1988-06-01

Characterization of cross-bridge elasticity and kinetics of cross-bridge cycling during force development in single smooth muscle cells

David M. Warshaw
University of Massachusetts Medical School

Et al.

Characterization of Cross-Bridge Elasticity and Kinetics of Cross-Bridge Cycling during Force Development in Single Smooth Muscle Cells

DAVID M. WARSHAW, DIANNE D. REES, and FREDRIC S. FAY

From the Department of Physiology, University of Massachusetts Medical Center, Worcester, Massachusetts 01605

ABSTRACT Force development in smooth muscle, as in skeletal muscle, is believed to reflect recruitment of force-generating myosin cross-bridges. However, little is known about the events underlying cross-bridge recruitment as the muscle cell approaches peak isometric force and then enters a period of tension maintenance. In the present studies on single smooth muscle cells isolated from the toad (*Bufo marinus*) stomach muscularis, active muscle stiffness, calculated from the force response to small sinusoidal length changes (0.5% cell length, 250 Hz), was utilized to estimate the relative number of attached cross-bridges. By comparing stiffness during initial force development to stiffness during force redevelopment immediately after a quick release imposed at peak force, we propose that the instantaneous active stiffness of the cell reflects both a linearly elastic cross-bridge element having 1.5 times the compliance of the cross-bridge in frog skeletal muscle and a series elastic component having an exponential length-force relationship. At the onset of force development, the ratio of stiffness to force was 2.5 times greater than at peak isometric force. These data suggest that, upon activation, cross-bridges attach in at least two states (i.e., low-force–producing and high-force–producing) and redistribute to a steady state distribution at peak isometric force. The possibility that the cross-bridge cycling rate was modulated with time was also investigated by analyzing the time course of tension recovery to small, rapid step length changes (0.5% cell length in 2.5 ms) imposed during initial force development, at peak force, and after 15 s of tension maintenance. The rate of tension recovery slowed continuously throughout force development following activation and slowed further as force was maintained. Our results suggest that the kinetics of force production in smooth muscle may involve a redistribution of cross-bridge populations between two attached states and that the average cycling rate of these cross-bridges becomes slower with time during contraction.

INTRODUCTION

Force development in smooth muscle is believed to reflect the recruitment of force-generating myosin cross-bridges that cyclically interact with neighboring actin fila-

Address reprint requests to Dr. David M. Warshaw, Dept. of Physiology and Biophysics, University of Vermont, Burlington, VT 05405.

ments (for review, see Murphy, 1980; Fay et al., 1981; Hellstrand and Paul, 1982). In skeletal muscle, cycling cross-bridges are envisioned as going through a series of at least one detached and two attached cross-bridge states (Huxley and Simmons, 1971; Ford et al., 1986). Thus, at peak isometric force, a steady state distribution of cross-bridge states exists that is determined by the rate constants for the transitions between states. Understanding of similar processes in smooth muscle would be greatly enhanced by information about the relative number of attached cross-bridges and rate constants for transitions between states throughout the development and maintenance of force.

In skeletal muscle, the relative numbers of attached cross-bridges can be estimated by measuring active fiber stiffness since the elasticity appears to reside entirely within the cross-bridge (Huxley and Simmons, 1971). A similar approach can be applied to single smooth muscle cells. Since these cells exhibit considerably less stiffness than skeletal muscle (Warshaw and Fay, 1983b), the possibility must be investigated that structures other than cross-bridges contribute to fiber stiffness. We therefore designed an experimental protocol to determine the origin of the cell's elasticity. The results suggest that this elasticity resides in both cross-bridges and structures external to the force-generators.

Information about cross-bridge kinetics can be obtained from the tension response that results from a small, rapid change in the length of a single skeletal muscle fiber (Ford et al., 1977; Kawai and Brandt, 1980). We studied the tension responses to changes in muscle length throughout the development and maintenance of force in single smooth muscle cells. At early times after activation, we observed a greater stiffness-to-force ratio than at the peak of contraction, which suggests that the mean force per attached cross-bridge is changing during force development. In addition, tension transients reveal a slowing of tension recovery throughout the periods of force development and maintenance, which may reflect modulation of transition rates between cross-bridge states. Preliminary accounts of these results have been presented (Warshaw et al., 1980; Warshaw and Fay, 1984).

METHODS

Experimental Protocols and Data Analysis

Detailed descriptions of the procedures for isolating single smooth muscle cells (Fay et al., 1982) and their preparation for mechanical studies (Warshaw and Fay, 1983b; Warshaw, 1987) have been presented previously.

Origin of cell elasticity: rationale. In order to probe the events underlying force generation in a single smooth muscle cell, we have investigated the mechanical response to small rapid changes in cell length (L_{cell}). The initial phase of this force response appears to be elastic in that the change in force is coincident with the applied length change (see Fig. 1). A protocol was designed to determine the origin of this elastic response, by measuring single smooth muscle cell stiffness during force development following activation and, in the same cell, immediately following quick (2.5-ms) releases of between 0.5 and 2.0% L_{cell} applied during the period of peak force maintenance (see Fig. 2). Thus, cells were electrically stimulated to contract (Warshaw and Fay, 1983b) and 0.5% sinusoidal length oscillations (250 Hz) were applied to the cell throughout the period of force development and maintenance, providing a continuous measure of stiffness with a 4.0-ms time resolution (see *Stiffness Determinations* below).

The rationale for this protocol is that if the elastic response originates entirely in the force-generators, then as force develops after activation because of recruitment of active cross-bridges, stiffness should increase proportionately with force (see Fig. 2 *B*; Warshaw and Fay, 1983*b*). By contrast, at peak isometric force, stiffness immediately following a quick release should not change, assuming that the release is rapid relative to the detachment or attachment rates of the cross-bridges and that the force-generators in smooth muscle have a linear length-force characteristic, as in skeletal muscle (see Fig. 2 *B*). At the other extreme, the elastic response could originate entirely outside the population of force-generators, that is, in a series elastic component. If this is the case, the relationship between stiffness and force (see Fig. 2 *B*) should be fixed by the properties of the series elastic component and should be unaffected by how force is modulated (i.e., recruitment of force-generators during activation vs. distortion of
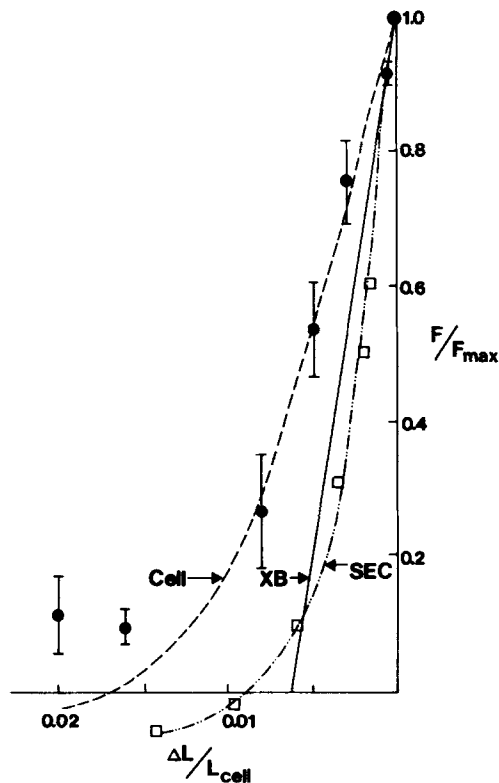

