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HYSTERESIS AND THE LENGTH DEPENDENCE OF CALCIUM SENSITIVITY IN CHEMICALLY SKINNED RAT CARDIAC MUSCLE

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SUMMARY

1. The relationship between pCa ($-\log_{10}[Ca^{2+}]$) and steady-state isometric tension has been investigated in saponin- or Triton-treated (chemically 'skinned') cardiac muscle of rat.

2. Hysteresis exists in the relationship such that the muscle is less sensitive to Ca^{2+} during increasing activation (as $[Ca^{2+}]$ is stepped upward) than during reducing activation (as $[Ca^{2+}]$ is stepped downward).

3. The extent of the hysteresis is insensitive to interventions that increase overall calcium sensitivity by chemical means, such as caffeine, carnosine or increased pH.

4. The extent of the hysteresis is sensitive to sarcomere length. The phenomenon is virtually absent above sarcomere lengths of about 2·2–2·3 μm but becomes progressively greater at shorter sarcomere lengths.

5. The effect of sarcomere length on calcium sensitivity is restricted to the upward-going (increasing activation) part of the pCa–tension loop below 2·2 μm. The downward-going (decreasing activation) part of the hysteretic relationship is virtually unaffected by sarcomere length up to 2·2 μm.

6. Significant alterations in sarcomere length do not occur during tension development in the experiments described here: the phenomenon is not attributable to experimental artifacts of this kind.

7. Hysteresis develops sufficiently rapidly to be consistent with a physiological relevance during the normal heart beat.

8. The effects of sarcomere length show that the phenomenon is not due to force *per se* since, for example, greater peak force produces less hysteresis as sarcomere length is increased towards 2·2 μm.

9. Tonicity increase (by high-molecular-weight dextran), which shrinks the myofilament lattice, increases calcium sensitivity but reduces the effect of sarcomere length on calcium sensitivity.

10. The results suggest that lattice shrinkage is the mechanism which accounts for hysteresis in, and the sarcomere length dependence of, calcium sensitivity in cardiac muscle.

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INTRODUCTION

The relationship between intracellular free calcium concentration ($[Ca^{2+}]_i$) and tension has been extensively investigated both in intact muscle cells by using calcium indicators such as aequorin (Blinks, Weir, Hess & Prendergast, 1982) and in 'skinned' muscle where the sarcolemma is removed by microdissection (e.g. Fabiato & Fabiato, 1978) or chemical means (e.g. Endo & Kitazawa, 1978). Knowledge of the form of this relationship is central to the interpretation of contraction and relaxation, particularly when experimental interventions are likely to influence $[Ca^{2+}]_i$. It has been tacitly assumed in the past that the relationship is unique for any given set of ionic conditions. However: (1) It has recently been reported that hysteresis exists; the contractile proteins are more sensitive to Ca^{2+} as $[Ca^{2+}]_i$ is being reduced than as it is being raised (Ridgway, Gordon & Martyn, 1983). (2) Sarcomere length affects calcium sensitivity (Endo, 1972; Hibberd & Jewell, 1982). (3) Phosphorylation can influence apparent calcium sensitivity (e.g. Silver, 1986).

Hysteresis, in the present context, means that a submaximal calcium level can maintain a higher tension than it can create. (As will be shown, these tension levels can be maintained indefinitely so that a time lag is not implied by the use of the term 'hysteresis'.) Consequently, $[Ca^{2+}]_i$ must fall further than previously supposed to account for the relaxation of muscle. We have observed this phenomenon in cardiac muscle. It is sensitive to sarcomere length, which may throw new light on the interaction between sarcomere length and the calcium sensitivity of the contractile apparatus. In contrast to the interpretation of Ridgway *et al.* (1983) and Gordon, Ridgway & Martyn (1984) we conclude that it is not force *per se* that alters the calcium sensitivity during hysteretic sequences; rather it seems to be dependent upon myofilament lattice spacing which, when reduced, increases calcium sensitivity. These findings reveal a further complication in the interpretation of tension levels in terms of $[Ca^{2+}]_i$ and vice versa, methods which are widespread in muscle physiology. Preliminary results have been reported to the Physiological Society (Harrison, Lamont & Miller, 1985).

METHODS

The methods used in this study are described here in some detail since they have only been published in outline to date (Miller, Sinclair, Smith & Smith, 1982; Miller & Smith, 1984, 1985; Smith & Miller, 1985). Most experiments were carried out on small, free-running trabeculae isolated from the right ventricle of the rat. The majority of preparations are taken from the base of the ventricle near the valves. The animals were killed by a blow to the head and the heart was rapidly excised and flushed with a saline solution (solution F, Table 1) at about 20 °C. A suitable trabecula (1–2 mm long when mounted and 80–130 μm, or exceptionally 180 μm, in diameter) was excised and mounted for isometric force measurement.

Mounting and force measurement. The muscles were snared at both ends with nylon monofilaments (25 μm diameter) emerging from stainless-steel tubes (100 μm i.d., 200 μm o.d) to give a final preparation length of 1–2 mm. The steel tubes were mounted on a force transducer (Akers AE 875, Horten, Norway) and a rigid rod, both carried on a micromanipulator which permitted fine adjustments to the length of the preparation. The overall compliance of the system is $< 0{\cdot}01$ μm/μN which is equivalent to a length change of $< 0{\cdot}15$% for a typical preparation 2 mm in length generating 30 mg wt in tension. The first resonant frequency of the transducer and mounting was 180–190 Hz. The mounting is schematically illustrated in Fig. 1*A*.

Solution exchange. The method is a development of that originally described by Ford & Podolsky

(1972) and Ashley & Moisescu (1977). The experimental solutions are carried in a series of wells (4·5 ml) in a Perspex block. A solution change is effected by lowering the block and sliding it horizontally to bring the new solution under the muscle. The block is then raised to re-immerse the tissue. These movements, made by two stepper motors operating under microprocessor control, took 1–2 s in total for most of the present experiments. Where necessary (see e.g. Fig. 9), this time can be reduced to less than 0·2 s. The emergence of the muscle through the solution meniscus, as

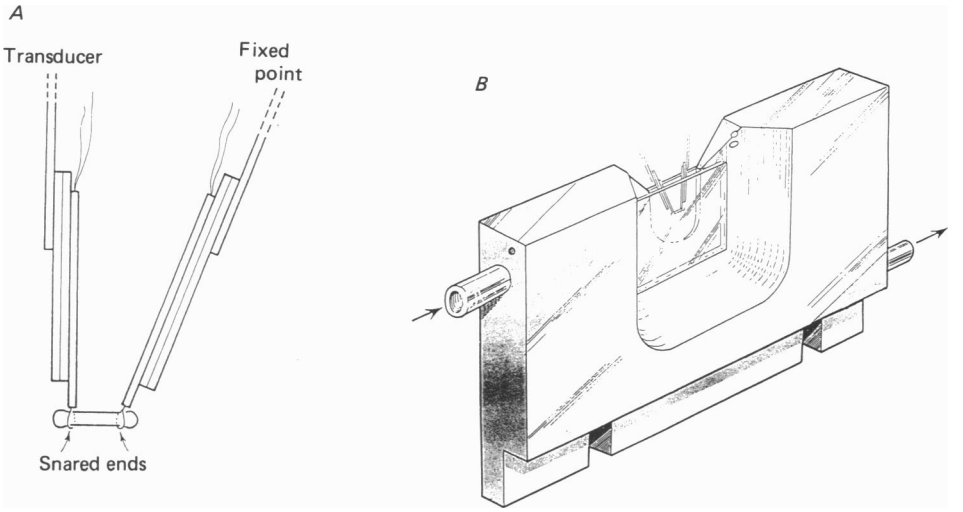

Fig. 1. *A*, schematic view of the mounting for muscle preparations. Nylon monofilament snares hold the muscle. The tube diameter (200 μm) is, in fact, generally twice that of the preparation. Additional lengths of tube in the same plane as the long axis of the muscle stiffen the whole assembly while keeping it thin enough to enter the microscope chamber. *B*, schematic view of the chamber used in the DIC microscope for sarcomere length measurements. The gap of 1·5 mm between the glass faces (two cover-slips) allows the mounting to gain access to the bath while being narrow enough for the optical requirements of high-magnification differential interference contrast. The stage can be temperature controlled by flowing water through the jacket (arrowed). The fluid contained in the observation chamber can be changed via tubes which open at its lip and base (not shown).

the bath is lowered, removes any significant adhering droplets. Checks confirm that the volume transferred from bath to bath, even after several repeats, is strictly negligible. The solutions are stirred continuously by a small paddle. The system permits accurate timing of solution changes in pre-programmed runs and ensures, for example, that the muscle is immersed to exactly the same depth in each bath which is essential for the reproducibility of tension levels.

Measurements of sarcomere length. Precise knowledge of sarcomere length is critical in these experiments. It is well-established that the contractile protein's calcium sensitivity increases as sarcomere length increases (e.g. Hibberd & Jewell, 1982). In addition, the extent of hysteresis is markedly sarcomere length dependent, as will be shown, as is the extent of calcium uptake and/or release by the sarcoplasmic reticulum in saponin-treated preparations (C. Lamont & D. J. Miller, unpublished observations).

A separate chamber is used to permit monitoring of sarcomere length. We have chosen to measure sarcomere length by differential interference contrast (DIC) light microscopy rather than laser diffraction. The microscope method allows a better check on local variations in sarcomere pattern, as well as enabling one to assess the dimensions of the muscle and the extent of any damage. The dimensions of the preparation were routinely determined by measuring the diameter near the middle and towards each end in two planes at right angles. Values quoted in the text are

averages of these measurements. A further advantage of direct microscopy is that no special selection of preparations, as seems to be necessary when laser diffraction pattern is used (see Hibberd & Jewell, 1982, Methods) was required. This, and other consequences of this approach, are considered further in the Discussion. The microscope used is a modified Vickers M-17 using the DIC system after Smith (1969). The microscope lies supine to facilitate access to the space between objective and condenser. The stage has been replaced with the chamber illustrated in Fig. 1*B*. The solutions in the chamber (volume 0·3 ml) can be changed by syringes connected to small tubes which open at its base and lip (not shown). The relatively low contrast of the DIC image obtained from cardiac muscle is conveniently enhanced by viewing the muscle on a monochrome TV monitor. In all experiments, the sarcomere length is set while viewing the muscle in its relaxed state in this chamber; the preparation is then transferred to the solution change system. From time to time during the experiment sarcomere length can be checked. Usually no significant adjustments are necessary. The graticule in the microscope was calibrated with diffraction gratings viewed and recorded at the same magnifications as the muscle.

In some experiments (e.g. Fig. 8), the sarcomere length was monitored continuously, together with tension, throughout a series of solution changes which were made in the observation chamber described above. The image was recorded on a standard domestic video recorder for subsequent study by computer-assisted image analysis (Magiscan 2, Joyce-Loebl Ltd, Moss, Miller & Lamont, 1986).

