Comparison between the Sarcomere Length-Force Relations of Intact and Skinned Trabeculae from Rat Right Ventricle

Influence of Calcium Concentrations on These Relations

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SUMMARY. To investigate the extent to which the properties of the cardiac myofibrils contribute to the length-force relation of cardiac muscle, we determined the sarcomere length-force relations for rat ventricular trabeculae both before and after the muscles were skinned with the detergent Triton X-100. Sarcomere length was measured continuously by laser diffraction. In the unskinned trabeculae stimulated at 0.2 Hz, the relation between active force and sarcomere length at an extracellular calcium concentration of 1.5 mm was curved away from the sarcomere length axis, with zero force at sarcomere length of 1.5–1.6 μm. At 0.3 mm calcium, the sarcomere length-force relation was curved toward the sarcomere length axis. Chemical skinning of the muscle with 1% Triton X-100 in a “relaxing solution” caused an increase in intensity and decrease in dispersion of the first order diffraction beam, indicating an increased uniformity of sarcomere length in the relaxed muscle. During calcium-regulated contractures in the skinned muscles, the central sarcomeres shortened by up to 20%. As the calcium concentration was increased over the range 1–50 μM, the relation between steady calcium-regulated force and sarcomere length shifted to higher force values and changed in shape in a manner similar to that observed for changes in extracellular calcium concentration before skinning. The sarcomere length-force relations for the intact muscles at an extracellular calcium concentration of 1.5 mm were similar to the curves at calcium concentration of 8.9 μM in the skinned preparations, whereas the curves at an extracellular calcium concentration of 0.3 mm in intact muscles fell between the relations at calcium concentrations of 2.7 and 4.3 μM in the skinned preparations. A factor contributing to the shape of the curves in the skinned muscle at submaximal calcium concentrations was that the calcium sensitivity of force production increased with increasing sarcomere length. The calcium concentration required for 50% activation decreased from 7.7 ± 0.52 μM to 3.77 ± 0.33 μM for an increase of sarcomere length from 1.75 to 2.15 μm. The slope of the force-calcium concentration relation increased from 2.82 to 4.54 with sarcomere length between 1.75 and 2.15 μm. This change in calcium sensitivity was seen over the entire range of sarcomere lengths corresponding to the ascending limb of the cardiac length-force relation. It is concluded that the properties of the cardiac contractile machinery (including the length-dependence of calcium sensitivity) can account for much of the shape of the ascending limb in intact cardiac muscle (Circ Res 58: 755–768, 1986).
the supply of Ca++ to the myofibrils is not changed immediately, and thus cannot account for the immediate change in developed force.

The second possible mechanism for length-dependent activation in intact cardiac muscles is that the sensitivity of the myofibrils to Ca++ may increase as muscle length increases. Such a length dependence of myofibrillar Ca++ sensitivity has been observed in studies with skinned fibers (muscle fibers with a disrupted sarcolemma), although in most of these studies only the sarcomere length range above 2.2 \( \mu \)m was investigated (see Allen and Kentish, 1985a; Stephenson and Wendt, 1984). The physiological range of sarcomere lengths during contraction in intact cardiac muscle under normal conditions is probably 1.6–2.3 \( \mu \)m (Page, 1974, Sonnenblick and Skelton, 1974; ter Keurs, 1983). Length-dependent variation of the sensitivity of the contractile system to Ca++ ions has been observed in mechanically skinned cardiac cell fragments over a range of sarcomere lengths between 1.8 and 2.3 \( \mu \)m (Fabiato, 1980). Subsequently, a quantitative study by Hibberd and Jewell (1982) has shown that Ca++ sensitivity of skinned cardiac trabeculae depends on resting sarcomere length between 1.9 and 2.5 \( \mu \)m. To what extent the length-dependence of myofibrillar Ca++ sensitivity observed by Hibberd and Jewell (1982) accounts for the length dependence of activation in intact cardiac muscle is not clear, for two reasons: (1) Hibberd and Jewell (1982) did not measure the sarcomere length during contraction of the skinned muscles, and so it is not known whether the length dependence of myofibrillar Ca++ sensitivity occurs over the entire range of active sarcomere lengths found in intact cardiac muscle; neither is the effect of shortening of the central sarcomeres that occurs in cardiac muscle (Julian and Sollins, 1975; Krueger and Pollack, 1975; ter Keurs et al., 1980) known. (2) Although comprehensive force-sarcomere length relations for the ascending limb have been determined in maximally activated skinned muscles, and so it is not known whether the length dependence of myofibrillar Ca++ sensitivity occurs over the entire range of active sarcomere lengths found in intact cardiac muscle; neither is the effect of shortening of the central sarcomeres that occurs in cardiac muscle (Julian and Sollins, 1975; Krueger and Pollack, 1975; ter Keurs et al., 1980) known. (2) Although comprehensive force-sarcomere length relations for the ascending limb have been determined in maximally activated skinned single cells (Fabiato and Fabiato, 1975, 1976) and in intact trabeculae (ter Keurs et al., 1980, Gordon and Pollack, 1980), the relations are not strictly comparable because of the different degrees of Ca++ activation in the two preparations and because of their different structural characteristics.

In the present experiments, we determined the force-sarcomere length relations in trabeculae at various concentrations of Ca++ before and after these muscles were skinned with a detergent. A preliminary account of these experiments has been published (Kentish et al., 1983).

**Methods**

**Apparatus**

Sarcomere length in the central part of the muscles was measured by laser diffraction, as described previously (ter Keurs et al., 1980). In short, the intensity distribution of the first order diffraction pattern was monitored by a photodiode array (Reticon 256 EC), which was scanned electronically every 0.5 msec. The median SL was computed electronically after a correction had been made for the contribution of light scattered from zero order. SL could usually be measured to a resolution of 0.02 \( \mu \)m. Muscle length was measured and controlled with a servo motor (Cambridge Technology 300 Dual Mode Servo) with a capacitive length transducer (overall compliance of motor + arm = 0.6 \( \mu \)m/mN). The force transducer was a semiconductor strain gauge (AE 801, AEM) with a short carbon fiber extension arm (sensitivity = 1.5 \( \mu \)V/mN, compliance = 1 \( \mu \)m/mN, natural frequency = 2.9 kHz). Muscle force, muscle length, and median SL were displayed on an oscilloscope with hard-copy unit (Tektronix 5103, 613, 4631) and were recorded on a Gould chart recorder. In addition, the intensity distribution of the corrected first order diffraction pattern was monitored on an oscilloscope. A position-sensitive photodiode (UDT 45C4) was also used in some experiments to measure the intensity of the first order diffraction band.

