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EFFECTS OF CALCIUM AND IONIC STRENGTH ON SHORTENING VELOCITY AND TENSION DEVELOPMENT IN FROG SKINNED MUSCLE FIBRES

BY FRED J. JULIAN AND RICHARD L. MOSS*

From the Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114, U.S.A.

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SUMMARY

1. The influence of Ca^{2+} concentration and ionic strength on the maximum velocity of shortening (V_{max}) and the tension generating capability of frog skinned muscle fibres has been studied at temperatures between 1 and 10 °C.

2. Fibre segments were mounted between a force transducer and servo motor, where they could be viewed and photographed through a microscope. Segments in which the striations became non-uniform during activation were discarded.

3. Velocity was obtained as a function of load by stepping the tension to values less than the steady isometric tension. V_{max} was then determined by an extrapolation technique. V_{max} was also obtained using a second, independent method by measuring the times required to take up various amounts of slack imposed on the segments.

4. V_{max} was significantly influenced by the Ca^{2+} concentration, decreasing by about one half when the Ca^{2+} concentration was reduced to give steady tensions less than half-maximal.

5. V_{max} was not influenced by changes in ionic strength, in the range 0·09–0·18 M. Steady tension was found to increase as ionic strength was decreased in the same range.

6. These results indicate that the effect of changes in ionic strength is to alter the numbers or stiffness of attached cross-bridges, while there is no apparent influence of ionic strength on the steady-state kinetics of the actin–myosin interaction during unloaded shortening. The mechanism responsible for the influence of Ca^{2+} on V_{max} is unknown, though possible sites of action for Ca^{2+} are discussed.

INTRODUCTION

There has been much effort devoted to the study of the effects of the calcium concentration on the unloaded speed of shortening, V_{max}, of skinned muscle fibres. Several investigators have found that reducing the Ca^{2+} concentration to sub-maximally activating levels results in a substantial decrease in V_{max}, both in briefly glycerinated muscle fibres of the frog (Julian, 1971) and in other skinned muscle

* Present address: Department of Physiology, School of Medicine, University of Wisconsin, Madison, WI 53706, U.S.A.

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preparations (Wise, Rondinone & Briggs, 1971; Honig & Takauji, 1976; de Clerck, Claes & Brutsaert, 1977). In contrast, studies on mechanically skinned frog fibres (Podolsky & Teichholz, 1970; Thames, Teichholz & Podolsky, 1974; Gulati & Podolsky, 1978) found that Ca^{2+} does not influence V_{max}. Thames *et al.* (1974) presented evidence suggesting that the difference between their results and Julian's (1971) could be explained on the basis of a small difference in the ionic strength of the solutions used in the two studies. They found that at 7 °C V_{max} remained constant as ionic strength was varied by changing the concentration of KCl from 140 to 280 mM . Under these conditions, changes in the concentration of Ca^{2+} did not influence V_{max}. Below, 140 mM-KCl, V_{max} measured during maximal activations declined progressively as the KCl concentration was reduced to 50 and then 0 mM. At sub-maximal levels of Ca^{2+}, Thames *et al.* (1974) found V_{max} to decline by a factor of about 3 when the concentration of KCl was reduced from 140 to 50 mM. Gulati & Podolsky (1978), on the other hand, found that at 1 °C, V_{max} was independent of ionic strength, and that changes in the amount of activating Ca^{2+} had no influence on V_{max} regardless of ionic strength.

In the present study, we report the effects of variations in solution Ca^{2+} concentration and ionic strength on V_{max} and the steady tension measured in briefly glycerinated frog skeletal muscle fibres (Julian, 1971) at temperatures ranging from 1 to 10 °C. The preparations were observed and photographed through a microscope, so that only those preparations having good striation uniformity during contraction were used. At 1, 7 and 10 °C, V_{max} was found to be unaltered by changes in ionic strength between 0·09 and 0·18 M. At an ionic strength of 0·14 M and 5 °C, V_{max} was found to depend markedly on the concentration of free Ca^{2+}, confirming and extending earlier results presented by Julian (1971). A similar dependence of V_{max} on Ca^{2+} concentration was found at 10 °C and ionic strengths of 0·09, 0·14 and 0·18 M. In agreement with previous work on mechanically skinned segments (Gordon, Godt, Donaldson & Harris, 1973; Thames *et al.* 1974), steady tension was found to vary inversely with ionic strength in the range studied.

Some aspects of this work have been presented at a meeting of the Biophysical Society (Moss & Julian, 1978).

METHODS

Preparation. Frogs (*Rana pipiens*) were obtained from northern Vermont during both the winter and summer months. Winter frogs were kept at room temperature (20–23°) and were fed live crickets and meal worms for a period of 2 weeks to several months before use. Summer frogs were kept in the cold (approximately 6 °C) and were usually sacrificed within 2 weeks of arrival. Single muscle fibres were isolated from either the medial head of the anterior tibialis or the dorsal head of the semitendinosus muscles. The fibres were chemically skinned using the procedure of brief glycerination described by Julian (1971). The skinned fibres were bathed for 30 min in relaxing solution (100 mMKCl, 4 mM-ATP, 1 mM-$MgCl_2$, 2 mM-EGTA, 10 mM-imidazole, pH 7·0) containing 0·5 % (w/v) of either Lubrol-WX or Brij 58 (Sigma Chemical Co.), both of which are non-ionic detergents.

A modification of this skinning procedure was frequently used. Bundles of about twenty fibres were isolated from the muscle and then depolarized in KCl wash solution (100 mM-KCl, 2 mM-EGTA, 10 mM-imidazole; pH 7·0; 20–23 °C). Bundles of five to eight fibres were cut free of the tendons and transferred to the glycerinating solution (Julian, 1971) for 15–45 min and next into the detergent-containing relaxing solution. Fibre segments, 3–5 mm long, could then be stripped free for transfer to the experimental chamber.

Solutions. The method for controlling the free Ca^{2+} concentration of the activating solutions with ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), have been described previously (Julian, 1971). The apparent stability constant for the Ca-EGTA complex was assumed to be $10^{6.68}$. Relaxing solution contained the following, in mM: EGTA, 2; Na_2ATP, 4; $MgCl_2$, 1; imidazole, 10; pH 7·00; KCl was varied as described below. In the present study, 'Fluorimetric Grade' imidazole (Sigma Chemical Co.), which is of very high purity, was used. The ionic strength of the solutions was adjusted by varying the concentration of KCl. The ionic strengths were as follows, with the total KCl concentration in mM indicated in parentheses: 0·18 M (140); 0·14 M (100); and 0·09 M (50). Ionic strength was calculated using the equation, ionic strength = $\frac{1}{2}\Sigma mZ^2$, where m was the molar concentration of an ion species and Z was its charge. Solution temperature was maintained at 1, 5, 7 or 10 °C (±0·2 °C), depending on the measurements to be made. In some cases, as described below, 1, 6-diaminohexane *N,N,N',N'*-tetraacetic acid (Fluka Chemical Co.; HDTA) was added to the relaxing solution, replacing part of the EGTA. The concentration of HDTA in these cases was 3·5 mM and that of EGTA was 0·5 mM. The presence of HDTA in the relaxing solution, along with the reduction in the amount of EGTA, resulted in a substantial increase in the rate of tension rise during activation with Ca^{2+}. This is a modification of a similar technique described by Moisescu (1976). The concentrations of HDTA and EGTA that were used in the present work were chosen to give a substantial increase in the rate of tension rise without changing the contribution of HDTA and/or EGTA to the ionic strength of the relaxing solution. The use of this technique greatly enhanced striation uniformity during activations to steady tension levels less than about 0·9 P'_0 (0·14), which is the maximum Ca^{2+}-activated tension in solution of ionic strength 0·14 M.

Chamber and apparatus. The experimental chamber was made from a flat, rectangular aluminium plate, which was temperature controlled with thermoelectric devices mounted along one edge. Experimental troughs were cut into the moveable centre portion of the plate and were coated with either Teflon or Parylene (Nova Tran Corp., No. Attleboro, MA). The floors of the troughs were made of Plexiglas. Solution changes were done by depressing the centre portion, sliding it laterally until another trough was in position beneath the fibre, and then allowing the centre portion to return to its original, elevated position.

