Slowing of Velocity during Isotonic Shortening in Single Isolated Smooth Muscle Cells

Evidence for an Internal Load

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ABSTRACT   In single smooth muscle cells, shortening velocity slows continuously during the course of an isotonic (fixed force) contraction (Warshaw, D. M. 1987. *J. Gen. Physiol.* 89:771–789). To distinguish among several possible explanations for this slowing, single smooth muscle cells were isolated from the gastric muscularis of the toad (*Bufo marinus*) and attached to an ultrasensitive force transducer and a length displacement device. Cells were stimulated electrically and produced maximum stress of 144 mN/mm². Cell force was then reduced to and maintained at preset fractions of maximum, and cell shortening was allowed to occur. Cell stiffness, a measure of relative numbers of attached crossbridges, was measured during isotonic shortening by imposing 50-Hz sinusoidal force oscillations. Continuous slowing of shortening velocity was observed during isotonic shortening at all force levels. This slowing was not related to the time after the onset of stimulation or due to reduced isometric force generating capacity. Stiffness did not change significantly over the course of an isotonic shortening response, suggesting that the observed slowing was not the result of reduced numbers of cycling crossbridges. Furthermore, isotonic shortening velocity was better described as a function of the extent of shortening than as a function of the time after the onset of the release. Therefore, we propose that slowing during isotonic shortening in single isolated smooth muscle cells is the result of an internal load that opposes shortening and increases as cell length decreases.

INTRODUCTION

The hyperbolic form of the relationship between isotonic force and shortening velocity, first described in skeletal muscle (Hill, 1938), is now a familiar aspect of smooth muscle mechanics (Murphy, 1976; Hellstrand and Paul, 1982; Warshaw, 1987) and has become a basis for testing theoretical models of crossbridge cycling (Huxley, 1957; Eisenberg et al., 1980). The force–velocity relationship, as defined by

Hill (1938), indicates that in living skeletal muscle each force level is associated with a unique and constant shortening velocity. However, this is not the case for all muscle preparations. We have previously observed progressive slowing of shortening velocity during a single isotonic shortening response in intact single isolated smooth muscle cells (Warshaw, 1987; Warshaw et al., 1987b). Since this phenomenon has also been reported in cardiac muscle tissue (Chiu et al., 1982), skinned skeletal muscle fibers (Gulati and Podolsky, 1981; Moss, 1982), skinned smooth muscle tissue (Arner and Hellstrand, 1985; Brenner, 1986), and intact smooth muscle tissue (Herlihy and Murphy, 1974; Mulvany, 1979), it appears that slowing during isotonic shortening is a property of many muscle types.

At least three explanations have been proposed to account for slowing of isotonic shortening velocity: (a) Shortening velocity may vary as a function of the extent of shortening as a result of either an internal load within the muscle that opposes shortening and increases as cell length decreases (Brenner, 1986) or a shortening-dependent alteration in the kinetics of crossbridge cycling (Moss, 1986). (b) Cooperative interactions involving contractile filament proteins may affect the affinity of myosin crossbridges for binding sites on actin (Bremel et al., 1972; Bremel and Weber, 1972). If this is the case, the reduction in the number of attached crossbridges thought to occur when isometrically contracting muscle is released and allowed to shorten against a fixed load (Huxley, 1957; Eisenberg et al., 1980) would reduce the number of attached crossbridges even further. The muscle would progressively slow (Podolin and Ford, 1983) as the crossbridges that remain bound bear progressively larger loads. Depending on the extent of the cooperative interactions, a steady state might eventually be reached, but initially at least, slowing would occur. (c) Slowing of crossbridge cycling may be time dependent (Dillon et al., 1981; Butler et al., 1986). This study is an attempt to distinguish among these explanations for slowing during isotonic shortening in single smooth muscle cells.

METHODS

Cell Isolation and Preparation

The procedure for isolation of single smooth muscle cells from the gastric muscularis of the giant toad (*Bufo marinus*) has been described in detail elsewhere (Warshaw and Fay, 1983). Briefly, single smooth muscle cells were enzymatically isolated from the tissue and suspended in amphibian physiological saline (APS). A 20-μl aliquot of cells was transferred to a glass slide containing a 0.5-ml bubble of APS with 10 μM isoproterenol added to keep the cells from contracting during the attachment procedure. The cells were then viewed through an inverted microscope magnified 250 times. Cells were picked up with a micromanipulator. Using microprobes produced in the laboratory, the cells were tied between an ultrasensitive force transducer (model 406, natural frequency = 98 Hz, sensitivity = 14 mV/μN with microprobe attached; Cambridge Technology, Inc., Cambridge, MA) and a piezoelectric length displacement device (model PZ-40, natural frequency = 1 kHz, maximum displacement = 40 μm; Physik Instrumente, Waldbronn, FRG).

Next, the cells were stretched with the micromanipulator until a transient passive force of 0.2 μN was obtained. This procedure served to tighten the knots and prevent them from slipping during cell activation. The length of the cell remaining between the attachment sites after the knotting procedure was defined as L_{cell}. Setting cell length in this way produced

similar length–tension relationships in all cells studied (see Fig. 7). Furthermore, linear regression analysis of the data shown in Fig. 7 predicts that a decrease in length to 0.41 L_{cell} should eliminate the isometric force generating capacity of the cell. This predicted length is similar to the 0.39 L_o length estimated for smooth muscle tissue in which the length–tension relationship has been well characterized (Mulvany and Warshaw, 1979). The consistent length–tension relationship found in single cells and the similarity between the cell and tissue data suggest that setting cell length in this manner may place cells at about the same point on their length–tension relationship and that this length is close to the optimum for force generation. All experiments were performed at room temperature (20°C).

Cell dimensions were measured with a calibrated eyepiece micrometer (see Table I). Then, cells were stimulated with a series (1 Hz) of transverse electrical field stimulations (60 mA, 0.1-ms duration) delivered by platinum electrodes. Either cell force (isotonic protocols) or cell length (isometric protocols) was controlled by a personal computer (IBM PC-XT). The feedback circuit required for control of cell force has been described in detail elsewhere (Warshaw, 1987). Cell length, as measured by the output of an eddy current sensor (KD2300-.5SU, natural frequency = 5 kHz, resolution = 0.03 μm; Kaman Instrumentation Corp., Colorado Springs, CO) and force were recorded simultaneously on FM tape. Length and force records were digitized at 1 kHz for later computer analysis.

TABLE I

Single Smooth Muscle Cell Dimensional and Mechanical Parameters

Cell parameters	Units	Value	(n)
Length (L_{cell})	μm	72.8 ± 5.0	(21)
Cross-sectional area (CSA)	μm^2	14.6 ± 1.2	(21)
Active Force (F_{max})	μN	1.98 ± 0.17	(21)
Active Stress ($P_{max} = F_{max}/$CSA)	mN/mm^2	144.2 ± 7.9	(21)
Active Young's Modulus E_{act} (at 50 Hz)	× 10^4 mN/mm^2	0.51 ± 0.07	(5)

Values are means ± SE. *n* is the number of cells.

Experimental Protocols

Isotonic protocols

Two isotonic protocols were used. Stretching cells produced a transient passive force which decayed back to zero over the course of several seconds. Therefore, passive force was always zero. The computer was signaled to record this baseline force level. Then the cell was stimulated, and at the peak of isometric force the computer was signaled with a keystroke to record the maximum active force value (F_{max}). The computer first calculated the reference signals required by the feedback circuit to control cell force at the desired levels, and then began the isotonic protocol. Since cells subjected to multiple isotonic releases to the same force level showed the same initial velocity of shortening during the first 15 s after peak force was reached (Warshaw, 1987), all protocols were completed within the first 15 s.

Force–velocity protocol. This protocol was used to determine: (a) the relationship between force and shortening velocity, and (b) the time course of slowing during isotonic shortening. Cell force was reduced (in 50 ms) to and maintained for 1.0 s at five preset fractions of F_{max} between 0.2 and 0.75 F_{max} (Fig. 1). At each force level the cell was allowed to shorten. Between shortenings, cell force was ramped back to F_{max} over a period of 1.0 s. The last force step was

similar to the first force step, providing an internal control to assess whether any reduction of initial isotonic shortening velocity had taken place over the course of the experiment.

