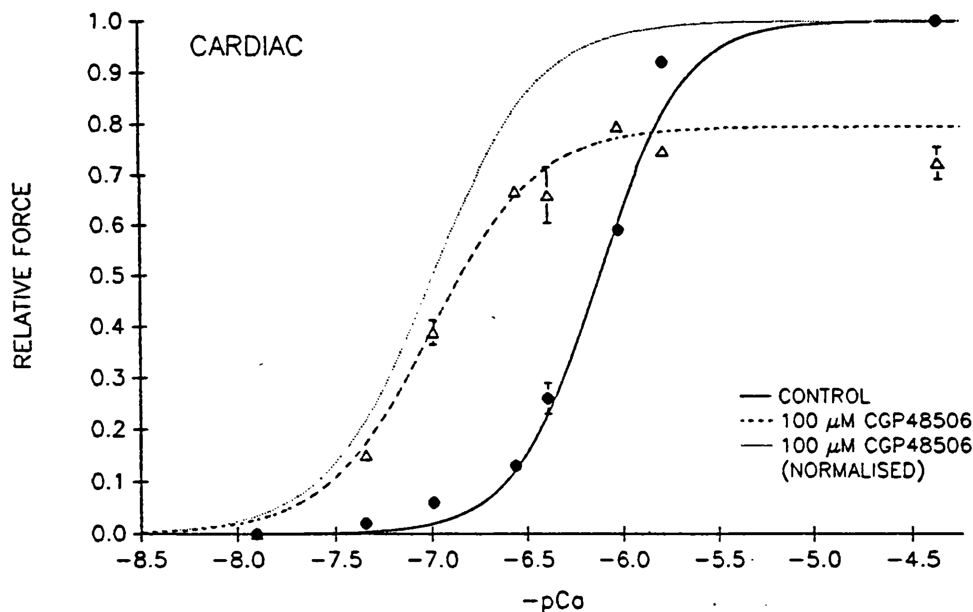


Fig.23 EFFECTS OF CGP48506 ON SKINNED RABBIT PSOAS AND PORCINE SEPTOMARGINALIS FIBRES. Experiments performed with UCL solutions (table 4) according to the UCL protocol described in section 4.2.4.a.ii. Points represent mean data ± SEM from 4 experiments. Curves were generated from mean pCa_{50} and Hill "n" values (table 13) and, where stated, normalised to control maximum force.

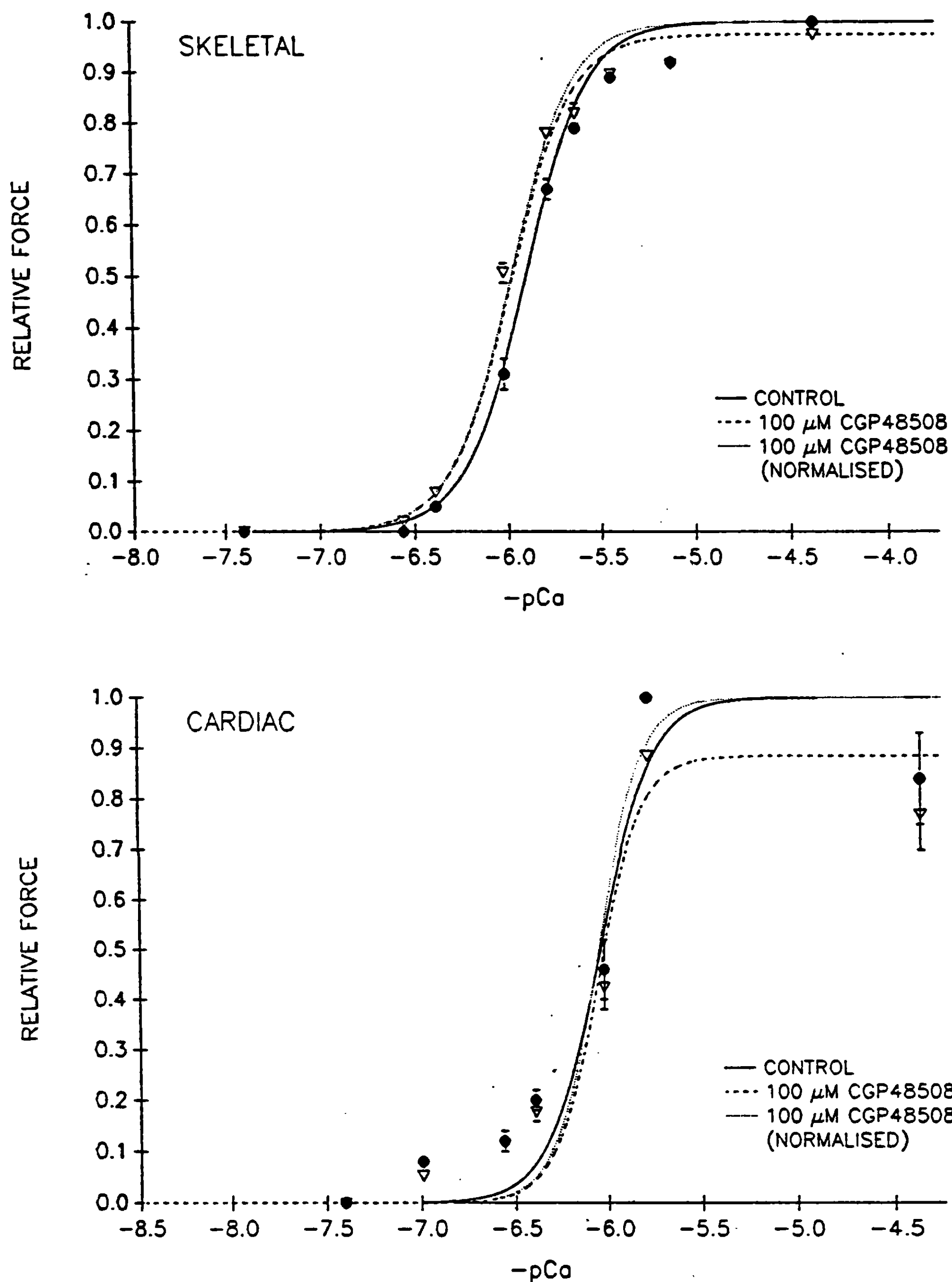


Fig.24 EFFECTS OF CGP48508 ON SKINNED RABBIT PSOAS AND PORCINE SEPTOMARGINALIS FIBRES. Experiments performed with UCL solutions according to the UCL protocol described in section 4.2.4.a.ii. Points represent mean data $\pm$ SEM from 6 psoas and 4 septomarginalis experiments. Curves were generated from mean pCa_{50} and Hill "n" values (table 13) and, where stated, normalised to control maximum force.

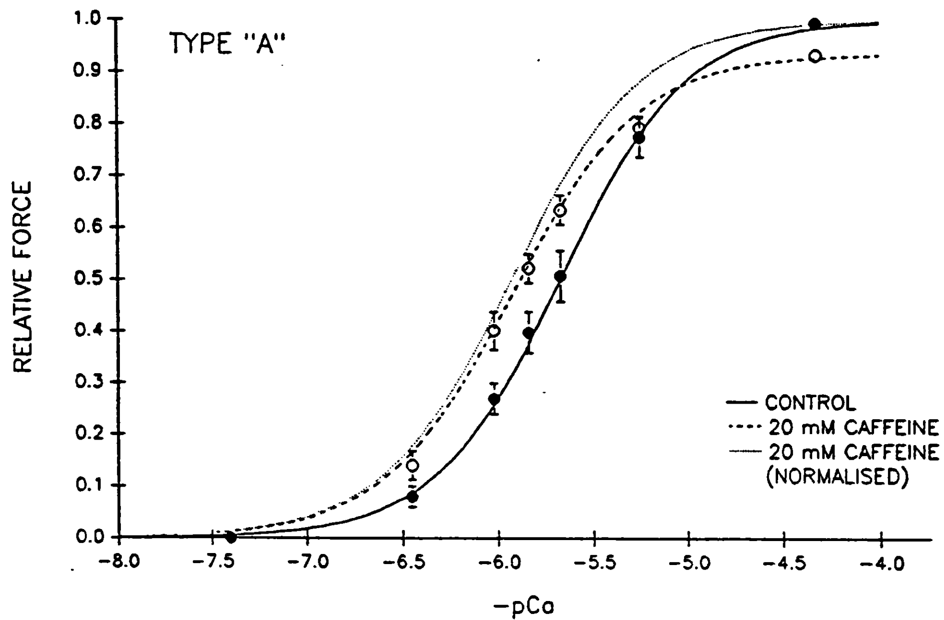
			CGP48506	CGP48508
pCa_{50}	Control	Sk	5.78±0.01(4)	5.90±0.01(6)
		C	6.12±0.01(4)	6.04±0.02(4)
	Test	Sk	6.13±0.02(4)	6.04±0.01(6)
		C	6.99±0.01(4)	6.05±0.02(4)
	Shift	Sk	0.35±0.01(4)*	0.14±0.00(6)*
		C	0.88±0.02(4)*	0.01±0.00(4)
Hill n	Control	Sk	2.10±0.18(4)	2.58±0.07(6)
		C	1.94±0.03(4)	3.08±0.35(4)
	Test	Sk	2.42±0.15(4)	2.62±0.04(6)
		C	1.58±0.07(4)	3.74±0.65(4)
	Shift	Sk	0.32±0.04(4)*	0.05±0.06(6)
		C	-0.36±0.10(4)	0.66±0.40(4)
Max F	% Fall	Sk	1.3±0.4(4)	2.5±0.4(6)*
		C	20.5±0.8(4)*	11.5±1.6(4)*

TABLE 13. EFFECTS OF CGP48506 AND CGP48508 ON SKINNED RABBIT PSOAS AND PORCINE SEPTOMARGINALIS FIBRES. Experiments performed with UCL solutions and according to the UCL protocol. Test compounds applied at 100 μM. All values expressed as mean±SEM(n). * denotes a significant difference from zero (Paired t-test). Suppression of maximum force expressed relative to force of 1st maximal activation. Sk = skeletal (rabbit psoas); C = cardiac (porcine septomarginalis).

c) Effects of Caffeine

The effects of 20 mM caffeine on types A and B porcine sacrospinalis fibres, septomarginalis and rabbit psoas fibres were also tested. Caffeine induced a 0.26 unit increase in pCa_{50} of type A sacrospinalis preparations but a corresponding shift of only 0.15 units in type B fibres (table 11, fig.25). The pCa_{50} of porcine septomarginalis was increased by 0.26 units by 20 mM caffeine which also increased the pCa_{50} of rabbit psoas by 0.11 units (table 12). Caffeine appeared to reduce the H_n of rabbit psoas whilst having no effect on the H_n values for the other fibre types.

In addition 20 mM caffeine suppressed the maximum force of type A pig sacrospinalis, rabbit psoas and pig septomarginalis preparations by 6.5%, 16.3%



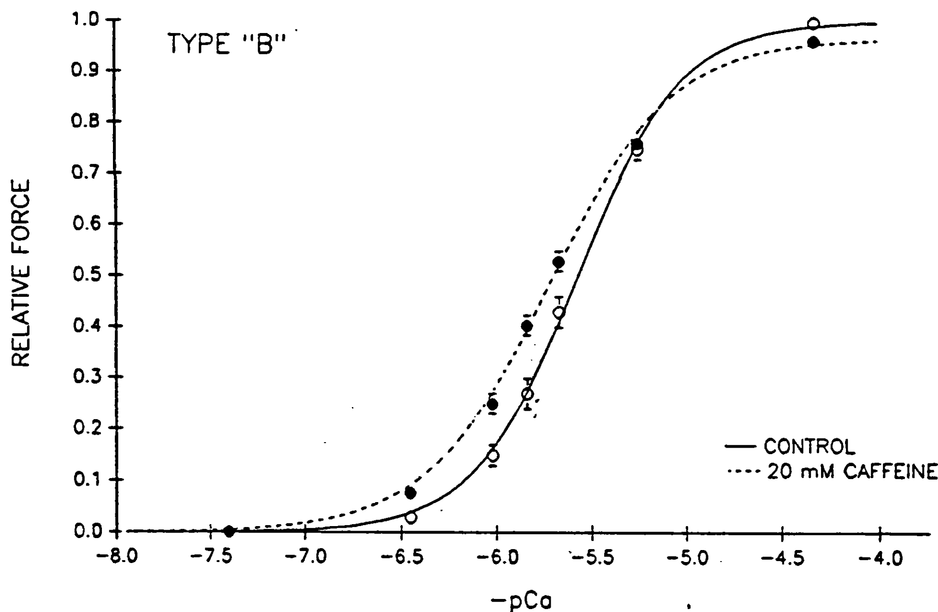


Fig.25 EFFECTS OF CAFFEINE ON TYPES A AND B PORCINE SACROSPINALIS SKINNED FIBRES. Experiments performed with Ciba-Geigy solutions according to the UCL protocol as described in section 4.2.4.a. Points represent mean data $\pm$ SEM from 4 experiments with type A and 5 with type B fibres. Curves were generated from mean pCa_{50} and Hill "n" values (table 11) and, where stated, normalised to control maximum force.

and 16.0% respectively. No effect of caffeine was observed on the maximum force of type B sacrospinalis fibres.

Thus the Ca^{2+}-sensitising effects of caffeine were most pronounced in cardiac and type A fibres and less so in type B fibres. These results were broadly similar to those observed in table 6, and suggest that type A fibres are not fast twitch like psoas but probably slow twitch.

4.4 DISCUSSION

4.4.1 C-G AND UCL METHODS OF Ca^{2+}-SENSITIVITY MEASUREMENT

Comparison of the C-G and UCL methods using rabbit skinned psoas (table 9) revealed the only significant difference to be the smaller rise in resting tension associated with the latter method. The prolonged activation intrinsic to the C-G method eventually results in a greater force at a given $[Ca^{2+}]$ than does the shorter UCL method activation. It may be that this greater force is due to slow contraction of the damaged ends of the fibres at their points of attachment. If such damage does cause the rise in tension then it would not be surprising that the increase in resting force is significantly greater using the C-G method. It is still debatable which measure of force represents the "true" level but, since this does not affect determination of Ca^{2+}-sensitivity, the C-G and UCL methods are equally effective in quantifying inotropic actions of novel compounds.

4.4.2 COMPARISON OF FIBRES FROM THE 3 SPECIES

Whilst bearing in mind that fibres from rat, rabbit and pig were prepared using slightly different methods, a tentative comparison can be made between species. The control pCa_{50} values measured for fibres from rat, rabbit and pig (both types A and B) were not significantly different. Thus the concentration of Ca^{2+} required for 50% activation is the same for all three species. There were also no significant differences between the steepnesses (as expressed by H_n) of the force-pCa relationships of rat, rabbit and pig type B fibres. However the H_n value of 1.38 for type A pig fibres was significantly lower than that of 1.88 for type B fibres. This implies that there is less cooperativity in the former i.e. type A fibres are more similar to slow twitch muscle and type B to fast-twitch muscle (Stephenson & Forrest, 1980) - a conclusion supported by further evidence obtained in studies of the effects of BA41899 and caffeine (section 4.3.2).

