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THE INFLUENCE OF FREE CALCIUM ON THE MAXIMUM SPEED OF SHORTENING IN SKINNED FROG MUSCLE FIBRES

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SUMMARY

1. The influence of $[Ca^{2+}]$ on the maximum velocity of shortening (V_{max}) was examined in mechanically skinned *Rana pipiens* and *Rana temporaria* fibres using improved force clamps and the slack test techniques. All measurements were made at 7·5 °C.

2. At low relative loads ($P/P_0 < 0·1$), maximally activated *R. pipiens* fibres shortened more rapidly than did submaximally activated fibres. At higher relative loads, however, little difference in the speed of shortening was observed.

3. V_{max} (determined by the slack test) of *R. pipiens* fibres increased as the level of activation increased. Over sarcomere lengths 1·8–2·1 μm it was 2·28 muscle lengths/s (m.l./s) (S.E. of mean ±0·25, $n = 5$) at 20–35 % activation, 2·89 m.l./s (±0·22, $n = 7$) at 40–60 % activation, and 4·18 m.l./s (±0·25, $n = 6$) at 100 % activation. At longer sarcomere lengths (2·2–2·6 μm), higher V_{max} values were observed at all levels of activation, but the influence of Ca^{2+} on V_{max} persisted. V_{max} was 3·54 m.l./s (±0·41, $n = 4$) at 20–30 % activation and 5·15 m.l./s (±0·22, $n = 5$) at 100 % activation.

4. In *R. temporaria* fibres, V_{max} (determined by force clamps over sarcomere lengths 1·8–2·1 μm) also increased as the level of activation increased, from 3·47 m.l./s (±0·06, $n = 6$) at 13–29 % activation to 5·62 m.l./s (±0·17, $n = 6$) at 100 % activation.

5. V_{max} was also determined (using the slack test) in mechanically and chemically skinned rabbit soleus fibres. V_{max} at 15 °C (1·05 m.l./s, ±0·11, $n = 5$) at full activation decreased by more than 3-fold as the level of activation was reduced to 10 %.

6. We conclude that the level of activation influences the V_{max} of skinned skeletal muscle fibres. This has now been demonstrated in three different preparations and by a variety of techniques. This effect is most pronounced at low relative loads, and might not be observed if there are experimental limitations which prevent making velocity measurements at low relative loads.

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INTRODUCTION

An important question concerning contraction kinetics of vertebrate skeletal muscle is whether or not the maximum velocity of shortening (V_{max}) is dependent on [Ca^{2+}]. This question has received considerable attention, but the conclusions of various studies have been conflicting: that V_{max} is dependent on [Ca^{2+}] (Julian, 1971; Wise, Rondinone & Briggs, 1971; Julian & Moss, 1981) and that V_{max} is independent of [Ca^{2+}] (Podolsky & Teichholz, 1970; Thames, Teichholz & Podolsky, 1974; Gulati & Podolsky, 1978; Brenner, 1980).

In a recent review, Podolin & Ford (1983) have suggested two possibilities to explain this difference in results. First, there may have been different levels of phosphorylation in the *chemically* skinned fibres of Julian and colleagues (Julian, 1971; Julian & Moss, 1981) compared to that in the *mechanically* skinned fibres of Podolsky and colleagues (Podolsky & Teichholz, 1970; Thames *et al.* 1974; Gulati & Podolsky, 1978). Secondly, a greater 'shortening deactivation' may have been induced by the 'triple release technique' used by Julian (1971) than by the 'single release technique' used by Podolsky and colleagues (Podolsky & Teichholz, 1970; Thames *et al.* 1974; Gulati & Podolsky, 1978).

In this study we have re-examined the effect of [Ca^{2+}] on V_{max} using *mechanically* skinned fibres and using only 'single releases'. We also used improved techniques that have recently been developed to determine V_{max} in skinned fibres (Julian, Rome, Stephenson & Striz, 1986) in conjunction with improved procedures for activating skinned muscle fibres (Moisescu, 1976; Moisescu & Thieleczek, 1978). The activating procedure used minimizes fibre deterioration and reduces development of residual force (Thames *et al.* 1974; Gulati & Podolsky, 1978). Our results obtained on twitch fibres of *Rana pipiens* and *Rana temporaria* clearly indicate that V_{max} is dependent on [Ca^{2+}]. This was reflected by the dependence of V_{max} on the level of activation.

Parts of this work have been presented at meetings of the (U.S.A.) Biophysical Society (Stephenson & Julian, 1982; Rome, Striz, Stephenson & Julian, 1985).

METHODS

Twitch muscle fibres isolated from the iliofibularis muscle of *Rana pipiens* and *Rana temporaria* were mechanically skinned under paraffin oil according to the procedure described by Moisescu & Thieleczek (1978). Slow-twitch rabbit soleus fibres were either chemically skinned in solutions containing 50% (v/v) glycerol according to the method of Julian & Moss (1981) or were mechanically skinned as described above for the amphibian fibres. Segments of fibres (1·5–5 mm in length) were attached to a servomotor and force transducer as described by Julian *et al.* (1986).

Experimental apparatus

The apparatus was the same as that described by Julian *et al.* (1986). The sensitivity of the Cambridge Technology force transducer (natural frequency = 2 kHz) was set at either 2, 1 or 0·2 V/mN depending on the force generated by the fibre. In some slack test experiments on *R. pipiens* and the rabbit soleus fibres, a force transducer consisting of a semiconductor strain gauge (AE 801, Mikroelektronik) and a short titanium wire (length 2–4 mm, diameter 0·15 mm) attached to the end of the silicon beam was used, with a natural frequency of 2–5 kHz and sensitivity of 0·1 V/mN. Passive sarcomere length was measured as described previously (Julian *et al.* 1986).

Solutions

The compositions of the bathing solutions used for this study are shown in Table 1. The solutions were prepared and analysed as described previously (Stephenson & Williams, 1981). In some

instances caffeine was added to all three solutions (A, B and H) (10 and 20 mM for *R. temporaria* fibres and 10 mM for *R. pipiens* fibres) in order to reduce Ca^{2+} movement associated with sarcoplasmic reticulum and thus to speed up the process of activation (Moisescu & Thieleczek, 1978). pCa was calculated using an apparent affinity for Ca^{2+} binding to EGTA of $10^{6.69}$ M^{-1} (Stephenson & Williams, 1981).