The rest of the apparatus was as described by ter Keurs et al. (1980), except that the volume of the flow-through muscle bath was decreased from 2 ml to 250 \( \mu \)l with Lucite inserts in order to accelerate solution changes.

**Experimental Protocols**

All experiments were performed at room temperature (22°C–24°C). In the first part of each experiment, the relationship between force and SL was determined in the unskinned trabeculae by a procedure similar to that described by ter Keurs et al. (1980). Briefly, unbranched trabeculae were dissected from the right ventricles of 12-week-old Wistar rats and were mounted in the muscle bath. A hook on the force transducer was passed through the tricuspid valve close to the muscle; the other end of the muscle was held by an oval ring of stainless steel wire attached to the motor. A piece of ventricular wall remaining at this end of the muscle prevented the end of the muscle from slipping through the ring. The dimensions of the muscle used were measured when muscle length had been set to a resting SL of 2.1 \( \mu \)m. These dimensions were (in mm): length, 3.29 ± 0.10, width, 0.277 ± 0.036; thickness, 0.096 ± 0.009 (mean ± se, n = 12). The muscles were superfused at 3 ml/min with an oxygenated saline (ter Keurs et al., 1980) containing 0.3 or 1.5 \( \mu \)M Ca++ and were stimulated at 0.2 Hz via platinum field electrodes. Muscles were discarded if they did not contract uniformly or if there was twisting of the diffraction pattern. After a 30-minute period for stabilization of the muscles, the force-SL relations were determined as follows. First, the muscle length was set to give a resting SL of 2.1 to 2.15 \( \mu \)m. Muscle length was then altered for four beats. The resting force and resting SL were measured just before the fourth beat, and the total force and active SL were measured at peak force during the fourth beat (Fig 1A). Active force in the muscle was calculated as the total force at the SL at the peak of contraction minus the resting force at the same SL; this assumes that the parallel elastic elements are in, or in parallel with, the sarcomere alone (cf ter Keurs et al., 1980, and see Results). The muscle was maintained at the control SL of 2.1 \( \mu \)m for 6–10 beats between each series of 4 test beats. The process was repeated for a range of test stretches or releases at two Ca++ concentrations: 0.3 \( \mu \)M and 1.5 \( \mu \)M.
The muscle then was skinned by 30-minutes of superfusion with "relaxing solution" (see below for details of solutions) to which had been added 1% Triton X-100 [procedure modified slightly from that of Kentish (1984)]. This "skinning solution" and all subsequent solutions were pumped through the bath at 1 ml/min. If the SL altered during the skinnng period, it was reset to 2.1 μm. The skinned muscle then was bathed in solutions containing 1 mM to 200 μM free [Ca++]. The method used to establish the relationships between force and SL at a given Ca++ concentration is illustrated in Figure 1B. First, a "reference" contracture was produced by changing from the relaxing solution (free [Ca++] < 0.3 μM) to an "activating solution" containing 4.3 μM free [Ca++] (Fig. 1B, panel A). To accelerate the attainment of a steady force in this and other contractures, the 10 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) remaining in the muscle from the relaxing solution was washed out with a "pre-activating solution" of low [EGTA] (Ashley and Moisescu, 1974; Moisescu, 1976; see below). During the reference contracture, we maintained the SL at 2.1 μm by stretching the muscle. The muscle then was relaxed for 2 minutes and was activated with an activating solution of the desired [Ca++] (up to 200 μM) for 2 or 3 minutes. In the last minute, by which time force and SL were steady (Fig. 1B panel B; see also Fig. 3), the muscle was given transient stretches or releases, each lasting for about 5 seconds. Force and SL were measured when they had reached new steady values during each stretch or release. As with the unskinned muscle, the active force was taken as the total force at a given SL minus passive force in the relaxed muscle at the same SL. After the series of length changes shown in Figure 1B, the muscle was returned to relaxing solution for 2 minutes. The reference contracture then was repeated (Fig. 1B, panel C). The forces in the first and second reference contractures were used to correct for the small but progressive loss of contractile force in the skinned preparations (14.3% ± 8.2% per hour, mean ± SE from six muscles). It was assumed that the maximum contractile performance of the muscle declined linearly with time between the reference contractures. Accordingly, a linear interpolation was used in which each value of force measured during the determination of the force-SL relation was multiplied by the appropriate factor to correct for the decline. Preparations in which force declined 50% (or more) during the first three control
contractures, and preparations in which the diffraction pattern disappeared during contractures were rejected.

The protocol shown in Figure 1B was repeated for a range of Ca++ concentrations from 1 μM to 200 μM. In some experiments, pairs of Ca++ concentrations were tested consecutively, with no relaxation of the muscle in between.

We point out that the activating solutions used to produce the reference contractures did not maximally activate the myofibrils. It is usual to employ activating solutions of optimal [Ca++] for the correction procedure, but these were not used in the present experiments because contractures at the optimal [Ca++] often resulted in irreversible degradation of the diffraction pattern (see Results). Theoretically, the use of suboptimal [Ca++] has the disadvantage that the force in the reference contractures would be altered if there were a permanent change in the Ca++ sensitivity of the muscle during the experiment. However, it has previously been shown that the deterioration of force occurs without any significant change in Ca++ sensitivity (Moisescu, 1976; Kentish, 1982).

The major technical problem in these experiments was that the diffraction pattern deteriorated irreversibly if the muscle was activated at high Ca++ concentrations and at long sarcomere lengths. Several changes in experimental protocol were tried in an attempt to reduce this deterioration. Previously reported methods for improving the retention of the pattern by slow activation with Ca++ (Iwazumi and Pollack, 1981) or by stretches and releases of the muscle (Brenner, 1983) proved to be of little value for the skinned cardiac muscle. To investigate whether the loss of the diffraction pattern resulted from a limitation in the supply of high-energy substrate to the myofibrils (cf Brenner, 1983), in one experiment we doubled the [Mg-ATP] from 5 mM to 10 mM and the [CP] from 15 mM to 30 mM and added 0.1 mM adenosine diphosphate (ADP). The [Mg+] was maintained at 3.0 mM by raising the [MgCl2], and the ionic strength was kept at 0.2 m by lowering the [potassium proprionate]. However, this change of solution composition affected neither the force-SL relationships (results not shown) nor the deterioration of the diffraction pattern.