Solutions. The rationale for the solution composition, the method of calculating free ion levels and ionic strength, the choice of ion-binding constants for the various ligands, the precautions for EGTA purity, calcium-contamination determination and the measurement of pH are described in detail elsewhere (Miller & Smith, 1984, 1985; Smith & Miller, 1985). To minimize the inconvenience associated with the accurate measurement of pH (see Illingworth, 1981; Miller & Smith, 1984), the electrode was routinely calibrated against a standard appropriate to our own media. This comprised a solution of 25 mM-HEPES, 197 mM-KCl and 4·765 mM-KOH (from fresh 1 M titration standard, BDH) which we calculate to have an ionic strength of 0·202 M, $pH_{concentration}$ (pH_c) of 6·866 and $pH_{activity}$ (pH_a) of 7·000. Ionic strength in this paper is defined as the total of ionic equivalents, I_e:

$$I_e = \tfrac{1}{2} \Sigma C_j z_j, \quad (1)$$

where C_j is the concentration of the jth ionic species, and z_j is its valency (for a full justification of this approach for solutions of this type containing several multivalent ionic species see Smith & Miller, 1985). In accordance with Bates, Roy & Robinson (1973), the zwitterion of HEPES is assumed to have no effect on ionic strength. The thermodynamic pK_2 ($-\log$ second H^+ dissociation constant) of 7·629 at 20 °C reported by Vega & Bates (1976) was used, giving a pK_{app} ($-\log$ apparent H^+ dissociation constant) of 7·494 at $I_e = 0{\cdot}2$ M. The correction of activity coefficients and affinity constants for ionic strength (as I_e) was as described by Smith & Miller (1985). The free Ca^{2+} concentration ($[Ca^{2+}]$) was established in the first instance by mixing solutions A and B (Table 1) in the desired ratios. Free $[Ca^{2+}]$ is also expressed as pCa ($= -\log_{10}[Ca^{2+}]$). In those experiments where tonicity was altered, dextran (T70, Sigma) was added to the final solutions (A and B, Table 1). This has the effect of maintaining Ca^{2+} volume molality (and that of all other ionic species) since the volume occupied by the aqueous phase essentially remains constant (see also Godt & Maughan, 1981).

Water used for the solutions was single distilled only. The supply in Glasgow is so low in mineral and organic content that it is not necessary to take special precautions, even with selectively skinned muscle where the intracellular organelles are directly exposed to the bathing media. Experiments were carried out at room temperature (20–22 °C).

Chemical skinning procedure. The mounted muscle was initially exposed to a 'relaxing' solution (B, Table 1) including either the cholesterol-precipitating agent saponin (50 μg/ml) or Triton X-100 (1 % v/v) for 20–30 min. The chemical skinning agent was then removed by washing in solution B before the first test $[Ca^{2+}]$ was applied. Most muscles were first treated with saponin; after completion of most of the manoeuvres, Triton was applied to check for significant alterations in responses. Calcium sensitivity is unaltered by this treatment but calcium-activated contractures develop (time to half-maximum tension) about 1·5 times faster (see Miller & Smith, 1985, and Harrison, 1985, for details). The caffeine-induced contracture (see e.g. Fig. 9) is, thereby, completely abolished since Triton rapidly destroys the integrity of the sarcoplasmic reticulum and mitochondrial membranes.

Data handling. The tension signal was routinely digitized (12 bit) at appropriate rates. The signal

was pre-filtered to avoid aliasing errors with an active filter (roll-off 12 dB per octave, 3 dB cut-off at 15 or 25 Hz) and amplified to use the full dynamic range of the A–D converter. Experimental data and calibration signals were stored on disc on a PDP-11 34 computer for subsequent analysis. A continuous chart recording (Linseis 1800, full-scale deflection frequency response flat from DC to 1 Hz, 3 dB down at 4 Hz) was also made. Tension responses are shown calibrated in either or both absolute force and relative force. For the latter, tension was normalized to maximum calcium-activated force (C_{max}) which was determined at intervals throughout the experiment. This was done using either, or both of, solutions A and E (Table 1). Provided that relatively short intervals

TABLE 1. Composition of solutions (in mM except where stated)

Solution	K*†	Mg‡	Total calcium	pCa	ATP	Creatine phosphate	EGTA	HDTA	HEPES
A	130	7·0	10·0	4·25	5·0	15·0	10·0	—	25·0
B	130	7·0	0·02	9·03	5·0	15·0	10·0	—	25·0
C	130	7·0	0·2	5·12	5·0	15·0	0·2	9·8	25·0
D	130	7·0	0·02†	7·29	5·0	15·0	0·2	9·8	25·0
E	130	7·0	10·1	3·99	5·0	15·0	10·0	—	25·0
F (Tyrode)	5	1·0	2·0	2·70	—	—	—	—	5·0

All solutions included 5 mM-glucose and (except F) 25 i.u. creatine kinase (Sigma), and sodium ions§; pH_a 7·00 (see above). Total chloride concentration (as KCl and HCl, or NaCl in F) varies from about 110 to 120 mM.

* Potassium ions added as KCl and KOH.

† Contamination levels in the final experimenal solution (see Miller & Smith, 1984).

‡ Magnesium added as 1 M-$MgCl_2$; free Mg^{2+} = 2·13–2·5 mM in all solutions including mixtures of A and B or C and D.

§ Sodium ions (40 mM) are present as Na_2ATP and Na_2CrP except in F (140 mM-NaCl).

elapsed between normalizing maxima, a linear interpolation between these test levels was found to describe adequately the decline of tension. The mean rate of decline of peak force for ten preparations in successive experiments was 0·178% per minute (range 0·024–0·45%); more rapid decline was taken as indicative of damage and the results not used for quantitative work. This figure compares very favourably with a mean of 1·20% per minute reported by Jewell & Kentish (1981) for Triton-treated rat ventricle preparations; the reduction in rate of decline they report with dithiothreitol (to 0·12%) suggests that our preparations maintain contractile performance well, despite the absence of sulphydryl-protecting agents.

RESULTS

Some of the basic features of the phenomenon of hysteresis in the calcium sensitivity of cardiac muscle are illustrated in Fig. 2. In this sequence, $[Ca^{2+}]$ was stepped to four suprathreshold concentrations and then stepped downward. At each new $[Ca^{2+}]$ tension was allowed to stabilize and was maintained for several minutes. It is clear that at each $[Ca^{2+}]$ producing submaximal tension, force is greater as $[Ca^{2+}]$ steps downward (C_{down}). The dashed lines indicate the 'expected' tension level based on that achieved on 'upgoing' $[Ca^{2+}]$ steps (C_{up}). The phenomenon is, for a given preparation, reproducible, which confirms that it cannot be explained by a change in the bathing $[Ca^{2+}]$ between the successive exposures to the same chamber.

It is emphasized that *steady-state* tension was compared for 'upgoing' and 'downgoing' conditions at any given pCa in each case analysed for this paper. In some instances, when tension levelled out, it was maintained and observed for over 10 min to confirm that C_{down} was greater than C_{up} at that steady-state. The stepwise activation used here results in relatively slow tension development, unlike the 'Ca^{2+}-clamp' technique employed for example in Fig. 9, since the calcium

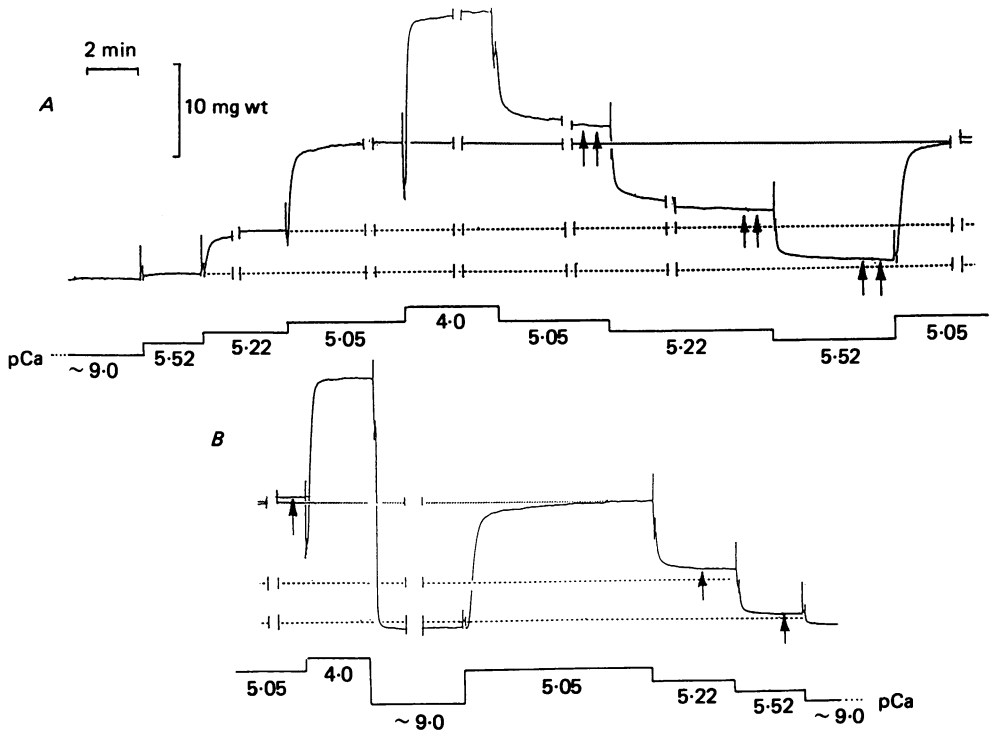

Fig. 2. Tension record from a rat ventricle trabecula (Triton treated) showing the basic phenomenon of hysteresis. The (continuous) trace is split into two panels, *A* and *B*. Tension was allowed to stablize at each of the test pCa levels. Breaks in the trace are of 4 min duration. The test $[Ca^{2+}]$ expressed as pCa ($pCa = -\log_{10}[Ca^{2+}]$) is indicated below the tension trace. Dashed lines mark the steady-state tension at each pCa on the initial 'upgoing' sequence. *A*, the muscle was initially exposed to four test calcium levels. Steady-state tensions would define an 'upgoing' curve. The calcium level was then stepped downwards to a calcium level which was just suprathreshold for tension production (pCa 5·52). The full extent of hysteresis (the extra tension at a given pCa) is indicated by the double arrows. The muscle was then exposed to a higher intermediate calcium level. *B*, as the muscle has not been completely relaxed, a little hysteresis remained (indicated by the single arrows). The preparation was then exposed to a maximally activating calcium level to check that tension was not declining and then allowed to relax fully (pCa ~ 9·0). Next it was exposed to a pCa of 5·05 producing about 50% of C_{max}; calcium sensitivity had now moved back onto the 'upgoing' curve and exactly matches that extrapolated from the beginning of panel *A*. The bathing calcium was then reduced. As with incomplete relaxation, incomplete activation produced a partial hysteresis (indicated by the single arrows), less than that seen in panel *A* (double arrows). Sarcomere length 2·2 μm, diameter 130 μm.

buffer must equilibrate fully at each new $[Ca^{2+}]$. Nevertheless, tension was almost always within 2 or 3% of its steady-state level within 2 min.