FIGURE 1. Length:force relationship ($L{:}F$) for single muscle cells and the contribution of cross-bridges and a series elastic component to the cell's $L{:}F$. The cell's $L{:}F$ was obtained by imposing a 2.0% release in cell length at peak isometric force and by plotting the resultant tension response against cell length during the length change (filled symbols). The data have been corrected for tension recovery that occurred during the length change (Warshaw and Fay, 1983*a*) and an exponential curve fitted to the data (Cell). The cell $L{:}F$ reflects the elastic properties of cross-bridges (*XB*) in series with an elastic component having an exponential $L{:}F$ (SEC) (see text). The data are from four experiments.

the attached cross-bridge population by a quick release). Should the observed elastic response of these single cells be intermediate between the behavior predicted for a pure series elastic component or for elasticity originating solely in cross-bridges, the observed pattern of response can be utilized to extract information about the elastic characteristics of the force-generators themselves.

As developed in detail in Appendix I, the stiffness of the cross-bridge population relative to the maximum active cell stiffness can be estimated by:

$$\{S_{tot}/[1 - (S_{tot}/S'_{tot})]\}/[F_m/(1 - F_m)],$$

where S_{tot} is cell stiffness at a given force level (F_m) during development of active force, and S'_{tot} is cell stiffness immediately following a quick release, which drops force to F_m from the peak of

A

Length $0.5 \updownarrow 2.0\%$ ΔL $\underline{\underline{I}}$ $0.5\% L_{Cell}$

                                                250Hz

$F \cdot F_{max}$

Force

E_{ACT} E_{QR} $\underline{I} \Delta F$

$F \cdot O$

STIM.

B

1.0 XB — — — — —

$XB + SEC$

E/E_{max}

SEC

——— Activation

— — Quick Release

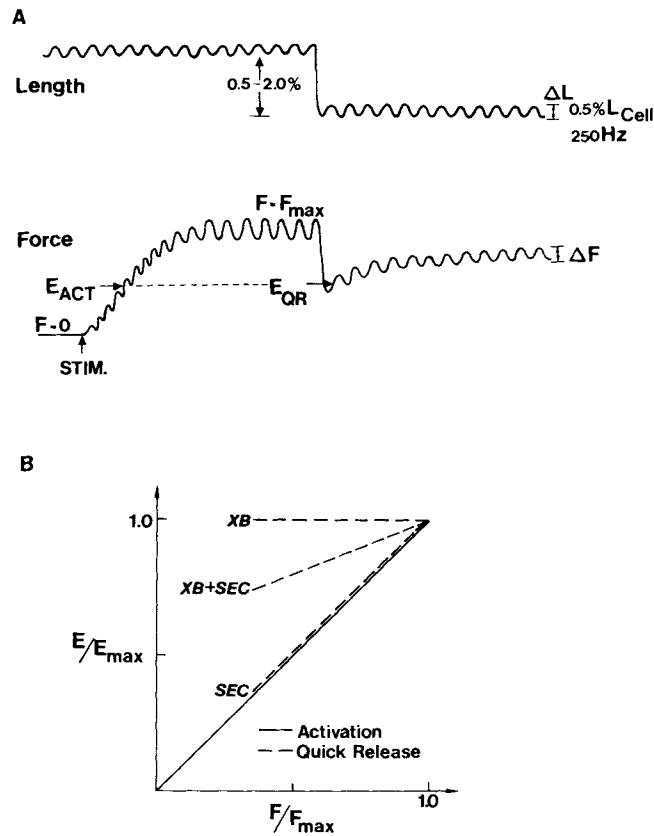   1.0

F/F_{max}

FIGURE 2. Determination of the origin of the elasticity within single smooth muscle cells. (*A*) Experimental protocol. Fiber stiffness (*E*) was determined by small (ΔL = 0.5% L_{cell}) sinusoidal (250-Hz) length perturbations that were continuously imposed during the development of force following activation and immediately following a quick release (0.5–2.0% L_{cell}). (*B*) Interpretation of results. Case 1: elasticity resides entirely within cross-bridges (*XB*). Recruitment of cross-bridges in parallel upon activation would result in normalized stiffness (E/E_{max}) proportional to force (F/F_{max}) (solid line). If at peak isometric force (F_{max}) as seen in *A*, a rapid release in length was imposed, force would drop suddenly. If bridges do not detach during the step, then stiffness at the completion of releases of varying magnitude (E_{QR}) will be independent of the force reached at the end of the step (dashed line, *XB*). Case 2: elasticity resides entirely within an exponential series elastic component (SEC). For this case, stiffness will always be proportional to force. Therefore, the proportional relationship between stiffness and force will be identical for both force development upon activation (solid line) and immediately following a quick release (dashed line, SEC). Case 3: elasticity resides within cross-bridge and series elastic component (*XB* + SEC). Both the recruitment of cross-bridges and the exponential nature of the series elastic component's length:force relationship dictate that stiffness and force be directly related during the development of force (see cases 1 and 2 above; solid line). However, at peak isometric force following a release, the slope of the stiffness:force relationship must lie somewhere between the extremes determined for cases 1 and 2 (dashed line, *XB* + SEC).

isometric force production. Note that stiffness and force are expressed relative to their value at peak force development (see Fig. 4). While this approach may determine the cross-bridge contribution to the cell's elastic properties, it does not allow insights into the properties of the series elastic component. Such insight can be obtained, however, by subtracting the length change absorbed by cross-bridge elasticity from the length:force relationship observed for the whole cell (see Fig. 1).

Stiffness determinations. Before the analysis of cell stiffness, the recorded force output was digitally filtered (Warshaw and Fay, 1983*b*) to remove oscillations superimposed upon the force trace owing to the force-transducer–damped resonance. Stiffness was then defined as the amplitude of the sinusoidal force change divided by the amplitude of the imposed sinusoidal length change (see Fig. 5, *A* and *B*). Thus, cell stiffness was determined on a cycle-by-cycle basis. Since stiffness measurements were obtained during force development after activation, when isometric force is changing most rapidly, a Fourier series analysis (Beauchamp, 1973; Kawai and Brandt, 1980) was used to estimate and extract the amplitude of the sinusoidal force change (see Appendix II) from the increasing steady state force upon which it was superimposed (see Fig. 5).

Stiffness was determined first while the cell was relaxed and then after activation. By subtracting relaxed stiffness from stiffness during isometric contraction, a value for active cell stiffness was calculated. Once stiffness values (S) were determined, these values were normalized to cell cross-sectional area (CSA) and length (L_{cell}) to provide an estimate of Young's modulus (i.e., $E = S \times CSA/L_{cell}$).

The contribution of cellular viscosity to cell stiffness was assessed by determining the phase angle between the applied sinusoidal length change and the observed change in force (see Appendix II). The phase angles of both the length and force sinusoids were calculated from the Fourier analysis. If the stiffness reflects purely elastic structures uninfluenced by cellular viscosity, the stiffness phase angle should be zero.