Cell stiffness is believed to be related to the number of attached crossbridges (Warshaw et al., 1988). To characterize changes in cell stiffness during isotonic shortening, the force–velocity protocol was also performed with sinusoidal oscillations (frequency = 50 Hz) superimposed on the force control signal (Fig. 2). The frequency response of the force feedback circuit (natural frequency of 75 Hz) limited the frequency at which stiffness during isotonic shortening could be measured.

Isotonic double step protocol. The purpose of this protocol was to determine whether isotonic shortening velocity is better described as a function of time after the beginning of the release or as a function of the extent of shortening. This protocol is similar to that used by Brenner (1986). Cell force was varied between two preset force levels in such a way that shortening responses were obtained at the same force level but starting from two different lengths (Fig. 3 A). First an isotonic release from F_{max} to 0.4 F_{max} (release 1) was imposed and shortening was allowed to proceed for ~1 s. Cell force was then returned to F_{max} within the
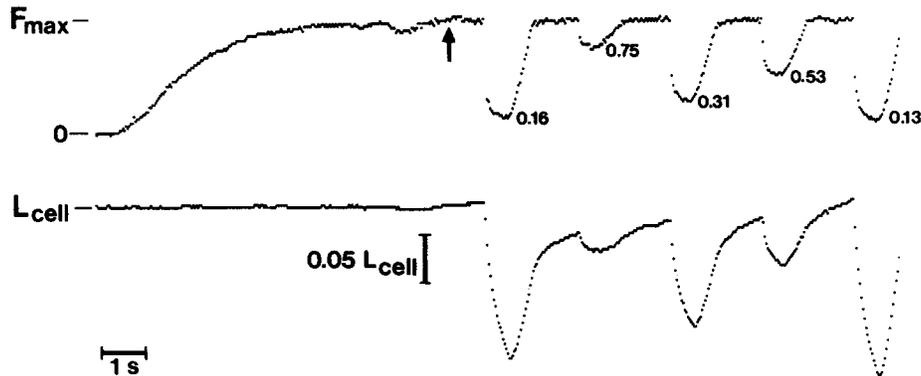

FIGURE 1.   Force–velocity protocol. Force (upper trace) and length (lower trace) are shown. Force control begins at the arrow. Isotonic releases are obtained at five preset fractions of maximum force (F_{max}). Resting force is zero. Numbers beside each isotonic release indicate the force level of that release. $L_{cell} = 70.4$ μm, $F_{max} = 2.56$ μN.

next 0.5 s. Next, cell force was stepped to 0.4 F_{max} for 250 ms (release 2), increased to 0.5 F_{max} for 250 ms, and stepped to 0.4 F_{max} again for another 250 ms (release 3). Using this protocol, three isotonic shortening responses were obtained at 0.4 F_{max}, however release 3 began from a reduced length.

The results expected from the isotonic double step protocol depend on whether isotonic shortening velocity is a function of the time after the onset of the release or the extent of shortening (Fig. 3, B and C). The length change vs. time and velocity vs. length change plots from releases 1 and 2 should superimpose regardless of whether velocity is a function of the extent of shortening or time after the step reduction in force, since these releases begin from the same length. However, isotonic release 3 will have a velocity vs. length change plot that overlies 1 and is continuous with 2 if velocity is a function of the extent of shortening, but not if velocity is a function of the time after the force step. Similarly, when the initial point of the length change vs. time trace of isotonic release 3 is placed over the point at which release 1 reaches the same length, release 3 will overlie release 1 only if shortening velocity is a function of the extent of shortening.
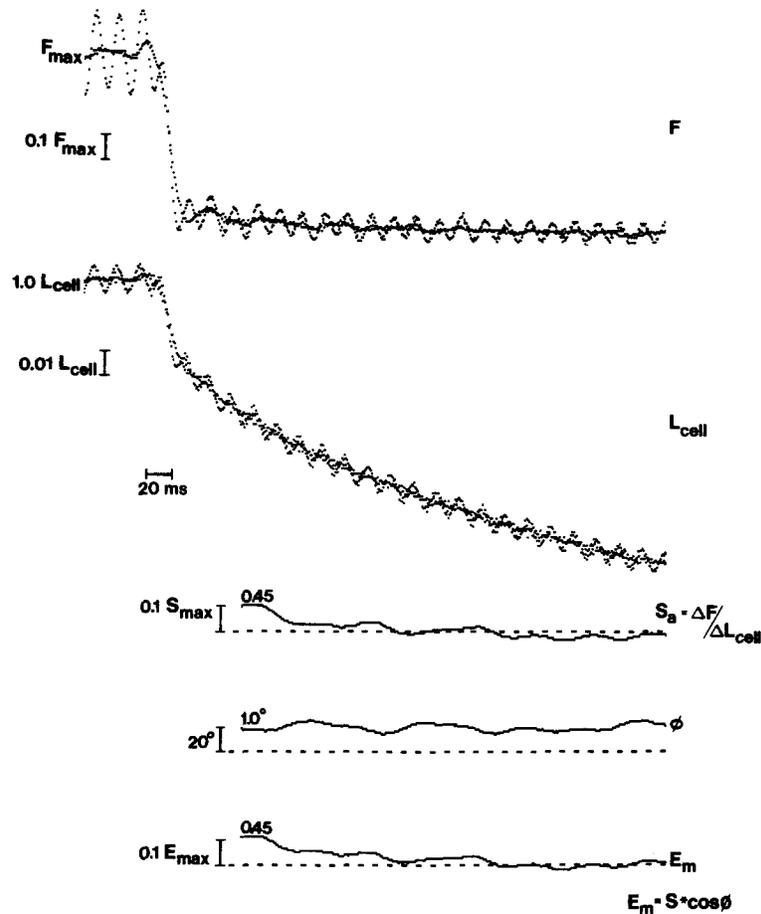
FIGURE 2. Isotonic shortening with stiffness measurements. An isotonic release obtained with the protocol shown in Fig. 1 has 50-Hz sinusoidal force oscillations added to allow continuous stiffness measurements during the isotonic response. Force (F) and length (L_{cell}) signals are shown before and after the extraction of the sine wave with a notch filter. Stiffness amplitude (S), phase angle (ϕ), and elastic modulus (E_m) for this isotonic shortening response are shown. Numbers at the beginning of the S and E_m traces are the fractions of S_{max} and E_{max} of the first point in the trace. The number at the beginning of the ϕ trace is the phase angle at the first point of the trace. $F_{max} = 3.02$ μN, $L_{cell} = 100.5$ μm, $E_{max} = 0.43 \times 10^4$ mN/mm^2. Dashed horizontal lines are placed for visual reference.

Isometric protocols

Two isometric protocols were performed. For both protocols, while the cell was relaxed, cell length was measured and input to the computer. The computer then calculated the control signals that drove the length displacement device to produce the desired length changes. The cell was then stimulated to contract. When F_{max} was reached, the computer was signaled with another keystroke and the length change protocol began.

Dynamic length–tension protocol. The purpose of this protocol was to determine the relationship between cell length and isometric force-generating capacity. If slowing during

isotonic shortening took place over a range of lengths in which isometric force-producing capacity decreased greatly, the decrease could provide a trivial explanation for slowing during isotonic shortening (see Discussion). In this protocol (Fig. 4) cell length was reduced in steps of ~0.1 L_{cell} until a length of 0.6 L_{cell} was reached. Then cell length was ramped back to L_{cell} in two ramps of equal size. Each step release was complete in 5 ms, while each return ramp was performed over 0.5 s. At each length 1.5 s was allowed for the cell to develop steady isometric force.

