*Biochimica et Biophysica Acta*, 546 (1979) 64—76
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BBA 47649

# SARCOMERE LENGTH EFFECTS ON THE $Sr^{2+}$- AND $Ca^{2+}$-ACTIVATION CURVES IN SKINNED FROG MUSCLE FIBRES

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(Received June 6th, 1978)

*Key words: Sarcomere length; $Ca^{2+}$-activation curve; $Sr^{2+}$-activation curve; (Skeletal muscle fiber)*

## Summary

1. A procedure recently described to produce rapid changes in $[Ca^{2+}]$ and $[Sr^{2+}]$ within the whole cross-section of skinned muscle preparations (Moisescu, D.G. (1976) Nature 262, 610—613, and Moisescu, D.G. and Thieleczek, R. (1978) J. Physiol. 275, 241—262) has enabled us to obtain whole $Ca^{2+}$- or $Sr^{2+}$-activation curves at different sacromere lengths with the same preparation.

2. The maximal isometric force response was found to be very similar in $Ca^{2+}$- and $Sr^{2+}$-buffered solutions for otherwise identical conditions.

3. The change in sarcomere length between approx. 2.2 and 2.6 $\mu$m reversibly shifted both the $Ca^{2+}$- and the $Sr^{2+}$-activation curves by approx. 0.1 log units towards lower concentrations of the activator, without affecting their shape.

However, the change in sarcomere length in the range above 2.6 $\mu$m did not have an effect upon the relative isometric force response-pCa (and -pSr) relationship.

4. All the $Ca^{2+}$- and $Sr^{2+}$-activation curves present a similar steepness and indicate that the relative isometric force increases from approx. 10 to 90% if the concentration of the activator is increased 3-fold.

5. The half time for force development in these experiments did not appear to be influenced by the length of the sarcomeres.

6. A potentiometric method for determining the apparent affinity constants of $Ca^{2+}$, $Mg^{2+}$ and $Sr^{2+}$ to EGTA and ATP under various conditions is described.

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Abbreviations: Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; HDTA, hexamethylendiamine-*N,N,N',N'*-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; pCa = $-\log[Ca^{2+}]$; pSr = $-\log[Sr^{2+}]$.

## Introduction

Many results in the literature suggest that the active force response of the intact muscle fibre can be increased at longer sacromere lengths and there are several possibilities to explain these observations (for a review, see Ref. 1).

One possibility is that the contractile system itself is more sensitive to the calcium ion at longer sarcome lengths and Endo [2,3] has reported results obtained with 'skinned' fibre preparations [4] from frog and toad muscles which are in line with this view. However, the physiological significance of these results has been brought into question by Endo himself [2,3] as well as by others [1]. This was because the sarcomere length effect observed by Endo was limited only to a narrow range of the $Ca^{2+}$-activation curve where the force responses were significantly smaller and slower than those in vivo.

It has been shown [5] that $Sr^{2+}$ can induce forces similar to those induced by $Ca^{2+}$ in the skinned mucle preparation of frog and recently we have developed a new technique [6,7] which reduces the time of activation in the skinned muscle fibre by at least one order of magnitude and minimises the concentration gradients for $H^+$, $Sr^{2+}$, $Ca^{2+}$, $Mg^{2+}$, and ATP in the activated preparation.

Under these circumstances we have considered it of interest to study the effect of sarcomere length on the $Sr^{2+}$-activation curves and also to reinvestigate the equivalent effect on the $Ca^{2+}$-activation curves by using the new activating procedure [6,7].

A preliminary account of these results has been published [8].

## Materials and Methods

The procedures for skinning the muscle fibres (iliofibularis, *Rana esculenta*), preparing the bathing solutions, activating the preparations and measuring the isometric force were described before [6,9].

The composition of solutions is shown in Table I and the employed apparent affinity constants of $Ca^{2+}$, $Sr^{2+}$ and $Mg^{2+}$ to various ligands in these solutions

TABLE I

COMPOSITION OF SOLUTIONS

All solutions contained 137 mM K, 36 mM Na, 60 mM Tes, 8 mM total ATP, 10 mM creatine phosphate, 1 mM $Mg^{2+}$, 20 mM Cl and 15 U/ml creatine kinase. The pH of the solutions was 7.10 ± 0.01 at 20°C. The solutions have been designed to minimise the gradients of pH, pCa, pSr, [ATP], [$Mg^{2+}$] which are associated with the ATPase activity of the preparation [8]. A solution of type H mixed with a solution of type B in a proportion higher than 300 : 1 is called a "low relaxing" solution.

| Solution | $Mg_{total}$ (mM) | HDTA (mM) | EGTA (mM) | CaEGTA (mM) | SrEGTA (mM) |
|---|---|---|---|---|---|
| A | 8.05 | — | — | 50 | — |
| A′ | 8.5 | — | 10 | — | 40 |
| A″ | 8.3 | — | 5 | — | 45 |
| B | 10.25 | — | 50 | — | — |
| H | 8.45 | 50 | — | — | — |