Segments, 3–5 mm long, were cut from the briefly glycerinated and detergent-treated fibres. These were transferred to an experimental trough and attached to two horizontal wires by means of miniature connectors, described below. As shown in Fig. 1, one wire was attached to a force transducer, and the other wire was attached to an arm extending from the rotor of a torque motor (described below). The transducer was of the capacitance change type (Julian & Sollins, 1973) and had a resonant frequency of 800 Hz or 2·8 kHz, depending on the stylus used. Capacitance changes were detected with an FM circuit (Cambridge & Haines, 1959), the output gain of which was adjusted to give a sensitivity of 2 mV/mg with peak-to-peak noise of less than 1 mV. Tension output was recorded using both a storage oscilloscope and a strip chart recorder (Hewlett-Packard, Model 7133A). The transducer and torque motor were each fixed to three-way translators to permit positioning of the preparation, and also changes in over-all segment length.

Torque motor and control circuitry. The torque motor (Model G100PD, General Scanning Corp., Watertown, MA) was stiffened and its frequency band width extended by application of displacement and velocity feed-back. A control and servo system similar to the one described by Julian (1971) was used in order to obtain force–velocity data. Length changes were measured with a capacitance change transducer element which was an integral part of the torque motor. The motor arm was fluid damped to allow the use of greater servo loop gain, resulting in improved force control while minimizing oscillations associated with the step changes in force.

Muscle segment connectors. The segments were attached to the experimental apparatus with miniature two-piece connectors (Moss, 1979). The main part of the connector consisted of a trough, 1·5 mm long, which was ground from thin-walled, stainless steel tubing (0·30 mm O.D.). One of these connectors was attached with Shellac to each of the two wires in the experimental trough. In later experiments, the connectors were modified to comprise an integral part of these wires as shown in the inset of Fig. 1. A fibre segment was placed in the connectors in such a way that a piece of the segment approximately 1 mm long remained exposed between the connectors. Stainless-steel pins (0·16 mm in diameter, 0·8 mm long) were placed upon the portions of the segment within the main bodies of the connectors, and were secured in place with loops of 10–0 nylon monofilament.

Microscopy. The fibre segments were continually viewed through a Zeiss WL microscope, using a 200 W mercury vapour lamp or a Zeiss microflash unit as the light source. A low power lens combination (Moss, 1979) was used, and this permitted a full end-to-end view of most fibre segments within a single field. Photomicrographs were usually obtained during the period of steady active tension development just before each velocity measurement and again immediately following the

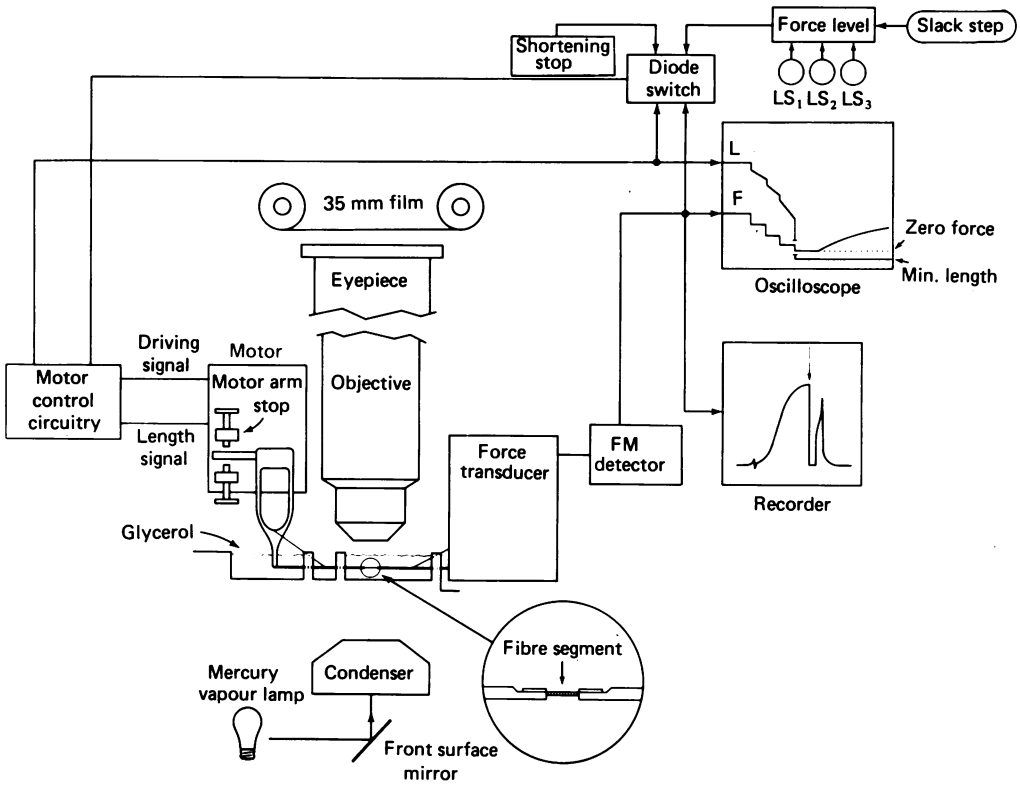

Fig. 1. Schematic diagram of the experimental apparatus. The fibre is bathed in a trough containing relaxing or activating solution; and this trough is separated from a second, glycerol-containing trough by an air gap. The relative sizes of the experimental trough, motor arm, transducer, microscope objective and fibre segment are accurate as drawn. The 'motor control circuitry' consists mainly of a commercially built unit (Scanner Control, CCX 101, General Scanning Corp., Watertown, MA). See Julian (1971) for operation of diode switch. The oscilloscope and recorder traces are drawn by hand. The arrow on the recorder trace indicates the instant at which the force steps and the step to slack length, shown in the oscilloscope traces, are applied. 'Min. length' represents the minimum length of the fibre segment following the step to slack length. LS_1, LS_2 and LS_3 represent load steps one, two and three. Inset: enlarged view of fibre segment showing methods of attachment to segment end. Threads which secure the pins in place are not shown. See Moss (1979) for more detail.

measurement. Photographs were taken using a 35 mm film format. The developed negatives were projected on a photo-enlarger yielding a final magnification of 110–350 × depending on the projection distance, and an average striation spacing was measured by counting several rows of striations which ran the entire length of the segment (Moss, 1979). This procedure permitted a direct verification of the sarcomere length range over which the segment shortened during the velocity measurements. Also, segments were in this way inspected both for the disappearance of striations, and for the appearance of gross striation non-uniformities during activation.

Procedure. Once the fibre segment was attached to the apparatus, the temperature of the bathing

solution was lowered to the appropriate level. Fixed-end contractions were then done, and between contractions the length of the segment was adjusted until an average sarcomere length of about 2·3 μm was obtained during steady force development, as determined from light micrographs. During these setting up procedures, the photomicrographs were, for convenience, obtained using a high power lens combination (approx. 460×) and a Polaroid film format (Moss, 1979). Force–velocity measurements were obtained using one of two methods. In the first of these, previously described by Julian (1971), the fibre segment was transferred from relaxing solution to an activating solution of the same ionic strength. During this time, the motor control network was set to maintain the segment at an over-all constant length, i.e. the segment was under length control. When a steady force had developed, the load on the segment was suddenly stepped to a value less than the maximally developed force, as shown in Fig. 4. An elastic recoil of the segment coincident with the force step was evident in the length trace. Force was then maintained constant (i.e. segment under force control) during which time the velocity of shortening was constant. At this point a second force step to a still lower load was applied and another velocity measurement was obtained. Frequently, a third load step followed. Shortening continued until the electronic shortening stop was reached and the segment was again under length control. A low-power photomicrograph of the segment was then taken, after which the segment was placed in relaxing solution of the same ionic strength. If the average sarcomere length measured from this photograph was less than about 2·0 μm, the velocity measurements were discarded. For any given fibre, velocity measurements made under conditions of high Ca^{2+} and/or high ionic strength were the last to be made because of the possible deleterious effects that large amounts of tension could have to increase end compliance.

Loads (P) were expressed relative to the steady isometric force (P') measured just prior to the load steps; velocities of shortening (V) were calculated as muscle (i.e. segment) lengths per sec (m.l./sec), where m.l. is the segment length at which the average striation spacing was 2·2 μm during steady isometric contraction. The velocity and relative force data were used in a linear form (Katz, 1939) of Hill's (1938) equation in which $(1-P/P')\,1/v$ was plotted against P/P'. A straight line was fitted to these points, and the unloaded speed of shortening (V_{max}) was calculated as the inverse of the intercept of the fitted line on the $(1-P/P')\,1/v$ axis. Examples of such plots are shown in Fig. 5.