  Length step vs. ramp protocol. The purpose of this protocol was to determine whether a history of constant velocity shortening reduces the subsequent isometric force-generating
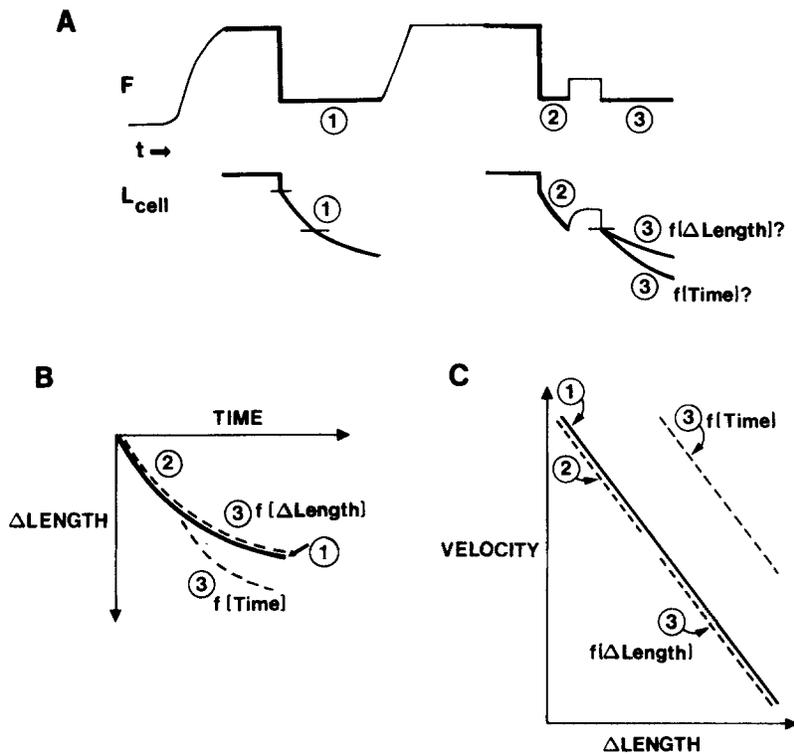

FIGURE 3.   Illustration of isotonic double-step protocol and predicted results. *A*, Force (*F*) and length change (Δ Length) traces are shown. Three isotonic releases are obtained at the same force level. Release 3 begins from a reduced length. Results predicted for length change vs. time (*B*) and shortening velocity vs. length change (*C*) plots if shortening velocity is a function of cell length change or of the time after the onset of the release.

capacity of a cell (i.e., shortening deactivation). If shortening at a constant velocity deactivates these cells, then isotonic shortening may deactivate them as well and cause slowing. Isovelocity shortening was used to approximate isotonic conditions because the control system cannot be switched back and forth between length and force control within a single protocol. Isovelocity shortening in these cells does not produce a constant force; however, it does allow the cell to shorten under load as in an isotonic contraction. In this protocol (Fig. 5) cell length was stepped to 0.94 L_{cell} in 2.5 ms, and the cell was allowed to redevelop force. Then cell length

was ramped back to L_{cell} over 0.5 s. Next, cell length was decreased by 0.015 L_{cell} in 2.5 ms to approximate the series elastic recoil of the load step and then immediately ramped to 0.94 L_{cell} over 2 s. Once again, the cell was allowed to redevelop force after the period of isovelocity shortening.

Data Analysis

Shortening velocity. The velocity of shortening (V) at any time (t) is the slope of the length (L) vs. time trace at that time point ($V = dL/dt$). Therefore, shortening velocity could be computed by one of two methods: (a) Length vs. time records, beginning at the time that the force step was 90% complete, were fitted with a single exponential using the nonlinear regression routine from the BMDP statistical software package as previously described (Warshaw, 1987). Shortening velocity was then computed from the first derivative of the equation of the fit. Only fits with $r^2 > 0.80$ were included in the data analysis. The rate constant calculated from the equation fit to the length vs. time record, also describes the rate
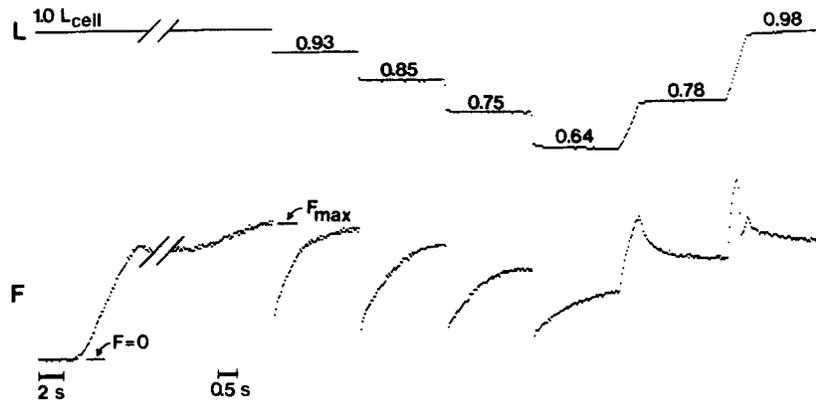

FIGURE 4.   Dynamic length–tension protocol. Length (L) and force (F) are shown. At peak isometric force (F_{max}) length is stepped to predetermined fractions of L_{cell}. The cell is allowed to develop isometric force at each length. Numbers on the length trace indicate the fraction of L_{cell} to which each step was made. Relaxed force is zero. $L_{cell} = 53.6$ μm, $F_{max} = 1.02$ μN.

of slowing of isotonic shortening velocity. (b) Length vs. time records were digitally differentiated using a 21-point convolution procedure (Savitsky and Golay, 1964). The high frequency noise produced by this routine was removed with a lowpass digital filter with a 10-Hz cutoff frequency. Velocity vs. time records after filtering were superimposed over and visually compared with unfiltered records in both the frequency and the time domains to ascertain that only high frequency noise had been removed.

Initial velocity of shortening for each isotonic response (V_i) was defined as the shortening velocity at the time that the force step was 90% complete. No difference was found between the initial velocities of shortening computed by these two methods.

Velocity vs. length change. Since shortening velocity, muscle length, and time are interrelated quantities, the empirical assumption of a mathematical form for the fitting of the length vs. time record (a requirement for determining shortening velocity by method *a*) also defines the relationship between shortening velocity and cell length. Therefore, the digital differentiation method (*b* above) of computing velocity, which does not require any empirical assumptions, was used for the analysis of shortening velocity vs. cell length change.

Force vs. velocity. For the theoretical analysis of slowing during an isotonic shortening response (see Appendix), it was necessary to define the force–velocity relationship. Initial velocity of shortening (computed by method *a* above) vs. force data were fitted with a rectangular hyperbola (Warshaw, 1987) using nonlinear regression analysis (BMDP statistical software). The equation describing the relationship between force (F) and initial shortening velocity (V_i) is: $[(F/F_{max}) + (a/F_{max})](V_i + b) = [1 + (a/F_{max})] \cdot b$.

Cell stiffness and elastic modulus. Cell stiffness during isotonic shortening was computed as follows. Length and force vs. time records were corrected digitally for transducer resonance (Ford et al., 1977) and for any phase angle produced by the measurement system. The imposed sinusoidal force oscillations and the resultant sinusoidal length oscillations (Fig. 2) were separated from the underlying force and length signals using a digital notch filter (cutoff frequencies = 45–55 Hz). Next, the force and length sinusoids were used to compute stiffness amplitude (S) and phase angle (ϕ) on a cycle-by-cycle basis (Warshaw et al., 1988). Stiffness amplitude is the amplitude of the force sinusoid divided by the amplitude of the length sinusoid. Phase angle is the phase difference between the length and force sinusoids. Finally, the elastic modulus ($E_m = S \cdot \cos \phi$) was computed from cell stiffness data. The elastic modulus
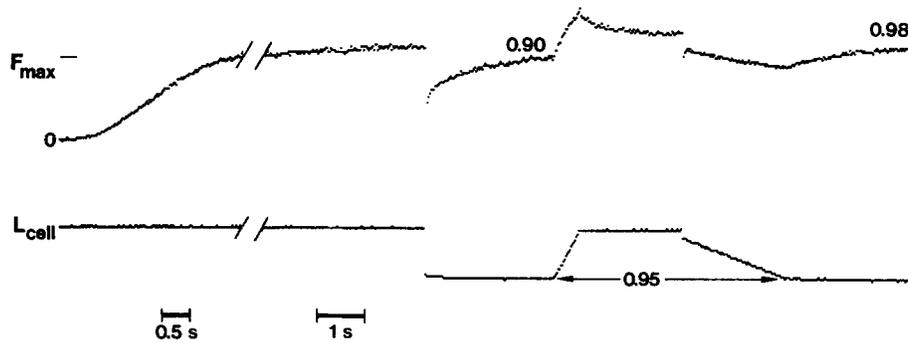

FIGURE 5.   Isometric step vs. ramp protocol. Force (upper trace) and length (lower trace) are shown. At peak force (F_{max}) cell length is stepped down to 0.95 L_{cell}, returned to L_{cell}, and then ramped down to 0.95 L_{cell} in 2 s. Resting force is zero. Numbers on the force trace indicate fractions of F_{max} redeveloped after a step and a ramp reduction in cell length. $L_{cell} = 67$ μm, $F_{max} = 2.49$ μN.

was normalized to cell length (L_{cell}) and cross-sectional area (CSA) to obtain the active Young's modulus ($E_{act} = E_m \cdot L_{cell}/CSA$).