4.4.3 MODULATION OF Ca^{2+}-SENSITIVITY BY INOTROPIC AGENTS

a) Effects of BA41899

i) *Resting Force*

BA41899 had no effect on the resting tension of rat or rabbit psoas or porcine septomarginalis. It may increase resting force of porcine sacrospinalis (both A and B type fibres) but only by a very small amount (1.43% original maximum force).

ii) *Maximum Force*

In contrast, 100 μM BA41899 suppressed maximum force of porcine type A sacrospinalis and septomarginalis by 18% (tables 11 & 12) but had no clear effect on the maximum force of either psoas or type B sacrospinalis. From these results (and those discussed below) it is clear that sacrospinalis muscle contains two populations of fibres tentatively identified as slow twitch (type A) and fast twitch (type B): since the sacrospinalis is a postural muscle it is probable that it consists of both slow and fast twitch fibres. It is possible that BA41899 suppresses maximum ATPase activity (and hence maximum force) in cardiac and slow twitch fibres but does not affect fast twitch maximum ATPase activity. Such a mode of action in suppressing maximum tension cannot be ascribed to 20 mM caffeine since the latter seemed to suppress maximum force in all types of muscle (as discussed in section 2.4.1.d) and possibly to a similar extent in fast and slow twitch fibres (Wendt & Stephenson, 1983) though c.f. Mounier et al (1989).

iii) *Ca^{2+}-Sensitivity*

BA41899 also sensitised skinned psoas fibres to Ca^{2+} in a dose-dependent manner (tables 10 & 11) without affecting H_n. This Ca^{2+}-sensitisation was identical in rat and rabbit with a shift in pCa_{50} of 0.21 units induced by 100 μM BA41899 at pH 6.7. The degree of sensitisation was also similar for rabbit psoas (0.23 units) tested at pH 7.00 and at an ionic strength of 200 compared to 100. A rise in pH increases the Ca^{2+} affinity of TnC (chapter 3), but the effect of BA41899 appears to be independent of pH. However, other differences between the Ciba-Geigy and UCL solutions make it difficult to draw any firm conclusions from such a comparison.

Interestingly, BA41899 enhanced the Ca^{2+}-sensitivity of type A sacrospinalis fibres to a greater extent than type B fibres (respective shifts in pCa_{50} of 0.40 and 0.25 units). Likewise cardiac muscle showed a large increase in pCa_{50} (0.64 units) in the presence of 100 μM BA41899 (table 12). This provides further

evidence that type A fibres are of the "cardiac like" slow-twitch variety. This should prove helpful in determining the mechanism of action of BA41899 as a Ca^{2+}-sensitiser since it appears to affect slow twitch and cardiac skinned fibres to a larger degree than fast twitch ones. Thus differences in fibre sensitivity to BA41899 could be explained by differences in the structures of cardiac, slow and fast twitch TnC (Collins et al, 1973; Wilkinson, 1980) or crossbridge cycling rates (Marston & Taylor, 1980), as is discussed further in chapter 6.

b) Effects of Enantiomers of BA41899

The discovery that BA41899 was composed of equal proportions of two enantiomers, which it was possible to separate, provided an opportunity to investigate further which features of the molecule were involved in its Ca^{2+}-sensitising action.

i) *Resting Force*

CGP48506 and CGP48508, behaved similarly to BA41899 in that neither affected the resting tension of rabbit psoas or porcine septomarginalis.

ii) *Maximum Force*

Although 100 μM CGP48508 suppressed the maximum force of rabbit psoas very slightly, CGP48506 having no effect, both compounds caused a marked decrease in maximum force of cardiac skinned fibres (table 13) with CGP48506 inducing a drop in force of 20.5% and CGP48508 of 11.5%. 100 μM BA41899 (= 50 μM each of CGP48506 & CGP48508) suppressed cardiac mximum force by 18%, so assuming such suppression varies with concentration of the enantiomers 18% is higher than expected if CGP48506 and CGP48508 acted independently. It has become clear that explaining decreases in maximum force is far from simple. The suppression is not always related to changes in Ca^{2+}-sensitivity: increasing ionic strength reduces maximum force without affecting Ca^{2+}-sensitivity (Kentish, 1984); Ca^{2+}-sensitisers may decrease (caffeine, BA41899) or increase (ORG 30029; Miller & Steele, 1990) maximum force and some (e.g. caffeine) affect skeletal and cardiac muscle equally whereas others (e.g. BA41899) suppress maximum force more in cardiac than skeletal. In addition, agents which desensitise skinned fibres to Ca^{2+} may also suppress maximum force by a greater extent in cardiac than skeletal fibres (e.g. Pi, acidic pH) or vice versa (BDM). It is clear that such a range of effects on maximum force are unlikely to be explained by a single mechanism. In addition, this is convincing evidence that effects on maximum force and Ca^{2+}-sensitivity have different underlying mechanisms.

iii) *Ca^{2+}-Sensitivity*

BA41899 and its enantiomers all caused a leftwards displacement of the force-pCa relationship of rabbit psoas (tables 10-12, figs.22-24). The increase in pCa_{50} was greatest in the presence of CGP48506 (0.35) followed by BA41899 (0.23) and CGP48508 (0.14). With cardiac fibres CGP48506 caused a 0.88 unit increase in pCa_{50} whereas BA41899 induced a rise of 0.64 units. Assuming the ΔpCa_{50} induced by 50 μM of either enantiomer is less than that induced by 100 μM, a 50 : 50 mixture of CGP48506 and CGP48508 (100 μM BA41899) produced greater shifts in pCa_{50} of skeletal and especially cardiac fibres than expected if the enantiomers acted independently. Thus one enantiomer apparently enhances the Ca^{2+}-sensitisation by the other.

Interestingly, CGP48508 had no effect on pCa_{50}: to my knowledge this is the first known Ca^{2+}-sensitiser which has a greater effect on skinned skeletal than cardiac fibres.

Such different behaviour of the enantiomers from each other and with skeletal and cardiac skinned fibres indicates that the sensitisers may interact with a protein which has different forms in cardiac and skeletal muscle. Since CGP48506 sensitises cardiac fibres to Ca^{2+} more than skeletal ones whereas CGP48508 only sensitises skeletal fibres it also appears that the enantiomers act by different mechanisms, either via different proteins or on different regions of the same protein. Possible mechanisms for the Ca^{2+}-sensitisation of myofilaments are discussed further in chapter 6.

c) Effects of Caffeine

Caffeine was applied to rabbit psoas and porcine septomarginalis preparations to check that it sensitised pig cardiac fibres (psoas was used as a reference to previous work - section 2.3.1). In view of the conflicting results reported on the effects of caffeine on maximum force in skinned fibres from different muscle types (section 2.4.1.d) it was also thought interesting to compare the actions of caffeine on (presumed) fast and slow-twitch (types B & A) fibres.

i) *Maximum Force*

It had previously been shown that 20 mM caffeine suppresses maximum force of rabbit psoas by 18% (table 6) which was in agreement with the 16% suppression obtained at Ciba-Geigy (table 12) using the same solutions. Caffeine had a similar effect in cardiac preparations, reducing maximum force by 16%. Under different conditions (i.e. using Ciba-Geigy solutions) caffeine suppressed the maximum force of porcine type A fibres by 7% but had no effect on type B sacrospinalis (fig.25). This may indicate that the degree of suppression in skeletal

fibres is muscle- or species-dependent since rabbit psoas was affected more than porcine sacrospinalis, though solution differences might also be the cause. Other studies have produced apparently conflicting results with regard to muscle type and caffeine suppression. Wendt & Stephenson (1983) found no marked difference between caffeine-induced depression of maximum force in rat EDL, soleus and cardiac fibres whereas Mounier et al (1989) observed a greater depression in rat soleus than plantaris skinned fibres. The effects of caffeine are therefore not solely species-dependent but also vary between muscles. Assuming type A sacrospinalis fibres are slow twitch it appears that caffeine affects the maximum force of these fibres more than fast twitch ones in pig. Cardiac preparations seem to be affected similarly to fast twitch fibres in rat (Wendt & Stephenson, 1983) though perhaps not in pig. Since TnC is highly conserved between species and slow twitch TnC resembles the cardiac form but differs from the fast twitch moiety (Collins et al, 1973; Wilkinson, 1980), the above variations in the suppressive effect of caffeine are unlikely to reflect variations in TnC. This in turn makes it unlikely that the depression of maximum force by caffeine involves actions on TnC alone, if at all.

ii) *Ca^{2+}-Sensitivity*

There is greater consistency between studies on the Ca^{2+}-sensitising action of caffeine in different fibre types: cardiac and slow twitch skinned fibres are clearly sensitised more than fast twitch skeletal fibres (table 6, Wendt & Stephenson, 1983; Mitsumoto et al, 1990). This provides further evidence that type A sacrospinalis fibres are slow twitch whereas type B are fast twitch since caffeine increased the pCa_{50} of the former by more than the latter (table 11, fig.25). Such effects may be linked to the TnC moiety present in the different fibres though caffeine does not act directly on TnC (section 3.3.4). The caffeine-induced rise in pCa_{50} therefore either requires the presence of other components of the Tn-Tm complex or may be the result of a direct action of caffeine on the crossbridges. This is discussed further in chapter 6.

PART 2: Ca^{2+}-SENSITIVITY OF ISOLATED TnC-DANZ

4.5 METHODS

4.5.1 BACKGROUND

Since TnC has different forms in cardiac and skeletal muscle, the possibility remained that CGP48506 and CGP48508 may affect Ca^{2+} binding to TnC directly. This was investigated using isolated skeletal TnC labelled with DANZ (section 3.1.3) to detect changes in Ca^{2+} affinity. Cardiac TnC-IAANS was not used as none was immediately available and there was insufficient time to prepare a fresh batch.

4.5.2 SOLUTIONS

Isolated TnC-DANZ solid which had been stored frozen for one month at -20°C was dissolved to a concentration of 20 μM in solution B which was similar in composition to the solution A used in skinned fibre work. Solution B consisted of 100 mM BES; 98 mM K Propionate; 5.1 mM $MgCl_2$ (1.25 mM Mg^{2+}); 5 mM ATP; 10 mM Creatine Phosphate and 2.0 mM K_2EGTA, pH 7.00 and IS 160. Stocks of BES and K Propionate had been passed through a column of chelex (BioRad) to remove contaminant Ca^{2+}. The TnC-DANZ stock was divided into 30 μl aliquots and stored at -20°C.

CGP48506 and CGP48508 were dissolved in 100% DMSO to give stock solutions of 20 mM of each.

4.5.3 PROTOCOL

A Shimadzu RF-540 spectrofluorophotometer was used to measure fluorescence with settings at: excitation wavelength 340 nm; emission wavelength 520 nm; excitation slit 10 nm; emission slit 5 nm; scan speed on fast and sensitivity high. 567 μl of solution B were mixed with 3 μl 100% DMSO and 30 μl 20 μM TnC-DANZ stock solution (to give 1 μM on dilution), in a 600 μl quartz cuvette, using a plastic pipette. Immediately after mixing, a reading of fluorescence was taken (time zero) then new readings were made every 5 seconds for 30 seconds. This enabled any drift in fluorescence or fluorimeter recording to be corrected for by taking an average reading (although this proved unnecessary). The excitation and emission spectra of the TnC-DANZ were measured by taking readings of fluorescence after successive 5 nm increments in wavelength (from 250 - 450 nm for excitation and from 400 - 600 nm for emission).

Ca^{2+} was titrated against TnC-DANZ in control solution as described in Ch2, section 3.2.3. Corrections were made in calculations of pCa for the effects of

measured changes in pH as Ca^{2+} was added (section 3.2.3).

In one experiment the 0.5% DMSO was replaced by 3 μl of solution B to check for any effects of DMSO on the Ca^{2+}-sensitivity of TnC-DANZ.

The effects of 100 μM CGP48506 and CGP48508 on Ca^{2+}-binding to TnC-DANZ were measured by replacing the DMSO in the control runs with 3 μl of either CGP48506 or CGP48508 20 mM stock solutions. Ca^{2+} binding to both test compounds was known to be insignificant.

4.5.4 STATISTICAL ANALYSIS

Results were analysed as described in section 3.2.4.

4.6 RESULTS

4.6.1 EXCITATION AND EMISSION SPECTRA

a) Experimental Solutions

None of the solutions used were fluorescent before the addition of TnC-DANZ.

b) TnC-DANZ

Skeletal TnC-DANZ was fluorescent even in the absence of Ca^{2+}, with a peak emission wavelength at 520 nm and peak excitation at 340 nm. On the addition of 437 μM Ca^{2+} (pCa 3.36), the optimum emission wavelength shifted to 505 nm whereas the excitation peak remained unchanged. Fluorescence measurements were therefore made at excitation and emission wavelengths of 340 nm and 520 nm respectively.

4.6.2 CHANGES IN ABSOLUTE FLUORESCENCE

As the pCa fell from 8.69 to 3.36 in control solution (+ DMSO) the fluorescence of TnC-DANZ increased by $80 \pm 2\%$ (n=5), slightly less than the 110% rise observed in previous experiments (section 3.3.3). Addition of 100 μM CGP48506 or CGP48508 did not seem to alter minimum or maximum absolute fluorescence, although the fluorescence enhancement as pCa dropped from 8.69 to 3.36 increased to $94 \pm 2\%$ in the presence of CGP48506 and to $91 \pm 2\%$ in CGP48508 solution. The presence of 0.5% DMSO in the control solution appeared to suppress both minimum and maximum fluorescence of TnC-DANZ, by 25% and 27% respectively. A pCa change from 8.69 to 3.36 caused fluorescence to increase by 79% in the absence of DMSO and $80 \pm 3\%$ (n=5) in its presence.

4.6.3 MEASUREMENT OF Ca^{2+}-SENSITIVITY OF TnC-DANZ

a) Controls

The relationship between pCa and fluorescence of TnC-DANZ was sigmoidal and very similar to that in section 3.3.4. Control pCa_{50} values were 5.54±0.03 and 5.55±0.03 in the CGP48506 and CGP48508 experiments respectively (table 14, fig.26). These values were close to the pCa_{50} of 5.56 obtained in previous work with skeletal TnC-DANZ (table 8). The H_n values of the controls in the enantiomer experiments were 1.31±0.06 and 1.28±0.08 (table 14) compared to the 1.36 observed in table 8.

		CGP48506	CGP48508
pCa_{50}	Control	5.54±0.03(4)	5.55±0.03(4)
	Test	5.57±0.02(4)	5.57±0.01(4)
	Shift	0.04±0.02(4)	0.02±0.02(4)
Hill n	Control	1.31±0.06(4)	1.28±0.08(4)
	Test	1.36±0.06(4)	1.32±0.02(4)
	Shift	0.02±0.05(4)	0.04±0.07(4)

Table 14. EFFECTS OF 100 μM CGP48506 AND CGP48508 ON Ca^{2+}-SENSITIVITY OF SKELETAL TnC-DANZ. All values expressed as mean±SEM(n). Solutions as described in section 4.5.2.

b) Effects of CGP48506 and CGP48508

As shown in table 14 and fig.26, neither CGP48506 nor CGP48508 had any effect on the Ca^{2+}-sensitivity of skeletal TnC-DANZ fluorescence.

c) Effects of DMSO

Removal of the DMSO from the control solution did not affect the pCa_{50} or H_n of the pCa-fluorescence relationship of TnC-DANZ, with respective shifts of 0.01 and 0.07.

