Natural and synthetic factors which influence the calcium sensitivity of chemically-skinned rat cardiac muscle

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Abbreviations

ATP.....	Adenosine triphosphate.
EGTA.....	thyleneglycol bis(Baminoethylether)-N-N-tetraacetic acid.
HDTA.....	Hexamethylene-NNN'N'-diamnine tetra-acetic-acid.
PDE.....	Phosphodiesterase.
P_i.....	Inorganic phosphate.
s.l.	Sarcomere length.
s.r.	Sarcoplasmic reticulum.
CICR	Calcium-induced calcium-release.
$[Ca^{2+}]$	Calcium concentration.
$[Ca^{2+}]_i$	Intracellular calcium concentration.
$[Ca^{2+}]_o$.....	Extracellular calcium concentration.
C_{max}	Maximum calcium activated tension.

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Summary

The work which comprises this thesis is concerned with natural and synthetic substances which influence the Ca^{2+}-sensitivity of the intracellular systems in chemically-skinned cardiac muscle. Preparations skinned with Triton-X100 retain only traces of membrane structure while the contractile proteins remain functional. This form of treatment is ideal for investigating factors which influence the Ca^{2+}-sensitivity of the contractile proteins. Selectively-(saponin-) skinned trabeculae retain the intracellular membranes associated with the s.r. and mitochondria, while the surface membrane is rendered permeable to the bathing solution. This type of preparation has been used to investigate interventions which influence the functioning of the s.r.

In chapter 3 the experimental protocol employed to study the functioning of the s.r. is described. This involved producing trains of caffeine contractures in selectively-treated preparations. Between exposures to caffeine, the muscle was 'Ca^{2+}-loaded' in a solution with a [Ca^{2+}] subthreshold for tension production. The contracture amplitude increased as the duration of Ca^{2+}-loading or the [Ca^{2+}] of the Ca^{2+}-loading solution was increased. Such trains of contractures proved reproducible over prolonged periods of time. Using this method, it was possible to study the effects of various substances which influence the functioning of the s.r. under strictly controlled Ca^{2+}-loading conditions. However, adequate interpretation of these results requires

an understanding caffeine's action on the s.r. The reported effects of caffeine on a variety of preparations are discussed during the introduction of this chapter. Following this, a method is described which allows a direct assessment of the ability of the s.r. to effect relaxation in saponin-skinned preparations. The action of caffeine on net s.r. Ca^{2+}-accumulation was studied using this method. The results suggest that millimolar concentrations of caffeine render the s.r. incapable of net Ca^{2+}-accumulation under these experimental conditions. This led to the suggestion that the s.r. is unlikely to contribute significantly to the relaxation of the caffeine contracture in selectively-skinned trabeculae. The possible involvement of the mitochondria in the caffeine-induced response was also studied. However, a mitochondrial contribution was excluded as the caffeine contractures were unaffected by addition of azide or changing the [Na] of the bathing solutions.

Chapter 4 addresses the possibility that enzymes which influence the intracellular levels of cAMP may persist following saponin treatment. This could potentially influence the results presented in the following chapters as many of the substances studied are known to affect phosphodiesterase (PDE) activity and other cellular systems which alter the levels of cyclic nucleotides in intact cells. The Effects of PDE inhibition, and stimulation of adenylate cyclase were studied. In addition, an attempt was made to characterise the response of the s.r. and contractile proteins to exogenous cAMP.

Caffeine-induced contractures were potentiated in the presence of cAMP. This finding is consistent with previous studies in a variety of preparations. However, the contractile proteins were unaffected by exogenous

cAMP unless the duration of skinning was reduced to an unacceptable level. Addition of forskolin toxin potentiated the caffeine contracture. This introduced the possibility that (i) cAMP production may continue following saponin-skinning and (ii) interventions which influence the adenylate cyclase activity may potentiate the caffeine response via a cAMP mediated mechanism. The results presented in the following chapters were interpreted with reference to these findings.

Chapter 5 is a comparative study of the effects of caffeine and sulmazole on the contractile proteins and s.r. Both substances induce s.r. Ca^{2+}-release and increase myofilament Ca^{2+}-sensitivity. The contribution of increased Ca^{2+}-sensitivity to transient sulmazole and caffeine contractures was assessed. The opposing effects of sulmazole and caffeine on C_{max} were also studied.

In chapter 6, the intracellular actions of ORG30029 (N-hydroxy-5,6-dimethoxy-benzo[b]thiophene -2-carboximidamide) were investigated. This compound is one of several novel PDE inhibiting cardiotonic compounds which also act at the level of the myofilaments to increase Ca^{2+}-sensitivity. The action of ORG30029 was compared with that of other well characterised PDE inhibitors such as milrinone & IBMX. ORG30029 proved to be a potent Ca^{2+}-sensitiser, increasing submaximal Ca^{2+}-activated force at concentrations less than 10μM, but had no apparent effect on the s.r. In common with most other Ca^{2+}-sensitisers higher concentrations of ORG30029 also potentiated C_{max}.

Chapter 7 considers the effects of various natural and synthetic modulators of Ca^{2+}-sensitivity on the development of rigor tension. Particular attention was paid to the effects of pH as this has relevance to the events occurring late during myocardial ischaemia. The effect of pH was compared with P_i which decreases Ca^{2+}-activated force and the Ca^{2+}-sensitising compounds sulmazole and caffeine. It was found that a decrease in pH over the range 8.5-5.5 was associated with a marked reduction in rigor tension. Sulmazole and caffeine, which increase Ca^{2+}-activated force in skinned preparations, similarly increased rigor tension. However, P_i which produces a marked depression in Ca^{2+}-activated force production had little or no effect on rigor tension. The implications of these results and possible differences in the underlying mechanisms of action are considered.

Numerous studies have linked taurine to the control of Ca^{2+}-metabolism in excitable tissues. In cardiac muscle, taurine is present at concentrations up to 40mM, however, its function remains unknown. Chapter 8 investigates the effect of taurine on s.r. Ca^{2+}-accumulation and myofilament Ca^{2+}-sensitivity. Taurine was found to produce a small increase in Ca^{2+}-sensitivity but a marked increase in the ability of the s.r to accumulate Ca^{2+}. An attempt was made to correlate the intracellular actions of taurine described in this chapter with the reported effects of the amino acid on intact cardiac preparations. The possible physiological role of taurine in the heart is discussed.

Chapter 1: **General Introduction**

Excitation contraction coupling in striated muscle

Ebashi & Endo (1968), demonstrated that the protein troponin, isolated from skeletal muscle, could confer Ca^{2+}-sensitivity on crude actomyosin preparations. This action of troponin was enhanced in the presence of another myofibrillar protein now called tropomyosin (Ebashi et al 1969). Troponin contains three subunits which act in concert to regulate contraction. Troponin-C contains four Ca^{2+}-binding sites and in effect senses the free Ca^{2+}-concentration. Troponin-I binds to actin and inhibits actomyosin ATPase. The inhibitory action of troponin-I is removed when Ca^{2+} is bound to the regulatory sites on troponin-C. Troponin-T connects the two other subunits to tropomyosin. It is now generally accepted that after the action potential, a rise in $[Ca^{2+}]_i$ and subsequent binding to troponin-C triggers contraction in striated muscle.

Despite vigorous research, the mechanism underlying the $[Ca^{2+}]$ release step in excitation-contraction coupling remains unclear. One possible mechanism which has received considerable attention is that of calcium-induced calcium-release (CICR). According to this hypothesis, the small amount of Ca^{2+} that crosses the sarcolemma during the action potential is insufficient by itself to activate the myofilaments but induces a release of Ca^{2+} from the s.r. sufficient for activation (Bianchi, 1968, for review, see Frank, 1980).

In skeletal muscle, the twitch persists for an hour or more in the absence of extracellular Ca^{2+}. Thus the rise in intracellular Ca^{2+} preceding the twitch must be derived from intracellular stores. This apparently precludes the possibility that Ca^{2+} entry through the surface membrane could be the physiological trigger for intracellular Ca^{2+}-release. However, it has been suggested that the release of Ca^{2+} from within the preparation, perhaps from the junction of the t-tubules and the s.r., may induce further release from the sarcoplasmic reticulum (Frank, 1980). The CICR mechanism has been extensively investigated in mechanically-skinned skeletal fibres (Ford & Podolsky, 1970, Endo, Tanaka & Ogawa, 1970). In these studies it was found that raising the Ca^{2+}-concentration triggered further release of Ca^{2+} from the s.r. However, it was necessary to elevate the $[Ca^{2+}]$ considerably above the threshold for tension before CICR was detected. Largely on the basis of these observations the CICR mechanism has been discounted in skeletal muscle and the trigger for s.r. Ca^{2+}-release remains unclear.

In contrast to the situation in skeletal muscle, there is considerable evidence that CICR is important in cardiac muscle. The CICR mechanism has been investigated extensively in mechanically-skinned cardiac cells (for reviews see Fabiato & Fabiato, 1977, Fabiato, 1983, Fabiato, 1986). Unlike skeletal muscle, large CICR contractures may be induced by raising the $[Ca^{2+}]$ to a level just subthreshold for tension production provided the change in $[Ca^{2+}]$ is achieve rapidly enough. Recently, experiments have been carried out using the caged Ca^{2+} chelator Nitr-5 in intact cardiac muscle. Ca^{2+}

can be rapidly released by flash photolysis of the chelator. The results provide evidence in support of Fabiato's work that CICR activates contraction in intact cardiac cells (Valdeolmillos et al 1989). The importance of CICR is now believed to be species dependent and roughly correlated with the relative cell volume of s.r. Thus CICR is well developed in the rat and absent in the frog (Fabiato, 1982).

The regulation of force in striated muscle

At the cellular level, there are two basic mechanisms by which contractile force may be influenced in striated muscle. Using the terminology adopted by Jewell (1977) these are; (1) activation factors and (2) physical factors. The general assumption that skeletal muscle cells are fully activated at the peak of the twitch has important consequences when considering the regulation of force in the whole muscle. At constant muscle length, a change in force production can only be achieved by increasing the number of active fibres. In cardiac muscle the situation is quite different as all cells are active during each beat of the heart. Thus as will be considered below, changes in the level of activation generally underlie the regulation of force in cardiac muscle.

Changes in contractile force with length

In both cardiac and skeletal muscle, contractile force is a function of muscle length. The length-tension relationship has been the subject of several

recent reviews (Jewell, 1977, Stephenson & Wendt, 1984, Allen & Kentish, 1985) and shall only be summarised below.

Physical factors underlying the length-tension relationship

One of the fundamental proposals of the sliding filament theory is that the plateau and descending limb of the length tension relationship in intact frog skeletal muscle could be explained by the extent of the interaction between thick and thin filaments, varying simply with overlap (Gordon et al, 1966). This required two basic assumptions; (1) force is produced by independently-acting crossbridges linking actin and myosin filaments and (2) that skeletal muscle is fully activated at the peak of the twitch. Interpretation of the ascending limb (s.l. $<2.0\mu$M) is not adequately explained by this theory. This may be a consequence of the overlap of opposing thin filaments at short muscle lengths and ultimately the interference between the thick filament and the and the Z-line.

Changes in activation with muscle length

It has long been known that cardiac muscle exhibits a much steeper length-tension relationship than skeletal muscle. This occurs despite an apparently similar structure at the level of the myofilament lattice. Studies on intact and skinned preparations suggest that cardiac muscle is only partially activated under physiological conditions (Allen & Kurihara, 1980, Fabiato, 1981a). Thus, it would appear that changes in activation rather than simple

myofilament overlap could underlie the steepness of the length-tension relationship in cardiac muscle.

An increase in the level of activation may occur via an increase in the supply of Ca^{2+} to the myofilaments or an increase in Ca^{2+}-sensitivity. The evidence regarding length-dependent changes in $[Ca^{2+}]_i$ are contradictory. In intact papillary muscles injected with aequorin, a sudden reduction in length caused either no change (Allen & Kurihara, 1982) or a small decrease (Allen & Smith, 1985) in the amplitude of the Ca^{2+}-transient. However, in mechanically-skinned cardiac cells, s.r. Ca^{2+}-release was potentiated by a sudden increase in length (Fabiato, 1980). It would appear that slower increases in contractility following a length change are associated with an increase in $[Ca^{2+}]_i$ (Allen & Kurihara, 1982).

A number of studies have investigated the length dependence of myofilament Ca^{2+}-sensitivity using skinned cardiac trabeculae. The apparent Ca^{2+}-sensitivity was found to increase with s.l. over the ascending and descending limbs of the length-tension relationship (Fabiato & Fabiato, 1978a, Hibberd & Jewell, 1982, Kentish et al, 1986).

Endogenous modulators of Ca^{2+}-sensitivity

As described above, it seems likely that an increase in myofilament Ca^{2+}-sensitivity underlies the steepness of the Frank-Starling relationship. However, in recent years it has become apparent that Ca^{2+}-sensitivity may also be influenced by a number of substances or interventions.

Ca^{2+}-sensitivity and the hydrolysis of ATP

Inorganic phosphate is a metabolite of ATP hydrolysis and depresses Ca^{2+}-sensitivity and C_{max} in chemically-skinned cardiac muscle (Kentish, 1986, Mekhfi & Ventura-Clapier, 1988). Similarly, in mechanically-skinned single cells Ca^{2+}-sensitivity and C_{max} are decreased at low pH (Fabiato & Fabiato, 1978b). NMR studies on Langendorff perfused hearts have demonstrated that under normal physiological conditions there is little change in metabolite concentrations. However, during ischaemia, there is an appreciable rise in P_i and a fall in pH. (for review see Allen & Orchard, 1986). The final consequence of ischaemia is the depletion of ATP and development of a rigor contracture. It is interesting to note that myofilament Ca^{2+}-sensitivity increases at low [ATP] which may offset the depressive effects of P_i and low pH on contractility (Bremel & Weber, 1972, Fabiato & Fabiato, 1975c).

Ionic strength

In chemically-skinned skeletal and cardiac fibres, increasing ionic strength by addition of NaCl or KCl, reduces Ca^{2+}-sensitivity and C_{max} (Kentish, 1984 and Fink et al, 1986). The effect on Ca^{2+}-sensitivity appears to be specific to K^+ and Na^+ as choline chloride salts were without effect on Ca^{2+}-sensitivity. It is likely that the type of inhibition exerted by K^+ and Na^+ can be described in terms of simple competition with Ca^{2+} for specific sites

on the regulatory system (Fink et al, 1986). In contrast, the action of ionic strength is less clear, as will be discussed below.

α and β-receptor stimulation

A number of studies have concluded that cAMP decreases, and cGMP increases, myofilament Ca^{2+}-sensitivity by altering the phosphorylated state of troponin-I. Most results in support of this view were obtained from three types of experiment; (1) ATPase measurements in isolated myofibrillar preparations (e.g. Ray & England, 1976) (2) EGTA-treated trabeculae (e.g. McClellan & Winegrad, 1978) and (3) aequorin-injected papillary muscles (e.g. Allen & Kurihara, 1980). If this is the case, then changes in Ca^{2+}-sensitivity may occur during β-adrenergic stimulation and indeed any other intervention which alters the levels of cyclic nucleotides (e.g. the activity of adenylate cyclase or phosphodiesterase).

However, it should be noted that there is considerable doubt concerning the validity of results obtained using EGTA-treated cardiac preparations (Kentish & Jewell, 1984, Miller & Smith, 1985). Furthermore, it has recently been observed that myofibrillar ATPase measurements may not be correlated with changes in Ca^{2+}-sensitivity in skinned or intact preparations (Smith & England, 1990). Finally, interpretation of aequorin signals is equivocal as cAMP also affects other cellular systems, notably the s.r. (Tada et al, 1974). For a more complete discussion of these points, see chapter 4.

In cardiac muscle, stimulation of α-receptors produces an increase in contractility although this effect is generally smaller than the maximum β-adrenergic response. There is some evidence to suggest that α-adrenergic stimulation is not associated with changes in the levels of cyclic nucleotides (Endoh, 1982). In experiments on aequorin injected rabbit papillary muscles, α-adrenergic stimulation produced changes in the relationship between $[Ca^{2+}]_i$ and tension, consistent with an increase in myofilament Ca^{2+}-sensitivity (Blinks & Endoh, 1986). However, the underlying mechanism remains unclear.

It has been reported that an increase in the intracellular concentration of inositol trisphosphate (IP_3) accompanies α-receptor stimulation (Schmitz et al 1987a). Experiments on skinned skeletal muscle have demonstrated that IP_3 may increase the Ca^{2+}-sensitivity of the contractile proteins and facilitate CICR. (Thieleczek & Heilmeyer, 1986). This has led to the suggestion that an increase in $[IP_3]$ may explain the apparent increase in Ca^{2+}-sensitivity which occurs with α-stimulation (Schmitz et al, 1987b). However, the reported effects of IP_3 on the Ca^{2+}-sensitivity of skinned fibres are not entirely consistent. Other studies on skinned skeletal and cardiac fibres failed to confirm that IP_3 increased myofilament Ca^{2+}-sensitivity (Lea et al, 1986, Isac et al, 1988, Noisek et al, 1986, Fabiato, 1986).

Endogenous imidazoles

Recent work in this laboratory has shown that carnosine and other naturally occurring imidazoles increase the Ca^{2+}-sensitivity of the contractile proteins, Ca^{2+}-uptake by the s.r. and facilitate CICR oscillations in chemically-skinned cardiac muscle (Lamont & Miller, 1990). Imidazoles such as carnosine additionally contribute to the total pH buffer capacity of the cell (Davey, 1960). Although these compounds are not particularly potent, the total cellular concentration may be as high as 20mM (House et al, 1989). While is likely that endogenous imidazoles influence Ca^{2+}-metabolism in intact muscle, their overall physiological importance remains unclear. Preliminary studies have demonstrated that their concentration falls in congestive heart failure (O'Dowd, personal communication). However, it is not clear whether this is a cause or a consequence of the disease process.

Taurine

The amino acid taurine is present in high concentrations (>20mM) in cardiac muscle. Numerous studies have associated taurine with the regulation of Ca^{2+}-metabolism in excitable tissue. The cellular concentration decreases in ischaemia and increases in congestive heart failure (Crass & Lombardi, 1977, Huxtable & Bressler, 1974). The amino acid produces complex changes in the contractility of isolated cardiac preparations which are critically dependent upon the species and $[Ca^{2+}]$ (Franconi et al, 1982). The intracellular action of taurine has been investigated in saponin-skinned

trabeculae (see chapter 8). Taurine causes a small increase in myofilament Ca^{2+}-sensitivity and pronounced increase in ability of the s.r. to accumulate Ca^{2+}.

Synthetic Ca^{2+}-sensitisers

Current treatment for congestive heart failure relies primarily on the use of cardiac glycosides and vasodilators and diuretics. Unfortunately, the cardiac glycosides have a narrow therapeutic window. In common with other interventions which increase $[Ca^{2+}]_i$, glycosides also have a tendency to produce arrhythmias. These problems prompted the search for novel inotropic agents. One attractive inotropic mechanism is to increase the Ca^{2+}-sensitivity of the contractile proteins. This has the theoretical advantage that contractility may be increased without alteration in $[Ca^{2+}]_i$.

Sulmazole (ARL115BS) was the first cardiotonic compound demonstrated to increase Ca^{2+}-sensitivity in skinned cardiac muscle (Herzig, Feile & Rüegg, 1981a, van Meel, 1987, see also chapter 5). Since then a number of compounds have been introduced with similar properties e.g. pimobendan, MCI 154, ORG 30029 (for review see Wetzel & Hauel, 1988). The methylxanthine caffeine was also shown to increase Ca^{2+}-sensitivity in striated muscle (Wendt & Stephenson, 1983).

Mechanisms underlying changes in myofilament Ca^{2+}-sensitivity

The definition of Ca^{2+}-sensitivity used in this discussion is purely functional. In skinned fibre experiments it simply refers to the relationship between $[Ca^{2+}]$ and tension relative to C_{max}. In aequorin-injected papillary muscles the definition is frequently even less precise as changes in Ca^{2+}-sensitivity may be inferred from the relative amplitude of the Ca^{2+}-signal and the tension transient, without reference to C_{max}. To say that two interventions increase Ca^{2+}-sensitivity does not necessarily suggest a similar mechanism of action or even a final influence on the affinity of troponin-C. Indeed, it has been postulated that the pCa-tension relationship could be altered by changing the crossbridge attachment time (Brandt et al, 1982). This mechanism could, in theory, alter the apparent Ca^{2+}-sensitivity without any change in Ca^{2+}-binding. In the following paragraphs I shall consider the possible mechanisms underlying changes in myofilament Ca^{2+}-sensitivity.

Length-dependent changes in Ca^{2+}-sensitivity

Isotopic measurements of Ca^{2+}-binding have demonstrated a length-dependent increase in the affinity of troponin-C in chemically-skinned cardiac muscle (Hofmann & Fuchs, 1987a). However, the underlying mechanism remains obscure. It has been suggested that force production rather than muscle length per se, affects Ca^{2+}-sensitivity (Allen & Kurihara, 1982). In support of this theory, the affinity of troponin-C was found to increase dramatically during the formation of rigor bridges (Bremel &

Weber, 1972). Furthermore, the length dependence of Ca^{2+}-binding was absent if Ca^{2+}-activated force production was inhibited by addition of vanadate (Hofmann & Fuchs, 1987b). However, as discussed by Hibberd & Jewell (1982), this mechanism could not explain the increase in Ca^{2+}-sensitivity observed on the descending limb of the length-tension relationship in skeletal and cardiac muscle (Endo, 1972, Fabiato & Fabiato, 1978a).

An alternative although related theory is that lattice spacing influences the Ca^{2+}-affinity of troponin-C (Hibberd & Jewell, 1982). Until recently this possibility has been considered but rejected, as early studies failed to demonstrate a change in force production when lattice spacing was reduced by osmotic compression with high molecular weight polymers such as PVP (Fabiato, 1978a). However, a number of recent studies have reported that dextran increases Ca^{2+}-sensitivity and C_{max} in chemically-skinned preparations. This effect is present in both skeletal and cardiac muscle and more pronounced at short sarcomere lengths (Stienen et al, 1985, Lamont, 1987, E.L. de Beer et al, 1988a).

X-ray diffraction experiments have demonstrated that lattice spacing is inversely related to s.l. (Matsubara & Elliot, 1972). Furthermore, lattice spacing decreases during rigor and Ca^{2+}-activated force production as a result of lateral forces exerted by the crossbridges (Matsubara et al, 1984). Thus force production and s.l. may influence Ca^{2+}-sensitivity via a common action on lattice spacing (Harrison et al, 1988). In theory, this mechanism could

explain most of the observed results. At constant s.l. an increase in Ca^{2+}-activated force would reduce lattice spacing and increase Ca^{2+}-sensitivity. Thus the Ca^{2+}-binding to troponin would exhibit positive cooperativity. A similar effect could occur during formation of rigor bridges. On the ascending limb of the length-tension relationship, lattice spacing would decrease because of the increase in muscle length and because the force exerted by the crossbridges increases as thin filament overlap is reduced. On the descending limb of the length-tension relationship, lattice spacing would continue to decrease explaining the increase in Ca^{2+}-sensitivity, despite a reduction in filament overlap.

Ionic strength and pH

It is interesting at this point to consider how other interventions which influence Ca^{2+}-sensitivity and C_{max} relate to this simple theory.

In striated muscle, repulsive forces between fixed negative charges located on the thick and thin filaments are an important factor determining lattice spacing (Rome, 1967). Consequently, an increase in ionic strength or $[H^+]$ would be expected to shield the negative charges thereby reducing the repulsive forces and lattice spacing. This has been confirmed by X-ray diffraction studies on skinned skeletal muscle (April, Brandt & Elliot, 1972). However, this is a paradox as a decrease in lattice spacing alone would be expected to increase Ca^{2+}-sensitivity and C_{max}.

One possible explanation for this has been provided by Matsuda & Podolsky (1986), again using X-ray diffraction. It was discovered that the intensity of the 1,1 reflection was reversibly influenced by pH and ionic strength. The 1,1 reflection is highly dependent upon the presence of the thin filament in the trigonal position. It was concluded that at high ionic strength and $[H^+]$, associated with the screening of the fixed charges, thin filaments move away from the trigonal positions. However, as described above, lattice spacing is also reduced under these conditions. To distinguish between these effects, osmotic compression of the lattice with dextran was studied. It was found that the equatorial reflections were unaffected by lattice spacing alone. Thus it would appear that factors which influence the negative charges on the lattice may influence force primarily by disrupting the thin filament arrangement. Presumably this inhibitory action outweighs the opposing effect on lattice spacing. It is likely that low pH has additional actions on force production. It is known that low pH reduces Ca^{2+}-binding by a direct effect on troponin-C (Kentish & Palmer, 1989).

Caffeine and related compounds

The mechanism of action underlying the increase in Ca^{2+}-sensitivity with caffeine or other synthetic compounds is unclear. For the purposes of this discussion it will be assumed that the action of endogenous compounds such as carnosine is via a similar mechanism. There is some justification in

this as the imidazole ring structure is common to most (but not all) sensitisers.

An increase in Ca^{2+}-binding to troponin-C has been demonstrated with sulmazole and pimobendan in chemically-skinned cardiac muscle (Solaro & Rüegg, 1982, Fujino et al, 1989). The most simple explanation is that these substances act directly on troponin-C to increase the Ca^{2+}-sensitivity of the regulatory site. Jaquet & Heilmeyer (1987) investigated the effect of a various Ca^{2+}-sensitising cardiotonics on the Ca^{2+}-binding properties of isolated cardiac troponin-C. It was found that the Ca^{2+}-bound by troponin-C decreased as the concentration or troponin increased. This was apparently due to the tendency of cardiac troponin-C to self-associate. Although some of the compounds did influence Ca^{2+}-binding, the direction of the effect was dependent upon the extent of self-association. The results could not be correlated with the action of these compounds on skinned muscle preparations. Indeed pimobendan, a potent Ca^{2+}-sensitiser in skinned muscle decreased the affinity of isolated troponin-C for Ca^{2+}. In contrast UDCG212Cl, the principle metabolite of pimobendan, has no effect on the Ca^{2+}-sensitivity of skinned muscle (personal observation $n>10$) but apparently produced a large increase in the Ca^{2+}-bound by isolated troponin-C. It was further suggested that the unique tendency of cardiac troponin-C to self associate may explain why the drugs affect the contractility of cardiac but not skeletal muscle preparations. However, caffeine, sulmazole, pimobendan and carnosine do increase the apparent Ca^{2+}-sensitivity of chemically-skinned skeletal muscle fibres (Wendt

& Stephenson, 1983, Piazzesi et al, 1987, Lamont & Miller, 1990). In unrelated experiments, Kentish & Palmer (1989) observed that Ca^{2+}-binding to cardiac troponin-C was unaffected by caffeine. Taken together these studies provide little evidence that such compounds influence myofilament Ca^{2+}-sensitivity by a direct action on troponin-C. It is possible that caffeine-like substances require the presence of other myofibrillar proteins, such as troponin-I to exert their Ca^{2+}-sensitising effect.

An alternative theory is that, like s.l., the increase in Ca^{2+}-sensitivity is associated with changes in lattice spacing. To take caffeine for example, there is no apparent reason why lattice spacing should change on addition of the drug. Caffeine is uncharged at neutral pH and thus should not influence lattice spacing or the thin filament structures in the same manner as ionic strength or pH. However, it was recently reported that addition of caffeine (10mM) causes swelling of the actin-myosin matrix in chemically-skinned cardiac muscle (de Beer et al, 1988a). This was supported by X-ray diffraction experiments in skinned skeletal muscle fibres which demonstrated an increase in lattice spacing with caffeine (de Beer et al 1989). Furthermore, de Beer et al (1988a) reported that caffeine was potent at short muscle lengths and ineffective at long s.l.s in freeze dried skeletal and cardiac fibres. These experiments provide evidence that caffeine may indeed act via changes in lattice-spacing.

There are, however, a number of problems with this interpretation; (1) The increase in lattice spacing with caffeine would be expected to

decrease Ca^{2+}-sensitivity and C_{max} (as with dextran-see above). (2) The negative charge on lattice measured with a microelectrode was found to decrease with caffeine in similar preparations (G. F. Elliot Personal Communication). The direction of this change is contrary to that expected from the work of de Beer et al. (3) The action of caffeine or sulmazole was not found to be length dependent in chemically-skinned cardiac muscle (unpublished observations and Kentish-personal communication). (4) The shift in the pCa-tension relationship with caffeine or sulmazole was unaffected by osmotic reduction in lattice spacing with dextran (C. Lamont, D.Steele unpublished observations).

Inorganic Phosphate

Again, there is no reason to suspect that the depression in C_{max} and Ca^{2+}-sensitivity with P_i is mediated via changes in lattice spacing. Furthermore, P_i has no effect on Ca^{2+}-binding to isolated bovine troponin-C (Kentish & Palmer, 1989). It has been suggested that its action is associated with the inhibition of P_i release subsequent to ATP hydrolysis. This effect is considered in more detail in chapter 7.

In Conclusion

This discussion illustrates the potential importance of changes in Ca^{2+}-sensitivity in the regulation of contractile force in the heart. However, interventions which produce an apparently similar effect on force may do so

by entirely different mechanisms. Despite considerable research efforts, the mechanism by which caffeine and similar compounds increase Ca^{2+}-sensitivity remains unclear. Perhaps the most interesting observations, despite their apparent contradiction, are those regarding lattice spacing and charge. Some of the discrepancies may result from the dual action of caffeine on Ca^{2+}-sensitivity and C_{max}. Caffeine (>10mM) decreases C_{max} while Ca^{2+}-sensitivity continues to increase (see chapter 5). In contrast, all other reported Ca^{2+}-sensitising compounds either increase C_{max} or have no effect. It is possible that the increase in lattice spacing with caffeine may explain the decrease in C_{max} observed at higher concentrations. Further X-ray diffraction studies, comparing the actions of caffeine and other Ca^{2+}-sensitisers which potentiate C_{max}, may yield valuable information regarding the mechanisms of action.

This discussion has been limited to the Ca^{2+}-sensitivity of the contractile proteins without reference to other important Ca^{2+}-regulatory systems such as the s.r. The work which comprises this thesis has involved the characterisation of natural and synthetic compounds which influence the Ca^{2+}-sensitivity of the contractile proteins. It will be demonstrated that many substances which influence myofilament Ca^{2+}-sensitivity also affect the s.r.

Chapter 2: **Materials and Methods**

General protocol

Male Wistar rats (200-230g) were killed by concussion and cervical dislocation. The heart was rapidly removed and flushed with Ringer solution comprising: NaCl 150mM, KCl 5mM, $MgCl_2$ 1mM, $CaCl_2$ 2mM, HEPES 5mM and glucose 10mM brought to pH7.0 with NaOH.

The atria were removed and the right ventricle opened by a single cut close to the interventricular septum. Most experiments were carried out on free running trabeculae (length 1-3mm diameter 70-120μm) isolated from the base of the right ventricle near the valve. Occasionally, small papillary muscles were used. Although these tended to be of less uniform dimensions, they behaved in a qualitatively similar manner to trabeculae. The preparations were mounted for isometric tension recording prior to chemical skinning. All experiments were carried out at room temperature (20-24°C).

Preparation mounting and force measurement

One end of the preparation was suspended between an Akers AE 857 transducer and a fixed point using snares (figure 2.1a). The transducer was encased in a brass tube (length 12cm, outside diameter 1cm) to reduce the fluctuations in temperature and light intensity to which the transducer is sensitive. The snare holders were made of stainless steel tubing, outside diameter 200μM, inside diameter 100μM (Goodfellows Metals Ltd., Cambridge). Lengths of tubing (3-4mm) were double threaded with nylon monofilament (diameter 25μM) to produce a snare at the bottom of the tube

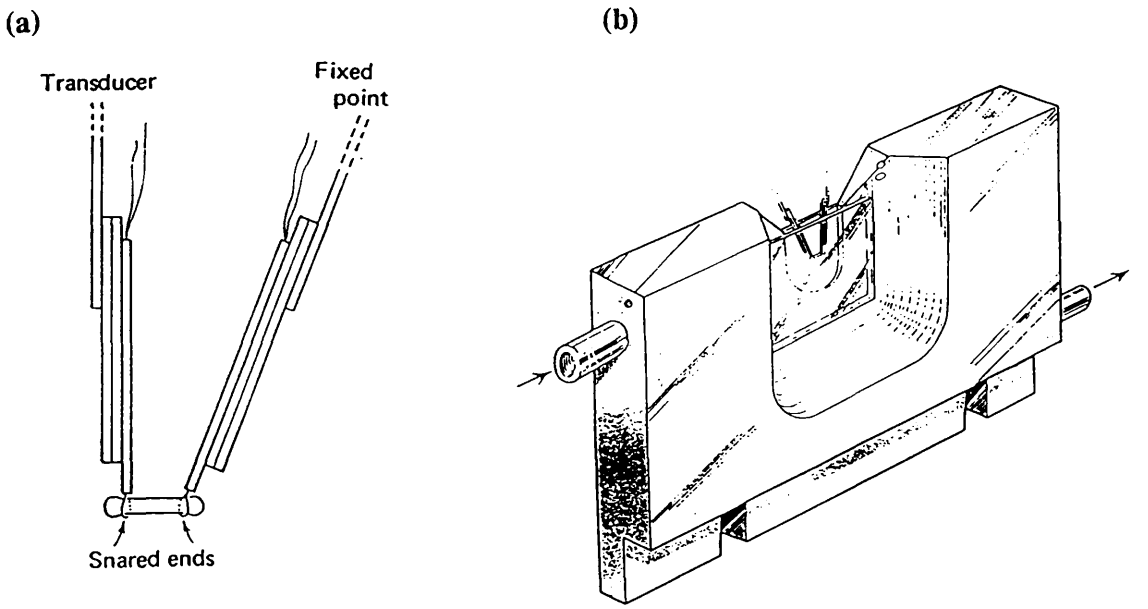


Figure 2.1 Panel (a) shows a schematic diagram of the muscle mounting system. The trabecula (100μm dia., 2-3mm long) is held by snares (nylon monofilament, 30μm dia.) running in stainless steel tubes (200μm outside dia., 100μm inside dia.). The tubes are glued to extensions from the force transducer and a fixed end. The separation between the preparation ends is adjusted by means of a micromanipulator. The whole assembly is mounted on a magnetic base to permit transfer from the dissection microscope to the bath-change system and the DIC microscope. Panel (b) shows a schematic view of the chamber used to determine and permit adjustment of s.l. The bath (vol. 0.3ml) is created by two standard coverslips approximately 2mm apart. This permits access from above for the preparation mounting assembly. The temperature of the chamber can be controlled by flushing the brass housing.

which could be tightened by pulling from the top. One snare holder was attached to the fixed point and the other to the extended transducer with Superglue3 (Loctite). Three additional pieces of tubing were glued to the side of the snares to provide extra rigidity.

The compliance of the transducer was measured by applying weights to the end of its arm in the plane of use. The displacement was measured using a microscope with a calibrated eyepiece. The compliance was found to be equivalent to a length change of less than 1% assuming a muscle length of 2mm and a force of 60mgwt.

The transducer and fixed point were mounted on a Narashige MM3 micromanipulator allowing fine movement in three planes. This was necessary to enable positioning of the muscle in the microscope chamber and subsequent adjustment of s.l.

The microscope system and measurement of sarcomere length

Accurate measurement of s.l. is important as a number of physiological parameters are dependent on length in striated muscle. In particular, the Ca^{2+}-sensitivity of the myofilaments increases with s.l. (Hibberd & Jewell, 1982). Although a matter of some controversy, s.r. Ca^{2+}-release may also increase with muscle length (Stephenson & Wendt, 1984).

Sarcomere length was measured by Differential Interference Contrast (DIC) light microscopy. This method was used in preference to the laser diffraction system employed in most other laboratories. Direct observation

allows local variations in s.l. to be observed, and enables the extent of damage to be assessed. In practice, fine adjustments of the snares were carried out at this point. In most preparations, the sarcomere pattern was maintained although distorted in the region of contact with the snares, indicating relatively little damage.

The diameter of the trabecula could be measured in two perpendicular planes, either directly from a calibrated eyepiece graticule or by focusing through the muscle and reading the distance from the scale on the microscope stage.

The microscope was a modified Vickers M-17, using the DIC system described by Smith (1969). The microscope was positioned horizontally to allow access to the space between the objective lens and the condenser. The microscope stage was replaced by the brass chamber illustrated in the figure 2.1b.

The solutions in the chamber (volume 0.3 ml) could be changed via two fine cannulae with attached syringes. The cannulae were glued round the metal edge of the bath terminating at the lowest point. This arrangement, together with the vaseline used to secure the glass coverslips forming the two sides of the chamber, prevented the solutions from coming into contact with the metal bath and any contamination that may result. The muscle was viewed on a monochrome monitor via a video camera (Panasonic) which further enhanced the contrast of the DIC image.

The s.l. was checked immediately after skinning and at intervals throughout the experiment. Changes in the resting s.l. rarely occurred although it is generally accepted that internal shortening does occur in preparations of this type during activation (Kentish et al, 1986). The microscope graticule was calibrated with diffraction gratings, viewed at the same magnification and position as the muscle.

Solution exchange

Many of the experiments described in this thesis require the duration of exposure to experimental solutions to be accurately controlled. Consequently, a computer-controlled solution exchange system was used. This has been described in detail elsewhere (Miller et al, 1982) and shall be described only briefly below.

The bath comprised a series of wells (5ml) in a perspex block. To exchange solutions the bath was lowered, moved horizontally and raised under the preparation. The bath was moved by two stepper motors controlled by an Apple IIe microcomputer. The speed of the stepper motors could be increased to produce a solution change in less than 200ms. This system and accompanying software allowed large numbers of accurately-timed solution changes to be programmed in advance. Solutions were continuously stirred with a small stainless steel paddle.

Chemical supplies

The chemicals and suppliers are listed in table 2.1 In most experiments disodium ATP and CrP were used as they are more stable than potassium or Tris salts commercially available. It has been demonstrated that in these preparations, mitochondrial Ca^{2+}-release is Na-sensitive (Harrison & Miller, 1984). Some experiments were carried out with physiological Na-levels to investigate the possible involvement of the mitochondria. In these experiments $Tris_2CrP$ and K_2ATP were used, also from Sigma.

Solution Composition

Stock solutions, comprising 1M KCl, 100mM $MgCl_2$, EGTA, CaEGTA, HDTA and 500mM HEPES were prepared on a regular basis. The final experimental solutions were made by mixing appropriate volumes of these stock solutions.

The EGTA and HDTA were made by dissolving the acids in twice the molar amount of KOH. The amount of EGTA added was calculated with reference to recent reports regarding the purity of EGTA obtained from Sigma (Bers, 1982, Miller & Smith, 1984) Solutions were then heated (60°C) and stirred vigorously until all the EGTA was dissolved before addition of distilled H_2O to the desired end volume. Ca_2EGTA was prepared in a similar manner but equimolar $CaCO_3$ was added with the EGTA and KOH. CO_2 is driven off leaving Ca_2EGTA. Finally, ATP and CrP were added as solids

Table 2.1

Compound	Supplier
ATP	Sigma Chemicals, Poole, Dorset, England.
CrP	"
EGTA	"
cAMP	"
cGMP	"
Saponin	"
Caffeine	"
Taurine	"
Papaverine	"
HEPES	"
HDTA	Fluka, Derbyshire, England.
Triton-X100	Pierce, Rockford, Illinois,USA.
KCl	BDH Chemicals, Poole, England.
NaCl	"
KOH	"
NaOH	"
$CaCl_2$	"
$MgCl_2$	"
NaN_3	"
Sucrose	Formachem, Strathaven, Scotland.
Glucose	"
Forskolin toxin	Calbiochem, California,USA.
Sulmazole	Dr. Karl Thomae GmbH. W. Germany.
Pimobendan	"
ORG 30029	Organon Laboratories, Newhouse, Scotland.

before the solution was adjusted to pH_a 7.0 with KOH. The composition of the three basic solutions is shown in Table 2.2.

A range of $[Ca^{2+}]$ was produced by mixing '10Activating' and '10Relaxing' solutions in various ratios. A rapid increase in $[Ca^{2+}]$, across the whole preparation radius, could be produced by moving the preparation from the '0.2Relaxing' solution (0.2 mM $EGTA_{total}$) to an activating solution with a higher Ca^{2+}-buffer capacity (10 mM $EGTA_{total}$). Ionic balance was maintained with HDTA. This 'calcium jump' technique has been described in detail elsewhere (Moisescu, 1976, Ashley & Moisescu, 1977).