TABLE 1. Solutions used for activating skinned muscle fibres

Solution	Total ATP (mM)	Total Mg (mM)	Mg^{2+} (mM)	CP (mM)	CK* (u./ml)	HDTA (mM)
A	8	8·0	1	10	15	—
B	8	9·2	1	10	15	—
H	8	8·4	1	10	15	49·85

Solution	EGTA (mM)	Ca EGTA (mM)	TES (mM)	Cl^- (mM)	Na^+ (mM)	K^+ (mM)
A	—	50	70	16	36	123
B	50	—	70	18·5	36	124
H	0·15	—	70	17	36	124

pH was carefully adjusted to 7·10±0·01 at 7·5 °C in all solutions used with frog fibres. Caffeine concentrations of 10 mM were used in *R. pipiens* experiments, and 0, 10, and 20 mM in *R. temporaria* experiments. The solutions used with slow-twitch rabbit fibres were adjusted to pH 7·10±0·01 at 15 °C and differed slightly with respect to the total Mg (8·05 mM for A, 9·95 mM for B and 8·50 mM for H), Cl^- (16·1 mM for A, 20·0 mM for B and 17·0 mM for H) and total K (128 mM for A, 129 mM for B and 128 mM for H) such as to maintain $[Mg^{2+}]$ at 1 mM at this temperature. Caffeine concentration was 10 mM.

HDTA, hexamethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate; EGTA, ethyleneglycol-bis(β-amino-ethylether)-*N*,*N*′-tetraacetate; TES, 2(2-hydroxy-1,1-bis(hydroxymethyl)ethyl) aminoethane sulphonic acid; CP, creatine phosphate; CK, creatine phosphokinase.

A mixed with B formed activating solutions. B is the relaxing solution. H is the pre-activating solution. pCa = 6·69 + log (EGTA excess/Ca EGTA).

* Activity taken from manufacturer's specifications (Sigma).

Force clamps

Problems associated with performing good force-clamp measurements and extrapolating to zero load have been described in considerable detail (Julian *et al.* 1986). The problems are of greater concern when trying to determine V_{max} in submaximally activated fibres. Since the force–velocity curves are non-hyperbolic, it is necessary to include the lowest relative loads possible to provide an accurate estimate of V_{max} (Julian *et al.* 1986). To perform load clamps at low relative forces, the measurements must be free of residual tension and resting tension, and an accurate zero base line must be determined. Residual tension was eliminated in the *R. pipiens* fibres and reduced in the *R. temporaria* fibres by using the rapid activation method of Moisescu & Thieleczek (1978). Resting tension was made negligible by working at relatively short sarcomere lengths. Finally, an accurate zero base line was measured by giving the fibre a rapid length step (inducing slack) at the end of shortening (*R. pipiens* and *R. temporaria*) or by giving the fibre a rapid length step and restretch prior to significant force generation (*R. temporaria* fibres) in the activation solution. Velocities were generally read over the 5–10 ms time period following the initiation of shortening since they tend to slow with time (Julian *et al.* 1986).

In the *R. temporaria* fibres, when sufficient points could be obtained, a Hill curve was fitted to the untransformed force–velocity data, thus avoiding major inaccuracies associated with using the linearized Hill curve (Julian *et al.* 1986).

Slack test

Problems associated with performing accurate slack tests have also been described recently in detail (Julian *et al.* 1986). As with the force clamps, these problems became of greater concern when trying to determine the V_{max} of submaximally activated fibres. We were especially concerned that

our experimental design might lead to a systematic error that would affect our determination of a possible influence of $[Ca^{2+}]$ on V_{max}.

There are three sarcomere-length-dependent parameters that could affect determination of V_{max}: (1) parallel elastic resting tension, (2) resistance to shortening at short sarcomere lengths (Gordon, Huxley & Julian, 1966; Edman, 1979) and (3) the tension-generating capacity of the fibre. In a perfect preparation there would be no resting tension, no resistance to shortening, and tension generation capacity would be constant over the distance the muscle shortened; however, there was no range of sarcomere lengths where the frog fibre preparation behaved in this manner.

In order to avoid systematic errors influencing our conclusions, we have done measurements both at short (1·8–2·1 μm) and longer (2·2–2·6 μm) sarcomere lengths and we performed the slack tests both starting at one sarcomere length and releasing to various sarcomere lengths and starting from various sarcomere lengths and releasing to a fixed length. In many of the contractions, multiple photographic exposures of the fibre were taken to determine the time to take up slack (t_s) for a particular release and to determine what characteristics of the force record corresponded to t_s (Julian *et al.* 1986).

We have also performed slack tests with skinned slow-twitch fibres from the rabbit soleus muscle. These fibres approximate an ideal preparation for the slack test more closely than the frog fibres, because they exhibit negligible resting tension and resistance to shortening over the sarcomere length range 2·3–2·65 μm.

RESULTS

R. pipiens fibres

Force clamps. Fig. 1 shows force clamps from a fibre at a pCa of 6·3, followed by force clamps at a pCa of 4·4. We found that at relative loads of greater than 0·1 P_0 there was little difference in the velocity of shortening. At lighter loads, however, this difference became quite marked. Thus in all fibres tested we observed an increase in shortening velocity at light loads as $[Ca^{2+}]$ varied from low to high. In experiments in which the fibre was first activated in high $[Ca^{2+}]$, however, we often observed spuriously high speeds at low loads on returning to low $[Ca^{2+}]$. Since the lowest absolute load that we could reliably clamp was about 1 mg (10 μN), it was not possible to clamp very low relative loads at low levels of activation. Julian *et al.* (1986) have demonstrated that force–velocity data deviate from the hyperbolic Hill equation at low relative loads and that inclusion of these light loads is essential to provide a reasonable estimate of V_{max}. We therefore used the slack test which Julian *et al.* (1986) have shown gives an accurate measure of V_{max} when properly calibrated.

Slack test. Slack tests were performed on *R. pipiens* fibres under the following conditions: at short sarcomere lengths, at long sarcomere lengths, and by using the two types of slack test described in the Methods. In all cases, we observed a significant influence of $[Ca^{2+}]$ on V_{max}.