Partial hysteresis

The second part of the trace (Fig. 2*B*) illustrates that hysteresis is only partially reversed or induced when muscles are not fully relaxed or activated, respectively. In this sequence $[Ca^{2+}]$ was raised again after the first upgoing and downgoing sequence

without returning the muscle to the fully relaxed state. The tension achieved in this second upgoing exposure to pCa 5·05 is significantly higher (arrowed, Fig. 2*B*) than that achieved initially, or at the end of the sequence under the standard conditions (started from the fully relaxed state). Peak force is unaffected by the route with which it is approached. Similarly, as the last part of the trace shows, when $[Ca^{2+}]$ is stepped downwards after submaximal activation, the extent of the hysteresis is less. The last two tension levels on the trace are not as far above the index line (arrowed, Fig. 2*B*) as those achieved at the same pCa immediately after full activation (double arrows, Fig. 2*A*).

In this instance, the hysteresis is small. However, as is illustrated later, the magnitude of the phenomenon can be altered experimentally. When results of this kind are translated into plots of $[Ca^{2+}]$ *vs.* tension, additional features are apparent. Figure 3*A* shows an example.

In these preparations, the pCa–tension relationship is well described by the Hill equation (e.g. Miller & Smith, 1985; Fry & Miller, 1985):

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

where C = steady-state tension, C_{max} is maximum calcium-activated force, K_{app} (units M^{-h}) is an apparent affinity constant and h is the Hill coefficient. The curves were fitted by a non-linear least-squares fitting procedure (after Levenberg and Marquat, see Brown & Dennis, 1972; Brown, 1972). For the purpose of comparison of calcium sensitivity for muscles where h differed, the $[Ca^{2+}]$ (or pCa) for half-maximal activation and relaxation were taken. This corresponds to the hth root of K_{app} (or $\log_{10}K_{app}/h$).

The reliability of the Hill equation as a fit to data of this kind and a detailed analysis of its application to experiments on skinned cardiac fibres are not considered here. No particular theoretical assumptions are made as a result of the success of the fitting procedure (see also the Discussion). However, this does provide a convenient and objective description of the pCa–tension relationship for the present purposes.

The median shift of log $[Ca^{2+}]$ for half-maximum tension ($\log_{10}(1/K_{app})$) between the upgoing ($-\log_{10}K_{app,up}$) and downgoing ($-\log_{10}K_{app,down}$) curves was 0·065 (range 0·047–0·085, $n = 6$) towards lower $[Ca^{2+}]$ (sarcomere length 2·1–2·2 μm). However, in addition to the reduced $[Ca^{2+}]$ required for half-maximum tension the steepness of the pCa–tension relationship is reduced on the downward limb of the hysteretic loop. The Hill coefficient gives a convenient measure of the steepness of the relationship. In the same six experiments for the ascending curve h was 2·75 (mean; range 2·40–2·98). For the descending curves h was reduced by 0·276 (range 0·0–0·427).

Hysteresis has also been observed in similar experiments on frog (*Rana temporaria*) and human cardiac muscle (not shown).

The variation in absolute calcium sensitivity between individual muscles makes it meaningless to present accumulated data in the form of Fig. 3*A*. In addition, we have chosen not to rely upon the pCa–tension relationship from those experiments where less than five pCa values were used to define it. Data from a total of twenty-two preparations are included in Fig. 3*B* which is devised to present otherwise valid data.

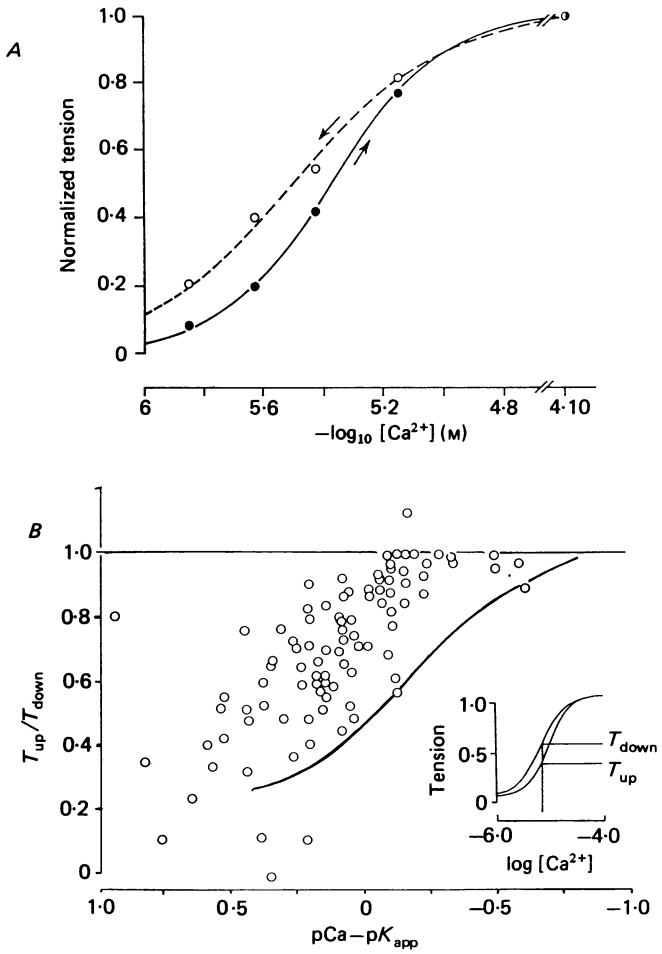

Fig. 3. *A*, pCa–tension relationship for a rat ventricle trabecula (Triton treated). Results similar to those obtained in Fig. 2 are plotted. The abscissa is pCa, the ordinate is steady-state relative force which has been normalized to maximum calcium-activated force (see text for further comments and details). Peak force is equivalent to about 60 mg wt (= 0·6 mN approximately) in this sequence. The arrows on the curves indicate whether data were obtained as $[Ca^{2+}]$ was stepped upward or downward. In this and subsequent Figures curves were fitted according to the Hill equation (eqn (2)), using the best-fit procedures described in the text adjacent to the equation. $\text{Log}_{10} K_{app} = 5{\cdot}375$ ($K_{app} = h$th root of K_{app}, eqn (1), with units M^{-1}, see text) and $h = 2{\cdot}4$ for the upward, and 5·5 and 1·8 for the downward limbs, respectively. Sarcomere length 1·9 μm; diameter 175 μm. *B*, accumulated data representing the extent of hysteresis of the type shown in *A*. The ratio of the tensions achieved in the upward (T_{up}) and downward (T_{down}) directions is plotted on the ordinate. In order to standardize the plot for the differences in absolute calcium sensitivity between individual preparations, the tension ratios are plotted against a calcium scale (abscissa) representing test pCa − pK_{app} ($= \log_{10}(1/K_{app})$) for the individual muscles. (K_{app} was established, by the best-fit procedure described in conjunction with eqn (2), for each experiment for both upward and downward curves, the latter being used for obtaining pK_{app} since it is almost insensitive to sarcomere length, as shown in Fig. 5.) Data from twenty-two preparations are represented here.

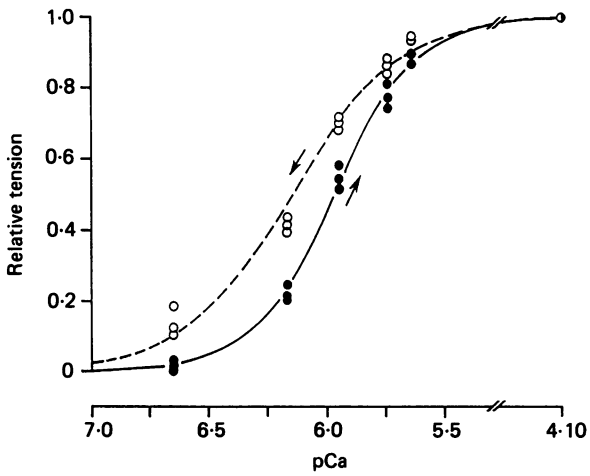

Fig. 4. pCa–tension relationship for a rat ventricle trabecula (saponin treated). Data from three separate runs on the same preparation are shown. The solutions all included 25 mM-imidazole, replacing HEPES. The pH_a was 7·2 instead of 7·00 used elsewhere. The values for $\log_{10} K_{app}$ and the Hill coefficient were 5·98 and 2·5 for the upward curve (●) and 6·15 and 1·9 for the downward curve (○). Sarcomere length = 2·1–2·2 μm.

For each experiment, points at a given pCa are expressed as the ratio of tensions achieved as [Ca^{2+}] is stepped upward (T_{up}) and downward (T_{down}). All the values are 1·0 or less confirming that when hysteresis occurs it is in the direction described above. The inset shows that data will tend to fall into a range between relatively little hysteresis, where the positions, and hence the slopes, of the descending and ascending curves are very similar, and greater hysteresis (see inset), where the slopes differ appreciably. The theoretical lines on the main plot are derived from this example. The lower line corresponds to a substantial hysteresis, and the upper to its absence, respectively. The cumulated results fall satisfactorily within the general area indicated by these extremes. There is additional variability because of differences in the absolute calcium sensitivity from one preparation to another. This tendency is compensated for by plotting the ratio T_{up}/T_{down} against $pCa - pK_{app,down}$. Values for $K_{app,down}$ were derived for each preparation as described above in connection with Fig. 3*A*. For the present purposes, this plot confirms that hysteresis is always in the direction that T_{up} is less than T_{down}.

Selective or complete membrane disruption

Hysteresis of this kind is found whether the muscle has been selectively skinned with saponin, which does not disturb the intracellular membrane systems and retains diffusible enzymes (e.g. Fig. 4), or with Triton, which removes all cellular membranes (e.g. Fig. 2). This observation allows us to conclude that cytoplasmic and membrane-bound enzyme systems are neither required for, nor modulate, the phenomenon. Hysteresis must be a feature of the contractile (and regulatory) proteins themselves.