**Solutions**

All solutions for the skinned muscles contained 100 mM potassium proprionate, 20 mM BES buffer (see below), 5 mM K2H2ATP, 8.17–9.00 mM MgCl2 (adjusted to give a calculated [Mg+] of 3.0 mM), 10 mM Na2HCP, 1 mM dithiothreitol, and 25 μg/ml creatinine kinase. In addition, relaxing solution contained 10 mM K2EGTA and 0 or 2.5 mM added Ca++, pre-activating solutions contained 9.85 mM K2-2,6-diaminohexane-N,N',N'-tetraacetic acid (HDTA) and 0.15 mM K2EGTA, and activating solutions contained 10 mM K2EGTA and 4–10 mM Ca++ (to give a calculated free Ca++ concentration of 0.3–50 μM) The [K2EGTA] was calculated by taking into account the impurity of EGTA [measured purity = 96.2% (see Kentish, 1984)] The pH was adjusted to 7.00 with KOH. The activating solutions of Ca++ concentrations up to 50 μM were made as described by Ashley & Moisescu (1977). An activating solution of 280 μM free Ca++ was used in two experiments. The free concentrations of the ions were calculated by an iterative computer program. Details of this calculation, of the measurement of the EGTA purity and the contaminant calcium, and of the calibration of the pH electrodes are given elsewhere (Kentish, 1984). The calculated ionic strength was 0.20 m.

In the first few experiments, relaxing solution contained 10 mM EGTA and no added Ca++. However, the calculated [Ca++] of this solution (1 μM) was much less than that which exists in cardiac cytosol during diastole [probably about 0.1 μM Ca++ (Fabriato, 1983)]. To produce a more physiological resting [Ca++], in most experiments we used a relaxing solution of 0.16 μM free Ca++ ([total calcium] = 2.5 mM) to achieve relaxation of the muscle. As a check that this [Ca++] was below the threshold for activation of the muscle at any SL studied, the force in this solution was compared with that in the solution of [Ca++] = 1 μM. The solution with 0.16 μM free Ca++ did not produce any activation of the muscle. Even at the longest SL studied (2.3 μm), at which the Ca++ sensitivity would be expected to be greatest (see Results), the forces developed in the two solutions were identical. A similar test proved that the preactivating solution (calculated [Ca++] = 0.1 μM) did not activate the muscle.

**Chemicals**

All chemicals were supplied by BDH or Merck, except for the following, (N,N-bis[2-hydroxyethyl]-2-aminoothane sulfonic acid; 2-[bis(2-hydroxyethyl)amino]ethane sulfonic acid (BES), Na2H2ATP, Na2HCP, K2EGTA, dithiothreitol, creatinine kinase (Sigma); H2HDTA (Fluorochem).

**Results**

**Force-SL Relations in the Unskinned Muscles**

The results from the unskinned muscles are shown in Figures 1A and 6. Figure 1A illustrates the protocol used to determine the relations between force and SL. The mean results from six muscles are shown in Figure 6A. The force-SL relations are shown both for the resting muscles and for the active muscles at the peak of contraction. These relations were determined at two concentrations of extracellular Ca++: 1.5 mM (the standard concentration in these experiments) and 0.3 mM. The mean resting force, which was the same for both Ca++ concentrations, was zero at a SL of 1.9–2.0 μm and increased rapidly as the SL was raised above this sarcomere length (Fig. 4 and Fig. 6A). Above a SL of about 2.2 μm, further stretch of the muscle produced little increase in the length of the sarcomeres in the center of the resting muscle. This behavior indicates that the elastic elements in parallel with the sarcomeres were extremely stiff (viz. ter Keurs et al., 1980).

As in the resting muscle, in the actively contracting muscle, sarcomere lengths above about 2.3 μm were never seen, even if the muscle was highly stretched. Active force development was zero below a SL of 1.5–1.6 μm. Because the force (F)-sarcomere length (SL) relations were frequently nonlinear, the data were fitted to F = a (SL- SL0) by nonlinear least squares analysis (see Fig. 5 and Table 1). The average force-sarcomere length data (Fig. 6) were fitted to the same relation, in which the average c of Table 1 was substituted.
SL-Force Relations in Cardiac Muscle

TABLE 1

| [Ca++] (mM) | SLo (µm) | c | P  
|--------------|----------|---|-----
| 1.5 mM*      | 1.58 ± 0.06 | 0.52 ± 0.17 | <0.01
| 0.3 mM*      | 1.60 ± 0.07 | 1.44 ± 0.44 | 0.1
| 1.9 µM*      | 1.84 ± 0.18 | 2.31 ± 0.55 | <0.005
| 2.7 µM*      | 1.78 ± 0.09 | 2.37 ± 0.38 | <0.02
| 4.3 µM*      | 1.68 ± 0.12 | 1.55 ± 0.42 | <0.01
| 8.9 µM*      | 1.62 ± 0.07 | 0.69 ± 0.12 | <0.01
| 50 µM*       | 1.14 ± 0.17 | 0.93 ± 0.13 | NS  

Data (mean ± sd) were from six intact* and subsequently skinned† trabeculae. Force-sarcomere length relations were fitted through the data according to \( F = a(SL - SL_0)^c \), where \( SL_0 \) is the intercept with the abscissa, \( c > 1 \) indicates curvature toward the abscissa, and \( c < 1 \) indicates curvature toward the ordinate. Departure from linearity (\( P \) of \( c = 1 \)) was tested by analysis of variance (Snedecor and Cochran, 1973).

