

Contraction Kinetics of Intact and Skinned Frog Muscle Fibers and Degree of Activation

Effects of Intracellular Ca²⁺ on Unloaded Shortening

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ABSTRACT This study addresses a long-standing controversy on the effects of the degree of activation on cross-bridge kinetics *in vivo*, by utilizing isolated intact and skinned fiber preparations. Steady force levels ranging from 0.1 to 0.76 P_0 were achieved at 0°C with temperature-step stimulation of intact fibers by varying the amount of caffeine in the bathing medium. The speed of unloaded shortening (by slack test) was found to be practically constant, which suggests that intracellular Ca²⁺ in the intact preparation has relatively little effect on isotonic shortening. Along with the results on tetanically stimulated fibers (force, P_0), we observed a minor but significant trend for the speed to decline with lowered force levels. This trend is explained by the presence of a constant internal load equaling ~1% P_0 . The effect of Ca²⁺ on the shortening behavior of skinned fibers was examined at 0 and 10°C. At 0°C, there was practically no effect of Ca²⁺ on the shortening response in slack tests. At 10°C, there was also no Ca²⁺ effect during the first activation cycle, but in subsequent cycles the speed of shortening was reduced during partial activation, which indicates that there were permanent changes in the fiber properties under these experimental conditions. The latter result could be explained if the internal load had increased to ~5% P_0 in the modified skinned fiber (compared with 1% P_0 in intact fiber). These findings show that isotonic contraction of frog fibers is intrinsically unaffected by the variations in intracellular Ca²⁺ that modulated the force over a nearly complete range. The results provide support for the idea that Ca²⁺ influences the force development *in vivo* by on-off switching mechanisms.

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INTRODUCTION

Calcium has a central role in the contraction mechanism of muscle. The concentration of free intracellular Ca^{2+} is low in the unstimulated muscle cell (Blinks et al., 1978), and the interaction of myosin cross-bridges with actin is blocked. Upon stimulation, as free Ca^{2+} in the myoplasm is elevated and binds to thin-filament regulatory proteins, the inhibition for cross-bridge attachment on thin filaments is removed (Ebashi, 1980). This suggests that Ca^{2+} is a switch for cross-bridge attachment. The attached cross-bridges exert force, but do so only during their stay in some of those configurations (H. E. Huxley, 1969; A. F. Huxley, 1980). The relative durations in the various configurations are influenced by the kinetic rate constants and affect the mean force of muscle. Therefore, to understand the cross-bridge mechanisms of force generation, it is important to establish whether or not Ca^{2+} influences the cross-bridge turnover kinetics. However, in spite of a great deal of effort, the issue has remained controversial.

Most previous studies measured force-velocity relationships and contraction transients on skinned fiber preparations, because the degree of activation of such preparations is altered in steady state simply in response to direct changes of free Ca^{2+} concentration in the bathing milieu. However, the results and conclusions varied, in part because the experimental protocols differed (see Table III in Gulati and Podolsky, 1981). Thus, with 190 mM ionic strength at 4–7°C, the relative force-velocity relationships and velocity transients were the same at various degrees of activation, which suggests that the cross-bridge kinetics underlying these properties are unaffected by Ca^{2+} (Podolsky and Teichholz, 1970; Gulati and Podolsky, 1978). However, with low ionic strength (100–150 mM) above 4°C and also with 190 mM ionic strength at 10°C, the mechanical kinetics of skinned fibers were altered by changes in $[\text{Ca}^{2+}]$ in the bath, which suggests a different action of Ca^{2+} (Julian, 1971; Thames et al., 1974; Julian and Moss, 1981). Thus, a question remains as to which mechanism operates in the physiological condition. The resolution of this controversy is important not only for establishing the cross-bridge theory of skeletal muscle contraction, but also for understanding the physiology of myocardium in which the level of calcium activation varies widely under normal conditions (e.g., Allen and Blinks, 1978).

The study of the effects of intracellular Ca^{2+} on contraction kinetics has also been attempted with intact fibers, but the efforts have been limited to non-steady state conditions by the difficulty of manipulating the levels of intracellular Ca^{2+} . Twitch force was depressed by including dantrolene in the bathing medium (Edman, 1979) or by replacing H_2O with D_2O (Cechhi et al., 1981). However, the isotonic shortening properties of the fibers were constant with these agents. Since a reduction in the amount of Ca^{2+} release was a probable cause of the decrease in force with D_2O (Allen et al., 1984) and dantrolene, the results supported the view that Ca^{2+} does not affect the shortening response during the twitch.

The present study extends the examination of the Ca^{2+} effect in intact fibers to the steady state conditions for the degree of activation. The activation level was modulated by applying a temperature step at various concentrations of caffeine in the bathing medium. The contraction speed of intact fibers is

practically independent of the degree of activation, which is in agreement with the Ca²⁺ effect in skinned fibers at 190 mM ionic strength at low temperatures as well as in intact fibers during the twitch. We also find a minor but significant trend for the speed to decline at the lowered activation levels, but this is explained as being the result of constant internal load. Furthermore, we present evidence on skinned fibers at 10°C that the effects of Ca²⁺ previously seen at this temperature were the result of permanently altered properties of the contractile system.

Brief reports of these results have appeared (Gulati and Babu, 1983, 1984b).

METHODS

Fiber Preparation

INTACT FIBER Isolated fibers from anterior tibialis muscles of *Rana temporaria* were used in most of the experiments. The isolation procedures and the experimental setups were the same as described recently (Gulati and Babu, 1982, 1984a). The mean sarcomere length for all intact fiber experiments was 2.2–2.3 μm, as determined by laser diffraction.

SKINNED FIBER In part of the study, we used preparations of skinned fibers from semitendinosus muscles of *Rana pipiens pipiens* (Gulati and Podolsky, 1981; Gulati, 1983). Some of the fibers were also briefly exposed (10 min) to a 50% (by volume) glycerol solution (solution 5, Table I) and then to a detergent solution (solution 6) for a similar duration in accordance with Julian's (1971) protocol. The mean sarcomere length for all skinned fiber experiments was ~2.4 μm. The experimental setup (transducers, chambers, etc.) for the skinned fibers was the same as that for the intact fibers (Gulati and Babu, 1984a).

Solutions

The various solutions used for the intact and skinned preparations are listed in Table I. Solution 1 was used during dissection of the muscle and solution 2 was used for most of the experiments on intact fibers, with both temperature-step and electrical stimulation. Whenever a modified solution was used, it is noted in the text. Caffeine was omitted for all electrical stimulations. In the case of skinned fibers, solutions 4 and 7 were used as relaxing solutions. For maximal and partial activations of the skinned fiber, EGTA in solutions 4 and 7 was replaced with Ca-EGTA. The various proportions of these amounts were calculated on a microcomputer as before (Gulati, 1983). Note that of solutions 4 and 7, only solution 4 contains creatine phosphate with kinase added just before the experiment.

The pH of each solution was adjusted to 7.00 ± 0.01 at room temperature just before the experiment. All chemicals were of the best grade available from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA).