To ensure that the zero-force base line was determined accurately, most force–velocity measurements of the type just described were modified in the following way. At the end of the phase of shortening under the lightest load, a large step decrease in length was imposed in order to introduce slack into the segment. In this way a true force zero was obtained. The records of Fig. 4 are typical of this procedure. In these cases, photographs of the segment could only be taken just prior to the force steps, since the segments were greatly shortened following the steps to lengths less than slack length.

An alternative method for determining V_{max} (Hill, 1970; Julian, 1971; Edman, 1979), which will be called the slack test, was used in a number of experiments. Segments were rapidly released (i.e. length step complete in 0·7 msec) to various slack lengths from an initial length corresponding to an average striation spacing of about 2·35–2·40 μm. Following the release, force remained at zero for some period of time, dependent on the extent of release, during which the segment shortened without load to take up the imposed slack. The duration of unloaded shortening was measured between the point at which force first reached zero and the point at which force just began to rise above zero, i.e. when the force deviated from a straight horizontal line superimposed on the force trace. In any one segment, for a given set of experimental conditions, various amounts of slack were introduced in from three to seven succeeding contractions. Examples of the records obtained are shown in Fig. 8. The amount of release was then plotted against the time of unloaded shortening as shown in Fig. 9. A straight line was fitted to these points by the least squares method and V_{max} was measured to be the slope of this line, expressed in m.l./sec.

Once a velocity measurement was made and the relaxation was complete, the tension of the segment in relaxing solution was measured by slackening the segment from its original length. The tensions which were measured in this way were generally less than 1% of the maximum active tensions obtained in activating solutions of similar ionic strength. At each of the ionic strengths studied, Ca^{2+} concentrations which produced tensions less than about 0·2 of the P'_0 at that ionic strength were not used, so that corrections for the effects of passive elements on the measured active tensions were not necessary.

RESULTS

The following abbreviations will be used in the presentation and discussion of the results of this study. P' is used generally to indicate the steady isometric tension developed by a fibre segment in solutions of a specified pCa, either maximally or sub-maximally activating, and a specified ionic strength. P'_0 is used specifically to indicate the steady tension developed in solutions of pCa 5·49 (i.e. maximally activating solutions) and of a specified ionic strength. P'_0 (0·14) is the steady tension

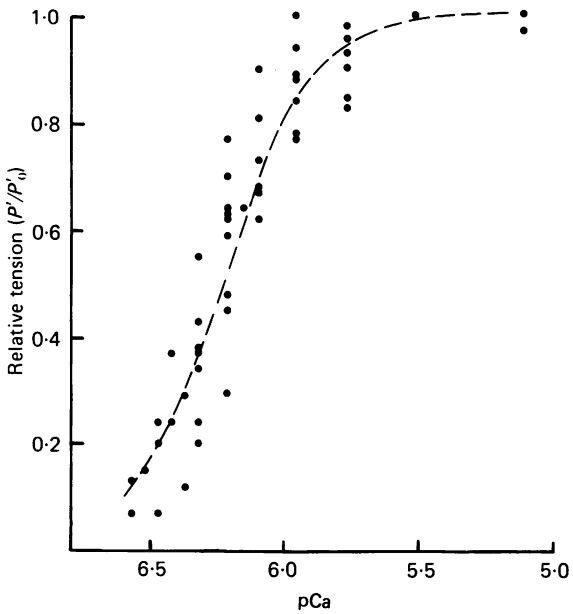

Fig. 2. Plot of relative isometric tension against pCa. Tensions for each segment are expressed relative to the tension developed by the same segment at pCa 5·49. Activations at pCa 5·49 were interspersed among those at sub-maximal pCa values to allow correction for fibre run-down (see Moss, 1979). The average tension at pCa 5·49 was 229 ± 57 kN/m^2. The average sarcomere lengths were between 2·2 and 2·3 μm during steady contraction. The dashed curve was calculated using the following linearized form of the Hill (1910) equation: relative force = $[Ca^{2+}]^n/(K^n + [Ca^{2+}]^n)$. A value for n of 2·6 was calculated as the slope of a straight-line fit to the data by the least-squares method. K is the $[Ca^{2+}]$ for a relative force of 0·5 P'_0 and was estimated to be $5{\cdot}9 \times 10^{-7}$ M. Temperature was 5° or 7 °C and ionic strength was 0·14 M.

developed in solutions of pCa 5·49 and of ionic strength 0·14 M. P is used to indicate the load on a fibre segment during a velocity measurement done using the load stepping technique. In the plotting of force–velocity results, load will be expressed as a relative tension, P/P'.

Relationship between tension development and pCa. Relative isometric tension is plotted against pCa in Fig. 2 for seventeen fibre segments activated in solutions at temperatures of 5 or 7 °C and an ionic strength of 0·14 M. The sarcomere length during steady force development was in the range 2·2–2·3 μm, as determined from

photomicrographs. The relationship between tension and pCa is S-shaped, a finding which has been reported previously by many investigators (for example, Hellam & Podolsky, 1969; Julian, 1971). The tension–pCa relationship was not studied extensively in solutions of ionic strength other than 0·14 M or of temperatures other than 5 or 7 °C. It was determined in several segments that a pCa of 5·49 produced tensions which were maximal at 1 and 10 °C and at the various ionic strength that were

TABLE 1. Effect of ionic strength on tension development at pCa 5·76, studied at 1 and 10 °C. All tensions at each temperature are expressed relative to the tension developed by the same segment at ionic strength 0·18 M. Measurements of tension at the ionic strengths less than 0·18 M were bracketed by tension measurements at 0·18 M, so that relative tensions could be corrected for fibre deterioration (see Moss, 1979). The absolute tensions at 0·18 M ionic strength were 181 ± 37 kN/m^2 ($n = 22$) at 10 °C, and 116 ± 49 kN/m^2 ($n = 10$) at 1 °C.

	Ionic strength		
	0·09 M	0·14 M	0·18 M
10 °C	$1{\cdot}97 \pm 0{\cdot}08$ ($n = 4$)	$1{\cdot}35 \pm 0{\cdot}14$ ($n = 3$)	1·0
1 °C	$1{\cdot}48 \pm 0{\cdot}10$ (n = 3)	1·31	1·0

studied. This was done by comparing the tensions generated by the segments in solutions of pCa 5·49 and approximately pCa 5·0, and these two tensions were similar in magnitude at each temperature and ionic strength.

Relationship between tension development and ionic strength. The influence of ionic strength on isometric tension development was studied in eight fibre segments activated in solutions of pCa 5·76, and the results are shown in Table 1. Even in the relatively small number of fibres sampled, it is apparent that the relative tension was greatly affected by moderate changes in ionic strength. At 10 °C, tension nearly doubled when ionic strength was lowered from 0·18 to 0·09 M. This result is similar to those of Gordon, Godt, Donaldson & Harris (1973) and Thames *et al.* (1974), who used skinned muscle fibres, and Edman & Hwang (1977) and Gordon & Godt (1970) who used living fibres. In the present study altering solution temperature from 10 to 1 °C reduced somewhat the effect of changes in ionic strength on tension development.

Effect of ionic strength on shortening velocity at 7 °C. An early observation of the present study was that fibre segments could rapidly develop areas of marked striation non-uniformity when maximally activated in solutions of ionic strength 0·14 M. At 0·09 M ionic strength, segments frequently pulled apart following even a single maximal activation. Because of these deleterious structural effects, the pCa was adjusted, following an initial maximal activation at 0·14 M, to 6·32, a sub-maximal level, for the comparison of the relative force–velocity properties of segments at ionic strengths of 0·14 and 0·09 M. Segments were selected for use only if they developed tensions of 0·5–0·6 P'_0 (0·14) when activated in solutions of pCa 6·32 and 0·14 M ionic strength. At 0·09 M, activations in solutions of pCa 6·32 resulted in tensions of about 0·9 P'_0 (0·14). Photomicrographs of segments in solutions of pCa 6·32 showed that striation uniformity was maintained at an ionic strength of 0·09 M, even during repeated contractions.