TABLE II

APPARENT AFFINITY CONSTANTS ($K_L^{'app}$) OF ATP AND EGTA FOR $Ca^{2+}$, $Sr^{2+}$, $Mg^{2+}$ MEASURED BY THE METHOD DESCRIBED IN THE APPENDIX

| Ligand | Cation | $K_L^{'app}$ ($M^{-1}$) | Experimental conditions | | | Temperature (°C) | Observations |
|---|---|---|---|---|---|---|---|
| | | | $K^+$ (mM) | $Na^+$ (mM) | pH | | |
| ATP | $Mg^{2+}$ | 7500 ± 500 | 90 | 30 | 7.10 | 20 | Agreement with the results indicating an 'absolute' affinity constant of $Mg^{2+}$, $H^+$ and $K^+$ to $ATP^{4-}$ of 2.2—2.6 · $10^4$ $M^{-1}$, 8.9 · $10^6$ $M^{-1}$ and 14 $M^{-1}$ respectively at 20°C (see Ref. 16 and 17). |
| | | 6500 ± 500 | 135 | 35 | 7.10 | 20 | |
| | | 6300 ± 400 | 90 | 30 | 7.10 | 0 | |
| | | 5800 ± 400 | 135 | 35 | 7.10 | 0 | |
| | | 4900 ± 400 | 160 | 35 | 7.10 | 20 | |
| | | 4800 ± 400 | 170 | 30 | 7.10 | 20 | |
| | $Ca^{2+}$ | 3900 ± 300 | 90 | 30 | 7.10 | 20 | Equivalent to an 'absolute' affinity constant of $Ca^{2+}$ to $ATP^{4-}$ of 1.3 · $10^4$ $M^{-1}$ at 20°C. |
| | | 3400 ± 300 | 135 | 35 | 7.10 | 20 | |
| | | 3280 ± 200 | 90 | 30 | 7.10 | 0 | |
| | | 2900 ± 300 | 135 | 35 | 7.10 | 0 | |
| | | 2500 ± 300 | 170 | 30 | 7.10 | 20 | |
| | $Sr^{2+}$ | 1400 ± 200 | 135 | 35 | 7.10 | 20 | |
| EGTA | $Mg^{2+}$ | 46 ± 6 | 50—200 | 20—50 | 7.10 | 20 | |
| | | 25 ± 5 | 50—200 | 20—50 | 7.10 | 0 | |
| | | 14 ± 2 | 50—200 | 20—50 | 6.60 | 20 | |
| | | 240 ± 30 | 50—200 | 20—50 | 7.60 | 20 | |
| | $Ca^{2+}$ | (5 ± 1) $10^6$ | 50—200 | 20—50 | 7.10 | 0—20 | Agreement with Schwarzenbach [18], Owen [19], Allen et al. [20], Moisescu and Ashley [21]. |
| | | (5 ± 0.5) $10^6$ | 50—200 | 20—50 | 6.60 | 20 | |
| | $Sr^{2+}$ | (2 ± 0.2) $10^4$ | 100—200 | 35 | 7.10 | 0—20 | |

are indicated in Table II. These constants have been determined in our laboratories using potentiometric methods, some of which are described in detail in the Appendix.

Sarcomere length has been measured with a Leitz UMK 50/0.6 long working distance objective. The average size of sacromeres has been determined in different parts of the preparation before and after the length of the skinned fibre was changes.

## Results

Fig. 1 illustrates that there is no significant difference between the maximum force responses induced by $Ca^{2+}$ and $Sr^{2+}$ in the same preparation for otherwise identical conditions.

In Fig. 2 are presented three sets of force responses obtained on the same preparation at three sarcomere lengths: 2.2, 2.65, and 3.1 $\mu$m, respectively, when the preparation was activated in $Sr^{2+}$-buffered solutions.

First one can see that the absolute values of the submaximal forces at pSr 5.05, 4.78, and 4.54 are smaller at 2.2 $\mu$m sarcomere length than at 2.65 and 3.1 $\mu$m, respectively, although the maximum force response at 2.2 $\mu$m sarcomere length was higher than at2.65 and 3.1 $\mu$m. Second, one can observe that at room temperature force develops quite rapidly, with half times lower than 2 s for the whole range of $Sr^{2+}$ concentrations at all three sarcomere lengths.

All the results obtained with this fibre have been plotted in Fig. 4A. Although the deterioration in force was relatively small for each set of responses at a given sarcomere length (see Fig. 2) we have always corrected the graph data such as in Fig. 4 by normalizing to interpolated control contractions using a method similar to that of Julian [10]. The points corresponding to the sarcomere lengths of 2.65 and 3.1 $\mu$m lie practically on the same curve, while those corresponding to a sarcomere length of 2.2 $\mu$m appear to lie on a similar curve, which is shifted by approx. 0.1 log units towards higher free $Sr^{2+}$ concentrations.

Similar results to those in Figs. 2 and 4A have been obtained with 15 other preparations.

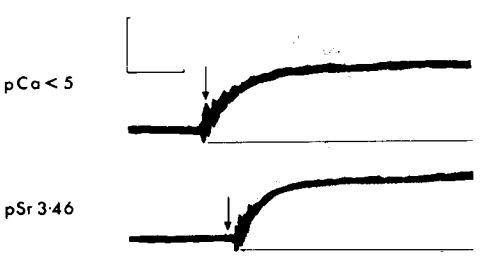


Fig. 1. Maximal force responses induced by $Ca^{2+}$ and $Sr^{2+}$ in frog bundles of myofibrils under otherwise identical conditions. The preparation (diameter 30 $\mu$m, length 0.9 mm, sarcomere length 2.4 $\mu$m has been initially equilibrated in a 'low relaxing' solution of type B/H = 1/666 and has then been separately activated into a solution of type A/B pCa 4.9 and A''pSr 3.46. The two force responses represent the 5th and 4th maximal force response, respectively. The base lines represent the 'zero' level for the active force in the solutions, and the arrows indicate the moments when the preparation was introduced into the respective solutions. Calibration bars: vertical: 0.06 mN; horizontal 0.3 s. Temperature = 20°C.