In some experiments, the $[Ca^{2+}]$ of the '0.2Relaxing' solution was increased by addition of $CaCl_2$. As the EGTA in the '0.2Relaxing' solution is not saturated, addition of Ca^{2+} results in the net release of approximately 2 Hydrogen ions per Ca^{2+}-bound at pH 7.0 (Smith & Miller, 1985). Consequently, the appropriate amount of KOH was added to maintain the solution at pH 7.0.

pH

Many factors of relevance to this study are known to be influenced by pH. The Ca^{2+}-sensitivity of the contractile proteins and maximum Ca^{2+}-activated tension (C_{max}) decreases with pH. The ability of the s.r. to accumulate Ca^{2+} is reduced at low pH (Fabiato & Fabiato, 1978b). Furthermore, the apparent binding constants of EGTA and ATP for Ca^{2+} and Mg^{2+} are highly pH sensitive.

Table 2.2

Solution	10Activating	10Relaxing	0.2Relaxing
KCl	100.0	100.0	100.0
$MgCl_2$	7.0	7.0	7.0
EGTA	-	10.0	0.2
HDTA	-	-	9.8
CaEGTA	10	-	-
Na_2ATP	5.0	5.0	5.0
Na_2CrP	15.0	15.0	15.0
HEPES	25.0	25.0	25.0
KOH	15.0	15.0	15.0

All concentrations mM

In order to minimise changes in pH during contraction the pH-buffer HEPES (25mM) was included in all solutions. The useful buffering range of HEPES is pH 6.5-7.5. The experiments described in chapter 7 were designed to investigate the effects of low pH on the development of rigor tension. In these experiments, citrate (25mM) was included in all solutions. citrate buffers pH effectively in the range pH 5.5-6.5.

Illingworth (1981) reported that liquid junction artefacts are a common source of errors in the measurement of pH. Ceramic plugs which are a feature of combination electrodes are the source of these errors. In this study, separate reference (Corning 003116029) and pH electrodes (Corning 00311101J) were used. This method gives a much more reliable measure of pH (Illingworth, 1981)

In practice, the pH of the experimental solutions was adjusted using a null point method. A standard solution was prepared which was calculated to have the same pH_a (7.0) and ionic strength (0.2M) as the solutions. The electrodes were allowed to equilibrate in the pH-standard and then all other solutions were adjusted to the same pH by the addition of KOH. This method should minimise the artefacts outlined by Illingworth, even if the electrodes are not functioning perfectly. For details of the composition of the pH standard solution see (Harrison et al, 1988).

Ionic strength

For the purposes of this study, the ionic strength (I) has been defined according to the equation shown below. It has been reported that this gives a better description of the experimental solutions than more commonly used definitions of I. For a more complete discussion see Miller & Smith (1985) and Smith (1983). The ionic strength is defined as the total of the ionic equivalents where c_j is the concentration of the jth ionic species and z_j is its valency.

$$I_e = \frac{1}{2}\Sigma C_j z_j$$

One important consequence of this is that the zwitterionic forms of HEPES and taurine (which are predominant at pH_a 7.0) are assumed to make no contribution to the ionic strength of the solutions. The solutions outlined in Table 2.2 have a calculated I_e of 0.19M.

Calculation of free metal concentrations

The calculation of the free metal concentrations in the experimental solutions is not a trivial consideration. EGTA binds metal ions other than Ca^{2+} to various degrees. The affinity constants of EGTA and the other ligands (ATP, CrP and HEPES) must be known in order to calculate free metal concentrations. Furthermore, the affinity constants are influenced by experimental variables such as pH, temperature and ionic strength. Thus, metal ion concentrations must be calculated for specific experimental conditions. Of primary importance to this study is the calculation of the free

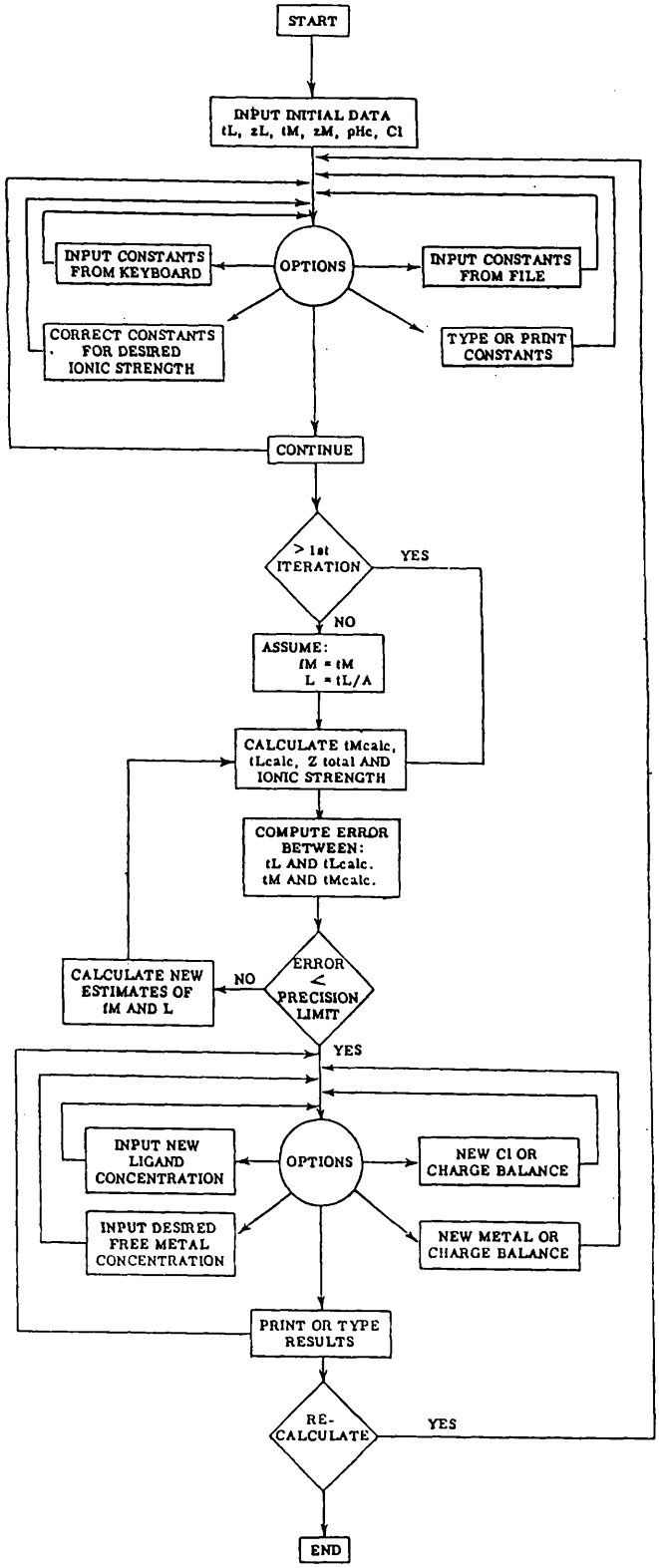


Figure 2.2 shows a flow diagram of the REACT program used to calculate the free metal ions in all the solutions used in this thesis (after Smith, 1983).

[Ca^{2+}], although changes in the concentration of other ionic species must be borne in mind. For example, an increase in the [Mg] has effects both on the s.r. and the contractile proteins (Fabiato & Fabiato, 1975c).

A computer program (REACT) written by G.L.Smith and D.J.Miller was used to calculate the free [Ca^{2+}] of all the solutions used in this study. A flow diagram is shown in figure 2.2. The affinities of EGTA and the other ligands for Ca^{2+} and the other metal ions have been incorporated. REACT can provide a complete profile of the free metal ion concentrations and ligand- metal concentrations in the experimental solutions. For details of binding constants and corrections for pH, ionic strength and temperature, see Smith & Miller, (1985).

In this study a range of [Ca^{2+}]s was produced by mixing '10Activating' and '10Relaxing' solutions. Each preparation was exposed to a range of [Ca^{2+}]'s to define the relationship between Ca^{2+} and tension. The free metal concentrations of the basic solutions (see table 2.2) and various commonly used ratios are given in table 2.3.

The chemical skinning procedure

The mounted trabecula was exposed to a '0.2Relaxing' solution containing either saponin (50μg/ml) or Triton-X100 (1% v/v) for 30 minutes. The chemical skinning agent was then removed by washing the muscle in fresh solution before proceeding with the experiment.

Table 2.3

Ratio 10A:10R	pCa
1:1	6.22
3:1	5.74
5:1	5.52
8:1	5.31
10:1	5.22
15:1	5.04
25:1	4.83
10Activating	4.19
10Relaxing	8.92
0.2Relaxing	7.18

I=0.19M
K^+=0.14M
Na^+=0.04M
Mg^{2+}=0.007M
Cl^-=0.114M
pH_a=7.0
T=20°C

Saponin is a glycoside of plant-origin which has been extensively used throughout this study to skin cardiac trabeculae chemically. This form of treatment was first described by Endo & Kitazawa (1978). Saponin's action is Ca^{2+}-sensitive. At normal extracellular $[Ca^{2+}]$ the glycoside causes a reversible positive inotropic effect which is associated with an increase in $[Na]_i$. However, if the $[Ca^{2+}]_o$ is decreased to intracellular levels, saponin combines with cholesterol in the surface membrane, causing irreversible perforations (Noireaud, et al, 1989). As the s.r. has a much lower cholesterol content than the surface membrane, the cells are rendered permeable while functionally retaining the contractile proteins and intracellular organelles (Endo & Kitazawa, 1978). In this manner, 'selectively' skinned multicellular preparations can be prepared with relatively little difficulty.

Treatment of the muscle with Triton X-100 effectively destroys all cellular membranes leaving the contractile proteins functional (Heleius & Simons, 1975). This method has an advantage over saponin treatment under some circumstances. Any intervention which influences the contractile response of a Triton-skinned muscle can confidently be assumed to act at the level of the myofilaments without the influence of other cellular systems. On the other hand, Triton-treatment will inevitably result in the loss of cellular emzymes which may be retained in the saponin-skinned state. Furthermore, it is well known that after skinning, the myofilament lattice of the muscle cell swells and no longer exhibits constant volume behaviour (Godt & Maughan, 1977). The degree of swelling is believed to be considerably less in the

saponin-skinned state than after Triton-treatment (G Elliot, personal communication)

Thus results obtained from each method must be viewed with reference to these considerations. However, with both treatments the principle is the same; after skinning the bathing solution becomes an extension of the intracellular environment. In this manner, ionic conditions of intracellular organelles can be manipulated simply by changing solution. Such preparations are ideal for investigating the functioning of the intracellular systems. For reference, figure 2.3 shows a schematic representation of a preparation after saponin and Triton skinning.

Data Handling

The output of the tension transducer (pre-amplified & filtered at 25Hz) was displayed on a chart recorder (Linseis 1800) and simultaneously digitised. During this study a number of digitising methods have been employed. The first method involved digitising the signal at an appropriate rate (50-200 ms per sample) and storing the data on a Digital PDP-11 34 computer for later analysis. This was superseded by an Apple Macintosh, with Mac Lab and appropriate software. However, this proved unsatisfactory as files longer than 750Kb could not be accommodated. As the experiments could on occasion last for 8-10 hours, it was necessary to split the data into a large number of files and transfer these later from the hard disc to floppy discs. While this was possible, it was by the nature of the experiments,

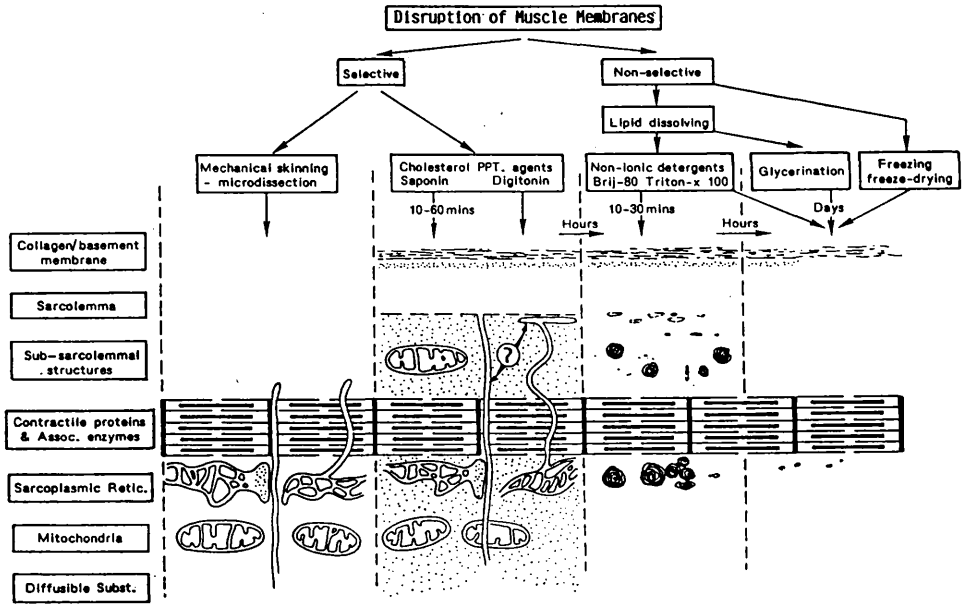


Figure 2.3 shows a schematic representation of the techniques commonly used to disrupt muscle membranes thereby rendering cells permeable to the bathing fluid. Of particular relevance to this study is the cholesterol-precipitating agent saponin and the non-ionic detergent Triton-X100.

inconvenient. The final and most satisfactory solution was to record the signal on videotape via an A/D VCR adapter (PCM 4/8, Medical systems corp. Greenvale, N,Y.) and domestic videorecorder. In effect the signal was recorded (±10V) at a much higher rate than necessary. At a later stage the relevant information was replayed back through the chart recorder or more commonly, re-digitised at an appropriate rate on to the Mac Lab system. At this stage the signal could be was transferred from the Mac Lab to a graphics package such as MacDraw II. Within MacDraw, the signal can quickly be scaled and annotated before obtaining a hard copy via an Apple Laser writer.

Data analysis

It is well known that the chemically-skinned cardiac preparations deteriorate with time (Jewell & Kentish, 1981). At the beginning of this study the rate of deterioration was rather variable and some preparations were rejected on this basis. In later experiments, the rate of deterioration was consistently small (0-5% per hour). The only experimental variable knowingly changed was the length of exposure to the '10Relaxing' solution, which was kept to a minimum. Instead, between contractures and hence for the majority of the time, the muscle was bathed in '0.2Relaxing' solution. It is not clear whether it is the low [Ca^{2+}] or the high [EGTA] itself which increases the rate of deterioration in '10Relaxing' solutions. However, it has been noted that prolonged exposure to a high [EGTA] (and low [Ca^{2+}]) causes the

myofilament lattice to become disorganised in skeletal muscle (G Elliot, personal communication). The underlying mechanism remains obscure.

The pCa-tension relationship was defined by exposing preparations to a range of $[Ca^{2+}]$. The general protocol involved maximally-activating the muscle before and after intermediate levels of Ca^{2+} were applied. Thus, in preparations which exhibited significant deterioration, the tension responses were normalised with respect to a corrected value of C_{max}. It was assumed that the rate of decline of C_{max} is linear with respect to time (Harrison, 1985).

Some experiments involved evoking trains of caffeine contractures in saponin-skinned trabeculae. In these experiments, the trabeculae rarely exhibited any deterioration within the time scale required to carry out an experimental intervention (20-30min).

The Hill Equation.

The relationship between Ca^{2+} and tension is described adequately by the Hill equation where $\mathbf{C_{max}}$ is the force at a saturating calcium concentration; $\mathbf{C_x}$ is the force at the actual calcium concentration $\mathbf{[Ca_x]}$; $\mathbf{EC_{50}}$ is the calcium concentration giving 50% of $\mathbf{C_{max}}$ and **n** is the Hill coefficient.

$$\frac{C_x}{C_{max}} = \frac{[Ca_x]^n}{EC_{50}^n + [Ca_x]^n}$$

The Hill coefficient is generally considered to be a measure of the cooperativity of Ca^{2+}-binding to the regulatory sites. While Ca^{2+}-binding to isolated troponin exhibits no cooperativity (ie n=1), Ca^{2+}-binding to skinned

cardiac muscle is highly cooperative (for a more complete discussion and references see Rüegg, 1986a). Indeed Hill coefficients greater than 4 have been measured (Kentish et al, 1986). The mechanism underlying cooperativity is poorly understood. Kentish et al (1986) reported that internal shortening in multicellular preparations causes an artifactual reduction in the measured Hill coefficient. As a result of these potential problems, no conclusions have been drawn regarding small changes in **n** which occur after various interventions. For a more complete discussion of these points see chapter 6.

Chapter 3: **Caffeine contractures in saponin-treated trabeculae**

Protocol for investigating s.r. Ca^{2+}-content with caffeine

The work which comprises this thesis has involved the use of caffeine to investigate factors which influence the ability of the s.r. to accumulate Ca^{2+}.

The protocol for these experiments is illustrated in figure 3.1a. A train of contractures was produced in response to caffeine (10mM) in a selectively (saponin) skinned trabecula. Between contractures, the muscle was exposed to a $[Ca^{2+}]$ subthreshold for tension production. During this period the s.r. can accumulate Ca^{2+} which is subsequently released by caffeine. All solutions were weakly Ca^{2+}-buffered (0.2mM $EGTA_{total}$) so that Ca^{2+} released from the s.r. could activate the myofilaments. The diagram illustrates that the magnitude of the contracture is dependent upon both the $[Ca^{2+}]$ in the preceding loading solution and the duration of loading. The time course of sample contractures is shown in panel 3.1b. Typically the contractures develop fully within 0.25s and relax in 1-2 seconds. The $[Ca^{2+}]$ in the bathing solution was routinely adjusted to initiate responses of 15-30% maximum Ca^{2+}-activated tension with a loading period of 1 min. The trains of contractures are very reproducible over prolonged periods (>1 hour) without significant deterioration.

Although it has been known for over a hundred years that caffeine can induce contractures in cardiac muscle, (Albers, 1851) its precise mechanism of action remains controversial. It is generally accepted that

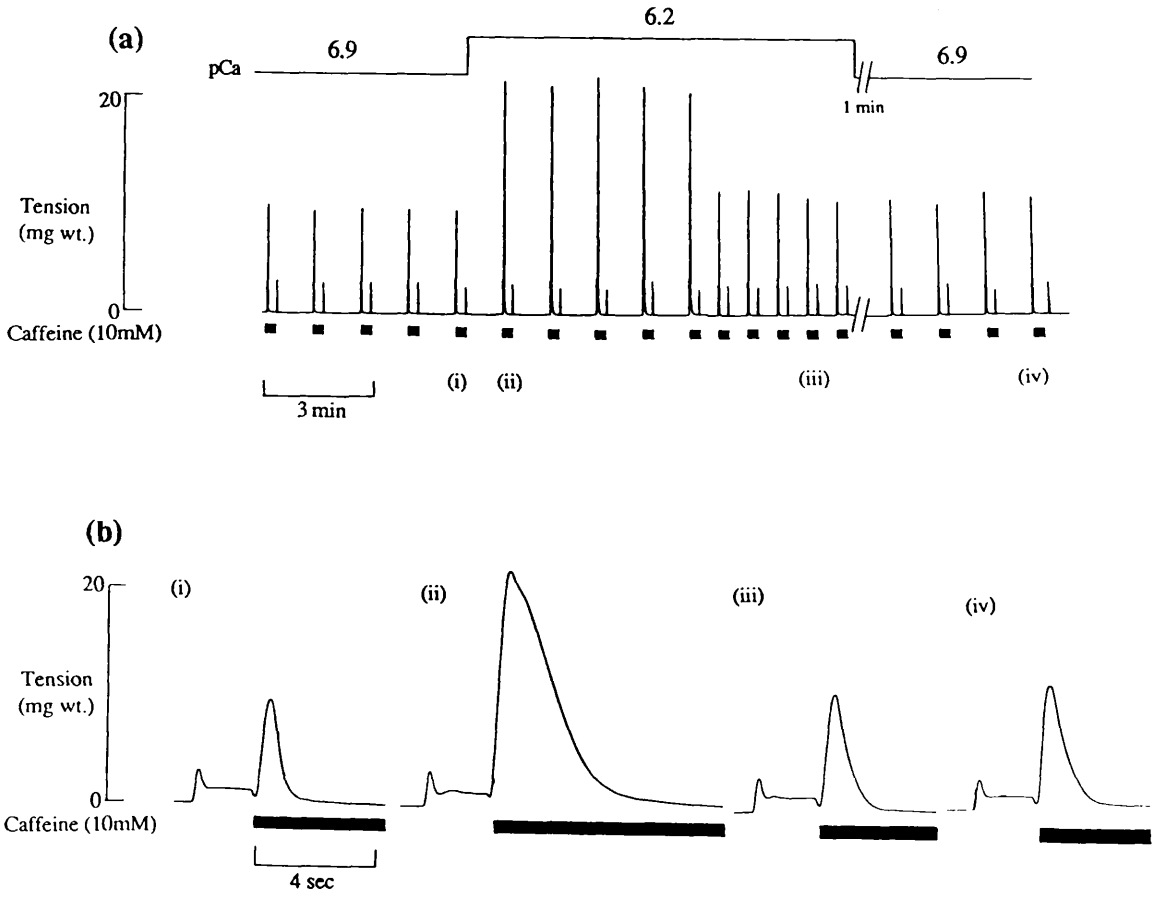


Figure 3.1 The effect of $[Ca^{2+}]$ and the duration of loading on the caffeine-induced contractures from a saponin-treated rat trabecula is shown. Each large upstroke is the caffeine-induced response, each small upstroke is the solution change artefact. Panel (a) shows a continuous record of isometric tension. 10mM caffeine was added (as indicated by the bar below the trace) for 15 sec. after a 60 sec. incubation in caffeine-free solution. As indicated by the bar above the trace, increasing the $[Ca^{2+}]$ from pCa 6.9 to pCa 6.3 potentiated the caffeine response. After several contractures the duration of exposure to the Caffeine-free solution was reduced to 30 sec. which reduced the amplitude of the contracture. Panel (b) shows example caffeine contractures selected from panel (a) on an expanded time scale. The solution-change artefact (during which time the preparation is in air) precedes the response initiated by caffeine.

caffeine releases Ca^{2+} from the s.r. (Weber & Herz, 1968). However, it has recently been questioned whether caffeine-induced contractures in intact cardiac muscle are the consequence of s.r. Ca^{2+}-release (Clusin, 1985). Some understanding of the initiation and relaxation of the caffeine contractures (figure 3.1) is necessary for the interpretation of the results described in the following chapters. Thus, I will briefly consider the reported effects of caffeine on a variety of different cardiac preparations.

Effects of caffeine on intact tissue

Many studies have investigated the effect of caffeine on the contractility of isolated intact cardiac preparations. Caffeine can be either positively or negatively inotropic depending on the species and preparation used. The results appear rather inconsistent. In some cases caffeine induced an initial rapid positive inotropic effect or a contracture, followed by an inhibition of force (Konishi et al, 1984, Hess & Wier, 1984, Chapman & Miller 1974a, Chapman & Léoty, 1976). Blinks et al (1972) found that millimolar concentrations of caffeine caused an increase in contractility in kitten papillary muscle but inhibited force at high concentrations. Alternatively, caffeine can simply induce a dose-dependent increase in contractility (e.g. Kimoto, 1972, Clark & Olson, 1973).

It has been suggested that both positive and negative inotropic effects may result from an inhibition of net Ca^{2+}-uptake, either by initiating release and/or by direct inhibition of the Ca^{2+}-uptake mechanism (Blinks et al 1972,

Chapman and Léoty 1976, Kitazawa, 1988, Hess & Wier, 1984). Blinks et al (1972) proposed that inhibition of net Ca^{2+}-uptake may result in a higher diastolic [Ca^{2+}] thereby facilitating the following twitch. The inhibition of Ca^{2+}-uptake is consistent with the finding that caffeine prolongs the time to peak tension and slows the relaxation of the twitch. This leads to an apparent paradox since inhibition of uptake would be expected to deplete the s.r. of Ca^{2+} and leave less available for subsequent CICR. Indeed this mechanism has been proposed to explain the negative inotropic effect seen in some preparations (Wier & Hess, 1984).

There is known to be considerable species variation in the contribution of s.r.-derived Ca^{2+} to the pool of cytosolic Ca^{2+} which initiates the twitch (Fabiato, 1982). This may contribute to the variation in response between species if caffeine inhibits net Ca^{2+}-uptake. However, it implies that if a maintained positive inotropic effect is observed, s.r.-derived Ca^{2+} does not contribute significantly to contraction and caffeine must increase contractility by an s.r.-independent mechanism. Indeed, it has been demonstrated that caffeine increases the Ca^{2+}-sensitivity of the contractile proteins and inhibits phosphodiesterase (Wendt and Stephenson, 1983, Kitazawa, 1988, Butcher and Sutherland, 1962). These mechanisms may underlie the positive inotropic effect seen in some intact preparations. Alternatively, enhancement of s.r.-calcium release, which appears to be important in skeletal muscle, may also occur in cardiac muscle (Miller 1973, Endo, 1977).

Experiments on isolated s.r.

Experiments on isolated skeletal s.r. provided a direct method of studying the action of caffeine on net Ca^{2+}-uptake. In most cases application of caffeine induced a transient release of Ca^{2+} which was followed by net Ca^{2+}-uptake (Thorpe & Seeman, 1971, Katz et al 1977, Miyamoto & Racker 1981, Su & Hasselbach, 1984), although reaccumulation of released Ca^{2+} was not always reported (Weber & Herz, 1968, Johnson & Inesi, 1969). The reasons for these discrepancies remain unclear.

Experiments on selectively skinned cardiac muscle

In isolated myocyte suspensions treated with digitonin, the caffeine-like compound sulmazole initiated a transient Ca^{2+}-release, followed by net Ca^{2+}-uptake as detected by Ca^{2+}-sensitive micro electrodes (Fry et al 1989). However, sulmazole is less potent than caffeine in its ability to induce Ca^{2+}- release in saponin skinned trabeculae (see chapter 5). This may explain why net Ca^{2+}-accumulation continued in the presence of the compound.

In saponin-treated guinea-pig trabeculae, the action of caffeine was found to be dependent upon the buffer capacity of the solutions. In the presence of high concentrations of EGTA, caffeine initiated a release of Ca^{2+} and the s.r. Ca^{2+} content was permanently reduced in the presence of the drug. In contrast, if the Ca^{2+} was weakly buffered, caffeine caused a much

larger release which was followed by some reaccumulation of Ca^{2+}, as seen in isolated s.r. (Kitazawa, 1988).

The effect of caffeine on isolated s.r. channels.

Single Ca^{2+}-channels have been isolated from skeletal and cardiac muscle s.r. and incorporated into lipid bylayers. In these preparations caffeine activates the s.r. Ca^{2+}-channel; no inactivation is seen in the presence of the drug (Rousseau & Meissner 1989, Sitsapesan & Williams 1989). However, these results should be assessed with some caution, as the channel is similarly activated by Ca^{2+} and also demonstrates no inactivation. This is at variance with the results of Fabiato (1985b) who has demonstrated (in mechanically skinned cardiac cells) that CICR involves a channel which is inactivated with use and time. The discrepancy may reflect the particularly unphysiological experimental conditions employed in the single channel experiments.

The aim of this chapter

It will be demonstrated in chapter 5 that the rising phase of the caffeine contracture (under conditions illustrated in figure 3.1) is primarily the consequence of s.r. release with little contribution from the concomitant increase in Ca^{2+}-sensitivity. However, it is also of relevance to this study to consider the nature of the relaxation phase of the caffeine contracture. On balance, the results described above suggest that, after the initial Ca^{2+}-release,

the s.r. may accumulate Ca^{2+} thereby effecting relaxation. This sequence of events could occur if caffeine acts by sensitising the s.r. to Ca^{2+}, thereby promoting CICR. (Endo, 1977). As with CICR, one might expect caffeine to produce a transient Ca^{2+}-release which inactivates with time (Fabiato, 1985b). Thereafter, net s.r. Ca^{2+}-uptake may occur and contribute to relaxation. Indeed it has been assumed that the s.r. can contribute to the relaxation under similar experimental conditions to those used in this study (Fabiato, 1974, Harrison, 1985).

Another possibility is that the relaxation is the result of mitochondrial Ca^{2+} sequestration. It is generally accepted that the mitochondria do not play a part in cellular Ca^{2+}-regulation under physiological conditions (Carafoli, 1982). However, in a detailed study carried out in this laboratory, is was concluded that the mitochondria were almost entirely responsible for the relaxation of the caffeine contracture in saponin treated trabeculae (Harrison et al. 1985). With the exception of the Ca^{2+}-loading procedure, the experimental conditions were similar to those used in this study. As a result the possibility of mitochondrial Ca^{2+}-regulation merits special attention.

The experiments to be described in the following chapter use a direct method to assess the effects of caffeine on Ca^{2+}-accumulation by the s.r. in saponin-skinned trabeculae. This method has the advantage over previous inherently unsatisfactory studies which used a combination of procaine and caffeine itself to assess the action of the compound on the s.r. (Endo, 1977, Kitazawa, 1988). The possible involvement of the mitochondria in the caffeine

contracture was investigated by experimental interventions which influence mitochondrial Ca^{2+}-uptake and release such as addition of azide and alteration of $[Na^{+}]$.

RESULTS

Oscillations in saponin-skinned trabeculae

In selectively (saponin) skinned preparations, exposure to a solution with a supra-threshold [Ca^{2+}] and a low Ca^{2+}-buffer capacity (0.2mM $_{total}$) causes tension oscillations. These are believed to involve the cyclic uptake and release of Ca^{2+} from the s.r. (Fabiato & Fabiato, 1972). Such oscillations are sensitive to pharmacological interventions which influence CICR. As it is technically difficult to study CICR directly in multicellular preparations (Fabiato, 1977) this method has been employed to investigate the action of compounds which influence the functioning of the s.r. (e.g. Lamont & Miller, 1987).

During such an experiment, it was noted that a partially-activated preparation, transferred to a similar solution with a subthreshold [Ca^{2+}], relaxed with a much faster time course than that expected from simple diffusion alone. This phenomenon also occurred in muscles treated with azide and in the absence of respiratory substrates, which excludes a mitochondrial contribution. It was concluded that the relaxation was largely the result of Ca^{2+}-uptake by the s.r. The experiments described below were designed to investigate the effects of caffeine on the rate of s.r. Ca^{2+}-uptake under these experimental conditions.

The influence of caffeine on the relaxation of submaximally activated trabeculae

This group of experiments were carried out in the absence of respiratory substrates and in the presence of 2mM azide to inhibit mitochondrial Ca^{2+}-accumulation. Before any experimental intervention, a reproducible train of contractures was initiated in response to 10mM caffeine (as in figure 3.1). This protocol ensured that the extent of s.r. Ca^{2+}-loading was standardised prior to any intervention. The preparation was then exposed to a suprathreshold [Ca^{2+}] (pCa 5.3). As illustrated in figure 3.2a, this resulted in a CICR followed by characteristic tension oscillations (for detail see inset). After several minutes, returning to pCa 7.3 caused a rapid relaxation. In 3.2b, introduction of the same high Ca^{2+} solution with 3mM caffeine, initiated a caffeine-induced Ca^{2+}-release. Thereafter, tension gradually increased to a higher level than that observed in the absence of caffeine, until a steady state was reached. In contrast to the previous trace, no significant tension oscillations were observed. After the same length of time the muscle was relaxed in a similar way. The final tension produced in the presence of caffeine (3mM) was similar to that achieved at the same [Ca^{2+}] after complete disruption of cellular membranes with Triton X-100, in the absence figure 3.2c and presence figure 3.2d of caffeine.

The relaxations of the contractures were normalised and are shown on an expanded time scale in figure 3.3. In the absence of caffeine, tension declines rapidly with a half time of 0.15s. In contrast however, in the

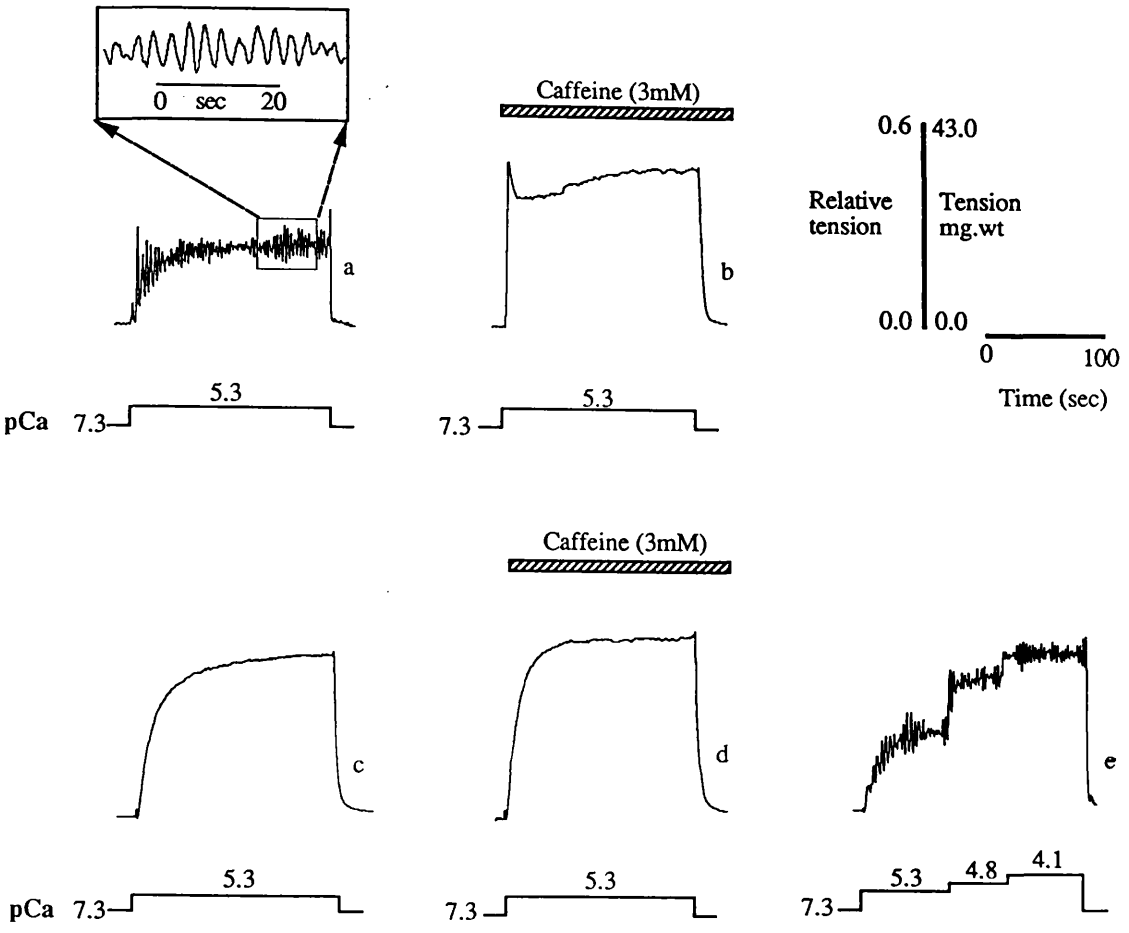


Figure 3.2. Tension responses from a skinned rat trabecula are shown. All solutions were weakly Ca^{2+}-buffered (0.2mM $EGTA_{total}$).In panel (a) the saponin-skinned muscle was transferred from a standard '0.2Relaxing' solution (pCa 7.3) to a similar solution with a subthreshold Ca^{2+}-concentration (pCa 5.3). This resulted in an increase in tension followed by CICR oscillations (for detail see inset). Returning to the '0.2Relaxing' solution caused a rapid relaxation. This was repeated in panel (b) but the muscle was activated and relaxed in the presence of 3mM caffeine (indicated by the bar above the trace). The procedure described for panels (a) and (b) was repeated following Triton-skinning (c) and (d). Panel (e) shows an activation and relaxation at a higher Ca^{2+}-concentration (pCa 4.1) in the saponin-treated state prior to Triton-skinning.

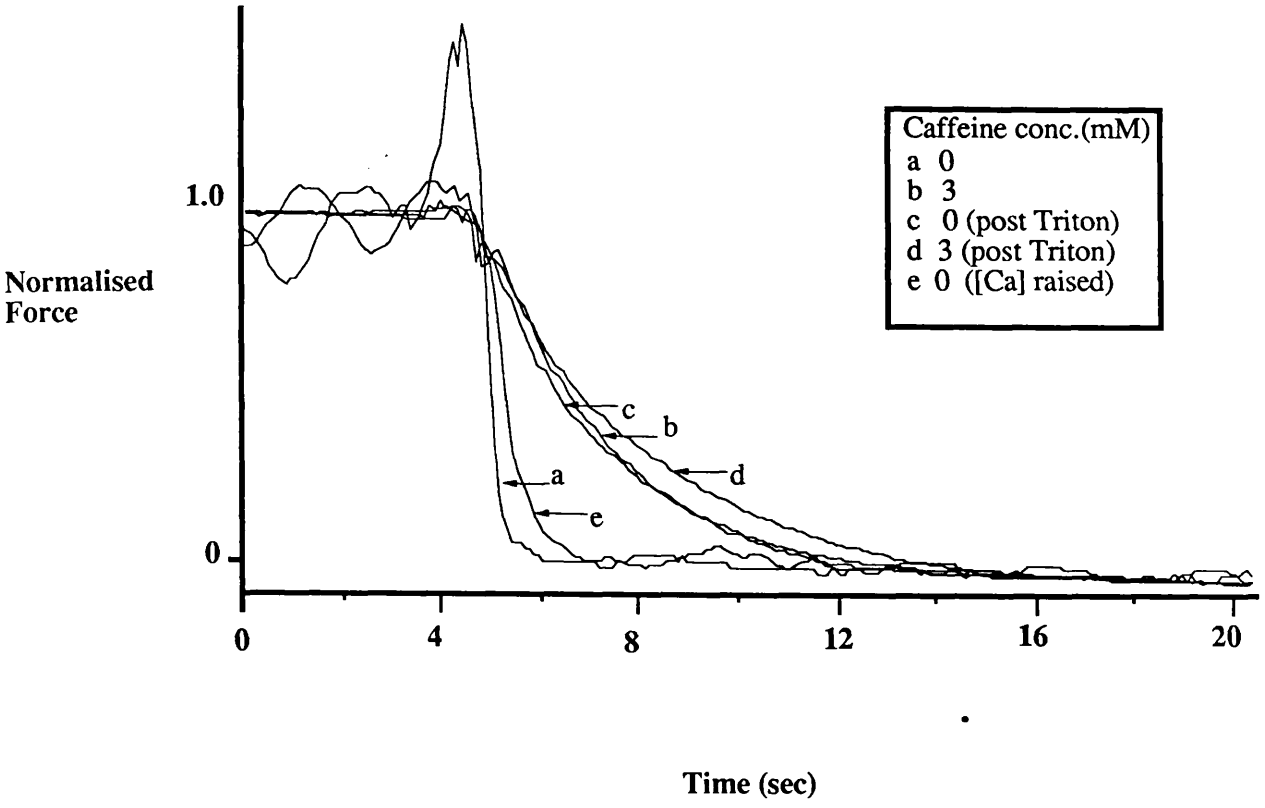


Figure 3.3 shows the relaxation phase of each contracture illustrated in figure 3.2 on a faster time base.

presence of caffeine (3mM) the time-course is much slower, with a half time of 1.8s and this curve approximates to those obtained after the destruction of the s.r. with Triton both with and without caffeine. The rate of relaxation after Triton-skinning is governed by diffusion and, to a good approximation, illustrates how fast the muscle would relax in the saponin treated-state without the influence of the s.r. The simplest explanation for this behaviour is that in the presence of 3mM caffeine, s.r. net-Ca^{2+}-uptake is inhibited. Thus under these experimental conditions the muscle behaves in many respects as it does after complete destruction of the s.r. with Triton. Similar results were obtained in 5 other preparations.