Cross-bridge kinetics and population distributions. Previous studies of tension transients in response to small step changes in length from single skeletal muscle fibers (Ford et al., 1977) and single smooth muscle cells (Warshaw and Fay, 1983*a, b*) suggest that the time course of tension recovery can be related to transitions between cross-bridge states (Huxley and Simmons, 1971; Ford et al., 1977; Eisenberg et al., 1980). To determine whether changes in cross-bridge kinetics occur between the period of force development following activation and the period of force maintenance, tension transients in response to 0.5% stretches and releases in cell length that were complete in 2.5 ms were studied at three time periods (see Fig. 7): (*a*) during force development, (*b*) at the moment peak isometric force was attained, and (*c*) after 15 s of maintained peak isometric force.

The time course of tension transients was analyzed to determine the number and characteristic rate of kinetically distinguishable processes by using a nonlinear least-squares regression analysis (Jennrich, 1981). Typically, the nonlinear least-squares fit used 750 points sampled at 2.5 kHz, starting at the moment force reached either a minimum or a maximum in response to a release or stretch in cell length (see Fig. 7). Although tension transients obtained during force development following activation were superimposed upon a changing force baseline, the rapid time course of the transients (i.e., tension recovery was >90% complete in <120 ms) meant that tension at any point during the transient was at most in error by 2.0% (i.e., 0.12 s for transient vs. 7.0 s to reach peak isometric force). Therefore, no force baseline corrections were made.

The time course of tension recovery in a given experiment was described best either by one or the sum of two exponential processes whose rate constants differ by an order of magnitude (Warshaw and Fay, 1983*a*). The best fit was chosen on the basis of a high coefficient of determination (R^2). The R^2 is an estimate of the variation within the tension data that can be

explained by the predicted exponential fit. Thus, an exponential fit was considered reasonable only if R^2 was >0.90, with 1.00 being the maximum.

Controls for Data Analysis

The data for active cell stiffness and active force will be presented as a modulus of the elasticity-to-force ratio (E/F) (see Fig. 6). Our initial studies of E/F during force development following activation (Warshaw et al., 1980) suggested that this property is not constant during force development but is greatest at the onset of force production. Since E/F required division by force, we were concerned that the enhanced E/F at low force levels resulted from an error in resolving active force at the onset of force development. Although the force-transducer was capable of resolving 0.2% of maximum force, we avoided any such errors by calculating E/F only at times when active force was at least 5.0% of its maximum.

To be certain that the methods for collecting and analyzing data were not the cause for an apparent enhancement of E/F, a simple electronic circuit was designed to mimic smooth muscle cell force and stiffness development, with the ratio of stiffness to force being constant. The circuit multiplied a sine wave of fixed amplitude by a ramp function so that the amplitude of the sine wave was constantly changing in proportion to the level of the ramp. The varying-amplitude sine wave was then added to the ramp to produce a mock cellular response where model force developed linearly with a superimposed sinusoidal force oscillation whose amplitude was exactly proportional to the force level. In addition, Poisson-distributed noise was superimposed upon the model force response to more accurately mimic real data. The model's composite signal (i.e., model force response) and that of the original fixed-amplitude sine wave (i.e., model length change) were digitized and analyzed in the same way as the cellular data.

Analysis of the electronic model's output revealed that the stiffness-to-force ratio was constant over the entire range of model force as expected from the known inputs. These results suggest that data handling and analysis did not introduce an error resulting in the observed enhancement of E/F at the onset of force development in the smooth muscle cell.

Solutions

All experiments were performed at room temperature (20°C) using amphibian physiological saline (Warshaw and Fay, 1983b). The solution was continuously bubbled with 95% O_2/5% CO_2 to pH 7.4.

Statistics

All data are presented as means ± standard error. In the analysis of the effect of time within the contraction upon the time course of tension recovery during the transients, recoveries were described by a single exponential process and compared using a repeated-measures analysis of variance (Jennrich et al., 1981). A trend within an experiment was considered significant at $p <$ 0.05.

RESULTS

Characterization of Cell Elasticity

At peak isometric force, a rapid 2.0% decrease in cell length results in a sudden decrease in force that coincides with the length step (Fig. 1). While the relationship between length and force appears to be linear for releases up to 0.8% L_{cell}, beyond this point the relationship clearly deviates from linearity. This deviation is due in part to superimposition of tension recovery processes on the elastic response (Warshaw

and Fay, 1983*a*). The observed length:force relationship (*L*:*F*) was corrected for superimposed tension recovery (dashed curve in Fig. 1) having rate constants and extents of tension recovery that were assumed to be identical to those observed following completion of the length step (see Warshaw and Fay, 1983*a*). The corrected *L*:*F* is described by the mathematical function $F/F_{max} = 1.11 \,[\exp\,(123dL/L_{cell})] - 0.11$; $R^2 = 0.99$. By taking the initial slope of the *L*:*F*, normalized cell stiffness is estimated as 92 F_{max}/L_{cell}. Since the cell *L*:*F* must reflect the elastic properties of structures associated with force generation (i.e., either cross-bridges or structures connected to the cross-bridges), experiments were carried out to assess the relative contribution of these structures to the observed elastic response.
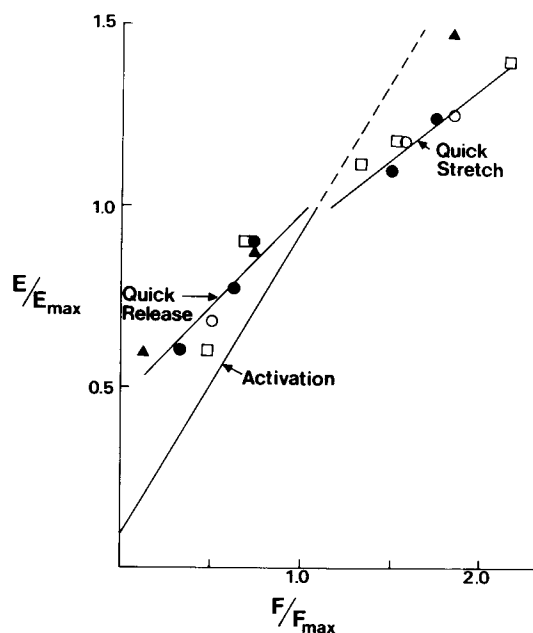

FIGURE 3. Stiffness data (*E*) throughout the development of force following activation (data from Warshaw and Fay, 1983*b*) and during force redevelopment following a release and stretch in cell length are plotted against force. Different symbols are data from four cells.

Origin of Cell Elasticity

The contribution of both cross-bridges and series elastic structures to the cell's elasticity was assessed by analyzing differences in the relationship between cell stiffness and force at two distinct times in an isometric contraction: during force development following activation, when the numbers of cross-bridges are presumably changing, and at the peak of isometric force immediately following a quick release, when the number of cross-bridges is assumed to be constant (see Fig. 2, Methods, and Appendix I for rationale). The relationship between stiffness and force during force generation and after a step length change at peak force is shown in Fig. 3. The modulus of elasticity is proportional to force both during force development and after a step length change at the peak of force production. However, the difference in slopes for the two conditions suggests that at any force level, cell stiffness is greater immediately after a step length change from the plateau of force production than at a similar force

obtained during force generation after activation. The observed relationship between stiffness and force is inconsistent with the elastic response originating either entirely within a series elastic component or entirely within the cross-bridges (Fig. 3). Rather, the data appear to indicate that the elasticity originates in cross-bridges that are in series with an exponential series elastic component (Fig. 2 B).