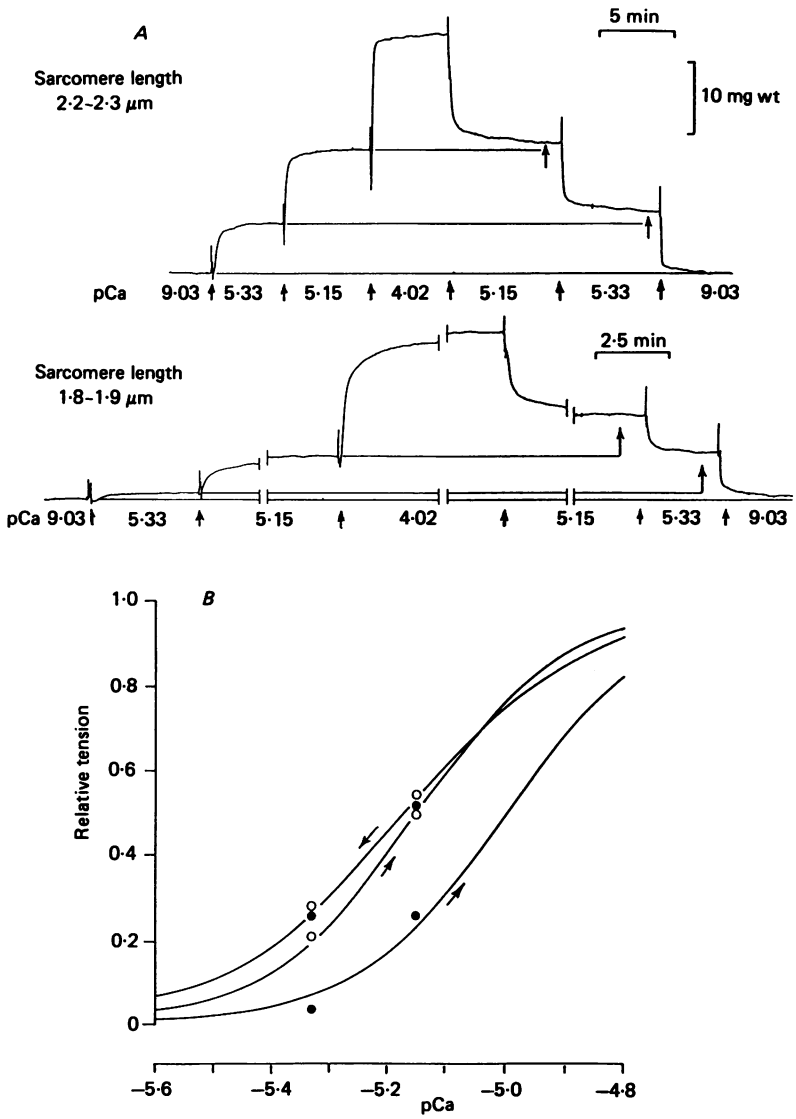

Fig. 5. *A*, tension responses from a rat ventricle trabecula (saponin treated). The upper trace was obtained at sarcomere length 2·2–2·3 μm, the lower trace at 1·8–1·9 μm. The same solutions were used for each of the sequences. Gaps in the lower trace are of 3 min duration to confirm the full equilibration of tension. *B*, the results from panel *A* are replotted as steady-state force, normalized with respect to maximum tension, at each sarcomere length. Open symbols for ~ 2·2 μm; closed symbols for ~ 1·8 μm. The arrows indicate whether calcium was being stepped upward or downward.

Effect of increased muscle calcium sensitivity

We were interested to test whether an alteration in the calcium sensitivity of the contractile proteins would influence the magnitude of hysteresis. Caffeine at millimolar levels increases calcium sensitivity (Wendt & Stephenson, 1983) and, as

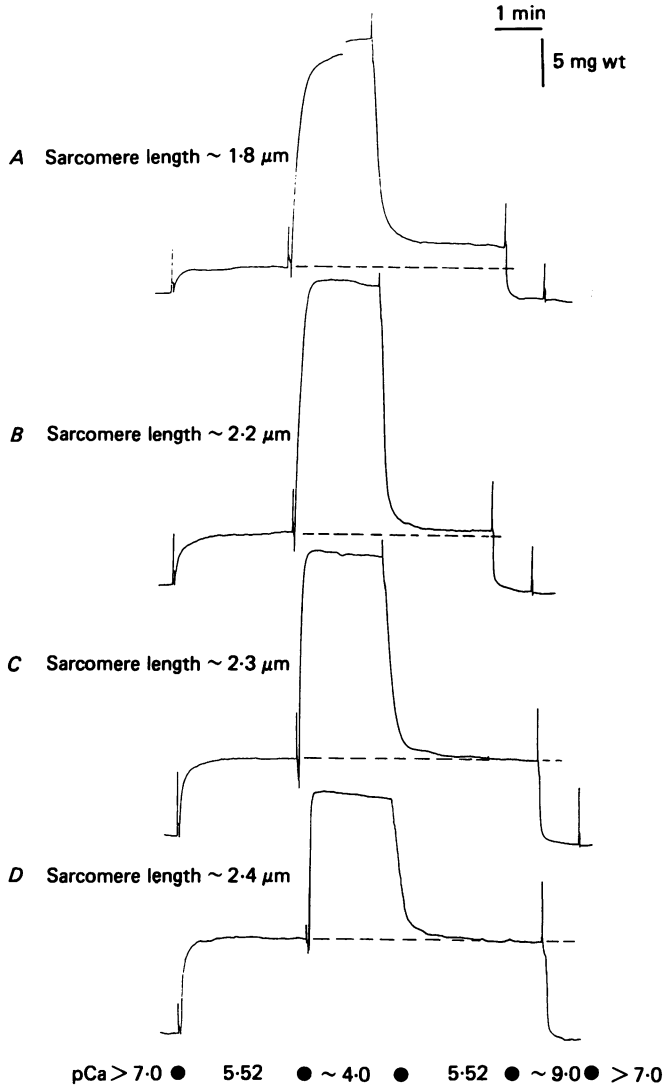

Fig. 6. Reduction of hysteresis at long sarcomere lengths. The same sequence of solution changes was repeated on the same preparation at three sarcomere lengths: panel *A*, ~ 1·8 μm; panel *B*, ~ 2·2 μm; panel *C*, ~ 2·3 μm; panel *D*, ~ 2·4 μm. The pCa values applying to each sequence are indicated below the traces. The gap in panel *A* was 1 min.

we have reported elsewhere (Harrison, Lamont & Miller, 1986), imidazole and other compounds have identical effects. Imidazole (10–25 mM) or caffeine produce a shift of the half-activation pCa by 0·2 units. Figure 4 shows the result of three separate runs in one experiment with imidazole. The calcium sensitivity was also increased by virtue of the higher pH_a employed: 7·2 in this case as opposed to 7·0 in most of the experiments (Table 1). As can be seen, hysteresis is still prominent and the shift in slope and K_{app} are quantitatively and qualitatively similar to those shown earlier.

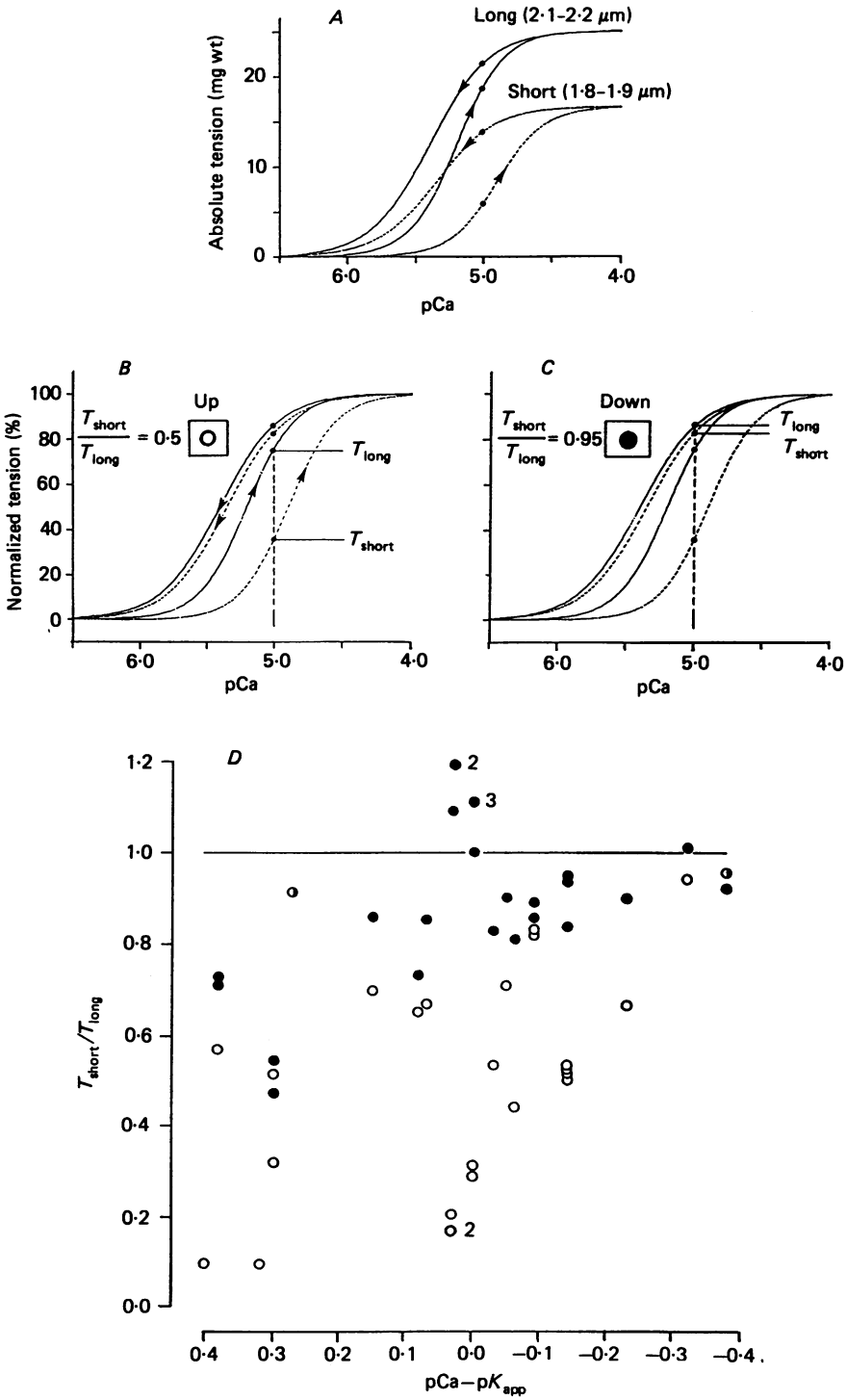

Fig. 7. For legend see opposite.

Effects of sarcomere length change

Gordon *et al.* (1984) have suggested from their experiments on barnacle skeletal fibres that it is tension production *per se* that produces the hysteresis. This conclusion might also be made on the basis of the 'partial hysteresis' results described above (p. 120). We have tested this hypothesis by altering sarcomere length. This allows us to explore a similar range of $[Ca^{2+}]$ and the full range of 'activation' but with less absolute force being developed at any given calcium level.

The means of observing and adjusting sarcomere length is described in the Methods section. Video recordings of the sarcomere pattern from randomly chosen parts of several preparations were subsequently analysed in detail. A report of some of these results has been presented elsewhere (Moss *et al.* 1986). This analysis reveals that, as others have noted, there is some variation of sarcomere lengths within a short length of muscle in the heart. The figures quoted throughout this paper indicate the range of sarcomere lengths encountered in the sections sampled. These points are considered further in the Discussion.

Figure 5*A* shows traces obtained from a typical preparation at two sarcomere lengths. The upper trace shows the control responses at a sarcomere length just at, or slightly beyond, the peak of the length–tension curve. A relatively small hysteresis develops. The lower trace shows the responses to the same solutions at a shorter sarcomere length. Note that the time base is faster than the upper trace. Peak tension has *decreased* to about 70% which is expected for this position on the length–tension curve; the extent of the hysteresis has, however, been substantially *increased*. Most strikingly, at pCa 5·33 very little tension develops as $[Ca^{2+}]$ is stepped up, but a higher, steady level is maintained as $[Ca^{2+}]$ steps down again. Results from this experiment are replotted in Fig. 5*B* as relative tension normalized to the peak achieved at each sarcomere length. The points representing peak tension are not shown on this expanded scale. The reduced calcium sensitivity at the lower sarcomere length shown has been reported by other authors; for the ascending limb of the length–tension relationship in cardiac muscle by Hibberd & Jewell (1982), Kentish, ter Keurs, Schouten, Noble & Ricciardi (1986), and for the descending limb of cardiac and skeletal muscle by Endo (1972), Fabiato & Fabiato (1978), Moisescu & Thieleczek (1979), Stephenson & Williams (1983) and Stephenson & Wendt (1984).