It is not clear whether this form of analysis is valid, not least because of the need to normalise the responses. In particular, the tension produced when the preparation is oscillating, is considerably less than during steady-state isometric conditions when the s.r. has been inhibited by caffeine or destroyed by Triton. There are at least two basic explanations for this phenomenon; (i) The average $[Ca^{2+}]_i$ may be lower than that of the surrounding medium (ii) The force produced by the muscle is reduced when the preparation is oscillating, despite an identical average $[Ca^{2+}]_i$.

The first possibility was investigated by increasing the $[Ca^{2+}]$ of the solution until the tension level was equal to that recorded in the presence of caffeine (figure 3.2e). As can be seen from figure 3.3e the rate of relaxation from pCa 4.1 was only slightly slower than observed at pCa 5.3 ($t_{½}$~0.35s). However. it seems unlikely that the lower tension produced during oscillations

is indicative of a lower [Ca^{2+}] in the vicinity of the myofilaments. As the volume of the bathing solution is effectively infinite, it is inconceivable that the s.r. could reduce the [Ca^{2+}] indefinitely. For this reason, it was concluded that tension was reduced because the preparation was oscillating and not because of the ability of the s.r. to accumulate Ca^{2+} (for a more complete analysis see Discussion).

The effect of caffeine on the relaxation from C_{max}

To avoid some of these problems, experiments were carried out at a higher [Ca^{2+}] so that the preparation was maximally activated. Obviously under these conditions all contractures were of equal magnitude and the myofilaments no longer exhibited tension oscillations (although the [Ca^{2+}] may still oscillate). Figure 3.4 shows the effects of a range of [caffeine] on the relaxation rate following maximal activation. The relaxation in the absence of caffeine was markedly slower than that seen at a lower [Ca^{2+}] in figure 3.3 with a half time of 3.7s. This may reflect the inhibition of Ca^{2+}-accumulation by the s.r. which is believed to occur at very high [Ca^{2+}]s (Fabiato, 1985a, Kitazawa 1988). Despite this, caffeine caused a dose-dependent slowing of the relaxation rate. Relaxations in the presence of 5mM and 10mM caffeine are superposable, indicating its effect saturated somewhere between 1mM and 5mM caffeine. After complete destruction of the intracellular organelles with Triton-X100, the rate is slightly faster than that seen in the presence of 5-10mM caffeine. This result is to be expected because it has been

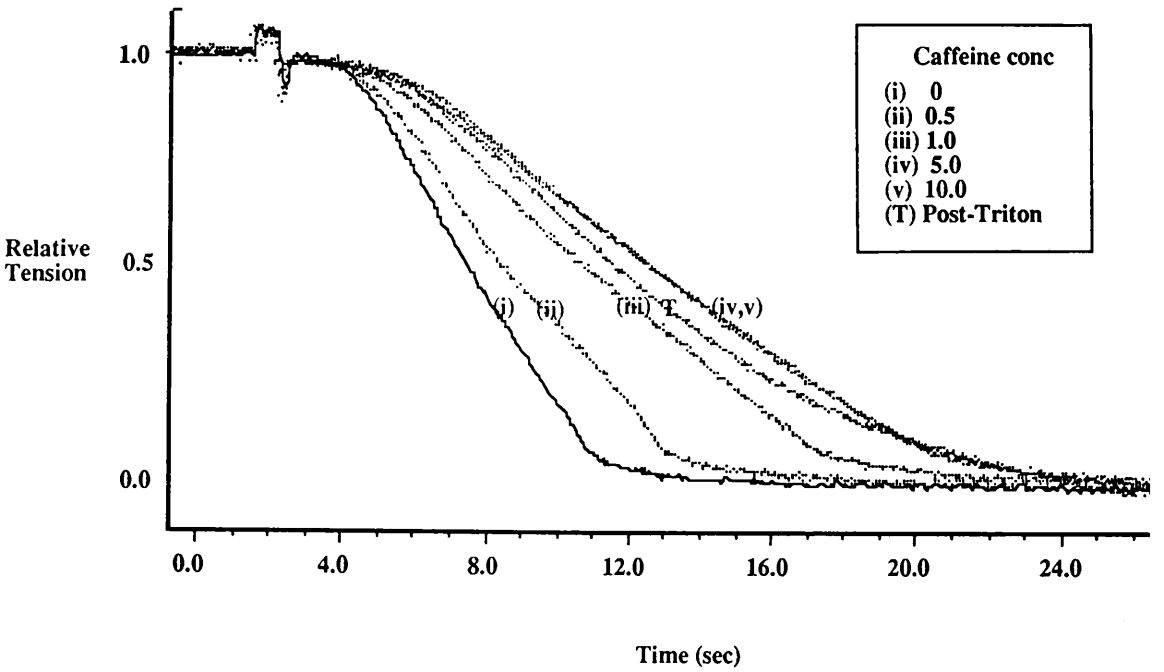


Figure 3.4 shows the relaxation of a trabecula in the presence and absence of various concentrations of caffeine (0-10mM), both after saponin and Triton-treatment. Protocol as described in figure 3.1 except the $[Ca^{2+}]$ of the suprathreshold solution was higher than required to produce C_{max}. Solutions were weakly Ca^{2+}-buffered (0.2mM $EGTA_{total}$).

demonstrated that the coefficient of diffusion is increased slightly when a saponin-treated preparation is subsequently Triton-skinned (Smith, 1983). This probably reflects the more complete destruction of the surface membrane by Triton. Thus the rate of relaxation after Triton-skinning may only be considered as an approximation to that which would occur in the saponin-treated state, without the influence of the s.r.; it is likely to give an overestimation of the rate of decline of force due to simple diffusion in saponin skinned trabecula.

Do the mitochondria contribute to the relaxation of the caffeine contracture under the conditions illustrated in figure 3.1 ?

The possible involvement of the mitochondria in the caffeine-induced response was investigated by the use of experimental interventions which influence mitochondrial Ca^{2+}-accumulation. For these experiments, 5mM glucose and 5mM pyruvate were included as respiratory substrates. The protocol involved first inducing a reproducible train of contractures, as shown in figure 3.1. Figure 3.5 shows superimposed contractures selected from a train of responses, before and after addition of 3mM azide. Azide (2mM) or cyanide (2mM) had no influence on the magnitude or time course of the caffeine-induced response in all of the 8 preparations tested. Similarly, changing the $[Na^+]$ over the range 0-20mM had no influence on the caffeine contracture (n=4 preparations). This excludes the possibility that the mitochondria contribute to the caffeine contracture under these experimental

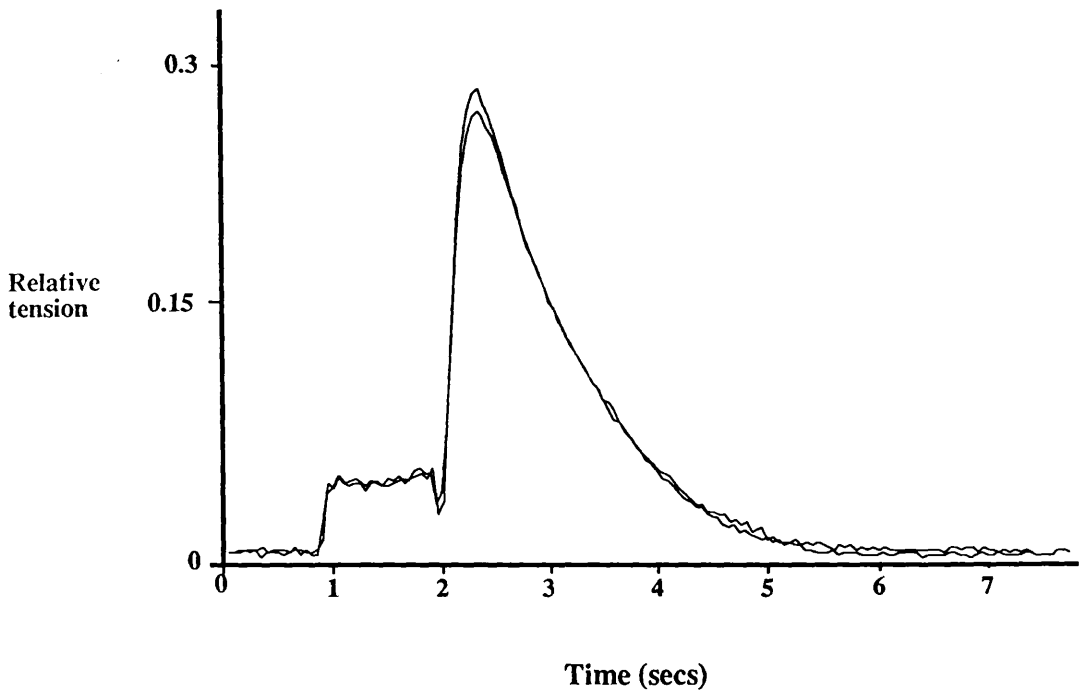


Figure 3.5 shows contractures selected from a train of caffeine-induced responses in a saponin-skinned trabecula (protocol as described in figure 3.1). Contractures before and after 10 minutes exposure to azide (2mM) are shown superposed.

conditions. This is at variance with previous observations (Harrison, 1985). Possible reasons for this discrepancy will be considered in the following section.

Discussion

Oscillating and steady-state tension production.

One obvious difference between contractures a and b figure 3.2 is that when the muscle is oscillating in the absence of caffeine, the average tension is considerably less than in the presence of caffeine or following Triton treatment. This phenomenon was consistently observed ($n>10$). Similar results were obtained on mechanically-skinned cardiac cells when the involvement of the s.r. was eliminated by raising the [EGTA] (Fabiato & Fabiato, 1975b). The Fabiatos concluded that the lower tension was due to the ability of the s.r. to compete with the contractile proteins and the EGTA, thereby reducing the intracellular [Ca^{2+}]. However, as illustrated in figure 3.2c, if the [Ca^{2+}] is raised until the tension is equal to that recorded in the presence of caffeine, the rate of relaxation is still considerably faster than when caffeine is present. Thus, this explanation of events cannot account for the slowing of relaxation seen with caffeine. Furthermore, the explanation proposed by the Fabiatos seems inappropriate because the muscle is bathed in an effectively infinite volume. It is unlikely that the s.r. could indefinitely reduce the average [Ca^{2+}] within the preparation, below that of the surrounding medium. Thus for the present experiments it is concluded that less tension is produced despite the same average intracellular [Ca^{2+}]. It seems most likely that the $[Ca^{2+}]_i$ is oscillating about an average level which is equal to the [Ca^{2+}] of the bathing solution. Averaged over several minutes

there need not be any net gradient of Ca^{2+} in to, or out of, the muscle and hence no need for an intracellular Ca^{2+}-store of infinite capacity.

Under steady-state isometric conditions, sarcomere length remains constant and relatively uniform, total muscle length remains constant, as does $[Ca^{2+}]_i$. In contrast the dynamic processes underlying tension oscillations are much more complicated. If the preparation is observed under the light microscope, localised waves of contraction may be seen to propagate along the muscle (Lamont & Miller, 1987). Under these conditions, changes in s.l. over the range 1.6-2.3μm have been measured using laser diffraction (C.Lamont-personal communication). As the total muscle length is kept constant, this probably reflects areas with a locally high $[Ca^{2+}]$ shortening and thereby stretching adjacent, less active sarcomeres. In many cases oscillations are unsynchronised, with several distinct focal points. Given the complexity of the situation, it is difficult to provide a simple explanation of why average tension is less when the muscle is oscillating. The following, much simplified model provides one possible partial explanation.

Consider that a preparation comprised just two sarcomeres (length 2.0μm) connected in series under isometric conditions, bathed in a solution capable of initiating half maximal activation (~4.3μM) (figure 3.6). For the reasons explained above, it will be assumed that there is no net Ca^{2+}-release or uptake by the muscle.

It is relevant to consider what would occur If the $[Ca^{2+}]$ in S1 increased by 2.6μM and simultaneously decreased by the same amount in S2.

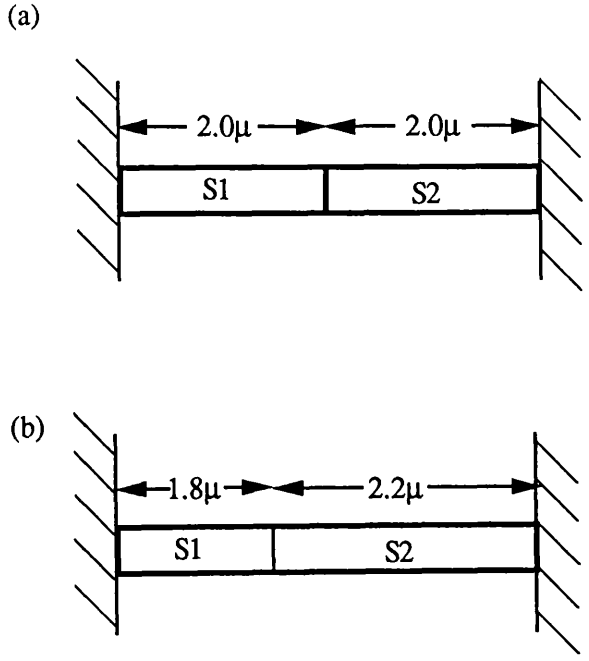


Figure 3.6 This figure shows a simplified model of a muscle preparation comprising two sarcomeres (S1 & S2) in series under perfectly isometric conditions. In panel (a), both sarcomeres are of equal length (2.0μm) and the $[Ca^{2+}]_i$ is 4.3μM in S1 & S2. In panel (b) the $[Ca^{2+}]_i$ in S1 has increase by 1.6μM and decreased by the same amount in S2. As a result, S1 has shortened to 1.8μm and S2 increased to 2.2μm.

S1 will shorten and S2 must lengthen by the same amount to maintain preparation length. Figure 3.7 shows the relationship which exists between absolute force and s.l. at various [Ca^{2+}]s (after Kentish et al, 1986). The tension in the two sarcomeres must tend towards equality and would therefore follow the paths shown in figure 3.7. The resulting tension will be smaller than if the two sarcomeres were activated at 5μM Ca^{2+} and the same s.l. It can be seen that this would hold true for a range of local changes in [Ca^{2+}] and s.l. The situation is, however, much more complex in the trabecula where a range of [Ca^{2+}]s, s.l.s and Ca^{2+}-sensitivities exist and unsynchronised oscillations may be occurring in different areas of the muscle. However, it should still be the case that the final tension level is dominated by sarcomeres with a low level of activation, because of the relationship between s.l. and Ca^{2+}-activated force and the logarithmic nature of the pCa tension relationship.

The uncertainty underlying interpretation of the oscillations of tension led to the use of fully-activated preparations. This has the advantage that contractures are of equal magnitude and presumably a comparable distribution of s.l.s (and hence Ca^{2+}-sensitivities). It has the disadvantage that the [Ca^{2+}] is above the range of most interest ie. 20-30% of maximum tension, as occurs at the peak of the caffeine contracture under the typical loading conditions employed in this study.

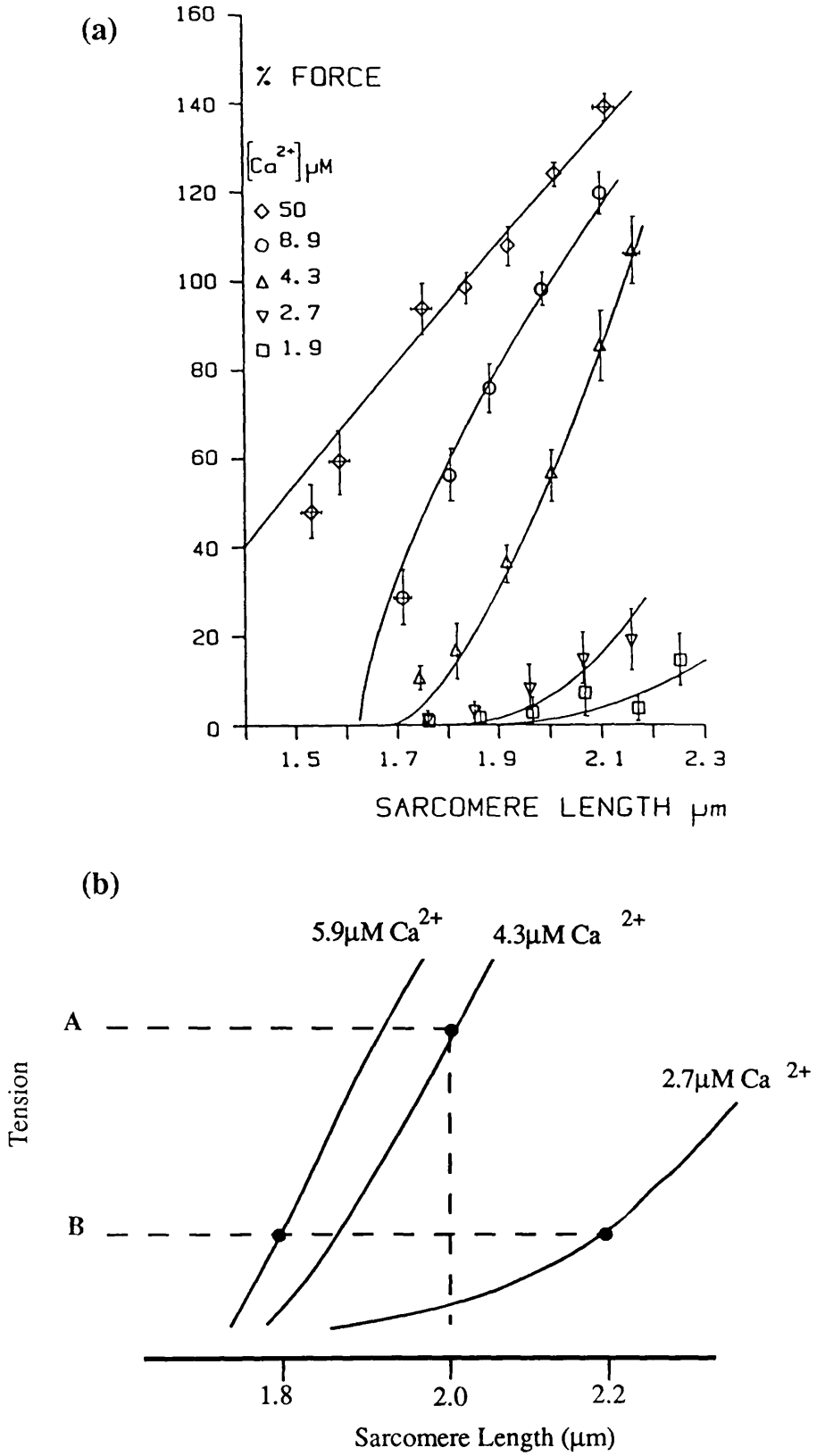


Figure 3.7a shows the relationship between absolute force and s.l. at various $[Ca^{2+}]$. Each point is the averaged response from 6 chemically skinned trabeculae. After Kentish, ter Keurs, Schouten, Nobel & Ricciardi (1986). Panel (b) shows a schematic representation of the predicted steady-state tension before (A) and after (B) the changes in sarcomere length and calcium under the conditions described in figure 3.6.

The effect of caffeine on the rate of relaxation

It is of relevance at this point to consider the state of the s.r. during oscillations, such as those illustrated in figure 3.2a. This phenomenon is frequently described as resulting from the "overload" of the s.r. This implies that the s.r. is full to capacity and, in effect, overflowing as it spontaneously releases Ca^{2+}. However, this would not appear to be the case, as when the $[Ca^{2+}]$ of the surrounding medium is lowered the s.r. can effect a rapid relaxation; tension falls much faster than the rate due to diffusion alone.

It has been demonstrated that the relaxation rate is slowed by caffeine in a dose-dependent manner. This might merely be the consequence of the increased Ca^{2+}-sensitivity of the myofilaments which is known to result from the presence of caffeine (Wendt and Stephenson, 1983). The action of a Ca^{2+}-sensitiser is similar in some respects to an increase in the $[Ca^{2+}]$. However, as demonstrated in figure 3.3e the rate of relaxation is little affected, even by a marked increase in the $[Ca^{2+}]$. Unlike a simple increase in $[Ca^{2+}]$, Ca^{2+}-sensitising compounds are believed to act by decreasing the rate of dissociation of Ca^{2+} from troponin-C. If the rate of dissociation is the rate limiting step in the relaxation then this could account for the slowing of the relaxation by caffeine. Indeed, it has been suggested that Ca^{2+}-sensitisers may be of limited use as inotropic agents because they may comprise the relaxation of the failing heart (Rüegg, 1986b).

Under the conditions employed in this study this explanation has been excluded for the following reasons; In figure 3.2c and d, after Triton

treatment, the contracture in the presence of caffeine (3mM) was only slightly larger than without the drug, indicating only a small effect on Ca^{2+}-sensitivity. However, a marked slowing of the relaxation rate was observed with caffeine in the saponin skinned state.

Further direct evidence was obtained from experiments comparing the action of pimobendan and caffeine. Pimobendan is a cardiotonic compound and its properties have been investigated in some detail using skinned muscle preparations. The drug has no direct effect on the s.r. but increases the Ca^{2+}-sensitivity of the contractile proteins in a much lower concentration range than caffeine (Harrison & Miller 1986). Figure 3.8 shows the comparative effects of caffeine and pimobendan on the Ca^{2+}-sensitivity of a Triton skinned trabecula. The muscle was activated to around 50% of maximum force and in 3.5a, the [caffeine] was increased cumulatively (0-3mM). This resulted in the expected increase in the Ca^{2+}-sensitivity. In 3.5b, the procedure was repeated, but in this case pimobendan (0.1mM) was added. The increase in Ca^{2+}-sensitivity caused by 3mM caffeine was approximately equal to that by 0.1mM Pimobendan. Figure 3.9 shows the rate of relaxation of the same saponin-skinned trabecula under the experimental conditions described for figure 3.4. The traces in the presence and absence of pimobendan (0.1mM) are superposable whereas caffeine (3mM) causes a marked slowing of the relaxation as shown in the figures 3.3 and 3.4.

In theory, if the preparation is maximally activated and the dissociation of Ca^{2+} from troponin is not the rate limiting factor during

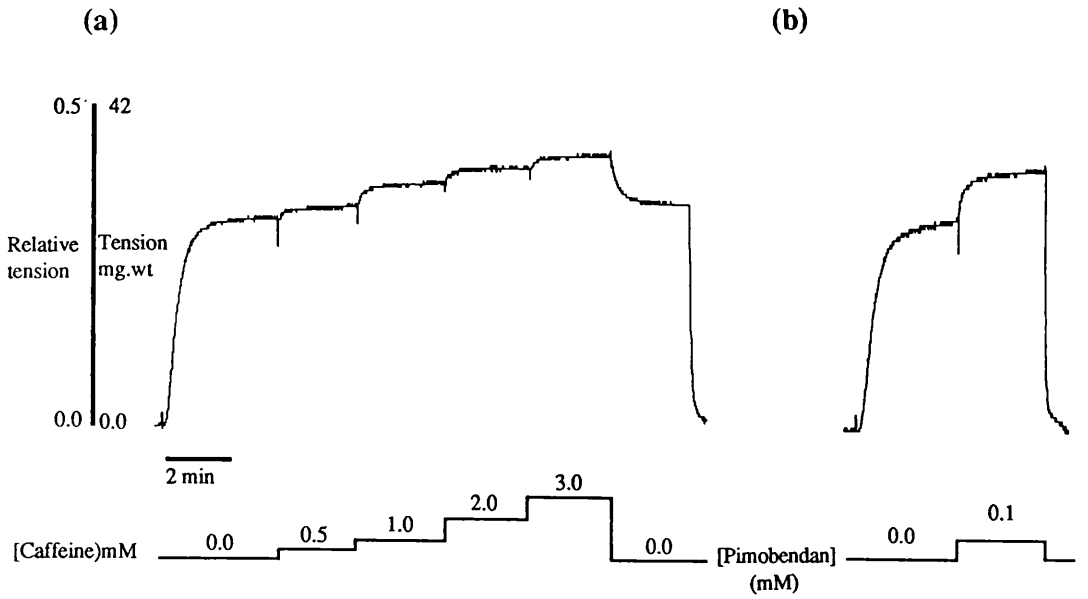


Figure 3.8 illustrates the effect of caffeine (0.5-3.0 mM) and pimobendan (0.1mM) on the Ca^{2+}-sensitivity of a Triton-skinned trabecula. All solutions were strongly Ca^{2+}-buffered (10mM $EGTA_{total}$). The increase in Ca^{2+}-sensitivity produced by 3mM caffeine (panel a) was approximately equal to that by 0.1mM caffeine pimobendan (panel b).

relaxation, then an increase in Ca^{2+}-sensitivity can have little influence on the relaxation rate. Caffeine causes a increase in Ca^{2+}-sensitivity, shifting the pCa tension relationship to the left. However, the magnitude of the range over which the contractile proteins are sensitive to Ca^{2+} remains unaltered (assuming there is no change in the Hill coefficient). It follows that during relaxation, the $[Ca^{2+}]$ must fall for slightly longer before it will result in a decrease in force. Thereafter, the rate of decline of force should be relatively unaffected, as the Ca^{2+} would have to fall by the same absolute amount to effect a complete relaxation. However, one would expect a longer delay, following the solution change, before a fall in tension is detected. In support of this, figure 3.4 illustrates that as the [caffeine] is increased (0-5mM) there is a slightly longer delay before the onset of relaxation. However, as caffeine continues to sensitise at concentrations above 5mM (Wendt & Stephenson, 1983) one would expect the relaxation with 10mM caffeine to be shifted further to the right. However this is not the case. The explanation for this remains unclear.

The relevance of these results to the caffeine contracture in saponin skinned trabeculae.

In conclusion, under the experimental conditions described above, caffeine induces a dose-dependent slowing of the relaxation in saponin skinned trabeculae, weakly Ca^{2+}-buffered with EGTA ($0.2mM_{total}$). Relaxations in the presence of caffeine have a similar timecourse to those following

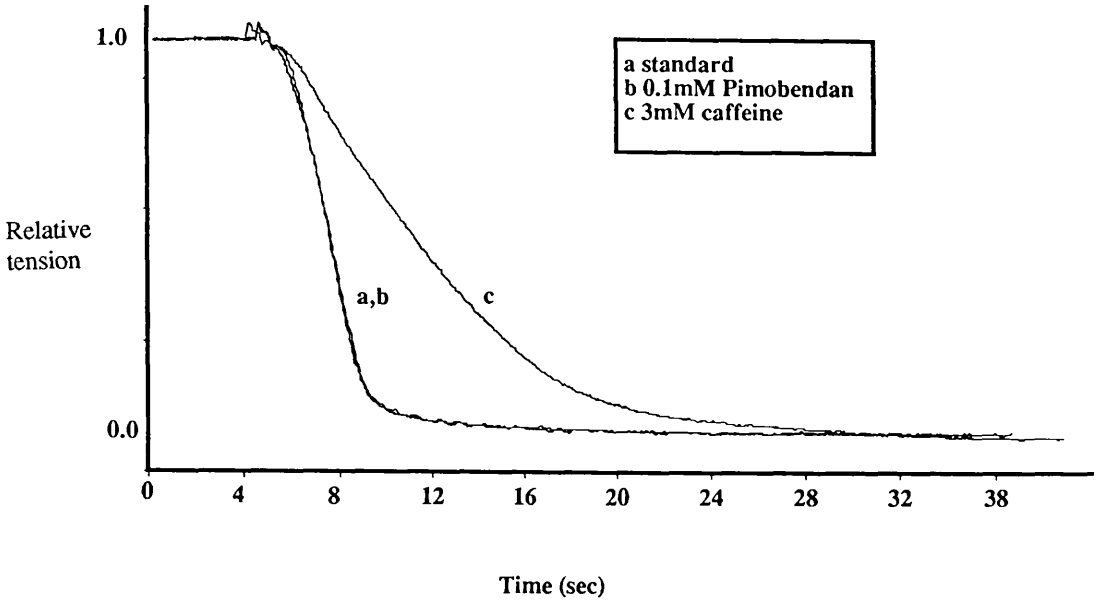


Figure 3.9 shows relaxations from C_{max} in the same preparation which produced the tension responses shown in figure 3.8. All solutions were weakly Ca^{2+}-buffered (0.2mM $EGTA_{total}$). Protocol as described in figure 3.4. Relaxations are shown in the absence and presence of both caffeine (3mM) and pimobendan (0.1mM).

destruction of the s.r. with Triton. The effect of caffeine is not due to the increase in the Ca^{2+}-sensitivity of the myofilaments. The results are consistent with the inhibition of net Ca^{2+}-uptake by the s.r. in the presence of caffeine.

The relevance of this understanding to the caffeine-induced contracture as illustrated in figure 3.1 will now be considered. During the caffeine contracture the drug concentration increases across the diameter of the preparation with a time course limited by diffusion. Thus the superficial [caffeine] will rapidly reach 10mM following the solution change while the cells at the centre of the muscle may not reach equilibrium by time the contracture has relaxed. It is reasonable to assume that, in order for Ca^{2+}-release to occur at any point across the diameter of the preparation, the concentration of caffeine at that point must be in the millimolar range (see chapter 5 for caffeine dose response relationship). Under these circumstances the results reported above suggest that the ability of the s.r. to effect relaxation would be compromised by the presence of caffeine.

In this study there was found to be no involvement of the mitochondria in this type of caffeine-induced response. This is at variance with a previous experiments carried out in this laboratory which led to the conclusion that the mitochondria were primarily responsible for the relaxation of the caffeine contracture (Harrison & Miller, 1984, Fry & Miller, 1985, Harrison, 1985). However, the experimental protocol employed by Harrison et al involved Ca^{2+}-loading the s.r. at maximally activating [Ca^{2+}] for many minutes, before exposure to caffeine. Under these conditions the

mitochondria do accumulate large amounts of Ca^{2+} (C.Lamont, personal communication). Thus it is not surprising that subsequent caffeine responses were sensitive to interventions which influence mitochondrial Ca^{2+}-uptake and release.

It seems likely that the relaxation of the caffeine contracture, as illustrated in figure 3.1, is mostly the consequence of the outward diffusion of Ca^{2+} from the trabecula. One other factor not yet considered which may contribute to the relaxation is the Ca^{2+}-buffer (EGTA). In a study by Fabiato (1985b) it was concluded that rate of Ca^{2+}-binding to EGTA was much slower than Ca^{2+}-binding to troponin-C. Thus, EGTA is a good buffer of steady state Ca^{2+}-levels but a poor buffer of rapid changes in $[Ca^{2+}]$. Indeed at the [EGTA] used by Fabiato (0.05mM) the Ca^{2+}-released from the s.r. was effectively unbuffered by the peak of a CICR contracture (0.7-1.0s). However, it is possible that Ca^{2+}-binding to EGTA may influence the relaxation phase of such contractures. Any such effects are likely to be more pronounced in the present study as the [EGTA] (0.2mM) was 4 times that used by Fabiato. The influence of EGTA on transient contractures and comparisons with buffers with faster Ca^{2+}-binding kinetics (e.g. BAPTA) is the subject of an ongoing study by A.Fabiato (referred to in Fabiato, 1985b).

Chapter 4:**Effects of cyclic-AMP on saponin and Triton-skinned trabeculae.**

INTRODUCTION

Catecholamines, cyclic nucleotides and contractility

Catecholamines increase contractile force, decrease the time to peak tension and speed relaxation rate in cardiac muscle. The mechanisms underlying this important response have been the subject of many studies. It is believed that catecholamines act primarily via β-adrenergic receptors to stimulate production of cAMP by membrane-bound adenylate cyclase. Activation of adenylate cyclase is achieved by way of a stimulatory GTP-dependent transducer protein subsequent to occupation of the β-receptor. The activated cyclase catalyses transformation of ATP to the second messenger cAMP. Raised cAMP levels are associated with the phosphorylation of specific intracellular regulatory sites (for a recent review see Schmitz et al, 1987a). As will be discussed in the following paragraphs, cAMP influences several interacting intracellular systems. It follows from the complexity of the situation that some details of the response under physiological conditions remain unclear.

The effect of cAMP sarcolemmal channels

The effects of cAMP on sarcolemmal Ca^{2+}-channels has been investigated by patch clamping and by incorporation of single channels into artificial lipid bilayers. In both studies, cAMP caused an increase in the open probability of the channel (Reuter, 1983, Flockerzi et al, 1986). The effect of

cAMP is associated with the phosphorylation of the Ca^{2+}-channels by a cAMP dependent protein kinase (Osterrieder et al, 1987). This increase in 'trigger' Ca^{2+} may contribute to the β-adrenergic positive inotropic effect.

The effect of cAMP on the sarcoplasmic reticulum

In isolated canine s.r., cAMP induced a 2-3 fold increase in the rate of Ca^{2+}-accumulation (Tada et al 1974). This was associated with the phosphorylation of the 22000-dalton sarcoplasmic protein, phospholamban by a cAMP dependent protein kinase. Phosphorylation causes an increase in the apparent Ca^{2+}-sensitivity of the s.r. Ca^{2+}-pump accompanied by a decrease in positive cooperativity between the two Ca^{2+}-binding sites (Tada et al 1975). The action of cAMP on the s.r. has been confirmed in mechanically-skinned cardiac cells. In this preparation, cAMP facilitated s.r. Ca^{2+}-uptake which resulted in the potentiation of CICR and a faster rate of relaxation (Fabiato & Fabiato, 1975a). It has been suggested that this action of cAMP may explain the increased relaxation rate characteristically found during β-stimulation.

Cyclic nucleotides and the contractile proteins

Studies on intact preparations have demonstrated that stimulation of β-receptors is associated with the phosphorylation of cardiac troponin-I (Solaro et al, 1976). Allen & Kurihara (1980) reported that the relationship between the intracellular Ca^{2+} transient and tension changed in a manner

consistent with a decrease in Ca^{2+}-sensitivity during β-adrenergic stimulation in cat cardiac muscle. This finding supported biochemical evidence that phosphorylation of troponin-I is associated with an decreased Ca^{2+}-sensitivity of actomyosin and myofibrillar ATPase in beef and pig heart (Ray & England 1976, Yamamoto & Ohtsuki, 1981). However, in guinea-pig and rat heart the Ca^{2+}-sensitivity of actomyosin was reportedly increased (Rubio et al 1975). Phosphorylation of troponin-I was also found to reduce the Ca^{2+}-sensitivity of isolated bovine troponin (Robertson et al 1982).

Confirmation of these findings has been sought in a number of skinned or permeabilised cardiac preparations, notably the work of Winegrad and his collaborators is frequently cited. Winegrad reported that cardiac muscle was rendered "hyperpermeable" by prolonged exposure to EGTA or EDTA. In these preparations, in the presence of a PDE inhibitor, cGMP increased and cAMP decreased the apparent Ca^{2+}-sensitivity of the contractile proteins. However, these effects were absent if the muscle was subsequently skinned with Triton-X100 or if the PDE inhibitor theophylline was not included in the solutions.

Theophylline increased the Ca^{2+}-sensitivity of the myofilaments. As there was no such effect following Triton-skinning, this was ascribed to changes in levels of cyclic nucleotides and a reaction which necessitated the presence of the surface membrane. This result is difficult to reconcile with many subsequent studies which demonstrated that such methylxanthines increase Ca^{2+}-sensitivity in mechanically and chemically-skinned muscle by a

direct action on the contractile proteins (e.g. Wendt & Stephenson, 1983). Furthermore, Winegrad reported that cAMP increased maximum calcium activated force, but only when added in the presence of a detergent such as Triton (Molpe et al, 1980, McClellan et al, 1980).

These reports caused some controversy as Winegrad's findings were not confirmed in subsequent studies. In particular, it was reported that: (i) Further treatment with detergent greatly enhanced the activation rate (ii) Maximum calcium activated force increased following Triton-skinning and this occurred in the absence or presence of cAMP. (iii) EGTA-treated cardiac muscle was not freely permeable to the ionic constituents of the bathing medium (Kentish & Jewell, 1984, Miller, & Smith, 1985).

These authors concluded that the ionic mileau in the vicinity of the myofibrils was not freely diffusible with that of the bathing solution and could vary in an unknown way throughout the experiment. Of most concern, it is possible that a change in the permeability of the sarcolemma or the activity of the intracellular organelles may be wrongly interpreted as an alteration in myofilament Ca^{2+}-sensitivity. While the conclusions drawn by Winegrad and his collaborators may not be without substance, the uncertainties surrounding the technique mean that this work is of little value in assessing the influence of cyclic nucleotides on the contractile proteins.

Studies carried out on mechanically-skinned cardiac cells to some extent support Winegrad's findings. Cyclic nucleotides failed to influence Ca^{2+}-sensitivity unless the enzyme phosphodiesterase (PDE) was inhibited

(Fabiato & Fabiato, 1981b). Under these conditions cAMP depressed and cGMP enhanced Ca^{2+}-sensitivity although the effect was small (<0.05 log units). In glycerinated porcine cardiac muscle, cAMP depressed Ca^{2+}-sensitivity by as much as 0.2 log units (Herzig et al, 1981b). Others have reported that cAMP induced no change in the Ca^{2+}-sensitivity of detergent-skinned cardiac muscle (Wendt & Stephenson, 1983) although it was associated with a large increase in troponin-I phosphorylation (Hoar et al 1980).

In conclusion, the majority of evidence would suggest that cAMP may induce a depression in the Ca^{2+}-sensitivity of the contractile proteins. While the biochemical evidence is largely consistent, it is unfortunate that a clear demonstration this effect in fully chemically-skinned cardiac muscle was not observed in most studies. It is possible that this may reflect the destruction or loss of cellular components associated with the action of cAMP.

Phosphodiesterase

Phosphodiesterase (PDE) hydrolyses cAMP to the inactive 5'-AMP and consequently it would be expected that inhibitors of this enzyme may share characteristics of β-adrenergic stimulation. However, the situation is complicated because many PDE inhibitors (e.g. methylxanthines) directly influence other cellular systems such as the s.r. and contractile proteins. The characteristics of the contractile response will therefore depend on which effect predominates at a given drug concentration, in a particular species.

Furthermore, it has been shown that multiple forms of PDE exist in cardiac muscle. The best characterised forms are the Ca^{2+}-calmodulin activated and the cGMP-stimulated PDE although at least two other types also exist each with its own unique physical and kinetic properties (Harrison et al 1986). It is not clear how these isozymes interact under physiological conditions to modulate the level of cyclic nucleotides and consequently, contractility. It has been suggested that there may be functionally distinct pools within the heart and possibly within each cell (Buxton et al, 1983).

The search for novel inotropic agents has led to the introduction of a number of PDE inhibitors, such as milrinone, currently undergoing clinical trials. These compounds must now be classified in terms of their ability to inhibit the individual isozymes. It would appear that the inotropic response may depend critically on the spectrum of action (Harrison et al, 1986).

It is of particular relevance to this study that the contractile proteins are now being considered as a target for putative inotropic agents. This will be dealt with in more detail in following chapters. Without exception, all reported Ca^{2+}-sensitising inotropes are also potent PDE inhibitors (Weishaar et al, 1986). The use of chemically-skinned muscle where the bathing solution has direct access to the contractile proteins, lends itself to the characterisation of these drugs. One obvious problem with this method is that persistence of enzymes associated with cAMP metabolism may be influenced by inhibition of PDE. Indeed several studies have assumed or deduced that significant PDE activity is present in mechanically or chemically-skinned

cardiac preparations (Fabiato, 1981b, McClellan & Winegrad, 1978, Wendt & Stephenson, 1983, Herzig et al 1981b). However, this is unlikely to compromise the interpretation of these results because exogenous cAMP been reported to have no effect on, or depress Ca^{2+}-sensitivity. Thus if anything, there would be a tendency to underestimate any Ca^{2+}-sensitising action (Wendt & Stephenson, 1983). Furthermore, after prolonged exposure to Triton X-100, little remains of the surface membrane (Miller, Elder & Smith, 1985). Thus, significant endogenous cAMP production seems unlikely and any that was produced would be free to diffuse from the muscle into the surrounding medium.

Saponin-treated trabeculae

The situation regarding saponin-treated trabeculae is less clear. Saponin acts by precipitating cholesterol from the surface membrane and consequently relatively large amounts of the membrane remain intact (Endo & Kitazawa, 1978). This leaves open the possibility that endogenous cAMP production may continue. Much of the work in described in the following chapters has made use of selectively-skinned preparations to investigate the effects of various compounds on the functioning of the s.r. Some are known to be potent PDE inhibitors (e.g. ORG30029) and the activity of others, including endogenous modulators of intracellular Ca^{2+} (e.g. carnosine) remain unknown. However, most of the compounds natural or synthetic, contain an

imidazole ring and this structural feature is frequently associated with PDE inhibition (Chapman & Miller, 1974b)

The aim of this chapter

As outlined above, a compound found to increase Ca^{2+}-accumulation by the s.r. in a saponin-treated trabecula may achieve this via at least three possible mechanisms; (i) Agonism of β-receptors remaining in the surface membrane leading to the production of cAMP. (ii) Stimulation of membrane-bound adenylate cyclase, thereby catalysing the production of cAMP. (iii) Inhibition of PDE and the consequent elevation of cAMP levels.