Statistics. Statistical comparisons of force, velocity, and stiffness measurements within cells were made with a Student's paired t test. When multiple comparisons were required (isotonic double-step protocol), ANOVA was used to determine if differences existed between groups. Once significance was determined by ANOVA, multiple comparisons were made between groups using Fisher's least significant difference test. All data are presented as means ± SE. A difference was considered significant at $P < 0.05$.

RESULTS

Force–Velocity Protocol

The force–velocity protocol (Fig. 1) provided length vs. time records, which allowed us to determine: (*a*) the initial velocity of isotonic shortening at each force level studied, (*b*) the rate of slowing of each isotonic shortening response, and (*c*) whether

TABLE II

Cell Elastic Modulus and Slowing of Shortening Velocity (n = 5)

F/F_{max}	0.235 ± 0.027	0.355 ± 0.007	0.499 ± 0.005	0.747 ± 0.005
V (start), L_{cell}/s	0.197 ± 0.027	0.147 ± 0.025	0.079 ± 0.010	0.025 ± 0.005
V (end), L_{cell}/s	0.044 ± 0.009*	0.029 ± 0.004*	0.012 ± 0.001*	0.003 ± 0.002*
E_m/E_{max} (start)	0.51 ± 0.03	0.62 ± 0.02	0.71 ± 0.02	0.88 ± 0.02
E_m/E_{max} (end)	0.41 ± 0.04 (NS)	0.55 ± 0.02 (NS)	0.68 ± 0.02 (NS)	0.88 ± 0.02 (NS)
L/L_{cell} (start)	0.949 ± 0.004	0.954 ± 0.007	0.954 ± 0.006	0.963 ± 0.007
L/L_{cell} (end)	0.893 ± 0.009	0.909 ± 0.006	0.929 ± 0.007	0.953 ± 0.007

Shown are velocity, elastic modulus, and cell length measurements for four force values at the earliest time point for which the elastic modulus can be accurately determined (start) and ~300 ms later (end). Force and elastic modulus are normalized to their maximum value (F_{max} and E_{max}). Length is normalized to L_{cell}. All values are means ± SE. n is the number of cells. V and E_m are compared at start and end (*, $P < 0.05$; NS, not significant).

slowing of shortening velocity occurred as a function of time after the initiation of stimulation.

At all force levels studied, the isotonic releases showed continuous slowing of shortening velocity as shortening progressed (Table II). For example, during the isotonic shortening response at 0.22 F_{max} shown in Fig. 6, shortening velocity declined from 0.48 L_{cell}/s to 0.29 L_{cell}/s as cell length decreased from 0.95 L_{cell} to 0.90 L_{cell} over the course of 130 ms.
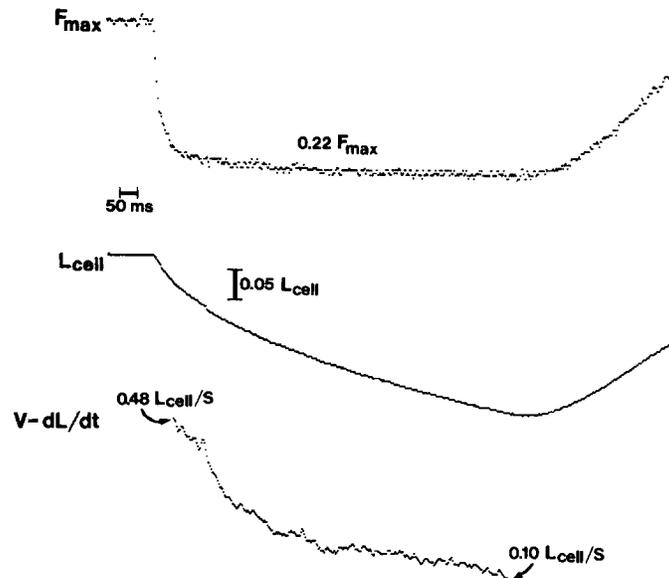

FIGURE 6. A single isotonic shortening response. Force (upper trace), cell length (middle trace), and shortening velocity (lower trace) vs. time are shown after a step reduction in force from peak isometric force (F_{max}). Note the continuous slowing as the shortening proceeds. The value of the force trace indicates the force, as a fraction of F_{max}, at which the shortening occurs. The shortening velocity (V) vs. time trace is obtained by digital differentiation of the length vs. time response. Numbers on the velocity trace indicate shortening velocity at the beginning and end of this isotonic shortening. $L_{cell} = 87.1$ μm, $F_{max} = 1.66$ μN.

TABLE III

Effect of Time after Stimulation on Isotonic Shortening Velocity (n = 8)

	First release	Last release
Force (F/F_{max})	0.21 ± 0.01	0.20 ± 0.02 (NS)
Initial shortening velocity V_i (L_{cell}/s)	0.30 ± 0.07	0.31 ± 0.04 (NS)
Slowing rate r (s^{-1})	−2.62 ± 0.26	−3.00 ± 0.43 (NS)

Time between first and last release = 9.5 s. The slowing rate (r) is the rate constant for the single exponential equation fitted to the length vs. time record. All values are means ± SE. n is the number of cells.

The first and last releases of the force–velocity protocol were made, as nearly as possible, to the same force level and began from the same starting length. Thus these two releases differed only in the time after the beginning of stimulation at which they were imposed. Comparison of these two sets of releases (Table III) showed that neither the initial velocity of shortening nor the rate of slowing changed during the time course of these experiments.

Isometric Control Protocols

To control for the possibility that a reduction in isometric force generating capacity at short lengths may be responsible for slowing during isotonic shortening, we
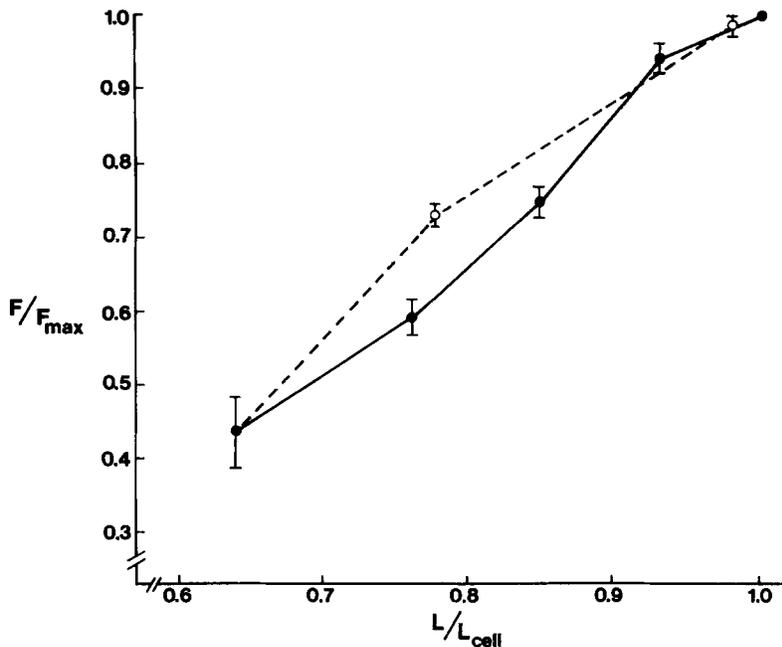

FIGURE 7. Length–tension relationship. Normalized isometric force (F/F_{max}) is plotted against normalized cell length (L/L_{cell}) . Solid symbols represent the force levels achieved after reductions in cell length using the protocol in Fig. 4. Open symbols are force levels after return ramp increases in cell length. For three cells, L_{cell} = 46.9 ± 6.7 μm and F_{max} = 1.53 ± 0.36 μN.

performed the dynamic length–tension protocol (Fig. 4). Fig. 7 shows the results obtained with this protocol. When cell length was reduced from 1.0 L_{cell} to 0.93 L_{cell}, isometric force-producing capacity was reduced by only 0.06 ± 0.02 F_{max}.