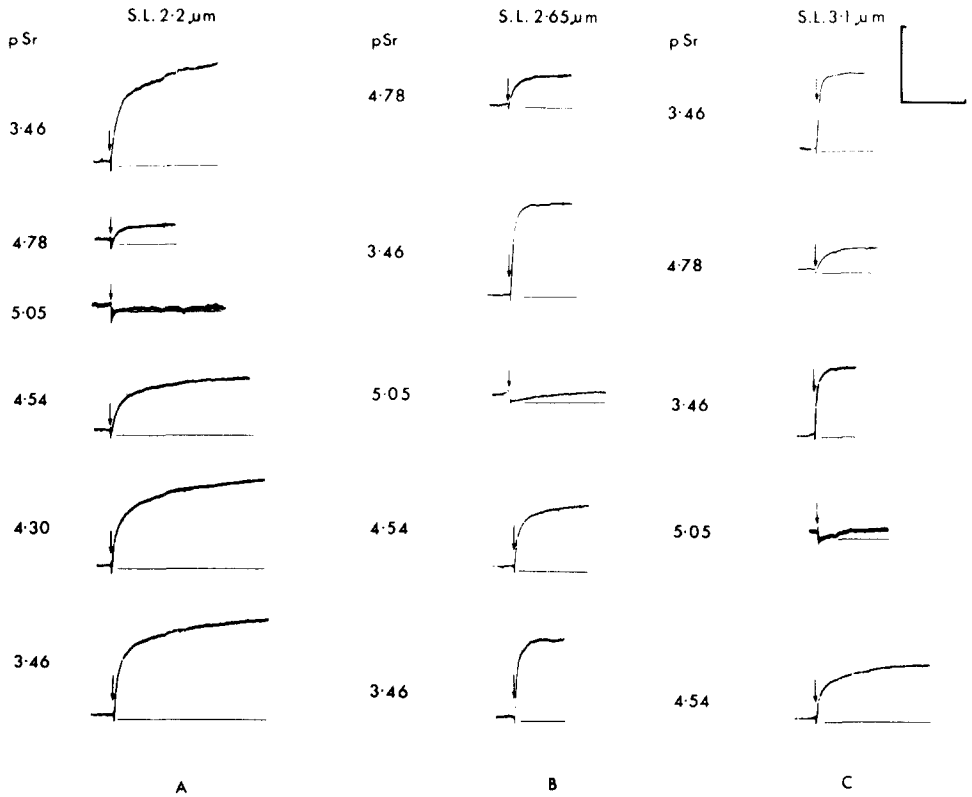


Fig. 2. Time course of isometric tension responses obtained in chronological order from the same myofibrillar preparation (diameter 60 μm) at three sarcomere lengths (2.2, 2.65 and 3.1 μm) when activated in $Sr^{2+}$-buffered solutions (of type A'/B up to pSr 3.7, and solution A'' for pSr 3.46). Before activation, the preparation has been equilibrated in a 'low relaxing' solution containing only about 0.1 mM EGTA (ratio B/H = 1/425). The base lines represent the 'zero' level for active force in the solutions and the arrows indicate the moment of immersing the preparation into the activating solutions. Calibration bars: vertical: 0.15 mN for pSr 5.05 and 0.3 mN for the rest; horizontal, 4 s for A, 5.5 s for B, 6 s for C. Temperature 20°C. All solutions contained 10 mM caffeine.

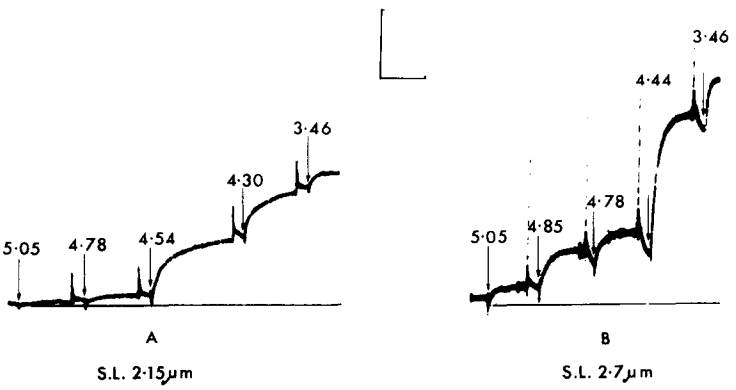


Fig. 3. Force responses from a preparation (diameter 35 μm) at two sarcomere lengths: 2.15 and 2.7 μm, respectively. The preparation has been first equilibrated at each sarcomere length in a low relaxing solution of type B/H = 1/600 and was subsequently activated in solutions of type A'/B up to pSr 3.7 and solution A'' for pSr 3.46. The arrows indicate the moment of changing the solutions around them. Calibration bars: vertical 0.1 mN for A; 0.05 mN for B; horizontal 15 s. Temperature 21°C.