Fig. 7. Differential effect of sarcomere length on the calcium sensitivity of saponin- and Triton-treated rat trabeculae on the upgoing and downgoing limbs of hysteresis curves. Panels *A*, *B* and *C* show hypothetical plots of the hysteretic pCa–tension relationships obtained at two sarcomere lengths. *A* represents the raw data of tension against pCa; *B* and *C* show the effect of normalizing to the maximum tension response (as in Fig. 5). *B* shows how the relative tensions at the longer (T_{long}) and shorter (T_{short}) sarcomere lengths were compared to give values for the upward, and, *C*, for the downward limbs. Actual data are plotted in *D*. The ordinate represents the ratio of the tensions achieved at each given pCa at one shorter and one longer sarcomere length (as T_{short}/T_{long}). The abscissa is standardized for the differences in absolute calcium sensitivity between individual preparations (but *not* between sarcomere lengths for a given preparation), by plotting test $pCa - pK_{app,down}$ ($= \log_{10}(1/K_{app,down})$) for the individual muscles (see Fig. 3*B* and relevant text for details). Results from the upgoing (○) and downgoing (●) parts of the curve can thereby be compared. Long sarcomere length was typically 2·1 or 2·2 μm; short sarcomere length was 1·7–2·0 μm. Cumulated data from six preparations.

However, it is striking that, in this example, the downward limb of the hysteretic relationship is almost the same at both sarcomere lengths. Thus, the major effects of sarcomere length (below 2·2 μm) on calcium sensitivity are restricted to the upward limb of hysteresis. This point is reinforced later (see Fig. 6 and text).

Hysteresis becomes undetectably small when the muscle is stretched beyond a sarcomere length of 2·3 μm though, as reported by others, calcium sensitivity continues to increase (Fabiato & Fabiato, 1975; Moisescu & Thieleczek, 1978). This is illustrated in Fig. 6. Four sarcomere lengths were investigated in this example: one below, one at, and two above the peak of the length–tension relationship. The steady increase in calcium sensitivity is exemplified by the increasing absolute force level in response to the first exposure to pCa 5·52 in Fig. 6*A*, *B*, *C* and *D*. Maximum calcium activated force rises between *A* and *B* and then falls again in *C* and *D* as sarcomere length is increased from 1·8 to 2·2, 2·3 and then 2·4 μm. The difference between the steady-state tension achieved during the first (upward) and second (downward) exposure to pCa 5·52 in Fig. 6*A*, *B*, *C* and *D*, i.e. the magnitude of hysteresis, falls to zero. The Figure reinforces that *absolute* force does not account for hysteresis: panels *A* and *C* or *D* represent similar absolute force levels with pronounced hysteresis in *A* but not *C* or *D*. Trace *B* has the highest force level but only an intermediate level of hysteresis.

For identical reasons to those given above for the pCa–tension relationships, collected data on the influence of sarcomere length cannot be usefully displayed in the form of a single plot. The results are accumulated in the form of Fig. 7.

Figure 7*D* plots the ratio of relative tensions (T) at two sarcomere lengths (2·2 μm or less: expressed as T_{short}/T_{long}) achieved on the upward (○) and downward (●) parts of sequences of [Ca^{2+}] changes from six preparations. The first three panels (Fig. 7*A*, *B* and *C*) schematically illustrate, and the subscript describes, how the values plotted in Fig. 7*D* were calculated. A ratio of one, near which most of the filled symbols are clustered, corresponds to no effect of sarcomere length on calcium sensitivity. The collected results confirm that there is little or no significant effect of sarcomere length on the calcium sensitivity of the *downgoing* curve.

The data show quite a wide scatter which merits further comment. The reproducibility of values between successive determinations of tension at a given pCa is generally good with these preparations. An example is provided by Fig. 4. The comparison between values T_{short}/T_{long} hinges upon such reproducibility. If it is postulated that the downward limb of the hysteretic curve is indeed independent of sarcomere length, then the 'expected' ratio for the open symbols will be unity and any real data should be scattered about 1·0. Inspection of Fig. 7*C* reveals that even a very small discrepancy between the downward curves at two sarcomere lengths will produce a ratio T_{short}/T_{long} that can differ markedly from 1·0, particularly at lower tension levels (higher pCa). The values obtained fall both above and below 1·0 but, given the sources of discrepancy noted here, seem satisfactorily close to 1·0. By contrast, for the upward curve, calcium sensitivity is expected to fall with reducing sarcomere length so that T_{short}/T_{long} is always expected to be less than 1·0, provided that the reduction in sarcomere length is sufficient to reduce calcium sensitivity appreciably. This is the case for the collected data. The fact that the curves for long sarcomere lengths lie appreciably to the left of those for short sarcomere lengths often results in there being a pCa where significant detectable tension is produced at the long length only. The tension ratio, therefore, becomes meaningless ($0·0/T_{long}$) and has been assigned an arbitrary value of 0·1 for the purposes of this plot.

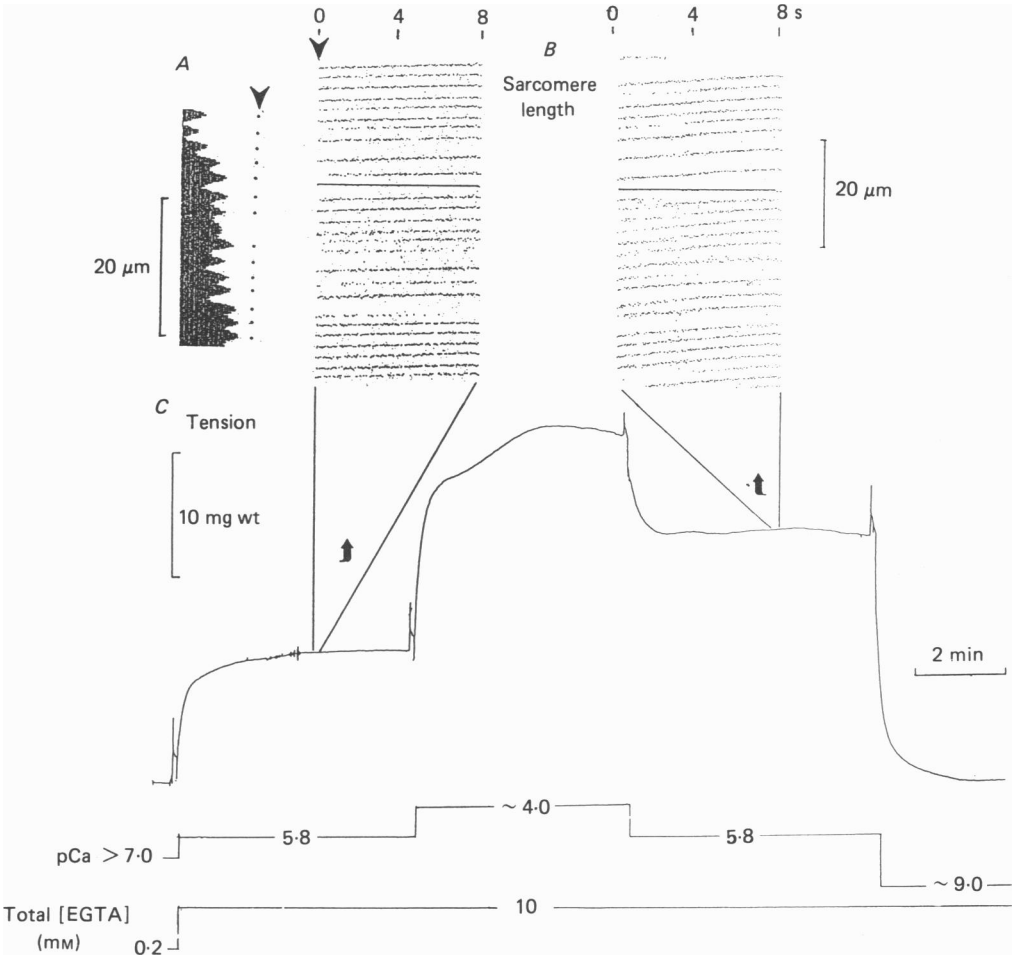

Fig. 8. Computer-assisted image analysis (Magiscan System; Joyce–Loebl Ltd) as a check on sarcomere length during force production. *A*, intensity analysis of a typical sarcomere pattern (in this case, mean sarcomere length = 1·8 μm approximately). The fifteen peaks represent the mid-point of adjacent I bands on the image under analysis; the troughs represent the A bands. The column of points to the immediate right (arrowed) indicate the position of these peaks as detected by the algorithm (see text for details). Where detection limits are not reached (e.g. 8th peak down), no 'sarcomere' is detected; the increment between points thus represents 2 × sarcomere length. *B*, two sequences of analyses of the type shown in panel *A* showing sarcomere length (ordinate) against time (abscissa). The columns of dots (arrowed) represent the I band mid-points, and each row represents the new position of each mid-point with respect to time (25 per second). The array to the left was determined on an 'upgoing', that on the right on a 'downgoing', hysteretic sequence, both at pCa 5·8. The continuous line indicates the position of a (stationary) graticule line in the field of view. Mean sarcomere length (excluding 'missed' sarcomeres) over the entire 8 s analysis period was 2·10 ± 0·25 (S.D.) ('upgoing') and 2·07 ± 0·17 (S.D.) μm ('downgoing'). *C*, the tension levels achieved by the muscle under analysis.

Checks on sarcomere distribution during force development

There are reports in the literature that sarcomere length can change appreciably during force production under 'preparation isometric' conditions (Kreuger & Pollack, 1975; Allen & Kentish, 1985; Kentish *et al.* 1986). It is clearly important to establish that the phenomenon of hysteresis cannot be explained as the result of such redistribution. We have made some continuous video recordings of the sarcomere pattern in the DIC microscope during hysteric sequences, using the chamber illustrated in Fig. 1*B*. The data have then been subjected to computer-assisted image analysis using the Magiscan system (Joyce-Loebl Ltd). The analyses were carried out in collaboration with Dr V. A. Moss. Figure 8 illustrates a typical sequence.