Experimental Protocol

ACTIVATION OF INTACT FIBERS The fibers were activated either electrically or by rapid cooling from 25 to 0°C in the presence of caffeine. The electrical tetanic stimulation was achieved with 1-ms pulses (duration, 1–2 s; frequency, 20 Hz of 15–26 V amplitude that was two to three times above rheobase). The rest period between tetani was ≥10 min. In temperature-step activation, the force levels were modulated by varying the concentration of caffeine.

A typical temperature-step activation of an isolated fiber for maximal force is shown in

Fig. 1A. The step was applied by using a four-chamber device as described earlier (Gulati and Babu, 1984a). The fiber was initially equilibrated in the caffeine-containing solution for 30–60 min at 25°C, removed from the solution (arrow *a* in Fig. 1A), and then plunged (arrow *b*) into an identical solution at 0°C. Force was produced. On return to 25°C (arrow *d* in Fig. 1A; arrow *c* indicates when the fiber was lifted out of the 0°C solution), the fiber was relaxed. If caffeine was omitted, there was no force following the temperature step. The first temperature-step contraction of a fiber was usually induced with 1 mM caffeine. Caffeine was then gradually increased in increments of 0.5 mM (allowing a 15-min equilibration in each new solution) until the force was maximal. This force level could be reproduced throughout the experiment. Caffeine for maximal force varied from fiber to fiber (1.5–2 mM).

To get partial activations, caffeine was lowered from the maximal level in increments of 0.1–0.01 mM. The pattern of force development with partial activation was slightly

TABLE I
Composition of Solutions*

	For intact fiber				For skinned fiber			
	1	2	3		4	5	6	7
KCl	2.5	2.5	2.5	KCl	95 [‡]	140	140	140
NaCl	115.6	115.6	100	MgCl ₂	6.06	5	5	1
CaCl ₂	0.7	1.8	10	Imidazole	10	10	10	10
MgCl ₂	0.12	—	—	ATP	5	5	5	4
Imidazole	—	—	10	EGTA	5	5	5	2
Phosphate buffer	3.2	3.2	—	Creatine phosphate (CP)	20	5	5	0
Dextrose	5.0	—	—	Glycerol (ml/ml)	—	0.5	—	—
Caffeine	—	0–3	0–3	Lubrol detergent (g/ml)	—	—	0.005	—
				CPK (IU/ml)	250	—	—	—
Osmotic pressure (mosmol/kg H ₂ O)	245	228	239	Ionic strength (estimated)	195	195	195	180

* The concentrations of all materials are given as millimolar except where noted otherwise.

[‡] In some experiments mentioned in the text, KCl in this solution was replaced with K-propionate (KPr).

different in that there was a significant delay in the appearance of force (second and third traces in Fig. 1B). (There was a similar delay in skinned fibers with submaximal Ca²⁺ [Hellam and Podolsky, 1969].) The entire caffeine-force relationship on the same fiber in Fig. 1B is shown in Fig. 1C (marked "intact fiber"). The threshold caffeine concentration, as well as the steepness of the caffeine-force response curve, varied from fiber to fiber. In some fibers, the maximal response was obtained with merely 0.1–0.2 mM above the threshold level of caffeine, but we were able to get partial activations on all fibers tested. The force plateau for a given partial activation was often, but not always, reproduced. The results of the slack test (see below) were used only when a given force level was reproduced (to within 10%) in at least three consecutive activations.

CONTROL STUDIES OF CAFFEINE EFFECT ON SKINNED FIBERS A number of recent reports suggest that the presence of caffeine increases the Ca²⁺ sensitivity of the myofilaments of both skeletal (rat fibers, Wendt and Stephenson, 1983) and cardiac muscles (Fabiato and Fabiato, 1976; McClellan and Winegrad, 1978). Although there is evidence that contractions of frog intact fibers with rapid cooling in the presence of caffeine are a direct result of intracellular Ca²⁺ release from the sarcoplasmic reticulum (Kurihara et al., 1984), a few controls were made with frog skinned fibers to see to what extent the caffeine effect on Ca²⁺ sensitivity itself might contribute toward partial activations in our experiments of living fibers. (Another study was made to check for the effect of maximal caffeine in slack tests; those results are given below.)

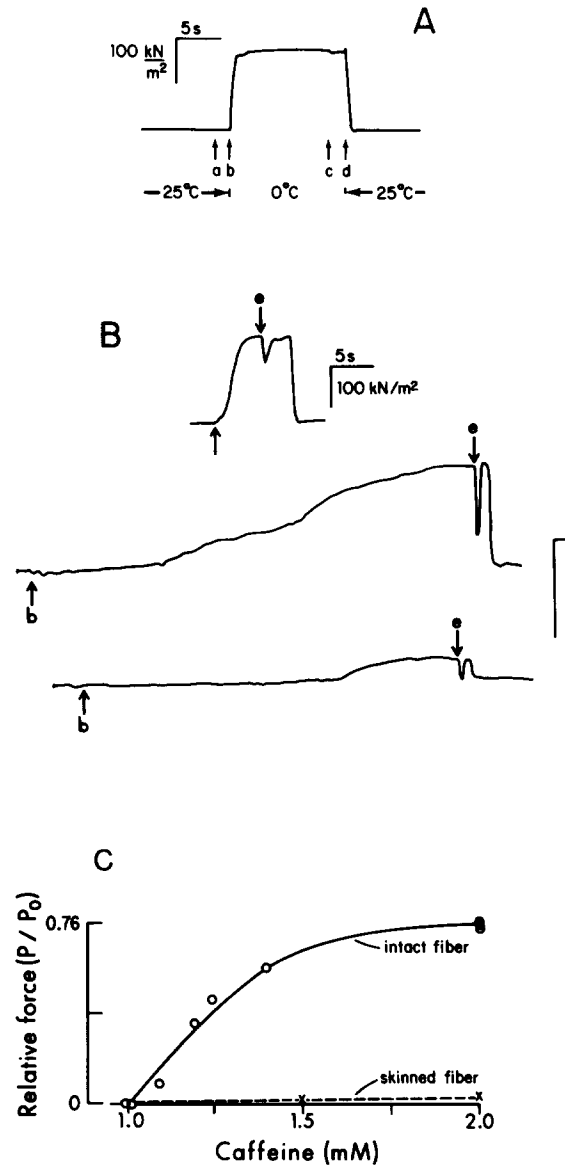


FIGURE 1. Contraction of isolated tibialis fibers with temperature step. (A) Maximal activation (experiment 26 xi 82; fiber diameter, 134 μ m). (B) Partial activation by varying caffeine (experiment 16 iv 82; fiber diameter, 146 μ m). The force is close to maximal (0.76 P_0) in the top trace, 0.36 P_0 in the second trace, and 0.09 P_0 in the third trace. Arrows marked *e* indicate where the length release was applied for the slack test (see Fig. 4). (Note that in these slow time base responses, the force does not fall to zero with the length releases because of the inability of the chart recorder to follow.) (C) Solid line: caffeine-force relationship from temperature steps on the same fiber as in B. Dashed line: the relationship estimated from direct caffeine effects on the pCa-force relations on skinned fibers (see Fig. 2 and the text).