To control for the possibility that shortening under load deactivated the cell to a greater extent than a step decrease in length and thus caused slowing during isotonic shortening, we performed the length step vs. ramp protocol (Fig. 5). In three cells, isometric force after a step decrease in cell length to 0.94 L_{cell} was 0.88 ± 0.02 F_{max}, while isometric force after a ramp reduction in cell length to 0.94 L_{cell} was 0.92 ± 0.04 F_{max}. Thus, there was no decrease in isometric force-generating ability of the cell after isovelocity shortening to 0.94 L_{cell} beyond that seen following a step reduction in length of the same magnitude.

Elastic Modulus during Isotonic Shortening

A continuous reduction in the numbers of attached crossbridges could provide an explanation for slowing during isotonic shortening (Podolin and Ford, 1983). Therefore, to determine if the relative number of attached crossbridges changed during isotonic shortening, we measured the elastic modulus of the cell (Fig. 2) and used this value to estimate the relative number of attached crossbridges (Warshaw et al., 1988). Table II shows the shortening velocity and elastic modulus at both the earliest time point for which the elastic modulus can be accurately determined (25–60 ms after the force step is complete), and at the end of the isotonic release. Even though significant slowing was seen at all force levels, no significiant change in the elastic modulus of the cell was observed during shortening.

Isotonic Double-step Protocol

To determine if the observed slowing of shortening velocity was dependent on the extent of shortening or on the time after the force step, we used the isotonic double-step protocol (Figs. 3 and 8). In six experiments in which isotonic releases were made to 0.4 F_{max}, V_i for release 2 was 0.191 ± 0.013 L_{cell}/s, while for release 3 V_i was 0.101 ± 0.013 L_{cell}/s. This difference was significant. By comparison, the average velocity for release 2 when this release reached the length change at which shortening began in release 3 was 0.110 ± 0.010 L_{cell}/s. This value showed no significant difference from the V_i for release 3. Furthermore, when shortening velocity was plotted against extent of shortening (Fig. 8 C) the values for release 3 superimposed those for release 1 and were continuous with those for release 2. The relationship between isotonic shortening velocity and the extent of shortening was approximately linear.

DISCUSSION

This study explored the phenomenon of slowing of shortening velocity during a single isotonic shortening response in isolated smooth muscle cells. The following discussion will attempt to distinguish among several possible explanations for this slowing.
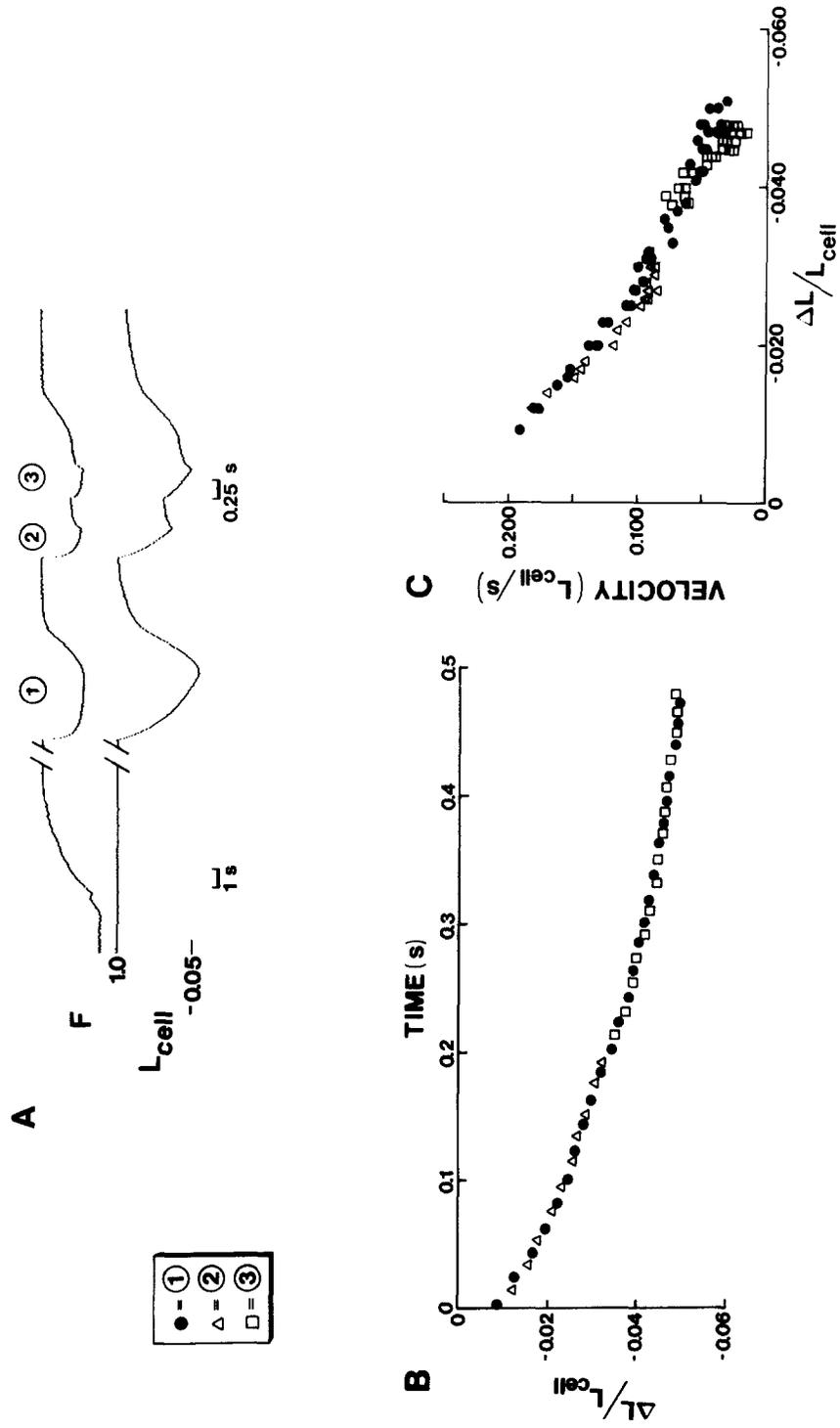
FIGURE 8.

Reduced Force-generating Capacity

A reduction in the isometric force-generating capacity, as a result of cell shortening, could lead to slowing of velocity during an isotonic shortening response. This dimunition in force production might be the result of length dependence of force generation, shortening deactivation, and/or nonuniformities in contractile unit lengths. Regardless of the mechanism, as the cell shortened against a fixed external load, its isometric force-producing capacity would be reduced. Therefore, at the shorter cell lengths, the fixed external load would become a progressively larger fraction of the maximum force-generating capacity of the cell. The cell would then be expected to slow as relative force increased, despite the fact that the absolute level of external force had remained constant. This possible explanation for slowing during isotonic shortening is considered below.

Length–tension relationship. Isometric force-producing capacity is known to decrease as muscle length is reduced below its optimum both in skeletal and in smooth muscle (Murphy, 1976). This effect of length on isometric force-producing capacity could provide a trivial explanation for slowing during isotonic shortening. However, at a load of $0.2\ F_{max}$, the amount of slowing observed as the cell shortened to $0.93\ L_{cell}$ is eight times greater than would be expected from the cell's dynamic length–tension relationship. These data show that length–tension effects could make only a small contribution to slowing during isotonic shortening.

Shortening deactivation. Shortening itself, either by a quick release or isotonically, can deactivate transiently stimulated skeletal muscle (Briden and Alpert, 1972). Step reductions in length can also deactivate tonically activated skeletal muscle (Edman, 1980) and smooth muscle tissue (Gunst, 1986). In striated muscle shortening deactivation is thought to be caused by the release of calcium from troponin-C (Ekelund and Edman, 1982), resulting in reduced numbers of attached crossbridges and decreased isometric force-generating capacity (Ridgway and Gordon, 1984). This could lead to slowing during isotonic contraction. The mechanism for shortening deactivation in smooth muscle is somewhat less clear but may also involve the release of calcium from regulatory proteins (Gunst, 1989).