The average values of \( c \) and the intercept with the abscissa (\( SL_0 \)) are given in Table 1. The relationship between force and SL was significantly curved away from the SL axis at 1.5 mM [Ca++] (\( c = 0.52 ± 0.17 \)) (cf. Table 1; Fig. 6A). At all sarcomere lengths, active force was greater at 1.5 mM [Ca++] than at 0.3 mM [Ca++]. Figure 5 shows the two experiments in which the decrease of \( F \) at any SL with a decrease of [Ca++] to 0.3 mM was the largest (Fig. 5A) and the smallest (Fig. 5C). The F-SL relation in a 0.3 mM [Ca++] was curved toward the abscissa (cf. Table 1; Fig. 6A) (\( c = 1.44 ± 0.44 \)). Departure from linearity (seen in five muscles) reached significance for two of the six muscles.

Changes in the Laser Diffraction Pattern during the Skinning Procedure

Figure 2 shows the force, SL, and first order intensity and distribution during the 30-minute perfusion of the muscle with skinning solution. Active force development ceased completely within a few seconds when the skinning solution was applied to the muscle (Fig. 2A). During the first few minutes of the skinning procedure, passive force often fell slightly and then remained constant. In some muscles, the median value of SL changed by up to 0.1 µm during the skinning procedure; if this occurred, the SL was reset to 2.1 µm at the end of the skinning period. The peak intensity of the first order light consistently increased, but in a complex fashion. Initially there was a rapid increase in intensity in the first minute or so after skinning was started. Over the next few minutes, the intensity decreased again, occasionally to its value in the resting un-

![Figure 2](http://circres.ahajournals.org/)

**FIGURE 2.** Panel A: Chart records of force, median sarcomere length, and first order light intensity in a trabecula immediately before and during the skinning procedure. Sarcomere length was measured with a photodetector array and intensity was measured with a position-sensitive photodetector. Panel B: the intensity distribution of light falling on the photodetector array (a) just before skinning, (b) 15 minutes after the start of skinning, and (c) before and during a test contraction at constant sarcomere length at [Ca++] = 4.3 mM. These records are superpositions of oscilloscope traces, Light intensity distribution was displayed on the oscilloscope after subtraction of light scattered from zero order and with automatic gain control for intensity so that the changes in peak intensity were eliminated.
skinned muscle. The decrease was associated with the development of an opaque appearance of the muscle. This was followed by a slower but sustained increase in the intensity, the final value of which was usually two or three times that in the unskinned muscle. This second increase in intensity was associated with a visible increase in the transparency of the muscle. The dispersion of the first order intensity pattern was decreased considerably by the skinning procedure (Fig. 2B), and most of this reduction appeared to occur in the first few minutes of skinning, i.e., it was associated with the first increase in peak intensity. The dispersion then remained constant for the remainder of the skinning period.

Muscle width (measured to the nearest 4 μm by a graticule in the inverted microscope) did not vary during the skinning procedure.

**Force Development in the Skinned Muscles**

Figure 3A shows the force development of a skinned trabecula superfused with an activating solution of [Ca++] = 4.3 μM and held at a constant muscle length; for comparison Figure 3B shows the force development in the same muscle, but with SL held constant by stretching the muscle. At constant muscle length, force rose slowly to reach a plateau in about 60 seconds. The development of force was accompanied by substantial change of SL. Initially, the SL in the central region of the muscle fell from the resting SL of 2.10 to 2.04 μm, but then slowly increased again. This slow increase accompanied the slow phase of force development. It seems likely that this was due to the central region of the muscle being stretched by the contraction of the ends. If the SL was held constant at 2.04 μm (the smallest value reached during the contracture at constant muscle length) force rose more rapidly to reach a plateau in 10 seconds. The level of force reached was the same as that in the previous contracture (Fig. 3A) at the point when the SL was 2.04 μm. In the examples of Figure 3 the [Ca++] was 4.3 μM; at higher concentrations of Ca++, the internal shortening during isometric muscle contraction was even more pronounced, and varied between 7% and 20% at [Ca++] of 8.9 μM in these experiments. This illustrates why it was necessary to measure the SL during contraction rather than just the SL in the resting muscle. In many preparations at a [Ca++] just above the threshold for activation, the muscle exhibited internal shortening rather than perceptible force development. For technical reasons, we chose to control muscle length and measure SL rather than try to maintain SL by stretching the muscle.

**Force-SL Relations in the Skinned Muscle**

The steady force and SL measured from recordings at various Ca++ concentrations (Fig. 1) were used to plot a family of force-SL relations at Ca++
concentrations from 1 nM to 50 μM. The relations between SL and passive force at Ca^{++} concentrations below 0.7 μM are shown in Figure 5: Ca^{++} concentrations from 1 nM to 0.7 μM were insufficient to produce any Ca^{++} activation of the skinned muscle. As the SL was increased above 2.0 μm, the passive force increased from zero, but this increase was less steep than in the same muscles before they had been skinned (Fig. 4). In addition, the muscle shown in Figure 4 could be stretched to a SL of 2.4 μm, although this had not been possible in the unskinned muscle.

At Ca^{++} concentrations above 0.7 μM active force was developed (Figs. 5 and 6). The absolute force at SL = 2.00 μm in the skinned preparations at 8.9 μM was 14.4% ± 6.9% (SD) higher than in the intact trabeculae (Fig. 6) at the same SL and [Ca^{++}] of 1.5 μM. No oscillations of force (FabiatO, 1978) or sarcomere length were observed in these muscles (Fig. 3).

One of the advantages of the protocol in the present experiments is that we were able to compare the force-SL relations in the same muscle before and after it had been skinned. Thus, such variables as the external geometry of the preparation and the number of myofibrils were constant throughout. Moreover, the diffraction patterns at [Ca^{++}] below 10 μM remained crisp during contraction (see Fig. 2C). It is clear from Figures 5 and 6 that the shape of the force-SL relation depended largely on the [Ca^{++}]. A direct comparison between the two types of preparation revealed that the curves for 8.9 μM [Ca^{++}] were similar in shape to, although slightly steeper than, those for the unskinned muscle at extracellular Ca^{++} concentrations of 1.5 μM (cf. Figs. 5 and 6). The force-SL relations at an extracellular