Fig. 2 shows the leftward shift in the pCa-force relationship with 5 mM caffeine at 0°C. Solution 4 (Table 1) was used, except that all the KCl was replaced by KPr. (The caffeine effect at 3 mM appears to be somewhat variable in KCl, and two fibers with Cl⁻ gave no effect at this concentration. Certain other differences in the results between KCl and KPr on skinned preparations were also noted [Kentish, 1984; Brenner et al., 1984].) The inset in Fig. 2 is a composite plot relating caffeine concentration to its effects on the amount (ΔpCa_{50}) of leftward shift in paired studies with 11 fibers. This was used to estimate the contribution of caffeine to the force in the temperature-step study (Fig. 1 C, dashed line

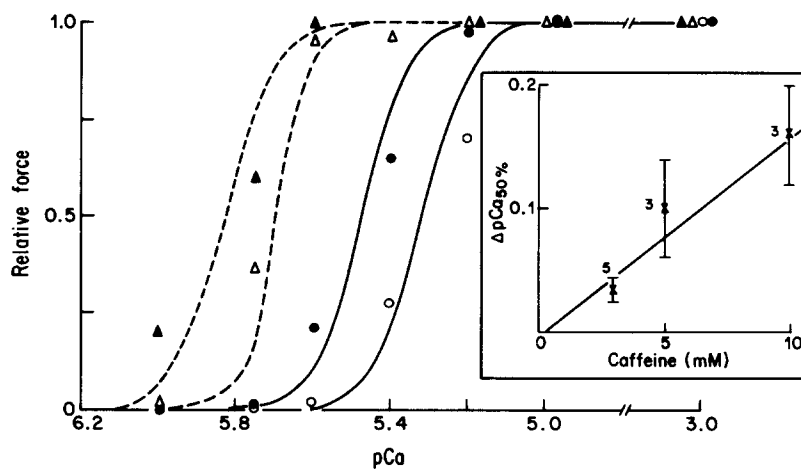


FIGURE 2. Effect of caffeine on pCa-force relationship of skinned fiber at 0°C. The results on two fibers are shown. O, 0 caffeine; ●, 5 mM; Δ, 1% Triton-X; ▲, 1% Triton-X + 5 mM caffeine. The curves were fitted by Hill equation of the form: relative force = $[Ca^{2+}]^n / (K^n + [Ca^{2+}]^n)$. The Hill coefficient n was in the range of 6–8 (without detergent), which can be compared with the value given by Godt and Lindley (1982). Note that ΔpCa_{50} values with caffeine are nearly the same with and without detergent. Inset: caffeine- ΔpCa_{50} relationship on 11 fibers, with a regression line through the points.

labeled "skinned fiber"). In making this estimation, we used the right-most curve in Fig. 2 as control; the curve was shifted to the left according to the caffeine concentration, and pCa 5.6 was taken as the resting value. The "skinned fiber" curve in Fig. 1 C shows that the effect of caffeine on Ca²⁺ sensitivity cannot contribute significantly to the force of intact fiber during the temperature step.

The possibility that the caffeine effect may be amplified (40–50 times) in intact fibers (because of the greater amount of membrane structure) is not supported by the result in Fig. 2, which was made in the presence of detergent (1–2% Triton-X). Triton-X shifted the pCa-force relationship, which is similar to the result of Hellam and Podolsky (1969) with another detergent (deoxycholate). The addition of 5 mM caffeine further shifted the relationship in the present study, but the amount of this shift (ΔpCa_{50}) was approximately the same as without the detergent.

If a similar direct effect of caffeine occurs in intact fibers, that should give rise to force in the unstimulated fiber. However, no tension with up to 3 mM caffeine at 25°C was

observed when checked carefully with the greatly increased sensitivity of the recorder. This raised the possibility that the effect of caffeine on Ca²⁺ sensitivity may be smaller, not greater, in intact preparations. (The lack of unstimulated force at 25°C with caffeine in intact fibers is unlikely to be due to a difference in the effect of caffeine itself at 0 and 25°C, since in rat skinned fibers the effect on Ca²⁺ sensitivity was the same at 25 and 5°C [Wendt and Stephenson, 1983].) Another possibility worth noting is that, based on earlier results (Stephenson and Williams, 1981; Godt and Lindley, 1982), also on skinned fibers, the control pCa-force relationship is itself shifted to the right at 22°C. The estimated leftward shift by caffeine (up to 3 mM), assuming that it occurs in the intact fiber, remains insufficient to give resting tension *in vivo*.

These control experiments and calculations, which show that caffeine effect on Ca²⁺ sensitivity does not make a significant contribution to the force development in intact fibers at 0°C, suggest that the role of caffeine in the temperature-step study is mainly confined to the effect on sarcoplasmic reticulum and that force is modulated with caffeine by Ca²⁺ levels in the myoplasm.

SLACK TESTS The unloaded speeds of shortening were determined by the slack test method for both the temperature-step and tetanic stimulations as described earlier (Gulati and Babu, 1984a). The speeds by this method on whole muscle and single fibers under various conditions are similar to the extrapolated estimates of unloaded speed from the force-velocity relations found from quick release or load steps in careful studies (A. V. Hill, 1970; Edman, 1979; Rome et al., 1985).

Calculations in which the thermal conductivity (k) of the fiber was taken as that of water (1.4×10^{-3} cal s⁻¹ cm⁻¹ °C⁻¹; Weast, 1972) at 20°C, and the largest fiber was assumed to be an approximate cylinder 200 μm in diameter, indicated that the average temperature should be near 0°C in slightly over 60 ms after the step (Carslaw and Jaeger, 1959).¹ For practical reasons, we usually waited at least 3 s (during maximal activations and much longer during partial activations) after the step to apply the slack release. Since with electrical stimulations the fibers were retained at 0°C throughout, the slack release in this case was applied sooner (~300 ms) after force reached the plateau. These precautions assured that the speeds were obtained at the desired temperature.

The computer analysis of the records to find the slack duration (Δt) was as described previously (Gulati and Babu, 1984a). By this method, the Δt values were reproduced within 1 ms with repeated analysis.

Data Analysis

HILL'S FORCE-VELOCITY RELATIONSHIP This relationship (A. V. Hill, 1938) is written in the following form to give speed of shortening, V_e , for an external load, P_e :

$$\frac{P_e}{P_0} = \frac{1 - (V_e/V_{\max})}{1 + \left(\frac{P_0}{a} \cdot \frac{V_e}{V_{\max}}\right)}, \quad (1)$$

where P_0/a is an empirical constant (= 4; A. V. Hill, 1970). Other symbols are listed below.

¹ The time to reach 0°C throughout the fiber was found by using the curve corresponding to $\kappa t/a^2 = 0.8$ in Fig. 24 of Carslaw and Jaeger (1959). κ is thermal diffusivity and equals $k/\rho \cdot c_p$, where $\rho = 1$ g/cm³ and c_p (specific heat) = 1 cal/g/0°C. t is in seconds and a (fiber radius for the calculation) = 100 μm.

To include the level of activation and the effect of internal load (F_R), two assumptions are made: (a) that the intrinsic relative force-velocity relation is unaffected by the degree of activation, and (b) that the internal load affects the isotonic motion in the same way as the external load. Thus,

$$\frac{F_R}{P} = \frac{1 - (V_0/V_{\max})}{1 + \left(\frac{P}{a} \cdot \frac{V_0}{V_{\max}}\right)} \quad (2)$$

Eq. 2 is used in the present study by taking $P/a = P_0/a = 4$. This treatment is similar to that described earlier (Gulati and Babu, 1984a).