Despite the finding that a step reduction in length to $0.93\ L_{cell}$ causes only a slight reduction in isometric force-producing capacity, it is still possible that shortening under load might deactivate single smooth muscle cells. However, there is no further reduction in isometric force-generating capacity caused by shortening under load beyond that resulting from a step reduction in muscle length (Fig. 5). Therefore, we conclude that although shortening deactivation has been observed by others in smooth muscle tissue (Gunst, 1986), shortening under load does not cause a

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FIGURE 8. (*opposite*) Isotonic double-step protocol and results. *A*, Isotonic shortenings take place at $0.4\ F_{max}$. See text for details of protocol. *B*, Length change vs. time plots obtained with this protocol. Circles indicate release 1, triangles indicate release 2, and squares indicate release 3. The initial point of release 3 has been placed over the point of release 1 which corresponds to the same cell length. *C*, Shortening velocity vs. cell length change plots. Symbols as for *B*. $L_{cell} = 50.2\ \mu m$, $F_{max} = 1.12\ \mu N$.

long-lasting deactivation of single smooth muscle cells greater than the effect of a step reduction in length. This finding does not rule out the possibility that isotonic shortening deactivates single isolated smooth muscle cells only during the period when the shortening is actually taking place. The stiffness measurements discussed below address this concern.

Nonuniformities in contractile unit lengths. In skeletal muscle, the development of sarcomere length heterogeneity, during shortening under load, reduces the subsequent isometric force-generating capacity at the shortened length (Julian and Morgan, 1979). Although the exact nature of the contractile unit in smooth muscle is not well defined, a similar phenomenon may occur in single smooth muscle cells and could explain slowing during isotonic shortening. However, as discussed above, in single smooth muscle cells shortening under load does not reduce subsequent isometric force-generating capacity more than a step reduction in length. Since the mechanical consequence of sarcomere heterogeneity developed during shortening under load in skeletal muscle is not observed in single smooth muscle cells, it is unlikely that nonuniformities in contractile unit lengths contribute significantly to slowing during isotonic shortening.

Slowing as a Function of Time after Stimulation

Many types of smooth muscle tissue exhibit reduced shortening velocities but maintained force production during prolonged stimulation (Siegman et al., 1985; Murphy, 1989). The reduced shortening velocity is presumably the result of a time-dependent slowing of the cycling rate of some (Dillon et al., 1981) or all (Butler et al., 1986) of the crossbridges. If such a phenomenon occurs in smooth muscle cells over the time course of a single isotonic release, it could account for the isotonic slowing reported here. However, we did not observe any slowing that is dependent on the duration of the stimulation over the time course of this experiment (Table III). Therefore, we conclude that while such time-dependent slowing is a well established phenomenon in smooth muscle tissue, it does not account for slowing within a single isotonic release reported in this study.

Slowing Caused by Cooperative Interactions

Evidence from in vitro experiments suggests that molecular cooperativity may affect the interactions of myosin heads with binding sites on actin either directly (Bremel et al., 1972) or via the binding of activator calcium to the thin filaments (Bremel and Weber, 1972). More recently, flash photolysis experiments have shown that similar interactions may occur in skinned smooth muscle tissue (Somlyo et al., 1988).

Many models of crossbridge cycling predict that the likelihood of a crossbridge being attached will be less in shortening muscle than in isometrically contracting muscle (Huxley, 1957; Eisenberg et al., 1980). If this is the case, when isometrically contracting muscle is allowed to shorten against a fixed load, the decrease in the number of attached crossbridges could decrease the opportunities for cooperative interactions and further reduce the number of attached crossbridges. Slowing would result as the crossbridges that remain active bear more force per crossbridge, resulting in lower velocities as predicted by the force–velocity relationship (Podolin and Ford, 1983). If crossbridges inactivated in this manner were inactive for the

remainder of the shortening, then this reduction in cooperative interactions could be a specific type of shortening deactivation.

If cooperativity does account for slowing during isotonic contraction, then the number of attached crossbridges must fall progressively as slowing during isotonic shortening proceeds. Muscle stiffness has been used extensively as a means of estimating relative numbers of attached crossbridges. In skeletal muscle virtually all of the elasticity is known to reside in the crossbridge (Ford et al., 1981). In smooth muscle tissue, the situation is considerably more complex because of the presence of
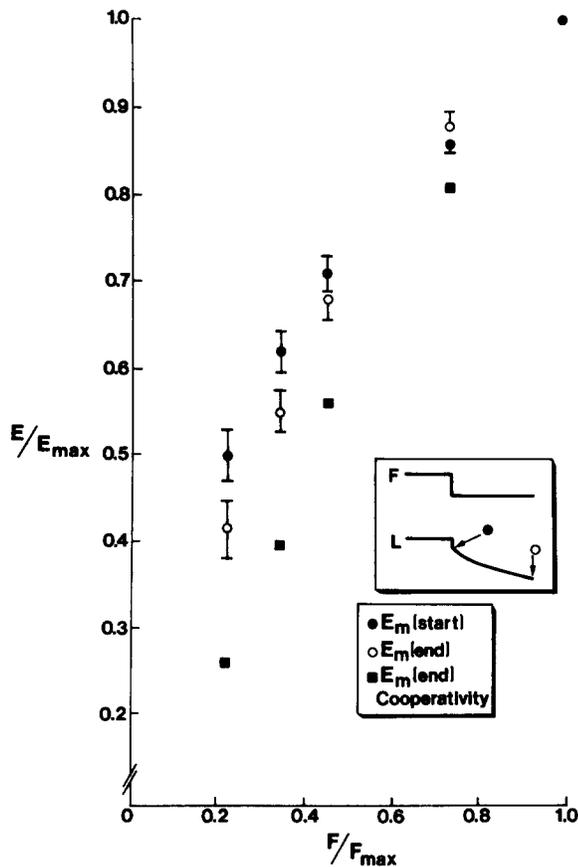

FIGURE 9. Elastic modulus vs. force. Normalized elastic modulus (E/E_{max}) is plotted against nomalized cell force (F/F_{max}). Solid circles indicate average elastic moduli values at the beginning of an isotonic shortening $(E_m[\text{start}])$ (see inset). Open circles are elastic moduli values at the end of an isotonic shortening $(E_m[\text{end}])$ for five cells (see inset). Solid squares indicate predicted stiffness values if slowing during isotonic shortening were caused by reduced numbers of attached crossbridges (see Appendix for calculations). Note that the reductions in cell stiffness necessary to explain slowing during isotonic shortening by reduced numbers of attached crossbridges are far greater than those observed.

elastic connective tissue both in series and in parallel with the muscle cells (Mulvany and Warshaw, 1981). Even in single isolated smooth muscle cells, where crossbridges are believed to contribute significantly to cell stiffness, there is an elasticity in series with the contractile machinery that has an exponential length vs. force relationship (Warshaw and Fay, 1983; Warshaw et al., 1988). This series elasticity must be considered in any attempt to estimate changes in relative numbers of attached crossbridges from stiffness measurements.

Fig. 9 compares the actual value of the elastic modulus at the beginning and end

of each isotonic release with the predicted value that would have occurred at the end of the release if isotonic slowing were caused by decreasing numbers of attached crossbridges (see Appendix). The decreases in cell stiffness necessary to explain slowing during isotonic shortening on the basis of cooperativity are at least three times the observed stiffness changes during isotonic shortening. Thus, although there is evidence for both shortening deactivation and cooperative interactions among contractile proteins in smooth muscle (Gunst, 1986; Somlyo et al., 1988), reduced numbers of attached crossbridges related to a cooperative mechanism (or any other mechanism such as shortening deactivation) cannot be the sole cause of slowing during isotonic shortening.

Slowing as a Function of the Extent of Shortening

It has already been demonstrated (Table III) that isotonic shortening velocity is not a function of the time after initiation of stimulation, at least over the time course of these experiments. Isotonic shortening velocity could still be a function of the time after the beginning of the isotonic release, however. The isotonic double-step protocol (Fig. 8) shows that isotonic shortening velocity is better described as a function of the extent of shortening than as a function of time after the beginning of the release.