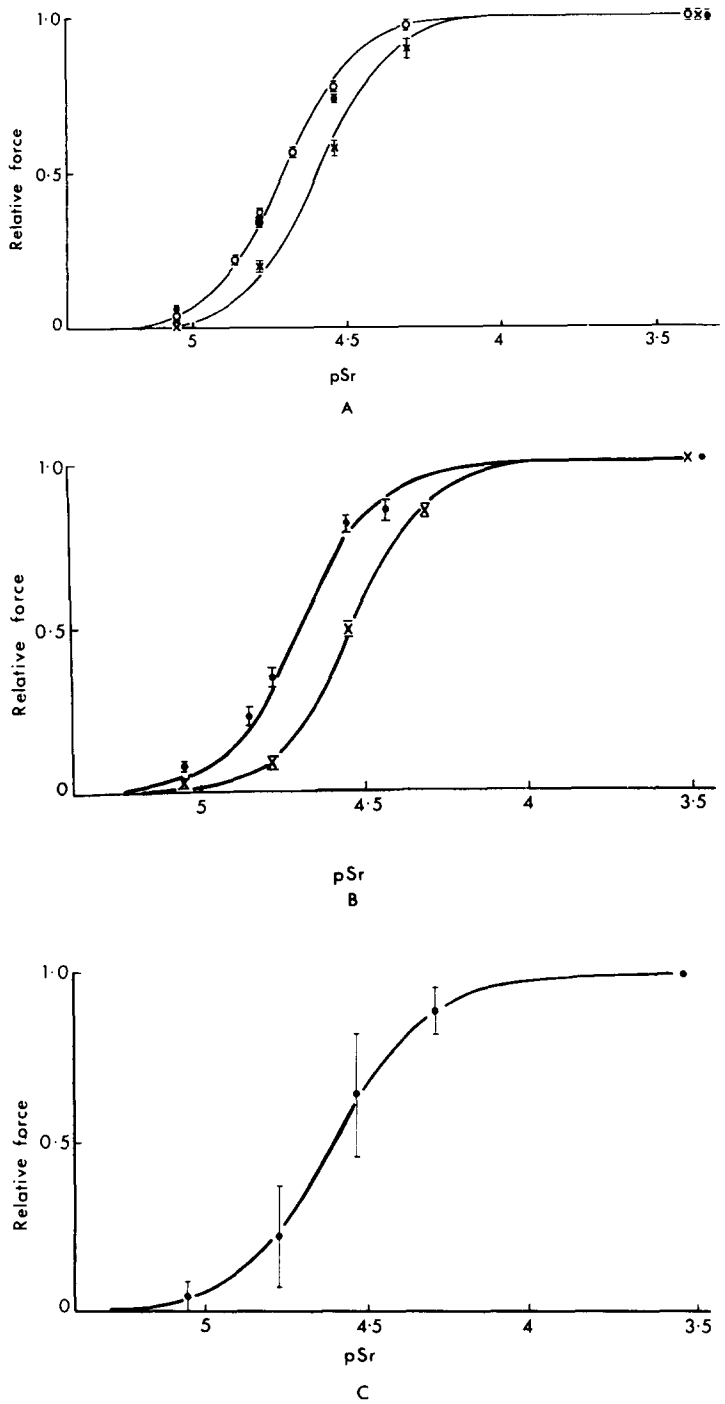


Fig. 4. Relative isometric force-pSr relationships for frog myofibrils as a function of sarcomere length. The solid lines are theoretical curves of type $P_r = K' \cdot [Sr^{2+}]^n/(1 + K' \cdot [Sr^{2+}]^n)$, where $P_r$ is the relative force. The constants $K'$ and $n$ are chosen such as to fit the results [15]. (A) $Sr^{2+}$-activation curves from all force responses obtained with the preparation in Fig. 2 (×, 2.2 μm; ○, 2.65 μm; ●, 3.1 μm). The solid curves were drawn for $n = 4$ and $K' = 2.3 \cdot 10^{18}$ $M^{-4}$ and $6.3 \cdot 10^{18}$ $M^{-4}$, respectively. (B) $Sr^{2+}$-activation curves from all force responses obtained with the preparation in Fig. 3 (×, 2.15 μm; ●, 2.7 μm). The solid curves were drawn for $n = 4$ and $K' = 1.5 \cdot 10^{18}$ $M^{-4}$ and $6 \cdot 10^{18}$ $M^{-4}$, respectively. (C) $Sr^{2+}$-activation curve based on all the results in (A) and (B) taken together. The solid curve was drawn for $n = 3$ and $K' = 6 \cdot 10^{13}$ $M^{-3}$. Note the less steep curve in (C) than in (A) and (B), although curve (C) is derived from the results in (A) and (B). The vertical bars indicate the range of the results.

We have also used another activation procedure and this is illustrated in Fig. 3. Here $[Sr^{2+}]$ was increased stepwise in the same preparation at two sarcomere lengths until maximum force was reached. One can again observe that the relative submaximal force is higher at 2.7 $\mu$m sarcomere length than at 2.15 $\mu$m for the same $Sr^{2+}$-concentrations. In Fig. 4B are presented the activation curves obtained from all the results on this preparation and one can also see that the curve for 2.7 $\mu$m lies about 0.1 log units towards lower $[Sr^{2+}]$ than the curve for 2.15 $\mu$m.