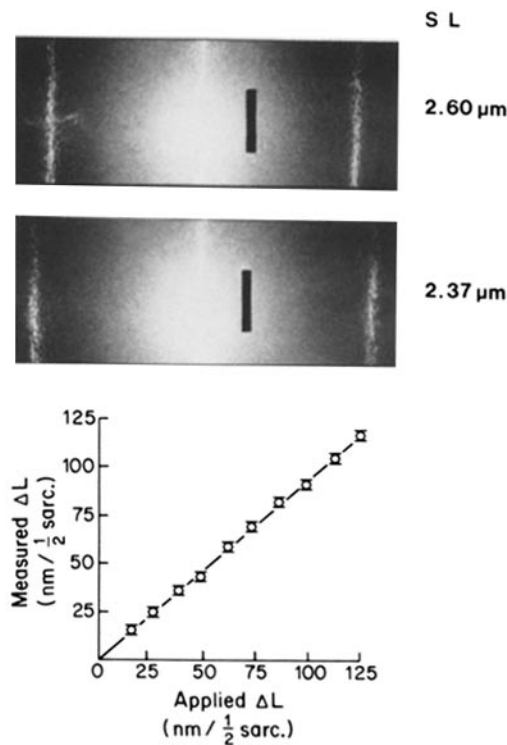


FIGURE 3. Laser diffraction patterns (first order) of a typical fiber at two lengths, for calibration of the motor arm. The centrally illuminated region is due to the main beam. The fiber length was altered by a signal of the known amount to the motor arm, and the distribution of the applied length change per half-sarcomere was calculated from the change in the distance between the first orders. The scale bars were photographed from the original screen and were useful for calibration. The relationship between applied and measured length changes gave a slope of 0.94.

STATISTICS Regression lines are found as best fits on the computer by least squares. Exponential best fits are computed similarly by using the equation: $A_1 \exp(A_2 x) + A_3$.

All data in the present study are reported as means \pm standard error. Statistical significance was assumed when Student's *t* test gave a *P* value of ≤ 0.05 . The paired *t* test was used whenever appropriate.

CALIBRATION OF THE SERVO-MOTOR ARM To translate the magnitude of an applied length step at one end of the fiber to the displacement within the sarcomeres, a standard calibration curve was made by direct measurements on a number of intact, relaxed fibers at 25°C. The excursion of the motor arm, 1.25 mm/V, was first determined under the microscope with an eyepiece micrometer ($\times 80$). Next, the fiber length was adjusted to make the sarcomere length 2.5–2.6 μm . The first-order laser diffraction pattern was projected onto a screen and photographed using Polaroid film (Fig. 3). Release steps of various amounts were applied with the motor arm, and the resulting changes in the diffraction patterns at three points along the length of the fiber (usually two near the ends and one in the middle) were photographed separately. The relation between the applied and the actual sarcomere length changes was plotted and the slope of the line from the least-squares fit was found to be 0.94. This factor is included in the conversion of applied length steps into nanometers per half-sarcomere in all experiments.

LIST OF SYMBOLS

F_R	internal load
L_0	resting intact fiber length (millimeters) between tendons, corresponding to a sarcomere length of 2.2–2.3 μm
ΔL	magnitude of the shortening step during a slack test (expressed as % L_0 or as nanometers per half-sarcomere)
P_0	tetanic force with electrical stimulation; P_1 , maximal isometric force level with temperature step; P , force level for partial activation
P_e	external load
t_0	time zero at which the slack release is initiated
Δt	slack time (from t_0 to the time at which the period of slack ends)
V_e	speed of shortening for an external load P_e
V_0	speed of shortening at zero external load as determined by the slack test; $V_0 = V_{\text{max}}$, for electrical stimulation

RESULTS

The slack test was used to determine the speed of unloaded shortening at 0°C. The temperature-step method with caffeine allowed an experimental control of the degree of activation under relatively steady state conditions (Fig. 1) and was used for the majority of studies. However, in the first few cases, the contraction properties under maximal activation by temperature step were compared with tetanus.

Fig. 4 shows typical results of slack tests on one fiber. Six slack releases varying from 49 to 110 nm per half-sarcomere were applied during the force plateau. With each release, force dropped to zero and stayed there for Δt ms (duration measured between t_0 and the time marked by the white arrowhead in each trace). Δt varied with the amount of release. Fig. 4C plots these values and yields estimates of the speeds of unloaded shortening: 2.5 and 2.7 μm per half-sarcomere per second with temperature-step activation and tetanus, respectively.

The results of such paired studies on four fibers in Table II gave: 2.7 ± 0.3 (temperature step) and 3.1 ± 0.3 μm per half-sarcomere per second (tetanus). The difference was significant (paired t test, $P < 0.02$).

Table II also gives the results of isometric force with the two types of activations for 13 fibers. Tetanic force was somewhat greater than the maximal force with