The elastic properties of the cross-bridge population can be obtained by analyzing the relationship between $S_{tot}/[1 - (S_{tot}/S'_{tot})]$ and $F_m/(1 - F_m)$, as indicated previously (Appendix I). According to our model, $S_{tot}/[1 - (S_{tot}/S'_{tot})]$ should be a linear function of $F_m/(1 - F_m)$. The slope of this relationship yields the cross-bridge stiffness relative to overall cell stiffness. The data were fitted by linear regression to the equation $S_{tot}/[1 - (S_{tot}/S'_{tot})] = (1.32 \pm 0.16) [F_m/(1 - F_m)] + (0.21 \pm 0.34)$; $R^2 = 0.94$ (see Fig. 4). The slope of this relationship suggests that the cross-bridge stiffness is between 1.2 and 1.5 times greater than the measured cell stiffness. Thus, cell stiffness underestimates cross-bridge stiffness by as much as 33%. Although alternative models for the location of the cell's elasticity were considered (e.g., parallel elastic compo-
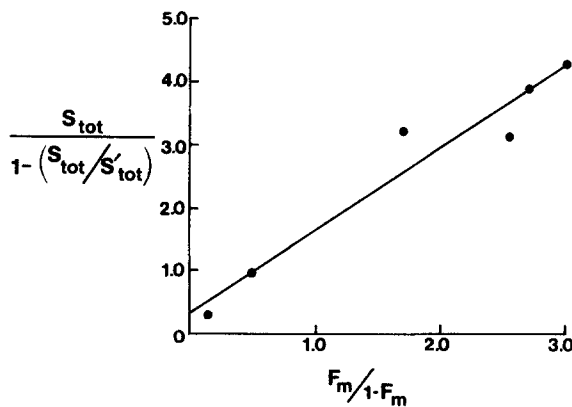

FIGURE 4. The relationship between $S_{tot}/[1 - (S_{tot}/S'_{tot})]$ and $F_m/(1 - F_m)$ in a single smooth muscle cell. The slope of this relation (see Appendix I) was used to estimate the cross-bridge stiffness relative to the observed cell stiffness.

nent), the model presented was chosen as the simplest model consistent with the existing single-cell mechanical data.

Knowing the cross-bridge contribution to cell stiffness and assuming a linear cross-bridge L:F whose slope (i.e., stiffness) is 1.32 times that of the initial portion of the cell's L:F (see above), one can derive the L:F for the series elastic component (see Fig. 1). Since the cross-bridges are in series with an elastic element, subtracting the cross-bridge compliance at every force level from the cell compliance should yield the L:F of the series elastic component. This L:F was fitted by nonlinear regression analysis to an exponential equation of the form $F/F_{max} = 1.03 [exp (554\Delta L/L_{cell})] - 0.03$; $R^2 = 0.95$.

Modulus of Elasticity during Force Development

The production of force upon activation and the corresponding stiffness change throughout development and maintenance of force in a typical single smooth muscle cell are shown in Fig. 5, A and B. Note that the cellular modulus of elasticity, though quite small in the relaxed cell ($E_{rest} = 0.21 \pm 0.06 \times 10^4$ mN/mm^2; $n = 5$), begins to
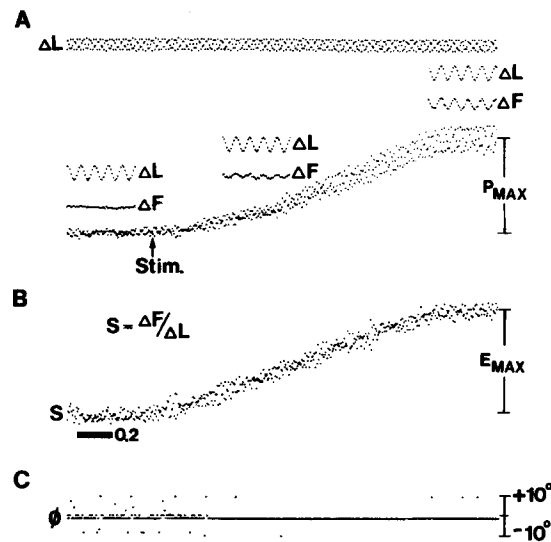
A

B

C

FIGURE 5.   Active stiffness and phase angle during force development upon activation. (*A*) Isometric force development with superimposed sinusoidal tension fluctuations (ΔF) in response to the imposed sinusoidal length change (ΔL = 0.5% L_{cell} at 250 Hz). The cell was stimulated electrically at the arrow (Stim.). The maximum active force (F_{max}) is normalized to muscle cross-sectional area and shown as maximum active stress (P_{max} = 140 mN/mm^2). (*B*) Stiffness time course. Stiffness increases with active force. Stiffness is normalized for cell length (173 μm) and cross-sectional area (9.6 μm^2) and is presented as Young's modulus (E_{max} = 6.1 × 104 mN/mm^2). (*C*) Phase angle (ϕ).

increase as force increases following activation (i.e., increased amplitude of force envelope owing to the constant length oscillation at 250 Hz). Both peak isometric force (F_{max} = 192 ± 32 μg; n = 7) and the modulus of elasticity (E_{ACT} = 1.83 ± 0.68 × 10^4 mN/mm^2; n = 7) reach their maxima between 4 and 7 s after stimulation (see Table I). To determine whether the changes in stiffness observed during force development reflect changes only in the elasticity of structures involved in force development, an estimate of the phase angle between the length change and the resultant force response was obtained (see Fig. 5 *C*) (Meiss, 1978). The phase angle was 10 ± 13 degrees for 26 cycles at rest and 3 ± 7 degrees for 27 cycles at the peak of force production. Since the phase angle during both the resting and active states was not different from zero degrees, we concluded that cell stiffness must have originated

TABLE I

Single Smooth Muscle Cell Data

Characteristic	Units	Value
Length (L_{cell})	μm	146 ± 21 (7)
Cross-sectional area (*CSA*)	μm^2	20.8 ± 4.7 (7)
Active force (F_{max})	μg	192 ± 32 (7)
Active stress (P_{max}) (F_{max}/CSA)	mN/mm^2	132 ± 39 (7)
Relaxed Young's modulus (E_{rest})	× 10^4 mN/mm^2	0.21 ± 0.06 (5)
Active Young's modulus	× 10^4 mN/mm^2	1.82 ± 0.68 (7)
Relaxed phase angle (250 Hz)	Degrees	6 ± 5 (4)
Active phase angle (250 Hz)	Degrees	2 ± 4 (4)

Values are means ± SE. Numbers in parentheses are the number of cells.