Fig. 2 shows results from a slack test done at pCa 6·05, followed by one at pCa 4·4. In this experiment the fibre was released to a fixed length from different starting lengths. When the fibre was exposed to low $[Ca^{2+}]$, it took a longer time to take up the additional slack imposed between the short and long steps than in the maximally activated case (Fig. 2*C*). Thus, when fully activated, the fibre shortened considerably faster than when partially activated.

As can be seen, this method of doing the slack test (i.e. releasing the fibre to the same final length) results in similar levels of force redevelopment, but the initial force development is larger at the longer sarcomere length (i.e. 15% between releases No.

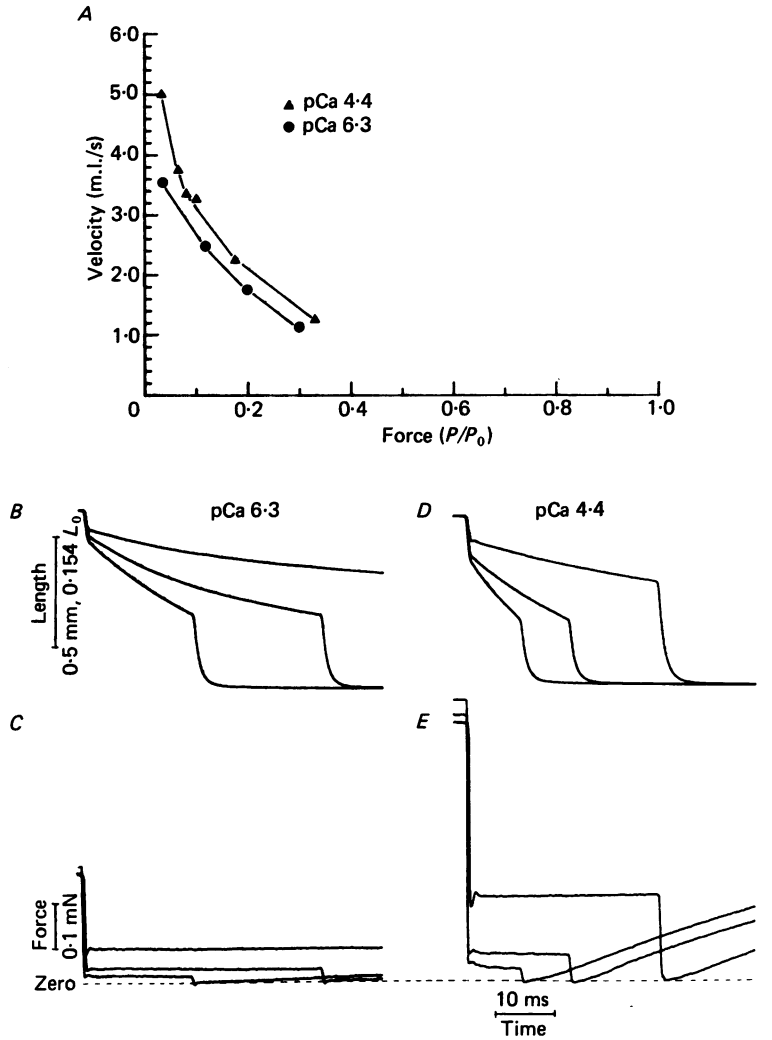

Fig. 1. The influence of $[Ca^{2+}]$ on the force–velocity relationship of a *R. pipiens* fibre. *A* shows the force–velocity data for a mechanically skinned fibre first activated at a pCa of 6·3 and later activated at a pCa of 4·4. Velocity is expressed in muscle lengths/s (m.l./s) and the force is expressed as a proportion of maximum isometric tension at the pCa under consideration. Note that there was little difference in the speed of shortening between pCa 4·4 and pCa 6·3 until the load was below 0·1 P_0. *B* and *C* show original length and force records respectively for the force clamps at a pCa of 6·3, and *D* and *E* show original length and force records for the force clamps at pCa 4·4. The initial sarcomere length for the force clamps was 2·05 μm. Note that at the end of the force clamps, the fibre was given a rapid length step to impose slack into fibre, so that an accurate zero-force level could be ascertained. Fibre 24/4/84. Fibre length was 3·25 mm at a sarcomere length of 2·1 μm.

2 and 1 and 27 % between releases No. 4 and 3, Fig. 2). This would mean that the series elastic element of the fibre would be more stretched for long steps than for short ones. The slack test, however, is based on the assumption that the length of the series elastic component remains constant. This would lead to slightly over-estimating V_{max}. Provided that the length of the series elastic element for short steps is well