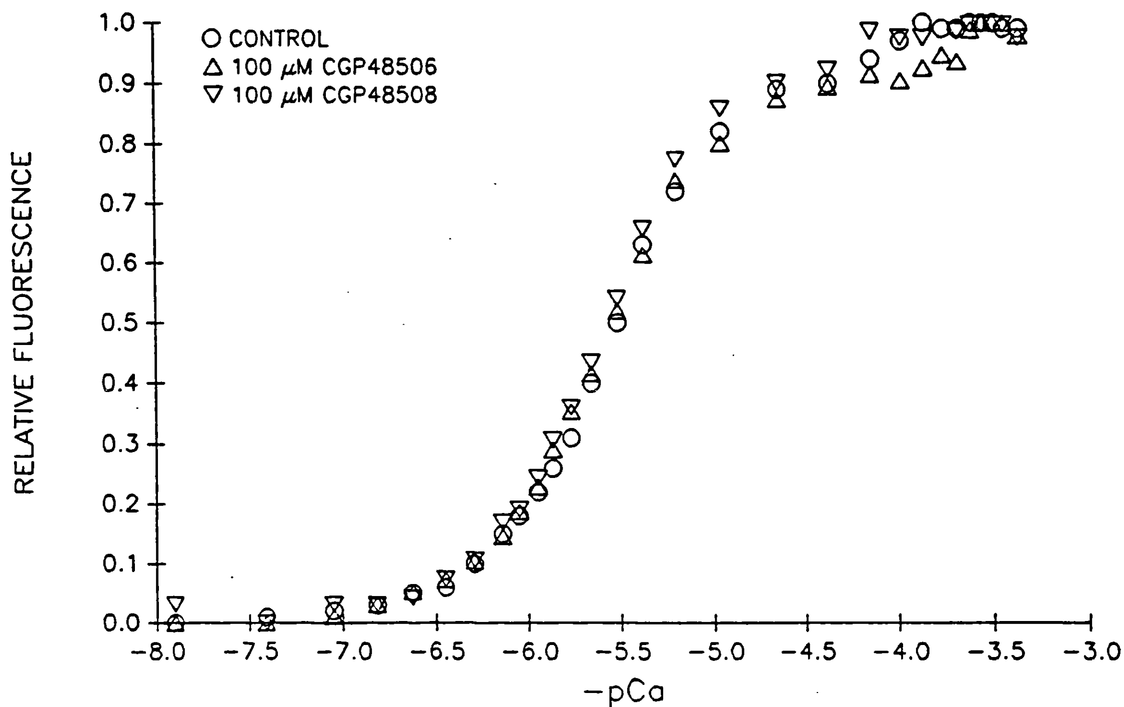


Fig.26 EFFECTS OF CGP48506 AND CGP48508 ON Ca^{2+} BINDING TO ISOLATED SKELETAL TnC-DANZ. Solutions and conditions as described in sections 4.5.2 and 4.5.3. Points represent mean data from 4 experiments.

4.7 DISCUSSION

4.7.1 Ca^{2+}-SENSITIVITY OF TnC-DANZ

a) Controls

The TnC-DANZ had similar fluorescence properties to those described for other batches of TnC-DANZ (section 3.3). Ca^{2+} binding to the two Ca^{2+}-specific sites could be monitored using the associated increases in fluorescence (fig.26).

b) Effects of CGP48506 and CGP48508

Use of DMSO as a solvent for the enantiomers did not affect the change in absolute fluorescence as pCa dropped from 8.69 to 3.36, although it decreased minimum and maximum fluorescence by 25% and 27% respectively. This was presumably due to a Ca^{2+}-independent quenching of the DANZ probe by DMSO. The Ca^{2+}-sensitivity of isolated skeletal TnC-DANZ was unaffected by 0.5% DMSO so any changes observed in the presence of the enantiomers could be attributed to them rather than their solvent.

Although neither enantiomer significantly altered minimum or maximum absolute fluorescence of TnC-DANZ, both increased slightly the fluorescence enhancement as pCa fell from 8.69 to 3.36. This probably reflected a very slight rise in maximum absolute fluorescence but none of the effects were large with the Ca^{2+}-induced enhancement of fluorescence increasing by $16.3 \pm 2.3\%$ in the presence of CGP48506 and $11.0 \pm 4.1\%$ with CGP48508.

Neither CGP48506 nor CGP48508 significantly changed the Ca^{2+}-affinity of TnC-DANZ, with respective shifts in pCa_{50} of 0.04 ± 0.02 and 0.02 ± 0.02. Thus the greater effect of CGP48506 (ΔpCa_{50} 0.35) than CGP48508 (ΔpCa_{50} 0.14) on skeletal skinned fibres (table 13) cannot be ascribed solely to a greater effect of the former on Ca^{2+} binding to TnC. It is anyhow likely that other myofilament proteins are involved in the Ca^{2+}-sensitising actions of the enantiomers because although CGP48506 has a greater effect on cardiac (ΔpCa_{50} 0.88) than skeletal fibres, the opposite is true for CGP48508 (which has no effect on the pCa_{50} of skinned trabeculae, table 13).

Neither compound changed the H_n of Ca^{2+} binding to TnC-DANZ, thus with both control and test values of approximately 1.3, there was little or no cooperativity in Ca^{2+} association with the two low affinity sites (see also section 3.4.2.a concerning the use of H_n as a measure of cooperativity). If CGP48506 or CGP48508 acted on only one of the Ca^{2+}-specific sites of TnC the cooperativity observed in skinned fibre studies would be expected to fall in the presence of the

enantiomer. This was not observed (table 14) and therefore cannot explain the lesser effect of CGP48508 on Ca^{2+}-sensitivity compared to CGP48506.

TnC does not have a direct role in the Ca^{2+}-sensitising action of either enantiomer but may be indirectly involved through interactions with other proteins, such as TnI. This was further investigated in experiments where TnC-DANZ was substituted into skinned fibres (chapter 5).

4.8 SUB-CONCLUSIONS

a) BA41899 sensitised skinned fibres to Ca^{2+}, having the greatest effect on cardiac muscle, followed by slow-twitch and then fast-twitch skeletal fibres. BA41899 also suppressed maximum force of cardiac and slow-twitch preparations similarly but did not reduce that of fast-twitch fibres, suggesting a different mechanism of action of the inotrope on shift in pCa_{50} and depression of maximum force.

b) BA41899s' enantiomers, CGP48506 and CGP48508, acted via different mechanisms on pCa_{50}: CGP48506 had a greater Ca^{2+}-sensitising action on cardiac than skeletal fibres whereas CGP48508 only sensitised skeletal muscle to Ca^{2+} (the first known example of a "skeletal-specific" Ca^{2+}-sensitiser). Although the enantiomers acted independently on the pCa_{50} of skeletal fibres, the Ca^{2+}-sensitising action of CGP48506 was enhanced in the presence of CGP48508. Neither compound affected Ca^{2+}-binding to isolated TnC and must therefore act either on another component of Tn or directly on the crossbridges.

c) CGP48506 and CGP48508 both suppressed maximum force in cardiac fibres, and CGP48508 had a similar but much smaller effect in skeletal fibres. The enantiomers acted via independent mechanisms on maximum force, perhaps involving a decrease in maximum ATPase activity.

CHAPTER 5: SIMULTANEOUS MEASUREMENTS OF FORCE AND CHANGES IN TnC CONFORMATION IN SKINNED FIBRES

5.1 INTRODUCTION

5.1.1 TnC EXTRACTION

TnC can be specifically extracted from myofibrils. This was first done by Cox et al.(1981), who used a solution containing 20mM TRIS/HCl and 5mM EDTA to extract up to 90% of the TnC present in rabbit skeletal myofibrils. No other components of the troponin complex were released by this method. Zot & Potter (1982) investigated the mechanism of TnC extraction using the same technique whilst controlling Ca^{2+} and Mg^{2+} binding to TnC by varying the concentrations of EDTA and EGTA in the extraction solution. TnC could only be extracted from the myofilaments when neither Ca^{2+} nor Mg^{2+} was bound to the Ca^{2+}-Mg^{2+} sites of TnC. The Ca^{2+}-specific sites were not involved in TnC extraction. Since it was known that divalent metal binding to the Ca^{2+}-Mg^{2+} sites caused large changes in secondary structure in TnC, Zot and Potter concluded that TnC extraction was the result of a weakened interaction between TnC and TnI induced by the removal of Ca^{2+} and Mg^{2+} from the Ca^{2+}-Mg^{2+} sites.

The extraction of TnC from skinned skeletal fibres was further shown to be reversible (Moss et al, 1982 & 1983; Brandt et al, 1984), i.e. lost TnC could be replaced. This enabled the substitution of native TnC by another form of TnC.

5.1.2 APPLICATIONS OF TnC SUBSTITUTION

It proved possible to monitor Ca^{2+} binding to TnC simultaneously with tension development (Zot et al, 1986) by replacing native TnC in rabbit myofilaments with skeletal TnC labelled with DANZ (section 3.1.3). Zot et al (1986) measured Ca^{2+} binding to TnC in reconstituted myofibrils and skeletal skinned fibres concurrently with ATPase activity and force production. They found the pCa-force relationship was the same in fibres with native TnC and in those reconstituted with TnC-DANZ. TnC-DANZ fluorescence (and hence Ca^{2+} binding to the Ca^{2+} specific sites) was more sensitive to Ca^{2+} than was force. It appeared that the fluorescence change depended on Ca^{2+} binding to either Ca^{2+} specific site of TnC-DANZ (Johnson et al, 1978) whereas tension required Ca^{2+} occupation of both low affinity sites. This conclusion was reinforced on incorporation of cardiac TnC-IAANS into skinned psoas fibres: the Ca^{2+} dependencies of force and fluorescence were the same presumably because cardiac TnC has only one Ca^{2+} specific site. Relative force and ATPase activity had the same Ca^{2+}-sensitivity in skinned fibres and the same

relationship with TnC-DANZ fluorescence (Zot et al, 1986), though the latter was in contrast to the findings of Grabarek et al (1983) who showed that ATPase activity was more sensitive to Ca^{2+} than fluorescence of TnC-DANZ in regulated actin.

TnC-DANZ substitution into skinned fibres also lends itself to the elucidation of the mechanism of action of various agents which alter the Ca^{2+}-sensitivity of skinned fibres. It should be possible to determine whether such agents act by altering Ca^{2+} binding to TnC in situ or whether they act by alternative means. The aim of the following experiments was thus to replace skeletal TnC in skinned psoas fibres with TnC-DANZ and thence to investigate the role of TnC in the mechanism of action of CGP48506, CGP48508, caffeine, pH and Pi (or as many of these as possible!).

5.2 METHODS

5.2.1 PREPARATION OF FIBRES

Skinned rabbit psoas fibres were prepared as described in section 2.2.2.b.

5.2.2 SOLUTIONS

Solutions used to determine maximum force and Ca^{2+}-sensitivity were as described in section 2.2.2.a. CGP48506 solutions were as described in section 4.2.2.

TnC was removed from skeletal fibres with an "extraction" solution based on that of Cox et al (1981). It comprised 20 mM TRIS/HCl and 5 mM EDTA, pH 7.30 at 22°C. Occasionally Cox's rigor "wash" solution was also used, consisting of 40 mM TRIS/HCl and 25 mM KCl, pH 6.50 at 22°C. The muscle was reconstituted with TnC using a solution of 50 μM TnC or 3 μM TnC-DANZ in relaxing solution.

Free Ca^{2+} concentration was calculated as described in section 2.2.1.

5.2.3 FORCE MEASUREMENTS AT UCL

Initial experiments to determine optimal conditions for TnC extraction were carried out at UCL using the system described in section 2.2.2.e. Attempts were made to adapt this system for simultaneous force and fluorescence measurements, but these were unsuccessful.

5.2.4 FORCE AND FLUORESCENCE MEASUREMENTS AT CIBA-GEIGY

Experiments were carried out using a system developed by Guth and Wojciechowski (1986) which enabled simultaneous measurement of force and fluorescence (fig.27). It consisted of a horizontal quartz tube (volume 20 μl), into each end of which protruded a pair of tweezers, one attached to a fixed stop and the other to a KG3 force transducer. The tweezers were accessed by moving the cuvette (tube) sideways. Solutions were passed from a series of storage wells through silicone tubing into the "fixed stop" end of the cuvette and flow rate was controlled by the opening frequency of an electric valve. Solution leaving the open "transducer" end of the cuvette was removed by suction. The force signals were amplified and recorded on a 2-channel chart recorder together with fluorescence. Fluorescence of the TnC-DANZ was detected by a photomultiplier system similar to that described by Guth and Wojciechowski. The exciting light was provided by a mercury arc lamp (HB0100), and filtered (UG11 narrow band width) to give an excitation wavelength of 340 nm. Emitted light was collected at 520 nm (KV500 wide band width filter). Variations in intensity of the exciting light were detected by a photodiode and were eliminated from the fluorescence signal by taking a ratio of the two. The amount of background light was reduced to a minimum by adjusting the size of a slit which selected the area from which fluorescence was detected. The slit was narrowed until it was occupied entirely by the fibre (even during contraction). All fluorescence measurements were made in the dark and at room temperature (23° C).

1-5 glycerinated rabbit psoas fibres were glued into the tweezers. The cuvette was positioned around the fibres and a flow of relaxing solution initiated to bathe the preparation. Activating and extraction solutions were applied to the fibres by changing to the appropriate solution storage well and transiently increasing the solution flow-rate to ensure a rapid changeover.

TnC-DANZ solution was introduced into the open end of the cuvette using a Hamilton syringe. The cuvette was first emptied of solution to facilitate injection of the TnC-DANZ, which was done as rapidly as possible to prevent dehydration of the fibres. The electric valve was closed for the duration of both introduction and incubation of TnC-DANZ with the fibres to conserve TnC-DANZ. The level of TnC-DANZ solution in the cuvette was occasionally topped up to compensate for evaporation.