in purely elastic structures and that any increase in cell stiffness with force production is the result of a true increase in cell stiffness and not of changes in the cell's viscous properties upon activation. Although active stiffness and force appear to rise together, a more sensitive index of their relationship can be obtained by calculating the ratio of the modulus of elasticity to force (E/F) (see Fig. 6). Note that E/F is greater at the onset of force development (i.e., at 0.05 F_{max}) and decreases monotonically to a constant value at peak force, which is maintained even after 15 s of contraction.
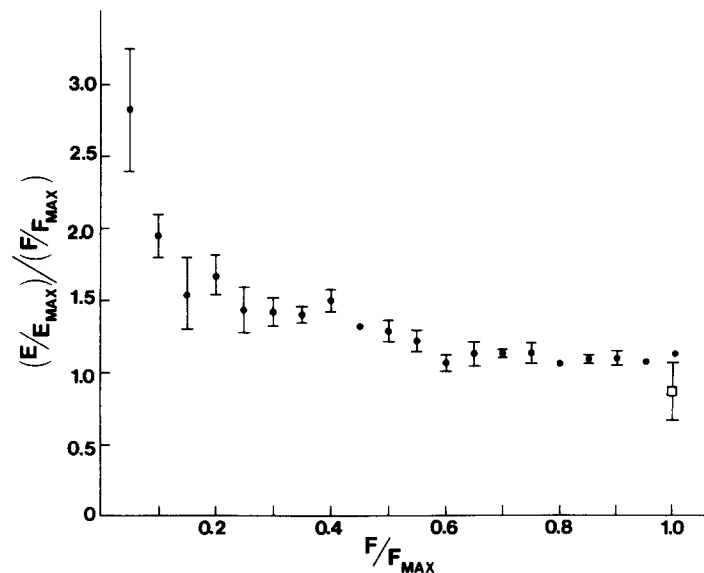

FIGURE 6. Relationship of modulus of elasticity-to-force ratio (E/F) vs. force during force development following activation. Mean values and standard errors $(n = 4)$ for E/F are plotted vs. the value of force at which E/F was calculated. Force and modulus of elasticity are normalized to the peak isometric force (F/F_{max}) and modulus (E/E_{max}).

Tension Transients vs. Contraction Time

To further characterize the processes responsible for an increased E/F at the onset of force development, tension transients were analyzed (a) throughout the period of force development, (b) at peak isometric force, and (c) after 15 s of maintained peak isometric force (see Fig. 7). Tension transient analysis revealed that, at all three times, 67% of the tension recoveries were fitted best by a single exponential, whereas the remaining 33% were described by two exponential processes (see Fig. 7). There was no apparent relationship between the ability to fit a single or double exponential and the time during which the transients were obtained. In order to compare the overall rate of tension recovery between several cells, the recovery process was fitted by a single exponential for all cells. In those cells in which two exponential processes could be discerned, a forced single-exponential fit required a rate constant intermediate between the fast and slow segments of recovery.

Comparison of transients at different times in the contraction revealed that the cell was capable of complete tension recovery within 1 s after a sudden change in length.
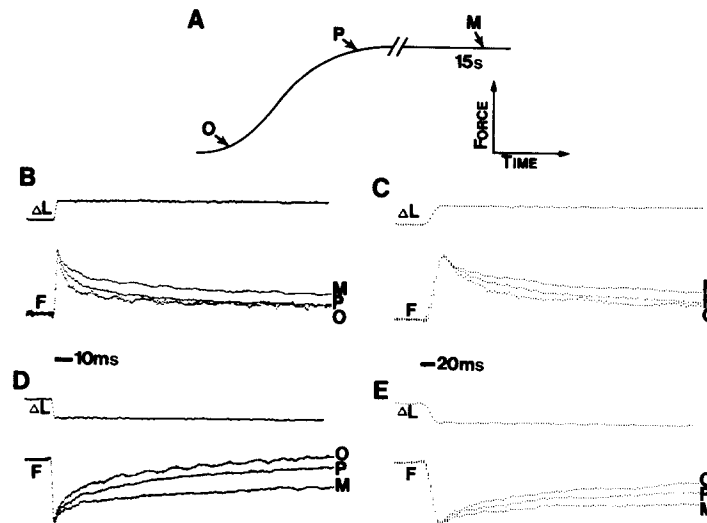
FIGURE 7. Tension transients during a contraction in a single smooth muscle cell. (*A*) Schematic of force production in a smooth muscle cell. At three time points during the contraction, at the onset of force development (O), at peak isometric force (P), and after 15 s of maintained tension (M), small (0.5% L_{cell}), rapid (2.5 ms) step stretches (*B* and *C* on a faster time scale) and releases (*D* and *E* on a faster time scale) were imposed. To facilitate comparison of the tension transients obtained at O, P, and M, responses were normalized so that the absolute value of the immediate force change in responses to the ΔL was considered to be 100%. All tension responses attained complete recovery of the initial tension change in response to ΔL; therefore, the apparent decrease in extent of recovery going from O to M is indicative of slower time constants for recovery.

However, the time constants for tension recovery after a release or a stretch were fastest immediately after activation. The mean time constant for tension recovery following a sudden length change was slowed about two times for a release, and almost six times for a stretch at peak isometric force relative to the early stages of force development. Comparison of the mean time constant for tension recovery following both releases and stretches 15 s into force maintenance revealed further slowing of recoveries relative to those seen immediately upon achieving peak isometric force (see Fig. 7 and Table II).

TABLE II

Tension Recovery Data

Length change	Tension recovery time constant			ANOVA
	Time in contraction*			
	Early	Peak	Late	
	ms	*ms*	*ms*	
Release	23.9 ± 3.2 (4)	50.8 ± 4.3 (4)	61.0 ± 5.1 (4)	$p < 0.03$
Stretch	5.3 ± 1.0 (4)	30.3 ± 4.1 (4)	41.0 ± 6.9 (4)	$p < 0.02$

Values are means ± SE. Numbers in parentheses are the number of cells.

*Three time periods during a contraction were studied: early – 2 s after the onset of force development; peak – the time when maximum active force was attained; late – 15 s after peak force was attained.

Changes in the characteristics of the tension transients were also evident in fibers where tension recovery could be resolved into two exponential processes. For example, in Fig. 7, D and E, both the fast and slow components of tension recovery following a release were slower during tension maintenance than during the onset of active force. Immediately after activation, the fast and slow recovery processes had time constants of 2.0 and 40 ms, respectively, which slowed to 7.1 and 67 ms after 15 s of maintained tension. In addition, the fast phase of recovery accounted for 64% of the total recovery early in the contraction but for only 50% after 15 s of force maintenance. These differences in the extent and rate of recovery may provide insight into variations in the distribution of cross-bridge states (Eisenberg et al., 1980; Warshaw and Fay, 1983a, b) as well as the kinetics of the cross-bridge cycle (Murphy et al., 1983) during the development and maintenance of force.

DISCUSSION

The generation of muscular force is accompanied by increased fiber stiffness, which, in skeletal muscle, is presumed to reflect the attachment of myosin cross-bridges to actin filaments (Huxley, 1980). Since both filamentous actin and myosin are found in smooth muscle (Cooke and Fay, 1972; Bond and Somlyo, 1982), it is assumed that processes similar to those in skeletal muscle are responsible for changes in force and stiffness upon activation in smooth muscle. However, although smooth muscles generate comparable forces per cross-sectional area, they require much longer times to achieve those forces, and in addition maintain those forces with far greater economy of ATP utilization (Paul et al., 1976; Siegman et al., 1980). The studies described in this article were directed at determining the origin of cell elasticity and characterizing the mechanical events leading to force development and maintenance.