The results of Fig. 4A and B have been pooled together in Fig. 4C, and there is a marked decrease in the steepness of the overall activation curve in Fig. 4C when compared with the individual activation curves in Fig. 4A and B (see legend and Discussion).

Changes in the sarcomere length have the same effect upon the $Ca^{2+}$-activation curves, and this is illustrated in Figs. 5 and 6. Fig. 5 shows the force response obtained in one preparation when activated in two solutions after increasing the sarcomere length from 2.25 to 2.6 $\mu$m. At pCa 4.9 the response is maximal at both sarcomere lengths.

Force developed quite rapidly in both solutions and at both sarcomere lengths (half time <0.6 s) but the force level reached at pCa 6.0 was higher when the average sarcomere length was 2.6 $\mu$m than when the sarcomere length was 2.25 $\mu$m.

In Fig. 6 results are obtained at 3 different sarcomere lengths with the same preparation, and these results are typical of 10 experiments. The relative $Ca^{2+}$-activation curve was also shifted by about 0.1 log units towards higher pCa values when the sarcomere length was changed between 2.25 and 2.6 $\mu$m, but remained essentially the same when the sarcomere length was increased from about 2.6 to 2.85 $\mu$m.

In order to see whether the apparent increase in $Ca^{2+}$ and $Sr^{2+}$ sensitivity of stretched muscle fibres is connected with the changes in the interfilament distance in the preparation, brought about by stretching, we have used the observation of Matsubara and Elliott [11] that the interfilament distances in frog skinned muscle fibres do not essentially change (changes within 0.5%)

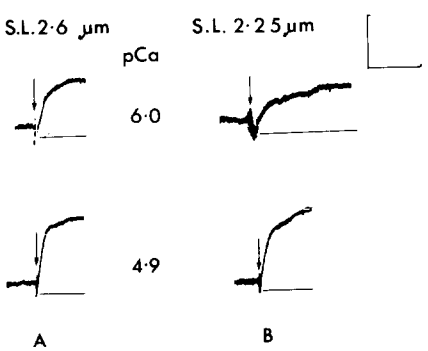


Fig. 5. Force responses from a preparation (diameter 30 $\mu$m) activated in $Ca^{2+}$-buffered solutions of type A/B at two sarcomere lengths 2.6 $\mu$m for A and 2.25 $\mu$m for B. The preparation was initially equilibrated in a 'low relaxing' solution with 0.1 mM EGTA (B/H = 1/500) before being activated. All solutions had 10 mM caffeine. Calibration bars: vertical 0.1 mN; horizontal 3 s. Temperature = 20°C.

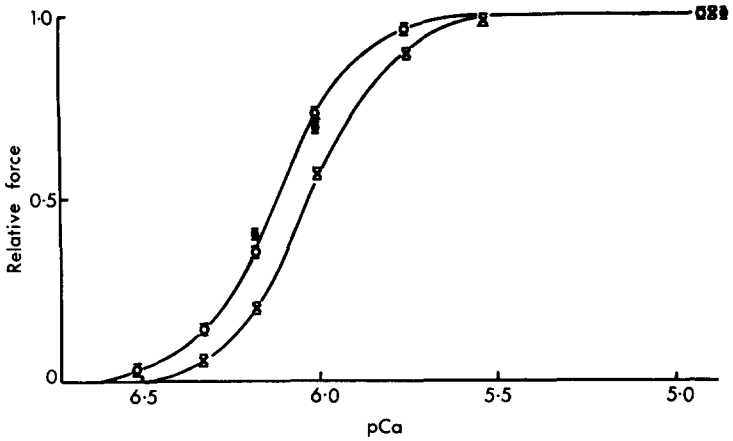


Fig. 6. The effect of varying sarcomere length upon the relative force ($P_r$)-pCa relationship in the same frog myofibrillar (as in Fig. 5) preparation (symbols: X, 2.25 $\mu$m; ○, 2.6 $\mu$m; and •, 2.85 $\mu$m). The solid lines are theoretical curves of the type $P_r = K \cdot [Ca^{2+}]^4 \cdot (1 + K \cdot [Ca^{2+}]^4)^{-1}$, with $K = 1.3 \cdot 10^{24}$ $M^{-4}$ and $2.9 \cdot 10^{24}$ $M^{-4}$, respectively. The small bars indicate the range of the results. Temperature 20°C. All solutions had 10 mM caffeine; the activating solutions were of type A/B.

when the sarcomere length varied between 2.2 and 2.65 $\mu$m if the ionic strength of the solution was under 0.09 M. Fig. 7 shows one set of results obtained with a preparation at an ionic strength of about 80 mM. As expected, the relative force responses in Fig. 7A and B are higher than the corresponding