Force–velocity data obtained from four fibre segments using the load stepping

method are shown in Fig. 3*A* for ionic strengths of 0·14 and 0·09 M. Data points from a given fibre segment were included in the plot only if measurements were made at both ionic strengths and if microscopic examination revealed no structural irregularities during activation. V_{max} was extrapolated to be 0·84 m.l./sec at an ionic strength of 0·09 M, and 0·87 m.l./sec at 0·14 M. It is evident that there is virtually no difference in the force–velocity data at these ionic strengths.

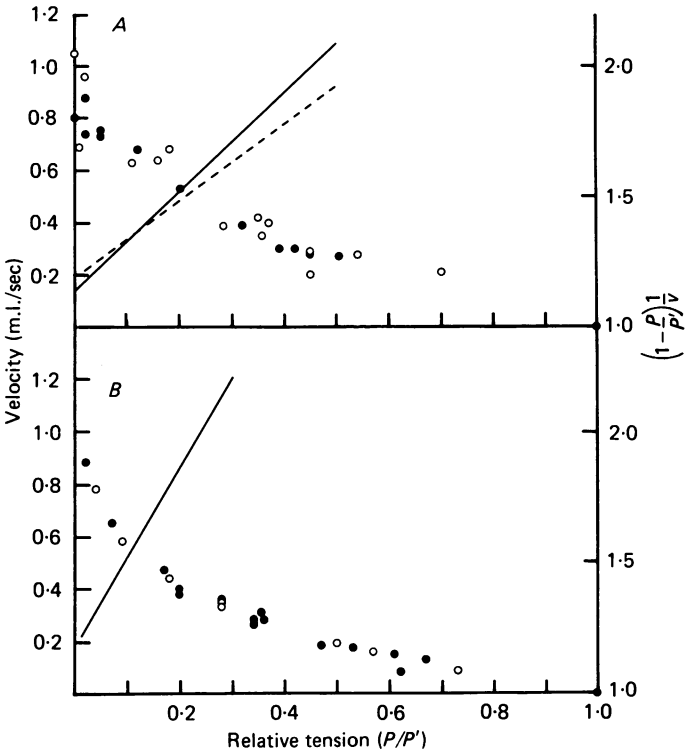

Fig. 3. Plots of velocity *versus* relative force, measured using the load stepping technique. In *A*, velocity measurements were made in four fibre segments at pCa 6·32 in solutions of ionic strength 0·14 M (filled circles) and 0·09 M (open circles). The straight lines are fitted to the data using a linearized form of the Hill (1938) force–velocity equation, in which $(1-P/P')\,1/v$ is plotted against relative tension. The continuous line is fitted to the data at ionic strength 0·09 M and the dashed line to that at 0·14 M. In *B*, velocity measurements were obtained from four additional segments at pCa 6·09 and ionic strengths of 0·14 M (filled circles) and 0·18 M (open circles). Both sets of data are fitted by the same straight line. The segments were pre-soaked in HDTA-containing relaxing solution (see Methods). Velocity is expressed in muscle lengths/sec.; temperature, 7 °C. Segments from October, 1977 to January, 1978.

At an ionic strength of 0·18 M, very small tensions, between 0·05 and 0·20 P'_0 (0·14), were developed in solutions of pCa 6·32. These small forces made difficult the adjustment of the control electronics, and also required that correction be made for the presence of even small amounts of resting tension. A pCa of 6·09 was therefore chosen for the measurements at ionic strengths of 0·14 and 0·18 M. At this pCa the segments developed tensions of 0·8–0·9 P'_0 (0·14) at an ionic strength of 0·14 M and about 0·5 P'_0 (0·14) at 0·18 M.

Force–velocity data from four fibre segments are shown in Fig. 3*B* for ionic strengths of 0·14 and 0·18 M. V_{max} was extrapolated to be 0·85 m.l./sec at both ionic strengths, and there is again little tendency for the force–velocity relationships to differ.

It is interesting to note that at an ionic strength of 0·14 M, the value of a/P_0 was 0·42 at pCa 6·09 and 0·63 at pCa 6·32. This indicates that the degree of curvature of the force–velocity relation is greater at the higher Ca^{2+} concentration. A similar finding was reported by Julian (1971). Changes in ionic strength apparently do not influence the value of a/P_0.

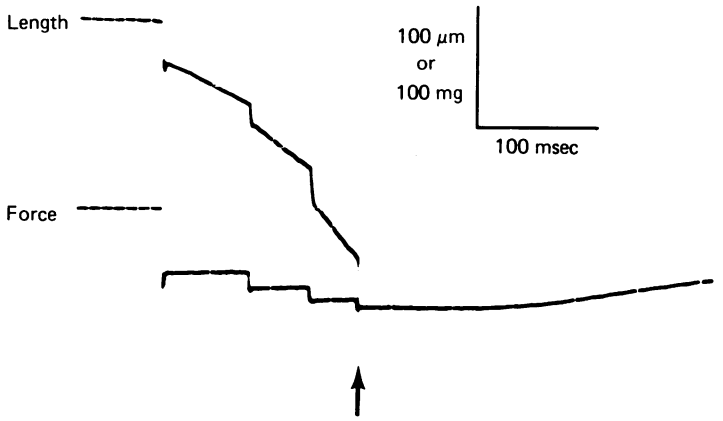

Fig. 4. Length (upper) and force (lower) traces of a force–velocity measurement made using the load stepping technique. Details of the technique are explained in Methods. Force was stepped in succession to 0·33 P'_0, 0·19 P'_0 and then to 0·07 P'_0 and velocities of 0·31, 0·49, and 0·79 m.l./sec respectively, were obtained. Following these velocity measurements, the fibre was quickly slackened (arrow) to obtain a zero-force base line. The minimum length achieved following the step to slacken the segment was just off the oscilloscope screen at this length sensitivity. Force began to redevelop once the imposed slack was taken up. A V_{max} of 1·08 m.l./sec was extrapolated from these data using a linearized version of the Hill (1938) force–velocity equation. Temperature, 5 °C; ionic strength, 0·14 M; pCa 5·49; over-all length, 1·50 mm. Segment from March 5, 1979.

The effect of Ca^{2+} *concentration on maximum shortening velocity.*

The maximum shortening velocities estimated on the basis of the data in Fig. 3 are quite similar at the ionic strengths and two pCa values which were studied. Julian (1971) previously reported that there was an approximate twofold increase in V_{max} when the tension generated by chemically skinned fibres was increased from 0·2–0·35 to 0·9 P'_0 or above by adjusting the free Ca^{2+} concentration. Wise *et al.* (1971) also saw a dependence of V_{max} on Ca^{2+} concentration. However, Podolsky & Teichholz (1970), Thames *et al.* (1974) and Gulati & Podolsky (1978) found no change in V_{max} when the isometric tension was varied from about 0·20 to 1·0 P'_0. The data of Fig. 3 show no change in V_{max} as tension was varied from about 0·5 to 0·9 P'_0. We have therefore re-investigated the influence of Ca^{2+} on shortening velocity.

Force–velocity data were obtained from twenty-two fibre segments at 5 °C using the load stepping technique. An example of the modified version of this technique, in which the tension base line is determined by a final step to slack length, is shown

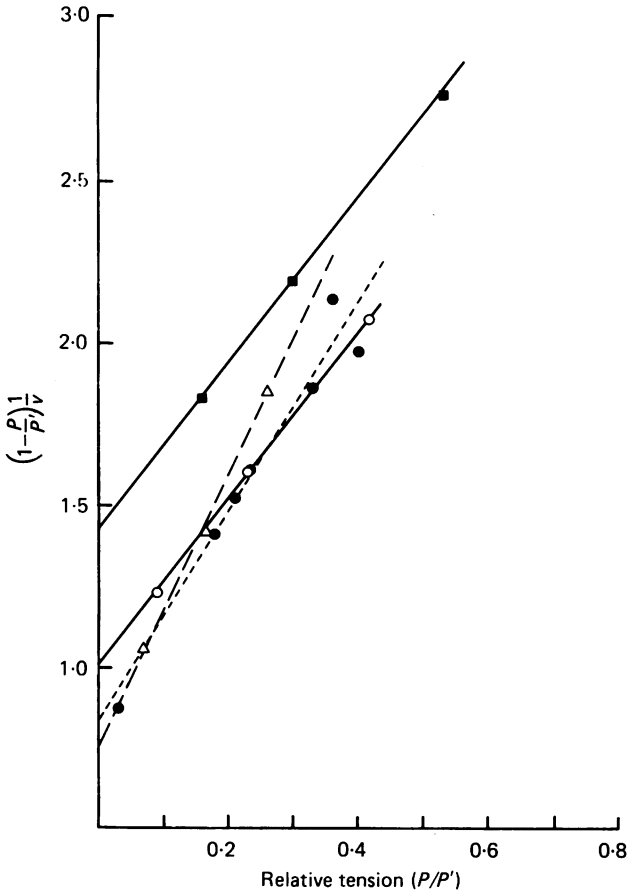

Fig. 5. Plots of linearized force–velocity data, obtained by load stepping, of a segment from March 8, 1979. Data are plotted using the linearized form of the Hill (1938) force–velocity equation described by Katz (1939). The intercepts of the plotted lines with the ordinate are the inverses of the extrapolated V_{max} values for the several sets of data. A summary of these data is as follows:

pCa	Symbol	P'/P'_0	V_{max} m.l./sec
5·49	●---●	1·00	1·18
5·95	△---△	0·70	1·31
6·09	○——○	0·52	0·99
6·16	■——■	0·29	0·70