An area of sarcomere pattern is defined for analysis with the sarcomeres running perpendicular to the TV raster lines. The intensity of the pixels (coded in sixty-four grey tones = 6 bit resolution) are averaged across the width of this area (typically five to ten raster lines, eight in this case) and expressed for each pixel along its length (in this case 65 μm amounting to 3% of the muscle length). This information can be displayed as a plot of intensity against distance along the muscle (Fig. 8*A*). The program then seeks the intensity peaks (or troughs), finds their mid-point and gives a value for their separation, corresponding to the distance between the middle of successive I (or A) bands. The mid-point positions are displayed as a series of points whose separation corresponds to sarcomere length (vertical scale in Fig. 8*B*). With the discrimination criteria adopted, some faint bands are not detected by the program so an integer multiple of the sarcomere length results (a gap in the array of dots). Successive analyses can be made of one section over a period of time to show any change in sarcomere length, length distribution, or any displacement overall during this time. Figure 8*B* shows two 8 s sections of analysis carried out on the same area of the muscle while tension was at a steady state on the upgoing and downgoing part of the sequence. The tension trace (Fig. 8*C*) shows that in this case T_{down} was 1·8 times T_{up} at pCa 5·75. The tension responses reached a steady-state less rapidly than those in the standard experimental chamber due to the relatively slow solution exchange. Inspection of Fig. 8*B* shows that there is little obvious difference in sarcomere length; between upward and downward sections a tiny lateral translation of the section under analysis occurred, amounting to a few micrometres displacement in 15 min. This can be judged relative to the exactly horizontal line in the middle of each panel corresponding to the position of a graticule line on the image, against which the I band centres drift upward. The mean sarcomere length ($\pm$S.D.) for the whole of the sample period of 8 s (at 25 frames per second) was $2{\cdot}10 \pm 0{\cdot}25$ μm (upgoing) and $2{\cdot}07 \pm 0{\cdot}17$ μm (downgoing). A larger sample including several regions of the same field as the sequence illustrated gave figures of $2{\cdot}11 \pm 0{\cdot}21$ μm (upgoing) and $2{\cdot}04 \pm 0{\cdot}19$ μm (downgoing). These figures compare with the resting sarcomere length which was $2{\cdot}11 \pm 0{\cdot}23$ μm at the start of the sequence and $2{\cdot}09 \pm 0{\cdot}20$ μm at the end. This shows that there is very little alteration in sarcomere length or distribution associated with force development or the substantial hysteresis.

Speed of development of hysteresis

The speed with which hysteresis develops is obviously important in establishing the physiological relevance of the phenomenon. The activation sequences described above are necessarily rather protracted; it often takes several minutes to complete a sequence of force developments and be convinced that steady tension is achieved at each level. We have taken advantage of the fact that hysteresis can be observed

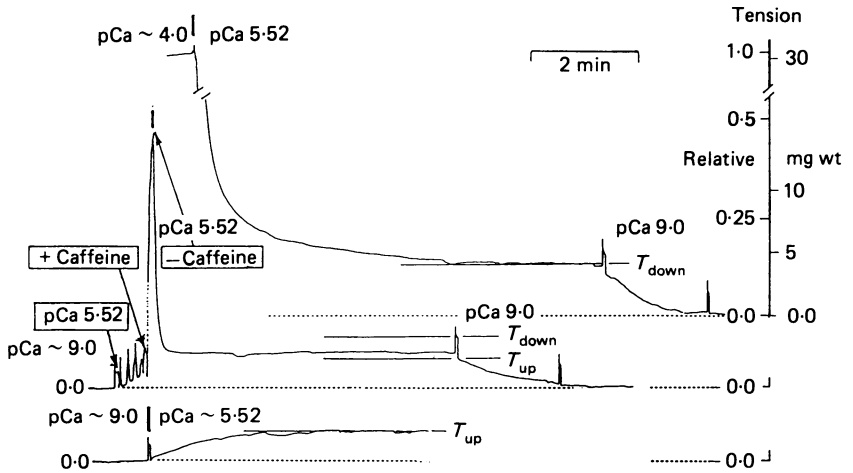

Fig. 9. Rapid development of hysteresis after a caffeine contracture. Middle trace, a saponin-treated preparation was equilibrated to a low concentration of total EGTA (0·2 mM) to minimize calcium buffering capacity. At the pCa chosen (~ 5·5) the muscle shows small tension oscillations. Addition of caffeine (10 mM) induces a rapid, transient contracture. At peak tension, the muscle was transferred to a high buffer capacity (10 mM-total EGTA) solution. This 'clamps' the [Ca^{2+}] throughout the preparation rapidly to the set level (pCa 5·52). The tension level achieved at steady state after the caffeine contracture can now be compared with that achieved in the same solution during standard upward (T_{up} level from lowest trace; pCa 9 to pCa 5·52) and downward (T_{down} level from uppermost trace; pCa 4 to pCa 5·52) sequences. The caffeine contracture only reaches about half-maximum tension and is very brief, but is sufficient to induce some hysteresis.

in 'selectively' (saponin-treated) skinned preparations to check this point. Caffeine contractures can be evoked under conditions of low calcium capacity in the saponin-treated muscle where sarcoplasmic reticulum and mitochondrial function are maintained (Endo & Kitazawa, 1978; Harrison & Miller, 1984; Fry & Miller, 1985). Tension peaks within about 1 s and, depending upon the conditions, can reach up to 100% of C_{max}. Under these circumstances, a test [Ca^{2+}] can be imposed very quickly on the muscle by the 'calcium-jump' technique (Miller, 1975; Moisescu, 1976; Ashley & Moisescu, 1977; Moisescu & Thieleczek, 1978): the buffer capacity is suddenly increased, e.g. from 0·2 to 10 mM-total EGTA. If hysteresis develops within the very short time of the caffeine contracture then it might occur in the intact cell and *in vivo*. The experiment is, however, greatly complicated by the fact that the size of the caffeine contracture (and hence the extent of calcium uptake and/or release) is also

markedly affected by sarcomere length. Greater hysteresis (described above), but lesser caffeine contractures (Fabiato & Fabiato, 1975; C. Lamont & D. J. Miller, unpublished observations), are produced at shorter sarcomere lengths. Within the experimental limitations, however, the result illustrated in Fig. 9 confirms that hysteresis will develop after as little as 1 s or so of half-maximal tension development.

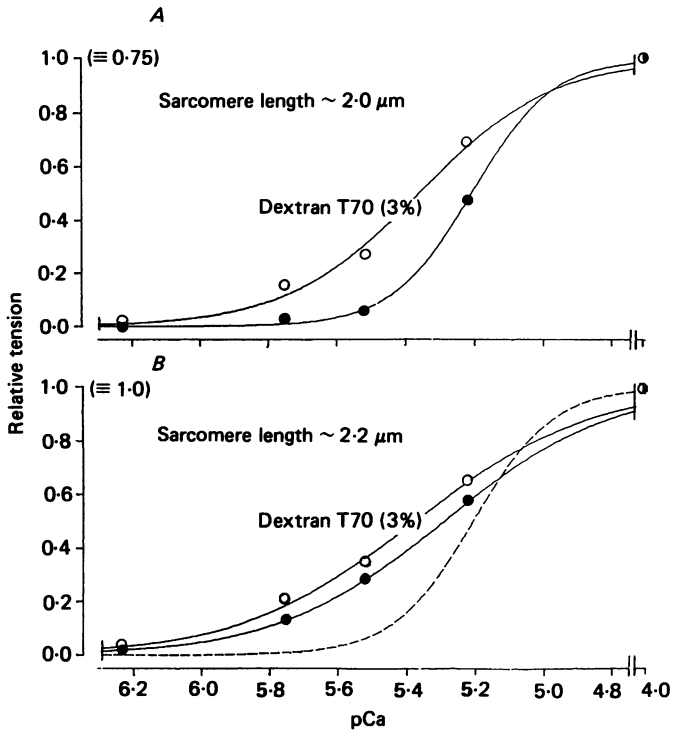

Fig. 10. The influence of hypertonic shrinkage on the calcium sensitivity and length dependence of calcium sensitivity in skinned cardiac muscle. Panels *A* and *B* show pCa–tension relations from a rat cardiac trabecula. In panel *A* the sarcomere length was 2·0 μm. Addition of 3% dextran T70 (○) increased the calcium sensitivity ($\log_{10} K_{app}$ from 5·21 to 5·36, h from 3·76 to 2·27) in a manner very similar to that produced by increasing the sarcomere length. In panel *B* the sarcomere length was increased to 2·2 μm. Addition of dextran produced a much smaller shift in the pCa–tension relation ($\log_{10} K_{app}$ from 5·30 to 5·38, h from 1·81 to 1·72).

The result provides another example of partial hysteresis (see Fig. 2 and associated text); when the muscle is submaximally activated, as here, the hysteresis ensuing after the caffeine contracture is less than that after full activation (T_{down}).

Effect of altering myofilament lattice spacing

Our hypothesis (developed in detail in the Discussion) to account for changes in calcium sensitivity associated with hysteresis and sarcomere length changes is that myofilament lattice spacing is the determinant. One direct test of this idea is to alter lattice spacing at constant sarcomere length by means of hypertonic shrinkage.

Molecules larger than about 40000 Da are excluded by the myofilament lattice (Godt & Maughan, 1977). This creates an osmotic gradient; water leaves the myofilament space and the lattice shrinks. The results in Fig. 10 (see also Lamont & Miller, 1987) reveal that such shrinkage results in an increase in calcium sensitivity and a reduced slope of the pCa–tension relationship, both effects resembling those seen in the 'downgoing' slopes of the hysteretic sequences, or as a result of increasing sarcomere length. Figure 10 shows further that the effect of increasing the sarcomere length is reduced when the lattice is shrunk by dextran.

DISCUSSION

Novelty of the phenomenon; sarcomere length effects

The suggestion that the pCa–tension relationship might show hysteresis has not found universal acceptance. The present results provide the first positive evidence for the phenomenon in cardiac muscle. It is greatest at short sarcomere lengths, and virtually absent above a sarcomere length of 2·2 μm which may help to explain why it has not been more widely observed. Some reports make no comment on sarcomere length (e.g. Gordon *et al.* 1984) and negative reports have generally been obtained at sarcomere lengths greater than 2·2 μm (Williams & Stephenson, 1983; Brandt, Gluck, Mini & Cerri, 1985; Pagani, Shemin & Julian, 1986). For the present data, local variations of sarcomere length during sequences of force development do not contribute to, or account for, hysteresis. The results in Fig. 8 confirm that significant changes in sarcomere length do not occur during the activation sequences. These findings do, however, require further comment since many workers have reported alteration in sarcomere length under 'preparation isometric' conditions (see Winegrad, 1974; Kreuger & Pollack, 1975; Jewell, 1977; Allen & Kentish, 1985). We believe that there are two contributing factors to this difference. First, our method of mounting with fine snares (see Fig. 1*A*) produces very little damage. The sarcomere pattern is often conserved to within a few micrometres of the snare, and the fraction of muscle length that is damaged is less than 1 %, even in the worst cases. Secondly, the use of light microscopy, rather than laser diffraction, as a method of measuring sarcomere length allows us to use preparations without having to select for optical characteristics. Hibberd & Jewell (1982) have reported that, in their study, many preparations failed to give reliable laser diffraction patterns over the range of tensions and sarcomere lengths that they employed. These preparations were rejected for their study. Informal comment from other workers who use the laser method suggests that this approach is widespread. However, we never have to reject preparations on these grounds. Trabeculae almost always show a clear sarcomere pattern, at least in a significant proportion of the tissue. However, there are often areas where the pattern is obscured by slight, superficial damage or endothelial cells. These preparations would presumably give unsatisfactory laser diffraction patterns. It is also striking that the preparations used by Hibberd & Jewell (1982) were slack at a sarcomere length of 1·85–1·9 μm. We find slack length to be at a sarcomere length of 1·7 μm in a significant number of preparations, though at longer sarcomere lengths in the remainder. Preparations selected by the requirements of good laser diffraction behaviour may be, on average, lower in their

complement of connective tissue and collagen fibres than those used here. In single 'calcium-tolerant' cardiac myocytes (from rat and other vertebrates) isolated by the use of collagenase, the resting sarcomere length is generally 1·9–2·2 μm (e.g. Fabiato & Fabiato, 1975, 1978), perhaps supporting this idea; others have reported minimum sarcomere lengths of 1·8 μm in such cells (Kreuger, Forletti & Wittenberg, 1980).