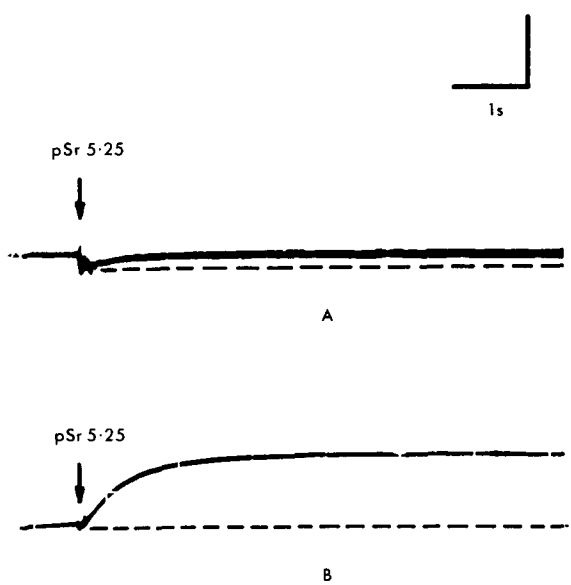


Fig. 7. Force responses from the same frog myofibrillar preparation activated in a $Sr^{2+}$-buffered solution having a low tonicity and ionic strength (ratio solution A'/solution B/distilled water, 125 : 875 : 2000) at two sarcomere lengths: 2.2 $\mu$m for A, and 2.65 $\mu$m for B. The preparation has been equilibrated in a 'low relaxing' solution (ratio solution B/solution H/distilled water, 1 : 500 : 1000) before being activated. The value of pSr in the activating solution is indicated above the arrows which show the moment of immersing the preparation in the activating solution. Calibration bar for force: 0.1 mN for A and 0.25 mN for B. Diameter 80 $\mu$m, temperature 20°C.

ones for the same [$Sr^{2+}$] as in Fig. 2 due to a lower concentration of monovalent cations in the medium and a lower ionic strength [9]. However, the effect of sarcomere length upon the tension response persisted.

The average drop in the maximal active force when increasing the sarcomere length from about 2.2 to 2.6 and 3.0 $\mu$m was close to 10 and 25%, respectively (15 $Sr^{2+}$-activated preparations and 10 $Ca^{2+}$-activated preparations) and these values are similar to those of Endo [2,3].

In separate experiments we have used a He-Ne laser (Spectra Physics 136-04) to follow changes in the sarcomere length during activation. The position of the first diffraction maximum did not usually change by more than 3% when force was approaching the steady-state in an activating solution as compared to that in the relaxing solution if the sarcomere length was above 2.2 $\mu$m.

## Discussion

The results presented here demonstrate that changes in the sarcomere length between about 2.2 and 2.6 $\mu$m affect in a similar manner the steady-state activation curves for both $Sr^{2+}$ and $Ca^{2+}$. However, the way in which the isometric force response was dependent upon the sarcomere length appeared to be essentially different from that reported by Endo [2,3].

Thus, our results indicate that the whole curve relating the relative isometric force to pCa or pSr is affected by sarcomere length changes in the range of 2.2—2.6 $\mu$m rather than only the part of the curve under 30% relative force. In addition force developed two orders of magnitude faster in our experiments than in those performed by Endo on the same muscle (iliofibularis) from *Rana pipiens*, *Rana Japonica* and *Xenopus laevis* [2,3].

It is very likely that these discrepancies are due to the differences in the procedures used for activation and in the composition of solutions, since when the pH-buffering capacity of our solutions was low (10 mM Tes buffer, pH 7.10), then the force responses were very slow, similar to those of Endo [2,3], and the longevity of the preparations was considerably shorter [12,13].

The effect of varying the sarcomere length upon the $Ca^{2+}$- and $Sr^{2+}$-activation curves as illustrated by Figs. 2–6 can be regarded as a change in the apparent affinity constants of Ca and Sr ions to the tension controlling sites. This does not exclude the possibility that only those reaction steps are modified, which follows the actual binding of Ca or Sr ions, since this could also result in a change of the overall apparent affinity constant for $Ca^{2+}$ and $Sr^{2+}$ [12]. In fact this latter possibility is more likely to be true since the activation curves for both $Ca^{2+}$ and $Sr^{2+}$ are identically affected by stretch.

At present we cannot find a simple explanation of this happening since a change in the sarcomere length does not appear to change the periodicity on the filaments [14] and the small associated change in the interfilament space does not seem to be essential (see Results).

Another conclusion to be drawn from these results is that the shape of all steady-state activation curves obtained by us for both $Ca^{2+}$ and $Sr^{2+}$ on skinned musle preparations from frog is very similar under various conditions of sarcomere length and temperature (Figs. 4A, B and 6; Ref. 7).

Thus the relative isometric force increases from 10 to 90% if the concentra-

tion of the activator increases about 3-fold. This alone suggests a minimum cooperativity of 4 activator ions per functional unit in the process of foce development [9,15], and the solid curves in Figs. 4A, B and 6 are theoretical predictions for 4 activator ions involved 'simultaneously' in the process of force development (see legends).

Donaldson and Kerrick [5] have recently reported less steep activation curves for $Ca^{2+}$ than for $Sr^{2+}$ in skinned muscle fibres from frog for similar conditions. This discrepancy can be partly explained by the much poorer buffering capacity for $Ca^{2+}$ than for $Sr^{2+}$ over the activation range in their experiments, particularly at relatively high $[Mg^{2+}]$ (see Methods; Ref. 9).