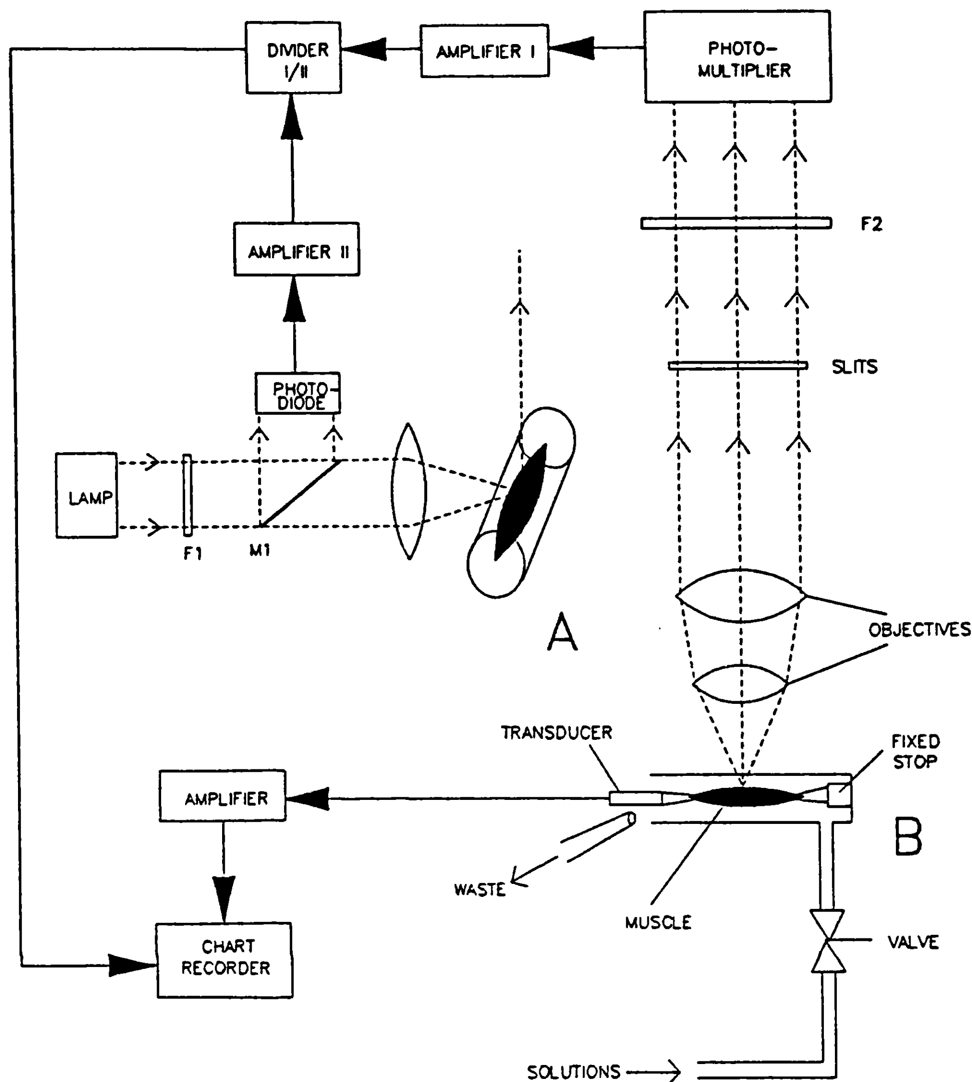


Fig.27 APPARATUS FOR SIMULTANEOUS MEASUREMENT OF FORCE AND FLUORESCENCE. Based on diagram in Guth & Wojciechowski (1986). Fibres were held by 2 pairs of tweezers attached to a transducer and fixed stop at opposite ends of a cylindrical quartz cuvette. The single cuvette is depicted twice (A & B) to enable illustration of the excitation system which lies perpendicular to the emission collecting system. Solution flow rate was regulated by an electric valve with waste droplets removed by vacuum suction. Light from a mercury arc lamp (HB0100) was filtered (F1) to give exciting light at 340 nm. This was split by a mirror (M1), 50% passing to a photodiode to detect changes in illumination intensity and 50% being focussed by a lens onto the fibre. Emitted fluorescence passed through an ULTRAFLUOR 6.3 objective, through a 520 nm filter (F2) and was detected by a Zeiss microscope photometer. Light paths indicated by dashed lines. Further details are given in section 5.2.4.

5.2.5 PROTOCOLS

a) Extraction of Skeletal TnC

Maximum force of the fibres was measured at room temperature (22° C). In order to find the optimal temperature for extraction, the fibres were incubated in relaxing solution while the temperature was changed to 6, 20, or 30° C. Wash solution was then applied for 5 minutes followed by extraction solution for one hour (Hoar et al, 1988), maintaining constant temperature. Maximum force was again measured after readjustment of the temperature to 22° C in relaxing solution. It was assumed that the size of this force, expressed as a percentage of the pre-extraction maximum, was a valid indicator of the extent of TnC extraction. Post-extraction resting tension was also expressed as a percentage of the pre-extraction maximum force.

In some experiments the duration of extraction was varied between 20 and 120 minutes though this had no consistent effect on the degree of TnC removal.

Omission of the wash solution step appeared to reduce the post-extraction rise in resting tension for reasons that are unclear. An additional change to the original extraction protocol was the raising of temperature in extraction solution (rather than HR), which was taken directly from the refrigerator (4° C), applied to the fibres and heated to 20, 25 or 30 ° C. The duration of extraction was reduced to 15-20 minutes. In experiments where TnC extracted from skeletal fibres was replaced by TnC-DANZ, extraction was performed with a rise in temperature from 4 to 20 ° C, duration 15 minutes, omitting the wash solution step. Force and fluorescence from TnC-DANZ appeared more stable when extraction was limited to only a 50% drop in force.

b) Time-course of TnC Extraction

Time-course of TnC extraction was measured by maximally activating a rabbit psoas fibre at 22° C, then transferring the preparation successively to relaxing, extraction (4-20° C), relaxing and maximal activating solutions as described above. The cycle was repeated with incubations in extraction solution increasing from 15 seconds (for 2 cycles) to 30 seconds (3 cycles) to 1 minute (3 cycles), giving a total extraction time of 5 minutes. The force decline was plotted against time and the time taken for force to fall by 50% was determined for each fibre.

c) Reconstitution of Fibres

Skeletal TnC was substituted into TnC-depleted rabbit psoas fibres by incubating the latter in a solution of 1 mg/ml (50 μM) rabbit skeletal TnC in relaxing solution, at 22° C, for 10-30 minutes. The degree of reconstitution was

estimated from the percentage recovery of maximum Ca^{2+}-activated force (expressed relative to its pre-extraction value). When reconstitution resulted in a recovery of maximum force to less than 50% of its pre-extraction value, the incubation of the fibre in the TnC solution was repeated.

To investigate whether the extraction procedure also removed myosin light chains (as has been reported by Moss et al, 1982), the effect on force recovery of incubating such fibres in 10 mg/ml bovine cardiac myosin light chains (Sigma plc) in relaxing solution for 15 minutes at 22°C was occasionally tested.

For work carried out at Ciba-Geigy, in which it was necessary to replace native TnC in rabbit psoas fibres with that labelled with DANZ, the TnC-DANZ was applied for 1 hour in relaxing solution at a concentration of 3 μM. This lowered concentration was chosen to reduce background fluorescence whilst providing sufficient labelled TnC to reconstitute the skinned fibres reasonably quickly. Reconstitution with TnC-DANZ was performed in darkness.

d) Effect of TnC Substitution on Ca^{2+}-Sensitivity

When at least 60% of the pre-extraction maximum force was reached after TnC extraction (4-20°C, no wash solution) and replacement, a force-pCa relationship was determined for the fibre according to protocol A outlined in section 2.2.2.f. These results were compared with the pre-extraction Ca^{2+}-sensitivity of the fibre. Occasionally the effects of caffeine on Ca^{2+}-sensitivity were also compared before and after TnC swapping.

e) Simultaneous Measurement of Force and Fluorescence

Following TnC extraction and replacement with TnC-DANZ, the fibres were transferred to relaxing solution and left to equilibrate for 10 minutes. Their force and fluorescence was measured from this point onwards.

First both maximum force and fluorescence were tested to check that TnC-DANZ incorporation had occurred. After relaxation for 2-3 minutes, activation at varying Ca^{2+} concentrations and in the presence and absence of 100 μM CGP48506 was carried out in the following solution sequence:

Relaxation + Test Compound
Relaxation
Sub-maximal Activation
Sub-maximal Activation + Test Compound
Maximal Activation
Maximal Activation + Test Compound

The fibres were left in each solution until force stabilised. The fluorescence baseline was still decreasing a little when measurements began: this was corrected for by assuming the decline was linear between the initial contraction and final relaxation of each activation cycle. In addition the decline in maximum fluorescence was corrected for by maximally activating the fibres at the end of each activation cycle, although the maximum fluorescence at the precise time the fibres were exposed to the test Ca^{2+} concentration a few minutes earlier would have been somewhat higher. It was impossible to correct for this since it was unknown to what extent the decrease in maximum fluorescence was time-dependent or Ca^{2+}-activation dependent.

To minimise the decline in fluorescence, the duration of the experiments was shortened by omitting to relax the fibres between the four activations in the above sequence. In addition, once it had been established that CGP48506 (the test compound) had no effect on either maximum force or fluorescence, the maximal activation in the presence of CGP48506 was omitted.

5.3 RESULTS

5.3.1 SKELETAL TnC EXTRACTION

Results from use of the extraction methods described in section 5.2.5.a are shown in tables 15 and 16.

EXTRACTION CONDITIONS	% DECLINE IN MAX.F.	% INCREASE IN RESTING F.
6° C Wash + Extraction	56±21 (5)	74±26 (5)
22° C Wash + Extraction	61±18 (4)	59±24 (4)
30° C Wash + Extraction	100±0 (3)	59±18 (3)
4-20° C Extraction only	61±4 (39)	19±3(39)
4-25° C Extraction only	66±3 (12)	9±1 (12)
4-30° C Extraction only	68±2 (29)	6±1 (28)

TABLE 15. CHANGES IN FORCE OF RABBIT SKINNED PSOAS FIBRES OBSERVED FOLLOWING DIFFERENT TnC EXTRACTION PROTOCOLS. Solutions and protocols as described in sections 5.2.2 & 5.2.5.

	% DECLINE IN MAX.F.	% INCREASE IN RESTING F.
Rabbit 1 Muscle Bundle 1	86±3 (12)	48±3 (12)
Rabbit 2 Muscle Bundle 1	47 (2)	7 (2)
Rabbit 3 Muscle Bundle 1	24±9 (6)	4±1 (6)
Rabbit 4 Muscle Bundle 1	56±5 (7)	11±3 (7)
Rabbit 4 Muscle Bundle 2	67±7 (3)	9±4 (3)
Rabbit 4 Muscle Bundle 3	42±7 (3)	4±1 (3)
Rabbit 4 Muscle Bundle 4	65±13 (3)	3±2 (3)
Rabbit 5 Muscle Bundle 1	66±4 (3)	5±3 (3)

TABLE 16. VARIATIONS IN THE DEGREE OF TnC EXTRACTION FROM RABBIT SKINNED PSOAS FIBRES. Extraction performed at 4-20°C without wash solution, as described in section 5.2.5.

Assuming loss of force to be a valid indicator of the amount of TnC extraction, it appeared that the greatest amount of TnC removed (100%) occurred after incubation in wash and extraction solutions at 30°C. Unfortunately attempts at reconstituting these fibres with TnC failed completely which indicated that the muscle had been damaged excessively by the extraction procedure. The use of a wash solution seemed to correlate with the development of a large (58-74%) rise in resting tension, although there was considerable variation between fibres. In the absence of wash solution, changing the temperature of extraction from 20°C to 25°C to 30°C made no difference to the results obtained (table 15). In general, between 61 and 68% of the TnC could be extracted using these methods, with less than 20% increases in resting tension.

However, the "extractibility" of the TnC varied both between muscles taken from different rabbits and to a lesser extent between different bundles of fibres taken from the same muscle (table 16). TnC extraction ranged from 24% to 86% in different rabbits and from 42% to 67% within one psoas muscle.

In the successful "force plus fluorescence in skinned fibres" experiments, an example from which is shown in fig.28, the mean force following TnC extraction was 53±11% (n=5).

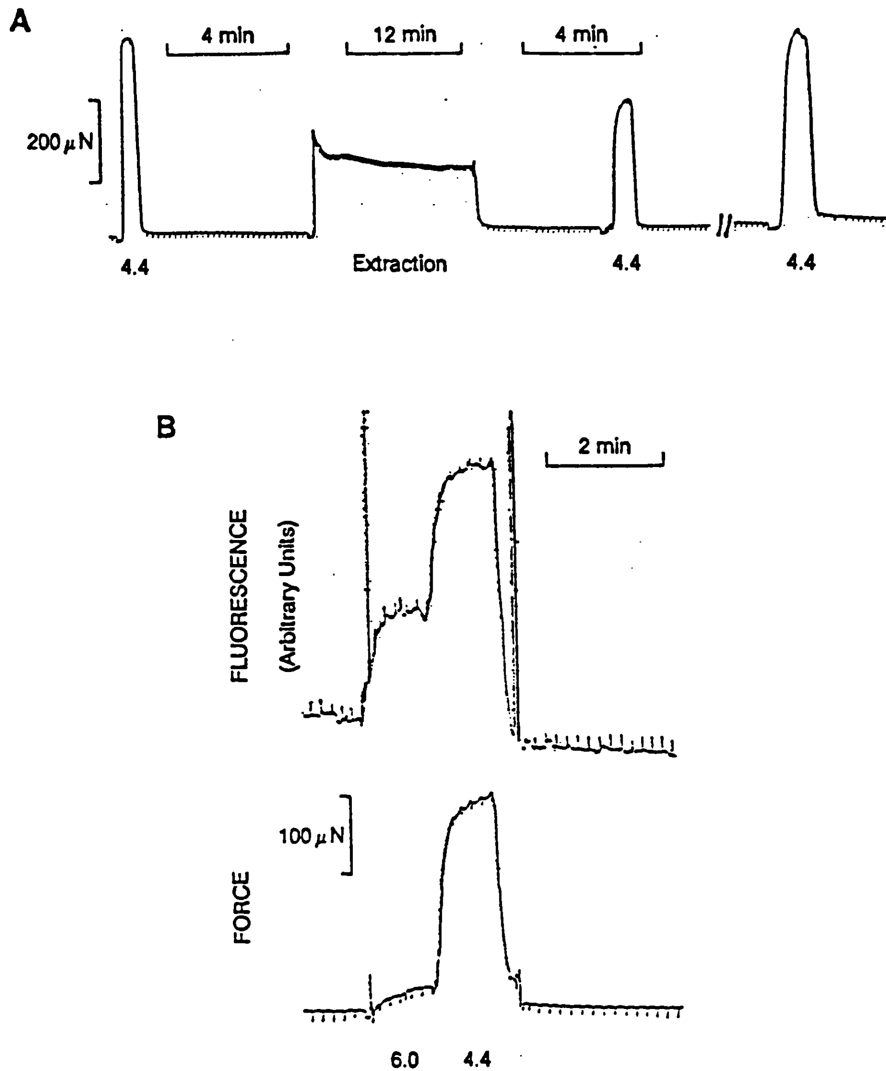


Fig.28 EFFECTS OF Ca^{2+} ON FORCE AND FLUORESCENCE OF RABBIT PSOAS FIBRES RECONSTITUTED WITH TnC-DANZ. (A) Following maximal activation, 4 fibres were extracted (4-20 °C) for 15 mins resulting in a fall in maximum force. // indicates reconstitution with 3 μM TnC-DANZ solution for 1 hour. Solutions and conditions as described in sections 5.2.2 & 5.2.5. Numbers below trace indicate pCa. (B) Simultaneous force and fluorescence traces from 2 TnC-substituted fibres. Measured in UCL solutions (section 2.2.2.a) under conditions described in section 5.2.5.e. Numbers below trace show pCa. Sub-maximal levels of Ca^{2+} produced greater increases in relative fluorescence than in relative force.

a) Time-course of Extraction

The rate of extraction of TnC, expressed as the time taken for 50% of the total decline in force to have occurred, varied slightly between fibres from the same psoas muscle (0.70 ± 0.08 (n=5) min). The relationship between percentage decrease in maximum force and time was an exponential fall and was fitted accordingly using a computer programme (GraphPad) to determine half time. Conversely, the increase in resting tension occasionally observed during extraction rose exponentially with time, the half time of which (0.63 ± 0.05 min, n=5) closely resembled that of the decline in maximum force. Fibres from different rabbits appeared to differ in their rates of extractions with half times from 0.70 ± 0.08 (n=5) minutes to 1.76 and 2.43 minutes (n = 1 for each).