The experiments described in the following chapter were designed to investigate the possibilities described above. In particular, the response of the s.r. and contractile proteins to exogenously applied cAMP was determined. Possible effects of PDE inhibition were determined using the opiate derivative papaverine. This compound is a potent non-selective inhibitor of all known types of PDE and has the advantage over theophylline or IBMX that it does not affect Ca^{2+}-sensitivity or induce s.r. Ca^{2+}-release (Kitazawa, 1988, Wendt & Stephenson, 1983, Weishaar et al, 1986). Adenylate cyclase activity was studied by application of forskolin toxin, a direct stimulator the catalytic subunit (Blinks & Endoh, 1986). And finally the existence of functional β-adrenergic receptors was investigated by addition of isoprenaline.

RESULTS

The activity of adenylate cyclase and the action of Forskolin Toxin

Forskolin toxin (10^{-6}M) was consistently found to produce an increase the amplitude of the caffeine contracture (n=6 preparations). As the effect of forskolin was usually small, considerable care was taken to ensure that experimental artefacts were not responsible for any apparent increase in response.

In these experiments, four baths of solutions were used; two caffeine and two Ca^{2+}-loading solutions. The experiment was started by alternately exposing the preparation to one pair of caffeine and Ca^{2+}-loading solutions until a reproducible train of contractures was induced. The muscle was then alternated between the other pair of identical caffeine and loading solutions. The standard sequence is shown figure 4.1a the change to the alternative pair of solutions is indicated by the bar above the trace. In practice changing to the alternative pair of solutions frequently resulted in a small increase or decrease in the amplitude of the responses (5-10%). This was usually caused by a difference in the level of one of the solutions and a consequent change in the efficiency of stirring. If this occurred the experiment was stopped and new solutions prepared. When no significant difference was observed between the two pairs of solutions (as in figure 4.1a), forskolin (10^{-6}M) was added to one pair and the sequence was repeated.

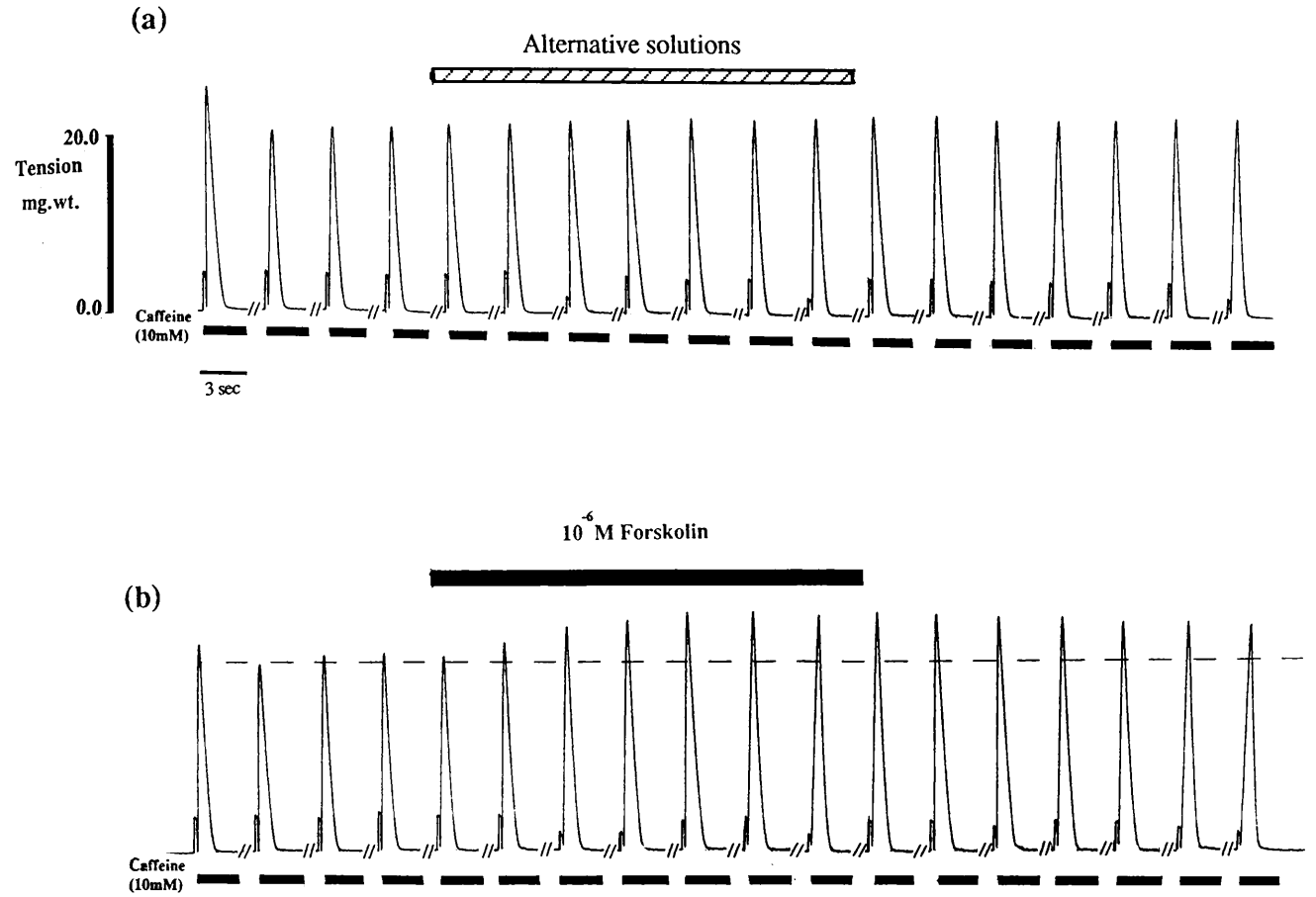


Figure 4.1 Panel (a) shows a train of caffeine-induced contractures in a saponin-treated trabecula (protocol as described in figure 3.1). All solutions were weakly Ca^{2+}-buffered (0.2mM $EGTA_{total}$). As indicated by the shaded bar above the trace the preparation was exposed to an alternative but identical pair of 'Ca^{2+}-loading' (pCa 6.2) and caffeine solutions (pCa 7.2). This resulted in no significant change in the amplitude of the caffeine contracture. Forskolin toxin (10^{-6}M was then added to one pair of 'Ca^{2+}-loading' and caffeine solutions and the sequence repeated. As shown in panel (b), after introduction of forskolin, the caffeine contracture gradually increased over 3-4 load & release cycles until a new steady-state was achieved. Removal of forskolin caused a slow decline in the amplitude of the caffeine transient over many minutes. One minute exposures to the 'Ca^{2+}-loading' solutions have been omitted at the points indicated by the breaks in the trace (-//-).

A typical response is shown in figure 4.1b. Frequently, a delay of several minutes was observed after introduction of forskolin, before a response was noted or a new steady state was reached. Furthermore, the caffeine contractures always remained potentiated for many minutes after removal of the drug. While the effect of forskolin (10^{-6}M) was typically small (22.2±5.1%, mean±SD, n=5), in one preparation, a much larger potentiation was observed (figure 4.2). Again the response was slow in onset and poorly reversible.

The effect of exogenous cAMP on the caffeine contracture.

The addition of isoprenaline (10^{-5}M) failed to influence the submaximal caffeine-induced contracture (n=4, data not shown). Presumably this indicates that significant endogenous cAMP accumulation does not result from stimulation of β-receptors which may remain in the sarcolemma following saponin-skinning.

Introduction of exogenous cAMP did influence the caffeine response. A typical result is shown in figure 4.3. A train of submaximal caffeine contractures was initiated in a saponin-skinned trabecula. Once a steady state was achieved, cAMP (1μM) was added to both loading and caffeine solutions as indicated by the shaded bar above the trace. Following the introduction of cAMP, the peak amplitude of the caffeine response gradually increased, reaching a new steady state within two load and release cycles. In this case the contracture was doubled in size. After removal of cAMP, the

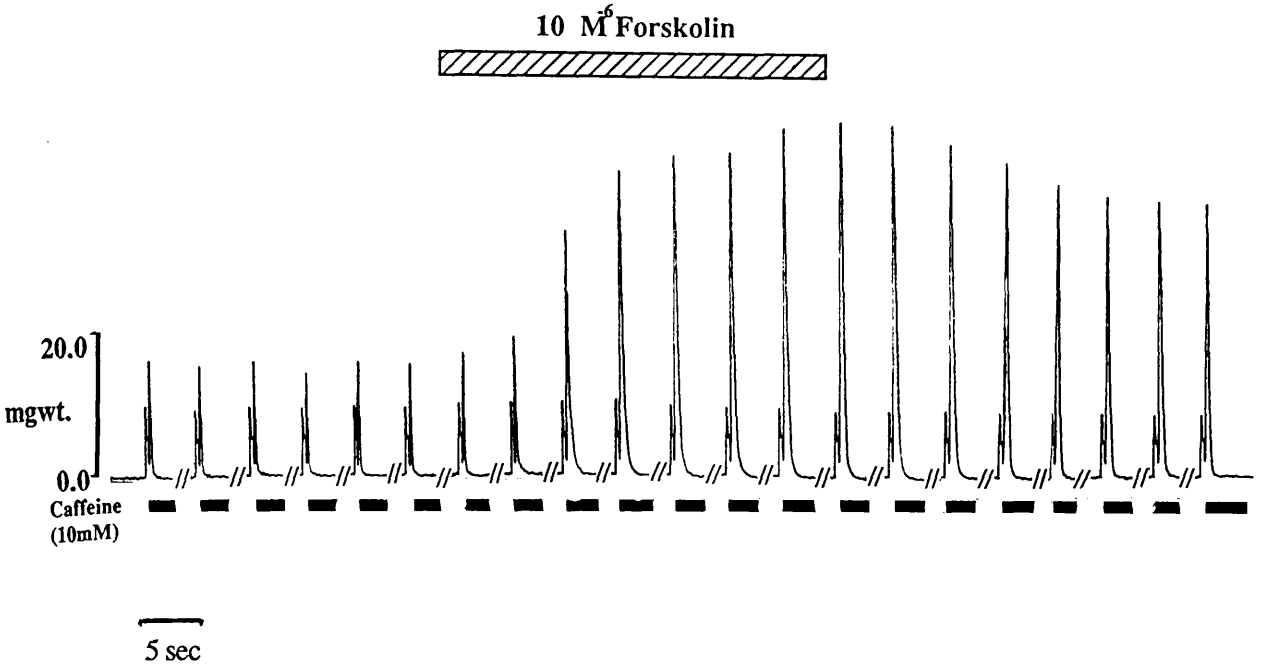


Figure 4.2 shows a train of contractures in a saponin-skinned trabecula (protocol as described in figure 4.1). All solutions were weakly Ca^{2+}-buffered (0.2mM $EGTA_{total}$). Introduction of forskolin (10^{-6}M) was followed by a gradual increase in the amplitude of the caffeine contracture over 10 load and release cycles until a new steady state was achieved. As in figure 4.1 the effect was only slowly reversible. At the points indicated by the breaks (-//-) in the trace a 1 minute exposure to a 'Ca^{2+}-loading' solution (pCa 6.5) has been omitted.

contracture remained potentiated, declining slowly over many minutes towards control levels. The slow return towards control levels was a consistent characteristic of the phenomenon (n=14).

The variability of the phenomenon

Attempts were made to define the relationship between [cAMP] and potentiation of the caffeine contracture. Experiments were carried out by initiating a train of submaximal contractures in a freshly saponin-treated muscle. cAMP was then introduced by adding aliquots of concentrated stock to the bathing solution, resulting in concentrations over the range 10^{-9}M to 10^{-5}M increasing by a factor of 10 each time.

The concentration at which a potentiation was first observed varied considerably between preparations and a meaningful dose response curve based on collected data could not be obtained. Furthermore, contractures rarely appeared to increase in a graded manner as the [cAMP] increased. More commonly, little or no response was observed from 10^{-9}M to 10^{-8}M however, as the concentration was raised over the range 10^{-8}M to 10^{-5}M contractures were potentiated at a concentration which varied from muscle to muscle. Whatever the concentration, contractures were not usually increased more by a further 10 fold increase in the [cAMP].

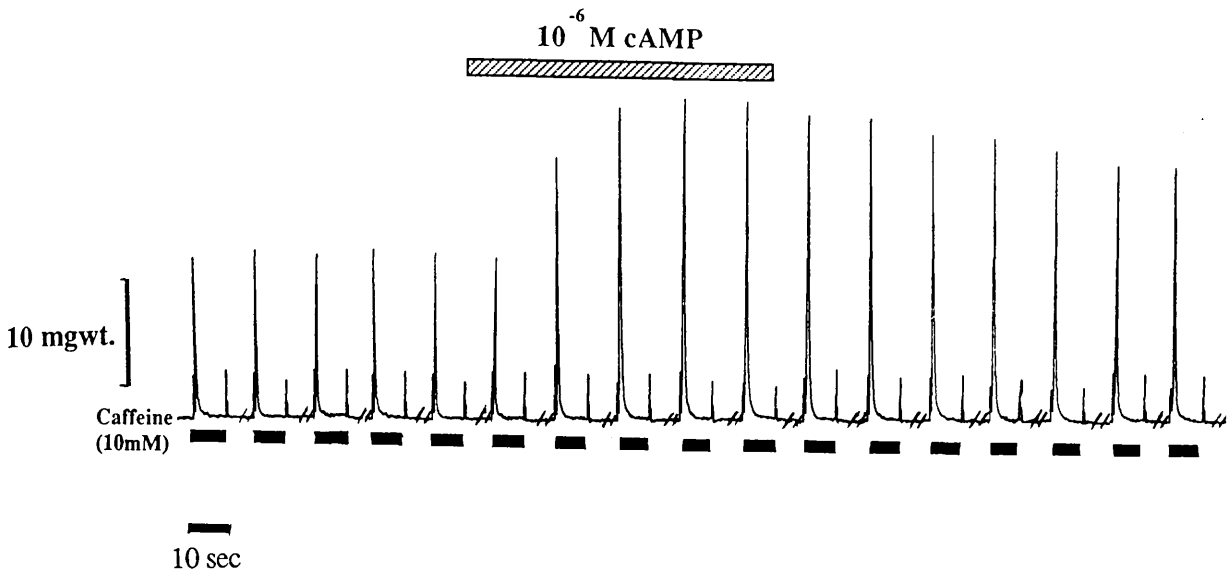


Figure 4.3 shows a train of contractures in a saponin-skinned trabecula (protocol as described in figure 4.1). All solutions were weakly Ca^{2+}-buffered (0.2mM $EGTA_{total}$). Introduction of cAMP (10^{-6}M) was followed by a gradual increase in the amplitude of the caffeine contracture until a new steady state was achieved. In common with the effects of forskolin toxin shown in the previous two figures, the potentiation produced by cAMP was only slowly reversible.

The effect of phosphodiesterase inhibition on the cAMP response.

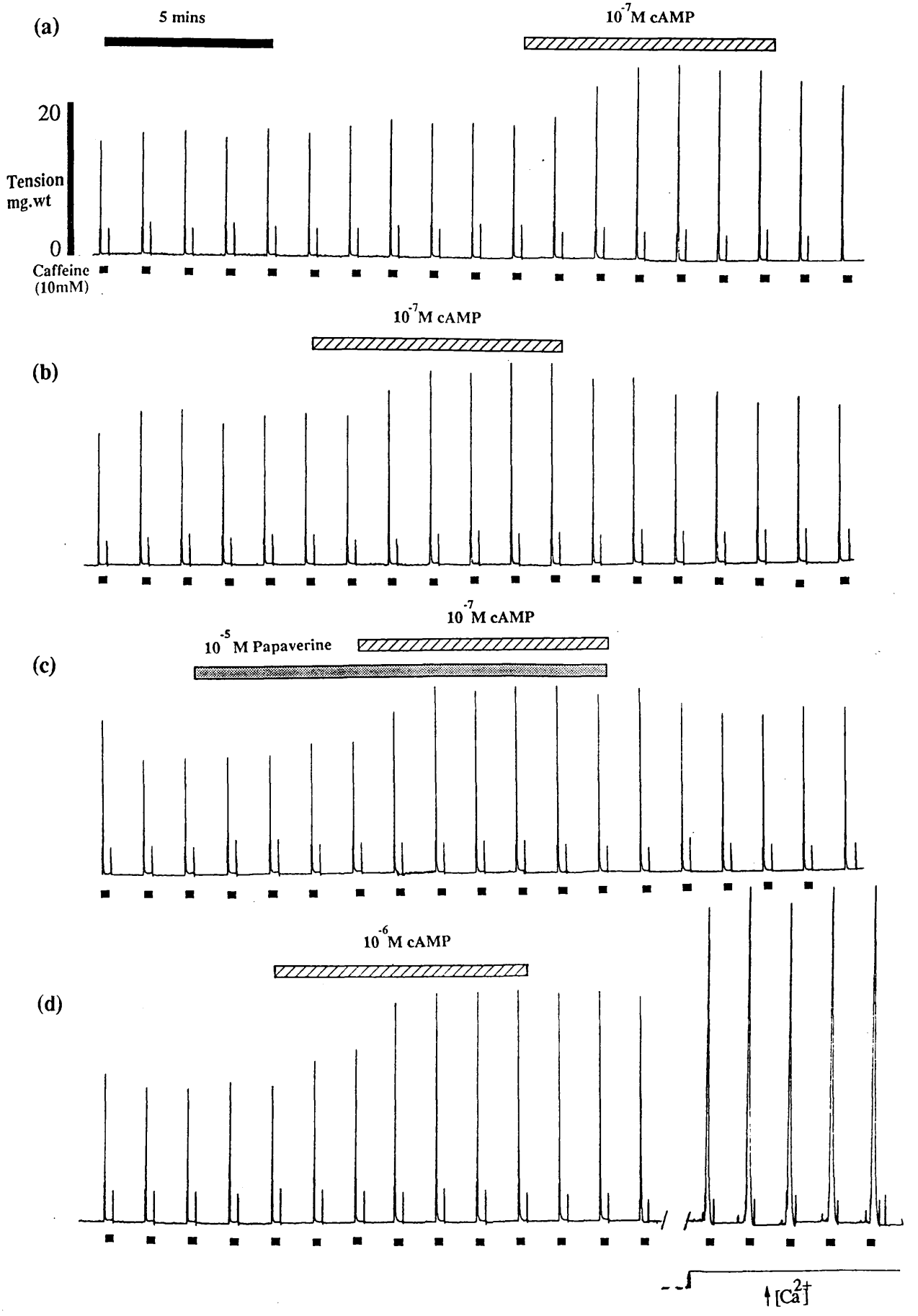
In 8 preparations, introduction of the PDE inhibitors papaverine (10μM) or amrinone (100μM) had no effect on the submaximal steady-state caffeine-induced contracture.

As outlined in the introduction, a number of studies have observed that inhibition of PDE can modulate the response of detergent-skinned cardiac muscle to exogenously applied cAMP. Consequently, it was one of the aims of the experiments reported in this chapter to determine whether PDE activity could be demonstrated in saponin-treated preparations. This was to be carried out by obtaining a submaximal response to cAMP and then adding the PDE inhibitor papaverine. This type of experiment was complicated by the unpredictable and poorly reversible nature of the response to cAMP.

The results of one such experiment in which the response of the preparation to cAMP was reversible within a short period of time, are shown in figure 4.4. Panels a-d are sections taken from a continuous trace in the same preparation. A train of submaximal contractures was initiated (figure 4.4a). After addition of cAMP (10^{-7}M), indicated by the shaded bar above the trace, the amplitude of the contracture increased by 57%. Removal of cAMP was followed by a characteristically slow return towards control levels.

Five minutes later the sequence was restarted (figure 4.4b). However, contractures were still significantly above control levels (22%). Reapplication 10^{-7}M cAMP returned the amplitude to that observed on first application

Figure 4.4 Panels (a-d) show sections from a continuous tension record from a saponin-skinned trabecula. A train of contractures was induced by alternately exposing the muscle to a subthreshold 'Ca^{2+}-loading' solution (pCa 6.5) and a '0.2Relaxing' solution with 10mM caffeine (details of experimental protocol are as described in figure 3.1). In panel (a), introduction of 10^{-7}M cAMP caused an increase in the amplitude of the caffeine contracture. Removal of cAMP caused a slow return towards standard levels. Five minutes after removal of cAMP the contractures were still potentiated (panel b). Reintroduction of cAMP 10^{-7}M caused the amplitude of the contractures to increase to a level near that observed in the presence of cAMP in panel (a). Again removal of cAMP was associated with a slow decline in the contracture amplitude towards standard levels. In panel (c), ~15 min. after removal of cAMP, the contracture amplitude had returned to standard levels. Introduction of papaverine had no significant influence on the contractures. Introduction of cAMP (10^{-7}M), in the continued presence of papaverine, increased the amplitude of the caffeine response to that observed in the presence of cAMP (10^{-7}M) in panels (a & b). In panel (d), introduction of cAMP (10^{-6} M) caused an increase in the caffeine response to a final level which was greater than that produced by 10^{-7} M cAMP (as shown in panels a-c). Also shown in panel (d), increasing the [Ca^{2+}] of the 'Ca^{2+}-loading' solution (pCa 6.22), increased the amplitude of the contractures above that observed with cAMP.


(a)
5 mins
10-7M cAMP
20
Tension
mg.wt
0
Caffeine
(10mM)
10-7M cAMP
(b)
10-7 M cAMP
10-5 M Papaverine
(c)
10-6 M cAMP
(d)
[Ca2+]

(4.4a) and again removal of cAMP produced a slow return towards control levels.

In figure 4.4c, ~15 minutes after removal of cAMP the amplitude of the contractures had returned to control levels. Under these conditions, papaverine (10μM) was added to the bathing solution This caused no obvious alteration in the amplitude of the caffeine contracture. cAMP (10^{-7} M) was then reapplied for the third time and again the amplitude increased to that seen in the presence of cAMP in (a) and (b). Thus it would appear that papaverine affected neither steady-state control contractures nor the degree of potentiation induced by 10^{-7}M cAMP.

Figure 4.4d illustrates that raising the cAMP concentration 10 fold to 10^{-6} M caused a further increase in the amplitude of the caffeine response above that observed at 10^{-7}M (ie 77% as compared to 57%). Therefore, in this preparation, 10^{-7}M cAMP produced a submaximal effect. Thus any significant increase in [cAMP] induced by PDE inhibition should have been associated with a greater potentiation of the caffeine response. Checks were made that the degree of s.r. loading in the presence of 10^{-6}M cAMP was not the maximum that could be achieved. Increasing the [Ca^{2+}] in the loading solution from pCa 6.2 to pCa 5.7 further increased the amplitude of the contracture (trace 4.4d). Similar results were obtained in 2 other preparations.

The effect of cGMP on the caffeine contracture

Figure 4.5 illustrates that introduction of cGMP (10^{-6} M -10^{-4}M) was without effect on the steady state caffeine contracture (n=4 preparations). Similarly, cGMP failed to influence the caffeine response following potentiation with cAMP in 2 preparations (results not shown).

Cyclic nucleotides and Ca^{2+}-sensitivity

Neither cAMP nor cGMP influenced the Ca^{2+}-sensitivity of Triton-skinned cardiac muscle (n=6 preparations results not shown). Similarly, no effect was found in 8 saponin-skinned trabeculae using the standard protocol described in chapter 2 (i.e. 30min exposure to 50μg/ml Saponin). The presence of a PDE inhibitor did not itself influence Ca^{2+}-sensitivity nor did it unmask a cAMP-mediated effect in any of the preparations. This is at variance with experiments carried out on cardiac muscle treated with glycerol (Herzig et al, 1981b).

It seemed possible that saponin and Triton-treatments were too 'harsh' and that cellular components associated with the phosphorylation of Troponin-I may be lost or inactivated. Consequently, experiments were carried out on preparations in which the duration of exposure to saponin was reduced arbitrarily by half (10-15min). Data from a typical experiment are shown in figure 4.6.

After brief saponin treatment (10 min), the muscle was cumulatively exposed to a range of [Ca^{2+}]s to determine the Ca^{2+}-sensitivity of the trabecula

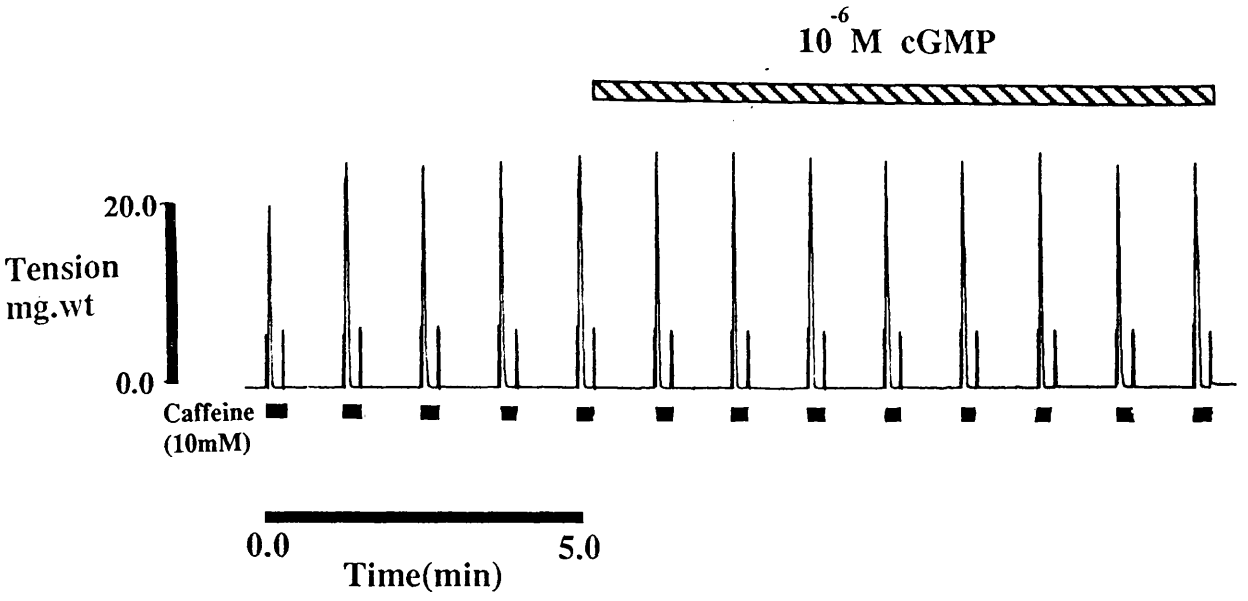


Figure 4.5 shows a train of contractures in a saponin-skinned trabecula (protocol as described in figure 4.1). All solutions were weakly Ca^{2+}-buffered (0.2mM $EGTA_{total}$). Introduction of cGMP (10^{-6}M) had no significant influence on the amplitude of the caffeine-induced contracture.

(figure 4.6a). Under these standard conditions, the threshold for tension was found to be at pCa 6.0 (not shown) and the [Ca^{2+}] required for maximum Ca^{2+}-activated force was ~pCa 4.0. The preparation was then partially activated (pCa 5.5) and cAMP (100μM) added, as shown in figure 4.6b. Under these experimental conditions, cAMP caused a slow decline in force which was not readily reversible on removal of the compound. Ca^{2+}-sensitivity was then determined in the presence of cAMP (4.6c). It is obvious that the Ca^{2+}-sensitivity of the trabecula was slightly reduced, as was maximum Ca^{2+}-activated force (~6%).

At face value these results are qualitatively very similar to those obtained by Winegrad and Herzig et al (see introduction). However, there are a number of problems regarding the present study. In particular, if the trabecula was observed by light microscopy, red blood cells were clearly visible in a central core but absent in capillaries closer to the surface of the preparation. Furthermore, if the [Ca^{2+}] in the bathing solution was increased, in the presence of a high concentration of EGTA, localised oscillations could be observed in the central core of the muscle. These were evidenced by the cyclic shortening and lengthening of sarcomeres and the movement of blood cells in capillaries. This phenomenon was occasionally, but not always, accompanied by oscillations in tension. The most obvious explanation for this behaviour is that the duration of exposure to saponin was only sufficient to perforate more superficial cells, leaving an unskinned core.

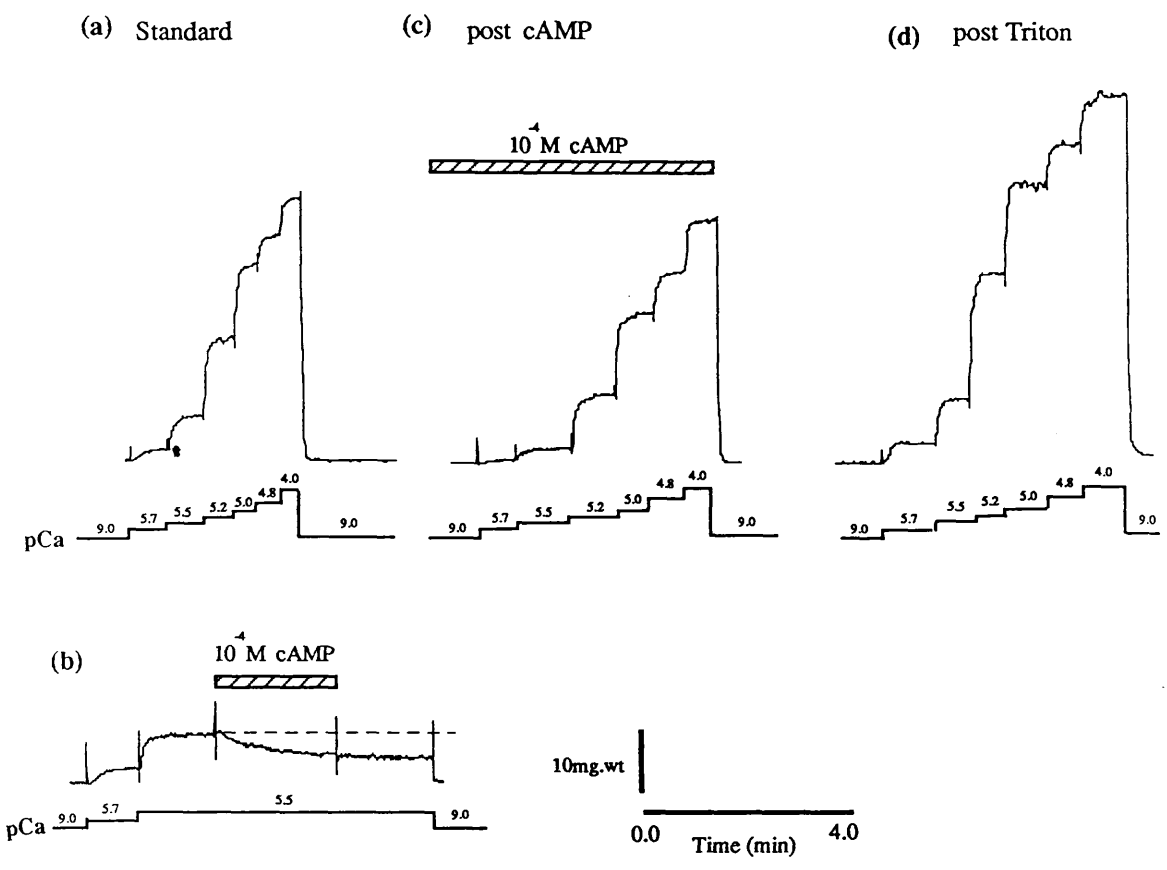


Figure 4.6 shows tension responses in a trabecula 'briefly' treated (10-15min) with saponin (panels a,b & c) and following Triton-skinning (panel d). All solutions were strongly buffered (10mM $EGTA_{total}$). In panel (a) the pCa-tension relationship was obtained by increasing the $[Ca^{2+}]$ over the range pCa 9.0-4.0. The preparation was then submaximally activated at pCa 5.7(panel b). At this point cAMP (10^{-4}M was added. This was associated with a decrease in tension which was not readily reversible on removal of cAMP. Panel (c) shows the pCa tension relationship observed in the presence of cAMP (10^{-4}M). Panel (d) shows the pCa-tension relationship after subsequent skinning with Triton (30 min).

In order to investigate this possibility, the trabeculae were treated with 1% Triton X-100 for a further 30 min. to disrupt all remaining cellular membranes. The pCa-tension relationship was determined again. After Triton treatment, there was a marked increase in C_{max} (29%) and Ca^{2+}-sensitivity (figure 4.6d). To illustrate this point more clearly, the raw data illustrated in figure 4.6 are shown plotted in figure 4.7a in absolute force and normalised in figure 4.7b. The data were well fitted by the Hill equation. Addition of cAMP increased the $[Ca^{2+}]$ required for half maximum activation from pCa 5.2 to pCa 5.09. However, after Triton-skinning, sensitivity increased slightly beyond that found under standard conditions (EC_{50}=pCa 5.24). The phenomena described above were reproducible although there was some variability in the apparent decrease in Ca^{2+}-sensitivity induced by cAMP and the potentiation of maximum Ca^{2+}-activated force after Triton-skinning. Results from 4 preparations are summarised in table 4.1.

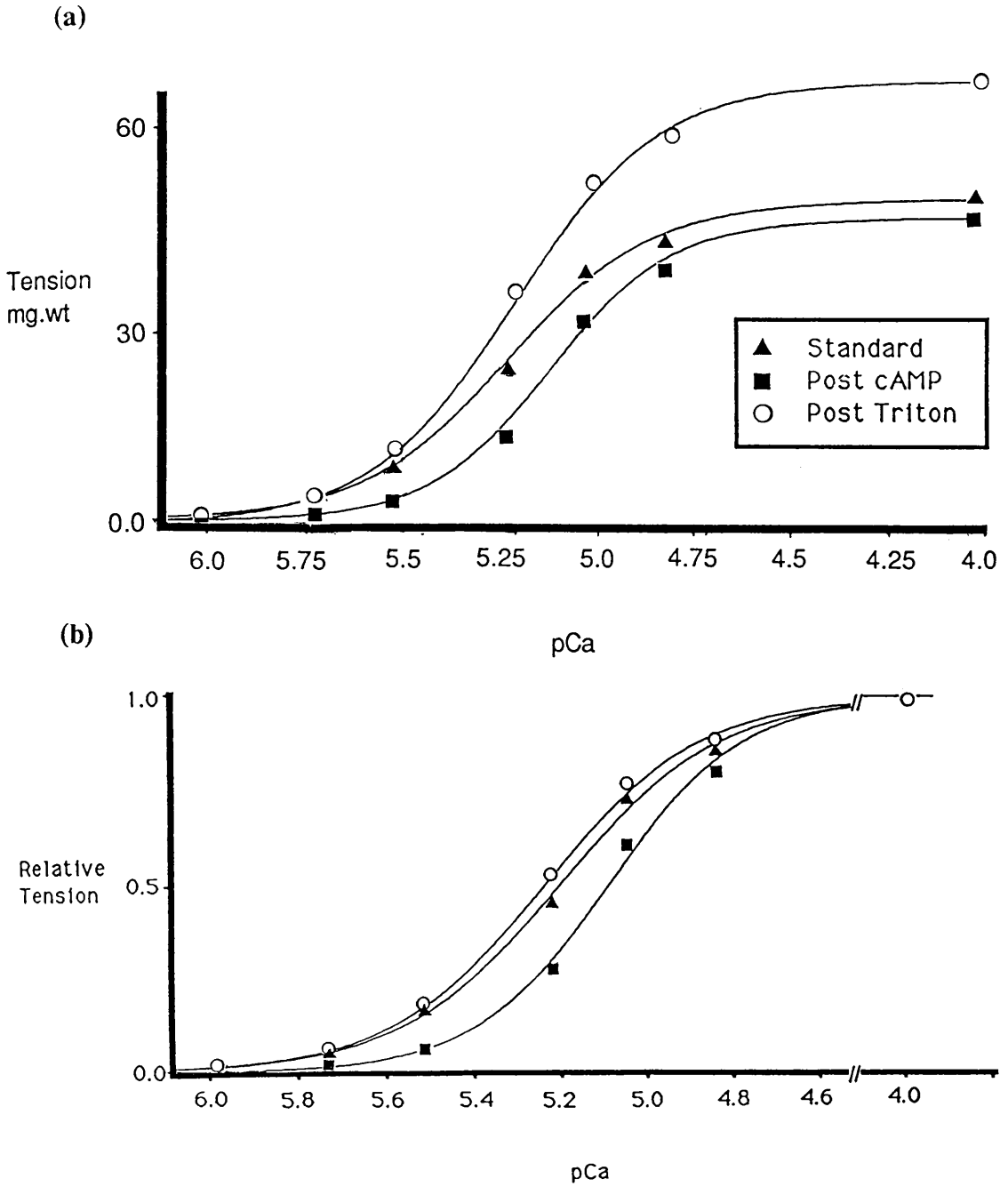


Figure 4.7 The raw data shown in figure 4.7 are plotted in absolute tension (panel a) and relative to C_{max} (panel b).

Table 2.1

Preparation number	Standard	post cAMP	post Triton	% Increase in Cmax post Triton
	pCa required for half Cmax			
(i)	5.20	5.09	5.24	29
(ii)	5.49	5.38	5.45	21
(iii)	5.1	5.05	5.18	35
(iv)	5.31	5.25	5.33	27

DISCUSSION

Forskolin Toxin and the activity of adenylate cyclase

Introduction of forskolin toxin (10^{-6}M) reproducibly increased the amplitude of the caffeine contracture. The effect usually represented around 20-30% (figure 4.1b) of the standard contracture although in one preparation a much larger response was observed (figure 4.2). Forskolin (10^{-6}M - 10^{-5}M) had no effect on the Ca^{2+}-sensitivity of the contractile proteins in saponin or Triton-skinned trabeculae, when the Ca^{2+}-concentration was strongly buffered with EGTA (10mM). Forskolin toxin is believed to activate membrane-bound adenylate cyclase by a direct action on the catalytic subunit. The simplest interpretation of this result is that a significant quantity of functionally-active adenylate cyclase persists after saponin-skinning. This is possible because saponin treatment retains the sarcolemma but perforates it by the precipitation of cholesterol (Endo & Kitazawa, 1978). Furthermore, it is believed that forskolin-stimulated cAMP production does not require the presence of guanine nucleotides or a functional stimulatory G-protein. Thus relatively little of the pathway leading to cAMP production need be intact for the reaction to proceed.

The action of forskolin on the s.r. requires sufficient cAMP production to influence the s.r. despite its outward diffusion. Indeed, a high basal level of cAMP has been measured in single cardiac cells skinned with the saponin-like compound digitonin (Fry et al, 1989). In this this preparation

[Ca^{2+}] uptake was unaffected by introduction of cAMP (10^{-4}M). The authors concluded that under these conditions the cells maintained a high [cAMP] and consequently, further addition of cAMP was without effect. However, it should be noted that the concentration of cells in the suspensions used by Fry et al was considerably higher than in this study. Thus in saponin-skinned trabeculae there is presumably lower basal [cAMP] and this could explain why an effect on the s.r. was observed.

cAMP and the caffeine contracture

Application of exogenous cAMP consistently (n=15 preparations) potentiated the caffeine contracture and was interpreted as an increase in the rate of Ca^{2+} accumulation by the s.r. This result confirms the biochemical evidence and experiments carried out on skinned cardiac cells described in the introduction (Tada et al, 1974, Fabiato & Fabiato, 1975a). However, there was considerable variation between preparations in the sensitivity and response to cAMP. This could be explained if there was a basal level of cAMP production which varied from muscle to muscle (see above). Under these circumstances, one would expect little response until the [cAMP] in the bathing medium was greater than that within the preparation.

An analogous situation occurred when the effect of inorganic phosphate (P_i) was investigated in Triton-skinned cardiac trabeculae (Kentish, 1986). P_i depressed Ca^{2+}-sensitivity in a dose dependent manner. As P_i is a metabolite of ATP, hydrolysis it accumulates within the trabecula during a

sustained contracture. As a consequence of the diffusion limited exchange with the bathing solution, it could be predicted that greater P_i accumulation would occur in preparations of larger diameter. It was proposed that this could explain the lower P_i sensitivity observed in large trabeculae. With P_i, the situation was relatively predictable and Kentish was able to calculate the theoretical mean [P_i] accumulated at a given muscle radius, ATPase rate and degree of activation.