Temperature was 5 °C; ionic strength was 0·14 M; over-all length was 1·54 mm; tension in the relaxed segment was less than 0·01 P'_0 preceding the velocity measurements and was less than 0·03 P'_0 following a control contraction at pCa 5·49 immediately following the measurements.

in Fig. 4. This modification allowed a high accuracy of determination of P' and three points of the force–velocity characteristic. In general, the loads used were less than 0·5 P'_0 (0·14) in order to avoid possible distorting effects of velocity transients which occur at higher loads (Armstrong *et al.* 1966; Civan & Podolsky, 1966; Podolsky *et al.* 1974). The shortening records that were obtained were usually quite linear, during

activations in solutions of both high (Fig. 4) and low Ca^{2+} concentrations. Linearized force–velocity curves are plotted in Fig. 5 for a fibre segment at four different Ca^{2+} concentrations. The extrapolated V_{max} for this fibre segment at a relative developed tension of 1·0 P'_0 (0·14) was 1·18 m.l./sec. At 0·29 P'_0 (0·14) extrapolated V_{max} was 0·70 m.l./sec, approximately 60% of the value obtained at the higher Ca^{2+} concentration.

Data from all the fibre segments studied are shown in Fig. 6, in which V_{max} is plotted against the relative isometric tension attained prior to the load steps. Relative tension rather than pCa was used as the independent variable due to the variation

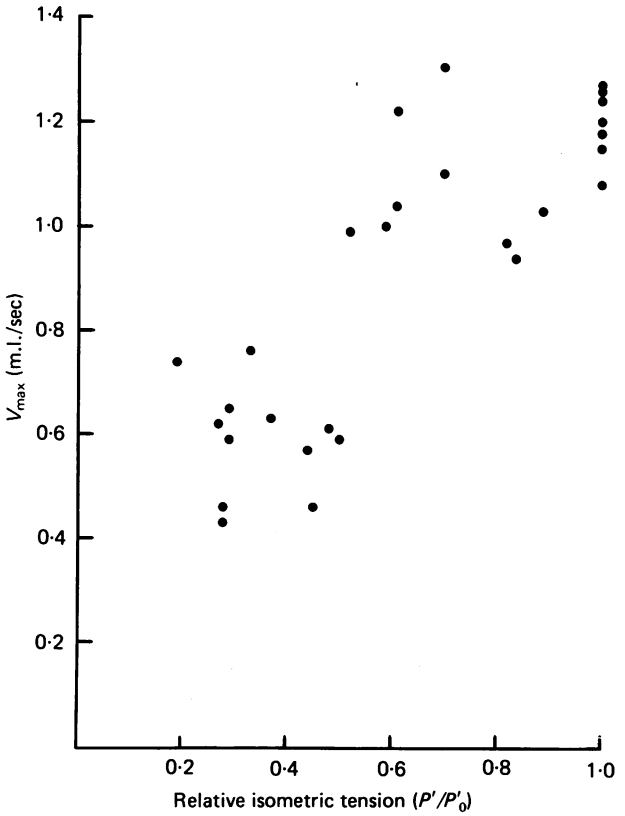

Fig. 6. Plot of V_{max}, obtained by load stepping, *versus* the relative isometric tension just preceding the velocity measurement. V_{max} was obtained by extrapolation of the linearized force–velocity data. Temperature was 5 °C in all cases.

in tension development at intermediate pCa values, as can be seen in the data of Fig. 2. The mean V_{max} at P/P'_0 (0·14) = 1 was 1·21 ± 0·09 (S.D.) m.l./sec (n = 8). The mean V_{max} for isometric tensions below 0·5 P'_0 (0·14) was 0·59 ± 0·10 m.l./sec (n = 12). Thus, V_{max} decreased by about one half when the Ca^{2+} concentration was altered to decrease the isometric tension from its maximal value to values less than about 0·5 P'_0 (0·14).

The data scatter of Fig. 6 lead us to believe that the compression of the ends of the fibre segments could possibly be distorting, to a small degree, the measured velocities of shortening. If it is assumed that a constant amount of series elasticity is introduced by the connectors, then the percentage of segment which is actively

shortening would be less for a short than for a long segment. The presence of such a lumped passive series elasticity would mean that sarcomere shortening velocity is underestimated by over-all segment shortening, and this effect would be greater for shorter segments.

We have therefore replotted, in Fig. 7, the velocity data of Fig. 6 for only those segments in which velocities were measured at both maximum and low relative isometric tensions. V_{max} is expressed as a fraction of V_{max} in the same segment

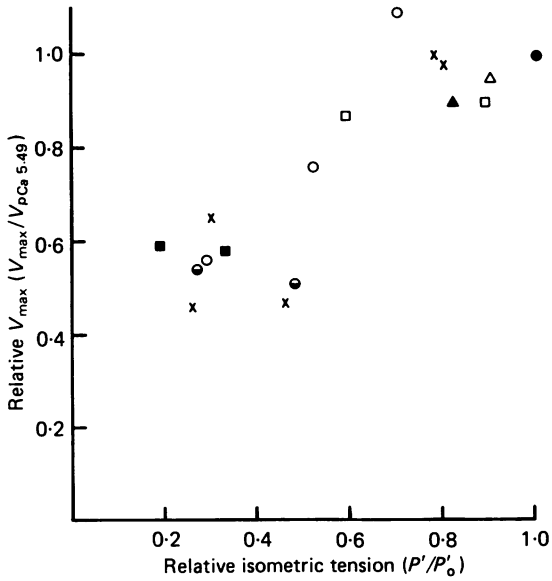

Fig. 7. Plot of relative V_{max} *versus* the relative isometric tension just preceding the velocity measurement. V_{max} is expressed as a fraction of the maximum velocity ($V_{pCa\ 5\cdot49}$) obtained in the same segment at pCa 5·49 (i.e. relative tension equals 1·0). The crosses are data obtained with the slack test; the other symbols represent data obtained from several fibre segments (each having a different symbol) using the load stepping technique. Temperature was 5 °C.

measured at P'_0 (0·14). The data scatter in this plot is somewhat reduced, and V_{max} for tensions less than 0·5 P'_0 (0·14) was $0\cdot55 \pm 0\cdot04$ ($n = 5$) of V_{max} at P'_0 (0·14).

Julian (1971) previously measured V_{max} to be about 2·4 m.l./sec in briefly glycerinated fibres. A major part of the disparity with the V_{max} values reported here is most likely a result of the different methods of attachment used in the two studies. In the present study, segment length probably over-estimated the actual length of the segment which was actively shortening (i.e. underestimated V_{max}) because the compliance introduced by the connectors was located at the ends of the segments just adjacent to the connectors. In Julian's study, segment length measurements probably underestimated the actively shortening length (i.e. over-estimated V_{max}) in that some portions of segments within the fibre loops tied to the end wires were contributing to over-all shortening.