5.3.2 RECONSTITUTION OF FIBRES WITH TnC

Incubation of extracted (4-20°C) rabbit psoas fibres with isolated skeletal TnC resulted in a recovery of maximum force to $70 \pm 3\%$ (n=19) of its original (pre extraction) value. At least $62 \pm 6\%$ (n=19) of the force generated by the reconstituted fibres was linked to the newly replaced TnC, the rest having been present after extraction and before reconstitution. It should, however, be noted that all percentages are minimum values since no correction was made for the decline in maximum force with time. The recovery of maximum force was accompanied by a fall in resting tension back to its pre extraction level in those fibres where resting tension had increased during extraction. The replacement of skeletal TnC with its DANZ-labelled form was equally successful to replacement with unlabelled skeletal TnC: maximum force recovered to $70 \pm 3\%$ of its pre-extraction value, $52 \pm 9\%$ of the recovered force being linked to TnC-DANZ. Thus the fluorescent probe did not hinder reconstitution. In the successful "force plus fluorescence" experiments (where TnC extraction had been limited to approximately 50%), the force following reconstitution with TnC-DANZ recovered to $89 \pm 9\%$ (n=5) of the pre-extraction maximum (fig.28).

No recovery of force was observed on incubation of 30°C or 4-30°C extracted fibres with TnC.

Unlike extraction of TnC, the degree of reconstitution of fibres with skeletal TnC was not related to the particular animal used nor did it seem to vary consistently between different muscle bundles. It is known that extraction can remove light chains (Moss et al, 1982). This was tested, after TnC extraction at 4-20°C, by adding 10 mg/ml light chains to fibres following reconstitution with TnC. The addition of myosin light chains, either between TnC extraction and reconstitution or after reconstitution with TnC, had no effect on force so it appeared that the loss of light chains during extraction was negligible.

a) Changes in Ca^{2+}-Sensitivity after TnC Substitution

Pilot experiments at UCL showed that the pCa_{50} of psoas fibres following reconstitution with unlabelled skeletal TnC shifted by 0.17 ± 0.02 (n=10) units from 5.86 ± 0.05 (n=10) to 5.69 ± 0.05 (n=10). Thus the extraction/substitution procedure desensitised the fibres to Ca^{2+}. The Ca^{2+} sensitising effect of caffeine was, however, unaffected by TnC removal and replacement, with 20 mM caffeine causing a shift in pCa_{50} of 0.10 ± 0.01 (n=6) units before extraction and 0.08 ± 0.03 (n=9) units following reconstitution. After reconstitution of psoas fibres with skeletal TnC, H_n decreased by 0.19 ± 0.07 (n=10), falling from 1.74 ± 0.11 (n=10) before extraction to 1.54 ± 0.09 (n=10).

5.3.3 Ca^{2+}-SENSITIVITY OF FORCE AND FLUORESCENCE

a) Controls

Attempts to measure TnC-DANZ fluorescence in situ in skinned fibres at Ciba-Geigy, were initially beset with problems. Rabbit psoas fibres had an intrinsic fluorescence at excitation 340 nm and emission 520 nm even prior to TnC extraction. The level of this fluorescence remained steady and was unaffected by the presence of Ca^{2+}. After reconstitution the fluorescence of the fibres in relaxing solution was far greater than before extraction and increased by approximately 50% in maximal activation solution . The level of the new baseline fluorescence was, however, extremely unstable, decreasing most rapidly during the first 10 minutes and then steadily for about an hour. At this point the amount of TnC-DANZ related fluorescence was considered too small to work with.

In some experiments the baseline fluorescence showed a steady increase! It was discovered that this change in fluorescence was due to spontaneous increases in the size of the slit from which fluorescence was measured. On the few occasions that both slit size variation and solution changing artifacts were absent, the following results were obtained.

Under control conditions it was clear that TnC-DANZ fluorescence was more sensitive to Ca^{2+} (pCa_{50} = 5.86 ± 0.06, n=5) than was the accompanying force (pCa_{50} = 5.71 ± 0.03, n=5) as shown in figs.28 & 29. The mean difference in pCa_{50} was 0.15 ± 0.08 (n=5). The fluorescence curve was also less steep than the force one with a H_n of 2.09 ± 0.46 (n=5) compared to 3.92 ± 0.40 (n=5) for force - the mean difference in H_n was 1.83 ± 0.52 (n=5).

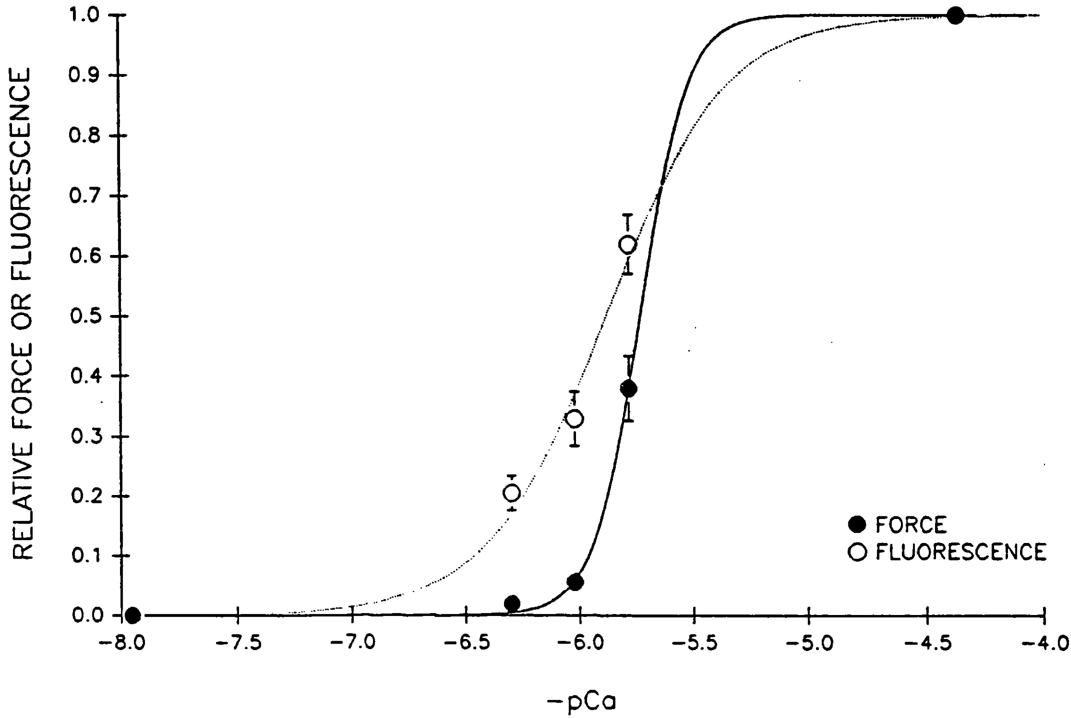


Fig.29 Ca^{2+}-SENSITIVITY OF FORCE AND FLUORESCENCE OF TnC-DANZ RECONSTITUTED SKINNED RABBIT PSOAS FIBRES. Experiments performed under conditions described in sections 5.2.4 and 5.2.5.e. Points represent mean data from 5 experiments. Curves were generated from mean pCa_{50} and Hill "n" values (section 5.3.3.a). TnC-DANZ fluorescence was more sensitive to Ca^{2+} than was the accompanying force.

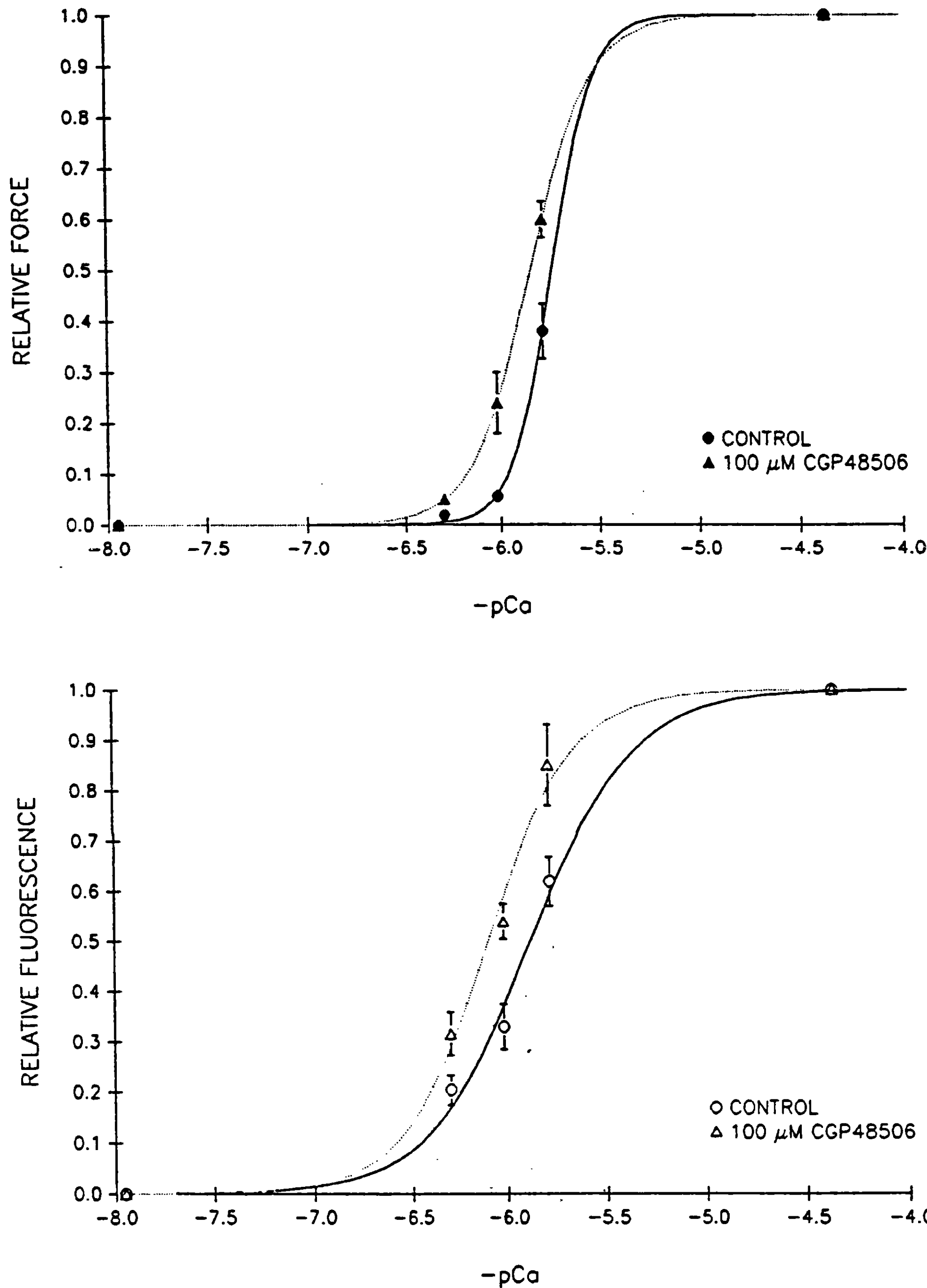


Fig.30 EFFECTS OF CGP48506 ON FORCE AND FLUORESCENCE OF TnC-DANZ RECONSTITUTED SKINNED RABBIT PSOAS FIBRES. Experiments performed under conditions described in section 5.2.5.e. Points represent mean data from 5 experiments. Curves were generated from mean pCa_{50} and Hill "n" values (section 5.3.3.b). CGP48506 had less of a Ca^{2+}-sensitising effect on force (top) than fluorescence (below).

b) Effects of CGP48506

100 μM CGP48506 increased the force developed at sub maximal levels of Ca^{2+} (as observed in fig.23) with no effect on maximum force (fig.30). pCa_{50} increased by 0.15±0.04 (n=5) units to 5.86±0.03 (n=5). 100 μM CGP48506 also sensitised the TnC-DANZ fluorescence to Ca^{2+} (fig.30) with no significant effect on maximum fluorescence. The shift in pCa_{50} of the fluorescence-pCa curve caused by CGP48506 was 0.25±0.04 (n=5) units to 6.11±0.03 (n=5). This shift was not significantly different to that induced for force. Thus CGP48506 had a similar Ca^{2+}-sensitising action on fluorescence and force, though it had no effect on the H_n of either, with values of 3.05±0.40 (n=5) for force and 2.26±0.37 (n=5) for fluorescence (neither significantly different from control values).

5.4 DISCUSSION

5.4.1 TnC EXTRACTION

Extraction of TnC from psoas fibres was less straightforward than had been anticipated. Use of the extraction method of Kerrick et al (1985) (with wash and extraction solutions at 6°C) resulted in a decrease in maximum force of only 56% accompanied by a 74% rise in resting tension (expressed relative to pre-extraction maximum force). This compared to the 87-100% reduction in maximum force, with a 20-30% rise in resting force, found by Kerrick et al. Attempts to improve the extraction of TnC by increasing the temperature of extraction to 22°C were only marginally successful (table 15). A significant potentiation of resting tension was still observed, the molecular basis of which was unknown. Moss et al (1983) found that resting force increased after treatment of psoas fibres with 20 mM EDTA solution for 4 hours but this tension was reduced from 25% to 5% of the pre extraction maximum force by extracting at 30°C instead of 37°C. It appeared from the current study that the omission of the wash solution stage reduced the change in resting tension following extraction (table 15). However, Babu et al (1986) included a rigor wash incubation without affecting resting tension of hamster psoas. Generally it seems that changes in resting force are best avoided by extracting at low temperatures (<10°C) for durations less than 90 minutes and in the absence of incubation in wash solution (Moss et al, 1983; Moss et al, 1985; Babu et al, 1986 & Gulati & Babu, 1988).

Use of temperatures of 30°C or above for extraction results in the removal of myosin light chain LC2 together with TnC (Moss et al, 1982). LC2 depletion would only have a small depressive effect on maximum force (approximately 10%, Moss et al, 1982). It is also possible that extraction at high temperatures

causes the loss of a protein cofactor required for switching from weakly to strongly bound crossbridge states (Gulati & Babu, 1988). This could explain the loss of contractile function observed both in the current study and by Moss et al (1983) and by Babu et al (1986) after extraction at 30° C followed by reconstitution.

Extraction at 4-20° C or 4-25° C in the current study resulted in 61% and 66% decreases in maximum tension respectively. Using similar conditions, Zot et al (1986) achieved decreases of between 53% and 92% and Moss et al (1985) a decline of 58%.