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**Figure 5.** Force-sarcomere length relations of two trabeculae before and after skinning. Panels A and C show active force taken as total force at the peak of contraction minus the resting force borne at the sarcomere length measured at peak contraction. The concentration of Ca^{++} was 1.5 μM (squares) or 0.3 μM (circles). Panels B and D show the force-sarcomere length relations of the same trabeculae after skinning in [Ca^{++}] 1.9 μM (squares), 2.7 μM (triangles pointing down), 4.3 μM (triangles pointing up), 8.9 μM (circles), and 50 μM (diamonds). Force was measured as in the intact muscles. The scales of the graphs are identical for each muscle before and after skinning. For further explanation, see text.
FIGURE 6. Mean force-sarcomere length relations of six trabeculae prior to and after skinning. Sarcomere length was averaged in bins of 0.1 μm. The points show the mean ± SEM, if 1 SEM was larger than the symbols. Panel A: mean active force (open symbols) and passive force (filled symbols) in the intact trabeculae. The concentration of extracellular calcium was 1.5 mM (squares) and 0.3 mM (circles). Panel B: mean active force in the trabeculae after skinning at five Ca++ concentrations (1.9 μM, squares; 2.7 μM, triangles pointing down; 4.3 μM, triangles pointing up; 8.9 μM, circles) between the threshold and the saturating concentration. Note the similarity of the force-sarcomere length relation of the intact trabeculae at an extracellular Ca++ concentration of 1.5 mM (panel A) and after skinning at a [Ca++] of 8.9 μM whereas the force-sarcomere length relation before skinning at 0.3 mM in panel A falls between those of 4.3 and 2.7 μM in the skinned muscle.

[Ca++] of 0.3 mM fell between the force-SL relations at free Ca++ concentrations of 2.7 μM and 4.3 μM after skinning (Figs. 5 and 6). Figure 5, B and D, shows the range of variation of force of the force-SL relations with variation of free [Ca++] that we found in the same muscles after skinning. At a free Ca++ concentration of 8.9 μM, the force-SL relationship tended to be curved toward the ordinate (Table 1; Figs. 5 and 6), whereas, at low free Ca++ concentrations, the force-SL relationship was curved toward the abscissa (cf. the increase of c in Table 1).

At maximally activating Ca++ concentrations (50 μM and above), the relation was approximately straight and appeared to differ fundamentally from the relationships for the intact muscle and for the skinned muscle at lower Ca++ concentrations, in that a considerable amount of force was generated at SL = 1.6 μM. Note that, in Figures 5D and 6, there are fewer data points at high Ca++ concentrations and high sarcomere lengths. This was the case because, under these conditions, the sarcomere diffraction pattern frequently disappeared or became too broad to allow an adequate measurement of the median sarcomere length. More seriously, these conditions also produced an irreversible deterioration of the diffraction pattern: although the pattern became sharper again as the muscle was made to relax, the pattern in subsequent contractures, even at suboptimal [Ca++], was compromised. In many muscles, this irreversible degradation of the diffraction pattern occurred even at low sarcomere lengths if the

<table>
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<tr>
<th>Sarcomere length (μm)</th>
<th>F MAX (mN/mm²)</th>
<th>n</th>
<th>[Ca++]50 (μM)</th>
<th>n</th>
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<tr>
<td>2.15</td>
<td>86.3 ± 3.4</td>
<td>4.54 ± 0.74</td>
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<td>2.05</td>
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<td>4.50 ± 0.60</td>
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<tr>
<td>1.85</td>
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<td>46.2</td>
<td>4.35</td>
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<td>2</td>
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</table>

Average F MAX, n, and [Ca++]50 (± SEM) calculated from six individual experiments by nonlinear multiple regression of the modified Hill equation through the F — [Ca++] data at different sarcomere lengths. n is the number of sigmoid relationships used for calculation of F MAX, n, and [Ca++]50.
[Ca++] was near saturation. Several changes in experimental procedure were tried to attempt to reduce the deterioration in the diffraction pattern, but with little success. Only two factors seemed to influence the loss of the diffraction pattern. First, there was considerable variability between muscles in the quality and durability of the diffraction pattern: only four of the muscles studied exhibited patterns that unequivocally gave a median SL at the optimal Ca++ concentrations of 50 μM. Thus, we could determine the force-SL relationship at optimal [Ca++] only for relatively few muscles. Second, if the SL was raised above about 2.0 μm at the optimal [Ca++], a usable diffraction pattern was lost in most muscles. For this reason, we did not routinely subject the muscles to a high Ca++ concentration and long SL simultaneously, and we determined the force-SL relation at maximally activating Ca++ concentrations only at the end of the experiment.

Relation between Force and [Ca++] at Different Sarcomere Lengths

The force-sarcomere length relations at different free Ca++ concentrations calculated (see ter Keurs, 1983) for the average of six muscles (Fig. 6) and for the individual muscles (Fig. 5) were used to derive the force-[Ca++] relationships at different sarcomere lengths shown in Figure 7 and summarized in Table 2. Because force was not measured at predetermined values of SL, we used the curves, rather than the data points, to estimate the active force development at selected sarcomere lengths. The derived [Ca++]-activation curves were approximately sigmoidal on a semilogarithmic scale (Fig. 7A). These curves showed that increases in SL shifted the [Ca++]-activation curves to the left, i.e., to lower Ca++ concentrations. This increase in Ca++ sensitivity with SL appeared to be present at all sarcomere lengths in the range 1.7-2.3 μm. To obtain an objective

![Figure 7](http://circres.ahajournals.org/)
estimate of the [Ca++] required for 50% activation at each sarcomere length, we fitted the curves by non-linear least squares analysis (Snedecor and Cochran, 1973) to the modified Hill equation:

\[ F = F_{\text{MAX}} \left( \frac{[\text{Ca}^{++}]}{K^* + [\text{Ca}^{++}]} \right) \times 100\% \]

where: \( F \) = developed force, \( n \) = the Hill coefficient, (cf. Table 2), \( K^* \) = a compound affinity constant, and \( F_{\text{MAX}} \) = maximal F at that sarcomere length.

The [Ca++] for 50% activation [Ca++]50 was then given by

\[ [\text{Ca}^{++}]_{50} = \left( \frac{\log_{10} K^*}{n} \right) \]

The [Ca++] for 50% activation decreased from 9.53 μM to 3.77 ± 0.32 (mean of individual experiments ± SEM) in proportion to an increase in sarcomere length between 1.65 and 2.15 μM. The Hill coefficient increased (Table 2) slightly from 2.82 ± 0.23 to 4.54 ± 0.74 (mean of six individual experiments ± SEM) but significantly (\( P \leq 0.02 \) when tested by linear regression) with increasing sarcomere length between 1.75 and 2.15 μM. \( n \) is the number of sigmoid relationships used for calculation of \( F_{\text{MAX}} \) and [Ca++]50.