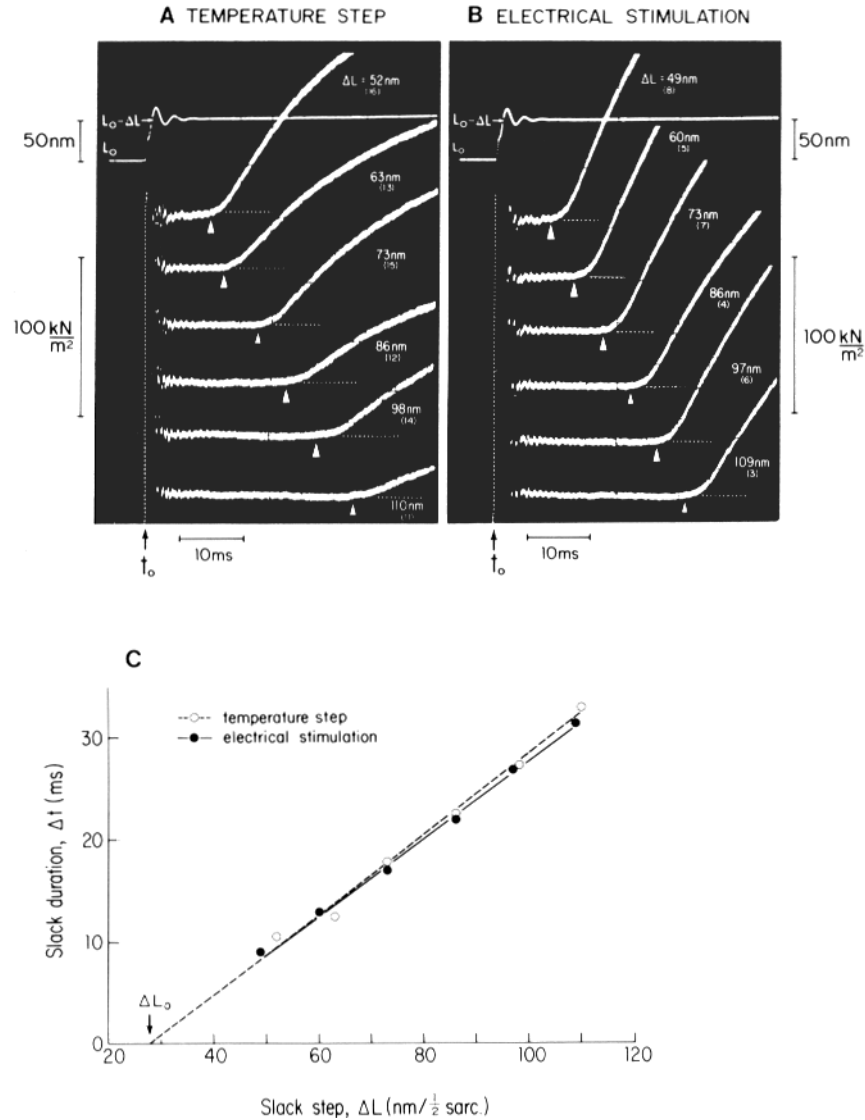


FIGURE 4. Speed of unloaded shortening with slack tests (experiment 21 v 82; fiber diameter, 116 μm ; L_0 , 7.12 mm). (A) Maximal activation with temperature step. (B) Tetanic stimulation. The top traces in A and B show typical length steps; upward deflection indicates shortening. The remaining six traces in each case show force responses to progressively larger shortening steps, indicated by ΔL values. The numbers in parentheses indicate the random order in the step sequence. The vertical dashed lines mark the start of step at t_0 , coinciding with arrow e in Fig. 1B; the arrowheads mark the end of the slack period computed by the method described earlier (Gulati and Babu, 1984a); the horizontal dashed lines mark the zero of force. Note that the slack duration Δt becomes progressively longer for larger shortening steps. (C) Plots of ΔL vs. Δt , with regression lines.

TABLE II
Contraction Parameters at 0°C with Different Modes of Activation

	Electrical stimulation	Temperature-step activation
Maximal isometric force per area (kN/m ²)*	312±16 (13)	238±12 (13)
Unloaded speed of shortening (μm/half-sarcomere/s)	3.07±0.32 (4)	2.70±0.31 (4)

* The results are from paired measurements for 13 fibers. Four of these fibers were used for the determination of speed in the second row.

temperature step. Sakai and Kurihara (1974) had also seen a similar difference in force of these types of stimulations on whole sartorius muscle.

To rule out the possibility that the lower force in temperature step was a transient effect following the step, we did a control experiment with electrical stimulation where the fiber at 25°C was similarly brought down to 0°C (without caffeine). Tetanic force after 3, 10, 15, and 20 s at 0°C was 239 ± 5 kN/m², nearly the same as after 15 min equilibration at 0°C (227 ± 5 kN/m²) in four different trials, which indicates that the temperature in the fiber was at 0°C soon after the step. The fact that force of temperature step is constant for >10 s (Fig. 1A) (in other cases, we had measured the same behavior up to 25 s [Fig. 1A in Gulati and Babu, 1984a; semitendinosus fiber]) further supports this view.

The difference in the forces of temperature-step activation and tetanus was maintained after Cl⁻ in the bathing medium was replaced by NO₃⁻ (Table III), which suggests that the above effect is not due to the anion. The possibility of caffeine having an indirect effect of selectively turning off a part of the contractile system is discussed later (p. 497).

Partial Activation

To determine the effects of the degree of activation on contraction speed, slack tests were next applied to partially activated intact fibers. Fig. 5 shows typical

TABLE III
Effect of Nitrate Anions on the Maximal Isometric Force at 0°C

	Force per cross-sectional area (kN/m ²)	
	Cl solution	NO ₃ solution
Electrical stimulation		
Twitch	192±12	239±11
Tetanus	300±11	300±11
Temperature-step activation	245±9	236±12

All data in this table were obtained on the same five fibers at a sarcomere length of 2.3 μm. The effects of NO₃⁻ in twitch and tetanus are comparable to the finding of Lopez et al. (1981) that twitch was greatly potentiated, but tetanic force was affected by <7%.

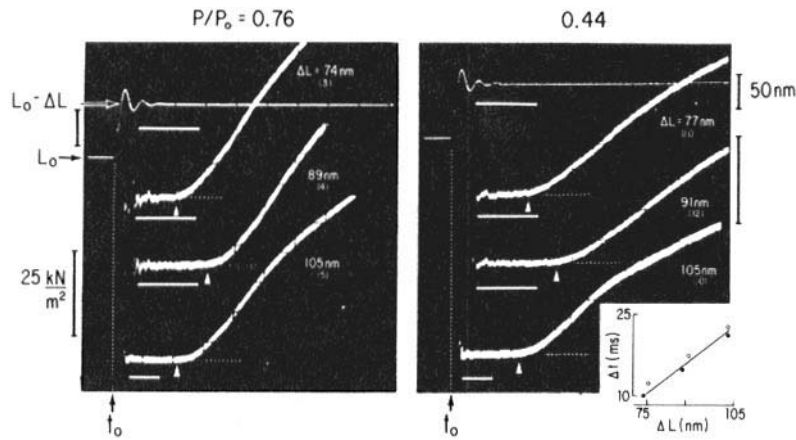


FIGURE 5. Effect of partial activation on a fiber. The solid white line below each force trace is a 10-ms time marker; note that the time scales for the third force trace in each panel are two times those for the other traces. For other notations, see Fig. 4. Inset: $\Delta L - \Delta t$ plots; closed circles: $P/P_0 = 0.76$; open circles: $P/P_0 = 0.44$ (experiment 28 iv 82; fiber diameter, 175 μm ; L_0 , 6.31 mm).

results on one fiber at activation levels, P/P_0 , of 0.76 and 0.44. The force responses to three releases, varying between 74 and 105 nm per half-sarcomere in increasing order, gave 2.6 μm per half-sarcomere per second for the speed of unloaded shortening for both the maximal and partial activations.

Fig. 6 shows the force response to the same release (112 nm, corresponding to $\sim 10\%$ of L_0) for another fiber, at three activation levels ($P/P_0 = 0.76$, 0.60, and 0.29). The slack durations for these records were found to be 33.3, 34.2, and 35.8 ms, respectively, which are nearly the same. The combined data ($\Delta L - \Delta t$ plots) for nine fibers in the range of 0.1–0.76 for P/P_0 are shown in Fig. 7. The data points are grouped into two batches; one batch is for maximal activations ($P/P_0 = 0.76$) and the second batch includes all the results for partial activations ($P/P_0 = 0.1-0.6$). The former gave a speed of shortening of 2.4 and

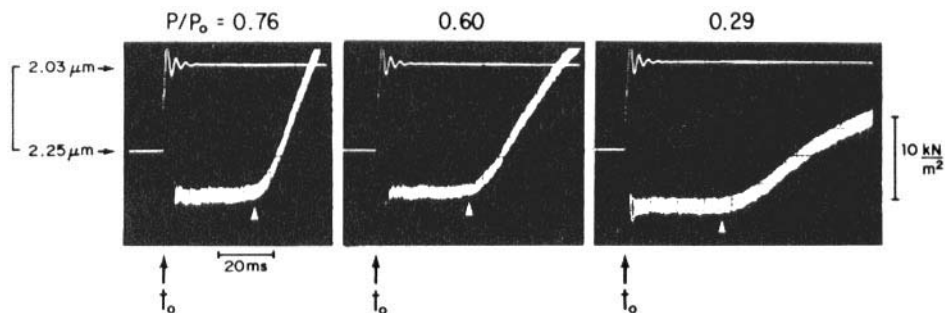


FIGURE 6. The slack response to a fixed length release ($\Delta L = 112$ nm) at various activation levels (experiment 27 iv 82; fiber diameter, 170 μm ; $L_0 = 7.62$ mm). See Fig. 4 for notations.