The absolute magnitudes of V_{max} at P'_0 (0·14) shown in Fig. 6 are low relative to the expected V_{max} in living fibres at 5 °C. For example, Julian & Sollins (1973)

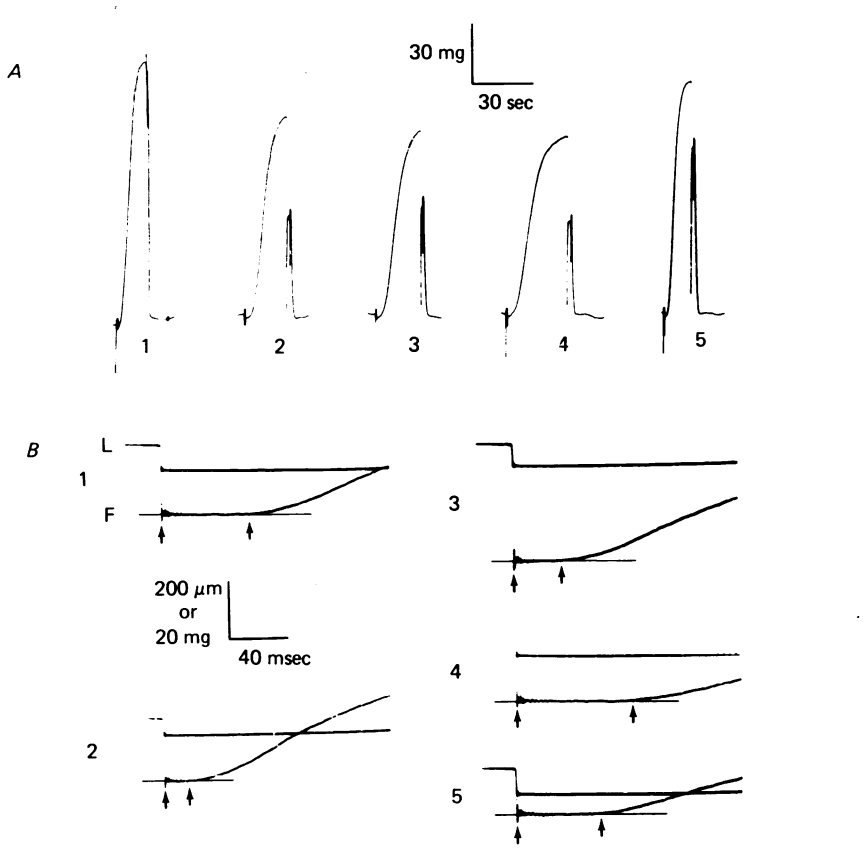

Fig. 8. Examples of the slack test method for V_{max} determination. The force traces for a sereis of velocity measurements are shown on a slow time base in parts *A*1–*A*5. The contractions in parts *A*1 *and A*5 are control contractions at pCa 5·49 and ionic strength 0·14 M and were obtained just before and after the velocity measurements which were done in parts *A*2–*A*4 in solution of pCa 6·21 and ionic strength 0·14 M. During each activation in parts *A*2–*A*4 the segment was slackened; and when force began to redevelop, the segment was transferred to relaxing solution and then re-extended to its original length. Photomicrographs of the active segment were obtained during steady tension development just prior to the length step. In parts B1 — B5, the length (*L*) steps and force (*F*) responses of another segment are shown on a much faster time base in the order in which they were obtained. Each length change required one cycle of activation and relaxation. The time of unloaded shortening was measured as the interval between the attainment of zero tension and the beginning of tension redevelopment, indicated by the pairs of vertical arrows on the force traces. The second arrow of each pair was placed at the point where the tension trace just deviated from a horizontal line drawn through the force trace, as shown. At this force sensitivity, the force signal just prior to the length steps were off the oscilloscope screen and therefore not visible. The following changes of length and times of unloaded shortening were measured: part *B*1, 166 μm and 58·8 msec; *B*2, 109 μm and 15·2 msec; *B*3, 141 μm and 31·9 msec; *B*4 195 μm and 78·4 msec; *B*5, 166 μm and 58·3 msec. Note the agreement in unloaded shortening times between the first and last measurements. A straight line was fitted to these data in the manner shown for another segment in Fig. 9. V_{max} = 0·88 m.l./sec; over-all length was 1·42 mm; average sarcomere length was 2·25 μm just prior to the length step; p_{Ca} 5·76 and ionic strength 0·18 M; 1 °C. A segment from February 5, 1979.

measured a V_{max} of 1·8 m.l./sec at 0 °C in anterior tibial muscle fibres of the frog. Some of the difference between skinned and living fibres may be due to the effect of the series elasticity discussed above. It is also possible that the skinned fibres shorten non-uniformly under load due to an initial slight dispersion in sarcomere length along the segment. The photographs which we have taken prior to and following the load steps suggest that if this is occurring, the effect is small. The sarcomere length measurements that were made generally indicated a greater sarcomere length non-uniformity, as indicated by the standard deviation, following the velocity

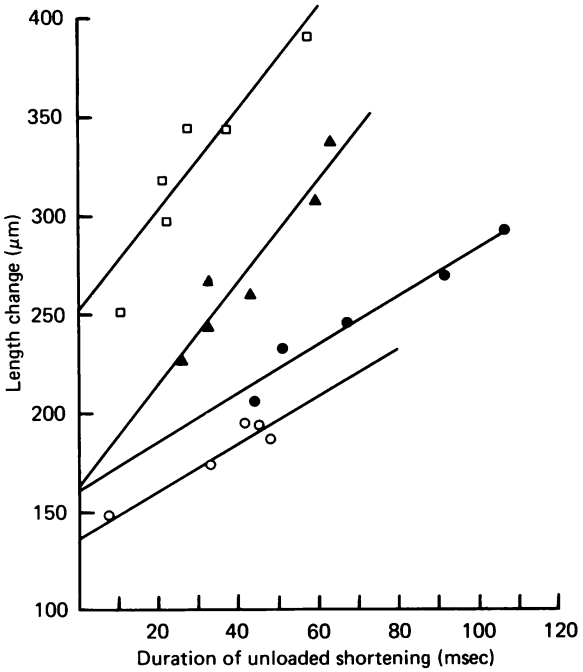

Fig. 9. Plots of length change *versus* duration of unloaded shortening, for one fibre segment from March 16, 1979. Straight lines were fitted to the four sets of data using the least-squares method. The following values for V_{max} were obtained; (□) pCa 5·49, $P'/P'_0 = 1$, $V_{max} = 1·95$ m.l./sec; (▲) pCa 5·95. $P'/P'_0 = 0·78$, $V_{max} = 2·01$ m.l./sec; (●) pCa 6·21, $P'/P'_0 = 0·46$, $V_{max} = 0·95$ m.l./sec; (○), pCa 6·26, $P'/P'_0 = 0·26$, $V_{max} = 0·92$ m.l./sec. Over-all segment length was 1·31 mm; average sarcomere length just prior to the length steps was 2·41 μm; 5 °C; ionic strength was 0·14 M; tension in the relaxed segment was less than 0·01 P'_0.

measurement than was observed prior to the measurement; however, upon scanning the segment, it was not possible to discover a discreet region which had shortened at the expense of the remainder of the segment.

We have therefore made measurements of V_{max} using a method, the slack test, in which the segment is without external load during shortening. Segments were allowed to develop steady isometric tension at a sarcomere length of 2·3–2·4 μm in activating solutions of various pCa values and 5 °C. The fibres were rapidly stepped to various shorter lengths below the slack length. Examples of the records obtained are shown for two segments in Fig. 8. The segments underwent unloaded shortening while taking

up the imposed slack, and this was followed by tension redevelopment once the segment became taut. The extent of fibre release was then plotted against the duration of unloaded shortening, as shown in Fig. 9, and V_{max} was calculated as the slope of a straight line fit to the data by the least squares method.

The data of Fig. 9, for one fibre segment, indicate that by this method, also, the ratio of the maximum shortening velocities measured at high *versus* low Ca^{2+} concentrations is nearly 2. However, in this instance, V_{max} at pCa 5·49 averaged $1·86 \pm 0·11$ m.l./sec ($n = 5$). this value is nearly $\frac{2}{3}$ the value of about 3·0 m.l./sec obtained by D. L. Morgan & F. J. Julian (personal communication) using a similar technique in living fibres at 5 °C. In order to compare directly the data obtained in the present study using the load stepping method and the slack test, the slack test data are plotted in Fig. 7 as relative velocity *vs.* relative isometric tension. It can be seen that despite the disparity, by a factor of about 1·5, in absolute V_{max} at pCa 5·49, there is good agreement in the results of the two methods as to the relative magnitude of the effect of Ca^{2+} concentration on V_{max}.