Two possible mechanisms could account for the dependence of slowing during isotonic shortening on the extent of shortening: (a) the kinetics of crossbridge cycling may be modulated by the extent of muscle shortening (Moss, 1986); or (b) slowing may be caused by an internal load which opposes shortening and increases as cell length decreases (Brenner, 1986). If such a load is present, the total force against which the cell must contract would be the sum of this internal load plus any external load. In the presence of a fixed external load, as the cell shortens and the internal load increases, shortening velocity would decline as the cell moves to higher forces on its force–velocity relationship. In this model, since the extent of shortening determines the magnitude of the internal load, shortening velocity would be a function of the extent of shortening. While it is not possible, on the basis of the results reported here, to distinguish between a change in crossbridge cycling kinetics and an internal load, an internal load may be the more likely alternative, as discussed below.

Evidence that the kinetics of crossbridge cycling may be modulated by muscle shortening is supported by slack test measurements in submaximally activated skinned skeletal muscle fibers (Moss, 1986; Metzger and Moss, 1988). Moss (1986) observed that beyond 5–7% sarcomere shortening, the rate at which the muscle took up its slack decreased (i.e., velocity slowed). Since the speed of unloaded shortening is thought to be limited by the rate of crossbridge detachment (Huxley, 1957), Moss (1986) attributed this finding to a shortening-dependent decrease in the rate constant for crossbridge detachment. It is unlikely that such a crossbridge-related mechanism, observed only in submaximally activated striated muscle, could be responsible for the continuous slowing during isotonic shortening in these single smooth muscle cells. Fay and co-workers have shown that toad stomach smooth muscle cells, activated in a manner similar to that used in this study, achieve and

maintain levels of cytosolic calcium many times greater than those necessary for maximum force production (Williams et al., 1987; Yagi et al., 1988).

For slowing during isotonic shortening to be the result of an internal load that opposes shortening, this load would have to originate within a structure that exists in parallel with the contractile apparatus. Such an internal load-bearing structure would have to contribute to cell stiffness. If this structure is characterized by a linear force vs. length relationship, then its contribution to cell stiffness would be fixed. However, if the internal load increased exponentially as this structure was compressed, then its contribution to cell stiffness would also increase exponentially as the cell shortened (Warshaw and Fay, 1983). The finding that cell stiffness does not increase as cell length falls during an isotonic shortening argues that the internal load-bearing structure may have a linear force vs. length relationship, at least over the range of cell lengths studied.

Two of the models proposed here as explanations for slowing during isotonic shortening are not mutually exclusive. It is possible that reduced opportunity for cooperative interactions and an internal load act together to produce the slowing reported here. If the internal load had an exponential force vs. length relationship it is even conceivable that it would contribute an increase in stiffness equal to the stiffness decrease from the cooperative reduction in the number of attached crossbridges, so that overall cell stiffness would remain unchanged during isotonic shortening. While this possibility would explain the stiffness data, it would not be consistent with the linear velocity vs. cell length change data (Fig. 8 C), since an exponentially increasing internal load with muscle shortening would cause an approximately exponential fall in shortening velocity as the extent of shortening increased.

The findings reported here are at odds with those of Arner and Hellstrand (1985), who found that slowing during isotonic shortening in skinned smooth muscle tissue was dependent on the time after the initiation of the release rather than on muscle length. The differences between our results and those of Arner and Hellstrand (1985) may be protocol related. In both our work and that of Brenner (1986), two isotonic shortening responses to the same force level were made in the course of the same release. Arner and Hellstrand (1985) performed an isotonic release, restretched the muscle to a different length, allowed the muscle to equilibrate while contracting isometrically, and then performed another isotonic release to the same force level. If the internal load exists within a viscoelastic element (Chiu et al., 1982), then the isometric equilibration period between releases may have been sufficiently long to allow the force of the internal load to dissipate within the viscous element, thus resetting the elastic element to its rest length. Then, during the second isotonic release the same internal load could only be reached at a shorter muscle length compared with the first isotonic response, which could account for the different results.

What structure or force could give rise to the internal load? In resting smooth muscle some contractile units may be at such short lengths that upon active shortening these units could no longer shorten and would thus impede shortening of the entire muscle. However, it is more tempting to speculate that the internal load might result from the compression of a physical structure within the cell, perhaps an

element of the cytoskeleton (Brenner, 1986; Warshaw et al., 1987a). Finally, the internal load does not necessarily have to reside in a specific structure. For instance, the decrease in cell volume thought to occur as single isolated smooth muscle cells shorten (Fay, 1976) could produce an increase in internal osmotic pressure which could impede further shortening.

APPENDIX

Slowing during isotonic shortening may be caused by a reduction in cooperative interactions among contractile proteins. If this is the case, slowing will be accompanied by a progressive reduction in the number of attached crossbridges and thus in cell stiffness. Given the crossbridge contribution to cell stiffness at F_{max} (Warshaw et al., 1988) and the force–velocity relationship, one can calculate the drop in cell stiffness that would occur if slowing during isotonic shortening were the result of reduced numbers of attached crossbridges. This calculation is shown below.

Assumptions (based on Warshaw et al., 1988):

1. Total cell stiffness (S_{cell}) is made up of the crossbridge stiffness (S_{xb}) and the stiffness of an elastic component in series with the crossbridges (S_{sec}).

$$1/S_{cell} = 1/S_{xb} + 1/S_{sec} \tag{1}$$

2. The crossbridges have a linear force vs. length relationship. Thus S_{xb} is directly proportional to the number of attached crossbridges.

3. The series elastic component has an exponential force vs. length relationship. Thus S_{sec} is a function of force.

4. Immediately after an isometric quick release, the number of attached crossbridges, and thus S_{xb}, is unchanged. The observed reduction in S_{cell} is the result of a reduction in S_{sec}.

Definitions:

1. Stiffness and force values are normalized so that $F = 1$ and $S_{cell} = 1$ at F_{max}.

2. S'_{xb}, S'_{sec}, S'_{cell} = stiffness values after an isometric quick release.

3. S'' and F'' = stiffnesses and force values at the beginning of an isotonic shortening.

4. S''' and F''' = projected stiffnesses and force values that would occur at the end of an isotonic shortening if slowing were caused by a reduction in the number of active crossbridges (i.e., reduced cooperativity). $F''' > F''$ since at F''' the fixed external force is normalized to a reduced F_{max}, which is the result of fewer attached crossbridges.

5. V''' = measured velocity at the end of an isotonic shortening.

First, we will write an expression for crossbridge stiffness at the beginning of an isotonic shortening in terms of known quantities (see Eq. 3).

Warshaw et al. (1988) have shown that at the peak of isometric tension $S_{xb} = 1.32$. Therefore, $S'_{xb} = 1.32$ as well. Substituting into Eq. 1 and solving for S'_{sec} yields:

$$S'_{sec} = 1.32 \cdot S'_{cell} /(1.32 - S'_{cell}). \tag{2}$$

Since S_{sec} is determined by cell force, $S'_{sec} = S''_{sec}$ at any fixed external force. Substituting Eq. 2 into Eq. 1 and solving for S''_{xb} yields:

$$S''_{xb} = \frac{[1.32 \cdot S'_{cell} \cdot S''_{cell}/(1.32 - S'_{cell})]}{[[1.32 S'_{cell}/(1.32 - S'_{cell})] - S''_{cell}]}. \tag{3}$$

Given the relationships between S'_{cell} and force (Warshaw et al., 1988), and between S''_{cell} and force (Table II), S''_{xb} can be computed at any force level.

Next, the force–velocity relationship is used to predict the reduction in the number of attached crossbridges (i.e., crossbridge stiffness) that is necessary to account for the slowing observed during isotonic shortening (see Eq. 5).

$$[(F/F_{max}) + (a/F_{max})] \cdot (V + b) = [1 + (a/F_{max})] \cdot b \tag{4}$$

From the fit of the relationship between cell force and initial velocity of isotonic shortening for the cells upon which stiffness measurements were performed ($a/F_{max} = 1.36$, $b = 0.46$ L_{cell}/s), we can calculate F''' from V'''. If reduced cooperativity causes slowing during isotonic shortening, F must increase and S_{xb} must decrease as the number of active crossbridges falls during isotonic shortening. Therefore:

$$F''/F''' = S'''_{xb}/S''_{xb}. \tag{5}$$

Since F'', F''', and S''_{xb} are known, S'''_{xb} can be computed.