In contrast, the situation regarding cAMP is less clear. If it is assumed that there is a basal level of cAMP production capable of influencing the functioning of the s.r. then there are a number of possible influences: (1) The rate of cAMP production by adenylate cyclase (2) the breakdown of cAMP by cellular enzymes (3) the amount of active adenylate cyclase retained after saponin treatment (4) the diameter of the muscle and hence the rate of loss of cAMP by diffusion to the bathing solution.

Considering the number of possible influences, it is perhaps not surprising that the trabeculae vary in responsiveness to exogenous cAMP. However, it does not explain why it proved difficult to obtain a graded response to cAMP. This could occur if the basal concentration of cAMP was comparatively high and hence the phosphorylation of phospholamban nearly complete. For practical convenience the [cAMP] was increased by a factor of ten each time. Such an increase could in theory change the degree of phosphorylation from say 80% to 100% rendering the muscle insensitive to another ten fold increase. Unfortunately because of the poorly reversible

nature of the effect, 'fine tuning' of the dose response relationship was rarely possible and this made the following experiments difficult to carry out.

Phosphodiesterase and the caffeine response

Application of papaverine (10μM) had no effect on steady state submaximal caffeine contractures in 6 saponin skinned trabeculae. Furthermore, as shown in figure 4.4 papaverine was similarly without influence on caffeine contractures potentiated with a submaximal dose of exogenous cAMP. The results are consistent with there being either no cAMP production or no residual PDE activity.

If it is assumed (see above) that there is a basal level of cAMP produced by the muscle then this would suggest that there is little PDE activity. There are several possible reasons why PDE activity may be absent in saponin-treated muscle. Cytosolic PDE may be lost to the surrounding medium through the perforations in the cell membrane. However, this seems unlikely because the sarcolemmal perforations produced by saponin are believed to be small enough to retain such enzymes (Endo & Kitazawa, 1978). Another possible explanation is that the apparent inactivity of PDE is a result of the experimental protocol employed in this type of experiment. The trains of contractures used to assay the s.r. Ca^{2+}-content are produced by the repeated application and removal of 10mM caffeine. This compound, in common with other methyxanthines is a PDE inhibitor and its presence may inactivate the enzyme.

Cyclic nucleotides and Ca^{2+}-sensitivity

Neither cAMP nor cGMP had any effect on the Ca^{2+}-sensitivity of trabeculae treated with saponin or Triton using the standard protocol described in chapter 2. However, if the length of exposure to saponin was halved, cAMP caused an apparent depression in Ca^{2+}-sensitivity while cGMP was still without effect. Under these circumstances, the characteristics of the response to cAMP were similar to those described by Winegrad et al (see introduction).

However, in this study at least, there is no doubt that the apparent depression in Ca^{2+}-sensitivity is mediated by a central unskinned core. Evidence of an unskinned region was obtained by microscopic observation of the trabecula which revealed the movement of blood cells in capillaries. The cells of the central core were also seen to oscillate when the $[Ca^{2+}]$ was increased, despite the presence of 10mM EGTA. Such oscillations only produced a noticeable effect on tension if they were synchronised. This indicates that core cells were partially permeable to $[Ca^{2+}]$ but not to EGTA. Furthermore, after Triton-skinning, maximum $[Ca^{2+}]$ activated force always increased significantly in such preparations.

It is difficult to reconcile these results with those of Winegrad et al who reported that, after detergent treatment, cAMP was without effect and attributed this to a response which necessitated the "lipid phase" of the surface membrane (McClellan & Winegrad, 1978). Similarly the present study

found no effect of cAMP in detergent-treated trabeculae. However, there was also no effect of cAMP, in preparations treated with saponin for ~30min. As saponin retains but perforates the sarcolemma, this suggests that the depression in Ca^{2+}-sensitivity observed by Winegrad requires that the membrane is not only present but intact. In the present experiments after brief saponin treatment, cAMP apparently depresses Ca^{2+}-sensitivity but peak force is considerably enhanced with Triton. This is not in agreement with the findings of Winegrad (McClellan & Winegrad, 1980) who observed that in EGTA-treated preparations peak force was not enhanced by further Triton-skinning, but this enhancement was observed by Jewell & Kentish (1984) on identical preparations.

One interpretation of the situation is that the unskinned core of the briefly saponin-treated trabeculae is in a similar state to that of EGTA-treated preparations as used by Winegrad. It is likely that these cells are only semi-permeable to Ca^{2+} and relatively impermeable to EGTA (Smith & Miller, 1985, Jewell & Kentish, 1984). Unfortunately, under these circumstances the modulation of force by cAMP cannot confidently be attributed to an effect at the level of the myofilaments. For example it could be explained equally well by a direct influence of cAMP on the s.r., thereby lowering the $[Ca^{2+}]$. Alternatively an indirect effect of cAMP on intracellular pH by stimulation of s.r. ATPase could also cause an apparent depression in Ca^{2+}-sensitivity (Kentish & Jewell, 1984).

However, the results obtained in this study cannot be reconciled with those of Herzig and his collaborators. Herzig et al (1981b) observed a cAMP-induced depression in Ca^{2+}-sensitivity in glycerol-extracted, Lubrol-treated pig septomarginalis fibres. It seems unlikely that tissue exposed to this skinning procedure could retain cells which are impermeable or unskinned. However, it should be noted that preliminary studies carried out in this laboratory found that Brij and Lubrol treatments required a considerably longer time for membrane disruption to occur.

In this study there was no effect of cAMP on the contractile proteins but a pronounced effect on the s.r. in trabeculae skinned with saponin for >30min. This is of interest because the positive action on the s.r. indicates that phosphorylation reactions can occur. This highlights the fact that there is little evidence in support of nucleotide regulation of the contractile proteins in preparations which are "unequivocally" skinned. There is, of course, evidence from other experimental techniques, in particular, experiments carried out on aequorin-injected papillary muscles. Changes in the relationship between intracellular [Ca^{2+}] and tension during α and β-receptor stimulation have been interpreted as changes in myofilament Ca^{2+}-sensitivity (Allen & Kurihara 1980, Blinks & Endoh, 1986, McIvor et al 1988). However, the intracellular Ca^{2+} transient may be influenced by many factors including Ca^{2+}-uptake by the s.r., Ca^{2+}-entry via the sarcolemma, myofilament Ca^{2+}-sensitivity and artefacts caused by Ca^{2+} gradients. Consequently the

interpretation of changes in the light transients under these conditions remains equivocal (McIvor et al, 1988).

In conclusion

The effects of forskolin toxin indicate that adenylate cyclase activity continues after saponin treatment and introduces the possibility that there is a basal level of cAMP production. The s.r. was sensitive to the application of exogenous cAMP but the contractile apparatus was not under standard skinning conditions (see chapter 2). Under no circumstances did inhibition of PDE in skinned fibres modify the responses of the s.r. At very least, the results demonstrate that inhibition of PDE is unlikely to contribute to the effects of natural and synthetic modulators of Ca^{2+}-metabolism described in the following chapters. However, substances which influence adenylate cyclase activity could modify the functioning of the s.r.

Chapter 5: **Effects of caffeine and sulmazole on saponin and Triton-skinned trabeculae**

INTRODUCTION

Sulmazole and the contractile proteins

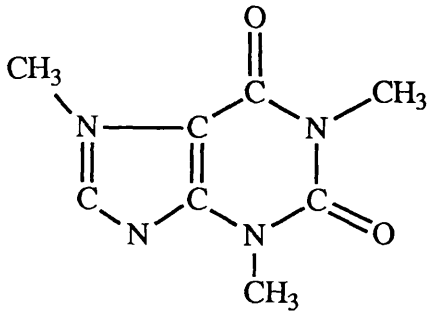
The benzimidazole derivative sulmazole (ARL115BS) belongs to a class of novel inotropic agents designed to act at the level of the myofilaments (Wetzel & Hauel, 1988). Sulmazole produces a dose-dependent increase in the Ca^{2+}-sensitivity of the contractile proteins in chemically-skinned cardiac muscle (Herzig, Feile & Rüegg, 1981, van Meel, 1987, van Meel et al, 1988). Herzig et al (1981) reported that the Ca^{2+}-sensitising action of sulmazole was unique as it was not mimicked by caffeine or other phosphodiesterase inhibitors. However, it was subsequently demonstrated that caffeine does increase Ca^{2+}-sensitivity in both skinned skeletal and cardiac preparations (Wendt & Stephenson, 1983). The reasons for this discrepancy remain unclear. The structures of sulmazole and caffeine are shown in figure 5.1a.

Sulmazole increased Ca^{2+}-binding and ATPase activity in isolated canine myofibrillar preparations (Solaro & Rüegg, 1982). Thus, the effect on Ca^{2+}-sensitivity may result from an increase in the affinity of troponin-C for Ca^{2+}. However, sulmazole and caffeine were without influence on the Ca^{2+}-affinity of isolated bovine cardiac troponin-C (Jaquet & Heilmeyer, 1987, Kentish & Palmer, 1989). This has led to the suggestion that the action of these substances on skinned muscle may require the presence of other myofibrillar proteins such as troponin-I or T.

(a)

(b)

Caffeine



Sulmazole

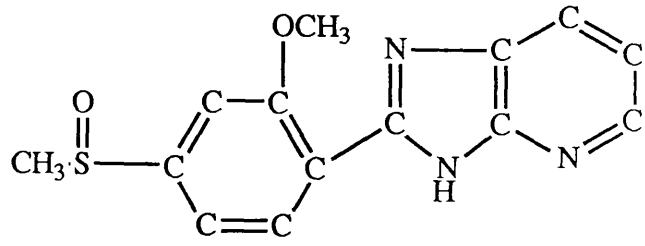


Figure 5.1 The structures of the methylxanthine caffeine (a) and the benzimidazole derivative sulmazole are illustrated (b).

Effects on cardiac sarcoplasmic reticulum.

Sulmazole induces transient contractures in intact (Walland, 1985) and mechanically-disrupted cardiac preparations (Trube & Trautwein, 1981). Such a result cannot necessarily be attributed to the release of Ca^{2+} from the s.r. when a compound also influences Ca^{2+}-sensitivity (Wendt & Stephenson, 1983). Experiments on isolated s.r. demonstrated that sulmazole (1mM) had little or no effect Ca^{2+}-release but, unlike caffeine in this preparation, inhibited Ca^{2+}-uptake (Hasselbach et al, 1981, Lichtner et al, 1981). On the other hand, sulmazole (10mM) caused a transient Ca^{2+}-release in suspensions of cardiac cells skinned with saponin (Fry et al, 1989). Furthermore, sulmazole (1mM) doubled the Ca^{2+}-current in isolated cardiac s.r. channels (Ashley & Williams, 1988). This was associated with an increase in the open probability without influence on the single channel conductance.

The aim of this study

It is well known that caffeine triggers release of Ca^{2+} from the s.r. (Weber and Herz, 1968). Consequently caffeine is widely used as a tool for estimating the Ca^{2+}-content of the s.r., in both skinned and intact muscle preparations. More recently, as a result of its greater solubility, sulmazole has been used as a caffeine substitute. Higher solubility represents a considerable advantage when concentrated aliquots of drug must be added to a fixed volume of solution. (Fry et al 1989, Williams et al 1988, Williams et al 1989).

While the results described above suggest that sulmazole and caffeine share certain characteristics, there is little information concerning their relative potencies on the intracellular systems and particularly the s.r. The object of this study was to compare directly the effects of caffeine and sulmazole on the s.r., using chemically-skinned cardiac preparations. Particular reference is paid to the problems which occur when such compounds are used to gauge s.r. Ca^{2+}-release in light of the concomitant increase in Ca^{2+}-sensitivity.

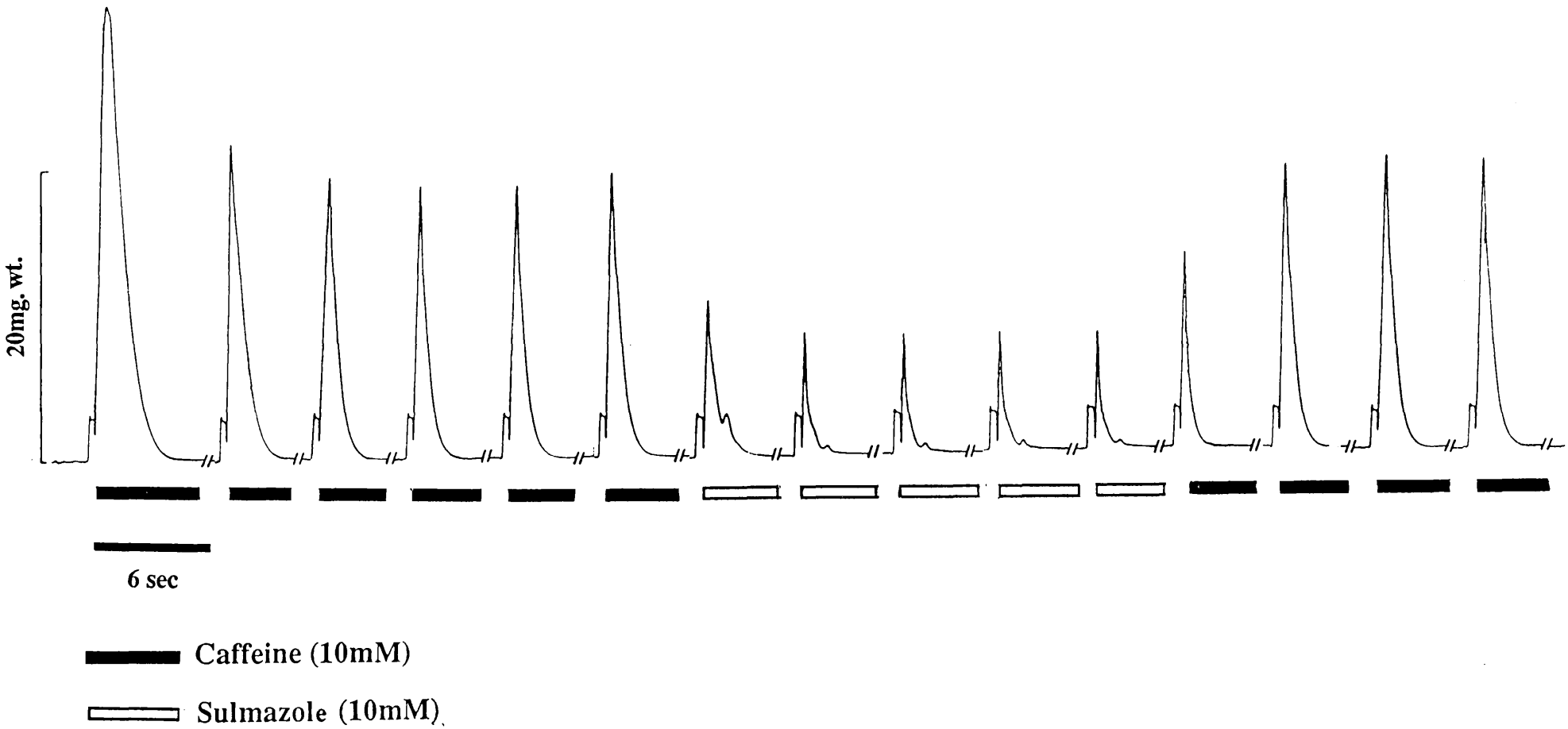


Figure 5.2 shows the protocol employed to compare the effiacy of caffeine and sulmazole to induce s.r. Ca-release. A train of contractures in response to caffeine was induced in a saponin-skinned rat trabecula (for protocol see figure 3.1). All soluutions were weakly Ca-buffered (0.2mM EGTA). At the point indicated by the break in the trace (-//-) a 55sec. exposure to a caffeine-free (or sulmazole-free) solution (pCa 6.5) has been omitted. The presence of caffeine (10mM) is illustrated by the shaded bar below the contractures and the presence of sulmazole (10mM) replacing caffeine in the sequence, is indicated by the plain bar.

RESULTS

Protocol to determine s.r. Ca^{2+}-release

Sulmazole induced caffeine-like contractures by promoting the release of Ca^{2+} from the s.r. Figure 5.2 illustrates the protocol used. A train of reproducible caffeine contractures was induced in a saponin-skinned trabecula. This involved alternately exposing the muscle to a Ca^{2+}-loading solution (pCa 5.7) and standard '0.2Relaxing' solution (as defined in chapter 2) with caffeine (10mM). All solutions were weakly Ca^{2+}-buffered with 0.2mM EGTA. For further details of this experimental protocol see chapter 3. At the point illustrated by the shaded bar above the trace, caffeine (10mM) was replaced by sulmazole (10mM).

The relative potencies of caffeine and sulmazole to induce Ca^{2+}-release

Sulmazole is apparently not as potent as caffeine in its ability to release Ca^{2+} from the s.r. (figure 5.2). In figure 5.3, contractures produced in response to 10mM caffeine and various concentrations of sulmazole (0.5-10mM) have been superimposed. All contractures are from the same preparation. This figure illustrates the dose-dependence of the sulmazole contracture. Tension oscillations were frequently observed during the relaxation phase of sulmazole contractures. This may be a characteristic of the less complete Ca^{2+}-release by sulmazole, as similar behaviour was

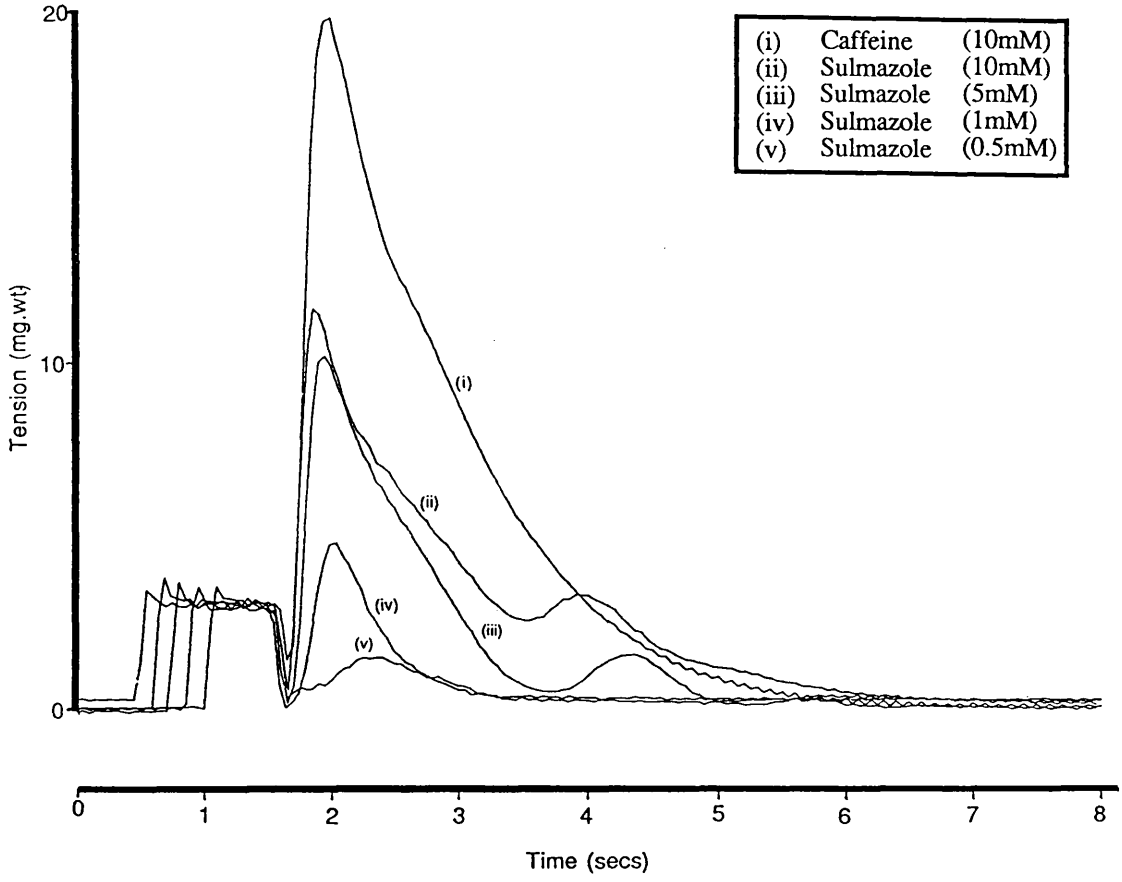


Figure 5.3 shows a tension record from a saponin-treated trabecula. Steady-state contractures selected from trains of responses (protocol as in figure 5.2) initiated by various concentrations of sulmazole and caffeine have been superposed with respect to the instant of immersion in the contracture evoking solutions.

observed in contractures produced by lower concentrations of caffeine (not shown).

Cumulated data on Sulmazole and caffeine-induced Ca^{2+}-release

Figure 5.4 shows accumulated data from 14 saponin-skinned trabeculae. Contraction amplitude is plotted on the ordinate and drug concentration on the abscissa. Each point represents the mean contracture amplitude ($\pm$ SD.), expressed as a percentage of the mean amplitude of contracture to 10mM caffeine during the preceding control sequence (protocol as in figure 5.2). To the right of each point, the second number in brackets represents the number of preparations and the first, the total number of contractures at that drug concentration.

This figure illustrates that the ability of caffeine to induce Ca^{2+}-release is almost saturated by around 5-10mM under these experimental conditions. This result is consistent with a previous study (Harrison, 1985) where saponin-skinned trabeculae were loaded at a suprathreshold [Ca^{2+}] and a high Ca^{2+}-buffer capacity (10mM EGTA). By comparison, sulmazole consistently induced a smaller contracture than caffeine at each concentration. Contractures in response to 10mM sulmazole were on average 57% of those produced by 10mM caffeine.

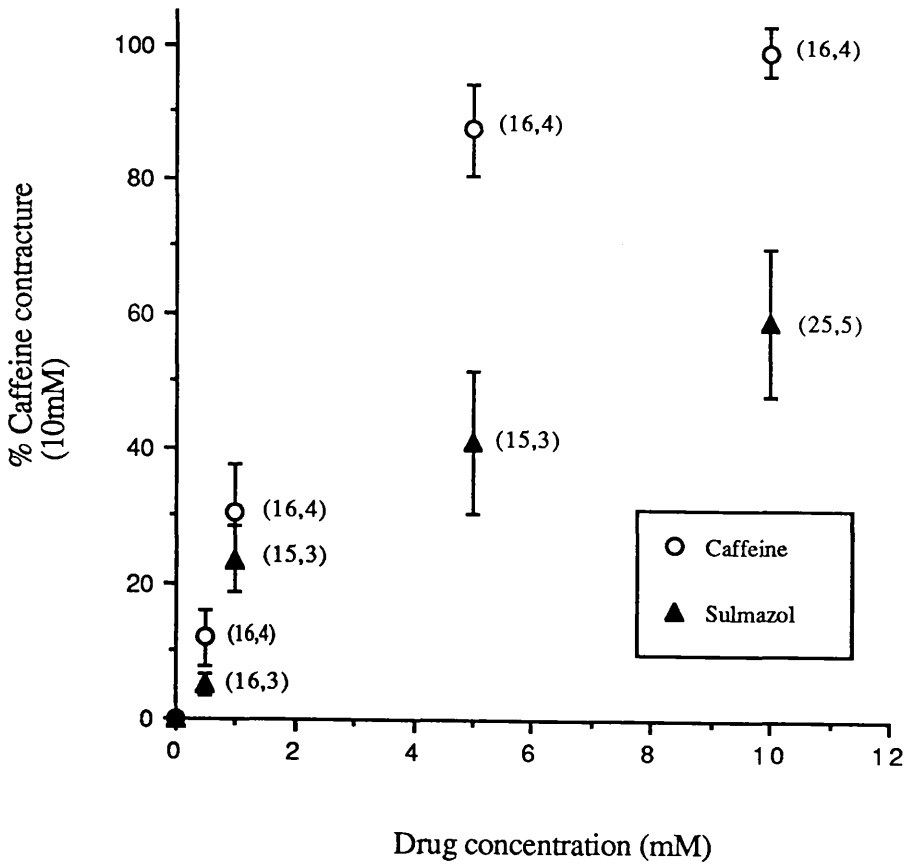


Figure 5.4 The diagram shows accumulated data from 14 saponin-skinned trabeculae. The dose dependence of sulmazole and caffeine induced Ca^{2+}-release is illustrated. Each point represents the mean contracture amplitude (± SD), expressed as a percentage of the mean amplitude of contracture to 10mM caffeine (protocol as in figure 5.2). To the right of each point, the second number in brackets represents the number of preparations and the first, the total number of contractures at that drug concentration.

Contribution of increased Ca^{2+}-sensitivity to contracture amplitude

Millimolar concentrations of caffeine and sulmazole produce transient contractures in saponin-skinned trabeculae (as illustrated above). However, as noted in the introduction, both compounds are known to increase the Ca^{2+}-sensitivity of the contractile proteins (Herzig et al, 1981a, Wendt & Stephenson, 1983, van Meel, 1987, van Meel et al, 1988). Thus, comparing the amplitude of caffeine and sulmazole contractures may not be an appropriate index of their relative ability to induce Ca^{2+}-release if they differ in potency as Ca^{2+}-sensitisers.

During a sulmazole or caffeine contracture, the drug concentration, and hence Ca^{2+}-sensitivity, increases with a time course limited by diffusion. Figure 5.5 shows contractures induced by the introduction of 10mM caffeine (a) or 10mM sulmazole (b) in the same saponin treated trabecula. In each case, the [EGTA] and pCa are shown to the right of each tension response.

The transient contractures were produced using the standard protocol illustrated in figure 5.2. In brief, the muscle was first exposed to a subthreshold Ca^{2+}-loading solution and then to a solution containing sulmazole or caffeine. The Ca^{2+}-buffer capacity was low throughout this procedure (0.2mM $EGTA_{total}$). Thus the resulting contracture has a large component of s.r.-Ca^{2+} release. Consistent with the results shown in figures 5.2, 5.3 & 5.4 the peak amplitude of the sulmazole contracture was approximately half that to caffeine.

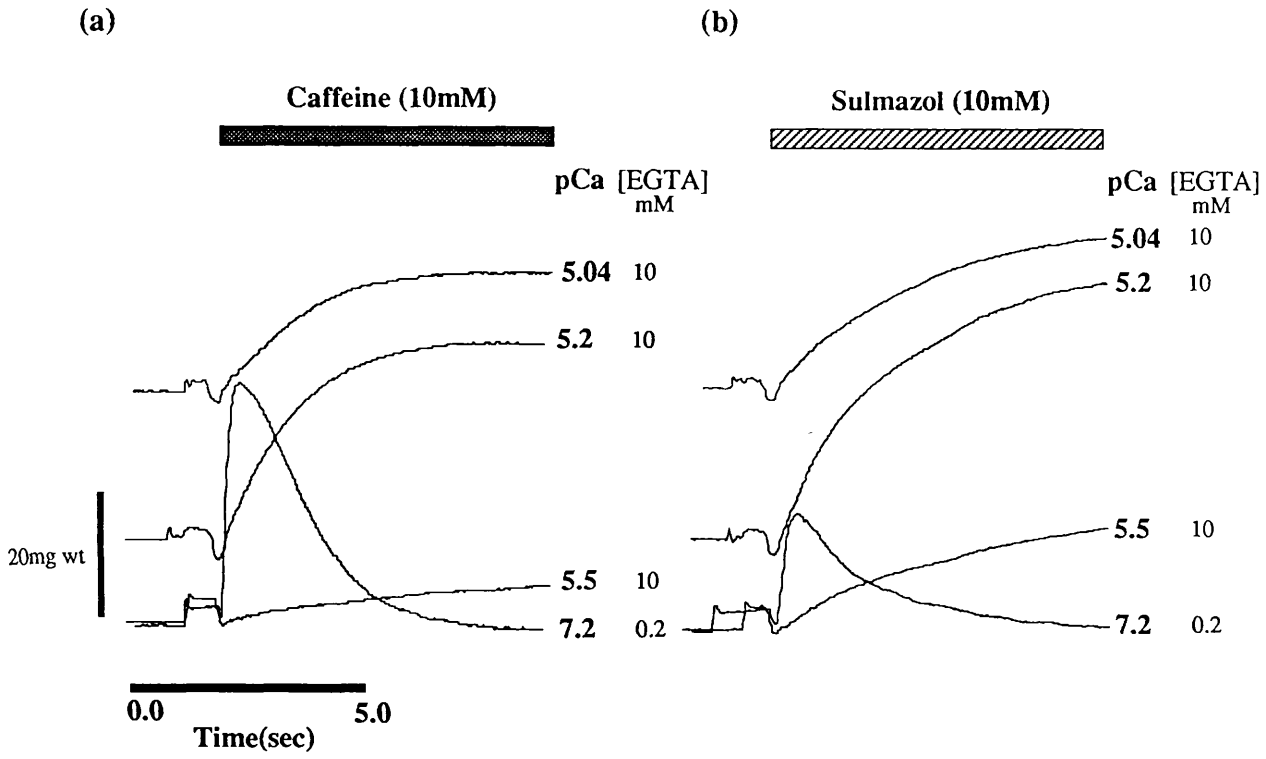


Figure 5.5 shows contractures in a saponin-skinned trabecula in response to 10mM caffeine and sulmazole. The presence of the drugs is indicated by the bar above each trace. In (a) and (b), transient responses were produced by application of the drugs following 'Ca^{2+}-loading' in a weakly Ca^{2+}-buffered solution (0.2mM $EGTA_{total}$) with a [Ca^{2+}] subthreshold (pCa 6.52) for tension production (protocol as for figure 5.2). In (a) and (b) the maintained responses were produced following introduction of the drugs in solutions with a high Ca^{2+}-buffer capacity (10mM $EGTA_{total}$) at various [Ca^{2+}] (shown to the right of each trace). Maintained responses are caused by the increase in the Ca^{2+}- sensitivity of contractile proteins as a contribution from s.r.-derived Ca^{2+} is eliminated by the high Ca^{2+}-buffer capacity. Transient responses in weakly Ca^{2+}-buffered solutions are the consequence of both s.r. Ca^{2+}-release and increasing Ca^{2+}-sensitivity.

Maintained tension responses were produced by exposing the muscle to a [Ca^{2+}] on (pCa 5.75) or above the threshold for tension. Once a steady state was reached, the preparation was transferred to an identical solution containing 10mM sulmazole or caffeine. Throughout this procedure the [Ca^{2+}] was strongly buffered with EGTA (10mM) to prevent Ca^{2+} released from the s.r. contributing to tension development. Thus, the slow increase in tension reflects a rise in myofilament Ca^{2+}-sensitivity. Although the drug concentration increases with the same time course in all contractures, the rate of rise of tension and the final tension level is dependent upon the [Ca^{2+}]. This phenomenon is consistent with the shape of the pCa-tension relationship and hence different at each level of activation. It should be noted that tension had not reached a steady state within the time scale shown in the diagram.

The figure illustrates that there is little increase in Ca^{2+}-sensitivity with either drug by the time the transient contractures reach their peak. Furthermore, the small increase in force (attributable to Ca^{2+}-sensitising) within this time scale is of a similar magnitude whether it is produced by the addition of sulmazole or caffeine. This occurs despite the much larger shift in sensitivity produced by sulmazole, demonstrated by the greater force enhancement seen once full equilibration of the drug has taken place. This is illustrated in figure 5.6 which shows the steady state pCa tension relationship under control conditions, in the presence of sulmazole (10mM) and caffeine (10mM) in the same preparation as for figure 5.5. Caffeine and

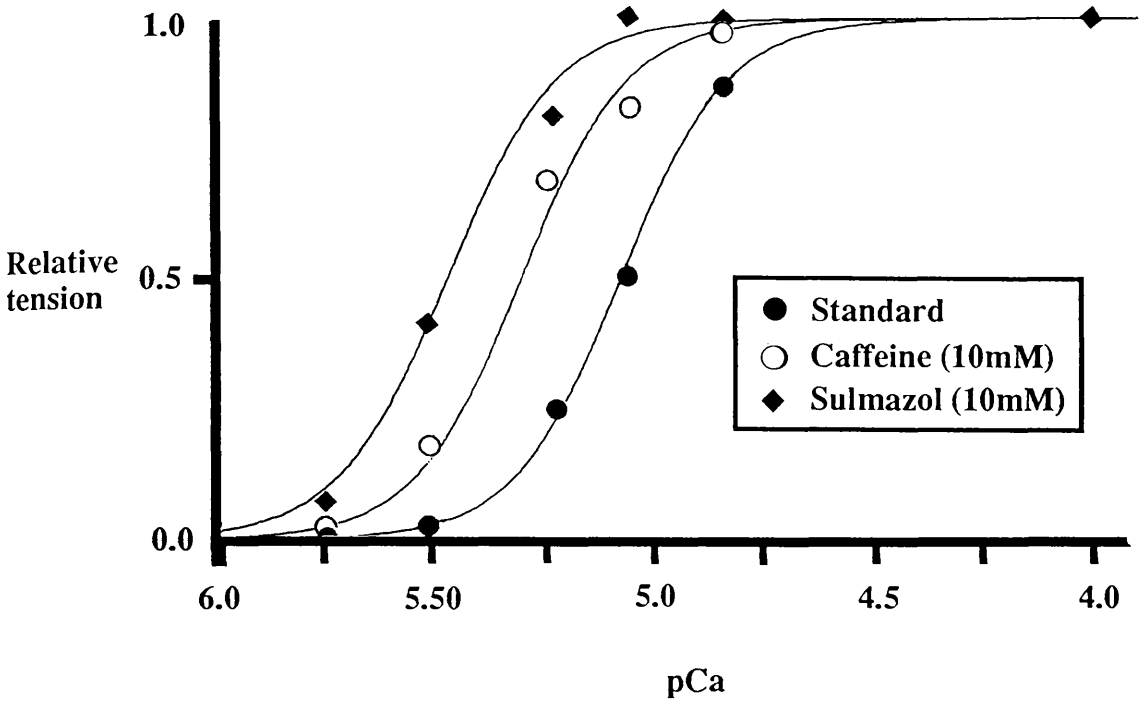


Figure 5.6 shows the steady-state increase in Ca^{2+}-sensitivity with 10mM caffeine and sulmazole in the same saponin-skinned trabecula used to produce figure 5.5. The solid lines are best fit curves to the Hill equation (see chapter 2). Caffeine and sulmazole decrease the $[Ca^{2+}]$ required for half maximal activation by 0.22 and 0.4 log units respectively.

sulmazole increased the pCa required for half-maximal activation by 0.23 and 0.40 pCa units respectively.

Effect of caffeine and sulmazole on CICR. oscillations.

If the [Ca^{2+}] of the bathing solution is raised above the threshold for tension, large oscillations occur which result from the spontaneous release of Ca^{2+} from the s.r. In figure 5.7, panel a shows that caffeine (0.1-1.0mM) reduces the magnitude but increases the frequency of the oscillations in a dose dependent manner. The lower panel illustrates that sulmazole is approximately equipotent with caffeine in its ability to inhibit the oscillations. Both compounds abolish the oscillations at 1mM. Similar results were obtained in 6 other preparations. This result is consistent with a previous study on skinned single cardiac cells (Trube & Trautwein, 1981). The dose dependence of this result is consistent with the inhibition of net Ca^{2+}-accumulation by caffeine (for discussion see chapter 3).

Effects on maximum Ca^{2+}-activated force

Figure 5.8 (a) and (b) illustrate the effect of cumulative doses of caffeine and sulmazole (0.1-40mM) on submaximal (pCa 5.2) and maximal (pCa 4.0) Ca^{2+}-activated force (C_{max}). All records are from the same Triton-skinned trabecula. Caffeine and sulmazole (0.1-10mM) produced a dose-dependent increase in submaximal force production. However, when the concentration was increased further to 40mM, sulmazole had little effect

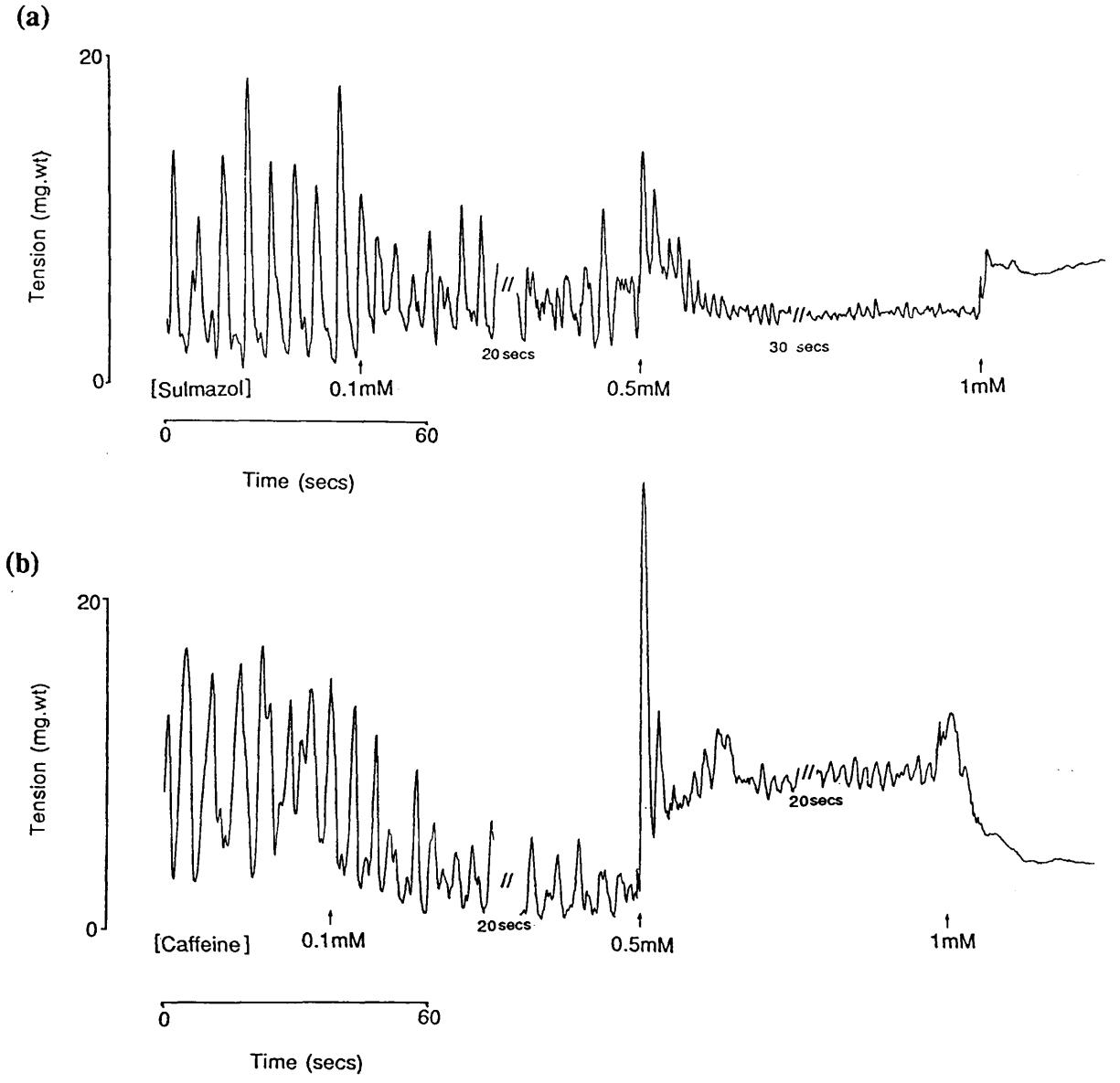


Figure 5.7 Panels (a) and (b) show the effects of caffeine and sulmazole (0.1-1mM) on CICR oscillations. Both traces are from the same saponin-skinned trabecula. The basic ('0.2Relaxing') solution was identical throughout the experiment (0.2mM EGTA $_{total}$, pCa 5.31). Drugs were added cumulatively as indicated by the arrows. Changes in the level of the trace are not of relevance as in this case they are largely the consequence of unequal solution levels.

while caffeine decreased force back to pre-drug levels. Up to 10mM caffeine had little or no influence on C_{max}. while 40mM caffeine depressed C_{max} by 37%. In contrast, sulmazole (0.1-10mM) increased C_{max} in a dose-dependent manner. The potentiation of C_{max} was reduced by ~5% in the presence of 40mM sulmazole.