The increased E/F during the early phase of force development reported in this study may well be a characteristic of all muscles, as similar results have been reported for whole smooth muscle (Dillon and Murphy, 1982; Kamm and Stull, 1986) and both single skeletal fibers (Cecchi et al., 1982; Ford et al., 1986) and whole skeletal muscle (Bressler and Clinch, 1974). If we assume that (a) all attached cross-bridges produce an equal amount of average force per cross-bridge, (b) all attached force-generating cross-bridges have equal stiffness, and (c) changes in stiffness in a single cell are due entirely to changes in the number of attached cross-bridges, then the enhanced E/F in smooth muscle cells in the period immediately after activation would suggest that one or all of these statements do not apply during the period of force development. Either the assumed relationship between stiffness and force is too simplistic a view in smooth muscle cells, or stiffness may be related in part to structures other than the cross-bridges. We must therefore ask: (a) What structures are being probed mechanically? (b) How are these structures being changed to account for the differences in E/F and tension transients during both force development and maintenance?

Location of Elasticity

By assuming that the generation of force following activation in single smooth muscle cells reflects the recruitment of force-generators and that at the peak of force production their numbers remain constant during a rapid release, it was possible to compare cell stiffness during two different physiological states where, at a given force

level, only the numbers of force-generators differ. The experimental design and the model used to analyze the data suggest that the elastic properties of a single smooth muscle cell reside within cross-bridges that are connected in series with an elastic component having an exponential L:F. Since this element must be physically linked to the cross-bridge and transmit force, possible sources of the elasticity are the myosin filament, the actin filament, dense bodies, and membrane-dense plaques. The extent to which any of these structures contribute to the series elasticity remains to be determined.

Because of the presence of a series elastic element, any attempt to equate cell and cross-bridge stiffness would underestimate the true cross-bridge stiffness. The present experimental approach allowed us to estimate the portion of the cell's elastic response that can be attributed to cross-bridges. Our results indicate that cross-bridge compliance ranges between 0.73 and 0.91% L_{cell}. In earlier studies of the mechanics of these single cells, we reported that cross-bridge compliance was 1.5% L_{cell} (Warshaw and Fay, 1983a, b), which assumed that the cell's elastic response originated entirely within the cross-bridge. This apparent twofold overestimate of cross-bridge compliance resulted from interpreting the lack of superposition of L:F from releases obtained during force development following activation onto the L:F at peak isometric force as indicating that the cell's elastic response could not be explained solely by a series elasticity (see Fig. 19 and pp. 186–187 in Warshaw and Fay, 1983b). As there were only limited data available to assess stiffness during releases of varying magnitudes at peak isometric force, we took the measured stiffness to fully reflect cross-bridge stiffness, since it was not possible to explain the results as reflecting a pure series elasticity. In the present studies, aimed at further probing the origin of the cell's elastic response, we find that a portion of the cell's elasticity must reside in structures outside the cross-bridge. While our resulting estimate of cross-bridge compliance is thus reduced, our present estimate of cross-bridge compliance in smooth muscle suggests that the smooth muscle myosin cross-bridge is still considerably (1.5–1.8 times) more compliant than cross-bridges in fast skeletal muscle (Ford et al., 1977), as we previously suggested (Warshaw and Fay, 1983b).

Enhanced E/F

One possibility that could account for the enhanced E/F at the onset of force development is the exponential nature of the cell's L:F.[1] The exponential form is probably

---

[1] If the cell's exponential elastic response is related to a series elastic component with a length:force characteristic of the form:

$$F = A[\exp(bdL) - 1].\tag{1}$$

The stiffness $(S = dF/dl)$ of this elastic component would be:

$$S = bF + Ab.\tag{2}$$

Thus, the stiffness-to-force ratio equals:

$$S/F = b + (Ab/F).\tag{3}$$

As force develops upon activation and is normalized to maximum force, F will have values that range between 0 and 1. Substituting these force values into Eq. 3, S/F approaches infinity at $F = 0$, whereas $S/F = b$ at $F = 1$. Thus, the enhanced S/F observed might be explained by the presence of a series elastic component having an exponential length:force characteristic.

related to the presence of an exponential series elasticity within the cell. However, the extent of the enhancement of E/F owing to the exponential nature of its elastic properties is considerably smaller than that actually observed. For example, one can calculate that at 9 and 24% of F_{max} during the onset of force development, the observed exponential elasticity would result in 26 and 8% increases in E/F, respectively, above that observed at F_{max}, whereas measurements of E/F revealed 95 and 50% enhancements, respectively. Clearly, then, other factors must also be responsible for the enhancement of E/F during the onset of force development.

The pathway of cross-bridge entry into the force-generating state also could contribute to the increased E/F. The cross-bridge cycle appears to involve transitions among at least three states (Huxley and Simmons, 1971; Eisenberg et al., 1980): (a) detached from actin, (b) attached but producing little or no force (weak binding), and (c) attached and producing significant force (strong binding). The stiffness associated with the two attached states is purportedly equivalent (Julian and Morgan, 1981). Thus, active force in a muscle fiber mainly reflects the sum of forces produced by attached cross-bridges in the high-force state, while stiffness reflects the contributions from both the low- and high-force cross-bridge populations. Thus, changes in E/F may reflect changes in the ratio of attached low- and high-force–producing cross-bridges. Our data, indicating a higher E/F during initial stages of force development, suggest that, during this period, the ratio of the low- to high-force–producing cross-bridges may be elevated relative to the population distribution that exists later, after activation.

This hypothesis is further supported by comparison of the tension transients obtained during the development of force (see Fig. 7). Data from both single smooth muscle cells (Warshaw and Fay, 1983a, b) and single skeletal muscle fibers (Julian and Morgan, 1981; Cecchi et al., 1982) suggest that the fast tension recovery phase following a release reflects the transition of attached cross-bridges from the low- to high-force states; the extent of this recovery is in large part a measure of the relative proportion of cross-bridges in the low- and high-force states (Eisenberg et al., 1980; Warshaw and Fay, 1983b). In single smooth muscle cells, the extent of tension recovery owing to this rapid process was always greatest at the onset of force development. Because of the small number of cells ($n = 2$), where both fast and slow phases could be clearly discerned throughout the development and maintenance of force, a quantitative assessment of the decrease in the extent of fast recovery is not possible. The changes in both E/F and the relative extent of the fast phase of tension recovery suggest, however, that shifts in the relative proportion of low- and high-force attached cross-bridges may have taken place during activation.

Why should such shifts in the population of attached cross-bridges take place during the period of force development? There are two likely hypotheses. (a) During the onset of force development, activated cross-bridges first enter the cross-bridge cycle, principally by going from the detached to the attached low-force–producing state, and then, after some time determined by the finite rate constants for transitions in the cycle, the cross-bridges achieve their steady state distribution. Or (b) during the process of activation, rate constants for transitions in the cross-bridge cycle are modulated and thus the population distribution of cross-bridges varies. We cannot say to what extent these two factors are responsible for the apparent shifts in the ratio of attached low- to high-force–producing cross-bridges in single smooth muscle cells.