the latter $2.1 \mu\text{m}$ per half-sarcomere per second, which shows that the speed is substantially constant over a wide range of activation levels with the possibility of only a small ($\sim 15\%$) effect on speed at lower force levels. The exponential fits to the data give similar results, and the conclusion is therefore independent of the type of the fit. The mean ΔL_0 intercept from the linear fits was slightly higher for the partial activations than for maximal activations ($\Delta L_0 = 47 \text{ nm}$ for

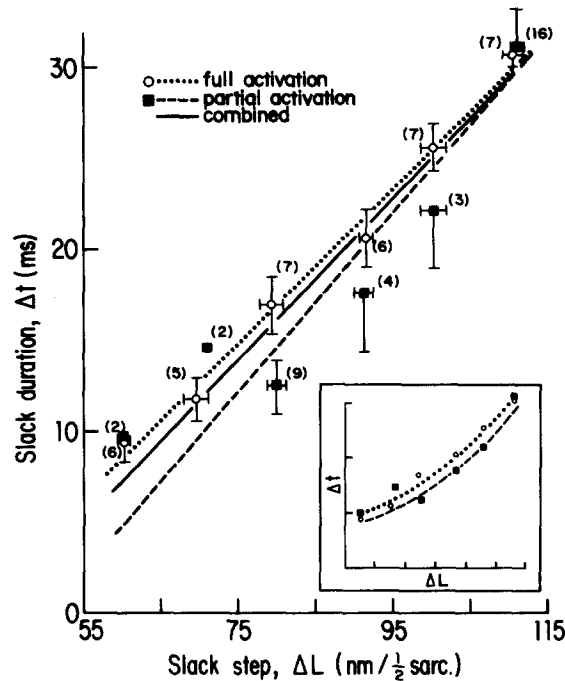


FIGURE 7. Combined results on nine fibers with temperature step (range of fiber diameter, $78\text{--}180 \mu\text{m}$; L_0 , $5.9\text{--}7.62 \text{ mm}$; caffeine, $0.85\text{--}2 \text{ mM}$). The regression lines give values for the speeds of shortening: 2.3 ± 0.2 (dotted line, $P/P_0 = 0.76$), 2.0 ± 0.3 (dashed line, $P/P_0 = 0.1\text{--}0.6$), and 2.2 ± 0.2 (solid line, $P/P_0 = 0.1\text{--}0.76$) μm per half-sarcomere per second. Inset: exponential best fit of the data. Fitted parameters are: full activation: $A_1 = 2.21 \pm 1.16$, $A_2 = 0.024 \pm 0.002$, $A_3 = 0$; partial activation: $A_1 = 1.85 \pm 1.40$, $A_2 = 0.025 \pm 0.003$, $A_3 = 0$.

$P/P_0 < 0.76$; 43 nm for $P/P_0 = 0.76$), but this was not a consistent finding from fiber to fiber (e.g., see inset to Fig. 5, where the ΔL_0 intercept is less in partial activation).

Fig. 8 gives the relationships between the speed of unloaded shortening and degree of activation, obtained by arranging the data in five batches. The dashed line is a fit through points up to $0.76 P_0$ and it has a small positive slope (0.29 ± 0.42). The full line in Fig. 8 includes the results of tetanus, which extends the force range, and the slope is still positive, which indicates that the downward trend for the speed with lower force levels may be real. A similar trend is seen also when the data for individual fibers are plotted as in the inset to Fig. 8. Each

point is a paired measurement normalized to the value at $P/P_0 = 0.76$. The regression line in this case gives an intercept of 0.8 at zero relative force.

Studies on Skinned Fibers

COMPARISON OF RESULTS AT 0 AND 10°C The previous studies on skinned fibers (ionic strength, 190 mM; 0–5°C) gave results that are similar to the present results on intact fibers. However, the results in low ionic strength were different: Ca^{2+} had an effect on shortening, but the skinned fibers in low ionic strength developed increasing amounts of irreversible residual tension on repeated acti-

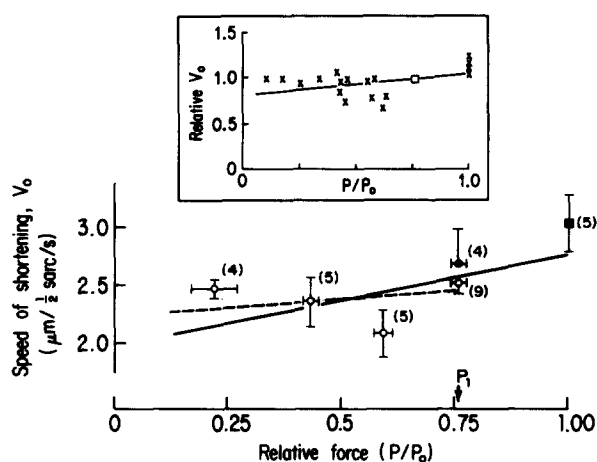


FIGURE 8. Speed of unloaded shortening as a function of the degree of activation. Speeds were determined for each fiber with its own $\Delta L - \Delta t$ plot as in Fig. 5. Symbols: \circ , data for nine fibers, used exclusively for temperature step; \bullet , four new fibers for temperature jump; \blacksquare , tetanus on the four new fibers as above and one additional fiber. Dashed line: $y = 2.24 (\pm 0.26) + 0.29 (\pm 0.42)x$; this is the least-squares fit for temperature jump only. Solid line: $y = 1.99 (\pm 0.25) + 0.79 (\pm 0.36)x$; this is the fit through all data points. Both fits show a decrease in speed with decreasing activation levels. Inset: relative speed on individual fibers normalized to the speed at $P/P_0 = 0.76$ for the same fiber.

vations (Thames et al., 1974; Gulati and Podolsky, 1981). A recent study at 10°C that employed 180 mM ionic strength still found a Ca^{2+} effect (Julian and Moss, 1981) and we wanted to check whether this condition also induced permanent changes in fiber properties.

The results on four chemically skinned frog fibers in Fig. 9 provide a test of their stability on activations at 0°C in solution 4 (another fiber was tested in a slightly modified solution with 1 mM total Mg), and at 10°C in solution 7 (two different fibers). The fiber was first contracted in pCa 5 for maximal force and then partially activated in pCa 5.3–6.2, selected to give about half-maximal force. In each contraction, during the plateau of force, a 20% L_0 length release was applied and the resulting force response was used for the slack test (inset to Fig. 9). One maximal contraction followed by one partial contraction is referred to as a cycle.