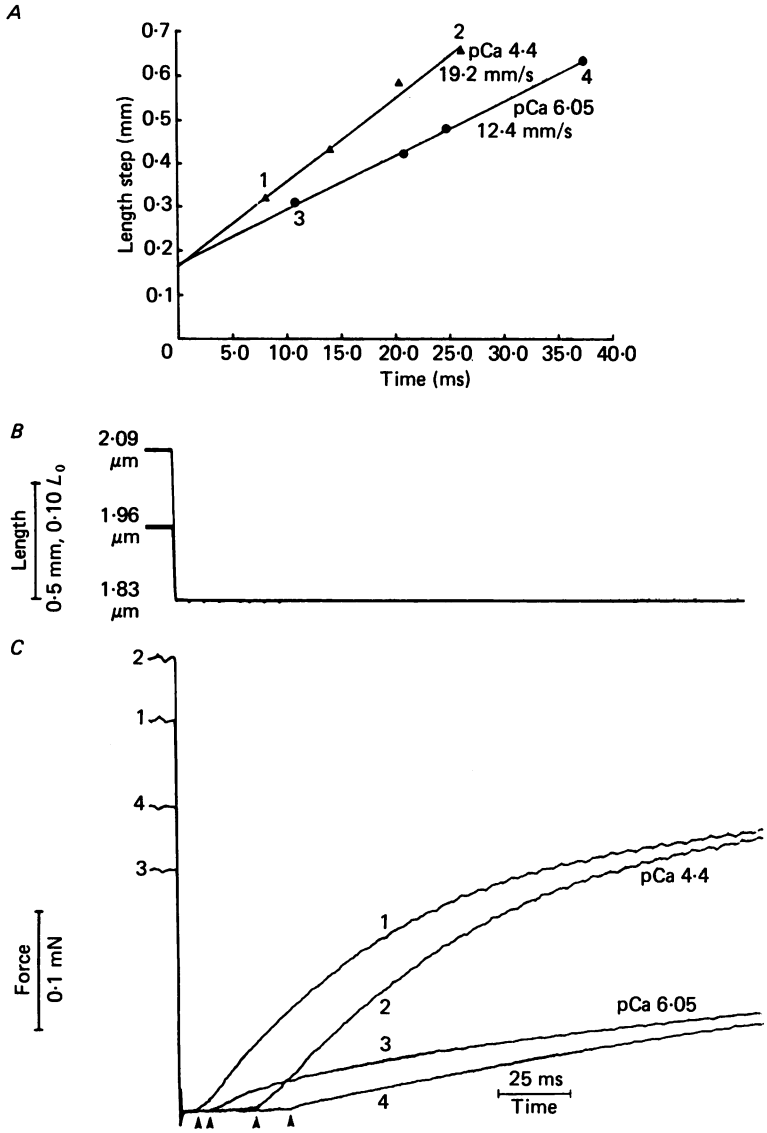

Fig. 2. The influence of $[Ca^{2+}]$ on V_{max} of a skinned *R. pipiens* fibre. Slack tests were performed on a fibre at pCa 6·05 and then at pCa 4·4. *B* and *C* show the original length and force records from two different releases at the two Ca^{2+} concentrations. The fibre was released to the same sarcomere length from different starting sarcomere lengths so that the recovery of tension was similar. Initial sarcomere length for releases 2 and 4 was approximately 2·09 μm, whereas for releases 1 and 3 it was approximately 1·96 μm. Photographs taken during the slack tests were very helpful in reading the time to take up the slack (marked by arrows), especially at low $[Ca^{2+}]$. As can be seen, there was about a 50% increase in speed as $[Ca^{2+}]$ was increased. Note that in this fibre the intercepts for the slack test determined at low and high $[Ca^{2+}]$ were approximately the same. Typically, the slack test determined for maximal activation had a higher intercept. Fibre 19/4/84. Fibre length was 5·0 mm at a sarcomere length of 2·1 μm.

estimated by the intercept of the slack test (i.e. 0·17 mm), then, assuming linear response, the length during long releases would be 0·196 mm (No. 2) and 0·216 mm (No. 4). Since the difference in fibre length between the long and short releases was about 0·33 mm, and the calculated difference in the length of the series elastic element between short and long releases is only 0·026 mm (Nos. 2 and 1) and 0·046 mm (Nos. 4 and 3) respectively, this would result in over-estimation of V_{max} by about 8 % at pCa 4·4 and 13 % at pCa 6·05. Typically, the intercept for the slack test at partial activation was smaller than at full activation, and thus the magnitudes of the over-estimation would be nearly independent of activation level. In any case, the magnitude of this type of error would be too small to lead to a false conclusion that $[Ca^{2+}]$ influenced V_{max}.

We also observed that force generation by different fibres varied even though activation was produced using the same pCa. This is probably due to variation in Ca^{2+} sensitivity of fibres, and its increase in the presence of caffeine (Stephenson & Williams, 1981; Wendt & Stephenson, 1983). Control experiments with fibre preparations from *R. pipiens* and *R. temporaria* showed that the sensitivity to $[Ca^{2+}]$ increased consistently in the presence of caffeine. 10 mM-caffeine caused a parallel left shift of the force–pCa curve along the pCa axis by not more than 0·1 pCa units. No effect of caffeine on V_{max} could be detected, although this was not rigorously tested (see Table 3). We therefore found it more reasonable to compare V_{max} values as a function of level of activation rather than of pCa. Table 2 shows a clear increase in V_{max} as the level of activation and $[Ca^{2+}]$ were increased. This effect was observed whether the fibre was released by various amounts from a given initial length (fibres 29/12/81–26/2/82) or released from various initial lengths to a final fixed length (fibres 19/4/84–2/5/84). Comparisons within a given fibre show a clear relationship between V_{max} and level of activation. The mean V_{max} values at different levels of activation were also statistically different from one another.

At longer sarcomere lengths (2·2–2·6 μm), fibres tended to have a higher V_{max} at all levels of activation, but the influence of the level of activation on V_{max} still persisted. V_{max} at full activation was 5·15 m.l./s (S.E. of mean $\pm$0·22, $n = 5$) whereas V_{max} at 20–30 % activation was only 3·54 m.l./s ($\pm$0·41, $n = 4$). The ratio of V_{max} at 20–30 % activation to that at full activation appeared to be slightly higher at longer sarcomere lengths (69 %) than at shorter sarcomere lengths (54 %). This result is expected if parallel elasticity increases V_{max} at long sarcomere lengths and this effect is more prominent at low levels of activation.

In a separate set of experiments, a V_{max} of 6·14 m.l./s has been reported at maximal activation at sarcomere lengths of 2·1–2·3 μm (Julian *et al.* 1986). It is not clear from our experiments whether the difference in V_{max} at short and long sarcomere lengths is due to an internal resistive force at short sarcomere lengths reducing V_{max}, or to resting forces at long sarcomere lengths increasing V_{max}. Nevertheless, a Ca^{2+} effect is observed at both long and short sarcomere lengths, and this makes it more likely that the effect is not the result of a systematic error in experimental procedure.

Sarcomere length effects on the force–pCa relation. Since the curvature of the shortening traces in Figs. 1 and 4 might be associated with changes in the force–pCa

TABLE 2. V_{max} of *R. pipiens* fibres as a function of level of activation. As the level of activation (act.) of fibres is increased, V_{max} also increases. V_{max} was determined by the slack test. The first five fibres were released by various amounts from a fixed sarcomere length and the last five fibres were released from different initial sarcomere lengths to a fixed sarcomere length. Experiments were conducted over sarcomere length 1·8–2·1 μm at 7·5 °C