The degree of TnC extraction varied between rabbits. A similar effect was observed by Ian Mulligan (pers. comm.) and the rate of skeletal TnC extraction also differed considerably between different rabbits and studies. . Although TnC removal from cardiac preparations is possibly species dependent (Hoar et al, 1988), it is unlikely that variations in ease of extraction are due entirely to genetic factors since extraction also varied in success between different fibres from a single psoas muscle (table 16). EDTA enhances TnC depletion of myofilaments by weakening the interaction between TnC and TnI, thus differences in the strength of this interaction under control conditions may reflect the ease of TnC removal.

Although percent decline in maximum force was used as an indicator of TnC removal from the myofilaments, force often decreases more than TnC content. Brandt et al (1984) observed that loss of tension was only proportional to loss of TnC up to approximately 35% TnC extraction. For extractions of 35% or more the percent reduction of tension was greater than the corresponding loss of TnC. Thus a 60% removal of TnC equated to a 72% drop in force, for example. Similarly Moss et al (1985) noted that tension and TnC content fell in proportion from 100 to 70%, after which force dropped to a greater extent than TnC content, at least in the range of 70-30% TnC left unextracted. A fall in maximum tension of 58% was accompanied by a 42% loss of TnC. Thus the percentage declines in maximum force quoted in the current study give an overestimation of TnC removal where force dropped by more than 35%.

5.4.2 RECONSTITUTION WITH TnC-DANZ

Reconstitution of extracted (4-20° C) psoas fibres with skeletal TnC caused maximum force to return to 70% of its pre-extraction value. This, however, assumed that no decline in maximum force occurred with time. Results in section 2.2.2.f indicated that over a period of 2.25 hours (chosen to represent the longest time between the start of extraction and the testing of force after reconstitution), tension decreased by 18%. Taking this to be the greatest possible decrease in

force, the corrected maximum force of the reconstituted fibres rises to 85% of the pre-extraction value. Thus the true percentage of control maximum force obtained after reconstitution with TnC ranged between the limits of 70% and 85%. This compared reasonably well with the values of $93 \pm 7\%$; >55% and 90-100% obtained by Moss et al (1983); Kerrick et al (1985) and Moss et al (1985) respectively.

In cases where a rise in resting tension occurred after TnC extraction this increase was reversed by reconstitution. Thus a non-rigor, Ca^{2+}-independent tension could be induced in some fibres by the removal of TnC. The rise in force cannot be attributed to partial extraction of TnI since it could be reversed on incubation with TnC, but it may be related to a slight conformational change in TnI occurring as a result of TnC removal.

The pilot experiments at UCL with unlabelled skeletal TnC showed that Ca^{2+}-sensitivity decreased by 0.17 pCa units during extraction and reconstitution, with pCa_{50} falling from 5.86 to 5.69. The pre-extraction pCa_{50} was similar to those observed previously under similar conditions (5.78-5.98, tables 6, 12 & 13). The pCa_{50} of 5.71 obtained at Ciba-Geigy following reconstitution of psoas fibres with TnC-DANZ indicates that a fall in Ca^{2+}-sensitivity had occurred here too. This was presumably related to the fact that force (and therefore TnC content) never returned to its pre extraction level but remained at 70-85% of this control value. Brandt et al (1984) showed that reconstitution to approximately 81% pre-extraction force resulted in a pCa_{50} 0.13 units lower than the original control pCa_{50} and a fall in H_n of 3.7 units. The degree of TnC extraction was inversely related to Ca^{2+}-sensitivity. Brandt et al proposed the loss of TnC reduced pCa_{50} and H_n as it decreased cooperativity between neighbouring TnCs on the thin filament. Moss et al (1985) found that a 68% decrease in maximum force on extraction of TnC was accompanied by a rightward shift of the tension-pCa relationship of 0.3 units. This, however, was fully reversed on reconstitution of the fibres to pre-extraction TnC levels (force increased back up to 93%).

The UCL experiments also demonstrated that Hill "n" decreased by 0.19 units during extraction and reconstitution, changing from 1.74 to 1.54. This pre-extraction control value is, however, somewhat lower than those obtained under similar conditions (2.10-3.75) in tables 12 and 13, although it is closer to the 1.9 observed in table 6. It appears that higher H_n values were achieved during work at Ciba-Geigy than at UCL - since the solutions used were of the same composition and the protocols employed were identical, the discrepancy may reflect differences between rabbit populations. Following reconstitution of psoas fibres with TnC-DANZ at Ciba-Geigy the H_n was 3.92 (greater than that in

controls of other experiments (see above)) - which is unlikely to result from a decrease in value during extraction. TnC extraction was limited to approximately 50% at Ciba-Geigy which resulted in a better (89%) recovery of maximum force after reconstitution - presumably this gentler treatment also preserved cooperativity though reasons for this are unclear.

5.4.3 SIMULTANEOUS FORCE AND FLUORESCENCE MEASUREMENTS

The initial (10 minute) decline in the fluorescence signal may be attributed to the washing out of excess unbound TnC-DANZ which had been trapped inside the myofilaments. Zot et al. (1986) showed that TnC-DANZ binds only to the TnC-depleted sites and to no other position on the fibres so it can be assumed that once all the unbound protein has been washed away, that left is incorporated successfully into the Tn complex. The remaining steady decline in baseline fluorescence has been noted by Guth & Potter (1987) and Morano & Ruegg (1991). It may be partly due to bleaching of the fluorescent probe and partly to a dissociation and washing away of TnC-DANZ (maximum force also decreases though at a slower rate). If the latter is the case then problems will arise in that Ca^{2+}-sensitivity of the fibres will also be changed (see above). This could be compensated for by incubating the fibres in TnC-DANZ solution between every set of activations but would mean a prolongation of the experiment; this would be undesirable as maximum force decreases with time.

a) Ca^{2+} Sensitivity of Force compared to Fluorescence

i) *Differences in* pCa_{50}

Once substituted into skinned fibres, TnC-DANZ fluorescence had a pCa_{50} of 5.86, which was higher than that of isolated TnC-DANZ (around 5.54, tables 8 & 14). The binding of TnC to TnI causes a 10-fold increase in the Ca^{2+} affinity of the former (Potter & Gergely, 1975) but this is offset by a 10-fold decrease on incorporation of TnC into thin filaments (Zot et al, 1983) (although these changes were measured in biochemical experiments where the myofilaments were not producing force). Thus the increase in Ca^{2+}-sensitivity of fluorescence of TnC-DANZ in situ in the myofilament lattice probably results from actin-myosin interactions, as discussed below.

Force was less sensitive to Ca^{2+} than was fluorescence (fig.28) with a difference in pCa_{50} of 0.15 units (fig.29). This matched reports by Zot et al (1986); Guth & Potter (1987) and Morano & Ruegg (1991) though the magnitude of the pCa_{50} difference varied between studies (0.6, 0.3 and 0.22 units respectively). This variation may reflect uncertainties in correcting fluorescence

decline during the experiments. In the current study the maximum fluorescence at the precise time of activation at submaximal Ca^{2+} was not known so the submaximal fluorescence values were corrected to the following maximal activation readings. If it had been assumed that maximum fluorescence decreased linearly with time, and sub maximal fluorescence had been expressed relative to the predicted maximum fluorescence at the time of activation, the calculated pCa_{50}s would have been approximately 0.05 units less than those using correction to the following maximal activation. Thus the difference between pCa_{50} of force and fluorescence would be smaller after a "time-based" correction. The variation between studies might reflect differences in experimental conditions though these were generally similar so it is difficult to determine the causative agent.

In addition, fluorescence depends not only on Ca^{2+} binding but also on crossbridge state (see below) so the possibility that the number of weakly attached crossbridges varies with Ca^{2+} concentration presents considerable problems for normalisation and interpretation of the results. It was suggested by Zot et al (1986) that the difference between pCa-force and fluorescence curves was due to the fact that the binding of Ca^{2+} to either low affinity site on TnC triggered the full increase in fluorescence (for a given Ca^{2+} concentration) whereas both sites had to be occupied for tension development. It now appears, however, that the presence of weakly bound crossbridges, that increase fluorescence but not force, may partially explain these results. The proportion of such crossbridges would decline towards lower pCa values which could explain the reduced steepness of the pCa-fluorescence curve compared to that of force.

The technique of using labelled TnC incorporated into skinned fibres to detect changes in Ca^{2+} binding directly was greatly complicated by the discovery (Guth & Potter, 1987; Zot & Potter, 1989) that changes in fluorescence could occur independent of Ca^{2+}. Fluorescence changes of TnC-DANZ in skinned fibres and reconstituted myofibrils were caused not only by direct Ca^{2+} binding to the low affinity sites of TnC but also by rigor, weakly bound and strongly bound crossbridges. Rigor crossbridges produced a different fluorescence change than Ca^{2+} binding, whereas the TnC structural changes induced by cycling crossbridges were in addition to those of Ca^{2+} binding but different from those produced by rigor. Weakly and strongly attached crossbridges appeared to have different couplings to conformational changes in TnC and crossbridge formation (Zot & Potter, 1989). Increasing SL from 2.3 to 3.8 μm (thereby reducing the number of crossbridge attachments) decreased maximum fluorescence by 39% but had no effect on the pCa_{50} of the pCa-fluorescence curve (Guth & Potter, 1987). Thus Guth & Potter concluded that small numbers of cycling crossbridges had the same effect on Ca^{2+}-sensitivity of TnC as large

numbers i.e. crossbridge mediated changes in TnC conformation were cooperative along the entire thin filament. In fact it may be that a reduced pCa_{50} due to the attachment of fewer crossbridges at SL 3.8 μm was offset by an increase in Ca^{2+}-sensitivity due to a length-dependence of pCa_{50}.

ii) *Differences in Hill "n"*

The steepness of the pCa-fluorescence curve was less than that of the pCa-force relationship (fig.29). This was in agreement with Zot & Potter (1987) but in contrast to the results of Zot et al (1986) and Guth & Potter (1987) who observed no significant difference between slopes for force and fluorescence. Zot et al (1986) noted that a difference in steepness between the curves for force and fluorescence would not be expected if fluorescence depended solely on Ca^{2+} binding to either low affinity site on TnC but force required occupation of both sites.

The H_n of the pCa-force relationship in the present study was 3.92 which indicated a large degree of positive cooperativity (despite the fact that reconstitution did not fully restore maximum force). The steepness of the pCa-fluorescence curve (H_n 2.09) was greater than those of isolated TnC-DANZ (1.28-1.43, tables 8 & 14). As discussed in section 3.4.2.a, the latter experiments indicated very little, if any, cooperativity was present in TnC-DANZ alone. However, a H_n of 2.09 shows apparent cooperativity of Ca^{2+} binding to TnC-DANZ in skinned fibres. These results support those of Grabarek et al (1983) who found H_n was 1.0 for isolated TnC-DANZ and 1.5 for TnC-DANZ in regulated thin filaments, rising to 2.4 on the addition of myosin. It seems likely that crossbridge formation promotes either Ca^{2+} binding to TnC or a Ca^{2+}-independent increase in TnC-DANZ fluorescence (or both) thereby giving an apparent increase in cooperativity (Guth & Potter, 1987). The fluorescence H_n was less than that observed for force which could reflect a greater effect of crossbridge attachment on actin-myosin interaction than on TnC conformation.

b) Effects of CGP48506 on Force and Fluorescence

100 μM CGP48506 had a smaller effect on pCa_{50} of force (increased by 0.15 units) in these experiments than in work performed on non-reconstituted psoas fibres (increase in pCa_{50} of 0.35, table 13). The pCa_{50} of the control curve was not significantly different from that in previous experiments (5.71 compared to 5.78 though the H_n was higher (3.92 instead of 2.10). Since the same solutions and psoas preparations were used for each study the different results presumably relate to the extraction/reconstitution procedure which may have reduced the sensitising action of CGP48506 (though not caffeine - section 5.3.2.a).

CGP48506 caused similar shifts in pCa_{50} of the force (0.15) and fluorescence (0.25) curves which indicates that it either increases the Ca^{2+} affinity of the low affinity sites of TnC or causes an increase in the TnC structural changes induced by cycling crossbridges (see section 5.4.3.a), thereby altering Ca^{2+} binding (reciprocal coupling - see Guth & Potter, 1987; Zot & Potter, 1989) or both. Unfortunately it was not possible to distinguish between these possibilities on the basis of the results obtained. If the former does hold true, the action of CGP48506 on TnC must be dependent on the presence of other myofibrillar proteins since no change in Ca^{2+}-sensitivity was induced by CGP48506 on isolated TnC-DANZ (table 14). It may be, for example, that TnC in fibres has a different conformation from the isolated form and this increases the efficacy of CGP48506 (or its affinity for TnC). Alternatively CGP48506 could bind to another myofibrillar protein (TnI perhaps) altering its interaction with TnC and thereby enhancing Ca^{2+} binding. Changes in TnI are known to affect the Ca^{2+}-affinity of TnC e.g. phosphorylation of TnI decreases Ca^{2+} binding to TnC (Robertson et al, 1982).

It is unlikely that CGP48506 causes a shift in crossbridge state from detached to weakly bound since the compound had no effect on the fluorescence at pCa 9.39. Other possible mechanisms for CGP48506 are discussed in chapter 6.

5.5 SUB-CONCLUSIONS

a) Force development and changes in TnC conformation were measured simultaneously by replacing native TnC in skinned psoas fibre with TnC-DANZ.

b) The relationship between pCa and TnC-DANZ fluorescence was more sensitive to Ca^{2+} and less steep than was that between pCa and force. This indicates either that occupation of only one Ca^{2+}-specific site induces the fluorescence response whereas both must be occupied for force generation, or that the number of weakly bound crossbridges varies with pCa.

c) Fluorescence and force were sensitised similarly to Ca^{2+} by CGP48506, suggesting that the compound may increase the Ca^{2+}-affinity of the Ca^{2+}-specific sites of TnC in the presence of other myofibrillar proteins, or that the conformation of TnC in fibres is such that its affinity for CGP48506 is increased. Alternatively CGP48506 could act directly on crossbridges, affecting Ca^{2+} binding by reciprocal coupling.

CHAPTER 6: OVERALL DISCUSSION

6.1 MAXIMUM FORCE

A fall in maximum force must result from a drop in the amount of force generated per attached crossbridge, or from a decrease in the number of force-producing crossbridges, or a combination of these. The number of strongly bound crossbridges could decrease due to either a fall in the apparent attachment rate (f_{app}) or a rise in the apparent detachment rate (g_{app}). Theoretically, ATPase activity measurements could help to distinguish between these possibilities: ATPase activity would remain unchanged if the force generated per crossbridge dropped but would probably be altered if the attachment or detachment rates changed.

6.1.1 DECREASE WITH ACIDOSIS

The mechanism by which maximum force is reduced by acidosis is unknown (section 2.4.1.b). It appears that acidosis suppresses maximum force by decreasing both force per crossbridge and the number of strongly bound crossbridges, since force decreases more than stiffness (section 2.4.1.b; Kentish & Palmer, 1989; Metzger & Moss, 1990). As yet it is still unclear how pH affects ATPase activity in skinned fibres and how decreases in ATPase activity and decreases in force are related.