Independent of these considerations are the following two points: (1) The fact that hysteresis is greater at shorter sarcomere lengths and absent above about 2·3 μm is difficult to reconcile with any model for hysteresis that involves changes in sarcomere length in the preparation as a consequence of force production. In force production, active sarcomeres would tend to shorten, reducing average sarcomere length, e.g. by stretching damaged regions. On the ascending limb of the length–tension curve (below 2·2 μm) this would result in negative hysteresis: as $[Ca^{2+}]$ was reduced, the undamaged muscle in the preparation would be at a lower sarcomere length and, therefore, less calcium sensitive. Hysteresis is diametrically opposite to this tendency. A preparation deliberately crushed with forceps part way along its length indeed showed 'negative' hysteresis, as predicted here. (2) In many cases (e.g. Figs 2, 5 and 6) though not all (Fig. 2, Harrison *et al.* 1985, their Fig. 1), we evoke forces first on the upward and then on the downward parts of the hysteretic loop. If anything, absolute force has a tendency to fall with time (see Methods section for details) again tending to reduce force as the test sequence proceeds. Thus, the experimental assessment of the magnitude of hysteresis is inherently an *under*estimate. We conclude that hysteresis is a phenomenon without an obvious artifactual origin. Cardiac trabeculae prepared with either Triton X-100 or saponin both show hysteresis and length dependence in their calcium sensitivity. Therefore, the phenomena are not due to enzymes or other diffusible elements lost from the cytoplasm after comprehensive skinning with Triton, but retained in the saponin-treated state. An alteration in the state of phosphorylation of the contractile mechanism, as a result of the activity of enzymes such as myosin light-chain kinase which are retained even in the Triton-treated muscle, cannot contribute. The steady-state nature of the hysteresis phenomenon and its sensitivity to sarcomere length change (rather than just $[Ca^{2+}]$) argue against this mechanism. Hysteresis and length dependence of calcium sensitivity reflect properties of the contractile proteins or the closely associated regulatory proteins.

Possible mechanisms for hysteresis in, and the length dependence of, calcium sensitivity

Hysteresis is an apparent increase in the calcium sensitivity of a muscle when it has immediately previously been exposed to a higher calcium level; i.e. a muscle can maintain a higher tension level than the given $[Ca^{2+}]$ can evoke. We propose that this form of increased calcium sensitivity has the same origin as that produced by increasing sarcomere length (Stephenson & Wendt, 1984; Allen & Kentish, 1985). Calcium sensitivity is measured by the steady-state tension achieved in response to single $[Ca^{2+}]$ steps, or as a sequence of increasing $[Ca^{2+}]$, starting from the fully relaxed state. These measurements are equivalent to the 'upgoing curve' of the hysteresis loops. A shift to the left of the pCa–tension relation, i.e. an increase in the 'apparent calcium sensitivity', can be explained in two ways. First, and most simply,

as an increase in the apparent affinity of troponin-C for calcium. This will result in more calcium binding and more tension at given subsaturation [Ca^{2+}]. Secondly, as a change at some stage later in the contractile process after the calcium has bound, for example if active cross-bridges alter the way in which tropomyosin transmits the effect of calcium binding, by troponin, to the contractile proteins.

Does force production increase the calcium affinity of troponin?

Allen & Kurihara (1982) suggested that troponin's calcium binding affinity was increased by tension production. This idea was based on biochemical work of Bremel & Weber (1972) showing a substantial increase in troponin's affinity for calcium when myosin heads are rigor-linked to actin. However, Fuchs & Planchak (1983) used force-producing (non-rigor) cross-bridges to show that force, unlike rigor, does not influence troponin's calcium binding affinity. Gordon *et al.* (1984) and Ridgway *et al.* (1983) suggested this 'force-dependent' mechanism to account for hysteresis in skeletal muscle, proposing that the immediate history of force determined the occurrence of hysteresis. This scheme would predict maximum hysteresis at 2·2 μm where force and the number of active cross-bridges is maximal. However, as results in Figs 5, 6, 7 and 8 reveal, hysteresis (as the difference between $K_{app,up}$ and $K_{app,down}$) is greatest at short sarcomere lengths when the absolute force is least. Finally, the 'force' model does not provide an explanation of the increase in calcium sensitivity over the full range of sarcomere lengths, since on the descending limb of the length–tension relation force is declining while the apparent calcium sensitivity is increasing (e.g. Stephenson & Wendt, 1984). The current evidence does not favour the idea that force *per se* increases calcium binding to troponin.

Does sarcomere length alter calcium affinity of troponin?

An alternative proposal is that sarcomere length itself is the determinant of calcium affinity. It has been suggested that changes in the geometric relationship between the thick and thin filaments might alter the physico-chemical environment of the calcium binding sites (see e.g. Stephenson & Wendt, 1984 and Allen & Kentish, 1985 for discussion of these points). This could be related to the changes in filament overlap and spacing which alter with sarcomere length. Fuchs (1984, 1985) tested the proposal by determining calcium binding in skeletal muscle at different sarcomere lengths. The calcium saturation curves were found to be independent of length over the sarcomere length range 1·6–3·8 μm. However, more recent evidence for cardiac muscle (Hofmann & Fuchs, 1987) does show an increase in calcium binding as sarcomere length is increased from about 1·8 to about 2·2 μm.

Overlap. At sarcomere lengths below 2·2 μm all the calcium binding sites are within the overlap region. Binding sites move out of the overlap region as sarcomere length is increased above 2·2 μm. Those binding sites which move out of the region of overlap experience a substantial change in their electrostatic environment. Since calcium sensitivity increases as sarcomere length increases, even from well below 2·2 μm where the overlap is complete, a simple change in overlap is not a convincing mechanism. The binding sites which have left the region of overlap would have to exert long-range, allosteric effects on those sites controlling the force-producing cross-bridges. There is some support for this view as the thin filaments of vertebrate

skeletal muscles are thought to activate co-operatively as a unit (Brandt, Diamond & Shachat, 1984). But, as noted above, and as with the force-dependent scheme, this mechanism does not account for the increase in calcium sensitivity on *both* limbs of the length–tension relation. Hysteresis results in an apparently similar increase in calcium sensitivity to that seen with sarcomere length increase. But hysteresis is observed at fixed sarcomere length suggesting that it cannot be due to overlap. Thus, overlap is unlikely to be the determinant of calcium sensitivity.

Lattice spacing. When an intact muscle fibre shortens it maintains a constant volume; the distance between the filaments is not fixed but varies with muscle length. The separation between the thick and thin filaments reduces as sarcomere length increases with concomitant calcium sensitivity increase. Filament separation is the only factor considered so far which alters monotonically over the full range of sarcomere lengths, providing a possible single mechanism for the increase in calcium sensitivity over both limbs of the length–tension relation. Hysteresis will be included in this mechanism because, in skeletal muscle at least, force production will reduce filament spacing (Shapiro, Tawada & Podolsky, 1979; Matsubara, Goldman & Simmons, 1984). This would explain why hysteresis was greatest at short sarcomere lengths, because here lattice spacing in the resting muscle is at its greatest and tension generation could reduce the spacing most. Provided that sufficient force is generated at short sarcomere lengths, and that the steric hindrance produced by the 'excess' of cross-bridges per half-sarcomere at sarcomere lengths below 2·2 μm is not too great, the lattice could reach its limiting value of about 37 nm ($d_{1,0}$ meridional reflection corresponding to the separation of the myosin filament planes). There are no data on calcium binding when lattice spacing is reduced (for example by hypertonic shrinkage). However, in Hofmann & Fuchs' work (1987), the use of different sarcomere lengths in skinned muscle almost certainly results in reduced lattice spacing at the longer sarcomere lengths.

Proposed mechanism

We propose, therefore, that the closer positioning of the filaments is the primary cause of an increase in calcium sensitivity. The shrinkage can be achieved in three ways: (i) First, as noted above, by force development. Tension generated by the cross-bridge has a resultant tending to draw the filaments closer as well as the other, functionally more obvious, resultant which tends to produce shortening. This will produce hysteresis since the lattice will be closed down during a previous high level of activation (see next paragraph). (ii) Second, by increasing sarcomere length. At short (1·7 μm) sarcomere length the separation of the filaments at rest is at its greatest, and calcium affinity at its lowest. The limit for nearing of the filaments (see section (i) above) is also shown to be reached at about 2·6 μm or so in amphibian skeletal fibres when sarcomere length is increased which could explain why calcium sensitivity fails to increase in these fibres above about 2·6 μm (Moisescu & Thieleczek, 1979). (iii) Third, shrinking the lattice with hypertonic solutions. It has been shown that the pCa–tension relationship is shifted to lower [Ca^{2+}] (Godt & Maughan, 1981, for skeletal muscle), and also made shallower (Lamont & Miller, 1987, for cardiac muscle) by lattice shrinkage. This effect is less at longer sarcomere lengths as shown in Fig. 10. The shifts in position and slope of the pCa–tension relationship by tonicity

change as shown in Fig. 10 are like those associated with hysteresis and the alteration of sarcomere length (compare Figs 3, 4 and 8).

There is good evidence from X-ray diffraction studies of skeletal muscle that the changes in filament spacing under the various conditions described above do occur (e.g. Matsubara & Millman, 1974; Matsubara, Umazume & Yagi, 1984, 1985). If these ideas are correct, the apparently disparate phenomena of the length, ionic strength, tonicity and hysteresis effects on calcium sensitivity of muscle may find a common explanation. The precise ionic strength and tonicity of the bathing solutions in skinned-fibre experiments will determine lattice spacing at a given sarcomere length (Matsubara *et al.* 1985). Thus, the extent of hysteresis, tonicity or sarcomere length effects on calcium sensitivity will vary with solution composition. For otherwise identical conditions, different muscle fibre types may also behave differently since myofilament lattice spacing may be influenced by myosin differences, e.g. in isoelectric point; this contributes to the discrepancies amongst reports of these phenomena. (The only evidence that is not readily accommodated in this model is the report by Stephenson & Williams (1983) that frog slow fibres show an increase in calcium sensitivity as sarcomere length reduces. X-ray data on lattice filament behaviour or the effect of tonicity increase in these fibres could resolve this point and provide a test of the hypothesis we propose.) On this scheme, the insensitivity of the 'downgoing' curve to sarcomere length would be the consequence of lattice spacing reduction during the preceding activation; for the ensuing 'downgoing' limb, calcium sensitivity is high regardless of the sarcomere length at which tension development had occurred. Thus, on 'downgoing' curves, calcium sensitivity starts out higher and force is greater at all Ca^{2+} concentrations *en route* from the maximum to the lower level of activation. (As $[Ca^{2+}]$ falls, it can be considered to pass all intermediate levels.) Since the force is higher at all times, the lattice cannot expand to the same size that it would have had at the same $[Ca^{2+}]$ (but lower affinity and hence lower force) in the 'upgoing' sequence. By this means, the calcium sensitivity can remain higher, though not constant, until force falls to zero. This scheme also explains why the shift in the pCa–tension relationship produced by increased tonicity is less at longer sarcomere lengths (Fig. 10) because the scope for lattice shrinkage is then reduced.