	20–35 % act. V_{max} (m.l./s)	40–65 % act. V_{max} (m.l./s)	80–100 % act. V_{max} (m.l./s)	$\frac{V_{max}\ 20–35\ \%}{V_{max}\ 80–100\ \%}$	$\frac{V_{max}\ 20–35\ \%}{V_{max}\ 40–65\ \%}$	$\frac{V_{max}\ 40–65\ \%}{V_{max}\ 80–100\ \%}$
29/12/81	1·72	—	4·01	0·43	—	—
7/1/82	—	—	4·20	—	—	—
8/1/82	—	2·16	—	—	—	—
11/1/82	—	—	5·30	—	—	—
26/2/82	—	2·60	4·20	—	—	0·62
19/4/84	—	2·4	3·79	—	—	0·63
25/4/84	2·30	2·94	3·57	0·64	0·78	0·82
30/4/84	2·83	3·64	—	—	0·78	—
1/5/84	2·84	3·68	—	—	0·77	—
2/5/84	1·74	2·79	—	—	0·62	—
Mean ± S.E. of mean	2·28 ± 0·25	2·89 ± 0·22	4·18 ± 0·25	0·54	0·74 ± 0·04	0·69 ± 0·07

relation at shorter sarcomere lengths (for a review see Stephenson & Wendt, 1984), we measured the magnitude of this effect in our preparation. We initially activated fibres at a sarcomere length of about 2·3 μm in solutions of varying [Ca^{2+}]. After the steady force generation was reached, the fibre was released to a sarcomere length of 1·95 μm and the force redeveloped at the shorter sarcomere length was measured. A set of force traces from such an experiment are shown in Fig. 3*A* and the relations between steady-state force and pCa at the average sarcomere lengths of 2·3 and

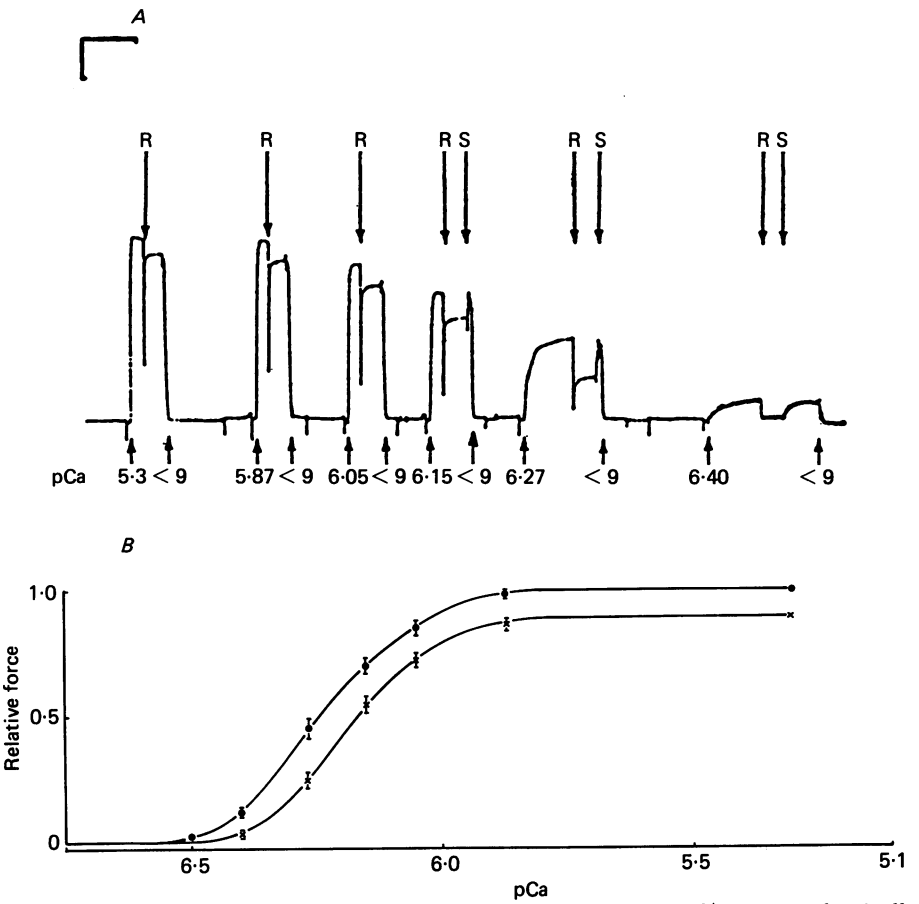

Fig. 3. Sarcomere length effect on isometric force sensitivity to [Ca^{2+}] in a mechanically skinned (*R. pipiens*) fibre. The preparation was initially activated in solutions of different pCa (see lower arrows in *A*) and was suddenly released (R, upper arrows in *A*) by a constant length step after steady force was reached. In several instances the preparation was re-stretched to the initial length while in the activating solution (S, upper arrows in *A*). Note that force level after re-stretching the preparation in this situation is close to the pre-release level. In *B* the steady values of the relative isometric force responses before and after release from twelve contractions were plotted against pCa. The vertical bars indicate the range of the results. The average sarcomere lengths corresponding to the conditions before and after release were 2·30 (●) and 1·95 μm (×) respectively. Calibration bars for *A* vertical 10^{-4} N, horizontal 25 s. The continuous lines in *B* were drawn by eye. Dimensions of the preparation: length 3·50 mm at sarcomere length 2·30 μm, apparent diameter in solution about 90 μm. Resting force at 2·30 μm remained constant throughout the experiment at 4×10^{-6} N.

1·95 μm are plotted in Fig. 3*B*. A larger percentage difference in force generation at the two lengths was observed at low levels of activation than at high ones.

R. temporaria fibres

R. temporaria fibres were used because they generate large forces, which makes it possible to clamp low relative loads at partial activation levels. Because of their large