However, even the $Sr^{2+}$-activation curves reported by Donaldson and Kerrick [5] are less steep than our individual curves in Fig. 4A and B. Their curves are based on results from more than one preparation and since there is always a certain variability in the $Ca^{2+}$- and $Sr^{2+}$-sensitivity among preparations, an apparent reduction in the steepness of these activation curves is expected. Indeed, as it appears from Fig. 4, the average curve (Fig. 4C) increases over a wider range of $[Sr^{2+}]$ (i.e., is less steep) than the individual curves in Fig. 4A and B. This can explain why the 'Hill coefficient' ($n$) for their $Sr^{2+}$-activation curves was between 2.6 and 2.9 and for our individual curves is between 3.3 and 4.

The great similarity in both the shape of the $Ca^{2+}$- or $Sr^{2+}$-activation curves and in the maximum force induced by $Ca^{2+}$ and $Sr^{2+}$ under otherwise identical conditions strongly suggests that in frog muscle these two ions are involved in the same steps of reaction to produce force.

## Appendix

*Determination of the apparent affinity constants of $Ca^{2+}$, $Sr^{2+}$ and $Mg^{2+}$ to EGTA and ATP in solutions of physiological importance.*

The apparent affinity constant of a divalent cation, $X^{2+}$, to a ligand L is defined as:

$$K'^{L}_{x} = ([L_t] - [L_f]) \cdot [X^{2+}]^{-1} \cdot [L_f]^{-1} \qquad A(1)$$

where $[L_t]$ is the total concentration of the ligand, $[L_f]$ is the total concentration of the ligand which is not complexed with the cation and $[X^{2+}]$ is the free concentration of the cation $Ca^{2+}$, $Sr^{2+}$ or $Mg^{2+}$.

A pH meter with a sensitivity of at least ±0.01 pH units is required for the determinations.

*Affinity constants of EGTA*

The following known information (see Refs. 16 and 17) has been used for the development of the potentiometric methods and for the derivation of the formulae: (i) the absolute affinity constants of $H^+$ to $EGTA^{4-}$ and $HEGTA^{3-}$ are both over $10^{8.8}\ M^{-1}$; and (ii) EGTA forms two complexes with each divalent anion considered: $X\text{-}EGTA^{2-}$ and $X\text{-}HEGTA^{-}$.

*EGTA affinity for $Ca^{2+}$*

A known molar fraction of EGTA ($\alpha$) (final concentration 0.5—2 mM) is

added to the solution which for simplicity should not contain another divalent ion or another ligand which binds $Ca^{2+}$. The pH is adjusted with NaOH or HCl to a pH value, $pH_i$, in the range of 5.8–6.0. The same molar fraction ($a$) of $CaCl_2$ is then added which results in a pH drop. The solution is titrated with NaOH (molar fraction $n_1$) back to the initial pH. An excess of about 10 mM $CaCl_2$ is then added to the solution resulting again in a pH drop and finally the initial pH value, $pH_i$ is restored with $n_2$ mol NaOH.

Knowing the values of $a$, $n_1$, $n_2$ and that of the final volume $V$, one can calculate the total apparent affinity constant of Ca to EGTA ($K'^{EGTA}_{Ca}$) at any pH value, $pH_f$ in the range 5.8–7.5 by using the following equations:

$$K'^{EGTA}_{Ca} = K'_{CaEGTA} + K'_{CaHEGTA} \qquad A(2)$$

$$\log K'_{CaEGTA} = \log K_{CaEGTA} + 2(pH_f - pH_i) \qquad A(3)$$

$$\log K'_{CaHEGTA} = \log K_{CaHEGTA} + pH_f - pH_i \qquad A(4)$$

$$K_{CaEGTA} = n_1 \cdot (n_1 + n_2) \cdot (n_1 + n_2 - a) \cdot a^{-2} \cdot n_2^{-2} \cdot V \qquad A(5)$$

$$K_{CaHEGTA} = n_1 \cdot (n_1 + n_2) \cdot (2a - n_1 - n_2) \cdot a^{-2} \cdot n_2^{-2} \cdot V \qquad A(6)$$

We recommend working at a pH between 5.8 and 6.0, since in this range the apparent affinity constant of Ca to EGTA is both high enough to be able to achieve essentially saturation with 10–20 mM free Ca, and low enough to be able to estimate accurately the free Ca and $EGTA_f$ concentrations where $a/V$ is between 0.5–2 mM.

The procedure described here should yield values differing by not more than approx. 10% from the correct total apparent affinity constant of Ca to EGTA. It is important to mention that the results obtained with this method are very similar to those obtained when using the $Ca^{2+}$-sensitive photoprotein aequorin [20,21] and the $Ca^{2+}$-sensitive electrodes [19] (see Table II).

*EGTA affinity for $Sr^{2+}$*

The procedure for determining the apparent affinity constant of $Sr^{2+}$ to EGTA was identical to that for $Ca^{2+}$. The determinations can be done directly at a more physiological pH (around 7.00), since the apparent affinity constant for $Sr^{2+}$ at this pH value is very similar to that for $Ca^{2+}$ at pH 5.8–6.0.

*EGTA affinity constant for $Mg^{2+}$*

The apparent affinity constant of EGTA for $Mg^{2+}$ is much smaller than that for $Ca^{2+}$, and therefore another procedure than that presented above must be employed for determining it at pH values lower than 9. The procedure is available on request.