It is not clear whether the reduced potentiation by 40mM sulmazole is genuine or artefactual. In this experiment no correction has been made for ionic strength. High ionic strength depresses C_{max} without effect on Ca^{2+}-sensitivity (Kentish, 1984, Fink et al, 1986). Figure 5.8(c) shows the effect of increasing the ionic strength by the addition of 40mM KCl. However, the contribution of sulmazole to ionic strength depends on the degree of dissociation under these experimental conditions. Unfortunately this is currently unknown. With caffeine, no correction for ionic strength is required as this drug is not ionised at neutral pH.

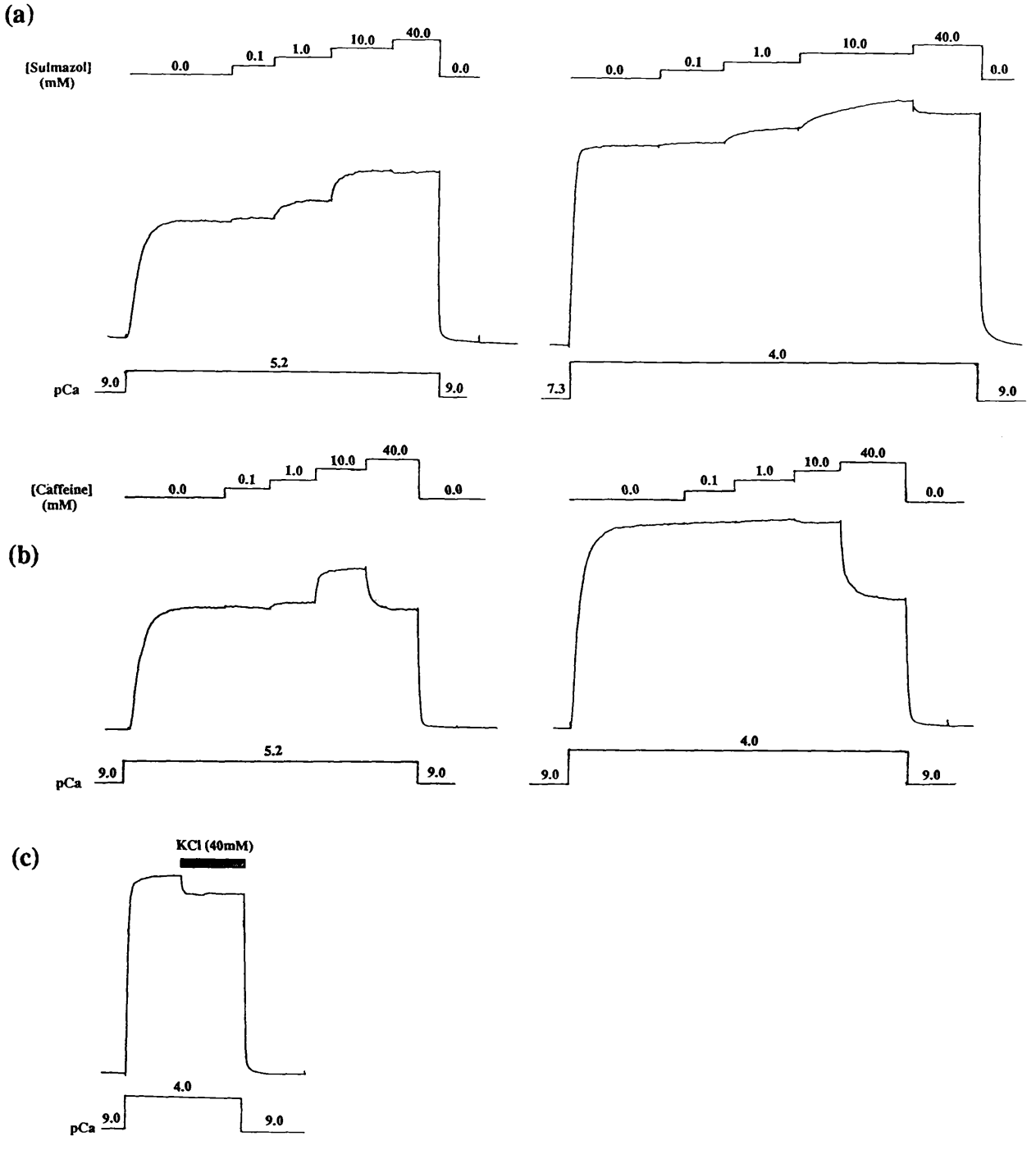


Figure 5.8 Panels (a) and (b) show the effects of increasing caffeine and sulmazole concentrations (0.1-40mM) on C_{max} and submaximal isometric tension. Panel (c) illustrates the depression in C_{max} produced by addition of KCl (40mM). All responses are in the same Triton-skinned rat trabecula. Solutions were strongly Ca^{2+}-buffered (10mM EGTA $_{total}$).

DISCUSSION

Effects on the sarcoplasmic reticulum

Figures 5.2,5.3,5.4 illustrate that sulmazole was consistently less potent than caffeine in its ability to induce a transient contracture in saponin-skinned trabeculae. As shown in figure 5.5, this difference reflects the relative ability of these substances to induce s.r. Ca^{2+}-release, with little influence from the concomitant increase in Ca^{2+}-sensitivity. This is at variance with a previous studies on cardiac cell fragments in which Sulmazole was reported to be equipotent with, or more potent than, caffeine (Trube and Trautwein, 1981). There are several possible explanations for this discrepancy;

(1) The sulmazole may be less pure than that used by Trube & Trautwein. This was discounted as the drug used in the present study was from three separate batches obtained directly from Boehringer Ingelheim GmbH.

(2) Differences in the state of the s.r. prior to contracture may explain the discrepancy. In this study considerable care was taken to standardise the loading conditions of the s.r. and reproducibility of responses prior to any intervention. It is not clear whether these factors were adequately controlled in the study by Trube and Trautwein. (3) It has been demonstrated that CICR. increases with the rate of rise of trigger-Ca^{2+} (For review see Fabiato, 1983). Caffeine and sulmazole may release Ca^{2+} by increasing the Ca^{2+}-sensitivity of the s.r. channel, thereby promoting CICR. Thus one would expect that the faster a drug is applied, the greater the Ca^{2+} release. The

equilibration of the drugs would be more rapid in the cell fragments, than in the multicellular trabeculae used in this study. Thus it is possible that under the conditions employed by Trube & Trautwein, 10mM sulmazole and caffeine produced a Ca^{2+}-release that was maximal. This would explain their apparent equipotence.

Sulmazole was at least as potent as caffeine in its ability to inhibit suprathreshold CICR. oscillations in saponin-skinned trabeculae. This is surprising as sulmazole is less able to induce s.r. Ca^{2+}-release under the conditions described above. This suggests that sulmazole may additionally inhibit the s.r. Ca^{2+}-uptake mechanism in agreement with results on isolated s.r. (Hasselbach et al, 1981, Lichtner et al, 1981).

Effects on submaximal force production

As illustrated in figure 5.8, caffeine and sulmazole (0.1-10mM) potentiated submaximal force production in Triton-skinned trabeculae. This effect of caffeine is in agreement with previous investigations (Wendt & Stephenson, 1983). However, it has been reported that the increase in Ca^{2+}-sensitivity with sulmazole saturated at 300μM (Herzig et al, 1981a, van Meel, 1987). In this study, Ca^{2+}-sensitivity continued to increase well into the millimolar range. The reasons for this discrepancy remain unclear. At the highest drug concentration tested (40mM), caffeine returned submaximal force to near pre-drug levels while sulmazole was without further effect. It

is relevant to consider this result in relation to maximum Ca^{2+}-activated force which will be discussed below.

Effects on maximum Ca^{2+}-activated force

All the results described above illustrate that while relative potencies differ, caffeine and sulmazole have qualitatively similar effects on skinned muscle preparations. However, as illustrated in figure 5.8, these drugs have opposite effects on C_{max}. Sulmazole (0.1-10mM) caused a dose-dependent increase in C_{max}. At 40mM this potentiation was slightly reduced. In contrast to the potentiation produced by sulmazole, up to 10mM caffeine had little influence on C_{max} while 40mM caused a marked depression in force. This effect of caffeine is in agreement with a previous study by Wendt & Stephenson (1983).

The consequence of these properties is that introduction of sulmazole (0.1-40mM) to muscle preparations can always be expected to potentiate absolute force production by increasing both Ca^{2+}-sensitivity and C_{max}. In contrast, introduction of caffeine can either decrease or increase force production depending upon the $[Ca^{2+}]$ and the [caffeine]. This conclusion should be borne in mind when considering the widespread use of caffeine in the investigation of E.C-coupling in intact muscle preparations. Ca^{2+}-sensitivity (ie force relative to C_{max}) continues to increase even at the highest concentrations of caffeine.

The opposite actions of sulmazole and caffeine on C_{max} illustrate that C_{max} can be modulated independently of Ca^{2+}-sensitivity. It terms of crossbridge theory, the simplest interpretation of an increase in C_{max} is an increase in the duration of bridge attachment without alteration of the total cycle duration. However the underlying mechanism remains obscure. It has been demonstrated recently that caffeine alters lattice filament spacing and the fixed charge on the lattice (de Beer et al, 1989 and G. Elliot personal communication). Compression of the myofilament lattice with dextran increases Ca^{2+}-sensitivity and maximum Ca^{2+}-activated force (Harrison et al, 1988). Thus changes in the lattice spacing may be involved in the actions of caffeine and sulmazole (see also chapter 1).

The ability of cardiotonic drugs to alter C_{max} is relatively common. Indeed in our hands, with the exception of caffeine, all Ca^{2+}-sensitisers tested (Pimobendan, MCI154, ORG30029, carnosine, taurine etc.) increased C_{max} to some extent. Although an increase in C_{max} has been observed with some compounds, (Kitada et al, 1987, Piazzesi et al, 1987) this effect has rarely been reported in the literature. Many factors of physiological relevance are known to influence C_{max} (e.g. phosphate, pH, ionic strength). Increasing C_{max} represents a method of increasing contractile force independently of Ca^{2+}-metabolism and thus merits further investigation as a potential mechanism of pharmacological and physiological importance.

Chapter 6: **The effects of ORG30029 on the Ca-sensitivity of skinned trabeculae.**

INTRODUCTION

Increased Ca^{2+}-sensitivity as an inotropic mechanism

Current treatment for congestive heart failure relies on the use of cardiac glycosides and vasodilators. Their narrow therapeutic window and tendency to arrhythmogenicity have prompted the search for other inotropic agents. Sulmazole (ARL115BS) was the first cardiotonic compound demonstrated to increase Ca^{2+}-sensitivity in skinned cardiac muscle (Herzig, Feile & Rüegg, 1981a). Unfortunately, as a result of toxicological problems, sulmazole proved unsuitable for clinical use. However, since then, a number of compounds have been introduced with similar pharmacological actions e.g. pimobendan and MCI 154 (for review see Wetzel & Hauel, 1988). The theoretical advantage of this inotropic mechanism is that contractility may be increased without the increase in $[Ca^{2+}]_i$ which can lead to arrhythmia.

For increased Ca^{2+}-sensitivity to be of any clinical relevance, it is necessary to produce drugs which are effective in the micromolar, if not the nanomolar concentration range. There is considerable variation in the potency of known Ca^{2+}-sensitising compounds. Caffeine has little influence below 1mM (see chapter 1) while one of the most potent compounds, pimobendan, has a small but statistically significant effect at $10\mu M$ (van Meel, 1987).

Increased Ca^{2+}-sensitivity and inhibition of PDE

One possible disadvantage is that an increase in Ca^{2+}-sensitivity may impair relaxation (Blinks & Endoh, 1986). This could be expected to occur if the rate of dissociation of Ca^{2+} from troponin is reduced (Rüegg, 1986b). However, with one possible exception (Allen & Lee, 1989) all Ca^{2+}-sensitising cardiotonic drugs also inhibit phosphodiesterase (PDE). It has been suggested that the rise in [cAMP] resulting from PDE inhibition favours s.r. Ca^{2+}-accumulation and so may offset any reduction in relaxation rate due to increased Ca^{2+}-sensitivity (Endoh et al, 1986). Furthermore, inhibition of PDE is generally associated with vasodilation, adding to the attractiveness of this combination of effects (Blinks & Endoh, 1986, Rüegg, 1986).

The aim of this study

The aim of this study was to investigate the intracellular actions of ORG30029 (N-hydroxy-5,6-dimethoxy-benzo[b]thiophene-2-carboximidamide HCL). This compound is a novel inotropic agent which inhibits phosphodiesterase (Shahid et al, 1989). However, it has been noted that effects on contractility cannot be correlated with changes in PDE activity at all drug concentrations (D. Nicholson personal communication). Consequently, a study was undertaken to determine whether an increase in Ca^{2+}-sensitivity or s.r. Ca^{2+}-uptake may contribute to the positive inotropic action of ORG30029. Experiments were carried out on saponin and Triton-skinned cardiac trabeculae. The effects of various concentrations of

ORG30029 were compared with those of milrinone and IBMX. The results illustrate that ORG30029 is a potent Ca^{2+}-sensitiser with no apparent direct effect on the s.r. It is likely that an increase in Ca^{2+}-sensitivity contributes to the positive inotropic action of this drug, at least in the micromolar concentration range.

RESULTS

Effects on Ca^{2+}-sensitivity

ORG30029 (0.01-1mM) increased the Ca^{2+}-sensitivity of the contractile proteins in a dose dependent manner. Figure 6.1 illustrates the shift in the pCa tension relationship produced by ORG30029 (100μM) in a Triton-skinned ventricular trabecula. Several determinations were made at each [Ca^{2+}] illustrating the reproducibility of responses.

Figure 6.2 shows original data from a Triton-skinned trabecula and illustrates the protocol used to obtain figure 6.1. In a and b, raising the [Ca^{2+}] from pCa 7.0 to pCa 5.63 produced around 10% of C_{max}. As indicated by the arrow, addition of 100μM ORG30029 (a) increased force production to around 40% of C_{max}. Addition of 10μM of the drug (b) caused a smaller but significant increase in tension.

Effects on maximum Ca^{2+}-activated force

In figure 6.3, a Triton-skinned trabecula was maximally Ca^{2+}-activated (pCa 4.0). As illustrated by the arrow introduction of the drug caused an increase in C_{max} (31%). The average potentiation produced by 1mM ORG30029 was 20.6 ± 1.1% (mean ± s.d. n=6). This effect was present although less pronounced at concentrations in the micromolar range (3.7 ± 1.1% n=6 at 100μM). It should be noted that tension responses as plotted

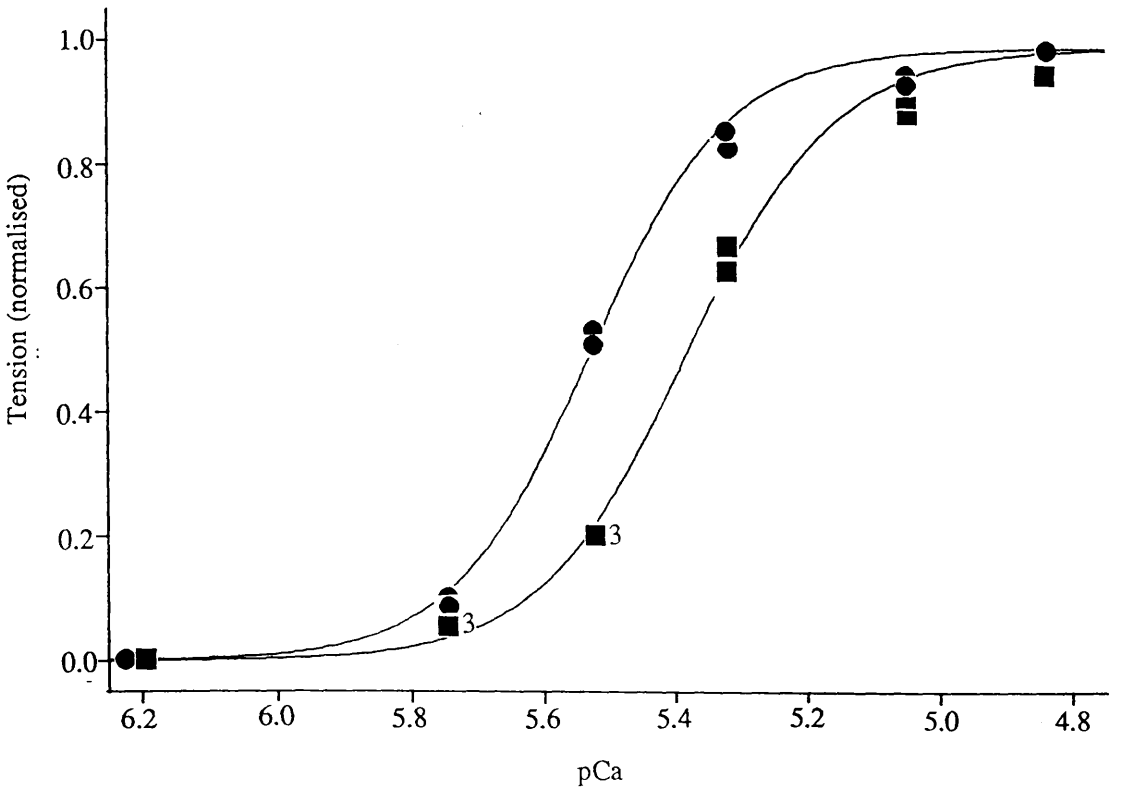


Figure 6.1 shows the pCa-tension relationship in a Triton-skinned rat trabecula with and without ORG30029. All solutions were strongly Ca^{2+}-buffered with EGTA ($10mM_{total}$). Curves were fitted by the Hill equation. Addition of ORG30029 ($100\mu M$) increased the pCa required for half maximal activation from pCa 5.34 to pCa 5.49 without change in the Hill coefficient (3.63).

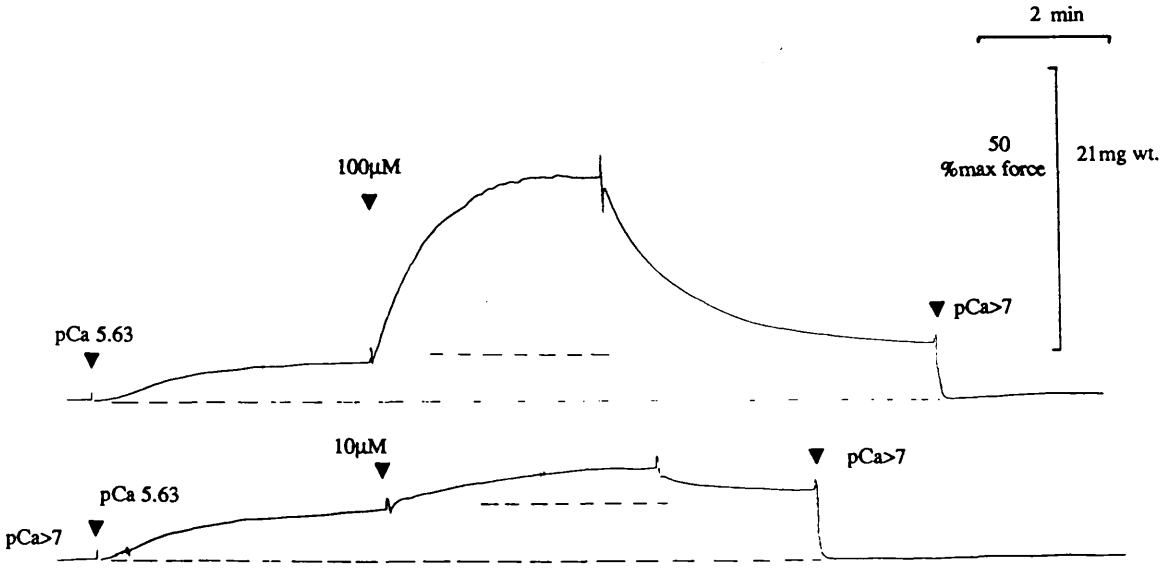


Figure 6.2 shows tension records obtained at a low level of activation (<10% C_{max}) in a Triton-skinned rat trabecula. All solutions were strongly Ca^{2+}-buffered with EGTA ($10mM_{total}$). The effect of adding and subsequently removing ORG30029 at two concentrations is shown.

in figure 6.1 have been normalised with respect to C_{max} to account for this effect.

Accumulated data on Ca^{2+}-sensitivity

Collected data from 9 preparations are shown in table 6.1. ORG30029 (100 & 50 μM) raised the pCa required for 50% maximum activation by 0.174 $\pm$0.053 and 0.05 $\pm$0.016 log units respectively (mean $\pm$s.d.). The variation in absolute Ca^{2+}-sensitivity between preparations generally makes the accumulation of data in this manner invalid. However, the collected data for absolute Ca^{2+}-sensitivity in the presence and absence of the drug show almost the same shift in the pCa-tension relationship. This probably reflects the fact that the present series of experiments was carried out on trabeculae of very similar dimensions and closely controlled s.l. The slope of the pCa-tension relationship characterised by the Hill coefficient, decreased in most experiments. However, the significance of this result remains in doubt (see discussion).

Comparison with IBMX and milrinone

For reference, the effects of ORG30029 have been compared with those of other known PDE inhibitors. In figure 6.4 (a-d) the $[Ca^{2+}]$ was increased (pCa 5.63) to produce around 20% of C_{max}. Addition of 1mM ORG30029 (figure 6.4a) caused a large increase in Ca^{2+}-activated force. Indeed as illustrated, the final level of force was greater than C_{max} in the

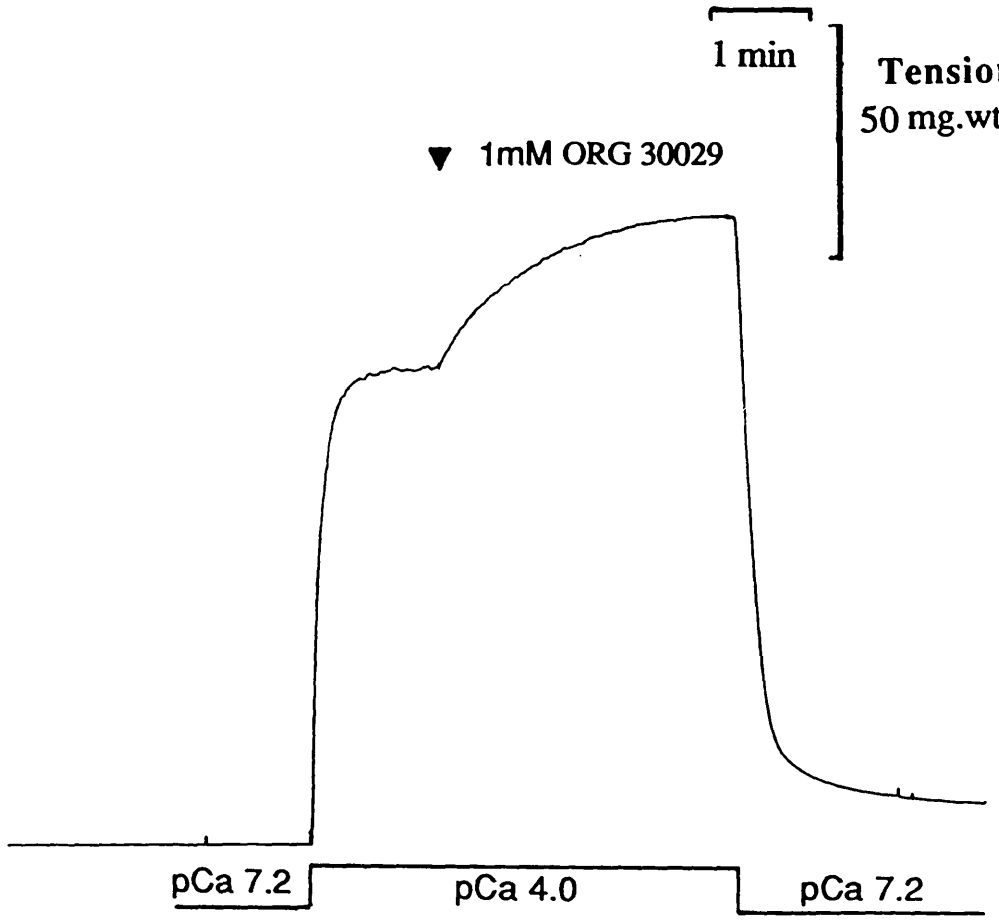


Figure 6.3 shows the effect of ORG30029 on C_{max} (evoked at pCa 4.0) in a Triton-skinned rat trabecula. All solutions were strongly Ca^{2+}-buffered with EGTA ($10mM_{total}$). Addition of ORG30029 (1mM) increased C_{max} by 31%.

Table 6.1

Ca sensitising effect of ORG30029 (mean ±s.e.m).

	$logK_{1/2}$	Hill coeff.	no. of preps
standard	5.401±0.026	3.61±0.1	
100μM	5.574±0.051	3.24±0.36	
mean shift	0.174±0.053		6
standard	5.407±0.011	4.32	
50μM	5.457±0.015	4.11	
mean shift	0.05±0.016		3
mean increase in C_{max}		(100μM) 3.69±1.12%	6
		(1mM) 20.6±4.6%	7

absence of the drug. This is consistent with the potentiation of C_{max} shown in figure 6.3. In contrast, an identical concentration of IBMX (figure 6.4b) caused only a small increase in force and milrinone (figure 6.4e) reversibly depressed Ca^{2+}-sensitivity. As shown in figure 6.4d, introduction of milrinone or IBMX at 100μM had no significant effect on Ca^{2+}-sensitivity.

Effects of ORG30029 upon Ca^{2+}-release and during Ca^{2+}-release by the s.r.

Figures 6.5 & 6.6 show results from selectively (saponin) skinned trabeculae. As illustrated in figure 6.5, ORG30029 (1mM) had no detectable ability to provoke Ca^{2+}-release from the s.r. This was demonstrated by direct application of the drug in conditions where caffeine (0.5-10mM) can produce large contractures. The experimental protocol is described in the figure 6.5 legend. An initial exposure to a just-subthreshold Ca^{2+}-concentration serves to load the s.r. Introduction of ORG30029 had no effect on tension. The drug was then removed and without further loading, introduction of caffeine (10mM) caused a transient contracture.

An alternative protocol was used to investigate the effect of ORG30029 on caffeine induced Ca^{2+}-release. A train of submaximal contractures was induced in a saponin-treated trabecula (figure 6.6a). For experimental details see chapter 2. As shown in figure 6.6b, when ORG30029 is included in both loading and release solutions, contracture amplitude increased from 33% to 49% of maximum Ca^{2+}-activated force. This potentiation is consistent with an increase in Ca^{2+}-sensitivity produced by the

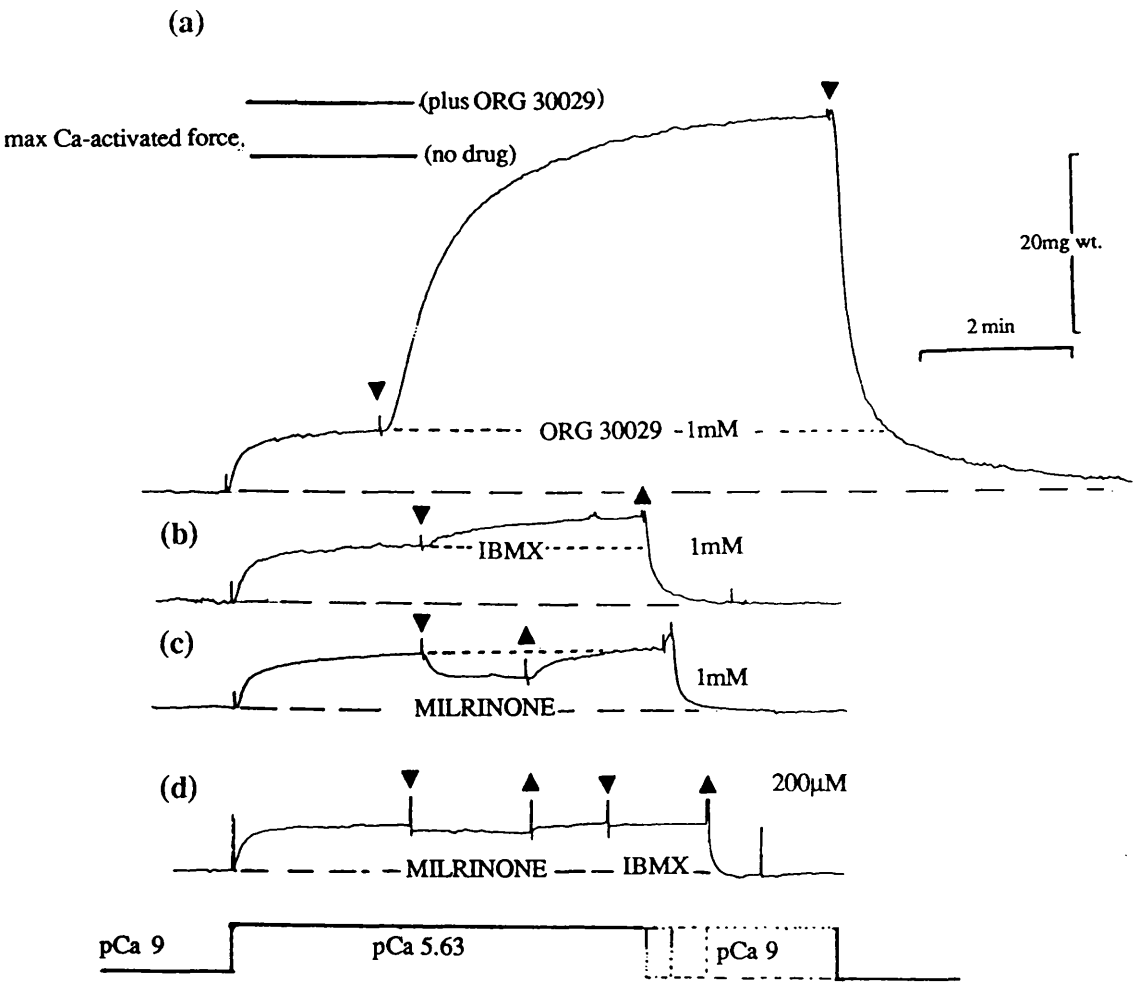


Figure 6.4 Direct comparison of ORG30029 and various other PDE inhibitors on the Ca^{2+}-sensitivity of a Triton-skinned trabecula is shown. All solutions were strongly Ca^{2+}-buffered with EGTA ($10mM_{total}$) 1mM of each compound was applied (▼) and removed (▲) at pCa 5.63 where the muscle was producing about 10% C_{max}. Above the top trace, two index lines indicate the levels of C_{max} (at pCa 4.0) with and without ORG30029. Milrinone (1mM) produced a small reduction in force at pCa 5.63 (lowest trace) and also C_{max} (not shown). The lowest trace shows the effects of 200μM milrinone or IBMX.

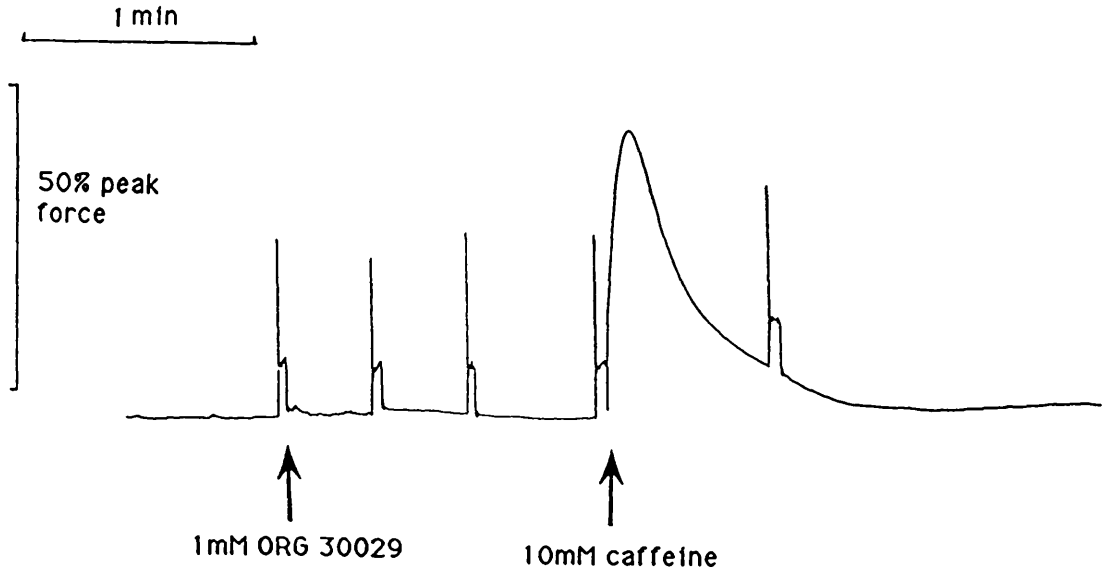


Figure 6.5 The lack of response of a saponin-treated preparation to ORG30029 (1mM) in contrast to that produced by caffeine (10mM) is illustrated. The s.r. was first loaded with Ca^{2+} by exposing the muscle to a just subthreshold pCa (10mM $EGTA_{total}$) for 2 minutes. The Ca^{2+}-buffer capacity was then reduced (0.2mM $EGTA_{total}$). The first solution change artefact indicates when ORG30029 was applied and the second its removal. In contrast introduction of caffeine (10mM) caused a contracture which peaked at nearly 50% C_{max}.

drug. When contractures are normalised (figure 6.6c) it would appear that the speed of tension development is little affected while relaxation is slightly delayed.

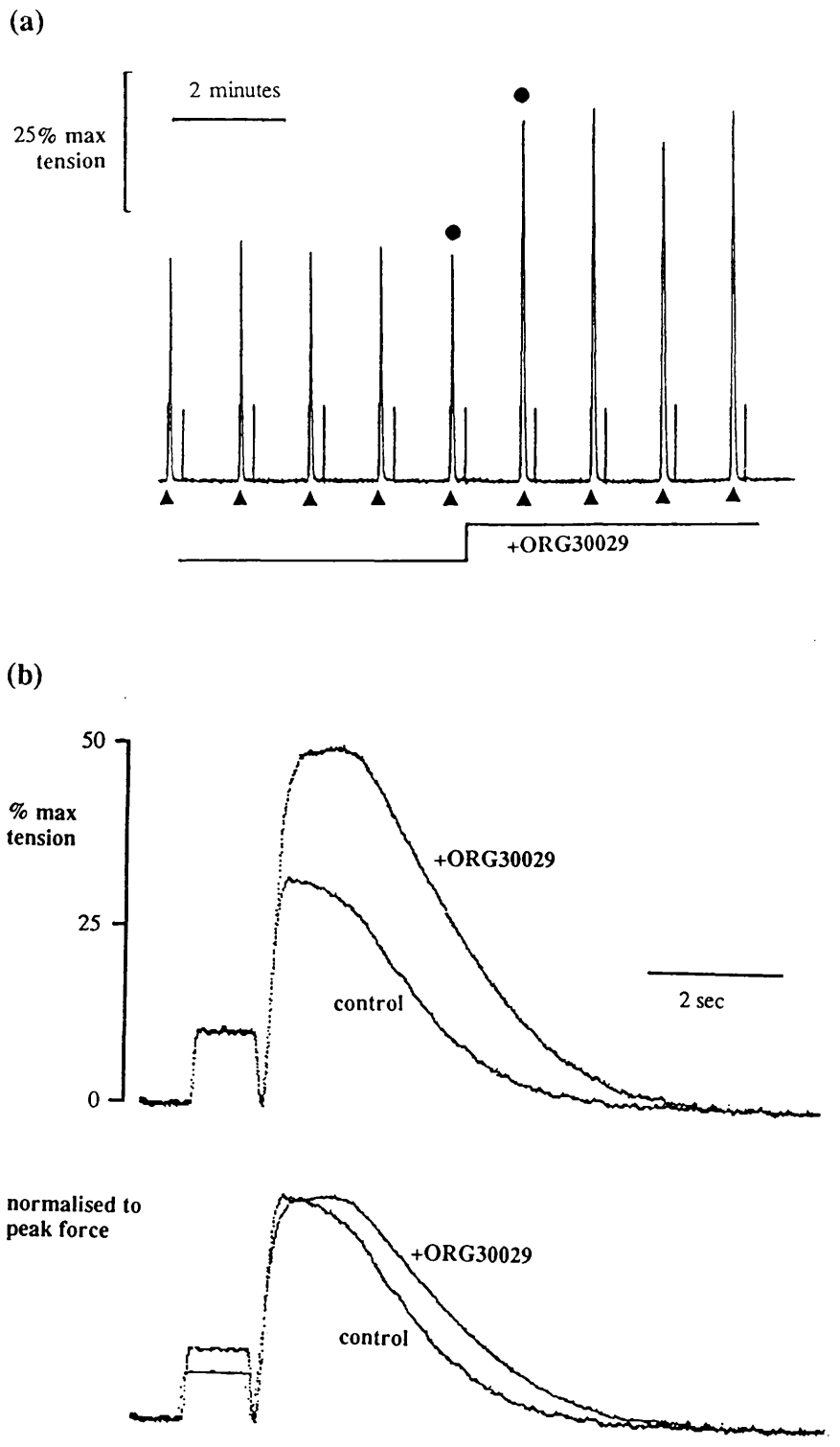


Figure 6.6 Panel (a) shows the effect of ORG30029 on a train of caffeine contractures in a saponin-skinned rat trabecula (for protocol see figure 3.1). All solutions were weakly Ca^{2+}-buffered (0.2mM EGTA $_{total}$). The large upstrokes are contractures resulting from the repeated application and removal of caffeine (for 15sec.). Between contractures the s.r. reloads with Ca^{2+} as the muscle is exposed to a $[Ca^{2+}]$ just subthreshold for tension production (pCa 6.22). Addition of ORG30029 (100μM) to both caffeine and caffeine-free solutions caused an increase in the amplitude of the contracture. Contractures with and without the drug are shown on a faster time-base in panel (b).

DISCUSSION

Effects on Ca^{2+}-sensitivity

The results illustrated in figures 6.1,6.2 & 6.4 suggest that an increase in Ca^{2+}-sensitivity may contribute to the positive inotropic action of ORG30029 at concentrations greater than 1μM. ORG30029 is effective over a similar concentration range to pimobendan and MCI154, the most potent Ca^{2+}-sensitisers yet reported (van Meel, 1987, Kitada et al, 1987).

The increase in force produced by ORG30029 (1mM) was much larger than with a similar concentration of IBMX (figure 6.4a & b). The effects of IBMX are in line with that expected for other methylxanthines such as caffeine and theophylline (Wendt & Stephenson, 1983). Indeed the increase in Ca^{2+}-sensitivity produced by IBMX (1mM) is of the same magnitude to that produced by caffeine under similar experimental conditions (see chapter 3).

Application of exogenous cAMP, or the potent PDE inhibitor papaverine, was without effect on the contractile response of saponin or Triton-skinned trabeculae (see chapter 3). Consequently it seems unlikely that changes in cAMP levels resulting from PDE inhibition play any part in the changes in Ca^{2+}-sensitivity observed in this study. Milrinone selectively inhibits PDE III with an EC_{50} of <30μM but has no influence on Ca^{2+}-sensitivity at 200μM (Weishaar et al, 1986). Thus the small decrease in Ca^{2+}-sensitivity produced by milrinone (1mM) is probably a direct action on

the contractile proteins. However, at such a high concentration, this is of no clinical relevance.

Changes in the Hill coefficient induced by ORG30029

ORG30028 caused a shallowing of the pCa tension relationship as indicated by the reduction in the Hill coefficient (Table 6.1). It is tempting to interpret this as a decrease in the cooperativity of the pCa tension relationship. However, it is necessary to consider possible artifactual explanations.

All experiments were carried out on multicellular preparations. It is inevitable that areas of the trabecula will be damaged during the dissection and mounting procedure. It has been demonstrated by other workers that during activation, undamaged regions can shorten by as much as 20%, stretching damaged areas. The extent of shortening increases with the degree of activation (Kentish et al, 1986). Ca^{2+}-sensitivity decreases with s.l. (Hibberd & Jewell, 1982). Consequently, the pCa tension relationship measured at constant muscle length is much shallower than in the same muscle at constant s.l. (Kentish et al 1986). It has been suggested that the 'snare' mounting technique used in this study causes less tissue damage and thus was not susceptible to these problems (Harrison et al, 1988). However, this was not confirmed in subsequent studies where s.l. was monitored during activation (C.Lamont personal communication).