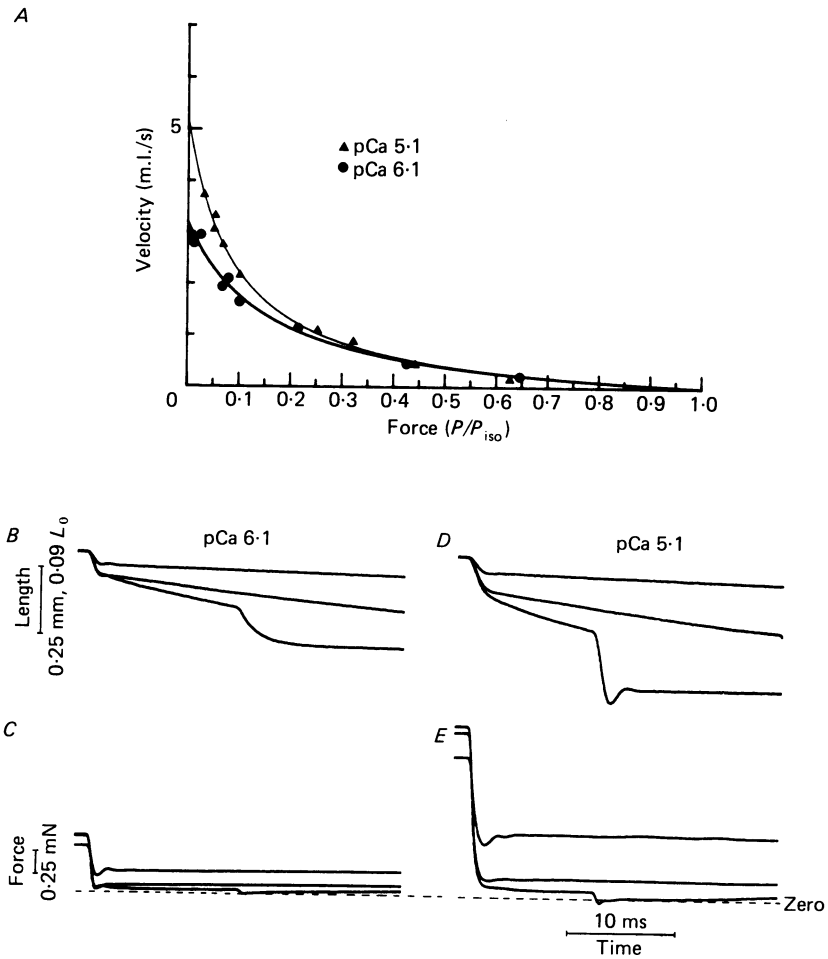

Fig. 4. The influence of $[Ca^{2+}]$ on the force–velocity relation of a skinned *R. temporaria* fibre. *A* shows the force–velocity data (force clamps) for a fibre first activated at pCa of 6·1 and later activated at a pCa of 5·1. Velocity is expressed in muscle lengths/s (m.l./s) and the force is expressed as a proportion of the maximum isometric tension generated at the pCa under consideration. Note that there is little difference in velocity at the two pCa values until the relative load drops below about 0·1 P_0. V_{max} determined by extrapolating the Hill curve to zero load was 5·23 m.l./s at a pCa of 5·1 and 3·2 m.l./s at a pCa of 6·1. The a/P_0 for the Hill curves were 0·09 and 0·16 respectively. *B* and *C* show original length and force records at a pCa of 6·1, and *D* and *E* show original length and force records at a pCa of 4·4. The initial sarcomere length for the force clamps was 1·97 μm. Fibre 20/11/84. Fibre length was 2·78 mm at a sarcomere length of 2·1 μm.

diameter, however, these fibres do not activate as quickly as the *R. pipiens* fibres. Also, relatively short sarcomere lengths were used together with a length step to even lower sacromere lengths at the end of clamps to determine the zero force level. This resulted in the formation of residual tension and more rapid decrement of isometric force than was observed in *R. pipiens*. During the eight to ten contractions necessary to determine force–velocity curves at one pCa, a decrement of 20% in isometric tension was usually observed as well as an increase in residual tension. Low loads were used first before the development of significant residual tension. Experiments were always done in the order of low $[Ca^{2+}]$ followed by high $[Ca^{2+}]$. After exposure to high $[Ca^{2+}]$, the residual force increased significantly, making accurate measurements at low levels of activation impossible. Fig. 4 shows the results of one experi-

TABLE 3. V_{max} of *R. temporaria* fibres as a function of level of activation (act.). V_{max} extrapolated from an unbiased fit of the Hill curves (five to eight points) to zero load. Each fibre was first activated at low $[Ca^{2+}]$ (pCa = 6–6·4, depending on level of caffeine which varied from 0 to 20 mM), and then activated at high $[Ca^{2+}]$ (pCa = 5·1–5·8, depending on caffeine concentration). Fibre 6/11/84 (not shown) had a V_{max} of 5·2 m.l./s at maximal activation and 0 mM-caffeine. Experiments were conducted over sarcomere lengths 1·8–2·1 μm at 7·5 °C

	Submaximal activation		Maximal activation	V_{max} low act.	Caffeine
	% act.	V_{max} (m.l./s)	V_{max} (m.l./s)	V_{max} max act.	(mM)
21/11/84	13	3·50	5·28	0·66	10
4/12/84	17	3·52	6·23	0·57	20
20/11/84	23	3·21	5·23	0·61	10
19/11/84	23	3·39	5·99	0·57	0
5/12/84	24	3·67	5·34	0·69	20
29/11/84	29	3·56	5·7	0·62	20
Mean ± S.E. of mean	21·5 ± 2·3	3·47 ± 0·06	5·62 ± 0·17	0·62 ± 0·02	

ment. As can be seen, pronounced differences in the force–velocity curves become apparent at only light loads, because the force–velocity curve at high $[Ca^{2+}]$ is more steeply curved than at low $[Ca^{2+}]$. On average, the Hill constant, a/P_0, was 0·147 (S.E. of mean ± 0·01, $n = 9$) at 13–29% activation and 0·092 (± 0·01, $n = 9$) at maximal activation.

As in the *R. pipiens* fibres, the V_{max} of the *R. temporaria* fibres is best expressed as a function of activation level rather than pCa. At low levels of activation (13–30%), the V_{max} of the fibres was only about 60% of that found at maximal levels of activation (Table 3). Fibres that were maximally activated without prior measurements at low $[Ca^{2+}]$ had slightly lower V_{max} values (4·93 m.l./s ± 0·18, $n = 3$) than those in which V_{max} was first measured at low $[Ca^{2+}]$, but the V_{max} was still significantly higher than that at low levels of activation. Interestingly, we did not observe any indication that V_{max} drops below 3 m.l./s at the levels of activation used in these experiments. We could not use the slack test reliably on these fibres at very low levels of activation because of the presence of residual tension. Also, the short, large diameter, fibre segments used exerted significant negative forces on the transducer following length steps, which made it difficult to determine t_s accurately.

Slow-twitch rabbit soleus fibres

In order to find a preparation which had a larger range of sarcomere lengths free of both resting tension and resistance to shortening, we used slow-twitch mammalian fibres obtained from the rabbit. In these fibres, the plateau of tension appears to be shifted to longer sarcomere lengths (2·4–2·6 μm) compared to amphibian fibres (Stephenson & Williams, 1982). In addition, we thought that the lower speed of shortening found in these fibres would provide better time resolution in reading the slack test.