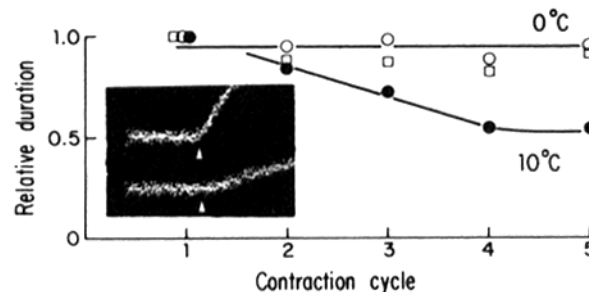


FIGURE 9. Behavior of the skinned fibers with repeated contraction cycles. Each point is the ratio of slack duration with maximal activation to the duration in partial activation for the same amount of length release (20% L_0). Note that the relative duration is close to unity at 0°C for all five cycles (open circles and squares; solution 4 used for \square ; for \circ , solution 4 was modified so that total $MgCl_2$ was 1 mM with the same ionic strength). At 10°C, this value is still one in the first cycle, but drops with each subsequent cycle (closed circles). Inset: the typical slack responses at 0°C for one cycle. The upper trace is the force response in pCa5 ($\Delta t = 81$ ms) and the force in the lower trace was 0.46 times maximal ($\Delta t = 83$ ms).

The results are shown in two parts. At 0°C, the slack duration is constant for all five cycles, which indicates that there is no Ca^{2+} effect on speed under these conditions throughout the experiment. In the second set of experiments at 10°C, the relative duration is still near 1 in the first cycle, which indicates that there is no Ca^{2+} effect in this cycle. However, the slack duration with partial activation increased as cycling was continued. By the fourth and fifth cycles, the estimated relative speed (i.e., the ratio of slack duration in partial to that in maximal activation) was close to 0.5. This factor is similar to that found by Julian and Moss (1981). Because in our study the effect of Ca^{2+} was not seen in the first cycle of contraction and appeared gradually with subsequent cycling, the results make clear that this apparent Ca^{2+} effect is not an original property of the fiber. Whether these alterations are the combined result of 10°C and the lack of regenerating system for ATP or of temperature alone was not further examined in this study.

FORCE AND THE SPEED OF SHORTENING WITH CAFFEINE In view of the fact that caffeine increased the Ca^{2+} sensitivity of the myofilaments in skinned fibers

TABLE IV
Effects of Caffeine on the Contraction Parameters of Frog Skinned Fibers
(0°C, pCa = 3)

	Caffeine	
	5 mM	10 mM
Relative force	1.00±0.01 (5)	0.96±0.02 (6)
Relative V_0	1.03±0.02 (4)	0.98±0.01 (4)

Values are the ratios of measurements in caffeine to those without caffeine. The numbers in parentheses refer to the fibers. The relative V_0 in these experiments was estimated from the 20% L_0 slack releases.

(see Methods), it was of interest to know whether this agent had effects on other contraction parameters of the frog fibers. We tested this by measuring the maximal force and by performing slack tests on skinned fibers in the presence of 5 and 10 mM caffeine (0°C). The results are given in Table IV.

Force in pCa 3 solutions (solution 4 with Pr) was the same with up to 10 mM caffeine. The slack duration following releases of up to 20% L_0 in paired studies is also found to be unaffected by caffeine in frog fibers. These results show that, aside from the effect in Fig. 2, there is no direct effect of caffeine on the force-generating properties and the turnover kinetics of the cycling cross-bridges (underlying the unloaded shortening). This gives greater confidence that the conclusions below regarding the degree of activation in intact fiber are valid.

DISCUSSION

The principal finding of this study is that the speed of unloaded shortening of isolated living fibers from frogs is practically independent of the degree of activation, with only a minor tendency for the speed to decrease at low force values. A wide range of force levels was attained in these experiments by employing rapid cooling at different caffeine concentrations, and this range probably reflects related changes in free Ca^{2+} concentrations in the myoplasm during activation. Therefore, the lack of a major effect on the intrinsic shortening speed at various force levels provides evidence that the contraction speed is insensitive to Ca^{2+} in the internal medium. This extends the conclusions of previous studies during twitch contractions with dantrolene (Edman, 1979) and D_2O (Cechhi et al., 1981) and during the rising phase of the tetanus (Lombardi and Menchetti, 1984). The results are expected on the basis of general steric blocking models (see Squire, 1981), where Ca^{2+} is an on-off switch for shifting the position of tropomyosin at the actin filament, and the modulation of force with Ca^{2+} is simply the result of change in the number of active bridges (Podolsky and Teichholz, 1970; Gulati and Podolsky, 1978). Furthermore, since the particular value of the intrinsic speed of shortening is determined by a rate-limiting step in the cross-bridge turnover cycle, and is also correlated with the V_{\max} of ATPase by actomyosin (Bárány, 1967), the present results indicate that the rate-limiting steps for both shortening and ATPase in the fiber are relatively unaffected by Ca^{2+} .²

There are also studies of actomyosin in solution, which suggests that Ca^{2+} has direct influence on a specific kinetic step (different from the rate-limiting step for V_{\max}) in the turnover cycle for ATPase. In these studies, at low ionic strength (Chalovich and Eisenberg, 1982), the regulatory proteins troponin-tropomyosin had little effect on the binding of actin and myosin (S-1) but, in the absence of Ca^{2+} , they still inhibited the rate of ATP hydrolysis by acto-S-1 (possibly by slowing the P_i release step). If this kinetic evidence is accepted for the range of physiological ionic strengths (150–200 mM) in the fiber, and because the cross-

² The exact molecular rate-limiting steps for the cross-bridge cycle are not yet determined. Some of the current possibilities included in a recent model of Eisenberg and Greene (see Eisenberg and Hill, 1985) were that the rate-limiting step for the ATPase cycle is an isomerization step ($\text{A} \cdot \text{M} \cdot \text{ADP} \cdot \text{P}_i \cdot \text{I} \rightarrow \text{II}$), and the motion is associated with a step after the P_i release.

bridge number is graded with partial activation (Yu et al., 1979; Brenner and Yu, 1983), a dual mechanism may be proposed for the regulation by Ca^{2+} . According to this, the Ca^{2+} effect on the above kinetic step in the fiber should also be essentially on-off, on top of steric blocking and unblocking by the structural shift in the tropomyosin position.

Since fewer cross-bridges are attached in the partially activated muscle, and since the turnover kinetics of the attached bridges are still the same, the results provide additional evidence that cross-bridges are independent force-generators in vivo (Gordon et al., 1966; A. F. Huxley, 1980).

Internal Load

In Fig. 8, there is a small but significant downward trend in speed at the lower activation levels, and a number of explanations may be considered for this effect.

One rests on the associated increase of the mean ΔL_0 intercept (an indicator in part of the overall fiber compliance) in the composite $\Delta L - \Delta t$ plot of partial activations (Fig. 7). We question this explanation because, in a previous study of compressed fibers (with 100 mM sucrose), a twofold decrease in speed was accompanied by a 20% decrease in ΔL_0 (see Fig. 5 in Gulati and Babu, 1984a).

Another possible explanation for the downward trend in speed is that it is a direct effect of Ca^{2+} ion. One way to imagine this is to consider that the Ca^{2+} effect on the kinetic rate-limiting step is slightly graded. This would complicate the interpretation of the steric blocking mechanism, but, even so, it is not a major effect.