With this possible artefact in mind, introduction of a compound which only increases Ca^{2+}-sensitivity would not necessarily be expected to change the slope of the pCa-tension relationship measured at constant muscle length. However, a drug which also increases C_{max} may increase the degree of internal shortening as a greater maximum force would be produced by undamaged areas. Consistent with this hypothesis, a reduction ionic strength is associated with an increase in C_{max} and a shallowing in the pCa-tension relationship at constant muscle length (Kentish, 1984, Fink et al, 1986). In contrast, caffeine (5-10mM) increases the Ca^{2+}-sensitivity but has no effect on C_{max} or the Hill coefficient Wendt & Stephenson, 1983). Thus, no reliable conclusions can been made concerning changes in the slope of the pCa tension relationship without measurement of s.l. during activation.

However, it is likely that changes in the apparent cooperativity do occur under some circumstances. If s.l. is controlled during contraction, Ca^{2+}-sensitivity increases with s.l. and the slope of the pCa-tension relationship also increases (Kentish, 1986). This was not observed in a previous study where muscle length but not s.l., was controlled during contraction (Hibberd & Jewell, 1982). Failure to observe the increase in the Hill coefficient could have been due to greater internal shortening as C_{max} increased with s.l.

It is unlikely that 'genuine' changes in the Hill coefficient reflect changes in the cooperativity of the three Ca^{2+}-binding sites on troponin. This is particularly the case as Hill coefficients greater than 4.0 have been

observed at constant s.l. (Kentish, 1986). Furthermore, there is evidence to suggest that only one of these sites may be involved in Ca^{2+}-regulation (Holroyde, 1980). Alternative explanations (such as an increased number of available crossbridges as $[Ca^{2+}]$ is increased or interactions between adjacent tropomyosin molecules) must be evoked to explain such slope changes.

Effects on maximum Ca^{2+}-activated force

ORG30029 increases C_{max} in a dose dependent manner (figure 6.3). This characteristic is typical of most other Ca^{2+}-sensitising compounds with the exception of caffeine (see also chapter 5). The simplest explanation of this phenomenon is that crossbridges remain attached for longer without alteration in the total cycle time. It is not known whether the increase in C_{max} is related to or independent from concomitant changes in Ca^{2+}-sensitivity.

Effects on the s.r.

As illustrated in figure 6.6 ORG30029 (100μM) potentiated submaximal caffeine contractures in saponin-skinned trabeculae. This procedure was carried out to determine whether the drug would potentiated contractile responses under dynamic (twitch-like) conditions rather than steady state force achieved over many minutes. The increase in force is consistent with an increase Ca^{2+}-sensitivity illustrated in figure 6.1. Again, it is unlikely that a change in [cAMP] is involved in the response (see above). However, it should be noted that the increase in the amplitude of the contracture

illustrated is equivalent to a shift in the $K_{0.5}$ of only 0.08 log units. This shift somewhat less than expected from the cumulated data shown in Table 6.1 (0.174 ± 0.053 log units). However, this assumes a uniform distribution of $[Ca^{2+}]$ throughout the muscle by the peak of the caffeine contracture which may not be the case. Alternatively, ORG30029 may decrease Ca^{2+}-accumulation by the s.r.

Chapter 7: **The effect of pH on the development of rigor tension.**

INTRODUCTION

This chapter is concerned with the effects of both natural and synthetic substances on rigor contractures. Although the effect of pH and P_i on Ca^{2+} activated force has been extensively studied (Fabiato, 1978b, Kentish, 1986), little is known about the influence these substances have on the development of a rigor contracture or the level of tension once a rigor contracture has developed. These issues are of particular relevance to the mechanical events late in myocardial ischaemia.

Early in ischaemia, anaerobic glycolysis maintains the supply of ATP to the muscle. However, for reasons that are not fully understood, glycolysis eventually ceases and the $[ATP]_i$ falls (for review see Allen & Orchard, 1986). By this stage the intracellular pH will have fallen below pH 6.0, and $[P_i]$ will have risen above 30 mM (Bailey et al, 1981, G.L. Smith personal communication).

There is indirect evidence that intracellular conditions may affect the development of a rigor contracture. Allen et al (1989), noted that the ischaemic contracture was smaller than that observed when the $[ATP]_i$ was reduced by the addition of agents that inhibit the aerobic and anaerobic metabolism (metabolic blockade). Previous work using NMR allows the comparison of the intracellular metabolism under conditions of ischaemia and metabolic blockade. Bailey et al (1981) noted a fall in $[CrP]_i$ and $[ATP]_i$ during ischaemia in isolated rat heart. These changes were accompanied by

a rise in P_i to approximately 30 mM and a marked fall in pH_i from 7.1 to close to pH 6.0. Similar changes were observed by Allen et al (1986a) in isolated ferret hearts during metabolic blockade. While P_i rose to similar levels, there was little change in pH_i. However, recent studies have observed pronounced pH changes during metabolic blockade in a variety of cardiac preparations. Ellis & Noireaud (1987) measured an acidosis of 0.25 pH units in isolated ferret papillary muscles during conditions similar to metabolic blockade; Eisner et al (1989) found an intracellular acidosis of approximately 0.5 pH units in isolated rat cells exposed to various forms of metabolic blockade; Bauer et al (1989) reported an intracellular acidosis of 0.25 pH units in isolated ferret hearts during metabolic blockade. Despite these results, the pH changes reported are still at least half that observed during ischaemia. If low pH inhibits rigor force as it does Ca^{2+}-activated force (Fabiato, 1978b), then this may account for the different final levels of rigor tension between ischaemia and metabolic blockade.

However, the mechanism underlying development of rigor tension is not fully understood. Rigor may be induced in chemically-skinned preparations under nominally Ca^{2+}-free conditions (pCa 9.0). Thus it would appear that the troponin-tropomyosin system may not be involved in the initiation of force production. As it is believed that one of the inhibitory actions of acidosis is a decrease in the Ca^{2+}-affinity of troponin-C, (Kentish & Palmer, 1989) the effects on rigor tension are unpredictable.

The aim of this study

The primary aim of this study was to investigate the influence of pH on rigor tension. The effect of pH was compared to that of P_i, caffeine and sulmazole, each of which is also known to influence Ca^{2+}-activated force production. The results demonstrate that low pH inhibits the development of rigor tension while sulmazole and caffeine potentiate force. However, P_i had no significant effect on the rigor contracture.

RESULTS

The effect of pH on rigor tension

Figure 7.1 illustrates the effect of acidosis on the development of rigor tension in a Triton-skinned trabecula. For details of changes to the standard solutions see figure subscript. After removal of ATP there was a short delay before development of a rigor contracture. Once tension had reached a steady level, the pH was changed from 7.0 to 6.0 (see bar above trace). This had no effect on developed tension. If however the preparation was relaxed again by addition of ATP, then reapplication of the rigor solution (still at pH 6.0) now produced a much smaller contracture. Thus *development* of rigor tension is inhibited by an acid pH. However, once the contracture has reached a steady state, it is insensitive to changes in pH.

Figure 7.2 shows rigor contractures developed at three different pHs in the same preparation. Rigor tension was greatly inhibited at pH 5.5 relative to that see at pH 7.0. In contrast, at pH 8.0 the contracture was almost twice that seen at neutral pH.

Cumulated Data

Figure 7.3 shows cumulated data from n=6 preparations. Rigor tension relative to that produced at pH 7.0 is plotted on the ordinate and pH on the abscissa. The results illustrate that rigor tension was always inhibited by an acid pH and almost abolished at pH 5.5. However, at alkaline pH,

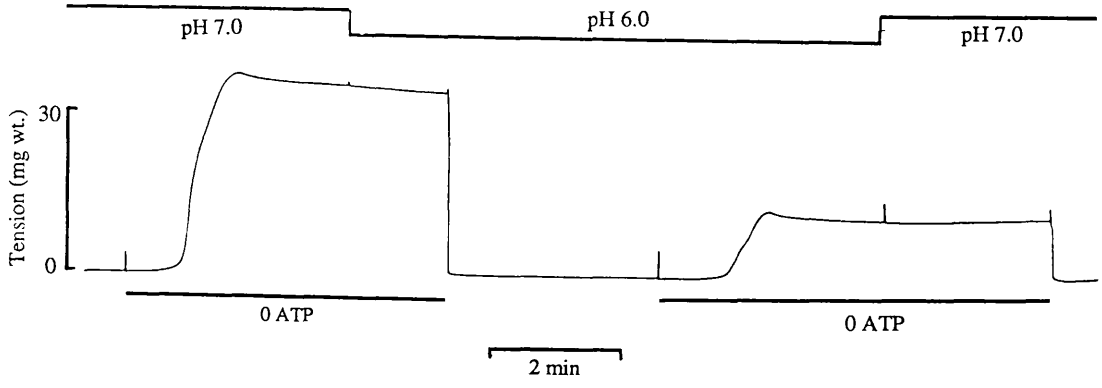


Figure 7.1 shows a record of tension in a Triton-skinned rat trabecula. The muscle was initially relaxed in a solution of the following composition; 5mM Na_2ATP, 10mM EGTA, 7mM $MgCl_2$, 130mM KCl, 25mM HEPES. At the point indicated by the bar under the trace, a rigor contracture was induced by transferring the preparation to a similar solution without ATP. The 'rigor' solution had the following composition; 10mM EGTA, 2mM $MgCl_2$, 130mM KCl, 25mM HEPES. The $MgCl_2$ solution was reduced in the 'rigor' solution to provide the same free [Mg] as the relaxing solution. The solutions were otherwise prepared in the standard manner described in chapter 2. The free $[Ca^{2+}]$ was calculated to be ~pCa 9.0 in both solutions. Once the rigor contracture had reached a steady-state the pH was reduced from 7.0 to 6.0. as indicated by the bar above the trace. The muscle was then relaxed again (still at pH 6.0) by readdition of the ATP-containing solution. After approximately three minutes in the relaxed state, another rigor contracture was induced by removal of ATP. The contracture developed at pH 6.0 was less than half the magnitude of that at pH 7.0. Again, once the rigor contracture was fully developed, tension was unaffected by a subsequent change in pH.

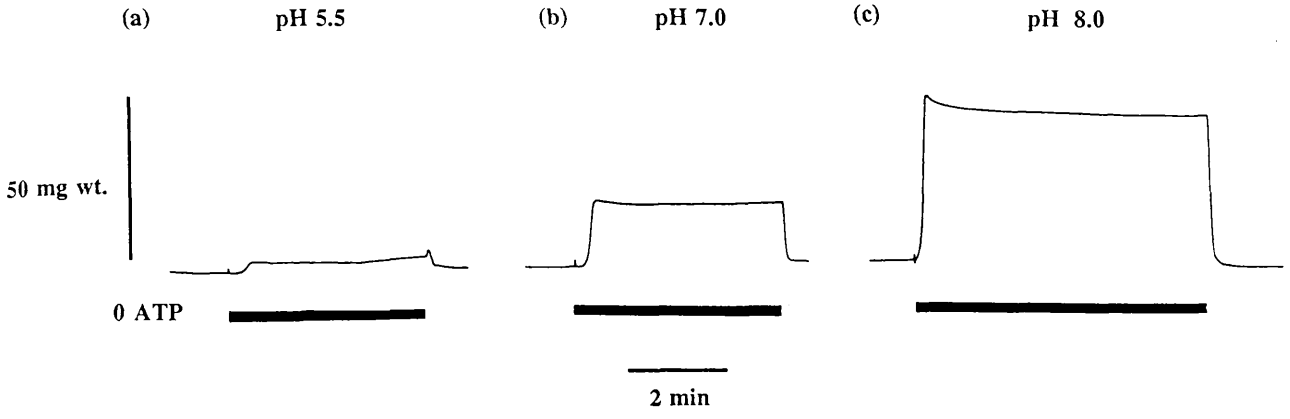


Figure 7.2 shows rigor contractures developed at three different pHs in the same Triton-skinned trabecula. Protocol and solutions are identical to those described in figure 7.1.

tension was consistently enhanced relative to that at pH 7.0. Some experiments were carried out outside this pH range but this resulted in irreversible loss of force production by the preparation.

Effects of inorganic phosphate

Like pH, inorganic phosphate (P_i) decreases C_{max} and the apparent Ca^{2+}-sensitivity of the contractile proteins. Indeed 20mM P_i decreased C_{max} to around 20-30% of control values (Kentish, 1986). However, P_i (10-20mM) had little or no effect on the development of rigor tension or steady state tension at pH 7.0 (figure 7.4a) or at pH 6.0 (figure 7.4b). Similar results were obtained in n=6 preparations.

Caffeine and sulmazole

Sulmazole and caffeine increase the Ca^{2+}-sensitivity of the myofilaments (Herzig et al, 1981a, Wendt & Stephenson, 1983, see also chapter 5). Figure 7.5 illustrates that after full development of the rigor contracture, introduction of 10mM caffeine (a) or sulmazole (b) had no influence on force production (the small decrease in force on addition of caffeine is a solution change artefact). However, if present during development of the contracture, force production was potentiated. Removal of the drugs after the full development of the contracture was without effect. Similar results were obtained in n=4 preparations.

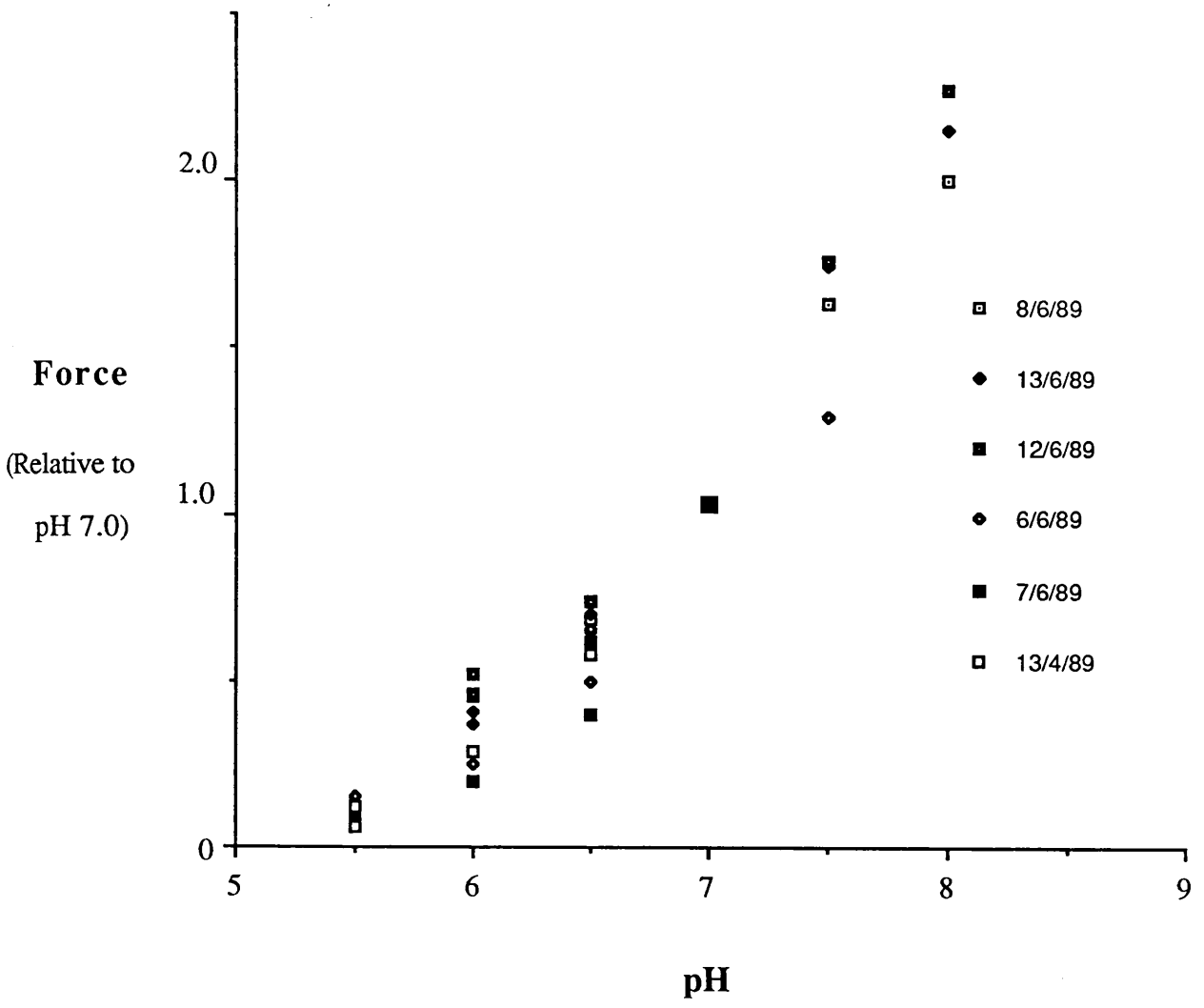


Figure 7.3 illustrates accumulated data on the pH dependence of rigor tension. Experimental protocol as described in figure 7.1. Each point represents the force relative to that developed at pH 7.0.

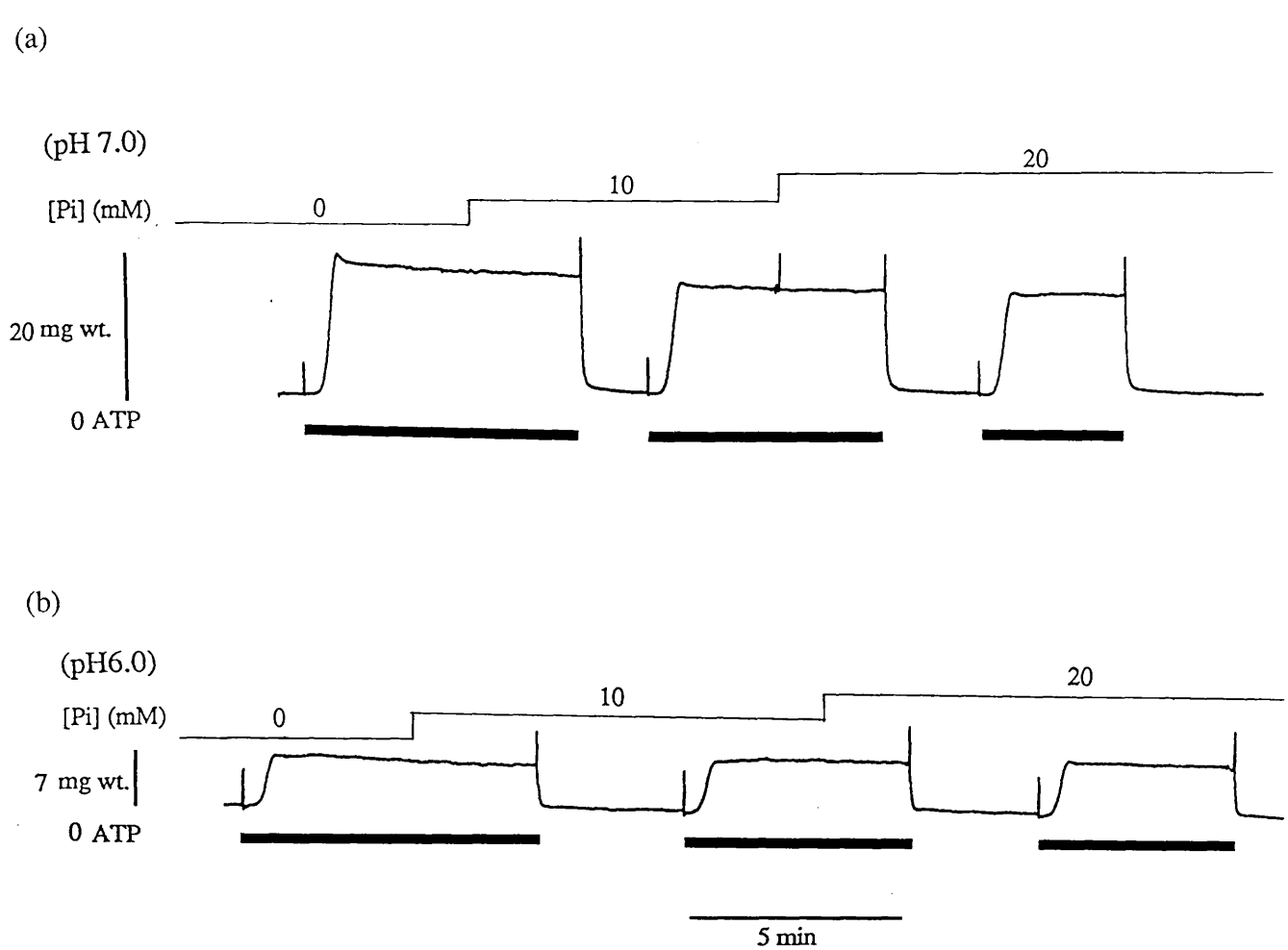


Figure 7.4 shows the lack of effect of inorganic phosphate (0-20mM) on the development of rigor tension and steady-state rigor tension in a Triton-skinned trabecula. Protocol as described in figure 7.1. The procedure shown in panel (a) was carried out at pH 7.0. This was repeated at pH 6.0 as illustrated in panel (b).

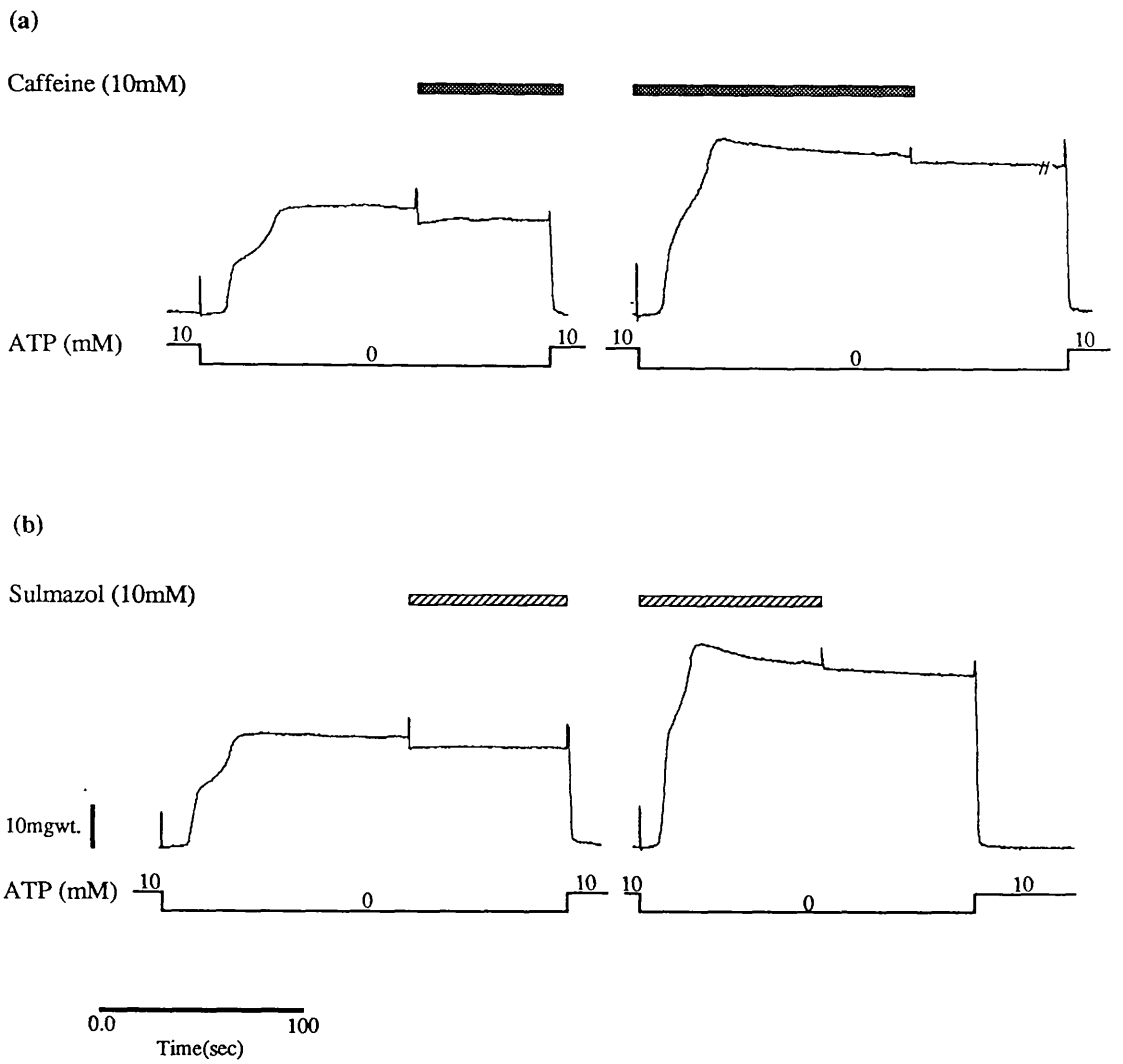


Figure 7.5 shows tension records in a Triton-skinned rat trabecula. In (a) and (b) rigor was induced by the removal of ATP. (Solutions and protocol are as described in figure 7.1.). Once the contractures had reached a steady state, 10mM caffeine (a) or sulmazole (b) was added. This resulted in no significant change in the level of tension. The preparation was then relaxed by reapplication of ATP. However, if a rigor contracture was induced in the presence of caffeine (a) or sulmazole (b) the final level of tension was potentiated by 33% or 88% respectively. The muscle was insensitive to the removal of the drugs once rigor was fully developed.

DISCUSSION

The development of rigor tension

In order to interpret these results, it is necessary to consider the events which occur during the development of a rigor contracture. It is generally accepted that under physiological conditions, contraction is initiated by Ca^{2+}-binding to troponin-C. Ebashi and his colleagues proposed that one troponin together with one tropomyosin acts on seven actin monomers making them incapable of forming complexes with myosin in the presence of ATP (for review see Ebashi, 1980). This arrangement of proteins was considered to act as a "functional-unit" to regulate contraction. On addition of Ca^{2+}, it seems likely that one troponin "turns on" all seven actin monomers (Bremel & Weber, 1972). However, in the present study, rigor tension was induced under nominally Ca^{2+}-free conditions (pCa 9.0). This brings into question whether the troponin-tropomyosin system is involved in this form of force production.

Bremel & Weber (1972) proposed the following model for the events occurring during the development of rigor in skeletal muscle; as the [ATP] falls, some of the actin molecules within each functional-unit form rigor complexes with nucleotide-free myosin. The remaining actin molecules not complexed with myosin are "turned on" even in the absence of Ca^{2+} and are capable of catalysing ATP hydrolysis. Ca^{2+}-binding studies demonstrated that

the formation of nucleotide free rigor bridges caused an increase in the Ca^{2+}-affinity of troponin. This raises the following obvious questions;

(1) does the formation of some rigor bridges within each functional unit increase the affinity of troponin to such an extent that Ca^{2+}-activated ATP hydrolysis occurs even at pCa 9.0? On the basis of Ca^{2+}-binding and ATPase measurements, Bremel & Weber suggested that while this may occur at higher $[Ca^{2+}]$ it does not occur when the $[Ca^{2+}]$ is as low as pCa 9.0. They concluded that under these conditions, the increased Ca^{2+}-affinity of troponin is a consequence of, but irrelevant to, actin-myosin interaction. If this is the case, then there is no apparent reason why compounds which act primarily by increasing the Ca^{2+}-sensitivity of troponin should influence rigor force production.

(2) Do crossbridges undergo repeated cycles of ATP hydrolysis and force production during the formation of a rigor contracture, before ending in the attached state in the absence of ATP? This consideration applies whether actin-myosin interaction is initiated by Ca^{2+}-binding to troponin-C or independently of this reaction as suggested by Bremel & Weber.

The results of this study will be interpreted with reference to the above considerations.

Effect of pH on rigor force

Figures 7.1,7.2 and 7.3 illustrate that rigor tension decreases dramatically with pH (8.0-5.5). This action of pH may explain the

observations that rigor tension is smaller after ischaemia than after metabolic blockade (see introduction).

There are several possible mechanisms by which this might occur. Recently the effect of low pH has been studied in relaxed skeletal muscle using X-ray diffraction. It was found that low pH altered the arrangement of the thin filament structures within the actin-myosin matrix as judged by the effect on the 1,1 equatorial reflection (Matsuda & Podolsky, 1986). Furthermore, and of particular relevance to this study, it was found that if the muscle was first put into rigor, the organisation of the lattice was maintained despite further changes in pH. It is reasonable to assume that disorganisation of lattice structure is associated with a reduction in force production. This accepted, the action of pH on the myofilament lattice could explain all of the effects of pH on rigor tension observed in this study.

It is also possible that other effects of acidity may explain or contribute to the inhibition of rigor force. In particular, low pH reduces the Ca^{2+}-sensitivity of the myofilaments by a direct effect on troponin-C (Kentish & Palmer, 1989). As described above, a form of Ca^{2+}-activated force production may occur during development of the rigor contracture even at very low [Ca^{2+}]. A decrease in Ca^{2+}-sensitivity could conceivably influence the final level of rigor force production by an action on Ca^{2+}-regulated crossbridges. However, in this instance it is unnecessary to invoke such an explanation as high ionic strength has similar effects to pH on lattice structure and rigor force but does not affect the apparent Ca^{2+}-sensitivity of

the myofilaments (Matsuda & Podolsky, 1986, Kentish, 1984, Fink et al, 1986).

Effects of caffeine and sulmazole

Sulmazole and caffeine increase the apparent Ca^{2+}-sensitivity of the contractile proteins in chemically skinned cardiac muscle (Herzig et al, 1981a, Wendt & Stephenson, 1983). With sulmazole this was associated with an increase in Ca^{2+}-binding to troponin-C, even above that observed during rigor (Solaro & Rüegg, 1982). Thus it is possible that the increase in Ca^{2+}-binding influences the final level of rigor force via crossbridges that are 'switched on' by Ca^{2+}.

However, this may not necessarily be the case. An alternative explanation is that any influence on Ca^{2+}-binding is secondary to a change in force production (Hofmann & Fuchs, 1987b). During Ca^{2+}-activated force production, this could occur via an increase in the duration of the attachment time. Another, possibility, of relevance to rigor force production, is that rigor bridges may attach in a number of different force-producing states (Huxley, 1974). Thus caffeine or sulmazole could act at the level of the crossbridge, to increase the duration of attachment in a state of high force production, during Ca^{2+}-activated force production. As the final level of rigor tension is influenced, this could mean that such compounds increase the probability of the crossbridges final attached state being one of high force production in the absence of ATP.

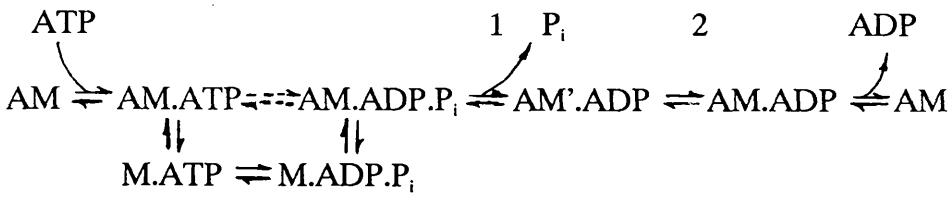
Effects of inorganic phosphate

Until this point all interventions which influence Ca^{2+}-activated force have been shown to have a similar effect on the development of rigor tension. It is known that 20mM P_i typically reduces C_{max} to around 20% of control levels in skinned cardiac muscle (Kentish, 1986). However, as illustrated in figure 4, 20mM P_i was found to have little or no effect on rigor tension.

Phosphate does not influence Ca^{2+}-binding by a direct effect on cardiac troponin-C (Kentish & Palmer, 1989). A decrease in Ca^{2+}-binding to troponin-C was observed on addition of vanadate (a phosphate analogue) during activation of skinned cardiac muscle (Hofmann & Fuchs, 1987b). Again, it is possible that this action may be secondary to its influence on Ca^{2+}-activated force production (see above and chapter 1).

Kentish (1986) suggested that the inhibitory action of P_i on Ca^{2+}-activated force could be explained with reference to a scheme in which the release of P_i from the actomyosin products complex occurs at a step which is close to equilibrium and which precedes ADP release. This is illustrated in the reaction scheme below where AM.ADP, AM and possibly AM'.ADP represent cross-bridge states that generate a large force. During Ca^{2+}-activated force production, reaction 1 is readily reversible whereas reaction 2, which is not reversible, may be linked to the generation of force (for detailed references regarding this model and assumptions therein see

Kentish, 1986). During normal Ca^{2+}-activated force production, increasing the $[P_i]$ will therefore reduce [AM'ADP] relative to AM.ADP.P_i. Thus an increase in P_i would decrease the number of actively cycling bridges at any given instant. Within this model, the action of P_i may only be considered relevant to a population of crossbridges, each of which spends a small proportion of the total cycle time attached and generating force.



With rigor, it is not clear whether the "switching on" of the crossbridges and the subsequent development of tension is dependent or independent of Ca^{2+} (see above). However, if a significant number of crossbridges **do** undergo repeated cycles of ATP hydrolysis during the development of rigor tension, then one would expect this to be influenced by P_i irrespective of the mechanism by which actin-myosin interaction was initiated. The simplest explanation for the lack of effect of P_i on rigor tension is that under these circumstances, most crossbridges attach and generate force but do not undergo repeated cycles of ATP hydrolysis before complete depletion of ATP. If this is the case, the final level of force could be modified by altering either the number of attached crossbridges or the final

attached state (if different force generating states are assumed to exist-see above)

In conclusion

The results of this study demonstrate that low pH inhibits the development of rigor tension while P_i is apparently without effect. These results are interesting since the rigor state gives useful clues about crossbridge mechanism. However, it is not clear to what extent the findings help to explain events occurring during ischaemia or hypoxia. In the current study [Ca^{2+}] was considerably below that found under such conditions. Furthermore, rigor tension was induced by complete removal of ATP.

It is generally accepted that a low [ATP] is required to produce the contracture observed in hypoxia and ischaemia. However, it is not possible to estimate the [ATP] accurately in intact tissue in these conditions. Low [ATP] it is likely that attachment of some rigor bridges will allow Ca^{2+}-activated force production as a result of the increase in Ca^{2+}-sensitivity (Bremel & Weber, 1972 Fabiato & Fabiato, 1975c). The situation would in effect be intermediate between normal contraction and the extreme conditions employed in this study. Consequently, it is likely that P_i would influence the development of force. Thus further experiments are required to investigate the effects of pH and P_i at higher [ATP] and Ca^{2+}.

This study does demonstrate that interventions which produce an apparently similar effect on Ca^{2+}-activated force may do so via different

mechanisms. Further, the ability of sulmazole and caffeine to potentiate rigor force under these conditions provides evidence that their primary mechanism of action may not be via an increase Ca^{2+}-binding to troponin-C.

Chapter 8: **The effect of taurine on skinned trabeculae.**

INTRODUCTION

Taurine levels in cardiac muscle

Taurine (2-aminoethansulphonic acid) is the most abundant free amino acid in cardiac muscle. The intracellular concentration of taurine is species dependent, commonly about 10 to 20mM but in rat it may be as high as 40 mM (Jacobson & Smith 1968). These intracellular levels are maintained despite a much lower plasma concentration of about 60μM (Perry & Hansen, 1969). Taurine uptake in cardiac muscle has a K_m of approximately 60μM (Schaffer et al, 1981, Bahl et al, 1981). High and low affinity taurine binding sites have been identified in cardiac sarcolemmal preparations. Estimates for the K_m of the high affinity site range from 40-200μM (Chovan et al 1980, Schafer et al, 1981, Bahl et al 1981, Awapara & Berg, 1976) while the K_m of the low affinity site is reportedly around 3mM (Schaffer et al, 1981). These authors have suggested that high affinity system may be associated with taurine transport because (i) the affinity of taurine binding to this protein is similar to the K_m of the uptake system and (ii) both uptake and binding are inhibited by β-alanine and hypotaurine.

Supranormal taurine levels have been found in heart tissue from patients with congestive heart failure and in experimental models of cardiac hypertrophy (Huxtable & Bressler 1974), while the taurine content of the heart decreases during ischaemia (Crass & Lombardi 1977).

The effect of taurine on the contractility of intact preparations

Many previous studies have investigated the action of taurine on cardiac muscle by superfusing intact preparations with taurine at concentrations ranging from 1 to 50 mM. These studies have shown that, in the presence of a normal extracellular [Ca^{2+}], addition of taurine will; (i) Increase Ca^{2+} binding to the sarcolemma (Chovan, Kulakoski, Sheakowski & Schaffer 1980) (ii) Increase Ca^{2+} content and twitch tension. However, if extracellular [Ca^{2+}] is raised above normal, taurine has the opposite effects on these variables (Franconi et al, 1982). Taurine antagonised the negative inotropic effect of lowered extracellular [Ca^{2+}] or the addition of verapamil (Chovan et al, 1979). In the hamster model of cardiomyopathy, there is a twelve-fold increase in total heart Ca^{2+} by sixty days of age. Administration of taurine reduced the magnitude of this elevated heart Ca^{2+} (McBroom & Welty, 1977).

Reperfusion of cardiac muscle exposed to low Ca^{2+}, hypoxia or ischaemia, causes arrhythmias and may lead to cell death. This "reperfusion paradox" is associated with the entry of Ca^{2+} through the sarcolemma which leads to overload and cellular damage. Preincubation with taurine was found to decrease the incidence of arrhythmias and protect cells against reperfusion damage (Takahashi et al, 1988, Crass & Lombardi, 1977, Franconi et al, 1985). A number of studies on superfused guinea-pig strips observed that (i) In a similar manner to taurine, α_1-antagonists protect against reperfusion

damage (ii) under conditions of ischaemia α-mediated responses dominate over β (iii) taurine inhibited the positive inotropic effect of α but not β-receptor activation in perfused guinea-pig ventricular strips (iv) taurine is lost from the myocardium during ischaemia (Franconi, et al 1985, Franconi et al, 1986, Takahashi et al, 1988). These findings have led to the suggestion that taurine is an endogenous modulator of α-mediated contractile responses.

Subcellular effects of taurine

The reported effects of taurine on the s.r. are contradictory. In isolated skeletal muscle s.r., taurine (15mM) increased the rate of Ca^{2+}-uptake by 25-30%. The maximum amount of Ca^{2+} accumulated also increased (Huxtable & Bressler, 1974). However, in subsequent studies on cardiac s.r. fractions, taurine produced only a small increase (not statistically significant) in Ca^{2+} binding and uptake (Whelty & Whelty 1981) or had no effect on these parameters (Khatter et al, 1981). More recently, the identification of specific taurine binding sites on isolated s.r. of rat, guinea-pig and rabbit has led to renewed speculation that taurine may regulate s.r. Ca^{2+}-accumulation (Quennedey et al, 1986). On the other hand, taurine appeared to increase Ca^{2+} binding to sarcolemmal and mitochondrial fractions of cardiac muscle (Entman at al, 1977, Chubb & Huxtable, 1978, Whelty & Whelty, 1981). It should be noted that despite the increase in mitochondrial Ca^{2+}-binding, uptake apparently remained unaffected (Khatter et al, 1981).

To date, there is no information regarding the direct effects of taurine on the contractile proteins of cardiac muscle. However, in mechanically-skinned crayfish and crab fibres taurine (5mM) increased tension at half-maximal activation by 40% while maximum Ca^{2+}-activated force remained unaffected. This action was mimicked to a lesser extent by glycine and serine while proline was without effect and alanine depressed tension by around 10% (Galler & Hutzler, 1988). These results were interpreted as changes in the Ca^{2+}-sensitivity of the contractile proteins.

The aim of this study

The role of taurine in cellular function is unknown, but it has been suggested that it may modulate Ca^{2+}transport in cardiac muscle (Schaffer et al, 1981). It has been demonstrated that taurine can influence the contractility of intact cardiac muscle in a manner which depends upon the inotropic state. In such experiments taurine (up to 50mM) was added to the perfusate, however, plasma levels are believed to be less than 1mM. Thus, it is not clear whether the amino acid is acting at unphysiologically high concentrations on the sarcolemma or by replenishing depleted intracellular taurine.