Temporal Modulation of Smooth Muscle Cell Mechanics

As the fast and slow phases of tension recovery are believed to reflect different steps in the cross-bridge cycle, the present results, indicating slowing of all phases of the tension transient, suggest that several steps in the cycle are slowing as force develops and is maintained. These results cannot be explained by any changes in the series elastic component since changes in tension recovery kinetics take place even in the absence of any stiffness changes during the period of force maintenance (Fig. 6).

When modulation of cross-bridge kinetics is considered, one usually refers to a homogeneous population of cross-bridges being modulated, which may occur in these single cells. However, if only a fraction of the cross-bridges were affected, then overall slowing of the cycle could be obtained. For instance, if a subpopulation of cross-bridges became rigor-like, more commonly known as latch-bridges (Siegman et al., 1976; Murphy et al., 1983), then the effect of this population would be to impose an internal load on the remaining normally cycling cross-bridges. However, Butler et al. (1986) do not find evidence for such an internal load from energetic measurements on rabbit taenia coli. It is thus noteworthy that the slowing of recovery during tension transients in single smooth muscle cells is similar to results during rigor induction in both smooth and skeletal muscles (Mulvany, 1975; Siegman et al., 1976; Guth and Junge, 1982).

In summary, then, the present results indicate that cell elasticity resides within cross-bridges connected in series with an elastic component. In addition, the progression from the resting state to maintained tension maintenance in smooth muscle involves not only attachment of cross-bridges but also at least one additional process characterized by the redistribution of attached cross-bridge states and slowing of all aspects of the cross-bridge cycle. This slowing of the cycle during periods of force maintenance agrees with studies revealing decreased energy utilization (Siegman et al., 1980) and slower velocity of shortening (Dillon et al., 1981) during force maintenance. The cause of these changes in the cycle kinetics in smooth muscle remains an intriguing puzzle whose solution awaits further investigation.

APPENDIX I

Characterization of the Cell Elasticity

An experimental protocol was designed (see Methods) that will test for the origin of the cell's elasticity (see Figs. 2 and 3). Since the stiffness vs. force relationship suggests that the cell's elastic response originates from cross-bridges in series with an elastic component having an exponential length:force relationship, the following analysis was used to characterize both the cross-bridge contribution to cell stiffness and the length:force relationship of the series elastic component.

Assumptions

(a) The cell's nonlinear length:force relationship (see Fig. 1) reflects the length:force relationships of both series elastic and cross-bridge elements.

(b) A series elastic component (SEC) exists that has an exponential length:force relationship (see Fig. 1) normalized to cell length (L_{cell}) and peak isometric force (F_{max}):

$$F_{SEC}/F_{max} = \{(A + 1) \exp [b(dL_{SEC}/L_{cell})]\} - A, \tag{1}$$

where A and b are constants, and the length change of the series elastic component, dL_{SEC}, is:

$$dL_{SEC} = L_{SEC}(\text{final}) - L_{SEC}(\text{initial}),\tag{2}$$

with $L_{SEC}(\text{initial})$ equal to L_{SEC} at F_{max}.

(c) The cross-bridge population (XB) has a normalized linear length:force relationship (see Fig. 1) normalized to L_{cell} and F_{max}:

$$F_{XB}/F_{max} = (F/F_{max})k(dL_{XB}/L_{cell}),\tag{3}$$

where, upon activation, F/F_{max} is force normalized to F_{max} during force development. In addition, F/F_{max} reflects the numbers of attached cross-bridges. The k is the cross-bridge stiffness constant. The cross-bridge length change dL_{XB} equals $L_{XB}(\text{final}) - L_{XB}(\text{initial})$, where $L_{XB}(\text{initial})$ is the cross-bridge length at F_{max}.

(d) The quick release in cell length is sufficiently rapid to prevent significant detachment or attachment of cross-bridges during the release itself.

Analytical Framework

Since cell stiffness (S_{cell}) is related to both series elastic (S_{SEC}) and cross-bridge (S_{XB}) stiffnesses, S_{SEC} and S_{XB} normalized to cell stiffness (S_{max}) at F_{max} are defined as dF/dL. Therefore:

$$S_{SEC}/S_{max} = b[(F_{SEC}/F_{max}) + A]\qquad(\text{derived from Eq. 1});\tag{4}$$

$$S_{XB}/S_{max} = (F/F_{max})k\qquad(\text{derived from Eq. 3}).\tag{5}$$

Since the series elastic component and cross-bridges are connected in series, their reciprocal stiffnesses add. Therefore, reciprocal cell stiffness normalized to S_{max} is:

$$1/(S_{cell}/S_{max}) = [1/(S_{SEC}/S_{max})] + [1/(S_{XB}/S_{max})].\tag{6}$$

Rearranging Eq. 6:

$$S_{cell}/S_{max} = (S_{SEC}S_{XB})/[(S_{SEC} + S_{XB})S_{max}].\tag{7}$$

Rearranging Eq. 7 to solve for S_{SEC}:

$$S_{SEC} = (S_{cell}S_{XB})/(S_{XB} - S_{cell}).\tag{8}$$

Substituting from Eqs. 4 and 5 for S_{SEC} and S_{XB}, respectively:

$$b[(F_{SEC}/F_{max}) + A] = S_{cell}(F/F_{max})k/[S_{max}(F/F_{max})k - S_{cell}].\tag{9}$$

The series arrangement of the series elastic and cross-bridge elements results in F_{SEC}, F_{XB}, and cell force (F_{cell}) being equal:

$$F_{cell} = F_{SEC} = F_{XB}.\tag{10}$$

Substituting F_{cell} for F_{SEC}, Eq. 9 becomes:

$$b[(F_{cell}/F_{max}) + A] = S_{cell}(F/F_{max})k/[S_{max}(F/F_{max})k - S_{cell}].\tag{11}$$

For the case where stiffness is obtained at different force levels during activation (see Methods and Fig. 2), the cross-bridge number is increasing (i.e., F/F_{max} is increasing). Therefore, Eq. 11 applies. However, at peak isometric force, where the maximum number of cross-bridges are attached (i.e., $F/F_{max} = 1$), when a quick release (0.5–5.0% L_{cell}) is applied, Eq. 11 becomes:

$$b[(F'_{cell}/F_{max}) + A] = S'_{cell}(k)/[S_{max}(k) - S'_{cell}],\tag{12}$$

where F'_{cell} and S'_{cell} are cell force and stiffness within 4 ms after completion of the quick release (see Fig. 2).