The most straightforward explanation rests on the idea of an internal load within the fiber. An estimate for the internal load is derived in Fig. 10. The upper shaded area represents the range of observed speeds at the various activation levels (obtained from ± 1 SE of the solid regression line in Fig. 8). The dashed lines in Fig. 10 were calculated from Eq. 2. An internal load as small as 1–2% P_0 adequately explains the present results.

It is interesting that a frictional force of similar size had been measured in unstimulated whole muscle (D. K. Hill, 1968) and isolated fibers (Ford et al., 1977; Haugen and Sten-Knudsen, 1981; Gulati and Babu, 1984c) from their characteristic force response to stretching over a wide range of speeds. It was suggested that this resting force is the result of unusual cross-bridges in the unstimulated fiber (D. K. Hill, 1968), but the evidence is conflicting over whether these bridges are also present in the stimulated fiber. For instance, Ford et al. (1977) used this property to make corrections in their force transients, but the results were uneven for stretching and shortening and this was taken to suggest that the factors underlying the characteristic resting tension may be altered during contraction. On the other hand, radially compressed fibers (with the addition of impermeant solutes NaCl or sucrose to the bathing medium) were found to have both a much slower shortening speed and a greatly increased, and nearly matching, frictional force (Gulati and Babu, 1984a, c). This suggested that the bulk of the resistive component arising from the resting bridges persisted during active shortening. Clearly, more work would be useful in examining this point.

The elaborate cytoskeletal matrix may also be a factor for the internal load, in analogy with the proposal for passive tension (Wang and Ramirez-Mitchell, 1983; Maruyama et al., 1984). However, parts of the matrix should be collapsed in radially compressed fibers, where the internal load was increased (Gulati and

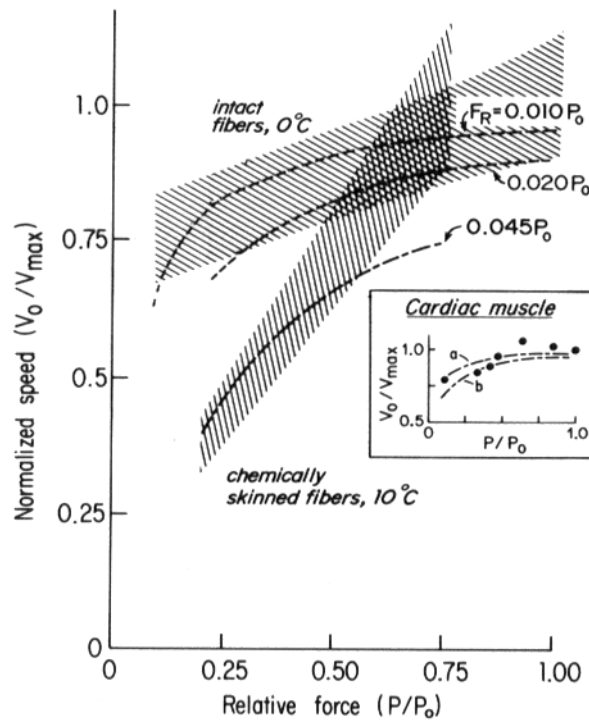


FIGURE 10. Theoretical relations between relative force (P/P_0) and normalized speed (V_0/V_{max}). The upper shaded area indicates the 95% confidence limits of data on intact fibers from Fig. 8. The skinned fiber data of Gulati and Podolsky (1978, 1981) at 0–4°C were within the same range. The lower shaded area indicates the skinned fiber data at 10°C combined from Figs. 2 and 10 of Julian and Moss (1981). The various dashed lines are found with different theoretical values for internal load by the use of Eq. 2. Note that 1–2% P_0 internal load covers the entire data at 0°C. The results at 10°C are fitted with an internal load of 4–5% P_0 for most of the data. Inset: the relation between speed and degree of activation of rat cardiac muscle. The theoretical lines to fit the data points (see text) are determined from Eq. 2 with $F_R = 0.5\% P_0$ (a) and 1% P_0 (b), which indicates for the myocardium, as for skeletal fibers above, that relatively small amounts of internal loads can explain the declining trends in speed at low degrees of activation.

Babu, 1984a), and any contribution of the cytoskeletal structures must therefore be small.

The explanation according to internal load (independent of its origin) for the drop in contraction speed at low activation levels is consistent with the main conclusion of this study that Ca^{2+} ions do not grade the cross-bridge properties but rather affect them in all-or-none manner.

Effect of Caffeine on the Contractile System of Intact Fiber

The force level in tetanic stimulation is slightly greater than the maximal force with temperature step (Table II). The speed is also lower with temperature step, but this follows the trend with partial activations (Fig. 8). A number of possibilities were considered for the force effect. One, that insufficient calcium was released from the sarcoplasmic reticulum, is not supported by the preliminary measurement with aequorin (Kurihara et al., 1984). Increasing the extracellular caffeine to a supramaximal level enhanced the light signal (produced by Ca^{2+} binding to aequorin), but this caused no additional force. Also, the lower maximal force with temperature step is not the result of direct inhibition by caffeine of force generation by the cycling cross-bridges (Table IV). An indirect action by the altered intracellular constituents (cAMP, cGMP, etc.) is still possible if, for instance, a fraction (~24%) of the cross-bridge population is selectively blocked. This can be examined separately by studying the effects of the above constituents on skinned fibers.

 Ca^{2+} Effect in Skinned Fibers at 10°C

The present study, by comparing the results of repeated contraction cycles at 0 and 10°C near physiological ionic strength, also helps resolve the controversy regarding Ca^{2+} effects in frog skinned fibers. Consistent with previous results (Table III in Gulati and Podolsky, 1981), as well as with the present intact fiber results (Fig. 8), Ca^{2+} has practically no effect on the unloaded shortening speed at 0°C (Fig. 9). We show that at 10°C (in solution 7), there is also no intrinsic Ca^{2+} effect on slack duration (in Fig. 9, the speed in the first cycle is same in partial and full activations), although here (10°C) the effect of Ca^{2+} was modified progressively over the course of the experiment, which indicates that the fiber properties were being permanently altered (see also Thames et al., 1974). By the fifth contraction cycle, the Ca^{2+} effect in our study was similar to that found by Julian and Moss (1981). Interestingly, Fig. 10 shows that the assumption of an increased internal load (to 4–5% P_0) could explain their results at 10°C over most of the force range, which suggests that the decrease in speed in partially activated fibers under those conditions is an indirect effect. A number of ways can be imagined that may cause such an effect when the fiber is modified (for instance, by the loss of troponin and/or myosin light chains), but a detailed discussion on this issue is outside the scope of the present study (see Podolin and Ford, 1983).

Relation to Cardiac Muscle

The present results have relevance to the study of Ca^{2+} effects in the heart muscle. The inset in Fig. 10 plots the results of Daniels et al. (1984; modified from their Fig. 6 by combining the $[Ca]_o$ -force and the $[Ca]_o$ -velocity plots) on rat trabeculae and includes fits of the data with Eq. 2. We assume that the internal load in the cardiac muscle is comparable to (or less than) that for intact fibers. The success of the present fits further supports the view that Ca^{2+} has relatively little effect on the intrinsic mechanism of shortening, and suggests that Ca^{2+} is a simple on-off switch in the intact myocardium as well.

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