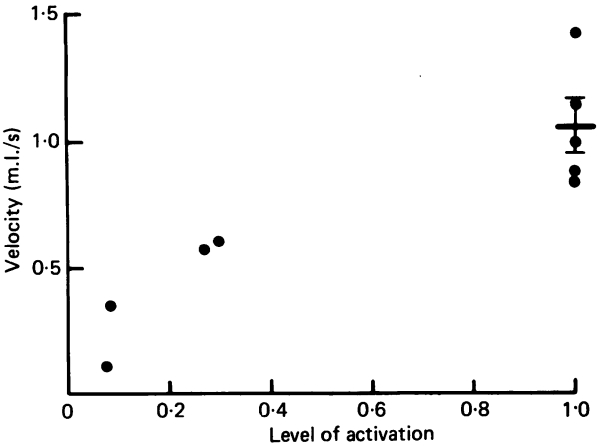

Fig. 5. V_{max} as a function of activation in skinned rabbit soleus fibres. V_{max} is plotted for five fibres against the proportion of maximum activation. V_{max} was measured by the slack test. The fibres were released from a fixed length (sarcomere length 2·52–2·65 μm) by various amounts ranging from 5 to 12%. The mean and S.E. of mean of V_{max} at full activation are denoted by the large and small bars respectively.

The slack test was used to determine V_{max} at 15 °C. The fibres were activated at different [Ca^{2+}] and relaxed by the same procedure as described for the amphibian fibres. The A, B and H solutions used for 15 °C were slightly modified with respect to total Mg, and to Cl^- and K^+ to maintain the [Mg^{2+}] constant (1 mM) at 15 °C (see Table 1). 10 mM-caffeine was used in all solutions. As can be seen from Fig. 5 there was a strong correlation between V_{max} and the level of activation. In fibres where V_{max} was measured at two levels of activation, there was always an increase in V_{max} with increased levels of activation.

DISCUSSION

Recently we have improved the stability of our fibre preparation by using the rapid activation technique, and greatly improved our ability to measure V_{max} (Julian *et al.* 1986). We have also become more cognizant of factors that might lead to errors in the determination of V_{max}, especially those (e.g. sarcomere length) whose magnitude might change systematically with the level of activation. By using these improved preparations and techniques and by specifically testing for the possibility of systematic errors, we conclude that [Ca^{2+}] has a significant influence on V_{max} of

skinned muscle fibres. This general conclusion is in agreement with those made by Julian and colleagues (Julian, 1971; Julian & Moss, 1981). Our results can also explain the findings of Podolsky and his colleagues (Podolsky & Teichholz, 1970; Thames *et al.* 1974; Gulati & Podolsky, 1978), who did not observe an influence of $[Ca^{2+}]$ on V_{max}.

In a recent review Podolin & Ford (1983) suggested that the difference in these conclusions was not due to any technical aspects of the work done by the different groups, but rather to other possibilities. One suggestion was that Julian and his colleagues used chemically skinned muscle fibres, whereas Podolsky and his colleagues used mechanically skinned fibres, and this may have led to a difference in the level of phosphorylation of the fibres which could influence V_{max}. In this study, we have observed an influence of $[Ca^{2+}]$ on V_{max} in fibres skinned in a similar fashion to that used by Podolsky and colleagues (Podolsky & Teichholz, 1970). Further, it has been recently shown that the level of phosphorylation does not influence the V_{max} of skinned mammalian fibres (Sweeney & Kushmerick, 1985). In agreement with this, we have not observed any significant differences between V_{max} in the presence (10–20 mM) and in the absence of caffeine when the levels of activation were similar. Caffeine is known to be a potent phosphodiesterase inhibitor (Butcher & Sutherland, 1962) and the state of phosphorylation of the myofilaments may have been altered in the presence of caffeine (Stull, 1980). It is therefore unlikely that the procedure used for skinning the fibre, or differing levels of phosphorylation, are responsible for the opposing conclusions with respect to the influence of $[Ca^{2+}]$ on V_{max}.

Podolin & Ford's (1983) other suggestion that 'shortening deactivation' might be responsible for these different conclusions also seems unlikely. In this study we used single releases as was done by Podolsky and his colleagues (Podolsky & Teichholz, 1970; Thames *et al.* 1974; Gulati & Podolsky, 1978). We clamped the loads much earlier (< 5 ms) and read the velocities much earlier (5–10 ms) in the shortening record than did Podolsky and his colleagues. We also read the velocities over a narrower range of average sarcomere lengths (within 0·05 μm for each fibre). Even though our force–velocity data would thus be less influenced by 'shortening deactivation', we still observed an influence of $[Ca^{2+}]$ on V_{max}.

In this study we did observe, however, curvature in the length traces during force clamps, which has been referred to in the past as 'shortening deactivation' (Gulati & Podolsky, 1978; Brenner, 1980). The reason shortening velocity changes with time and/or length during force clamps in skinned fibres, but not in living fibres, is unknown. One contributing factor may be the change in the force–pCa relationship with sarcomere length presented in Fig. 3. Essentially, as the fibre shortens the P_0 decreases. During a force clamp, the P/P_0 would thus increase, and a decrease in velocity would be expected. This effect can be estimated from the information contained in Fig. 3*B* and using the Hill equation with the assumptions that the values for V_{max} and a/P_0 do not change during shortening. At full activation, the speed of shortening is predicted to drop only by a factor of 1·04 and 1·10 over the sarcomere range between 2·3 μm and 1·95 μm when the initial relative load at 2·3 μm was 0·05 and 0·2 respectively. The value for a/P_0 used in these calculations was 0·09 according to the results in Fig. 4. At 40 % activation, the speed of shortening would decrease by a factor of 1·27 and 1·94 for the same relative loads ($a/P_0 = 0{\cdot}16$ is

assumed, Fig. 4). Furthermore, the fibre would stop shortening altogether before reaching a sarcomere length of 1·95 μm when the initial load at 2·3 μm is higher than the isometric force which the fibre can generate at 1·95 μm. The qualitative prediction from these calculations that the degree of curvature of the length traces increases at low levels of activation and at higher relative loads, appears to agree with our data as well as that of others (Gulati & Podolsky, 1978; Brenner, 1980). This suggests that 'shortening deactivation' can be explained to some extent by the decline in isometric-force-generating capability of the fibre which accompanies shortening.