The present study was designed to investigate the intracellular action of taurine on the s.r. and contractile proteins, using saponin or Triton-skinned cardiac muscle. The experiments show that taurine causes a small increase in myofilament Ca^{2+}-sensitivity with little effect on maximum Ca^{2+}-activated force. Furthermore, taurine produced a marked increase in the ability of the

s.r. to accumulate calcium. These results may explain some of the apparently disparate effects of taurine previously reported in intact cardiac muscle.

RESULTS

The effect of taurine on caffeine-induced contractures.

The record shown in figure 8.1 was obtained from a saponin-treated trabecula in the presence of 0.2mM EGTA. A train of reproducible caffeine contractures was induced (for further details see chapter 3). Figure 8.1 illustrates that taurine (5mM) will increase the size of the caffeine contracture when it is included in the loading solution. In this experiment the $[Ca^{2+}]$ was maintained at 130 nM, this gave a caffeine-induced contracture that was 20% of maximal Ca^{2+}-activated force. Taurine was first added to the caffeine solution. This had no immediate effect on the size of the caffeine contracture. However, taurine was then included in the loading solution which increased the magnitude of the caffeine contracture. The figure shows that the potentiating effect of taurine stabilises within one or two load-release cycles. It was concluded that taurine was able to increase the ability of the s.r. to accumulate Ca^{2+}. Another intracellular site for taurine's action could be the mitochondria (Dolora et al 1970). However, in this study all mitochondrial substrates were absent from the bathing solution. Furthermore under these loading conditions it is thought that the mitochondria do not contribute to the caffeine-induced response as they are unaffected by azide or changing the Na^+ concentration of the bathing solution (see also chapter 3).

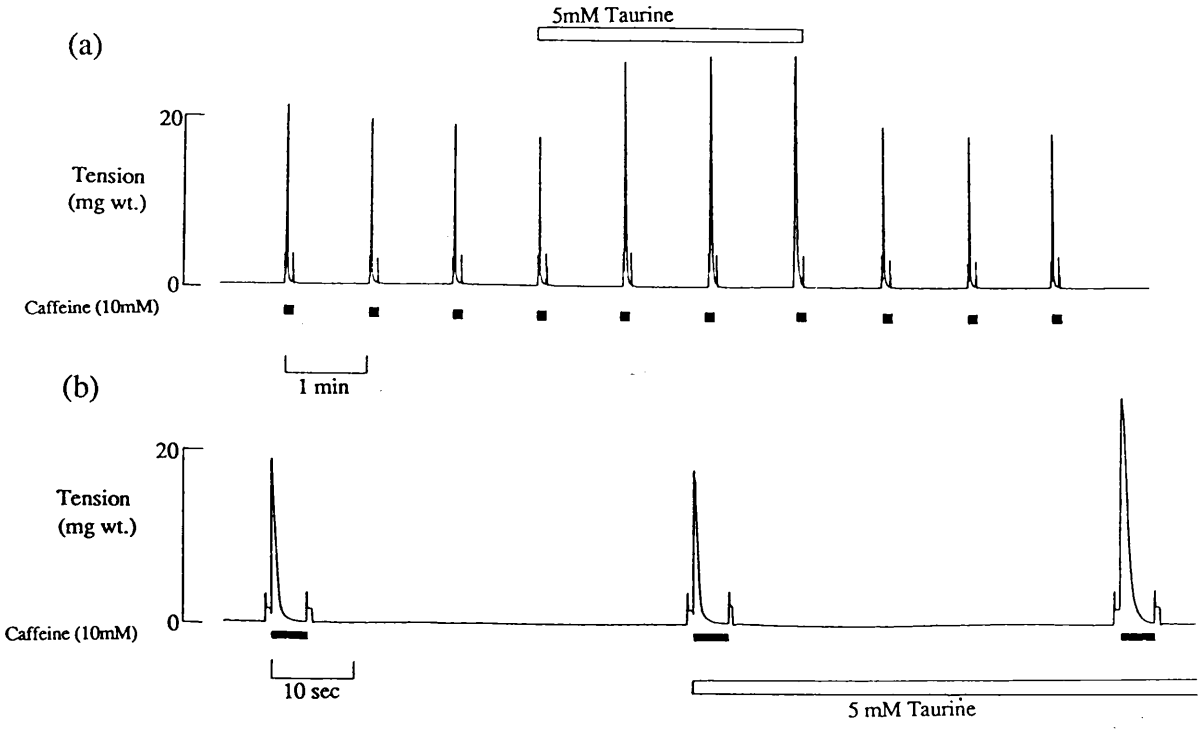


Figure 8.1 shows train of caffeine contractures in a saponin-skinned rat trabecula (for protocol see figure 3.1). All solutions were weakly Ca^{2+}-buffered (0.2mM EGTA $_{total}$). The presence of caffeine is indicated by the bar below each contracture. The $[Ca^{2+}]$ of the caffeine-free solution was 0.12μM. At the point indicated, taurine (5mM) was added to both caffeine and caffeine-free solutions. Panel (b) shows a section of this record on a faster time base.

The dose dependence of taurine's action on the caffeine-contracture

The individual contractures shown in figure 8.2 are representative of the steady-state effects of taurine at the concentrations shown. As little as 10μM taurine in the loading solution produced a small but consistent potentiation of the contracture. The response was dose-dependent with the maximum effect seen at about 5mM. Higher concentrations of taurine caused no further increase in the size of the caffeine contracture and at concentrations above 5 mM the effect apparently decreased. In this preparation 40mM taurine caused a small depression of the caffeine-contracture relative to the standard response.

The osmotic action of taurine on the sarcoplasmic reticulum

Taurine is predominantly in its zwitterionic form at pH 7.0 and therefore does not contribute to the ionic strength. However, addition of taurine will increase the osmolarity of the solutions. To assess the purely osmotic effects, its actions were compared with those of similar concentrations of sucrose in 4 preparations.

At concentrations of 10mM and above, sucrose was found to decrease the amplitude of the caffeine-induced contracture in a dose-dependent manner. Similar effects were seen with the pH buffer TAPS. An example of the depressive effects of the higher concentrations of sucrose is shown in figure 8.3(b). A small potentiation obtained with 30mM taurine is illustrated for comparison in figure 8.3(c). Thus the reduced potentiation

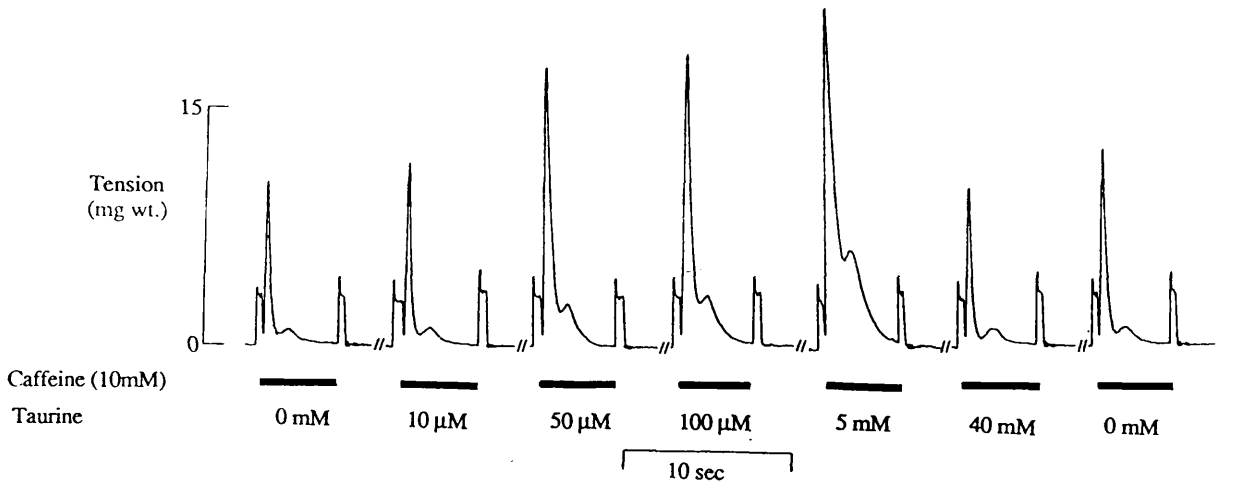


Figure 8.2 shows the effect of a range of taurine concentrations on caffeine contractures in a saponin-skinned rat trabecula (protocol as in figure 3.1). All solutions were weakly Ca^{2+}-buffered (0.2mM $EGTA_{total}$). The presence of caffeine is indicated by the bar below each contracture. The $[Ca^{2+}]$ of the caffeine-free solution was 0.12μM. The transients represent steady-state caffeine contractures, selected from a train of responses, during incubation in various taurine concentrations. In each case the [taurine] is shown below the contracture.

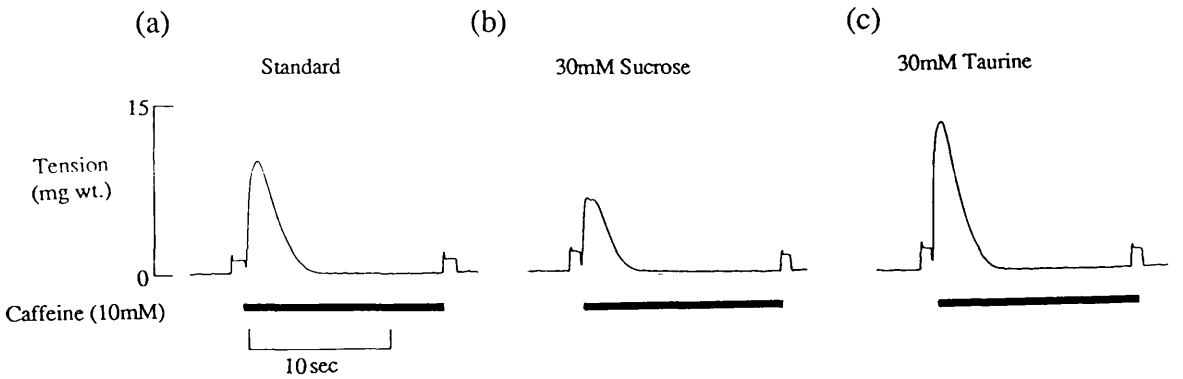


Figure 8.3a shows the steady-state caffeine-induced response selected from a train of contractures in a saponin-skinned rat trabecula (protocol as in figure 3.1). All solutions were weakly Ca^{2+}-buffered (0.2mM $EGTA_{total}$). The presence of caffeine is indicated by the bar below each contracture. Panel (b) shows the caffeine response following addition of sucrose (30mM) to Ca^{2+}-loading and caffeine solutions and panel (c) following addition of taurine (30mM) in the absence of sucrose.

by high concentrations of taurine may result from an inhibition of s.r. function resulting from the increased osmolarity of the solutions. Sucrose reportedly has no effect on the Ca^{2+}-sensitivity of cardiac myofibrils (Kentish, 1987) and this was confirmed in the present study (data not shown).

Accumulated data

Figure 8.4 summarises data accumulated from 14 saponin-treated preparations. The taurine concentration is shown on a log scale on the abscissa and the peak amplitude of the caffeine contracture, expressed as a percentage of the standard contracture, is plotted on the ordinate. In these experiments the Ca^{2+}-loading was chosen so that the standard contracture was close to 20% of maximum Ca^{2+}-activated force. Variation in the size of the standard contracture in any one preparation is small (solid circles). Several successive contractures were averaged to define 100% in each case.

The potentiation was typically greatest at 1-5mM taurine but reduced nearer to control levels from 10 to 40 mM. However, when the effect of osmolarity was corrected for by sucrose substitution no depressive effects were observed at 30 mM taurine (filled circle) which then potentiated to a similar extent as 1 to 5mM taurine. As illustrated in figure 8.4 (open and filled squares, the effects of taurine are completely reversible.)

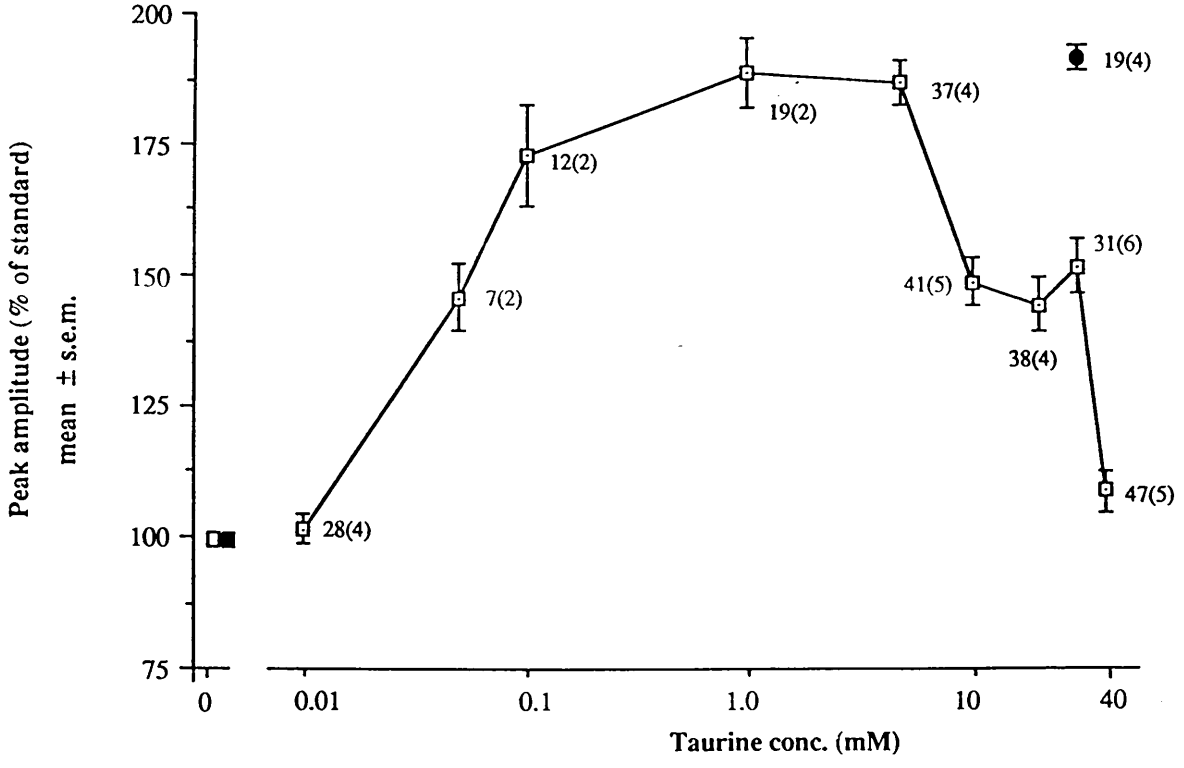


Figure 8.4 The effect of taurine on the amplitude of caffeine-induced contractures. The points represent the mean steady-state response at different taurine concentrations as a percentage of the mean standard response (error bars indicate the standard error of the mean). The numbers beside each point indicate the number of observations, the numbers in brackets are the number of preparations used. The points on the left hand side of the graph represent the mean standard contracture before (□) and after (■) taurine (s.e. is smaller than points). Results obtained with 30mM taurine when compared with those in taurine free + 30mM sucrose are also illustrated (●).

The effect of s.r. Ca^{2+}-loading on the taurine response

Another factor that can influence the effect of taurine on the caffeine contracture is illustrated in figure 8.5(a). Under these circumstances the $[Ca^{2+}]$ in the loading period was increased to 0.47 μM. The resulting caffeine-induced contracture reached approximately 80% of maximal Ca^{2+}-activated force. On addition of 30mM taurine, the size of the caffeine-induced contracture decreased and showed a large variation between successive responses. This variation in the amplitude may be the consequence of the spontaneous release of Ca^{2+} from the s.r. occurring in the interval between the caffeine contractures. Evidence for this is provided by the small fluctuations in tension during the period of incubation in the loading solution. The normal effect of taurine, detailed earlier, was obtained from the same preparation if the $[Ca^{2+}]$ in the loading and caffeine solutions was reduced to 0.2 μM which gave a caffeine-induced-contracture below 50% of maximal Ca^{2+}-activated force (figure 8.5(b)). Under these conditions no spontaneous fluctuations in tension were observed whilst the preparation was in the loading solution.

Taurine and myofilament Ca^{2+}-sensitivity.

To investigate the effects of taurine on Ca^{2+}-sensitivity, trabeculae were skinned with Triton-X100 to remove the remaining diffusional barriers between the myofilaments and the bathing solution. A typical result is shown in figure 8.6. Taurine (30mM) produced only a small increase in force when

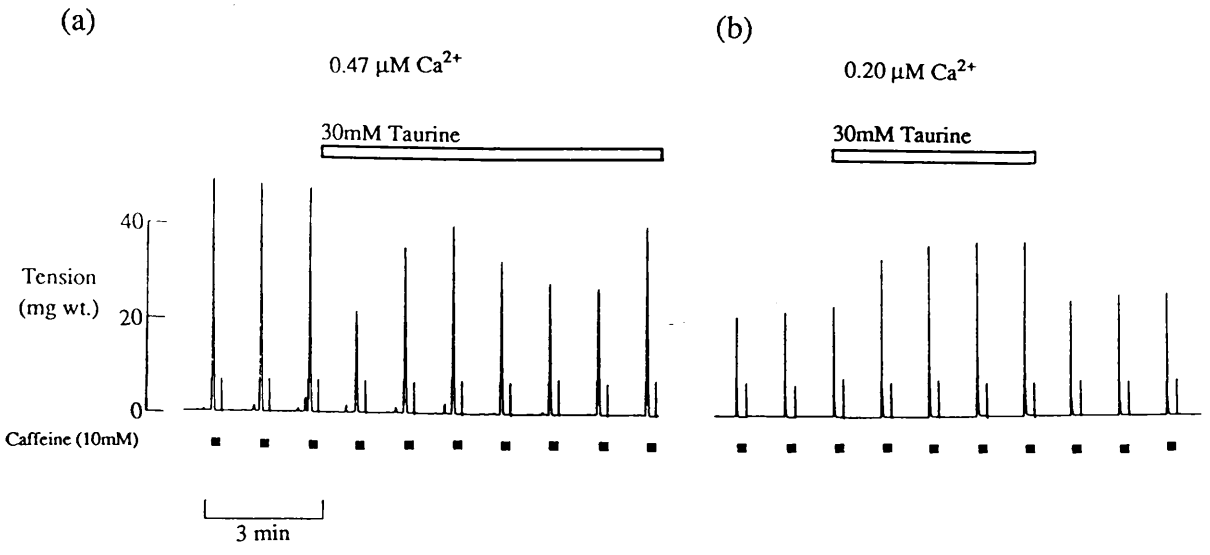


Figure 8.5 The effect of taurine on caffeine-induced contractures in a saponin-skinned rat trabecula (protocol as for figure 3.1). All solutions were weakly Ca^{2+}-buffered (0.2mM $EGTA_{total}$). The presence of caffeine is indicated by the bar below each contracture. In panel (a) the muscle was exposed to 0.47 μM Ca^{2+} for 1 min. prior to each caffeine contracture. Panel (b) illustrates tension recordings from the same muscle in the presence of 0.2 μM Ca^{2+}. In panels (a) and (b) the presence of taurine (30mM) is indicated by the bar above each trace.

added to a bathing solution with 57 μM Ca^{2+} (maximal Ca^{2+}-activated) force. However, the effect of taurine was greater when applied at a [Ca^{2+}] evoking approximately 50 % of maximal Ca^{2+}-activated force. In general, taurine (30 mM) caused only a slight increase in maximal Ca^{2+}-activated force (0.8%,$\pm$0.4% n=4). The overall effect of taurine on myofilament Ca^{2+}-sensitivity is illustrated in figure 8.7. Taurine decreased the [Ca^{2+}] required for half maximal tension from 3.02 μM to 2.51 μM. In four experiments, half maximal tension was achieved at a range of [Ca^{2+}] from 3 to 8 μM, 30 mM taurine decreased the [Ca^{2+}] needed for half maximal tension by an average of 10.5% ($\pm$3% S.D.).

The concentration dependence of this effect is shown in figure 8.8. Approximately half Ca^{2+}-activated force was achieved in the presence of 3.8 μM Ca^{2+}. The addition of 10 μM taurine had no effect on force, but there was a small increase in force on addition of 1 mM taurine. The addition of taurine at 20 mM increased Ca^{2+}-activated force by 15%, however, the effect of 40 mM was not significantly greater.

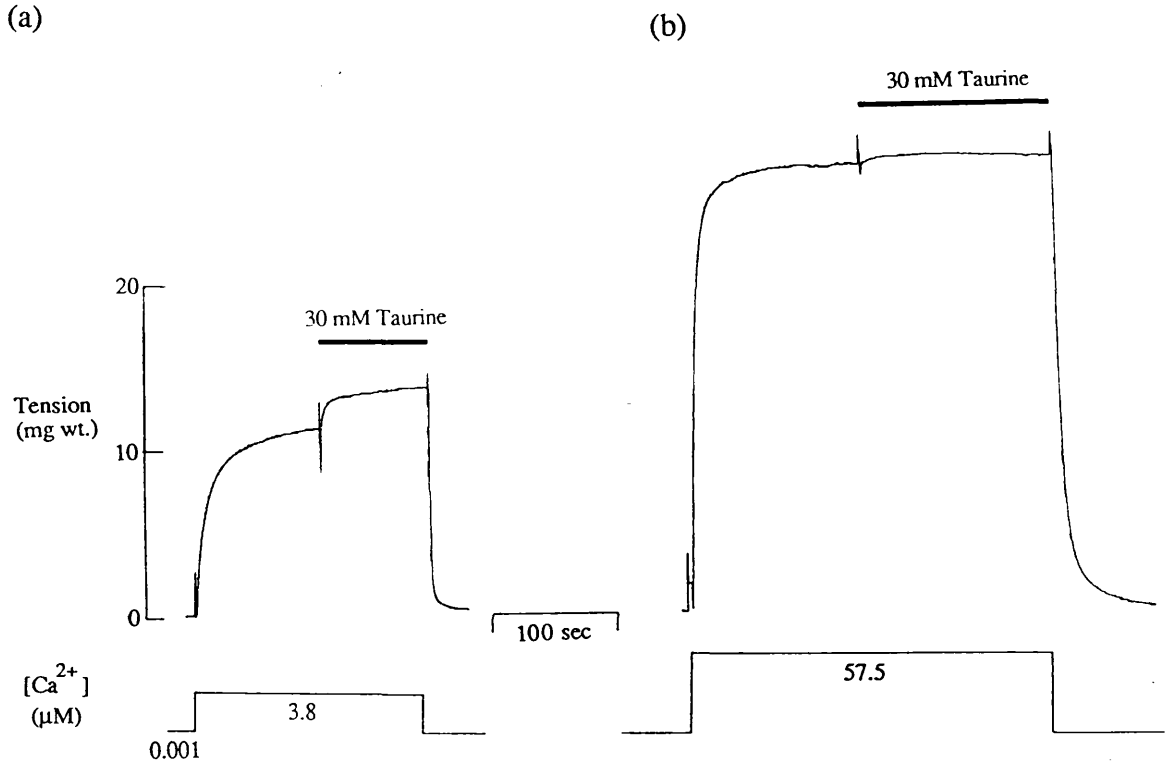


Figure 8.6 The effect of taurine on Ca^{2+}-activated force in a Triton-skinned trabecula. All solutions were strongly Ca^{2+}-buffered (10mM $EGTA_{total}$). In panel (a) the tension recording was obtained by raising the $[Ca^{2+}]$ in the bathing solution from 0.001 μM to 3.7 μM. In panel (b), maximum Ca^{2+}-activated force was achieved by raising the $[Ca^{2+}]$ to 57.3 μM. In panels (a) and (b) the presence of taurine (30mM) is indicated by the bar above each trace.

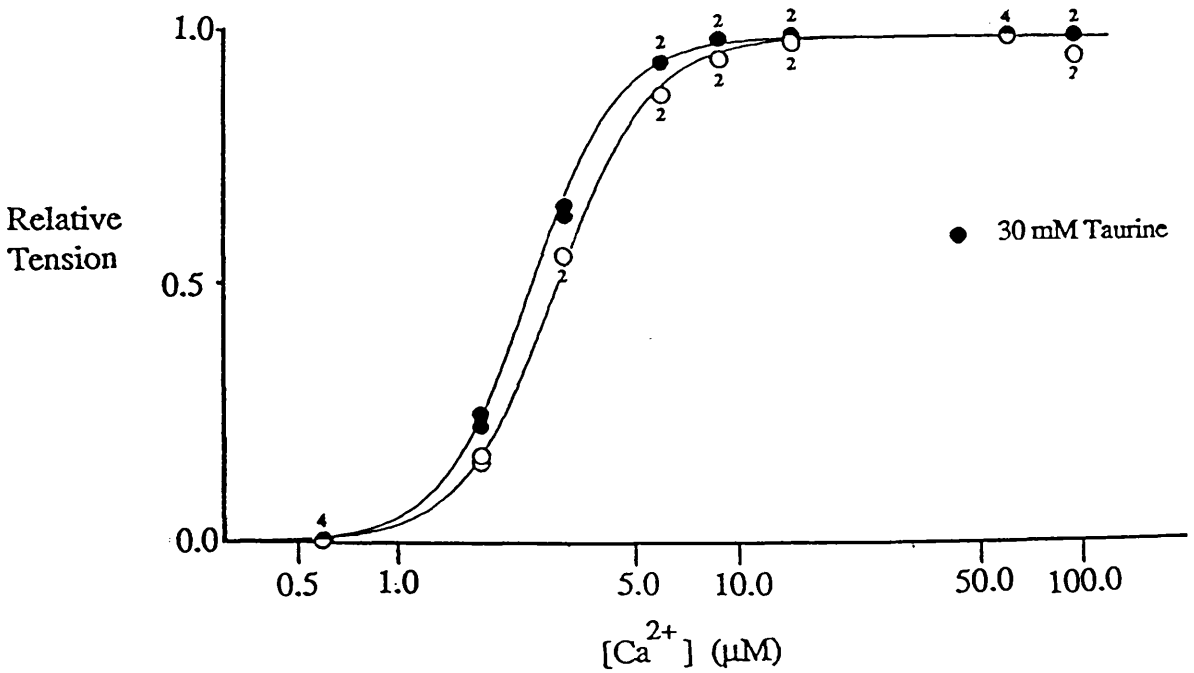


Figure 8.7 The relationship between $[Ca^{2+}]$ and tension in a Triton-skinned trabecula in the absence (○) and presence (●) of 30 mM taurine. The numbers beside each point represent the number of observations. The solid lines are best fit curves to the Hill equation (see chapter 2).

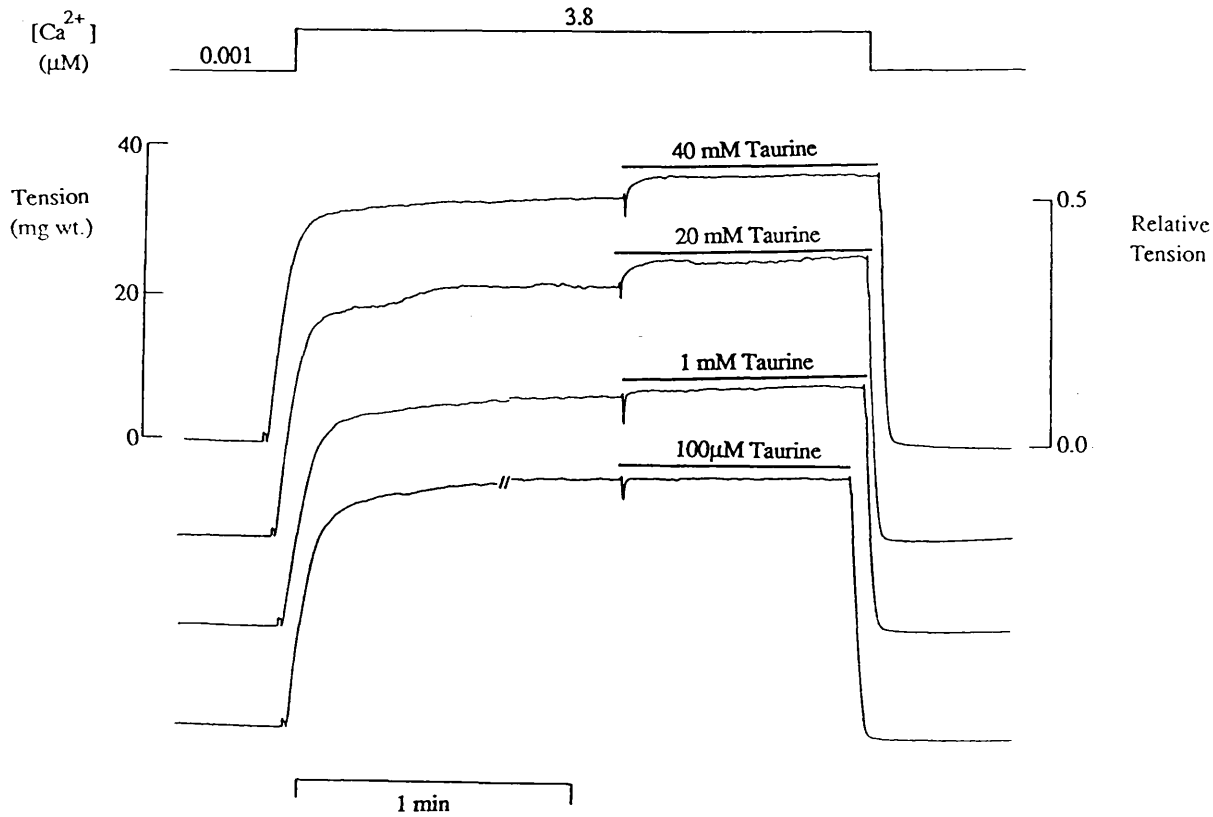


Figure 8.8 illustrates the dose dependence of taurine's action on submaximal force production in a Triton-skinned trabecula. All solutions were strongly Ca^{2+}-buffered (10mM $EGTA_{total}$). Each of the four tension records represents the force in response to raising the $[Ca^{2+}]$ from 0.001 μM to 3.8 μM which evokes approximately half maximal Ca^{2+}-activated force. Taurine concentrations ranging from 40 mM to 100 μM were introduced as indicated by the bar above each trace. Tension relative to maximal Ca^{2+}-activated is force shown on the right hand ordinate. A 1 min break in the trace before applying 100μM taurine is indicated (-//-).

DISCUSSION

Effects on Ca^{2+}-sensitivity

Figures. 8.6,8.7 & 8.8 illustrate that taurine increased the apparent Ca^{2+}-sensitivity of the contractile proteins. This effect is much smaller than that observed in crustacean fibres. In crab muscle, half maximum Ca^{2+}-activated force was potentiated by 40% at 5mM taurine (Galler & Hutzer, 1988), whereas in this study, under similar conditions 40mM taurine produced only 15% more force.

At this point it is interesting to consider the effects of known Ca^{2+}-sensitisers on different muscle preparations. Caffeine, sulmazole and the endogenous imidazole carnosine increase the Ca^{2+}-sensitivity of the contractile proteins (Herzig, Feile & Rüegg, 1981a, Wendt & Stephenson, 1983, Lamont & Miller, 1990). However, the caffeine-induced sensitivity shift is of a similar magnitude in cardiac and slow skeletal muscle but much smaller in fast skeletal fibres (Wendt & Stephenson, 1983). Furthermore, and of particular relevance to this study, none of caffeine, sulmazole or carnosine influence the Ca^{2+}-sensitivity of crustacean muscle (Ashley & Griffiths, 1985, Lamont & Miller, 1990).

These results may reflect subtle differences in the structure of the troponin subunits. There are known to be differences in the Ca^{2+}-binding properties of troponin-C from different muscle types and species. For instance, fast twitch vertebrate skeletal muscle has two low affinity

Ca^{2+}-binding sites and two high affinity Ca^{2+}-binding sites which also bind Mg^{2+}. In contrast, cardiac muscle has only one Ca^{2+}-specific site in addition to two Ca^{2+}/Mg^{2+}-binding sites and crustacean troponin has only one Ca^{2+}-binding site (Rüegg, 1986a).

Thus it is possible that the different characteristics of the troponin molecule underlie the much smaller taurine-induced sensitivity shift in cardiac muscle as compared with that reported for crustacean fibres. However, it should be noted that there is some doubt whether troponin-C is the primary site of action of caffeine-like compounds (for discussion see general introduction).

The effect of taurine on the caffeine-induced contracture

Taurine increased the magnitude of submaximal caffeine contractures. One possible explanation of the phenomenon is that taurine increased the free $[Ca^{2+}]$ of the solution. This could occur either through Ca^{2+}-contamination of taurine itself or by altering Ca^{2+}-binding to other constituents of the bathing solution (e.g. EGTA or ATP). However, the $[Ca^{2+}]$ was checked in solutions using the fluorescent indicator Fura-2 (1 μM) in a fluorometer (Perkin Elmer). In the range of solutions used in the study, taurine (up to 50 mM) did not alter the $[Ca^{2+}]$ significantly (these measurements were carried out by Dr G.L.Smith in this department). With this possibility excluded this, it can be concluded that taurine increased the ability of the s.r. to accumulate Ca^{2+} under these experimental conditions.

It seems unlikely that taurine produces this effect via a cAMP-mediated mechanism for the following reasons; (i) There is no evidence of β-receptor responses after saponin-skinning (ii) phosphodiesterase activity cannot be demonstrated in these preparations (iii) while forskolin toxin does increase the caffeine contracture, taurine reportedly has no effect on cardiac adenylate cyclase (iv) unlike the response to cAMP the potentiation produced by taurine was reversible (Khatter et al, 1981 see also chapter 4).

Results from previous studies have suggested that taurine acts by increasing Ca^{2+}-binding to sites on the sarcolemma (Chovan et al 1980). More recently, specific taurine binding sites have been found in rat cardiac s.r. (Quennedey et al, 1986). Thus an increase in Ca^{2+}-binding to the s.r. and the contractile proteins could explain the results from this study. If the overall effect of taurine is to increase Ca^{2+}-affinity for Ca^{2+}-binding sites throughout the muscle cell, the influence of taurine on contractility will depend on the relative effects of taurine on the various intracellular $[Ca^{2+}]$ regulating mechanisms.

Taurine (at millimolar concentrations) increased the Ca^{2+}-sensitivity of the contractile proteins. This may contribute to the larger caffeine-induced contractures seen at concentrations greater than 1 mM. In an attempt to quantify this, the effect of 30mM taurine on the caffeine-induced contraction was measured in a saponin-treated trabecula. The muscle was then treated with Triton-X 100 and the change in Ca^{2+}-sensitivity due to 30 mM taurine

was measured. The results indicated that taurine caused a doubling of the size of the caffeine contracture, (osmotic strength compensated), but if the increased Ca^{2+}-sensitivity was taken into account, the amplitude of the caffeine contracture would still be increased by a factor of 1.76. The complex shape of the relationship between contracture amplitude and taurine concentration (figure 8.4) above 10mM may reflect the conflicting influences of increasing Ca^{2+}-sensitivity and increasing osmolarity.

Physiological relevance of changes in taurine concentration

In ischemic heart disease, the taurine concentration decreases while in congestive heart failure the opposite occurs (Huxtable & Bresner, 1974, Crass & Lombardi, 1977). In the case of ischaemia, it seems likely that the fall in taurine levels is a result of cell damage. Indeed it has been observed that an increase in taurine excretion is a general symptom of cellular damage (Baldetorp & Martensson, 1980). In congestive heart failure it is at least possible that the increase in taurine may represent a compensatory mechanism.

In this study, taurine concentrations greater than 1mM increased the Ca^{2+}-sensitivity of the contractile proteins although the effect was small even at 40mM (0.05pCa units see also figures 8.6 & 8.7). Taurine was much more potent in its effect on the s.r. This was evident at 10 μM taurine, and maximal at 5 to 10mM with an apparent K_m of about 50 μM (figure 8.4). If the osmotic strength is compensated with sucrose, the influence of taurine on

the caffeine contracture apparently saturates at around 1mM. Thus taken at face value, any increase in cellular taurine such as occurs in congestive heart failure would be without effect.

However, there are a number of factors which may influence the shape and position of the dose response curve. In the first case, it is not clear whether sucrose is a suitable substitute for taurine. Apparently there is no information about the relative s.r.-permeabilities of these substances. Thus, in figure 8.4, the reduced potentiation of the caffeine-contractures at high concentrations of taurine may be a genuine phenomenon rather than an artefact due to osmolarity changes.

If this is the case then the increase in intracellular taurine which occurs in congestive heart failure may serve to decrease s.r., and hence, cellular Ca^{2+}. Furthermore, whether sucrose compensation is appropriate or not, it seems likely that the position of the curve may depend upon the $[Ca^{2+}]$. The interaction between taurine and Ca^{2+} will be considered in the next section.

The Ca^{2+}-dependence of taurine's action on the s.r.

In intact preparations the inotropic effect of taurine is Ca^{2+}-sensitive. In guinea-pig ventricle, taurine increased the size of twitch in a dose-dependent manner, but was negatively inotropic if applied at a high external calcium concentration (Franconi et al 1982). The effect of taurine on rat muscle is more complex. At normal external calcium concentrations

taurine can cause a negative inotropic response (Dietrich & Diacono 1971), or a small positive inotropic effect (Schaffer, Chovan & Werkman 1978). However, in the presence of low external calcium the taurine caused a marked positive inotropic response (Khatter, Soni, Hoeschen Alto & Dhalla, 1981).

In the current study the effect of taurine on the s.r. exhibited a similar Ca^{2+}-dependence. Under the standard conditions used in this study, the Ca^{2+}-loading was adjusted until the contracture in response to 10mM caffeine produced 15-40% of maximum Ca^{2+}-activated force. Under these conditions, introduction of up to 40mM taurine caused a potentiation of the caffeine response. However, as illustrated in figure 8.5, if the Ca^{2+} load on the s.r. was sufficiently high, taurine reduced the amplitude of the caffeine contracture, which also became variable in size.

One possible explanation for these observations is that, under these circumstances, the s.r. becomes overloaded with Ca^{2+} in the presence of taurine. Studies on aequorin-injected papillary muscles have shown a comparable change in the amplitude of the Ca^{2+} transient when the extracellular [Ca^{2}] was raised to well above physiological levels (Allen, Eisner, Pirolo & Smith, 1985a). The increased influx of Ca^{2+} is taken up by the s.r. which quickly becomes overloaded. In this state, the s.r. releases Ca^{2+} spontaneously and as a result, less is available to be released during a twitch. The highly variable twitch amplitude arises from the differing amounts of Ca^{2+} remaining in the s.r. after spontaneous release (Allen et al, 1986b). In

support of this theory, fluctuations in base line tension are obvious in figure 8.5(a) indicating loss of Ca^{2+} from the s.r. However, these oscillations do not apparently increase in size or frequency after addition of taurine although it is possible that much of the Ca^{2+}-release may be subthreshold for tension production. Furthermore, promotion of spontaneous Ca^{2+}-release is inconsistent with the reported antiarrhythmic action of taurine.

Another, albeit more speculative, possibility is that taurine may increase calcium uptake by the s.r. at low $[Ca^{2+}]$s and decrease uptake at high $[Ca^{2+}]$. This may seem unlikely. However in a study by Serbring & Huxtable (1985) taurine was found to increase Ca^{2+}-binding to sarcolemmal proteins at low extracellular $[Ca^{2+}]$ (<2mM) and decrease binding at $[Ca^{2+}]$ above 2mM. If taurine had a similar Ca^{2+}-dependent effect on s.r. Ca^{2+}-binding (and presumably uptake), this would be consistent with most results on intact tissue. In hypodynamic hearts, taurine would increase s.r. Ca^{2+}-uptake and hence contractility. In contrast, in high extracellular Ca^{2+} or in conditions leading to overload (such as ischaemia) taurine would tend to decrease s.r. Ca^{2+} and reduce the probability of spontaneous release and arrhythmia. It is not obvious how such a Ca^{2+}-dependent relationship could occur. It is possible that taurine influences conflicting parameters such as Ca^{2+} and Mg^{2+} binding and the end result is a bell shaped response.

In conclusion

Most previous studies have dismissed the action of taurine as pharmacological and have concluded that taurine is acting at high concentrations on the sarcolemmal membrane. However, more recently, it has been suggested that some of the effects of taurine require the maintenance of intracellular levels although the mechanism of action was unknown (Franconi et al, 1982). This study is the first to demonstrate that taurine has a direct effect on the s.r. and contractile proteins in cardiac muscle. These findings may explain some of the apparently disparate effects of taurine observed in cardiac muscle.

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