*Affinity constants of ATP*

The published physico-chemical data about ATP (see Refs. 16 and 17) indicate that the following set of reactions are sufficient to describe accurately the ionic species in a solution containing $Na^+$, $K^+$, $X^{2+}$ and ATP over a pH range of 6.6–7.1:

$$ATP^{4-} + H^+ \overset{K_1^{ATP}}{\rightleftharpoons} HATP^{3-} \qquad A(7)$$

$$ATP^{4-} + X^{2+} \overset{K_X^{ATP}}{\rightleftharpoons} XATP^{2-} \qquad A(8)$$

$$ATP^{4-} + Na^{+} \overset{K_{Na}^{ATP}}{\rightleftharpoons} NaATP^{3-} \qquad A(9)$$

$$ATP^{4-} + K^{+} \overset{K_K^{ATP}}{\rightleftharpoons} KATP^{3-} \qquad A(10)$$

where $K_1^{ATP}$, $K_X^{ATP}$, $K_{Na}^{ATP}$, $K_K^{ATP}$ are the absolute binding constants of $H^+$, $X^{2+}$, $Na^+$ and $K^+$ to $ATP^{4-}$, respectively.

*ATP affinity for $Mg^{2+}$*

The apparent affinity constant of $Mg^{2+}$ for ATP can be directly estimated in a medium containing $Na^+$ and $K^+$ at any pH in the range of 6.6—7.1 with the procedure described below:

A known molar fraction of ATP ($c$) is added to the $Na^+$ and $K^+$ containing solution such as the final concentration of ATP to remain in the range of 1—3 mM. The pH is adjusted with NaOH or HCl to the required pH. Then a similar, but not necessarily equivalent, amount of $MgCl_2$ ($d$) is added and the pH is restored to the initial value with $p_1$ mol NaOH. Subsequently an excess of $MgCl_2$ is added to the solution (final concentration 15 -20 mM) and the pH is brought to the initial value with $p_2$ mol NaOH. If the amount of $MgCl_2$ was sufficient to saturate the ATP, then one should notice only an insignificant release of protons by doubling the total Mg concentration in solution. This point must be generally checked.

Considering that ATP was saturated with Mg after the second addition of $MgCl_2$ one can calculate the apparent affinity constant of Mg to ATP, $K'^{ATP}_{Mg}$ from the following equation derived from Eqns. A(7)—A(10):

$$K'^{ATP}_{Mg} = p_1 \cdot (p_2)^{-1} \cdot V^{-1} \cdot [d - c \cdot p_1/(p_1 + p_2)]^{-1} \qquad A(11)$$

where $V$ is the volume of the solution after the addition of $p_2$ mol NaOH. This equation can be used with good results over the pH range 6.6—7.1.

*ATP affinity for $Sr^{2+}$ and $Ca^{2+}$*

Similar procedures to that described for $Mg^{2+}$ can be successfully used for $Sr^{2+}$ and $Ca^{2+}$.

## Acknowledgements

We are grateful to Professors H.Ch. Lüttgau and P.C. Caldwell, F.R.S., for continuous interest and support. This work was supported by the Deutsche Forschungsgemeinschaft and the Australian Research Grant Committee.

## References

1 Taylor, S.R. (1974) in The Physiological Basis of Starling's Law of Heart, CIBA Symp. 24, 93—109
2 Endo, M. (1972) Nature N. Biol. 237, 211—213
3 Endo, M. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 505—510
4 Natori, R. (1954) Jikeikai med. 1, 119—126
5 Donaldson, S.K.B. and Kerrick, W.G.L. (1975) J. Gen. Physiol. 66, 427—444

6 Moisescu, D.G. and Thieleczek, R. (1978) J. Physiol. 275, 241—262
7 Moisescu, D.G. (1976) Nature 262, 610—613
8 Moisescu, D.G. and Thieleczek, R. (1978) Proc. Austr. Physiol. Pharmacol. Soc. 9, 24P
9 Ashley, C.C. and Moisescu, D.G. (1977) J. Physiol. 270, 627—652
10 Julian, F.J. (1971) J. Physiol. 218, 117—145
11 Matsubara, I. and Elliott, G.F. (1972) J. Mol. Biol. 72, 657—669
12 Moisescu, D.G. (1973) Ph.D. Thesis, University of Bristol, U.K.
13 Moisescu, D.G. (1975) Pflügers Arch. 355, R-62
14 Huxley, H.E. and Brown, W. (1967) J. Mol. Biol. 30, 383—434
15 Moisescu, D.G., Ashley, C.C. and Campbell, A.K. (1975) Biochim. Biophys. Acta 396, 133—140
16 Sillén, L.G. and Martell, A.E. (1964) Stability Constants of Metal-Ion Complexes, Vol. 17, Chemical Society Publication, London
17 Sillén, L.G. and Martell, A.E. (1970) Stability Constants of Metal-Ion Complexes, Vol. 25, Supplement I, Chemical Society Publication, London
18 Schwarzenbach, G. (1960) Die Komplexometrische Titration, F. Enke Verlag, Stuttgart
19 Owen, J.D. (1976) Biochim. Biophys. Acta 451, 321—325
20 Allen, D.G., Blinks, J.R. and Prendergest, F.G. (1977) Science 195, 996—998
21 Moisescu, D.G. and Ashley, C.C. (1977) Biochim. Biophys. Acta 460, 189—205