Force-[Ca++] relations were also studied in two muscles at constant muscle length (resting SL was 2.15 μM) (see Fig. 7B). They were less steep (\( n = 2.64 \) and 2.70) than those at constant sarcomere length; [Ca++]50 was 5.9 and 3.3 μM, respectively.

Discussion

Intact Muscles

The active force-SL relations in the intact muscle in 1.5 mM [Ca++] were very similar to those previously found with the same type of preparation (Gordon and Pollack, 1980; ter Keurs et al., 1980; ter Keurs, 1983). It is pointed out that the experimental protocol used to determine the force-SL relation did not allow any time for the slow changes in activation that occur with a time course of minutes following a length change (Parmley and Chuck, 1973; Lakatta and Jewell, 1977). Thus, the force-SL relation was an "instantaneous" rather than a steady state relation (viz. Lakatta and Jewell, 1977). Because the contractions were at constant muscle length (ML) rather than constant sarcomere length, there was considerable internal shortening during muscle contraction. Theoretically, this could have produced "shortening deactivation," but it has been shown by previous studies that the force-SL relation obtained from contractions at constant ML is the same as in those at constant SL (Pollack and Krueger, 1976; ter Keurs et al., 1980).

The influence of extracellular [Ca++] on the shape of the force-SL relation has been reported in detail elsewhere (ter Keurs, 1983). The change in shape reproduces the effect of extracellular [Ca++] on the force-length relation of papillary muscles (Allen et al., 1974) and the effect of postextrasystolic potentiation on the force-SL relation of rat trabeculae (ter Keurs et al., 1980).

Skinning Procedure

Apart from the rapid loss of muscle excitability, the most striking changes during the skinning procedure were those relating to the intensity and dispersion of the first order diffraction pattern. It is not unlikely that the initial increase in intensity and decrease in dispersion were due to a true increase in the homogeneity of sarcomere length. Possibly, this rapid increase in SL homogeneity upon skinning resulted from spontaneous, uncoordinated contractions of individual sarcomeres in the intact muscle during diastole. Under conditions of a raised resting intracellular [Ca++], cardiac muscle preparations often show spontaneous contractile activity, which is visible as uncoordinated contractions in individual cells or strings of cells and which can be recorded as fluctuations in the intensity of scattered light (Stern et al., 1983) and as oscillations of cytosolic [Ca++] (Orchard et al., 1983). Although visible spontaneous activity died away completely during the stabilization period at the start of each experiment, it is possible that there were still random sarcomere movements that were too small and uncoordinated to be manifested as force development or as intensity fluctuations, but which nevertheless produced uncoordinated shortening of individual sarcomeres and thereby increased the dispersion of sarcomere length.

The second, slow increase in first order intensity occurred simultaneously with a further decrease of the width of the first order of the diffraction pattern, and probably was due to the dissolution of mitochondria and sarcoplasmic reticulum and to the loss of cytosolic proteins, such as myoglobin (Kentish, 1982). In the intact cells, all these structures would tend to absorb or scatter laser light. The transient decrease in intensity that preceded the second slow increase was associated with a visible turbidity (as seen through the binocular microscope), and therefore may have been due to the disruption of membranous organelles, which later dissolved.

Skinned Muscles

Passive Properties

A comparison of the force-SL relations for the resting muscle before and after skinning (Figs. 4 and 6) showed that the skinned muscle was more compliant, in that resting force increased less steeply as SL was increased above 2.0 μM. It is possible that the greater resting force in the intact muscles was due to the presence of some residual activation by Ca++ (see above), although various lines of evidence argue against this possibility: (1) there were no light intensity fluctuations in the intact muscles by the time the force-SL relationship was determined, (2) the extracellular [Ca++] did not affect the resting...
force in the intact muscles (Fig. 4) and (3) resting force first was seen at the same SL (1.9-2.0 \( \mu \)m) in both preparations. It seems more likely that the greater stiffness of the intact muscle compared with the skinned muscle was due to the contribution of a parallel elastic element, which was altered by the skinning procedure. The sarcolemma could have provided this parallel element, either directly because the membrane itself bore some resting force at SL > 2.0 \( \mu \)m (although this is unlikely, as the sarcolemma is compliant) or indirectly because the membrane conferred constant-volume behavior upon the cells of the intact muscle. Constant-volume behavior causes the negatively charged myofilaments to be forced closer together at longer sarcomere lengths, and mutual repulsion between the filaments conceivably could produce a force that opposes lengthening of sarcomeres above 2.0 \( \mu \)m, although this force is not manifest in skeletal muscle. This force would not be seen in skinned cells, which lack constant-volume behavior (e.g., Matsubara and Elliott, 1972). Another possibility is that an elastic stroma of nonmyofibrillar filaments, which may bear much of the passive force (Wingrad and Robinson, 1978; Price and Sanger, 1983; Magid et al., 1984), suffered a change in its physicochemical properties during the skinning procedure. This could have occurred either as a result of the dissolution of organelles that were enveloped in the stroma or as a result of a direct effect of the skinning solution on the stroma.

**Active Properties**

After the muscles had been skinned, it was initially much easier to measure the SL because of the increase in intensity and decrease in dispersion of the first order diffraction pattern compared with those in the unskinned muscle. However, this benefit was gradually offset by the major problem with the skinned muscles: the first order diffraction pattern deteriorated in successive Ca++-regulated contractures, especially if the muscle was subjected to a high [Ca++] at a long SL. A similar loss of the first order diffraction pattern in the resting skinned muscle was distinct throughout the experiment, the resting pattern could not be used as an index of the active SL because, in these preparations, the SL sometimes decreased considerably during activation by Ca++ (e.g., Fig. 3).