The slack test had two unexpected advantages over load stepping for the measurement of V_{max}, resulting in an increase in the number of activations each segment could undergo without significant deterioration. Fibre segments usually maintained a lower resting tension in relaxing solution for several more cycles of activation and relaxation than did the segments subjected to load stepping. There was also a tendency during repeated activations for the striation patterns to maintain better uniformity.

Relationship between V_{max} and Ca^{2+} concentration at 10 °C. The slack test was used to measure V_{max} as a function of pCa in six fibre segments at 10 °C. At this temperature, the fibre segments did not tolerate repeated contractions at pCa 5·49; therefore, V_{max} was measured at pCa 5·76 or above. The data obtained in solutions of ionic strength 0·14 M are plotted as filled circles in Fig. 10. Once again, V_{max} was found to decrease as the free Ca^{2+} concentration was reduced.

Effect of ionic strength on the relationship between V_{max} and Ca^{2+} concentration. Slack test measurements of V_{max} at ionic strengths of 0·18 and 0·09 M were also obtained at 10 °C in fourteen additional segments. These results, plotted in Fig. 10, indicate that the relationship between V_{max} and pCa is not significantly influenced by moderate changes in ionic strength at 10 °C. The average V_{max} for all ionic strengths at pCa 5·76 was $2·09 \pm 0·12$ m.l./sec ($n = 6$). The average V_{max} at pCa 6·42 was $0·83 \pm 0·16$ m.l./sec ($n = 6$).

Effect of ionic strength on V_{max} at 1 °C. V_{max} was determined by the slack test in ten fibre segments in solutions of varied ionic strength at 1 °C. The results of these measurements, which were done only at pCa 5·76, are plotted as the open symbols in Fig. 10. At this temperature, also, V_{max} is unaffected by changes in ionic strength in the range 0·09–0·18 M. The average V_{max} for all ionic strengths was $0·91 \pm 0·08$ m.l./sec ($n = 12$). Based on this value and that at 10 °C, V_{max} at pCa 5·76 has a Q_{10} of about 2·5, close to, for example, the value of 2·6 obtained by Julian (1971) for V_{max} in living frog muscle fibres.

Effect of ionic strength on the tension in the relaxed segment. Thames *et al.* (1974) found that V_{max} measured in mechanically skinned fibres decreased as ionic strength was reduced from the control value, and that following relaxation, the resting tension was

significantly greater than the amount present just preceding the contraction. At low ionic strengths, V_{max} was found to depend on the Ca^{2+} concentration. The authors suggested on the basis of these results that at low ionic strength abnormal cross-bridges form which act as an internal load to retard shortening during activation. On the basis of the elevated resting tension, these cross-bridges were thought to persist following relaxation.

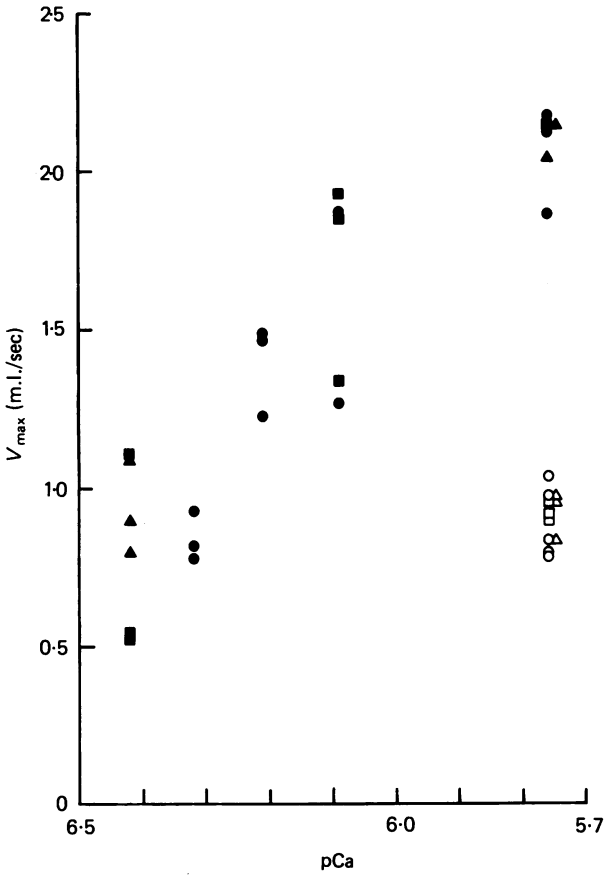

Fig. 10. Plot of V_{max} against pCa, showing the effect of temperature and ionic strength. Measurements were done using the slack test. The triangles are data obtained at ionic strength 0·09 M; the squares at 0·14 M; and the circles at 0·18 M. The filled symbols are data obtained at 10 °C; the open symbols are data at 1 °C. Some of the data points at pCa 5·76 are displaced laterally to avoid complete overlap by other symbols.

In the present study, following each velocity measurement the segments were relaxed in solutions of similar ionic strength. The tensions in the relaxed segments were measured by slackening the segments and recording the change in base-line tension. Segments which were activated in solutions of ionic strength 0·14 or 0·18 M usually showed no change in the relaxed tension from that measured just following mounting, even after several cycles of activation. Moreover, segments which were activated in solutions having these ionic strengths showed little change in developed tension during successive activations.

Activations which took place in solutions of ionic strength 0·09 M were commonly

followed by a small increase in the resting tension once the segment was relaxed. The time course of change in the resting tension following activation in the low ionic strength solution is shown for two segments in Fig. 11. The increase in resting tension following the first cycle of activation and relaxation in solutions of ionic strength 0·09 M was generally quite small, i.e. less than about 2 % of the total tension developed by the same segment in activating solution of the same ionic strength. Cycles of

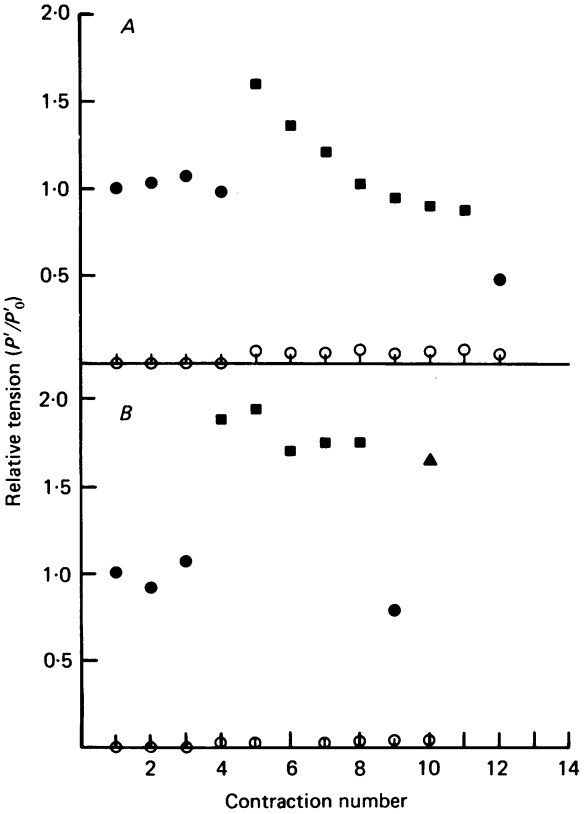

Fig. 11. Plots of active tension and resting tension *versus* contraction number for two different fibre segments. Segments from January 10 (*A*) and January 13 (*B*), 1978. The first several contractions were done in solutions of ionic strength 0·14 M and pCa 6·32 (filled circles), followed by contractions in solutions of 0·09 M and pCa 6·32 (filled squares) and then a return to the original activating solution. In *B* a final control contraction was done in solution of pCa 5·49 and ionic strength 0·14 M (filled triangles). Resting tensions (open circles) were measured just following each activation in solutions of similar ionic strength. The active and resting tensions are in each case expressed as a fraction of the steady tension developed during the first contraction.

activation and relaxation at this ionic strength did not result in any consistent changes in the resting tension. At the same time, the amount of active tension developed by the segment might remain about constant or even decline substantially, as shown in the two parts of Fig. 11. There was no consistent effect of the presence of small amounts of increased resting tension on V_{max}. The resting tension of the segments declined a variable amount when the segments were returned to relaxing solution of ionic strength 0·14 M.