One possible mechanism is that protons (which, like Pi, are a product of ATP hydrolysis) might promote transition from strongly to weakly bound crossbridges i.e. reverse the force-generating step of the crossbridge cycle. Kentish (1991) proposed the existence of a weakly attached AM.ADP.Pi state (fig.2) where H^+ was bound, which converted to a strongly attached AM.ADP.Pi state on the dissociation of H^+. Acidosis would therefore reduce force (which depends on strongly-attached crossbridges), but by more than stiffness (which may depend on both weakly and strongly bound crossbridges).

It is not clear why cardiac fibres are more susceptible than skeletal muscle to acidosis-induced suppression of maximum force and ATPase activity (present results; Godt & Kentish, 1989). This may reflect differences in intrinsic ATPase activity (which is greater in fast-twitch skeletal than cardiac muscle; Kentish & Nayler, 1979). A difference in ATPase activity between the muscle types must reflect differences in the rate constants in the crossbridge cycle. The greater acidosis-induced suppression of cardiac fibres cannot be explained by the effects of Pi accumulation in fibres (section 2.4.1.b).

6.1.2 DECREASE WITH Pi

Pi reduces maximum force by reducing the number of strongly attached crossbridges and the force generated per crossbridge (section 2.4.1.c), as demonstrated by the findings that maximum ATPase activity decreased in the presence of Pi but by less than force, and force fell by more than stiffness (Kawai et al, 1987). It has been shown that Pi increases the rate of change from strongly bound (force-generating) to weakly bound crossbridges (Hibberd et al, 1985), presumably by binding to AM.ADP to form AM.ADP.Pi (fig.2). This can account for the above changes.

It is unclear why Pi has a greater effect on maximum force of cardiac than skeletal muscle. As in the case of acidosis this might be due to the higher ATPase activity of fast-twitch skeletal fibres or it might reflect a greater accumulation of Pi in skeletal fibres such that addition of further Pi has a smaller apparent effect (changes in [Pi] at low concentrations have greater effects on maximum force than changes at high concentrations, section 1.1.11).

6.1.3 DECREASE WITH SENSITISERS

The mechanism(s) by which caffeine, BA41899 and its enantiomers suppress maximum force is unknown, though one possibility is that they could alter myofilament lattice spacing (section 2.4.1.d). Previous work has shown that BA41899 reduces maximum ATPase activity (Herzig, pers. comm.) though it is unclear whether there is a fall in force generated per crossbridge as well as a decrease in the number of force-producing crossbridges. No information is available concerning the effects of caffeine, CGP48506 or CGP48508 on ATPase activity. A decrease in maximum force without a drop in maximum ATPase activity would be less satisfactory from a therapeutic standpoint: a fall in maximum force may itself be disadvantageous and if the latter is not accompanied by a drop in ATPase activity, the increased energy cost to the failing heart may negate the usefulness of a Ca^{2+}-sensitiser as an inotropic agent. Substances which suppress maximum force but increase pCa can still be useful as cardiotonic agents since the heart does not reach maximal activation during a beat but normally operates at around 50% of its maximal potential (Fabiato, 1981).

The effects of caffeine and the BA41899-related compounds on maximum force are unlikely to be related to their effects on pCa_{50} (section 4.4.3.b) since Ca^{2+}-sensitivity was generally increased whereas maximum force was decreased. Exceptions were CGP48508, which had no effect on pCa_{50} of cardiac fibres but suppressed their maximum force considerably, and CGP48506, which sensitised psoas fibres to Ca^{2+} whilst having no effect on maximum force. The

mechanism(s) of the maximum force reduction by caffeine is not the same as for acidosis or Pi since the maximum forces of cardiac and skeletal fibres were equally suppressed by caffeine. Thus it may be expected that caffeine would interact with a different stage of the crossbridge cycle, whereas BA41899 and its enantiomers (all of which reduce maximum force more in cardiac fibres than skeletal ones) might act at the AM.ADP or AM.ADP.Pi stage (fig.2).

6.2 Ca^{2+}-SENSITIVITY

As discussed in section 1.1.13.c, an increase in pCa_{50} (Ca^{2+}-sensitisation) of skinned fibres could result from a direct increase in the Ca^{2+}-affinity of TnC. By the same argument it follows that a decrease in pCa_{50} could be caused by a direct decrease in the Ca^{2+}-affinity of TnC. Since the rate of Ca^{2+} binding to TnC is fast and diffusion limited (Johnson et al, 1979), Ca^{2+}-affinity is most likely to be altered by a change in the dissociation rate of Ca^{2+} from TnC.

The Ca^{2+}-sensitivity of skinned fibres is not necessarily the same as the Ca^{2+}-sensitivity of TnC (section 5.4.3.a). The binding of TnI to TnC enhances the Ca^{2+}-affinity of the latter 10-fold (Potter & Gergely, 1975; Holroyde et al, 1980). It has been suggested that the interaction of Tn-Tm with actin causes a 10-fold decrease in Ca^{2+}-affinity of the regulatory sites of TnC (Zot et al, 1983). In addition, other factors such as sarcomere length also contribute to the Ca^{2+}-sensitivity of skinned fibres. Zot et al (1983) used reconstituted myofibrils where mechanical restrictions occurring in skinned fibres were absent. In addition, increases in TnC Ca^{2+}-affinity caused by interaction between actin and myosin (Guth & Potter) were not taken into account. The current study enabled direct comparison of the Ca^{2+}-affinity of TnC (section 5.4.3.a) both isolated ($pCa_{50} \approx 5.54$) and in skinned fibres ($pCa_{50} \approx 5.86$). TnC had a higher affinity for Ca^{2+} once incorporated in the myofilaments, which can be explained if bound crossbridges increase the Ca^{2+}-affinity of TnC (section 5.4.3.a; Guth & Potter, 1987). In the present study, however, the extent of Ca^{2+}-sensitisation of TnC by crossbridges via reciprocal coupling is unknown.

A change in Ca^{2+}-sensitivity could therefore result from changes in the following: the Ca^{2+}-affinity of TnC; interactions within the Tn complex; interaction between Tn-Tm and actin; or from a direct action on the crossbridges; or from a combination of any of these.

6.2.1 DECREASE WITH ACIDOSIS

Approximately 50% of the decline in Ca^{2+}-sensitivity of skinned fibres during acidosis is due to a decrease in Ca^{2+}-affinity of the TnC. Thus acidosis probably increases the "off rate" of Ca^{2+} from TnC. This could be tested in further

experiments by measuring the rate of dissociation of Ca^{2+} from TnC (c.f. Smith & England, 1990). The 2-fold greater pH-sensitivity of cardiac compared with skeletal fibres could be explained by the greater pH-sensitivity of cardiac compared with skeletal TnC (section 3.4.2.b).

The remaining part of the decline in Ca^{2+}-sensitivity of skinned fibres during acidosis can be attributed to an acidosis-induced halving of the TnI-induced enhancement of Ca^{2+} binding to TnC (El-Saleh & Solaro, 1988), as discussed in section 3.4.2.b. Alternatively, Ca^{2+}-sensitivity could be decreased by a fall in the degree of reciprocal coupling between TnC and crossbridges: acidosis reduces the number of force-generating crossbridges (see above) and since strongly bound crossbridges increase the affinity of TnC for Ca^{2+} (Guth & Potter, 1987) an accompanying drop in this affinity would be expected during acidosis.

6.2.2 DECREASE WITH Pi

The Pi-induced desensitisation of skinned fibres to Ca^{2+} was not due to a direct decrease in the Ca^{2+}-affinity of TnC (section 3.4.2.c).

It is possible that some other component of the Tn-Tm complex is required for Pi to affect Ca^{2+} binding to TnC and that once TnC is incorporated into the myofilament lattice its properties are altered such that Pi does directly reduce its Ca^{2+}-affinity. Alternatively Pi may affect Ca^{2+}-sensitivity by direct actions on the crossbridges (section 3.4.2.c), as it decreases the number of strongly bound crossbridges (similar to acidosis - see above), though in this case a greater change in pCa_{50} might be expected in cardiac compared to skeletal fibre as the Pi-induced depression of maximum force is greater in the former. Since weakly attached crossbridges might increase the Ca^{2+}-affinity of TnC by less than strongly attached ones (Morano & Ruegg, 1991), agents which promote weakly bound or unattached states may cause an apparent reduction in Ca^{2+}-sensitivity. Pi, for example, could shift the crossbridge cycle equilibria (fig.2) "backwards" from AM.ADP such that the M.ADP.Pi and AM.ADP.Pi forms are favoured. Alternatively, Chalovich et al (1981) showed that the Ca^{2+}-regulated step in the crossbridge cycle may be the transition from AM.ADP.Pi to AM.ADP (rather than M.ADP.Pi to AM.ADP.Pi). In this case Pi might compete with Ca^{2+} at this stage, the two acting in "opposite directions". Here more Ca^{2+} would be required to generate force than in the absence of Pi, thus the Ca^{2+}-sensitivity of force would decrease.

6.2.3 INCREASE WITH SENSITISERS

The Ca^{2+}-sensitising actions of caffeine, BA41899 and its enantiomers were not due to direct increases in the Ca^{2+}-affinity of TnC (sections 3.4.2.d & 4.7.1.b), although studies using TnC-DANZ in skinned fibres showed that TnC Ca^{2+}-affinity was probably altered by CGP48506 (section 5.4.3.b). TnC is likely to have modified physico-chemical properties when incorporated into the myofilament lattice. However studies using isolated proteins have provided valuable insights into the mechanisms of action of various Ca^{2+}-sensitisers. Although most of these agents (e.g. sulmazole, isomazole and perhexiline) had no effect on the rate of Ca^{2+} dissociation from isolated cardiac TnC (Smith & England, 1990), pimobendan and bepridil both decreased the "off rate" for Ca^{2+} binding to the Ca^{2+}-specific site.

The failure of sensitisers to affect Ca^{2+} binding to isolated TnC indicates that other components of the Tn-Tm complex might be required for the Ca^{2+}-sensitising action. This was demonstrated by Smith & England (1990) with isomazole and perhexiline which had no effect on TnC alone but increased the Ca^{2+} affinity of the Tn-Tm complex, thus implying that interactions between TnC and the other proteins were necessary for Ca^{2+}-sensitisation. It is possible, for example, that isomazole and perhexiline may act in the opposite way to acidic pH by enhancing the interaction of TnC and TnI. In contrast, sulmazole and pimobendan had no effect on the Ca^{2+}-affinity of Tn-Tm (Smith & England, 1990).

Radioactive labelling of caffeine and BA41899 and the study of their binding to isolated protein complexes such as TnC-TnI, Tn and Tn-Tm may help determine the compounds' binding site, as may comparative studies between the effects of Ca^{2+}-sensitisers on skeletal and cardiac, or adult and neonate, skinned fibres, which have different forms of several muscle proteins.

Alternatively, the sensitisers might act directly on the crossbridges. Ca^{2+}-sensitisers such as caffeine, BA41899 and its enantiomers might be expected to facilitate the transition from AM.ADP.Pi to AM.ADP or AM thus promoting the formation of force-generating crossbridges (and increasing the Ca^{2+}-affinity of TnC). An alternative suggestion is that Ca^{2+}-sensitising agents increase the concentration of weakly bound crossbridges even in the absence of Ca^{2+} (i.e. the transition from M.ADP.Pi to AM.ADP.Pi is facilitated). On the addition of Ca^{2+}, a greater number of weakly attached crossbridges would then be available for conversion to the force-generating form. Such a mechanism could be tested by measuring the stiffness of relaxed muscle (at low ionic strength) in the presence and absence of a Ca^{2+}-sensitiser: an increase in stiffness would indicate a rise in concentration of weakly bound crossbridges. These mechanisms, however, would

be expected to increase maximum force instead of causing the observed suppression. It is possible that the sensitisers increase the number of attached crossbridges but decrease the amount of force generated per crossbridge.

Although their mechanism(s) of Ca^{2+}-sensitisation remain unclear, it appears that the agents do not all act at the same site since CGP48508 had greater effects on the pCa_{50} of skeletal fibres than cardiac ones whereas the reverse was true for caffeine, BA41899 and CGP48506. At least for caffeine it was shown that the different susceptibilities of skeletal and cardiac muscles to Ca^{2+}-sensitisation were not related to their different sarcomere lengths.

6.2.4 CHARACTERISTICS REQUIRED FOR Ca^{2+}-SENSITISATION

It would be useful to determine the active regions of the Ca^{2+}-sensitisers e.g. by testing modified versions of the molecules or the effects of isomers (where these exist). Some work has already been carried out in this field: although early sensitisers (caffeine, sulmazole, pimobendan) were based on the imidazole ring, many of the later compounds have completely different structures (fig.31). A common feature to most, however, is the presence of lone pairs of electrons usually associated with nitrogen atoms but occasionally (e.g. sulmazole) also with sulphur. The importance of these electrons in the Ca^{2+}-sensitisation mechanism has been established at least for BA41899 where methylation of the lone pair nitrogen causes the compound to switch from a sensitiser to a desensitiser (Herzig, pers. comm.). Clearly, however, the environment of the lone pair is also important in determining the effects on Ca^{2+}-sensitivity: this is apparent firstly from comparisons of molecules which are not Ca^{2+}-sensitisers but have similar structures to compounds which are, and secondly from differences between the degree of Ca^{2+}-sensitisation induced by isomers of the same molecule. As shown in fig.31, amrinone and milrinone (neither of which sensitise the myofilaments to Ca^{2+} (Salzmann et al, 1985; van Meel, 1987)) are closely related to APP 201-533. The Ca^{2+}-sensitising action of APP 201-533 presumably depends on the orientation of the methyl group with respect to the rest of the (planar) ring. The CH_3 group is absent in amrinone and may be orientated differently in milrinone which has the strongly electrophilic CN group. It seems likely that a lone pair nitrogen with a higher than average electron density is required for Ca^{2+}-sensitisation to occur, and for this reason the relative distances of electronegative and elecropositive groups from the nitrogen are of paramount importance.