It is not clear why the sarcomere striation pattern deteriorates in the skinned muscle. One possibility is that the force-generating capabilities of individual sarcomeres or half-sarcomeres decrease non-uniformly during the course of an experiment. Thus, during activation of the muscle, when each sarcomere in a series must bear the same force, the sarcomeres will be at different degrees of overlap, with the sarcomeres of poorer contractile performance at longer sarcomere lengths (i.e., higher up the ascending limb) than those of better contractile performance. Another possible explanation is that the arrays of thick and thin filaments lose their regular structure during contraction of the skinned muscle: Iwazumi (personal communication, 1983) has observed "smearing" of the A- and I-bands in single cardiac myofibrils during activation at near-maximal Ca++ concentrations, although he studied only the SL range above 2.2 \( \mu \)m. However, neither explanation provides a reason for the loss of striations in skinned muscle but not in intact muscle. One likely cause of this difference is the Ca++ concentration, because maximally activating Ca++ concentrations, as used in the present study, probably are never attained in intact cardiac cells (Fabiato, 1983). Other possibilities are that the sarcomere disruption is caused by the prolonged nature of the Ca++-regulated contractures in the skinned muscle, by a loss of some vital proteins as a result of skinning, or as a result of prolonged exposure to low calcium concentrations between contractions or by the loss of the constant-volume behavior.

To our knowledge, the present study is the first in which the force-SL relations were determined in the same cardiac muscle before and after skinning. Factors such as the geometry of the preparation, the number of myofibrils, and the amount of connective tissue were therefore the same in the two types of preparation. This is a prerequisite if meaningful comparisons are to be made between the force-SL relations of intact and skinned muscles.

For the following discussion, it is important to bear in mind that, in the skinned muscles, the concentration of the activating Ca++ (in the bathing solution) was constant during the determination of the force-SL relationship. Thus, any observed length dependence in the contractile characteristics of the muscle must have been due to the properties of the sarcomeres plus connective tissue. In the unskinned muscle, a length dependence of the Ca++ supply could have been an additional factor.

The force-SL relationships for the intact muscle and for the skinned muscle seemed to have the same basic shape (Figs. 5 and 6). The only major difference was that, whereas in the intact muscles force development was zero at a SL of 1.6 \( \mu \)m and below, in the skinned muscle a considerable force could be produced at these sarcomere lengths if the [Ca++] was raised to 50 \( \mu \)M (Figs. 5 and 6). A considerable force has also been observed in maximally activated fragments of single skinned cells from rat ventricle at sarcomere lengths as low as 1.2 \( \mu \)m (Fabiato and Fabiato, 1975). The force-SL relation at maximal activation was however steeper for the trabeculae than for fragments of skinned single cells: extrapo-
loration of the data in Figure 6 indicates that zero force would have occurred at a sarcomere length of about 1.2 μm, whereas, force in fragments in single cells at this SL was 60% of maximum (Fabiato and Fabiato, 1975). This difference was not due to incomplete activation of the myofibrils at the lowest sarcomere lengths in our experiments, because the force was not increased if the [Ca++] was raised further to 280 μM (results not shown). The apparent discrepancy probably represents a true difference between the two types of preparation: trabeculae, unlike single cells, contain intercellular connections (Winegrad and Robinson, 1978) and extracellular connective tissue (Kentish, 1982) that could produce forces that oppose shortening at the shorter sarcomere lengths. These forces would act to decrease the force measured at the shorter sarcomere lengths and would thus make the force-SL relation steeper. Alternatively, the myofilament lattice spacing of the mechanically skinned cell fragments may have been greater than that of trabeculae described here. This would lead to steeper force-SL relations in the present study (see below).

One of the main findings in the present study is the similarity of the force-SL relations in the skinned muscle compared to those of the intact muscle if the [Ca++] was below maximally activating levels. For example, the force-sarcomere length relation of the intact trabeculae at an extracellular Ca++ concentration of 1.5 mM was quite similar (of Table 1 and Fig. 6) to the relation after skinning at a Ca++ concentration of 8.9 μM. The mean force-sarcomere length relationship at an extracellular Ca++ concentration of 0.3 mM tended to be convex toward the abscissa (Table 1; Fig. 6) and fell between the relations at [Ca++] = 2.7 and 4.3 μM in the skinned muscle, which were also convex toward the abscissa. The force-sarcomere length relationships of individual muscles at an extracellular Ca++ concentration of 0.3 mM varied between relationships similar to those at [Ca++] = 2.7 μM (Fig. 5A) or at [Ca++] = 4.3 μM (Fig. 5C) after skinning.

At first sight, this comparison suggests that the shape of the instantaneous force-SL relationship in the intact muscle can be accounted for by the properties of the myofibrils (plus a contribution from extracellular tissue; see above). However, underlying this conclusion is the assumption that the properties of the myofibrils in the skinned muscle accurately reflect the properties of the myofibrils in the intact muscle. For this assumption to be justified, two conditions must have been met: the chemical environment of the myofibrils in the two preparations should have been similar, and the properties of the myofibrils should not have been altered by the skinning procedure. We chose the ionic conditions of the solutions so that they resembled those in intact cardiac cells (e.g., pH = 7.0, Poole-Wilson, 1978; [Mg++] ~ 3.0 mM; Hess et al., 1982; see Kentish, 1982, for further details). The cytosolic [Ca++] attained during the twitch of cardiac muscle of course varies with the inotropic status of the muscle, but evidence from aequorin-injected cardiac muscle suggests that under the conditions of our study the peak cytosolic [Ca++] is likely to have been about 5–10 μM at 1.5 mM extracellular [Ca++] (Allen and Kurihara, 1980; Fabiato, 1981). In any case, the [Ca++] range we studied must have encompassed that found in the intact muscle under almost all conditions. However, it should be noted that the solutions used for the skinned muscles were of necessity only simple models of the cytosol, because they lacked the soluble proteins and metabolic intermediates present in normal cytosol. The influences these substances might have on the force-SL relation are unknown.

It is conceivable that the second condition—that skinning did not alter myofibrillar properties—was not met, because, at least in skeletal fibers, skinning causes the myofibrils to swell and lose constant-volume behavior (Matsubara and Elliott, 1972; Godt and Maughan, 1977). Although the detergent-skinned trabeculae did not swell visibly during skinning, it is likely that swelling of the myofibrils occurred, but that it was compensated by some dissolution of intracellular organelles (Kentish, 1982), which account for more than 40% of cell volume in intact cells. The spacing between the myofilaments in skinned muscle can be reduced by adding to the solutions large polymers such as polyvinylpyrrolidone (PVP) that are excluded from the myofilament lattice (Godt and Maughan, 1977). However, Fabiato and Fabiato (1976) found that PVP increased the slope of the force-SL relationship in skinned cardiac cells at optimal [Ca++] . This indicates that any increase in myofilament spacing during skinning in the present experiments would have tended to make the force-SL relationships less steep than they were in the intact muscle. It is also possible that sarcomeres in the skinned muscle may have been in a damaged state (see above). If there had been nonhomogeneity of SL in the skinned muscle, this too would have tended to flatten the force-SL curve. Thus, in both cases, our results for skinned cardiac muscle may have underestimated rather than overestimated the steepness of the force-SL relationship for the myofibrils (plus extracellular tissue) per se in the intact muscle.