If one compares Eqs. 11 and 12 at equivalent force levels during activation and following a quick release (i.e., $F_{cell} = F'_{cell}$), then the left-hand sides of Eqs. 11 and 12 are equal. Setting the right-hand sides of Eqs. 11 and 12 equal to each other and rearranging results in the following relationship:

$$k = (S_{cell}/S_{max})/[1 - (S_{cell}/S'_{cell})]/\{(F/F_{max})/[1 - (F/F_{max})]\}. \tag{13}$$

Since all terms on the right-hand side of Eq. 13 are measurable, the cross-bridge stiffness constant (k) can be determined from the slope of the assumed linear relationship between $(S_{cell}/S_{max})/[1 - (S_{cell}/S'_{cell})]$ and $\{(F/F_{max})/[1 - (F/F_{max})]\}$. This relationship is plotted in Fig. 4 with $(S_{cell}/S_{max})/[1 - (S_{cell}/S'_{cell})]$ represented by $S_{tot}/[1 - (S_{tot}/S'_{tot})]$ on the y axis and $\{(F/F_{max})/[1 - (F/F_{max})]\}$ by $[F_m/(1 - F_m)]$ on the x axis. Once the value for the cross-bridge stiffness relative to the cell stiffness is obtained from k, one can plot the derived cross-bridge length:force relationship and the experimentally obtained cell length:force relationship on the same graph (see Fig. 1). By subtracting the cross-bridge length:force relationship from the cell length:force relationship, the resultant nonlinear length:force relationship will be that of the series elastic component (see Fig. 1).

APPENDIX II

The Fourier series describes a given waveform, $X(t)$, in terms of a series of sine and cosine waves whose frequencies are an integral multiple ($k = 1, 2, \ldots n$) of a fundamental frequency (f_0):

$$X(t) = a_0/2 + \Sigma[a_k \cos (k2\pi f_0 t) + b_k \sin (k2\pi f_0 t)].$$

Since the sinusoidal length changes were specified and generated by computer, we could assign the fundamental frequency for both length and force changes precisely. This was evident in that coefficients (a_k, b_k) of the first five harmonics were <5% of the fundamental coefficients (a_1, b_1). Using the Fourier series analysis, the amplitude of both length and force changes (A_l, A_f) were calculated from the coefficients of the fundamental [$A_{l,f} = SQRT(a^2 + b^2)$] on a cycle-by-cycle basis. The estimated force amplitude change was then divided by the length amplitude change to obtain stiffness ($S = A_f/A_l$) on a cycle-by-cycle basis. The phase angle (ϕ) between length (ϕ_l) and force (ϕ_f) was calculated as follows:

$$\phi_l = \tan^{-1}[a_1(l)/b_1(l)] \text{ and } \phi_f = \tan^{-1}[a_1(f)/b_1(f)].$$

The stiffness phase angle is then $\phi_f - \phi_l$.

REFERENCES

Beauchamp, K. G. 1973. Fourier series and analysis. *In* Signal Processing Using Analog and Digital Techniques. John Wiley & Sons, New York, NY. 231–279.

Bond, M., and A. V. Somlyo. 1982. Dense bodies and actin polarity in vertebrate smooth muscle. *Journal of Cell Biology.* 95:403–413.

Bressler, B. H., and N. F. Clinch. 1974. The compliance of contracting skeletal muscle. *Journal of Physiology.* 237:477–493.

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.

Cecchi, G., P. J. Griffiths, and S. Taylor. 1982. Muscular contraction: kinetics of cross-bridge attachment studied by high frequency stiffness measurements. *Science.* 217:70–72.

Cooke, P. H., and F. S. Fay. 1972. Correlation between fiber length, ultrastructure, and the length-tension relationship of mammalian smooth muscle. *Journal of Cell Biology.* 52:105–116.

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

Dillon, P. F., and R. A. Murphy. 1982. High force development and cross-bridge attachment in smooth muscle from swine carotid arteries. *Circulation Research.* 50:799–804.

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

Fay, F. S., R. Hoffman, S. Leclair, and P. Merriam. 1982. Preparation of individual smooth muscle cells from the stomach of *Bufo marinus. Methods in Enzymology.* 85:284–292.

Fay, F. S., D. D. Rees, and D. M. Warshaw. 1981. The contractile mechanism in smooth muscle. *In* Membrane Structure and Function. E. E. Bittar, editor. John Wiley & Sons, New York, NY. 4:79–130.

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

Ford, L. E., A. F. Huxley, and R. M. Simmons. 1986. Tension transients during the rise of tetanic tension in frog muscle fibers. *Journal of Physiology.* 372:595–609.

Guth, K., and J. Junge. 1982. Low Ca^{++} impedes cross-bridge detachment in chemically skinned taenia coli. *Nature.* 300:775–776.

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, Inc., New York, NY. 1–35.

Huxley, A. F. 1980. *In* Reflections on Muscle. Princeton University Press, Princeton, NJ. 68–79.

Huxley, A. F., and R. M. Simmons. 1971. Proposed mechanism for force generation in striated muscle. *Nature.* 233:533–538.

Jennrich, R. 1981. Nonlinear regression. *In* BMDP Statistical Software 1981. W. J. Dixon, editor. University of California Press, Los Angeles, CA. 290–304.

Jennrich, R., P. Sampson, and J. France. 1981. Analysis of variance and covariance including repeated measures. *In* BMDP Statistical Software 1981. W. J. Dixon, editor. University of California Press, Los Angeles, CA. 359–388.

Julian, F. J., and D. L. Morgan. 1981. Variation of muscle stiffness with tension during tension transients and constant velocity shortening in the frog. *Journal of Physiology.* 319:193–203.

Kamm, K. E., and J. T. Stull. 1986. Activation of smooth muscle contraction: relation between myosin phosphorylation and stiffness. *Science.* 232:80–82.

Kawai, M., and P. W. Brandt. 1980. Sinusoidal analysis: a high resolution method for correlating biochemical reactions with physiological processes in activated skeletal muscles of rabbit, frog and crayfish. *Journal of Muscle Research and Cell Motility.* 1:279–303.

Meiss, R. A. 1978. Dynamic stiffness of rabbit mesotubarium smooth muscle: effect of isomeric length. *American Journal of Physiology.* 234:C14–C26.

Mulvany, M. J. 1975. Mechanical properties of frog skeletal muscles in iodoacetic acid rigor. *Journal of Physiology.* 252:319–334.

Murphy, R. A. 1980. Mechanics of vascular smooth muscle. *In* Handbook of Physiology. American Physiological Society, Bethesda, MD. II:325–351.

Murphy, R. A., M. O. Aksoy, P. F. Dillon, W. T. Gerthoffer, and K. E. Kamm. 1983. The role of

myosin light chain phosphorylation in regulation of the cross-bridge cycle. *Federation Proceedings.* 42:51–56.

Paul, R. J., E. Gluck, and J. C. Ruegg. 1976. Cross-bridge ATP utilization in arterial smooth muscle. *Pflügers Archiv.* 361:297–299.

Siegman, M. J., T. M. Butler, S. V. Mooers, and R. E. Davies. 1976. Cross-bridge attachments, resistance to stretch, and viscoelasticity in mammalian vascular smooth muscle. *Science.* 191:383–385.

Siegman, M. J., T. M. Butler, S. V. Mooers, and R. E. Davies. 1980. Chemical energetics of force development, force maintenance, and relaxation in mammalian smooth muscle. *Journal of General Physiology.* 76:609–629.

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. 1983a. Tension transients in single isolated smooth muscle cells. *Science.* 219:1438–1441.

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

Warshaw, D. M., and F. S. Fay. 1984. Cross-bridge elasticity in single smooth muscle cells. *Biophysical Journal.* 45:346a. (Abstr.)

Warshaw, D. M., D. D. Rees, and F. S. Fay. 1980. Active stiffness measurement of single smooth muscle cells. *Federation Proceedings.* 39:1730. (Abstr.)