Although the decline of P_0 with sarcomere length appears to fit the data qualitatively, we do not think that it is a complete explanation for the curvature of the length records. For instance, even at very high [Ca^{2+}], in which the fibre would be maximally activated over the whole range of lengths it shortens, we still observed curvature in the length record. This may possibly result from the greater sarcomere non-homogeneity found in skinned fibres compared to that found in living fibres (Julian & Moss, 1980). We have found, for instance, that conditions which would lead to increases in sarcomere non-homogeneity also leads to spuriously high velocities of shortening. We have previously reported (Julian *et al.* 1986) that after about ten contractions at maximal activation, especially at high loads, the P_0 generated by a fibre decreased, but the velocity of shortening at low loads measured shortly after the release was actually higher than in the fresh fibre.

In this study we observed that at low levels of activation, fibres that were first activated at high levels of Ca^{2+} (which may lead to the development of irreversible sarcomere non-homogeneity) had much higher speeds of shortening when measured shortly after release (close to those found at high [Ca^{2+}]) than fibres not previously exposed to high [Ca^{2+}]. Curvature of the length record and spuriously high shortening velocities immediately following release have also recently been observed in living fibres at near 0 °C which have failed to activate uniformly (F. J. Julian, unpublished results). Sarcomere non-homogeneity could lead to spuriously high velocities if a proportion of the sarcomeres are stretched to a length where part of the total force produced is borne by passive elastic structures. At an effectively reduced P/P_0, these sarcomeres will be shortening faster than the ones in which the total load is borne by active force-generating mechanisms, and thus the speed of the whole fibre will be greater than expected. Of course, as these sarcomeres shorten the influence of the passive elastic component would be reduced, leading to a slowing of the over-all speed of shortening of the fibre and a curvature in the length record.

Until the mechanism underlying the curvature of the length records is fully understood there will be some question as to the most appropriate time after a release at which to measure the velocity of shortening of a fibre for use as an indicator of cross-bridge cycling rate. This important issue may be responsible to some extent for the seemingly qualitative difference between our results and those of Julian (1971). In the previous study, the shortening velocity at all relative loads was lower during contractions at partial activation than at full activation, whereas in this study the shortening velocities were nearly the same at all relative loads above 0·2 P_0. Since the length records at partial activation are more curved that those at full activation, reading the present records at a later time following release, as was done in the case of Julian (1971), would result in lower velocities of shortening at partial activation

relative to that at full activation over the full range of loads. Since a significant influence of $[Ca^{2+}]$ is still observed when velocity is measured as early as possible after release, and the magnitude of the Ca^{2+} effect increases as the time increases, it is reasonable to conclude that $[Ca^{+2}]$ does influence V_{max}.

It is our belief that the differences in conclusions concerning the influence of $[Ca^{2+}]$ on V_{max} can be adequately explained by the inherent technical difficulties in the determination of V_{max} in skinned fibres. Essentially, we were able to measure velocities at sufficiently low loads (including zero load) to observe a $[Ca^{2+}]$ effect. We found, for instance, over most of the range of relative loads greater than 0·2, that the velocities of shortening at high levels of activation were about the same as those at low levels. This result is in agreement with that found by Podolsky and his colleagues (Podolsky & Teichholz, 1970; Thames *et al.* 1974; Gulati & Podolsky, 1978), and if we made our extrapolation to V_{max} from just these data our conclusion would have been the same, i.e. that V_{max} is independent of $[Ca^{2+}]$. As we have reported previously, however, the force–velocity relationship is not hyperbolic at low loads, so that an accurate determination of V_{max} requires measurements at low relative loads. To attain sufficiently low loads to quantify the effect of activation on V_{max} in *R. pipiens*, it was necessary to use the slack test. The fact that we observed similar influences of the level of activation on V_{max} under different conditions provides strong evidence for believing our conclusion is correct. Further, by using a fibre that generates greater forces (*R. temporaria*), we were able to demonstrate a large influence of activation level on V_{max} by using force clamps as well.

As mentioned earlier in the Methods, the experiments using frog muscle were made more difficult because there was a very limited range of sarcomere lengths available in which to perform measurements which were free from both resting tension and resistance to shortening. Using relatively long sarcomere lengths causes resting tension to increase shortening speed (Edman, 1979), and it would be anticipated that this increase would be larger at low levels of activation, where fewer cross-bridges would be attached to resist the shortening produced by the elastic force. At shorter sarcomere lengths, where no resting tension is present, however, internal resistive forces (Gordon *et al.* 1966; Edman, 1979) might slow the speed of shortening, and it would be anticipated that the decrease in speed would be greater at low levels of activation, where there would be fewer cross-bridges attached to overcome the resistive force.

A combination of these effects may have been largely responsible for differences in speeds measured at short and long sarcomere lengths, and provides an explanation for why the ratio of the V_{max} at lower levels of activation to V_{max} at full activation was higher when working at long compared to short sarcomere lengths. It is unlikely that resistance to shortening significantly influenced V_{max} in these experiments. While working at short sarcomere lengths, velocity was typically measured at a fibre length that was equivalent to a passive sarcomere length of about 1·9 μm. Assuming internal shortening of 0·1–0·15 μm per sarcomere upon activation (Julian & Moss, 1980), the active sarcomere length at which velocity was measured would be above the value of 1·6 μm where myosin filaments collide with the Z line producing a marked slowing of speed in intact fibres (Gordon *et al.* 1966; Edman, 1979). We cannot completely exclude the possibility that some other mechanism causes a resistance to shortening at sarcomere lengths longer than 1·6 μm in skinned fibres. The important

finding of our study, however, was that at both short and long sarcomere lengths, similar influences of $[Ca^{2+}]$ on V_{max} were observed. Furthermore, the large influence of $[Ca^{2+}]$ on V_{max} observed in the rabbit soleus fibres, which were free of these possibly confounding influences, further supports this conclusion.

We conclude that the level of activation influences the maximum speed of shortening. This has been shown in three different preparations, at different sarcomere lengths, and by using force clamps and two variations of the slack test. Obviously, further work is necessary in order to elucidate the molecular mechanism of the Ca^{2+} effect on V_{max}.

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