With these considerations in mind, we conclude that much, if not all, of the instantaneous force-SL relationship in the intact trabeculae can be explained by the inherent properties of the myofibrils (plus a possible contribution from the mechanical properties of the extracellular tissue). However, this does not exclude the possibility that part of the instantaneous force-SL relationship results from a length-dependence in the supply of Ca++ to the myofibrils (Fabiato and Fabiato, 1975), although a major contribution from this factor seems unlikely, because the amplitude of the [Ca++] transient in intact cells injected with aequorin is not altered in the right direction to account for the alteration of developed
force in the first few beats after a change in muscle length (Allen and Kurihara, 1982; Allen and Smith, 1985). On the other hand, a change in the Ca ++ supply to the myofibrils is probably responsible for the slow changes in active force development seen in the few minutes after the length change (Allen and Kurihara, 1982).

The steepness of the force-SL relationships in the skinned muscle at suboptimal [Ca ++ ] was partly a consequence of the fact that Ca ++ sensitivity of the myofibrils increased with SL (Fig. 7). A similar length dependence of Ca ++ sensitivity has been observed in many studies on the descending limb of the force-SL relation in skinned fibers (for references, see Allen and Kurihara, 1985a; Stephenson and Wendt, 1984). It also confirms the results of Fabiato (1980) in mechanically skinned cell fragments and of Hibberd and Jewell (1982), who found that the Ca ++ sensitivity of detergent-skinned trabeculae increased as the SL in the relaxed muscle was increased from 1.9–2.0 μm to 2.3–2.5 μm. However the length dependence of Ca ++ sensitivity in our experiments was almost twice as large as in the study by Hibberd and Jewell (1982). The reason for this discrepancy is not clear. The only major difference between the solutions used in the two studies was that we used a [Mg ++ ] of 3 mM (viz., Hess et al., 1982) whereas Hibberd and Jewell used 1 mM. It is possible that the discrepancy could merely be due to the fact that Hibberd and Jewell (1982) measured the SL only in the relaxed muscle, whereas we were able to measure the SL throughout Ca ++ activation of the muscle; internal shortening during contraction can cause the active SL to be considerably less than the resting SL (Fig. 3). The results of the present study show for the first time that the length dependence of myofibrillar Ca ++ sensitivity occurs over the entire range of active sarcomere lengths (1.6–2.3 μm) that corresponds to the ascending limb of the length-tension relationship in cardiac muscle (Page, 1974; Sonnenblick and Skelton, 1974; ter Keurs, 1983) Thus, it is likely that this phenomenon contributes to the Frank-Starling relation under all physiological conditions.

Our experiments provide no clue as to the mechanism of the length dependence of Ca ++ sensitivity. One plausible mechanism is that the affinity of troponin for Ca ++ increases with SL. Recent experiments using skinned muscles loaded with photoproteins (Allen and Kentish, 1985b; Stephenson and Wendt, 1984) have provided evidence in favor of this hypothesis.

All the force-[Ca ++ ] relations at known sarcomere lengths (Fig. 7A) were considerably steeper than has previously been found for detergent-skinned cardiac muscle during contractions at constant muscle length (e.g., Hibberd and Jewell, 1982; Kentish, 1984). The Hill coefficient n of around 4 was almost twice as great as n at constant muscle length in our study (n = 2.7 and 2.6; cf. Fig 7B) and in other studies (mean n = 2.48–2.94; Hibberd and Jewell, 1982, and n = 2.14; Kentish, 1984). A decreased slope of the force-[Ca ++ ] relationship for muscle isometric contractions compared with SL isometric contractions could arise from a combination of the influence of SL on Ca ++ sensitivity and the internal shortening that occurs in the cardiac trabeculae (Kentish, 1984): as the muscle is activated with progressively greater concentrations of Ca ++ , more force is generated and more internal shortening occurs; the SL in the central part of the muscle decreases progressively and the appropriate force-[Ca ++ ] relationship for the sarcomeres shifts to the right (as in Fig. 7A). The overall force-[Ca ++ ] relationship for isometric muscle contractions is therefore flatter than it would have been if the SL had been held constant.

The observed n of about 4.5 at a sarcomere length of 2.15 μm is also twice the n derived from studies on mechanically skinned cell fragments (Fabiato and Fabiato, 1978; Fabiato, 1981) at the same sarcomere length. This discrepancy remains unexplained, but may be related to the absence of force and sarcomere length oscillations in these skinned muscles (see Figs. 1 and 3) at suboptimal calcium concentrations, whereas such force oscillations usually occur in cell fragments during partial activation by calcium ions (Fabiato, 1978).

The force-[Ca ++ ] relation at known sarcomere lengths is too steep to be explained by positive cooperativity between the three Ca ++ -binding sites on cardiac troponin, especially since there is evidence that only one of these sites is directly involved in Ca ++ regulation of contraction (Holroyde et al., 1980). If, indeed, Ca ++ sensitivity depends upon the number of crossbridges (see above), the steepness of the relationship can be explained by an increase in the number of crossbridges as the [Ca ++ ] is raised (cf. Brandt et al., 1980). However there are several other explanations, such as interactions between adjacent tropomyosin molecules, that could explain positive cooperativity (see also Hibberd and Jewell, 1982). The observed tendency of the Hill coefficient n to increase with sarcomere length would be consistent with a model (Brandt et al., 1980) in which an increase of the sarcomere length increases the number of possible crossbridges and by virtue of a cooperative process both increases n and decreases [Ca ++ ]s.
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Comparison between the sarcomere length-force relations of intact and skinned trabeculae from rat right ventricle. Influence of calcium concentrations on these relations.
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