Caffeine, pimobendan, sulmazole and BA41899 can exist in more than one configuration, with one form having a greater Ca^{2+}-sensitising action than the other. Caffeine sensitises rat atrial myofilaments to Ca^{2+} to a larger extent than isocaffeine (author's unpublished observations) which presumably reflects the

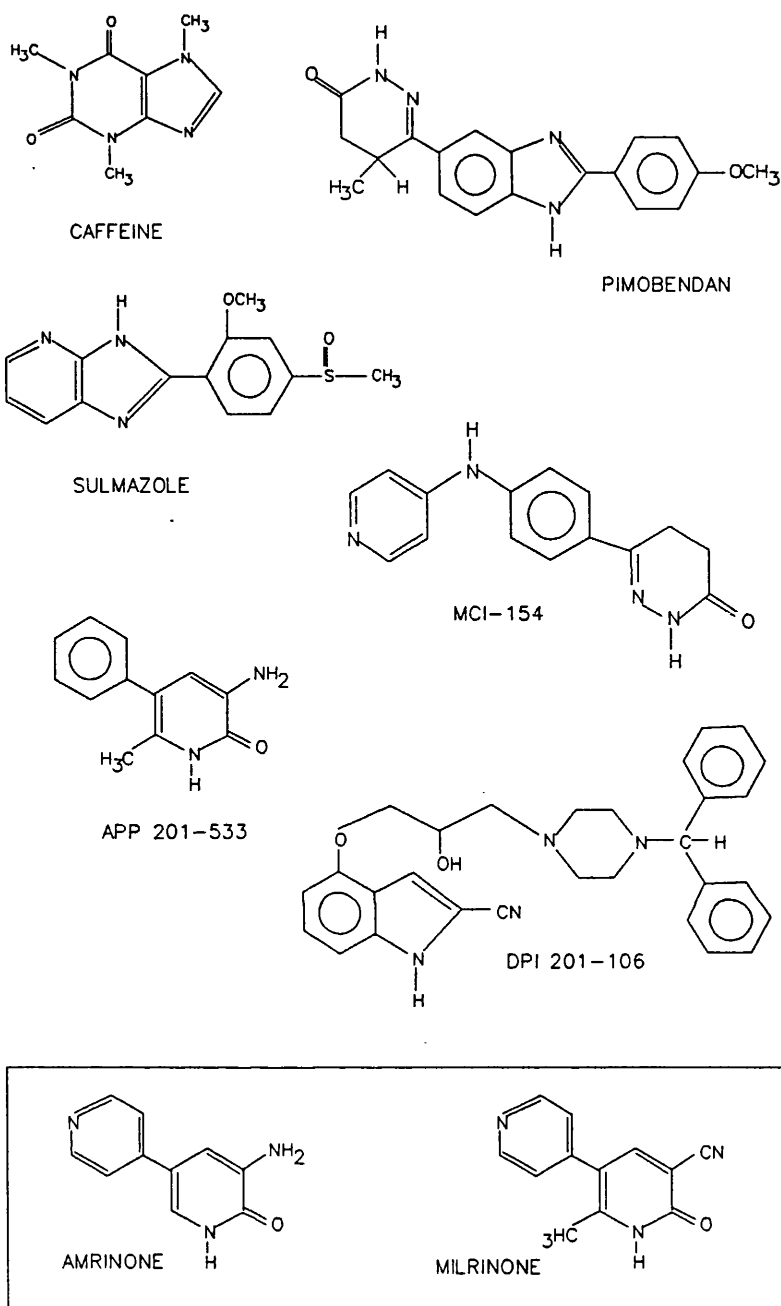


Fig.31 CHEMICAL STRUCTURES OF SIX Ca^{2+}-SENSITISING AGENTS. The imidazole ring occurs in many Ca^{2+}-sensitisers such as caffeine, pimobendan, sulmazole and DPI 201-106 but is absent in MCI-154 and APP 201-533. Of the compounds shown only DPI 201-106 is not a PDE inhibitor. Despite structural similarities to APP 201-533, amrinone and milrinone (drawn in box) are not Ca^{2+}-sensitising agents.

altered environment of the relevant lone pair nitrogen. It might be expected that the shape of isocaffeine would differ considerably from that of caffeine as a methyl group has shifted from one side of the molecule to the other and will therefore have a totally new spatial relationship to the other groups. In contrast the enantiomers of pimobendan, sulmazole and BA41899 differ within a pair only in the orientation of one group. Despite these relatively subtle differences, the Ca^{2+}-sensitising effects of l-pimobendan, (+)sulmazole and CGP48506 were much greater than the corresponding effects of d-pimobendan (Fujino et al, 1988), (-)sulmazole and CGP48508. Indeed (-)sulmazole did not sensitise skinned myocardial fibres to Ca^{2+} at all (van Meel et al, 1988). It is therefore clear that the site(s) on the myofilaments which interact with Ca^{2+}-sensitisers can distinguish between relatively subtle conformational differences between molecules. This in turn implies that the myofilament sites themselves have a clearly defined specific structure (though the site of interaction with one sensitiser may differ from that with another).

6.3 CONCLUSIONS

In conclusion, therefore, it has been shown that the effects of acidosis, caffeine, Pi, BA41899, CGP48506 and CGP48508 on Ca^{2+}-sensitivity of skinned fibres vary between skeletal and cardiac muscle and, with the exception of acidosis, are not due to direct alteration of the Ca^{2+} affinity of TnC. This was the first study in which the effects of such agents on skeletal and cardiac isolated TnC and skinned fibres were demonstrated in comparable physiological solutions. In addition the greater effects of caffeine on cardiac compared to skeletal fibres were shown not to be related to the different sarcomere lengths of the two muscle types. Finally changes in fluorescence of TnC-DANZ in situ in skinned psoas fibres were measured simultaneously with force in an attempt to determine the role of TnC in the Ca^{2+}-sensitisation of force by CGP48506. The results were complicated by Ca^{2+}-independent increases in TnC-DANZ fluorescence generated by weakly and strongly attached crossbridges, thus the technique has only limited application in the elucidation of mechanisms of action of Ca^{2+}-sensitising agents.

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[*From the Proceedings of the Physiological Society*, 4–5 *November* 1988
Journal of Physiology, **410**, 67*P*, 1989]

Effect of pH on force and stiffness in skinned muscles isolated from rat and guinea-pig ventricle and from rabbit psoas muscle

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A decrease in pH is known to depress the maximum force production of cardiac and skeletal myofibrils (Fabiato & Fabiato, 1978), though the mechanism of this inhibitory effect is unclear. It could be due to either a decrease in the number of attached force-producing crossbridges or a decrease in the mean force produced by each crossbridge. To try to distinguish between these possibilities, we measured force and instantaneous stiffness of detergent-skinned cardiac muscles and glycerinated skeletal fibres during maximal Ca^{2+} activation in solutions of different pH (7·4–6·2). Stiffness, which is a measure of the number of attached crossbridges (Huxley & Simmons, 1971), was determined by imposing rapid (< 1 ms) length changes on the muscles and measuring the extreme tension reached (T_1); the slope of the line relating length change to T_1 is the instantaneous stiffness (Fig. 1).

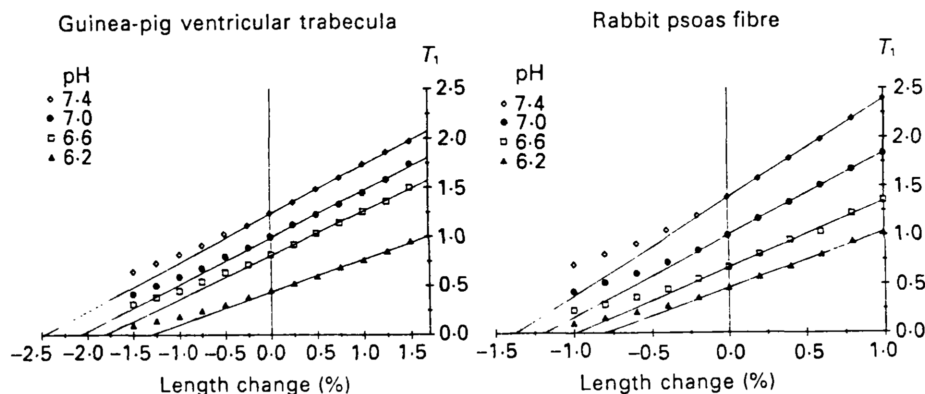

Fig. 1. Stiffness measurements in typical skinned cardiac and skeletal muscles at four pH values. T_1 values are relative to isometric force at pH 7·0. The solid lines are regression fits to the data for stretches. Temperatures: 20 °C (cardiac), 14 °C (skeletal). Solution composition as in Kentish (1986).

Stiffness fell as the pH was lowered, indicating that some of the fall in isometric force was due to fewer attached crossbridges. However, as shown by the reduced x-axis intercept, stiffness fell relatively less than isometric force. Part of this could have been artifact resulting from variable series elasticity, but it is likely that there was also a true decrease in the mean force per crossbridge.

Supported by the BHF. S.P. is a SERC/CASE student.

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[*From the Proceedings of the Physiological Society*, 30 *June*–1 *July* 1989
Journal of Physiology, **417**, 160*P*, 1989]

Calcium binding to isolated bovine cardiac and rabbit skeletal troponin-C is affected by pH but not by caffeine or inorganic phosphate

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Caffeine increases the Ca^{2+}-sensitivity of cardiac or skeletal myofibrils (Wendt & Stephenson, 1983), whereas acidity or phosphate (P_i) decreases it (Fabiato & Fabiato, 1978; Kentish, 1986). These effects may be mediated by changes in the affinity of troponin C (TnC) for Ca^{2+}. To test this, we measured Ca binding to isolated cardiac and skeletal TnC labelled respectively with 2-((4′-iodoacetamido)-anilino)-naphthalene-6-sulphonic acid (IAANS) and dansylaziridine (DANZ), whose fluorescence increases when Ca^{2+} binds to the regulatory Ca-specific site(s).

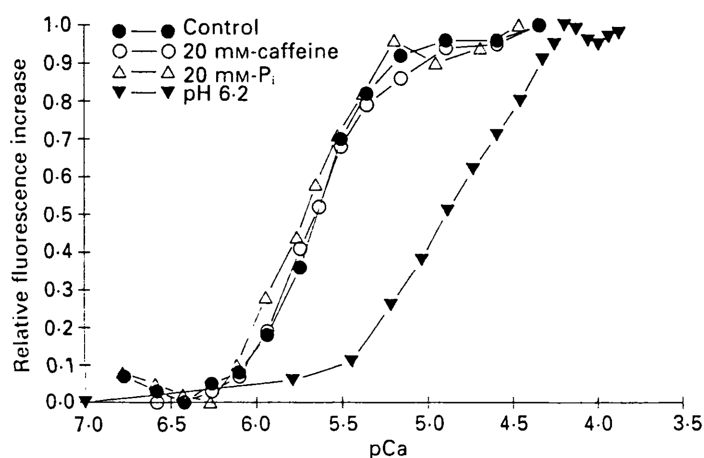

Fig. 1. Fluorescence increase of cardiac TnC–IAANS as a function of [Ca^{2+}]. Solutions as in Kentish (1986), except [EGTA] was 2 mM, Mg^{2+} 1 mM, and [TnC] 3 µM. Ca added as $CaCl_2$. pH buffer was 100 mM-BES (pH 7·0) or MES (pH 6·2). Ionic strength 0·2 M, 20 °C.

Neither caffeine (20 mM) nor P_i (20 mM) affected Ca^{2+} binding to cardiac TnC (Fig. 1) or to skeletal TnC (not shown). However, lowering pH to 6·2 reduced the Ca^{2+} affinity of both cardiac and skeletal TnC. These results suggest that the effect of pH on Ca^{2+} sensitivity of skinned fibres may be partly due to a direct effect on Ca^{2+} binding to TnC. In contrast the effects of caffeine and P_i on the Ca^{2+} sensitivity of skinned fibres may require the presence of other myofibrillar proteins.

Supported by the British Heart Foundation. S.P. is a SERC/CASE student.

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Biophys. J. 57, 147a (1990)

EFFECT OF CAFFEINE AND pH ON Ca BINDING TO ISOLATED TROPONIN C.

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The Ca sensitivity of skinned fibres is increased by caffeine and decreased by low pH. To investigate whether these effects are related to direct actions on the Ca affinity of troponin C (TnC), we measured the Ca binding to isolated TnC (rabbit skeletal and bovine cardiac), labelled with DANZ and IAANS, respectively. The fluorescence of these labels increases when Ca binds to the regulatory site(s) (Johnson et al., J. Biol. Chem. 253, 6451, & 255, 9635). The solutions were similar to those used for skinned muscles (1 mM Mg^{2+}, ionic strength 0.2 M, 25°C).

At pH 7.0, the pCa for 50% increase in fluorescence (pCa50) was 5.55 ± 0.02 (S.E, n=4) for skeletal TnC and 5.26 ± 0.07 (n=4) for cardiac TnC. Reducing the pH to 6.2 decreased the pCa50 for skeletal TnC by 0.34 ± 0.02 (cf. El-Saleh & Solaro, J. Biol. Chem. 263, 3274) and for cardiac TnC by 0.50 ± 0.07. However, 20 mM caffeine did not significantly affect the pCa50 for either type of TnC at pH 7.0. Thus the effect of pH on the Ca sensitivity of skinned fibres may be due, at least in part, to a direct action on the Ca affinity of TnC, whereas the effect of caffeine cannot be explained by this mechanism.

Supported by the BHF, SERC and Ciba-Geigy plc.

11P EVIDENCE THAT THE SENSITISING ACTION OF CAFFEINE ON MYOFIBRILS IS NOT DUE TO A DIRECT EFFECT ON THE Ca^{2+} AFFINITY OF TROPONIN C

J.C. Kentish* & S. Palmer, Department of Pharmacology, U.M.D.S., St. Thomas's Hospital, London SE1 7EH. (Introduced by S.J. Paterson).

The "Ca^{2+}-sensitizing" drugs such as caffeine and sulmazole (AR-L 115 BS) act directly on myofibrils of striated muscle to increase the sensitivity to Ca^{2+} (e.g. Wendt & Stephenson, 1983). This may partly explain the action of these drugs to increase the force of contraction. However, we do not know the mechanism of this sensitization, or why the effect of caffeine on cardiac myofibrils is about 3 times greater that on skeletal myofibrils (Wendt & Stephenson, 1983).

To examine whether the sensitization of force by caffeine is due to an increase in the Ca^{2+} affinity of troponin C (TnC), we used isolated cardiac and skeletal TnC labelled with fluorescent probes IAANS (2-((4'-iodoacetamido)-anilino)-naphthalene-6-sulphonic acid) and DANZ (dansylaziridine), respectively. The fluorescence of these labels increases when Ca^{2+} binds to the "Ca^{2+}-specific" sites that are involved in the regulation of contraction. Ca^{2+} binding curves were produced by measuring the increase in fluorescence as $CaCl_2$ was added sequentially to a solution containing (mM): 100 BES (N,N-bis[2-hydroxyethyl]-2-aminoethane sulphonic acid), 85 K propionate, 4.4 $MgATP^{2-}$, 1 Mg^{2+}, 10 creatine phosphate, 1 dithiothreitol, 2 EGTA, 3 uM labelled TnC, pH 7.0 at 25°C. Addition of 20 mM caffeine produced no significant change in the pCa_{50} ($-\log[Ca^{2+}]$ for 50% fluorescence increase) for cardiac TnC (5.26 $\pm$ 0.07 in zero caffeine, mean $\pm$ S.E., n=4) or skeletal TnC (5.55 $\pm$ 0.02, n=4). This is in marked contrast to the effects of 20 mM caffeine on force production of skinned muscles, in which the pCa_{50} for force increased by 0.31 $\pm$ 0.04 (n=4) in detergent-skinned cardiac muscles from rat and 0.09 $\pm$ 0.01 (n=4) in glycerinated skeletal fibres from rabbit. However, we confirmed the finding of El-Saleh & Solaro (1988) that low pH (6.2) reduces the Ca^{2+} affinity of TnC, as it does for the Ca^{2+}-sensitivity of force production. Thus some interventions, such as acidity, change the Ca^{2+} sensitivity of force by directly altering the Ca^{2+} affinity of TnC, whereas caffeine does not act in this way. It seems likely that the effect of caffeine on myofibrils is via an indirect mechanism, in which caffeine acts on a site other than TnC.

Supported by the B.H.F. S.P. is a CASE student (SERC/Ciba-Geigy plc).

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