DISCUSSION

Influence of ionic strength on tension and shortening velocity. The results that have been presented indicate that in briefly glycerinated fibre segments, the actively developed tension is inversely related to the ionic strength of the bathing solution in the range 0·09–0·18 M. This result is in agreement with similar previous measurements which have been done using mechanically skinned (Gordon *et al.* 1973; Thames *et al.* 1974; Gulati & Podolsky, 1978) and living (Gordon & Godt, 1970; Lännergren & Noth, 1973; Edman & Hwang, 1977) skeletal muscle fibres.

Further findings of the present study show that V_{max} and the force–velocity relations obtained at 1, 7 and 10 °C using briefly glycerinated segments are virtually unaffected by changes in ionic strength from 0·09 to 0·14 M, and from 0·14 to 0·18 M. Other investigators have obtained similar results using skinned skeletal muscle fibres at 1 °C (Gulati & Podolsky, 1978) and myofibrils from rat heart at 27 °C and an ionic strength range similar to the highest studied here (Honig & Takauji, 1976). The present results in the ionic strength range 0·14–0·18 M are also similar to the results at 7 °C reported by Thames *et al.* (1974) using mechanically skinned fibre segments. However, at an ionic strength similar to the lowest studied here, Thames *et al.* observed a substantial decrease in shortening velocity. An important difference in the two studies is the use by Thames *et al.* of maximally activating solutions in which to make velocity measurements at low ionic strength. Experiments which were preliminary to the present work indicated that at high Ca^{2+} concentrations and low ionic strength, skinned fibre segments frequently developed regions of striation non-uniformity and areas in which the striations were observed to disappear. These parts of the segments, if they are relatively stiff, may be able to bear the forces generated by the portions of the segment having uniform striations. As a result the velocity of shortening measured at the ends in segment lengths per second would underestimate the sarcomere shortening velocity.

Some of the briefly glycerinated segments exhibited a significant, though small, tension while in relaxing solution following a cycle of activation and relaxation in solutions of ionic strength 0·09 M. The amount of this tension was variable from preparation to preparation, and we do not know whether this was the residual tension reported by Thames *et al.* (1974); however the presence of small amounts of resting tension did not result in force–velocity relations which were different from those obtained from segments having negligible amounts of tension when relaxed. The source of the increased base-line tension at low ionic strength was not determined. In this regard, Goodno, Wall & Perry (1978) found that the ATPase activity of relaxed myofibrils increased as the ionic strength was reduced below physiological levels. This suggests that the increase in resting tension at an ionic strength of 0·09 M may result from cyclic interactions of cross-bridges with actin sites.

Measurements of V_{max} in living, tetanically stimulated muscle fibres indicate that V_{max} is inversely related to bathing solution tonicity in the range 0·62–1·44 times the normal tonicity (Edman & Hwang, 1977). It is difficult to know just what the effects of changes in the tonicity of the extracellular medium may have on the ionic strength of the intracellular fluid of living fibres. For this reason, comparisons of the force–velocity data of living fibres to that of skinned fibres, in which ionic strength and Ca^{2+} concentration are directly controlled, are difficult to justify. There exists the possibility that the variation in the V_{max} of living fibres as solution tonicity is altered is a result of a secondary effect of ionic strength on excitation–contraction coupling, and is not an effect of ionic strength *per se* on the interaction of actin and myosin. For example, changes in intracellular ionic strength may alter the amount of Ca^{2+} which is released from the sarcoplasmic reticulum during stimulation. Experiments on mechanically skinned fibre segments (Endo & Thorens, 1975; R. L. Moss, unpublished) and on isolated sarcoplasmic reticulum vesicles (Kasai &

Miyamoto, 1976) indicate that skeletal muscle sarcoplasmic reticulum is stimulated to release Ca^{2+} when the solution ionic strength is lowered. Also, there is evidence that solutions of high ionic strength may partially inhibit the release of Ca^{2+} from the sarcoplasmic reticulum of living frog muscle fibres during tetanic stimulation (Shlevin & Taylor, 1979) and of barnacle muscle (Ashley & Ridgeway, 1970). Thus, it is possible that in living frog fibres, the alteration in V_{max} which is observed when the ionic strength is changed could be the result of an effect of an altered myoplasmic Ca^{2+} level on the mechanical V_{max} of the fibres. If this were the case, the variation of P_0 with ionic strength in living fibres would be due to changes in both the ionic strength *per se*, and also the amount of Ca^{2+} bound to regulatory sites.

In the present study, the tension generated by the skinned segments at pCa 5·76 was markedly increased when the ionic strength was lowered. Such an increase might be brought about by either an increased number of cross-bridges attached to thin filament sites or an increase in the tension-generating capacity of each attached cross-bridge, with no change in the total number attached. The available biochemical data relating the actomyosin (Burke, Reisler, Himmelfarb & Harrington, 1974) and acto-heavy meromyosin (Rizzino, Barouch, Eisenberg & Moos, 1970) ATPase activities to actin concentration at various ionic strengths are conflicting and do not allow a choice between alterations in the numbers of attached cross-bridges or in the tension generated by each cross-bridge.

Effect of Ca^{2+} *concentration on shortening velocity.* The results of this study show that the concentration of activating Ca^{2+} has a substantial influence on V_{max}: at 5 °C, in Fig. 6, confirming and extending the results of Julian (1971); and at 10 °C and ionic strengths of 0·09, 0·14, and 0·18 M in Fig. 10. Similar findings have been reported for rat heart myofibrils at 37 and 27 °C and ionic strengths of about 0·14 and 0·18 M (Honig & Takauji, 1976) and for skinned rat heart cells at room temperature and an ionic strength similar to the highest level studied here (de Clerck *et al.* 1977).

The relationship between V_{max} and the relative isometric tensions measured at submaximal Ca^{2+} concentrations is perhaps best seen in the plot of Fig. 7. For relative isometric tensions (P'/P'_0) less than about 0·5, relative V_{max} was about 0·55; and between 0·5 and 0·7 P'_0, V_{max} was measured to have intermediate values, although data in this range are sparse.

These results are not in agreement with those of Podolsky & Teichholz (1970), Thames, *et al.* (1974) and Gulati & Podolsky (1978) who used mechanically skinned fibres. The experimental conditions of those studies and ours are otherwise similar except that in the present study the segments were observed and photographed through a microscope. It is possible that in the studies of Podolsky and his colleagues, undetected irregularities along the segments somehow masked the very distinct effect of Ca^{2+} on V_{max} reported here. Also, the force–velocity data obtained in those earlier studies were generally measured at relative loads of 0·2–0·8 P'. If non-uniformities are present in the segments, these are no doubt accentuated at such low shortening velocities, thereby distorting the velocity measurements (see Julian & Morgan, 1979 for a discussion of the effects of sarcomere length non-uniformity on over-all fibre shortening). The V_{max} extrapolations of the present study were obtained from velocity measurements at relative loads less than 0·5 P', to minimize the problems of non-uniformity. The results were verified using an independent technique, the slack test, for velocity measurement. This technique should reduce the possible effects of non-uniformity on shortening since all sarcomeres are unloaded, and this would

minimize the series interaction of sarcomeres, resulting in a more accurate measure of V_{max}. The V_{max} values measured using the slack test were about 1·5 times greater than those determined using load stepping. This difference in V_{max} by the two methods is not a characteristic only of the skinned fibre preparation. Edman (1979) has reported that in living single fibres V_{max} obtained by the slack test is 5–7 % greater than that by load stepping; and in a similar preparation, D. L. Morgan and F. J. Julian (unpublished results) found an approximately $\frac{1}{3}$ greater V_{max} by the slack test method. It is possible, for example, that sarcomere length non-uniformity may distort velocity measurements obtained under load, or that the force–velocity relation is steeper near zero load than is predicted by a simple hyperbola fitted to velocity data at higher loads (Hill, 1970).

Our finding that V_{max} remained approximately constant at values of P' less than about 0·5 P'_0 argues strongly against the presence of a significant internal load acting to retard shortening. If such a load were present, as suggested by Thames *et al.* (1974), V_{max} would be expected to decrease as the relative isometric tension was lowered by increasing the solution pCa. Further results of the present study indicate that V_{max} is not influenced by the presence of small amounts of resting tension at low ionic strengths. We conclude, therefore, that the effect of Ca^{2+} concentration (expressed as relative isometric tension) on V_{max} shown in Figs. 6 and 7 does not result from an increased internal load brought about by alterations in the solution ionic strength or the Ca^{2+} concentration.

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