Knowing S'''_{xb}, S'''_{cell} can be calculated as follows: $S'''_{sec} = S'_{sec}$ since the absolute force level is constant during the isotonic response. Therefore, S'''_{cell} can be obtained from Eq. 1:

$$S'''_{cell} = S'_{sec} \cdot S'''_{xb}/(S'_{sec} + S'''_{xb}). \tag{6}$$

S'''_{cell} can be compared with the measured value of S_{cell} at the end of an isotonic release to ascertain if a large enough fall in cell stiffness occurred to explain slowing during isotonic shortening on the basis of reduced cooperative interactions.

REFERENCES

Arner, A., and P. Hellstrand. 1985. Effects of calcium and substrate on force-velocity relation and energy turnover in skinned smooth muscle of the guinea-pig. *Journal of Physiology.* 360:347–365.

Bremel, R. D., J. M. Murray, and A. Weber. 1972. Manifestations of cooperative behavior in the regulated actin filament during ATP hydrolysis in the presence of calcium. *Cold Spring Harbor Symposia on Quantitative Biology.* 37:267–275.

Bremel, R. D., and A. Weber. 1972. Cooperative behavior within the functional unit of the actin filament in vertebrate skeletal muscle. *Nature New Biology.* 338:97–104.

Brenner, B. 1986. The necessity of using two parameters to describe isotonic shortening velocity of muscle tissues: the effect of various interventions upon initial shortening velocity (v_i) and curvature (b). *Basic Research in Cardiology.* 81:54–69.

Briden, K. L., and N. R. Alpert. 1972. The effect of shortening on the time-course of active state decay. *Journal of General Physiology.* 60:202–220.

Butler, T. M., M. J. Siegman, and S. U. Mooers. 1986. Slowing of cross-bridge cycling in smooth muscle without evidence of an internal load. *American Journal of Physiology.* 251:C945–C950.

Chiu, Y., E. W. Ballou, and L. E. Ford. 1982. Velocity transients and viscoelastic resistance to active shortening in cat papillary muscle. *Biophysical Journal.* 40:121–128.

Dillon, P. F., M. O. Aksoy, S. P. Driska, and R. A. Murphy. 1981. Myosin phosphorylation and cross-bridge cycle in arterial smooth muscle. *Science.* 211:495–497.

Edman, K. A. P. 1980. Depression of mechanical performance by active shortening during twitch and tetanus of vertebrate muscle fibers. *Acta Physiologica Scandinavica.* 109:15–26.

Eisenberg, E. T., T. L. Hill, and Y. Chen. 1980. Cross-bridge model of muscle contraction. Quantitative analysis. *Biophysical Journal.* 29:195–227.

Ekelund, M. C., and K. A. P. Edman. 1982. Shortening induced deactivation of skinned fibers of frog and mouse striated muscle. *Acta Physiologica Scandinavica.* 116:189–199.

Fay, F. S. 1976. Structural and functional features of isolated smooth muscle cells. *In* Cell Motility, Book A. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 185–201.

Ford, L. E., H. E. Huxley, and R. M. Simmons. 1977. Tension responses to sudden length change in stimulated frog muscle fibers near slack length. *Journal of Physiology.* 269:441–515.

Ford, L. E., H. E. Huxley, and R. M. Simmons. 1981. The relation between stiffness and filament overlap in stimulated frog muscle fibers. *Journal of Physiology.* 331:219–249.

Gulati, J. T., and R. J. Podolsky. 1981. Isotonic contraction of skinned muscle fibers on a slow time base. *Journal of General Physiology.* 78:233–267.

Gunst, S. J. 1986. Effect of length history on contractile behavior in canine tracheal smooth muscle. *Ammerican Journal of Physiology.* 250:C146–C154.

Gunst, S. J. 1989. Effects of muscle length and load on intracellular Ca^{2+} in tracheal smooth muscle. *American Journal of Physiology.* 256:C807–C812.

Hellstrand, P., and R. J. Paul. 1982. Vascular smooth muscle: relations between energy metabolism and mechanics. *In* Vascular Smooth Muscle: Metabolic, Ionic, and Contractile Mechanisms. M. F. Crass III and C. D. Barnes, editors. Academic Press, New York. 1–35.

Herlihy, J. T., and R. A. Murphy. 1974. Force-velocity and series elastic characteristics of smooth muscle from the hog carotid artery. *Circulation Research.* 34:461–466.

Hill, A. V. 1938. The heat of shortening and the dynamic constraints of muscle. *Proceedings of the Royal Society of London, Series B.* 126:136–195.

Huxley, A. F. 1957. Muscle structure and theories of contraction. *Progress in Biophysics and Biophysical Chemistry.* 7:255–318.

Julian, F. J., and D. L. Morgan. 1979. The effect on tension of non-uniform distribution of length changes applied to frog muscle fibers. *Journal of Physiology.* 293:379–392.

Metzger, M., and R. L. Moss. 1988. Thin filament regulation of shortening velocity in rat skinned skeletal muscle: effects of osmotic compression. *Journal of Physiology.* 398:165–175.

Moss, R. L. 1982. The effect of calcium on the maximum velocity of shortening in skinned skeletal muscle fibers of the rabbit. *Journal of Muscle Research and Cell Motility.* 3:295–311.

Moss, R. L. 1986. Effects on shortening velocity of rabbit skeletal muscle due to variations in the level of thin filament activation. *Journal of Physiology.* 377:487–505.

Mulvany, M. J. 1979. The undamped and damped series elastic components of a vascular smooth muscle. *Biophysical Journal.* 26:401–414.

Mulvany, M. J., and D. M. Warshaw. 1979. The active tension-length curve of vascular smooth muscle related to its cellular components. *Journal of General Physiology.* 74:85–104.

Mulvany, M. J., and D. M. Warshaw. 1981. The anatomical location of the series elastic component in rat vascular smooth muscle. *Journal of Physiology.* 283:501–521.

Murphy, R. A. 1976. Contractile system function in mammalian smooth muscle. *Blood Vessels.* 13:1–23.

Murphy, R. A. 1989. Special topic: contraction in smooth muscle cells. *Annual Review of Physiology.* 51:275–284.

Podolin, R. A., and L. E. Ford. 1983. The influence of calcium on shortening velocity of skinned frog muscle cells. *Journal of Muscle Research and Cell Motility.* 4:263–282.

Ridgway, E. B., and A. M. Gordon. 1984. Muscle calcium transient: effect of post-stimulus length changes in single fibers. *Journal of General Physiology.* 83:75–103.

Savitsky, A., and M. Golay. 1964. Smoothing and differentiation of data by simlified least squares procedures. *Analytical Chemistry.* 36:1627–1639.

Siegman, M. J., T. M. Butler, and S. U. Mooers. 1985. Energetics and regulation of crossbridge states in mammalian smooth muscle. *Experientia.* 41:1020–1025.

Somlyo, A. V., Y. E. Goldman, T. Fujimori, M. Bond, D. R. Trentham, and A. P. Somlyo. 1988. Cross-bridge kinetics, cooperativity, and negatively strained cross-bridges in vertebrate smooth muscle. *Journal of General Physiology.* 91:165–192.

Warshaw, D. M. 1987. Force:velocity relationship in single isolated toad stomach smooth muscle cells. *Journal of General Physiology.* 89:771–789.

Warshaw, D. M., and F. S. Fay. 1983. Cross-bridge elasticity in single smooth muscle cells. *Journal of General Physiology.* 82:157–199.

Warshaw, D. M., W. J. McBride, and S. S. Work. 1987a. Corkscrew-like shortening in single smooth muscle cells. *Science.* 236:1457–1459.

Warshaw, D. M., D. D. Rees, and F. S. Fay. 1988. Characterization of cross-bridge elasticity and kinetics of cross-bridge cycling during force development in single smooth muscle cells. *Journal of General Physiology.* 91:761–779.

Warshaw, D. M., S. S. Work, and W. J. McBride. 1987b. Effect of low extracellular calcium on shortening velocity in isolated smooth muscle cells. *Pflügers Archiv.* 410:185–191.

Williams, D. A., P. L. Becker, and F. S. Fay. 1987. Regional changes in calcium underlying contraction of single smooth muscle cells. *Science.* 235:1644–1647.

Yagi, S., P. L. Becker, and F. S. Fay. 1988. Relationship between force and Ca^{2+} concentration in smooth muscle as revealed by measurements on single cells. *Proceedings of the National Academy of Sciences USA.* 85:4109–4113.