Variability of the phenomenon

The magnitude of hysteresis is variable; some preparations show a large hysteresis, others very little. The variability at a given sarcomere length might be due to the variable swelling after chemical 'skinning'. The increase in lattice spacing seen when the sarcolemma is removed or disrupted (Rome, 1967) has been attributed to the removal of the osmotic constraint upon fibre volume (Matsubara & Elliott, 1972). In preliminary observations we note that our cardiac preparations increase in diameter by about 10–20% upon skinning (see also Matsubara & Elliot, 1972). At least a part of the phenomenon might be a direct consequence of the methodology: i.e. what is generally termed an experimental artifact. However, data of Matsubara & Millman (1974) show that lattice spacing does reduce with sarcomere length increase in *intact* cardiac muscle as constant-volume behaviour would predict. Skinned fibres might, therefore, tend to exaggerate the lattice spacing changes that can account for the phenomena of length dependence of, and hysteresis in, calcium sensitivity.

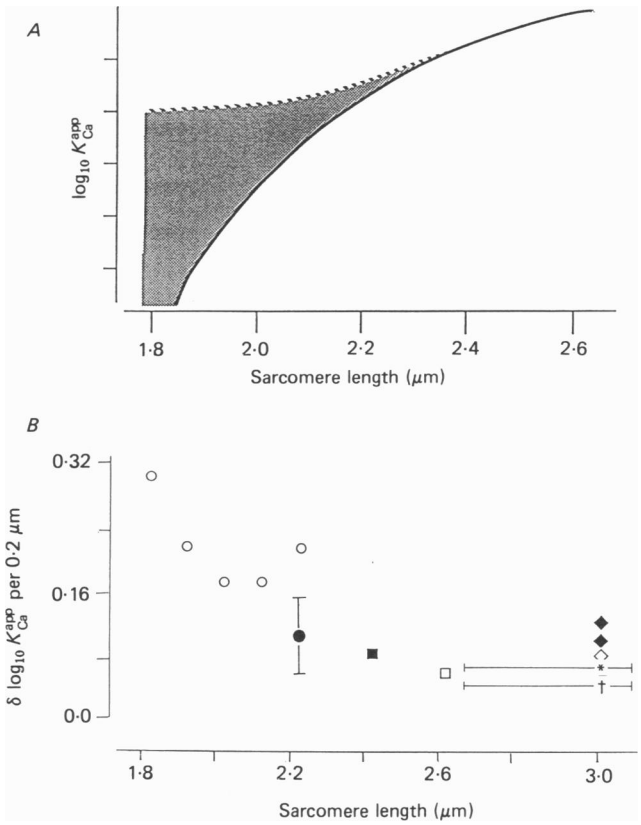

Fig. 11. Diagrammatic representation of the effect of sarcomere length on calcium sensitivity. *A*, the ordinate represents log of the reciprocal of the $[Ca^{2+}]$ required for half-maximum tension ($K_{app,Ca}$; numerically equivalent to the hth root of K_{app}, see eqn (2)), arbitrary scale. K_{Ca} is determined on upward (continuous line) and downward (dashed line) limbs of hysteretic sequences such as those shown in Fig. 3*A*. K_{Ca} increases as sarcomere length (abscissa) increases at all sarcomere lengths over the range shown for the upward limb (see e.g. Fig. 7) but not for the downward limb below about 2·2 μm (see e.g. Fig. 8). The area between the two curves (shaded) is that available for different calcium sensitivities between upward and downward limbs: 'hysteresis'. *B*, the change of $\log_{10} K_{app,Ca}$ with sarcomere length is itself sarcomere length dependent, as this plot reveals. Data are taken from Fabiato & Fabiato (1978, ◆), Kentish *et al.* (1986, ○), the present results (●, mean ± S.D.), Hibberd & Jewell (1982, ■) and Stephenson & Williams (1982, □, and regression line values for fast (†), and slow (*) fibres, respectively (20 °C); the absolute sarcomere length for the last values is not available, so the range is indicated). All values are expressed as the change of $\log_{10} K_{app}$ per 0·2 μm change in sarcomere length (ordinate). See text for further details.

Sarcomere length and calcium sensitivity

Figure 11*A* represents diagramatically the effect of sarcomere length (abscissa) on calcium sensitivity (ordinate). (Actual data are shown in Fig. 11*B*: see below.) It has been well established that calcium sensitivity of striated muscle increases with increasing sarcomere length across the range from 1·7 μm to about 2·6 μm in most amphibians, or to 3·6 μm in mammalian skeletal fibres (Stephenson & Wendt, 1984;

Allen & Kentish, 1985), with the sole exception of amphibian slow fibres whose calcium sensitivity has been reported to decrease with increasing sarcomere length (Stephenson & Williams, 1983). The method of determination in each case corresponds to what we term the 'upgoing' curve: steady-state force determined after stepping [Ca^{2+}] from a low to one, or more, higher values. The present results reveal that the calcium sensitivity on the ascending limb of the length–tension relationship is essentially restricted to the 'upgoing' curve of the hysteretic relationship. To a good approximation, the calcium sensitivity is constant at sarcomere lengths below 2·2–2·3 μm for the 'downgoing' curve (see Fig. 7). There is virtually no hysteresis above 2·2 μm. Evidence for the 'upgoing' curve in Fig. 11*A* is provided in Fig. 11*B*. The results plotted suggest that the effect of stretch is greater at shorter sarcomere lengths. These data have been obtained under 'upgoing curve' conditions. The affinity of the contractile system for Ca^{2+} is an apparent one: it is affected by pH, ionic strength and other conditions. This makes it difficult to compare rigorously the values obtained in different laboratories and preparations. However, it is possible to express the *change* in K_{app} (as $\delta \log_{10} K_{app}$/unit length change) from the various published reports. The results show that $\log K_{app}$ does not increase by a constant amount (i.e. K_{app} does not increase by a constant proportion) for a given increase in sarcomere length. Thus, it might be more appropriate to express the change as the *fractional* change in $\log K_{app}$. As the extent of the change in K_{app} with sarcomere length is sarcomere length dependent, it has been assessed as $\log K_{app,long}/\log K_{app,short}$ for a 0·2 μm increment in sarcomere length from 'short' to 'long'. Data from some work other than our own can be expressed in this way (e.g. Kentish *et al.* 1986) but others have used larger increments of sarcomere length (e.g. Fabiato & Fabiato, 1978; Stephenson & Williams, 1982). These have been scaled (to $\delta \log K_{app}/0{\cdot}2$ μm) to our own absolute values for this plot.

Stephenson & Williams (1982) had already plotted $\delta \log K_{app}$ *vs.* δ sarcomere length with linear regression lines; this has the implicit assumption that δ sarcomere length is an independent variable. The slope of the line in Fig. 11*B* shows that this is probably not the case even within the range of sarcomere length studied; the fact that K_{app} ceases to increase at some point (sarcomere length > 2·6 μm in amphibian muscle, Stephenson & Williams, 1982) shows that strictly, sarcomere length probably cannot be considered independent even in mammalian skeletal muscle where calcium sensitivity reportedly increases right up to a sarcomere length of 3·8 μm. The scatter of data about the regression lines in Stephenson & Williams (1982) suggests that an other than linear relationship might be equally satisfactory. Therefore, we have recalculated some of their data from the individual experimental plots which gave information on absolute as well as δ sarcomere length to produce the points included in Fig. 11*B*. Their mean figures for fibres at 22 °C, which can most appropriately be compared with other available data, are also included.

The collected results reveal that the effect of sarcomere length on calcium sensitivity is probably greater at shorter than at longer sarcomere lengths. However, some data (e.g. Stephenson & Williams, 1982, their Fig. 4; Fabiato & Fabiato, 1978, their Fig. 5) show an opposite tendency in some instances. Whether there is a smooth fall in K_{app} against sarcomere length until the limiting sarcomere length (beyond which $\delta K_{app} = 0$) is reached, or if the relationship alters slope abruptly at 2·2 μm, or elsewhere, cannot be decided on the basis of the data available. The former possibility has been assumed in constructing the schematic curves in Fig. 11*A*. If the mechanism of hysteresis and sarcomere length dependence of calcium sensitivity are the same, as we suggest, in principle there is some scope for hysteresis at any

sarcomere length at which the effect of sarcomere length is still evident (but in contrast compare Fig. 6*C* and *D*). Under the present hypothesis, hysteresis can only manifest itself if sufficient lateral force is produced to reduce the lattice spacing appreciably. Active tension falls when sarcomere length is $> 2{\cdot}2$ μm, the geometry of the cross-bridge linkage predicts a greatly reduced lateral resultant at longer sarcomere length, and lattice spacing is already appreciably reduced at these sarcomere lengths. This may explain why significant hysteresis fails to appear above $2{\cdot}2$ μm (see Fig. 6). This notion is allowed for in the schematic curves in Fig. 11*A*.

Physiological consequences

With the proviso that hysteresis and the length dependence of calcium sensitivity may be exaggerated in skinned muscle preparations the possible consequences for the physiological state can be considered. It would mean that sarcoplasmic [Ca^{2+}] would have to fall further for relaxation to occur than would have been previously supposed from work on skinned fibres. The short sarcomere length of cardiac fibres *in vivo* would tend to enhance the extent of any hysteresis. This could affect the interpretation of relaxation in terms of intracellular [Ca^{2+}] changes. Our finding that the 'downgoing' pCa–tension curve is relatively insensitive to sarcomere length implies that, regardless of the sarcomere length achieved during systole, the range of [Ca^{2+}] over which relaxation occurs might remain much the same. The extent of activation (when the 'upgoing' curve applies) is, however, strongly length dependent. Similar logic would apply to those skeletal muscles undergoing substantial length change during the physiological range of action if this brings their sarcomere length below $2{\cdot}2$ μm. Recent evidence reveals that the extent of skeletal muscle shortening during limb movement is considerably greater than previously supposed (Fellows & Rack, 1987). An analysis of the sarcomere length dependence of hysteresis in skeletal fibres is clearly required. An additional, complicating factor for heart muscle is that as the degree of activation changes (during any change in inotropic state) so too will the extent of hysteresis (e.g. Figs 2*B* and 9). A muscle which is only partially activated, as the heart is widely assumed to be and as skeletal fibres may be during partially fused tetanic responses, will operate over different hysteretic loops depending on the extent of this activation. Additionally, heart muscle is normally not completely *in*activated: (i) the delay between successive beats is usually too brief to allow tension to subside fully, (ii) quiescent heart shows a small component of resting tension which can be altered by changing bathing [Ca^{2+}] (e.g. Patmore, 1986). Possible dynamic aspects of the shift between hysteretic loops remain to be investigated.

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